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„Regulation and function of conserved *Platynereis*  
hormones“

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## Table of contents

|                                                                                       |           |
|---------------------------------------------------------------------------------------|-----------|
| <b>1. Introduction.....</b>                                                           | <b>8</b>  |
| 1.1 <i>Platynereis dumerilii</i> as a model organism .....                            | 8         |
| 1.1.1 The life cycle of <i>Platynereis</i> .....                                      | 9         |
| 1.1.2 Pigmentation of <i>Platynereis</i> .....                                        | 11        |
| 1.2 Circadian rhythms .....                                                           | 13        |
| 1.2.1 Input pathways.....                                                             | 14        |
| 1.2.2 Output pathways.....                                                            | 14        |
| 1.2.3 Fly and mammalian circadian clocks .....                                        | 15        |
| 1.3 Role and function of pigment dispersing hormone-like peptides in invertebrates... | 18        |
| 1.3.1 Function of PDHs in crustaceans .....                                           | 18        |
| 1.3.2 Function of PDF in insects .....                                                | 19        |
| 1.3.3 Function of PDF in nematodes .....                                              | 22        |
| 1.4 Function and regulation of melatonin .....                                        | 23        |
| 1.4.1 Functions of melatonin .....                                                    | 24        |
| 1.4.2 Melatonin receptors.....                                                        | 26        |
| <b>2. Aim of the project .....</b>                                                    | <b>29</b> |
| <b>3. Results .....</b>                                                               | <b>30</b> |
| 3.1 Melatonin receptors .....                                                         | 30        |
| 3.1.1 Cloning of a second melatonin receptor candidate (MelR2) .....                  | 30        |
| 3.1.2 BAC screening of MelR1 and MelR2.....                                           | 30        |
| 3.1.3 Expression pattern of melatonin receptor candidate 2 in larvae .....            | 31        |
| 3.1.4 Receptor-ligand binding assays (Impedance assay).....                           | 33        |
| 3.2 PDF receptor .....                                                                | 38        |
| 3.2.1 Elongation of the pdfr candidate fragment .....                                 | 38        |
| 3.2.2 Expression quantification in tails of immature worms .....                      | 39        |
| 3.3 Pigment dispersing factor in <i>Platynereis</i> .....                             | 41        |
| 3.3.1 Spatiotemporal expression pattern of PDF in immature <i>Platynereis</i> .....   | 41        |
| 3.3.2 Function and regulation of PDF in <i>Platynereis</i> .....                      | 43        |
| <b>4. Discussion.....</b>                                                             | <b>55</b> |
| 4.1 Target tissues of melatonin in <i>Platynereis</i> .....                           | 55        |

|           |                                                                            |           |
|-----------|----------------------------------------------------------------------------|-----------|
| 4.1.1     | Embryonic localisation of melatonin receptor 2 in <i>Platynereis</i> ..... | 55        |
| 4.1.2     | Ligand-binding assays .....                                                | 57        |
| 4.2       | Function and regulation of PDF in <i>Platynereis</i> .....                 | 59        |
| 4.2.1     | Elongation of PDF receptor.....                                            | 59        |
| 4.2.2     | Uncovering the role of PDF in <i>Platynereis</i> .....                     | 59        |
| <b>5.</b> | <b>Materials and Methods .....</b>                                         | <b>65</b> |
| 5.1       | <i>Platynereis dumerilii</i> culture.....                                  | 65        |
| 5.2       | Cloning and subcloning.....                                                | 65        |
| 5.2.1     | RNA extraction and cDNA synthesis .....                                    | 65        |
| 5.2.2     | PCRs .....                                                                 | 66        |
| 5.2.3     | Gel electrophoresis and gel extraction.....                                | 67        |
| 5.2.4     | Ligation and transformation .....                                          | 68        |
| 5.2.5     | Restriction digests.....                                                   | 68        |
| 5.2.6     | Sequencing.....                                                            | 69        |
| 5.3       | Mini-/Maxiprep .....                                                       | 69        |
| 5.4       | Smart RACE (Rapid Amplification of cDNA Ends) .....                        | 70        |
| 5.4.1     | Southern blot and colony lift .....                                        | 71        |
| 5.5       | BAC library screening.....                                                 | 73        |
| 5.5.1     | BAC filter hybridization.....                                              | 73        |
| 5.5.2     | Probe generation .....                                                     | 73        |
| 5.5.3     | BAC purification .....                                                     | 75        |
| 5.5.4     | Southern blot .....                                                        | 75        |
| 5.6       | Quantitative Real-Time PCR (qRT PCR).....                                  | 76        |
| 5.6.1     | RNA extraction and cDNA synthesis .....                                    | 76        |
| 5.6.2     | qRT-PCR reaction .....                                                     | 76        |
| 5.7       | Mammalian cell culture.....                                                | 77        |
| 5.7.1     | Thawing of cells .....                                                     | 77        |
| 5.7.2     | Counting of cells .....                                                    | 77        |
| 5.7.3     | Transfection optimization.....                                             | 78        |
| 5.8       | Impedance assay .....                                                      | 78        |
| 5.8.1     | Day1 .....                                                                 | 78        |
| 5.8.2     | Day2 .....                                                                 | 79        |
| 5.8.3     | Day3 .....                                                                 | 79        |

|           |                                                           |            |
|-----------|-----------------------------------------------------------|------------|
| 5.9       | Immunostaining .....                                      | 79         |
| 5.9.1     | Antibodies .....                                          | 79         |
| 5.9.2     | Antibody staining procedure.....                          | 80         |
| 5.10      | Whole Mount In Situ Hybridisation (WMISH).....            | 81         |
| 5.10.1    | Antisense probe generation .....                          | 81         |
| 5.11      | Drug treatment .....                                      | 81         |
| 5.12      | Injection of immature worms .....                         | 82         |
| 5.12.1    | Anaesthetizing animals .....                              | 82         |
| 5.12.2    | Injection capillaries .....                               | 82         |
| 5.12.3    | Injection setup.....                                      | 82         |
| 5.13      | Quantification of pigments .....                          | 83         |
| 5.14      | Microscopy.....                                           | 83         |
| 5.14.1    | Fluorescence microscopy and bright light microscopy ..... | 83         |
| 5.14.2    | Confocal Laser Scanning Microscope (CLSM).....            | 83         |
| 5.15      | Sequence analysis .....                                   | 83         |
| <b>6.</b> | <b>References.....</b>                                    | <b>84</b>  |
| <b>7.</b> | <b>Appendix .....</b>                                     | <b>93</b>  |
| 7.1       | Primers .....                                             | 93         |
| 7.2       | Sequences and sequence analysis .....                     | 95         |
| 7.3       | Counted PDF positive middle cells.....                    | 101        |
| 7.4       | <i>Platynereis dumerilii</i> immunostaining protocol..... | 103        |
| 7.5       | <i>Platynereis</i> whole-mount in situ hybridization..... | 107        |
| 7.6       | Miniprep.....                                             | 114        |
| 7.7       | BAC miniprep protocol.....                                | 115        |
| 7.8       | <i>Platynereis</i> qPCR analysis of gene expression ..... | 117        |
| <b>8.</b> | <b>Curriculum vitae .....</b>                             | <b>124</b> |

**Abstract**

*Platynereis dumerilii* has become an important model system for studying evolution, neurobiology or comparative genomics. It depends on external fertilization via synchronous mass spawning which is controlled by the circalunar clock. Besides, they possess a circadian rhythm to cope with daily changes of the environment. Circadian rhythms consist basically of three parts: 1)input pathways 2)clock or oscillator 3)output pathways.

Several molecular components of the circadian system have already been identified in *Platynereis*. Among those are two major output molecules of the circadian clock: Melatonin and pigment dispersing factor (PDF). PDF was originally discovered in crustaceans, where it controls colour change. Later it was also found in *Drosophila* where it is an output factor of the circadian clock but also feeds back on it.

The aim of this study was to investigate the receptors of melatonin and PDF as this might shed more light into the regulation and target tissues of these hormones. Additionally the function of PDF in *Platynereis* was analysed to see if it influences pigmentation or if it is an important key player of the circadian rhythm.

I cloned a second melatonin receptor candidate (MelR2) and analysed it together with melatonin receptor candidate 1 (MelR1) its ligand-binding properties. However, no clear statement can be made concerning their binding properties. In situ hybridization revealed that the second melatonin receptor candidate is expressed in the ciliary tissues in embryonal stages. As it is not expressed in nervous tissue, the second receptor candidate might mediate different functions of melatonin than MelR1. Additionally, I elongated the pdfr sequence and via qPCR I could show that there is no daily cycling on RNA level.

Immunostaining of immature worms gave first hints that there are circadian differences in PDF protein levels, which lead us to the assumption that it might be regulated by the circadian clock. Synthetic PDF was injected into immature worms to see if it feeds back on clock genes or influences pigment size. No direct effects of the injected PDF on clock genes or pigmentation could be detected. However, it is unclear

if the injected PDF is taken up by the blood stream and if the concentration was high enough to cause effects. Nevertheless subsequent studies showed that the pigmentation is influenced by the circadian clock and not just by light.

Summarizing, the role of PDF in *Platynereis* is still not completely clear and further experiments have to be performed to shed more light into its function and regulation.

## **Kurzfassung**

*Platynereis dumerilii* ist zu einem wichtigen Modellsystem geworden um Evolution, Neurobiologie oder vergleichende Genomik zu studieren. Die Fortpflanzung erfolgt durch externe, synchrone Massenbefruchtung, welche durch die zirkalunare Uhr gesteuert wird.

Außerdem besitzen sie einen zirkadianen Rhythmus um tägliche Umweltveränderungen zu bewältigen. Dieser besteht im Wesentlichen aus drei Teilen: 1)Input Wege 2)Uhr oder Oszillator 3)Output Wege.

Etliche molekulare Bestandteile dieses zirkadianen Systems wurden bereits in *Platynereis* identifiziert, unter anderem auch zwei wichtige output Moleküle der zirkadianen Uhr: Melatonin und „pigment dispersing factor“ (PDF). PDF wurde ursprünglich in Crustaceen entdeckt, wo es den typischen Farbwechsel steuert. Später wurde es auch in *Drosophila* gefunden, wo es ein wichtiger output Faktor der zirkadianen Uhr ist und auch auf diese rückwirkt.

Das Ziel dieser Studie war es, die Rezeptoren von Melatonin und PDF zu untersuchen, da dies Aufschluss über die Regulation und Zielgewebe dieser Hormone geben kann. Außerdem wurde die Funktion von PDF in *Platynereis* analysiert um zu sehen, ob es einen Einfluss auf die Pigmentierung hat oder ob es ein wichtiger Faktor im zirkadianen System ist.

Ich habe einen zweiten Melatoninrezeptorkandidat (MelR2) kloniert und ihn gemeinsam mit dem Melatoninrezeptorkandidat 1 (MelR1) auf seine Liganden-Bindungseigenschaften untersucht, aber es konnten keine eindeutigen Ergebnisse ermittelt werden. Mittels in situ Hybridisierung konnte ich MelR2 in Ziliargeweben in embryonalen Stadien nachweisen. Da er aber nicht im Nervengewebe exprimiert wird, könnte er für andere Funktionen zuständig sein als MelR1.

Zusätzlich konnte ich die Sequenz des PDF Rezeptors (pdfr) verlängern und durch qPCR zeigen, dass der Rezeptor auf RNA Ebene konstant exprimiert wird.

Immunfärbung unreifer Würmer gab erste Hinweise, dass es zirkadiane Unterschiede in der Expression von PDF gibt, was zu der Annahme führt, dass es wahrscheinlich durch die zirkadiane Uhr gesteuert wird. Synthetisches PDF wurde in Würmer injiziert

um zu sehen, ob es auf die zirkadiane Uhr wirkt oder die Pigmentierung beeinflusst. Es konnten aber keine direkten Effekte auf die Expression der „clock“ Gene oder auf die Pigmente festgestellt werden. Unklar ist jedoch, ob das injizierte PDF vom Blutsystem aufgenommen wurde und ob die Konzentration hoch genug war um Veränderungen zu bewirken.

Es ist daher noch weitgehend unklar welche Rolle PDF in *Platynereis* hat und es benötigt weitere Experimente um mehr über die Funktion und Regulation dieses Hormons herauszufinden.

# 1. Introduction

## 1.1 *Platynereis dumerilii* as a model organism

In the last decades, the marine bristleworm *Platynereis dumerilii* (Polychaeta, Annelida, Lophotrochozoa, Nereidida) has emerged as a model organism for evolutionary, neurobiological, ecological as well as developmental studies<sup>1,2</sup>.

It belongs to the lophotrochozoa - a clade of protostome animals which together with deuterostomia form the bilateria<sup>3</sup>. As depicted in Figure 1 protostomia comprise two branches: ecdysozoa (e.g. arthropods, nematodes) and lophotrochozoa (e.g. annelids, mollusks). Most molecular model organisms belong to the ecdysozoa (e.g. *C. elegans*, *D. melanogaster*) or deuterostomes (*M. musculus*, *D. rerio*, chicken), whereas the lophotrochozoans are rather under-represented despite containing the largest number of animal phyla and their relevance to reconstruct the urbilaterian genome<sup>4</sup>.

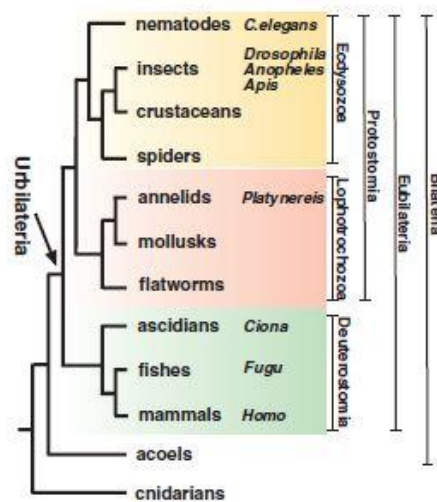


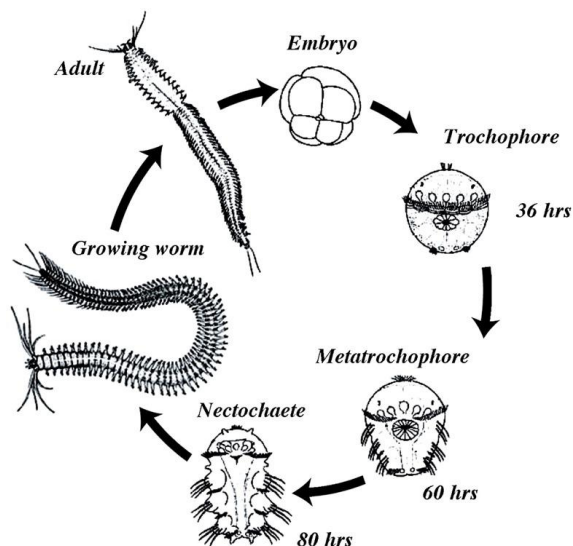
Figure 1: Evolutionary tree of Bilateria. Taken from Raible et al.<sup>3</sup>

As it exhibits many ancestral type features it makes this model organism well suitable for comparative studies and to understand animal evolution, as conventional animal models are often highly specialized<sup>3</sup>. By analyzing 30 *Platynereis* genes with orthologs

in other Bilateria, Raible et al. found that on intron level *Platynereis* is more similar to vertebrates than to invertebrates.

On top of that, the worms can be easily bred and maintained in the laboratory and produce offspring throughout the year with one batch containing more than 2000 eggs. It has been kept in the laboratory since 1953 resulting in generations of inbred strains<sup>2</sup>. Due to their transparency, worms as well as larvae can be easily visualized using microscopic techniques. Moreover a set of molecular standard techniques is available like whole mount in situ hybridization (WMISH) and immunocytochemistry<sup>5</sup>. Genomic and transcriptomic resources have been generated and a partially assembled *Platynereis* genome has recently become available (unpublished EST resources). Improved microinjection techniques made it possible to generate transgenic lines (Tessmar-Raible lab, personal communication). Additionally, *Platynereis* also has a huge regeneration capability, which makes it a good candidate to perform transplantation or ablation experiments.

### 1.1.1 The life cycle of *Platynereis*



**Figure 2:** Life cycle of *Platynereis dumerilii*. Drawing taken from Hauenschild et al., 1969

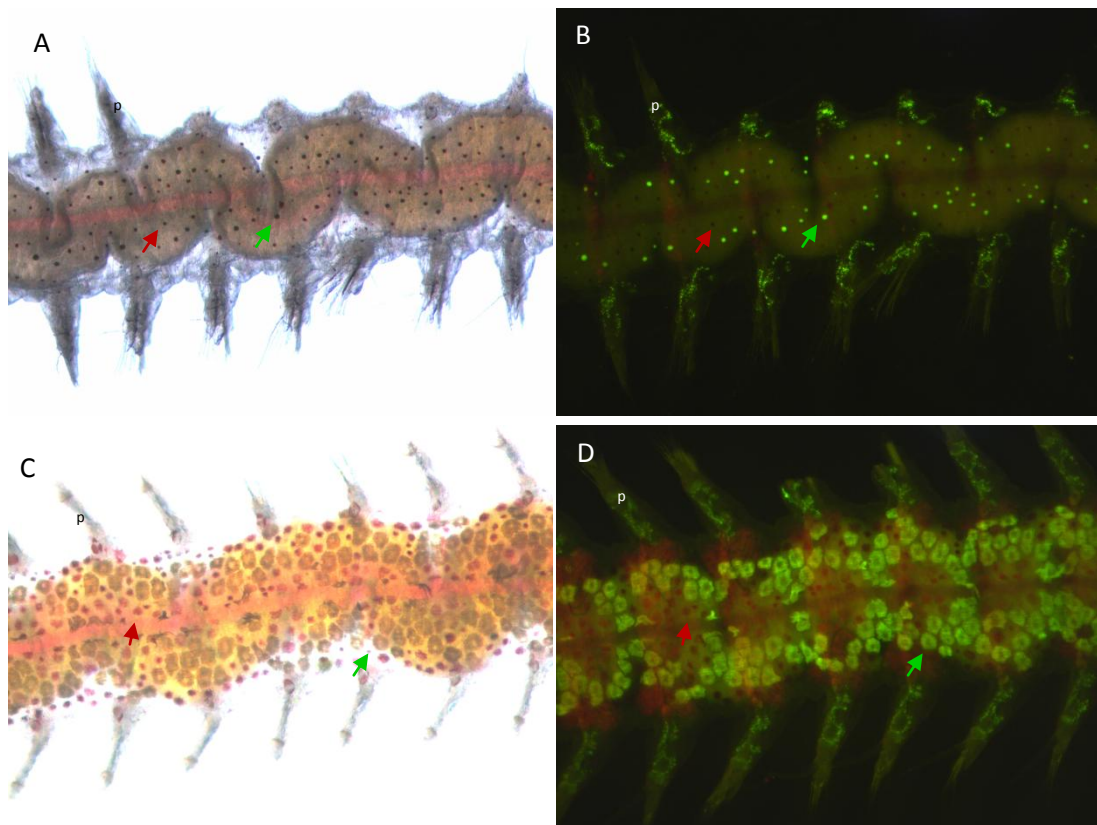
The maturation of *Platynereis* is highly synchronous, which is required for external fertilization. Most worms spawn during new moon, with a decline during full moon phase. Once the oocytes are fertilized, they undergo a series of spiral cleavages, which happens in a protective jelly mass. Eventually the larvae hatch from the surrounding jelly at the trochophore stage and start swimming using their ciliary belt (Figure 2). As the trunk starts to grow, the larval morphology gradually changes from spherical to conical. At the metatrochophore stage (60 hpf) already three segments are visible and two additional eyes – the adult eyes – appear. The larvae further grow, looking more torpedo like and parapodia start to form. As the gut becomes functional at the late nectochaete stage, the larvae start to feed on algae and detritus and it changes its lifestyle from pelagic to benthic. An additional fourth segment is formed by the posterior growth zone and the errant juveniles develop spinning glands in their parapodia, which are necessary to form their characteristic tubes. Cephalic metamorphosis takes place incorporating the first chaetigerous larval segment into the head and transforming the first pair of parapodia to tentacular cirri. Further segments bud from the posterior growth zone and the worms start to build tubes on the bottom of the tank, only occasionally leaving them. Gametes start to form in the sexually immature atokus worm, maturing inside the body cavity. During sexual metamorphosis, several morphological and physiological changes take place: food uptake stops, eye size increases and the parapodia change their shape. At this stage it is easy to distinguish male and female worms as they have characteristic body colours with males being red/white and females turning yellow as their body is full with eggs. Synchronized by the lunar light, males and females start releasing their gametes, swimming in close circles around each other.

As a semelparous organism, males and females die soon after spawning. In culture, the lifespan of *Platynereis* from fertilization until maturity is on average six to seven month but can take up to 18 months<sup>6</sup>.

### 1.1.2 Pigmentation of *Platynereis*

*Platynereis dumerilii* has 3 different kinds of chromatophores which are present on the dorsal side of the body<sup>7</sup> (Figure 3):

1. Erythrophores on the surface
2. deep erythrophores
3. Iridocytes



**Figure 3: Pigmentation of *Platynereis* tails.** (A, B) shows worm with contracted pigmentation at 4 am, (C, D) expanded pigments of a different worm at 4 pm. Pictures taken with reflected light and 40x magnification. Iridocytes display strong green auto fluorescence (FITC filter), whereas erythrophores are red and show a weaker auto fluorescence. (A, C) bright field pictures (B, D) fluorescent pictures. All pictures show dorsal view. Red arrows indicate erythrophores, green arrows iridocytes. p=paraodia

The appearance as well as the intensity of pigmentation varies between different individuals.

Erythrophores on the surface are of brick red colour and located closely to the dorsal ring muscles<sup>7</sup>. Compared to deep-set erythrophores they are able to expand more strongly and both are only distinguishable when looking at them under reflected light.

Expanded, both show the same colour which implies that both types of erythrophores contain pigment of the same colour (Figure 3).

Deep-set erythrophores are rather brown-violet and occur more frequently on the anterior region of the worm (pharynx muscle) and get less in posterior direction<sup>7</sup>.

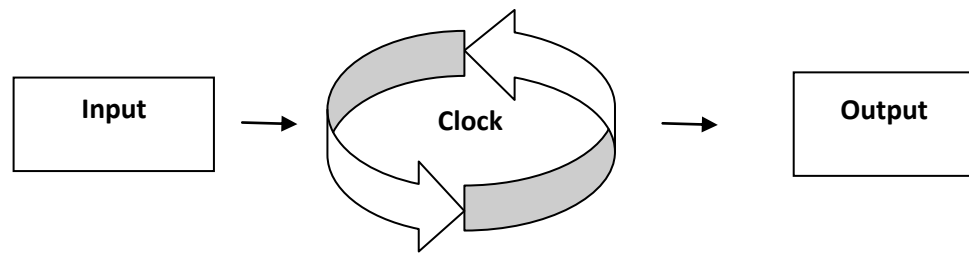
Iridocytes – which show the strongest fluorescence– are present in the parapodia, close to the dorsal blood vessel and seem to get more towards the posterior end of the worm (Figure 3). When looking at them with incident light they appear white, whereas with transmitted light their colour is more brownish-yellow<sup>7</sup>. Moreover in the head only iridocytes are present.

Fischer has shown in 1964, that there is a daily rhythm of expansion and contraction of pigments with a maximum during the day and a minimum in the night, with all pigments reacting the same way. Interestingly, this rhythm is also maintained in the tails of decapitated animals. As the number of chromatophores is more or less constant, the pigmentation changes mainly due to expansion and concentration of pigment<sup>7</sup>. Studies in our lab have also shown that pigments size changes in a daily rhythm which is comparable to Fischer's results. Besides, he found that this rhythm continues for some time in whole worms as well as decapitated worms when put under constant light (LL) or constant darkness (DD), indicating an endogenous oscillator that controls the pigment dispersion<sup>7</sup>.

Further studies conducted by Röseler showed that each segment is able to keep up its endogenous oscillator under LL<sup>8</sup>. Moreover she could demonstrate that there is a humoral factor that influences pigmentation, as coelomic fluid taken from animals at the beginning of the dark phase applied to pieces of skin derived from worms kept in LL led to a contraction of these pigments<sup>8</sup>.

Even nowadays, many years after these first experiments, little is known about the factor that regulates pigment dispersion in the worms.

## 1.2 Circadian rhythms



**Figure 4: General concept of the circadian system:** The input pathway receives environmental information and transmits it to the clock or oscillator which then activates output pathways.

Most organisms possess a circadian system to cope with daily changes in their environment. A central pacemaker has been identified for many organisms, which includes the suprachiasmatic nuclei (SCN) in the hypothalamus of mammals or the optic lobes of cockroach, cricket and *Drosophila*<sup>9–12</sup>. The era of chronobiology began in 1971 with the discovery of period (*per*) mutants in *Drosophila*, which showed abnormal circadian behaviour<sup>13</sup>. Since then, many more clock genes have been identified and it has become clear that the generation of circadian rhythms is based on molecular feedback loops.

Circadian rhythms exhibit three basic properties: 1)periodicity 2)robustness 3)entrainment<sup>14</sup>. This means they persist (free-run) with a period of around 24 hours in the absence of external cues (or zeitgeber), can be reset by the daily light-dark or temperature cycles and have a certain period length.

Early models described the clock mechanism as a simple transcription-translation oscillator loop (TTO), with positive and negative transcription factors forming a negative feedback loop<sup>15,16</sup>. Additional clock genes were characterized in *Drosophila melanogaster* (*vri* and *PAR* domain protein 1), which form interlocked loops with period (*per*) and timeless (*tim*)<sup>17</sup>. This challenged the simple single loop cycle and initiated the theme of a multilayered network that operates on many different levels. Moreover, it was recently been demonstrated that even red blood cells, which lack a nucleus, possess a self-sustained circadian oscillator<sup>18</sup>.

The circadian system consists basically of three parts as depicted in Figure 4: an input pathway that receives environmental cues and transmits them to the circadian clock/oscillator, which keeps circadian time and activates output pathways, and output pathways that control various metabolic, physiological and behavioral processes<sup>19</sup>.

### 1.2.1 Input pathways

In most organisms light is the most important entraining agent for the circadian system to synchronize to environmental conditions and when given during the early or late night it can induce phase delays or advances<sup>20–22</sup>. To entrain or reset the clock, photoreceptors are necessary. In crickets or cockroaches the compound eye is the most important photoreceptor<sup>23,24</sup>. Additionally, in *Drosophila* the blue light receptor molecule CRY1 and rhodopsins are known to play a role in photic entrainment<sup>25,26</sup>. Concerning mammals, melanopsin (OPN4) is the key molecule for transducing the light signal to the SCN for clock entrainment<sup>27,28</sup>.

Temperature is also an important cue which helps synchronizing the clock, especially in dark places. However, the molecular mechanism that underlies it is hardly understood. In *Drosophila* temperature increase up-regulates the clock (clk) gene and a decrease down-regulates it under constant light. Per, tim, vrille (vri) and Pdp1ε are down-regulated and up-regulated by step-up and step-down, respectively<sup>29</sup>.

### 1.2.2 Output pathways

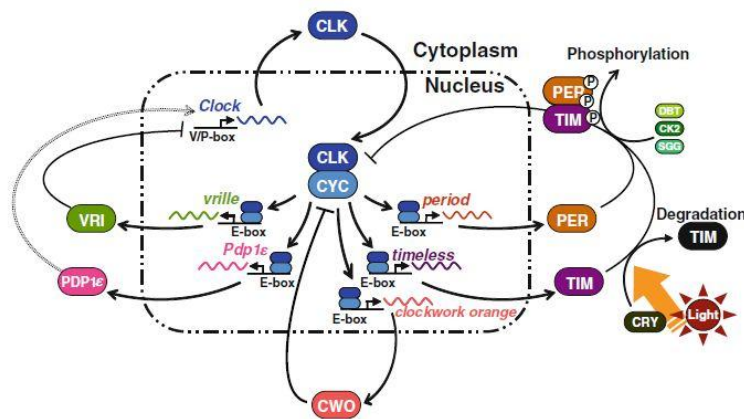
It is not well understood through which pathway the clock regulates rhythms. For several animals, it has been shown that the central pacemaker sends projections to certain areas of the brain<sup>30–32</sup>.

An important output molecule in mammals is melatonin, which is rhythmically secreted from the SCN and distributes the circadian message within the whole body via the general circulation<sup>33–37</sup>. It is influenced by the light/dark cycle and primarily synthesized at night. Numerous potential binding sites of melatonin have been

reported in the brain as well as in the periphery<sup>38</sup>. Since melatonin receptors are also present in the SCN, endogenous melatonin is also able to feed back onto the master clock<sup>39</sup>.

In *Drosophila* the pigment dispersing factor (PDF) is the best characterized output molecule of the circadian clock. Its involvement was first notified by Helfrich Förster et al. as it is expressed in the vLN, colocalising with PER<sup>40</sup>. Since then it is thought to be an output molecule in a variety of insects. Melatonin and PDF will be described in detail later.

### 1.2.3 Fly and mammalian circadian clocks



**Figure 5: Scheme of *Drosophila* circadian clock.** It consists of several interlocked feedback loops. For more information see text. Taken from Tomioka et al.<sup>41</sup>

The two best-studied animal systems for understanding circadian pacemakers are *Drosophila* and mammals. Both exhibit striking similarities in components of the clock and the overall mechanism. Several feedback loops constitute the rhythm-generating machinery in *Drosophila*<sup>42–44</sup> (Figure 5).

The first loop is formed by per, tim, clk and cycle (cyc)<sup>45</sup>. Around mid-day CLK and CYC form heterodimers and bind E-box regulatory elements, activating the transcription of per and tim. During the day TIM remains at low levels as it is susceptible to light. Without TIM, PER is not stabilized and gets phosphorylated by DOUBLE-TIME (DBT)

kinase and is targeted for degradation<sup>46</sup>. During night TIM accumulates, forming a complex with PER and therefore stabilizing it. This heterodimeric complex can enter the nucleus and represses its own transcription by inhibiting CLK-CYC. Reduced levels of PER and TIM, eventually release CLK-CYC from the repressed state and they can reactive the transcription of *tim* and *per*.

A second loop controls the cycling levels of CLK<sup>17,47</sup>. Besides *per* and *tim*, *vri* (*vri*) and PAR domain protein1ε (*pdp1ε*) are activated by CLK-CYC during late day. *VRI* enters the nucleus and inhibits the transcription of CLK by binding to the V/P promoter region, which leads to lower levels of *clk* mRNA during the night. *Pdp1ε* translation is rather delayed, with the protein increasing during late night. *PDP1ε* and *VRI* are thought to bind the promoter of *clk* competitively and as *VRI* levels decline *PDP1ε* can bind and activate the transcription of *clk*. Therefore the levels of *clk* increase during the day, leading to a higher amount of CLK protein.

A third feedback loop has also been proposed including clockwork orange (*cwo*), which is a transcriptional repressor<sup>48–51</sup>. It is rhythmically expressed and forms its own negative feedback loop under the control of CLK-CYC. By binding E-box elements it can repress the expression of other clock genes, such as *tim* and *per*.

The mammalian circadian oscillator is also composed of interlocked transcriptional feedback loops, with many components having orthologs or functional equivalents. By making minor changes the *Drosophila* oscillator can be easily converted to a mammalian one<sup>52</sup>.

### ***Peripheral clocks in mammals and Drosophila***

The central clock of mammals is located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus, whereas in flies the central clock is located in a group of 5-6 bilaterally symmetric small ventral lateral neurons (sLN<sub>v</sub>s) situated in the lateral brain

close to the optic lobes<sup>12,30</sup>.

Many isolated tissues of mammals or *Drosophila* exhibit circadian rhythms in clock gene expression. In addition to the central clock, numerous subsidiary clocks exist outside the SCN in peripheral tissue such as liver, heart, lung and muscle<sup>53</sup>.

A decade ago it was thought that the mammalian SCN is the master clock, whereas the peripheral clocks are “slave” oscillators driven by the SCN. In vitro circadian rhythms in liver, lung or skeletal muscle dampen after two to seven days, whereas the SCN keeps its rhythmicity for up to 30 days<sup>53</sup>. This data strongly suggests a hierarchical control of peripheral clocks by the central one. However, SCN lesioned mice did not abolish circadian rhythmicity in peripheral tissues<sup>54</sup>.

In *Drosophila*, peripheral oscillators could be found in the antennal neurons and in the prothoracic gland (PC), which is important to generate a rhythm for adult emergence<sup>55</sup>. Interestingly, the peripheral clock in the antennae does not require the central one<sup>56,57</sup>.

In *Platynereis dumerilii*, possible molecular components of the circadian and the circalunar system have already been identified and are currently studied eagerly. Not only has the worm a circadian clock, but also a circalunar one, which is necessary to synchronize mass spawning to lunar cycles<sup>58</sup>.

As a slow evolving organism which has still many ancestral features it provides a better chronobiological model than other frequently studied model organisms<sup>3</sup>. In addition, it is a great model system to understand the function of peripheral clocks, as the decapitated worms can be studied for several days, which is not possible in most other organisms. *Platynereis* will therefore play an important role in uncovering fundamental properties of circadian rhythm.

### 1.3 Role and function of pigment dispersing hormone-like peptides in invertebrates

The pigment dispersing hormones (PDHs) and the pigment dispersing factor (PDF) are octadecapeptides which are conserved in a broad range of insects, crustaceans and nematodes. They bind to class B (secretin type) G protein-coupled receptors which are present on clock neurons themselves<sup>59–63</sup>. As the effects of hormones are mainly mediated through specific receptors on the surface of target cells, a detailed knowledge about these receptors helps us to understand the target tissues of certain hormones. Moreover the localization of the receptor might shed more light into the function of the hormone.

#### 1.3.1 Function of PDHs in crustaceans

The first pigment dispersing hormone was found in the eyestalk of the shrimp *Pandalus borealis*<sup>64,65</sup> and later a second peptide was identified in the eyestalk of the fiddler crab *Uca pugilator*<sup>66</sup>. As the two PDHs differ from each other, peptides with similar sequence to that of *P. borealis* are now referred to as  $\alpha$ -PDH and that of *U. pugilator* as  $\beta$ -PDH. Additional isoforms of PDH were found in subsequent studies which can be grouped as  $\alpha$ - or  $\beta$ -family members based on sequence similarity<sup>67</sup>.

The mature peptide originates from a precursor peptide that contains a signal peptide, followed by a precursor related peptide and mono- or dibasic cleavage sites that flank the mature PDH<sup>67–71</sup>.

All PDHs are involved in colour changes related to pigment dispersion of integumental pigments and the distal migration of retinal screening pigment during the day to protect photoreceptor cells from light<sup>64</sup>. This endogenous oscillator of colour change with dispersed pigments during the day and contracted during the night is due to a cyclic synthesis and release of PDH and red pigment-concentrating hormone (RPCH). In some crustaceans it has been shown that melanophores and erythrophores continue

to alternate dispersion and concentration of their pigments under constant darkness indicating that it is clock controlled<sup>72</sup>.

Apart from the pigment dispersion, crustaceans display circadian locomotor activity but the function of the circadian clock still remains unclear<sup>73</sup>. Recently, PDH was linked to circadian entrainment in two crustaceans<sup>74</sup>. Furthermore, PDH-neurons close to the medulla show similarities to PDF neurons in insects, suggesting that PDH plays a similar role as in insects in controlling behavioural circadian rhythms<sup>75,76</sup>.

### 1.3.2 Function of PDF in insects

Peptides, which are highly similar to  $\beta$ -PDHs exist also in insects. When applied to the optic stalk of crabs, these peptide extracts were able to induce pigment dispersion and were therefore named pigment dispersing factors<sup>77,78</sup>.

In all insects the somata of PDF neurons are located at the anterior margin of the medulla and arborize in the accessory medulla (aMe), sending projections towards the central brain and the compound eye<sup>79-91</sup>. Therefore, PDF is well suited to transmit circadian signals. In *Drosophila* PDF is expressed in the ventrolateral neurons, which are divided into small (s-LNvs) and large ventrolateral neurons (l-LNvs)<sup>92</sup>.

The function of PDF is tightly associated with the visual system, but no involvement of PDF in eye pigment migration has been reported so far. As these animals have no chromatophores in the epithelia, the neuropeptide must have a different function<sup>93,94</sup>.

#### ***PDF as an output factor of the circadian clock***

Most of the early work on circadian rhythms was performed on cockroaches and this gave the first physiological evidence that PDH/PDF is involved in the circadian rhythm. Cutting of the optic stalks of *L. maderae* causes a degeneration of PDH-immunoreactive (ir) neurons, accompanied by loss of circadian locomotor rhythmicity, which is restored after several weeks after the PDF fibers are regrown<sup>83</sup>.

Whereas the pdf mRNA seems to be produced in a constant fashion in the fruit fly, the protein oscillates with a daily rhythm<sup>95,96</sup>. This rhythmic release of PDF is under clock control, as *per<sup>s</sup>* mutants show short periods of PDF accumulation and *per<sup>o</sup>* mutants lack this rhythm of accumulation and release. Recently, studies argue that PDF cycling might not be as important for daily rhythm as previously suggested, since flies without cycling of PDF remain rhythmic under DD<sup>97</sup>.

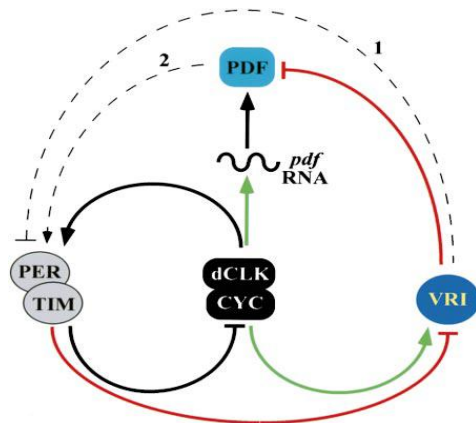
### ***PDF as a network coordinator***

PDF might not just act as a mere output factor but also couples the oscillations between different clock neurons, as *tim* mRNA cycling dampens in *Pdf<sup>01</sup>* mutants under constant darkness<sup>98</sup>. Further studies revealed that it is crucial to coordinate phase and amplitude of circadian protein oscillations under DD<sup>99</sup>. Moreover, it was shown that PDF lengthens the period of some clock genes, while shortening the period of others with increasing PDF level<sup>100</sup>. This led to the assumption that PDF feeds back on the clock to alter its pace. For some clock neurons PDF might not be necessary for rhythmicity or internal synchronization but they are strongly influenced by PDF regarding their period. As demonstrated by immunocytochemistry and in situ hybridization the PDF receptor is present on clock genes, confirming further the feedback of PDF on the clock<sup>59–61</sup>.

### ***PDF as light input factor***

Besides the already mentioned roles of PDF, it is also involved in the light-input pathways of the clock, as arousal effects of the I-LNV are mediated by PDF<sup>101</sup>. Moreover, injected PDF was able to elicit phase-dependent resetting of the circadian locomotor rhythm, showing that it could be either a nonphotic input signal or an integral part of the pacemaker<sup>102</sup>.

### Molecular control of PDF in *Drosophila*



**Figure 6: Interlocked feedback loop of *Drosophila* clock.** For more information see text. Picture taken from Blau and Young, 1999

As depicted in Figure 6, pdf expression in the fruit fly is stimulated by CLOCK and posttranscriptionally inhibited by VRILLE. It has not been ascertained yet whether VRI regulates per and tim expression independently of PDF (1) or via PDF (2)<sup>103</sup>. Moreover, PDF peptide levels are influenced by mutations in either per or tim, demonstrating a posttranslational, circadian regulation of PDF<sup>96</sup>.

### PDF receptor in flies

In 2005 a breakthrough for functional PDF research was achieved, when three independent groups cloned the PDF receptor in the fruit fly<sup>59–61,104</sup>. The PDFR is a class B G-Protein coupled receptor (GPCR), which has an extracellular binding domain, seven transmembrane domains and a G-protein-binding cytoplasmic domain. These hormone receptors use Gs (stimulatory G-proteins) to couple to adenylate cyclases and therefore regulate cAMP levels. PDFR displays sequence homology to the mammalian VIP receptor (VPAC<sub>2</sub>) and to the calcitonin receptor, with both of them being expressed in the mammalian master clock<sup>59</sup>.

Several studies described the expression pattern of the PDFR in clock neurons as well as in neurons outside the clock. However, there were differences in the precise location<sup>59–61</sup>. Shafer et al. measured PDF receptivity within the clock network, showing that all clock neurons (except the large LNVs) respond to PDF with an increase in cAMP<sup>63</sup>. Since the receptor is expressed on clock neurons themselves, PDF must feedback on these neurons<sup>105</sup>. Later experiments showed that most of the PDFR product is present in the brain and only a small fraction is located in the body<sup>60</sup>. Interestingly, pdf expression could also be detected in non-clock brain cells and in the visual system, suggesting that PDF might use non-neuronal cells to regulate the visual input to the circadian system<sup>105</sup>.

Pdfr null mutants show similar behavioural rhythms as Pdf<sup>0</sup> mutants<sup>61</sup>. However, this phenotype could be rescued by expressing the PDFR gene in clock neurons of pdf deficient flies, indicating the importance of PDF signaling for normal clock function. Moreover, rhythmicity of pdf null mutants in constant darkness could be restored by using membrane-tethered PDF molecule<sup>106</sup>.

### **1.3.3 Function of PDF in nematodes**

*C. elegans*, the best studied representative among nematodes, exhibits a circadian rhythm in activity and its genome contains homologues of many clock genes<sup>107</sup>. However, the core clock seems to be different to that of insects<sup>108,109</sup>. Recently, three PDF homologues were found (PDF-1a, PDF-1b and PDF-2) with all of them able to activate the three receptors<sup>62,110</sup>.

The *C. elegans* PDFs have important functions in sensing and integrating environmental stimuli (oxygen sensing and chemosensation) and are necessary for normal locomotor behaviour<sup>110</sup>. Although pdf-1a and b mutants display altered locomotor behaviour it has not been demonstrated that PDF peptides are involved in the circadian system.

### ***PDF receptors in nematodes***

*C. elegans* contains three PDF receptors which are isoforms of one gene – pdfR-1. All of them belong to the class B type of G protein-coupled receptors and are expressed in several sensory- and motoneurons<sup>62</sup>.

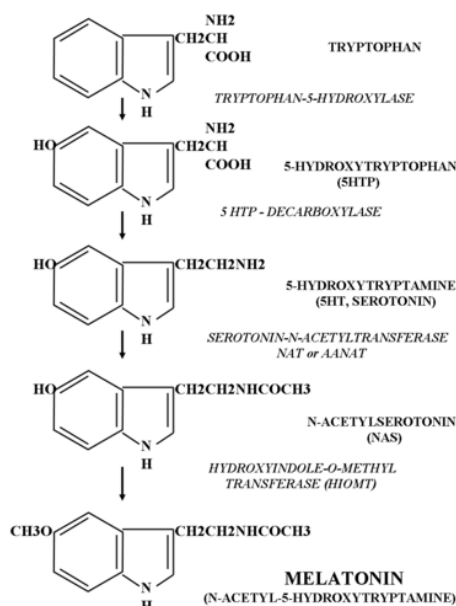
#### **1.3.4 PDF in *Platynereis***

PDF as well as its receptor have already been identified in *Platynereis*. However, the function and regulation of it is still not clear. Recent studies done in our lab suggest that it might show circadian fluctuations on protein level in the head of the worms. On mRNA level, PDF seems to be expressed in a constant fashion in the head as well as in the tails of worms, as qPCR data showed (Enrique Arboleda, personal communication). It is also not yet known if it can act on pigmentation, changing its size or distribution. So far, little is known about it and therefore it is important to further analyse it and find out if it has similar roles as in other species mentioned above.

### **1.4 Function and regulation of melatonin**

Melatonin was originally discovered as a skin-lightening molecule concentrating melanocytes of amphibia and fish<sup>111</sup>. In vertebrates, the hormone is rhythmically secreted by the pineal gland<sup>112</sup>. However, it is also synthesized by other organs and cells: retina, bone marrow, platelets, gastrointestinal tract, skin, lymphocytes<sup>113–120</sup>. Subsequently it was also found in many organisms including bacteria, macroalgae, plants, fungi and in various invertebrate animals<sup>121</sup>.

Pineal melatonin production exhibits a circadian rhythm, with a peak during the night and is suppressed during the day by light<sup>121–124</sup>. Its effects are mediated via high-affinity melatonin receptors, which will be described later.



**Figure 7: Melatonin synthesis pathway.** Tryptophan is the precursor which is converted to serotonin via 5HTP. Serotonin is subsequently transformed into melatonin by the main enzymes AANAT and HIOMT. Taken from: <http://www.endotext.org/neuroendo/neuroendo15/neuroendo15.htm>, version January 2011

As depicted in Figure 7 the major pathway of melatonin (N-acetyl-5-methoxytryptamine) biosynthesis starts with the conversion of tryptophan to serotonin. Serotonin is subsequently acetylated by arylalkylamine N-acetyltransferase (AA-NAT) to form N-Acetylserotonin (NAS), which is then converted to melatonin via hydroxyindole O-methyltransferase (HIOMT)<sup>124,125</sup>. However, other enzymes can be involved as has been shown for other organisms<sup>126</sup>.

In most cases the rate-limiting enzyme is AA-NAT, which is under the control of the SCN, the circadian pacemaker<sup>127</sup>.

#### 1.4.1 Functions of melatonin

Melatonin is an extremely versatile molecule and countless studies have analysed the protective function of it. One is that of antioxidative protection. Since the discovery that melatonin is capable of scavenging hydroxyl radicals, investigation of its antioxidant actions was strongly stimulated<sup>128</sup>. The antioxidant effects of melatonin also includes indirect ways of action such as up-regulating antioxidative enzymes and

down-regulating pro-oxidants<sup>129,130</sup>. As the antioxidant properties of melatonin are species-independent it might represent an evolutionary early function of melatonin<sup>131</sup>.

There is also evidence that melatonin has oncostatic effects against a variety of tumor cells<sup>132–134</sup>. Moreover, it confers immune-enhancing actions as exogenous melatonin counteracts immunodeficiencies caused by stress or drug treatment and protects mice from encephalitogenic viruses<sup>135</sup>. Double blind studies have shown that it can reduce blood pressure in patients with hypertension<sup>136,137</sup>. Melatonin might also regulate the reproductive function in seasonal mammals as it has been shown to have inhibitory effects on the hypothalamic-pituitary–gonadal axis and down-regulates gonadotropin-releasing hormone (GnRH)<sup>138–140</sup>. Especially in seasonal breeders reproduction is timed by the photoperiod, which is mediated by changes in melatonin<sup>141–143</sup>.

Melatonin also exhibits chronobiological effects. It has been shown that exogenous melatonin leads to phase advances of the circadian rhythm in humans and produces early sleepiness<sup>144</sup>. The phase shifting effect of melatonin is attributed to its binding on MT2 receptors which are present on the SCN<sup>145</sup>. Moreover it seems to entrain circadian rhythms in mammals, keeping the circadian rhythm in phase with environmental conditions<sup>146</sup>.

In non-vertebrates, melatonin is not always peaking at night although maxima were frequently found<sup>131</sup>.

### ***Function of melatonin in *Platynereis****

Melatonin has been studied quite intensively in vertebrates. However, little is known about its function in invertebrates.

In *Platynereis*, the two main enzymes involved in the melatonin pathway - AANAT and HIOMT – could be identified and were tested in our lab via WMISH for their expression pattern. This supports the hypothesis that melatonin and its biosynthesis pathway are evolutionarily conserved in animals.

Furthermore antibodies have now become available which are currently tested in our lab for their specificity (personal communication). Additionally, its role in reproduction in *Platynereis* is also investigated and might help to uncover the function of melatonin in *Platynereis*.

#### **1.4.2 Melatonin receptors**

The effects of melatonin are mainly mediated by melatonin receptors. Therefore it is necessary to study both ligand and receptor. Binding sites of melatonin were originally identified using the radioligand 2- $^{125}\text{I}$ iodomelatonin ( $^{125}\text{I}$ -Mel), which allowed anatomical localization of melatonin receptors<sup>147–150</sup>. This showed that high affinity binding sites for  $^{125}\text{I}$ -Mel were present in the SCN and the pars tuberalis (PT) of the pituitary gland was most intensively labeled in most mammalian species<sup>39</sup>. While the melatonin receptors in pituitary PT are thought to regulate reproductive effects of melatonin, the receptors in the SCN might be responsible for the circadian functions of melatonin<sup>39,151</sup>.

Originally the melatonin binding sites were classified into two subtypes – ML-1 and ML-2 - due to kinetic and pharmacological differences<sup>148,152</sup>. They bind  $^{125}\text{I}$ -Mel with picomolar or low nanomolar affinity<sup>152</sup>.

The first melatonin receptor was cloned from *Xenopus* dermal melanophores, by transiently expressing cDNA in COS-7 cells<sup>153</sup>. Subsequently three types of melatonin receptors were identified in vertebrates, as now homology based screening methods could be applied<sup>154</sup>.

In mammals, two high-affinity melatonin receptors exist, which were originally termed Mel<sub>1a</sub> and Mel<sub>1b</sub><sup>155,156</sup>. Later a third subtype was cloned from chicken brain and named Mel<sub>1c</sub><sup>157</sup>. So far, no mammalian homolog of the Mel<sub>1c</sub> receptor could be identified.

The identification of several receptor agonists (e.g. 2-Iodomelatonin and 6-Chloromelatonin) and selective antagonists (e.g. Luzindole, 4-P-PDOT) led to further knowledge about the function of the receptors<sup>158</sup>.

The nomenclature of the melatonin receptors was later changed, renaming Mel<sub>1a</sub> to MT<sub>1</sub> and Mel<sub>1b</sub> to MT<sub>2</sub><sup>159</sup>. Moreover the ML-2 binding site was renamed to MT<sub>3</sub> receptor. The mammalian MT<sub>1</sub> and MT<sub>2</sub> receptors are G protein-coupled receptors (GPCRs), signaling mainly via heterotrimeric Gi proteins, which leads to a decrease of cAMP levels<sup>160,161</sup>. In contrary, the MT<sub>3</sub> receptor does not belong to the GPCRs but instead has been characterized as quinone reductase 2, which play a role in the protection against oxidative stress<sup>162,163</sup>.

Using in situ hybridization and autoradiography, the MT<sub>1</sub> receptor could be identified in the rodent SCN, paraventricular thalamus and in the pituitary pars tuberalis (PT)<sup>156,164</sup>. In the SCN and the PT the expression of the MT<sub>1</sub> receptor exhibits daily changes, with elevated levels during daytime, supporting the hypothesis that ligand availability regulates receptor levels<sup>164,165</sup>. As both MT<sub>1</sub> and MT<sub>2</sub> are expressed in the SCN, pineal melatonin is believed to feed back onto the master clock, regulating neuronal activity and circadian rhythms<sup>166</sup>.

Nocturnal secretion of melatonin suppresses the expression of the clock gene *Per1* in the PT by activating the MT<sub>1</sub> receptor, which inhibits cAMP-dependent signaling<sup>167</sup>. Simultaneously, endogenous melatonin inhibits prolactin release in the PT during night, through binding to MT<sub>1</sub> receptors<sup>167</sup>. Melatonin might therefore regulate gene expression to link the central pacemaker and peripheral tissues to modulate circadian and seasonal rhythms.

Due to activation of different receptors, melatonin can exert distinct or opposite physiological responses. It has been shown that melatonin inhibits neuronal firing in the SCN via activating MT<sub>1</sub> receptors, whereas binding to MT<sub>2</sub> receptors leads to phase shifts in neuronal firing<sup>145,168,169</sup>.

The receptors could also be identified in other tissues such as reproductive organs, cerebral and peripheral vasculature, retina, choroid plexus, Harderian gland and the adrenal cortex, regulating a variety of physiological processes<sup>170,171</sup>.

### ***Melatonin receptors in Platynereis***

In *Platynereis*, two melatonin receptor candidates were identified in our lab by Stefan Hammer. By doing WMISH the first receptor candidate showed expression in the ventral nerve plate and in single cells in the head of *Platynereis* larvae and close to the neuropil, where also clock genes are located (S. Hammer, unpublished data). This might mean that melatonin can act on clock genes as one of its possible receptor candidates is expressed in this region.

Ligand-binding studies performed in our lab using the first melatonin receptor candidate gave first hints that it might bind to melatonin (S. Hammer, unpublished data). However, more experiments need to be performed to corroborate this preliminary data and to verify it further.

## 2. Aim of the project

As mentioned above, several molecular components of the circadian system have already been identified in *Platynereis*. Among those are two major output molecules of the circadian clock: Melatonin and pigment dispersing factor (PDF). PDF was originally discovered in crustaceans, where it controls retinal and integumental colour change. Subsequently it was also found in *Drosophila* where it has a completely different function. In this system it is an important output factor of the circadian clock, which also feeds back on it. Previous experiments performed in our lab suggest that in *Platynereis* PDF might also be involved in the circadian rhythm. However, its involvement in pigment regulation has not been studied so far.

To distinguish between these possibilities, I analysed its role in pigmentation via injecting the peptide into adult worms. Moreover, I studied the mRNA expression level of certain clock genes in injected worms, to see if it can influence their cycling. Immunohistochemistry using an antibody against PDF should give insights into its expression pattern and if it is involved in the circadian rhythm. With the help of these experiments, we should be able to find out if it has similar functions as in *Drosophila* or as in crustaceans or if acts both on pigmentation as well as in the circadian rhythm. As *Platynereis* is a slow evolving organism, these experiments can also help to identify the ancestral function of PDF. Additionally, I studied the PDF receptor as this might shed more light into the signaling pathway and downstream targets of PDF.

The second part of my project was focusing on the two melatonin receptors of *Platynereis*. Melatonin is an extremely versatile molecule, which has been identified in many organisms. Most of its actions are mediated by binding to its receptor. After cloning a second melatonin receptor candidate, I analysed its localization by doing whole mount in situ hybridisation. This should help to find out more about the target tissues of melatonin and to compare the differences between the two receptor candidates. Furthermore, the ligand-binding properties of both melatonin receptor candidates were assessed by doing impedance assays to verify that it truly binds melatonin.

### 3. Results

#### 3.1 Melatonin receptors

As outlined in the introduction, two melatonin receptor candidates were identified in *Platynereis*. To gain more knowledge about the target tissues of melatonin and for further ligand-binding studies, I analysed these receptors. The first candidate (MelR1) was found via blast search, cloned and further analysed in our lab by Stefan Hammer. This receptor candidate showed expression in the ventral nerve plate and in single cells in the head of *Platynereis* larvae (Stefan Hammer, unpublished data). Later in his research he was able to identify a second candidate (MelR2) with the new sequence database (4dlx, unpublished EST resources).

##### 3.1.1 Cloning of a second melatonin receptor candidate (MelR2)

In order to obtain the second receptor candidate, I performed PCR with specific primers (chapter 5.2) using larval cDNA as template for amplification. Moreover the sequence was modified using special primers, so that the sequence also consisted of restriction sites (EcoRI and NotI), a Kozak sequence and start/stopcodons. This would be important for cloning the sequence into mammalian expression vectors and for cell culture assay.

Specific PCR resulted in one clear major band at the size of ~1.4 kb and sequencing verified the gene as melatonin receptor candidate 2 (Appendix 7.2).

##### 3.1.2 BAC screening of MelR1 and MelR2

To identify regulatory sequences of both receptors, which would be important to generate reporter constructs, I performed a BAC library screening of both receptors (chapter 5.5). After BAC filter hybridization 18 clones were positive for melatonin

receptor candidate 1 (MelR1) and 7 for MelR2. These clones were ordered and then further analysed to find out which BACs contains the right sequence. By doing radioactive hybridization, 2 BAC clones (CH305-92K2, CH305-135K13) could be identified for MelR1 and 1 clone (CH305-185B17) for MelR2, which were afterwards sent for sequencing. These sequences were then further analysed by Florian Raible.

| BAC clone     | gene      | Obtained sequence (LGC)         |
|---------------|-----------|---------------------------------|
| CH305-P92K2   | Pdu_melR1 | 23 pieces, total length: 161032 |
| CH305-P135K13 | Pdu_melR1 | 15 pieces, total length: 184781 |
| CH305-P185B17 | Pdu_melR2 | 8 pieces, total length: 160624  |

**Table 1: Sequencing results of BACs containing melatonin receptor candidate sequences**

### 3.1.3 Expression pattern of melatonin receptor candidate 2 in larvae

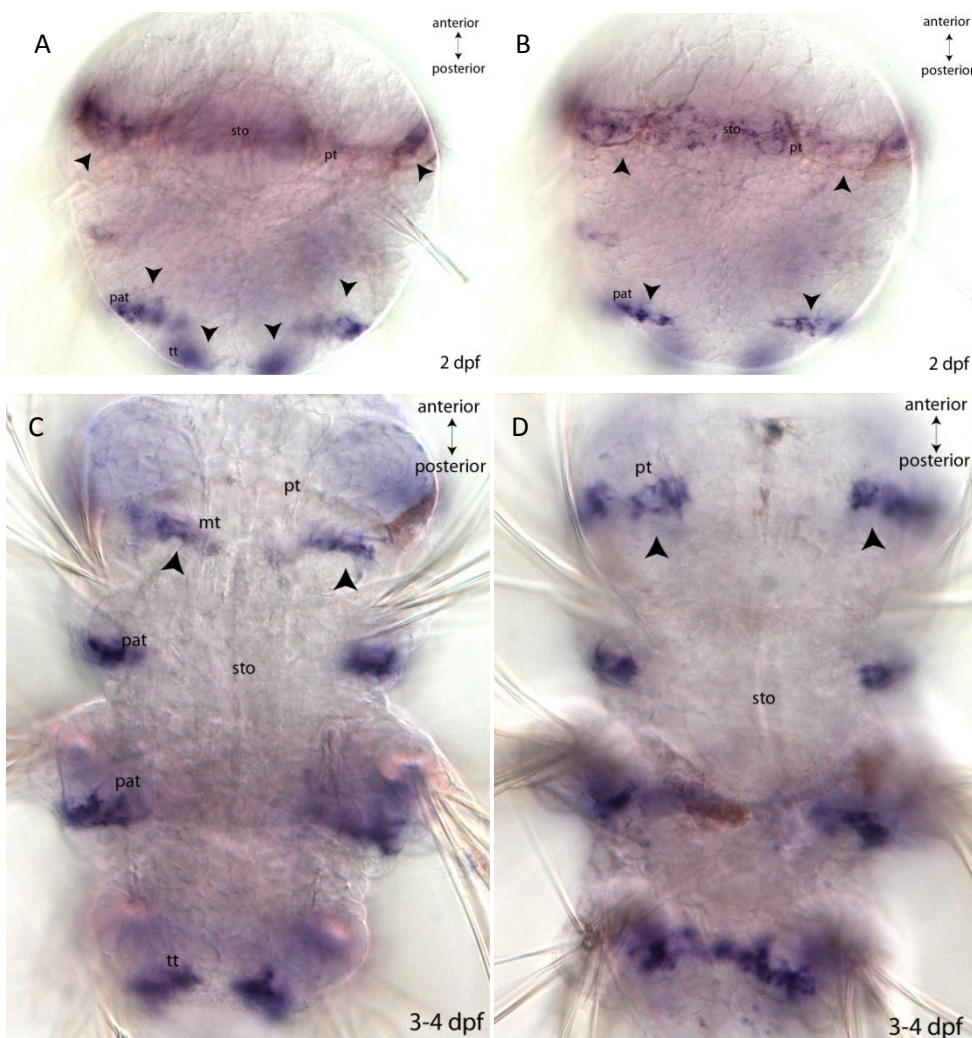
In order to characterize the newly identified melatonin receptor candidate further and find out where it is expressed, I performed whole mount in situ hybridisation as described in chapter 5.10 using alkaline phosphatase and the substrates NBT/BCIP to localize cells producing the mRNA. This might help to identify target tissues of melatonin.

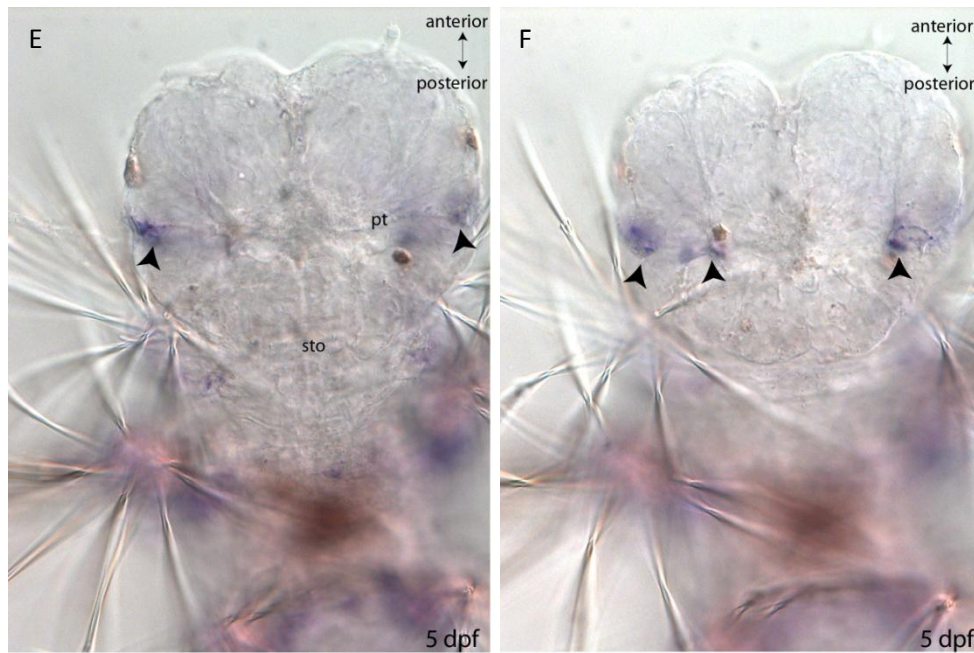
MelR2 positive cells could be detected from stage 2 dpf onward in ciliary structures (Figure 8). Two days old embryos showed a strong staining in the region of the prototroch (pt) and also symmetric staining in the paratroch (pat) and telotroch (tt) (Figure 8 A,B).

In 3-4 days old larvae there was still a strong symmetrical staining at the prototroch visible and also another staining came up in the region of the metatroch (mt). Moreover on the tip of each parapodia in the paratroch and again in the telotroch many MelR2 positive cells could be found (Figure 8 C,D).

At the stage of 5 days post fertilization, the staining in the ciliary belt and in the body decreased enormously, with only two or three cells on each side of the head (in the prototroch) being weakly stained (Figure 8 E,F).

However, no cellular staining could be seen in later developmental stages prior to 5 dpf and adult worms, which is consistent with the qPCR data that showed a decrease in expression levels of the melatonin receptor candidates the older the animals get (Claudia Lohs, chapter 4.1.1).





**Figure 8: Expression pattern of melatonin receptor candidate 2 (MelR2) in *Platynereis* larvae.** Black arrows indicate MelR2 expressing cells. All pictures show ventral view of the larvae. (A, B) 2 days old embryos (B) ciliary band in focus (C, D) 3-4 days old embryos (E, F) 5 days old embryos. sto=stomadeum, pt=prototroch, pat=paratroch, tt=telotroch, mt=metatroch, dpf=days post fertilisation

### 3.1.4 Receptor-ligand binding assays (Impedance assay)

In order to verify both receptors and to show that they can bind melatonin, I performed several ligand-binding assays using both melatonin receptor candidates.

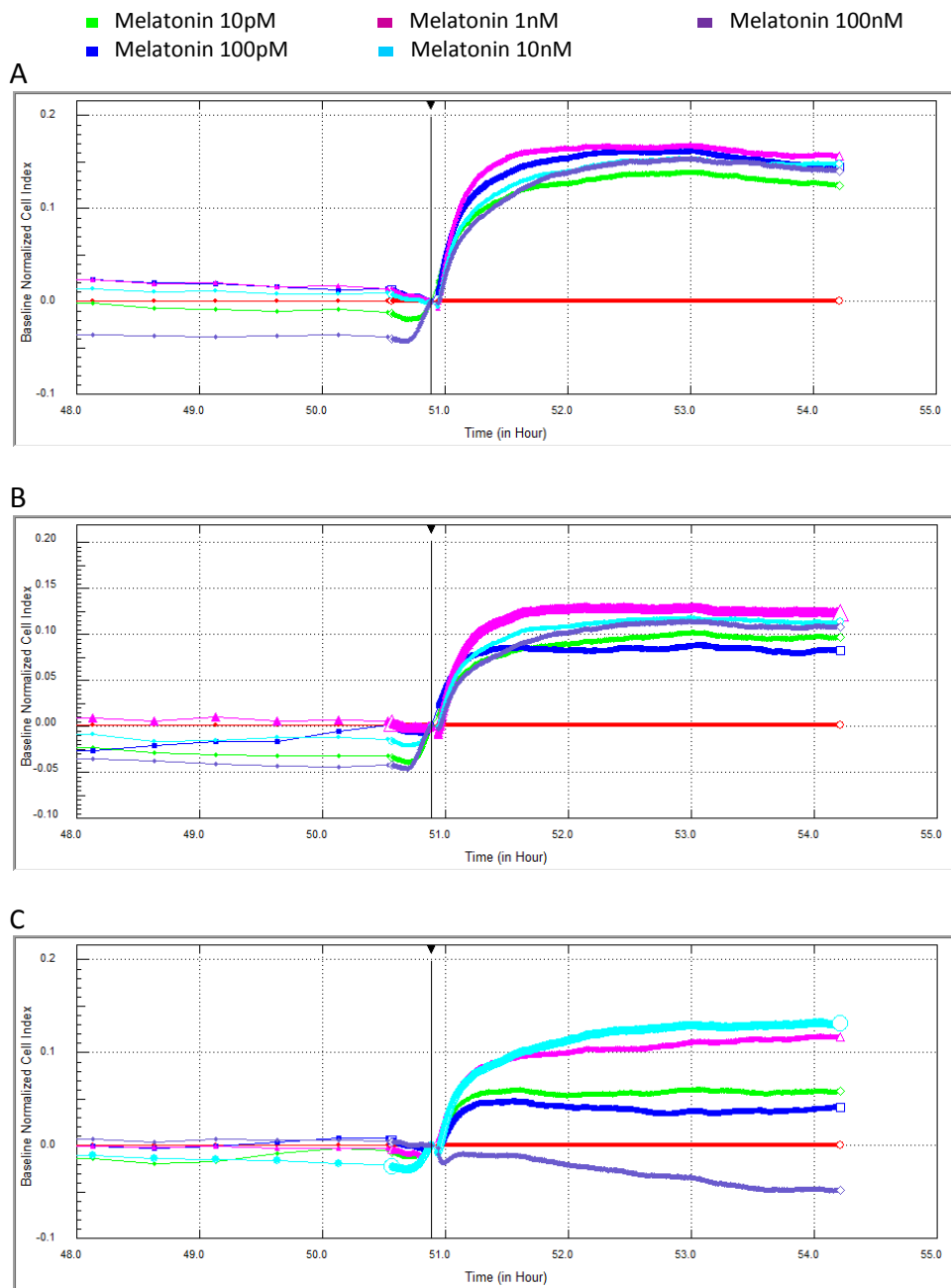
A previous ligand-binding assay showed that the first receptor candidate reacts to melatonin by increasing the cell impedance value (CI value) to 0.03, when adding the compound at a certain concentration (S. Hammer, unpublished data). To reproduce the finding that the MelR1 candidate binds melatonin and to verify the second receptor candidate, further cell impedance assays (xCELLigence System, Roche Applied Science) were performed. These assays were carried out on three days: On the first day the cells were seeded out on the 96 well E-plate until they reached confluency and on the subsequent day transfected with receptors of interest. The actual assay to test the binding of the ligand to the receptor was performed on the third day by adding melatonin to the transfected cells. The cell impedance is measured immediately while adding the compound. These changes in impedance give information if the receptors

react to the added substrate. As a positive control, cells were transfected with the high affinity mouse melatonin receptor 1A (Mm\_MtnR1A), which should show a response to melatonin. Values were normalized to the time point of melatonin administration.

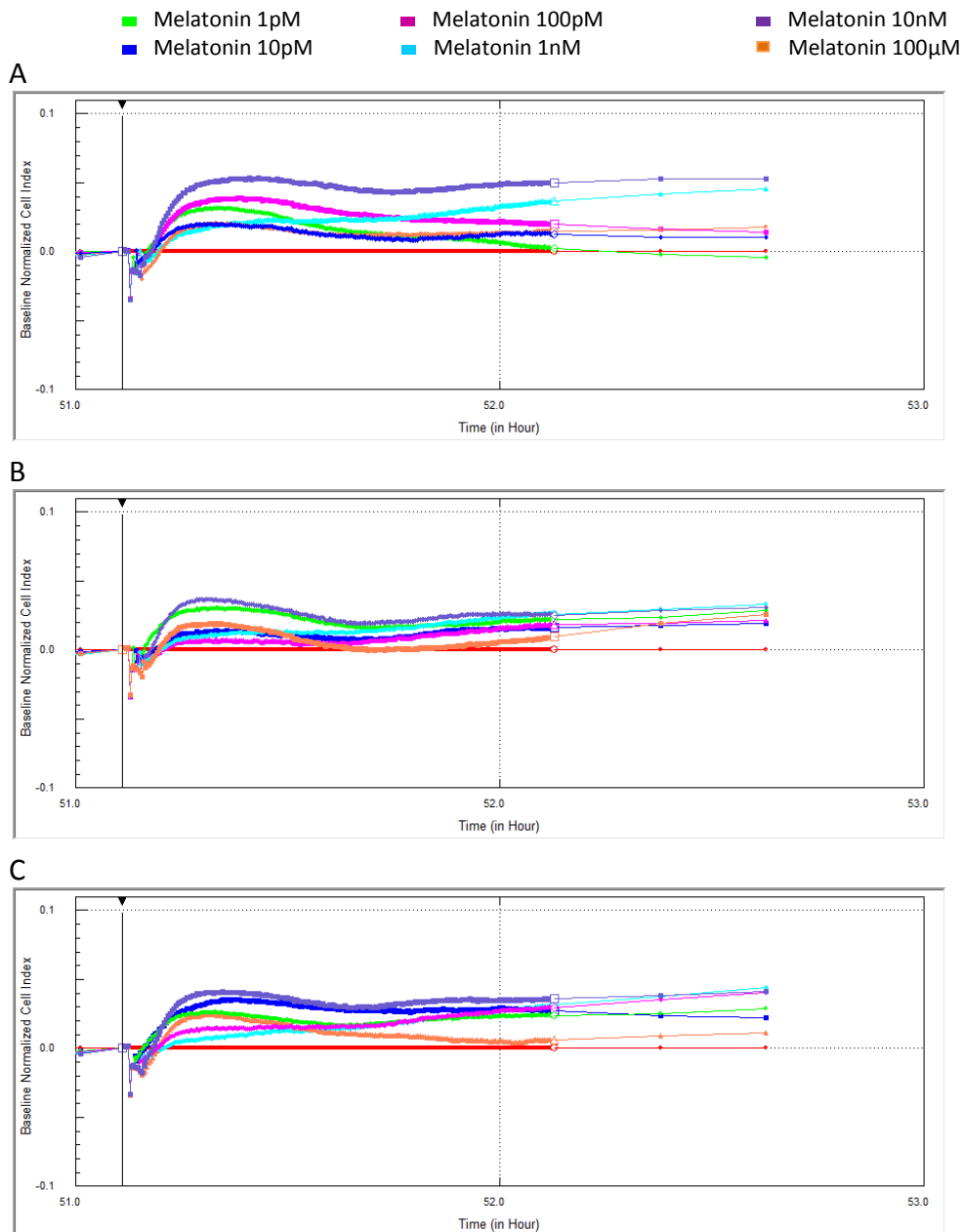
### ***First impedance assay***

To find out if there is a concentration dependant activation of the receptors, melatonin was added to the cells in 5 different concentrations (10 pM, 100 pM, 1 nM, 10 nM, 100 nM). As baseline mouse MtnR1A transfected cells treated with PBS were used. All receptors reacted most to lower melatonin concentrations. Both the Mm\_MtnR1A and the Pdu\_MelR1 showed the highest response to 1 nM melatonin with CI values of 0.17 (Mm\_MtnR1A) and 0.14 (Pdu\_MelR1) (Figure 9 A and B). In contrary, the second melatonin receptor candidate (Pdu\_MelR2) reacted strongest to 10 nM melatonin but also the reaction to 1 nM (CI value of 0.14) was not that much lower (CI=0.12) (Figure 9 C). Untransfected cells, which also served as a negative control, only showed slight changes in impedance, which could be due to adding of PBS.

All 3 receptors reacted immediately after adding melatonin with an increase in cell impedance and the CI values stayed high for ~3 hours after activation. Only the second receptor showed a decline in impedance when adding the highest melatonin concentration.



**Figure 9: Melatonin receptor stimulation by melatonin in COS-7 cells.** Cells were stimulated with melatonin (10 pM to 100 nM) and the cellular response was detected by the xCELLigence System. All data were normalized to unstimulated cells transfected with Mm\_MtnR1A (red line). The black vertical line indicates the time point of melatonin administration. (A) COS-7 cells transfected with Mm\_MtnR1A stimulated with melatonin (B) COS-7 cells transfected with *Platynereis* MelR1 candidate stimulated with melatonin (C) COS-7 cells transfected with *Platynereis* MelR2 candidate stimulated with melatonin.

**Second impedance assay**

**Figure 10: Melatonin receptor stimulation by melatonin in COS-7 cells.** Serum starved cells (3 hours in 0.5 % FCS) were stimulated with melatonin (1 pM to 100 μM) and the cellular response was detected by the xCELLigence System. All data were normalized to unstimulated cells transfected with Mm\_MtnR1A (red line). The black vertical line indicates the time point of melatonin administration. (A) COS-7 cells transfected with Mm\_MtnR1A stimulated with melatonin (B) COS-7 cells transfected with *Platynereis* MelR1 candidate stimulated with melatonin (C) COS-7 cells transfected with *Platynereis* MelR2 candidate stimulated with melatonin.

As previous data suggested that the response of the impedance assay can be increased by starving the cells before adding the compound, the medium was changed and cells

starved for 3 hours before adding melatonin in 6 different concentrations (1 pM, 10 pM, 100 pM, 1 nM, 10 nM, 100  $\mu$ M) (Roche xCELLigence System, Application note no. 6/December 2008). As baseline mouse MtnR1A transfected cells treated with PBS were used.

Only for the mouse receptor a dosage-dependent reaction could be seen but all three receptors showed the highest response to 10 nM melatonin with cell index values of 0.05 (Mm\_MtnR1A) and 0.04 for both *Platynereis* melatonin receptor candidates (Figure 10 A-C). However, given general concerns about the reproducibility of the assay (see below), a solid statement on this cannot be made.

To find out if there is a dose-response of the receptors, further assays were performed with conflicting outcomes. As the transfection efficiency was only 39 % (data not shown) different conditions were tested to optimize this. Also a second cell line (N2A) which showed high transfection efficiency (90 %) in other experiments performed in our lab with the impedance machine was used. This could also not verify the data gained in the first two assays, as also the negative controls reacted to melatonin.

#### ***Transfection optimization for COS-7 cells***

To increase the transfection efficiency for COS-7 cells, three different cell concentrations and different ratios of transfection reagent to Plasmid-DNA were analysed via FACS. The highest efficiency of 57 % was achieved using  $17,3 \times 10^4$  cells/ml and a 4:1 ratio of TransIT-LT1 to Plasmid-DNA. Moreover a cell concentration of  $11,5 \times 10^4$  cells transfected in a ratio of 3:1 gave the second highest efficiency of 56 %. As these cells looked better and were not overgrown, these conditions were afterwards used for further experiments.

## 3.2 PDF receptor

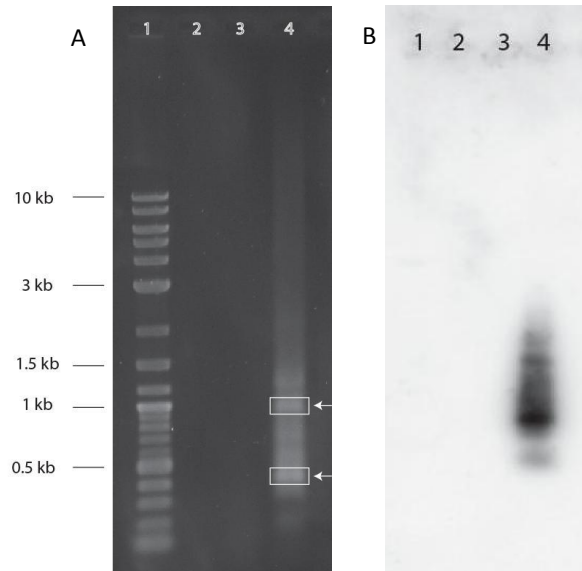
The pdf receptor candidate (pdf<sub>r</sub>) was identified and cloned by S. Hammer from larval cDNA. However, he was not able to get the whole sequence, which would be important for receptor-ligand binding studies to verify that it is a true pdf receptor. Therefore I tried to extend the already known 709 bp fragment via BAC-library screening and 5'RACE and performed WMISH to see where it is expressed in adult worms to get an idea about the target tissues of PDF. Additionally performed qPCR should give us information if the receptor shows circadian fluctuations on mRNA level.

### 3.2.1 Elongation of the pdf<sub>r</sub> candidate fragment

To find regulatory regions in the sequence, which can be later used to generate reporter constructs, a BAC-library screening was performed (chapter 5.5). BAC filter hybridization identified 2 BACs (CH305-53K17, CH305-91N19) which were positive for the pdf<sub>r</sub>. These clones were then further analysed to find out on which BACs the genes are located. By doing radioactive hybridization 1 BAC clone (CH305-53K17) could be identified, which was afterwards sent for sequencing. The obtained sequence consisted of 26 pieces with a total length of 149168 bp. After bioinformatical analysis done by Florian Raible, several PCRs were performed which could elongate the 709 bp fragment to 1.401 kb (Appendix 7.2).

As the 5' end of the sequence was still missing after doing BAC screening, a 5'RACE (chapter 5.4) was performed. The nested RACE-PCR gave two strong positive signals after radioactive hybridization at around 500 bp and at 1 kb (Figure 11). These were then cloned and filter lifts performed to pick clones that contain the right insert. The clones were analysed through analytical restriction digests and sent for sequencing. The short ~500 bp sequence contained the specific pdf<sub>r</sub> sequence but it consisted mainly of the probe and could only extend the 5' region to some basepairs. Moreover

the longer identified fragment of 1 kb contained only intronic sequence prior to the reverse primer, which means that the 5' UTR as well as the 3' end are still missing.



**Figure 11: pdfr 5'RACE agarose gel (A) and southern blot of the gel (B).** (1) 2-log ladder (2) empty lane (3) 5'RACE product (4) nested 5'RACE product. Arrows indicate bands that were cut out after radioactive hybridization.

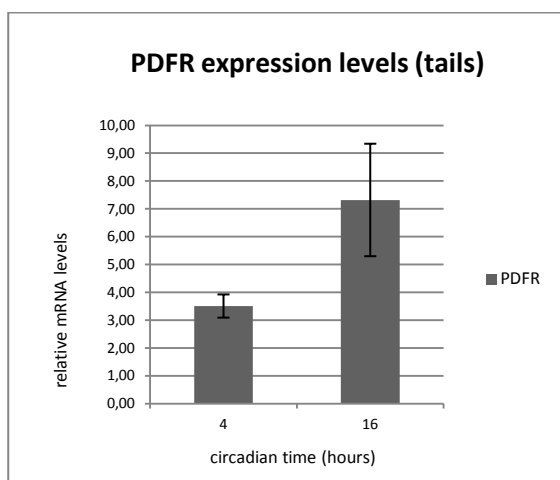
### 3.2.2 Expression quantification in tails of immature worms

As we could detect PDF expression not only in the head but also in the tails of immature worms by doing immunostaining, we were interested in the expression pattern and levels of its presumptive receptor in these tails. By doing whole mount in situ hybridization (WMISH) of *Platynereis* tails, the gene could not be detected. Therefore qPCR was performed, to get a first idea how strongly the gene is expressed in tails. From previous quantitative real-time PCRs of the pdfr candidate, it was known that the gene is expressed in the head of adult worms and by doing WMISH the pdfr RNA could be detected in brain regions of 2 to 14 days old embryos (S. Hammer, unpublished data).

Two time points were chosen to see if there are differences in expression levels. As 4 am/4 pm were previously used for comparing differences in PDF protein expression levels, these time points were also taken for pdfr qPCR analysis. Four biological replicates were sampled for each time point, whereas each replicate consisted of one

tail. Differences between samples were corrected using the endogenous reference gene *cdc5* for normalization. Unpaired Student's T-Test was used to find out if there is a statistically significant difference between 4 am and 4 pm.

The qPCR data showed that the *pdfR* candidate is also expressed in the tails of immature worms but the transcript levels were low at both time points taken for analysis (Figure 12). Despite higher relative mRNA levels at the day time point, the Student's t-test performed showed that there is no significant difference with a p-value of 0.1137.



**Figure 12: pdfR expression in tails of immature worms.** Four biological replicates were taken for each time point. Error bars represent the standard error. Transcript levels are displayed as mean  $\Delta C_t$  values and samples were normalized to *cdc5*. No significant difference could be detected between the time points.

### 3.3 Pigment dispersing factor in *Platynereis*

As mentioned in the introduction, PDF exerts different functions in different organisms but little is known of its role in *Platynereis*. In *Drosophila*, PDF is an output factor of the circadian clock and is thought to help synchronizing clock neurons, whereas in crustaceans, PDHs are known to be involved in pigment dispersion<sup>64,100</sup>. Furthermore, PDF might also be part of the input pathway of the circadian clock, which has been suggested for *Drosophila*<sup>101</sup>. Therefore we hypothesized that it might have similar functions in *Platynereis* and to test these hypotheses, several experiments were performed to gain more knowledge about its role of PDF in *Platynereis*.

#### 3.3.1 Spatiotemporal expression pattern of PDF in immature *Platynereis*

As the pigment dispersing factor is a major output factor of the circadian clock in many organisms, we wanted to see if PDF shows circadian changes on protein level by doing immunostaining at different time points<sup>40</sup>. This data could then be compared to RNA expression levels of its presumptive receptor. First of all, we wanted to characterize the expression pattern of PDF in tails of adult worms at different time points. As the main focus was on the peripheral clock of the worm, the tail was the main subject of analysis. Furthermore the expression pattern has already been extensively studied by S. Hammer (unpublished data).

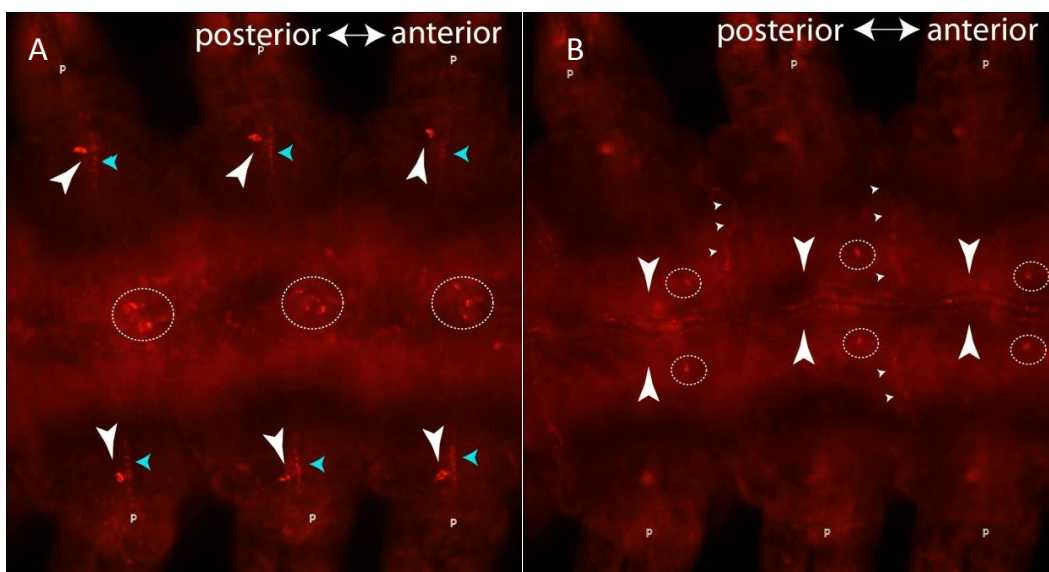
To be sure that the staining is specific, animals were also stained with pre-immune serum, which didn't give any specific staining (data not shown). For the characterization two to three month old worms were used, which were fixed around new moon, which means there is no nightly light exposure that could interfere with the regulation of PDF.

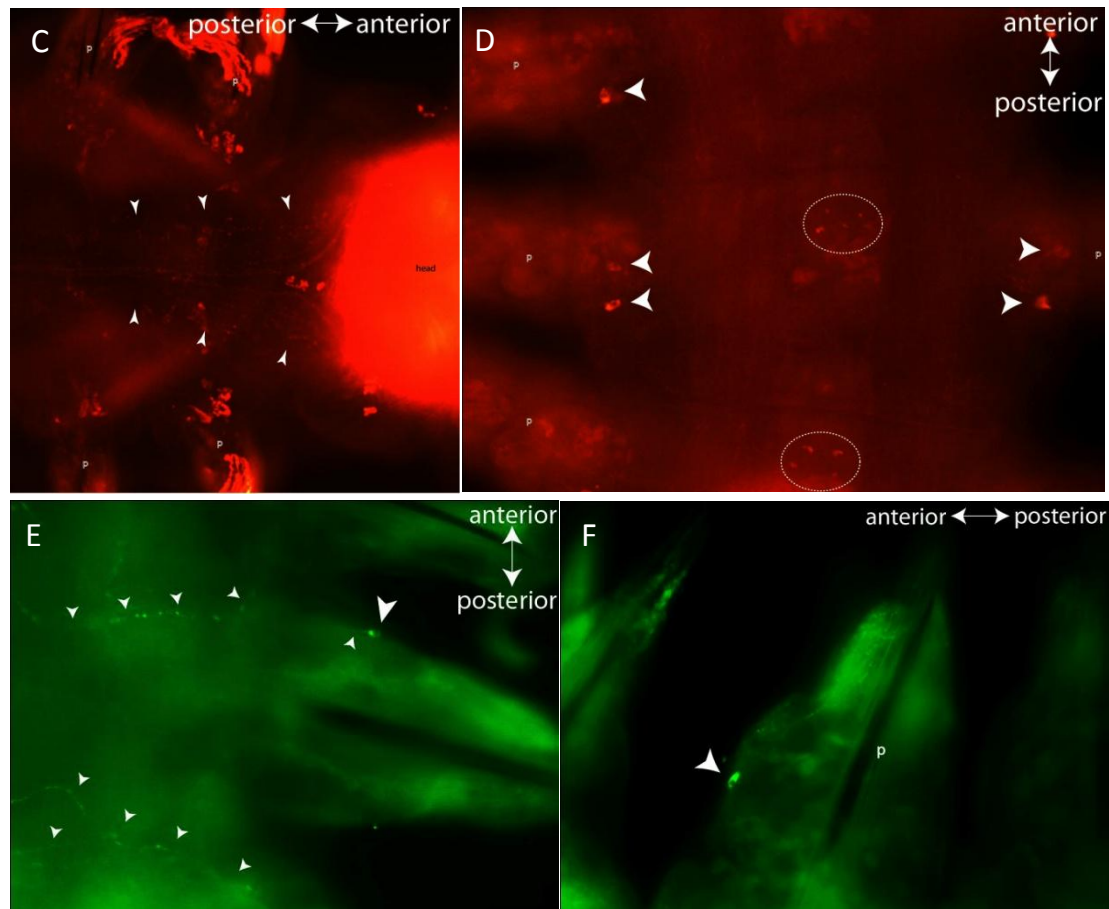
Huge projection networks along the ventral nerve chord can be seen that start at the head, going posterior through each segment of the worm (Figure 13 C arrows). In some segments there are even projections coming from cells in the parapodia that connect to these ventral nerve chord projections (Figure 13 A blue arrow, B small arrows).

As seen in Figure 13, PDF is present in each segment in three distinct regions. There is usually one strongly stained cell expressing PDF at the base of the ventral parapodia that has projections going toward the tip of the parapodia but also going in the opposite direction, towards the middle the worm (Figure 13 A big arrow). These projections do not necessarily go in both directions, with some cells sending fibers just in one direction. Interestingly, the position of this cell can change as depicted in Figure 13 E, F (big arrow), where it is located more lateral/distal at the parapodia compared to its usual position. However, this was only seldom the case. In very rare cases even two cells could be detected (Figure 13 D white arrows).

Additionally there are two big cells on each side next to the ventral nerve chord projections with small fibers going out (Figure 13 B circles). They are not present in every segment and show a stronger staining closer to the head, which gets less towards the posterior of the worm, vanishing around the 13<sup>th</sup> segment.

Besides these regions expressing PDF, there are also cell clusters of 2-7 cells that are located deeper in the worm, underneath the ventral nerve chord projections (Figure 13 A circles). The amount of the cells, size and strength of the staining can vary quite a lot between the segments but also between the worms. Moreover these cells show a stronger staining in the middle segments of the animal, getting less towards the posterior and are sometimes not even detectable anymore.





**Figure 13: PDF antibody staining of immature worms.** All pictures show the ventral side of the worm. White arrows and circles indicate PDF expressing cells (A) blue arrows show projections of PDF positive cells in the ventral parapodia (A-D) Cy3 coupled secondary antibody was used to detect PDF antibody (E,F) Alexa Fluor 488 coupled antibody was used to detect the PDF antibody. For more information see text. p=parapodia

### 3.3.2 Function and regulation of PDF in *Platynereis*

After characterizing the expression pattern of PDF in tails of adult worms, the next step was to find out what kind of function it has in *Platynereis*. The pigment dispersing factor is a well studied neuropeptide in *Drosophila melanogaster*, where it is an important output factor of the circadian clock and recent studies showed that it also feeds back on clock neurons influencing their period<sup>100</sup>. Moreover in crustaceans, where it was originally discovered, it was found to regulate retinal and integumental pigmentation changes<sup>64</sup>.

In order to differentiate between these options, several experiments were performed to dissect its role in this species.

***Circadian changes in PDF expression in immature worms***

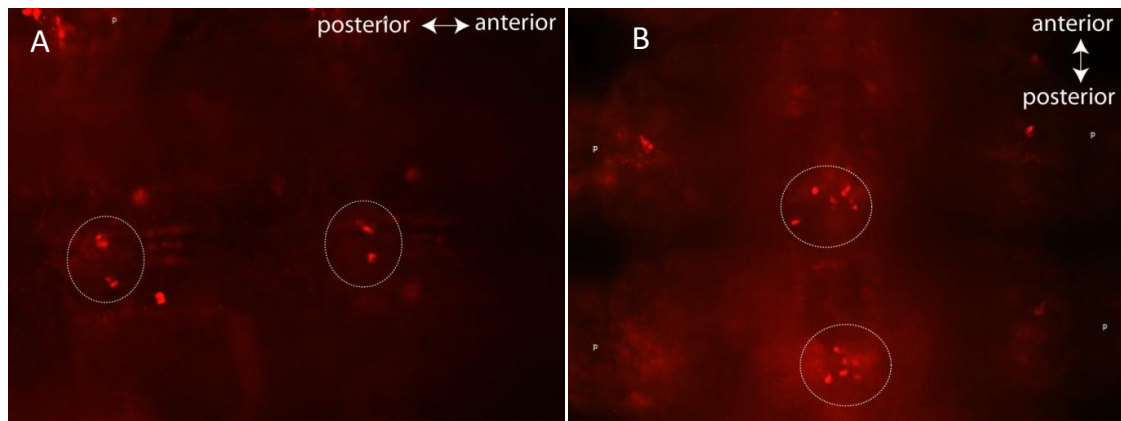
In *Platynereis* there is a central clock in the head but also a peripheral in the tail of the worm exists, which is independent to the central one as qPCR has shown that clock genes in tails of decapitated worms still cycle (Enrique Arboleda, personal communication). As depicted in Figure 13, the neuropeptide is also expressed in the tail of worms, in each segment in three distinct regions. That's why worms fixed at two time points (4 am and 4 pm) around new moon were analysed by doing antibody staining to find out if there are circadian changes in the expression level of the PDF peptide. To have less variation, worms of the same box were used and inverted for approximately 7 days for the night time point.

As each worm and each segment had a slightly different staining pattern it was hard to find differences that could indicate circadian changes. After comparing the staining pattern of several worms, the middle staining underneath the ventral nerve chord seemed to show most variation, with fewer cells during the nighttime (Figure 14 A circles). The other cells were present at both time points, constantly expressing PDF. To be sure that these are significant differences, these middle cells were counted in the segments 5 to 10 in 6 worms per time point (see Appendix 7.3). This confirmed the assumption that there are less cells in this region expressing PDF at nighttime. Although not every segment had the same number and there were also differences between worms, the average number of PDF expressing cells per segment (only including middle cells) was 3 at 4 am and 5 at 4 pm (Table 2). In most worms fixed at 4 pm the cells were quite big and present throughout the whole body, whereas at nighttime in some of the counted segments no PDF positive cell was visible. Moreover, they were usually weakly stained and only present in the anterior segments. To be sure that this difference between time points has a statistical significance an unpaired student's t-test was performed. With a p-value of 0.0111 the difference between the time points is considered to be statistically significant.

This means that there are circadian changes in the expression of PDF in some cells, with quite some variation between the biological replicates.

| timepoint | Average number of cells per segment<br>(middle cells) |
|-----------|-------------------------------------------------------|
| 4am       | 3                                                     |
| 4pm       | 5                                                     |

**Table 2: Average number of PDF positive middle cells per segment.**  
6 segments were counted per worm

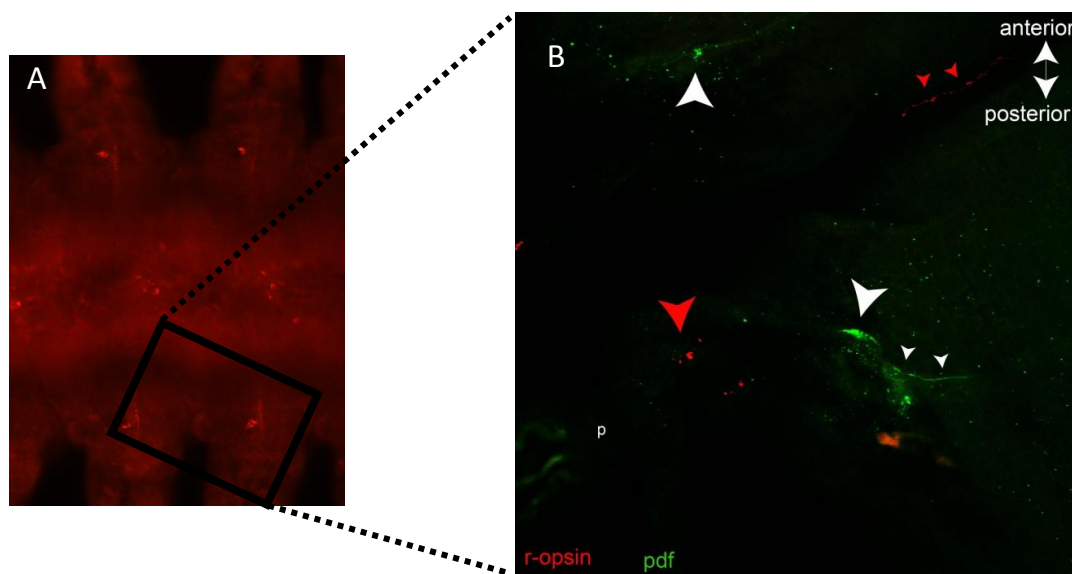


**Figure 14: Comparison of PDF expressing cells in tails of immature worms.** Ventral side of the worms is shown. Worms were fixed at two different time points (4 am/4 pm). Circles indicate differences in PDF expression level in the middle region of the worm. (A) expression pattern at 4 am (B) expression pattern at 4 pm. p=parapodia

### ***Expression localisation of PDF and r-opsin cells***

As a transgenic line expressing GFP under the control of the r-opsin locus generated in our lab showed that this gene is also expressed in the tails of worms on the ventral side in the middle region and in the parapodia, we wanted to find out if there is co-expression of r-opsin and PDF. This would give more information about possible regulation of PDF and how it is connected to other molecules which are involved in the circadian rhythm. R-opsin is a main light sensor in *Platynereis* and might also be involved in the circadian clock. Therefore two fixation methods and several anti-GFP antibodies were tested to visualize r-opsin in transgenic animals to be able to perform co-stainings with PDF. Best results could be achieved using Bouins fixative and the mouse anti-GFP5G4 antibody. Fixing worms with PFA and Methanol, which is used for the PDF antibody, resulted in no specific r-opsin staining. Therefore the Bouins fixative was used for double staining. Transgenic worms used for antibody staining were beforehand screened for r-opsin-GFP expressing cells.

As depicted in Figure 15 B no co-expression could be detected, although the GFP positive cells are located in close vicinity to the PDF cells. The PDF positive cell in the base of the parapodia had huge projections going towards the middle of the worm, but also towards the r-opsin cell which is more distally expressed. Moreover, the r-opsin expressing cell seemed to have small projections, but no direct connection with the PDF cells could be seen. However, r-opsin seemed to be quite broadly expressed with huge projections going along the whole body of the worm and also in the parapodia. As in these transgenic worms no medial r-opsin cells were found, it is still unclear if there might be co-expression with PDF in this region.



**Figure 15: Co-expression study of r-opsin and PDF in tails of immature worms.** (A) Overview picture, ventral view. PDF expressing cells are stained red (B) Confocal laser scanning microscopy scan, ventral view, section of 2 segments is shown, small red arrows indicate r-opsin expression in segment borders. Red arrows indicate r-opsin positive cells or projections, white arrows PDF positive cells and projections. p=parapodia

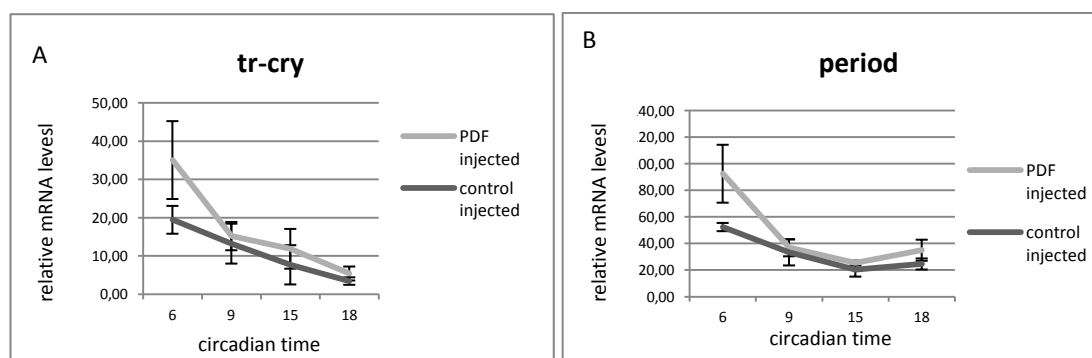
### ***Expression quantification of clock genes in PDF injected worms***

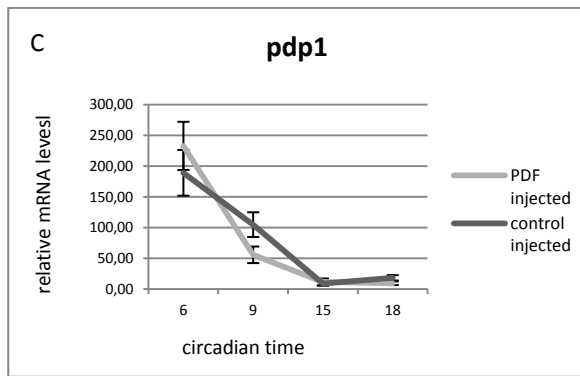
Studies in *Drosophila* have shown that the pigment dispersing factor does not only act as an output factor of the clock but also feeds back on the clock synchronising clock neurons and changing their period<sup>100</sup>. To see if that is also true for *Platynereis* the expression level of three clock genes (tr-cry, period, pdp1) was quantified via qPCR in worms injected with synthetic PDF peptide or PBS (as negative control). Only tails were taken for qPCR measurements and the samples were normalized to cdc5. Three tails

were analysed per time point with one tail representing one biological replicate. As the injection procedure required some time, only four time points were chosen per injection round. Worms were injected at 3 am or midnight, since this time point was also used for other injections described below. To begin with, a concentration of 800 nM PDF was injected in immature worms.

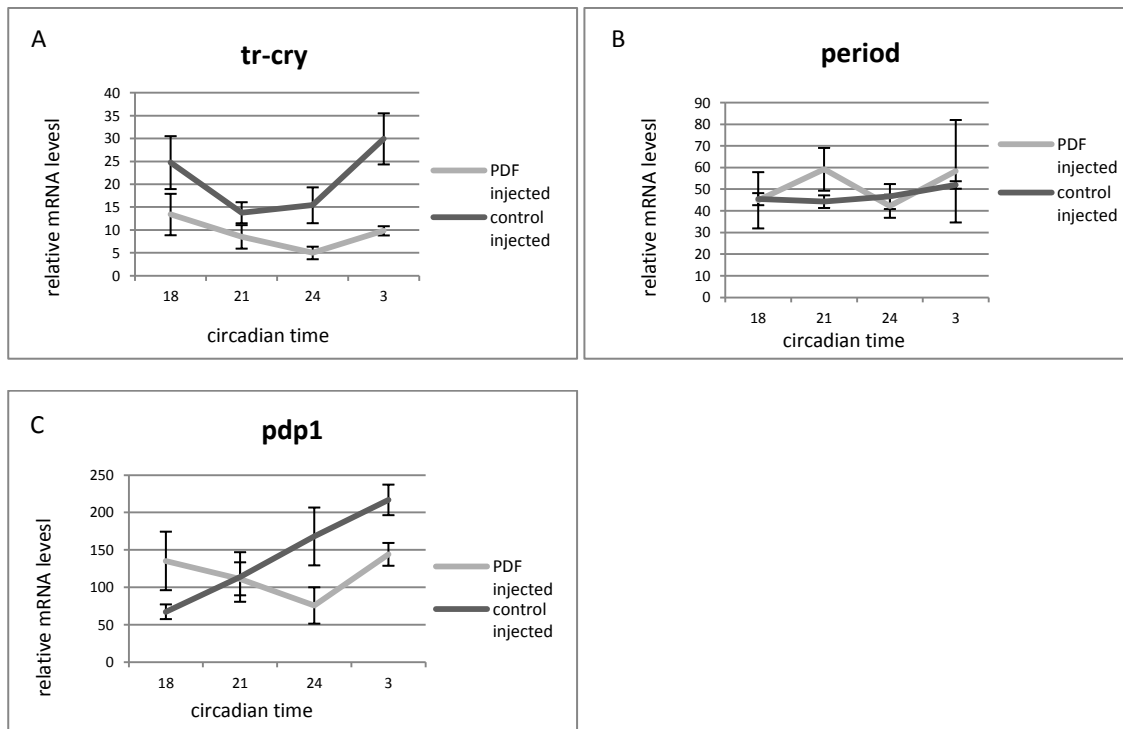
As can be seen in Figure 16, there was no big difference in the expression level in all three analyzed clock genes compared to their control. Relative mRNA levels as well as the shape of the curves were comparable to the control. Only at the 6 am time point there were slight differences in the relative mRNA levels of *tr-cry* and *period* with the PDF injected worms having a higher value.

To corroborate the data, the experiment was repeated but different time points were chosen for the expression analysis. These time points were taken as we wanted to see how the gene expression might get affected at later time points by the injection. The expression level of *pdp1* looked quite different between injected and control injected worms, as the PDF injected worms showed a slight decrease in expression until midnight, whereas in the controls the expression increased steadily (Figure 17 C). Additionally, in the control injected *tr-cry* had higher mRNA levels at all time points compared to the values of injected worms but the shape of the curves were the same (Figure 17 A). Although the values slightly varied from each other, there was no significant difference in expression levels compared to the controls in all of the analysed genes (Figure 17).





**Figure 16: Expression quantification of clock genes in PDF injected worms.** 800 nM PDF was injected into worms and tails were afterwards frozen at time points 6, 9, 15, 18 for expression analysis. As a control PBS was injected. Three tails were taken per time point, whereas one tail represented one biological replicate. Error bars represent the standard error and samples were normalized to *cdc5* (A) *tr-cry* expression levels (B) *period* expression levels (C) *pdp1* expression levels.



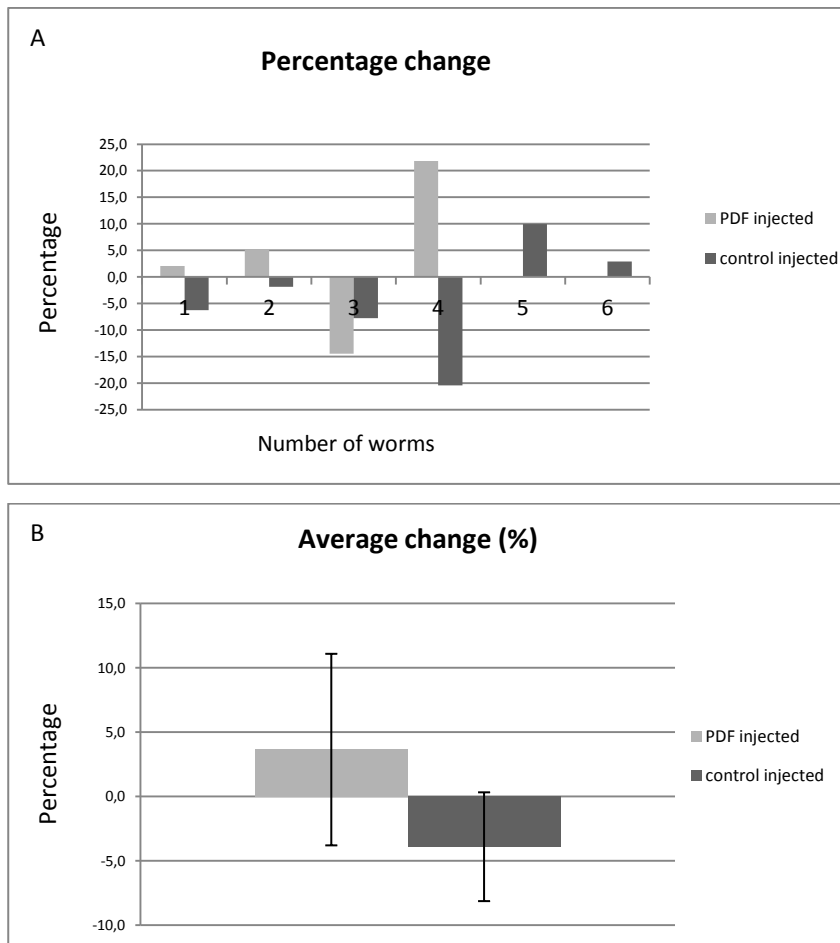
**Figure 17: Expression quantification of clock genes in PDF injected worms.** 800 nM PDF was injected into worms and tails were afterwards frozen at time points 18, 21, 24, 3 for expression analysis. As a control PBS was injected. Three tails were taken per time point, whereas one tail represented one biological replicate. Error bars represent the standard error and samples were normalized to *cdc5* (A) *tr-cry* expression levels (B) *period* expression levels (C) *pdp1* expression levels.

***Influence of PDF on pigmentation***

In crustaceans – where the peptide was discovered – the pigment dispersing hormone (PDH) is involved in colour changes related to pigment dispersion of integumental pigments and shielding pigments in the compound eye<sup>64</sup>. As *Platynereis* also have chromatophores on the dorsal side of the body and on the head, which show a daily rhythm in dispersion and concentration, we wanted to test if PDF could be the factor that regulates these pigmentation changes. Therefore I injected PDF peptide into the coelom of premature worms at nighttime as the pigments are usually contracted at this time. In order to ensure where the peptide was injected, TRITC was used as marker to visualize the injected solution. To see if there are changes in pigmentation, pictures were taken some days before injection and two hours after injection always at the same time point and using the same setup and magnification. Using Photoshop the pixel size of the iridocytes in the same segments was calculated and therefore a dispersion or contraction of the respective pigments determined.

In order to find out if changes of pigmentation are not caused by the injection procedure itself, some worms were injected with PBS as a control.

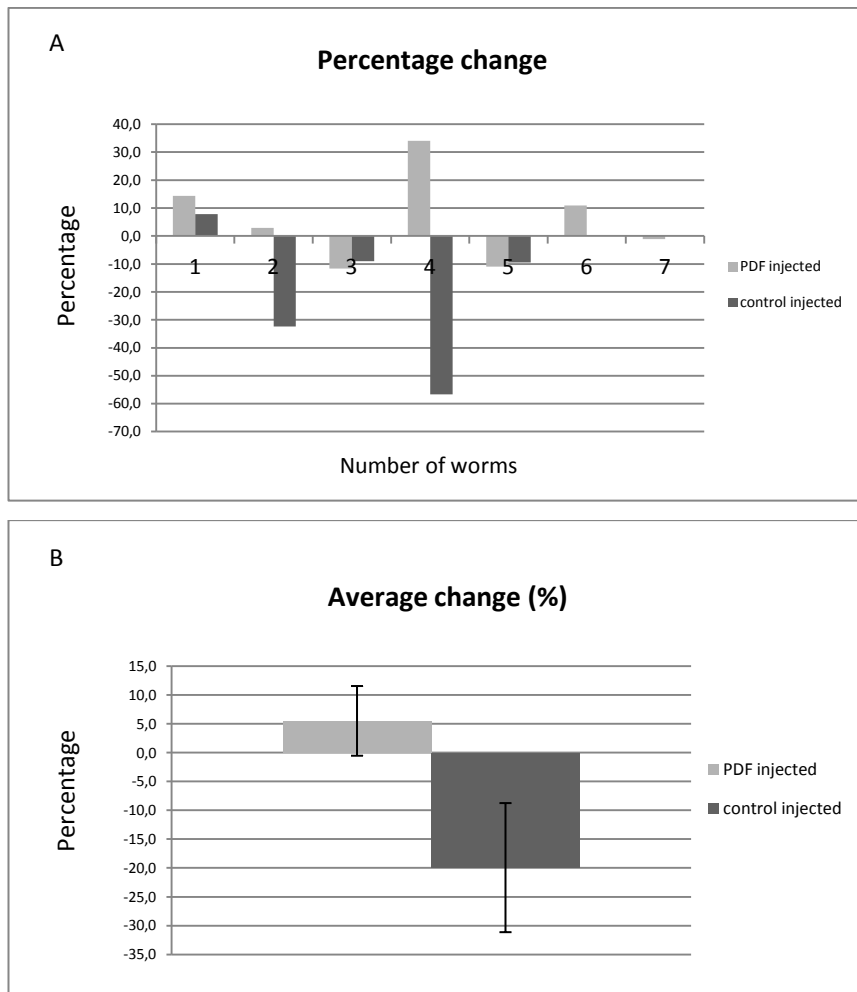
The first injection round was done using a concentration of 800 nM PDF and the peptide was injected at 3 am, as the pigments are still contracted at this time. Not all PDF injected worms survived the procedure, that's why only four were analysed. As can be seen in Figure 18 A there is hardly an increase in pigment size after injecting PDF. Three out of four worms showed a slight expansion of pigments, whereas in the control where PBS was injected only two of six worms had slightly expanded pigments, while in the other worms the values dropped. Looking at the average change in pigmentation there is no significant difference between control and PDF injected worms (Figure 18 B).



**Figure 18: Pigmentation changes in PDF injected worms.** Worms were injected with 800 nM PDF or PBS as a control at 3 am, pictures were taken at the same time point before the injection and afterwards and the pixel size calculated. The error bar corresponds to the Standard error. (A) Percentage change of PDF and control injected worms (B) Average change of PDF and control injected worms. For more information see text.

Another round of injection was performed using a higher concentration of PDF (1 mM) and a different injection time point (midnight), as the concentration of the previously used PDF might be too low to cause dispersion.

Four out of seven PDF injected worms showed an increase after injection, whereas in the control only one worm had enlarged pigments afterwards (Figure 19 A). In the control the pigmentation seemed to get lower in most worms, whereas in the PDF injected only three worms had iridocytes that behaved like this. However, looking at the average percentage increase there was no significant change in pigmentation size after injecting PDF (Figure 19).

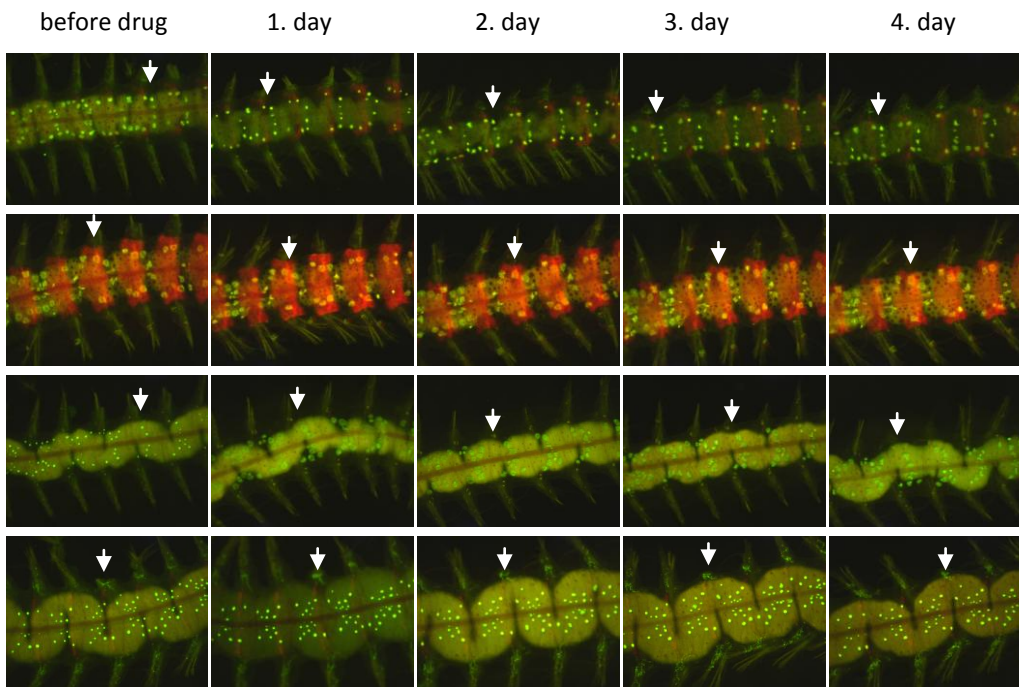


**Figure 19: Pigmentation changes in PDF injected worms.** Worms were injected with 1 mM PDF or PBS at midnight, pictures were taken at the same time point before the injection and afterwards and the pixel size calculated. The error bar corresponds to the Standard error. (A) Percentage change of PDF and control injected worms (B) Average change of PDF and control injected worms. For more information see text.

### ***The pigmentation is influenced by the circadian clock***

In 1964 Fischer analysed the pigmentation in *Platynereis* and found that the size of the pigments changes throughout the day. He also found out that worms put into constant light for some days maintain this daily rhythm of colour change<sup>7</sup>. This led us to the assumption that the pigmentation might be influenced by the circadian clock. Therefore we put worms into NSW containing a casein kinase 1 $\epsilon$  (CK1 $\epsilon$ ) and CK1 $\delta$  inhibitor (PF 670462), which dampens the cycling of the circadian clock. Casein kinases are involved in phosphorylating period, which subsequently leads to its degradation and are therefore necessary for normal clock function. The pigmentation was checked

before drug exposure and each consecutive day for four days. Two time points were analysed (3 am and 3 pm) and the changes were evaluated using Photoshop. For each time point different worms were taken.



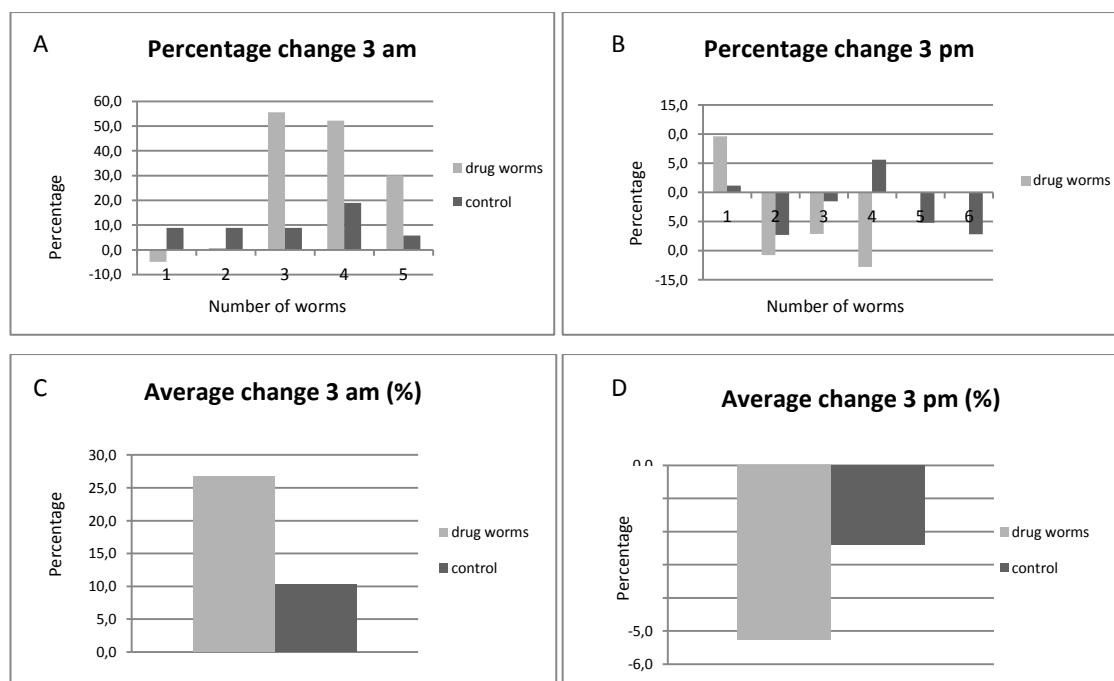
**Figure 20: Pigmentation change of worms put into casein kinase inhibitor drug for four days.** First picture of each column is taken before drug exposure, pictures in following columns were taken each consecutive day the worm is exposed to the drug. Two different time points were chosen to take pictures: 3 am (row 3 and 4) and 3 pm (row 1 and 2). Every row shows a different worm. Row 2 and 4 displays control worms, row 1 and 3 drugged worms. White arrows indicate the same region of the worm. Dorsal view, fluorescent pictures were taken using the FITC filter. One or several parapodia were cut off for orientation and the same magnification and setup was used for all pictures. Green spots represent the iridocytes, which were used for measurement.

In Figure 20 the pigmentation of different worms can be seen and how it changed after putting the worms into the drug compared to control worms. Only the iridocytes were analysed, as they display high autofluorescence, which are depicted as green/yellow spots on the pictures. One or several parapodia were cut off to retrieve the studied segments.

As depicted in Figure 20 even after one day in the drug the pigmentation already looked different. At 3 pm, when the pigmentation is usually spread out it got smaller, whereas at 3 am (when it should be contracted) the pigmentation was expanded. Moreover the pigmentation changed every day the worm is subjected to the drug. As

qPCR data has shown that clock genes get flat after 4 days in drug, the experiment was stopped then. The pigment size was calculated before putting worms into the drug and after 4 days of drug exposure and the two different values compared.

After four days in drug three out of five worms showed a big increase in pigment size at 3 am whereas the control stayed more or less the same (Figure 21 A). At 3 pm most of the drugged worms showed a decrease in pigmentation but the change was smaller than at 3 am. As can be seen in Figure 20 C and D the pigmentation of the drugged worms increased about 25 % at 3 am and decreased slightly at 3 pm (5 %), whereas the control only showed minimal changes.

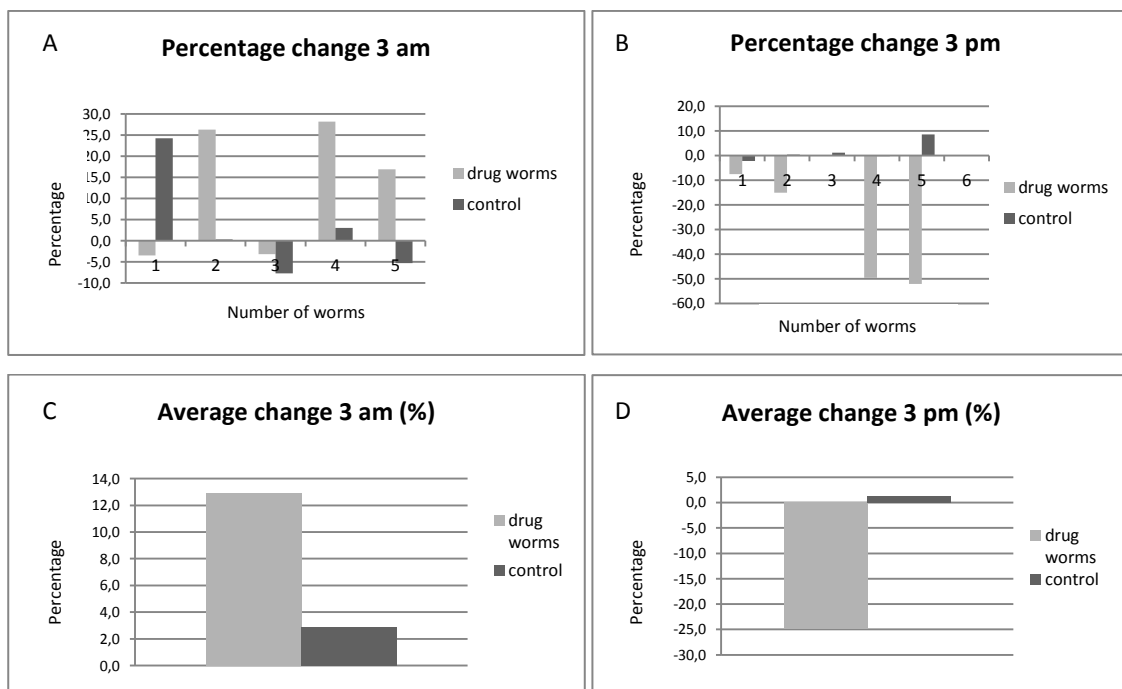


**Figure 21: Pigmentation changes in the tails of worms after 4 days of drug exposure (PF 670462).** Worms were put for 4 days into the PF 670462 drug. Pictures were taken at the same time point before drug exposure and after 4 days in drug and the pixel size was calculated. (A, B) Percentage change of drug and control worms at 3 am and 3 pm (C, D) Average change of drug and control worms at 3 am.

Also the pigmentation in the head was analysed, to see if it gets influenced by the drug the same way as the body pigmentation. Identical worms were used for quantifying head and tail pigmentation after four days of drug exposure. As a control, worms were left in the normal light-dark cycle and iridocytes were measured after 4 days. Figure 21 A shows that 3 out of 5 worms had more expanded pigments at 3 am after being 4

days in the PF 670462 drug, whereas in the control the pigmentation stayed nearly stable, with only 1 worm showing an increase. At 3 pm most of the drugged worms (4 out of 5) had more contracted iridocytes than in the beginning when no drug was still present (Figure 21 B,D). In the control the chromatophores hardly changed after 4 days, showing that the pigmentation in the head is still at its normal level. Interestingly, the head and body pigmentation behaved the same way after 4 days drug exposure, with an increase at 3 am and a decrease at 3 pm, although the head pigmentation showed a bigger change at 3 pm.

This illustrates that the pigmentation is not just light regulated but that by inhibiting the circadian clock the daily rhythm of chromatophore dispersion and contraction gets changed in a way that both high and low values of pigmentation seem to converge with each other.



**Figure 22: Pigmentation change in the head of worms after 4 days of drug exposure (PF 670462).** Worms were put for 4 days into the PF 670462 drug. Pictures were taken at the same time point before drug exposure and after 4 days in drug and the pixel size was calculated. (A, B) Percentage change of drug and control worms at 3 am and 3 pm (C, D) Average change of drug and control worms at 3 am.

## 4. Discussion

### 4.1 Target tissues of melatonin in *Platynereis*

Melatonin is a ubiquitous molecule that regulates a number of physiological and neuroendocrine processes. Classical effects are attributed to its binding to G protein-coupled membrane receptors. To find out more about its signaling pathway and downstream targets it is important to not only analyse melatonin itself but also to have a look at its receptors. This can give further knowledge about the function of this versatile molecule.

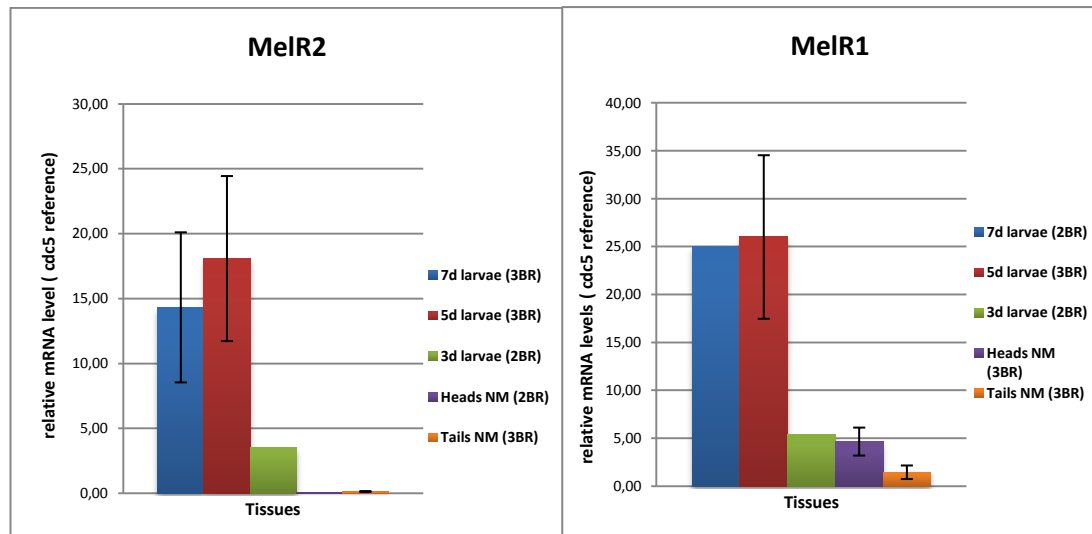
#### 4.1.1 Embryonic localisation of melatonin receptor 2 in *Platynereis*

By doing whole mount in situ hybridisation the target tissues of the second melatonin receptor candidate could be identified in embryonic stages. In all studied larval stages it is mainly expressed in ciliary structures such as prototroch, paratroch, metatroch or telotroch (Figure 8). However, the staining got weaker from 5 dpf on with only about four cells in the ciliary belt being positive for the receptor candidate. Furthermore it was not possible to locate these cells in immature worms, which is consistent with the qPCR data conducted by Claudia Lohs (Figure 23, adapted from Claudia Lohs). This shows that both receptors are expressed at very low levels in the tail and head of immature worms, with the second candidate being hardly detectable. This means, that there could be many cells producing little amount of the melatonin receptor RNA or few cells expressing higher amounts. As the staining could not be detected in adults, this argues for more cells expressing the melatonin receptor candidates at a low level. When comparing the expression pattern of the two receptor candidates, there doesn't seem to be any co expression with candidate 1, as MelR1 is expressed in the ventral nerve plate and close to the neuropil (S. Hammer, unpublished data). This indicates that the two receptors could have different roles in *Platynereis* with receptor

candidate 1 perhaps being more important in the signal transduction of the circadian effects of melatonin as it is expressed in nervous tissue of the head of embryos, where also clock genes are located. In contrary, for the second candidate no expression in the nervous tissue could be seen. However, this could mean that the receptors exert different functions in *Platynereis*. As melatonin is a molecule with numerous functions, this receptor candidate might be involved in other effects mediated by melatonin.

Aside from that, the two enzymes important for melatonin production (AANAT and HIOMT) have been shown to be expressed posterior of the neuropil (S. Keplinger, unpublished data). This means, that they are more closely located to the first receptor candidate, which is expressed anterior of the neuropil.

To gain more knowledge about the downstream targets of melatonin and its involvement in the circadian clock it would be important to find out where its receptors are expressed in adult worms and if there is a co-localisation with clock genes. This could be achieved by improving the in situ protocol or if an antibody against the receptor candidates were available. Melatonin is also thought to be involved in regulating reproduction in seasonal animals by inhibiting GnRH<sup>138–140</sup>. Therefore the receptors should also be expressed in GnRH-producing cells. As GnRH has also been identified in *Platynereis*, it might be interesting to see if there is a connection between melatonin and reproduction. However, as both receptors could not be verified via ligand-binding assays (see below), it would be necessary to prove that these are the real melatonin receptors before speculating more about their function.



**Figure 23: Expression quantification of MelR1 and MelR2 in different tissues.** Adapted from Claudia Lohs. Error bars represent Standard Error. Transcript levels are displayed as mean  $\Delta C_t$  values and samples were normalized to *cdc5*.

#### 4.1.2 Ligand-binding assays

After identifying two receptor candidates and performing first expression analysis it was important to validate their functionality by testing if they bind melatonin. Therefore several cell impedance assays (Roche) were performed using COS-7 cells. In a preceding experiment performed in our lab the melatonin receptor candidate 1 has already been shown to react to melatonin by increasing the cell impedance value (CI value) to 0.03. Two subsequent cell impedance assays, which I performed, showed a positive reaction of the receptors after stimulation with different melatonin concentration with CI values of above 0.1 for both receptor candidates. This was much higher than the previously performed assays, where only 0.03 could be reached for MelR1. A reason for this could be that no codon-optimized sequences were available for the previous experiment, which could have led to a lower expression rate in the mammalian cells.

A dose-dependent reaction could only be determined for the mouse melatonin receptor in one experiment, by testing different melatonin concentrations. This would have been important to see how they differ from each other and if they are high or low affinity receptors. However, in both assays the receptor candidates reacted best to

quite low (1 nM and 10 nM) concentrations of melatonin. As the curves and values are similar to the mouse MtnR1A, which is a high affinity receptor, this could give a first hint that they also have a high affinity for melatonin.

In the second assay shown, cells were starved by changing the medium prior to melatonin application. This didn't lead to an enhanced reaction of the cells, but reduced the response of the cells. After the medium change the cells were completely disturbed by this, which could have led to the diminished reaction of the cells.

These first assays showed a high response of both receptors to the administered melatonin. In further assays performed (data not shown), also the negative controls where PBS was added showed a reaction, which is in contrast to the data I obtained in the first 2 experiments. Therefore another cell line was tested and also a transfection optimization for the COS-7 cells was performed. Despite this, the negative controls still showed an increase of the CI value comparable to that of the tested receptors after adding melatonin. Moreover the transfection rate for COS-7 cells was still not optimal, with only 57 % expressing the receptor.

What might be a big issue when doing these assays is that we don't know how much melatonin is already in the medium. Depending on the FCS there could be sometimes more, sometimes less melatonin in the medium, already binding to the receptors. This could mean that the receptor is already saturated with serum melatonin, when melatonin is applied on the last day of the experiment. Therefore the reaction to the added compound could be in the same range as the negative control.

As different media were used in the beginning and in later assays this could be a cause for these differences. That's why it would be important to test the used serum beforehand for its melatonin concentration to exclude an influence of serum melatonin on the experimental outcome.

Moreover in the original experiments in which they verified melatonin receptors they usually measured the binding of radioactive melatonin (2-iodomelatonin) to the presumptive receptor<sup>152</sup>. This method could lead to a more robust statement to corroborate the results of the first assays.

As only the first two experiments showed a reaction of the receptor candidates to melatonin and the subsequently performed ones didn't confirm these positive results, a solid statement on their ligand-binding properties cannot be made.

## **4.2 Function and regulation of PDF in *Platynereis***

The second part of my project was focusing on another important *Platynereis* hormone - PDF - and its receptor. Former lab members have already been able to clone PDF and showed that it is expressed in the larvae as well as in immature worms. Also its receptor could be detected in embryonic stages via in situ hybridization.

### **4.2.1 Elongation of PDF receptor**

To elicit changes in the target tissue the neuropeptide PDF has to bind its receptor. Therefore we tried to elongate the already known fragment of the PDFR, which would be important for binding assays or creating transgenic lines, to see where it is expressed in adult worms. The receptor sequence could be elongated to 1.4 kb by doing a BAC screening. Unfortunately none of the positive BACs contained the whole sequence and the 5'UTR (Appendix 7.2). Moreover the subsequently performed 5'RACE could also not elongate the already known fragment, as the sequence which was positive after hybridization only contained intronic sequence (data not shown). To finally get the whole sequence, repeating the RACE or blasting the already known fragment against the 4dlx database (unpublished EST resources) might prove useful.

### **4.2.2 Uncovering the role of PDF in *Platynereis***

After determining the regions where the peptide is expressed in adult worms we also wanted to find out more about its ancestral function, by studying the marine bristleworm. In crustaceans, PDHs are known to be involved in pigment dispersion,

whereas in *Drosophila* it is an important output factor of the circadian clock and might also help synchronizing clock neurons<sup>64,100</sup>. Therefore different experiments were performed to figure out if it exerts the same functions as in *Drosophila* or if it might be the factor that regulates pigmentation. Moreover the pigmentation in *Platynereis* was studied to get a better knowledge about it and to find out if it is regulated by the circadian clock. Now I want to summarize the results of the different experiments and discuss some unresolved questions.

### ***PDF might be an output factor of the circadian clock***

By doing immunostaining I was able to detect PDF in many regions of the body of the worm. It is expressed basically in three regions: At the base of each parapodia, underneath the ventral nerve chord and next to the ventral nerve chord (Figure 13). Each worm showed a slightly different staining, which made it difficult to detect circadian fluctuations in PDF expressing cells. However, circadian differences could be seen in the middle cells underneath the ventral nerve chord, with significantly more cells at 4 pm. This data indicates that PDF might be translationally regulated as the protein shows a daily cycling but not the mRNA as qPCR data has shown (Enrique Arboleda, personal communication). In *Drosophila* PDF abundance is also not controlled on a transcriptional level but posttranslational by the timing of its release, which underpins the findings in *Platynereis*<sup>95,96</sup>. Former lab members also analysed the expression of PDF in the head, which also showed some daily fluctuations between different time points, but no solid statements could be made (S. Hammer, unpublished data).

As only the antibody against the amidated form of PDF was available, it might also be important to have one against the non-amidated form, as this might show more circadian fluctuations. In addition, more time points should be analysed and free running experiments should be performed to corroborate these results.

In adult animals, only the antibody staining of PDF has been described in detail so far. To find out more about the regulation and target tissues of PDF it is necessary to find

out where its receptor is expressed in adult worms. Unfortunately no antibody against PDFR is available and I wasn't able to detect it via in situ hybridization in immature worms. It could only be detected by qPCR, which showed that it is expressed at low levels and that there are no significant differences between the chosen time points. This data suggests that the receptor is constantly expressed and that its ligand shows fluctuation.

PDF in *Drosophila* is also thought to be involved in the input pathway of the circadian clock<sup>101</sup>. Double staining of PDF and the light receptor r-opsin showed that the cells are expressed in close proximity at the base of the parapodia, although no direct connections could be detected. In some segments r-opsin is expressed ventrally in the middle region, where also PDF is expressed. This argues that there might be some crosstalk between the cells and that these regions are important centers of the peripheral clock. However, no other clock genes could be detected yet in the body of the worm, due to technical problems with either WMISH or antibody staining. To find out more about the peripheral clock itself and the regulation and signaling of PDF, it would be crucial to find out where other clock genes are expressed and if there are projections towards PDF.

PDF cells in the head are also located closely to the eyes and to r-opsin cells, which is another good indication that there might be some communication between these cells (S. Hammer, unpublished data).

Besides that, prohormone convertase 2 could be detected via WMISH (Benjamin Backfisch, data not shown) in the same regions where PDF is expressed. As most peptides need to be processed to be functional, this could indicate the importance of these ventrally located areas in the tail of the worms.

Concluding, PDF seems to be involved in the circadian clock, although more experiments are necessary to unravel its role in the circadian system.

***Does PDF feedback on the circadian clock?***

Another hypothesis we wanted to test, was if there is a feedback of PDF on the circadian clock, as studies in *Drosophila* suggest that PDF might shorten or lengthen the period of some clock neurons<sup>100,172</sup>. Observations indicate that in the fruit fly it might be crucial for adjusting cycling period, amplitude and the phase of certain components in the circadian clock<sup>100</sup>. To see if it has similar effects, synthetic PDF was injected into adult worms and qPCR was performed on these tails to see if that changes the expression of certain clock genes. This proved rather inconclusive as there was hardly any difference compared to the control injected worms. However, little is known about the regulation and targets of PDF in *Platynereis* and how it acts on individual clock neurons. On top of this it is also unclear if the injected concentration of PDF is high enough to cause any effects and if it reaches the target cells. Interestingly, the controls also looked strange in some cases (compared to non injected worms), which could mean that the worms were not entrained yet to the new rhythm or the whole injection process disturbed the cycling of the clock genes. Therefore we cannot be sure yet if there is a feed back or not but we cannot exclude it, as we don't know if the injected concentration is high enough to cause an effect and the time point of injection might also be not the best. Moreover more biological replicates and more time points are necessary to corroborate the data. If PDF feeds back on the clock, its receptor should be expressed on clock neurons. Unfortunately, pdf<sub>nr</sub> could not be detected via WMISH in adult tails nor do we know yet where clock neurons are located in these tails.

***Does injected PDF change pigment dispersion?***

*Platynereis* worms have chromatophores on the dorsal side of the body and on the head that show a daily rhythm of expansion and concentration. Now we tested if PDF might influence this daily fluctuations the same way as it does in crustaceans<sup>64</sup>. Therefore different concentrations of PDF were injected into adult worms and the

iridocytes were monitored before and afterwards. Altogether different time points and concentrations were tested to find the optimal conditions to which the pigments might react more strongly.

Although some worms showed a small increase after injection, no significant change could be detected when compared to the control injected worms. This does not necessarily mean that PDF is not involved in pigment dispersion, as the peptide might be degraded or the concentration might be too low to cause any effect. Moreover the solution was injected into the coelom of worms, so we cannot tell if it is taken up by the blood system and gets eventually transported to the pigments. Worms have a closed blood system that might not take up the injected solution completely. Therefore only a small amount might reach the pigments in the end, which might not be enough to cause any changes. Maybe a different kind of injection setup would help to inject into the blood system as the one we used is not accurate enough to hit the blood vessels.

When injecting into worms I also noticed that only a part of the injected solution stays inside of the worm, which could also influence the outcome. Furthermore different time points of injection should be tested and we also don't know how soon after injection the pigments might react to it to the peptide, hence this should also be further investigated.

### ***Disruption of the circadian clock influences pigmentation***

The circadian clocks consist of several interlocked feedback loops that drive rhythmic expression of clock genes<sup>42–44</sup>. Previous work in our lab has shown that the casein kinase I  $\delta/\epsilon$  inhibitor (PF 670462) successfully dampens the cycling of the clock genes (Juliane Zantke, Enrique Arboleda, personal communication).

As the injection experiment didn't prove that PDF is the factor that controls pigmentation, further experiments were performed to find out if the chromatophores might be influenced by the circadian clock at all. This showed that the pigmentation of the worms strongly reacts to the casein kinase I  $\delta/\epsilon$  inhibitor. As they were monitored

every day, one could see that the iridocytes behaved differently each subsequent day after drug exposure. However, head and body pigmentation did not always respond the same way to the drug, with the head pigmentation being sometimes more contracted or dispersed compared to the tail. This indicates that they might be regulated independently from each other. To prove this, decapitated worms should be exposed to the drug, but this is already subject of ongoing studies.

After four days in drug – when there should be no more cycling of the clock – the usually expanded pigments at 3 pm were smaller and the usually contracted pigments at 3 am increased in size, which is true for tail as well as head pigmentation (Figure 21 and 22). This could reflect that the cycling of the circadian clock dampens, as the pigmentation of both time points seems to come to the same level. However, a different set of worms was used to measure both time points. Therefore it is still unclear if the pigmentation doesn't change throughout the whole day and to confirm this, the same set of worms should be used for all analysed time points.

But still these results are a first indication that the pigmentation is under control of the circadian clock and is not only light regulated. Nonetheless, the factor that regulates it is still unknown and needs further investigation.

Summarizing, the role of PDF in *Platynereis* is still not fully understood and further experiments have to be performed to shed more light into its function and regulation. There is good evidence that it is involved in the circadian rhythm. However, these first experiments are just the beginning of uncovering possible functions of this peptide.

What would also be interesting is to find out if PDF can influence the behavior of worms, as it has been shown to do in *Drosophila*<sup>173</sup>. Hence, we first have to find out more about the natural behavior, which has not been studied so far. This could then give more information about the function of PDF in *Platynereis*.

## 5. Materials and Methods

### 5.1 *Platynereis dumerilii* culture

*Platynereis dumerilii* cultures have been held in laboratory since 1953 starting with C. Hauenschild and the cultures in our lab are derived from these original breedings<sup>2</sup>.

Animals were kept in transparent plastic boxes in a 1:1 mixture of natural sea water (from the North Sea) and artificial salt water in a shelving system in 2 separate rooms with inverted lunar cycle. The temperature in these rooms is held at a constant level of 18 °C and a light-dark cycle of 16 hours darkness and 8 hours light is kept. This means that the light is turned on between 7 am and 11 pm. To imitate full moon conditions a light bulb of lower intensity is turned on for 7 days during the night after 21 days of normal light-dark conditions. Worms were fed two times a week with algae (*Platymonas*) and spinach leaves.

To invert worms for experiments they were put in boxes with an inverted light-dark cycle for 4 to 7 days.

### 5.2 Cloning and subcloning

#### 5.2.1 RNA extraction and cDNA synthesis

RNA of *Platynereis* larvae (2d, 3d, 5d old) or *Platynereis* tails was extracted using the QIAGEN RNeasy Mini Kit following the protocol “Purification of Total RNA from Animal Tissues” using a tissue homogenizer (QIAGEN, Tissue LyzerII; 20U/sec for 30 min) to disrupt the tissue. The RNA was eluted in 30 µl of RNase-free water and subsequently the concentration was measured with a spectrophotometer NanoDrop (Thermo Scientific, USA). RNA was stored at -80 °C.

Afterwards cDNA was generated using the Roche Transcriptor High Fidelity cDNA synthesis kit. Both random hexamers and oligodT primers were used to synthesize

complementary cDNA. The resulting cDNA was then used to clone the MelR2 candidate and to extend the PDFR.

### 5.2.2 PCRs

For all PCRs performed the Thermal Cycle Quattro Chassi from VWR was used. Several polymerases were used with their according buffers and protocols for different purposes. The concentration of dNTPs in H<sub>2</sub>O and of the specific primer was always 10 mM each. The calculation of primer melting temperatures was done using OligoCalc (Oligo Calculator version 3.26, last modified by WAKibbe 07/15/2010).

Cloning of the pdu-melR2-candidate was accomplished using following PCR reagents and programme. As template served 1-7 days old *Platynereis* larvae.

#### PCR reagents:

|                 |                                                          |
|-----------------|----------------------------------------------------------|
| 0,25 µl         | Phusion High Fidelity DNA Polymerase (Thermo Scientific) |
| 5 µl            | 5x Phusion HF Buffer                                     |
| 0,5 µl          | primer #1444 (10mM)                                      |
| 0,5 µl          | primer #1445 (10mM)                                      |
| 3 µl            | cDNA (pdu larvae)                                        |
| 0,5 µl          | dNTPs                                                    |
| <u>15,25 µl</u> | <u>dH<sub>2</sub>O</u>                                   |
| 25 µl           | total                                                    |

#### PCR programme:

|              |                           |                              |
|--------------|---------------------------|------------------------------|
| 98 °C 2 min. | 98 °C 15 sec Denaturation | 72 °C 5 min final Elongation |
|              | 82 °C 1 min Annealing     |                              |
|              | 72 °C 50 sec Elongation   |                              |
|              | 37x                       |                              |

The *Platynereis* MelR2 candidate was afterwards subcloned in the mammalian expression vector pcDNA3.1(+) to use it for cell culture assays. Therefore the vector

was cut with the restriction enzyme *SacI* (NEB), gel purified and the previously cloned *MelR2* was ligated into it.

Genomic PCRs were performed to extend PDF.

PCR reagents:

|                 |                                                          |
|-----------------|----------------------------------------------------------|
| 0,25 µl         | Phusion High Fidelity DNA Polymerase (Thermo Scientific) |
| 5 µl            | 5x Phusion HF Buffer                                     |
| 0,5 µl          | primer #1404 (10mM)                                      |
| 0,5 µl          | primer #1405 (10mM)                                      |
| 1 µl            | genomic DNA (pdu larvae)                                 |
| 0,5 µl          | dNTPs (10mM)                                             |
| <u>17,25 µl</u> | <u>dH<sub>2</sub>O</u>                                   |
| 25 µl           | total                                                    |

PCR programmes:

|              |                           |                              |
|--------------|---------------------------|------------------------------|
| 98 °C 2 min. | 98 °C 15 sec Denaturation | 72 °C 3 min final Elongation |
|              | 64 °C 1 min Annealing     |                              |
|              | 72 °C 5 min Elongation    |                              |
|              | 35x                       |                              |

|              |                                   |                              |
|--------------|-----------------------------------|------------------------------|
| 98 °C 2 min. | 98 °C 15 sec Denaturation         | 72 °C 5 min final Elongation |
|              | 55-62 °C gradient 1 min Annealing |                              |
|              | 72 °C 5 min Elongation            |                              |
|              | 35x                               |                              |

### 5.2.3 Gel electrophoresis and gel extraction

The resulting PCR product was afterwards loaded on a 1-2 % TAE-agarose-gel and ran at a constant voltage of 100 V for at least 1 hour together with the 2-log DNA ladder (New England Biolabs) as size marker. Afterwards the gels were stained for about 30 min. in an Ethidium Bromide bath (1:10000 dilution in 1xTAE) to check if the amplified product had the right size.

If this could be confirmed the remaining product was loaded on a gel containing SYBRSafe (Invitrogen; 1:10000 dilution in 1xTAE) and the band of the right size was cut out. Subsequently the DNA was extracted using the QIAquick Gel Extraction Kit (QIAGEN) and eluted in 30 µl ddH<sub>2</sub>O.

#### **5.2.4 Ligation and transformation**

Depending on the Polymerases used in the PCR different cloning vectors were used. PCR products with a PolyA overhang were cloned using the pGEM®-T Easy Vector Systems Kit (Promega) following the “Protocol for Ligations Using the pGEM®-T and pGEM®-T Easy Vectors and the 2X Rapid Ligation Buffer” from the pGEM®-T and pGEM®-T Easy Vector Systems Technical Manual TM042. If the PCR generated blunt-end products the CloneJET™ PCR Cloning Kit (Fermentas) was used and the overall reaction volume was halved to 10 µl.

The ligation reaction was then transformed into competent *E. coli* cells (XL1-blue, selfmade by lab technicians). Therefore 50 µl cells were thawed up on ice, 5 µl ligation mix added and the cells incubated on ice for 30 minutes. After a heat shock at 42 °C for 30 seconds the cells were placed on ice for 2 minutes. 200 µl SOC medium was added and the cells were shaken at 350 rpm for 50 minutes to 1.5 hours at 37 °C. Transformed *E. coli* cells were then streaked out on LB plates with the right selective antibiotics. Usually ampicillin (1:1000) was used for selecting positive clones.

#### **5.2.5 Restriction digests**

To determine if the vectors have the right insert the plasmids were digested using either restriction enzymes from Fermentas or New England Biolabs (NEB) with the corresponding buffers. For the pJET 1.2 blunt vector a digest with BglII (NEB) was done to cut out the insert and for the pGEM®-T Easy vector EcoRI (NEB or Fermentas) was used.

Usually single digests were performed:

|               |                    |
|---------------|--------------------|
| 6,5 µl        | ddH <sub>2</sub> O |
| 1 µl          | 10xBuffer          |
| 2 µl          | plasmid (~1 µg)    |
| <u>0,5 µl</u> | <u>enzyme</u>      |
| 10 µl         | total              |

Depending on the enzymes also 1 µl BSA (NEB 1:10 diluted in ddH<sub>2</sub>O) was added to the reaction. If a double digest was required 0,5 µl of a different enzyme was added and only 6 µl ddH<sub>2</sub>O.

### 5.2.6 Sequencing

Clones with insert of the right size were sent for sequencing (100 ng/µl plasmid concentration) to LGC genomics.

## 5.3 Mini-/Maxiprep

For a standard Miniprep with the alkaline lysis method the common lab protocol (see Appendix, chapter 7.6) was used. The QIAGEN Plasmid Maxi Kit together with QIAfilter Maxi Cartridges were used following the protocol “Protocol: Plasmid or Cosmid DNA Purification Using QIAfilter Plasmid Midi and Maxi Kits” from the QIAfilter Plasmid Purification Handbook.

To receive endotoxin free DNA plasmids for cell culture assays the protocol “Protocol: Plasmid or Cosmid DNA Purification Using the EndoFree Plasmid Maxi Kit” from the Endofree Plasmid Purification Handbook using the EndoFree Plasmid Buffer Set was used.

## 5.4 Smart RACE (Rapid Amplification of cDNA Ends)

For obtaining the 5' end of the candidate gene (pdf) 5'-RACE-PCR was performed. Therefore larval RNA (2d, 3d, 5d old embryos) from the previous RNA extraction was used (described in chapter 5.2.1) to generate 5'-RACE-Ready cDNA.

Subsequently, 5'-RACE-Ready cDNA was generated using the SMART RACE cDNA Amplification Kit (Clontech) following the user manual "VII. First-Strand cDNA Synthesis".

Nested gene-specific primer (NGSP) and gene specific primer (GSP) were designed using the webtool PRIMER3 (<http://frodo.wi.mit.edu/>, Rosen and Skaletsky, 2000) following the SMART RACE User Manual.

### PCR reagents

|          |                                      |
|----------|--------------------------------------|
| 5 µl     | 10xBuffer (QIAGEN)                   |
| 1 µl     | dNTPs (10mM)                         |
| 0,25 µl  | HotStar Taq Plus Polymerase (QIAGEN) |
| 5 µl     | Universal Primer Mix (UPM, Clontech) |
| 1 µl     | GSP (10 µM) # 1842                   |
| 3 µl     | 5'-RACE-Ready cDNA                   |
| 37,75 µl | ddH <sub>2</sub> O                   |
| 50 µl    | total                                |

For the nested PCR 1 µl of the first PCR reaction was used as a template, 5 µl of Nested Universal Primer (NUP, 10 µM, # 93) instead of UPM and 1 µl NGSP (10 µM, # 1841) instead of the GSP.

### PCR programme:

|             |                             |                             |                                  |
|-------------|-----------------------------|-----------------------------|----------------------------------|
| 95 °C 5 min | 94 °C 1 min<br>Denaturation | 94 °C 1 min<br>Denaturation | 72 °C 10 min final<br>Elongation |
|             | 65 °C 30 sec Annealing      | 69 °C 30 sec Annealing      |                                  |
|             | 72 °C 2 min Elongation      | 72 °C 2 min Elongation      |                                  |
|             | 5x (precycle)               | 25x                         |                                  |

### 5.4.1 Southern blot and colony lift

After loading an aliquot of the PCR products onto a 1 % agarose gel, a southern blot was performed. The DNA was blotted overnight on a nylon membrane (PALL) using the "Dry Blot" technique. First, the gel was put into a denaturation solution (0.5M NaOH, 1.5M NaCl) for 30 min., then the gel was turned upside down (with the slots facing down) onto a clean surface and a nylon membrane was carefully put on the gel. On top of this membrane 4 layers of Whatman blotting paper and multiple layers of paper towels were placed. For a better transfer weight was put on top.

The next day the positions of the loading slots were marked with a pencil and the DNA was UV-crosslinked to the membrane (UV Stratalinker, STRATAGENE, set to autocrosslink) while the membrane was still wet.

For the radioactive hybridisation of the membranes a probe of 160 bp was generated.

#### PCR reagents

|              |                                                            |
|--------------|------------------------------------------------------------|
| 2,5 µl       | 10xBuffer                                                  |
| 1,5 µl       | MgCl <sub>2</sub> (25 mM)                                  |
| 1 µl         | primer # 1804                                              |
| 1 µl         | primer #1844                                               |
| 0,5 µl       | dNTPs (10 mM)                                              |
| 0,5 µl       | FirePol Polymerase (Solis Biodyne)                         |
| 1 µl         | template (pJET 1.2 blunt with PDFR fragment 1:10 dilution) |
| <u>17 µl</u> | <u>ddH<sub>2</sub>O</u>                                    |
| 25 µl        | total                                                      |

#### PCR programme:

|             |                           |                              |
|-------------|---------------------------|------------------------------|
| 94 °C 2 min | 94 °C 30 sec Denaturation | 72 °C 5 min final elongation |
|             | 59 °C 1 min Annealing     |                              |
|             | 72 °C 30 sec Elongation   |                              |
|             | 35x                       |                              |

The RadPrime labelling kit (Invitrogen) was used to generate the radioactive probe containing  $^{32}\text{P}$  labelled dCTP which will be used in later steps to visualize fragments that contain hopefully longer regions towards the 5' end of the gene. This probe was then purified (ProbeQuant G-50 Micro Columns, GE Healthcare) according to the manual and afterwards hybridized with the blotting membrane (Rapid-hyb Buffer, GE Healthcare). All these steps were performed by Florian Raible.

Bands which gave a strong radioactive signal on the nylon membrane after hybridization were cut out from the gel and subsequently cloned into the pGEM<sup>®</sup>-T Easy vector and transformed into XL1-blue cells (as described in chapter 5.2.4) or TOP10 chemical competent cells (Invitrogen) where I didn't get colonies with the XL1-blue cells. The bacteria were grown on selective LB+amp plates at 37 °C over night.

A colony lift was then performed to identify clones that carry the right fragment. Therefore a membrane (OPTITRAN, BA-S 85, Whatman) was carefully put onto the plate (to avoid smearing of the colonies) with edges cut out to mark the orientation. This membrane (colonies upwards) was transferred to 3 different membranes soaked with denaturation solution (0.5M NaOH, 1.5M NaCl; 5 min.), neutralisation solution (1.5M NaCl, 1M Tris-HCL; 5 min.) and 2xSSC for 2 minutes. To permanently bind the DNA, the membrane was UV-crosslinked and then air dried. The plates were afterwards put on 37 °C for 3 hours to regrow the colonies.

The same radioactive probe was used again to identify positive clones on the plate. As soon as they were identified, colonies were picked, prepped and the vectors digested using the restriction enzymes EcoRI-HF (NEB) to cut out the insert and HinfI (NEB) together with EcoRI-HF to compare the restriction patterns of the different clones. Clones with the right insert size were sent for sequencing.

## 5.5 BAC library screening

To identify regulatory sequences of the candidate genes PDF, PDFR, MelR1 and MelR2 Bacterial Artificial Chromosomes (BACs) containing the genomic DNA of *Platynereis dumerilii* were screened.

### 5.5.1 BAC filter hybridization

Filters spotted with BAC clones were hybridized with radiolabelled probes of MelR1, MelR2, PDF and PDFR to find out which of the BAC clones contain sequences of the candidate genes. The RadPrime labelling kit (Invitrogen) was used to generate the radioactive probes also used later (see SMART RACE, chapter 5.4) and the radioactive work was performed by Florian Raible.

As the BAC clones are spotted in duplicates in a certain pattern on the filter you can identify the ones that contain the sequences of the candidate genes as double signals by putting a grid on the filter.

A total of 28 BAC clones were ordered. For MelR1 18 clones (CH305-50G7, CH305-58I11, CH305-59I11, CH305-67P14, CH305-90E15, CH305-92K2, CH305-94B18, CH305-96O3, CH305-103O1,8 CH305-120A5, CH305-129G17, CH305-134K16, CH305-135K13, CH305-135L16, CH305-141J23, CH305-205A10, CH305-205F21, CH305-221K4), for MelR2 7 clones (CH305-27M12, CH305-96H16, CH305-104J4, CH305-117A8, CH305-181C10, CH305-185B17, CH305-187I15), for PDF 1 clone (CH305-167K21) and for PDFR 2 clones (CH305-53K17, CH305-91N19) were further analyzed.

### 5.5.2 Probe generation

**MelR1 probe:** A restriction digest with EcoRI was performed to cut out the MelR1 fragment from the vector (pGEM®-T Easy #786). This was then run on a 1 % agarose gel, cut out and gel eluted and eventually used for radioactive labeling.

**MelR2 probe:** For the MelR2 probe generation see Cloning and subcloning (Chapter 5.2).

### **PDFR probe**

#### PCR reagents:

0,25 µl Phusion High Fidelity DNA Polymerase (Thermo Scientific)  
5 µl 5x Phusion HF Buffer  
0,5 µl primer #1443/1149 (10mM)  
0,5 µl primer #1151 (10mM)  
3 µl cDNA (pdu larvae)  
0,5 µl dNTPs (10mM)  
15,25 µl ddH<sub>2</sub>O  
25 µl total

#### PCR programme:

|              |                           |                              |
|--------------|---------------------------|------------------------------|
| 98 °C 2 min. | 98 °C 15 sec Denaturation | 72 °C 3 min final Elongation |
|              | 63 °C 1 min Annealing     |                              |
|              | 72 °C 5 sec Elongation    |                              |
|              | 35x                       |                              |

### **PDF probe**

#### PCR reagents

2,5 µl 10xBuffer  
1,5 µl MgCl<sub>2</sub> (25 mM)  
1 µl primer # 1443  
1 µl primer #1442  
0,5 µl dNTPs (10 mM)  
0,5 µl FirePol Polymerase (Solis Biodyne)  
1 µl template plasmid #699 (pGEM®-T Easy with PDF fragment 1:10000 dilution)  
17 µl ddH<sub>2</sub>O  
25 µl total

PCR programme

|             |                           |                              |
|-------------|---------------------------|------------------------------|
| 94 °C 2 min | 94 °C 30 sec Denaturation | 72 °C 5 min final elongation |
|             | 65 °C 30 sec Annealing    |                              |
|             | 72 °C 1 min Elongation    |                              |
|             | 35x                       |                              |

**5.5.3 BAC purification**

Single colonies were produced by streaking out the clones on LB plates containing chloramphenicol (12,5 µg/ml). Afterwards three colonies were picked per plate and a BAC Miniprep was performed of the 28 ordered clones following the common lab protocol “BAC purification, version: FR 09/09” (Appendix, chapter 7.7). For diagnostic analysis of the clones a single digest using EcoRI-HF (NEB) and HindIII (NEB) and a double digest using both enzymes was performed and subsequently loaded on a 0,8 % TAE-agarose gel.

**5.5.4 Southern blot**

The DNA was blotted overnight on a nylon membrane as described in chapter 5.4.1 and the membrane was afterwards hybridized with radioactive probes, which was done by Florian Raible or Benjamin Backfisch.

BAC clones CH305-92K2, CH305-135K13 (MeIR), CH305-53K17 (PDFR), CH305-185B17 (MeIR2) were sent for sequencing to LGC genomics.

## 5.6 Quantitative Real-Time PCR (qRT PCR)

### 5.6.1 RNA extraction and cDNA synthesis

To analyse the effect of PDF injection on clock genes, the head of premature adult worms was cut off at a certain time point after injection and the tails (without jaw) were frozen immediately in liquid nitrogen. For each time point 3 biological replicates were collected, meaning one tail represented one biological replicate. Two technical replicates of each biological replicate were run on the same plate.

The same was done when analyzing *pdf* expression in the tails, but only 2 time points were chosen and 4 biological replicates per time point were taken.

RNA was extracted using the QIAGEN RNeasy Mini Kit following the protocol using the metal bead homogenization protocol and the Tissue LyzerII (Quiagen) at a frequency of 20 for 3 minutes as described in chapter 5.2.1. The RNA was stored at -80 °C until usage. cDNA was synthesized using the Quantitect Reverse Transcription kit (QIAGEN) following the protocol from Julianne Zantke and Enrique Arboleda (Appendix, chapter 7.8). 0,4 µg RNA served as a template for the cDNA generation and the resulting cDNA was diluted in 30 µl RNase-free water.

### 5.6.2 qRT-PCR reaction

Immediately after cDNA synthesis the qRT-PCR reaction (see Appendix, chapter 7.6) was set up consisting of a SYBRGreen PCR MasterMix (Applied Biosystems), forward and reverse primer (final concentration of 0,5 pmol/µl) and RNase-free water. 15 µl of this mastermix was applied in each well of the plate and afterwards 5 µl of cDNA was added and the plate carefully sealed. Subsequently the reaction was started using the StepOnePlus Real Time System (Applied Biosystems).

A complete run for 2 hours (10 min at 95°C; 40 cycles of (15 sec at 95°C and for 1 min at 60°C)) was performed usually including a melting curve analysis. The housekeeping gene *cdc5* served for normalization and as an internal control. Microsoft Excel 2007

was used to further analyse the obtained data and to calculate standard error and standard deviation.

To find out the statistical significance p-values of an unpaired student's t-test were calculated.

## **5.7 Mammalian cell culture**

COS-7 cells were grown under sterile conditions at 37 °C and 5 % CO<sub>2</sub>. As cultivation medium DMEM High glucose with L-Glutamine (PAA Cell Culture Company) containing 10 % fetal calve serum, Penicillin (100 U/ml) and Streptomycin (100 µg/ml) were used. To passage the cells medium was removed, cells washed with sterile 1xPBS and detached from the plate by adding 1 ml Trypsin-EDTA (PAA Cell Culture Company) per 10 cm cell culture dish for several minutes. After this the cells were resuspended in prewarmed full DMEM medium and split in the appropriate ratio.

### **5.7.1 Thawing of cells**

COS-7 passage number 3 cells were thawed quickly in the waterbath at 37 °C and immediately resuspended in 9 ml warm DMEM. This was followed by centrifugation of the cells at 1200 rpm for 5 minutes. Medium was removed, the pellet resuspended in warm DMEM and the suspension transferred to a 10 cm tissue culture dish.

### **5.7.2 Counting of cells**

A Neubauer chamber was used for counting the cells. 12 µl trypsinized cells, resuspended in DMEM were mixed with 12 µl of trypan blue solution and the cell number was determined using a light microscope.

### 5.7.3 Transfection optimization

The transfection efficiency was analysed using the Zytofluorometer FACSCalibur (BD BioSciences). COS-7 cells were grown on a 24 well plate at different concentrations, transfected on the second day with EGFP tagged oxytocin receptor and analysed via FACS on the third. Untransfected cells were used for gating.

| LT1:DNA<br>(transf.<br>efficiency) | LT1:DNA<br>(transf.<br>efficiency) | LT1:DNA<br>(transf.<br>efficiency) | LT1:DNA<br>(transf.<br>efficiency) | LT1:DNA<br>(transf.<br>efficiency) | LT1:DNA<br>(transf.<br>efficiency) |
|------------------------------------|------------------------------------|------------------------------------|------------------------------------|------------------------------------|------------------------------------|
| 2:1 (52 %)                         | 2:1 (51%)                          | 2:1 (52 %)                         | 6:2 (43 %)                         | 6:2 (50 %)                         | 6:2 (49 %)                         |
| 3:1 (50 %)                         | 3:1 (56 %)                         | 3:1 (56 %)                         | 8:2 (51 %)                         | 8:2 (52 %)                         | 8:2 (49 %)                         |
| 4:1 (47 %)                         | 4:1 (54 %)                         | 4:1 (57 %)                         | 3:2 (51 %)                         | 3:2 (55 %)                         | 3:2 (53 %)                         |
| 4:2 (50 %)                         | 4:2 (54 %)                         | 4:2 (55 %)                         | negative                           | negative                           | negative                           |
| 5,8x10 <sup>4</sup> cells/ml       | 11,5x10 <sup>4</sup> cells/ml      | 17,3x10 <sup>4</sup> cells/ml      | 5,8x10 <sup>4</sup> cells/ml       | 11,5x10 <sup>4</sup> cells/ml      | 17,3x10 <sup>4</sup> cells/ml      |

**Table 3: Transfection optimization of COS-7 cells.** Different cell concentrations and different ratios of LT1 to DNA were tested to improve efficiency.

## 5.8 Impedance assay

To measure the activation of the pdu-MelR-candidates by melatonin the xCELLigence Real-Time Cell Analyzer (RTCA) SP (Roche) was used. For each condition 4 technical replicates were analysed.

### 5.8.1 Day1

On the first day 50 µl COS-7 cells at a certain concentration were seeded on an E-96 well plate (Roche Applied Science). Prior to seeding, the background was measured by applying 50 µl full DMEM to the plate.

### 5.8.2 Day2

18-24 hours after seeding, some cells were transfected with pdu-MelR1 candidate, pdu-MelR2 candidate (both codon-optimized for expression in mammalian cells), Mm-MtnR1A (as positive control) and Oxytocin receptor pEGFP-N3. As a transfection reagent *TransIT*<sup>®</sup>-LT1 Transfection Reagent (Mirus) was used in a ratio of 3:1 (TransIT-LT1:DNA). 0,3 µl/well of transfection reagent and 10 µl/well OptiMEM (Invitrogen) were mixed and 0,1 µg DNA was added. After incubating this solution for 15 minutes 10 µl were carefully added to each well.

### 5.8.3 Day3

On the last day of the experiment (24-72h after transfection) 90 µl melatonin (Bachem, 5g Lot-No. 1019057, dissolved in PBS) in different concentrations or just PBS was added to the wells while the plate was still in the RTCA SP Station to measure changes immediately when adding the compound.

When the reaction was finished the data was analyzed using the RTCA Software version 1.2.1.1 (ACEA Biosciences Inc.).

## 5.9 Immunostaining

### 5.9.1 Antibodies

The PDF antibodies were produced by the PRIMM company against the amidated C-terminus of PDF (nh<sub>2</sub>-NPGTLDAVLDPDLMSL-coNH) conjugated to the carrier protein ovalbumin.

As the rabbit 2 antibody gave the best results in previous testing done in the lab only this one was used in my experiments in a 1:500 dilution.

To localize r-opsin expressing cells in transgenic animals (r-opsin fused to GFP) and to see if PDF and r-opsin are co-expressed three different GFP antibodies were tested. As the mouse5G4 anti-GFP antibody (antibody facility in house) worked best, this was used for the double staining (1:75 diluted). To visualize the axonal projections, dendrites etc. a mouse anti-acetylated tubulin (AT) was used in a 1:200 dilution. All antibodies used (primary and secondary) were diluted in 2,5 % sheep serum.

| Specificity       | Species | Dilution |
|-------------------|---------|----------|
| $\alpha$ -PDF     | Rabbit  | 1:500    |
| $\alpha$ -GFP-5G4 | Mouse   | 1:75     |
| $\alpha$ -GFP-2B6 | Mouse   | 1:75     |
| $\alpha$ -GFP     | Rabbit  | 1:5000   |
| $\alpha$ -AT      | Mouse   | 1:200    |

Table 4: Primary antibodies used during the thesis.

### 5.9.2 Antibody staining procedure

The immunostainings were performed according to our lab protocol “*Platynereis dumerilii* Immunostaining Protocol” (Appendix, chapter 7.4) with slight changes. As the PDF antibody was shown to work best with animals fixed in 4 % PFA and methanol (MeOH) this fixation method was chosen (see WMISH, Appendix, chapter 7.5). Only for r-opsin (anti-GFP antibody) and PDF double staining the Bouins fixative was selected. Embryos were fixed with Bouins fixative for ~20 minutes and afterwards washed with 1xPTW several times, whereas adult worms were fixed for 2 hours at 4 °C.

#### Bouins fixative:

| Substance         | ratio |
|-------------------|-------|
| Pricric acid      | 15    |
| 35 % formaldehyde | 5     |
| Acetic acid       | 1     |

Worms fixed in PFA+MeOH were rehydrated in 75 % MeOH, 50 % MeOH and 25 % MeOH for ~ 5 minutes before washing 2 times in 1xPTW. The proteinaseK digestion

time used for adult worms was 3 minutes. As secondary antibody to detect PDF either Alexa Fluor 488 goat anti-rabbit or Cy3 goat anti-rabbit (both Invitrogen) were used.

Samples were stored and mounted in Glycerol with DABCO (250 mg DABCO dissolved in 10 ml PBS and 90 ml Glycerol).

## **5.10 Whole Mount In Situ Hybridisation (WMISH)**

WMISH was carried out following the common lab protocol “*Platynereis* whole-mount in situ hybridization, June 2011” using the new staining protocol with staining buffer 1 and 2 (see Appendix, chapter 7.5).

If crystals appeared during the staining procedure the staining buffer was exchanged. The reaction was stopped by washing the larvae or worms several times in 1xPTW. Afterwards they were stored in Glycerol + DABCO and analyzed using the Axioplan microscope (Zeiss).

### **5.10.1 Antisense probe generation**

DIG-labeled antisense probes were generated from vectors containing the gene of interest using either SP6 or T7 promoters following the protocol (Appendix, chapter 7.5) and afterwards stored in Hyb-buffer at -20 °C.

## **5.11 Drug treatment**

To see if the pigmentation is controlled by the circadian clock, worms were put into NSW containing PF 670462 drug (Tocris, 800µM final concentration). This drug is a selective casein kinase 1ε (CK1ε) and CK1 δ inhibitor, therefore blocking PER protein nuclear translocation. Pictures of worms were taken before drug exposure and for 4 days in drug using the Lumar microscope (Zeiss) at a 40x magnification.

## 5.12 Injection of immature worms

### 5.12.1 Anaesthetizing animals

To stun worms prior to injection or later observation with the microscope they were put into NSW mixed 1:1 with 75 %  $\text{MgCl}_2$  for 15 to 30 minutes.

### 5.12.2 Injection capillaries

As injection capillaries served self pulled needles usually used for injecting zebrafish eggs (Science products GB 100, TF-10: 0,78x1.00x100mm with filament). They were pulled using the micropipette puller (Sutter instruments: model P 97; Flaming/Brown micropipette puller) at following parameters: Heat = 520, Pull = 90, Vel. = 80, Time = 160.

After loading the needles with a microloader pipette tip (Eppendorf) they were opened using forceps.

### 5.12.3 Injection setup

- Zeiss Stereo CL 1500 EW
- Eppendorf InjectMan NI2
- Eppendorf FemtoJet express

Anaesthetized worms were put on a glass slide and injected with 5  $\mu\text{l}$  PDF (+TRITC) or PBS (+TRITC). PDF peptide (amidated form, Bachem AG, Lot-No.: 4068586, H-Asn-Pro-Gly-Thr-Leu-Asp-Ala-Val-Leu-Asp-Met-Pro-Asp-Leu-Met-Ser-Leu-NH<sub>2</sub> acetate salt) was injected at different concentrations (800 nM or 1 mM) dissolved in sterile PBS. The injection station was set to manual with a constant pressure of 0 Pa, and an injection pressure of 800 Pa.

When the injection was finished worms were put back into NSW to recover and wrapped in aluminum foil to keep them in darkness until later observation with the Lumar microscope (Zeiss).

### **5.13 Quantification of pigments**

After taking pictures of the chromatophores of adult worms with the Lumar microscope (Zeiss) the iridocytes of certain segments or of the head were quantified using Photoshop CS5 (Adobe). Always the same setup and magnification was used for quantifying pigments.

### **5.14 Microscopy**

#### **5.14.1 Fluorescence microscopy and bright light microscopy**

Images were taken using an Axioplan 2 microscope (Zeiss) with the Axiovision software (AxioVS40 V4.8.1.0, Carl Zeiss Imaging Solutions). Embryos and adult worms were therefore mounted in 87 % glycerol plus DABCO to avoid bleaching.

The Lumar microscope (Zeiss) was used to analyse the pigmentation changes. Microscope pictures were processed either using ImageJ or Photoshop CS5 (Adobe).

#### **5.14.2 Confocal Laser Scanning Microscope (CLSM)**

In some cases the Zeiss LSM-510 Meta confocal microscope was used with a 40x oil objective together with the Zeiss LSM software to detect fluorescence antibody staining. To avoid bleaching adult worms were mounted in 87 % glycerol plus DABCO. Pictures were processed using ImageJ.

### **5.15 Sequence analysis**

For analyzing sequences CLC Bio Main workbench (v6) was used and blast search was performed using the web-interface on NCBI (<http://www.ncbi.nlm.nih.gov/>).

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## 7. Appendix

### 7.1 Primers

Primers were ordered by Sigma-Aldrich.

| ID # | Name                            | Sequence (5' -> 3')         | Length (bp) | Organism    |
|------|---------------------------------|-----------------------------|-------------|-------------|
| 1    | <b>pduc5_L1</b>                 | CCTATTACATGGACGAAGATG       | 29          | Platynereis |
| 2    | <b>pduc5_R1</b>                 | TTCCCTGTGTGTTTCGCAAG        | 20          | Platynereis |
| 140  | <b>pJETreverse</b>              | AAGAACATCGATTTTCCATGGCAG    | 24          | pJET blunt  |
| 141  | <b>pJETforward</b>              | CGACTCACTATAGGGAGAGCGGC     | 23          | pJET blunt  |
| 246  | <b>Pdu_period_L2</b>            | GGTCAACATGAAGTCGTACAGG      | 22          | Platynereis |
| 247  | <b>Pdu_period_R2</b>            | CACTGGTTTTTCGGCTCAAG        | 19          | Platynereis |
| 270  | <b>Pdu_cryvbtL3</b>             | TGTTCAAATTCATGGGACA         | 20          | Platynereis |
| 271  | <b>Pdu_vbtcryR3</b>             | TGTTTTAGCCTCAGCCATT         | 20          | Platynereis |
| 913  | <b>Pdu_MelR_lo</b>              | CAAAACAAACGGAATGGATGATC     | 23          | Platynereis |
| 924  | <b>Pdu_MelR seq</b>             | GGCATGCATGACCTCTTG          | 18          | Platynereis |
| 1050 | <b>pdu_pdfR_race_up2</b>        | CAGTCTTGGTGTATTCCACCATTGTC  | 26          | Platynereis |
| 1050 | <b>pdu_pdfR_race_up2</b>        | CAGTCTTGGTGTATTCCACCATTGTC  | 26          | Platynereis |
| 1051 | <b>pdu_pdfR_race_up3_</b>       | CTTTAGTACTTCACATAATTGGAG    | 26          | Platynereis |
| 1149 | <b>GSP2_3'RACE_pdfR_720bp</b>   | CCGGATGCATCGGAATTTGTTCTGGC  | 28          | Platynereis |
| 1151 | <b>GSP1_5'RACE_pdfR_720bp</b>   | GCCGTGAAGAAGCAGTAGAGCAGAG   | 27          | Platynereis |
| 1841 | <b>Pdfr_RACE_GSP_lo2</b>        | CGGCCAACATGTCTTGAAGTCTCAGC  | 25          | Platynereis |
| 1842 | <b>Pdfr_RACE_lo1</b>            | CTGGCCGAGAGTAGTCCCAGGTTC    | 25          | Platynereis |
| 1844 | <b>Pdfr_RACE_GSP_lo3</b>        | TTAGTGTCCAACCCGTGTAGAGGAG   | 25          | Platynereis |
| 1152 | <b>NGSP1_5'RACE_pdfR_720bp</b>  | CTTGGAAGGAGGCTAGGAAGTGTGAG  | 27          | Platynereis |
| 1171 | <b>pdu_melR_qPCR_4_forward</b>  | GTTGGTAATTGCAGCACTGG        | 20          | Platynereis |
| 1172 | <b>pdu_melR_qPCR_4_reverse</b>  | AAGAACAAAAATATTTCCAAGTCTC   | 26          | Platynereis |
| 1173 | <b>pdu_melR_qPCR_1_forward</b>  | TGATCTGGTTGTACGGCTTT        | 21          | Platynereis |
| 1174 | <b>pdu_melR_qPCR_1_reverse</b>  | AATGATGTCCCCTTGACTGC        | 20          | Platynereis |
| 1177 | <b>pdu_pdfR_qPCR_1_forward</b>  | TGCTGGTCACCAAGTTAAGAGA      | 22          | Platynereis |
| 1178 | <b>pdu_pdfR_qPCR_1_reverse</b>  | CGCCAAGTAACGAAGAAGA         | 20          | Platynereis |
| 1404 | <b>Pdu_pdf_genomic_test_lo</b>  | TTGCAGCATAGCGAAAATTTCTCG    | 25          | Platynereis |
| 1405 | <b>Pdu_pdf_genomic_test_up</b>  | ATAGTGAGTCGTATACGGCCGG      | 22          | Platynereis |
| 1441 | <b>Pdu_PdfR_up_VLYFFT</b>       | GTTCTGTATTTTTTACAAGGAGTCTAA | 32          | Platynereis |
| 1442 | <b>Pdu_Pdf_lo_212</b>           | TCTGTCTGCGTCAGTGAGTGTTC     | 26          | Platynereis |
| 1443 | <b>Pdu_Pdf_up_33</b>            | GAGAAAGTCAGCCAGTGTTCATC     | 25          | Platynereis |
| 1444 | <b>EcoRI_Kozak_Pdu_MelR2_AT</b> | TCAGAATTCGCCGCCACCATGGCAAAC | 46          | Platynereis |
| 1445 | <b>NotI_Pdu-melR2_TGA_lo</b>    | TATGCGGCCGCTCATACTGTGTCTCG  | 33          | Platynereis |
| 1781 | <b>Pdu_PdfR_test_up1</b>        | ATGCAGAAGCTTACAAATCCTCAACTG | 27          | Platynereis |
| 1782 | <b>Pdu_PdfR_test_up2</b>        | TATTGCAATGCCACATGGGACAAGG   | 25          | Platynereis |
| 1783 | <b>Pdu_PdfR_test_up3</b>        | ATGATCCACGTCATGATTCAACTC    | 24          | Platynereis |
| 1784 | <b>Pdu_PdfR_test_lo4</b>        | GGAAACAGTAGAGCAGAGCTATGAAG  | 26          | Platynereis |

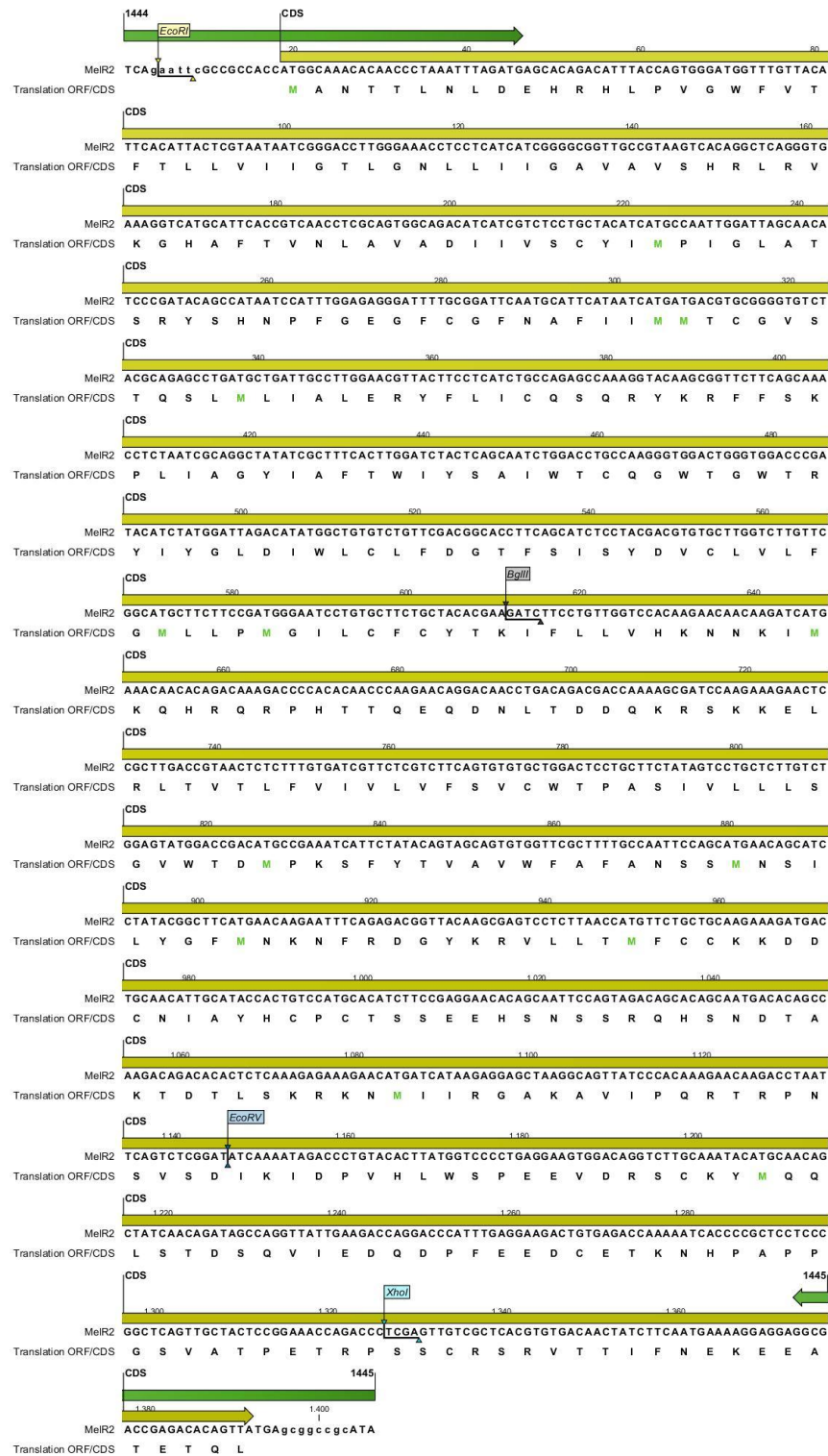
|      |                                |                               |    |             |
|------|--------------------------------|-------------------------------|----|-------------|
| 1785 | <b>Pdu_PdfR_test_lo3</b>       | CTGGTGAGTGGGCACTTCCG          | 20 | Platynereis |
| 1786 | <b>Pdu_PdfR_test_lo2</b>       | ACGTTGGTCACAAGGGCGTCTCAC      | 24 | Platynereis |
| 1787 | <b>Pdu_PdfR_test_lo1</b>       | CATTATCGGTCTTCTGTCCCTATG      | 24 | Platynereis |
| 1803 | <b>Pdu_PdfR_test_up_0a</b>     | CACTTTTTCATCTCACTTGCTCAACTGAT | 29 | Platynereis |
| 1804 | <b>Pdu_PdfR_test_up_0b</b>     | GCAGAAAGGCCCATCTGGGGTC        | 22 | Platynereis |
| 1805 | <b>Pdu_PdfR_test_up_0c</b>     | CTGGAGGACTGCATCAAGTTCTTGG     | 25 | Platynereis |
| 1833 | <b>Pdu-pdp1R3</b>              | TCCTCCTTAACTCCACAGTCTTC       | 24 | Platynereis |
| 1834 | <b>Pdu-pdp1L3</b>              | GAAACTGCGTCTCGAAATGA          | 20 | Platynereis |
| 1841 | <b>Pdfr_RACE_GSP_lo2</b>       | CGGCCAACATGTCTTGAAACTCAGC     | 25 | Platynereis |
| 1842 | <b>Pdfr_RACE_lo1</b>           | CTGGCCGAGAGTAGTCCCCAGGTTC     | 25 | Platynereis |
| 1177 | <b>pdu_pdfR_qPCR_1_forward</b> | TGCTGGTCACCAAGTTAAGAGA        | 22 | Platynereis |
| 1178 | <b>pdu_pdfR_qPCR_1_reverse</b> | CGCCAAGTAACGGAAGAAGA          | 20 | Platynereis |

**Table 5: Primer list.** Sequence, internal identification number and length of all primers used.

## 7.2 Sequences and sequence analysis

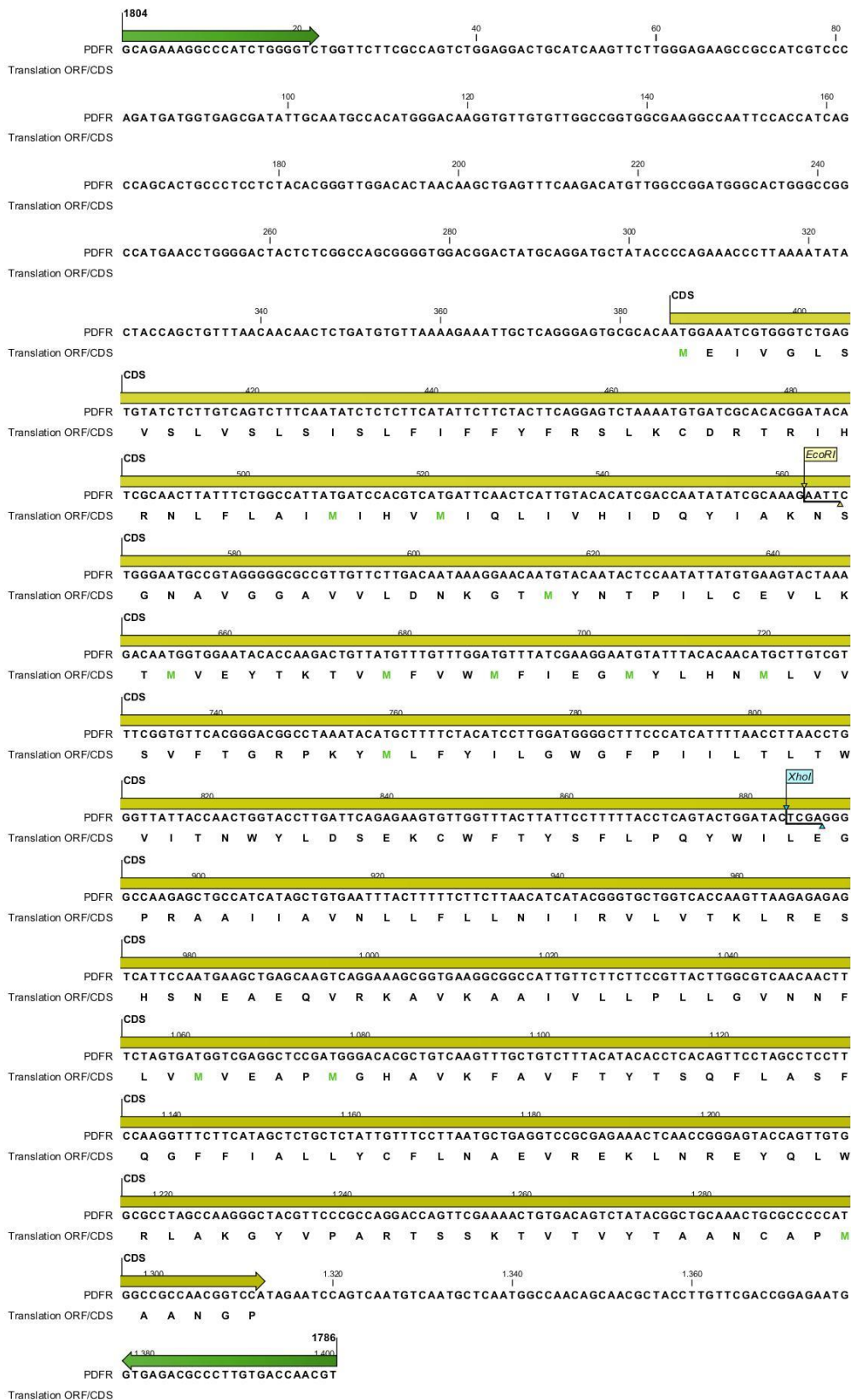
### Pdu\_MelR2:

length: 1.406 bp



Pdu\_PDFR

elongated sequence: 1.401 bp





**Figure 24: PDFR elongated sequences mapped to BAC 53K17. Analysis done by F. Raible. Green parts correspond to exons of elongated sequence.**

## Alignment of the elongated PDFR sequence onto the BAC 53K17

### Output Sim 4

(<http://pbil.univ-lyon1.fr/members/duret/cours/inserm210604/exercise4/sim4.html>)

```
seq1 = PDFR elongated sequence, 1401 bp
seq2 = genomic sequence of PDFR on BAC (Seq2), 45021 bp

>PDFR elongated sequence (1401 nucleotides)
>genomic sequence of PDFR on BAC (45021 nucleotides)

1-94 (935-1027) 98% ->
95-200 (12199-12304) 100% ->
201-352 (19287-19438) 100% ->
353-456 (20176-20279) 100% ->
457-625 (23000-23168) 100% ->
626-782 (23278-23434) 98% ->
783-843 (24660-24720) 91% ->
844-913 (27747-27816) 98% ->
914-999 (27897-27982) 97% ->
1000-1180 (30671-30851) 93% ->
1181-1307 (31947-32073) 99% ->
1308-1401 (33273-33366) 100%

      0      .      :      .      :      .      :      .      :
      1 GCAGAAAGGCCCATCTGGGGTCTGGTTCTTCGCCAGTCTGGAGGACTGCA
      |||||
     935 GCAGAAAGGCCCATCTGGGGTCTGGTTCTTCGCCAGTCTGGAGGACTGCA

      50      .      :      .      :      .      :      .      :
     51 TCAAGTTCTTGGGAGAAGCCGCCATCGTCCCAGATGATGGTGAG
      |||||
     985 TCAAGTTCTTGGGAGAAGCCGCCATCGTCCCAGATGATGGTGA GTA...

     100      .      :      .      :      .      :      .      :
     95  CGATATTGCAATGCCACATGGGACAAGGTGTTGTGTGGCCGGTGGC
      >>>|||||
    12196 GAGCGATATTGCAATGCCACATGGGACAAGGTGTTGTGTGGCCGGTGGC

     150      .      :      .      :      .      :      .      :
    142 GAAGGCCAATTCCACCATCAGCCAGCACTGCCCTCCTCTACACGGGTGG
      |||||
   12246 GAAGGCCAATTCCACCATCAGCCAGCACTGCCCTCCTCTACACGGGTGG

     200      .      :      .      :      .      :      .      :
    192 ACACTAACA AGCTGAGTTTCAAGACATGTTGGCCGGATGGG
      |||||>>>...>>>|||||
   12296 ACACTAACAGTA...CAGAGCTGAGTTTCAAGACATGTTGGCCGGATGGG

     250      .      :      .      :      .      :      .      :
    233 CACTGGGCGGCCATGAACCTGGGGACTACTCTCGGCCAGCGGGGTGGAC
      |||||
   19319 CACTGGGCGGCCATGAACCTGGGGACTACTCTCGGCCAGCGGGGTGGAC

     300      .      :      .      :      .      :      .      :
    283 GGACTATGCAGGATGCTATACCCAGAAACCCTTAAAAATATACTACCAGC
      |||||
   19369 GGACTATGCAGGATGCTATACCCAGAAACCCTTAAAAATATACTACCAGC

     350      .      :      .      :      .      :      .      :
    333 TGTTTAACAACAACCTCTGAT GTGTAAAAAGAAATGCTCAG
      |||||>>>...>>>|||||
   19419 TGTTTAACAACAACCTCTGATGTA...CAGGTGTAAAAAGAAATGCTCAG

     400      .      :      .      :      .      :      .      :
    374 GGAGTGCGCACAATGGAAATCGTGGGTCTGAGTGTATCTCTTGTCAGTCT
      |||||
   20197 GGAGTGCGCACAATGGAAATCGTGGGTCTGAGTGTATCTCTTGTCAGTCT

     450      .      :      .      :      .      :      .      :
```

```

424 TTCAATATCTCTCTTCATATTCTTCTACTTCAG          GAGTCTAA
|||||>>>...>>>|||||
20247 TTCAATATCTCTCTTCATATTCTTCTACTTCAGGTG...AAGGAGTCTAA

500 . : . : . : . : . :
465 AATGTGATCGCACACGGATACATCGCAACTTATTTCTGGCCATTATGATC
|||||
23008 AATGTGATCGCACACGGATACATCGCAACTTATTTCTGGCCATTATGATC

550 . : . : . : . : . :
515 CACGTCATGATTCAACTCATTGTACACATCGACCAATATATCGCAAAGAA
|||||
23058 CACGTCATGATTCAACTCATTGTACACATCGACCAATATATCGCAAAGAA

600 . : . : . : . : . :
565 TTCTGGGAATGCCGTAGGGGGCGCCGTTGTTCTTGACAATAAAGGAACAA
|||||
23108 TTCTGGGAATGCCGTAGGGGGCGCCGTTGTTCTTGACAATAAAGGAACAA

650 . : . : . : . : . :
615 TGTACAATACT          CCAATATTATGTGAAGTACTAAAGACAATG
|||||>>>...>>>|||||
23158 TGTACAATACTGTG...CAGCCAATATTATGTGAAGTACTAAAGACAATG

700 . : . : . : . : . :
656 GTGGAATACACCAAGACTGTTATGTTTGGTTGGATGTTTATCGAAGGAAT
|||||
23308 GTGGAATACACCAAGACTGTTATGTTTGGTTGGATGTTTATCGAAGGAAT

750 . : . : . : . : . :
706 GTATTTACACAACATGCTTGTCTGTTTCGGTGTTCACGGGACGGCCTAAAT
|||||
23358 GTATTTACACAATATGCTTGTCTGTTTCGGTGTTCACGGGACGGCCAAAAT

800 . : . : . : . : . :
756 ACATGCTTTTCTACATCCTTGATGCG          GCTTTCCCATCATT
|||||>>>...>>>|||||
23408 ACATGCTTTTCTACATCCTTGATGCGGTG...CAGGCTTTCCCATCATT

850 . : . : . : . : . :
797 TTAACCTTAACCTGGGTTATTACCAACTGGTACCTTGATTACAGAGAA
|||||>>>
24674 TTAACCTTAACCTGGGCTATTACCAACTGGTTCCTTGATTACAGAGTA

900 . : . : . : . : . :
844          GTGTGGTTTACTTATTCCTTTTACCTCAGTACTGGATACTCG
...>>>|||||
24724 ...CAGGTGTTGGTTTACTTATACCTTTTACCTCAGTACTGGATACTCG

950 . : . : . : . : . :
888 AGGGGCCAAGAGCTGCCATCATAGCT          GTGAATTTACTTTTT
|||||>>>...>>>|||||
27791 AGGGGCCAAGAGCTGCCATCATAGCTGTA...CAGGTGAATTTATTTTT

1000 . : . : . : . : . :
929 CTTCTTAACATCATACGGGTGCTGGTCACCAAGTTAAGAGAGAGTCATT
|||||
27912 CTTCTTAACATCATACGGGTGCTGGTCACCAAGTTAAGAGAGAGTCATT

1050 . : . : . : . : . :
979 CAATGAAGCTGAGCAAGTCAG          GAAAGCGGTGAAGGCGGCCA
|||||>>>...>>>|||||
27962 CAATGAAGCTGAGCAAGTTAGGTA...CAGGAAAGCGGTGAAGGCGGCCA

1100 . : . : . : . : . :
1020 TTGTTCTTCTTCCGTTACTTGGCGTCAACAACCTTCTAGTGATGGTCGAG
|||||
30691 TTGTTCTTCTTCCGTTGCTTGGCGTCAACAACCTTCTAGTGATGGTCGAC

1150 . : . : . : . : . :
1070 GCTCCGATGGGACACGCTGTCAAGTTTGCTGTCTTTACATACACCTCACA
| | |||||
30741 CTTAAGCTGGGACACGCTGTCAAGTTTGCTGTCTATATGTACACCTCAA

```

```

1200      .      :      .      :      .      :      .      :
1120 GTTCCTAGCCTCCTTCCAAGGTTTCTTCATAGCTCTGCTCTATGTTTCC
      |||||
30791 GTTCCTAGCCTCCTTCCAAGGTTTCTTCATAGCTCTGCTCTACTGTTTCC

1250      .      :      .      :      .      :      .      :
1170 TTAATGCTGAG      GTCCGCGAGAACTCAACCGGGAGTACCAG
      |||||>>>...>>>|||||
30841 TTAATGCTGAGGTA...TAGGTCCGCGAGAACTCAACCGGGAGTACCAG

1300      .      :      .      :      .      :      .      :
1211 TTGTGGCGCCTAGCCAAGGGCTACGTTCCCGCCAGGACCAGTTCGAAAAC
      |||||
31977 TTGTGGCGCCTAGCCAAGGGTTACGTTCCCGCCAGGACCAGTTCGAAAAC

1350      .      :      .      :      .      :      .      :
1261 TGTGACAGTCTATACGGCTGCAAACCTGCGCCCCATGGCCGCCAACG
      |||||>>>
32027 TGTGACAGTCTATACGGCTGCAAACCTGCGCCCCATGGCCGCCAACGGCG

1400      .      :      .      :      .      :      .      :
1308      GTCCATAGAATCCAGTCAATGTCAATGCTCAATGGCCAACAGCA
      ...>>>|||||
32077 ...TAGGTCCATAGAATCCAGTCAATGTCAATGCTCAATGGCCAACAGCA

1450      .      :      .      :      .      :      .      :
1352 ACGCTACCTTGTTTCGACCGGAGAATGGTGAGACGCCCTTGTGACCAACGT
      |||||
33317 ACGCTACCTTGTTTCGACCGGAGAATGGTGAGACGCCCTTGTGACCAACGT

```

EXONS

```

> 935 33366 Seq1
935 1027
12199 12304
19287 19438
20176 20279
23000 23168
23278 23434
24660 24720
27747 27816
27897 27982
30671 30851
31947 32073
33273 33366

```

### 7.3 Counted PDF positive middle cells

#### PDF positive middle cells counted at 4 pm

##### Worm Nr1

| Segment-NR | Number of cells | Average: 5,6 |
|------------|-----------------|--------------|
| 5          | 6               |              |
| 6          | 6               |              |
| 7          | 4               |              |
| 8          | 7               |              |
| 9          | 5               |              |
| 10         | 0               |              |

##### Worm Nr2

| Segment-NR | Number of cells | Average: 5,5 |
|------------|-----------------|--------------|
| 5          | 7               |              |
| 6          | 4               |              |
| 7          | 4               |              |
| 8          | 6               |              |
| 9          | 5               |              |
| 10         | 7               |              |

##### Worm Nr3

| Segment-NR | Number of cells | Average: 5,1 |
|------------|-----------------|--------------|
| 5          | 5               |              |
| 6          | 5               |              |
| 7          | 7,              |              |
| 8          | 4               |              |
| 9          | 6               |              |
| 10         | 4               |              |

##### Worm Nr 4

| Segment-NR | Number of cells | Average: 4,8 |
|------------|-----------------|--------------|
| 5          | 4               |              |
| 6          | 5               |              |
| 7          | 4               |              |
| 8          | 7               |              |
| 9          | 4               |              |
| 10         | 5               |              |

##### Worm Nr 5

| Segment-NR | Number of cells | Average: 4,8 |
|------------|-----------------|--------------|
| 5          | 6               |              |
| 6          | 5               |              |
| 7          | 5               |              |
| 8          | 6               |              |
| 9          | 4               |              |
| 10         | 3               |              |

Average number of PDF cells (middle): 5,16

**PDF positive middle cells counted at 4 am**

## Worm 1

| Segment-NR | Number of cells | Average: 4,8 |
|------------|-----------------|--------------|
| 5          | 4               |              |
| 6          | 5               |              |
| 7          | 4               |              |
| 8          | 6               |              |
| 9          | 6               |              |
| 10         | 4               |              |

## Worm 2

| Segment-NR | Number of cells | Average: 4 |
|------------|-----------------|------------|
| 5          | 4               |            |
| 6          | 4               |            |
| 7          | 5               |            |
| 8          | 4-              |            |
| 9          | 4               |            |
| 10         | 4               |            |

## Worm 3

| Segment-NR | Number of cells | Average: 3,5 |
|------------|-----------------|--------------|
| 5          | 3               |              |
| 6          | 4               |              |
| 7          | 3               |              |
| 8          | 4               |              |
| 9          | 4               |              |
| 10         | 3               |              |

## Worm 4

| Segment-NR | Number of cells | Average: 0,5 |
|------------|-----------------|--------------|
| 5          | 0               |              |
| 6          | 0               |              |
| 7          | 0               |              |
| 8          | 0               |              |
| 9          | 2               |              |
| 10         | (1)             |              |

## Worm 5

| Segment-NR | Number of cells | Average: 1,2 |
|------------|-----------------|--------------|
| 5          | 2               |              |
| 6          | 0               |              |
| 7          | 2               |              |
| 8          | 0               |              |
| 9          | 0               |              |
| 10         | 3               |              |

## Worm 6

| Segment-NR | Number of cells | Average: 2,2 |
|------------|-----------------|--------------|
| 5          | 3               |              |
| 6          | 3               |              |
| 7          | 3               |              |
| 8          | 2               |              |
| 9          | 2               |              |
| 10         | 0               |              |

Average number of PDF cells (middle): 2,7

## 7.4 *Platynereis dumerilii* immunostaining protocol

(Tessmar/Raible lab)

### Reagents:

**2xPTW:** dilute 10x PBS to 2x PBS, add Tween20 to 0.1%.

**16% PFA (common stock!):** dissolve 16% paraformaldehyde in water by stirring and heating to 65°C, add dropwise 1 M NaOH until the solution gets clear (check pH  $\approx$  7.5), cool to room temperature and store at 4°C.

**4% PFA/PTW (to prepare):** For 10ml, mix 2,5ml 16%PFA with 7,5ml 2xPTW.

### Procedure:

#### PFA Fixation:

- Pour larvae batch through big filter.
- Wash with sea water (keeping the embryos wet - in cup with NSW!!).
- With a pipette, transfer them to a small filter, invert it upon an Epqi and rinse with 4%PFA/PTW into 2ml tube.

**Note:** Use 2x PTW + PFA for the fixation!

- Put on shaker for 2hrs at room temperature.
- Wash 5 x 5 min at room temperature, on shaker.
- Store at 4°C.

**Note:** The storage of fixed larvae for immunostaining should not be longer than 3 days.

### Proteinase digestion

#### Reagents:

**ProteinaseK:** 20 mg/ml Stock

#### Reagents you have to prepare before you start:

- **Proteinase K Solution:** 150µl ProtK in 40ml PTW
- **2mg/ml Glycine/PTW:** 80mg/40ml PTW

#### Procedure:

- All steps are performed at room temperature, in hand-made nets submerged into 40ml volumes (lids of pipette boxes).
- Note:** If you put the embryos onto a shaker, don't put more than 32ml (embryos could fall out of the nets).
- Transfer larvae of each different stage into nets submerged in fresh 1x PTW – rinse 2 x 5 min (transfer the nets between boxes).
- digest with ProteinaseK, 150µl in 40ml PTW, without shaking for several minutes depending on the stage of the embryos (**see below**).
- rinse 2 x shortly in freshly prepared 2 mg/ml glycine/PTW (also in boxes).
- wash 5 x 5 min in PTW – transfer nets in boxes (on shaker).

**Comments:** the ProteinaseK-digestion is a critical step of this protocol and will substantially influence the quality of the whole procedure, thus, times of digestion have to be optimized. The following list of digestion times worked well in our hands and can be used as guideline.

| stage           | Time of ProteinaseK digestion |
|-----------------|-------------------------------|
| 1h-15h          | use 'early WMISH-protocol'!   |
| 16h-72h         | 45'' - 1 min                  |
| 72h-5d          | 1 min 30 sec                  |
| 1week to 6weeks | 2 min 30 sec                  |

**Blocking****Reagents:**

Sheep serum

1xPTW

**Procedure:**

- Using a 1ml pipette, transfer the larvae in 1.5 or 2 ml Eppendorf tubes. Label the tubes, indicating the stage of the embryos. Let them sink (it takes maximum 5 minutes) and aspirate the PTW out.
- Immediately, add 1ml of sheep serum 5%. Leave them shaking at room temperature for 1 hour.
- Meanwhile, start preparing the tubes and antibody dilutions for the next step.

**Primary Antibody incubation and wash****Reagents:**

Pre-Immune serum (rabbit 1)

Primary antibody: anti c-Op sin2 (rabbit 1)

Primary antibody: anti acetylated tubulin

Sheep serum

1xPTW

**Procedure:****Preparation of the antibody dilutions**

You will incubate the larvae in:

- Pre-immune serum (PIS): dilution 1:50 in a total volume of 200µl containing 2.5% sheep serum.
- 2 primary antibody dilutions: anti-c-Op sin2 (dilution 1:50) and anti-acetylated tubulin (dilution 1:200), both in 200 µl containing 2.5% sheep serum.
- Label the tubes you will use during the procedure and after it, to keep the primary antibodies for further uses.
- Prepare the dilutions you will use.

**After the blocking step: Antibody incubation**

- With a pipette, mix the embryos in the same tubes where they are, so they are more or less equally distributed in the 5% sheep serum.
- Immediately, distribute them among the empty labeled Eppis you will use. Try to add similar amounts of embryos in the different tubes.
- Let them sink. Aspirate as much as you can the remaining sheep serum. Immediately, add the corresponding AB or PIS dilution from the Eppi you already prepared.

- Let them incubate during 1 hour at room temperature, shaking. (Alternatively: overnight at 4°C, shaking).
- Let them sink. With a pipette, aspirate as much liquid as you can. (NOTE: The antibody solutions can be re-used, so KEEP the liquid you remove in the empty tubes you already labelled. Store them at 4°C)
- Immediately, add 1ml of PTW in each Eppi. Do 5 x 10 min washes. On shaker.
- Meanwhile, start preparing the secondary antibodies dilutions for the next step.

### **Secondary Antibody incubation and wash**

#### **Reagents:**

Secondary antibody: Alexa Fluor 488 goat anti-rabbit  
Secondary antibody: Cy3 goat anti-mouse  
DAPI (4',6-diamidino-2-phenylindole)  
2.5% sheep serum  
1xPTW

#### **Procedure:**

You will incubate the larvae in 2 secondary antibody dilutions: Alexa Fluor 488 goat antirabbit (dilution 1:200) and Cy3 goat anti-mouse (dilution 1:200), both in a volume of 200µl containing 2.5% sheep serum. It will also contain DAPI (300 ng/ml).  
Mix everything and wrap the tube in aluminium paper.

- After the last wash of the previous step, add 200µl of your secondary antibodies solution in each tube.
- Incubate during 1 hour at room temperature, shaking. (Alternatively: overnight at 4°C, shaking). Keep your samples covered (darkness).
- Let them sink, and aspirate as much liquid as you can. (You don't have to keep this solution).
- Immediately, add 1ml of PTW in each Eppi. Do 5 x 10 min washes. On shaker, covered.

### **Samples mount**

#### **Reagents:**

Glycerol  
ProLong Gold antifade reagent

#### **Procedure:**

- Mounting in glycerol, in 6-wells plates: add your larvae in PTW in a well, aspirate the PTW, cover the larvae with glycerol. Keep wrapped at 4°C. Label the plates!
- The day before doing the observation under the confocal microscope, mount in antifade solution:
  - o Prepare cover slides taped with 2-3 stripes of tape at the edges. (For fixed embryos, 2 tapes for animals up to 50hpf and 3 tapes for animals 50hpf to 5dpf usually work well).
  - o With a pipette, pick one animal from the ones you mounted in glycerol and place it on the middle of a slide.

**Note:** When choosing the animals, you can check under the fluorescence microscope which larvae have a better signal. Those will be the ones you will check under the confocal microscope.

- o Fill up space with some drops of antifade reagent, so that the embryos are spatially fixed.

- o Put one clean cover slip on top.
- o Embryos can be moved into different position for viewing by moving cover slip (under binocular). Under the slide, mark where is the animal located, so it will be easier to find.
- o Keep them covered (darkness) at 4°C.

## 7.5 *Platynereis* whole-mount in situ hybridization

Tessmar/Raible Lab version January 2012

(contributed by Benjamin Backfisch & Juliane Zantke)

Fixation and storage of embryos / adult worms

### **Reagents:**

**2x PTW:** dilute 10x PBS, pH7,5 to 2x PBS, add Tween20 to 0.1% and sterile filter

**16% PFA (common stock!):** dissolve 16% paraformaldehyde in water by stirring and heating to 65°C, add dropwise 1 M NaOH until the solution gets clear (check pH  $\approx$  7.5), cool to room temperature and store at 4°C (note from Medaka protocol: store for up to 4 weeks; if Medaka embryos do not stay clear but turn "milky" in MeOH prepare fresh 16% PFA stock).

**4% PFA/PTW:** For 10ml, mix 2,5ml 16%PFA with 7,5ml 2xPTW, just prior to use

### **Procedure:**

#### **PFA Fixation (*Platynereis*):**

- Pour embryo batch through big filter
- Wash with natural sea water (NSW)
- Transfer embryos to small net, invert it upon an Eppi and rinse with 4%PFA/PTW into 2ml tube

**Note:** Use 2x PTW + PFA for the fixation!

**Put on a shaker for 2hrs at room temperature (RT)**

#### **Methanol transfer:**

- wash **3 x 5 min** in **100% MeOH** at RT
- replace MeOH and store embryos prior use **at least over night at -20°C**

COMMENTS: MeOH treatment enhances probe and antibody penetration, longer washes in 100% MeOH at RT and longer storage of embryos /adult worms at -20°C in MEOH is advantageous.

## **RNA probe preparation**

### **Reagents (prepared):**

- **NTP-Mix:** ATP, CTP, GTP 15.4 mM each, UTP 10.0 mM (all Roche)
- **Digoxigenin-11-UTP** 10 mM (Roche)

-> **NTP/DIG-UTP-Mix: 1.3 vol NTP-Mix, 0.7 vol Dig-11-UTP**

- **Fluorescein-12-UTP** 10 mM (Roche)
- **RNasin** 20-40 U/μl (Promega, Pharmacia)
- **T7-/SP6-/T3-RNA-Polymerase** 20 U/μl (Roche)
- **5xTranscriptionbuffer** (Stratagene)
- **DNaseI** RNase-free 10U/μl (Roche)
- **STE:** 100 mM NaCl/20 mM TrisCl, pH 7.5/10 mM EDTA
- **NucTrap™ push-columns** (Stratagene) or **RNeasy** (Qiagen)

**Hybridization Mix-H2O:** prepared and stored at -20°C in ISH drawer  
**stock HYB-H2O**

- Formamide 100 % 5 ml  
SSC 20 x 2.5 ml  
Heparin 50 mg/ml 10 µl  
Torula-RNA (Sigma) solid 50 mg  
Tween20 10 % 10 µl
- **Nylon-membrane** (HybondN, Amersham)
  - **P1**: 100 mM TrisCl, pH 7.5/150 mM NaCl
  - **P1I**: PBS/0.1% TritonX-100
  - **P1II**: 100 mM TrisCl, pH 9.5/100 mM NaCl/50 mM MgCl<sub>2</sub>
  - **Anti-Digoxigenin-Fab fragments/Anti-Fluoresceine-Fab fragments** (Roche)
  - **BCIP** (Roche): 50 mg/ml in 100% DMF
  - **NBT** (Roche): 75 mg/ml in 70% DMF/H<sub>2</sub>O

**Procedure:**

- **linearize 1-2 µg of template** with a suitable enzyme allowing transcription (blunt or 5-prime overhang should be preferred to avoid snap back effects)
- **purify template** from enzyme and digestion buffer (QIAquick PCR purification kit, or use gel purification, followed by QIAquick gel extraction kit) elute in **50ul of H<sub>2</sub>O**
- **control for a complete digest on an 1% Agarose gel**
- add - **in the following order** – to a total volume of **20 µl** in a 1.5ml tube:

(DO NOT heat the components)

|                                     |            |
|-------------------------------------|------------|
| linearized template (0.5- 1 µg) max | 12.5µl     |
| 100 mM DTT                          | 2 µl       |
| NTP/ <b>DIG-UTP</b> -Mix            | 2 µl       |
| RNase inhibitor                     | 0.5 µl     |
| 10xTranscriptionbuffer              | 2 µl       |
| RNA-Polymerase                      | 1 µl       |
| H <sub>2</sub> O                    | add x µl   |
|                                     | 20µl total |

- incubate for **2-4 hours at 37°C**
- **add 1 µl DNaseI (RNase free)** and incubate for another **15 min at 37°C**
- store at -20°C or
- **purify RNA using the Qiagen RNeasy kit – RNA clean up**
- **elute in 50ul RNase free ddH<sub>2</sub>O**
- take an aliquot of **2 µl**, mix with **4µl RNA loading dye**, heat to **80°C for 1 minute**, snap cool, and load and run on a **1% Agarose/ TAE gel**
- **For Quality test: blot gel for 1 hr to a nylon-membrane (HybondN, Amersham)**
- dilute the remaining probe by **adding 75 µl Hyb-buffer and store at -20°C**, like this probes remain usable for several years

**OPTIONAL: Quality test:**

incorporation of DIG-/Fluorescein-UTP is controlled on the blotted membrane (above) or by 1µl aliquots of a dilution series (1:1, 1:10, 1:50, 1:100, 1:500) on a nylon-membrane (HybondN, Amersham)

- dried membrane are equilibrated for **1-2 min** in **P1**
- block membrane for **30 min** in **P2** by gentle shaking
- incubate membrane **30 min** in 1:2000 dilution of antibody (Anti-Digoxigenin or Anti-Fluoresceine respectively) in **P2** (≥ 0.2 ml/cm<sup>2</sup>)

- wash 2x15 min in **P1** ( $\geq 1$  ml/cm<sup>2</sup>)
- incubate membrane 2 min in **P3**
- stain membrane for 5 min in **4.5µl/ml NBT and 3.5µl/ml BCIP in P3**
- wash 1-2 min in **P2** and take picture
- a good probe should give a signal at 1:500 dilution after 2-3 min

**Comments:**

As a rule of the thumb 12µl of the probe in 200µl of Hyb-mix will give a good staining.

Probes from 600bp- 2.5 kb were used successfully with this protocol, short probes below 0.5 kb will generate only faint staining although this varies with the specificity of each probe and may be worth being tried.

**Proteinase digestion and postfixation**

**Reagents:**

- **ProteinaseK**: prepare a stock solution of **20 mg/ml in PBS** and store frozen aliquots at -20°C, dilute 1:200 just prior to use to a **final concentration of 100 µg/ml in PTW (use 150µl ProtK (20 mg/ml) in 30ml 1x PTW)**
- **Glycine stock (100x): 200mg/ml in PTW (50g/250ml) or freshly prepared 2mg/ml Glycine/PTW**
- **4% PFA see above**

**Comments: all steps are performed at RT**, in hand-made nets submerged into **40ml** volumes (lids of pipet boxes).

**Alternatively**, perform all steps in 6 well-plate, volumes of solutions per well are 3ml.

**Reagents you have to prepare before you start:**

- **75% MeOH** (30ml MeOH + 10ml PTW)
- **50% MeOH** (20ml MeOH + 20ml PTW)
- **25% MeOH** (10ml MeOH + 30ml PTW)
- **Proteinase K Solution**: 150µl ProtK in 30ml PTW (final concentration: 100µg/ml)
- **2mg/ml Glycine/PTW**: 80mg/40ml PTW
- **4% PFA/PTW** : 24ml PTW + 8ml 16%PFA

**Procedure:**

- transfer embryos of different stages **into nets submerged in 100% MeOH**

Make sure that embryos/ larvae are always submerged well.

- rehydrate 3 min in **75% MeOH/PTW**
- rehydrate 3 min in **50% MeOH/PTW**
- rehydrate 3 min in **25% MeOH/PTW**
- rinse 2 x 5 min each in fresh **1x PTW**
- digest with **ProteinaseK** (100µg/ml), without shaking for several minutes depending on the stage of the embryos (see below)
- rinse 2 x shortly in **freshly prepared 2 mg/ml glycine/PTW**
- fix in **4% PFA/PTW** for 20 min, shaking
- wash 5 x 5 min in **1x PTW**

**Comments:** the ProteinaseK-digestion is a **critical step** of this protocol and will substantially influence the quality of the whole mount in situ, thus, times of digestion have to be optimized. The following list of digestion times worked well in our hands and can be used as guideline.

| Stage of development          | Time of ProteinaseK digestion |
|-------------------------------|-------------------------------|
| 1h-15h                        | use 'early WMISH-protocol'!   |
| 16h-24h                       | 45 sec                        |
| 48h                           | 1 min                         |
| 72h                           | 1 min 30 sec                  |
| 4d-7d                         | 2 min                         |
| 8d to 6weeks and older        | 3 min                         |
| adult immature / mature heads | 5 min                         |

**Comments:****Proteinase digestion for adult tails:****Reagents and digestion time:**

- **ProteinaseK:** prepare a stock solution of **20 mg/ml in PBS** and store frozen aliquots at -20°C, dilute 1:2000 just prior to use to a **final concentration of 10 µg/ml in PTW (use 15µl ProtK (20 mg/ml) in 30ml 1x PTW)**
- **Digest for 10 min**
- **Glycine stock (100x): 200mg/ml in PTW (50g/250ml) or freshly prepared 2mg/ml Glycine/PTW**
- **4% PFA see above**

**Procedure:**

- see above Proteinase digestion and postfixation

**Comments: Heat treatment can be used as an alternative to Proteinase K treatment**

- rehydrate embryos
- transfer embryos to 1ml 1x PTW in glass scintillation vial
- heat waterbath to 100°C
- when boiling, turn off waterbath
- gentle agitate embryos and incubate in waterbath for 5 min
- gentle agitate every 5 min while incubating to avoid sticking together of the embryos
- incubate on ice until RT is reached

**Hybridization****Reagents (prepared):**

**Heparin:** make a stock of 50 mg/ml in H<sub>2</sub>O, store at -20°C

**Hybridization Mix:** 50% formamide (Fluka, ultra pure), 5xSSC, 50µg/ml heparin, 0.1%Tween20, 5 mg/ml torula RNA, store at -20°C, for 50 ml of Hyb-Mix:

|                    | <u>stock</u> | <u>Hyb-mix</u> |
|--------------------|--------------|----------------|
| Formamide          | 100 %        | 25 ml          |
| SSC                | 20 x         | 12.5 ml        |
| Heparin            | 50 mg/ml     | 50 µl          |
| Torula-RNA (Sigma) | solid        | 250 mg         |
| Tween20            | 10 %         | 500 µl         |
| H <sub>2</sub> O   | add          | 50 ml          |

**Procedure:**

all steps are performed in a water bath preheated to 65°C:

- transfer embryos to **2 ml safe lock** Eppendorf tubes
- let embryos settle down and **remove PTW** and **add 1 ml HybMix**
- **prehybridize 1-2 hours at 65°C**
- in between, **denature probe** (5-10 µl/200µl Hyb-Mix, as a rule of the thumb 8 µl of the probe will give a good staining) in 200 µl of Hyb-Mix for **10 min at 80°C**
- **remove pre hybridization** solution leaving embryos slightly covered to avoid their desiccation, the embryos are very sensitive at 65°C
- **quickly add 200µl of hybridization probe** and hybridize at 65°C **overnight**

Washes

**Reagents:**

**SSCT solutions:** dilute from 20xSSC, and add Tween20 to 0.1% (= 1:200)

**Formamide**

**Reagents to prepare:**

**50ml 4x SSCT** – from 20x SSC stock solution plus add 20%Tween to 0.1% (1:200)

**50% formamide/2xSSCT** – dilute 4x SSCT 1:1 with Formamide

**comments:** prepare 50% formamide/2xSSCT prior to use, remaining solution can be stored at – 20°C

**2x SSCT** – dilute from 4x SSCT

**0.2x SSCT** – dilute from 4x SSCT

**Procedure:**

all steps are performed in a water bath, all wash solutions are preheated to 65°C

- wash embryos **2 x 20 min in 1 ml 50% formamide/2xSSCT at 65°C**
- wash embryos **20 min in 1 ml 2xSSCT at 65°C**
- wash embryos **2 x each 20 min in 1 ml 0.2xSSCT at 65°C**

Antibody incubation

**Reagents to prepare:**

**5% sheep serum** in 1x PTW (Stock: 100% sheep serum)

**2.5% sheep serum** in 1x PTW

**ANTIBODY:** dilute **5% sheep serum** to **2.5%** and add the following amount of Ab:

|                                         |               |
|-----------------------------------------|---------------|
| <b>Anti-DIG-AP, Fab fragments</b>       | <b>1:2000</b> |
| Anti-fluo-AP, Fab fragments             | 1:1000        |
| Anti-acetylated tubulin, mAB (optional) | 1:200         |

**Procedure:**

- block embryos **1-2 hours with 1ml of 5% sheep serum at RT, shaking**
- incubate embryos in **200 µl of ANTIBODY (2.5% Sheep + Ab)** for **1-2 hrs at RT, shaking;** (alternatively, overnight at 4°C)

**Comments:** For adult worms it is recommended to incubate the antibody overnight at 4°C.

- **wash 5 x 10 min in PTW at RT**
- do last washing step over night at 4°C, (or directly continue protocol to step “Staining”)

Detection

**Reagents:**

**BCIP** (Boehringer): 50 mg/ml in 100% DMF

**NBT** (Boehringer): 75 mg/ml in 70% DMF/H<sub>2</sub>O

### NEW STAINING PROTOCOL

**Comments:**

The staining reaction can be speed up by the use of the following staining procedure

**Reagents to prepare:**

**Staining Buffer 1 (SB1)**, used for equilibration:

100 mM TrisCl, pH 9.5, 100 mM NaCl, 0.1% Tween20

|                  | <u>stock</u> | <u>1xSB</u>     | <u>1xSB</u>      |
|------------------|--------------|-----------------|------------------|
| TrisCl, pH 9.5   | 2 M          | 2.5 ml          | 5 ml             |
| NaCl             | 5 M          | 1 ml            | 2 ml             |
| Tween20          | 20 %         | 250 µl          | 500 µl           |
| H <sub>2</sub> O |              | <b>ad 50 ml</b> | <b>ad 100 ml</b> |

**Reagents to prepare:**

**Staining Buffer 2 (SB2)**, used for staining:

100 mM TrisCl, pH 9.5, 100 mM NaCl, 50 mM MgCl<sub>2</sub>, 0.1% Tween20, 10% PVA (Polyvinylalcohol) in H<sub>2</sub>O

|                   | <u>stock</u> | <u>1xSB</u>     | <u>1xSB</u>     |
|-------------------|--------------|-----------------|-----------------|
| TrisCl, pH 9.5    | 2 M          | 0.5 ml          | 1 ml            |
| NaCl              | 5 M          | 0.2 ml          | 0.4 ml          |
| MgCl <sub>2</sub> | 1M           | 0.5 ml          | 1 ml            |
| Tween20           | 20 %         | 50 µl           | 0.1 ml          |
| 10% PVA           |              | <b>ad 10 ml</b> | <b>ad 20 ml</b> |

5% PVA works as well and is less viscose.

**Preparation of 10% PVA:** use PVA powder (Sigma-Aldrich), heat while stirring at 80°C in a water bath (beaker filled with water on the thermo plate)

**SB2+NBT+BCIP:** to be added per 1 ml SB (= per sample)

|                                          |              |
|------------------------------------------|--------------|
| <b>NBT (75mg/ml; final: 337.5 µg/ml)</b> | <b>4.5µl</b> |
| <b>BCIP (50mg/ml; final: 175 µg/ml)</b>  | <b>3.5µl</b> |

**Procedure:**

- **equilibrate** embryos **2 x 5 min** in **SB1** in the tubes
- **prepare SB2+NBT+BCIP**, calculate the amount of staining solution you need (1ml/tube)
- apply **1ml** of **SB2+NBT+BCIP** into the wells of a **6-well-dish (one well per tube)**
- suck of as much **SB1** as possible, leaving embryos slightly covered
- transfer embryos to the wells already containing **SB2+NBT+BCIP**
- **stain in the dark without shaking** for up to 72 hrs

**Comments:** If the lab temperature rises above 25°C, staining in the incubator is recommended.

- check embryos for the first time after 30-45 min, but **do not expose embryos too long to light!** (check every 45-60 min)
- if necessary, replace solution with fresh staining solution **next morning**
- wash 3 x 5 min in PTW **to stop reaction**
- store at least **1hr in PTW/4%PFA** for postfixation
- **remove PTW/4%PFA**
- **add 2 ml of 87% glycerol**
- **leave in 87% glycerol** for at least **1-2 hrs** for complete equilibration

**Comments:** use 2.5mg/ml DABCO/glycerol for embryos counterstained with Acetylated  $\alpha$ -tubulin

- afterwards you can proceed to mounting and microscopy

## 7.6 Miniprep

(Tessmar/Raible lab)

- pour 2ml of culture into 2ml safe lock Eppendorf tube
- spin culture for 1 minutes at 13000rpm
- add 200 ul buffer P1 and resuspend
- add 200ul buffer P2, mix by inverting the tube 4-6x
- add 200ul buffer P3, mix by inverting the tube 4-6x
- spin 10 minutes at 13000rpm
- take supernatant to another tube
- add 450ul 2-Propanol, mix by inverting the tube
- spin for 10 minutes at 13000rpm
- wash pellet with 1ml 70% EtOH
- spin 5 minutes at 13000 rpm
- air dry and resuspend in 50ul H<sub>2</sub>O

### Resuspension buffer P1 (steril filter and store at 4°C) 1000 ml

- |                              |                         |
|------------------------------|-------------------------|
| - 10 mM Glucose              | 10ml of our 1M stock    |
| - 25mM Tris (pH 8.0) 12,5 ml | 25 ml of our 1M stock   |
| - 10 mM EDTA (pH 8.0) 10 ml  | 20 ml of our 0,5M stock |
| - add 100mg RNase A (1 vial) |                         |

### Alkaline Lysis buffer P2 (mix fresh of both components, use no longer than 1 month old)

- 0,2 M NaOH
- 1% SDS

### Neutralization buffer P3 (store at RT) 500 ml

- |                               |                                     |
|-------------------------------|-------------------------------------|
| -5M Potassium acetate         | 300ml from 5M stock                 |
| - Glacial acetic acid 57,5 ml | 57,5ml from 100 % stock             |
| - H <sub>2</sub> O            | 142,5ml of sterile H <sub>2</sub> O |

## 7.7 BAC miniprep protocol

(Tessmar/Raible lab version FR 09/09)

### Yield: 50-125 ug/3ml liquid culture

Solutions:

Chloramphenicol stock: 100mg/ml

P1, P2, P3: as for ordinary miniprep

2-Propanol

70% EtOH in H<sub>2</sub>O

### Procedure

**Overview:** *The procedure is reminiscent of an ordinary plasmid miniprep, but the samples need to be treated more carefully (no strong shaking, some longer incubations, incubation on ice etc.). The material obtained from 3 ml of liquid culture should suffice to run several digests for Southern analyses.*

1. Single out BAC clones and grow mini cultures
  - Using a sterile needle, scratch off some frozen material (try not to thaw plate or tube)
  - Stir in 20ul LB, transfer to LB plate with 12.5ug/ml Chloramphenicol (Cm)
  - Use sterile bacteria loop to streak out LB on selection plates (3-4 rounds)
  - Grow o/n
  - Pick 3 clones per plate
  - Grow o/n in 5ml LB containing 12.5ug/ml Cm
2. Harvest Bacteria
  - Pellet 2ml in tabletop centrifuge in 2ml tubes: 5000 rpm, (2700 rcf), 8min
  - Remove supernatant, add another 2ml culture, repeat
  - Note: temperature of this spin is not critical (RT or 4°C)
  - After removal of supernatant, pellets can be frozen in N<sub>2(l)</sub> and placed at -80°C for storage. Else, directly continue.
3. Release BAC DNA from bacteria
  - Resuspend pellet in 600ul of P1
  - Add 600ul P2, leave on RT for 5' (solution should clear)
  - Gently add 600ul P3, close tube, invert gently
  - Place tubes on ice for at least 5 minutes
  - Spin full speed for 10 minutes in a cooled centrifuge (4°C)
4. Precipitation of BAC DNA
  - While spinning, prepare – on ice – 2 Eppendorf tubes per sample. Each tube should contain 700ul 2-Propanol
  - Transfer spun tubes back on ice
  - Carefully transfer the supernatant of each sample to the two tubes (820 ul each). Avoid any white precipitate when transferring.
  - Right upon transfer, close each tube, gently invert. Precipitate the DNA by letting the tubes sit on ice for at least 5 min

- Alternatively (this has worked very well), leave the tubes at -20°C o/n

5. Cleanup of BAC DNA

- Spin first tube of each sample in cold centrifuge (4°C), full speed, 20 min
- Aspirate off supernatant, add content of second tube, repeat
- Aspirate off supernatant, add 1ml 70% EtOH
- Spin at RT in centrifuge for 20 min (full speed)
- Carefully aspirate off supernatant (pay attention not to suck off pellet, can get loose)
- Optional: repeat 70% EtOH wash
- Wait until pellets turn glassy
- Add 50ul EB (or TE)
- Dissolve at RT (let rest, tap occasionally, if you pipet, pipet very gently)
- Measure 1.5 ul on a Nanodrop to determine yield. I typically had 1.2-2.5 ug/ul

6. Diagnostic BAC digests

- For diagnostic digests with 6bp-cutters, use 0.8% Agarose gels and around 5ug DNA per lane. Reasoning: if the BAC insert size is around 130kB, an average 4kB band will show up as ca. 150ng (well visible), and a 400bp band will still be 15ng (just visible with EtBr)
- Maximize the amount of your digest you put on the gel. No need to set up 20ul reactions if you only load 5!
- When comparing different BACs with each other, use more than one enzyme and their combination to maximize the chance of finding differences.

- Example setup for digest:

|            | EcoRI (1x)  |                              | HindIII (1x)  |
|------------|-------------|------------------------------|---------------|
| BAC DNA    | 4.5ul       | DNA (4.5ul)                  | 4.5ul         |
| H2O        | 2.7ul       | Buffer component<br>(3.5ul)  | 2.7ul         |
| 10x buffer | 0.8ul       |                              | 0.8ul         |
| H2O        | 1.3ul       | Enzyme<br>component<br>(2ul) | 1.3ul         |
| 10x buffer | 0.2ul       |                              | 0.2ul         |
| Enzyme     | 0.5ul EcoRI |                              | 0.5ul HindIII |

- Note: After setting up, I pooled **for each BAC** 3.3ul EcoRI reaction with 3.3ul HindIII reaction. So, after all, there were **three 6.6ul reactions** (two single digests, one combined digest), each was fully loaded on a gel
- Also note that yellow and green components can be conveniently scaled up for multiple BACs. Green component (enzyme) is itself buffered to 1x reaction buffer, so that the enzyme is not harmed while you pipet the samples.
- After running the samples, the gels can be blotted and hybridized with gene-specific probes to reconstruct exon numbers and overlap between BACs.

## 7.8 *Platynereis* qPCR analysis of gene expression

(Tessmar/Raible lab version january 2012)

### Outline

- Primer design
- RNA extraction
- Reverse transcription
- Set up the SYBR green reaction Master Mix
- Analysis of qPCR data
- Testing qPCR primers

### *Platynereis* qPCR analysis of gene expression

(contributed by Enrique Arboleda & Juliane Zantke)

#### Primer Design

Theoretical Considerations:

**Primer size:** Between 18bp and 27bp, with an optimal of 20bp

**Melting Temperature (T<sub>m</sub>) of primers:** 59°C to 61°C, with and optimal of 60°C

**Amplicon Size:** Between 70bp and 150bp (primers included). We use the smallest possible.

**Note:** Smaller amplicons are more efficient in our PCR thermal profile because they are amplified quicker. Longer amplicons on the other hand can be useful to easily distinguish primer dimers by comparing melting curves (a ~40bp dimer will have a clearly different melting temperature than a ~150bp amplicon).

**Intron Spanning Primers:** When possible, design primers that span an intron. For this, the Forward primer is placed at the end of an exon and the Reverse primer at the beginning of the subsequent exon (*i.e.* spanning the intron in between).

**Comments:** Genomic DNA is considered a contaminant agent in a q-PCR reaction since it can be amplified and mask the signal coming from the cDNA amplification. Primers designed to span an intron will not produce a specific amplicon (because of the long intron in between), **BUT** DNA contamination will affect the efficiency of the reaction! Be clean and use negative controls.

**Primers complementary and self-complementary sequences:** Designed primers should not be complementary, as otherwise primer dimers will be synthesised preferentially to any other product. Self-complementary primer (ability to form secondary structures such as hairpins) should also be avoided.

Design Primers Online:

**We use the program primer3 through the web page of Roche Applied Science.** The default settings use the theoretical considerations mentioned before. However, they can be changed if necessary. A PDF with detailed information on the method and on Roche's web interface can be found at [https://www.roche-applied-science.com/sis/rtpcr/upl/upl\\_docs/universal\\_probelibrary.pdf](https://www.roche-applied-science.com/sis/rtpcr/upl/upl_docs/universal_probelibrary.pdf)

- Go to the web page <https://www.roche-applied-science.com>
  - Click on “PCR quantitative essays” at the bottom of the page.
  - Click on “Design real time PCR assays” on the right side of the page.
  - See the options under “select organism” and choose “Other Organism (paste sequence only)”
  - Alternatively (as long as this link is active) go to <https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=UP030000> and “select organism”

**Procedure:**

- Paste your sequence.
- **Option1:** If you are using a cDNA sequence, uncheck the “Automatically select an intron spanning assay” option.
- **Option2:** If you are using a genomic DNA sequence and you are unsure about intron/exon composition, leave the “Automatically select an intron spanning assay” option activated. **BUT** be careful if you find an intron! Remember this is just a probability essay and therefore check if the given intron is real (e.g. by PCR on cDNA and/or comparing to homologous sequences).
- **Option3:** If you know the intron/exon composition, choose the “Automatically select an intron spanning assay” option. To design intron-spanning primers, use opening and closing square brackets [ ] to indicate the position of exon-exon boundaries. The square brackets may be “empty” or contain a number to show intron size in the overview graphics, but must not contain characters (e.g. intron sequence information).
- The software will give you the best possible pair of primers. To see additional primers, click on “More essays” at the bottom of the page. Scroll down in the newly opened page and look for the top ranked primer-pairs.
- Test your primers before using them on the q-PCR assay! See the correspondent section on “Testing q-PCR Primers” ahead.

**Storage of Primer**

- Dilute primers (final concentration 10µM)
- Make aliquots and store them at –20°C
- Do not thaw primers more often than three times (affects the primer efficiency of the qPCR reaction)

**RNA extraction from frozen tissue (embryos, heads, tails...)**

**Reagents:**

**RNeasy Mini Kit**

**Metal beads and Tissue Lyser** for homogenization of tissue / **alternatively** you can use **plastic Pestles**

**80% Ethanol (to prepare)**

**Procedure:**

**RNA extraction using RNeasy Mini Kit (Animal Tissues protocol):**

**Metal bead homogenization protocol**

- Add **350µl Buffer RLT** containing **β-Mercaptoethanol** onto frozen tissue sample in 2ml tube containing one metal bead
- Homogenize tissue (**Tissue Lyser**) settings: **frequency 20, time 3min**
- Samples can be stored at -80C or continue with RNA extraction

Comments: For the extraction of larvae it is recommended, after homogenization, to centrifuge tubes at max speed for 2 min at RT. Non homogenized particles like chaetae or pharynx will form a pellet. Take the supernatant to fresh tube and continue extraction.

- Add **350µl of 80% Ethanol** to the sample, mix immediately by pipetting
- Apply the **700µl** of the sample to an RNeasy spin column, discard the metal bead
- Centrifuge for **15 s at 10 000 rpm**, discard the flow-through
- Add **700µl Buffer RWI**, centrifuge for 15 s, 10 000rpm, discard the flow-through
- Add **500µl Buffer RPE**, centrifuge for 15 s, 10 000rpm, discard the flow-through
- Add **500µl Buffer RPE**, centrifuge for 2 min, 10 000rpm, discard the flow-through
- **Elution step:** place RNeasy spin column in a new 1.5ml tube, add **30µl** of RNase-free water
- Centrifuge for **1 min** at 10 000 rpm
- Store RNA on ice
- Measure concentration on Nano Drop, proceed with cDNA synthesis or store RNA at -80°C

**Pestle homogenization protocol**

- Add **350µl Buffer RLT** containing **β-Mercaptoethanol** onto frozen tissue sample
- Disrupt the tissue and homogenize in Buffer RLT using a pestle
- Store sample on ice while homogenizing additional samples (note: **do not** extract more than 6 samples = 6 timepoints at once)
- Add **350µl of 80% Ethanol** to the sample, mix immediately by pipetting
- Continue with extraction protocol (see above)

**Reverse Transcription with Elimination of Genomic DNA for Quantitative Real-Time PCR**

Note: For detailed description please read the QuantiTect Reverse Transcription Handbook (Qiagen)

**Reagents (stored at -20°C) QuantiTect Reverse Transcription Kit (Quiagen):**

- **gDNA Wipeout Buffer, 7x**
- **Quantiscript Reverse Transcriptase** (store on -20°C block)
- **Quantiscript RT Buffer, 5x**
- **RT Primer Mix**
- **RNase-Free Water**

**Procedure:**

**Prepare the genomic DNA elimination reaction on ice according to Table 1**

**Note:** The protocol is for use with 10pg to 1µg RNA. A RNA concentration of 0.4µg works well for *Pdu* adult haeds as well as larval stages.

- **Calculate the amount of RNA** required for each sample (final concentration **0.4µg** total RNA)

- Add RNase-free water to a final volume of **12µl**
- Add **2µl gDNA Wipeout Buffer**

**Table1. Genomic DNA Elimination Reaction Components**

| Components              | Volume/ reaction |
|-------------------------|------------------|
| gDNA Wipeout Buffer, 7x | 2µl              |
| Template RNA            | 0,4µg            |
| RNase-free water        | variable         |
| <b>Total volume</b>     | <b>14µl</b>      |

- Incubate **6 min at 42°C** (thermo block), **place immediately on ice**
- Prepare the **reverse-transcription master-mix on ice** according to **Table 2**

**Table 2. Reverse-Transcription Reaction Components**

| Components                              | Volume/ reaction |
|-----------------------------------------|------------------|
| Reverse transcription master mix        |                  |
| Quantiscript Reverse Transcriptase      | 1µl              |
| Quantiscript RT Buffer, 5x              | 4µl              |
| RT Primer Mix                           | 1µl              |
| Template RNA                            |                  |
| Entire genomic DNA elimination reaction | 14µl             |
| <b>Total volume</b>                     | <b>20µl</b>      |

- Add 6µl of the master-mix to each reaction, mix
- Incubate **15 min** at 42°C (thermo block)
- Incubate **3 min** at 95°C to inactivate Quantiscript Reverse Transcriptase (thermo block)
- Store on ice for 1 minute
- Dilute sample with 30-40µl of RNase-free water, depending on the expression of the target gene
- Spin down (in micro-centrifuge)
- Store on ice, proceed directly with real-time PCR

**Set up the SYBR Green Reaction Master Mix**

Reagents:

**Power SYBR Green PCR Master Mix** (Applied Biosystems)**Primer forward and reverse** (10µM each)

Comments: Two internal reference genes are used for normalization.

1. ***Pdu-cdc5*** – cell division cycle 5 (Oligo number 1 and 2)
2. ***Pdu-rps9*** – ribosomal gene (Oligo number 902 and 903)

The expression of your candidate genes will be normalized by the amount of the internal control gene.

**Procedure:**

**Set up the SYBR Green Master mix** (one reaction per gene) at room temperature containing: water, Primer F, Primer R, Sybr Green mix (final volume 396,0µl) / see set up scheme below. It is highly recommended to perform technical replicates to rule out pipetting mistakes. To calculate the amount of Master Mix use the Q-RT-PCR Plate set up excel file (see example below).

| Q-RT-PCR Plate set up              |     |     |     |     |     |      |     |                  |            |     |     |                | Primer |
|------------------------------------|-----|-----|-----|-----|-----|------|-----|------------------|------------|-----|-----|----------------|--------|
|                                    | 1   | 2   | 3   | 4   | 5   | 6    | 7   | 8                | 9          | 10  | 11  | 12             |        |
| A                                  | 9   | 12  | 16  | 21  | 24  | 4    | 9   | 12               | 16         | 21  | 24  | 4              | bmal   |
| B                                  | 9   | 12  | 16  | 21  | 24  | 4    | 9   | 12               | 16         | 21  | 24  | 4              | bmal   |
| C                                  | 9   | 12  | 16  | 21  | 24  | 4    | 9   | 12               | 16         | 21  | 24  | 4              | hiomt  |
| D                                  | 9   | 12  | 16  | 21  | 24  | 4    | 9   | 12               | 16         | 21  | 24  | 4              | hiomt  |
| E                                  | 9   | 12  | 16  | 21  | 24  | 4    | 9   | 12               | 16         | 21  | 24  | 4              | aanat  |
| F                                  | 9   | 12  | 16  | 21  | 24  | 4    | 9   | 12               | 16         | 21  | 24  | 4              | aanat  |
| G                                  | 9   | 12  | 16  | 21  | 24  | 4    | 9   | 12               | 16         | 21  | 24  | 4              | cdc5   |
| H                                  | 9   | 12  | 16  | 21  | 24  | 4    | 9   | 12               | 16         | 21  | 24  | 4              | cdc5   |
|                                    | BR1 | BR1 | BR1 | BR1 | BR1 | BR1  | BR2 | BR2              | BR2        | BR2 | BR2 | BR2            |        |
| Calculation of the qPCR Master Mix |     |     |     |     |     |      |     |                  |            |     |     |                |        |
| Sample Number:                     |     | 24  |     |     |     | 26,4 |     |                  | MasterMix: | 15  |     | µl each        |        |
| Sample volume:                     |     | 20  |     |     |     |      |     |                  |            |     |     |                |        |
|                                    |     |     |     |     |     |      |     |                  | 79,2       |     |     | water          |        |
| Template in µl:                    |     | 5   |     |     |     |      |     |                  | 26,4       |     |     | Primer F 10µM  |        |
|                                    |     |     |     |     |     |      |     |                  | 26,4       |     |     | Primer R 10µM  |        |
|                                    |     |     |     |     |     |      |     |                  | 264,0      |     |     | Sybr Green mix |        |
| Final Primer concentration         |     |     |     |     |     |      |     |                  |            |     |     |                |        |
| Primerconcentration F              |     | 0,5 |     |     |     |      |     |                  |            |     |     |                |        |
| Primerconcentration R              |     | 0,5 |     |     |     |      |     |                  |            |     |     |                |        |
|                                    |     |     |     |     |     |      |     | 396,0            |            |     |     | Final Volume   |        |
| calculation with                   |     | 10  |     |     |     | %    |     | pipetting losses |            |     |     |                |        |

- **Apply 15µl of master-mix** into the appropriate wells
- **Add 5µl of cDNA** of each time point into the corresponding wells
- **Seal the plate** using MicroAmp optical Adhesive Film
- **Centrifuge plate**
- **Start q-PCR run** (StepOnePlus Real Time System, Applied Biosystems)

Amplification thermal profile:

10 min at 95°C

40 cycles of (15 sec at 95°C, for 1 min at 60°C)

A dissociation curve analysis (melting curve) is performed as the last step of the PCR reaction by slowly raising the temperature of the sample from 60°C to 95°C.

**Analysis of q-PCR data****Adjust the Threshold:**

Before analysing the data you have to **adjust the Threshold** for all genes you have run on the plate. Therefore choose the lowest given Threshold and apply it to the other genes. By exporting the data you can create an excel file to use for further analysis.

**Analysis:**

The **delta cP** values and corresponding **fold change** values will be calculated using a precast excel-form. Relative mRNA levels will be plotted against the circadian time points.

**Comments:** The StepOne Software v2.2. for PC can be downloaded for free!

**Testing q-PCR Primers****Purpose:**

To check if the chosen primers amplify efficiently and to ensure that primer-dimer is not happening.

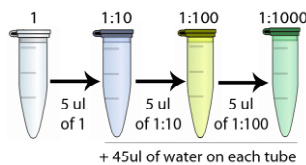
**Strategy:**

Run and analyze a standard q-PCR run as stated in the previous sections. Using **serial dilutions of the same cDNA** you should find higher cP values (stably increasing) in more diluted samples. Also use a **negative control** with water instead of cDNA to monitor primer-dimers. **Melting curves** will be also useful to check for more than one product after the q-PCR reaction cycles.

**Comments:** If possible, and in order to have a positive control for comparison, always use in parallel a pair of primers you have already tested.

**cDNA Dilutions, Negative Controls and q-PCR Run:**

- Take your cDNA and make serial dilutions 1:10, 1:100, 1:1000.

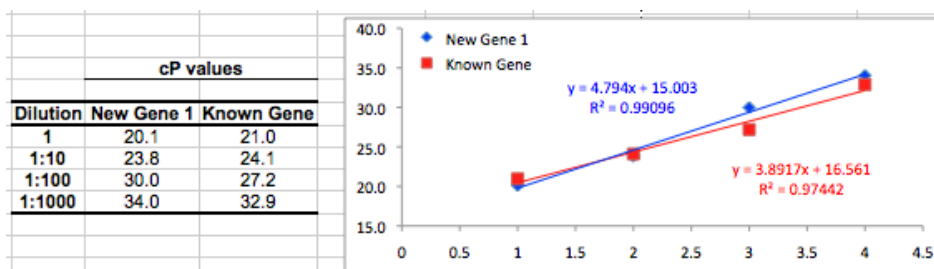


- Prepare a negative control of –RT (add water instead of RT during cDNA preparation)
- Prepare a second negative control by adding 5µl of water instead of cDNA (Master mix with Sybr Green Mix and Primers are still present)
- Load the plate and run the q-PCR program as already stated

|   | Primers to Test |        | Primers Already Tested |        |   |   |   |   |   |    |    |    |
|---|-----------------|--------|------------------------|--------|---|---|---|---|---|----|----|----|
|   | 1               | 2      | 3                      | 4      | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | 1               | 1      | 1                      | 1      |   |   |   |   |   |    |    |    |
| B | 1:10            | 1:10   | 1:10                   | 1:10   |   |   |   |   |   |    |    |    |
| C | 1:100           | 1:100  | 1:100                  | 1:100  |   |   |   |   |   |    |    |    |
| D | 1:1000          | 1:1000 | 1:1000                 | 1:1000 |   |   |   |   |   |    |    |    |
| E | Water           | Water  | Water                  | Water  |   |   |   |   |   |    |    |    |
| F | -RT             | -RT    | -RT                    | -RT    |   |   |   |   |   |    |    |    |
| G |                 |        |                        |        |   |   |   |   |   |    |    |    |
| H |                 |        |                        |        |   |   |   |   |   |    |    |    |

**Analysis:**

- Negative controls of Water and -RT should not have any detectable signal. However, as a rule of thumb, cP values above 34 (or 7 cycles more than the correspondent cDNA synthesis sample) are still considered good results.
- Take the cP values for each dilution and plot them on Excel (Do NOT include the negative controls).
- Generate a linear regression (linear trendline in Excel) and check for the equation and the value of  $R^2$



- A perfect linear regression ( $R^2 \sim 1$ ) implies a better amplification efficiency. The rate of amplification can also be compared among genes by using the slope on the equation

(i.e. the number before the x). **Keep in mind** that for some lowly expressed genes the 1:1000 dilution may already give values of cP above 34. If that is the case, avoid that last point in the graph.

- **Note:** As a standard rule, cP values of the non-diluted cDNA (Tube 1) should be between 18 and 28. For lower or higher values a different cDNA concentration and/or a different reference gene might be needed.
- Always check the melting curves for a second peak indicating the formation of another amplicon in the reaction. Smaller amplicons (such as primer dimers) usually have lower melting temperatures.

## 8. Curriculum vitae

### Personal details

|                |                              |
|----------------|------------------------------|
| Name           | Elisabeth Maria Steinkellner |
| Date of birth  | May 16, 1985                 |
| Place of birth | Grieskirchen                 |
| Citizenship    | Austria                      |

### Education

|                        |                                                                                  |
|------------------------|----------------------------------------------------------------------------------|
| 1991-1995              | elementary school Neumarkt i. H.                                                 |
| 1995-1999              | secondary school Neumarkt i. H.                                                  |
| 1999-2004              | Bundeshandelsakademie I Wels                                                     |
| October 2004-June 2005 | Study of Biology (University of Vienna)                                          |
| October 2005-June 2006 | Study of Medicine (Medical University Vienna)                                    |
| since October 2006     | Study of Biology specialising in Genetics/Microbiology<br>(University of Vienna) |

### Scientific career

|                     |                                                                                                                                                                      |
|---------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| since February 2011 | diploma thesis with the title “Regulation and function of conserved <i>Platynereis</i> hormones” in the group of Dr. Florian Raible at the University of Vienna/MFPL |
|---------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------|

## Lebenslauf

### Persönliche Daten

|                     |                              |
|---------------------|------------------------------|
| Name                | Elisabeth Maria Steinkellner |
| Geburtsdatum        | 16. 05. 1985                 |
| Geburtsort          | Grieskirchen                 |
| Staatsangehörigkeit | Österreich                   |

### Ausbildung

|                        |                                                                                   |
|------------------------|-----------------------------------------------------------------------------------|
| 1991-1995              | Volksschule Neumarkt i. H.                                                        |
| 1995-1999              | Hauptschule Neumarkt i. H.                                                        |
| 1999-2004              | Bundeshandelsakademie I Wels                                                      |
| Oktober 2004-Juni 2005 | Biologiestudium an der Universität Wien                                           |
| Oktober 2005-Juni 2006 | Medizinstudium an der Medizinischen Universität Wien                              |
| seit Oktober 2006      | Biologiestudium an der Universität Wien mit dem Schwerpunkt Genetik/Mikrobiologie |

### Wissenschaftlicher Werdegang

|                   |                                                                                                                                                                 |
|-------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------|
| seit Februar 2011 | Diplomarbeit mit dem Titel „Regulation and function of conserved <i>Platynereis</i> hormones“ in der Gruppe von Dr. Florian Raible an der Universität Wien/MFPL |
|-------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------|