Ispitivanje uloge L-tipa Kriptokoroma na cirkalunarno i svjetlom uvjetovano ponašanje u morskom kolutićavom crvu Platynereis dumerilii

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Diplomski rad obranjen je dana: _____

pred povjerenstvom:

1. _____

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Rad ima ____ stranica, ____ slika, ____ tablica i ____ literaturnih navoda.

Summary

In order to maximize their chances of survival and procreation animals have to be able to predict and adapt to a range of periodic changes in their environment. To be able to anticipate environmental rhythms, like day-night illumination, they developed an endogenous timekeeping oscillators (circadian clocks), with approximately the same period length and phase as the external cycle they are subjected to. Endogenous clocks use external cues to entrain to the environment and produce behavioral and physiological outputs to help animals adjust accordingly. Light is the most prominent entrainment cue (zeitgeber) and light-responsive cryptochromes have been proven to facilitate entrainment in species like *Arabidopsis thaliana*, *Soganus luridus* and *Drosophila melanogaster*.

Since marine habitats harbor a multitude of natural rhythms, marine organisms often have more than one type of clock. Marine annelid *Platynereis dumerilii* possess two clocks entrained by light: circadian clock, controlling their daily locomotor activity, and circalunar clock, controlling the timing of their sexual maturation. Considering *P. dumerilii* possess a light responsive Drosophila-type cryptochrome (*L-cry*), I hypothesized *L-cry* acts as a putative blue-light receptor facilitating clock function, so loss-of-function animals (*L-cry$^{-/-}$*) should display disruptions in circalunar and circadian behavioral and physiological outputs.

I tested the hypothesis by performing a maturation pattern analysis to see how *L-cry* influences the circalunar rhythm, and multiple circadian experiments to test its circadian role. To confirm unpublished laboratory observations of *L-cry$^{-/-}$* animals developing slower than their *L-cry$^{+/+}$* and *L-cry$^{+/-}$* siblings I also performed a regeneration experiment. The experiments showed *L-cry$^{-/-}$* exhibit a major maturation delay likely due to overall developmental delay especially in segment addition, regeneration and gametogenesis. *L-cry* likely does not play a dominant role in maturation

itself, but mechanisms involved could be under circalunar or circadian influence. *L-cry*^{-/-} also exhibited changes in timing of circalunar maturation as well as increase in arrhythmic circadian behavior and susceptibility to masking effects of light, which confirms my hypothesis that *L-cry* has a crucial role in circadian and circalunar clocks of *P. dumerilii*.

Sažetak

Kako bi si povećali izglede za preživljavanje i reprodukciju životinje trebaju predvidjeti i prilagoditi se nizu periodičkih promjena u svom okruženju. Da bi mogle predosjetiti prirodne ritmove, poput promjena intenziteta svjetlosti tijekom dana i noći, razvile su endogene oscilatore za mjerenje vremena (cirkadijalne satove), koji imaju približno jednaki period i fazu kao i vanjski ciklus pod čijim su utjecajem. Biološki sat koristi signale iz okoline kako bi se sinkronizirao s uvjetima okoliša te uzrokuje fiziološke i bihevioralne promjene u životinja kao odgovor. Svjetlost je najjači okolišni signal koji može sinkronizirati endogene satove (zeitgeber signal), a kriptokromi osjetljivi na svjetlost dokazano omogućuju sinkronizaciju bioloških satova putem svjetla u vrsta poput *Arabidopsis thaliana*, *Soganus luridus* i *Drosophila melanogaster*.

S obzirom da su morska staništa pod utjecajem niza prirodnih ciklusa, morski organizmi često imaju više vrsta bioloških satova. Morski kolutićavac *Platynereis dumerilii* ima dva biološka sata pod utjecajem svjetla: cirkadijalni sat, koji kontrolira lokomociju tokom dana i noći, te circalunarni sat, koji kontrolira temporalni aspekt spolnog sazrijevanja. S obzirom da *P. dumerilii* posjeduje tip kriptokroma srodan onom kod *Drosophilae* (*L-cry*) postavila sam hipotezu prema kojoj je *L-cry* potencijalni receptor plavog svjetla koji sudjeluje u radu bioloških satova. Prema hipotezi $L\text{-}cry^{-/-}$ mutanti, bez funkcionalnog L-Cry proteina, trebali bi pokazati poremećaje u cirkadijalnom i cirkadijalnom ponašanju te povezanoj fiziologiji.

U svrhu testiranja hipoteze provela sam analizu obrasca spolnog sazrijevanja kako bi istražila utjecaj *L-cry* gena na cirkalunarni sat te niz cirkadijalnih eksperimenata, kako bi istražila njegovu ulogu u cirkadijalnom ponašanju. Kako bi potvrdila neobjavljena zapažanja unutar laboratorija prema kojima se $L\text{-}cry^{-/-}$ životinje razvijaju sporije od $L\text{-}cry^{+/+}$ i $L\text{-}cry^{+/-}$ potomaka provela sam analizu sposobnosti regeneracije. Eksperimenti su

pokazali da *L-cry*$^{-/-}$ jedinke sazrijevaju znatno kasnije, vjerojatno zbog generalnog zastoja u razvoju, u fazi dodavanja segmenata, regenereacije i gametogeneze. *L-cry* vjerojatno nema dominantnu ulogu u samom procesu spolnog sazrijevanja, ali uključeni mehanizmi mogu biti pod utjecajem cirkalunarnog ili cirkadijalnog sata. *L-cry*$^{-/-}$ jedinke su također pokazale promjene u tempiranju cirkalunarnog sazrijevanja, kao i povećanje u aritmičnom cirkadijalnom ponašanju i podložnosti „masking" efektu svjetlosti, što potvrđuje moju hipotezu da *L-cry* ima ključnu ulogu u cikalunarnim i cirkadijalnim satovima *P. dumerilii*.

Ključne riječi: circadijalni sat, cirkalunarni sat, L-kriptokrom, *Platynereis dumerilii*, sinkronizacija putem svjetla

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1 Introduction

In order to optimize procreation and survival odds animals need to adapt to a range of reoccurring changes in their habitat. If they are able to predict fluctuations in light intensity, temperature, food availability etc., they can select the optimal time to mate, feed and rest safely. To achieve temporal adaptability they have to possess a sort of an internal clock, capable of anticipating environmental changes. Extensive investigation of endogenous rhythms revealed organisms commonly achieve temporal adaptability through use of biological oscillators (1). Interdisciplinary nature of "biological timekeeping" research called for uniform experimental guidelines and well defined research principles which gave rise to a new branch of science- Chronobiology.

Due to similarities with oscillators in physics, chronobiology adopted waveform terms like period, amplitude and phase to describe behavioral output of biological oscillators (2). Duration of a full cycle of rhythmic phenomena (a single wave) is a period. Frequency is inversely proportional to period, specifying how many cycles fit in a time interval. While in trigonometry amplitude is defined as half a distance from the maximum value of a wave (peak) to the minimum (trough), behavioral outliers can greatly influence the amplitude. To reduce the outlier contribution, in chronobiology amplitude is calculated from fitted curves rather than raw signals. Phase is a momentary state of an oscillator in relation to a reference point. Reference point most commonly used in a circadian period is the daily onset of activity, which labels animals as nocturnal or diurnal depending on their predominant activity period. As principles specific to chronobiology somewhat differ from classical biology I will explain the ones necessary for further discussion.

1.1 Fundamental concepts of chronobiology on the example of circadian clock

Circadian clocks are a dominant topic in chronobiology and the best understood biological oscillators thus far. Circadian comes from the latin word *circa* which means "approximately" and *dian* meaning "day". As implied by the name, circadian clock is a timing mechanism, comprised of cell autonomous oscillators, that helps animals anticipate the daily periodic changes in their environment. For biological oscillators to be considered circadian they have to possess three crucial features: 1) ability to entrain to periodic external stimuli, 2) daily period of approximately 24 h that persists under constant conditions and 3) compensation for acute changes in the environment (weather) (1,3).

Unlike fixed periodic changes in nature, periods of endogenous oscillators are not as stable so to mirror external conditions they have to be adjusted accordingly- entrainment. On a cellular level, oscillators rely on molecular networks to keep a robust track of time and use outside cues to entrain them, which is evident by behavioral or physiological output. Since endogenous clocks are considered "time keepers" entrainment stimuli are "time givers", but term used in chronobiology is German zeitgeber. Several different cues can act as zeitgebers, most prominent of them being light, which is why circadian research focuses on light-dark dependent phenotypes. In chronobiological research time is denoted on a zeitgeber scale (ZT0-ZT24) rather than local time. This approach labels onset of daily (24h) illumination as 'zeitgeber time zero' (ZT0).

When observing animals in natural habitats or laboratory cultures their daily activity will often have an average period of approximately 24 h. Since, animals can react through mechanisms that bypass the circadian clock rhythmicity observed under periodic external stimuli (day-night illumination) is not an endogenous rhythm or even an indicator of one. This phenomena is referred to as "the masking effect". When masked by periodic

external stimuli animal behavior is synchronized to it (behavioral entrainment), but their endogenous clock is not necessarily entrained to it (circadian entrainment). In order to examine a true endogenous period animals have to be observed in constant conditions, constant variable being the zeitgeber. To determine the circadian period animals are monitored under constant darkness or constant light. If under constant conditions rhythmicity persist over several cycles (days), we can conclude animals possess an endogenous clock. Endogenous period exhibited under constant conditions is referred to as the free-running period so these terms are used interchangeably. Since in nature illumination can randomly vary due to weather, etc.; animals use light onset (dawn) and offset (dusk) for entrainment so daily fluctuations (weather conditions) do not significantly influence the period. In laboratory conditions researchers utilize bright light pulses to induce period phase shifts (3). Light pulse can cause a phase advance or delay depending on its relative timing, which is specific to the species. Therefore, two crucial properties in entrainment research are the free-running period and period phase shift.

Detailed description of molecular oscillators constituting the circadian clock is available for number of models from the simple microorganisms to mammals (4,5). Although proteins involved in circadian control may vary, protein interactions are well preserved between species. In its essence, circadian oscillator is a network of positive and negative transcription elements that form a self-sustained transcriptional-translational feedback loop (3). Positive elements of the loop activate transcription of so called 'clock genes'. In turn, proteins encoded by clock genes act as negative elements suppressing positive element function. Over time, decay of negative elements lowers their concentration which enables positive elements to rise again, closing the loop. All of these interactions cause time delays within the loop resulting in an approximately 24 h molecular cycle. Apart from influencing negative elements, positive element activate transcription of so called 'clock-controlled genes' (CCGs). Many CCGs are

hormones, signaling molecules or transcription factors which is how the circadian clock produces its behavioral and physiological output.

To take a closer look at circadian clock elements we can examine the fruit fly *Drosophila melanogaster*, whose detailed report significantly contributed to understanding molecular oscillators in other species. During the day *Drosophila* CLK (clock) and CYC (cycle) proteins form heterodimers that bind to E-box domains to drive the transcription of negative elements *per* (period) and *tim* (timeless)*,* as well as CCGs (5). In the early evening PER and TIM levels rise and together with DBT (double-time) enter the nucleus to remove CLK-CYC complex from E-box elements. At dawn, light induces conformational changes in CRY (cryptochrome) which results in CRY-TIM heterodimers that lead to TIM degradation. In absence of TIM, PER and CLOCK are degraded by DBT. Eventually, CLK levels rise again forming CLK-CYC heterodimers and the cycle begins again. There are additional steps, but this is the backbone, with *tim* and *per* being crucial components since their removal abolishes the circadian function.
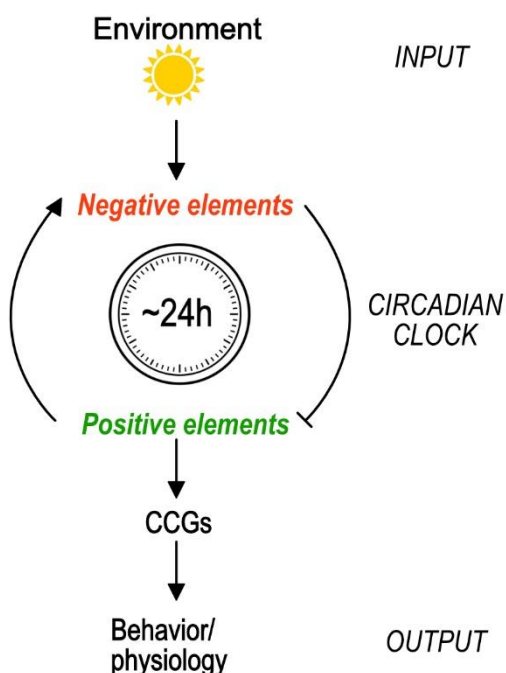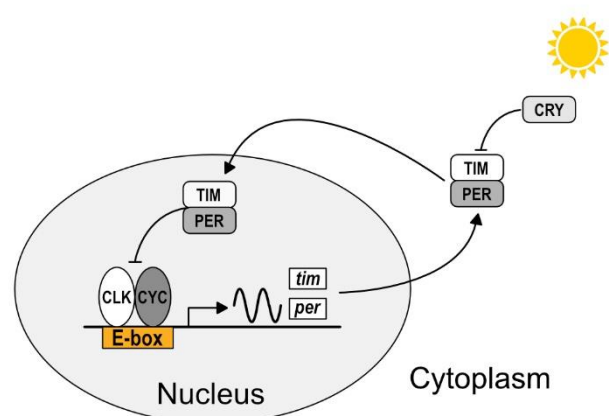
1.2 Data analysis in chronobiology

Signal is a function carrying information attributes of a phenomenon. In my research of circadian phenomena I focused on locomotor output signals. Visual inspection is a good way to evaluate nature and reliability of data before proceeding with further analysis. Common way to visually inspect data in chronobiology is utilizing actograms (2). Actogram is a double plotted time series where pairs of consecutive days are plotted on top of each other with last day of the pair repeating in the next row. Vertical alignment of consecutive days makes period shifts easier to detect by eye. Also, light conditions are graphically represented in order to easily interpret the actogram.
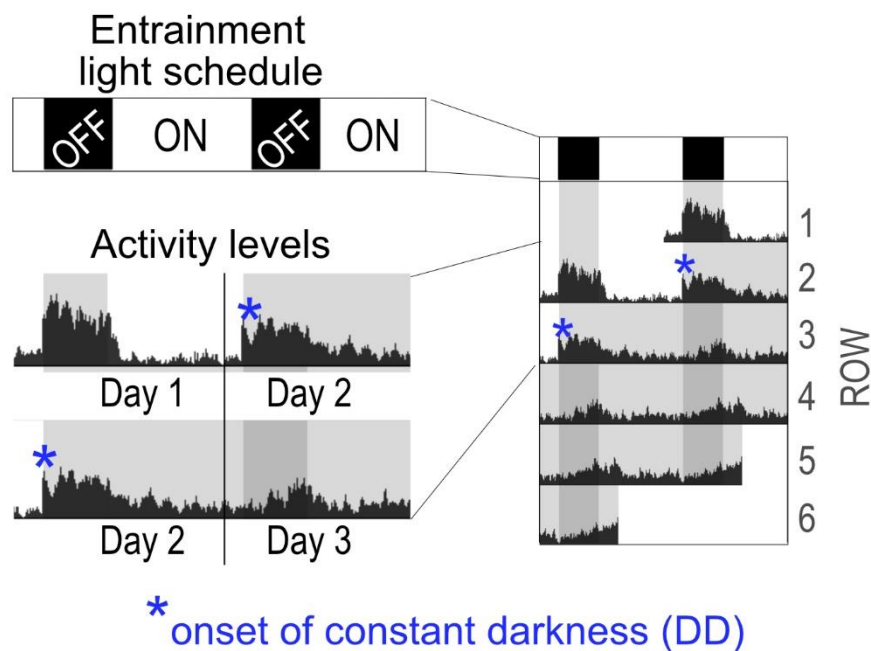
Behavioral data can be noisy due to individual variations, technical constraints or stochastic errors. Noise makes it very hard to see rhythmicity by eye so to alleviate bias and quantify the behavior mathematical

algorithms are applied. Lomb-Scargle periodogram is one of the most robust algorithms in rhythmic pattern analysis, particularly resistant to missing data points in time series (2,6). Result of a Lomb-Scargle period analysis is a 'frequency power spectrum'. Frequency power spectrum is a real-valued function of a transformed signal analyzed. Since every signal can be broken down to a sum of sine waves, power spectrum quantifies the contribution of each frequency within a chosen interval to the resulting signal. In chronobiology frequency is transformed into period length so result of a Lomb-Sargle analysis is a graph (periodogram) with period power (hereafter 'power') plotted against a chosen range of period lengths. Since power determines the level to which a certain period contributes to the resulting signal, periodogram peak represents a predominant rhythm of the observed behavior. Robust rhythms have higher power values and sharper peaks then weak periods. To discriminate significant peaks from arrhythmic noise, threshold with a chosen significance level (usually p= 0.05) is specified. Peaks above the threshold indicate rhythmicity, while periodograms without significant peaks indicate arrhythmic behavior.

Lomb-Scargle periodogram example
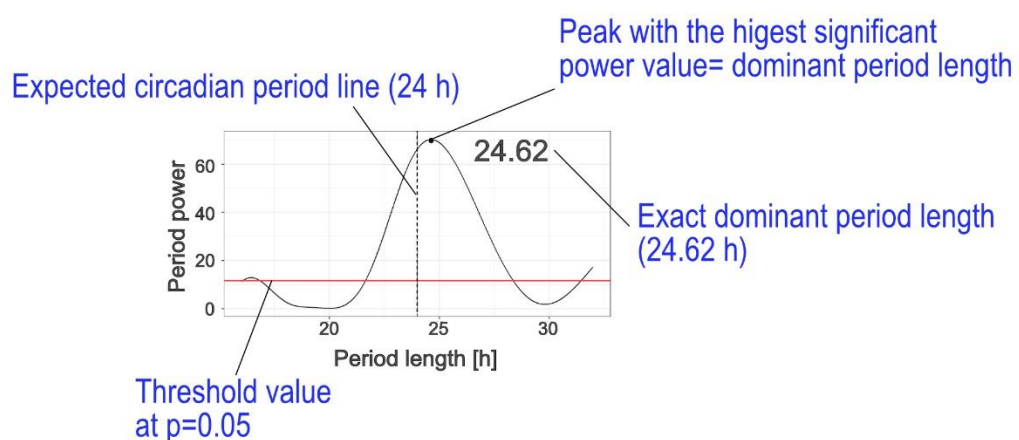
1.3 Circalunar rhythms in marine organisms

Episodic changes of environmental conditions in nature like illumination intensity, temperature or pressure are directly or indirectly controlled by the Sun and the Moon. While rhythms governed by the Sun are well understood by now (circadian/ seasonal), details of lunar induced rhythmicity are still elusive. Since life pioneered in the sea, under influence of variety of environmental cycles, marine organisms had millions of years to adapt, exhibiting circadian as well as non-circadian rhythms. Tidal, circalunar and circasemilunar are examples of rhythms under lunar control and commonly found in marine organisms (7,8). As such, marine species are a key component in understanding the evolution and interaction of different levels of rhythms of life.

The Moon's full journey around Earth takes 29.5 days (lunar month). Depending on the position of the Moon relative to Earth and the Sun different portions of the moon disc can be seen in the night sky. Lunar month is roughly divided in 4 moon phases, depending on the intensity of sunlight reflected from the Moon surface and portion of the moon disc visible in the night sky. Full moon is a moon phase when the most sunlight is reflected from the Moon surface and the moon disc looks whole. On the other hand, new moon appears when sunlight is minimally reflected, meaning the moon disc is not visible. The remaining two phases are transitional and named first quarter and last quarter moon. Since water strongly absorbs light marine animals are profoundly under influence of light spectrum and intensity alterations compared to terrestrial animals (9). A lot of marine animals procreate by means of external fertilization so entraining to a specific lunar phase enables male and female specimens to robustly synchronize with each other and increase the odds of procreation. Reef corals *Acropora millepora* procreate for only few days a year and, despite their lack of specialized visual sensory organs, synchronously spawn during

full moon (10). Reef fish *Soganus luridus* spawn during first quarter moon (11) and bristle worm *Platynereis dumerili* during new moon (12).

Though there are many instances of circalunar spawning rhythms in marine animals (7), in given examples blue-light receptors cryptochromes were proposed as instruments of circalunar entrainment. In terrestrial organisms, like *Arabidopsis thaliana* and *Drosophila melanogaster,* cryptochromes have already been confirmed to mediate input of blue-light signals to the circadian clock (13,14). Cryptochromes are proteins from an ancient Cryptochrome/Photolyase Family (CPF), often possessing photo-reactive properties. Since water absorbs most of the visible light spectrum, blue light due to its short wave length and high energy is the most permeable to ocean water (9). Hence, blue-light reactive cryptochromes are widely distributed in marine animals (15). CPF cryptochromes are categorized as vertebrate-type, Drosophila-type, plant-type and cry-DASH, but nomenclature was based on organisms initially discovered in so they appear in other phyla as well (16).

1.4 *Platynereis dumerilii* as a model organism in search of blue-light receptors

Ideal model organism for elucidating circalunar light entrainment has to possess a light entrained circalunar rhythm, be easily maintained in a laboratory culture and molecularly accessible. All of this can be found in a bristle worm *Platynereis dumerili, a* widely spread marine polychaetous annelid from Nereididae family (17). It has a mixed benthic/pelagic life cycle (lives at bottom as well as open sea) easily mimicked in laboratory conditions that spans from around 3 to 18 months. *Platynereis* procreate through means of external fertilization and have an amazing ability to regenerate most of their fairly transparent trunks, which makes molecular accessibility that much easier (18,19). Finally, *P. dumerilii* spawning is

synchronized to new moon phase by nocturnal light and in addition possess a circadian clock as well (12).

Platynereis life cycle is characterized by two major metamorphosis events, accompanied by change of habitat (17). *Platynereis* embryos hatch from eggs to start as zooplanktons at open sea. At 3-4 days of age larvae settle on the bottom and begin the benthic phase of their life cycle transforming into juvenile worms. At this point, *Platynereis* trunks are elongated with 3 identical segments containing paired lateral outgrowths (parapodia) enabling them to crawl. Juvenile worms also have a differentiated head (prostomium) with two pairs of eyes, two sensory appendages, four pairs of tentacle-like extensions (perisomal cirii) and strong jaws at the anterior end of the trunk, while the posterior end contains anal opening (pygidium) with a pair of anal cirii. Throughout juvenile development, segments are continuously added from a segment addition zone anterior to the pygidium up to approximately 75 segments. Juvenile worms start to feed on algae around 7-8 days post-fertilization so segment proliferation becomes tremendously variable, even between siblings. At this stage, worms mostly crawl, but they can also swim in a slow undulatory manner. Pre-mature worms (juveniles >2 months of age) spend most of their time in self-produced silky tubes, exiting mostly to search for food. Juvenile *Platynereis* possess high capacity for posterior trunk regeneration, while anterior trunk cannot be regenerated. As they approach maturation worms stop growing, lose the ability to regenerate and focus all their energy on the last metamorphosis from pre-mature juveniles to sexually mature adults. The metamorphosis is very dramatic as many cells are eliminated in order to make room or provide energy for the transformation. Adults even change their color form grey-brown of juvenile animals to red or yellow of adult males and females respectively. At the end, sexually mature worms are skilled swimmers equipped to do one last thing before they die. Under the dark of new moon they swim to the open sea at dusk, look for mates and take part in the nuptial dance, a mass spawning event. Worms changing color in the process of sexual maturation and fast circular swimming on the

day of peak sexual maturity makes it easy to distinguish the maturing individuals, separate them from the batch and harvest when mature. Since *P. dumerilii* spawning in nature takes place at night, in laboratory maturing males and females are kept in separate cups to ensure selective breeding during the day.

P. dumerili nuptial dance (spawning) is a behavioral event synchronized by circalunar (new moon) as well as circadian (at dusk) clock. Although molecular aspects of *Platynereis* rhythmicity are not described in detail, they possess a full complement of fruit fly and mouse circadian clock gene orthologs, with circadian as well as circalunar expression profiles in pre-mature worm heads (12). Circalunar changes in circadian gene expression, as well as behavior, indicate circalunar clock of *P. dumerilii* is under circalunar influence, but opposite is not the case. Research also showed *Platynereis* possess two types of cryptochromes, vertebrate-type *tr-cry* and Drosophila-type *L-cry*. *Platynereis tr-cry* acts as a transcriptional suppressor in the circadian transcription/translation loop and has no proven photo-responsive properties. However, Drosophila-type *L-cry* is strongly degraded under influence of 6 h light pulse when expressed in *Drosophila* S2 cells (established expression system), proving it can act as a light receptor. *L-cry* is also co-expressed with clock gene orthologs inside the oval-shaped forebrain regions and around eyes (in the eyes not confirmed yet). On the basis of L-Cry light induced degradation, co-expression with circadian genes and orthology to previously confirmed blue-light receptors I propose L-Cry acts as a blue-light photoreceptor responsible for circadian and/or circalunar entrainment in bristle worm *Platynereis dumerilii*. I hypothesize loss-of-function *L-cry* mutant animals ($L\text{-}cry^{-/-}$) have disruptions in circalunar and circadian behavioral and molecular outputs.

2 Research goals

In providing insight to *L-cry*$^{-/-}$ chronobiological phenotypes I focused on 3 main goals: 1) circalunar behavior, 2) regeneration capacity and 3) circadian behavior. To investigate if *L-cry*$^{-/-}$ maturation pattern differs from *L-cry*$^{+/-}$ and *L-cry*$^{+/+}$ controls, I analyzed maturation logs from animal batches in-phase with natural lunar and daily cycles (*Chapter 5.1.*), keeping in mind previous research done on *Platynereis* (12). To confirm unpublished observations of *L-cry*$^{-/-}$ animals developing slower than their batch siblings, supported by age upon maturation analysis, I performed a regeneration assay comparing *L-cry*$^{-/-}$ regeneration capacity to *L-cry*$^{+/+}$ controls (*Ch.5.2.*) taking notes from previous research (20,21). Due to no previous investigation of *L-cry*$^{-/-}$ *P. dumerilii* circadian behavior, I first compared *L-cry*$^{-/-}$ and *L-cry*$^{+/+}$ locomotion under periodic LD conditions (*Ch.5.3. and 5.4*) and followed it by measuring their endogenous circadian rhythm in constant darkness (*Ch.5.5.*) to finally attempt to disrupt their circadian clock with continuous 426 nm blue light pulsing (*Ch.5.6.*).

3 Materials and Methods

3.1 Animals

Experimental animals I used, homozygous and heterozygous mutants ($L\text{-}cry^{-/-}$ and $L\text{-}cry^{+/-}$ respectively) and wild types ($L\text{-}cry^{+/+}$), come from intercross and in-cross *L-cry* batches with an inbred *vio* and *pin* background. Mutants were generated with TALEN technology by causing a 34 bp deletion in exon 2 and a 9 bp deletion in exon 3 of *L-cry* gene (18)*. The deletion resulted in 43 bp shorter *L-cry* amplification product which can be screened using agarose gel electrophoresis. All the animals I used in behavioral experiments were pre-mature (sex unknown) and the animals that matured within a week after the experiment were excluded to control for changes in locomotion that come with different stages of maturity. The animals I used in regeneration experiments were of strictly controlled size defined under *4.2. Regeneration capacity*.

3.1.1 Animal culture

I kept all the experimental animals in the same room, under constant temperature of 18 ± 2°C. They were raised under white light, with daily illumination ratio of 16 hours artificial light and 8 hours of complete darkness (LD 16:8; lights ON 5:00 UTC, OFF 21:00 UTC). 8 nights a month, in phase with natural moon cycle, in addition to the LD illumination they also experienced a dim nocturnal light, mimicking full moon (FM). I stored the animals in plastic containers filled with a 1:1 mixture of natural sea water (NSW) and 0,03% saline solution- artificial sea water (ASW), changing the water every two weeks. On Mondays I fed them pesticide-free minced spinach and on Wednesdays and Fridays a mixture of algae and TetraMin® flakes by Tetra.

3.2 Pre-mature worm gDNA extraction

In order to maintain mutant lines by making specific in-crosses and for experimental purposes, I continuously genotyped pre-mature animals. Prior to sampling I anesthetized the worms in 1:1 solution of 7.5% $MgCl_2$ and ASW for minimum of 5, but not more than 30 minutes at a time. Using Stemi 2000 Stereo Microscope I sampled 2-5 posterior segments of their trunks for gDNA extraction.

I performed genomic DNA extraction using one of the following protocols:

3.2.1 gDNA extraction protocol 1

MATERIALS
- Extraction Buffer:
  Tris 200 mM
  EDTA 25 mM
  NaCl 250 mM
  SDS 0,5 %
  *pH 7.5; keep at room
  temperature (RT)

- TissueLyser, blocks at room temperature
- Isopropanol, cooled at -20˚C
- 70% ethanol

PROCEDURE
I added 200 µl of Extraction Buffer to a 2 ml tube with a single metal bead and sampled segments. Tube was shaken using TissueLyser at 30 Hz for 30 s. After 10 minutes of centrifugation at 13000 rpm I transferred the supernatant to a clean 1.5 ml tube, added 200 µl of cold isopropanol and mixed by inverting. After an additional centrifugation step at 13000 rpm for

10 min, I discarded the supernatant and washed a remaining pallet with 500 µl of 70% ethanol. I dried the pellet from all remaining ethanol, re-suspended it in 30 µl of dH$_2$O and stored at 4˚C or -20˚C until further processing.

3.2.2 gDNA extraction protocol 2

MATERIALS

- Standard PCR buffer 10X:

    15 mM MgCl2

    500 mM KCl

    100 mM Tris-HCl, pH 8.3 at 25ºC

    *sterilized by filtering

- Digestion Buffer:

10x Standard PCR Buffer	4 µl
Protainase K 20mg/ml	4 µl
dH2O	32 µl
TOTAL	40 µl

- Water bath
- PCR machine

PROCEDURE

While sampling, I put the cut trunk segments directly in a 50 ul PCR tube with 40 ul of Digestion Buffer kept on ice. After sampling all the worms I took the tubes off ice, briefly spun them down and incubated at 55˚C water bath for 2-2.5 hours. After the incubation step, I inactivated the enzymatic reaction at 95˚C for 10 minutes using a PCR machine.

3.3 *L-cry* amplification

I set up all the *L-cry* PCRs using HotStarTaq Plus DNA polymerase from Qiagen. Primers I used were previously designed in our laboratory,

forward primer being AGACTGACGATTGGGACAACAAG and reverse TTGTCATACCTCAATCAGCTGCTTG.

The reaction was as follows:

HotStarTaq Plus	0.125 μl
10X Qiagen PCR buffer	2.5 μl
25mM MgCl$_2$	1.5 μl
dNTPs (10mM)	1.0 μl
Forward primer (5uM)	1.0 μl
Backward primer (5uM)	1.0 μl
gDNA	2.0 μl
dH$_2$O	15.875 μl
TOTAL	25.0 μl

PCR conditions

95 ˚C	5 min

95 ˚C	30 s	
67 ˚C	1 min	x35
72 ˚C	1 min	

72 ˚C	5 min

Described reaction results in a 580 bp product in wild type animals.

3.4 L-cry mutation screening by agarose gel electrophoresis

In order to detect a 43 bp deletion in the L-cry gene I used a 1.5% agarose gel with GelGreen Nucleic Acid Gel Stain from Biotium added to it

(20 μl dye per 100 ml gel). I also added 2 μl of Orange DNA Loading Dye (6x) from Thermo Fischer to 8 μl of amplified DNA, loaded it on a gel and run at 110-120 V for approximately 1.5 h, i.e. until clearly separated bands could be detected under blue light. Alongside the samples I run 5 μl of 2-log DNA Ladder from NEB, ranging from 0.1 to 10 kb.

3.5 Sexual maturation data accumulation

Approaching maturation, which is evident by worms changing color from gray-brown to red or yellow, depending on the sex, I took them out of the common boxes and put them into smaller cups without any food. In these cups they reached maturity which I recorded daily. After mating I washed the worms with ASW 2-3 times, put them in a 2 ml tube and stored at -20˚C for an additional genotyping step. If there were no suitable partners available upon maturation, I still stored the worms for additional genotyping, following the same washing step.

3.5.1 Whole worm gDNA extraction

MATERIALS
- Heat Block, with a shaking function
- NucleoSpin® Tissue kit (Machery Nagel)

I processed the samples according to manufacturer's instructions, provided with the kit.

3.6 Regeneration capacity assay

I selected intact pre-mature worms (segments and trunk without visible trauma) with 42 ± 6 segments, 0.35 ± 0.06 mm distance between anterior pair of eyes and approximately 13.2 ± 3.98 mm long and photographed in two batches of 24 using Zeiss Stereomicroscope. To initiate regeneration, I cut off 4-5 posterior trunk segments to induce physical

trauma and utilized them for genotyping. After genotyping I excluded heterozygous individuals from further processing. I also excluded all the animals that obtained additional trauma throughout the course of the experiment which left me with n(*L-cry*$^{-/-}$) =7 and n(*L-cry*$^{+/+}$) = 8. On days 3,8,14 and 21 post-amputation (dpa) I photographed the worms using the same stereomicroscope and settings to track the regeneration progress.

For the duration of the experiment, I kept the animals separately in numbered 6-well dishes filled 2/3 of the height with ASW and fed them according to their regular schedule. To keep the wells in optimal conditions, I changed the water before feeding (3 times a week).

3.7 Behavioral experiment recording and analysis

3.7.1 Recording

I performed all behavioral experiments with a Kastl behavioral arena, equipped with Motif recording system, provided by Loopbio. Kastl has an infrared (IR) light-emitting diode (LED) on top of which I placed a 36- or 25-arena Plexiglas plate, while above the plate and perpendicular to the IR source a camera with an IR light high pass filter is mounted. Each arena, when filled to the brim with ASW, can hold an individual animal. To prevent animals from transferring to neighboring arenas I placed a plastic grid on top of the plate to act as a physical barrier. In addition, Kastl is equipped with white light and monochromatic LEDs, that I programed to go ON and OFF at a desired schedule, and light/temperature sensor to monitor the set up running conditions. In this set up camera only detects IR light coming from underneath the Plexiglas plate holding the animals regardless of the type of light used at a particular time, while animal photoreceptors are not additionally stimulated since IR spectrum does not overlap with their perceivable light spectrum. This approach provided me with a uniform video recording (uniform contrast parameters crucial for video analysis) of all the worm arenas at once.

3.7.2 Video analysis

I processed videos with Loopy, video tracking software by Loopbio. The software subtracts the image of each individual animal from the background based on the contrast between the two. The camera is set to record 15 frames per second (fps) and to smoothen noise, I applied a rolling mean over 60 consecutive frames. For easier data handling, I downsampled 15 fps to 60 data points per hour (each point is representative of a 1 minute interval), using mean values. At this point of data export I applied an additional smoothening step with a rolling mean function. As a result, I acquired a quantitative locomotion output over time documented for each animal as a Euclidean distance traveled between two consecutive data points.
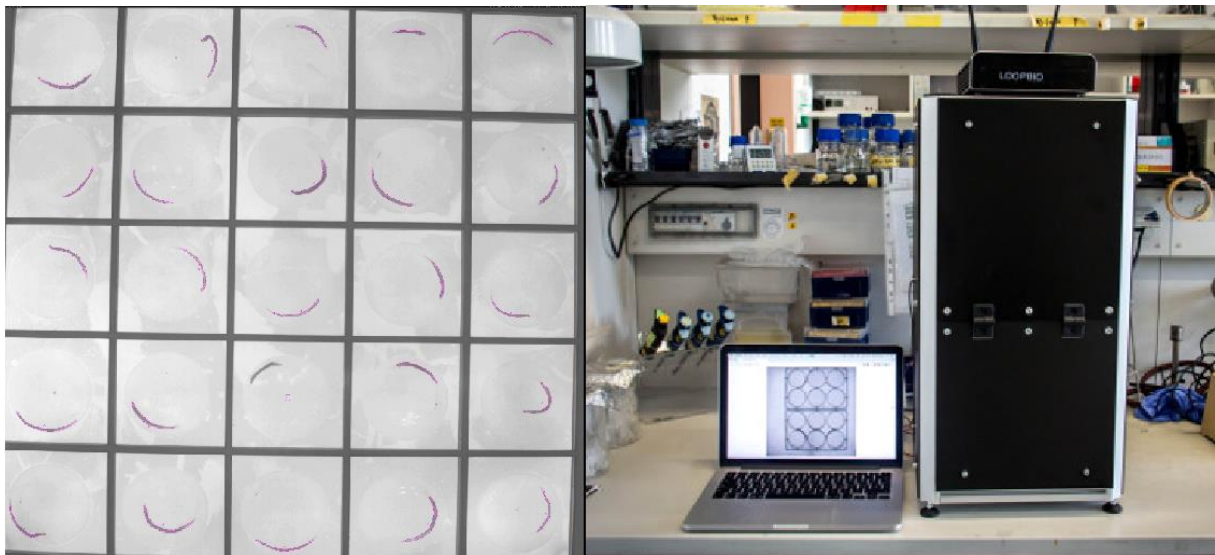
3.7.3 Data analysis

I performed data management, analysis and visualization with 64-bit R studio 1.0.143 for Windows in R-3.4.4 environment. I produced the actograms using Actogram J extension for ImageJ 1.46 R software and normalized activity data of individual worms.

3.8 Behavioral experiment design

I took the worms entrained to in-phase circadian and circalunar light regimes, fed them according to the aforementioned schedule (*3.1.1. Animal culture*) up until the initiation of the recording when I omitted feeding for the duration of the experiment. Due to observations, that point to worms moving less when satiated, towards the end of the project I switched to a regime where all feeding was omitted 4 days prior to the experiment.

Since there was no lunar light stimulus inside the experimental set up, all experiments conducted during the full moon are representative of free-running full moon (FR-FM) conditions. For the purpose of FR-FM experiments that were initiated after the first night of nocturnal illumination in the animal culture, animals were transferred to an out-phase culture room (same LD/temperature conditions, but different lunar conditions) in time to prevent them from receiving the moon stimulus. Considering new moon (NM) is the absence of any nocturnal light, no additional preparation steps were necessary for NM experiments.

To initiate the experiment I placed the animals into individual arenas with ASW for at least a couple of hours, to settle down and produce tubes, after which I recorded them over several days, while exposing to different light conditions.

3.8.1 Constant darkness free-run

After recording animals under usual 16 h white light and 8 h dark, for at least 24h, I switched them to complete darkness for the following 3 or more days, allowing them to free-run.
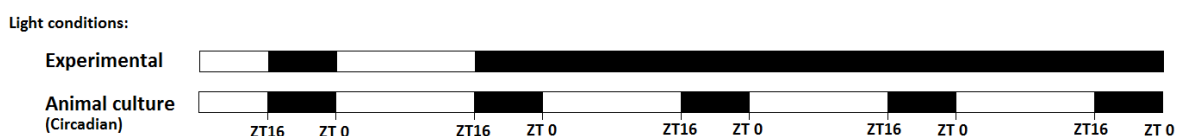
3.8.2 Blue light phase shift

After recording animals under their usual LD conditions for at least 24h, I prolonged the illumination period by 12 hours of blue light (426 nm), before the usual 8 hours of darkness. From this point on, I recorded the animals under 16:8 LD for at least 2 days straight, with light stimulus being 426 nm blue light.
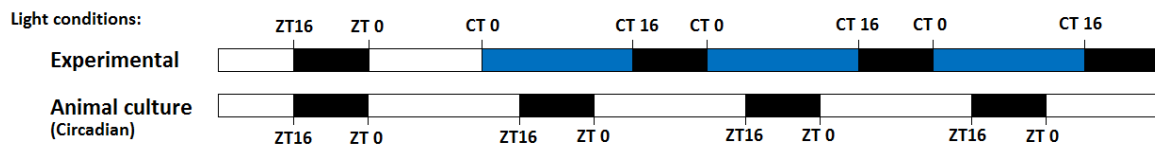
3.8.3 Blue light pulsing

Pulsing was performed using blue light (BL) that was turned ON and OFF in 2 hour intervals. Experiments were initiated with an interval of darkness at ZT 16, when lights in the animal culture go off. This way, circadian period was not shifted by the first pulse of light, but influenced specifically by subsequent pulsing. I continuously pulsed and recorded the worms for minimum of 56 h.
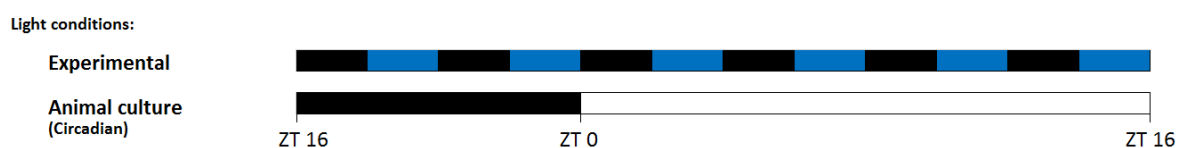
3.9 Statistical analysis

I performed all the statistical analysis using 64-bit R studio 1.0.143 for Windows in R-3.4.4 environment.

4 Results

4.1 Sexual maturation

Since *P. dumerilii* is a marine animal that spends most of its lifespan at sea bottom within self-produced transparent tubes it is subjected to diurnal illumination of sunlight and moonlight. *P. dumerilii* spawning is regulated by an endogenous circalunar clock synchronized by nocturnal illumination (moonlight) in a process still not completely understood, but likely reliant on *L-cry* gene. In order to explore *L-cry* homozygous mutant (*L-cry$^{-/-}$*) maturation I analyzed animal culture spawning logs and compared *L-cry$^{-/-}$* spawning patterns to their heterozygous (*L-cry$^{+/-}$*) and homozygous (*L-cry$^{+/+}$*) controls. I logged maturing animals daily, recognized by fast circular swimming, and stored them for a subsequent genotyping step. Compiling spawning data by using only finished batches (all worms matured and genotyped) provided me with a total of n(*L-cry$^{-/-}$*)= 116, n(*L-cry$^{+/+}$*)= 189 and n(*L-cry$^{+/-}$*)= 305 animals.

By comparing age upon maturation with one-way ANOVA (Figure 1a) I deduced *L-cry$^{-/-}$* animals are maturing at a significantly older age than *L-cry$^{+/+}$* (p < 1·10^{-7}) or *L-cry$^{+/-}$* animals (p < 1·10^{-7}). Although spawning started as soon as 33 days post fertilization in the case of *L-cry$^{+/+}$* and 97 days for *L-cry$^{+/-}$* animals, first *L-cry$^{-/-}$* animals did not spawn until 121 days post fertilization (Figure 1b). This makes *L-cry$^{+/+}$* and *L-cry$^{+/-}$* spawning curves skewed to the left with peak values at 206 and 153 days respectively, while *L-cry$^{-/-}$* curve is bell shaped peaking at 312 days of age.

Considering lunar month (29.5 days) was adjusted to laboratory conditions (30 days, hereafter 'lunar month') it cannot be divided in 4 equal parts of full days representing moon phases. For analysis, I divided the lunar month to FM light ON phase (FM-ON) of 8 days and FM light OFF phase (FM-OFF) of the remaining 22 days. I additionally divided the FM-OFF in two equal groups of 11 days to analyze distinct double peak spawning patterns
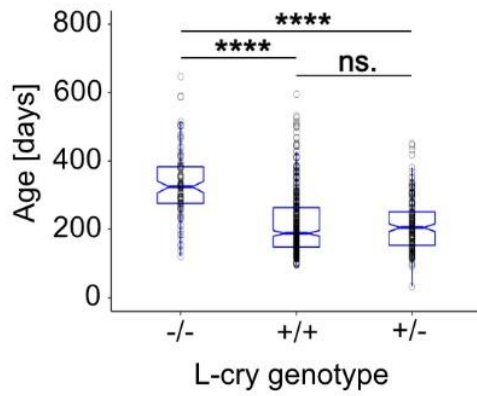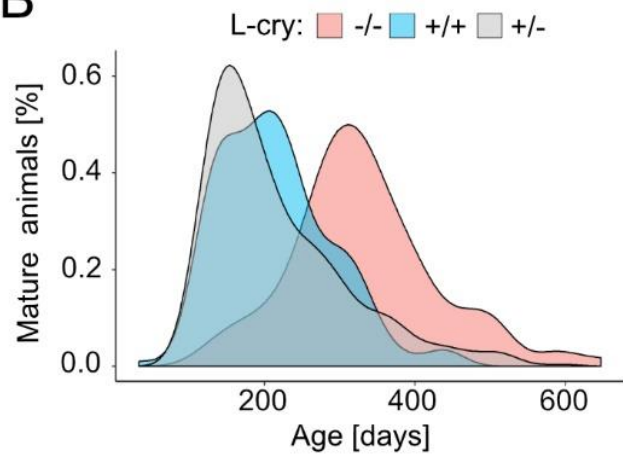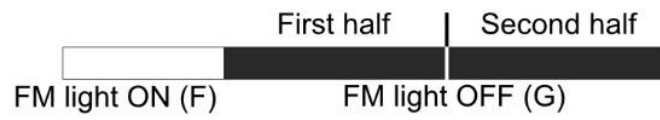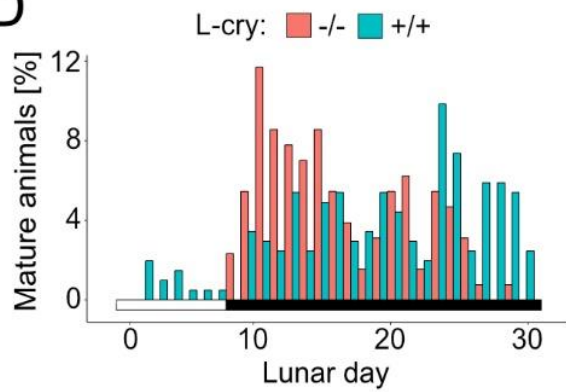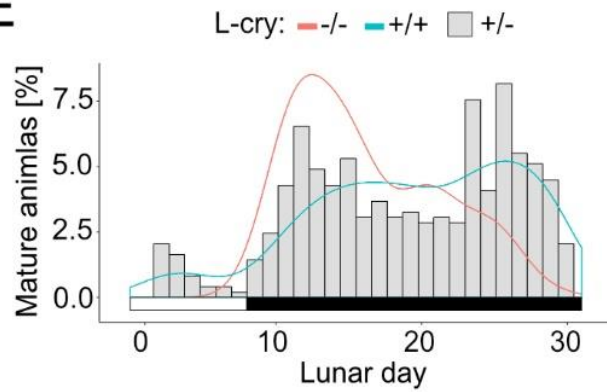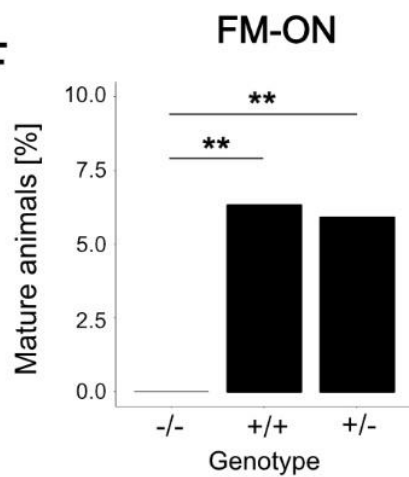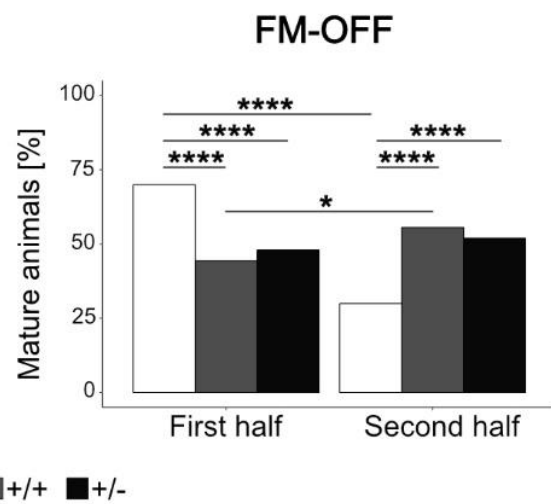
Figure 1. Circalunar sexual maturation patterns. (A) *Median age upon maturation between L-cry$^{-/-}$ and L-cry$^{+/+}$ genotypes. Hollow grey dots represent individual animals (**** p ≤ 0.0001, ns. p> 0.05- one-way ANOVA analysis).* **(B)** *Kernel's density estimate of age upon maturation between L-cry$^{-/-}$ (pink), L-cry$^{+/-}$ (grey) and L-cry$^{+/+}$ (blue) animals.* **(C)** *Ribbon representing the nocturnal illumination and experimental lunar month phases taken for statistical analysis.* **(D)** *Proportion of L-cry$^{-/-}$ (pink bars) and L-cry$^{+/+}$ (blue bars) animals maturing per day of a lunar month. Ribbon on the x-axis represents nocturnal illumination in the animal culture mimicking natural moonlight (FM-ON= white, FM-OFF= black).* **(E)** *Comparison of Kernel's density estimate for L-cry$^{-/-}$ (pink line) and L-cry$^{+/+}$ (blue line) mature animals per day of a lunar month and proportion of mature L-cry$^{+/-}$ animals (grey bars).* **(F)** *Ratio of mature animals in the FM light ON phase (FM-ON)−− and total number of animals per genotype (L-cry$^{-/-}$, L-cry$^{+/-}$ and L-cry$^{+/+}$); (** p ≤ 0.01- Chi-squared test for equality of proportions).* **(G)** *Ratio of mature animals in subgroups of the FM light OFF phase (FM-OFF) to a total number of FM-OFF animals pre genotype (L-cry$^{-/-}$ = white, L-cry$^{+/+}$ = grey, L-cry$^{+/-}$ = black); (* p ≤ 0.05, **** p ≤ 0.0001- Chi-squared test for equality of proportions).*

presented by animals (Figure 1c). FM-ON is representative of a full moon phase, when in nature moon light is at its maximum intensity. FM-OFF phase does not mimic the gradual waxing and waning of the natural moonlight but instead nocturnal illumination is completely omitted.

When I visualized proportions of mature animals per day of a lunar month the most prominent difference was no *L-cry$^{-/-}$* animals maturing during the FM-ON phase, contrary to small percentage of L-cry$^{+/-}$ and L-cry$^{+/+}$ maturing animals (Figure 1d-e). Instead, *L-cry$^{-/-}$* animals start maturing on the very first day of the FM-OFF and peak soon after. To contrast, L-cry$^{+/+}$ animals mature during the FM-ON, continue maturing after the FM lights go OFF to peak in the second part of the FM-OFF phase. Interestingly, *L-cry$^{+/-}$* have distinct peaks in first and second part of the FM-OFF phase. Since FM-ON and -OFF phases are of different lengths I performed separate statistical tests on the groups. According to Chi-squared test for equality of proportions, during the FM-ON phase

significantly less L-$cry^{-/-}$ animals mature than L-$cry^{+/+}$ (Figure 1f; p= 4.87x10^{-3}) or L-$cry^{+/-}$ (p= 6.48x10^{-3}). Also, significantly more L-$cry^{-/-}$ animals mature in the first as opposed to the second part of FM-OFF phase (Figure 1g, p= 5.76x10^{-10}). In contrast, differences in maturation of L-$cry^{+/+}$ animals between the two parts of FM-OFF are less severe (p= 2.6x10^{-3}) and there is no difference for L-$cry^{+/-}$ animals (p = 0.25). This data shows L-$cry^{-/-}$ animals have a strikingly different spawning pattern than L-$cry^{+/+}$ and L-$cry^{+/-}$.

4.2 Regeneration capacity

Significant sexual maturation delay I observed in L-$cry^{-/-}$ animals could be due to an interruption in some of the 16 phases of *P. dumerilii* life cycle. In animal culture, 24 h after fertilization jelly encapsulating unfertilized or misdeveloped eggs is removed, but *L-cry* homozygous in-cross batches did not significantly defer in size (data not shown) from heterozygous or wild type batches which leads me to believe the interruption occurres later in development. *Platynereis* spend most of their lifespan as juvenile worms growing in size (segments) so I assumed the interruption had occurred in post-larval stage. Pre-mature (>2 months of age) *P. dumerili* segment numbers do not directly correlate with animal's level of maturity therefor I could not simply compare sibling L-$cry^{-/-}$ and L-$cry^{+/+}$ animals to detect a maturation delay. However, *Platynereis* possess an extraordinary ability to regenerate most of their posterior trunks. Even though developmental and regeneration pathways do not necessarily use identical cellular mechanisms they likely overlap so regeneration of posterior injury (amputation) is often the approach in post-larval *Platynereis* development research (22,23). To test the hypothesis that sexual maturation delay is a caused by impairment of developmental mechanisms mirrored in regeneration capacity, I performed a controlled amputation by making a cut perpendicular to the anterior-posterior axis, 4-5 segments above a terminal pygidium (posterior

end of the trunk containing anus) and followed their recovery over several days post-amputation (dpa). For statistical purposes I treated the terminal pygidium as one of the segments (5-6 segments cut).
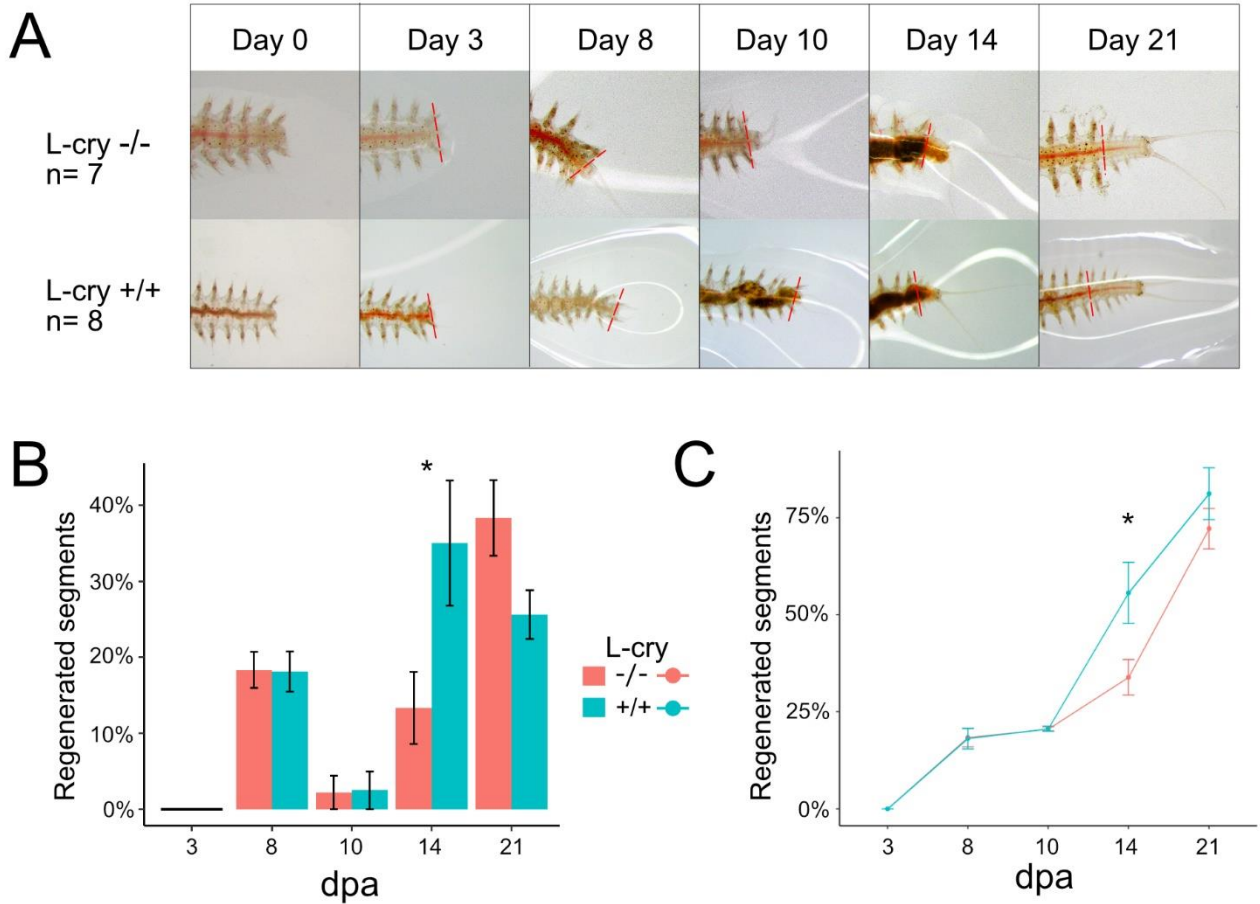
Figure 2. Comparison of regeneration capacities following a posterior trunk amputation. (A) *Representative progression of regeneration over time for L-cry$^{-/-}$ (top) and L-cry$^{+/+}$ (bottom) animals.* **(B)** *Cumulative proportion of regenerated segments over time in L-cry$^{-/-}$ (pink) and L-cry$^{+/+}$ (blue). Error bars signify SEM (*p < 0.05; Welch two sample t-test).* **(C)** *Proportion of regenerated segments between observed days post-amutation (dpa). Error bars signify SEM (L-cry$^{-/-}$ = pink, L-cry$^{+/+}$ = blue; *p < 0.05; Welch two sample t-test).*

There was no difference in regeneration rates up to 10 days post-amputation (10dpa), but between 10 and 14dpa *L-cry$^{-/-}$* regeneration rate dropped significantly. At 3dpa both *L-cry$^{-/-}$* and *L-cry$^{+/+}$* animals closed the

injury and generated outgrowth with a slight posterior indentation (Figure 2a). 8dpa revealed most of the animals regenerated the terminal pygidium, growing small anal cirri (tentacle-like extensions attached to pygidium). Anal cirri grew in length by 10dpa and well defined pygidium was distinguishable from the anterior segment addition zone showing new segments in formation. Some of the 10dpa segments developed paired parapodia (lateral outgrowths necessary for crawling), which I regarded as regenerated, while others were only visible as undifferentiated tissue formations separated by developing segmental grooves. However, 14dpa revealed a significant delay in regeneration of L-$cry^{-/-}$ animals where between 10 and 14dpa L-$cry^{-/-}$ regenerated 13.33 ± 4.71 % of the segments cut, while L-$cry^{+/+}$ animals regenerated 35 ± 8.23 % of segments (Figure 2c; p= 4.28x10^{-2}; Welch two sample t-test). By 21dpa L-$cry^{-/-}$ animals were able to compensate for the delay. At the end of the experiment (21dpa) L-$cry^{-/-}$ animals regenerated a total of 72.22 ± 5.21 % of segments, whereas L-$cry^{+/+}$ regenerated 81.25 ± 6.64 %, which at n(L-$cry^{-/-}$)= 7 and n(L-$cry^{+/+}$)= 8 is not significantly different (p=0.813; Welch two sample t-test).

4.3 Circadian rhythmicity under 16:8 light-dark (LD) regime

After noticing L-$cry^{-/-}$ animals exhibit a change in circalunar behavior I wondered if consequently their circadian rhythmicity is affected as well. In order to see if animals have any steady locomotor patterns in standard culture room conditions I recorded L-$cry^{-/-}$ and L-$cry^{+/+}$ animals under 16 h white light followed by 8 h of dark for 56 h and analyzes the locomotor output. To account for circalunar influence, I performed the experiment during new moon (NM) and free-running full moon (FR-FM) phase. In astronomy, NM is a moon phase in which moon disc is not visible in the sky (dark moon) which in laboratory conditions translates to complete darkness. FR-FM is a time frame (8 days) when in the culture room animals would be subjected to a dim nocturnal light (full capacity of lunar illumination), but

in an experimental set up the "moonlight" is omitted to avoid the masking effect, making it free-running (reliant solely on the endogenous circalunar clock and not external cues). On inspecting individual locomotor activity data (Figure 3b/d; representative individuals), I noticed there were no extreme deviations which could potentially skew the average. I visualized the locomotor activity by comparing average distances traveled between consecutive time points for *L-cry*$^{-/-}$ and *L-cry*$^{+/+}$ animals under NM (Figure 3a) and FR-FM (3c). Considering activity average is not suitable for quantifying the behavior, I used individual actograms (data not shown) to analyze locomotion and perform Lomb-Scargle period analysis. For statistics, I pooled individual values for distance traveled per hour (distance/h) of light or dark, Lomb- Scargle period and power.

A

B

MUTANT 1   25.77

MUTANT 2   28.5

MUTANT 3   23.75

MUTANT 4   19.63

WILD TYPE 1   24.92

WILD TYPE 2   24.32

WILD TYPE 3   24.92

WILD TYPE 4   24.98

L-cry: -/-   +/+

FR-FM

C

D

MUTANT 1		WILD TYPE 1	
MUTANT 2	27.12	WILD TYPE 2	24.45
MUTANT 3	26.33	WILD TYPE 3	27.12
MUTANT 4	24.78	WILD TYPE 4	28.58
	23.8		25.47
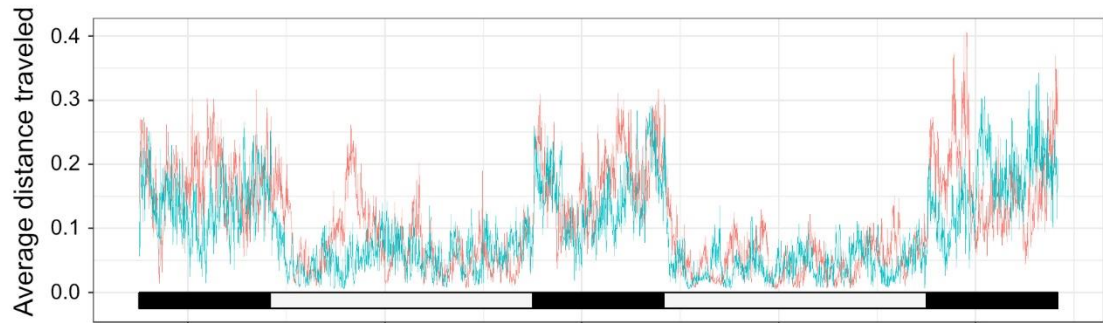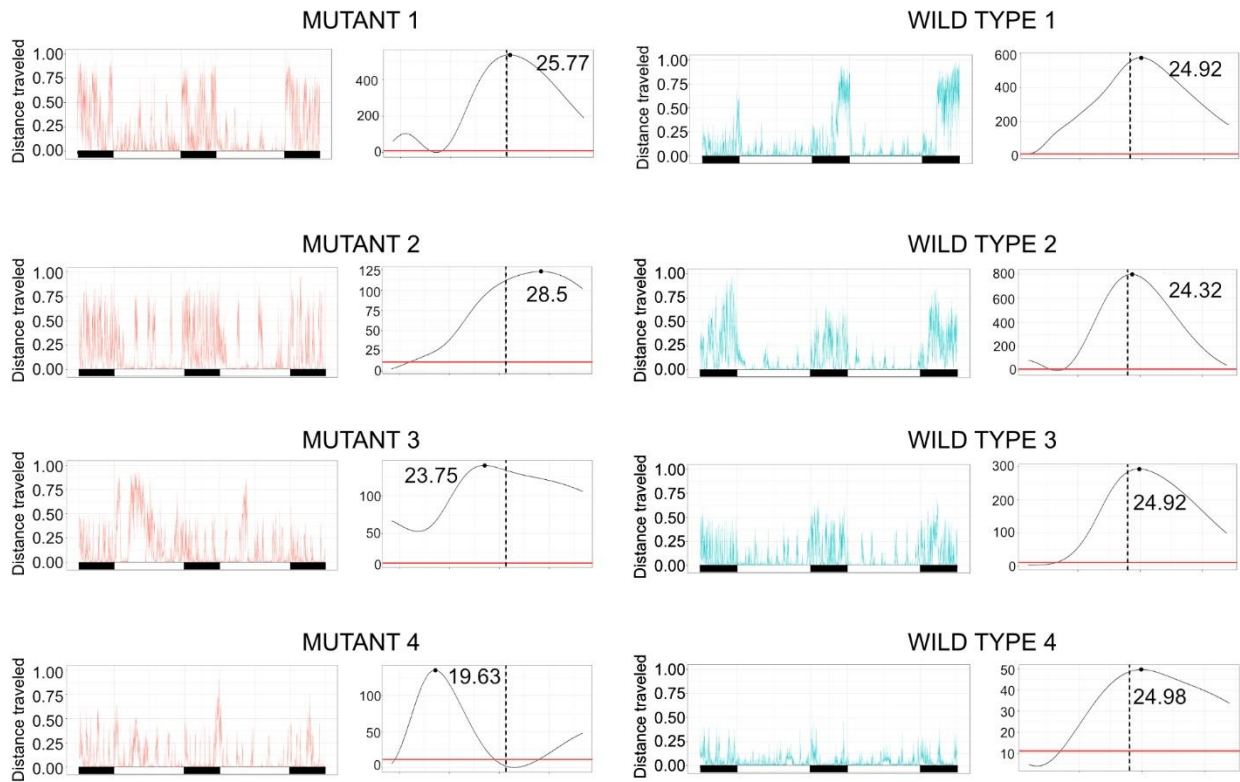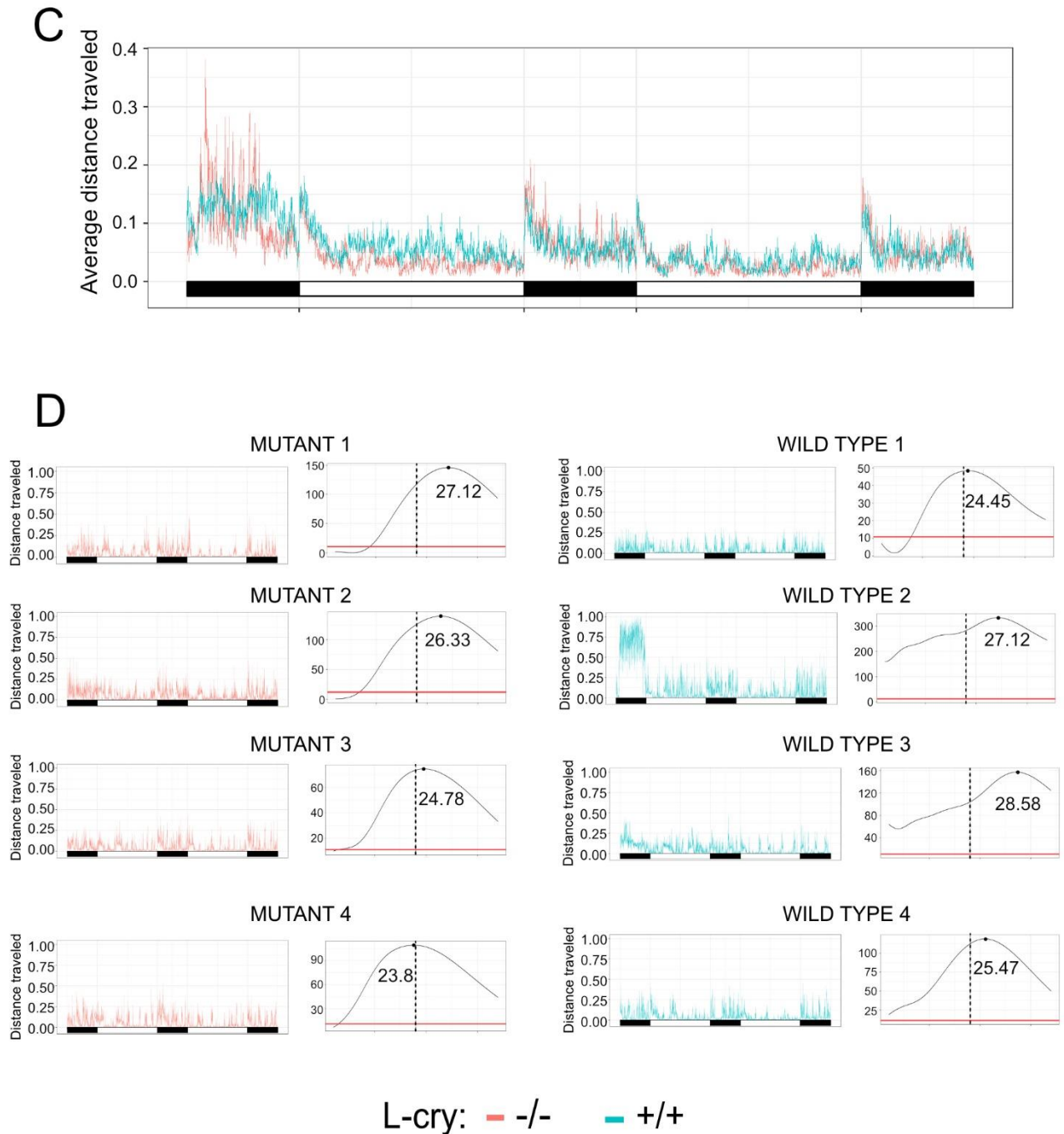
L-cry: — -/-   — +/+

Figure 3. 16:8 LD locomotor activity under new moon (NM) and free-running full moon (FR-FM). *L-cry^{-/-}= pink lines, L-cry^{+/+}= blue lines.* **(A)** *Average locomotor activity under NM.* **(B)** *Representative individual activity plots and their respective Lomb-Scargle periodograms.* **(C)** *Average locomotor activity under FR-FM.* **(D)** *Representative individual activity plots and their respective Lomb-Scargle periodograms.; Ribbons on the x-axis of activity plots depict lighting*

By using a paired rank sum test to compare individual distance/h traveled during light versus dark I discovered *L-cry^{-/-}* animals are considerably more nocturnal (mostly active during the dark) than *L-cry^{+/+}*, in both NM (p(*L-cry^{-/-}*)= $4.883 \cdot 10^{-3}$, p(*L-cry^{+/+}*)= $1.061 \cdot 10^{-2}$; Figure 4c) and FR-FM experiments (p(*L-cry^{-/-}*)= $4.883 \cdot 10^{-4}$, p(*L-cry^{+/+}*)= $4.883 \cdot 10^{-3}$; Figure 4d). Although, both *L-cry^{-/-}* and *L-cry^{+/+}* individuals increase the nocturnal to diurnal activity ratio under FR-FM the difference of one order of magnitude stays constant between moon phases. This shows *L-cry^{-/-}* animals are consistently more nocturnal than their *L-cry^{+/+}* controls in 16:8 LD, possibly due to increased sensitivity to masking effects of light.

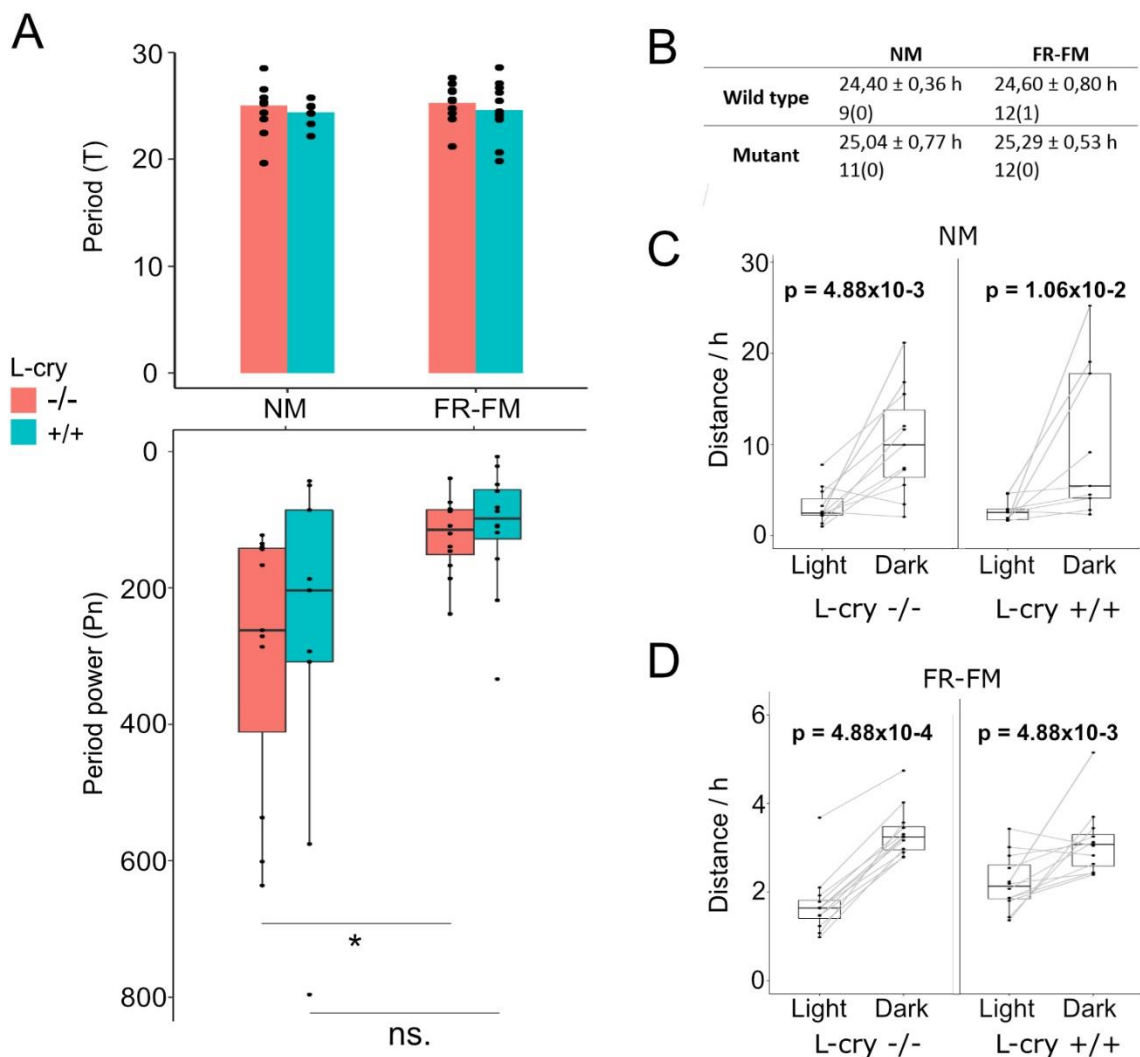|	NM	FR-FM
Wild type	24,40 ± 0,36 h 9(0)	24,60 ± 0,80 h 12(1)
Mutant	25,04 ± 0,77 h 11(0)	25,29 ± 0,53 h 12(0)

Figure 4. Quantification of 16:8 LD locomotor activity under new moon (NM) and free-running full moon (FR-FM). (A) *Average Lomb-Scargle period (T) of L-cry$^{-/-}$ (pink bars) versus L-cry$^{+/+}$ animals (blue bars) under NM and FR-FM (top); corresponding Lomb-Scargle power (Pn) in a box and whisker plot (bottom). Black dots represent individual animals (*p ≤ 0.05- Welch two sample t-test).* **(B)** *Summary table of Lomb-Scargle period analysis over 56 h. [period ± SEM h; total number of animals (number of arrhythmic individuals)].* **(C-D)** *Comparison of distances traveled per hour (distance/h) of light and dark under NM (C) and FR-FM (D). Black dots represent individual animals and gray lines connect paired measurements (* p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001; paired rank sum test).*

In both experiments, animals were able to synchronize to the 24 h LD cycle (except one *L-cry$^{+/+}$* individual under FR-FM; Figure 4b), but in *L-cry$^{-/-}$* Lomb-Scargle power significantly dropped under FR-FM compared to NM (Figure 4a). Under NM *L-cry$^{-/-}$* had a 25.04 ± 0.77 h activity period while *L-cry$^{+/+}$* period was 24.40 ± 0.36 h. FR-FM did not significantly change the activity period length with *L-cry$^{-/-}$* being 25.29 ± 0.53 h and L-cry$^{+/+}$ 24.60 ± 0.8 h (Figure 4b). Although Lomb-Scargle period did not change, Welch two sample t-test confirmed power significantly decreased in *L-cry$^{-/-}$* under FR-FM (Figure 2a; p= 1.46x10^{-2}), while in *L-cry$^{+/+}$* rhythm weakening is evident, but not statistically significant (p= 8.59x10^{-2}). This data shows *L-cry$^{-/-}$* ability to behaviorally entrain to a periodic 24 h LD significantly weakened under FR-FM.

## 4.4 Blue light (426 nm) LD phase shift

Since L-Cry is a putative blue light receptor, it is possible ability of *L-cry$^{-/-}$* animals to respond to monochromatic blue light is impaired. To investigate how animals react to monochromatic blue light, I designing an experiment that released them from white light 16:8 LD into a 426 nm light 16:8 BLD (blue light- dark) after a 12h phase shift. This design not only

produced a 12h phase advance in lighting conditions, but also switched the illumination from broad spectrum white light to monochromatic blue light which should minimize exciting too many light receptors in different parts of the spectrum. I conducted the experiment three times under NM and once under FR-FM. In order to simplify data managing I developed a nomenclature for LD intervals relative to CT0 i.e. the moment illumination switched from white to blue light (Figure 5a). This way each interval was given a name consisting of abbreviation of light conditions utilized and a numerical suffix signifying the distance from CT0. Light interval abbreviations were as follows: WL- white light, BL- blue light and D- dark; while numerals were negative (-n) or positive (n) depending on weather the interval occurred prior to or following the light switch. I analyzed individual activity levels by distance traveled per hour (distance/h) of each LD interval (Figure 5b).

Both $L\text{-}cry^{-/-}$ and $L\text{-}cry^{+/+}$ animals showed a transitional activity pattern under NM, meaning their activity was increased at the time they anticipate darkness (BL1), but under FR-FM only $L\text{-}cry^{-/-}$ animals exhibited this behavior. Transitioning from WL-1 to BL1 animals showed no significant changes ($p \geq 0.05$) in distance/h traveled, regardless of genotype ($L\text{-}cry^{-/-}$/$L\text{-}cry^{+/+}$) or lunar conditions (NM/FR-FM; Figure S2a-b). However, comparing BL1 to BL2 under NM (Figure 5c) both $L\text{-}cry^{-/-}$ and $L\text{-}cry^{+/+}$ were significantly more active during BL1 ($p < 1\cdot10^{-3}$), while under FR-FM only $L\text{-}cry^{-/-}$ showed an increase in BL1 activity ($p= 0,042$; Figure 5d).

To see if animals retain their nocturnal behavior in 16:8 BLD conditions I compared BL to D interval distance/h traveled. On average $L\text{-}cry^{-/-}$ and $L\text{-}cry^{+/+}$ animals were able to stay significantly nocturnal ($p < 1\cdot10^{-5}$; Figure 5e/g) under NM. Under FR-FM only $L\text{-}cry^{-/-}$ animals were nocturnal in the first ($p= 2.93\text{x}10^{-3}$; Figure 5f) and the second ($p= 1.95\text{x}10^{-3}$; Figure 5h) BLD cycle. This experiment showed $L\text{-}cry^{-/-}$ animals stay predominantly nocturnal in a periodic BLD illumination even if it is shifted from the LD cycle they are entrained to.
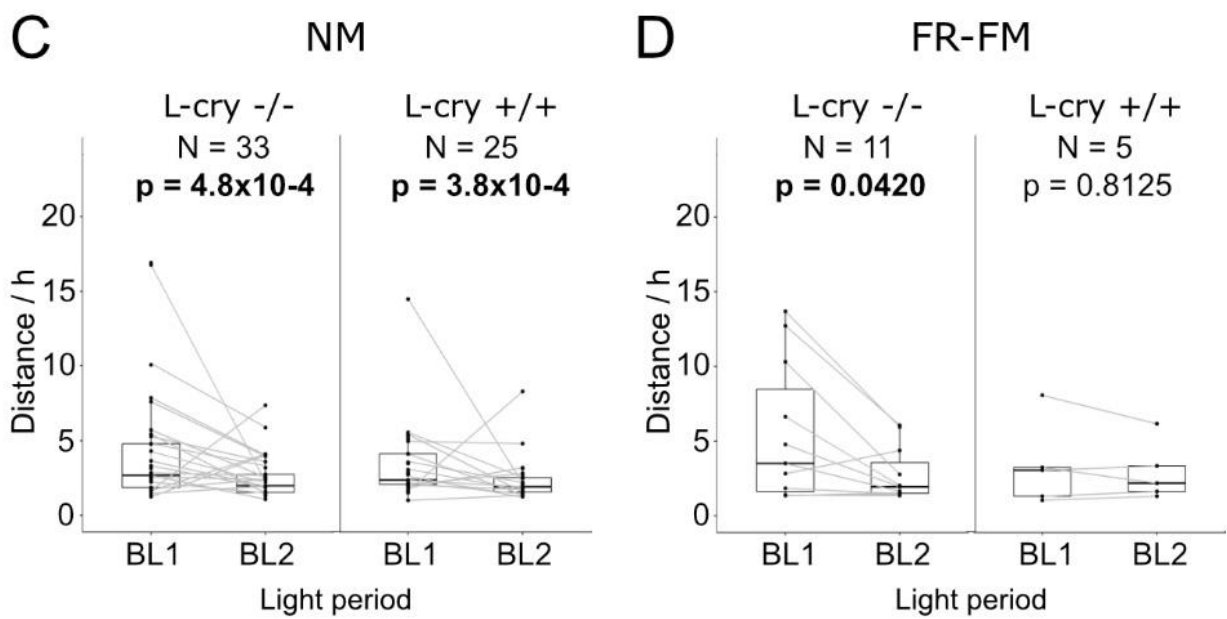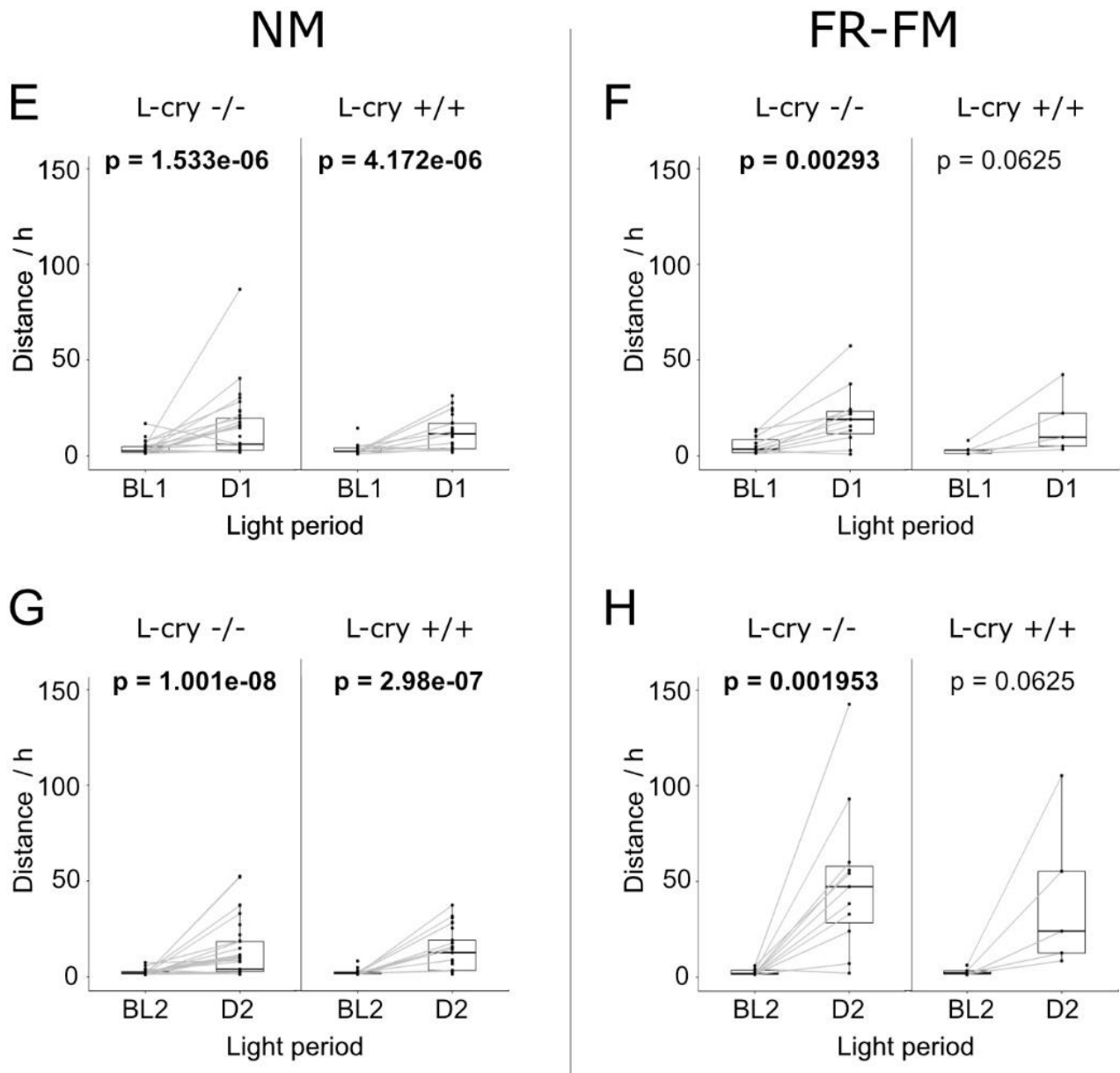
Figure 5. Quantification of phase shifted 426 nm light cycle (BLD) locomotor activity under new moon (NM) and free-running full moon (FR-FM). (A) *Ribbons depicting light conditions animals were entrained to in the culture room and experimental conditions they were exposed to, as well as nomenclature (white light= WL, blue light= BL and dark= D) of different light periods relative to CT0 (-n= prior to CT0, n= following CT0).* **(B)** *Individual distances traveled per hour of a light period between L-cry$^{-/-}$ (pink) and L-cry$^{+/+}$ (blue) animals under new moon (NM) or free-running full moon (FR-FM). Red arrows indicate the time of a phase shift (ns. p> 0.05, * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001; paired rank sum test).* **(C-H)** *Comparison of*

individual distances traveled per hour of BL1 to BL2 under NM (C) and FR-FM (D),
BL1 to D1 under NM (E) and FR-FM (f), BL2 to D2 under NM (G) and FR-FM (H).
Black dots represent individual values and gray lines connect paired measurements
(same individuals).

4.5 Constant darkness free-running period

Since previously addressed daily rhythmicity under periodic LD (Figure 4) is not direct evidence of a functional circadian clock (masking effect), I measured *P. dumerilii* endogenous period under constant conditions. I conducted constant darkness (DD) free-running experiments by releasing *L-cry$^{-/-}$* and *L-cry$^{+/+}$* animals kept in 16:8 LD into DD for a minimum of 72 h. From the collected data I constructed actograms (Figure 6c-d; representative individuals) to perform Lomb-Scargle period analysis. Individuals without a Lomb-Scargle period (power < threshold at p-value= 0.05) or with periods ≥29 h I considered arrhythmic.

Upon repeating the experiment 3 times under NM I observed a significant increase (p < 0.01) in arrhythmic behavior of *L-cry$^{-/-}$*, where 25.71% of *L-cry$^{-/-}$* and 0% of *L-cry$^{+/+}$* animals were arrhythmic (Figure 7b). However, in a FR-FM experiment there was no difference in the percentage of arrhythmic animals between genotypes (33.34% of *L-cry$^{-/-}$* and *L-cry$^{+/+}$* were arrhythmic). Visual inspection of average DD actograms indicated period amplitude decreased under FR-FM (Figure 6). Moreover, Welch two sample t-test revealed a significant decline in individual Lomb-Scargle power under FR-FM for both *L-cry$^{-/-}$* and *L-cry$^{+/+}$* (only rhythmic individuals; Figure 7a). This data shows a significant increase in arrhythmic *L-cry$^{+/+}$* DD behavior under FR-FM, as well as substantial increase (≥ 25%; NM and FR-FM) of *L-cry$^{-/-}$* arrhythmicity overall. In spite of arrhythmicity, majority of *L-cry$^{-/-}$* and *L-cry$^{+/+}$* animals were entrained to the 24 h LD cycle they were raised in, but their circadian rhythm became weaker in anticipation of full moon (FR-FM).
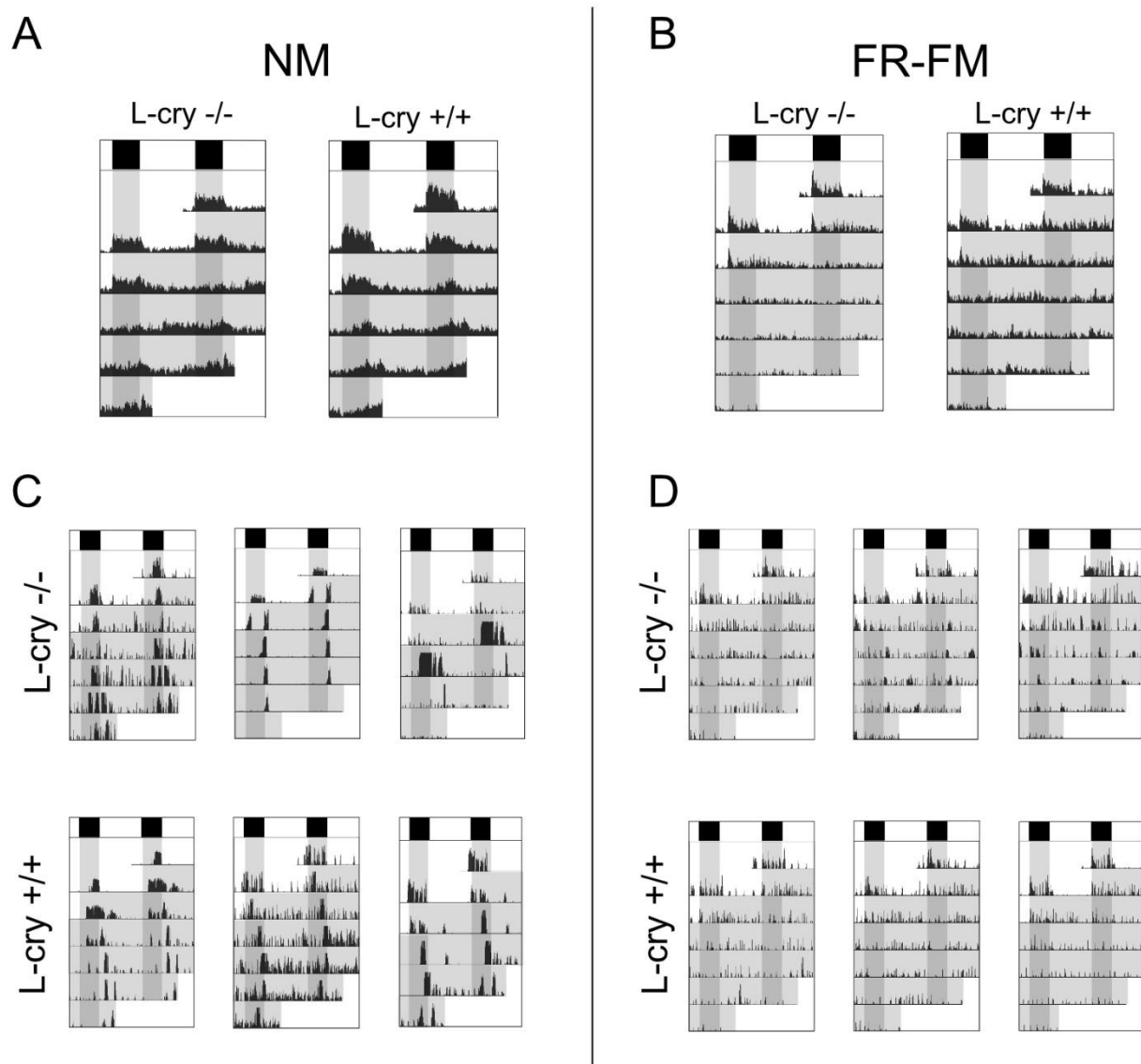
Figure 6. Periodograms from new moon (NM) and free-running full moon (FR-FM) constant dark (DD) free-run. (A-B) Average DD free-running periodograms under NM (A) and FR-FM (B) *(C-D)* Representative individual DD free-running periodograms under NM (C) and FR-FM (D). Ribbons on top of actograms represent 16:8 LD conditions animals were released from into DD (dark= black, white light= white). For easier visual inspection of actograms, light grey areas represent time animals spent in dark and dark grey areas represent time in DD when they would experience 8 h of dark if not released from LD.
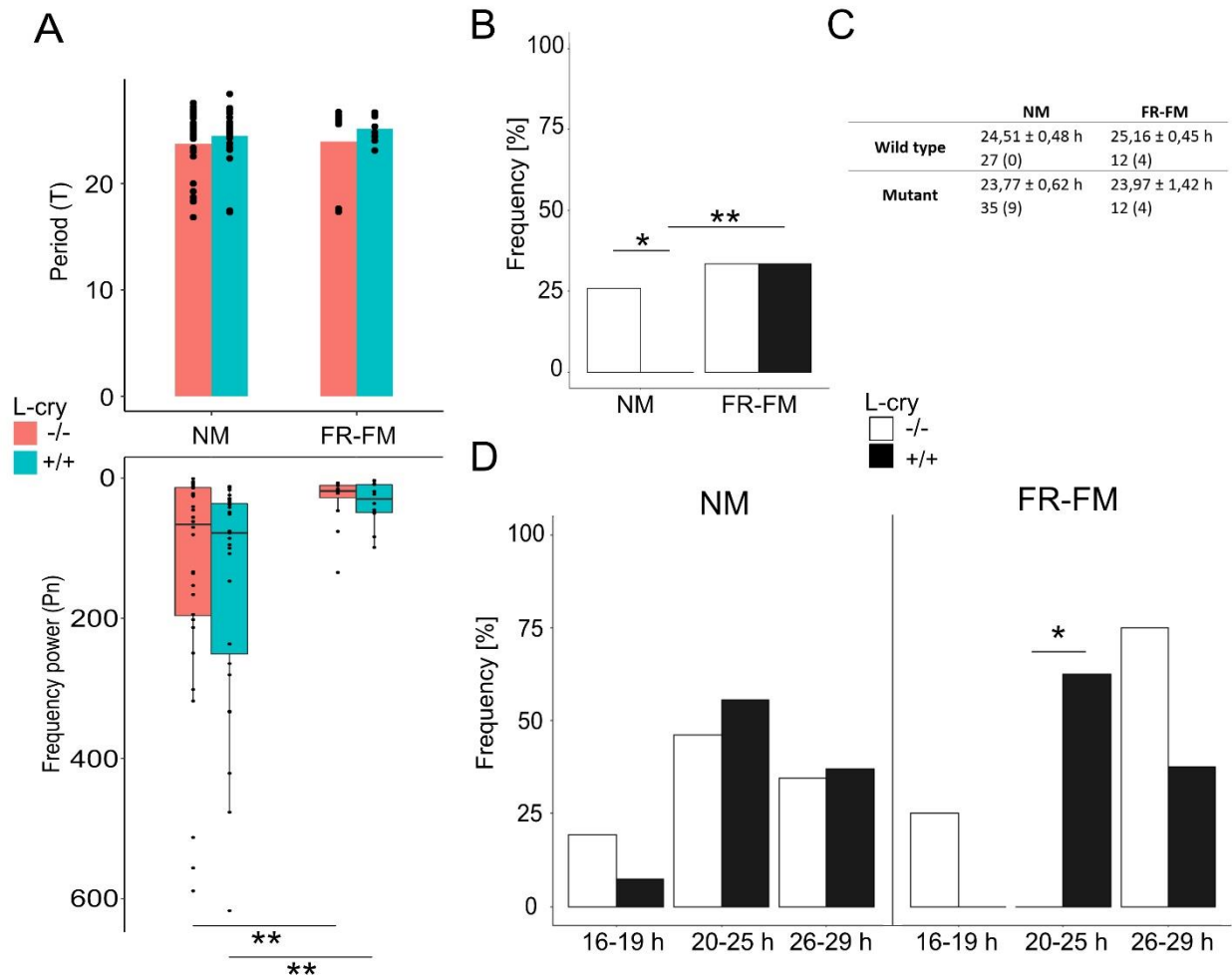
Figure 7. Quantification of constant dark (DD) free-running locomotor activity under new moon (NM) and free-running full moon (FR-FM). (A) *Average Lomb-Scargle period of L-cry^-/- (pink bars) versus L-cry^+/+ (blue bars) under NM and FR-FM (top); respective Lomb-Scargle power in a box and whisker plot (bottom). Black dots represent individual animals (**p ≤ 0.01- Welch two sample t-test).* **(B)** *Proportion of arrhythmic L-cry^-/- (white) versus L-cry^+/+ animals (black) in DD under NM and FR-FM (* p ≤ 0.05, ** p≤ 0.01- Chi-squared test for equality of proportions).* **(C)** *Summary table of Lomb-Scargle period analysis over 72h [period ± SEM h; total number of animals (number of arrhythmic individuals)].* **(D)** *Frequency of individual L-cry^-/- (white) and L-cry^+/+ (black) free-running period lengths per 16-19 h, 20-25 h and 26-29 h group under DD/NM (left) and DD/FR-FM (right); (* p ≤ 0.05- Chi-squared test for equality of proportions).*

Among rhythmic subjects, average period length in DD under NM was 25.77 ± 0.62 h for *L-cry*$^{-/-}$ and 24.51 ± 0.48 h for *L-cry*$^{+/+}$ animals, whereas under FR-FM it was 23.97 ± 1.42 h and 25.16 ± 0.45 h respectively (Figure 7c). While there was no significant differences in period length between genotypes under NM or FR-FM I noticed a peculiar pattern in individual period distributions (Figure 7a). To take a closer look at the rhythmic data structure I divided individuals in 3 distinct groups by period length (Figure 7d). Although intervals were arbitrary, I aimed to have a group that includes the approximate circadian period (20-25 h) so by default, the rest of the data span fell into two remaining groups of 16-19 h and 26-29 h. This approach revealed a similar distribution pattern between *L-cry*$^{-/-}$ and *L-cry*$^{+/+}$ periods under NM, with least animals falling into the first group (16-19 h). As evident from NM average periods, majority of animals were in the 20-25 h group (*L-cry*$^{-/-}$= 46.15% and *L-cry*$^{+/+}$= 55.56%). Though distribution of period lengths did not significantly differ under NM, under FR-FM all the *L-cry*$^{-/-}$ animals occupied period groups shorter or longer than 20-25 h, which is significant compared to 62.50% of *L-cry*$^{+/+}$ animals with a 20-25 h period (p= 4.41x10^{-2}). It is interesting to note that during FR-FM there was 25% of *L-cry*$^{-/-}$ to 0% of *L-cry*$^{+/+}$ in the 16-19 h group. However, due to low numbers of rhythmic animals in the FR-FM experiment (n= 8 for *L-cry*$^{-/-}$ and *L-cry*$^{+/+}$) the difference was not statistically significant (p= 0.388). This analysis of data structure revealed a significant splitting of period lengths in rhythmic *L-cry*$^{-/-}$ animals under FR-FM.

4.6 Blue light (426 nm) constant pulsing

After seeing *L-cry*$^{-/-}$ animals are entrained to the culture room 24 h LD cycle and can adjust to a 24 h BLD cycle under NM, as do *L-cry*$^{+/+}$, I intended to utilize 426nm light to disrupt the circadian clock of *P. dumerilii*. It is common to use light pulses in phase shift experiments, which restart the circadian clock by causing a phase advance or delay, depending on timing

of the pulse (24). Using this notion I aimed to test the robustness of *Platynereis* circadian clock by continuously exposing animals to 2 h 426nm light pulses intermitted by 2 h of darkness during NM. I chose the 2 h interval because I assumed it would give animals enough time to exhibit an acute locomotor reaction and potentially influence endogenous machinery via light induced/suppressed transcription, as well as due to technical constrains of the experimental set-up.
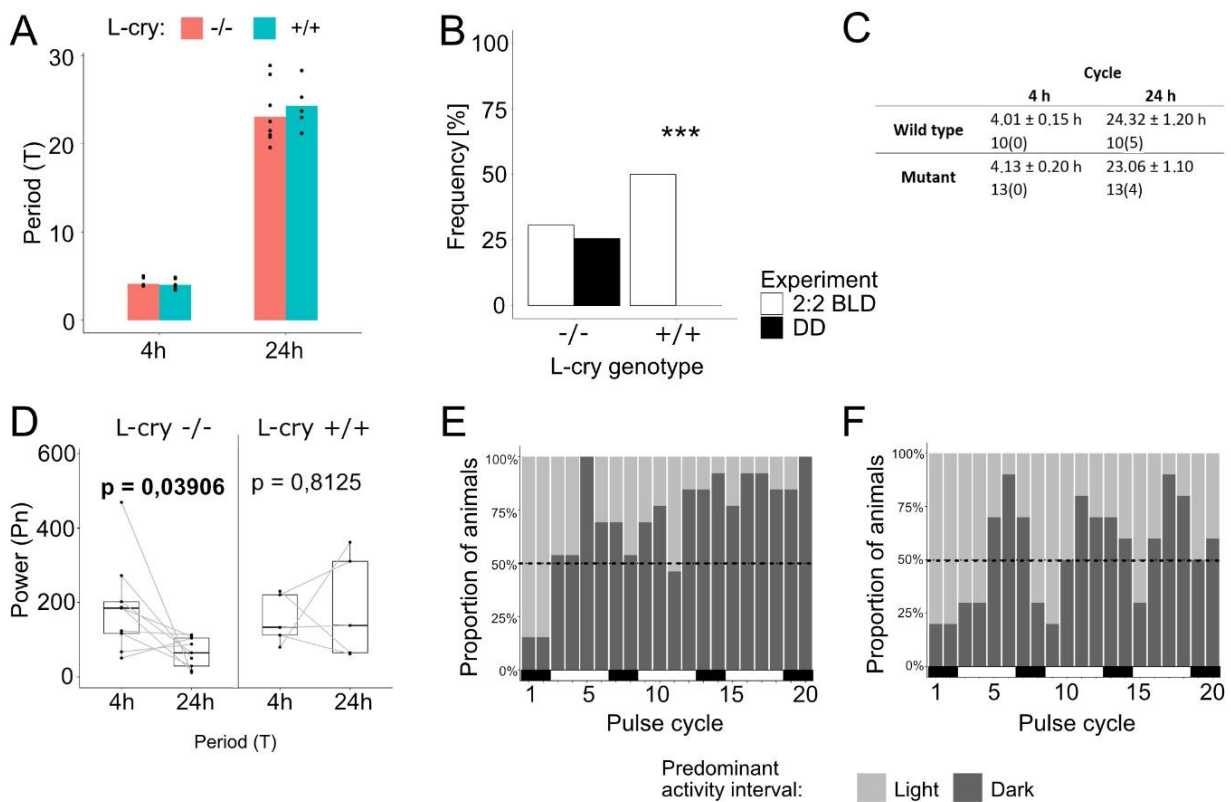
Figure 8. Blue light (426 nm) pulsing- circadian disruption. (A) *Lomb-Scargle period for 4 and 24h cycles between L-cry$^{-/-}$ (pink bars) and L-cry$^{+/+}$ (blue bars). Black dots represent individual animals.* **(B)** *Proportion of arrhythmic animals in 2:2 BLD pulsing (white bars) versus DD free-run (black bars) experiment; (*** p ≤ 0.001- Chi-squared test for equality of proportions).* **(C)** *Summary table of Lomb-Scargle period analysis over 80h [period ± SEM h; total number of animals (number of arrhythmic individuals)].* **(D)** *Comparison of Lomb-Scargel power for 4 and 24 h cycles. Black dots represent individual values and grey lines connect paired measurements (ns. p> 0.05, * p ≤ 0.05; paired rank sum test).* **(E-F)** *Comparison of L-cry$^{-/-}$ (E) and L-cry$^{+/+}$ (F) animals according to*

First, I placed the animals in the experimental set-up to settle down while mimicking the 16:8 LD culture room conditions. 2 h after white lights went off (ZT18) I initiated the 2:2 BLD protocol and pulsed the animals for the following 78 h. First 2 h of dark were included in my data analysis which gave me a time series of 80 h, subdivided into 20 pulsing cycles (2 h dark + 2 h light pulse= 4 h BLD cycle). I analyzed their rhythmicity with Lomb-Scargle period analysis for 4 h (pulsing) and 24 h (circadian) cycles, as well as calculated ratios of distance/h traveled between consecutive dark and blue light intervals (Figure 8).

All the animals were behaviorally entrained to the 4 h pulsing cycle administered (T4(*L-cry$^{-/-}$*)= 4.13 ± 0,20 h; T4(*L-cry$^{+/+}$*)= 4.01 ± 0.15 h; Figure 8a/c), meaning they were adjusting to a 4 h pulsing cycle by an equally long locomotor activity cycle. Although this is not a classical free-running protocol, there were no indicators of a 24 daily cycle so I assumed constant pulsing will allow animals to free-run, as well as challenge their circadian clocks through constant re-entrainment. In fact, 50% of *L-cry$^{+/+}$* and 69.23% of *L-cry$^{-/-}$* animals still showed a circadian period of approximately 24 h. Among the rhythmic animals there was no significant difference between *L-cry$^{-/-}$* and *L-cry$^{+/+}$* animals in Lomb-Scargle 24 h period length (T24(*L-cry$^{-/-}$*)= 23.06 ± 1.10 h; T24(*L-cry$^{+/+}$*) 24.32 ± 1.20 h; Figure 8a/c). While rhythmic *L-cry$^{+/+}$* animals displayed no difference between 4 h and 24 h Lomb-Scargle power, *L-cry$^{-/-}$* 24 h period power was significantly weaker than 4 h period power of same animals (p= 3.91×10^{-2}; Figure 8d). This data shows portions of *L-cry$^{-/-}$* and *L-cry$^{+/+}$* are able to behaviorally entrain to a 4 h LD cycle (pulsing) while still exhibiting background circadian rhythmicity.

To see if animals were able to maintain their predominantly nocturnal locomotion pattern during the 2:2 BLD experiment I utilized ratios of consecutive BL:D distance/h and followed the changes over 20 cycles. To explain, if animal was given a value < 1 it moved mostly during the dark interval and if value was ≥ 1 it was mostly active during the blue light pulse, of a given pulsing cycle. Visually inspecting proportions of *L-cry*$^{-/-}$ animals based on BLD intervals by predominant activity (Figure 8e) it is clear that as the pulsing progresses majority of *L-cry*$^{-/-}$ (>50%) are nocturnal, with 100% of nocturnal *L-cry*$^{-/-}$ animals during the last pulsing cycle. To contrast, *L-cry*$^{+/+}$ nocturnal behavior fluctuates over time and only 60% of animals are nocturnal by the end of the experiment. This data shows *L-cry*$^{-/-}$ animals were more precise at following the 4 h BLD cycle, maintaining their nocturnal behavior. This is likely due to the masking effect of periodic BLD rather than endogenous *L-cry*$^{-/-}$ mechanisms, since some *L-cry*$^{+/+}$ animals (data not shown) clearly become diurnal over the course of experiment but still follow the 4 h cycle.

5 Discussion

Platynereis dumerilii possess light entrained circalunar and circadian clocks controlling lunar timing of sexual maturation and daily locomotor activity respectively (12). Cryptochromes were confirmed to act as blue light receptors mediating light entrainment in animals like *Soganus luridus* and *D. melanogaster* (11,25). *Platynereis* possess a light responsive drosophila-type cryptochrome ortholog *L-cry* (12), so I hypothesized that *L-cry* loss-of-function *Platynereis* mutants (*L-cry$^{-/-}$*) exhibit difficulties in circalunar and circadian function as well as possible downstream mechanisms like development, maturation and regeneration. My experiments confirmed *L-cry$^{-/-}$* exhibit a maturation delay likely caused by an overall developmental delay, altered lunar timing of sexual maturation and circadian rhythmicity issues indicating they are more susceptible to masking effect of light.

5.1 Sexual maturation delay and the circalunar clock

In spite of the circalunar clock advising animals to spawn shortly after new moon, in our laboratory culture approximately 6% of *L-cry$^{+/-}$* and *L-cry$^{+/+}$* animals (figure 1d-e) spawned during the full moon (FM-ON). To contrast, none of the 116 *L-cry$^{-/-}$* animals matured during FM-ON (p< 7·10^{-3}; Figure 1f). Even though *L-cry$^{-/-}$* maturation seems to have a circalunar rhythm in periodic lunar conditions it is significantly different from *L-cry$^{+/-}$* and *L-cry$^{+/+}$* maturation patterns. *L-cry$^{-/-}$* maturation peaks soon after the FM lights go off while *L-cry$^{+/+}$* maturation peaks towards the end of FM-OFF, which in nature translates to 'soon after NM'. *L-cry$^{+/-}$* spawning pattern looks like a sum of both *L-cry$^{-/-}$* and *L-cry$^{+/+}$* patterns (Figure 1e), which indicates that one functioning *L-cry* allele is influencing the circalunar periodicity. Both *L-cry$^{-/-}$* and *L-cry$^{+/-}$* spawning patterns suggest a dominant role of *L-cry* in timing of sexual maturation. Since *L-cry$^{-/-}$* spawn shortly

after FM-ON this suggests they react directly to FM-ON, which is possible without an endogenous clock.

To test the endogenous L-$cry^{-/-}$ circalunar period the experiment should be performed in free-running lunar conditions. L-$cry^{+/+}$ could serve as positive control, as they show free-running period in constant lunar conditions (12). Since *Platynereis* are nocturnal by nature, the best way to test their free-running circalunar period would be to observe them under constant darkness. Animals would have to entrain to standard culture room conditions (16:8 LD, 8 days FM-ON) for at least 2 lunar cycles and then released into 16:8 LD without any nocturnal illumination. If their circalunar rhythmicity would persist over several consecutive free-running lunar cycles (months) this would indicate a circalunar clock driving the rhythmicity. Furthermore, circalunar phase shift experiment would tell us if their circalunar clock can be entrained to nocturnal light. Phase shift can be achieved by changing the timing of the 8 day FM-ON phase to occur either sooner or later than scheduled during the entrainment. In case of the lunar phase shift experiment, if the free-running circalunar period would be in phase with the altered FM-ON illumination schedule this would indicate that circalunar clock is fully functional (free-running circalunar period and entrained by FM-ON). Based on the observed L-$cry^{-/-}$ spawning pattern I suspect this would not be the case.

Although circalunar clock controls the temporal aspect of *P. dumerilii* spawning its influence on maturation itself has not been observed yet. In fact, compared to L-$cry^{+/+}$, L-$cry^{-/-}$ take 3 additional lunar cycles to start maturing (Figure 1b). L-$cry^{-/-}$ population matures more gradually so animal age upon spawning curve is bell shaped while L-$cry^{+/-}$ and L-$cry^{+/+}$ curves show predominant maturation at the younger age (curves are left skewed). Significant maturation delay ($p < 1 \cdot 10^{-7}$, Figure 1a) is not necessarily a direct result of changes in the circalunar clock. Interestingly, I did not observe a maturation delay in L-$cry^{+/-}$ since they peak around the same age as L-$cry^{+/+}$ (Figure 1a-b), suggesting L-cry does not have a dominant role in the process of maturation. Downstream mechanisms influenced by the

circalunar clock or the circadian clock, proven to be under circalunar control (12), can affect growth and maturation. Previous unpublished observations from the laboratory suggested that pre-mature L-$cry^{-/-}$ animals (>2 months of age) develop slower that their L-$cry^{+/+}$ siblings. Due to unpredictable nature of pre-mature development, measuring animal's size and comparing it to the stage of sexual maturity was not a viable option in elucidating the maturation delay. To bypass the issue of developmental variability, I utilized regeneration capacity as an alternative protocol. Although molecular details are not fully clarified, *P. dumerilii* segment growth and regeneration, after formation of pygidium and reestablishment of a segment addition zone, are morphologically identical (17,21) and activate some of the same homeobox (Hox) genes (22,23).

Since regeneration is an accelerated form of growth, regeneration after amputation of a caudal trunk is a common way to study post-larval development in *P. dumerilii*. I compared L-$cry^{-/-}$ and L-$cry^{+/+}$ regeneration capacity by amputating 4-5 caudal segments and following their recovery on 3,8,14 and 21 days post-amputation (dpa). Upon inducing posterior caudal amputation, I observed a delay in regeneration of L-$cry^{-/-}$ between 10 and 14 dpa (Figure 2b-c), which they were able to compensate with time (21 dpa). I performed the regeneration assay blindly, by using the amputated trunk portions to genotype the animals in retrospect, and used animals from *L-cry* heterozygous in-cross batches. This way I wanted to eliminate experimenter's bias, but this resulted in large reduction of L-$cry^{-/-}$ and L-$cry^{+/+}$ genotypes as majority of animals were heterozygous (L-$cry^{+/-}$) or damaged during the course of experiment (excluded). Animal numbers (n(L-$cry^{-/-}$)= 7, n(L-$cry^{+/+}$)= 8) are a potential issue when assessing reliability of my data. To confirm the phenotype, additional experiments with larger number of animals are necessary. Detected regeneration delay resulting in full recovery over time is consistent with unpublished observations of L-$cry^{-/-}$ animals developing slower that their siblings as well as L-$cry^{-/-}$ animals maturing and producing viable offspring, but on a delayed schedule.

Though current body of literature offers no link between *P. dumerilii* regeneration, development and chronobiological mechanisms, Hox genes widely involved in *P. dumerilii* regeneration and anteroposterior-axis development (22,23,26) could be influenced by circadian or circalunar clocks causing discrepancies in *L-cry⁻/⁻*. Previous research showed that stem cells located at segment addition zone involved in *P. dumerilii* posterior regeneration and development that express Hox genes are related to primordial stem cells (20), giving rise to gametes. Thus, maturation delay could be a result of an overall developmental delay with discrepancies in growth through segment addition, regeneration and gametogenesis.

5.2 Circadian rhythmicity in periodic light-dark conditions

After detecting changes in *L-cry⁻/⁻* circalunar behavior I hypothesized that *L-cry⁻/⁻* might consequently show defects in circadian rhythmicity. Considering there was no circadian research done on *P. dumerilii L-cry⁻/⁻,* I first tried to establish if *L-cry⁻/⁻* exhibit rhythmic locomotion patterns similar to *L-cry⁺/⁺* (12) when reacting to periodic LD conditions. I recorded them in 16:8 LD, utilizing white light, during NM and FR-FM and compared nocturnal behavior, Lomb-Scargle circadian period and power between *L-cry⁻/⁻* and *L-cry⁺/⁺*.

L-cry⁻/⁻ are significantly more nocturnal than *L-cry⁺/⁺* (by an order of magnitude under NM and FR-FM; Figure 4c-d), however there is no significant difference in period power between genotypes (Figure 4a). Lomb-Scargle period power reflects the strength of the rhythm, but activity amplitudes do not directly influence it. Stronger nocturnal behavior suggests *L-cry⁻/⁻* animals are more reactive to masking effect of periodic illumination. With 16:8 LD experiments I successfully demonstrated that both *L-cry⁻/⁻ and L-cry⁺/⁺* animals are behaviorally entrained to 24 h periodic

LD and predominantly nocturnal, which are *P. dumerilii* intrinsic characteristics.

Contrary to current literature (12), in my experiments $L\text{-}cry^{+/+}$ animals do not exhibit significantly weaker periodicity under FR-FM 16:8 LD (p= 8.59×10^{-2}) while L-cry$^{-/-}$ do (p= 1.46×10^{-2}). This could simply be due to stochastic variability between experiments so repeating 16:8 LD experiment under FR-FM would be beneficial. Previous research also detected that average period becomes shorter under FR-FM, while in my experiments there is no significant difference in period length between moon phases in either *L-cry$^{-/-}$* or *L-cry$^{+/+}$*, with average periods ranging from 24 to 26 h. When searching for circadian periodicity, previous study (12) applied a much wider time interval (12-29 h) while my interval was more stringent (16-29 h). Different data analysis could explain the period discrepancies, but it does not explain why $L\text{-}cry^{+/+}$ animals do not exhibit a weaker rhythm (period power) in 16:8 LD FR-FM conditions. However, I have observed dampened period power in $L\text{-}cry^{-/-}$ under FR-FM (p= 8.59×10^{-2}) compared to NM.

Since *L-cry$^{-/-}$* were able to behaviorally entrain to a periodic 24 h white light-dark schedule I followed with a similar protocol switching animals from a 16:8 white light-dark (LD) to 16:8 blue light-dark (426 nm, BLD), with a 12 h phase advance (Figure 5a). By switching the light from broad spectrum white light to monochromatic blue light I wanted to minimize redundant photoreceptor interference by avoiding the excitation of photoreceptors activated by other parts of the light spectrum. As the experiment was not long enough to perform Lomb-Scargle analysis I quantified animal locomotion by comparing individual distance traveled per hour of each light or dark interval (WL- white light, BL- 426 nm blue light, D- dark).

Due to L-Cry being a putative blue-light receptor I expected *L-cry$^{-/-}$* to have difficulties in adjusting to blue light compared to *L-cry$^{+/+}$*, especially in the first blue light interval (BL1). However, that was not the case under NM since *L-cry$^{-/-}$* exhibited a transitioning activity pattern in BL1 (active in

anticipation of entrainment schedule darkness; Figure 5c) and remained nocturnal in BLD to the same extend as *L-cry*$^{+/+}$ (Figure 5e/g). Under NM, both *L-cry*$^{-/-}$ and *L-cry*$^{+/+}$ animals were significantly more active during BL1 in anticipation of darkness than during BL2 (Figure 5c; NM/BL1-BL2: p(*L-cry*$^{-/-}$)= 4.8·10^{-4}, p(*L-cry*$^{+/+}$= 3.8·10^{-4}). This indicates both genotypes are able to behaviorally entrain to BLD. Considering there are both rhabdomeric (r-opsins) and ciliary photoreceptors (c-opsins) confirmed in *Platynereis* (27) it is not completely surprising that pre-mature *L-cry*$^{-/-}$ are able to perceive blue light even in the absence of a functional L-Cry. Circadian clock can be entrained through more than one type of receptor. In *Drosophila* CRY was confirmed to function as a blue light receptor mediating circadian light entrainment, but it is not the sole pathway of entrainment since fruit flies can also entrain through extracellular rhabdomeric photoreceptors (14,25). This was behaviorally displayed via cry^b flies (cry loss-of-function mutants) possessing an endogenous circadian period in DD and even being able to entrain to blue light, but double mutants for cry and r-opsin receptors (norpA) failed to entrain (14).

Interestingly, under FR-FM *L-cry*$^{+/+}$ did not exhibit a transitioning activity pattern in BL1 while *L-cry*$^{-/-}$ did, although weaker compared to NM (Figure 5d; FR-FM/BL1-BL2: p(*L-cry*$^{-/-}$)= 0.042, p(*L-cry*$^{+/+}$)= 0.8125). This is probably the consequence of very low *L-cry*$^{+/+}$ animal numbers (n=5) due to technical issues, so repeating the FR-FM experiment would be beneficial. What I successfully demonstrated with this experiment design is under NM *L-cry*$^{-/-}$ animals are able to perceive 426 nm blue light to the same extend as *L-cry*$^{+/+}$. In order to alleviate the masking effect of periodic blue light and test if *L-cry*$^{-/-}$ can entrain their circadian clock to monochromatic blue light one would have to cause a blue light phase shift followed by constant darkness. This could be achieved by subjecting animals to 16:8 white light-dark schedule, for a several days and then prolonging the 16 hours of white light with additional 4 h of blue light before releasing them into complete darkness to free-run. If *L-cry*$^{-/-}$ can entrain to blue light, their free-running period would be advanced by 4 hours.

5.3 Endogenous circadian period

After confirming *L-cry$^{-/-}$* animals are behaviorally entrained to 16:8 LD cycle, predominantly nocturnal and remain nocturnal even when illumination is switched to blue light (426 nm) I wanted to measure endogenous circadian period of *L-cry$^{-/-}$*. To examine the endogenous period I performed a constant darkness (DD) free-running experiment. When comparing *L-cry$^{-/-}$* circadian period to *L-cry$^{+/+}$* in DD, using Wilcox rank sum test, I noticed no significant differences of NM/DD and FR-FM/DD individual period lengths (Figure 7a/c). However, I noticed a peculiar pattern of *L-cry$^{-/-}$* period distribution with all the individuals showing periods ether longer or shorter from 20-25 h interval. Since a circadian period is expected to fall into 20-25 h, I refer to this phenomena as period splitting. I observed period splitting in *L-cry$^{-/-}$* under FR-FM/DD, with 0% of *L-cry$^{-/-}$* exhibiting period in 20-25 h range in comparison to 62.5% L-cry$^{+/+}$ periods (p= 4.41x10^{-2}; Figure 7d). Such distribution can have two explanations. Firstly, it could be that *Platynereis* endogenous period is a product of two separate oscillators working together to produce daily rhythmicity with the help of L-Cry photoreceptor (1), so in *L-cry$^{-/-}$* these oscillators de-synchronize under FR-FM/DD and drift apart. Since I only reported the dominant period (strongest power value) this could have created an overall impression of period splitting. However, most of the *L-cry$^{-/-}$* FR-FM/DD periodograms do not show a significant weaker peak (*7. Supplement data*, Figure S1b), but instead consist of single peaks so this is likely not the case. Second scenario supporting period splitting is that it is the result of background oscillations, of non-circadian origin. Period power of rhythmic *L-cry$^{-/-}$* and *L-cry$^{+/+}$* animals is significantly weaker under FR-FM/DD than NM/DD (p < 0.01). Decline of period power under FR-FM is consistent with current literature (12). However, *L-cry$^{+/+}$* arrhythmic DD behavior significantly increased between moon phases, form 0% under NM to 33.34% under FR-FM (p= 9.46x10^{-3}), while in *L-cry$^{-/-}$* it stayed consistent with 25.71% and 33.34%

respectively. The persistent rhythmicity in some $L\text{-}cry^{-/-}$ animals under FR-FM might be a consequence of background oscillations that mask the dampened circadian clock.

There is a discrepancy between arrhythmic behavior in DD between my experiments and previously published data (12). Zantke et al, working with wild type strains of *P. dumerilii* in the same laboratory as I, reported that under NM 41% (n_{total}=17) and under FR-FM 37% (n_{total}=24) of animals were arrhythmic. Although I performed the FR-FM/DD experiment only once, with 12 animals per genotype, in NM/DD experiments I had a total of 35 $L\text{-}cry^{-/-}$ and 27 $L\text{-}cry^{+/+}$ animals. Discrepancies can be explained by difference in experimental set-up and data analysis. In previous research all the animals were recorded in the same box, free to interact with each other and feed, which directly affects their behavior, while my set-up had individual wells for single animals with no food and all the animals that crawled to neighboring wells were excluded. Previous research used manual scoring of locomotor activity per minute of a 10 minute interval out of each hour, based on visual inspection of infrared camera recordings. Manual scoring decreases sensitivity and objectivity of locomotor analysis. I used computational tools for locomotor activity detection and analysis of behavior with one minute resolution. However, due to more than quarter of animals exhibiting no circadian period under FR-FM/DD, from the 12 animals per genotype I recorded, number of rhythmic individuals was reduced to 8 per genotype. Therefore, conclusions I drew from the FR-FM period splitting should be confirmed with additional experiments.

5.4 Circadian clock robustness and entrainment

Although FR-FM phase shift data was not reliable due to low number of $L\text{-}cry^{+/+}$ animals (n=5), it is apparent under NM both $L\text{-}cry^{-/-}$ and $L\text{-}cry^{+/+}$ were responsive to blue light. Also, in NM/DD experiments majority of animals exhibited an endogenous circadian period of approximately 24 h.

In chronobiology, it is quite common to use light pulses to either advance or delay a period phase of an endogenous oscillator (24). I used this approach to disrupt the circadian clock of *Platynereis dumerilii* by continuously pulsing animals with 2 h pulses of 426 nm blue light intermitted with 2 h of dark. Since no data from such experiment was published yet, my reference was *L-cry*$^{+/+}$ data.

Over 78h of BLD pulsing all the *L-cry*$^{-/-}$ and *L-cry*$^{+/+}$ were able to behaviorally entrain to the 4 h pulsing cycle, exhibiting an approximately 4 h locomotor activity period. In addition to 4 h periodicity, 50% of *L-cry*$^{+/+}$ and 69.23% of *L-cry*$^{-/-}$ animals maintained a functional circadian clock in the background, exhibiting a circadian period as well. I observed that blue light pulsing significantly increased *L-cry*$^{+/+}$ circadian arrhythmicity, from 0% under NM/DD to 50% in BLD pulsing conditions (p= 6.51×10^{-4}), while *L-cry*$^{-/-}$ rhythmicity was unaffected (Figure 8b). In addition, *L-cry*$^{-/-}$ with both rhythmic profiles (4 and 24h) exhibited weaker circadian period (Figure 8d), and were more successful at maintaining nocturnal activity under challenging lighting conditions (Figure 8e-f). Although this is not an established circadian disruption protocol I think it is a relevant design as continuous pulsing presents a recurring entrainment signal that challenges the robustness of the circadian clock and at the same time creates approximation of a free-running environment (no reliable 24 h cues). Taking all this into consideration, my pulsing experiment shows that *L-cry*$^{+/+}$ animals are more susceptible to circadian disruption via blue light (426 nm) pulsing over 80 h, while *L-cry*$^{-/-}$ are more influenced by masking effects of light. This result supports the hypothesis that L-Cry is a blue light receptor involved in circadian entrainment.

		L-cry +/+		L-cry -/-	
		NM	FR-FM	NM	FR-FM
Constant darkness	T	24.51 ± 0.48 h	25.16 ± 0.45 h	23.77 ± 0.62 h	23.97 ± 1.42 h
	Pn	149.63	50.77	166.52	43.84
	N total	27	12	35	12
	AR (%)	**0%**	33.33 %	**25.71 %**	33.33 %
	T(20-25 h) %	55.56 %	62.5 %	**46.15 %**	**0%**
Blue light pulsing	T4	4.01 ± 0.15 h	-	4.13 ± 0.2 h	-
	Pn(T4)	134.45	-	169.4	-
	AR(T4) %	0%	-	0%	-
	T24	24.32 ± 1.2 h	-	23.06 ± 1.1 h	-
	Pn(T24)	89.88	-	51.68	-
	AR(T24) %	**50%**	-	30.77 %	-
	N total	10	-	13	-

Table 1. Summary table of constant darkness free-run and 426 nm light pulsing experiments. Abbreviations: T= circadian period length, Pn= normalized period power, AR= arrhythmic animals, T(20-25h) %= proportion of animals with period length 20-25 h, T4= period length for a 4 h periodic cycle, T24= period length for a 4 h periodic cycle, N total= total number of animals used for the analysis. Figures in bold show distinct phenotypes previously discussed.

6 Conclusion

Through my chronobiological experiments on *Platynereis dumerilii* I confirmed that this organism is a valuable model for elucidating functions of multiple clocks and their influence on downstream mechanisms. *L-cry* loss-of-function in *P. dumerilii* (*L-cry^{-/-}*) leads to a significant maturation delay likely caused by inadequate stem cell function that give rise to gametes and facilitate regeneration and post-larval development. Stem cell function could be under indirect circalunar or circadian control through homeobox genes, but evidence to support this are still lacking. *L-cry* showed dominant role in controlling the lunar timing of sexual maturation in *P. dumerilii,* with *L-cry^{-/-}* mostly spawning shortly after FM, while animals with a single functional *L-cry* allele (*L-cry^{+/-}*) spawned both after FM and NM.

In addition to changes in the timing of sexual maturation, *L-cry^{-/-}* animals exhibited circadian changes with higher arrhythmicity under NM/DD, while under FR-FM/DD some animals displayed splitting of circadian periods from the expected 20-25 h period, suggesting circadian clock is additionally dampened and background oscillations leak through. Contrary to my hypothesis, *L-cry^{-/-}* animals showed no difficulties in perceiving and adjusting to monochromatic blue (426nm) light, which can be explained by existence of other ocular and extra-ocular photoreceptors (opsins) in this organism. In fact, *L-cry^{-/-}* were more susceptible to masking effects of light, with stronger nocturnal activity throughout circadian experiments and no *L-cry^{-/-}* animals matured during FM. Increased susceptibility to masking effects of light suggests *L-cry^{-/-}* light entrainment mechanism is disrupted, so L-Cry likely plays a crucial role in light entrainment of circadian and circalunar clocks in *P. dumerilii*. Light entrainment in *L-cry^{-/-}* should be additionally examined by circadian and circalunar phase shift experiments.

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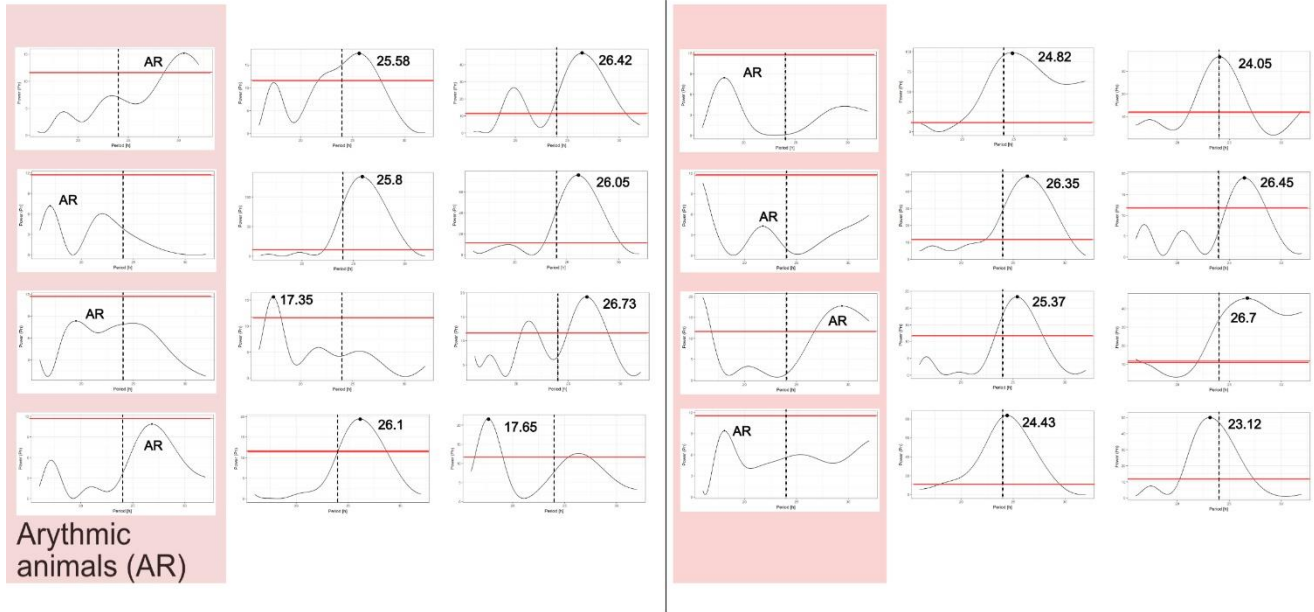
8 Supplement data

Figure S1. Individual DD Lomb-Scargle periodograms:

*Lomb-Scargle 1periodograms for individual mutant and control animals under DD conditions during NM **(A)** and FR-FM **(B)**. Horizontal red lines indicate a p-value= 0.05 and dashed vertical lines are set to 24 h. Arrhythmic animals (AR) showing periods longer than 29h or p-value < 0.05 are arranged on the left and are indicated by a pink rectangle.*
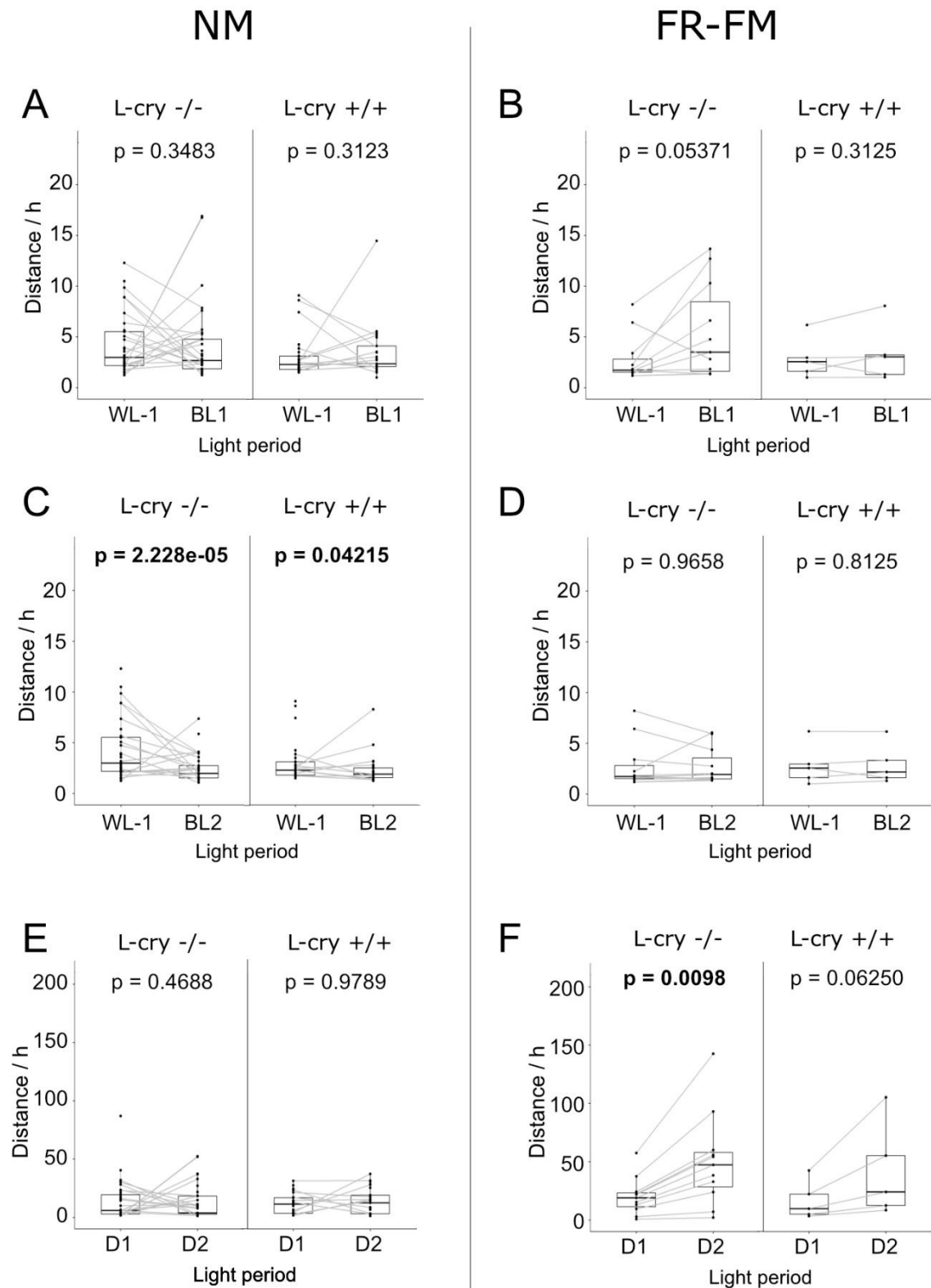
Figure S2 Comparison between distances traveled per hour of different light periods. *Black dots represent individual worms and gray lines connect paired measurements.* ***(A)*** *WL-1/BL1 under NM.* ***(B)*** *WL-1/BL1 under FR-FM.* ***(C)*** *WL-*

*1/BL2 under NM. **(D)** WL-1/BL2 under FR-FM. **(E)** D1/D2 under NM. **(F)** D1/D2 under FR-FM.*

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PERSONAL STATEMENT

Highly motivated master student seeking research opportunities in pursuit of an international career. Future aspirations include getting a PhD degree in the field of nervous system or behavioural genetics through application of transgenic techniques.

EDUCATION

2014– present

Master of Biotechnology in Medicine

University of Rijeka - Department of Biotechnology
Radmile Matejčić 2, 51000 Rijeka (Croatia)
http://www.biotech.uniri.hr/en/

- *Specific coursework: Gene therapy, Cell therapy, Tissue Engineering, Personalized Medicine, Systemic Biomedicine, Protein Research Methods, DNA Technology Methods, etc.*

2010–2014

Bachelor of Biotechnology and Drug Research

University of Rijeka - Department of Biotechnology
Radmile Matejčić 2, 51000 Rijeka (Croatia)

- *General coursework: Chemistry, Biochemistry, Physics, Informatics, Cellular and Molecular Medical Microbiology, Basic Physiology and Pathophysiology*
- *Specific coursework: Research Bioethics, Pharmacology, Toxicology, Gene Therapy , Bioassays in Parmaceutical Research, Drosophila as a Model Organism in Neuroscience, Behavioural Genetics*

WORK AND TRAINING

2016- 2017

Master student

MFPL, Tessmar-Raible group
Dr. Bohr-Gasse 9, 1030 Vienna (Austria)
SUPERVISOR: Univ. -Prof. Dr. Kristin Tessmar- Raible

- Taking care of transgenic animals (feeding, breeding, genotyping, logging maturation…)
- Conducting short and long term behavioural and molecular experiments

2015

Erasmus+ Summer Trainee

The Center for Brain Research- Medical University Vienna
Spitalgasse 4, 1090 Vienna (Austria)
SUPERVISOR: Assist. -Prof. Dr. Margot Ernst, PhD

- Generating site-specific mononucleotide mutations (design, sequence analysis)
- Cloning mutant DNA (PCR, bacterial culture, extraction/ purification)
- Computer modelling (structure prediction, computational docking).

2013–2014

Student Assistant in Cellular and Molecular Biology Practicum

Department of Biotechnology
Radmile Matejčić 2, 51000 Rijeka (Croatia)
SUPERVISOR: Prof. Dr. Sc. Saša Ostojić

- Preparing and supervising practical laboratory course of first year students

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PERSONAL SKILLS

General
- Highly motivated and passionate about research work
- Strong problem solving skills and natural curiosity
- Very keen of time/ project management apps and methods (Google Calendar, Todoist etc.)
- Flexible about my methods, but stubborn about my goals
- Quick learner, especially through practical experience

First language | Croatian

Other languages

UNDERSTANDING		SPEAKING		WRITING
Listening	Reading	Spoken interaction	Spoken production	
English				
C1	C1	C1	C1	B2
IELTS				
German				
A1	A1	A1	A1	A1

Levels: A1 and A2: Basic user - B1 and B2: Independent user - C1 and C2: Proficient user
Common European Framework of Reference for Languages

Laboratory skills
- Nucleic acid techniques (extraction, purification, amplification, cloning, gel electrophoresis...)
- Statistical data analysis (R studio, Excel)
- White light microscopy
- Spectrophotometry measurements (SpectrlLight III)
- Animal care (*Platynereis dumerilii*)
- Conducting behavioural experiments
- Basic microbiological techniques

Computer skills
- Excellent command of Microsoft OfficeTM tools and OS
- Proficient in e-mail correspondence
- Good command of R programming language
- Basic command of ClustalX, GOLD, VMD, Vector NTI, Chromas and Noldus

Driving licence | AM, B

SCHOLARSHIPS

- "Okrug district annual scholarship" based on academic record (4 years in a row)
- "Erasmus+ Mobility for Traineeship" scholarship 2015/16 (2 months)
- Master's project student salary funded by Austrian Academy of Science 2016/17 (1 year and 3 months)