



# The role of the Glucocorticoid Receptor in the Regulation of Diel Rhythmicity

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## ABSTRACT

Virtually all organisms have adapted to the earth's day-night cycles by the evolution of endogenous rhythms that regulate most biological processes. Recent research has highlighted the role of glucocorticoids and the Glucocorticoid receptor (GR) in coordinating clock function across various levels of biological organisation. In the present study, we have explored the role of the GR in the rhythmicity of the biological clock, by comparing 5 day old wildtype zebrafish larvae ( $gr^+$ ) with mutant larvae with a non-functional GR ( $gr^{s357}$ ). The mutants display a weaker rhythmicity in locomotor activity in wildtypes than in mutants, while the rhythmicity of the angular velocity was higher for wildtypes. The melatonin production of the mutants showed a weaker rhythmicity, but surprisingly, there were no differences in the rhythmicity of clock-related gene expression between genotypes that could explain a mechanism for GR functionality at the transcriptional level. Furthermore, our results show that  $gr^{s357}$  larvae have a more erratic swimming path, and cover more distance during locomotor activity than wild type larvae, in line with previously described behaviour of this mutant. Therefore, these results suggest that GR affects the diel rhythmicity of zebrafish larvae at the behavioural and endocrine level, but that these effects are not mediated by changes in the expression of clock-related genes.

## 1. Introduction

Virtually all organisms are exposed to day-night cycles as a consequence of the earth's planetary rotations around the sun [33]. The ability to anticipate and adapt to such diel changes in the environment imparts an evolutionary advantage to most species [26]. In vertebrates, this has resulted in the development of a complex network of autonomously functioning central and peripheral clocks interacting through molecular and hormonal signalling to coordinate and regulate endogenous circadian (circa 24h in the absence of external cues) rhythms [44]. This internal rhythm is entrained by ambient environmental cues or "Zeitgeber" such as light and temperature [44]. The oscillation of the internal circadian clock and its entrainment by external cues has been found to establish diel rhythmicity in several important processes such as locomotor and activity, food intake, sleep and reproductive activity, energy metabolism, hormone secretion, immune function and cell-cycle progression in various vertebrates [44].

A common molecular mechanism underlying the functioning of the biological clock has been elucidated in vertebrates, and it comprises a delayed auto-regulatory feedback loop [12, 37]. Briefly, a heterodimer of two members of the bHLH-PAS family of proteins, namely CLOCK (circadian locomotor output cycles kaput) and BMAL1 (brain and

muscle ARNT-like protein 1) activates the transcription of the period (*PER1*, *PER2* and *PER3*) and cryptochrome (*CRY1*, *CRY2*) genes upon binding to specific E-box regulatory sequences in the promoter regions of these genes. This leads to the increased production of the PER and CRY proteins, and their subsequent nuclear localisation, which takes several hours and peaks at the end of diel day time. The PER and CRY proteins then inhibit the transcriptional activity of the CLOCK/BMAL1 heterodimer, thereby effecting the termination of their own transcription. This core negative feedback loop is reinforced and stabilised by accessory loops involving other clock proteins like REV-ERB and ROR, and effects the oscillating transcription of several target genes regulating various processes [6, 17, 22, 31, 35]. Recent research efforts have focussed on identifying the additional regulatory factors involved in the coordination of clock rhythmicity across various levels of biological functioning.

This search has brought glucocorticoids to the forefront [13]. Glucocorticoids play a predominant role in the stress response [47]. Upon stress, activation of the Hypothalamus-Pituitary-Adrenal axis (Hypothalamus-Pituitary-Interrenal axis in fish) results in the release of corticotropin-releasing hormone (CRH) in the hypothalamus, which promotes the release of adrenocorticotropic hormone (ACTH) from the pituitary gland into the circulation. ACTH stimulates the production of

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glucocorticoids (cortisol in humans and fish) in the adrenal (or interrenal) gland. Glucocorticoids bind to the Glucocorticoid Receptor (GR), which then acts as a transcription factor, regulating transcription of a wide variety of genes by binding to specific glucocorticoid response elements (GREs) or through interaction with other transcription factors. As a result of these transcriptional changes, they regulate various physiological processes like glucose homeostasis, the immune response, and water and ionic balance [27].

Besides their role in the stress response, glucocorticoids are secreted in a diel rhythm, with high levels during daytime and low levels during night time. They can also influence the clock and interact with other clock outputs in the circadian regulation of physiology [42]. Recent studies have identified pathways for the regulation of certain clock genes by glucocorticoids and the GR. Gene expression of *Per1* could be affected in Rat-1 fibroblasts by dexamethasone, a GR agonist [5]. Glucocorticoids were found to regulate the expression of many other clock genes, including *Tim*, *Rev-erb $\beta$*  and *Dec2* [42]. In addition, GREs have also been identified in the promoter region of *Per1* and *Per2* genes [8, 48]. Although these findings suggest a role for cortisol and the GR as a 'permissive cue' for circadian rhythmicity through regulation of clock gene transcription [12], the exact mechanism has not been elucidated yet.

Zebrafish (*Danio rerio*) represent a valuable complementary model system for biomedical research as they share many homologies with other vertebrates, including the circadian clock and stress response systems ([2, 38, 43, 45]). In the present study, a genetically modified zebrafish line lacking a functional GR (*gr<sup>s357</sup>*) was used [49]. In this line, a point mutation in the GR disrupts the transcriptional activity of the receptor, so this fish lacks the negative feedback of HPI axis activity by the GR, resulting in chronically elevated levels of cortisol and depression-like behaviour [19, 49].

To investigate the role of glucocorticoid signalling in circadian rhythmicity using the zebrafish model system, we have compared the 24 hour rhythm of locomotor activity, melatonin concentration and expression of genes involved in the biological clock of wildtype larvae (*gr<sup>+</sup>*) to those of *gr<sup>s357</sup>* mutant larvae at 5 days post fertilization (dpf). We found differences between the strains in the rhythm and quantity of activity and the locomotor path, which are reflected in the production of melatonin over time, but not in the underlying clock gene expression. These data suggest that the GR affects circadian rhythms at early developmental stages, but not at the level of transcriptional regulation of clock-related genes.

## 2. Materials and methods

### 2.1. Maintenance and breeding of zebrafish

Zebrafish (*Danio rerio*) were maintained and handled according to the guidelines from the Zebrafish Model Organism Database (ZFIN, <http://zfin.org>) and in compliance with the directives of the local animal welfare committee of Leiden University. The *gr<sup>s357</sup>* mutant zebrafish line was provided by Dr. H. Baier (Max Planck Institute of Neurobiology, Martinsried, Germany; generated in TL/WIK background; [32]), and heterozygote fish were maintained in our fish facility for >5 generations at 28 ± 1°C in densities of 40 ± 5 individuals (male:female ~ 1:1) in 7.5 l tanks in standardised recirculation systems (Fleuren & Nooijen, Nederweert, The Netherlands). Ambient conditions were maintained as 14/10 h day:night cycles, with light periods 7:00 (0 hours Circadian Time, hCT) to 22:00 (14 hCT). Fish were fed twice daily, at 1 ± 1 hCT and at 8 ± 1 hCT, with dry food (DuplaRinM, Gelsdorf, Germany) and frozen artemia (Dutch Select Food, Aquadistri BV, Klundert, The Netherlands). For the creation of the homozygous wild type (referred to as *gr<sup>+</sup>*) and mutant (referred to as *gr<sup>s357</sup>*) larvae, *gr<sup>+</sup>* or *gr<sup>s357</sup>* adult fish (previously obtained by genotyping offspring from crossings of heterozygote fish and separating *gr<sup>+</sup>* and *gr<sup>s357</sup>* fish from the heterozygotes), were incrossed. Fertilization was performed by

natural spawning at the beginning of the light period. Eggs were collected and transferred to Petri dishes filled with egg water (60 µg/ml 'Instant Ocean' sea salts, Blacksburg, VA, USA, and 0.0005 % methylene blue), in densities of 50-80 eggs per petridish. The petridishes were kept in 28 °C climate rooms with 14/10 h day-night cycle. Dead and unviable eggs were removed from the plates and egg water was refreshed every day.

### 2.2. Analysis of locomotor behaviour

In order to evaluate differences in swimming behaviour between the two strains, the kinematics of locomotor activity was estimated. At 2 dpf, wildtype and mutant larvae were transferred to individual wells of 24 well plates (12 larvae per genotype), filled with 1.5 ml egg water per well (Blacksburg, VA, USA). At 4 dpf, the well plates were checked for any dead or malformed larvae, which were then removed. At 5 dpf, the well plate was placed in a Danio Vision observation system (Noldus BV, Wageningen, the Netherlands) by 01:00 hCT for acclimation. Light intensities varied between 0 lux during night-time and 10000 lux during daytime, with a gradual increase and decrease over a period of 15 minutes during 'dawn' and 'dusk'. Recordings of locomotor activity were started at 03:00 hCT and continued for 24 hours, after which experimental larvae were euthanized. This procedure was performed in triplicate to yield a total of 36 replicates per genotype (N=36). Kinematic parameters of locomotor activity were analysed using tracking software EthoVision XT10 (Noldus Information Technology, Wageningen, The Netherlands). Parameters assessed were: (a) Maximum velocity ( $V_{max}$  in  $mm\ s^{-1}$ ), i.e. the maximum velocity attained by the centre of mass of individual larvae over a time span of one minute, measured over a period of 24 hours. This parameter is a measure for maximum locomotor activity. (b) Time spent in outer zone (in %), i.e. the relative time spent in an 'outer zone', a 4 mm (ca. 1 body length) wide ring at the edge of the well. This parameter is calculated as the time spent in the outer zone, as a percentage of the total testing time (1 minute), and it is an indicator for thigmotaxis behaviour [39]. (c) Average distance moved (D, in mm), i.e. the distance moved by the centre of mass of individual larvae, averaged over one minute, measured over a time period of 24 hours. This parameter is a measure for the quantity of locomotor activity. (c) Mean angular velocity ( $\Omega$  in  $^{\circ}\ s^{-1}$ ), i.e. the rate of change of angular position of the centre of mass of individual larvae over a span of one minute, measured over a time period of 24 hours. This parameter is a measure for the directionality of the swimming path and qualifies the swimming mode, with high values indicating an erratic swimming mode [46].

### 2.3. Sampling of larvae for melatonin ELISA and qPCR analysis

Five dpf larvae were transferred to petridishes (ten individuals per petridish) between 1 to 2 hCT to determine differences between the two strains in the endocrine regulation of the diel rhythmicity and the expression of clock-related genes. At six different time points of the circadian cycle, 03:00 hCT, 07:00 hCT, 11:00 hCT, 15:00 hCT, 19:00 hCT and 23:00 hCT, duplicate samples were obtained by transferring sixteen larvae for melatonin ELISA assay and eight larvae for the qPCR analysis from one petridish to an Eppendorf tube, removing excess egg water, and flash-freezing the tubes containing the samples in liquid nitrogen (done twice, once for the melatonin ELISA and once for the qPCR analysis). This procedure was done in triplicate (on three separate experimental days) to yield a total of six replicates per time point (N=36, for both melatonin ELISA and qPCR analysis). Samples were then stored at -20 °C until analysis.

### 2.4. Analysis of melatonin concentrations

Samples were thawed on ice prior to tissue homogenisation. 150 µl of RLT buffer (Qiagen) and 5 sterile glass beads were added to each

sample. Samples were then homogenised using a MM400 tissue lyser (Retsch, Germany) at 30 Hz for 1 min. Homogenised samples were then centrifuged at 13200 rpm for 1 min at 4°C, and supernatants were used for ELISA.

The ELISA-based procedure for determination of melatonin concentrations was performed according to the manufacturer's instructions (Melatonin Elisa kit RE54021, IBL International, Hamburg, Germany). In short, 50 µl of supernatant was pipetted in duplicate into the wells of the Microtiter Plate, 50 µl of Melatonin Biotin and 50 µl of Melatonin Antiserum were added, the plate was covered and incubated overnight. The next day, the plate was washed three times, and 150 µl of Enzyme Conjugate was added. After incubation for two hours on an orbital shaker (500 rpm), the plate was washed again three times. Then, 200 µl of PNPP Substrate Solution was added and the plate was again incubated for 40 minutes on an orbital shaker (500 rpm). Finally, 50 µl of PNPP Stop Solution was added and extinction was measured at 405 nm (Reference-wavelength: 600-650 nm). The obtained optical densities (ODs, average of the duplicate OD values) of the standards (y-axis, linear) were plotted against their concentration (x-axis, logarithmic, Graphpad Prism, Version 7.0) A curve fit was performed with a 4 Parameter Logistics distribution. The concentrations of the samples were then calculated from the standard curve.

## 2.5. Quantitative PCR analysis of clock gene expression

RNA isolation was carried out on previously sampled larvae in accordance with the manufacturer's instructions (RNeasy Mini Kit, Qiagen). Briefly, 350 µl of 70% ethanol was added to the tissue homogenate before they were transferred to individual spin columns. The spin columns were centrifuged for 15 s at 10,000 rpm, and the flow through was discarded. The spin columns were then centrifuged serially after adding RW1 buffer and RPE buffer. Finally, the RNA was eluted by adding 30 µl RNase-free water, centrifuging at 10,000 rpm for 15 s and collecting the eluent. The RNA concentrations of extracts were quantified by Nanodrop spectrophotometry (Nanodrop Technologies, Oxfordshire, UK) and diluted to a final concentration of 33.33 ng µl<sup>-1</sup> in RNase free water. cDNA was synthesised using iScript cDNA Synthesis Kit (BIO-RAD technologies) in accordance to the manufacturer's instructions. Briefly, 4 µl of iScript 5x master mix was added to 15 µl RNA (33.33 ng µl<sup>-1</sup>) along with 1 µl of iScript reverse transcriptase. A S1000 Thermal Cycler (BIO-RAD technologies) was used, and the cDNA was diluted ten times with RNase free water and stored at -20°C.

The cDNA samples were used to quantify the expression levels of 4 clock genes, *per1a*, *per2*, *clock1* and *nr1d2a*. The housekeeping gene *ppia* was used as a reference gene. Briefly, 0.5 µl of the forward and reverse primers (supplemental Table 1), 4 µl of nuclease free water and 10 µl of GoTaq™ qPCR mastermix (Promega technologies, USA) were added to individual wells (triplicate per sample) of 96-well qPCR plates along with 5 µl of cDNA sample. Real time qPCR was carried out using a CFX96 Touch - Real time qPCR system (BIO-RAD technologies) programmed for 40 amplification cycles. Analysis of the obtained C(t) values and melt curves was done using CFX software (BIO-RAD technologies). The qPCR data were analysed using the  $\Delta\Delta C_t$  method [29] to obtain expression of target clock genes relative to the housekeeping gene *ppia*.

## 2.6. Data analysis

### 2.6.1. Statistical analysis of locomotor parameters

The raw locomotor data output from EthoVision XT10 software (Noldus Information Technology, Wageningen, The Netherlands) consisted of locomotor parameters ( $V_{max}$ , D and  $\Omega$ ) and the time spent in the outer and inner zone of a well (thigmotaxis), of individual larvae (N=36) tracked for 24 hours and averaged over 1 minute intervals. These sets of values were used for further statistical analyses. All data

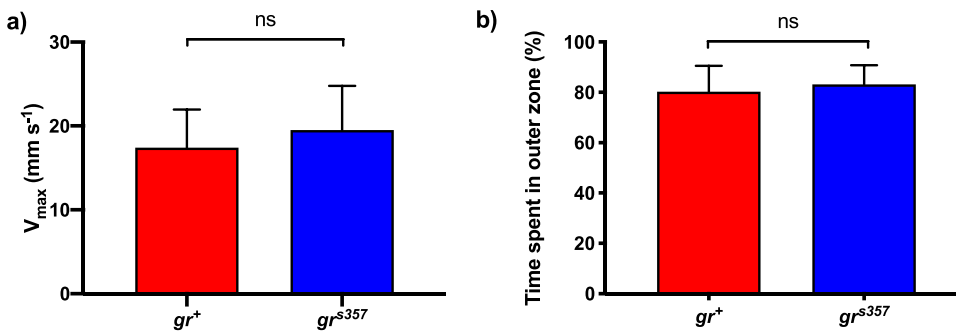
were subjected to an outlier elimination (ROUT, Q = 10%). Data were further checked for normality and homoscedascity using Bartlett's t-test, and the Brown Forsythe test respectively (significance accepted at  $p < 0.05$ , N=36). To determine the capacity for locomotor, individual values of  $V_{max}$  over 24 hours were compared between the different genotypes using a t-test (significance accepted at  $p < 0.05$ , N=36). To determine the locomotor activity and the locomotor path, values for D and  $\Omega$ , respectively, were averaged for 30-minute intervals over 24 hours, resulting in values at 48 time points. In addition, to determine the baseline thigmotaxis, individual values of the time spent in the outer zone as % of the testing time (1 minute), averaged over 24 hours, were compared between the different genotypes using a t-test (significance accepted at  $p < 0.05$ , N=36). Subsequently, Repeated Measure (RM) Two-way ANOVAs with Geisser-Greenhouse corrections in case of equal sample sizes, or Mixed-effects analyses in case of unequal sample sizes due to outlier elimination, were used to test for a significant effect of time and genotype. As a post hoc test, a Sidack's multiple comparison test was used (significance accepted at  $p < 0.05$ , N=36). In order to determine the diel rhythmicity of locomotor behaviour, a sine wave (non-zero baseline, phase = 24 hours) was fitted over the data points of D and  $\Omega$ . The resulting amplitudes were then compared between the genotypes using a t-test (significance accepted at  $p < 0.05$ , N=36). For the evaluation of the net amount of locomotor activity, the areas under the curve (AUC) of D and  $\Omega$  were compared between the genotypes over the entire 24 hour periods of observation, using a t-test (significance accepted at  $p < 0.05$ , N=36). Finally, to determine whether the light conditions during the 24-hour period may have an effect on locomotor parameters, values averaged over and the 14:10 h light and dark phase were compared (RM Two-way ANOVA with Geisser-Greenhouse corrections or Mixed-effects analyses, time and genotype as factors, Sidack's post hoc test,  $p < 0.05$ , N=36).

### 2.6.2. Statistical analysis of Melatonin concentrations

As was done for locomotor activity data, melatonin concentrations at six time points, i.e. 3:00, 7:00, 11:00, 15:00, 19:00 and 23:00 hCT, were cleaned of outliers (ROUT, Q=10%), checked for normality and homoscedascity (Bartlett's t-test, and Brown Forsythe test, respectively) and analysed for the effect of time and genotype (Two-way ANOVA for equal sample sizes, Mixed-effects analysis for unequal sample sizes; significance accepted at  $p < 0.05$ , N=36) during the light and dark phase. In a second analysis, a sine wave (non-zero baseline, phase = 24 hours) was fitted over the data points, and the amplitudes as a measure for diel rhythmicity were compared between the genotypes, using a t-test (significance accepted at  $p < 0.05$ , N=36). Finally, the AUC as a measure for net production of melatonin over the 24 hour period of sampling, was compared between the genotypes (t-test, significance accepted at  $p < 0.05$ , N=36). Finally, to determine whether melatonin concentrations were affected by light conditions, values were averaged over the light and the dark phase and compared with each other (RM Two-way ANOVA with Geisser-Greenhouse corrections or Mixed-effects analyses, time and genotype as factors, Sidack's post hoc test,  $p < 0.05$ , N=36).

### 2.6.3. Statistical analysis of RT-qPCR data

For the relative expression of *per1a*, *per2*, *clock1*, *nr1d2a* and *reverb- $\beta$*  at six time points, data were cleaned of outliers (ROUT, Q=10%) checked for normality and homoscedascity (Bartlett's t-test, and Brown Forsythe test, respectively) and analysed for the effect of time and genotype during the light and dark phase (Two-way ANOVA for equal sample sizes, Mixed-effects analysis for unequal sample sizes; significance accepted at  $p < 0.05$ , N=36). Subsequently, a sine wave (non-zero baseline, phase = 24 hours) was fitted over the data points, and the amplitudes were compared between the genotypes (t-test, significance accepted at  $p < 0.05$ , N=36). Finally, the AUC was compared between the genotypes (t-test, significance accepted at  $p < 0.05$ , N=36). Finally, to determine whether expression levels were affected



**Fig. 1. Maximum locomotor capacity and thigmotaxis do not differ between genotypes.** a) Maximum swimming speed ( $V_{max}$ , mm s<sup>-1</sup>) and b) Time spent in outer zone (% total time tested) do not differ between the larvae (5 dpf) of the wild type ( $gr^+$ , red) and mutant ( $gr^{s375}$ , blue) strain. Data shown are means  $\pm$  SEM, ns = not significant,  $p > 0.05$ , t-test; N = 36.

by light conditions, values were averaged over the light and the dark phase and compared with each other (RM Two-way ANOVA with Geisser-Greenhouse corrections or Mixed-effects analyses, time and genotype as factors, Sidack's post hoc test,  $p < 0.05$ , N = 36).

All statistical analyses were performed using Graphpad Prism 8.0 (GraphPad Software, San Diego, California, USA).

### 3. Results

#### 3.1. Locomotor behaviour parameters

Locomotor activity was determined by tracking individual fish over time and analysing the quantity of locomotor activity, path, and relative time spent in the outer zone, using automated tracking software. In order to assess basic differences in locomotor capacity between the two genotypes we first estimated the maximum swimming velocity ( $V_{max}$ ) of  $gr^+$  and  $gr^{s375}$  larvae, and the time spent in the outer zone (% total time tested). There were no significant differences between the two genotype groups, indicating a similar locomotor capacity (t-test,  $p = 0.7642$ , N = 36; Fig. 1a) and a similar thigmotaxis index (t-test,  $p = 0.1658$ , N = 36, Fig. 1b).

Subsequently, to determine differences in quantitative locomotor activity between the genotypes during the diel cycle, we assessed the distanced moved (D) over 24 hours, in 30 minute intervals. Two-way ANOVA revealed a significant effect of the genotypes but no effect of the time points ( $F_{genotype}(1, 3360) = 21.48$ ,  $p_{genotype} < 0.0001$ ;  $F_{time}(47, 3360) = 1.219$ ,  $p_{time} = 0.1468$ ;  $F_{interaction}(47, 3360) = 0.7074$ ,  $p_{interaction} = 0.9343$ ; Fig. 2a). In order to assess differences in diel rhythmicity between the genotypes, we fitted a sine wave (non-zero baseline, 24 hour period) over the data and compared the resulting amplitudes (mm) with each other. This analysis revealed a smaller amplitude in the wild type than in the Gr-deficient larvae, reflecting a less pronounced diel rhythmicity (t-test,  $p < 0.0001$ , N = 36; Fig. 2b). Using a similar type of analysis we compared the areas under the curve (AUC, dimensionless) to each other, as a measurement for net activity of the two genotypes, and a t-test revealed a lower net activity in the wild type than in the Gr-deficient strain (t-test,  $p = 0.0012$ , N = 36; Fig. 2c). Finally, to determine whether the light conditions may have an effect on D, we averaged the values during the light and the dark phase. Two-way ANOVA revealed a significant effect of the genotypes but no effect of the time points ( $F_{genotype}(1, 116) = 5.71$ ,  $p_{genotype} = 0.0184$ ,  $F_{phase}(1, 116) = 0.3580$ ,  $p_{phase} = 0.5508$ ,  $F_{interaction}(1, 116) = 0.3561$ ,  $p_{interaction} = 0.5519$ ; Fig. 2d).

In order to assess differences between the genotypes in the quality of the swimming path, we analysed the angular velocity ( $\Omega$ , °s<sup>-1</sup>).  $\Omega$  differed significantly between the genotypes and also varied significantly among time points (Two-Way ANOVA,  $F_{genotype}(1, 3360) = 32.74$ ,  $p_{genotype} < 0.0001$ ,  $F_{time}(47, 3360) = 8.819$ ,  $p_{time} < 0.0001$ ;  $F_{interaction}(47, 3360) = 0.6537$ ,  $p_{interaction} = 0.9675$ , Fig. 2d). Fitting a sine wave (non-zero baseline, 24 hour period) resulted in a larger amplitude (°s<sup>-1</sup>) in the wild type than in the Gr-deficient larvae, reflecting a more pronounced diel rhythmicity (t-test,  $p < 0.0001$ ,

N = 36; Fig. 2e). We then compared the AUCs (dimensionless) to each other, as a measurement for the quality of the swimming path, and a t-test revealed a less erratic activity in the wild type than in the Gr-deficient strain (t-test,  $p < 0.001$ , N = 36; Fig. 2f). Finally, to determine whether the light conditions may have an effect on  $\Omega$ , we averaged the values during the light and the dark phase. Two-way ANOVA revealed a significant effect of both, the genotypes and the light phases ( $F_{genotype}(1, 116) = 14.0$ ,  $p_{genotype} = 0.0003$ ,  $F_{phase}(1, 116) = 77.37$ ,  $p_{phase} < 0.0001$ ,  $F_{interaction}(1, 116) = 2.314$ ,  $p_{interaction} = 0.1309$ ; Fig. 2h).

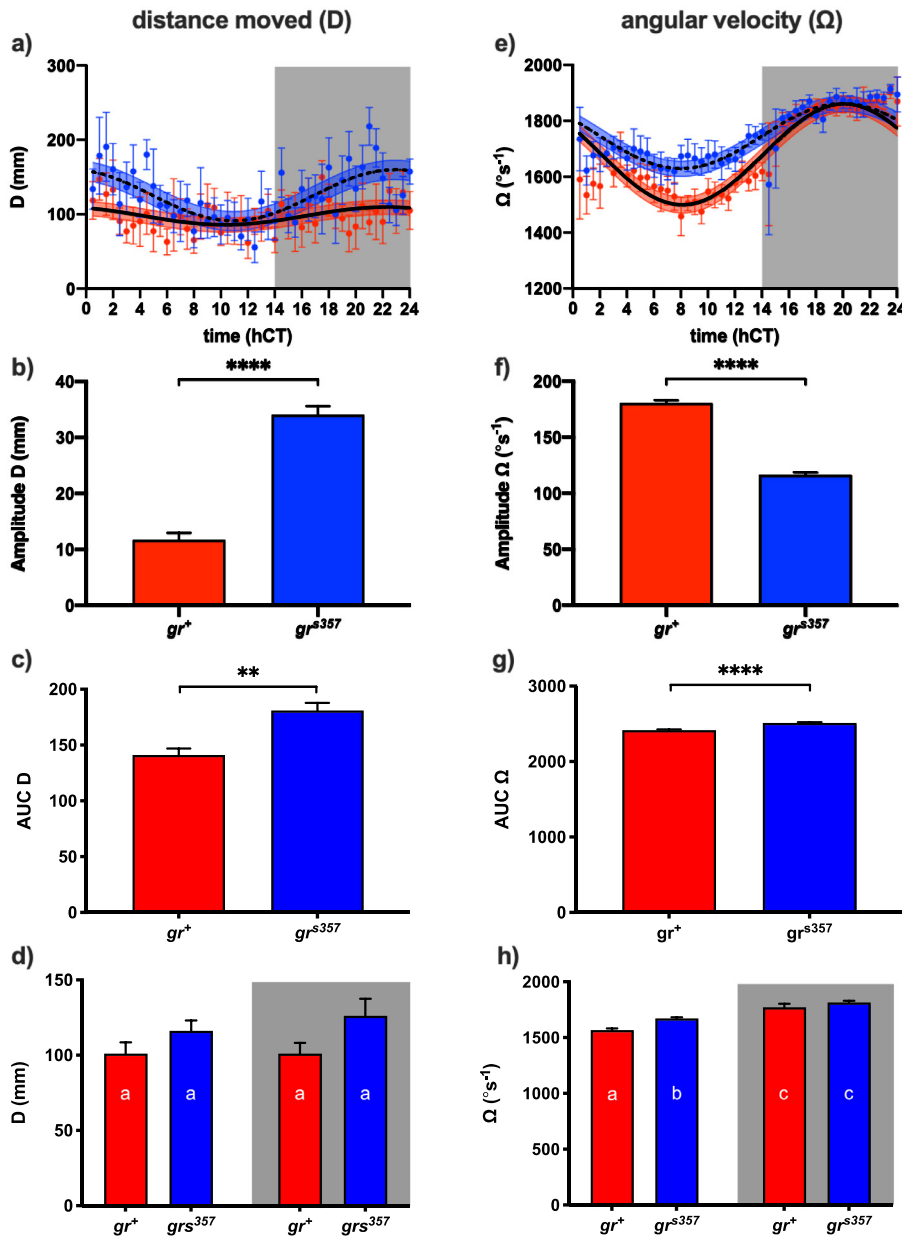
#### 3.2. Melatonin concentrations

At the endocrine level, we evaluated the full body melatonin concentrations at 6 time points during a 24 hour period in  $gr^+$  and  $gr^{s375}$  larvae. Melatonin concentrations showed no significant differences between genotypes but did show significant variation among time points (RM Two-Way ANOVA:  $F_{genotype}(1, 10) = 2.642$ ,  $p_{genotype} = 0.1351$ ,  $F_{time}(2, 049, 20, 49) = 11.04$ ,  $p_{time} = 0.0005$ ;  $F_{interaction}(5, 50) = 1.585$ ,  $p_{interaction} = 0.1816$ ). Sidack's post-hoc analysis ( $p < 0.05$ , N = 36) revealed significant differences between day and night time values for wildtype fish, but not for mutants (Fig. 3a). When comparing the resulting amplitudes of the plotted sine waves between the genotypes (Fig. 3b), the wild types had a significantly higher amplitude, reflecting a higher diel rhythmicity than the mutant (t-test,  $p = 0.0198$ , N = 36). When comparing the AUC, as a measure for net production of melatonin between genotypes (Fig. 3c), no significant differences were apparent (t-test,  $p = 0.3172$ , N = 36), indicating a similar net melatonin production for  $gr^+$  and  $gr^{s375}$  larvae over 24 hours. Finally, to determine whether the light conditions have an effect on melatonin concentrations, we averaged the values during the light and the dark phase. Two-way ANOVA revealed a significant effect of the light phases ( $F_{genotype}(1, 20) = 0.3175$ ,  $p_{genotype} = 0.5795$ ,  $F_{phase}(1, 20) = 18.39$ ,  $p_{phase} = 0.0004$ ,  $F_{interaction}(1, 20) = 2.523$ ,  $p_{interaction} = 0.1279$ ; Fig. 3d).

#### 3.3. Clock gene expression

Analysis of clock-related gene expression over 24 hours by qPCR revealed that expression patterns did not differ between genotypes. Mixed effects analyses of mRNA expression levels of *per1a* revealed no effect of genotypes but significant variation over time ( $F_{genotype}(1, 10) = 0.002416$ ,  $p_{genotype} = 0.9618$ ,  $F_{time}(1.852, 14.81) = 8.177$ ,  $p_{time} = 0.0046$ ,  $F_{interaction}(5, 40) = 1.105$ ,  $p_{interaction} = 0.3730$ , Fig. 4a). For mRNA expression levels of *per2*, mixed effects analyses revealed neither genotype nor time to have a significant effect ( $F_{genotype}(1, 10) = 0.5505$ ,  $p_{genotype} = 0.4752$ ,  $F_{time}(2.027, 16.62)$ ,  $p_{time} = 3.420$ ,  $F_{interaction}(5, 41) = 1.138$ ,  $p_{interaction} = 0.3558$ , Fig. 4b). Results for *clock1* revealed no effect of genotype but significant variation for time ( $F_{genotype}(1, 50) = 0.001913$ ,  $p_{genotype} = 0.9653$ ,  $F_{time}(1.716, 17.16) = 8.364$ ,  $p_{time} = 0.0040$ ,  $F_{interaction}(5, 50) = 0.8652$ ,  $p_{interaction} = 0.5111$ , Fig. 4c). Finally, for mRNA expression levels of *nr1d2-β*, neither genotype nor time had an effect ( $F_{genotype}(1,$





**Fig. 2.** Diel patterns of swimming behaviour are associated with genotype. Swimming parameters a – d) distance moved (D) and e – h) angular velocity ( $\Omega$ ), indicative for the quantity of locomotor activity and the quality of the locomotor path, resp., in wildtype ( $gr^+$ , red) and mutant zebrafish larvae ( $gr^{s375}$ , blue). 30-minute averages of a) D (mm) and e)  $\Omega$  ( $^{\circ}s^{-1}$ ) over 24 h during a 14:10 light to dark cycle (in hCT, indicated by white and grey background) were fitted with a sine wave (non-zero baseline, 24 hour period  $\pm$  95% confidence interval, shaded) over the  $gr^+$  (continuous line) and  $gr^{s375}$  (dotted line) data. b) The resulting amplitude for D (mm) is significantly lower for  $gr^+$  than for  $gr^{s375}$  larvae. c) Similarly, the area under the curve for D (AUC, dimensionless) is significantly lower for  $gr^+$  than for  $gr^{s375}$  larvae. These results indicate a lower quantity of locomotor activity and rhythmicity for D in  $gr^+$  than in  $gr^{s375}$  larvae. f) The Amplitude ( $^{\circ}s^{-1}$ ) of the sine wave for  $\Omega$  data over time is higher for  $gr^+$  than for  $gr^{s375}$  larvae. g) However, the AUC for  $\Omega$  is lower for  $gr^+$  than for  $gr^{s375}$  larvae. These results are indicating a stronger rhythmicity, and a lower degree of erratic swimming mode. Averages of d) D (mm) and h)  $\Omega$  ( $^{\circ}s^{-1}$ ) during the light and dark phase of a 14:10 cycle as an indicator for light as a stimulus differ between light and dark phase for both genotypes, but between wildtype and mutant during the light phase only for  $\Omega$ , indicating a larger difference between light and dark for mutant larvae for  $\Omega$ . All data shown are means  $\pm$  SEM. Significant differences are indicated by \*\*  $p < 0.01$  and \*\*\*\*  $p < 0.001$  and letters (d, h). T-test (a - c and e - g) and two-way ANOVA with Tukey's post-hoc test (d, h). N = 36.

10) = 0.8268,  $p_{\text{genotype}} = 0.3846$ ,  $F_{\text{time}}(0.8239, 7.086) = 0.8234$ ,  $p_{\text{time}} = 0.3696$ ,  $F_{\text{interaction}}(5, 43) = 0.8083$ ,  $p_{\text{interaction}} = 0.5502$ , Fig 4d).

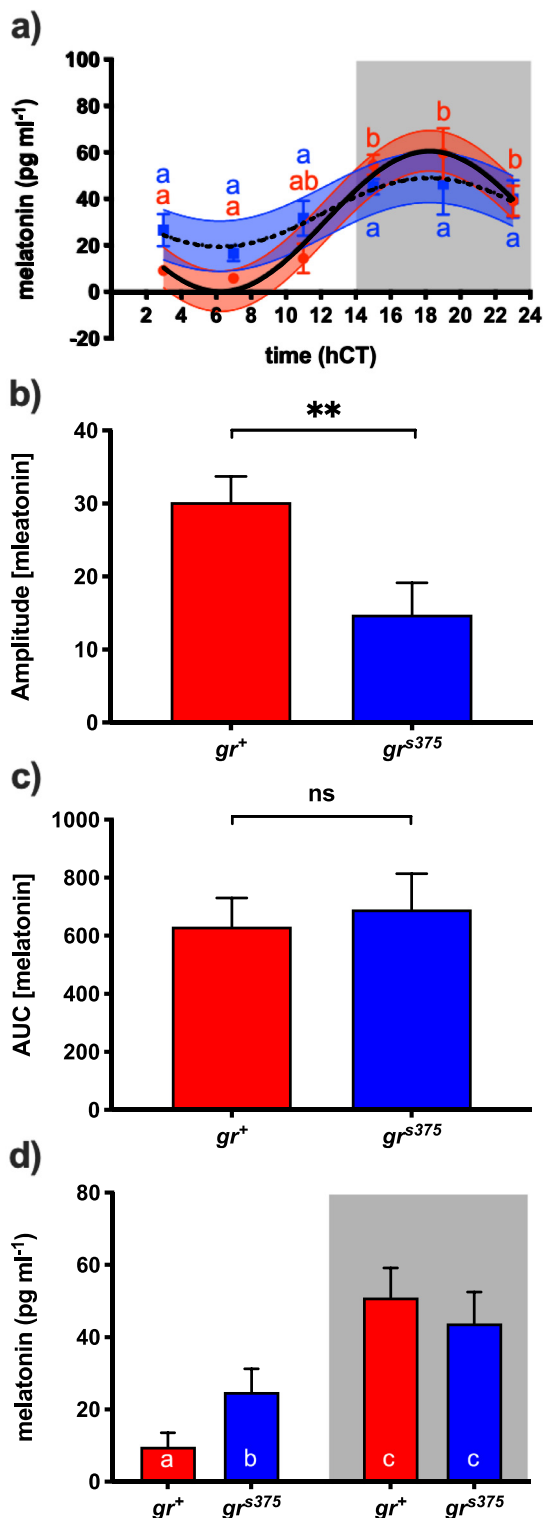
The amplitudes of the sine waves plotted over the expression values over time were not different between genotypes for any of the genes (t-test,  $p > 0.05$ , N = 36; Fig. 4b, e, h, k). The AUC values were not different either for any of the genes (t-test,  $p > 0.05$ , N = 36; Fig. 4c, f, i, l). These results indicate that there were no differences between genotypes in diel rhythmicity or net expression levels for any of the genes tested. Finally, to determine whether the light conditions have an effect on clock gene expression, we averaged the values during the light and the dark phase. Two-way ANOVA revealed no effect at all (*per1*:  $F_{\text{genotype}}(1, 20) = 0.08944$ ,  $p_{\text{genotype}} = 0.7680$ ,  $F_{\text{phase}}(1, 20) = 0.1398$ ,  $p_{\text{phase}} = 0.7124$ ,  $F_{\text{interaction}}(1, 20) = 0.3692$ ,  $p_{\text{interaction}} = 0.5503$ ; *per2*:  $F_{\text{genotype}}(1, 20) = 0.5421$ ,  $p_{\text{genotype}} = 0.4701$ ,  $F_{\text{phase}}(1, 20) = 0.1694$ ,  $p_{\text{phase}} = 0.2079$ ,  $F_{\text{interaction}}(1, 20) = 0.6364$ ,  $p_{\text{interaction}} = 0.4344$ ; *clock*:  $F_{\text{genotype}}(1, 20) = 0.05889$ ,  $p_{\text{genotype}} = 0.8107$ ,  $F_{\text{phase}}(1, 20) = 1.191$ ,  $p_{\text{phase}} = 0.2880$ ,  $F_{\text{interaction}}(1, 20) = 0.1288$ ,  $p_{\text{interaction}} = 0.7384$ ; *nr1d2*:  $F_{\text{genotype}}(1, 20) = 0.1493$ ,  $p_{\text{genotype}} = 0.2360$ ,  $F_{\text{phase}}(1,$

20) = 0.0021,  $p_{\text{phase}} = 0.9643$ ,  $F_{\text{interaction}}(1, 20) = 0.0571$ ,  $p_{\text{interaction}} = 0.8122$ ).

#### 4. Discussion

In the present study, we have compared diel patterns in locomotor behaviour, melatonin concentration and clock gene expression between wildtype ( $gr^+$ ) and mutant larvae with a dysfunctional GR ( $gr^{s375}$ ). Our study showed, at the behavioural level, a weaker rhythmicity in locomotor activity in terms of distance moved in wildtypes than in mutants, while the rhythmicity of the swimming path, measured as angular velocity, was higher for wildtypes. At the endocrine level, the rhythmicity of the melatonin levels was stronger in wildtype than in mutant larvae. At the molecular level, however, there were no differences between the genotypes in clock-related gene expression rhythmicity.

Thus, the observed differences between the genotypes in rhythmicity at the behavioural and endocrine level were not found at the level of clock-related gene expression, indicating a limited role of the GR in regulating diel rhythmicity of the expression of these genes in 5 dpf



zebrafish larvae. This was surprising, since it has previously been shown that glucocorticoids, such as corticosterone and dexamethasone, activate the oscillation clock gene expression in rodents [4, 42, 48], zebrafish [12] and even isolated cell lines [8].

This discrepancy between levels of biological function can be explained by glucocorticoids acting on alternative pathways which play a role in the regulation of the clock rhythmicity. It has been shown that in constant darkness conditions, the circadian clock of blind cavefish is

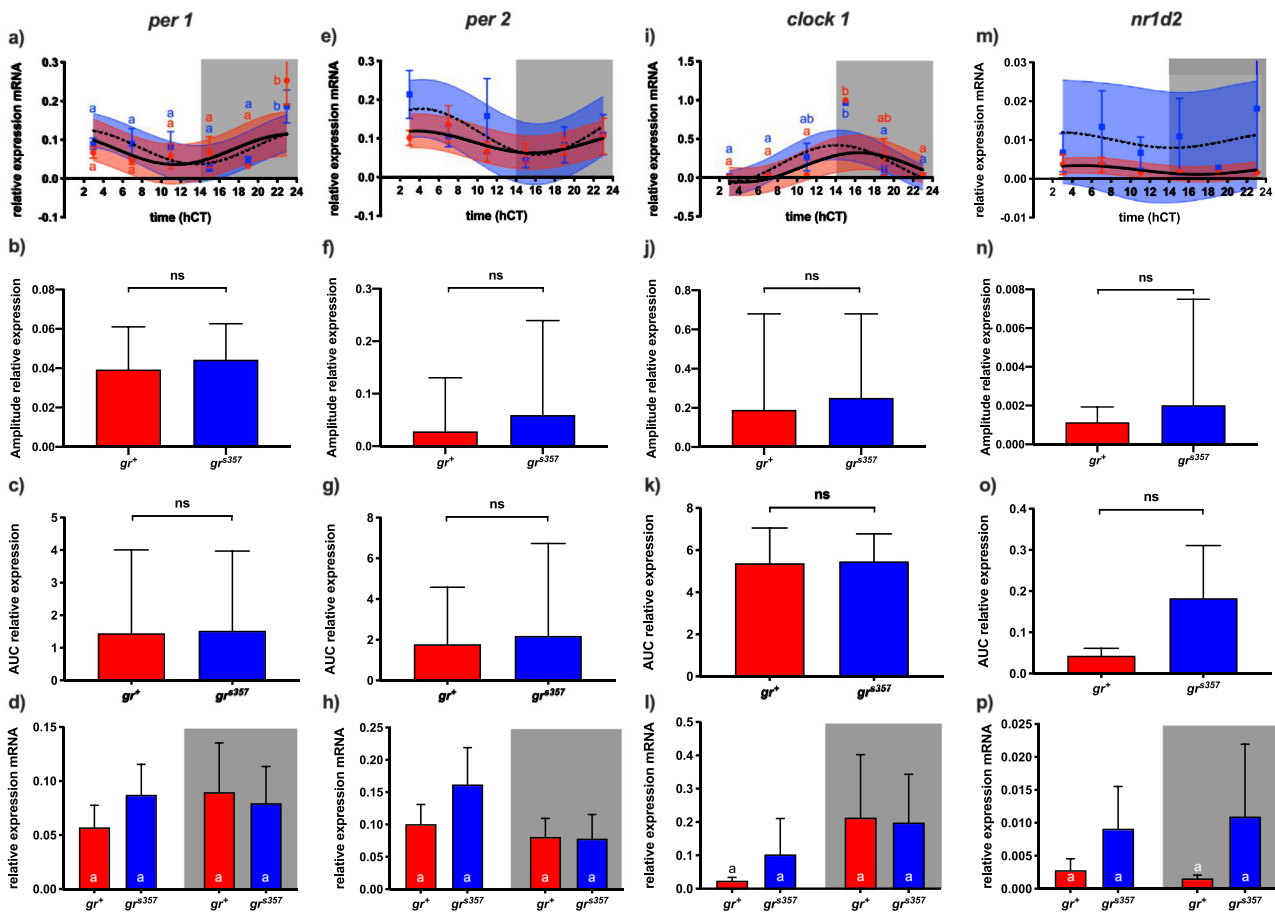
**Fig. 3.** Diel patterns in melatonin levels are associated with genotype. **a)** Full body melatonin concentrations over time (pg ml<sup>-1</sup>) in wildtype (*gr*<sup>+</sup>, red) and mutant zebrafish larvae (*gr*<sup>s375</sup>, blue). Larvae of different genotypes were collected every three hours over 24 h during a 14:10 light to dark cycle (in hCT, indicated by white and grey background). A sine wave (non-zero baseline, 24 h period  $\pm$  95% confidence interval, shaded) was fitted over the data of *gr*<sup>+</sup> (continuous line) and *gr*<sup>s375</sup> (dotted line). **b)** The resulting amplitude (pg ml<sup>-1</sup>) is significantly higher in wildtype than in mutant zebrafish larvae. **c)** The area under the curve (AUC, dimensionless) in wildtype does not differ significantly from mutant zebrafish larvae. **d)** Full body melatonin concentrations during the 14:10 h light and dark phase differ between light and dark phase for both genotypes, and between wildtype and mutant during the light phase, but not during the dark phase, indicating and larger difference between light and dark for wildtype than for mutant larvae. All data shown are means  $\pm$  SEM. Significant differences are indicated by letters (a, d) or \* (b): \*\*  $p < 0.01$ , ns  $p > 0.05$ . T-test (a, b, c) and two-way ANOVA with Tuckey's post-hoc test (d). N = 36.

maintained by food entrained oscillators (FEO), in contrast to light entrained oscillators (LEO; [7]). As our larvae are not yet feeding, and therefore do not depend on an external and oscillating food source, a difference in rhythmicity between mutant and wild type fish might therefore only be visible at a later stage, when FEO is also responsible for entraining the biological clock, together with LEO, especially since the GR also regulates feeding and digestion ([28]; Shen et al, 2017; Kuo et al., 2015). Our data on the differences in light responsiveness between the genotypes at the various functional levels seem to support this hypothesis.

Similarly, another explanation for not observing an effect of the dysfunctional GR on the biological clock is the age of the zebrafish larvae used. In larval zebrafish, endogenous cortisol starts to be produced only after 3 to 4 dpf, and a distinct cortisol stress response is observed only after 4 dpf [1]. It is therefore possible that a GR-mediated functionality of the molecular clock via GREs is not fully established yet in our larvae at 5 dpf. Indeed, when showing the regulating effect of glucocorticoids on clock gene expression, Dickmeis et al. [12] used the zebrafish larvae of 6 – 7 dpf, an age difference to our fish which can be significant for the functionality of the clock system in such a fast-developing organism [25]. However, an onset of the molecular clock regulation by GR can be assumed in the much higher variance in gene expression for *per2* and *nr1d2* over time in the *gr*<sup>+</sup> than in the *gr*<sup>s375</sup> fish. The GR may therefore have a gain setting effect on the expression of these genes at this early developmental stage, and will define their oscillation patterns later in life.

Also, we investigated only the diel regulation of the biological clock, under dark and light periods, and not the circadian regulation under constant conditions, which might be GR functionality dependent. To do this, an oscillation of the molecular clock has to be established under oscillating light and dark conditions during the first 6 days, and subsequently free running circadian rhythms can be observed after three days of constant light conditions [2, 24]. Larvae of 5 dpf are too young to be entrained and subsequently produce a freely oscillating circadian rhythmicity, which would allow to observe an effect by a dysfunctional GR.

Alternatively, the mutant GR may still have effects on the swimming performance of *gr*<sup>s375</sup> fish through a non-genomic action of the GR, since the *gr*<sup>s375</sup> comprises a point mutation in the second zinc finger of the DNA-binding domain, prohibiting DNA binding and interactions with other transcription factors like NF- $\kappa$ B, which are required for transcriptional regulation [49]. Non-genomic actions of GR have been described in neuronal cells (reviewed by [20]), and it has been shown that this activity involves membrane localization of GR requiring palmitoylation [34, 43], where they interfere with other intracellular signalling cascades, like the PI3K-Akt pathway [21]. In further studies, available CRISPR-Cas9-generated GR mutant zebrafish [15, 16] could be used to investigate whether this type of action may be involved,



**Fig. 4.** Diel patterns in clock gene expression are not associated with genotype. qPCR analysis of gene expression of **a - d** *per1*, **e - h** *per2*, **i - l** *clock1* and **m - p** *nr1d2* in wildtype ( $gr^+$ , red) and mutant zebrafish larvae ( $gr^{s357}$ , blue). **a, e, i, m**) relative gene expression ( $\Delta CT$ ) per gen. Larvae of different genotypes were collected every three hours over 24 h during a 14:10 h light to dark cycle (in hCT, indicated by white and grey background). A sine wave (non-zero baseline, 24 hour period,  $\pm$  95% confidence interval, shaded) was fitted over the  $gr^+$  (continuous line) and  $gr^{s357}$  (dotted line) data. **b, f, j, n**) The resulting amplitudes of the sine waves (dimensionless) were not statistically different between genotypes for any of the genes tested. **c, g, k, o**) The area under the curve (AUC, dimensionless) in wildtype does not differ significantly from mutant zebrafish larvae for any of the genes tested. **d, h, l, p**) The relative gene expression during the 14:10 h light and dark phase as an indicator for light as a stimulus, does not differ statistically between genotypes for any of the genes tested. These results indicate that the GR has no effect on the regulation of the clock gene expression. All data shown are means  $\pm$  SEM. Significant differences are indicated by letters (a, e, i, m, d, h, l, p) and ns  $p > 0.05$ . T-test and two-way ANOVA with Tuckey's post-hoc test (d, h, l, p). N = 36.

since differences between such a complete knockout and the  $gr^{s357}$  fish have already been observed [15].

Interestingly, the direct effect of light and dark periods during the 14:10 h cycle, were only significant for the quality of the swimming path, measured as angular velocity, and the melatonin production. In contrast to previous work [9, 10], expected difference between locomotion values during the dark and light phase were not observed here (Fig. 2d). Our data show relatively high locomotion activity during both phases, indicating either a reduced reactivity to external stimuli (but see also [41]), a shift in wake and rest phase which do not coincide with light and dark, or a generally high activity of the background strain used.

However, the observed effects of the GR on rhythmicity of melatonin production and locomotion behaviour can be explained in terms of direct interactions of glucocorticoids with the melatonin producing pineal gland [11] or the regulation of the melatonin MT2 receptor [40] by glucocorticoids. Chronically elevated cortisol levels, due to a dysfunctional GR in the feedback mechanism of the HPI axis [19, 49], can lead to a lack of oscillation of melatonin production between night and day and may therefore dampen its diel regulation. Similarly, a dampened oscillation of behavioural parameters can be explained by chronically elevated cortisol levels and subsequent constant and non-oscillating activation of mineralocorticoid receptors (MR; [36]), which

has a high affinity for cortisol [3], and plays a major role in stress and depression (de Kloet et al., 2016).

Finally, the overall higher locomotor activity quantified by the distance moved (D, mm), and the higher level of angular velocity ( $\Omega$ ,  $^\circ s^{-1}$ ) indicate a less calm and more erratic swimming behaviour in the  $gr^{s357}$  larvae [30]. Indeed,  $gr^{s357}$  adults have previously been shown to display longer freezing bouts and stronger thigmotactic responses to a novel tank challenge after a repeated stressor than their wild type counterparts [49], and stronger startle responses as larvae [19]. These anxiety-like behavioural responses in  $gr^{s357}$  fish which could be reversed by treatment with diazepam (GABA antagonist), an anxiolytic drug, and fluoxetine, an antidepressant (selective serotonin reuptake inhibitor; [19, 49]), have been previously deemed depression-like, since they mainly involved a reduced habituation to adverse stimuli, suggesting long-lasting, experience-dependent effects showing similarity to learned helplessness [19, 49]. They coincide with chronically high cortisol levels, due to a dysfunctional GR in the feedback mechanism of the HPI axis [19, 49]. Here we show that the anxiety or depression-like symptoms observed in  $gr^{s357}$  fish also include a dampened endocrine rhythm [14, 18, 23], with less pronounced differences of melatonin production between day and night in  $gr^{s357}$  individuals compared to wildtype fish.

## 5. Conclusions

Our data show a strong effect of GR deficiency on the diel rhythmicity of behavioural activity and melatonin production in 5dpf larval zebrafish, but surprisingly no effect on the rhythmicity of clock-related gene expression. This suggests that GR is involved in the regulation of diel rhythmicity at this early stage of development, without affecting the regulation of clock-related gene expression.

## Declaration of Competing Interest

The authors declare no conflict of interest.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.physbeh.2020.112991.

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