The effects of melatonin on circadian and developmental gene expression in *Danio rerio*

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Abstract

The circadian clock is an intracellular transcriptional-translational feedback loop that drives circadian rhythms. In zebrafish, these circadian clocks are found throughout the body and can be directly entrained by light. Melatonin synthesis begins early in zebrafish development (~22 hours post fertilization) and nocturnal melatonin biosynthesis is one of the first signs of the circadian system. Our data suggest that melatonin does not play a role in establishment of daily gene expression rhythms in developing zebrafish. We found that melatonin or a melatonin receptor antagonist did not impact expression of circadian clock-controlled genes (*irbp* and *aanat2)* or genes encoding components of the circadian clock (*period3* and *clock1)*. However, consistent with research by others suggesting that melatonin accelerates development, our preliminary work suggests that melatonin may advance gene expression onset of *dbx2* and *irbp* in the first 24 hours of development.
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CHAPTER 1: INTRODUCTION

Melatonin

Melatonin is an indoleamine hormone commonly associated with sleep in diurnal animals. However, melatonin exhibits a wide range of functions and abilities not related to sleep, such as cancer inhibition (Hill and Blask 1988), immune system modulation (Carrillo-Vico et al., 2013) and protection from reactive oxygen species (Sewerynek et al., 1996). Further highlighting the diverse nature of melatonin, it can be found in organisms not typically associated with sleep, such as microbes and plants (Reiter et al., 2014). Additionally, melatonin can act through membrane receptors, nuclear receptors, direct binding to proteins, or inhibition of proteasomal lysis (Reiter et al., 2014). The many properties of melatonin are further facilitated by the ability of melatonin to freely cross all known morphophysiological barriers such as the blood brain barrier and the placenta (Reiter et al., 2014). Although organisms have evolved to use melatonin in diverse modes, the most well studied functions of melatonin involve circadian rhythms.

As a central component of the circadian system in photosensitive organisms, melatonin synthesis in the pineal and subsequent circulation throughout the bloodstream confers time information within an organism.

For example, during mammalian gestation melatonin conveys phase and day length information to the fetus (von Gall et al., 2000). Melatonin also conveys seasonal information that is used to determine annual rhythms such as breeding (Reiter et al., 2014). The secretion of melatonin is highly conserved across vertebrates, being secreted by the pineal and retina at night in all vertebrates, including diurnal and nocturnal animals (Lima-Cabello et al., 2014).

In mammals, the Suprachiastmatic nucleus (SCN) is connected to the pineal gland through a multisympathitc pathway (Schomerus and Korf, 2005). The synthesis of melatonin in the mammalian pineal gland is controlled through night time release of norepinephrine by sympathetic efferents that innervate the pineal (Schomerus and Korf 2005). In contrast to mammals, melatonin synthesis in zebrafish is not regulated by sympathetic neurons via norepinephrine (Lima-Cabello et al., 2014). Melatonin synthesis is instead directly regulated by circadian clock proteins in zebrafish (Lima-Cabello et al., 2014).

In zebrafish, pineal melatonin synthesis begins at approximately 22 hours post fertilization (hpf) (Fig.1), as tryptophan is taken up by pineal cells (Lima-Cabello et al., 2014). Through several enzymatic steps, tryptophan is

transformed into serotonin (Lima-Cabello et al., 2014) . Serotonin then undergoes two more enzymatic steps, resulting in melatonin (Lima-Cabello et al., 2014). One of these steps involves Arylalkylamine-N-acetyltransferase (AANAT), a required enzyme in the synthesis of melatonin. At night, increased cAMP levels lead to the phosphorylation of AANAT, thereby activating and protecting AANAT from proteasomal proteolysis (Hastings, 2003). High AANAT levels reflect high melatonin levels. During the day, light leads to a decreased cAMP levels and this results in reduced phosphorylation of AANAT (Hastings, 2003). Subsequently, rapid proteolytic destruction of dephosphorylated AANAT occurs and as a result, daytime melatonin levels are low (Lima-Cabello et al., 2014) .

Two arylalkylamine-N-acetyltransferase genes are found in zebrafish: *aanat1* and *aanat2* (Appelbaum et al., 2006). Expressed solely in the retina, the enzyme AANAT1 is responsible for retinal melatonin synthesis in zebrafish (Appelbaum et al., 2006). The function of retinal localized melatonin in vertebrates is not well understood but may contribute to visual sensitivity at night (Wiechmann and Sherry, 2013). The zebrafish gene, *aanat2,* is expressed primarily in the pineal but is expressed to a lesser extent in the retina (Appelbaum et al., 2009). A component of the

intracellular circadian clock mechanism, the protein heterodimer CLK/BML along with OTX5 transcriptionally regulate *aanat2* by binding to e-box elements (Gamse et al., 2002; Pierce et al., 2008). Through binding of the CLK/BML heterodimer to e-box elements, the circadian clock directly regulates expression of *aanat2* and therefore melatonin synthesis (Pierce et al., 2008).

In recent years, an increase of melatonin research in zebrafish has highlighted the diverse functions of the hormone. Most notably, Danilova et al. 2004 demonstrated that zebrafish embryos treated with melatonin experienced increased cell proliferation and accelerated development. Zebrafish melatonin receptors are more widely expressed in developing embryos than adults, pointing to a possible developmental role of melatonin (Lima-Cabello et al., 2014; Shang and Zhdanova, 2007). Specific positive effects of melatonin have been established in more recent research. For example, melatonin has been found to increase neuronal differentiation in embryos (de Borsetti et al., 2011), induce follicle maturation in adult zebrafish (Carnevali et al., 2011) and even counteract the negative effects of cocaine on neuronal development in zebrafish embryos (Shang 2007).

Circadian Rhythms

Circadian rhythms are biochemical, behavioral, or physiological rhythms that maintain an approximately 24 hour cycle; as a result of the environmental changes that occur with the rotation of the Earth. These daily rhythms are driven by an intracellular transcriptional-translational feedback loop called the circadian clock (Fig. 2). These molecular circadian clocks are maintained by feedback loops and reset by input signals from the environment. As such, the circadian rhythms that molecular clocks regulate can confer an adaptive advantage by coordinating an organism to its environment and allowing an organism to anticipate daily or seasonal changes. For example, circadian rhythms of rest/activity allow an organism to conserve energy at beneficial times (Vaze and Sharma, 2013). This alignment with the environment occurs through information gathered from the zeitgebers (external cues) that entrain circadian clocks (Ben-Moshe et al., 2014). Changes in light and temperature are the primary zeitgebers of circadian clocks in zebrafish (Lahiri et al., 2014).

Entrainment is resetting of the circadian clock by a zeitgeber to maintain synchronization between the environment and the circadian clock (Ben-Moshe et al., 2014). Daily entrainment ensures that the period length

and phase of circadian rhythms remains steady and the rhythms are maintained on a 24 hour cycle (Fig. 3). Without daily entrainment, circadian rhythms become asynchronous with the surrounding environment (Lahiri et al., 2014). However, even if an organism is transferred to constant conditions with no entrainment signal, their circadian rhythms will persist but these rhythms may be distorted (Keneko 2004, Fig.3).

Circadian Organization in Mammals

The mammalian circadian system is organized in a hierarchal manner, with a master pacemaker controlling peripheral clocks. The master circadian pacemaker in mammals is in the suprachiasmatic nucleus (SCN), located within the hypothalamus (Hastings, 2003). Retinal ganglion cells that innervate the SCN relay photic information to the SCN, which in turn releases humoral and neuronal signals that coordinate clocks in peripheral tissues (Hastings, 2003). When the SCN is ablated in mammals, peripheral clocks become asynchronous with one another and with the environment (Yoo et al., 2004). It was long thought that peripheral circadian clocks in mammals were completely controlled by the SCN (Yoo et al., 2004). However, more recent findings suggest that mammals do not have

completely centralized circadian organization. After developing a mouse line expressing a real time luciferase reporter for the circadian gene *period2*, Yoo et al. 2004 found that tissue specific gene expression rhythms were maintained after SCN lesion. Furthermore, they found that these tissue specific gene expression rhythms were often different when compared to the SCN rhythm (Yoo et al., 2004). Surprisingly, some of these independent clocks in mammalian tissues and cells were as robust as the SCN clock (Yoo et al., 2004). Therefore, mammals can be viewed as a mixture of decentralized control, where peripheral clocks can maintain independent rhythms but also centralized control, where these clocks require signals from the SCN to maintain phase alignment.

Circadian Organization in Zebrafish

The zebrafish suprachiastmic nucleus (SCN) does not appear to aid in establishment of circadian clocks in developing zebrafish (Noche et al., 2011). Circadian rhythms from mutants lacking an SCN have normal phase length for gene expression of *cry1b*, *cry3*, *exorh*, and *aanat2* but amplitude is diminished (Noche et al., 2011). Reminiscent of the SCN, the zebrafish pineal gland contains all the components of a master pacemaker; including

an intrinsic circadian clock, classic photoreceptor cells, and the release of humoral signals (melatonin) starting early in development (Vatine et al., 2011). Further differentiating the circadian system of zebrafish and mammals, the pineal gland in mammals is not photoreceptive (Hastings, 2003).

In contrast to the centralized system of mammals that requires photic information from the retina; all tested zebrafish tissues, organs, and cultured cells have been shown to contain circadian clocks that can be directly entrained by light either *in vivo* or *in vitro* (Tamai et al., 2005), Elbaz 2005). The mechanism of light perception in the peripheral tissues of zebrafish has not been defined but CRY and PER proteins have been indicated as mechanism components (Vatine et al., 2011). In 2000, Whitmore et al. found that rhythmic gene expression of the circadian gene *clock* in cultured kidneys and hearts could be reset by light. In zebrafish cell lines housed in constant conditions, circadian clocks in individual cells run asynchronous to each other but rhythmic expression can be reset by light (Carr et al., 2006). This suggests that zebrafish have a decentralized system. However, additional unknown mechanisms to coordinate and regulate peripheral clocks could be present in zebrafish (Krznarich 2017 Chapter 3).

Zebrafish Intracellular Clock Mechanisms

The intracellular clock mechanism in zebrafish is reminiscent of the mammalian circadian clock; with core components being *clock, bmal* and *period* genes (Vatine et al., 2011). However, zebrafish experienced a whole genome duplication event at some point in their evolution (Vatine et al., 2011); as a result, zebrafish have more copies of circadian clock genes than mammals. The "core" feedback loop in the zebrafish clock expresses Period (PER) and Cryptochrome (CRY) proteins and is driven by the protein heterodimer CLK: BMAL (Fig.4). The CLK:BMAL complex binds to e-box elements on period and cryptochrome genes to drive transcription and subsequent translation of PER and CRY proteins. In turn, PER and CRY mechanically prevent CLK and BMAL proteins from forming a heterodimer, rendering them inactive (Vatine et al., 2011). This is a negative feedback loop of the zebrafish circadian clock, where the PER and CRY proteins are negatively regulating their own expression (Vatine et al., 2011).

The "stabilizing" loop expresses CLK and BMAL proteins, with Rev-Erbα and Rorα directing rhythmic expression of *clock* and *bmal* (Fig.4). Once translated, CLK: BMAL heterodimers then positively regulate their own transcription, with REV-ERBα protein blocking transcription through

competitive interactions with RORα at receptors. At the center of the two smaller loops is the CLK:BMAL protein pool, that drives expression of *clock*, *bma,l period* and *cryptochrome* genes (Vatine et al., 2011). These loops cycle in ~24 hours to maintain circadian clocks throughout the zebrafish.

Light and Zebrafish

Zebrafish begin life as transparent embryos, heavily affected by light conditions. Larvae raised in constant light exhibit developmental malformations and larvae raised in constant dark die before 18 days post fertilization (Villamizar et al., 2014). Embryos raised in constant dark and constant light experience delayed and absent melatonin secretion, respectively, in comparison to embryos raised on light:dark cycle (de Borsetti et al., 2011). Interestingly, visible wavelengths of light can have drastic effects on developing zebrafish as well (Villamizar et al., 2014). In a 2014 study, zebrafish larvae housed in red light decreased feeding to such an extent that a 100% mortality rate was observed by 20 days post fertilization (Villamizar et al., 2014). Even small changes in wavelengths can impact developing zebrafish; in the same 2014 study, expression of a growth and

stress related gene was found to be significantly different in developing zebrafish housed in violet light compared to blue light (Villamizar et al., 2014).

Light and the Zebrafish Pineal

The pineal gland in zebrafish is located dorsally in the brain and develops by 22 hours post fertilization (Lima-Cabello et al., 2014), relatively early in development (Fig.5). Unlike the mammalian pineal that is not photoreceptive, cell types similar to retinal rod and cone photoreceptor cells and opsin proteins are found in the zebrafish pineal (Li *et al.* 2012). These cells make the zebrafish pineal gland a classic photoreceptive organ, sharing homologies with the vertebrate retina (Ben-Moshe et al., 2014). A key opsin gene in pineal photoreception is *exo-rhodopsin*. Before eyes have formed, rhythmic expression of the photopigment gene *exo-rhodopsin* (*exroh*) begins in the zebrafish pineal at approximately 18 hpf (Vuilleumier 2006), illustrating the importance of early light perception in the pineal. Light induced expression of *Period 2 (per2*), a gene that possibly initiates the circadian clock in zebrafish is likely mediated by *exo-rhodopsin* (Ziv 2005). Furthermore, zebrafish mutants with reduced *exorh* have reduced *aanat2*

expression, suggesting a role for *exroh* in regulation of melatonin synthesis (Pierce et al., 2008).

Mammalian and Zebrafish Melatonin Receptors

Melatonin receptors are seven pass transmembrane G-protein coupled receptors that are known to homodimerize or heterodimerize in most vertebrates (Wiechmann and Sherry, 2013). The mammalian melatonin receptor MT1 (homologous to zebrafish *mtnr1aa, mtnr1a1, mtnrab*) is highly expressed in the brain, while MT2 (homologous to zebrafish *mtnr1bb, mtnr1ba*) is localized to the retina (Shang and Zhdanova, 2007). MT1 is found most abundantly expressed in areas associated with circadian function such as the par tuberalis and SCN in mammals (Danilova et al., 2004). In MT1 knockout mice, where only MT2 melatonin receptors are expressed, development is accelerated (Danilova et al., 2004). When MT1receptors are overexpressed in mice, development is slowed (Danilova et al., 2004).

The actions of melatonin in zebrafish are mediated by 6 melatonin membrane receptors: *mtnr1ab,mtnr1aa, mtnr1a1, mtnr1c, mtnr1bb*, and *mtnr1ba.* Expression of zebrafish melatonin receptors begins at 18 hpf

(Danilova et al., 2004), shortly after at 22 hpf, melatonin synthesis begins (Lima-Cabello 2014). It has been shown that *mtnr1ba* (a homolog of the MT1 receptor) and *mtnr1bb* (a homolog of the MT2 receptor) are expressed widely in developing zebrafish but widespread expression begins to decline at 18hpf; by 36hpf melatonin receptors have localized to the brain and retina (Danilova et al., 2004).

Expression patterns of *mtnr1ba*, *mtnr1bb*, *mtnr1aa*, 17hpf, 18hpf, 19hpf, 24hpf, 36hpf zebrafish were detailed by Danilova 2004 using RNA *in situ* hybridization; widespread expression was seen in embryos until approximately 36hpf when receptor expression began to localize to the head region of the embryo. Appelbaum 2009 (Fig.5) characterized the expression pattern of *mtnr1ba* and *mtnr1bb* in adult fish brains (6 months old) using RNA *in situ* hybridization noting overlapping expression of melatonin receptors and *hcrt* (hypocretin/orexin neuropeptides associated with sleep) expression in the periventricular gray zone of the optic tectum, and the periventricular thalamus and hypothalamus. Hernandez de Borsetti 2011 detailed expression pattern of *mtnr1ba* and *mtnr1bb* in 24hpf, 36hpf and 48hpf zebrafish and found results in line with previous work showing the wide distribution of melatonin receptor mRNA during development.

However, Hernandez de Borsetti 2011 published photos only of embryos anterior dorsal view so drawing spatial expression conclusions is difficult.

When considering methods to inhibit melatonin function, the melatonin receptor antagonist luzindole; a molecule that blocks melatonin receptors without stimulating downstream signaling is an attractive option. Previous studies using luzindole on zebrafish embryos have found melatonin function has been successfully inhibited using luzindole (Danilova et al., 2004; Zhdanova et al., 2001). While drugs specific to certain melatonin receptor types are available, luzindole has been the most widely used drug to block melatonin receptors in zebrafish (Danilova et al., 2004; Zhdanova et al., 2001).

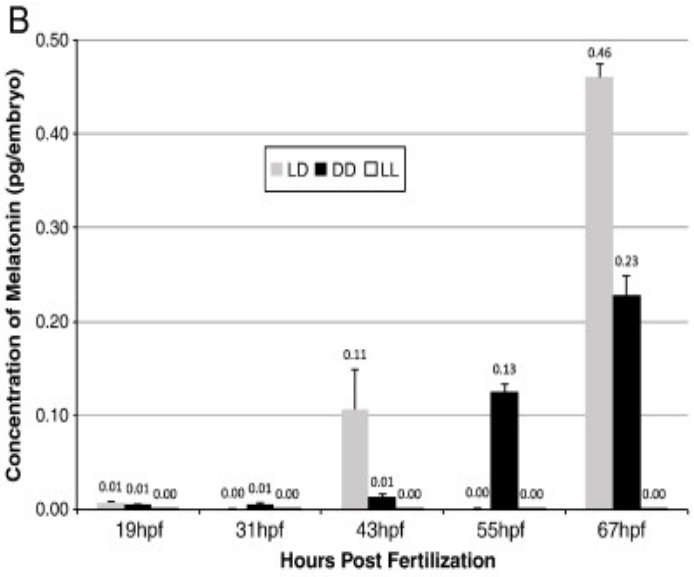

Figure 1. **Melatonin concentrations found in zebrafish embryos from 19hpf- 67hpf**. Zebrafish were housed in either constant light, constant dark or a 14 hour light: 10 hour dark cycle. Note the delayed secretion of melatonin in DD conditions and no secretion in LL conditions.

Hernandez de Borsetti, N. et al. (2011) Light and melatonin schedule neuronal differentiation in the habenular nuclei. *Developmental Biology*. 358: 251-261.

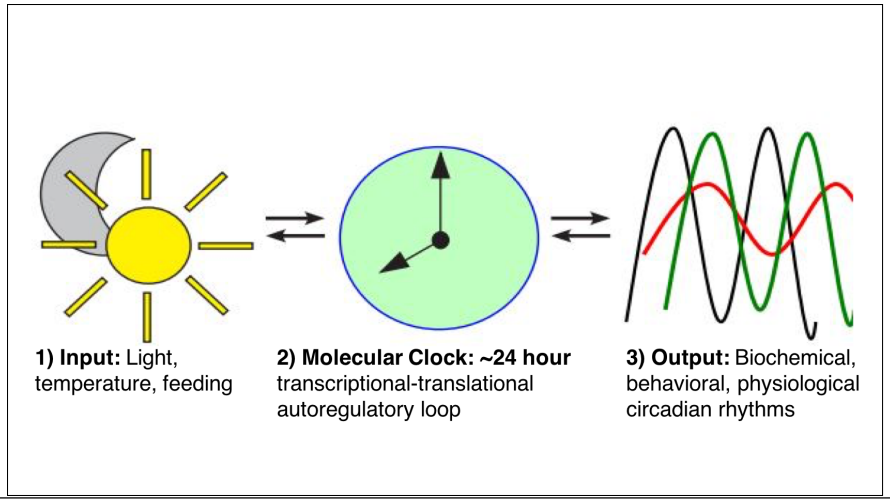

Figure 2: The circadian system. Environmental signals entrain the intracellular circadian clock mechanism, typically to a 24 hour time period. Circadian rhythms are the output of the circadian clock.

Image by J.O.Liang

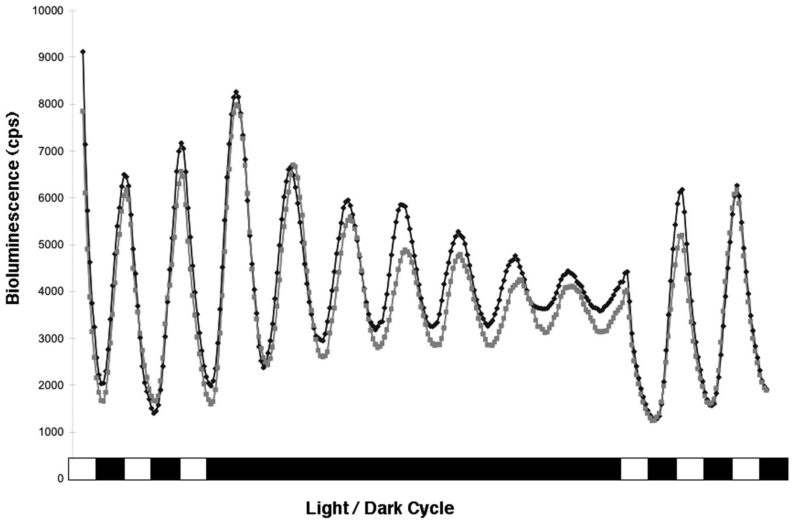

Figure 3. Rhythmic expression of the circadian *period* gene persists when zebrafish is transferred to constant dark but expression is dampened. Once placed back into a Light:Dark cycle, robust rhythmic gene expression is restored.

Image from: Tamai, T., Carr, A., Whitmore, D. (2005) Zebrafish Circadian Clocks: cells that see light. *Biochemical Society Transactions*. Vol 33: 5, 962-966

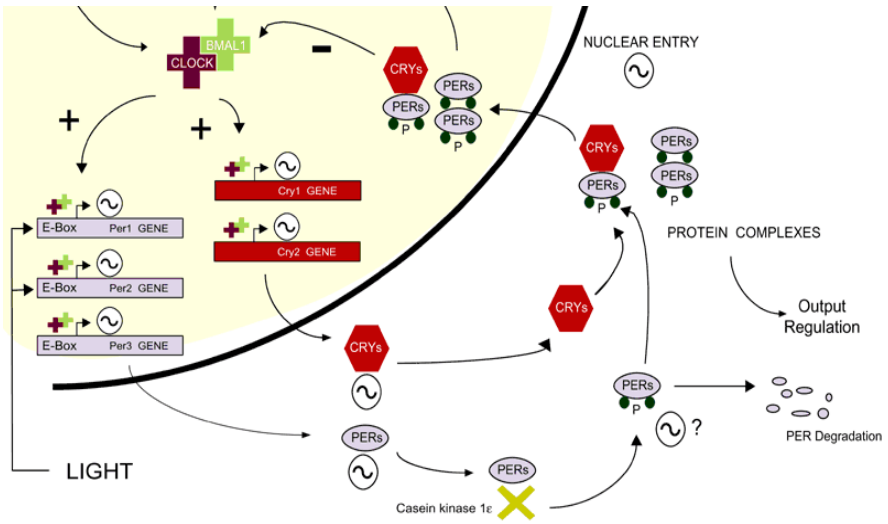

Figure 4. **Model of circadian clock molecular mechanism.** *clock* and *bmal* support a protein pool of CLK:BMAL heterodimers. CLK:BMAL then drive expression of *period* and *cryptochrome* genes. PER and CRY proteins down regulate their own expression by rendering CLK:BMAL inactive by mechanically preventing heterodimerization of CLOCK and BMAL proteins.

Itg.kit.edu Foulkes laboratory website

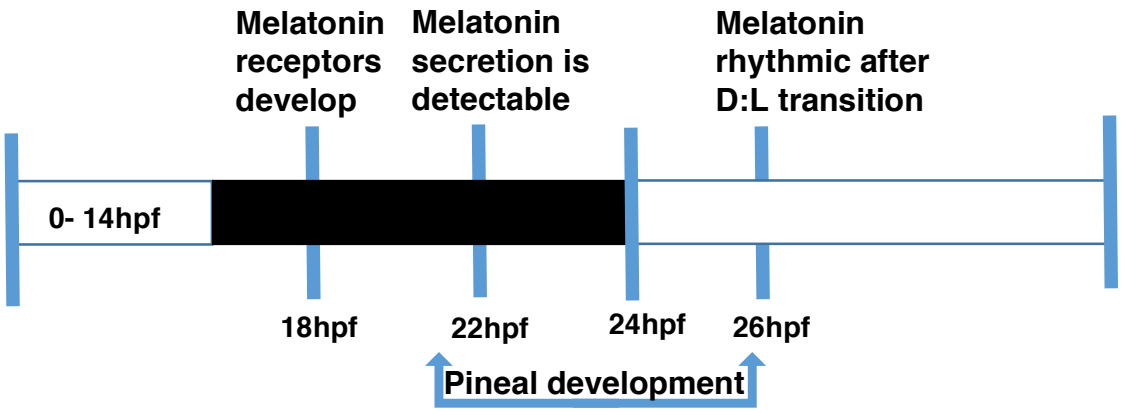

Figure 5. Key events in zebrafish development in relation is melatonin synthesis.

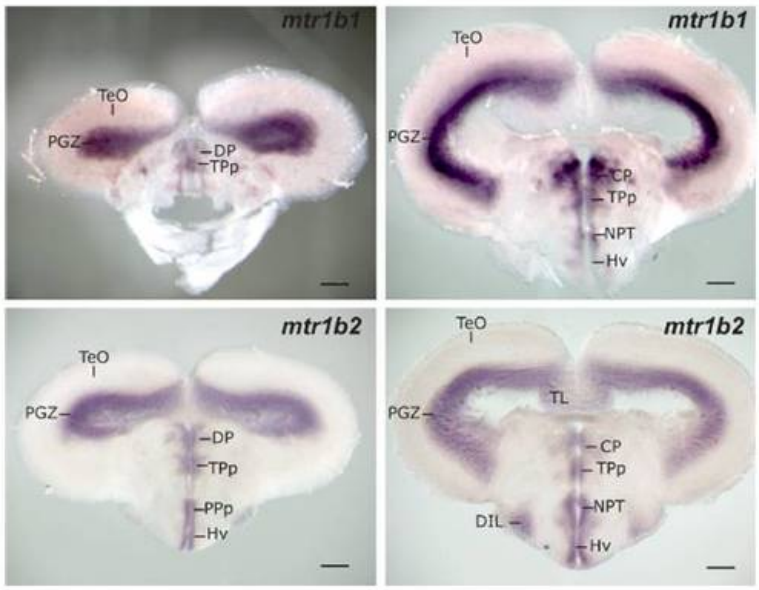

Figure 6: Expression of melatonin receptor genes *mtnr1ba* and *mtnr1bb* in adult zebrafish brains. CP, central posterior thalamic nucleus; DIL, diffuse nucleus of the inferior lobe; DP, dorsal posterior thalamic nucleus; Hv, ventral zone of the periventricular hypothalamus; NPT, posterior tuberal nucleus; PGZ, periventricular gray zone of the optic tectum; PPp, parvocellular preoptic nucleus, posterior part; TeO, optic tectum; TL, torus longitudinalis; TPp, periventricular nucleus of posterior tuberculum. (Scale bar, 200 μm.)

Appelbaum, L., Wang, G.X., Maro, G.S., Mori, R., Tovin, A., Marin, W., Yokogawa, T., Kawakami, K., Smith, S.J., Gothilf, Y., *et al.* (2009). Sleep-wake regulation and hypocretin-melatonin interaction in zebrafish. Proc Natl Acad Sci U S A *106*, 21942

CHAPTER 2: MELATONIN RECEPTOR CHARACTERIZATION

INTRODUCTION

Melatonin is a versatile hormone that is known to have developmental (Danilova et al., 2004), circadian (Gandhi et al., 2015), and reproductive (Carnevali et al., 2011) effects in zebrafish. Embryonic and early larval zebrafish in particular depend on melatonin to aid in neuronal differentiation (de Borsetti et al., 2011), regulate sleep (Gandhi et al., 2015), and potentially regulate their development (Danilova et al., 2004). Recent research has illustrated the diverse functionality of melatonin but the mechanisms behind these effects in zebrafish are not well understood. Thus, defining the spatial and temporal expression patterns of melatonin receptor genes is key to further understanding the function of melatonin within zebrafish.

The effects of melatonin on zebrafish development and sleep are thought to be mediated by G-protein coupled transmembrane melatonin receptors homologous to mammalian melatonin receptors (Danilova et al., 2004; Gandhi et al., 2015). Melatonin receptor subtypes are known to homodimerize and heterodimerize with one another in most vertebrates (Wiechmann and Sherry, 2013).

Since melatonin receptors have predominantly been in characterized in mammals and melatonin is highly conserved, we turn to the mammalian literature for a basic understanding of melatonin receptors. In mammals, there are two melatonin receptor subtypes, MT1 and MT2; with MT1 being more highly expressed than MT2 (Reppert et al., 1994). MT1 is found most abundantly in areas of the brain known to be involved in circadian rhythms (the pituitary par tuberalis and suprachiasmatic nucleus), suggesting a circadian role for this receptor type (Danilova et al., 2004; Reppert et al., 1994). The MT2 subtype, in contrast, is expressed mainly in the retina and to a lesser extent the brain (Danilova et al., 2004; Reppert et al., 1994). Similar to the findings in zebrafish, mammalian melatonin receptors have been found to be more widely expressed in embryos than adults (Rivkees and Reppert, 1991).

Zebrafish have undergone a genome duplication event at some point in their evolution and as such, have six known melatonin membrane receptor genes (Shang and Zhdanova, 2007). The mammalian melatonin receptor subtype MT1 is homologous to zebrafish *mtnr1aa, mtnr1a1, mtnrab* and the receptor subtype MT2 is homologous to zebrafish *mtnr1bb, mtnr1ba.* A third

subtype, *mtnr1c* falls into a new class by itself (Shang and Zhdanova, 2007). The MT2 melatonin receptor subtype is of particular interest to our studies because zebrafish MT2 homologs are thought to mediate the developmental role of melatonin in zebrafish (Danilova et al., 2004) and this is the focus of our work.

Relatively little work has been done to characterize spatial and temporal melatonin receptor expression in whole zebrafish. For example, to date *mtnr1aa* has only been characterized up to 36 hours post fertilization (hpf). Using whole mount RNA *in situ* hybridization (WISH) to characterize spatial expression of whole embryos (Danilova et al., 2004) found *mtnr1ba*, *mtnr1bb*, and *mtnr1aa* were widely expressed from between 18 hpf to 24 hpf. At 36 hpf, expression began to localize to the head region of the embryo (Danilova et al., 2004). Hernandez de Borsetti 2011 found wide distribution of *mtnr1ba* and *mtnr1bb* mRNA in 24 hpf, 36 hpf and 48 hpf zebrafish. However, the Hernandez de Borsetti 2011 paper only contains photos of embryos anterior dorsal view and not whole embryos, making conclusions about the range of spatial expression difficult. In 2007, Shang *et al.* used RT-PCR to report relative expression levels of all six zebrafish melatonin receptors at 5 dpf and 6 dpf; finding that melatonin receptors were

expressed rhythmically at 5 days post fertilization (dpf), but these results have not evaluated by spatial expression techniques.

Two receptor subtypes were chosen for this study, *mtnr1ba* and *mtnr1bb,* because they are homologous to the mammalian MT2 subtype and the most likely mediators of the developmental effects of melatonin in zebrafish (Danilova et al., 2004). This prediction is partially based on studies of splenocytes from MT1 knockout mice, where only MT2 receptors are expressed; these splenocytes have been found to proliferate in response to melatonin (Drazen and Nelson, 2001). In stronger support of *mtnr1ba* and *mtnr1bb* mediating developmental responses in zebrafish; when an MT2 selective agonist is applied to zebrafish embryos, they experience accelerated development (Zhdanova et al., 2001).

Our work set out to determine the spatial and temporal gene expression patterns of *mtnr1ba* and *mtnr1bb* during developmental time points from 24 hpf to 120 hpf using WISH. We found that *mtnr1bb* and *mtnr1ba* are widely expressed before 48 hpf then localize to the anterior portion of the larvae. However, in contrast to earlier studies that found melatonin receptor expression to be rhythmic at 5 days post fertilization (Shang and Zhdanova, 2007) we did not find rhythmic expression of

mtnr1ba and *mtnr1bb* from 24 hpf- 120 hpf. This work has characterized *mtnr1ba* and *mtnr1bb* expression at previously unpublished developmental times; finding that both receptor subtypes are located in almost all cells or all cells and then localize to the brain during zebrafish embryogenesis.

METHODS

Zebrafish care and maintenance

Adult and embryonic ZDR strain wildtype zebrafish were housed at 28.5 C on a 14 hour light:10 hour dark cycle unless otherwise noted. To obtain embryos, adult fish were placed in spawning tanks the day prior to collection. Embryo collection occurred within one hour of lights turning on after the last dark phase. All animal use and care was performed under UMN IACUC guidelines.

Whole Mount in situ Hybridization (WISH) and RNA probe construction

Spatial and temporal expression patterns of two zebrafish melatonin receptor genes, *mtnr1ba* and *mtnr1bb,* were characterized. Sense and antisense RNA WISH probes for *mtrn1ba* and *mtnr1bb* were constructed from PCR product (Thisse and Thisse, 2008). Primers were designed to

construct a PCR product that has a T7 RNA polymerase promotor on the 5' end and T3 RNA polymerase promotor on the 3' end (Thisse and Thisse, 2008). Synthesis of RNA antisense probe was completed using T3 polymerase and synthesis of sense RNA probe was completed using T7 polymerase. RNA probes were labeled with Digoxigenin.

Prior to WISH, chorions were removed manually. Zebrafish were fixed with 4% paraformaldehyde in 1X PBS at alternating light (L) and dark (D) time points: 24 hpf (D), 32 hpf (L), 48 hpf (D), 56 hpf (L), 72 hpf (D), 80 hpf (L), 96 hpf (D), 104 hpf (L), and 120 hpf (D). Embryos were processed for WISH according to Thisse and Thisse, 2008.

Table 1. Primer sequences* for constructing RNA probes used in WISH

Gene	Primer sequence
mtrn1ba	Forward primer 5'-cattaaccctcactaaagggaaGACCTGCTGGTGGTTTGCTA-3' Reverse primer 5'- taatacgactcactatagggCAGCGTTCAGGCAGGAGTTA-3'
mtnr1bb	Forward primer 5'-cattaaccctcactaaagggaaCTATCCATACCCGCTGGTGC-3' Reverse primer 5'-taatacgactcactatagggCACAAACCTCCGAGGAATCCA-3'

* lower case indicates T3 or T7 RNA polymerase promotor sequence

RESULTS

mtnr1bb is more widely expressed in zebrafish before 48 hpf than after

To determine spatial and temporal expression of MT2 subtype

homologous melatonin receptors in developing zebrafish, WISH was used to evaluate expression of *mtnr1bb*. Expression of *mtnr1bb* was characterized at alternating light and dark time points between 24hpf -120 hpf (Fig.7). At all time points, a subset of embryos were processed in parallel with a sense RNA probe that should not hybridize to *mtnr1bb* mRNA. These embryos served as controls for background staining.

At 24 hpf, expression of *mtnr1bb* was seen across almost the entire embryo, with high expression towards anterior and lower expression a posterior. By 56 hpf, expression of *mtnr1bb* localized to the head of the embryo; at 48 hpf and before, expression was found across most of the embryo (Fig.7). Between 96 hpf (D), 104 hpf (L), 120 hpf (D), slight variation expression occurred; with 104 hpf (L) having less expression than the two dark time points on either side (96 hpf and 120 hpf) (Fig.7). Sense probe treated embryos were always lighter in comparison to antisense probe treated embryos. (Fig.7).

mtnr1ba is more widely expressed in zebrafish before 48hpf than after

To determine spatial and temporal expression of MT2 subtype homologous melatonin receptors in developing zebrafish, WISH was used to

evaluate expression of *mtnr1ba*. Expression of *mtnr1ba* was characterized at alternating light and dark time points between 24hpf -120 hpf (Fig. 8). At all time points, a subset of embryos were processed in parallel with a sense RNA probe that should not hybridize to *mtnr1ba* mRNA. These embryos served as controls for background staining.

At 24 hpf, *mtnr1ba* is expressed across almost the entire embryo, with high expression towards anterior and lower expression in the posterior (Fig. 8). By 56 hpf, expression of *mtnr1ba* localized to the head of the embryo. No significant variation in expression was found between light and dark time points (Fig. 8). Sense probe treated embryos were always lighter in comparison to antisense probe treated embryos (Fig.8).

DISCUSSION

In this study, we characterized spatial and temporal expression of two melatonin membrane receptor genes, *mtnr1ba* and *mtnr1bb,* in developing zebrafish. Our findings support previous work (Danilova et al., 2004; de Borsetti et al., 2011) documenting the widespread expression of *mtrn1ba* and *mtnr1bb* throughout the developing zebrafish embryo until approximately 48 hpf (Fig. 7, 8). We have characterized expression of

mtnr1ba and *mtnr1bb* at eight time points in development that were previously not characterized. Analysis of these later time points indicates that transcripts localized to the head at approximately 48 hpf and after.

A previous study evaluating whole embryo gene expression using RT-PCR concluded that all six melatonin receptors in zebrafish were expressed rhythmically at 5 and 6 days post fertilization (dpf) (Shang and Zhdanova, 2007). Specifically, *mtnr1ba* was expressed over one fold higher at night than during the day and *mtnr1bb* was expressed half fold higher at night on than day by 5 dpf. Our study evaluated expression of melatonin receptors during the middle of the 14 hour light phase and middle of the 10 hour dark phase to try and capture the fluctuating expression that was found in Shang and Zhdanova 2007. Interestingly, we did not see a significant difference in expression levels of light or dark time points *mtrn1bb* or *mtrn1ba.*

There are several reasons why we may not have found rhythmic expression of *mtrn1bb* or *mtrn1ba.* First, it is possible that we did not see a gene expression rhythm because *mtrn1bb* or *mtrn1ba* were not yet rhythmic. The Shang and Zhdanova 2007 study was conducted from 5 dpf (120 hpf or later) to 6 dpf (later than 144 hpf) while our study ended at 120 hpf. A slight change in expression can be noted for *mntn1ba* between 96 hpf (D), 104 hpf

(L), 120 hpf (D), with 104 hpf (L) having less expression than the two dark time points on either side (96 hpf and 120 hpf) (Fig.7). It is possible this expression change was an early gene expression rhythm.

Another reason why we may not have seen a gene expression rhythm, is that WISH was not sensitive enough to detect small changes in expression levels. Furthermore, mRNA expression levels observed via WISH in single embryos varies slightly between each embryo and expression differences can be easily missed. In contrast, Shang et al. 2007 experiments used pooled mRNA from whole homogenized groups of 20-50 embryos and qRT-PCR. Pooling mRNA may compensate for individual expression variation. Lastly, our data is based on whole embryo expression observed without dissection. It is possible that expression differences in internal tissues are not easily visible and it should be considered that specific areas of the embryo may exhibit widely varied rhythmic expression not visible from whole mount observations. Further work is needed to determine if zebrafish melatonin receptors are rhythmic in specific tissues.

The two receptors in this study , *mtnr1ab* and *mtnr1bb*, were chosen because they are homologous to the mammalian MT2 subtype and present the most likely mediators of the developmental effects of melatonin in

zebrafish (Danilova et al., 2004). Although an MT2 selective agonist accelerated zebrafish development in Zhdanova et al. 2001, this effect should be interpreted with caution as selective agonists also activate MT1 receptors to some extent. However, our characterization has further established that MT2 homologous receptors are expressed widely in developing embryos and therefore, could play a role in zebrafish embryogenesis or other biological process regulated at this time in development.

Further systematic spatial and temporal characterization of zebrafish melatonin membrane receptors is needed to enable researchers to more accurately assess melatonin and melatonin receptor function in embryonic zebrafish. Since sequences of melatonin receptor genes are well known, knock out of melatonin receptors with CRISPR/Cas9 presents an accessible and affordable option to determine melatonin and melatonin receptor function in zebrafish. However, without fully understanding of the spatial and temporal expression of melatonin receptor mRNA in developing zebrafish embryos, these studies may be missing critical information, such specific tissues or developmental time points with high melatonin receptor expression.

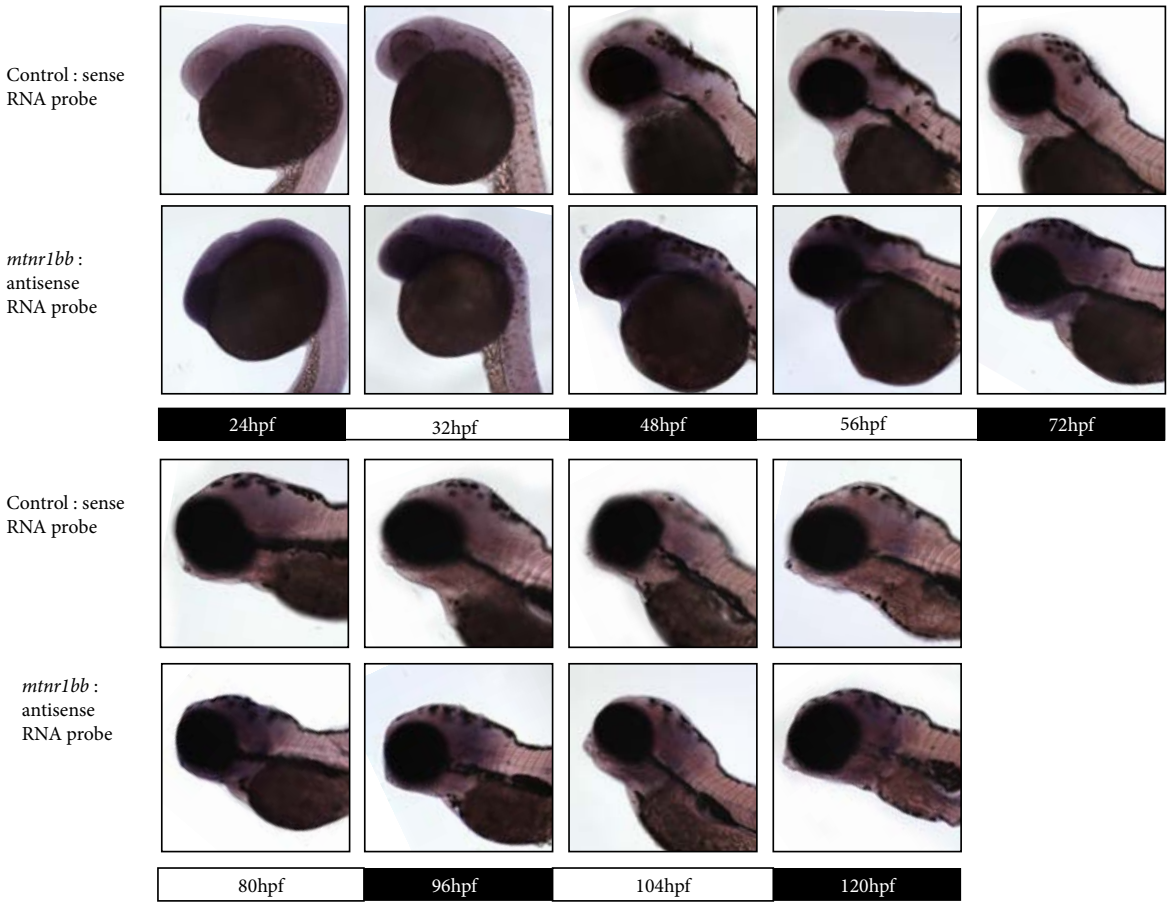

Figure 7. Expression of *mtrn1bb* is found throughout the zebrafish embryo before 48 hpf. Sense probe control at top, antisense probe below. WISH

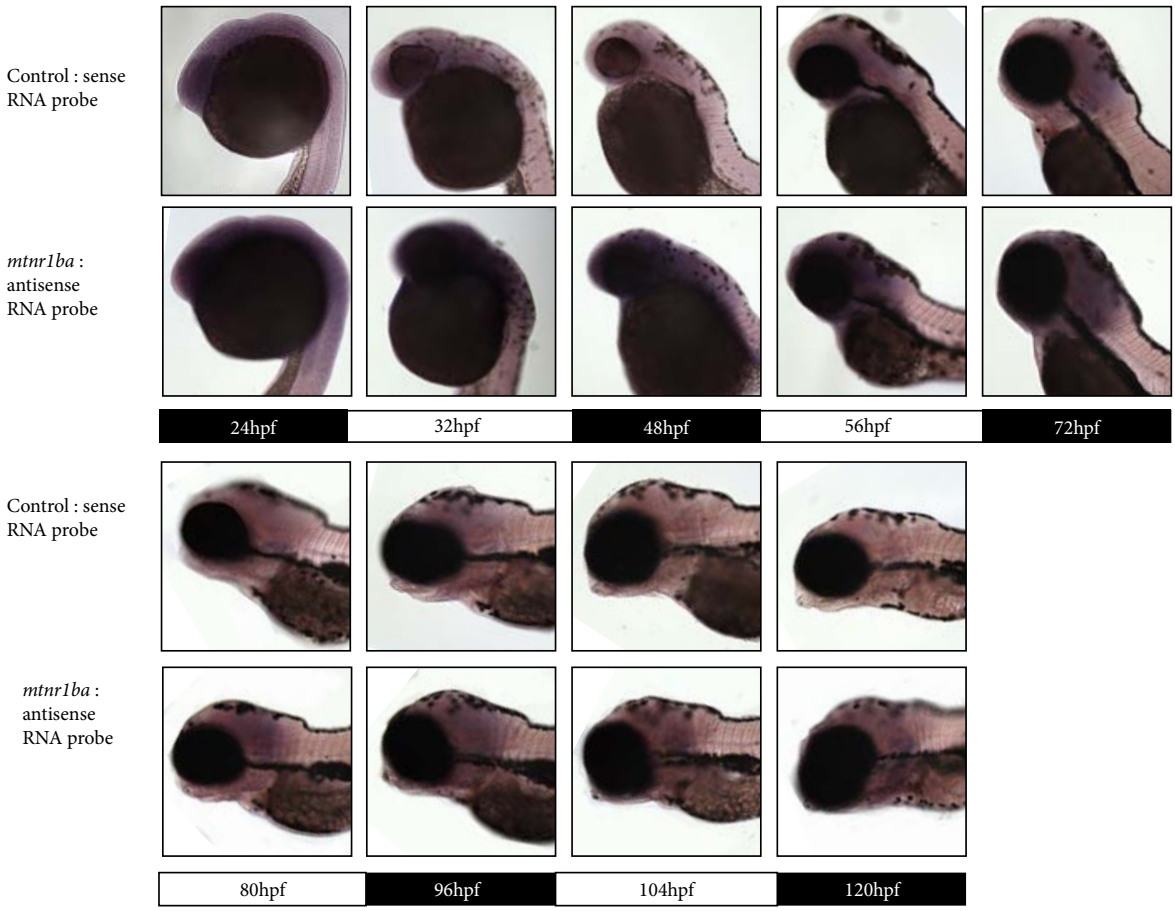

Figure 8. Expression of *mtrn1ba* is found throughout the zebrafish embryo before 48 hpf. Sense probe control at top, antisense probe below. WISH

CHAPTER 3: THE EFFECTS OF MELATONIN ON ZEBRAFISH CIRCADIAN RHYTHMS AND DEVELOPMENT

INTRODUCTION

The central component of the circadian clock is a transcriptional and translational feedback loop that drives rhythms with approximately 24 hour periods (Vatine et al., 2011). Although circadian rhythms are found across many taxa, circadian system organization varies among species (Vatine et al., 2011). The mammalian circadian system is organized in a largely centralized manner, with a master pacemaker located in the suprachiasmatic nucleus (SCN), which synchronizes peripheral clocks in the body. In contrast, the circadian system in zebrafish is thought to be decentralized; all zebrafish tissues tested to date have directly light entrainable clocks (Tamai et al., 2005).

It was long thought that mammals have a completely centralized circadian system, with the SCN exhibiting total control over phase, amplitude, and period of clocks throughout the body (Reiter et al., 2014). More recent research has challenged this notion, as many mammalian organs have been shown to contain autonomous clocks that can be entrained and maintain rhythms independent of the SCN (Schroeder and Colwell, 2013;

Yoo et al., 2004). However, ablation of the SCN leads to asynchronous clocks, illustrating that the mammalian SCN coordinates peripheral clocks (Yoo et al., 2004). The relationship of the SCN to independent peripheral clocks indicates that mammals have facets of both decentralized and centralized circadian organization.

Consistent with the model that the zebrafish circadian system is completely decentralized, the SCN in zebrafish embryos has not been found to regulate circadian clocks. Gene expression rhythms from zebrafish mutants lacking an SCN have normal phase length of the circadian regulated genes, *cry1b*, *cry3*, *exorh*, and *aanat2* but amplitude was diminished when the developing fish were transferred to constant conditions (Noche et al., 2011). If zebrafish exhibit some level of centralized circadian organization, melatonin and the pineal are appealing candidates for central mechanisms to coordinate circadian clocks in zebrafish. The zebrafish pineal gland contains all the components of a master pacemaker; including an intrinsic circadian clock, photoreceptor-like cells, and the circadian release of a humoral signal (melatonin) starting early in development (Vatine et al., 2011).

Melatonin synthesis is one of the first signs of the circadian system in zebrafish, being secreted by 22 hpf (de Borsetti et al., 2011; Kazimi and

Cahill, 1999). Furthermore, melatonin exhibits a circadian clock controlled rhythm after a single dark to light transition (Kazimi and Cahill, 1999); this is early in comparison to core circadian clock genes such as *bmal* that are not rhythmic until 3 days post fertilization (dpf) (Pando and Sassone-Corsi, 2001). Even before detectable melatonin levels, widespread melatonin receptor expression can be observed throughout the developing embryo at approximately 18 hpf (Danilova et al., 2004). This is in contrast to melatonin receptor expression in adult zebrafish, which is most highly expressed in parts of the brain (Appelbaum et al., 2009; Danilova et al., 2004). Developmentally early melatonin synthesis and widespread receptor expression suggest melatonin is playing a biological role during the first days of zebrafish development.

Supporting the idea that melatonin aids in establishing the circadian clock, maternal melatonin sets circadian rhythms during mammalian fetal development (Davis and Mannion, 1988). In mammals, melatonin has the ability phase shift circadian rhythms and speed re-entrainment of circadian clocks (Schroeder and Colwell, 2013). It has not yet been established if melatonin can phase shift or alter circadian clocks in zebrafish. During the time our study was in progress, another group published evidence that

zebrafish mutants lacking melatonin had regular circadian gene expression rhythms of three clock genes (Gandhi et al., 2015). Our work described here and the Gandhi 2015 study, suggest that melatonin does not play a role in regulating the circadian clock. To determine if melatonin could be acting as a central pacemaker during development, we tested the hypothesis that melatonin can alter gene expression rhythms of circadian clock and circadian controlled genes in zebrafish. We found that exogenous melatonin or melatonin receptor inhibition was not able to alter expression of circadian clock genes *clock1* and *per3* or genes downstream of the circadian clock, *irbp* and *aanat2*, in developing zebrafish embryos and larvae. However, the abundant expression of melatonin receptors in the first two days of zebrafish development suggests that the hormone may have a role in development.

It has been established that melatonin can accelerate certain aspects of development such as hatching and cell proliferation but the full role of melatonin in zebrafish development has not been explored (Danilova et al., 2004). We tested the hypothesis that exogenous melatonin accelerates development in zebrafish embryos. We found preliminary evidence, through analysis of the gene *clock1* and *irbp* and the developmental genes *dbx2* and *flh*, that melatonin accelerates the timeline of gene expression in developing

zebrafish. In support of our gene expression data, we found morphological differences in melatonin treated embryos versus controls.

METHODS

Zebrafish Care and Maintenance

Adult and embryonic ZDR wildtype zebrafish were housed at 28.5 C on a 14 hour light: 10 hour dark cycle unless otherwise noted. To obtain embryos, pairs of adult fish were placed in spawning tanks the day prior to collection. Embryo collection occurred within one hour of lights turning on in the morning and embryos were staged according to Kimmel 1995. All animal use and care was performed under University of Minnesota IACUC guidelines.

Drug Treatment

Embryos were placed in E3 zebrafish embryo medium with final concentrations of 20 nM, 100μM melatonin (Sigma) or 10 μM luzindole (Sigma) unless otherwise noted. Stock solutions consisted of 100mM melatonin and 100 mM luzindole in ethanol. Stock solutions of drugs were added to embryo media to final concentrations and control group embryos

were treated equal volume of ethanol.

Whole Mount RNA in situ Hybridization (WISH)

Prior to WISH, chorions were removed and embryos stored in methanol at -20C. Whole mount RNA in situ hybridization was completed according to Thisse 2008. Probes used were *per3, irbp, aanat2, clock1, flh*.

Constant Melatonin or Luzindole Exposure

0-2.5 hpf zebrafish embryos were exposed to either 20nM melatonin, 100μM melatonin, 10μM luzindole or ethanol (control). All embryo media contained 0.003% 1-phenyl 2-thiourea (PTU) to prevent melanocyte formation (Karlsson et al., 2001). Treatment and control water were changed every 24 hours. Embryos were raised in three lighting conditions; light: light (LL), 14 hour light: 10 hour dark (LD), and dark: dark (DD). Embryos were fixed with 4% PFA at light and dark time points each day relative to the LD cycle, starting at 24 hpf and continuing up to120 hpf.

Circadian Luzindole Experiment

Zebrafish embryos were collected within one hour of lights on and

sorted according to developmental stage to ensure all embryos were 256-cell (Kimmel et al., 1995) or earlier in development (~2.5 hpf). After staging and sorting, zebrafish embryos were placed on a 14 hour light: 10 hour dark cycle for 3 dark cycles then transferred to constant dark at 69 hpf and treated with 10μM luzindole. Embryos were fixed with 4% PFA at 70 hpf, 74 hpf, 94 hpf, 98 hpf, and 118 hpf.

Rhythmic exposure to melatonin

Embryos were housed and all work was conducted in constant dark. Embryo media contained 0.003%1-phenyl 2-thiourea (PTU) to prevent melanocyte formation (Karlsson et al., 2001). After collection, 1 hpf zebrafish embryos were placed in one of two groups: melatonin group or control group. Embryos were kept in specially designed dish with screen material on bottom to allow transfer of embryos from one treatment dish to another. Melatonin group embryos were exposed to 100 μM melatonin in embryo media for 10 hours to mimic nighttime then transferred to embryo media for 14 hours to mimic daytime. Control group embryos were exposed to embryo media and ethanol for 10 hours; then transferred to embryo media for 14 hours. Zeitgber time (ZT) refers to time after Zeitgeber signal, in this

case, ZT0 is beginning exposure of embryo media, which is meant to simulate daytime. Both treatment and controls group embryos were fixed with 4% PFA at 2 dpf during ZT 12.5, ZT 17.5, ZT 3, at approximately 3 dpf during ZT12.5, ZT17.5, ZT3 and at approximately 4 dpf during ZT12.5, ZT17.5.

Gene onset experiments

Embryos were collected within one hour of lights turning on. Embryos at 2-cell and 8-cell stages were used for experiments. Once staged, embryos were placed in constant dark; all work was conducted in constant dark until embryo fixation. After removal from incubator, lights were turned on and embryos were quickly pipetted into cryotubes and flash frozen in liquid nitrogen. Embryos were frozen and stored at -80 °C.

***RNA isolation and purifica*tion**

Frozen embryos were transported on liquid nitrogen until trizol reagent was added and homogenized for 30 sec. Following homogenization, RNA was isolated according to Lan 2009. RNA clean-up was completed with the Qiagen RNeasy mini kit.

Reverse Transcription Quantitative PCR

Reverse transcription of 4μg of RNA was completed using the Qiagen Omniscript Reverse Transcription Kit and random hexamers. A serial dilution of 1:4 was completed to produce standard curves and all wells were duplicated. Primer sequences for *irbp*, *clock1*, and *rpl13a* were graciously provided by the Brenton lab. Primer sequences for *dbx2* were obtained from Gribble 2007. The gene *rpl13a* was used as a normalization gene. All expression levels from melatonin treated embryos were normalized to corresponding ethanol treated embryos at the same time point.

Table 2. Primer sequences used for RT-qPCR

Gene	**Forward (5' to 3')**	**Reverse (5' to 3')**
dbx2	TCTGCCTGCTGCTCTGGAT	GTTGCGCTCCGTCTTGCTGA
clock1	GATCAGCAGACCCACACACA	GTAAAAGACGTGGGGCCTGA
irbp	TTGAACAGCTCACAGGCAGG	ATCATTGCACGTCCCAACCT
rpl13a	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG

RESULTS

Melatonin Concentration Curve

To identify effective concentrations of melatonin for our studies, we carried out three concentration curves. The first tested the range 10 nM to 100 μM, the second 10 nM to 10 μM and the third 1000 μM to 1 nM melatonin. Treatment started at 2.5 hpf and ended at 48 hpf, when the embryos were fixed and analyzed for expression of the pineal gene *irbp* (Fig. 9).

Unfortunately, melatonin had no effect on *irbp* expression. There were no observable differences in gene expression between embryos treated with melatonin and controls at any of the concentrations tested (Fig. 9). However, the melatonin was biologically active, as embryos treated with 20 nM or above failed to respond to touch, suggesting they were in a sleep light state (data not shown). Based on this behavioral result, subsequent experiments used a concentration of 20 nM or 100 μM melatonin.

Constant melatonin exposure had no effect on embryonic gene expression of per3, irbp, and aanat2 in the first 5 days of development.

Before the circadian clock becomes functional in zebrafish embryos,

circadian controlled genes and circadian clock genes are rhythmically expressed but will not continue to be rhythmic if embryos are transferred to constant conditions. If melatonin is acting as a mechanism to coordinate circadian clocks in zebrafish, then we expected these early gene expression rhythms to be dampened or eliminated in embryos exposed to constant exogenous melatonin.

To determine if melatonin can alter gene expression rhythms before clock function has started, embryos were treated with constant melatonin or ethanol (control) shortly after fertilization and placed in differing light conditions. To evaluate melatonin's impact on the circadian clock itself and downstream circadian controlled genes, three genes were chosen for WISH*:* *interphotoreceptor retinoid binding protein (irbp)* and *arylalkylamine N-acetyltransferase-2 (aanat2),* which are circadian clock controlled genes expressed in the pineal and *period3 (per3),* which is a circadian clock gene expressed throughout the embryo (Gothilf, 1999; Stenkamp, 1998; Delaunay, 2000).

Constant melatonin exposure did not alter gene expression of *per3, irbp, or aanat2* (Fig.10-14). Embryos that were exposed to constant melatonin and housed in a LD cycle from 24 hpf- 80 hpf did not have

significantly different *per3* expression when compared to controls (Fig. 11). Embryos exposed to melatonin from 24 hpf- 80 hpf housed in LD, LL, and DD light conditions did not have any difference in gene expression of *irbp* (Fig.12,13,14) or *aanat2* (Fig. 10) when compared to controls. *irbp* expression was slightly higher in melatonin treated embryos housed in constant light conditions in comparison to controls (Fig. 14).

In a separate experiment, expression levels of *irbp* were compared in zebrafish mutants lacking eyes (*chohk* mutants) and WT siblings from to 72 hpf. *irbp* expression did not differ between *chohk* mutants and WT (data not shown).

Constant luzindole exposure had no effect on gene expression of aanat2 or irbp in the first 5 days of zebrafish development

To determine if melatonin inhibition could alter circadian rhythms of gene expression before the onset of the circadian system, embryos were treated with a constant level of the melatonin receptor antagonist luzindole starting by 2.5 hours post fertilization and placed in differing light conditions (LD or LL). Constant luzindole exposure had no effect on *aanat2* or *irbp* expression embryos raised in LD conditions. Treated embryos were

indistinguishable from controls treated with ethanol (Fig. 15,16). Luzindole and ethanol treated embryos exhibited similar expression levels of *irbp* in LL; 32hpf- 56hpf luzindole treated embryos had slightly higher expression levels at all time points when compared to ethanol treated embryos (Fig. 17).

Luzindole eliminated circadian rhythms of clock1 expression in zebrafish larvae

To determine if inhibiting melatonin receptor function alters established circadian gene expression rhythms, embryos were exposed to a 14 hour light: 10 hour dark cycle for approximately three days then transferred to constant conditions and exposed to luzindole (Fig. 18). In embryos treated with vehicle (ethanol), expression of *clock1* decreased towards 94 hpf, increased at 99 hpf, and then decreased at 118 hpf; mirroring the expected expression of *clock1* if the gene was entrained sufficiently prior to transfer to constant conditions. In contrast, luzindole treated embryos expressed *clock1* at a steady level that was lower than ethanol treated embryos. Luzindole treated embryos also did not exhibit notable expression changes between time points.

Rhythmic exposure to melatonin had no effect on clock1 expression in developing zebrafish

If melatonin can alter circadian rhythms of gene expression in zebrafish, then rhythmic exposure to melatonin should be able to initiate a rhythm of gene expression in developing zebrafish larvae. To determine if periodic melatonin exposure can initiate a gene expression rhythm, embryos were exposed to 14 hour ethanol: 10 hours melatonin starting at 1 hpf. Exposure occurred in constant dark to avoid any environmental entrainment signals.

No difference in *clock1* expression was observed between the control treatment group and melatonin treatment group from 48.5 hpf to 81 hpf (Fig.19). *clock1* expression was not rhythmic in the melatonin treated group and ethanol treated group. A no probe WISH control was run simultaneously and did not develop purple color beyond normal background.

Exposure to melatonin had no effect on clock1 expression but may have accelerated the timeline of dbx2 expression

To verify our WISH findings that constant melatonin did not alter gene expression of circadian clock genes, *clock1* expression was evaluated

using RT-qPCR in embryos exposed to constant melatonin. We tested the nhypothesis that melatonin can accelerate the expression timeline of developmental genes by evaluating expression of *dbx2* in melatonin treated embryos at time points near known gene expression changes. Embryos were exposed to either ethanol or melatonin and then flash frozen at 24 hpf, 25 hpf, 26 hpf, 29 hpf to evaluate gene expression onset or alteration using RT-qPCR.

clock1 expression did not differ between ethanol and melatonin treated embryos (Fig.21). When abundance of *dbx2* expression in melatonin treated embryos was evaluated relative to ethanol treated embryos; expression of *dbx2* was 0.5 times higher in melatonin treated embryos at 23 hpf. At 24 hpf and 25 hpf *dbx2* expression was almost equal between melatonin and ethanol treated groups, and by 29 hpf expression in melatonin treated embryos was lower than ethanol treated embryos (Fig.22) Differences were not statistically significant, possibly due to natural variation among replicates and small sample size.

DISCUSSION

Constant melatonin or luzindole does not impact gene expression of irbp, per3, clock1 and aanat2 in developing zebrafish.

If melatonin aids in establishment of circadian rhythms in developing zebrafish, we have found no evidence for this. Experiments exposing zebrafish embryos to constant and timed addition of melatonin or luzindole during the first 5 days of development suggest that melatonin is not aiding in the establishment of circadian rhythms in zebrafish. Since melatonin could be acting directly on the clock or downstream of the clock to establish circadian rhythms; we evaluated components of the circadian clock mechanism (*per3* and *clock1*) and downstream circadian controlled genes (*irbp*, *aanat2)*. Melatonin is not likely to regulate gene expression of *irbp*, *aanat2*, and *per3* in developing zebrafish, as we found no significant change in gene expression when embryos were exposed to melatonin or a melatonin receptor antagonist. We also evaluated *clock1* expression in response to constant melatonin exposure until 29 hpf using RT-qPCR and found that melatonin did not alter *clock1* expression. Further supporting these findings, in an experiment designed to create a circadian rhythm using only melatonin

and no other environmental signals; embryos did not develop gene expression rhythms of *clock1* (a circadian clock gene) when exposed to timed addition of melatonin.

While our study was in progress it was shown that *aanat2* $^{-/-}$ mutants that lack pineal secreted melatonin have rhythmic gene expression of the circadian genes *period1 (fluorescent ISH and RT-qPCR)*, *period3 (luciferase reporter)* and *bmal1(RT-qPCR)* all matching that of their wild type counterparts (Gandhi et al., 2015). These genes are representative of both loops of the circadian clock. This contradicts the hypothesis that melatonin regulates circadian clocks in zebrafish, as mutants lacking melatonin should have perturbed circadian clock gene expression if this hypothesis is correct. However, there are limitations to the Gahndi 2015 study: only three circadian clock genes were tested; no downstream circadian regulated genes such as *irbp* were tested. Additionally, *aanat1*, a gene that largely contributes to retinal melatonin production was not knocked out for the circadian portions of these experiments (Gandhi et al., 2015). However, the use of RT-qPCR, FISH, and LUC:*per3* transgenic fish in the Gandhi 2015 study is of value. Using a range of techniques, they did not find any difference related to circadian clock gene expression between melatonin

deficient mutants and wild type fish. Furthermore, their results evaluating *per3* expression using a luciferase reporting transgenic line are in line with our findings that suggest exogenous melatonin had no effect on *per3*. While the Gandhi 2015 paper concludes that a lack of melatonin does not affect circadian rhythms in zebrafish; our preliminary data evaluating *clock1* suggests that exogenous melatonin may be able to alter established rhythms.

Preliminary circadian work suggest melatonin can alter established circadian gene expression rhythms.

Our circadian work suggests that blocking melatonin receptors with luzindole after the circadian clock is independently rhythmic, may have an impact on *clock1* gene expression in zebrafish. Embryos treated with luzindole had dampened amplitude of *clock1* rhythmic expression in comparison to control embryos that had a high-to-low gene expression rhythms. In this study, *clock1* was entrained to a rhythm before treatment with luzindole and *clock1* expression rhythms were expected to continue in control groups.

The finding that *clock1* rhythmic expression was damped after treatment with luzindole was unexpected, because constant melatonin

application had no effect on other circadian gene expression tested in this study. However, the genes evaluated in constant melatonin experiments were not part of the *clock/bmal* feedback loop within the circadian clock mechanism. It is possible that melatonin may alter expression of particular parts of the molecular circadian clock while not altering expression of the entire clock and the genes we evaluated were, by chance, not genes that are impacted by melatonin. Due to the interdependent nature of the circadian clock mechanism though, this seems unlikely. More likely, melatonin does not play a role in initiating the circadian clock but may be able to impact established rhythms.

In support of this idea, exposure to constant luzindole before the circadian clock has been established, did not alter gene expression of *irbp* and *aanat2* in embryos. Conversely, exposure to luzindole after the circadian clock has been established, did result in altered gene expression of *clock1* in embryos. Furthermore, when embryos were exposed to constant melatonin before the circadian clock had been established, *clock1* expression was not altered but when embryos were exposed to luzindole after the circadian clock had been established, *clock1* gene expression was altered. This illustrates a main conclusion of this work; melatonin most likely does not aid

in establishment of the circadian clock in zebrafish but melatonin may be able to regulate established rhythms.

Melatonin may alter irbp expression in the zebrafish pineal

Of interest, in constant light and constant melatonin conditions, embryos experienced increased pineal *irbp* expression (Fig. 14). Melatonin is not synthesized during constant light conditions (de Borsetti et al., 2011) so we can reliably conclude this effect was due to melatonin. The increase of *irbp* expression is an interesting finding because melatonin did not increase *aanat2* expression in constant light, a gene that is also expressed in the pineal and under circadian clock control (Fig. 10).

However, it has been suggested that melatonin increases visual sensitivity at night (Harsanyi and Mangel, 1992). Indicative of this effect, melatonin increases light induced photoreceptor cell death (Wiechmann and O'Steen, 1992) and luzindole protects photoreceptors from light induced damage (Sugawara et al., 1998) in rats. In zebrafish, IRBP protein levels remain constant during the day, despite rapid turnover and *irbp* mRNA is expressed much more highly during the day (Cunningham and Gonzalez-Fernandez, 2000)(Gamse et al., 2002). In 2000, Cunningham and Gonzalez-

Fernandez suggested a model in which *irbp* mRNA is expressing during the day at levels that compensate for IRBP protein degradation. If this model is correct, it is possible that in our study melatonin exposure increased photosentivity in our treatement embryos during constant light conditons, resulting in increased damage to photoreceptor machinery and *irbp* mRNA was expressed at a higher level to compensate for increased IRBP degradation. While these ideas are speculative, the finding that melatonin increased *irbp* expression should be investigated further as it may have significant insight into the role of melatonin in photoreception in the pineal

Melatonin may accelerate gene expression onset in developing zebrafish

Our preliminary work evaluating the ability of melatonin to accelerate zebrafish development suggests that melatonin can stimulate gene expression onset to occur earlier in development. Early in our study we found that embryos raised in constant dark and treated with a melatonin receptor antagonist were markedly less developed than controls. Conversely, melatonin treated embryos were further along in development when compared to controls (Fig. 23,24). Furthermore, we found that *irbp* was expressed sooner in development within melatonin treated embryos than

ethanol treated embryos (Fig.13).

No differences in gene expression onset or level of expression were found for the gene *floating head* (*flh*) between melatonin treated embryos and controls. This result was expected as *flh* expression begins by 4 hpf in zebrafish, much sooner than melatonin receptors are initially expressed (~18hpf). This is consistent with previous studies suggesting the developmental effects of melatonin are receptor mediated (Danilova et al., 2004).

While the RT-qPCR data gathered for this study does have limitations due to replicate variation, the observed reduction in *dbx2* over 5 hours is in line with the hypothesis that melatonin can accelerate development in zebrafish. The expression of *dbx2* begins by 24 hpf but ceases by 48 hpf (Gribble et al., 2007), this developmentally late onset of expression and dynamic change in expression makes *dbx2* a good gene to assess in zebrafish. The melatonin treated embryos experienced reduced expression of *dbx2* sooner than ethanol treated embryos (Fig. 22). RT-qPCR has proven to be a valuable method to evaluate gene expression onset and this experimental design could be used in future studies for other zebrafish genes.

Study Limitations

When evaluating gene expression using WISH, studies can only be performed up to 5 dpf, as WISH reliability is greatly reduced by 5 dpf in zebrafish due to inability of the RNA probe to penetrate older tissues (Thisse and Thisse, 2008). While WISH is a valuable tool to evaluate gene expression in specific tissues such as the pineal, it is difficult to compare subtle expression level differences. Visualizing and measuring gene expression using WISH is even more challenging for circadian genes expressed throughout the entire embryo. It is possible that exogenous melatonin or melatonin receptor blocking does have an effect on gene expression rhythms or gene onset in zebrafish but the effect is so slight that it is not detectable by WISH. As such, RT-qPCR presents a viable alternative to evaluate gene expression, since expression can be compared on a quantitative level.

Although, RT-qPCR also presents caveats for interpreting circadian data as well. To obtain enough mRNA to complete RT-qPCR, embryos need to be pooled and homogenized. This eliminates the ability of the researcher to evaluate gene expression on an induvial basis and view spatial expression.

Furthermore, embryos must be snap frozen so assessing the development or mortality of embryos is impossible.

Additionally, many circadian genes in zebrafish have tissue specific expression in zebrafish (Whitmore 2014). Further complicating matters, the spatial patterns of many circadian genes have not been mapped. While melatonin may not appear to be altering expression of genes tested in this study, their expression may be altered in a tissue specific manner that is beyond our detection with WISH or whole embryo RT-qPCR.

Conclusion

The potential developmental role of melatonin is an area that many zebrafish scientists have not explored but could result in major findings. It is hypothesized that melatonin evolved more than 2.5 billion years ago, making it an ancient component of biological systems and it is highly conserved across species (Reiter et al., 2014). If melatonin is found to have a significant developmental role in zebrafish, this role may be conserved across taxa, and as such, could represent a promising area of biomedical study.

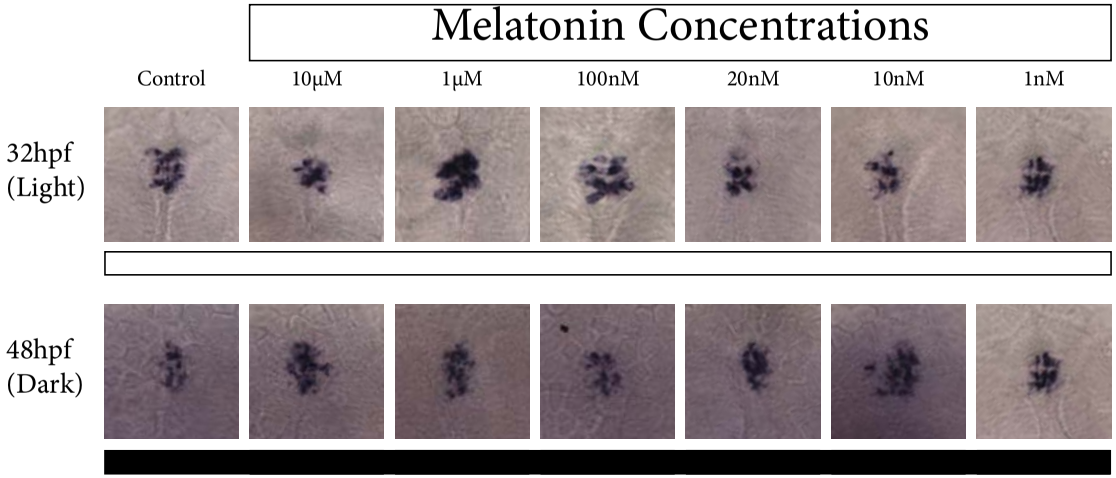

Figure 9. Embryos exposed to constant melatonin of various concentrations exhibited similar expression of *irbp*. Pineal expression pictured. Embryos were raised on 14hr Light: 10hr Dark cycle and fixed at 32hpf in light (first row) and 48hpf in dark (second row).
Whole mount *in situ* hvbridization.

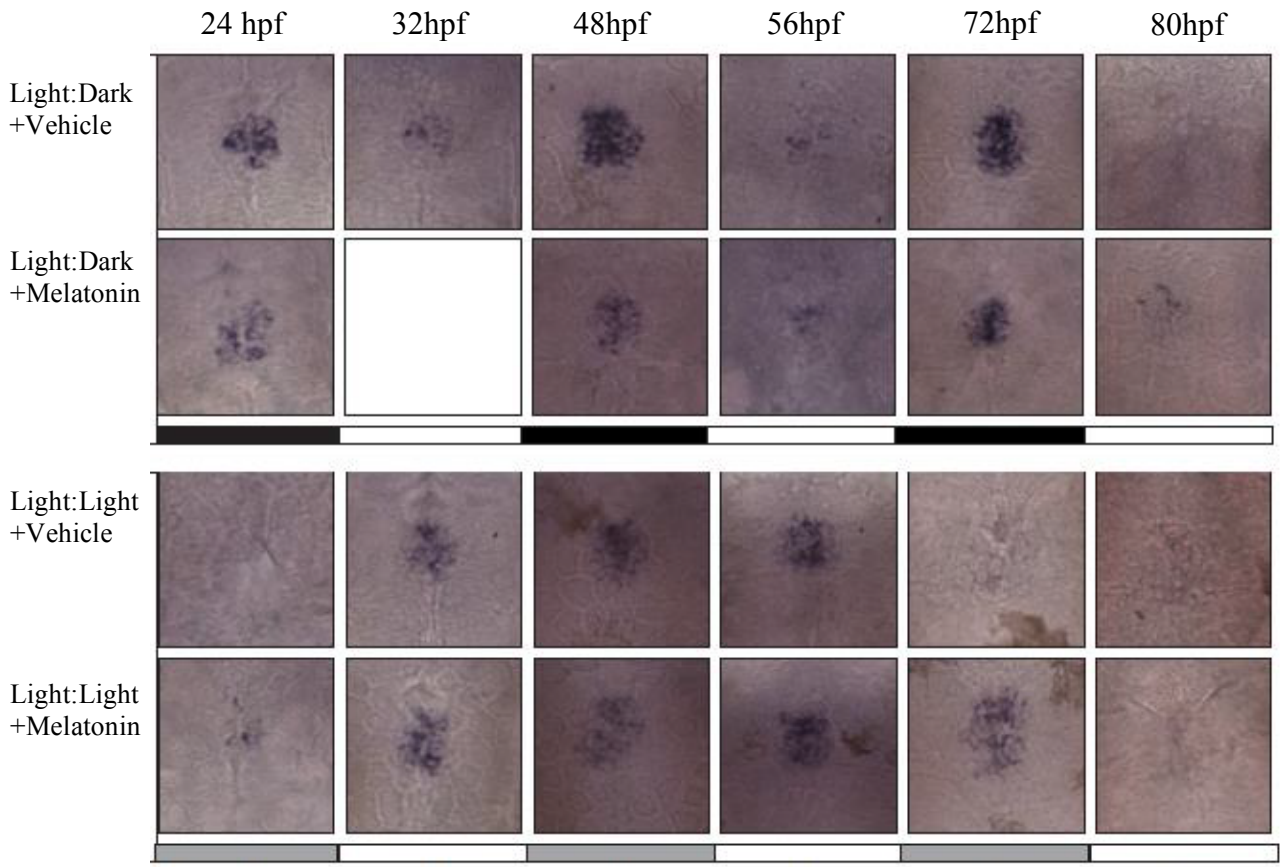

Figure 10. Constant melatonin had no effect on *aanat2* expression in Light:Dark or constant light conditions. Embryos exposed to melatonin had similar expression of *aanat2* in comparison to controls in L:D (Top) and L:L (Bottom) conditions. Pineal

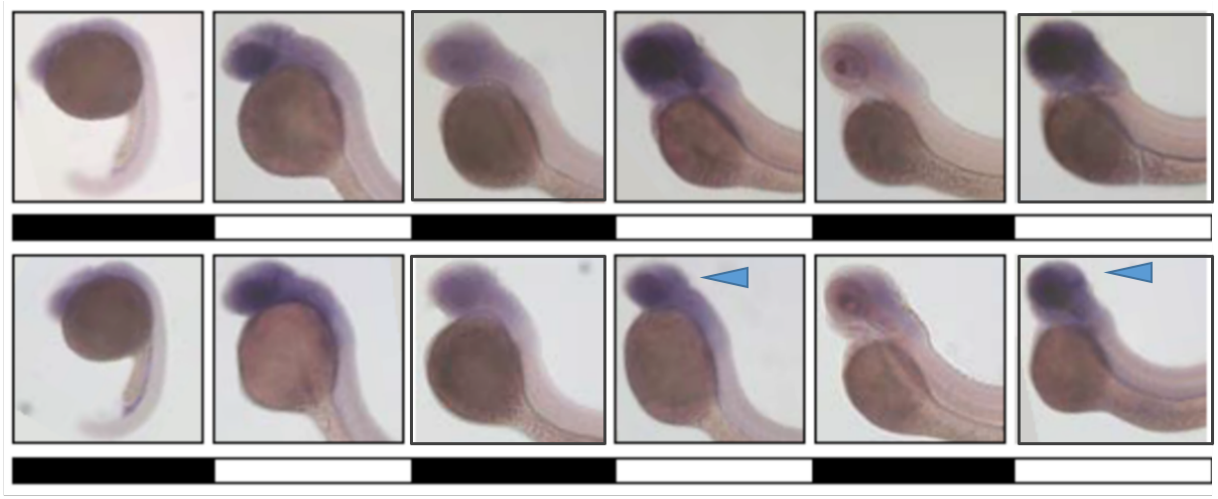

Figure 11. Constant melatonin had no affect on *per3* expression in comparison to controls. Expression of *per3* is similar in melatonin treated and ethanol treated embryos housed in L:D conditions. At 56hpf and 80hpf at light exposed time points, *per3* expression in the anterior appears to be slightly lower in melatonin treated embryos (blue arrows). Whole mount *in situ* hybridization.

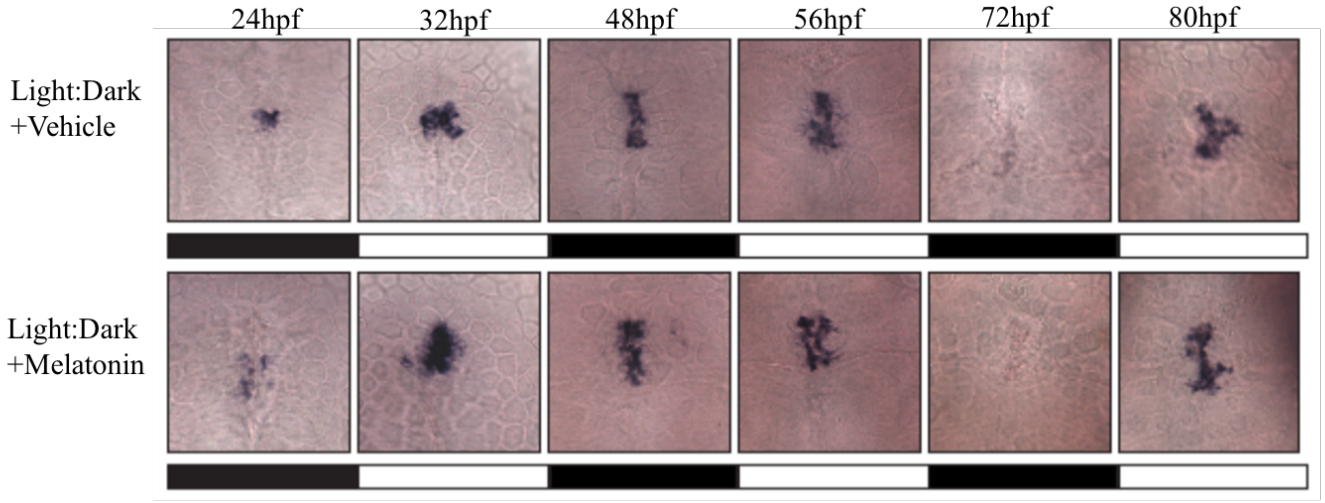

Figure 12. Constant melatonin had no effect on *irbp* expression in light:dark conditions. Expression of *irbp* does not differ between melatonin treated and ethanol treated embryos housed in Light: Dark conditions. Pineal expression pictured. Whole mount *in situ* hybridization.

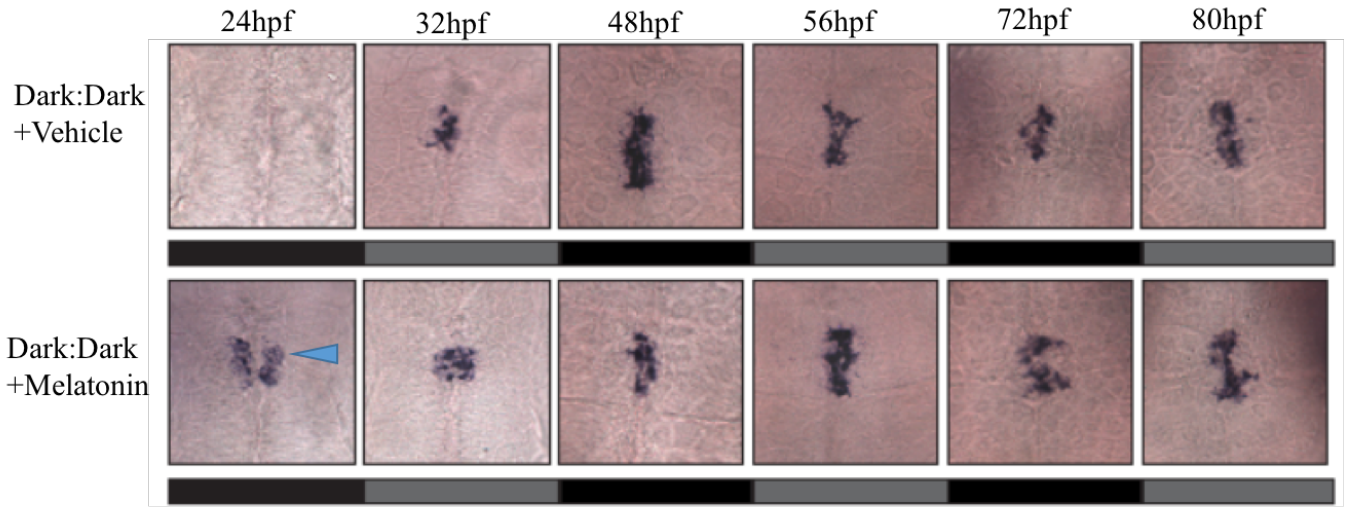

Figure 13. Constant melatonin had no effect on *irbp* expression in constant dark. Expression of *irbp* is similar in melatonin treated and ethanol treated embryos housed in constant dark conditions. At 24hpf *irbp* expression began in melatonin treated embryos (blue arrow) but not ethanol treated embryos. Whole mount *in situ* hybridization. Pineal expression pictured

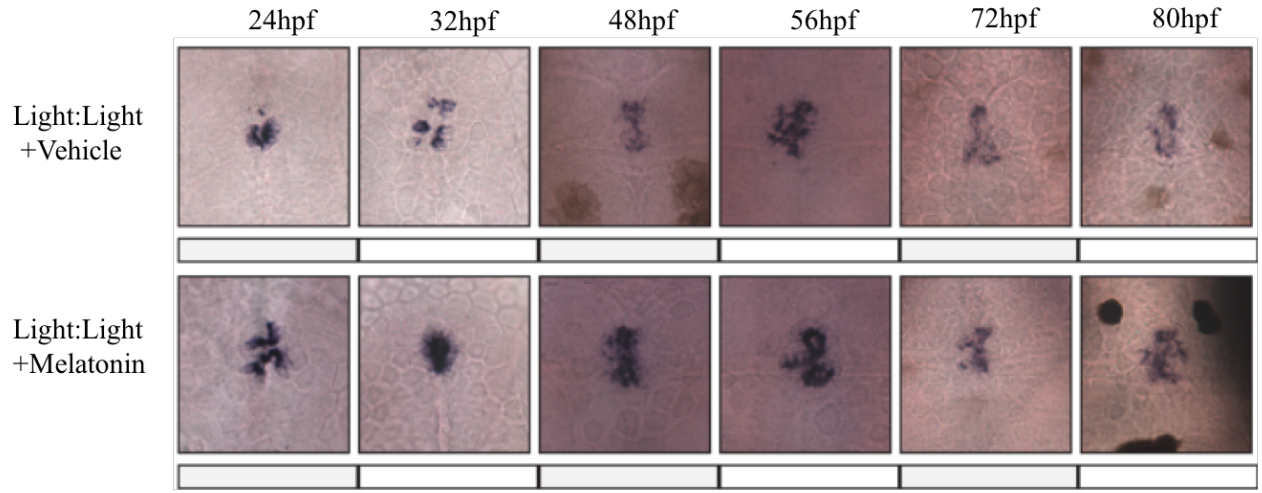

Figure 14. Constant melatonin had minimal effect on *irbp* expression; slightly increasing *irbp* expression in constant light up to 56hpf. Expression of *irbp* is similar in melatonin treated and ethanol treated embryos housed in constant light conditions. Whole mount *in situ* hybridization. Pineal expression pictured

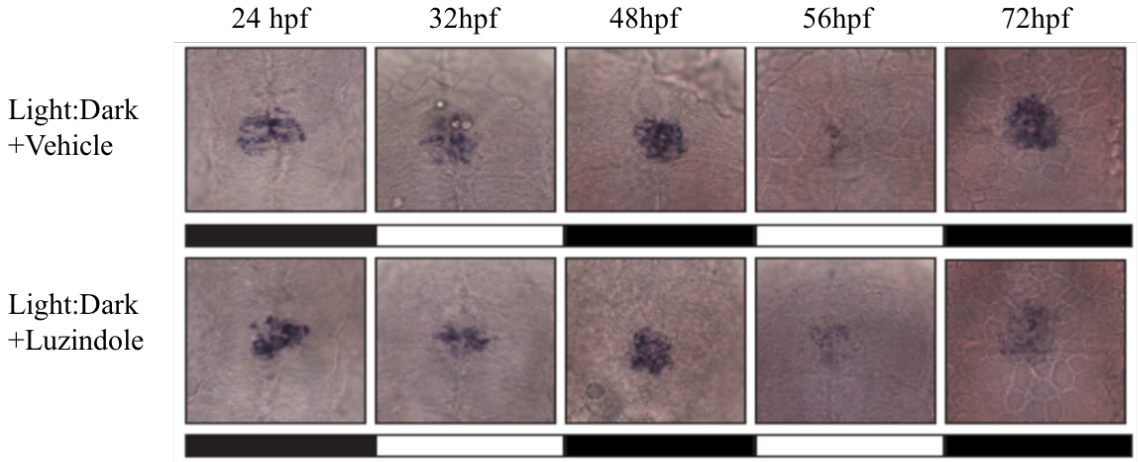

Figure 15. Constant luzindole had no effect on *aanat2* expression in Light: Dark. Embryos treated with constant ethanol and embryos treated with constant luzindole exhibited similar expression of *aanat2* in Light:Dark conditions. Whole mount *in situ* hybridization**.** Pineal expression pictured

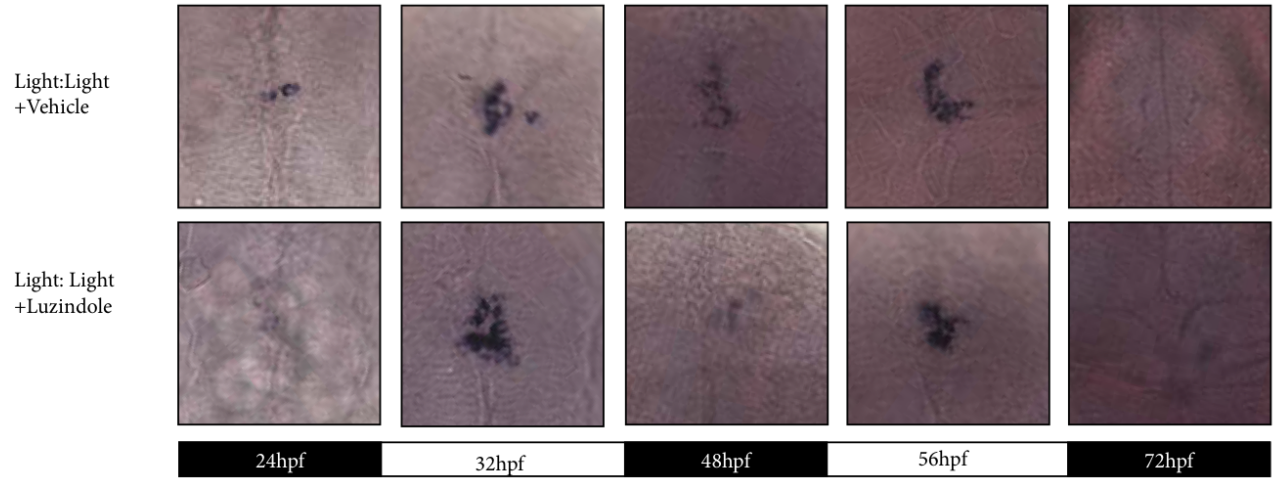

Figure 16. Constant luzindole had no effect on *irbp* expression in Light:Dark conditions. Embryos treated with constant ethanol and embryos treated with constant luzindole exhibited similar expression of *irbp* in Light:Dark conditions. Whole mount *in situ* hybridization. Pineal expression pictured

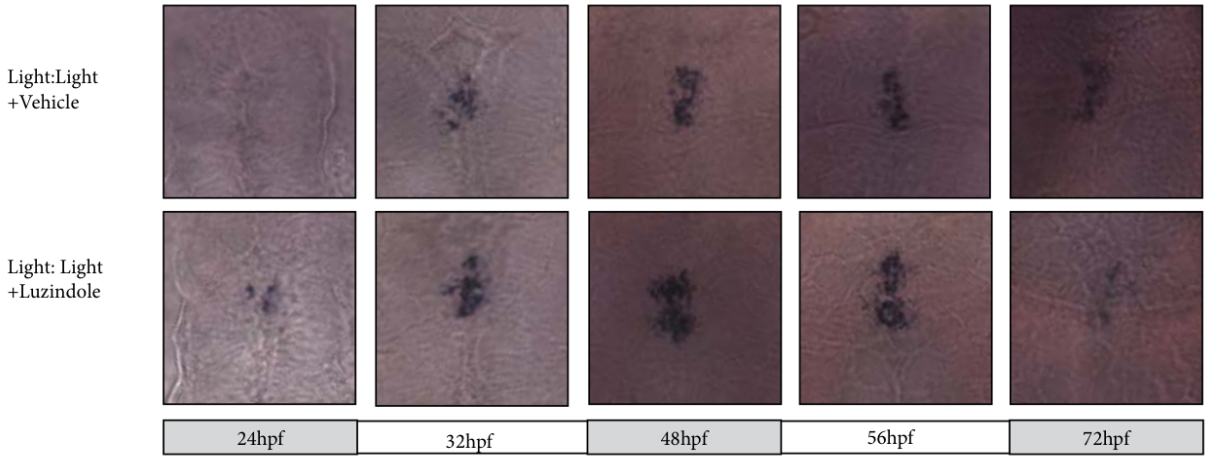

Figure 17. Constant luzindole had no effect on *irbp* expression in constant light conditions. Embryos treated with constant ethanol and embryos treated with constant luzindole exhibited similar expression of *irbp* in Light:Light conditions. Whole mount *in situ* hybridization**.** Pineal expression pictured

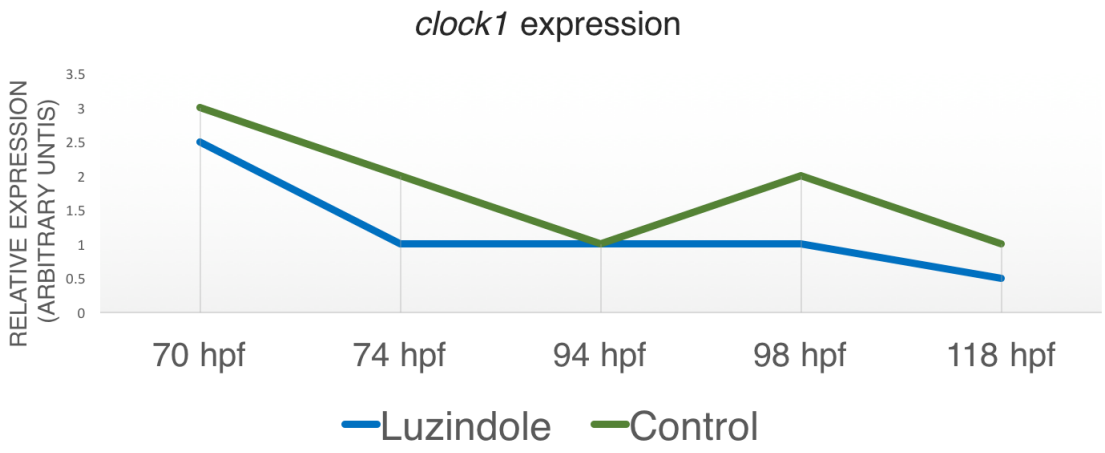

Figure 18. In a circadian time course, luzindole reduced *clock1* expression rhythm in comparison to controls. After exposure to light:dark cycles for 69 hours, embryos were transferred to constant darkness and treated with luzindole. Note the lower- higher- lower expression pattern of control embryos; this reflects the previous light/dark cycle. In contrast, luzindole treated embryos did not exhibit this variation. 70hpf- 118hpf embryos housed in constant dark. Whole mount *in situ* hybridization**.**

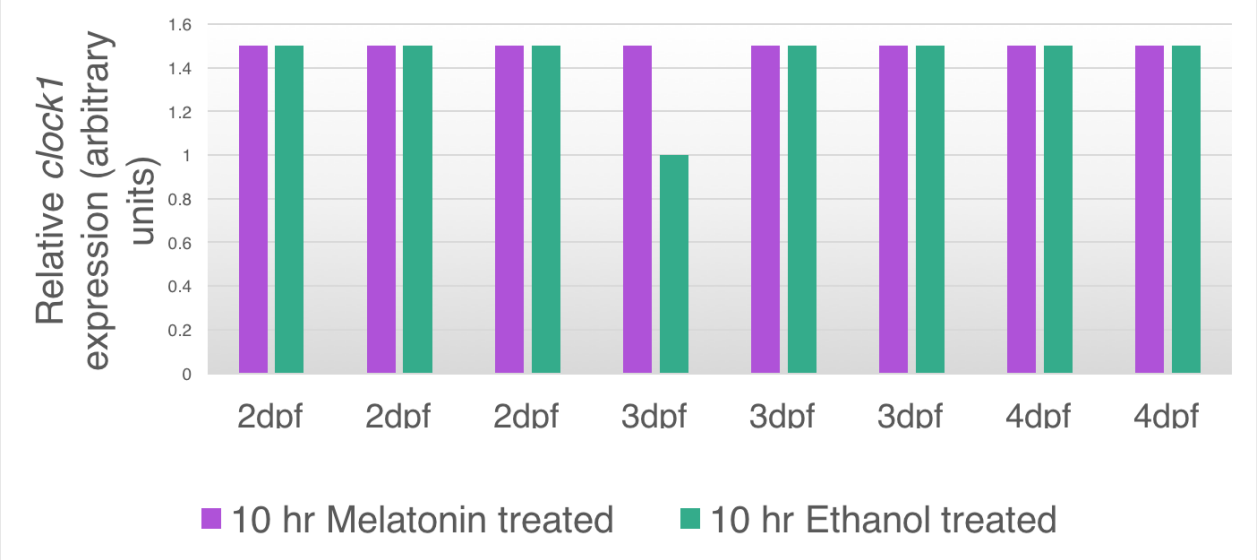

Figure 19. Rhythmic exposure to melatonin had no effect on *clock1* expression in developing zebrafish. Embryos housed in constant dark and exposed to either 10 hr melatonin: 14 hr no treatment or 10 hour ethanol : 14 hr no treatment
dpf: days post fertilization. Whole mount *in situ* hybridization.

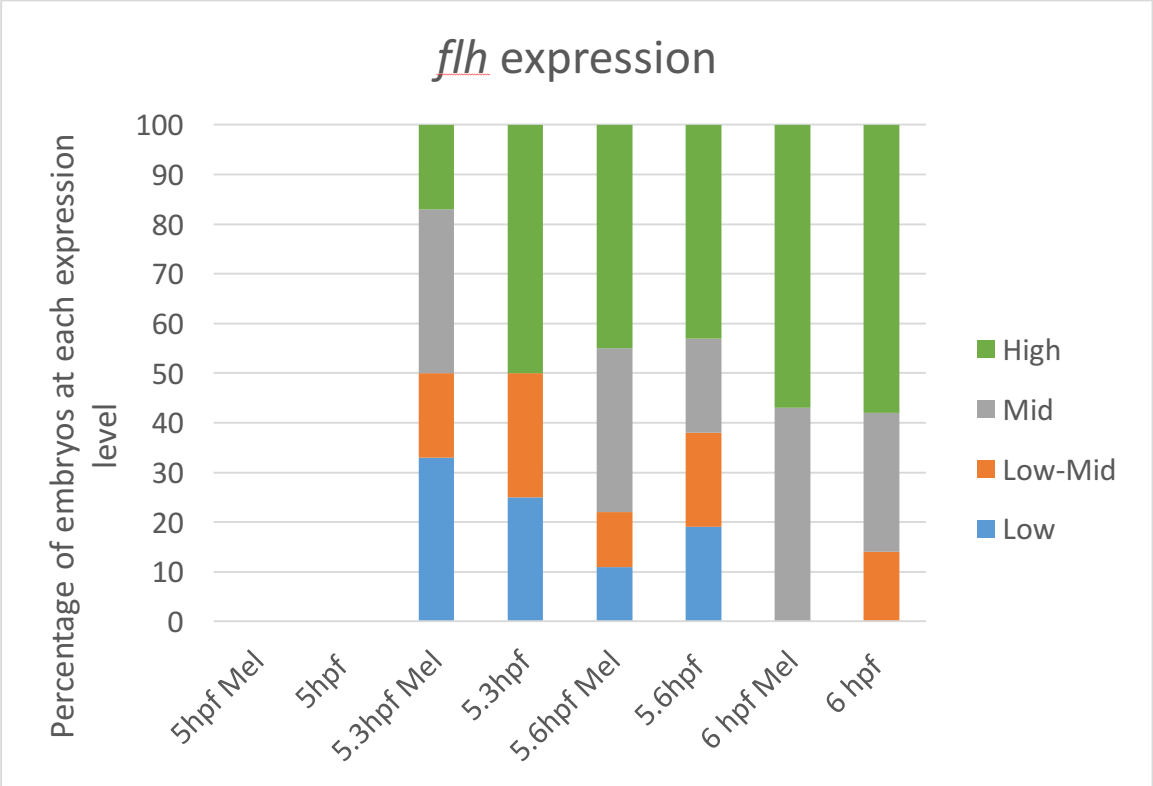

Figure 20. *flh* expression was not altered in response to exogenous melatonin application. Expression levels categorized as low, low-mid, mid or high. No significant difference is noted between melatonin treated embryos and ethanol treated embryos. Whole mount *in situ* hybridization**.**

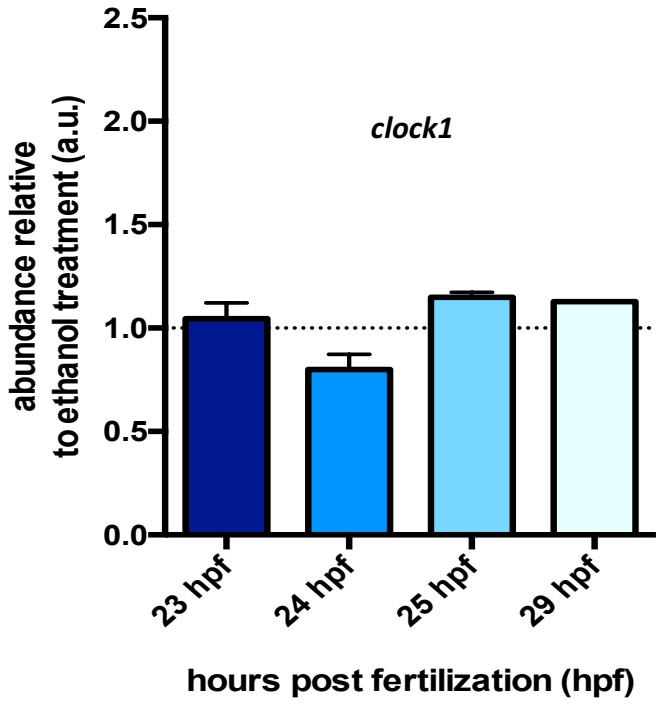

Figure 21. *clock1* expression did not differ significantly between control and melatonin treated embryos. RT-qPCR, expression of *clock1* in melatonin treated embryos relative to ethanol treated embryos

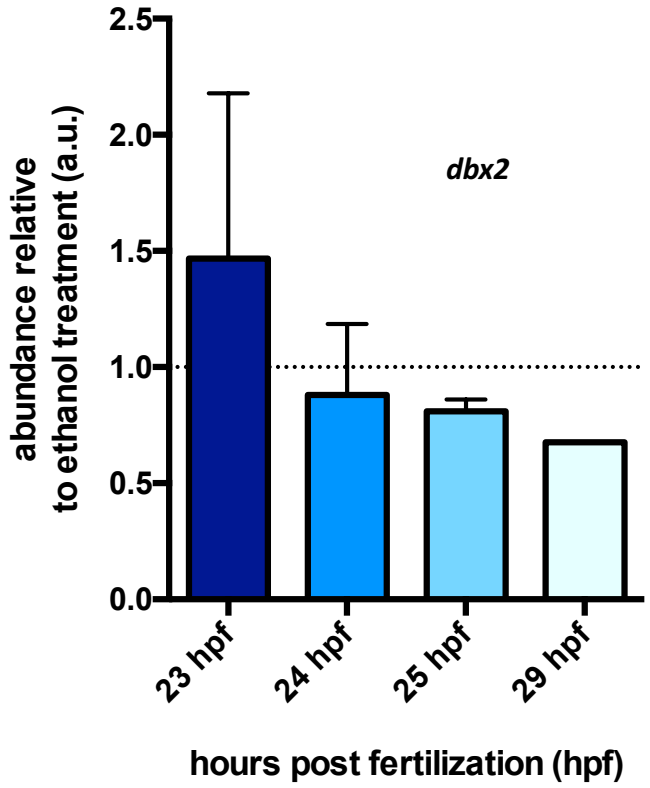

Figure 22. In a timecourse, melatonin treated embryos express *dbx2* at higher levels than controls during early time points and experience reduced expression by 29hpf in comparison to controls. RT-qPCR, expression of *dbx2* in melatonin treated embryos relative to *dbx2* expression in ethanol treated embryos.

Figure 23. **Phenotype comparison of 48 hour post fertilization zebrafish embryos; blocking of melatonin receptor function in embryos appears to slow development.** Left: Control embryo treated with ethanol vehicle. Right: embryo treated with luzindole from 3hpf-48hpf. Whole mount RNA in situ hybridization for *irbp*. Note the absence of eye and somite formation in luzindole treated embryo.

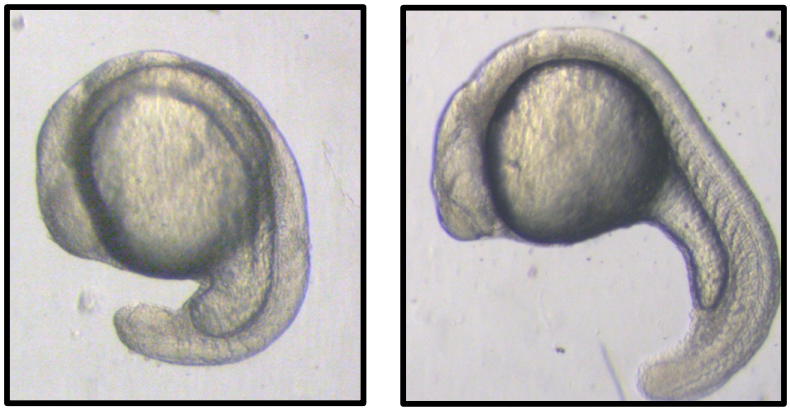

Figure 24. Phenotype comparison of 21 hour post fertilization embryos; melatonin exposed embryos developed faster than controls. Left is ethanol treated embryo, right is melatonin treated. Image of live embryos. Photo by Ababa Morke and J.O. Liang

CHAPTER 4: DISCUSSION AND FUTURE DIRECTIONS

Exploring the effect of melatonin on established circadian rhythms

To further assess if melatonin is a coordinating signal for circadian clocks in zebrafish, I propose first establishing if melatonin can phase shift the circadian clock in zebrafish. Our work strongly suggests that melatonin does not play a role in establishment of circadian rhythms in zebrafish embryonic development. However, our experiments evaluating *clock1* expression after clock establishment imply that melatonin may be able to alter established gene expression rhythms (Fig. 18). Moreover, the ability of melatonin to phase shift circadian rhythms has been documented in mammals (Schroeder and Colwell, 2013). If melatonin can impact established rhythms in zebrafish, this would be a novel finding that could bring significant alterations to our understanding of the zebrafish circadian system.

This work could be completed by utilizing adult WT zebrafish kept on a light dark cycle for several days to ensure entrainment of circadian genes, then transferred to constant conditions. These gene expression rhythms should persist with approximately the same phase of expression. Once fish are transferred to constant conditions, they could be treated with melatonin

for 10 hours starting at 3-4 hours after the expected onset of darkness from the original light: dark cycle. Treating with melatonin for 10 hours starting 3 hours after expected darkness is meant to mimic a dark cycle but shift this "nighttime" phase by 3 hours.

The limitations to our current study that relied primarily on WISH, was the inability to quantify gene expression and to evaluate gene expression after 5 days post fertilization (dpf). As such, using adult fish and RNA sequencing would extend experiment times and produce quantifiable data. Longer circadian experiments are necessary to investigate phase shifting, as some circadian genes take multiple days to re-entrain (Cahill, 2002). Furthermore, phase shifting rhythms with melatonin may take longer than studies using traditional environmental cues such as light. Designing experiments with an assay that allows for evaluating expression over a longer period of time would give a more accurate picture of melatonin's effects on the zebrafish circadian system.

If melatonin can phase shift the circadian clock in zebrafish, this may suggest that melatonin is a coordinating mechanism for peripheral clocks to maintain synchronization in zebrafish, much as the SCN coordinates peripheral clocks in mammals (Yoo et al., 2004). In this proposed

experiment, RNA could be extracted from whole zebrafish and from various tissues in the zebrafish to determine if melatonin is phase shifting circadian clocks in the periphery. In particular, it would be interesting to determine if timed melatonin addition impacts the pineal clock differently than peripheral clocks. Data from such an experiment could be used to support or refute the hypothesis that the pineal is a master pacemaker in zebrafish.

Knock out of melatonin receptors to determine developmental and circadian role of melatonin in zebrafish

Six melatonin receptors are widely expressed in the embryonic phases of zebrafish development until 36 hpf (Zhdanova et al., 2001). Although a genome duplication event most likely led to multiple melatonin receptors within zebrafish, it has not been established if there is functional redundancy between receptor sub-types or if each receptor sub-type has a unique role in zebrafish biology (Lima-Cabello et al., 2014). Our work characterizing expression of *mtnr1ba* and *mtnr1bb* illustrated the dynamic expression of melatonin receptors in zebrafish up to five days post fertilization. More importantly, this work highlights a key biological puzzle: why are melatonin receptors expressed so widely in zebrafish embryos before 36 hpf? To

answer this question, I propose utilizing loss of function studies to determine the function of each receptor sub-type in zebrafish.

When designing loss of function studies, a precise method must be used to eliminate individual receptor sub-type functionality as amino acid and gene sequences between sub-types are similar. CRISPR/Cas9 presents a specific, quick, and affordable option for double strand knock out of genes in zebrafish (Hwang et al., 2013). Based on mammalian findings and their work with a MT2 selective agonist, Danilova 2004 suggest that MT2 receptors play a major role in the effect of melatonin on zebrafish development. Alternatively, MT1 receptors in mammals have been implicated in regulating *per1* (a circadian clock gene) and shown to be highly expressed in the SCN of mammals (von Gall et al., 2000). These studies suggest that the MT1 and MT2 subtypes of melatonin receptors may have distinct functions; circadian system regulation and development, respectively. Additionally, the largest body of work on melatonin receptor function has been completed in mammals and framing our studies on this previous knowledge may help to determine function in zebrafish.

Initially, I suggest separate construction of a MT1 homologous *(mtnr1aa, mtnr1a1, mtnr1ab*) knock out mutant line and a MT2 homologous

(mtnr1bb, mtnr1ba) knock out mutant line. Knocking out sub-types homologous to mammalian receptors in tandem would allow for initial screens to determine function of subtypes. For example, knocking out MT1 subtype homologous receptors (*mtnr1a1*, *mtnr1aa*, and *mtnr1ab)* would be the quickest way to determine function due to their potential shared function and would allow for comparison to previous findings in mammals. After characterizing MT1 and MT2 homolog subtype function, systematic construction of single gene knock-out mutants could be used to elucidate individual melatonin receptor function in zebrafish.

Moreover, construction of these knock out lines could be used for circadian and developmental studies. Zebrafish melatonin receptor mutant lines in would be especially useful in determining which effects of melatonin are receptor mediated or receptor independent. Since melatonin has such diverse functions in vertebrates, establishment of melatonin receptor knock-out lines in zebrafish would open a wide range of experiments encompassing many areas of study.

Developmental rescue experiments using melatonin

The developmental aspects of this project have focused on the effect of melatonin on gene expression onset in developing zebrafish. Our WISH data has shown that *irbp* is expressed sooner in melatonin treated embryos. Moreover, the morphological observations made during these experiments illustrate the effect that melatonin or luzindole have on the development of zebrafish embryos (Fig. 23, 24). Luzindole in particular, dramatically delays the predicted zebrafish developmental phenotype (Fig. 23). While gene expression onset presents an interesting area to explore, it is very difficult to capture the exact moment when melatonin treated embryos begin expressing a gene sooner than controls and there is no evidence to suggest that melatonin increases gene expression once the gene has started expression in development. Additionally, the relevance of earlier gene expression onset in zebrafish is not as substantial as many other potential melatonin projects, such as the positive effects of melatonin following injury (Reiter et al., 2014).

Therefore, I think further work to investigate the role of melatonin in zebrafish development should focus on projects that could eventually transition into biomedical applications of melatonin. Melatonin has been

shown to have a high safety margin in humans and can cross the placental barrier. If melatonin can advance development, it could possibly be used for a variety of developmental problems in humans, including premature birth. Furthermore, since melatonin has been shown to rescue neuropil density after developmental delays (de Borsetti et al., 2011) and counteract the effects of cocaine on neuronal development (Shang and Zhdanova, 2007) in developing zebrafish; it is possible that melatonin may be able to counteract negative effects on development. If melatonin can accelerate development in developmentally delayed zebrafish (essentially rescuing this delay) and/or counteract negative effects on development; these are both findings that could impact future biomedical studies in higher vertebrates.

Prior to any rescue experiments, it should first be established when in development melatonin has the greatest effect on whole zebrafish development. Using WT embryos treated with melatonin, experiments should be designed to map the temporal aspects of melatonin application in relation to overall developmental outcomes. These experiments should establish the developmental time period zebrafish embryos are most susceptible to exogenous melatonin.

Following these preliminary findings, I think that experiments focused on the development of specific organs or tissues would be the most beneficial. For example, to determine if melatonin can rescue development in developmentally delayed zebrafish, experiments could focus on eye development or neural tube closure exclusively. In these experiments, melatonin could be applied to zebrafish embryos previously treated with drugs that delay particular aspects of development. Using developmentally delayed zebrafish embryos explores the possibility that melatonin can rescue developmental delays by speeding up developmental timelines. In a complementary approach to drug treatment, mutants could be constructed that experience delayed development of organs such as the lungs that are commonly affected by premature birth. Experiments focused on one organ or tissue could provide the framework for human biomedical studies.

Alternatively, research could focus on the potential protective or therapeutic effects of melatonin on zebrafish embryos. This research could be used to establish melatonin as a prophylactic or treatment for embryos under stress or drug exposure. Unlike the experiments previously mentioned that focus on accelerating development, this work would focus on damage repair or preventing damage by a particular agent. Melatonin is a potent

antioxidant and may be able to repair disrupted circadian systems (Shang and Zhdanova, 2007). In zebrafish embryos, melatonin has been found to counteract the negative effects of cocaine on neuronal development, a drug sometimes used by human mothers that impacts fetal brain development (Shang and Zhdanova, 2007). Experiments utilizing zebrafish embryos could be used to investigate melatonin's ability to counteract the negative effects of other illicit and prescribed drugs that commonly disrupt human fetal development.

In summary, melatonin may present an affordable and easily delivered potential treatment for developmental delays in vertebrates but much work remains. Zebrafish represent a well-studied developmental model that could be used to establish the role of melatonin in rescuing vertebrate development. Most importantly, significant findings in zebrafish could someday be used for biomedical research that leads to improve outcomes for developmentally delayed humans.

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