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Light and Feeding Cues: Entraining Circadian Rhythms in Chinook Salmon Tissues

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by

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Declaration of Co-Authorship / Previous Publication

I. Co-Authorship

I hereby declare that this thesis incorporates material that is result of joint research, as follows: Chapter 2 of the thesis was co-authored with Maha Hammoud, Dr. Daniel Heath and my advisor, Dr. Phillip Karpowicz, and I am the sole author of the remaining chapters, with editing and feedback from Dr. Phillip Karpowicz. In the published chapter, primary contributions, experimental designs, data analysis, interpretation, and writing were performed by the author, and the contribution of co-authors was primarily through helping with data generation (Maha Hammoud), data interpretation and writing (Dr. Phillip Karpowicz), and feedback (Drs. Daniel Heath and Phillip Karpowicz).

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II. Previous Publication

This thesis includes one original paper that have been previously published/submitted for publication in peer reviewed journals, as follows:

Thesis Chapter	Publication title/full citation	Publication status*

Chapter 2	Maryam Thraya, Maha Hammoud, Daniel Heath & Phillip Karpowicz (2019): Testing the expression of circadian clock genes in the tissues of Chinook salmon, Oncorhynchus tshawytscha, Chronobiology International, DOI: 10.1080/07420528.2019.1614019	Published

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Abstract

The circadian clock is known to help organisms synchronize their behaviour and physiology with the external environment. Present in almost all cells, the clock is made up of a transcription-translation feedback loop that is responsive to cues such as light and feeding. This clock functions to influence the timing of various processes within the cells by promoting gene expression at optimal timing. Chinook salmon (*Oncorhynchus tshawytscha*) is an important aquaculture species whose clock has not been characterized yet. The aim of the work presented here is to probe for the expression of circadian clock genes in some tissues of Chinook salmon. Since the clock is normally entrainable to light and feeding, manipulations of these two factors were carried out to explore the effects of this on the clock. Expression of clock genes is present in all tissues assayed (liver, heart, intestine, colon, retina, and skeletal muscle); however, the tissues differ in the phases of the daily rhythms, and some tissues do not display any rhythmicity. In the intestine, rhythmic daily expression is seen in various processes ranging from digestion to inflammation and cell regeneration. While it is still not conclusive whether the clock drives the rhythmicity seen in these processes, this serves as an indicator of possible circadian control. Overall, this thesis serves as a gateway to studying the role of the circadian clock in the life course of Chinook salmon, where it can be utilized to maximize growth in aquaculture and to uncover new facts about its life history in the wild.

Dedication

I would like to dedicate this thesis to each individual who devoted their life to research in the name of expanding our breadth of knowledge and improving human life.

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I would like to thank my advisor, Dr. Phillip Karpowicz, from the bottom of my heart for his guidance over the past four years, both with my project and career plans. I always felt like he had my best interest in mind. He is also the type of person who monitors their email afterhours and on weekends, which helped this project tremendously. If it were not for his genuine encouragement, my career in research would have ended during my undergraduate degree.

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I cannot thank each member of my lab enough for their support throughout my time in the lab, especially Kyle, Kathyani, Daniela, Zainab, Chantelle, Sharon, Colin, Vania and Jannatun; through all the shared experiences, we quickly became like family. I would also like to thank the dedicated undergraduate research assistants whose hard work resulted in much of the data presented here (Hanna, Maha, Alyssa, Kaitlin, and Heidi).

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Finally, I am forever indebted to my loving parents to whom I will owe every success. This is not to say I am not grateful to each family member who happens to see this page and wonder why they are not on here. I am blessed to have you all in my life!

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Chapter 1: General Introduction

Recurring changes in an organism's environment play an important role in allowing it to determine time of day. The duration of light exposure in a day, known as photoperiod, and the timing of food intake serve as cues for an endogenous circadian clock. A shift in the timing of these changes often results in a shift in the phase of the clock. The existence of such a time organizer allows organisms to anticipate environmental changes and prepare behavioural and physiological responses. The period of each cycle is about 24 hours, referred to as "circadian", and is not affected by fluctuations in temperature. Even though this clock is entrainable by external stimuli, it persists under constant environmental conditions, rendering the clock as "free-running" (Allada and Chung 2010; Bell-Pedersen et al. 2005; Hardin 2011; Pittendrigh 1954).

The circadian clock consists of a group of genes that respond to external cues and control the expression of downstream pathways. A master circadian clock is found in the suprachiasmatic nucleus (SCN) in the brain, where it receives light information from the eye, and uses this to entrain peripheral clocks of other tissues throughout the body. The clock is composed of transcription-translation feedback loop; transcription factors of the two positive regulators Clock and Bmal heterodimerize to drive the expression of the negative regulators *Period* (*Per*) and *Cryptochrome* (*Cry*) by binding to E-box elements in their promoters (Vatine et al. 2011). The translated Per and Cry proteins dimerize and translocate into the nucleus, where they inhibit the transcriptional activation that Clock and Bmal perform, causing a new cycle to begin (Vatine et al. 2011). In mammals and in zebrafish, an additional feedback loop gives stability to the core clock by directing the rhythmic expression of *Bmal* through the transcriptional regulators, Rorα and Rev-erbα. These two proteins work on activating and repressing the transcription of *Bmal*,

respectively (Vatine et al. 2011; Emery and Reppert 2004). In zebrafish, the *Clock* gene might also be under the control of Rorα and Rev-erbα (Vatine et al. 2011).

The circadian clock has been characterized in model organisms like Drosophila, mouse, and zebrafish, where the function of the clock molecular machinery is conserved (Kathleen G. O'Malley, Camara, and Banks 2007). The peripheral clocks of Drosophila and zebrafish can be directly entrained by light (Whitmore et al. 1998; Ito and Tomioka 2016). Zebrafish have homologues of both mammalian and invertebrate clock genes, suggesting that the teleost clock may be an evolutionary link between these two systems (Pando and Sassone-Corsi 2002). Zebrafish possess extra copies of many of the clock genes due to a genome duplication that took place during the evolution of teleosts (Postlethwait et al. 1998). Compared to the mammalian system, zebrafish have one extra copy of the Clock and Bmal genes, and four extra copies of the Cry gene (Looby and Loudon 2005; Hirayama et al. 2005). These extra copies either have redundant functions, or are more specialized versions of an original gene that performed multiple functions (Vatine et al. 2011). Salmonids, also being teloests like zebrafish, evolved from an additional whole genome duplication (Allendorf and Thorgaard 1984). Considering this complexity in the salmonid genome and the diversity of their life histories, studying the evolution of the clock genes in salmonid fishes is challenging but compelling.

Chinook salmon migration patterns, hence spawning times, are believed to be influenced by their circadian clocks (Kathleen G. O'Malley et al. 2013). These organisms inhabit areas ranging from central California to Alaska, and from Northern Japan to the Anadyr River in northern Siberia. Young salmon spend roughly the first year of its life in freshwater, and then the fry migrate into the sea, and do not return to their natal streams

until they are ready to spawn and die. Their migration patterns vary with the latitude they inhabit; Populations in North America living south of 56°N migrate to sea much later than those living further up North, but they do not migrate as far into the ocean as the Northern group does. Moreover, they return to their natal rivers in the fall several days prior to spawning, whereas the Northern populations return in the spring and summer, several months before spawning (Healey 1991). Thus, depending on the latitude, Chinook salmon experience varying photoperiods that could range from 14 hours of light and 10 hours of dark (14:10 LD) to 24 hours of constant light. These seasonally varying day lengths are thought to dictate their time of migration by providing a temporal cue that separates fall-run and spring-run fish. Run timing appears to be responsible for over forty per cent of the overall genetic variance among migrating populations (K. G. O'Malley and Banks 2008b). The zebrafish *Clock1* gene has two orthologs in Chinook salmon, which also differ from one another by a 1,200 bp non-coding segment, termed the polyQ domain (K. G. O'Malley and Banks 2008b). This polyQ domain displays a length polymorphism in *Clock1b* that correlates with the photoperiod experienced at each latitude (K. G. O'Malley and Banks 2008a). Because fish from Northern regions migrate to sea earlier than those in Southern regions, it is thought that the length polymorphism seen in Clock1b may link the circadian clock with different migratory behaviours. Clock gene motifs are also being used to resolve fall and spring migratory runs of Chinook salmon (Kathleen G. O'Malley et al. 2013).

Aquaculture is an evolving industry that is relied upon to meet the needs of a growing world population. Current practices in fish farms might not necessarily be ones that ensure maximal utilization of resources to enhance fish growth; for instance, some

farms only feed their animals during the working hours of employees rather than during the natural feeding time of a species. This might be resulting in lower feeding efficiency (Kotani and Fushimi 2011). Other poor practices involve raising fish larvae under constant light, which may prevent hatching if the larvae normally restrict this event to a particular time of the day (Frøland Steindal et al. 2018). Given that feeding time and lighting conditions have an impact on fish yields, knowing the pathways by which they act on the organism is key. The circadian clock may likely be the connection between rearing conditions and fish growth.

In Chinook salmon, the species of interest in this thesis, very little is known about the clock components and how they function in a given environment. Quantification of expression levels of the various components of the circadian clock is used to determine the state of the clock relative to the time of the day. Given that the clock controls many cellular pathways, it may be utilized to maximize fish yields by uncovering the optimal rearing settings composed of a specific duration of light exposure and a time at which feeding administration leads to the highest growth output. In this thesis, Chinook salmon tissues are studied to explore the activity of the circadian clock in this organism. Chapter 2 is a novel investigation of circadian clock function in tissues ranging from the nervous system, to the digestive and cardiovascular systems. Investigation of effects of lighting and feeding time manipulation on the clocks of some tissues is explored in chapter 3. Finally, in chapter 4, special focus is given to the intestine to explore how various physiological processes respond to changes in rearing conditions, which are potentially mediated by the circadian clock. The findings may be utilized in the future to improve Chinook salmon growth in aquaculture.

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Chapter 2:
Testing the expression of circadian clock genes in the tissues of Chinook salmon, *Oncorhynchus tshawytscha*

Introduction

Organisms have adapted their physiology and behavior to regular 24 h environmental changes in light and temperature present on Earth. For example, most animals manifest endogenous circadian rhythms that temporally organize biological processes with a 24-h period (Allada and Chung, 2010; Bell-Pedersen et al. 2005). This timing is driven by the circadian clock, a highly conserved molecular system that can be entrained by cues such as photoperiod and food (Hardin 2011), and whose 24-h period of molecular oscillations persists under varying temperatures (Pittendrigh 1954). In mammals, a central clock is present in the suprachiasmatic nucleus (SCN) in the brain, where it receives signals from the retinal ganglion cells that detect light entering the eye (Golombek and Rosenstein 2010). This central clock entrains peripheral clocks throughout the body via humoral signals (Dibner et al. 2010). However, in other animals, light can be detected cell-autonomously. For instance, both Drosophila and zebrafish (*Danio rerio*) cells are capable of receiving light input directly (Plautz et al. 1997; Whitmore et al. 2000). The circadian clock allows diverse animals, in diverse environments, to anticipate daily photoperiod cycles with appropriate physiological and behavioral responses (Allada and Chung, 2010).

The circadian clock has been well characterized in Drosophila, mouse, and zebrafish, which indicate that the basic clock molecular machinery in these different species is very similar. The core of the vertebrate circadian clock is comprised of the two positive regulators Circadian Locomotor Output Cycles Kaput (Clock) and Brain and muscle Arnt-like protein (Bmal) that heterodimerize to drive the expression of the negative regulators Period (*Per*) and Cryptochrome (*Cry*) by binding to the E-box elements in their

promoters (Vatine et al. 2011). The translated Per and Cry proteins then also heterodimerize and translocate into the nucleus, where they inhibit the transcriptional activation performed by Clock and Bmal, causing a new cycle to begin. In mammals and in zebrafish, an additional feedback loop gives stability to the core clock by directing the rhythmic expression of *Bmal* through the transcriptional regulators, *Rorα* and Rev-*erbα* (Emery and Reppert 2004; Vatine et al. 2011). Zebrafish have homologues of both mammalian and invertebrate clock genes, suggesting that the teleost clock may be an evolutionary link between these two systems (Pando and Sassone-Corsi 2002). Zebrafish also possess extra copies of many of the clock genes due to a genome duplication that took place during the evolution of teleosts (Postlethwait et al. 1998). Hence, compared to the mammalian system, zebrafish have one extra copy of both the *Clock* and *Bmal* genes, and four extra copies of the *Cry* gene (Kobayashi et al. 2000; Wang 2008, 2009). This has led to some differences in clock gene regulation. For instance, unlike in mammals, in zebrafish, photoperiod itself is capable of directly regulating the expression of *Cry1a* and *Per2* in cells to subsequently direct clock entrainment (Tamai et al. 2007; Vatine et al. 2009). Salmonids, a separate group of teleosts, evolved from an additional whole genome duplication, thus have an even more complex genome (Allendorf and Thorgaard 1984) and may possess further complexity in clock function. These events make salmonids a challenging organism to study circadian clock function in, but a rich system for asking questions about the evolution and diversification of clock function due to this redundancy.

Chinook salmon have a complex life history. Young salmon spend roughly the first year of life in freshwater, and then migrate into the sea and do not return to their natal streams until they are ready to spawn and die. The migration patterns of Chinook salmon

vary with latitude: populations in western North America living south of 56°N, which falls in the southern regions of Alaska, migrate to sea much later, but they do not migrate as far into the ocean as those in Alaska living north of 56°N. Moreover, southern populations return to their natal rivers later in the season before spawning, whereas the northern populations return earlier in the season before spawning (Healey 1991). These distinct life histories are thought to rely on photoperiod cues to determine appropriate migration time. For instance, northern populations experience drastic photoperiods in the summer and winter, consisting of nearly 24 h of constant light or dark, respectively. These fish prefer to migrate back to streams in the spring, earlier than southern populations that receive moderate photoperiods and return in the fall when the photoperiod is changing (O'Malley and Banks 2008a). Seasonally varying day lengths are thought to dictate the time of migration by providing a temporal cue that separates fall-run and spring-run fish.

The circadian clock has been proposed to be a factor in the divergence of Chinook salmon across the two migratory life histories (O'Malley et al. 2007). Two orthologous genes in Chinook salmon, named *Clock1a* and *Clock1b*, differ from one another by a 1,200 bp non-coding segment termed the polyQ domain (O'Malley and Banks 2008b). The polymorphism in the *Clock1b* polyQ domain length corresponds with photoperiod experienced at each latitude, especially during spawning (O'Malley and Banks 2008a; O'Malley et al. 2010). Considering the complexity of the salmonid genome and the diversity of their life histories, studying the evolution of the clock genes in salmonid fishes is compelling. Clock gene polymorphisms may drive salmon divergence, and ultimately may be the genetic substrate for speciation events. However, the daily expression rhythms of clock genes in Chinook salmon have not been studied to date. It is thus unclear

whether observed polymorphisms among populations are associated with differences in rhythmicity in clock function, or how rhythmicity differs in the different tissues of these animals. Because selection operates on any physiological process thought to be present in most if not all the tissues of the body, information about tissue-specific clock function would provide insight into how the circadian machinery in Chinook salmon operates. This will help in understanding how the clock of these fish changes during light-dark cycles, and may reveal new mechanisms that the clock uses for entrainment to exert its control over whole genome function.

The aim of our study was to test the daily expression of circadian clock genes in Chinook salmon. We hypothesized that Chinook salmon maintained under a regular photoperiod and feeding schedule would have 24 h changes in transcription throughout their tissues. To test this, RT-qPCR assays were developed for this non-model organism. These assays were validated to accurately detect clock gene transcription, and we then used them to quantify the expression of the circadian clock genes. We found substantial differences in clock function among different tissues. Certain clock genes show rhythms in a tissue-specific manner, in which one component may be arrhythmic in some tissues but rhythmic in others. Our results suggest that the clock functions throughout the body of Chinook salmon to drive distinct molecular rhythms in different tissues. This suggests that the divergence among Chinook salmon populations by clock gene evolution may occur in a complex tissue-specific fashion, rather than by evolution of an animal-wide synchronous clock mechanism.

Materials and Methods

Designing the primers

PCR primers were developed to quantify the transcription of various orthologs of the Bmal, Clock, Cry and Per genes, as well as the two housekeeping genes, *βActin*, and *Ef1a*. For genes that had not been sequenced in Chinook salmon, we used sequences from related organisms (see results) and aligned them in Geneious (Geneious Biologics). The region of strongest homology was used to generate PCR primers using NCBI Primer Design Tool, and/or Primer3 software. Up to two degenerate nucleotides were allowed in primers if the species had nucleotide variation in the region of homology. In all cases, a universal BLAST was done on the 20 or 21 base pair primers to ensure that they do not have homology with unintended regions in the genome. Primers for *Rev-erbα* and *Rorα* were later developed from the published Chinook salmon genome and thus were specific (by BLAST) to the intended genes.

Table 1: Details of primer sequences. Primer names, sequences, % efficiency, predicted amplicon sizes, % identity to expected sequences, and sequences of amplicons from the various genes examined.

Target Gene	Primer Sequences (5' → 3')		Expected Product Size	Identity to Chinook Salmon Sequences on NCBI (%)	Amplicon Sequence
βActin	Forward Reverse	TGACCCACACAGTACCCATC TGTCTCGTACGATTTCCCTCT	159	98	TGACCCACACAGTACCC ATCTACGAGGGTTACGC TCTGCCCCACGCCATCC TGCGTCTGGATCTGGCC GGCCGCGACCTCACAGA CTACCTGATGAAGATCC TGACGGAGCGCGGCTA CAGCTTCACCACCACGG CCGAGAGGGAAATCGTA CGAGACA

Ef1a	Forward Reverse	ATGCCCCTGTACTGGATTGC TGGGGGCATCCTCAAGTTTC	105	100	ATGCCCCTGTACTGGATTGCCACACTGCTCACATCGCCTGCAAGTTCAGCGAGCTCAAGGAGAAGATTGACCGTCGTTCCGGCAAGAAACTTGAGGATGCCCCCAG
Bmal1	Forward Reverse	AAGAGRCGCAGRGACAAGATG GCGYAGGACTGTKAGTTTGT	105	94	AAGAGACGCAGRGACAAGATGAACAGCTTCATAGACGAGCTGGCTGCACTAGTGCCTACATGCAACGCTATGTCCCGTAAACTGGACAAACTMACAGTCCTRCGC
Clock1a	Forward Reverse	CCAKKGTMCAGTTTTCCACCC GATGTTGGCCTCGATCATCCT	89	98	CCAKKGTMCAGTTTTCCACCCAGCTGGACGCAATGCAGCACCTGAAGGATCAGCTGGAGCAGAGGACCAGGATGATCGAGGCCAACATC
Cry3	Forward Reverse	CAGACTGGAGTGTGAACGCA AGCAGTGGAARAACTGCTGG	75	99 (*Cry2b*) 96 (*Cry-1-like)*	CAGACTGGAGTGTGAACGCAGGCAGCTGGATGTGTCACTCCTGCAGTTCCTTCTTCCAGCAGTTCTTCCACTGCT
Per1	Forward Reverse	GTGTGAAGCAGGTGCGAGCC GTRTRTACAGACAGATCCAGG	91	100	GTGTGAAGCAGGTGCGAGCCAACCAGGAGTACTACCACCAGTGGGGTGTGGAGGAGTGTCACGGCTGCAGCCTGGATCTGTCTGTACAYAC

Experimental design

Chinook salmon parr (6 months old, mixed sex) at the Freshwater Restoration Ecology Centre in LaSalle, Ontario, were housed in tanks under natural ambient photoperiod during the month of July (corresponding to a 15:9 LD cycle) and placed on a scheduled feeding regime (one feeding per day at 8 AM). Every 3 h, 10 animals were humanely sacrificed in a 0.04% clove oil bath (New Directions Aromatics Inc., Indonesia) followed by decapitation. This was repeated for a total of eight-time points over a 24-h period. Tissues sampled included the intestine, colon, liver, heart, and retina – all tissue

samples were preserved in RNAlater® RNA Stabilization Reagent (QIAGEN) and stored at −80°C for future analysis. In the case of the intestine, only the proximal quarter was used to extract RNA. In the liver, only a small portion was used for RNA extraction, and while the entire eye was preserved, the retina was isolated and used for RNA extraction.

Quantifying gene expression

RNA was extracted using the RNeasy® Mini kit (QIAGEN) following the manufacturer's protocol for "Purification of Total RNA from Animal Tissues", including the DNase step (QIAGEN). RNA concentration was measured by 260/280-nm absorbance ratio using a Thermo Scientific™ NanoDrop™ One Microvolume UV-Vis Spectrophotometer. A total of 1 µg of cDNA was synthesized using the iScript cDNA Synthesis Kit (BIO-RAD, Catalog #: 1708891BUN), following the manufacturer's instructions.

PCR primers (Eurofins Genomics) were tested using Chinook salmon cDNA on the Viia7 qPCR system (Applied Biosystems). Primers that generated RT-qPCR amplicons with lowest threshold cycles (Ct) and highest efficiency (~100%) were used in this study (calculated from ViiA™7 qPCR system). We validated amplicon identity for all genes except *Rev-erbα* and *Rorα* by comparing amplicon sequence with those we collected from closely related species (i.e. Coho salmon and Rainbow trout). Massively parallel ("Next Generation") sequencing was performed using an Ion PGMTM System with an Ion 318TM Chip (Thermo Fisher Scientific, Burlington, ON, Canada) following the protocol described in He, Chaganti and Heath (2018). The resulting sequences were quality filtered (He et al. 2018) and blasted against the NCBI database in a universal

search to confirm that the amplicon sequences align with the intended closely related species and do not align with any non-targeted genes.

The validated primers were used in RT-qPCR to quantify the transcription of the genes of interest. The expression of each clock gene was normalized to the two control genes, βActin and *Ef1a*. The double normalization was done by calculating the geometric mean for the Ct values of the two control genes using the following equation:

$$Geometric\ mean = SQRT\ (POWER(2, Ct_{Ef1a} - Ct_{gene}) \times POWER(2, Ct_{b1Act} - Ct_{gene}))$$

A one-way ANOVA (GraphPad Prism Version 7.0c for Mac OS X) was used to test the statistical significance of the variation in expression of each clock gene over time (significance is reported in figure legends). Two-way ANOVA was used to compare *Rev-erbα* and *Rorα* expression as indicated in Figure 3.

Results

Development of RT-qPCR for chinook salmon clock genes

The core circadian clock consists of a transcription/ translation feedback cycle. Chinook salmon circadian clock gene expression has not been measured to date; hence, we first developed assays to quantify clock gene mRNA levels by RT-qPCR. We targeted two control genes (βActin and *Ef1a*) and the clock components *Bmal1*a, *Bmal1*b, *Bmal2*, *Clock1a*, *Clock1b*, *Cry1*b, *Cry2a*, *Cry2b*, *Per1*a, *Per1*b, *Per2*, *Rev-erbα*, and *Rorα*. The sequences for the clock genes in Chinook salmon were not available at the outset of this study, so the zebrafish database (zfin.org) was searched to find orthologs. Zebrafish genes of interest were then compared to Coho salmon (*Oncorhynchus kisutch*), Rainbow

trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), and Nile tilapia (*Oreochromis niloticus*) by BLAST. Of these five teleost species, Coho salmon is the most closely related to Chinook salmon, followed by Rainbow trout, then Atlantic salmon (Kitano et al. 1997). On the other hand, zebrafish and Nile tilapia belong to different taxonomical orders (Nelson et al. 2017). We reasoned that a region of homology between salmonids and either zebrafish or Nile tilapia would mean that this region would be highly conserved across these distantly related species and hence present in *O. tshawytscha*.

The sequences for each gene ortholog were aligned, and a consensus sequence of 200–300 base pairs showing the highest homology was selected. PCR primers were designed to target these regions, and in all cases, we selected for primer sequences most faithful to Coho salmon or Rainbow trout sequences because they would be most likely to resemble Chinook salmon genes. A total of 26 sets of primers that was initially selected was narrowed down to six sets encompassing the genes *βActin*, *Ef1a*, *Bmal1*, *Clock1a*, *Cry3* and *Per1* (Table 1). Even though primers for *βActin* and *Ef1a* have been constructed in previous studies (Julin et al. 2009; Piorkowski et al. 2014), we built our own primers using the same method as the clock gene primers. Many primer sets were rejected either due to poor target amplification, or suboptimal efficiency in RT-qPCR reactions, but the final six amplicons used in this study were verified by sequencing to be identical to the target homolog sequences (Table 1). As this study was being prepared for publication, the Chinook salmon genome was published, which included the genes: *Bmal1*, Bmal2, *Clock1a*, *Clock1b*, *Cry1*-like, Cry2b, *Per1*-like, *Per2-like*, *ROR alpha*, *ROR beta-like*, and *Rev-erb alpha* (Christensen et al. 2018). This dataset reveals that the region targeted by our *Cry3* primers is shared between *Cry1* and Cry2b as well (Table 1), so at present, our

Cry3 primers do not specifically detect this gene but detect all three *Cry* genes. In all other cases, our primers are specific to the target gene of interest. In short, we have developed specific primers to detect clock gene expression in this non-model organism.

Clock gene expression is tissue-specific

Chinook salmon parr (6 months old) were entrained to a 15:9 light:dark (LD) photoperiod and morning feeding (8 AM). Tissue samples from the intestine, colon, liver, heart, and eye were collected every 3 h over 24 h (n = 10 individuals per time point). We sampled these tissues as they are subject to entrainment cues from the central pacemaker in the SCN. The intestine, colon, and liver pacemakers are peripheral clocks that receive cues from the master clock and are also thought to receive entraining factors based on the time of feeding. The heart pacemaker is thought to only receive cues from the master clock rather than from feeding. To quantify the transcription of clock genes in each tissue, RNA extraction, cDNA synthesis, and RT-qPCR were performed. Diurnal variation in transcription was assessed by normalizing the expression of four clock genes (*Bmal1*, *Clock1a*, *Cry3*, and *Per1*) at each time point to the geometric mean of two housekeeping genes (βActin and *Ef1a*).

Clock genes were detected in all of the examined Chinook salmon tissues; however, there are notable differences among the tissues. The liver exhibits circadian rhythms consistent with those in Rainbow trout, goldfish (*Carassius auratus*) and Nile tilapia (Costa et al. 2016; Hernández-Pérez et al. 2017; Velarde et al. 2009). In the liver, the transcription of the positive regulators *Bmal1* and *Clock1a* peaks synchronously in the early evening at 7 PM, whereas the expression of the negative regulator *Per1* peaks 12

h later, in the morning at 7 AM (Figure 1). These two positive and one negative clock components are thus anti-phasic as has been reported in animals such as Drosophila and mice (Hardin 2005; Takahashi 2015); however, the other negative component, *Cry3*, did not vary significantly over time.

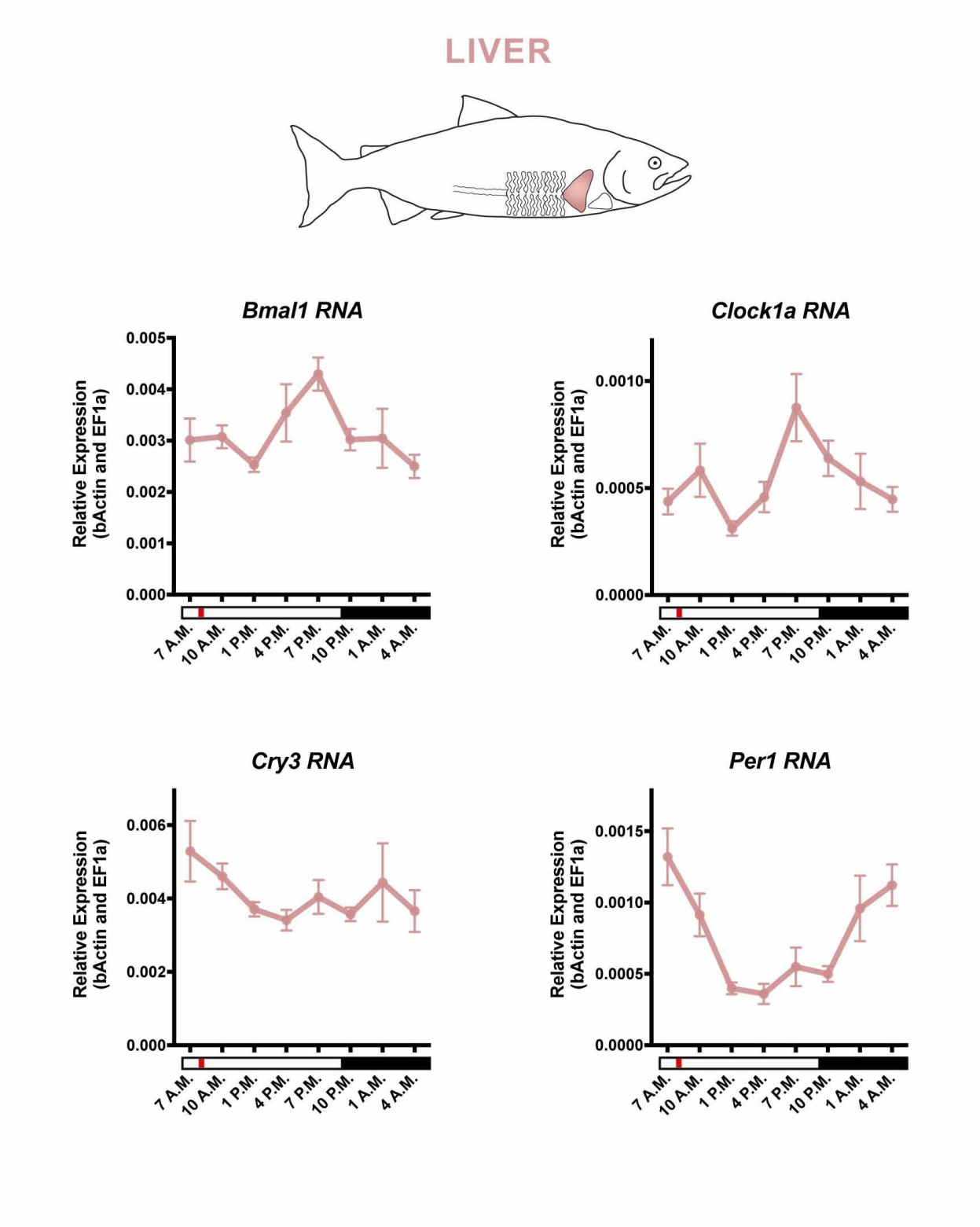

Figure 1: Expression of circadian clock genes in Chinook salmon liver. The horizontal open and solid bars along the X-axis show light and dark phases, respectively,

and the red square shows feeding time (8 AM). Each data point shows the mean of target gene expression relative to two control genes, error bars are ± SEM for ten individuals. Significant rhythms are present in *Bmal1*, *Clock1a* and *Per1*. ANOVA: *Bmal1* ($F(7,72) = 2.444$, $P = 0.0263$); *Clock1a* ($F(7,72) = 3.013$, $P = 0.0078$); *Cry3* ($F(7,72) = 1.259$, $P = 0.2827$); *Per1* ($F(7,72) = 6.37$, $P < 0.0001$).

The heart exhibits nearly identical phases of negative circadian clock components relative to the liver but differs in the phasing of the positive components. In the heart, the expression of the positive regulator *Clock1a* peaks during the night at 1 AM and that of the negative regulators *Cry3* and *Per1* peaks at 4 AM, thus showing a 3-h difference in phase between these (Figure 2). This is much shorter than the 12-h phase difference in the liver. In the heart, *Bmal1* expression was not significantly different over time, unlike the liver. This indicates that the exact same genes, from the same animals, are regulated in a dissimilar fashion in the liver and heart in a 15-h light, 9-h dark photoperiod with one morning feeding per day.

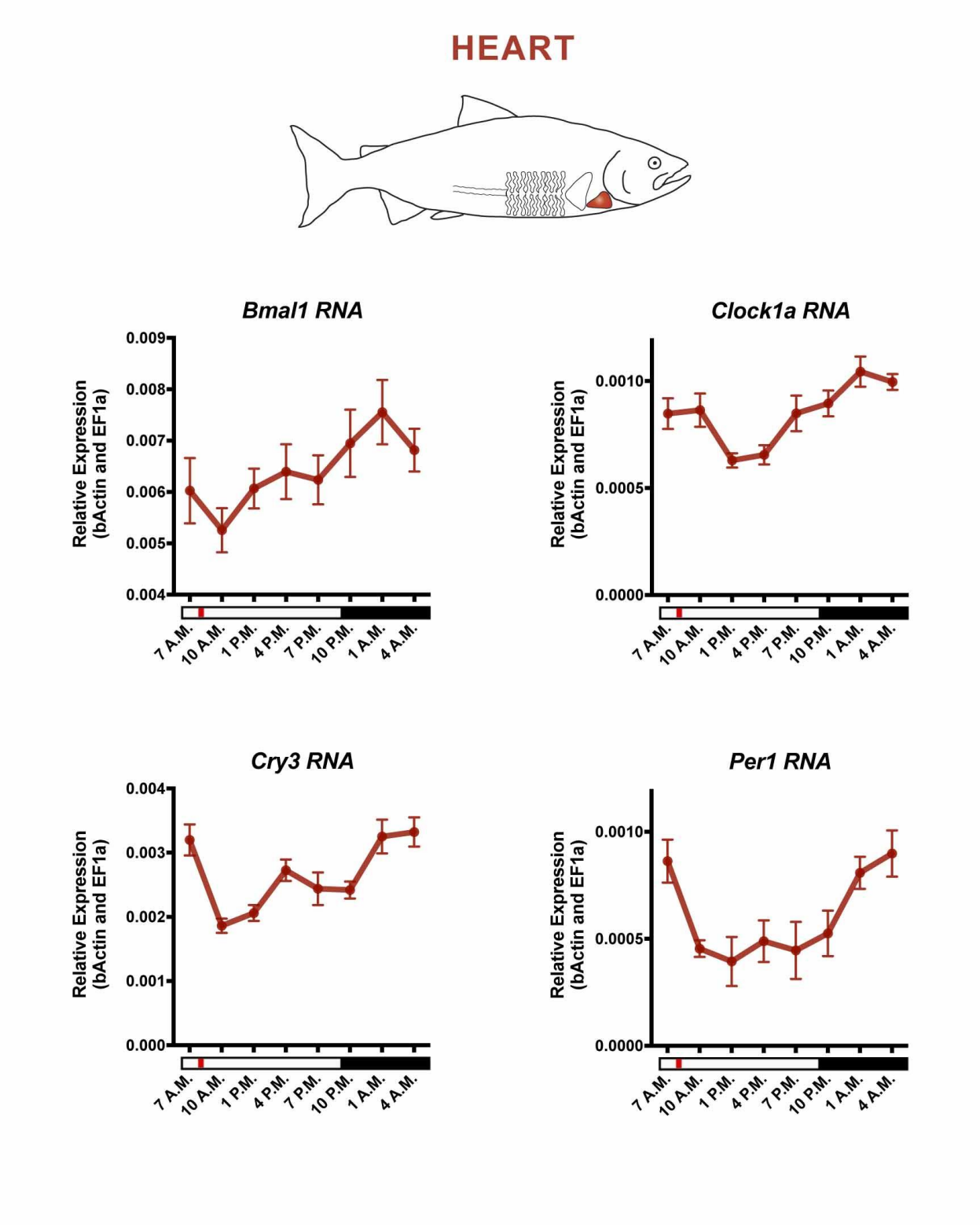

Figure 2: Expression of circadian clock genes in Chinook salmon heart. The horizontal open and solid bars along the X-axis show light and dark phases, respectively,

and the red square shows feeding time (8 AM). Each data point shows the mean of target gene expression relative to two control genes, error bars are ± SEM for ten individuals. Significant rhythms are present in *Clock1a*, *Cry3* and *Per1*. ANOVA: *Bmal1* ($F(7,72) = 1.726$, $P = 0.1164$); *Clock1a* ($F(7,72) = 5.407$, $P < 0.0001$); *Cry3* ($F(7,72) = 7.897$, $P < 0.0001$); *Per1* ($F(7,72) = 4.348$, $P = 0.0005$).

It is possible that *Rev-erbα* and *Rorα*, negative and positive regulators of *Bmal1* expression, respectively, are expressed differently in the liver and the heart and thus are responsible for the differences in clock gene expression observed in these two tissues. We examined the expression of *Rev-erbα* and *Rorα* in the heart versus liver and indeed found that the expression of both genes was significantly different between the two tissues (Figure 3; $P < .0001$). The daily timing of *Rorα* is different in these tissues; however, the timing of the maxima and minima of *Rev-erbα* was similar.

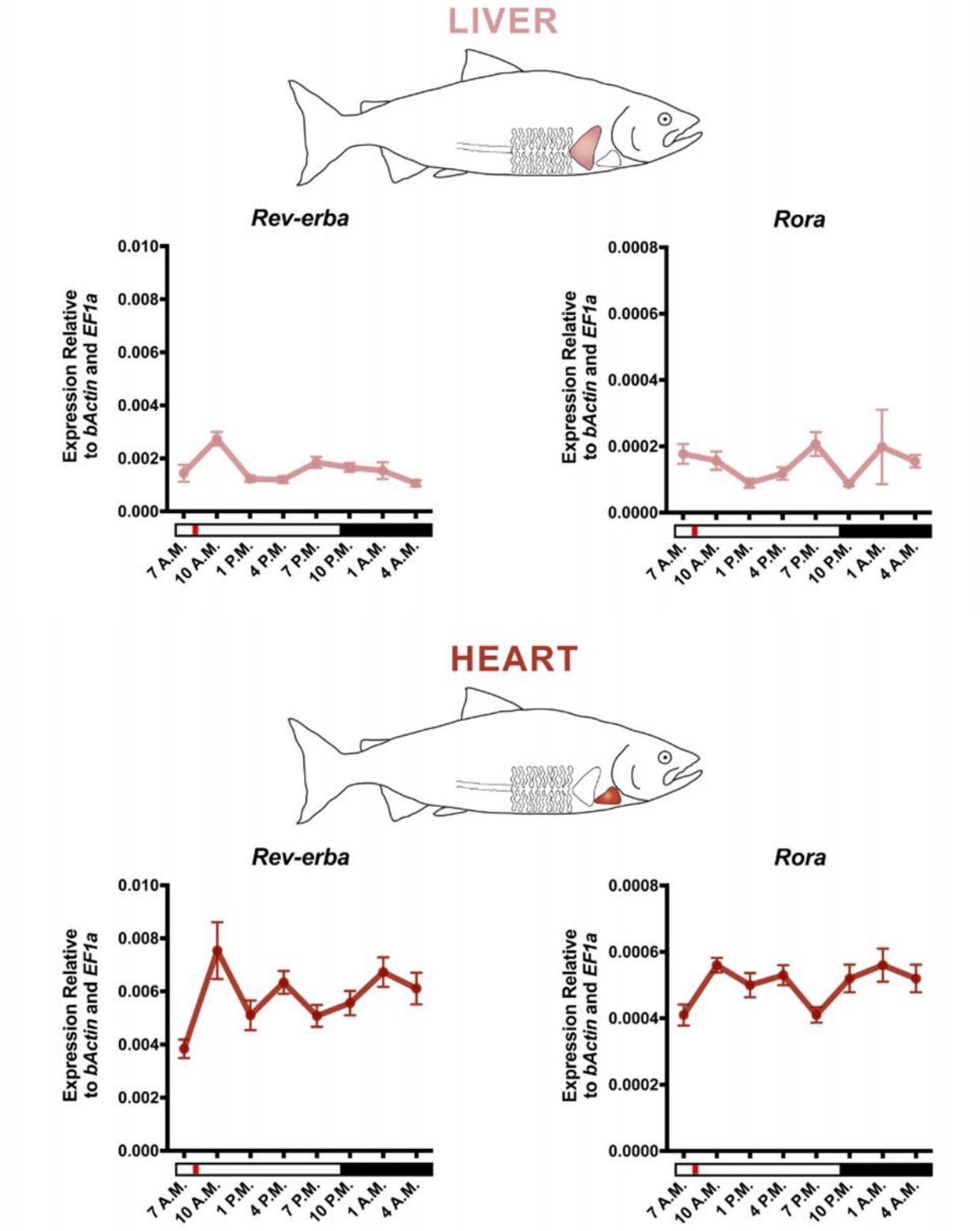

Figure 3: Relative expression of *Rev-erbα* and *Rorα* in Chinook salmon liver and heart. The horizontal open and solid bars along the X-axis show light and dark phases,

respectively, and the red square shows feeding time (8 AM). Each data point shows the mean, and the error bars are ± SEM for ten individuals. Significant rhythms are present in liver *Rev-erbα*, heart *Rev-erbα* and heart *Rorα*. ANOVA: Liver *Rev-erbα* ($F(7,72) = 5.917$, $P < 0.0001$); Liver *Rorα* ($F(7,72) = 1.052$, $P = 0.4030$); Heart *Rev-erbα* ($F(7,72) = 3.729$, $P = 0.0017$); Heart *Rorα* ($F(7,72) = 2.808$, $P = 0.0121$). A significant difference between the heart and liver was also determined by Two-way ANOVA: *Rev-erbα* ($F(1,144) = 352.6$, $P < 0.0001$); *Rorα* ($F(1,144) = 298.6$, $P < 0.0001$).

We next examined two tissues of the digestive tract, the proximal small intestine, and the colon. The anti-phasic rhythms present in positive and negative circadian clock components do not occur in these tissues. In the small intestine, simultaneous peaks in the expression of the positive and negative components – *Bmal1*, *Clock1a*, *Cry3*, and *Per1* – are together in-phase at 10 AM (Figure 4). Although high variance was noted at this time between salmon individuals, the increase in expression of all of these genes was noted in all individuals tested. This suggests positive and negative clock components are in-phase in the intestines of Chinook salmon: we therefore conclude that in the small intestine RNA levels of *Bmal1*, *Clock1a*, *Cry3*, and *Per1* are synchronously expressed under these conditions. In the other digestive tract tissue examined, the colon, an increase in expression of these same four genes may also be occurring at 1 PM, although due to high variance among the individual samples, this was not statistically significant (Figure 5, $P > .05$ in all cases).

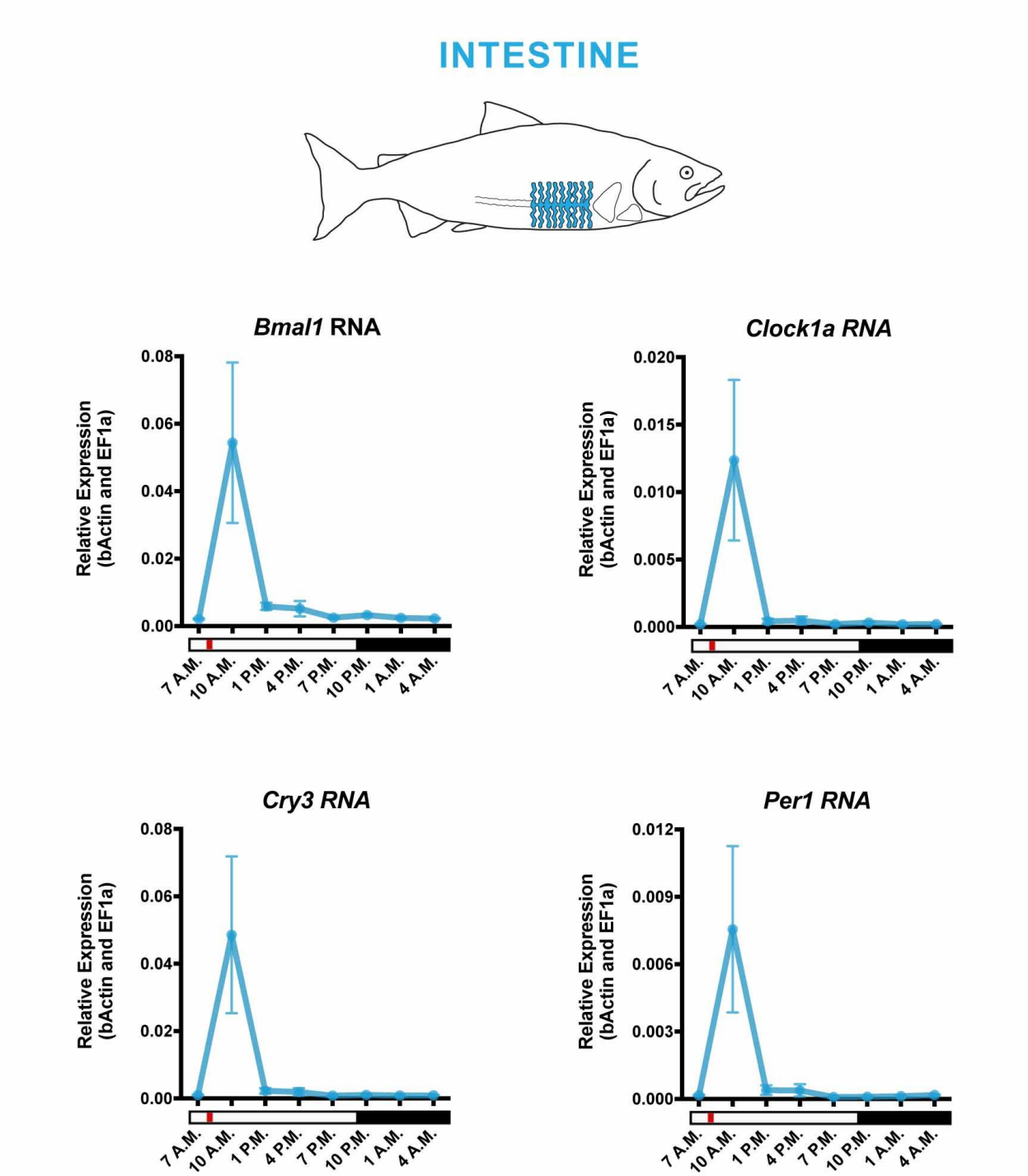

Figure 4: Co-expression of circadian clock genes in Chinook salmon intestine. The horizontal open and solid bars along the X-axis show light and dark phases, respectively, and the red square shows feeding time (8 AM). Each data point shows the mean of target gene expression relative to two control genes, error bars are ± SEM for ten individuals.

Significant rhythms are present in *Bmal1*, *Clock1a*, *Cry3* and *Per1*, with all these genes peaking at the same time. ANOVA: *Bmal1* ($F(7,72) = 4.573$, $P = 0.0003$); *Clock1a* ($F(7,72) = 4.102$, $P = 0.0008$); *Cry3* ($F(7,72) = 4.121$, $P = 0.0007$); *Per1* ($F(7,72) = 3.916$, $P = 0.0011$).

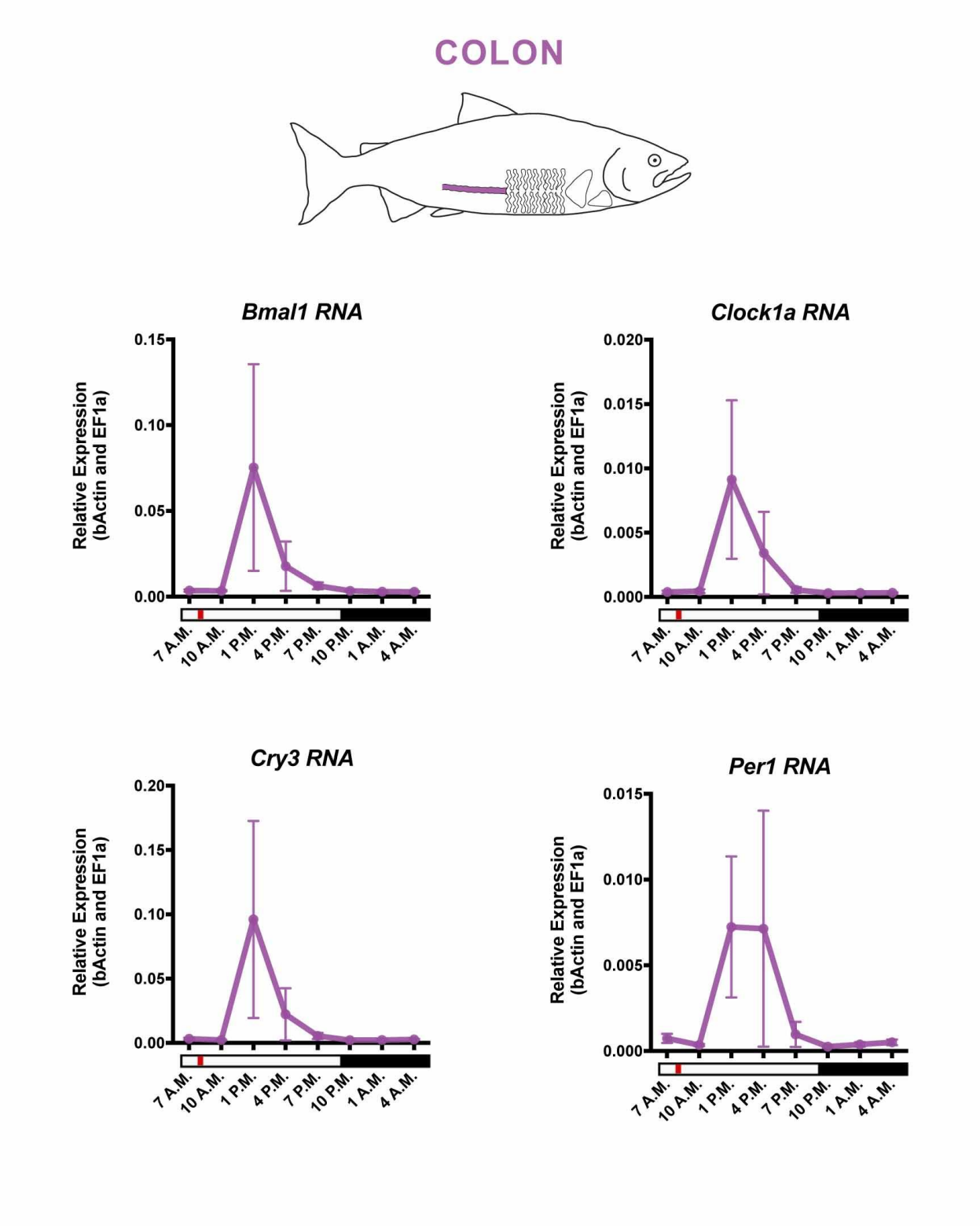

Figure 5: The Chinook salmon colon circadian clock does not exhibit significant time of day changes in expression. The horizontal open and solid bars along the X-

axis show light and dark phases, respectively, and the red square shows feeding time (8 AM). Each data point shows the mean of target gene expression relative to two control genes, error bars are ± SEM for ten individuals. No significant rhythms appear in any of the assayed genes (P > 0.05). ANOVA: *Bmal1* ($F(7,72) = 1.314$, $P = 0.2562$); *Clock1a* ($F(7,72) = 1.615$, $P = 0.1449$); *Cry3* ($F(7,72) = 1.356$, $P = 0.2372$); *Per1* ($F(7,72) = 1.174$, $P = 0.3284$).

Finally, we examined the retina, where in mammals, light is transduced to the SCN to entrain the master clock timekeeper. Similar to the colon, the retina does not show any statistically significant rhythms in the expression of any of the four assayed genes (P > .05 in all cases); however, a great deal of variance was observed at two times where there may be increases in expression (Figure 6: 4 AM and 4 PM). We thus conclude that the colon and retina do not show diurnal rhythms in *Bmal1*, *Clock1a*, *Cry3*, or *Per1*.

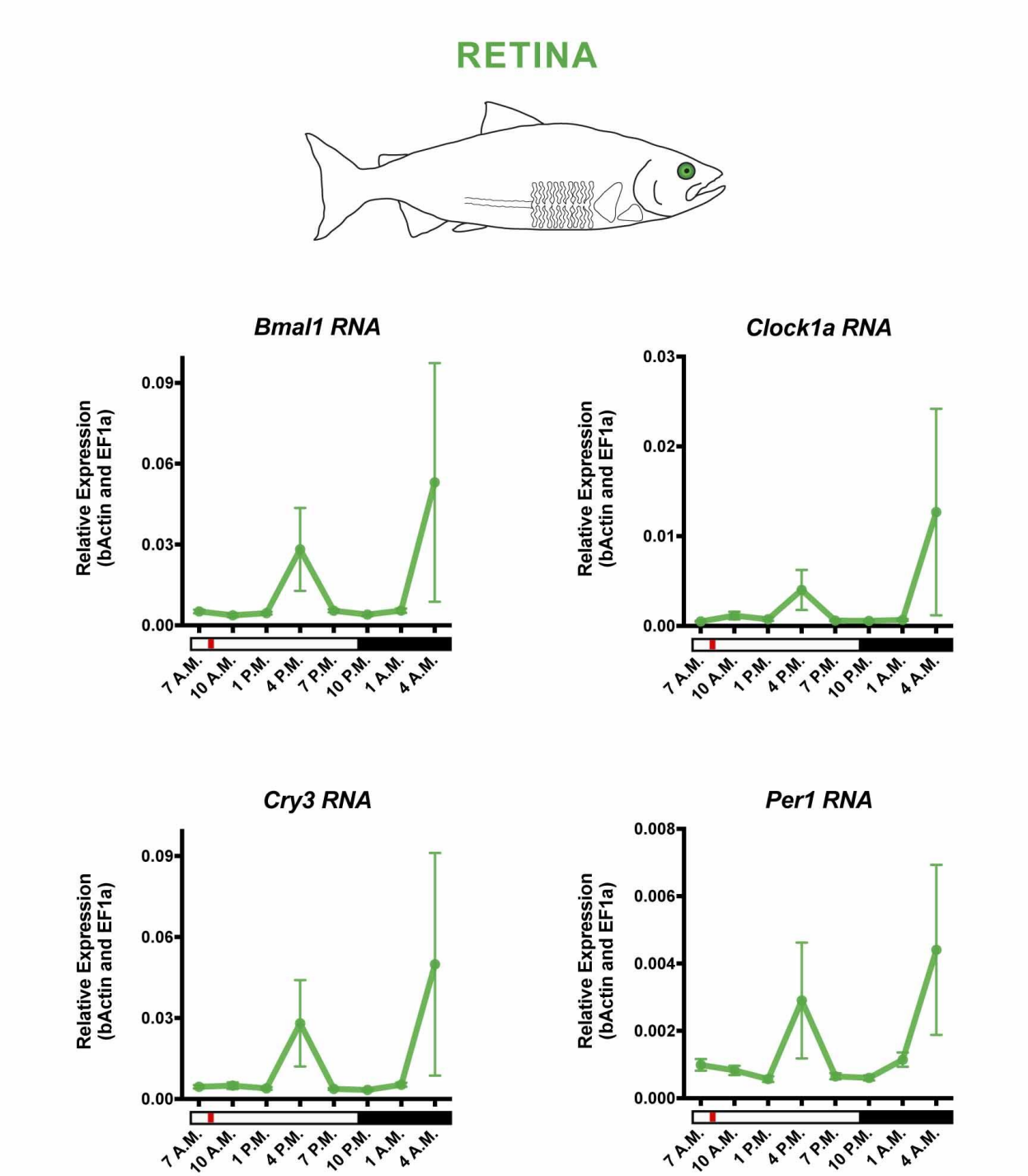

Figure 6: The Chinook salmon retina circadian clock does not exhibit significant time of day changes in expression. The horizontal open and solid bars along the X-axis show light and dark phases, respectively, and the red square shows feeding time (8 AM). Each data point shows the mean of target gene expression relative to two control

genes, error bars are ± SEM for ten individuals. No significant rhythms appear in any of the assayed genes (P > 0.05). ANOVA: *Bmal1* ($F(7,72) = 1.165$, $P = 0.3335$); *Clock1a* ($F(7,72) = 1.044$, $P = 0.4087$); *Cry3* ($F(7,72) = 1.192$, $P = 0.3185$); *Per1* ($F(7,72) = 1.649$, $P = 0.1355$).

Discussion

Chinook salmon circadian clock genes show tissue-specific patterns

In the present study, we successfully developed RT-qPCR assays to quantify transcription for four core circadian clock genes and we tested for transcription rhythms across five different tissues in Chinook salmon. We specifically tested whether a functional circadian clock is present in the five tissues – liver, heart, intestine, colon, and retina. The four clock genes examined show differences among the five tissues in terms of the diurnal rhythm phase, and differences between the time of maximal expression of the negative and positive circadian clock components. The heart, liver, and small intestine exhibit diurnal changes in clock gene expression. Unexpectedly, we found that in the colon and retina, no significant 24 h rhythms are present.

The rhythms present in Chinook salmon liver and heart are similar to those reported in many model organisms, where a phase difference exists between the maxima of positive and negative regulators. However, in the liver, the peaks in transcription of negative and positive clock components differ by 12 h, whereas in the heart they differ by 3 h. The liver of Atlantic salmon in 16:8 LD and ad libitum feeding displays peak expression in *Bmal1* near the end of the light phase, consistent with our data (Betancor et al. 2014). The liver of Rainbow trout, in 12:12 LD photoperiod with a morning feeding

also exhibits peak expression of *Bmal1* and *Clock1a* during the evening, while *Per1* expression peaks during the morning (Hernández-Pérez et al. 2017). This is consistent with our data that shows that *Bmal1* and *Clock1a* expression peaks 2 h before sunset, and that of *Per1* peaks 1 h into the light phase (Figure 1). The goldfish and Nile tilapia also show similar peaks in the expression of positive and negative regulators in the liver; however, we note that in those two species and in Atlantic salmon some of the genes examined are arrhythmic, as we observe for *Cry3* in Chinook salmon (Betancor et al. 2014; Costa et al. 2016; Velarde et al. 2009). This arrhythmicity is also seen in some clock genes in the pituitary gland of Coho salmon (Kim et al. 2015). We observe later expression peaks in the heart (Figure 2), similar to the peak expression time previously reported in Clock expression in zebrafish (Whitemore et al. 2000), but different from the peak time of *Cry1* and Cry2 expression in European seabass (*Dicentrarchus labrax*), which happens during the day (Del Pozo et al. 2012). *Bmal1* in the pituitary gland of Coho salmon is also consistent in its peak time with our results (Kim et al. 2015). Thus, the heart and liver appear to have tissue-specific differences in clock gene expression across multiple fish species.

In the case of Chinook salmon, it is not yet clear why the tissue-specific expression of clock-related genes is present; this may be simply due to tissue-specific differences in the expression or activity of the trans-activators *Bmal1*/Clock, and the transcriptional regulators *Rorα* or *Rev-erbα*. For instance, the negative clock components, *Cry3* and *Per1*, peak at the same time in the liver and heart which could be attributed to the synchronous trans-activation by the Bmal-Clock dimers in these two tissues. On the other hand, *Bmal1* and *Clock1a* in the liver peak at a different time from *Clock1a* in the heart.

Because *Rev-erbα* and *Rorα* are responsible for the rhythmicity in the expression of *Bmal* and *Clock* (Vatine et al. 2011), it is possible that *Rev-erbα* and *Rorα* are expressed or regulated differently in the liver and heart of Chinook salmon. Indeed, we found that *Rev-erbα* and *Rorα* are expressed at higher levels in the heart compared to the liver. The daily overall expression pattern of *Rorα* was different in both tissues but did not display obvious peaks or troughs of expression. On the other hand, the expression of *Rev-erbα* was similar, in that it peaked at 10 AM in both tissues. The timing of the RNA expression of these genes in the heart and liver does not seem to explain why the expression peaks in *Bmal1* and *Clock1a* happen at different times in the two tissues, but it would account for the higher levels of all clock genes in the heart (compare Figures 1 and 2). Expression at the protein level, and measurement of the transcriptional activity of *Rev-erbα* and *Rorα* would resolve this issue. Of note, the phase difference that we observed between *Rev-erbα* respective to *Bmal1* and *Clock1a* is consistent with that observed in zebrafish skeletal muscle at 12:12 LD (Amaral and Johnston 2012).

Overall our data support other clock gene expression studies and suggest that the different orthologs and paralogs of clock component genes can have unique expression patterns in different species and different tissues. We note that in some cases the relationship between positive and negative regulators is not anti-phasic like the model organisms such as Drosophila and mice (Hardin 2005; Takahashi 2015). At this time, it is not clear whether this is a general feature of different fish species, which may have multiple paralogs of clock genes and their regulators, or a result of environmental influence on core circadian clock entrainment.

Feeding as an entrainment cue in fish tissues

It is curious that, in the digestive tract, both the positive and negative regulators that we examined peak at the same time (Figure 4). However, our findings are not unprecedented. *Bmal1*, *Clock1a*, *Per1*, and *Rev-erbβ-like* peak in-phase in the liver of Rainbow trout in a timed feeding schedule under free-running constant dark (DD) conditions (Hernández- Pérez et al. 2017). Timed feeding also results in rhythmicity in the expression of several *Cry* and *Per* genes in the hindgut and liver of goldfish placed under free-running constant light (LL) conditions (Feliciano et al. 2011; Nisembaum et al. 2012). In tilapia, shifting the feeding time to the middle of the dark phase results in phase changes in certain clock genes and arrhythmicity in others (Costa et al. 2016). These studies reveal that feeding is an important entrainment cue in digestive tissues in different fish species.

In this study, fish were fed a standard aquaculture feed and food content in the digestive tract varied throughout the day, being highest in the intestine at the first time point following feeding (10 A.M), and decreasing in this tissue thereafter, as the food passed through to the colon (1 AM). We suspect that the driving cue that generates the simultaneous peaks in clock gene expression in Chinook salmon is the regular single feeding time which precedes the peak by 2 h (Figure 4). Feeding cues could also explain the difference in peak times between the liver and heart, since the liver is part of the digestive system and is more likely to be entrained by feeding. Although it is clear that photoperiod is an entrainment cue across fish species (Del Pozo et al. 2012; Hernández-Pérez et al. 2017), it seems likely that tissues can integrate both light and food entrainment signals to alter clock phase. Whether this is shared by all species, or even

life stages, is not clear. Our experiments were conducted on Chinook salmon parr, which would be exposed to almost constant photoperiod during this stage of their life cycle in northern locations. We thus speculate that certain tissues in Chinook salmon may have adjusted clock entrainment to cues other than photoperiod to maintain clock function across widely varying photoperiod cycles. Understanding the role of the clock in the evolution and adaptive fitness of Chinook salmon may have to consider both the seasonal effect of photoperiod and life cycle stage during which this effect takes place.

The colon and retina of Chinook salmon are arrhythmic

We do not find significant rhythms in either the colon or retina of Chinook salmon. Our conclusions are thus that these tissues are arrhythmic under 15:9 LD, either due to the photoperiod, or feeding time, or an integration of both of these entrainment cues. However, an important caveat to our study is that there is high variability in gene expression observed among the individual salmon examined. These fish were obtained from an introduced population from the Credit River in Ontario, Canada; therefore, it is expected that more genetic variability is present in this Chinook salmon population compared to model organisms that have lower genetic variability, and are maintained under constant lab conditions for many generations. We note that if the outliers are removed from the colon gene transcription dataset using the ROUT method for eliminating outliers (GraphPad Prism Version 7.0c for Mac OS X), all four clock genes show significant diurnal rhythms in the colon peaking at 1 PM (Figure 5), 3 h after those in the intestine (Figure 4). It is tempting to speculate that this could be a result of the lag time required for food to move from the intestine to the colon, and that both intestine and

colon tissues are directly entrained by feeding as material passes through the digestive tract.

The four clock genes we examined in the Chinook retina are similarly arrhythmic. This does not seem to be the case in Rainbow trout retina (López Patiño et al. 2011), zebrafish eye (Zhdanova et al. 2008), and goldfish retina (Velarde et al. 2009), which were reared under very similar conditions to those in this study (regular LD photoperiod and morning feeding). At present we cannot account for this discrepancy, only that we again note the high variance in retinal transcription levels in our study (e.g., note the error bars in Figure 6). Future work using a more natural population of Chinook salmon will resolve whether this is indeed due to the Credit River genetic population being genetically diverse, or to actual disruption of circadian clock function under 15:9 LD photoperiod. Day length has been shown to affect the amplitude of clock gene expression or phase (Lincoln et al. 2003). For instance, Atlantic salmon that exhibit rhythms in clock genes during a short day (8:16 LD) lose this rhythmicity during a longer day (16:8 LD) (Davie et al. 2009). Similarly, Atlantic salmon clock genes that are rhythmic in the brain and pineal gland under 12:12 LD become arrhythmic under LL conditions (Huang et al. 2010). Because our fish were maintained at very similar long day photoperiod, the arrhythmicity may be simply due to the photoperiod entrainment which has a direct impact on the retina.

Studying the circadian clock in Chinook salmon

Chinook salmon play significant ecological and economic roles. They contribute to the diet of various fish, birds, and bears, while also being top predators in the ocean (Scott 2003). Chinook salmon are key recreational fishing species and are growing in

importance as a production species in aquaculture (Scott 2003), and research that helps improve performance and yields is critical for their conservation and management. Because the circadian clock is likely to be regulating various behavioral and physiological traits in Chinook salmon, it is important to uncover how the clock is functioning in this organism. In our study, we note that the fish that have significant clock gene diurnal rhythms in the liver, heart, and small intestine, do not have significant rhythms in the colon or retina. Overall, our data thus show that each tissue possesses a unique circadian clock. The RT-qPCR assays we developed in this study will be useful in testing the relative contribution of photoperiod, feeding time, and genetic variability to clock function in Chinook salmon in future studies. These species have a unique and photoperiod-challenging life cycle which would drive the evolution of unique circadian clock adaptations. Even though we ensured that our PCR primers were amplifying a single target amplicon that matched the recently published Chinook salmon genome (Christensen et al. 2018), it is not clear at present how many paralogs and isoforms are present for these genes in Chinook salmon. The signal we used in our analyses is best viewed as an average of the expression of the paralogs for that gene of interest. In most cases, this strategy works to determine diurnal rhythms in the clock genes targeted, which suggest clock function is present in certain Chinook salmon tissues at this stage of its life cycle, and under long day photoperiod. Our work contributes to our understanding of how this species interact with their environment to achieve optimal circadian-driven physiology and behavior.

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Chapter 3:
The effects of photoperiod and feeding cues on circadian clock function in salmon intestine, liver, and muscle

Introduction

The clock system is composed of a master clock located in the suprachiasmatic nucleus (SCN) in the brain, and peripheral clocks found throughout the body (Ralph et al. 1990). Briefly, a circadian clock is encoded in a group of genes, composed of a positive arm encoded by *Bmal* and *Clock*, and a negative arm encoded by *Cry* and *Per*. For optimal clock function, components of the positive and negative arms of the clock are usually on different phases; that is, the positive arm drives the expression of the negative one, which in turn represses the positive arm. The master clock receives light input from retinal cells and entrains peripheral clocks throughout the body, allowing an organism to synchronize itself with the solar day (Dibner, Schibler, and Albrecht 2010; Golombek and Rosenstein 2010). Peripheral clocks receive entrainment cues from the master clock in the SCN via neuronal and humoral signals, as well as from food (Cailotto et al. 2009; Vujović, Davidson, and Menaker 2008; H. Guo et al. 2005). In zebrafish, unlike most other animals, light is also capable of directly inducing the expression of *Cry1a* and *Per2* as a way for light to directly regulate the function of the clock (Tamai, Young, and Whitmore 2007; Vatine et al. 2009; Peyric, Moore, and Whitmore 2013).

Depending on the latitude that these fish inhabit, and hence the photoperiod they receive, Chinook salmon choose to migrate to the ocean at different times in the season; those living higher up north (Alaska and northern regions) receive longer photoperiods migrate earlier in the season, whereas ones in the south (between southern Alaska and California) receive shorter photoperiods and generally migrate to sea later in the season (Healey 1991). It is thought that a domain of the *Clock1b* gene, which correlates in length with the latitude that the fish are found at, may be linked with the choice to migration

earlier versus later (K. G. O'Malley and Banks 2008). In birds like the Asian short-toed lark (*Calandrella cheleensis*), this is thought to happen by action on the endocrine hypothalamus–pituitary–gonad axis (Zhang et al. 2017). Such length polymorphisms in circadian clock genes are not uncommon. A latitudinal cline ranging from the Mediterranean to Scandinavia is observed in the threonine-glycine motif of the *Per* gene in drosophila melanogaster (Costa et al. 1992). Two alleles of the circadian gene *Timeless* vary between Scandinavia and the Mediterranean, and affect the organisms' response to light and temperature, further implicating these polymorphisms in the response to changes in photoperiod (Tauber et al. 2007). In the blue tit (*Cyanistes caeruleus*), a nonmigratory bird, positive correlations were found between polyQ length in the Clock gene and breeding latitude; these correlations were absent in the bluethroat (Luscinia svecica), a migratory bird (Johnsen et al. 2007). It is thought that length polymorphisms in the polyQ domain of the *Clock* genes enable them to compensate for variable photoperiods. A longer domain, such as in Chinook salmon found in the north, helps shorten the period of the oscillations by binding downstream genes such as Per more efficiently (Darlington et al. 1998). Moreover, it is thought that the polyQ domain may help maintain the amplitude of the oscillations under longer photoperiods (K. G. O'Malley and Banks 2008). In the long run, establishing whether these alleles are the true link between the environment and the organism's choice to migrate and reproduce is critical for our knowledge. Studying this is compelling, knowing that the *Clock* gene in salmonids is found in regions of the genome that control reproduction (Leder, Danzmann, and Ferguson 2006).

Temporal restriction of feeding is able to entrain peripheral clocks in various tissues, but not the master clock in the SCN (Damiola et al. 2000; Polidarová et al. 2011). The duodenum and liver seem to be more susceptible to feeding entrainment rather than SCN entrainment in mice, unlike the colon, where both cues compete (Polidarová et al. 2011). There are many proposed pathways for communication of entrainment cues with the clock. Corticosterone synchronizes itself with rhythmic food intake (Ventura, Gardey, and D'Athis 1984). Glucocorticoid signaling regulates expression of core clock genes in the liver (Reddy et al. 2007). Adrenaline is capable of restoring rhythmicity of some clock genes in livers of SCN-lesioned mice (Terazono et al. 2003). Glucose downregulates *Bmal1*, *Per1* and *Per2* expression in various peripheral clocks (Oike et al. 2010; Hirota et al. 2002). It is thought that an oscillator outside the SCN is responsible for food anticipation, as food anticipatory activity is documented in rats with an SCN ablation. This activity persists in the absence of scheduled feeding and resets its phase following a shift in feeding time (Mistlberger 1994; Stephan 2002; Marchant and Mistlberger 1997). Because the circadian clock controls a plethora of cellular processes, the influence of feeding on the clock is an important avenue to explore.

Being visual feeders, Chinook salmon must either expose themselves during the day in favor of finding visible prey, or only forage in the dark when predators cannot see them but making it harder for Chinook salmon to find their prey. Both patterns of activity have been seen in individuals at this life stage. Juvenile Chinook salmon in the Bridge river in British Columbia were seen to display nocturnalism, especially in colder winter temperatures, where being an ectotherm allows them to lower their energy demand, also helping them to cope with lower prey encounters in the dark. They are seen to become

even more nocturnal as they mature to parr and later stages, where protecting their acquired resources outweighs efficient foraging during the day (Bradford and Higgins 2001). Late-fall run Chinook salmon smolts were seen to be active only at night during migration to the ocean (Chapman et al. 2013). On the other hand, juvenile fall Chinook salmon from the Hanford Reach of the Columbia River in the United States were observed during spring feeding only during the daytime, largely found in schools and near the top of the water column (Tiffan, Kock, and Skalicky 2010). Similar feeding patterns were noted in juveniles of the lower Big Creek in the Salmon River in the United States, but not in the upper Big Creek where nighttime foraging was preferred. The overall maximum consumption happened in the afternoon in both groups (Cromwell and Kennedy 2011). Thus, there is some complexity when it comes to determining a definitive feeding time for juveniles of this species. In fish reared in aquaculture facilities, the advantage of foraging in the dark is lost, because the threat of predators is absent. Individuals within one population may display different diel behaviours and may even choose to switch over from one diel behaviour to another. Moreover, the competition between individuals, the variable distribution of prey among habitats and the change in preference for prey type add another layer of complexity. A long summer day may also force salmon to feed during the day, as their energy levels from the previous night decline, and temperatures can also dictate metabolic needs in these ectotherms. Salmon are also likely to display diurnal feeding if they have a great need to grow, such as when they are preparing for migration. In general, smaller fish tend to forage during the daytime due to a larger need to grow, whereas bigger fish forage at night since their main concern is protecting their acquired energy reserves from predators (Cromwell and Kennedy 2011). Similar patterns of activity

were seen with juvenile Atlantic salmon, where the decision to migrate is made about nine months in advance. Individuals who choose to migrate opt for efficient diurnal feeding to prepare themselves for the journey, while others spending the winter in the streams maintain a smaller body size and forage more safely at night (Metcalfe, Fraser, and Burns 1998). In general, diurnalism prevails when the risk of starvation is high, while nocturnalism is more common when avoiding predation is a priority.

In this chapter, the effects of different housing conditions are explored to determine the ability of circadian clock genes in peripheral tissues to synchronize with the light cycle and feeding time. Since these fish are raised in barrels in the absence of predators, their priority at this life stage would be to grow in size, hence daytime feeding is expected. While restricting food to either the morning or evening was tested, feeding throughout the day is likely more natural, as these fish likely cannot control the time that prey appears in the wild. Moreover, lighting was manipulated such that some fish received natural photoperiod consisting 16:8 LD, another group was reared under constant light, which is less natural for fish at the latitude where the experiments were done, but typical for populations living at high northern latitudes, to test the ability of the clock to free-run in the absence of an LD cycle giving it cues.

Hypothesis

I hypothesize that the circadian clock is active in Chinook salmon intestine, liver, and skeletal muscle and is responsive to photoperiod and feeding cues in a tissue-specific manner.

Objectives

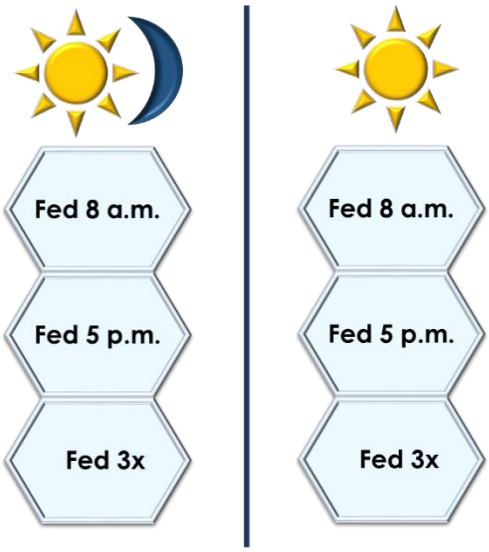
To test this hypothesis, I set up experiments where fish were reared under normal alternating long day-night, and ones under constant light, to test the circadian clock under photoperiod cues versus in free-running conditions. A fundamental property of the circadian clock is its ability to continue oscillating in the absence of cues (known as free-running). In order to probe for the effect of feeding time on the circadian clock, I reared fish under once-per-day feeding schedules and under feeding-throughout-the-day schedules to identify the condition that is most optimal for the circadian clock, knowing that feeding is an entraining cue of circadian clocks. Normally, fish are not likely to restrict their feeding to a single feeding event in a day, so feeding throughout the day is more natural; however, it is possible that a single feeding event may be advantageous for clock function, so this was explored. Within the once-per-day feeding schedules, one group received morning feeding, and another received evening feeding to test whether time of day influenced the ability of feeding to entrain the clock. RNA was extracted from the intestine, liver, and skeletal muscle and used to quantify the expression of components of the positive and negative arms of the clock; *Clock1b* is linked to migration timing so it was chosen to assay the positive arm and to search for patterns that could support such a role. *Per1* was chosen to assay the negative arm. These experiments tested the function of the circadian clock under varying environmental cues to identify the most optimal clock behaviour relevant to Chinook salmon biology and aquaculture.

Methods

Experimental setup

Juvenile Chinook salmon (6 months old, mixed sex) were reared at Yellow Island Aquaculture Ltd (YIAL) (Quadra Island, BC). These fish are highly inbred and thus are much more genetically homogenous than the Credit river fish in Chapter 2, reducing the effect that variation in individuals might have on the detection of rhythmicity (Komsa, 2012). The fish were divided into six groups housed in opaque barrels with open tops, each containing about 200 fish in 200 liters of water. Half of the groups were housed under ambient June photoperiod (16:8 LD) and half under constant light (LL), for the duration of ten to 14 days. Under each lighting condition, three different feeding schedules were established, either at 8 AM, 5 PM, or three feedings: at 8 AM, 12 PM and 5 PM (Figure 7). The total amount of feed received by each feeding group in one day was identical. Ten fish were sampled every 3hrs for the duration of 24hrs by decapitation after being anesthetized in a 0.04% clove oil bath (New Directions Aromatics Inc., Indonesia). The intestine, liver, and a sample of skeletal muscle anterior to the dorsal fin were preserved in approximately 7 mL of a concentrated salt buffer made of 0.5M EDTA disodium dehydrate (Fisher Scientific, CAS: 6381-92-6) at pH 8.0, 1M sodium citrate (Produits Chimiques ACP Chemicals Inc, Catalog #: S-2990), ammonium sulfate (Alpha chem, Catalog #: AM7210), sulfuric acid (Fisher chemical, Catalog #: A300) to bring the pH to 5.2. The tissues were stored at -80 ^{o}C for later RNA extraction and qPCR.

Figure 7: Experimental Setup



Primer design

Chinook salmon sequences were identified by running a BLAST of known zebrafish transcript sequences for genes of interest against the Chinook salmon sequences available on NCBI. In the cases where multiple isoforms of a transcript were found, the isoforms were aligned using Geneious (Geneious Biologics) to find a region of homology from which to develop primers. Primers for the transcripts of interest (Table 2) were developed using NCBI's primer designing tool. The guidelines from Primer Express Software 3.0 Getting Started Guide (Applied Biosystems) were used to manually generate the primer sequences (Table 3). The primers (Eurofins Genomics) were validated with the iTaq™ Universal SYBR® Green Supermix (BIO-RAD, Catalog #: 1725125), using standard dilution qPCR on the ViiA™ 7 qPCR system (Applied Biosystems). Primers with efficiency values around 100% ± 15% were selected for further use. Gel electrophoresis was used to verify the absence of multiple bands to ensure primer specificity.

Table 2: Primer sequences and reaction efficiencies.

Assay Name	Primer	Sequence (5'-->3')	Reaction Efficiency (%)
Gapdh	Forward	TGTCAGTGGTGGACCTAACC	93.790
	Reverse	GCCTTCTTGACAGCCTCCTT	
Tubα	Forward	AGACGACTCCTTCAACACCTT	104.879
	Reverse	CAGTGGGCTCCAGATCCA	
Clock1b	Forward	CAGCAGCACACGGTTCAA	104.660
	Reverse	AGGACAGAGCTGGTGTCTTG	
Per1	Forward	GAGTGGGAAGCACCAATGAA	94.082
	Reverse	ATTGGCTGCCATGGTTGTTG	

Table 3: Parameters followed during primer sequence development.

Parameter	Details
Amplicon size	70-90 bp
Primer length	18-22 bp (optimal 20 bp)
Primer Tm	58-60^{o}C (optimal 59^{o}C)
Specificity check database	Chinook salmon (taxid: 74940)
Specificity check stringency	At least 2 mismatches within the last 5 bps at the 3' end
Primer GC content	30-80%
Special notes	1) Avoid a G in the second position of the 5' end 2) Fewer than four consecutive G residues in the primer
All other parameters on NCBI	Default

RNA extraction

Between 5 – 30 mg of tissue were used for RNA extraction. Only the anterior (proximal) third of the intestine was used, whereas regional differences were disregarded when taking tissue samples form the liver and skeletal muscle. RNA was extracted using

the RNeasy® Plus Mini kit (QIAGEN, Catalog #: 74136) following the manufacturer's protocol for "Purification of Total RNA from Animal Tissues". The suggestion to use 50% ethanol instead of 70% ethanol for liver tissue was followed. RNA concentration was measured by 260/280-nm absorbance ratio using a Nanodrop and used to make 1 µg of cDNA using the iScript cDNA Synthesis Kit (BIO-RAD, (Cat. No. 170-8891), using the following 20 µL reaction mixture: 4 µL of iScript Buffer, 1 µL of Reverse trascriptase, 15 µL of RNA (1 µg) and RNase-free H_2O (Ambion™, Catalog #: AM9932). The reactions were incubated in the T100™ thermal cycler for 5 minutes at 25 ^oC, followed by 30 minutes at 42 ^oC, followed by 5 minutes at 85 ^oC. The synthesized cDNA was stored at -20 ^oC for later use in qPCR.

Control gene choice

Using intestinal cDNA from the LD 8 AM feeding group, the expression stability of seven housekeeping genes (*18S*, *G6pd*, *Gapdh*, *Tubα*, *bAct*, *Hprt*, and *Ef1a*) was assessed using geNorm analysis in qbase+ (Biogazelle, version 3.2). The genes with the lowest geNorm M value were selected to serve as control genes in all the experimental conditions and tissues. The two most stable genes, *Gapdh* and *Tubα*, were used for clock gene expression normalization.

qPCR

Validated primers were used in RT-qPCR to quantify the transcription of two circadian clock genes, *Clock1b* and *Per1*, along with the two control genes, *Gapdh* and *Tubα*. 10 µL reactions: 5 µL SYBR® green, 3.2 µL nuclease-free H_2O, 0.4 µL of each of forward and reverse primers) were run in duplicates using MicroAmp™ Optical 384-Well

Reaction Plate (Applied Biosystems, Catalog #: 4309849) incubated in the ViiA7 qPCR machine at 95 °C for 20 seconds, followed by 40 cycles of denaturation at 95 °C for 3 seconds and annealing and extension at 56 °C for 30 seconds. This was followed by a final step consisting of 15 seconds at 95 °C and 60 seconds at 65 °C. If the standard deviation between the duplicates exceeded 0.5 threshold cycles (Ct), the reactions were repeated to ensure accurate readings.

Correction of gene expression levels

LinRegPCR version 2020.0 was used to calculate the efficiency of each individual reaction in order to accurately estimate the expression levels of each gene in a sample. The Ct value was used to estimate the starting concentration of a sample, known as “N0”. Samples having individual efficiencies within 15% of the gene’s mean efficiency were included in the calculation of the gene’s mean efficiency in each plate. Factor-qPCR version 2020.0 was used to correct for inter-plate variation. The expression levels of the clock genes were normalized to the geometric mean of all the control genes according to the following equation: $Expression\ of\ clock\ gene = \frac{N0_{circadian\ clock\ gene}}{\sqrt[2]{N0_{Gapdh} \times N0_{Tuba}}}$.

Graphing and statistics

To assess the rhythmicity of each gene, the expression levels at each time point were fitted to a sinusoidal curve, using non-linear regression on GraphPad Prism Version 7.0c for Mac OS X. The following equation was used: $f(t) = M + Acos(t\pi/12 - \phi)$, where f(t) is the gene expression level at a specific time, the mesor (M) is the mean value, A is the amplitude, t is time in hours, and ϕ is the time of peaking in expression, known

as the acrophase. The acrophase was also confirmed by inputting the data into Chronos-fit version 1.05. A one-way ANOVA was done in Prism to test the statistical significance of the variation in expression of each clock gene over time (P-values are reported in figure legends). The noise-to-signal ratio was used to determine the significance of the curve fit, where the ratio was calculated from the standard error of the amplitude divided by the amplitude value, denoted by SE(A)/A (Halberg and Reinberg 1967). Gene expression was considered to be rhythmic if SE(A)/A< 0.3 and P<0.05 from the one-way Anova. Circular plots were developed in Oriana version 4.02 (Kovach Computing Services) to show phase relationships between the genes that displayed rhythmicity.

Results

The intestinal clock is responsive to photoperiod

In order to determine the optimal combination of lighting and feeding cues for the intestinal circadian clock, Chinook salmon fry were reared under either LD cycles, or constant light. In each of the two settings, three feeding schedules were established: fish were fed either in the morning (at 8 AM), evening (at 5 PM), or throughout the day (at 8 AM, 12 PM, and 5 PM). Tissues were harvested from ten different individuals every three hours, for a total of 24 hours. After RNA extraction, qPCR was performed on the two arms of the clock; *Clock1b* was chosen from the positive arm, because of its connection to migration time (K. G. O'Malley and Banks 2008). To assay the negative arm, *Per1* was chosen, as choosing *Cry1* or *Per2* would complicate the analysis due to the possibility that these two genes are directly induced by light, as seen in zebrafish (Vatine et al. 2009).

Expression levels were normalized to *Gapdh* and *Tubα*, which were determined to be the most stable of the seven housekeeping genes tested. The intestine displays rhythmic clock expression under normal LD photoperiod (Figure 8 – left-hand side, and Table 4). While expression of the two circadian clock genes is present in all conditions, only the groups reared under LD and fed either at 5 PM or throughout the day show significant rhythmicity (noise-to-signal ratio <0.3, and ANOVA P-value <0.05). No rhythms were observed in LL (Figure 8 – right-hand side, and Table 4), indicating that LD photoperiod was needed for circadian rhythms; in the absence of a recurring photoperiod cycle, the clock is unable to synchronize itself using feeding time only, hence it needs alternating LD. In the intestine, *Clock1b* peak expression is in the late morning, while that of *Per1* happens slightly earlier in the morning. *Per1* is known to be driven by *Clock* in other organisms, but it is unusual for *Per1* expression peak to be nearly synchronous with *Clock*. Similar synchronous peaking was found in Chinook salmon intestine previously (Thraya et al. 2019), so perhaps synchronous peaking is an intestine-specific characteristic. No dramatic differences are seen between the two feeding conditions in terms of their effect on peaking time, which supports the dominance of light over feeding as an entraining cue of the intestinal circadian clock. Overall, because these rhythms are only present under LD, and their phase of peaking is not affected by the change in feeding time, this indicates that the intestinal clock is most likely driven by photoperiod cues rather than feeding.

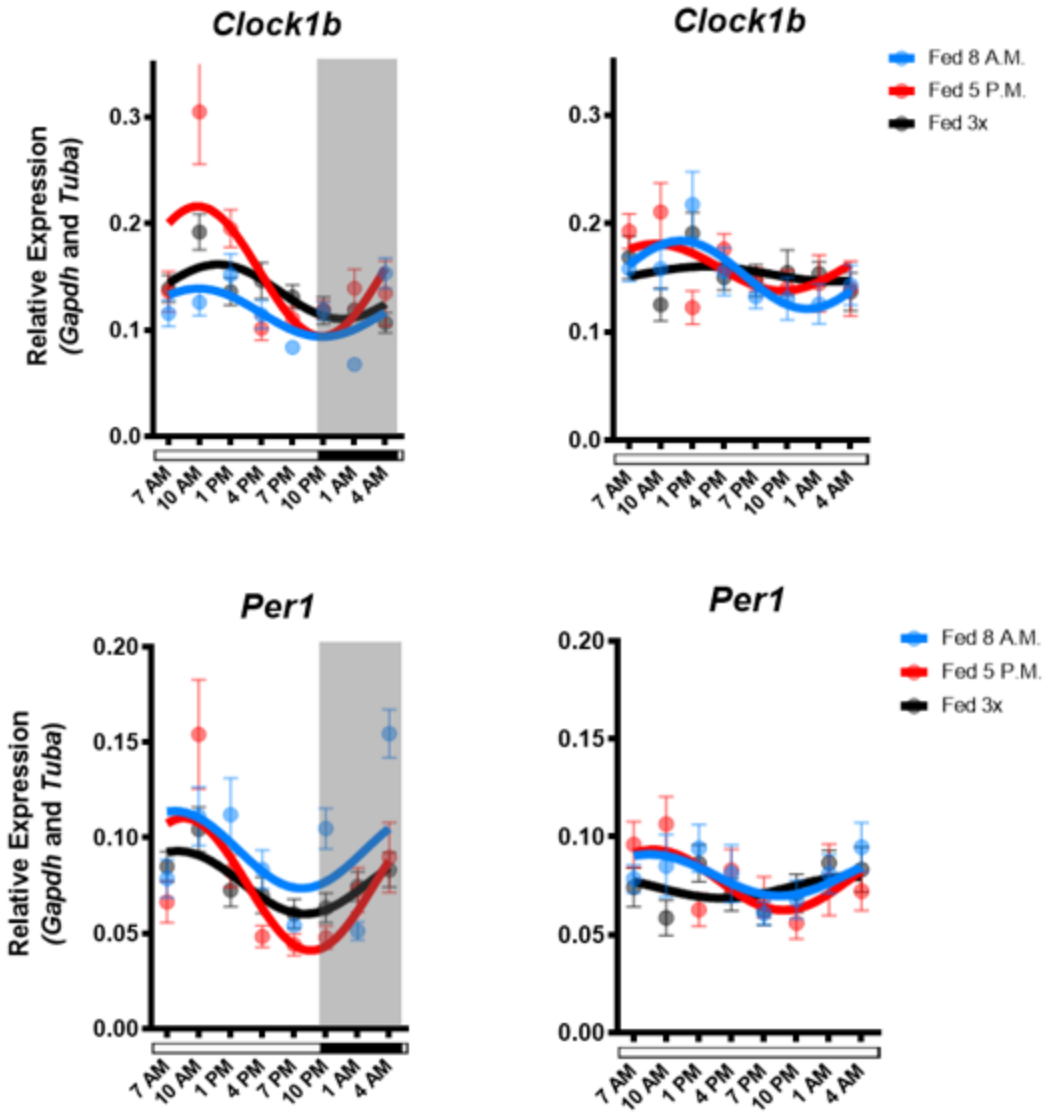


Figure 8: Expression of circadian clock genes in Chinook salmon intestine. Significant rhythms are present in both *Clock1b* and *Per1* under LD, fed 5 PM and fed 3X. The curves shown are the nonlinear regressions of the line graphs connecting the eight data points. The horizontal open and solid bars along the X-axis show light and dark phases, respectively. The shaded areas in the graphs represent the dark phase. Each data point shows the mean of target gene expression relative to two control genes. Error bars are ± SEM for 10 individuals.

Table 4: Parameters defining the daily variation in expression of clock genes in Chinook salmon intestine. An asterisk is used to denote rhythm significance.

Gene	Experimental Condition	Acrophase	Amplitude ± SE	Noise/ signal ratio	Anova P-value	Significance of Rhythm
Clock1b	**LD - Fed 8 AM**	-	0.0226 ± 0.0069	0.3067	<0.0001	
	LD - Fed 5 PM	9:40 AM	0.0609 ± 0.0138	0.2263	<0.0001	*
	LD - Fed 3X	11:40 AM	0.0255 ± 0.0067	0.2611	0.0006	*
	LL - Fed 8 AM	-	0.0313 ± 0.0099	0.3143	0.0435	
	LL - Fed 5 PM	-	0.0215 ± 0.0103	0.4784	0.0311	
	LL - Fed 3X	-	0.0068 ± 0.0085	1.2452	0.2005	
Per1	**LD - Fed 8 AM**	-	0.0202 ± 0.0073	0.3632	<0.0001	
	LD - Fed 5 PM	8:32 AM	0.0347 ± 0.0077	0.2229	<0.0001	*
	LD - Fed 3X	8:01 AM	0.0164 ± 0.0044	0.2655	0.0189	*
	LL - Fed 8 AM	-	0.0106 ± 0.0057	0.5379	0.4110	
	LL - Fed 5 PM	-	0.0154 ± 0.0060	0.3910	0.0641	
	LL - Fed 3X	-	0.0055 ± 0.0046	0.8441	0.2215	

Photoperiod results in rhythmicity in both circadian genes of the liver clock

Circadian clock function was explored in the liver to determine the effects of variable combinations of photoperiod and feeding time. Rhythmic expression is present in the liver in both the positive arm of the clock, represented by *Clock1b*, and the negative arm, represented by *Per1*. Rhythmicity is present under the two conditions determined for the intestine, LD fed 5 PM and LD fed throughout the day (Figure 9 – left-hand side, and Table 5). *Per1* expression is rhythmic under all combinations of lighting and feeding schedules. No drastic differences are exhibited by *Per1* when comparing the two lighting conditions for groups fed in the morning and for those fed throughout the day (Figure 9 – lower half, and Table 5); however, constant lighting results in a delayed peak in the 5 PM feeding group. This indicates that the liver clock is responsive to photoperiod, since switching from LD to LL delays the expression peak. This clock is also responsive to

feeding cues, as demonstrated by the difference in *Per1* peaking time among the morning and evening fed groups housed under LD.

Comparing the two most optimal conditions (LD fed 5 PM and LD fed 3X – Table 5) shows that *Per1* peak is delayed by about 5 hours relative to *Clock1b* in the first condition, and by 8 hours in the second. The second situation is more typical of the clock, where positive regulators (*Clock1b*) peak anti-phasic to negative regulators (*Per1*), ideally 12 hours apart. On the other hand, this relationship disappears in the remaining four conditions, because *Clock1b* is arrhythmic in those. This leads to the conclusion that LD photoperiod with feeding throughout the day is the most optimal condition of all six conditions.

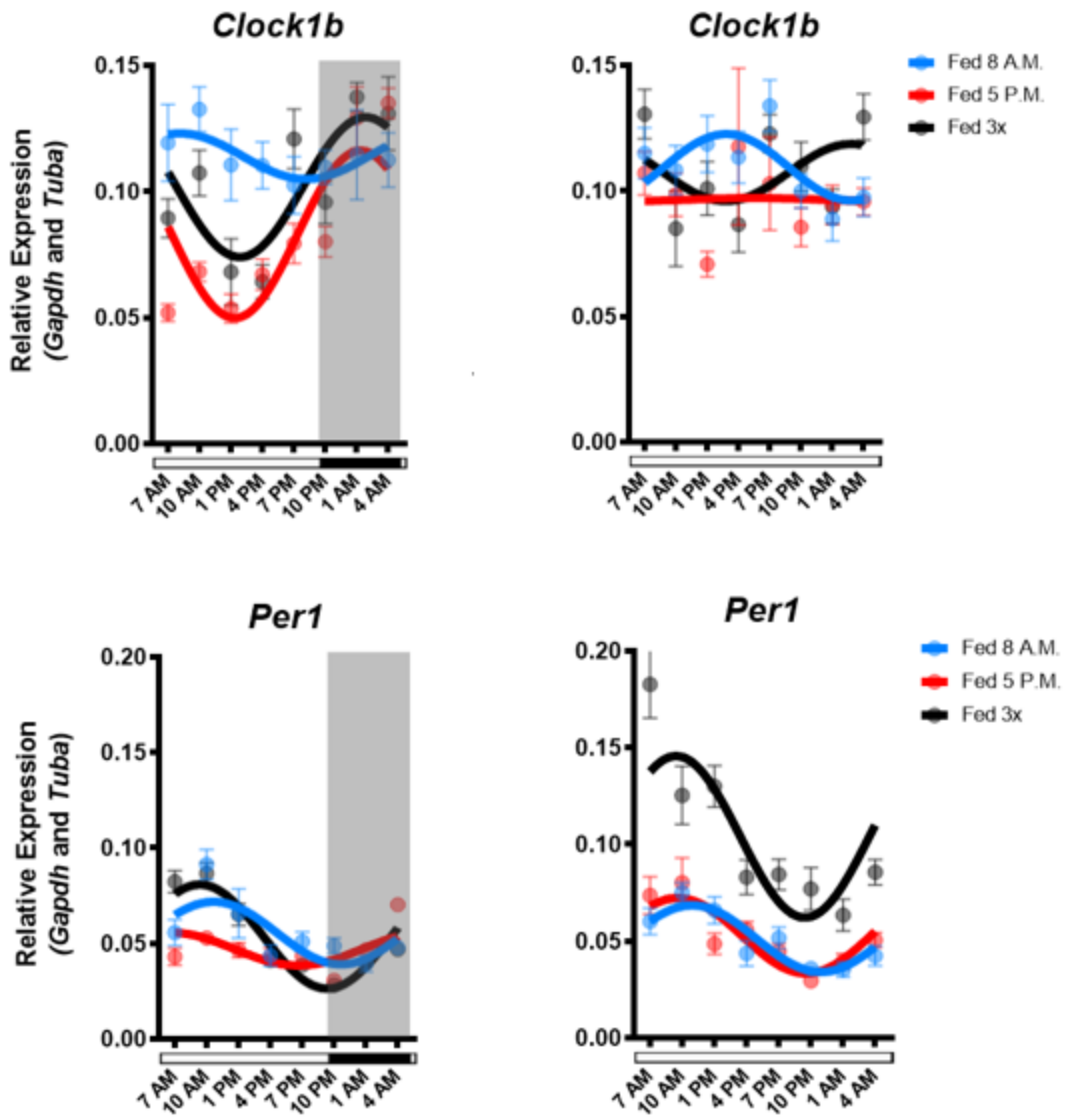


Figure 9: Expression of circadian clock genes in Chinook salmon liver. Significant rhythms are present in *Clock1b* under LD, fed 5 PM and fed 3X, and in *Per1* under all experimental conditions. The curves shown are the nonlinear regressions of the line graphs connecting the eight data points. The horizontal open and solid bars along the X-axis show light and dark phases, respectively. The shaded areas in the graphs represent the dark phase. Each data point shows the mean of target gene expression relative to two control genes. Error bars are ± SEM for 10 individuals.

Table 5: Parameters defining the daily variation in expression of clock genes in Chinook salmon liver. An asterisk is used to denote rhythm significance.

Gene	Experimental Condition	Acrophase	Amplitude ± SE	Noise/ signal ratio	Anova P-value	Significance of Rhythm
Clock1b	**LD - Fed 8 AM**	-	0.0090 ± 0.0060	0.6654	0.8163	
	LD - Fed 5 PM	1:21 AM	0.0332 ± 0.0045	0.1355	<0.0001	*
	LD - Fed 3X	1:49 AM	0.0277 ± 0.0056	0.2005	<0.0001	*
	LL - Fed 8 AM	-	0.0132 ± 0.0049	0.3689	0.0462	
	LL - Fed 5 PM	-	0.0006 ± 0.0072	12.0533	0.4632	
	LL - Fed 3X	-	0.0113 ± 0.0056	0.4895	0.0044	
Per1	**LD - Fed 8 AM**	10:39 AM	0.0164 ± 0.0037	0.2259	<0.0001	*
	LD - Fed 5 PM	6:37 AM	0.0086 ± 0.0023	0.2649	<0.0001	*
	LD - Fed 3X	9:21 AM	0.0272 ± 0.0024	0.0872	<0.0001	*
	LL - Fed 8 AM	10:55 AM	0.0172 ± 0.0029	0.1663	<0.0001	*
	LL - Fed 5 PM	9:34 AM	0.0194 ± 0.0035	0.1797	<0.0001	*
	LL - Fed 3X	9:25 AM	0.0417 ± 0.0065	0.1563	<0.0001	*

Skeletal muscle is rhythmic under constant light only

The clock has never been studied in the skeletal muscle of Chinook salmon, so clock function was explored for the first time, along with the effects of photoperiod and feeding time. Rhythmic expression is detected in both the positive arm (*Clock1b*) and negative arm (*Per1*) of the skeletal muscle clock (Figure 10, Table 6); however, *Clock1b* is rhythmic under LL instead of LD, unlike the intestine and liver where *Clock1b* is only rhythmic under LD. Expression peak of *Clock1b* in skeletal muscle precedes that of *Per1* by about 11 hours in both conditions where the two arms display rhythmicity (constant light, fed 8 AM and fed 5 PM). This anti-phasic rhythmic expression between the positive and negative regulators indicates that the clock is likely functional in skeletal muscle tissue under these two settings.

There are indications that the clock in skeletal muscle is entrained to both photoperiod and feeding cues. Differences in peaking time are seen among the different feeding groups, where the 5 PM group deviates from the other two groups, indicating that the skeletal muscle circadian clock is entrainable by feeding cues. A three-hour phase difference is also seen in *Per1* across the two lighting settings in the group fed at 5 PM, indicating that photoperiod entrains the muscle clock. However, as stated in the previous paragraph, only *Per1* is rhythmic in LD photoperiod, whereas *Clock1b* loses rhythmicity under LD. This could either be pointing to the dominance of feeding entrainment, or to the integration of both light and feeding, as in the case of *Clock1b*, where the rhythms are lost when cues are coming from both photoperiod and feeding, as if the clock becomes confused.

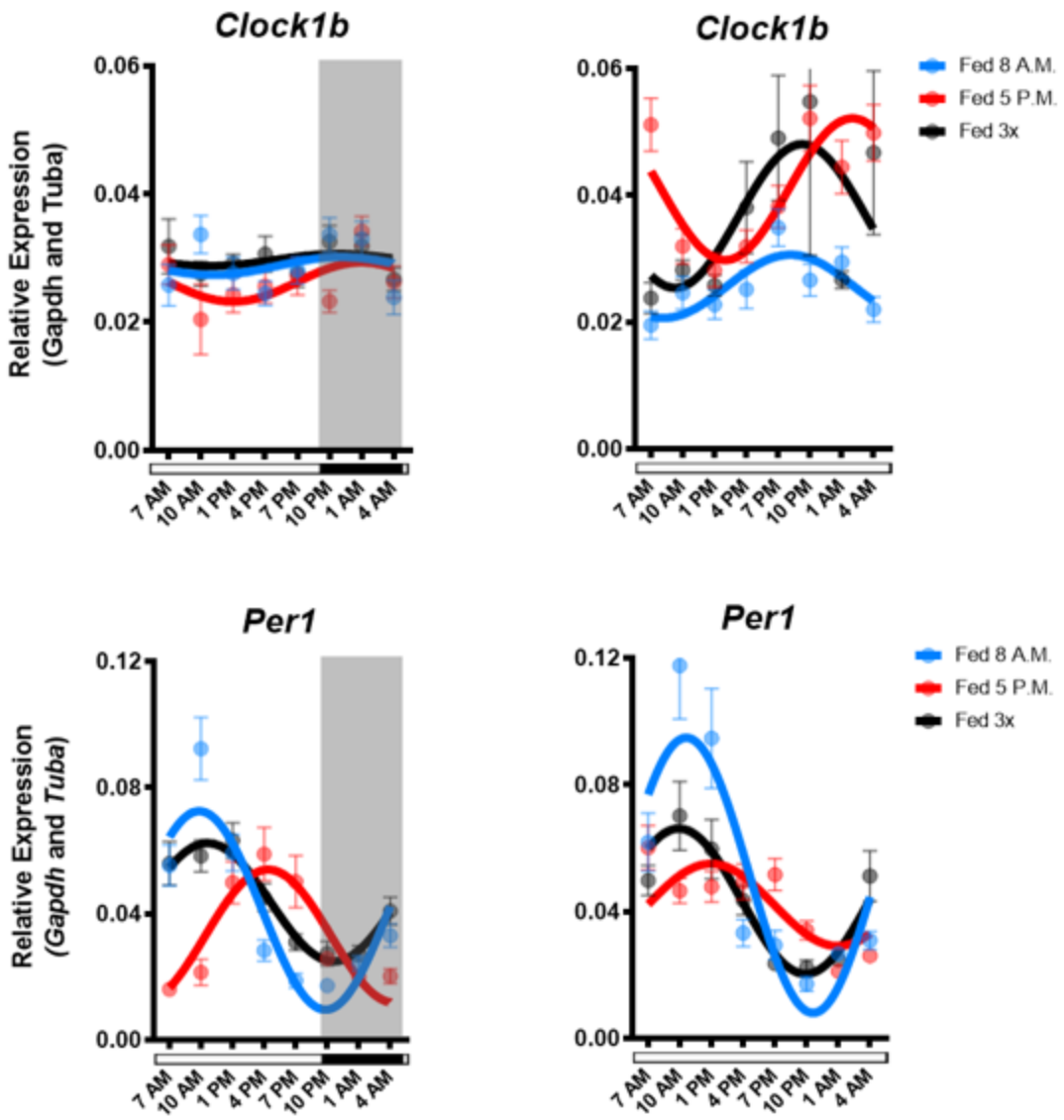


Figure 10: Expression of circadian clock genes in Chinook salmon skeletal muscle. Significant rhythms are present in *Clock1b* under LL, fed 8 AM and 5 PM, and in *Per1* under all experimental conditions. The curves shown are the nonlinear regressions of the line graphs connecting the eight data points. The horizontal open and solid bars along the X-axis show light and dark phases, respectively. The shaded areas in the graphs represent the dark phase. Each data point shows the mean of target gene expression relative to two control genes. Error bars are ± SEM for 10 individuals.

Table 6: Parameters defining the daily variation in expression of clock genes in Chinook salmon skeletal muscle. An asterisk is used to denote rhythm significance.

Gene	Experimental Condition	Acrophase	Amplitude ± SE	Noise/ signal ratio	Anova P-value	Significance of Rhythm
Clock1b	**LD - Fed 8 AM**	-	0.0014 ± 0.0014	1.0064	0.0264	
	LD - Fed 5 PM	-	0.0031 ± 0.0016	0.5277	0.1365	
	LD - Fed 3X	-	0.0009 ± 0.0013	1.3458	0.5661	
	LL - Fed 8 AM	8:09 PM	0.0050 ± 0.0013	0.2599	0.0020	*
	LL - Fed 5 PM	1:58 AM	0.0112 ± 0.0020	0.1758	<0.0001	*
	LL - Fed 3X	-	0.0113 ± 0.0055	0.4829	0.3002	
Per1	**LD - Fed 8 AM**	9:49 AM	0.0314 ± 0.0030	0.0940	<0.0001	*
	LD - Fed 5 PM	4:20 PM	0.0211 ± 0.0027	0.1293	<0.0001	*
	LD - Fed 3X	10:37 AM	0.0187 ± 0.0023	0.1238	<0.0001	*
	LL - Fed 8 AM	10:36 AM	0.0434 ± 0.0051	0.1168	<0.0001	*
	LL - Fed 5 PM	12:56 PM	0.0130 ± 0.0026	0.1995	<0.0001	*
	LL - Fed 3X	9:54 AM	0.0229 ± 0.0032	0.1379	<0.0001	*

The three tissues display different circadian phase relationships with one another

To determine how the clocks of the three tissues behave relative to one another, the phase of expression maxima of the clock genes was compared across the tissues. Natural photoperiod (LD) coupled with feeding throughout the day appears to be the most optimal condition in two out of the three tissues examined here. Under this setting, *Clock1b* peaks at noon in the intestine and in the middle of the night in the liver (Figure 11), implying that the two tissues are entrained differently. On the other hand, *Per1* peaking is somewhat conserved across the three tissues (one- to three-hour differences), happening in the morning.

In the second-best condition (LD fed at 5 PM), entrainment happens differently across the tissues. The intestine and liver are on opposite phases of *Clock1b* expression,

with peaks falling in the morning and at midnight, respectively (Figure 11). *Per1* also displays different phases across the three tissues, with maximal expression happening at 9 AM in the intestine, 4 AM in the liver, and 4 PM in the muscle.

Under LL, differences between tissues are even greater. The intestine shows no rhythms under all three feeding settings (Figure 11). The liver only shows rhythms in *Per1*, while skeletal muscle shows rhythms in most conditions in both *Clock1b* and *Per1* (Figure 11). This further demonstrates that the clocks of the three tissues are not in synchrony with one another, possibly receiving a mixture of different cues from the SCN and their local environment.

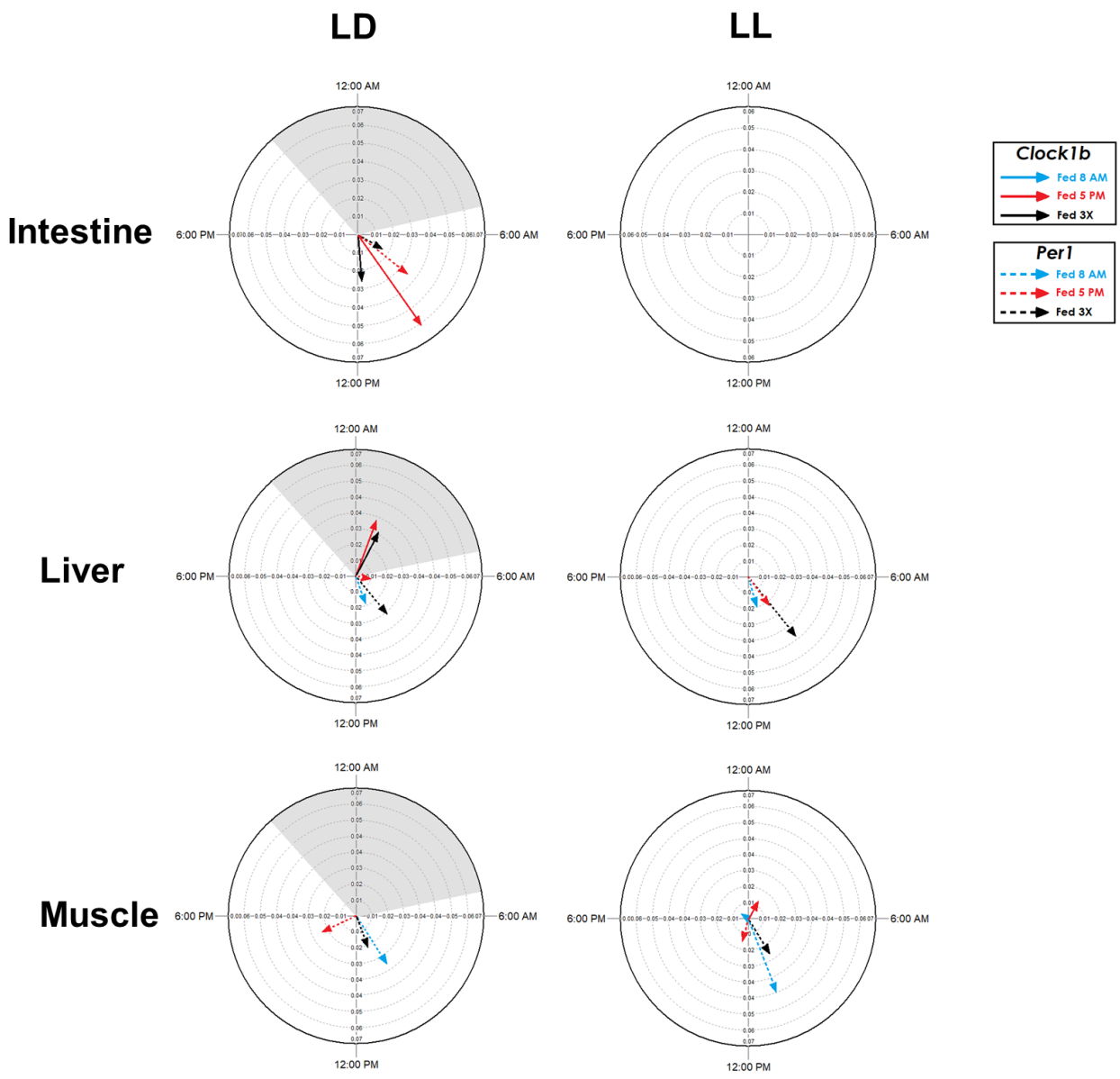


Figure 11: Circular graphs showing expression amplitude and peak times of *Clock1b* and *Per1* in the intestine, liver, and skeletal muscle. The tissues are on different phases of peak expression under many of the rearing conditions. The circular axis represents the acrophase of a rhythm, and the linear axis represents the amplitude of the rhythm. The three tissues are on different phases of peak expression, and some tissues do not display rhythmicity under some conditions.

Discussion

Feeding as an entrainment cue in the three tissues

The circadian clock has been studied in tissues of various fish species, where circadian clock function has been established. For instance, circadian clock rhythmicity is seen in the intestine in zebrafish, goldfish, and turbot (*Scophthalmus maximus*) (Peyric, Moore, and Whitmore 2013; Nisembaum et al. 2012; Ceinos et al. 2019). The liver displays circadian clock rhythmicity in animals such as mouse, zebrafish, Rainbow trout, Nile tilapia, goldfish, and gilthead sea bream (*Sparus aurata*) (Hernández-Pérez et al. 2017; Feliciano et al. 2011; Gómez-Boronat et al. 2018; Damiola et al. 2000; Costa et al. 2016; López-Olmeda et al. 2010; Vera et al. 2013). Circadian rhythms have also been established in the skeletal muscle clock of zebrafish, goldfish, Atlantic cod (*Gadus morhua*), and Crucian carp (*Carassius carassius*) (Amaral and Johnston 2012; Bao et al. 2018; Lazado et al. 2014; Wu et al. 2018).

In this study, circadian clock function was tested in three tissues (intestine, liver, and skeletal muscle) under two lighting schedules (LD and LL), and three feeding schedules (once in the morning, once in the evening, or throughout the day). It was found that the intestinal clock is only rhythmic under LD, and never under LL, and is most likely entrained to photoperiod rather than feeding (Figure 8 and Table 4). Similar results were also seen in the liver, although *Per1* in this tissue is rhythmic under all experimental conditions, including LL, and shows a different peaking time in morning versus evening feedings, indicating food entrainment as well (Figure 9 and Table 5). In the muscle, the clock performs better under LL, where both *Clock1b* and *Per1* are rhythmic, however,

Per1 is rhythmic in the muscle under all conditions (Figure 10 and Table 6). There are indications of both feeding and photoperiod entrainment in the liver and muscle clocks, but the liver is more likely reliant on photoperiod, since *Clock1b* is only rhythmic in LD.

Consistent with the findings in the intestine, *Per1a* does not respond to a change in feeding schedule in goldfish hindgut reared under constant light, supporting the conclusion that the Chinook salmon intestine is not entrained by feeding cues (Nisembaum et al. 2012). However, this finding is contradicted in the intestine of zebrafish reared in constant darkness, where the intestinal clock shows responsiveness to feeding; there is a 12-hour difference in peaking time between midday versus midnight feeding groups, in all of *Per1*, *Per2*, and *Cry1a* (Peyric, Moore, and Whitmore 2013). This suggests that the intestinal clock's response to feeding is different across teleosts.

In the liver and skeletal muscle, there is evidence of feeding entrainment, as demonstrated by the different peaking times depending on the timing of food administration (Tables 5 and 6). Feeding entrainment is seen in the livers of zebrafish, goldfish, gilthead sea bream, Rainbow trout, Nile tilapia and mouse, consistent with the findings in the present study; however, it is thought that the combination of photoperiod and feeding entrains trout liver, similar to Chinook salmon liver here (Hernández-Pérez et al. 2017; Feliciano et al. 2011; Gómez-Boronat et al. 2018; Damiola et al. 2000; Costa et al. 2016; López-Olmeda et al. 2010; Vera et al. 2013). *Clock1b* and *Per1* peak at different times within each tissue under the same feeding schedule. Consistent with this finding, the same feeding schedule elicits different responses in various clock genes, as seen in zebrafish intestine and Rainbow trout liver (Peyric, Moore, and Whitmore 2013; Hernández-Pérez et al. 2017).

Rhythmicity is seen in both *Clock1b* and *Per1* in the skeletal muscle clock when a single feeding event is the main entraining cue under LL (Figure 10 – right-hand side, and Table 6). This suggests a significant role for feeding in entraining clock rhythms in this tissue. Rhythmic oscillations are seen in clock activity in skeletal muscle of various species including Atlantic cod, goldfish, and under constant darkness in zebrafish (Amaral and Johnston 2012; Lazado et al. 2014; Bao et al. 2018). Furthermore, feeding is thought to entrain some circadian clock genes, as seen when Crucian carp and goldfish are starved for a week or two (Wu et al. 2018; Bao et al. 2018). Starvation has detrimental effects on some of the clock genes in these two species, further highlighting the importance of feeding as a cue for normal clock rhythmicity.

Overall, the feeding schedule that resulted in the most rhythmicity in the three tissues is feedings throughout the day. This is no surprise, as fish in the wild are more likely to be on a random feeding schedule, where they eat whenever prey is available.

Photoperiod as an entrainment cue

It is far more difficult for fish to predict feeding time in the wild than it is to predict the light-dark cycles that have been conserved on this earth for a long time. It would be further problematic if every feeding event caused a shift in circadian clock rhythms, especially if Chinook salmon are opportunistic feeders. Thus, it makes more sense for clocks to entrain themselves to a more reliable cue, such as photoperiod.

Indeed, zebrafish experiments have shown that the intestinal clock is far more receptive to photoperiod entrainment than to feeding, similar to what is observed here (Peyric, Moore, and Whitmore 2013). Because the clock genes assayed in the intestine

did not maintain rhythmicity under constant light, it is difficult to conclude that the intestinal clock is self-driven, rather than a simple response to photoperiod cycles. However, it is promising to know that *Per1* in zebrafish intestine displays free-running rhythmicity in constant darkness (Peyric, Moore, and Whitmore 2013). Perhaps testing whether the Chinook salmon intestinal clock maintains rhythmicity in constant darkness would help answer whether the rhythmicity seen in the clock is intrinsic or is a response to LD cycles.

I suspect that the liver clock is also under photoperiod control, as *Clock1b* is only rhythmic in the presence of LD cycles (Figure 9 – left-hand side, and Table 5). This is observed in other fish species, where rainbow trout liver clock is thought to be controlled by photoperiod (Hernández-Pérez et al. 2017). I also suspect light responsiveness in liver clocks since the amplitude of *Per1* rhythms is higher under constant light (Figure 9 – lower half, and Table 5). However, the ability of photoperiod to result in functional clock activity in the absence of regular feeding schedules is not reflected in Atlantic salmon liver; *Clock*, *Per1*, *Per2*, and *Rev-erbα* were found to be arrhythmic (Betancor et al. 2014). So perhaps the combination of both photoperiod and feeding cues drives the liver clock. It is difficult to comment on the autonomy of the rhythms seen here because none of the experimental conditions were designed to be devoid of feeding and photoperiod cues to the clock, such as rearing the fish in constant darkness and starvation. However, free-running rhythmicity is seen in Nile tilapia liver clock genes when the animals are switched to constant light on the day of sampling and starved (Costa et al. 2016); thus it is possible to find free-running rhythmicity in the liver when a similar experiment is done in Chinook salmon in the future.

Clock1b peaking in the liver is found in the middle of the night, and *Per1* peaking in the morning (Figure 9 and Table 5). While these two peaking times roughly coincide

with those of *Bmal1* and *Per1* in rainbow trout liver under LD and morning feeding (Hernández-Pérez et al. 2017), notable differences in acrophases are observed in other fish compared to the findings here. Under LD and a midday feeding, Nile tilapia liver *Clock1* peaks in the second half of the day, while *Per1b* peaks in the second half of the night (Costa et al. 2016). Similar afternoon peaking was seen in *Bmal1* in Atlantic salmon liver under a long day and ad libitum feeding (Betancor et al. 2014). The acrophases in Nile tilapia and Atlantic salmon circadian genes are relatively advanced compared to Chinook salmon and rainbow trout, but the phase-difference relationships, i.e. peaking times, between the positive regulators (*Clock* or *Bmal*) and the negative regulator (*Per1*) seem to be conserved across these species. This indicates that the liver clock is functional, but at a different phase depending on the species.

In skeletal muscle, only slight differences are seen in clock genes between the two lighting conditions. One notable difference is observed in *Per1* under the evening feeding schedule, where constant light results in a three-hour advancement in the phase of the peak (Figure 10 and Table 6). Zebrafish muscle clock genes display rhythmicity under constant darkness when fed to satiety twice a day, similar to the findings under constant light here, indicating that feeding may be providing the muscle clock with cues (Amaral and Johnston 2012). The acrophases of *Clock1a/b* and *Bmal1a/b* are similar to the ones seen in *Clock1b* with morning feeding, and the acrophase of *Per1b* is similar to that of *Per1* in some of the experiments. Similar acrophase is also observed in *Clock* in Atlantic cod muscle (Lazado et al. 2014). Moreover, it is possible that other clock components not assayed here are rhythmic under photoperiod cycles; thus, at this time, I cannot conclude

that the muscle circadian clock is not entrained to photoperiod based on the results from *Clock1b* failing to show rhythmicity under LD.

Phase-relationships between the tissues

Compared to other fish species, evening peaks are expected in *Clock1b* expression and morning peaks in *Per1*. This is a typical anti-phasic relationship, where the positive arm (*Clock1b* in this case) drives the expression of the negative arm (*Per1*), which leads to the inactivation of the positive arm proteins. Overall, this timing of peaking is observed here in all the tissues (Figure 11), with the exception of *Clock1b* in the intestine, which surprisingly peaks in the morning, similar to *Clock* in Crucian carp skeletal muscle after prolonged fasting (Wu et al. 2018). Moreover, *Per1* in zebrafish possesses light-inducible elements in its promoter (Vatine et al. 2011). However, *Per1* levels here start to rise well in advance of lights coming on (Figures 8, 9, and 10), which makes it unlikely that *Per1* is light-driven in Chinook salmon. Findings similar to this were documented in zebrafish intestine and skeletal muscle, Rainbow trout liver, turbot gut and liver (Clock1 and *Per1*), and with *Per2* gilthead sea bream (*Sparus aurata*) liver under normal photoperiod (Peyric, Moore, and Whitmore 2013; Vera et al. 2013; Hernández-Pérez et al. 2019; Amaral and Johnston 2012; Ceinos et al. 2019). At this life stage, the relatively large fish size makes it unlikely that enough light can penetrate the body and reach internal organs to entrain peripheral clocks directly without the involvement of the master clock that resides in the brain and receives light through the eyes. Thus, entrainment of peripheral clocks like the ones in the tissues assayed here is likely happening through neural and hormonal signals coming from the master clock.

Even though the entire animal was exposed to the same lighting and feeding schedule, the clocks of various tissues are running on different phases. The intestine and liver are on almost opposite phases when it comes to *Clock1b* peak expression under LD photoperiod (Figure 11 – left-hand side). Moreover, *Per1* expression in muscle is delayed by a few hours relative to the other two tissues. This is consistent with observations in goldfish, where some clock genes are on different phases of expression between the gut and liver (Velarde et al. 2009). However, in turbot, synchrony is seen between the clocks of the gut, liver, and muscle, contrary to what is observed here in Chinook salmon (Ceinos et al. 2019). Entrainment to feeding schedule happens at different rates in various mouse tissues, which could explain the differences seen here (Damiola et al. 2000). The animals here were given a few months to entrain to the feeding schedules, so I find it interesting that the three tissues are still not synchronized with one another. Goldfish tissues lose synchrony with one another when feeding is shifted from midday to midnight, thus perhaps the feeding times in the present study are causing the desynchrony observed here. The goldfish also display elevated levels of plasma cortisol, indicating that the lack of alignment between feeding and photoperiod may drive the organism further from homeostasis (Gómez-Boronat et al. 2018). Different degrees of entrainment from the SCN, direct light detection, and feeding could account for the different phases observed in this study. For instance, in gilthead sea bream, the liver is robustly responsive to feeding, while the brain does not respond to changed feeding time (Vera et al. 2013).

For optimal clock function, components of the positive and negative arms of the clock are usually on different phases; that is, the positive arm drives the expression of the negative one, which in turn represses the positive arm. On the contrary, somewhat

synchronous peaking is observed between the positive and negative arms of the clock in the intestine under the two most optimal rearing conditions (Figure 8 and Table 4), similar to the findings from our previous work and those from skeletal muscle in zebrafish, Chinese perch (*Siniperca chuatsi*), and Crucian carp (Thraya et al. 2019; Amaral and Johnston 2012; Wu et al. 2016, 2018). The clock is not only reliant on transcription rhythms, however. One explanation for the co-phasic transcription of *Clock1b* and *Per1* is that the anti-phasic property is conserved at the protein level, through different rates of translation and posttranslational modifications of the two arms of the clock. Clock1b proteins must be made in advance of Per1 proteins, since the former drive the expression of the latter. Thus, even if *Clock1b* mRNA does not peak until the time when *Per1* mRNA is maximal, all that is needed for a functional circadian clock is for Clock1b proteins to peak in advance of *Per1* mRNA. Rhythms in the RNA of the various clock components correspond with the rhythms of the protein counterparts in mouse liver in terms of level of expression and acrophase, with some lag seen in Cry and Per protein peaks relative to the RNA peaks, about 6-12 hours (Lee et al. 2001). Moreover, Cry proteins cannot enter the nucleus in Per1/Per2 double mutants, indicating that there is more complexity to the circadian clock than simple transcriptional rhythms (or in the case of my study, RNA abundance rhythms). Future analysis of protein levels throughout the day will shed light on this question. If this relationship between Clock1b proteins and *Per1* mRNA is not found, perhaps a different circadian clock gene (such as *Bmal1*, or *Clock1a*) is responsible for driving *Per1* expression.

Another possibility is that the remaining paralogs of the genes that make up the clock are oscillating with different phases and the ones assayed in this study happened

to be on similar phases with each other. This is seen in zebrafish intestine, where components of the negative arm (*Cry1a* and *Per2*) peak at different times (Peyric, Moore, and Whitmore 2013). It is even more clear in the zebrafish skeletal muscle, where *Cry2a* and *Cry2b* peak at the same time as *Bmal1a*, *Bmal1b*, *Clock1a* and *Clock1b*; whereas *Per1a*, *Per1b*, *Per2* and *Cry1a* peak much later (Amaral and Johnston 2012). Furthermore, there are far more paralogs for each core clock component in zebrafish than what has been annotated in the Chinook salmon genome present on NCBI. If the primers used in this study are complementary to the different paralogs of a clock component, and those components are oscillating with different phases, then the readout is an average of the unintended targets and the known paralog. This is problematic given that different paralogs can have different responses to feeding and photoperiod (Nisembaum et al. 2012).

Furthermore, the tissue samples used here are relatively heterogeneous in terms of the cell types that make up the tissue. A tissue sample may include epithelial cells, neural cells, smooth muscle cells, blood cells, etc. All of these are homogenized together during the gene expression protocols. If each cell type is oscillating at a slightly different phase, then this would lead to ambiguous readings. This is a possibility since this study shows how tissues (intestine, liver, and skeletal muscle) are on different phases of clock expression. Thus, if different cell types process entrainment signals differently, then it would make it possible for each cell type to be on a different phase of circadian clock expression.

Stress may explain why muscle clocks respond mostly to feeding

Skeletal muscle helps fish swim and avoid predators, and its activity is needed throughout the day and night; at the life stage sampled, fry are likely to be swimming against the current, as they spend several months in the streams where they hatch before migrating downstream towards the ocean. The fish are also exposed to long bouts of light in the summer, which makes determining time of day a challenge if the clock were only driven by light cues. Locomotor rhythms disappear under constant darkness in turbot, suggesting that lengthened exposure to constant conditions such as long days or long nights diminishes rhythmicity in muscle function (Ceinos et al. 2019). Thus, it is likely that muscle clocks are relying on feeding cues rather than photoperiod during such long summer days to entrain to their environment.

Glucocorticoids are candidates for communicating feeding cues to the clock and also serve a role in opposing the effects of sudden changes in food intake to prevent disadvantageous phase shifts in peripheral clocks in a tissue-specific manner (Patton and Mistlberger 2013). The *Per1* gene in both mice and zebrafish is inducible by glucocorticoids, and its promoter contains glucocorticoid response elements (Sánchez-Bretaño et al. 2016; Yamamoto et al. 2005). Glucocorticoids are secreted in response to stressful events, such as a potential predator approaching, and can reduce food intake, indirectly affecting the clock. It makes sense for the clock in this tissue to synchronize itself to feeding as a method of knowing when stressful events happen. Glucocorticoid levels are also responsive to feeding outside of the daytime, which elevates cortisol levels in goldfish (Gómez-Boronat et al. 2018). If predators are encountered outside the light period, the rise of cortisol levels may shift the clocks in a direction different from that cued

by light. Similarly, the mechanical activity of muscle fibers is also a cue for the clock. The fish muscle can become activated at night as the organism attempts to flee from a predator, further confusing the circadian clock in this tissue. Therefore, it may be better to uncouple the muscle clock from lighting cues to preserve homeostasis.

Insufficient evidence of PolyQ role in amplitude compensation

The polyQ tail length in the *Clock* gene is correlated with latitude, hence photoperiod, in Chinook salmon and other species, like birds and insects, and it maybe be linked to migration and reproduction timing (K. G. O'Malley and Banks 2008; Zhang et al. 2017; Costa et al. 1992; Johnsen et al. 2007; Tauber et al. 2007); Even though the present study was not designed to specifically explore this role, the ability of the polyQ domain to help maintain the clock's amplitude under constant light can be addressed by comparing the amplitudes of the rhythms under LD compared to LL; normally, the amplitude declines with increasing photoperiod (K. G. O'Malley and Banks 2008). It is not possible to compare the amplitude of *Clock1b* under LD versus LL, because within all three tissues, its rhythmicity happens in only one of the two lighting conditions (Tables 4, 5 and 6). On the other hand, *Per1* amplitude does not decline under constant light in the liver and muscle, under all feeding conditions except 5 PM feeding in the muscle (Tables 4, 5, and 6). This points to the existence of a photoperiod-compensation mechanism within the clock, opening the doorway to *Clock1b*-polyQ as a candidate for this.

There is evidence from the threespine stickleback (*Gasterosteus aculeatus*) and the bluethroat bird showing that polyQ polymorphisms are not correlated with photoperiods experienced at each latitude (O'Brien et al. 2013; Johnsen et al. 2007). This

threespine stickleback study stands out because the authors tested the photoperiodic response, measured by sexual maturation, rather than just drawing correlations as in the previous studies listed here. Therefore, the contribution of the polyQ domain to migration timing remains to be uncovered in Chinook salmon.

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Chapter 4: Rhythmicity in intestinal processes

Introduction

The intestine is critical for nutrient absorption and metabolism, thus it must be able to respond to changes within the gut and throughout the organ system. Rhythmic daily changes have been documented in gastrointestinal functions such as gastric emptying, nutrient uptake, digestion, colonic motility, and epithelial cell regeneration (M. Saito et al. 1975; Pan and Hussain 2009; Stokes et al. 2017; Karpowicz et al. 2013; Goo et al. 1987; Hoogerwerf et al. 2008, 2010). This rhythmicity allows for the optimization of organism fitness by coupling various processes to the most advantageous time of the day. Many of these changes are a result of circadian clock control and they lose rhythmicity in the absence of a functional clock. This is seen with nutrient uptake and digestion (Pan and Hussain 2009), as well as colonic motility (Hoogerwerf et al. 2010). Moreover, key signaling pathways involved in metabolism and immune functions contain genes that are known to be under clock control (Turek 2016). Rhythmic expression of the different clock genes has been described in the intestine of mammals, insects, and fish (Karpowicz et al. 2013; Parasram et al. 2018; Sládek et al. 2007; Velarde et al. 2009). The intestine thus harbors many important circadian processes that are not fully understood, nor studied in Chinook salmon. The goal of this chapter is to establish whether a subset of the genes involved in appetite regulation, nutrient uptake and digestion, inflammation and regeneration are under circadian control. Because the circadian clock is known to regulate many of these processes, manipulation of the rearing conditions that entrain the clock could be a gateway to increasing fish health and growth in aquaculture.

The regulation of appetite is likely under circadian control in Chinook salmon. Ghrelin is an important enteric hormone for inducing hunger and ultimately controlling the

time of feeding and metabolic changes that affect body weight (Cummings, Foster-Schubert, and Overduin 2005). Even though ghrelin-producing cells are most abundant in the stomach, they are also found in the intestine and colon (Sakata et al. 2002). Rats entrained to meal schedules show peaks in plasma ghrelin that start to form two hours prior to meal time (Drazen et al. 2006). Generally, plasma ghrelin peaks around light onset in nocturnal species and around dark onset in diurnal species (Kumar Jha, Challet, and Kalsbeek 2015). In addition, reduced stomach ghrelin expression and circulating ghrelin levels in Atlantic salmon (*Salmo salar*) is involved in inducing anorexia as a response to elevated water temperatures (Hevrøy et al. 2012). Thus, testing whether ghrelin expression in Chinook salmon intestine is under circadian regulation is important to my project.

Appetite suppressors that are expressed in the intestine include cholecystokinin (*Cck*) and glucagon-like peptide-1 (*Glp-1*) (Gelineau and Boujard 2001; Himick and Peter 1994; Dockray 2004). Cck is secreted by the small intestine and it induces exocrine secretions from the pancreas (Chandra and Liddle 2011). *Cck* expression is robustly rhythmic in the duodenum of rats (Xu et al. 2017). Glp-1 is an incretin hormone that promotes insulin secretion and is synthesized by intestinal L-cells (Mojsov, Kopczynski, and Habener 1990; Reimann and Reddy 2014). Circadian rhythms exist in Glp-1 secretion from intestinal L-cells, and this seems to be driven by the peripheral clocks in these cells (Gil-Lozano et al. 2014; Brubaker and Gil-Lozano 2016). Plasma Glp-1 levels show diurnal rhythmicity in normal weight humans, and in anticipation of food in rats (Muñoz, Rodríguez, and Morante 2015; Vahl et al. 2010). Vasoactive intestinal polypeptide (Vip) is another food intake regulator that is common in neurons of the

stomach, intestine, and brain (Inoue et al. 1984; Lam 1991; Vu et al. 2015). It is rhythmically expressed in the colon in the absence of light and feeding cues, and its rhythmicity is likely maintained by the circadian clock (Hoogerwerf et al. 2008). Whether it is orexigenic or anorexigenic is debatable. *Vip*-/- mice show reduced food intake (orexigenic role), reduced body fat and increased lean mass. This is accompanied by altered secretion of key appetite regulators (Vu et al. 2015). On the other hand, Vip administration in rats results in anorexigenic effects (Ghourab et al. 2011). Vip also plays an important role in synchronizing neurons in the SCN, and is thus another important candidate gene to investigate for clock function (Aton et al. 2005). Overall, determining whether these genes are expressed rhythmically helps uncover the times at which appetite is at its maximum and minimum, which aids in devising efficient feeding schedules.

Key pathways involved in nutrient uptake and digestion are thought to be under circadian control. Several genes acting in carbohydrate processing in the intestine show this property. The gene for the sodium glucose co-transporter, *Sglt1*, oscillates at the mRNA and protein levels, likely peaking in anticipation of food intake (Pan et al. 2002; Rhoads et al. 1998). Hexokinase (Hk), one of the early enzymes in glycolysis, which also facilitates glucose diffusion and uptake into the cell, displays diurnal rhythmicity in muscle tissue (Berg, Tymoczko, and Stryer 2002; Hodge et al. 2015). Maltase-glucoamylase (Mgam) acts in the last steps of starch digestion, aiding in the release of glucose (Nichols et al. 2003, 1998). In catfish intestine, the mRNA of this enzyme shows increased expression at night (Qin et al. 2019). Assaying these genes in Chinook salmon would

explain whether there is a time window during which these animals are better at utilizing carbohydrates from their diet.

Enterokinase (Ek*)*, also known as enteropeptidase, is produced in the small intestine and plays a key role in proteolysis by converting trypsinogen to its active form, trypsin (Abumrad, Nassir, and Marcus 2016). H+-coupled peptide transporter (Pept1) is responsible for uptake of small peptides at the brush-border of the intestine, and it is under circadian regulation in mammalian intestinal cells (Leibach and Ganapathy 1996). Similar to the behaviour of *Sglt1*, *Pept1* transcript and protein levels peak at the beginning of the night in rats, which corresponds with food anticipation (Pan et al. 2002). The breakdown of proteins also appears to be under circadian regulation. Amino acids are released from the N-terminal of various proteins by the enzyme leucine aminopeptidase (Lap), which has preference for leucine residues (Spackman, Smith, and Brown 1955; E. L. Smith and Spackman 1955). Lap activity shows rhythmicity in rats, peaking during the night (M. Saito et al. 1975). Knowing the time at which these genes are upregulated helps inform future feeding protocols.

Fatty acid uptake is facilitated by several classes of molecules, among which are intestinal fatty acid-binding protein (Fabpi) and long-chain fatty acid transport protein 4 (Fatp4). In the intestine, Fabpi is involved in the uptake of fatty acids and recruitment towards triacylglycerol synthesis (Alpers et al. 2000). Fatp4 is involved in fatty acid uptake in many tissues but is especially abundant in the intestine, where high levels of the Fatp4 protein are present on the apical side of enterocytes (Pan and Hussain 2012; Stahl et al. 1999). *Fabpi* is expressed in a diurnal manner in mouse and catfish (*Pelteobagrus vachellii*) intestine (Qin et al. 2019; Pan and Hussain 2009). Evidence regarding *Fatp4*

rhythmicity is scarce, but fatty acid uptake in the intestine shows a rhythmic activity in mouse intestine, and *Clock* mutants lose this diurnal property and absorb higher amounts of fat overall (Pan and Hussain 2007, 2009). Ppara is a receptor for a family of lipid signaling molecules known as N-acylethanolamines that regulate energy homeostasis and have links to satiation (Gómez-Boronat et al. 2019). This gene is thought to be a bridge between the circadian clock and metabolism. On one hand, interactions exist between *Ppara* and various genes of the circadian clock such as *Clock*, *Bmal1*, *Per2*, and *Reverb*; interestingly, the *Ppara* gene has an E-box for Clock/Bmal binding, but Ppara can also drive the expression of certain clock genes. (Canaple et al. 2006; Schmutz et al. 2010; Mukherji et al. 2013; Oishi, Shirai, and Ishida 2005). On the other hand, Ppara participates in intestinal lipid absorption and beta oxidation of fatty acids and is upregulated during the fasting phase (Liu, Alexander, and Lee 2014). *Ppara* displays diurnal rhythmicity in Atlantic salmon, zebrafish and gilthead seabream livers, and it maintains rhythmicity under constant conditions in seabream (Betancor et al. 2014; Juan Fernando Paredes et al. 2014; J. F. Paredes et al. 2015). This makes *Ppara* likely a circadian gene in Chinook salmon intestine as well. To make matters more interesting, Ppara also regulates other genes involved in digestion, such as *Pept1* (H. Saito et al. 2008). Furthermore, Ppara helps maintain the intestinal epithelial barrier by promoting the differentiation of intestinal stem cells into enterocytes, through increasing number of peroxisomes, making it a great therapeutic target for gut illnesses (Du et al. 2020). Knowing whether lipid uptake and digestion genes are under circadian control would help maximize food utilization by matching feeding time with the timing of highest gene expression.

Crosstalk exists between the circadian clock and the immune system. The circadian clock contributes to the rhythmicity of immune responses and upregulates proinflammatory genes during infections. On the other hand, infections dampen the rhythmicity of clock and metabolic genes in infected tissue (Bellet et al. 2013). Bmal1 plays a key role in linking these two systems together and displays anti-inflammatory functions (Curtis et al. 2014).

Tumor necrosis factor (Tnf) is an inflammatory cytokine involved in cytotoxicity against tumor cells (Wajant, Pfizenmaier, and Scheurich 2003). Interleukins (Ils) are also cytokines that induce proinflammatory responses by communicating information between leukocytes (Akdis et al. 2016). *Tnfα, Il-1β,* and *Il-6* are expressed rhythmically in intestinal epithelial cells of normal mice (Mukherji et al. 2013). *Tnf* rhythmicity seems to be regulated by the clock (Chen et al. 2003; Stokes et al. 2017). Furthermore, *Il-6* induction is suppressed by *Rev-erbα*, a negative clock regulator (Curtis et al. 2014). Knowing that inflammation is a clock-controlled process in many organisms, it is compelling to study this in Chinook salmon.

Our lab has shown that regenerative cell division in the intestine is rhythmic in mice and drosophila following stress (Stokes et al. 2017; Karpowicz et al. 2013). This is thought to occur through clock-regulated *Tnf* signaling, but some studies have shown that the Wnt pathway, a critical pathway that regulates intestinal stem cells, is under clock control (Soták, Sumová, and Pácha 2014; Komiya and Habas 2008). Targets of the Wnt pathway such as the *Myc* oncogene are also capable of attenuating the clock while promoting cell proliferation (Altman et al. 2015; Shostak et al. 2016). c-*Myc* shows rhythmic expression in mouse liver (Fu et al. 2002). Mouse Per2 and Cry2 downregulate c-*Myc*, while the

Clock gene may be upregulating c-*Myc* (Fu et al. 2002; Huber et al. 2016; Peng et al. 2018). Another Wnt target gene, Axis inhibition protein 2 (*Axin-2*), also appears to be rhythmic in multiple mouse tissues (Soták, Sumová, and Pácha 2014). Knockdown of *Bmal1* decreases the expression of *Axin2* in mouse embryonic cells (B. Guo et al. 2012). Another regulator of regeneration, *P21*, which inhibits proliferation, might be under circadian control in the mouse intestinal epithelium (Stokes et a. 2017). Because of the importance of tissue regeneration for organism health, exploring these pathways would provide critical information about the state of the tissue.

In this chapter, appetite regulation, various aspects of digestion, inflammation and regeneration in the intestine are explored to test for the presence of rhythmicity in gene expression. This is a pilot study of the circadian regulation of these processes in Chinook salmon and will inform future studies in other fish species. Furthermore, the effects of housing conditions are explored to determine whether lighting conditions and feeding time have any influence on the rhythms in these genes.

Hypothesis

I hypothesize that appetite regulation, digestion of carbohydrates, lipids and proteins, inflammation and regeneration within the Chinook salmon intestine display diurnal rhythmicity.

Objectives

To test this hypothesis, I used intestinal cDNA from the six experiments described in Chapter 3 to assess the rhythmicity of a suite of genes involved in appetite regulation,

nutrient uptake, digestion, inflammation and regeneration. Instead of the SYBR® green qPCR above, Taqman™ OpenArray™ gene expression assays were used as a semi high-throughput technique to investigate 28 genes simultaneously in each individual sample.

Methods

Experimental setup

Intestinal cDNA from the experiments in Chapter 3 was used to probe the expression of various clock and non-clock genes to assess rhythmicity under the experimental conditions illustrated previously in Figure 7 of Chapter 3. 28 genes were quantified simultaneously in each sample using the Taqman™ OpenArray™ gene expression assays. Three genes were reserved for control genes, which were chosen from the three most stable genes after running the geNorm stability analysis in Chapter 3. These were *bAct*, *Gapdh*, and *Tubα*.

Primer design

Chinook salmon sequences were identified by running a BLAST analysis of known zebrafish transcript sequences against the Chinook salmon sequences available in the NCBI database. In the cases where multiple isoforms of a transcript were found, the isoforms were aligned using Geneious (Geneious Biologics, version 10.0.5) to find a region of homology for which to develop primers. Primers for the transcripts of interest (Table 7) were developed using NCBI's primer designing tool. The guidelines from Primer Express Software 3.0 Getting Started Guide were used to generate the primer and

Taqman™ FAM™-MGB probe sequences (Table 8). The primers (Eurofins Genomics) were validated with SYBR® green standard dilution qPCR, and those with efficiency values around 100% ± 15% were selected for further use. Gel electrophoresis was used to verify the absence of amplicons that differ in size, to verify primer specificity. To ensure that the results achieved with SYBR® green qPCR predict successful assays when using Taqman™ chemistry later, two of the assays were also validated with the Taqman™ Fast Advanced Master Mix (Applied Biosystems, Catalog #: 4444553) using the ViiA™ 7 qPCR system (Applied Biosystems).

Table 7: Taqman™ primer and probe sequences with their SYBR® green efficiencies and the mean efficiencies from Taqman™ assays.

Assay Name	Sequences (5'-->3')		Mean Taqman™ Efficiency	SYBR® Green Efficiency (%)
bAct	Forward primer	GGCCGTACCACCGGTATC	1.888	91.887
	Reverse primer	AGCCCTCGTAGATGGGTACT		
	Probe	TCCGGTGACGGCGTGA		
Gapdh	Forward primer	TGTCAGTGGTGGACCTAACC	1.873	93.790
	Reverse primer	GCCTTCTTGACAGCCTCCTT		
	Probe	CCGGCAGCTACGCT		
Tubα	Forward primer	AGACGACTCCTTCAACACCTT	1.886	104.879
	Reverse primer	CAGTGGGCTCCAGATCCA		
	Probe	CTGGCAAGCACGTCC		
Bmal1	Forward primer	GACCAGGCCTCGGTATCC	1.893	98.020
	Reverse primer	CCCAGGTTGGCATCAGTCT		
	Probe	AACGACGAGGCTGCCAT		
Clock1b	Forward primer	CAGCAGCACACGGTTCAA	1.861	104.660
	Reverse primer	AGGACAGAGCTGGTGTCTTG		
	Probe	CAACCTCAGCAGCAGG		
Cry1	Forward primer	GCAGGGTGCATCGTAGGTAA	1.857	95.683
	Reverse primer	TCCTCTGGATGTTCTTCTTGCT		
	Probe	CCCATAGTGGAGCACGA		

Per1	Forward primer	GAGTGGGAAGCACCAATGAA	1.935	94.082
	Reverse primer	ATTGGCTGCCATGGTTGTTG		
	Probe	AAGAGGAGGAGGCGGGA		
Rev-erbα	Forward primer	ATCGCGTCTGGCTTCCATT	1.712	95.810
	Reverse primer	TGGATGTTCTGCTGGATGCT		
	Probe	ACGGCGTTCACGCC		
Rorα	Forward primer	AAGACACTGTCGGGAAAATGG	1.863	89.773
	Reverse primer	GGGTGAGGGACAGAGGAGATA		
	Probe	ACGGGCGGTGGTG		
Cck	Forward primer	ACACGACAGGATGCAAAAGC	1.847	87.645
	Reverse primer	GGTGCAGGACTGACCCTTAT		
	Probe	ACCGGCCCTCCCA		
Ghrl	Forward primer	ACCACAGGTAAGACAGGGTAAA	1.829	114.151
	Reverse primer	AGCTCAGCAAAGCTCTCAATG		
	Probe	CCTCGGGTTGGTCG		
Glp-1	Forward primer	GCAGAGATCTACCTACGCACA	1.830	97.660
	Reverse primer	TGACCTCCTCTGTTCCCTCT		
	Probe	CAGCCAGGCACAGAC		
Vip	Forward primer	ACGCAGACGGTCTCTTTACA	1.883	100.399
	Reverse primer	CGCTTTCCAATCAAGGATTCCA		
	Probe	ACAGCTGTCGGCGCG		
Sglt1	Forward primer	CCCTCTTTGCGAGCAACATT	1.887	95.010
	Reverse primer	AATCCGCCAATGGCAAGTC		
	Probe	ATCGCAGGGACTGCA		
Mgam	Forward primer	GAGGTAGAGCTCACACACAAC	1.957	98.153
	Reverse primer	ACCCATTGTGGTGTCAAACAG		
	Probe	TTCCAAGTACGCAGGGC		
Hk1	Forward primer	CACATCCACAGTACCCCAAGA	1.877	87.648
	Reverse primer	ACTCTCAGACAGCACAAAGC		
	Probe	CCAGTTGGTGCCAGAG		
Pept1	Forward primer	GGTATCCACTCCCAACAGAAGT	1.948	91.191
	Reverse primer	GCCCATAATGAACACGATGAGA		
	Probe	CTTCGGCGTCCCTG		
Lap	Forward primer	TCAACAACGTCGGCAAATACAG	1.764	86.639
	Reverse primer	CAATGAGGAGCCGTCACAAAC		
	Probe	CTGCACGGCGGCAG		
Ek	Forward primer	GTGGACAGTACGAGTGTCAGA	1.905	89.395
	Reverse primer	TCCAGTGTTGACCACAGGTT		
	Probe	CAGCTCCAGGTCCAGA		
Fatp4	Forward primer	AGGGTAAACACAAGGTCCGTAT	1.899	95.450
	Reverse primer	CGTTGAAGCGAGAGGTGAAC		

	Probe	TCGGTAACGGCCTGC		
Fabpi	Forward primer	GGAAAGACAACAGTAAGGTGCT	1.911	96.444
	Reverse primer	GCATCCACCCCATCGTAGTTA		
	Probe	CACCACTCGGGCCGT		
Ppara	Forward primer	GTGACCTGGCTCTGTTTGTG	1.903	102.066
	Reverse primer	TCGATGTGGGTCACGTTCA		
	Probe	CTGTGGAGACCGCC		
Tnf	Forward primer	GGACATGGTTCACCTGAGTAAC	1.875	92.539
	Reverse primer	GAGTTGAGCAGCGTCTGGTA		
	Probe	TCCCCAAGCTACGGCA		
Il1b	Forward primer	TGGAGAGTGCTGTGGAAGAA	1.834	107.533
	Reverse primer	TGTGATGTACTGCTGAACCCT		
	Probe	CAACAAGGAGGAGGGCA		
Il6	Forward primer	GGAGCTACGTAACTTCCTGGTT	1.831	97.892
	Reverse primer	TGGAAGTCTTTGCCCCTCTTT		
	Probe	ACCAAGAGAGCCCTC		
P21	Forward primer	CCAAGCTGCCTCTCCTCTAC	1.863	103.052
	Reverse primer	CCTGACCCTCCCTGTGATG		
	Probe	CAGAGGGAGGGAGGC		
Myc	Forward primer	GGAGAAAGTAGTGTCCGAGAGA	1.894	97.054
	Reverse primer	TCGTGCTGGTTACTGTGCTA		
	Probe	ACTCTAGCGTGGCCGG		
Axin2	Forward primer	ATACAAGCTGGGCACCAAGA	1.858	94.946
	Reverse primer	AGACCAGGCCGTTGATCTTA		
	Probe	CTCCAGCGGGAGATG		

Table 8: Parameters followed during primer and probe sequence development.

Parameter	**Details**
Amplicon size	70-90 bp
Primer length	18-22 bp (optimal 20 bp)
Primer Tm	58-60^{o}C (optimal 59^{o}C)
Specificity check database	Chinook salmon (taxid: 74940)
Specificity check stringency	At least 2 mismatches within the last 5 bps at the 3' end
Primer and probe GC content	30-80%
Probe length	13-18 bp

Probe Tm	68-70^{o}C
Probe details	1) 5’ end cannot be a G residue 2) fewer than four consecutive G residues (this applies to the primers too) 3) fewer than six consecutive A residues 4) Avoid 5'-...GGG-MGB-3' or 5'-...GGAG-MGB-3' 5) Avoid two or more CC dinucleotides in the middle (one of the probes unavoidably has CCC in the middle) 6) Avoid a G in the second position on the 5' end
All other parameters on NCBI	Default

Taqman™ OpenArray™ qPCR

After determining that the primer sets were successful at amplifying their target sequences, ten Taqman™ OpenArray™ gene expression chips (Applied Biosystems) were used to quantify the expression of 28 genes, run in duplicate reactions. The chips were preloaded with primer and FAM-MGB probe sequences. Each chip holds 48 samples, and a total of 479 samples were analyzed (about 80 samples from each experimental condition). The QuantStudio™ 12K Flex Real-Time PCR System (ThermoFisher Scientific) was used for expression quantification according to the manufacturer’s run method. The master mix was prepared in 384 well plates and then transferred onto the chips using the OpenArray™ AccuFill™ system. Each reaction was composed of 2.5 µL of Taqman™ OpenArray™ Real-Time PCR Master Mix (Applied Biosystems, Catalog #: 4462156), 1.2 µL of cDNA and 1.3 µL of nuclease-free H2O.

Correction of gene expression levels

LinRegPCR version 2020.0 was used to calculate the efficiency of each individual reaction in order to accurately estimate the expression levels of each gene in a sample. The baseline estimation criteria used by LinRegPCR were relaxed and jumps and droops were allowed in the log-linear phase from which the efficiency values are calculated. This was necessary because the high background fluorescence in a Taqman™ reaction would result in the exclusion of a large number of the samples. Samples having individual efficiencies within 15% of the gene's mean efficiency were included in the calculation of the gene's mean efficiency in each plate. The Ct value was used to estimate the starting concentration of a sample, known as "N0". Factor-qPCR version 2020.0 was used to correct for inter-plate variation. Samples were eliminated if one or more of the control genes were excluded by LinRegPCR. The expression levels of target genes were normalized to the geometric mean of all the control genes according to the following equation: $Expression\ of\ target\ gene = \frac{N0_{(target\ gene)}}{\sqrt[3]{N0_{(bAct)} \times N0_{(Gapdh)} \times N0_{(Tuba)}}}$.

Graphing and statistics

Similar to Chapter 3, the rhythmicity of each gene was assessed by fitting the expression levels at each time point to a sinusoidal curve, using non-linear regression on GraphPad Prism Version 7.0c for Mac OS X. The following equation was used: $f(t) = M + Acos(t\pi/12 - \phi)$, where f(t) is the gene expression level at a specific time, the mesor (M) is the mean value, A is the amplitude, t is time in hours, and ϕ is the time of peaking in expression, known as the acrophase. The acrophase value was also confirmed by inputting the data into Chronos-fit version 1.05. A one-way ANOVA was done in Prism

to test the statistical significance of the variation in expression of each clock gene over time (P-values are reported in figure legends). The noise-to-signal ratio was used to determine the significance of the curve fit, where the ratio was calculated from the standard error of the amplitude divided by the amplitude value, denoted by SE(A)/A (Halberg and Reinberg 1967). Gene expression was considered to be rhythmic if SE(A)/A< 0.3 and P<0.05 from the one-way anova. Circular plots were developed in Oriana version 4.02 (Kovach Computing Services) to show phase relationships between the genes that displayed rhythmicity.

Results

Taqman™ assays confirm SYBR® green findings

Using the semi-high-throughput Taqman™ qPCR, I wanted to confirm that the findings from the SYBR® green circadian clock assays in Chapter 3 are consistent with ones from the new qPCR method. In the six conditions assayed, three conditions show significant rhythmicity in four of the clock genes (LD fed 5 PM, LD fed 3X, and LL fed 5 PM), whereas the other three conditions show rhythmicity in only three clock genes (Figure 12, Figure 13, and Table 9). Of the rearing groups with the most rhythmicity, LD photoperiod coupled with feeding throughout the day displays the most optimal phase-relationships between the rhythmic genes (Figure 13); *Bmal1* and *Clock1b* peak simultaneously in the morning, followed by *Rorα* and *Rev-erbα* which peak in the afternoon. On the other hand, LD coupled with 5 PM feeding results in the synchronous peaking of *Bmal1* and *Cry1*, which belong to the positive and negative arms of the clock,

respectively. This is not ideal, as the *Cry1* gene is normally driven by Bmal1 transcription factors, therefore peaks much later. Similarly, LL fed 5 PM rearing results in synchronous peaking of *Bmal1* and *Rorα*, which is odd since Rorα is known to help drive *Bmal1* expression. Therefore, rearing under LD photoperiod and feeding throughout the day results in optimal circadian clock activity among all six conditions. This further confirms the conclusion from the SYBR® green assays in the previous chapter.

In the previous two chapters, synchronous peaking in the expression of positive (*Bmal/Clock*) and negative regulators (*Cry/Per*) was observed. This is not typical since the positive regulators drive the expression of the negative regulators; hence their peak must precede that of the negative regulators. There is some evidence of this here with the Taqman™ assays under two of the conditions (LD fed at 5 PM, and LL fed at 8 AM), where *Cry1* is synchronous with either *Bmal1* or *Clock1b.* On the other hand, under LL fed 5 PM, *Cry1* expression lags behind *Bmal1*, showing for the first time thus far signs of anti-phasic relationships between the positive and negative clock components in Chinook salmon intestine. This demonstrates that rearing conditions may be responsible for the unusual synchrony between the positive and negative regulators of the intestinal circadian clock.

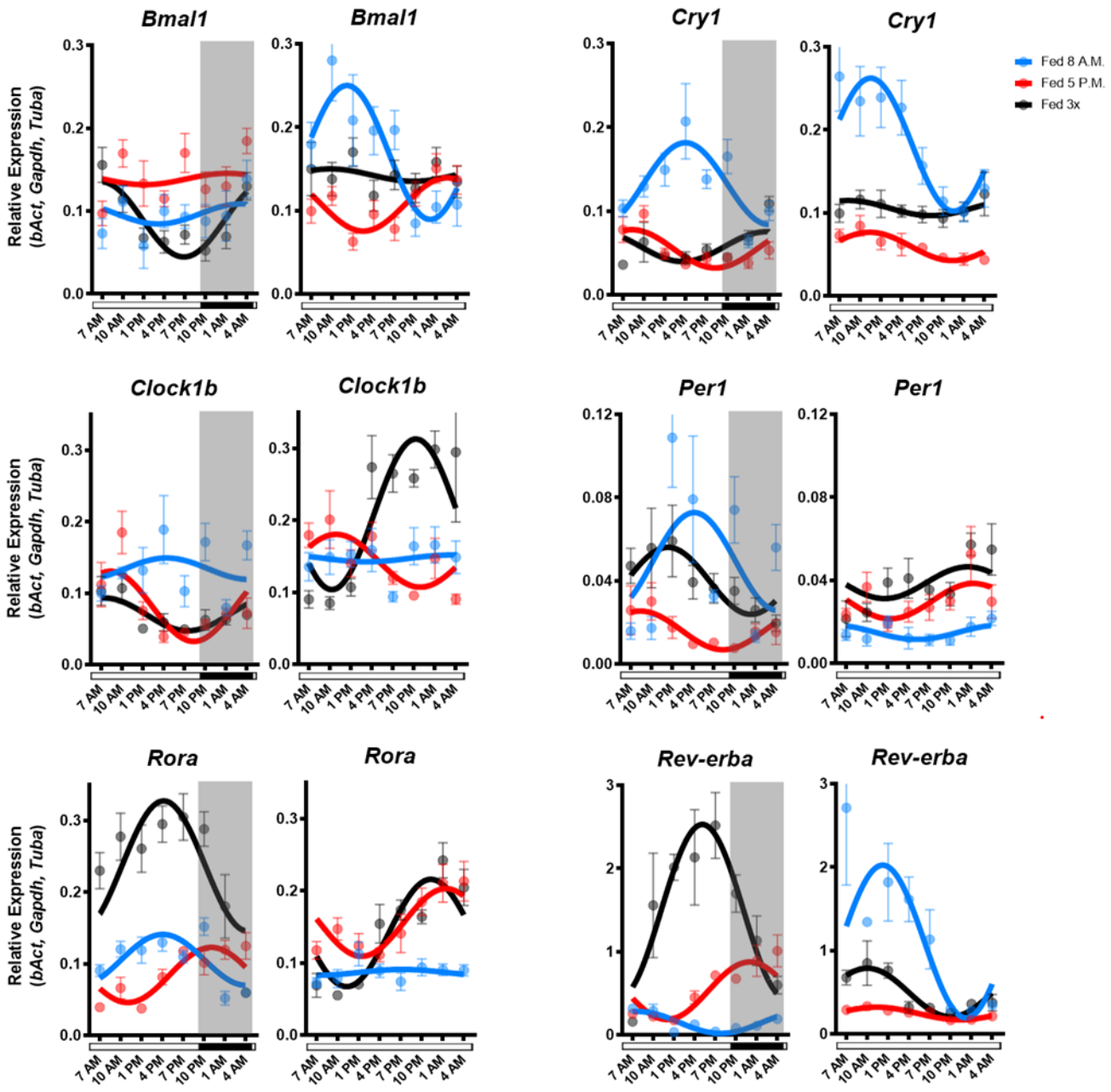

Figure 12: Expression of circadian clock genes in Chinook salmon intestine using Taqman™ OpenArray™ gene expression assays. The curves shown are the nonlinear regressions of the line graphs connecting the eight data points. The horizontal open and solid bars along the X-axis show light and dark phases, respectively. The shaded areas in the graphs represent the dark phase. Each data point shows the mean expression of a clock gene relative to three control genes. Error bars are ± SEM.

Table 9: Parameters defining the daily variation in Taqman™ OpenArray™ expression of clock genes in Chinook salmon intestine.

Gene	Experimental Condition	Acrophase	Amplitude	SE of Amplitude	Noise/ signal ratio	Anova P-value	Signific -ance of Rhythm
Bmal1	**LD - Fed 8 AM**	-	0.0121	0.0113	0.9337	0.3104	
	LD - Fed 5 PM	-	0.0068	0.0108	1.5806	0.0205	
	LD - Fed 3X	6:54 AM	0.0454	0.0081	0.1785	<0.0001	*
	LL - Fed 8 AM	12:09 PM	0.0803	0.0161	0.2002	0.0006	*
	LL - Fed 5 PM	2:29 AM	0.0320	0.0076	0.2375	0.0013	*
	LL - Fed 3X	-	0.0074	0.0097	1.3085	0.6547	
Clock1b	**LD - Fed 8 AM**	-	0.0150	0.0131	0.8723	0.0118	
	LD - Fed 5 PM	8:32 AM	0.0490	0.0105	0.2140	<0.0001	*
	LD - Fed 3X	7:27 AM	0.0229	0.0067	0.2931	0.0112	*
	LL - Fed 8 AM	-	0.0047	0.0116	2.4877	0.5812	
	LL - Fed 5 PM	-	0.0366	0.0111	0.3030	0.0029	
	LL - Fed 3X	10:19 PM	0.1045	0.0199	0.1903	<0.0001	*
Cry1	**LD - Fed 8 AM**	3:57 PM	0.0488	0.0103	0.2106	0.0002	*
	LD - Fed 5 PM	8:37 AM	0.0228	0.0046	0.2028	<0.0001	*
	LD - Fed 3X	-	0.0178	0.0062	0.3504	0.0002	
	LL - Fed 8 AM	11:31 AM	0.0800	0.0157	0.1963	0.0007	*
	LL - Fed 5 PM	11:22 AM	0.0170	0.0039	0.2290	0.0025	*
	LL - Fed 3X	-	0.0091	0.0068	0.7495	0.8220	
Per1	**LD - Fed 8 AM**	-	0.0235	0.0092	0.3930	0.0003	
	LD - Fed 5 PM	-	0.0093	0.0031	0.3347	0.2272	
	LD - Fed 3X	-	0.0161	0.0052	0.3237	0.1228	
	LL - Fed 8 AM	-	0.0034	0.0019	0.5464	0.1783	
	LL - Fed 5 PM	-	0.0085	0.0037	0.4387	0.0570	
	LL - Fed 3X	-	0.0076	0.0038	0.4925	0.0206	
Rev-erbα	**LD - Fed 8 AM**	7:46 AM	0.1309	0.0251	0.1914	0.0002	*
	LD - Fed 5 PM	12:07 AM	0.3453	0.0520	0.1506	<0.0001	*
	LD - Fed 3X	5:06 PM	1.0390	0.1663	0.1601	<0.0001	*
	LL - Fed 8 AM	12:13 PM	0.9153	0.1550	0.1693	<0.0001	*
	LL - Fed 5 PM	11:21 AM	0.0752	0.0145	0.1926	0.0011	*
	LL - Fed 3X	10:01 AM	0.2911	0.0611	0.2100	0.0042	*
Rorα	**LD - Fed 8 AM**	4:09 PM	0.0355	0.0067	0.1897	<0.0001	*
	LD - Fed 5 PM	11:02 PM	0.0384	0.0067	0.1733	<0.0001	*
	LD - Fed 3X	4:03 PM	0.0906	0.0166	0.1829	<0.0001	*
	LL - Fed 8 AM	-	0.0040	0.0053	1.3247	0.3049	
	LL - Fed 5 PM	1:24 AM	0.0468	0.0104	0.2227	0.0009	*
	LL - Fed 3X	11:13 PM	0.0743	0.0099	0.1338	<0.0001	*

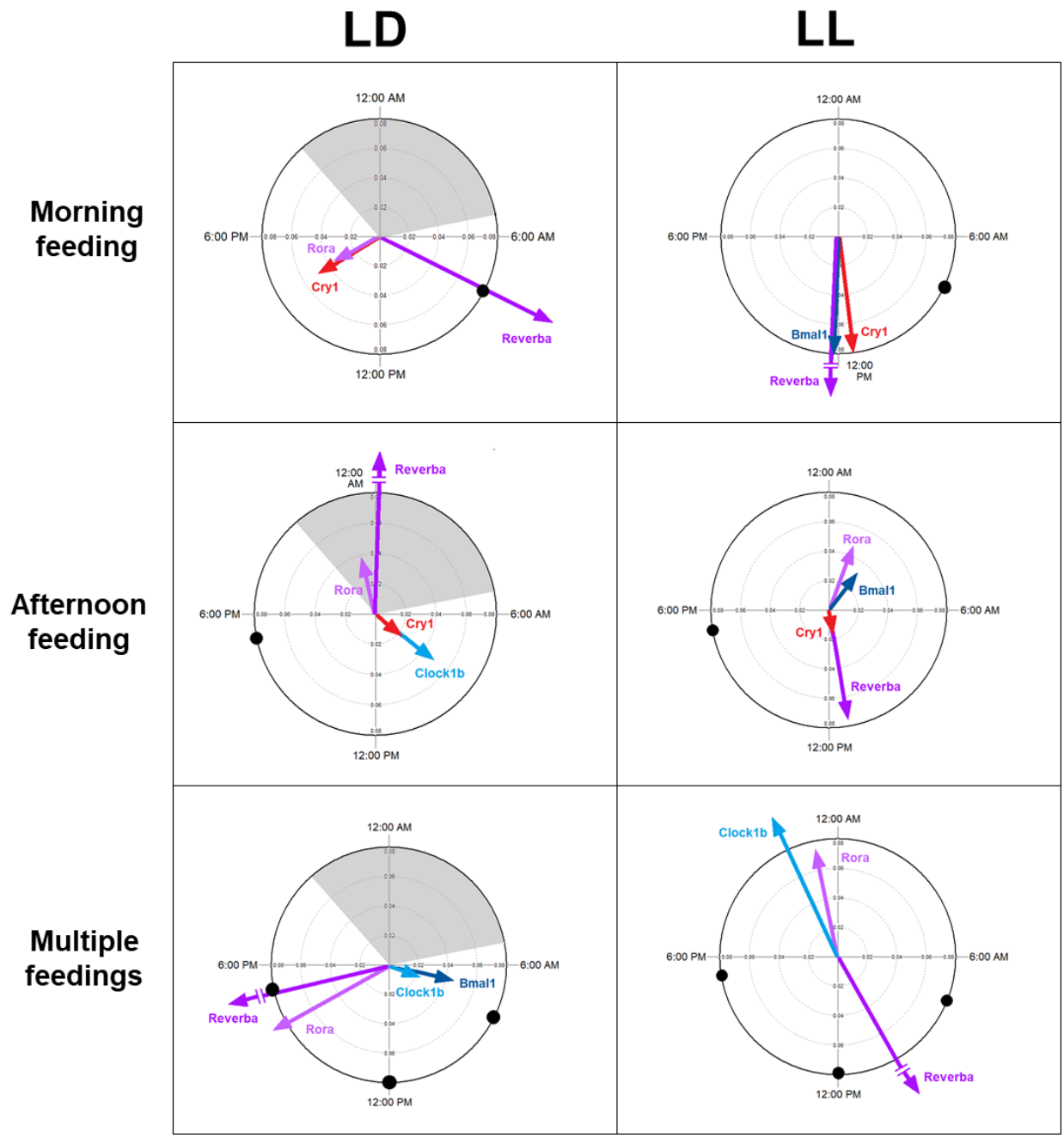

Figure 13: Circular plots depicting the phase relationships between various clock genes under the different lighting and feeding conditions. The circular axis represents the acrophase of a rhythm, and the linear axis represents the amplitude of the rhythm. When the amplitude exceeded the linear axis' limits, a break was introduced to show the remainder of the expression vector. Shaded regions represent the dark phase, and the solid black circles show the feeding times. The set up that shows the best clock function is LD receiving multiple feedings throughout the day, since the positive regulators, *Bmal1* and *Clock1b* peak simultaneously, followed by *Rev-erba* and *Rora*. The two next-best set ups are those with 5 PM feeding, both LD and LL, but both have overlap in genes that do

not normally peak together (eg. *Clock1b* and *Cry1* peak together in LD, and *Bmal1* and *Rora* peak together in LL). The remaining three conditions are less ideal, because they only have three rhythmic genes rather than four.

Rhythmicity is detected in three appetite-regulating genes

In order to find out whether the intestine produces appetite regulators in a rhythmic manner, daily expression of four appetite regulating genes was assayed. Early peaking is observed in three of the appetite regulators, between early morning and noon (Table 10). The exception to this is the LL fed 5 PM group for which the acrophases are around midnight. The early peaking in *Cck* is surprising, as it is an appetite suppressor, and one would expect lowest feeding during the night if these fish are diurnal; however, it is possible that it is not made into the mature protein until much later. This gene is synchronized by the LD cycle, since shifting to LL results in a change in peaking time from morning to midnight (feeding conserved at 5 PM). Overall *Cck* is entrained to photoperiod, and to only evening feeding in the absence of LD cycles (Table 10). The fourth gene, *Ghrl*, was omitted from the analysis due to failure of the qPCR in most samples.

Glp-1 peaking precedes feeding under LD when food is administered in the evening, but lags behind feeding when it is administered in the morning under constant light (Table 10). The former observation is surprising, because *Glp-1* is an appetite suppressor, like *Cck*. *Glp-1* might be responding to feeding, as it peaks five hours following an 8 AM feeding under constant light. It is difficult to conclude whether *Glp-1* is synchronized by light, given that when it is rhythmic under LD, a 5 PM feeding is a

confounding variable; however, it is synchronized by feeding under LL, as seen in the 8 AM feeding group (Table 10).

Vip seems to be responsive to both feeding and light; for instance, a six-hour phase difference is seen between the morning versus evening feeding groups under LD, indicating an influence of feeding time on the acrophase (Table 10). Moreover, an approximately eight-hour difference exists between the 5 PM feeding groups reared under LD versus LL, however, when food is administered at multiple intervals, the difference is diminished between the two lighting conditions. This suggests that *Vip* is synchronized by both lighting and feeding.

Table 10: Parameters defining the daily variation in Taqman™ OpenArray™ expression of appetite regulating genes in Chinook salmon intestine.

Gene	Experimental Condition	Acrophase	Amplitude	SE of Amplitude	Noise/ signal ratio	Anova P-value	Signific -ance of Rhythm
Cck	**LD - Fed 8 AM**	-	0.0896	0.0304	0.3393	<0.0001	
	LD - Fed 5 PM	7:49 AM	0.1376	0.0379	0.2755	0.0010	*
	LD - Fed 3X	7:34 AM	0.0659	0.0134	0.2037	0.0001	*
	LL - Fed 8 AM	-	0.1122	0.0356	0.3174	0.0239	
	LL - Fed 5 PM	12:12 AM	0.0958	0.0210	0.2191	0.0004	*
	LL - Fed 3X	-	0.0694	0.0222	0.3196	0.0324	
Glp-1	**LD - Fed 8 AM**	-	0.0105	0.0068	0.6511	0.1817	
	LD - Fed 5 PM	9:42 AM	0.0280	0.0052	0.1877	<0.0001	*
	LD - Fed 3X	-	0.0296	0.0209	0.7045	0.0134	
	LL - Fed 8 AM	12:55 PM	0.1436	0.0251	0.1747	0.0006	*
	LL - Fed 5 PM	-	0.1569	0.1017	0.6482	0.2895	
	LL - Fed 3X	-	0.0101	0.0151	1.4995	0.8695	
Vip	**LD - Fed 8 AM**	3:40 AM	0.0841	0.0141	0.1674	<0.0001	*
	LD - Fed 5 PM	9:49 AM	0.1271	0.0238	0.1873	<0.0001	*
	LD - Fed 3X	11:46 AM	0.0494	0.0122	0.2466	0.0002	*
	LL - Fed 8 AM	-	0.0167	0.0110	0.6605	0.5069	
	LL - Fed 5 PM	1:26 AM	0.0894	0.0127	0.1422	<0.0001	*
	LL - Fed 3X	10:13 AM	0.0332	0.0086	0.2580	0.0044	*

All digestive enzymes and transporters display rhythmicity

The intestine was studied to determine whether the uptake and digestion of food at the level of the small intestine happens rhythmically, by testing daily expression levels of genes involved in these processes. The peak in *Sglt1* mRNA appears to be predictive of feeding time when a single feeding is administered, but this property is lost with feedings throughout the day, where the peak happens right after the last feeding (Table 11). Under LD setting, peaking in *Mgam* tends to follow feeding time; however, a drastic difference is seen when reared under constant light, where the peak in the morning feeding group precedes feeding by eight hours, peaking at midnight (Table 11). This suggests a possible influence of lighting on this gene, possibly through the circadian clock. Under the condition found to be most optimal for the clock (LD fed 3X), the acrophases of *Sglt1* and *Mgam* occur well after those of *Bmal1* and *Clock1b* (Table 9), suggesting that they may be possible targets of the clock, especially since their rhythms display strong amplitudes. Rhythmicity is less common in *Hk1*, occurring in only two out of the six experimental settings (Table 11). The peak under LD precedes the morning feeding, suggesting a possible anticipation of feeding, but the peaking times of circadian clock genes (Table 9: 12 hours prior or four hours later) under that condition does not suggest such a control. Under LL fed 3X, the peak occurs about two hours after the first feeding. The peak in this case may simply be a response to the first feeding event.

Among the three protein digestion genes assayed, *Ek* displays the least rhythmicity, only under one condition, LD with feedings throughout the day (Table 11). This makes it likely that *Ek* is entrained to the LD cycle and that single feeding events somehow disturb the synchronization to the LD cycle. On the other hand, there are

indications that *Pept1* and *Lap* are responsive to both lighting schedule and feeding cues (Table 11). *Pept1* peaks very early on, between midnight and midmorning, possibly in anticipation of morning feeding. The peaking becomes delayed by six hours when shifted from LD to LL, indicating a role for the LD cycles in synchronizing this gene. Among the LL experiments, a nine-hour delay in peaking happens when the final feeding event of the day is delayed by nine hours, implicating feeding in the timing of expression peaks. *Lap* shows light responsiveness, where the peaks occur between early morning and early afternoon, in LD versus LL groups, respectively (Table 11). The entrainability to feeding is less clear, because small differences are seen between the feeding groups under one lighting setup.

Significant rhythms are seen in all three genes involved in lipid digestion, with variations depending on the housing conditions (Table 11). *Fatp4* displays rhythmicity under all conditions except for LD coupled with morning feeding. Differences in peaking time are seen when comparing single feeding schedules to feeding throughout the day under both LD and LL, which indicates a role for feeding in synchronization. The conservation of peaking time between the 8 AM and 5 PM feeding groups under constant light suggests that feeding time only has a minor role in entrainment. Peaking time changes when switching from LD to LL but keeping feeding time constant (eg. 5 PM or feeding throughout the day), indicating a role for the LD cycle in synchronizing the rhythms of *Fatp4*. *Fabpi* is only rhythmic under LD if one feeding was administered, indicating a potential role for feeding in entraining this gene (Table 11); however, both *Fatp4* and *Fabpi* are rhythmic under constant light and feeding throughout the day, which suggests that these genes might be free-running, making them a possible target of the circadian

clock. Finally, *Ppara* is rhythmic under two of the LD conditions, and only under single feeding events in LL (Table 11). This gene seems to be responsive to both lighting cues (five-hour difference between LD and LL acrophases for the 8 AM feeding group) and feeding cues (demonstrated by the 11-hour delay between 8 AM and 5 PM feeding groups under constant light).

Table 11: Parameters defining the daily variation in Taqman™ OpenArray™ expression of digestive enzymes and transporters in Chinook salmon intestine.

Gene	Experimental Condition	Acrophase	Amplitude	SE of Amplitude	Noise/ signal ratio	Anova P-value	Signific -ance of Rhythm
Sglt1	**LD - Fed 8 AM**	7:24 AM	0.1960	0.0511	0.2606	<0.0001	*
	LD - Fed 5 PM	-	0.0313	0.0276	0.8836	0.8249	
	LD - Fed 3X	5:42 PM	0.3121	0.0611	0.1959	0.0003	*
	LL - Fed 8 AM	-	0.1409	0.0451	0.3202	0.0929	
	LL - Fed 5 PM	12:58 PM	0.2548	0.0563	0.2210	0.0001	*
	LL - Fed 3X	-	0.0127	0.0177	-1.3951	0.0077	
Mgam	**LD - Fed 8 AM**	9:46 AM	1.0460	0.2955	0.2825	<0.0001	*
	LD - Fed 5 PM	11:36 PM	0.1983	0.0594	0.2996	0.0227	*
	LD - Fed 3X	6:35 PM	1.6080	0.2165	0.1346	<0.0001	*
	LL - Fed 8 AM	11:55 PM	1.0720	0.1432	0.1336	<0.0001	*
	LL - Fed 5 PM	-	0.1620	0.0556	0.3433	0.0068	
	LL - Fed 3X	-	0.1955	0.0708	0.3622	0.1771	
Hk1	**LD - Fed 8 AM**	3:42 AM	0.1345	0.0382	0.2839	0.0039	*
	LD - Fed 5 PM	-	0.0891	0.0486	0.5460	0.0105	
	LD - Fed 3X	-	0.0381	0.0288	0.7557	0.8016	
	LL - Fed 8 AM	-	0.0998	0.0406	0.4069	0.0196	
	LL - Fed 5 PM	-	0.0632	0.0273	0.4321	0.0126	
	LL - Fed 3X	9:45 AM	0.1587	0.0253	0.1594	<0.0001	*
Pept1	**LD - Fed 8 AM**	-	0.2724	0.1978	0.7261	<0.0001	
	LD - Fed 5 PM	-	0.1217	0.0864	0.7099	0.0174	
	LD - Fed 3X	3:10 AM	0.3684	0.0578	0.1568	<0.0001	*
	LL - Fed 8 AM	12:31 AM	0.9087	0.0944	0.1039	<0.0001	*
	LL - Fed 5 PM	-	0.1031	0.0523	0.5077	0.0605	
	LL - Fed 3X	9:39 AM	0.3538	0.0717	0.2026	<0.0001	*
Lap	**LD - Fed 8 AM**	5:04 AM	0.0719	0.0213	0.2967	0.0122	*
	LD - Fed 5 PM	-	0.1194	0.0492	0.4121	0.0558	
	LD - Fed 3X	6:19 AM	0.1360	0.0357	0.2626	0.0039	*
	LL - Fed 8 AM	1:20 PM	0.4451	0.0685	0.1539	<0.0001	*
	LL - Fed 5 PM	11:34 AM	0.1956	0.0476	0.2433	0.0011	*

	LL - Fed 3X	-	0.1243	0.0703	0.5656	0.2603	
Ek	**LD - Fed 8 AM**	-	0.1571	0.0841	0.5355	0.0031	
	LD - Fed 5 PM	-	0.0840	0.0589	0.7012	0.0398	
	LD - Fed 3X	6:30 PM	0.7869	0.1216	0.1545	<0.0001	*
	LL - Fed 8 AM	-	0.0743	0.0585	0.7869	0.0649	
	LL - Fed 5 PM	-	0.1113	0.0363	0.3262	0.0044	
	LL - Fed 3X	-	0.1323	0.0485	0.3667	0.0038	
Fatp4	**LD - Fed 8 AM**	-	0.0970	0.0336	0.3463	0.0055	
	LD - Fed 5 PM	10:15 AM	0.1463	0.0337	0.2303	0.0002	*
	LD - Fed 3X	3:36 PM	0.1504	0.0331	0.2203	0.0002	*
	LL - Fed 8 AM	12:32 PM	0.5145	0.0623	0.1210	<0.0001	*
	LL - Fed 5 PM	12:52 PM	0.2447	0.0379	0.1549	<0.0001	*
	LL - Fed 3X	9:01 PM	0.1803	0.0370	0.2050	0.0005	*
Fabpi	**LD - Fed 8 AM**	4:43 AM	2.1800	0.6181	0.2835	0.0032	*
	LD - Fed 5 PM	9:35 AM	4.2150	0.8094	0.1920	<0.0001	*
	LD - Fed 3X	-	0.6106	0.6112	-1.0010	0.8733	
	LL - Fed 8 AM	-	0.8348	0.5226	0.6260	0.1110	
	LL - Fed 5 PM	-	1.1380	0.4671	0.4105	0.0458	
	LL - Fed 3X	6:47 AM	1.6160	0.3956	0.2448	0.0204	*
Ppara	**LD - Fed 8 AM**	7:34 AM	0.2296	0.0372	0.1619	<0.0001	*
	LD - Fed 5 PM	-	0.0193	0.0235	1.2174	0.0863	
	LD - Fed 3X	3:43 PM	0.1859	0.0334	0.1796	<0.0001	*
	LL - Fed 8 AM	12:41 PM	0.2598	0.0376	0.1448	<0.0001	*
	LL - Fed 5 PM	11:51 PM	0.1562	0.0204	0.1306	<0.0001	*
	LL - Fed 3X	-	0.0442	0.0216	0.4878	0.1677	

Rhythmicity in inflammation and regeneration is gene-specific

In order to explore whether inflammation and regeneration happen rhythmically in the intestine, some of the genes involved in these two processes were quantified throughout the day. *Tnf* is rhythmic only under LD photoperiod and not under constant light, with peak expression happening in the early morning (Table 12). This indicates that this gene is likely synchronized to the LD cycles, possibly through the circadian clock. Some differences in peak timing exist between the different feeding conditions, pointing to a possible inclusion of feeding cues in entraining these rhythms. Because the rhythms peak in anticipation of feeding, this further demonstrates a link to the clock. On the other hand, *Il6* is only rhythmic under constant light, and peaked in the midmorning period

(Table 12). If feeding is entraining this gene, it seems that only late feeding events result in rhythmicity. Unfortunately, *Il1b* was eliminated from this study because the expression assays were unsuccessful in the majority of the samples (Table 12).

Rhythmicity was only detected in two of the three genes involved in regeneration. *P21* shows maximal expression in the morning, whereas *Myc* does so in the afternoon (Table 12). *P21* rhythmicity depends on the 5 PM feeding schedule and is slightly affected by the shift from LD to LL (Table 12). This indicates that light and feeding have an influence on the rhythms of *P21* and implicates the clock in this. It appears that *Myc* prioritizes light over feeding cues under LD but relies on feeding cues under LL; if a single feeding cue is present in combination with LD cycles, this disrupts the rhythms, whereas when feeding is the only cue under LL, a single feeding event in the morning generates rhythms in *Myc*. Axin2 did not display rhythmicity under any of the rearing conditions (Table 12).

Table 12: Parameters defining the daily variation in Taqman™ OpenArray™ expression of digestive enzymes and transporters in Chinook salmon intestine.

Gene	Experimental Condition	Acrophase	Amplitude	SE of Amplitude	Noise/ signal ratio	Anova P-value	Signific-ance of Rhythm
Tnf	**LD - Fed 8 AM**	4:34 AM	0.0643	0.0193	0.2996	<0.0001	*
	LD - Fed 5 PM	6:38 AM	0.0902	0.0236	0.2618	0.0024	*
	LD - Fed 3X	8:06 AM	0.0329	0.0067	0.2025	0.0003	*
	LL - Fed 8 AM	-	0.0637	0.0312	0.4903	0.5077	
	LL - Fed 5 PM	-	0.0108	0.0127	-1.1772	0.5916	
	LL - Fed 3X	-	0.0182	0.0111	0.6076	0.0665	
Il6	**LD - Fed 8 AM**	-	0.0045	0.0078	-1.7351	0.0003	
	LD - Fed 5 PM	-	0.0196	0.0075	0.3803	0.1210	
	LD - Fed 3X	-	0.0170	0.0142	0.8338	0.2957	
	LL - Fed 8 AM	-	0.0078	0.0115	1.4674	0.5494	

	LL - Fed 5 PM	10:41 AM	0.0271	0.0076	0.2795	0.0027	*
	LL - Fed 3X	9:46 AM	0.0628	0.0120	0.1918	0.0002	*
P21	**LD - Fed 8 AM**	-	0.0460	0.0145	0.3147	<0.0001	
	LD - Fed 5 PM	8:34 AM	0.0207	0.0055	0.2682	0.0108	*
	LD - Fed 3X	-	0.0142	0.0049	0.3425	0.1168	
	LL - Fed 8 AM	-	0.0083	0.0080	0.9654	0.8951	
	LL - Fed 5 PM	11:25 AM	0.0304	0.0072	0.2365	0.0007	*
	LL - Fed 3X	-	0.0089	0.0046	0.5203	0.4050	
Myc	**LD - Fed 8 AM**	-	0.0024	0.0066	2.7604	<0.0001	
	LD - Fed 5 PM	-	0.0247	0.0102	0.4142	0.0155	
	LD - Fed 3X	4:12 PM	0.0233	0.0060	0.2570	0.0149	*
	LL - Fed 8 AM	1:35 PM	0.0534	0.0123	0.2308	0.0044	*
	LL - Fed 5 PM	-	0.0058	0.0058	0.9931	0.3465	
	LL - Fed 3X	-	0.0149	0.0050	0.3338	0.0005	
Axin2	**LD - Fed 8 AM**	-	0.0325	0.0131	0.4033	0.0002	
	LD - Fed 5 PM	-	0.0323	0.0106	0.3296	0.0102	
	LD - Fed 3X	-	0.0064	0.0062	0.9618	0.0288	
	LL - Fed 8 AM	-	0.0223	0.0134	0.6017	0.1292	
	LL - Fed 5 PM	-	0.0093	0.0052	0.5609	0.3155	
	LL - Fed 3X	-	0.0075	0.0104	1.3874	0.0435	

Discussion

Best rearing conditions for appetite and metabolism

Rhythmicity was detected in all genes, albeit under various housing conditions. The condition that results in the highest number of appetite and digestion genes being rhythmic is LD coupled with feeding throughout the day, where 75% of the genes from these two categories are rhythmic, compared to 42-50% of the genes in the remaining experimental conditions (Figure 14, genes in blue, green, red, and orange). This finding is consistent with the conclusion from the clock assays (Table 4 and Table 9), where the most optimal condition for the clock was found to be LD and feeding throughout the day. The reason why rhythmicity in these processes is favorable is because it is advantageous to prepare physiological responses to feeding in advance of food arrival and attenuate

these once food has passed through and these factors are no longer needed. Having a functional clock facilitates the anticipation of feeding, hence why the best condition for the clock is also the best one for appetite and digestion. LD coupled with feeding throughout the day is also likely the most natural of all six conditions, as fish in the wild are generally exposed to alternating light-dark (other than summer and winter in the north), and their feeding is dictated by prey availability, making it more random throughout the day, rather than at a fixed and short interval.

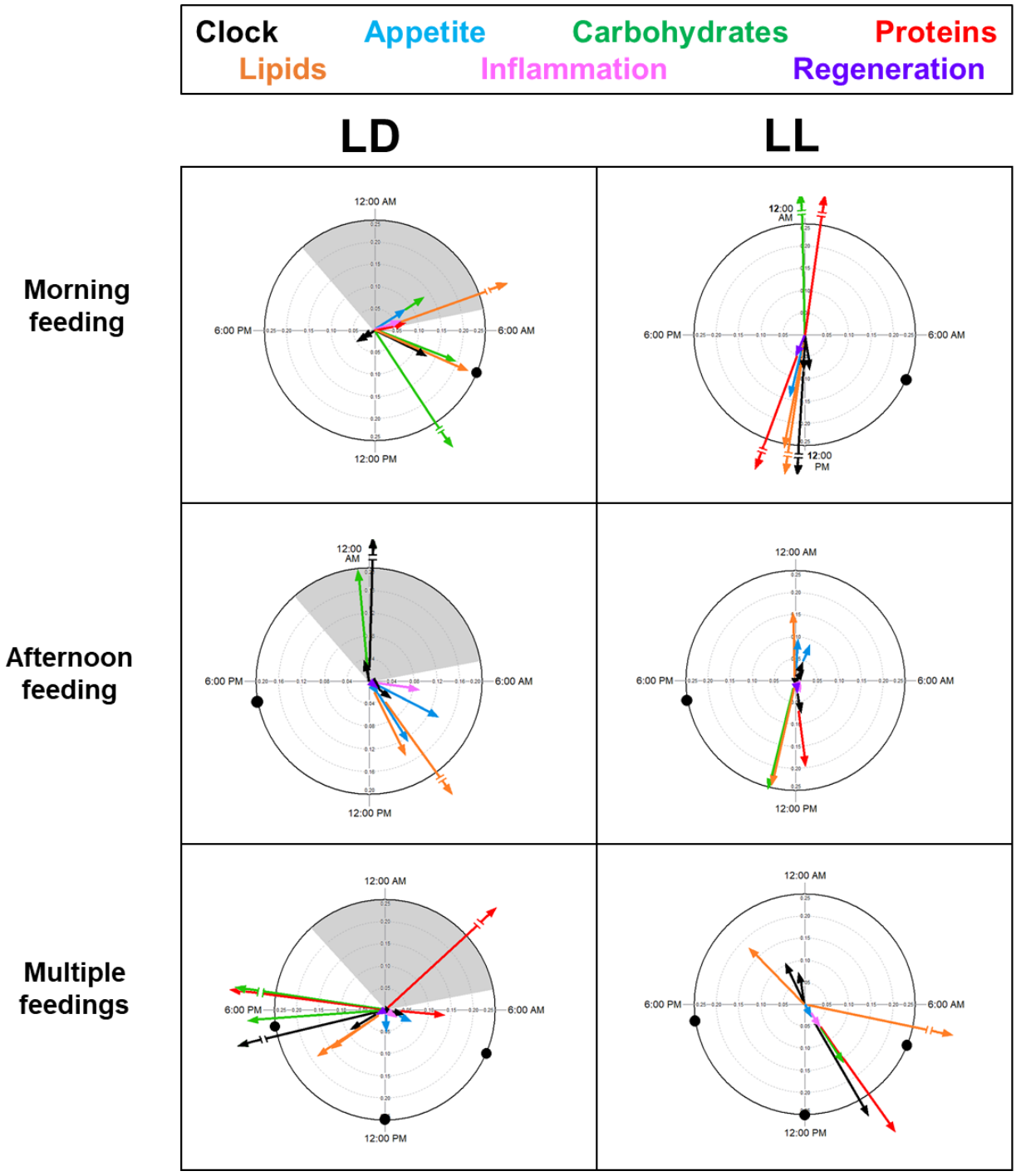

Figure 14: Circular plots depicting the acrophases of non-clock genes under different lighting and feeding conditions. The circular axis represents the acrophase of a rhythm, and the linear axis represents the amplitude of the rhythm. Each class of genes is coded with the color shown in the legend. Shaded regions represent the dark phase, and the solid black circles show the feeding times. LD coupled with feeding throughout the day shows rhythmicity in the highest number (75%) of metabolic and appetite genes. This

condition is the one that also shows the most optimal rhythmic relationships in the clock genes, as illustrated in Figure 13.

Appetite-regulating genes might be controlled by the clock

Unfortunately, the only appetite inducing hormone (Ghrl) assayed was not detected successfully, thus the data here is restricted to appetite suppressors; however *Vip* may serve as an appetite inducer as seen in some studies (Vu et al. 2015). Rhythmic expression was detected in *Cck, Glp-1,* and *Vip* in the intestine, which occurred early- to midday in most conditions, and around midnight in the group reared in constant light and fed at 5 PM (Table 10). Similar to how the phase of *Cck* expression responds to a difference in lighting schedules, when switched from LD to LL, this gene loses its rhythmicity in rats when the LD schedule is reversed, such that feeding occurs during the light phase (Xu et al. 2017). This indicates a role for light in directing *Cck* rhythms in both Chinook salmon and mice. Like the findings in some conditions here, the peak in this gene followed that of *Bmal1*, thus if *Cck* were under clock control, perhaps Rorα is acting on both *Cck* and *Bmal1*. The peaking that occurs in the LL group suggests two possibilities: This gene could either be responding to feeding by peaking eight hours later, or that the clock is free-running and maintaining the rhythms. This could be resolved by repeating this experiment under LL without feeding; if the rhythms persist, then they are true circadian rhythms, and if not, then it is likely that they are a food response.

Glp-1 rhythmicity in rats also seems to be dependent on LD cycles, while also being entrainable to feeding time (Gil-Lozano et al. 2014). While I cannot conclude whether *Glp-1* is light-entrainable from the findings here due to the possibility that feeding

is entraining the rhythms seen in LD fed 5 PM (Table 10), the finding in rat supports such a role for light. In rats, plasma Glp-1 levels rise in anticipation of feeding, similar to what is seen in the mRNA levels of the LD groups here receiving either morning or afternoon feedings (Vahl et al. 2010). This suggests a food entrainment through the clock. Perhaps the suppression of appetite does not take effect until translation, posttranslational modifications, and delivery of the hormone to the relevant perception centers in the body. In such a case, the rise of transcript levels prior to feeding would not be problematic.

Very strong rhythms are seen in *Vip* under most experimental conditions (Table 10). *Vip* rhythmicity in mouse colon is maintained under constant light and in the absence of feeding (Hoogerwerf et al. 2008). This suggests a strong link to the circadian clock. In the fish here, under the condition where I predicted the clock to be most functional (LD with feeding throughout the day), peaking time of *Vip* follows *Bmal1* and *Clock1b*, making it a potential target of these two genes. *Vip* is also implicated in synchronizing neurons of the SCN, where the master clock resides, so perhaps it could have a function in informing the master clock about the phase of peripheral clocks (Aton et al. 2005). This dual feedback between *Vip* and the clock makes it a great candidate for further studying in Chinook salmon to reveal how the master clock communicates with peripheral clocks, and all the other potential genes involved in this network.

Rhythms in digestive enzymes and transporters in other organisms

With the known association between the circadian clock and metabolism in many organisms, I wanted to investigate whether metabolic genes are under diurnal regulation to be utilized in enhancing fish growth down the road. *Sglt1* and *Mgam* are seen to be

upregulated in catfish (*Pelteobagrus vachellii*) intestine during the night, and meanwhile food was administered in the morning, it is possible that these fish are nocturnal (Qin et al. 2019). Similarly, nocturnal mice display nocturnal peaking of *Sglt1* RNA and proteins, rats show peaking of maltase activity at night, while diurnal rhesus monkeys (*Macaca mulatta*) show diurnal peaking in RNA (Pan et al. 2002; Rhoads et al. 1998; M. Saito et al. 1975). Similar results are observed here in the fish groups reared under LD here, where late feeding causes a delay in peaking of *Sglt1* and *Mgam* to the evening compared to morning feeding, indicating a link to feeding time (Table 11); the findings from the nocturnal species listed above and Chinook salmon here suggest that feeding maybe taking place later in the day and into the dark period. This is also confirmed in European seabass, where amylase activity in the mid-intestine shifts from daytime to nighttime in diurnal versus nocturnal fish (del Pozo et al. 2012). However, under LL, expression peaks in *Sglt1* and *Mgam* become predictive of feeding, happening four and eight hours prior to feeding, respectively. While there is an advantage to preparing for digestion in advance of feeding, an advancement of eight hours might not be beneficial, unless the production of the proteins is delayed such that it takes place closer to feeding time. Such a scenario is seen in Cry and Per proteins lagging 6-12 hours in their peaking time relative to the RNA peaking time (Lee et al. 2001). Moreover, both genes display rhythmicity in a greater number of experiments under LD compared to LL. Therefore, LD rearing is more favorable for these genes. The early peaking time seen in *Hk1* is suggestive of food anticipation (Table 11), but more evidence is needed to determine whether the clock participates in this rhythm. Similar to the findings here, daily rhythms are seen in HK activity in human red blood cells and in *Hk2* expression in mouse skeletal muscle, at the

onset of the active phase (Brok-Simoni et al. 1976; Hodge et al. 2015). This suggests that *Hk1* is a circadian gene, especially since it maintains its rhythmicity under constant light, but more evidence is needed to make this conclusion, because feeding is a confounding factor. Overall, there is evidence of rhythmicity in all of the carbohydrate genes assayed, but further experiments are needed to establish whether they are under circadian control, such as rearing in constant conditions, and comparing the mRNA levels of these genes to the protein levels of the circadian clock genes, which act as transcription factors that communicate circadian signal to these potential target genes.

Rhythmicity in protein-digestive genes is detected in the intestine. It is likely that *Pept1* is responsive to both feeding and light schedules, as seen here (Table 11). *Pept1* in rat intestine is strongly responsive to feeding schedule, where a shift to daytime feeding causes a similar shift in the gene's acrophase (Pan et al. 2004). mRNA and protein levels of this gene reach a maximum at dark onset, which is typical for a nocturnal animal (Pan et al. 2002, 2004). This supports a role for the clock in entraining *Pept1*, such that it peaks in anticipation of feeding. Maximal RNA expression of *Lap* is seen here ranging between 5 AM and 1:20 PM depending on the experiment (Table 11). In most cases, these peaks precede feeding time, suggesting an anticipation of feeding that may be guided by the circadian clock. For instance, the *Pept1* peaking in the LL fed 8 AM condition happens 12 hours following *Bmal1* peaking, making it a potential target of *Bmal1* (Tables 9 and 11). Its peaking under LL fed throughout the day follows that of *Clock1b*, also making it a *Clock1b* potential target. This possibility is supported by the fact that Lap activity follows a circadian pattern in the small intestine of rat fed ad libitum (eating freely), with maximal activity happening late at night (M. Saito et al. 1975). Variable peaking times between the

LD and LL conditions are noted here when feeding is administered in the morning, such that the peak is no longer predictive of feeding under LL, indicating a link to the LD cycle and to the clock. *Lap* peaking time in the LD fed 8 AM condition follows that of *Rora* and *Cry1* (Tables 9 and 11), making it potential target of either of these two (not both, since they perform opposite functions at the protein level, where the former drives *Bmal1* RNA expression, while the latter inactivates the Bmal1 protein). In the LL fed 5 PM condition, there is also evidence that *Lap* could be a *Rora* target, and a *Bmal1* target, since it peaks much later after these two. On the other hand, *Ek* rhythmicity is almost lacking, except under LD with feeding throughout the day (Table 11). Since its peaking follows that of *Bmal1* and *Clock1b* (Tables 9 and 11), *Ek* may be a circadian clock target; however, the fact that it fails to maintain rhythmicity under LL makes this questionable. Overall, as anticipated, the protein uptake and digestion genes are rhythmic in the Chinook salmon intestine and are potential targets of the circadian clock.

Similar to the findings here, the mouse intestine displays rhythmicity in *Fabpi* expression under normal LD cycles and ad libitum feeding (Pan and Hussain 2009). This indicates that *Fabpi* may be entrained to both photoperiod and feeding. The amplitude of the rhythms seems to become stronger when subjected to restricted feeding and weaker when exposed to constant light, indicating a role for feeding and light in entraining this gene. Though the role of light is not as clear in the results here, I suspect a feeding influence as demonstrated by the differences observed between the morning and evening fed groups (Table 11). *Fabpi* gene is upregulated during the night in nocturnal animals (catfish and mice) and during the day if feeding restricted is restricted to daytime, such that the expression starts increasing prior to feeding and peaks at the end of feeding (Qin

et al. 2019; Pan and Hussain 2009). This suggests feeding entrainment. The acrophases seen here occur in the morning in all the conditions where rhythmicity was detected, possibly indicating that these fish are diurnal, since the peak in catfish and mice happens during the feeding period. The observation of rhythmicity in *Ppara* is not unprecedented; Goldfish intestinal bulb shows peaking in *Ppara* one hour before feeding when reared in LD and fed two hours after light onset, similar to the findings here, suggesting food anticipation via the circadian clock (Gómez-Boronat et al. 2019). Goldfish are diurnal, which means that the Chinook salmon here are likely diurnal too under the LD with 8 AM feeding schedule. This gene was observed peaking towards the end of the day in Atlantic salmon liver housed under 16:8 LD and ad libitum feeding, which is similar to the delay of peaking to the afternoon in the group of salmon receiving feeding throughout the day in the present study, but it is difficult to conclude whether it is responding to the early feeding events or anticipating the later events (Betancor et al. 2014). Ppara can drive *Bmal1* expression in mouse liver through binding to its promoter (Canaple et al. 2006); however, the acrophases observed in the Chinook salmon intestine do not suggest such a role, similar to the simultaneous peaking of *Bmal1* and *Ppara* in Atlantic salmon liver (Betancor et al. 2014). Conversely, *Bmal1* regulates *Ppara* expression in mouse liver, but this is only seen here in the LD with feeding throughout the day group where *Ppara* peaking lags behind *Bmal1* (Canaple et al. 2006). This indicates that *Ppara* is a potential circadian clock target. Although, Ppara has the ability to activate *Rev-erb* in other organisms, the results here do not support this in Chinook salmon intestine, as both *Ppara* and *Rev-erbα* peak together in most conditions except LL fed 5 PM (Mukherji et al. 2013).

Overall, these results show that lipid uptake and digestion genes, similar to the carbohydrate and protein genes, are potentially circadian genes.

There is a deficiency in studies concerning the rhythmicity of *Fatp4*, and its expression is not rhythmic in mouse white adipose tissue (J. Smith et al. 2015). Therefore, the robust rhythmicity seen in this gene across most conditions is promising, especially for utilization in determining the optimal rearing schedule for maximal uptake of lipids from food. A relative of Fatp4 known as Fatp1 involved in fatty acid transport is downregulated in a myocyte-specific knockout of *Bmal1*, which indicates that it is somehow controlled by the circadian clock through *Bmal1* and implicating Chinook salmon *Fatp4* in such a regulation as well (Dyar et al. 2014). *Fatp1* is also highly responsive to the administration of a Rev-erbα/β agonist at light onset, which causes a peak in *Fatp1* mRNA around dark onset in mouse skeletal muscle, further indicating that it is a clock target (Solt et al. 2012). From these two examples, it appears that there might be a link between fatty acid uptake and the circadian clock in the intestine too.

It is possible that some of the studied genes are direct or indirect targets of the circadian clock. For instance, *Sglt1, Pept1* and *Fabpi* lose rhythmicity in *Clock* mutant mice, suggesting that the *Clock* gene is a contributor to their rhythms (Pan and Hussain 2009). Furthermore, Bmal1 binding was detected in mice immediately upstream of the *Sglt1* gene and at its transcription initiation site, making it likely a target of the clock (Iwashina et al. 2011). *Bmal1* conditional knockout in mouse muscle lowers the protein levels of HK2, suggesting a circadian control over this gene (Dyar et al. 2014). *Pept1* may also be under indirect clock control through the binding of albumin D site-binding protein (Dbp), a clock-controlled gene, to its distal promoter in rats (H. Saito et al. 2008).

Knockout of the various clock genes in the Chinook salmon intestine, as well as fully sequencing these genes to determine whether they have binding sites for clock transcription factors (E-boxes for Bmal/Clock binding, and RREs for Rev-erb/Ror binding) would address whether these genes are truly circadian targets. Even if such tests yield negative results, the clock may still indirectly control the expression of these genes by controlling feeding time, which may direct the rhythms in the digestion genes. These observations highlight the importance of studying the circadian clock in favor of optimizing nutrient uptake and digestion in Chinook salmon based on rearing conditions.

Rhythms in inflammation and regeneration suggest that the intestine may be under stress

Tnf appears to be only entrainable to LD cycles and loses rhythmicity under LL (Table 12). Similar findings are seen in mouse intestine where entrainment to LD and ad libitum feeding results in rhythmic oscillations in *Tnf* (Mukherji et al. 2013; Stokes et al. 2017). This suggests that *Tnf* is entrained to photoperiod. It is hard to fully establish whether feeding entrains these rhythms, but approximately two-hour differences are seen between the peaking times in the three feeding conditions. The rhythms in *Tnf* are lost in *Bmal*1 mutant mice, indicating that the clock drives these rhythms. In my results, the condition under which both *Bmal1* and *Tnf* are rhythmic is LD fed throughout the day, where *Bmal1* peaks at 7 AM and *Tnf* peaks at 8 AM. This indicates that *Bmal1* may not be directly driving *Tnf* expression, because one hour may not be long enough for this to happen; instead, *Bmal1* maybe needed to ensure that other components of the clock are able to drive the rhythms in *Tnf*. *Rev-erb* and *Rora* maybe the components that control *Tnf* rhythms, as the expression of the former two genes peaks 15-16 hours prior to *Tnf*

peaking. No rhythms were detected in *Il6* under any of the LD rearing conditions (Table 12). Similar to the findings here, *Il6* does not display rhythmicity under LD in mouse intestine (Stokes et al. 2017). This makes it unlikely to be a circadian gene. Even though it is thought that *Il6* is suppressed by *Rev-erbα*, no indications of this are present, as both *Il6* and *Rev-erbα* peak around the same time in the conditions where rhythmicity was detected in *Il6* (Curtis et al. 2014). The current study provides evidence that *Tnf* may be a circadian gene entrained by photoperiod, and possibly feeding, whereas *Il6* might not be a circadian gene in Chinook salmon.

It is thought that the circadian clock drives rhythms in intestinal regeneration as demonstrated by the loss of such rhythms in *Bmal1*-/- mice (Stokes et al. 2017). On the other hand, circadian rhythms in the drosophila gut are dependent on stem cell signaling pathways such as Wnt and Hippo (Parasram et al. 2018). In the absence of intestinal damage, no rhythmicity is expected in intestinal regeneration (Stokes et al. 2017). Moreover, *Bmal1* and *Tnf* are known to regulate cell regeneration. *P21*, an inhibitor of proliferation, peaks in the morning in fish receiving evening feeding in the results here (Table 12). Similarly, the zebrafish intestinal clock regulates *P21* expression, and LD coupled with timed feeding results in highest rhythms (Peyric, Moore, and Whitmore 2013). This suggests that feeding may have an impact on the entrainment of P21 rhythms. Tnf is implicated in driving this gene which peaks two hours following *Tnf* peaking in LD with 5 PM feeding. It is thought that the clock acts to delay proliferation for several hours during stress responses involving *Tnf* by decreasing entry into S-phase of the cell cycle (Stokes et al. 2017). Currently, it is unknown whether the intestine in these fish was under any stressful events to make any conclusions linking inflammation to proliferation. Overall,

an evening feeding regardless of light setting causes rhythmicity in either *P21* or *Myc*, which could indicate a stress response as seen in mice (Stokes et al. 2017). Perhaps these fish are expecting to be fed in the morning, and when this does not happen, the intestine becomes irritated.

Myc is an oncogene involved in the Wnt pathway that regulates intestinal stem cells. It is thought to have effects on both the clock and the cell cycle by inhibiting the clock and promoting proliferation (Altman et al. 2015; Shostak et al. 2016). In mouse colorectal cancer cell lines, Myc protein expression declines when the *Clock* gene is knocked down, indicating a potential circadian regulation of the *Myc* gene (Peng et al. 2018). Though this does not necessarily mean direct regulation by the Clock protein on the *Myc* promoter due to the potential involvement of other clock components, the timing of peak expression in *Myc* seen here in the LD with feeding throughout the day experiment suggests that this is possible (Table 12); *Myc* peaking lags behind *Clock1b* peaking by 8.5 hours, which allows for directing *Myc* expression. Similarly, *Myc* peaking lags behind *Bmal1* peaking, thus it could be a Bmal1/Clock1b target. However, the rhythm seen under LD fed 8 AM does not correspond with circadian control due to coinciding in peaking time with the rhythmic clock genes. Overall, there is evidence that *Myc* may be a circadian gene, possibly driven by the Bmal1/Clock1b transcription factors.

Indicators of both nocturnal and diurnal feeding

Midnight peaking of *Cck* and *Vip* is observed in the group reared in constant light and fed at 5 PM (Table 10). Since these genes are among the appetite suppressors, it is surprising that they peak this early, given that food was administered around that time in

many of the experimental groups. This could be an indicator of possible nocturnal feeding. For instance, *Cck* peaks in the morning in rat duodenum, while feeding happens at night (Xu et al. 2017). This gene also loses its rhythmicity in rats when the LD schedule is reversed, such that feeding occurs during the light phase, indicating a role for light in directing *Cck* rhythms. Moreover, late feeding causes a delay in peaking of *Sglt1* and *Mgam* to the evening compared to morning feeding, suggesting a link to feeding time. Nocturnal animals share a similar nocturnal peaking time in these genes too. Similarly, *Pept1* shows midnight peaking under the LL fed 8 AM rearing. These results suggest that the fish in these experiments might be nocturnal feeders.

On the other hand, lipid digestion seems to begin in the morning with genes like *Fabpi* and under some condition in *Ppara* (Table 11). This shows that anticipation of diurnal feeding exists. The rhythms seen in P21 only exist under 5 PM feeding (Table 12), and they are indicators of stress in the mouse intestine (Stokes et al. 2017). Thus, perhaps it is more natural for the intestine to receive food in the morning and intestinal stress happens if morning feeding is lacking. This points towards diurnal feeding behaviour. All in all, perhaps these fish are nocturnal under some of the conditions and diurnal under others.

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Chapter 5: Conclusion

Overall, this thesis serves as a pilot study of the Chinook salmon circadian clock across six different tissues and establishes the presence of rhythmic clock gene expression in four of these tissues (the intestine, liver, heart, and skeletal muscle). The retina and colon were found to be arrhythmic. The phase relationships between the components of the clock are still under debate and more tools are needed to uncover the true timing of activity of each clock component. For instance, uncovering all of the paralogs of each clock component in this organism having a complex genome (potentially possessing eight copies of each gene) would aid in determining whether the paralogs functions differently across the tissue. This would help better deduce the effects of rearing conditions on the clock. The method of entrainment of each tissue to the rearing conditions also needs to be elucidated to better understand how the rearing conditions can be used to result in optimal clock function.

The intestine was further assessed to explore rhythmicity outside the clock. Rhythmic transcript levels were detected in genes that regulate appetite, nutrient uptake and digestion, inflammation and cell regeneration. This rhythmicity is dependent on rearing condition to some extent, since many genes lose rhythmicity under some of the rearing conditions. It is not yet known whether the rhythms detected are an output of circadian clock regulation of such genes, or simply a response to cyclic changes in the environment. Sequencing the promoters of these genes to determine whether they possess binding sites for circadian clock proteins is one way to answer this. By comparing a small set of rearing conditions, it was found that normal LD photoperiod and feeding throughout the day result in the best circadian clock rhythms overall, and in intestinal processes specifically. These findings will help inform future practices in aquaculture and

in preservation of this species in the wild. Overall, this thesis is a novel presentation of circadian clock activity at the transcript level in various tissues of Chinook salmon.

This study established the presence of circadian clock function in Chinook salmon liver, heart, intestine, and skeletal muscle. The findings of rhythmic clock function were consistent across all the chapters. However, some variations are seen in peaking time; the liver expression peaks of *Per1* in the Credit river-derived fish (Figure 1: 7 AM) are advanced relative to those of the YIAL fish under the same rearing condition (Figure 9 and Table 5: 10:39 AM). While *Clock1a* is rhythmic in Chapter 2, *Clock1b* turned out not to be rhythmic under similar rearing conditions in Chapters 3 and 4. Furthermore, the intestine of the YIAL fish is arrhythmic compared to the Credit river fish under similar rearing conditions, where peak expression falls after feeding. These differences may be attributed to many factors. First, the two fish strains evolved in different environments and hence have likely adapted their clocks to their respective environments; the Credit river population was introduced to Lake Ontario over 50 years ago, whereas the YIAL population is native to the west coast of North America. Moreover, the YIAL fish are derived from hermaphrodites (a hermaphrodite produces both male and female gametes). The British Columbia population is also more genetically homogenous because male and female gametes were derived from a small number of parents, and are highly inbred, adding to the differences between the two populations (Komsa 2012). Moreover, the two populations evolved under different environmental conditions, and thus the clock may be behaving in a population-specific manner. Furthermore, the DNA primers used to quantify gene expression in the two populations are different. For the Credit river population, most primers were developed from sequences of other fish species, before the majority of

Chinook salmon sequences became available; whereas for the YIAL fish, the primers are specific to Chinook salmon, hence might be more accurate at detecting expression. Finally, the statistical tools used to determine the significance of the rhythms in a gene are different; Curve-fitting and noise-to-signal ratios were incorporated with the YIAL samples to better screen for rhythmicity, and hence why many genes that were previously rhythmic in the Credit river fish are no longer are rhythmic in the YIAL fish. Such differences may account for the discrepancies observed across the chapters of this thesis.

The phases of maximal expression differ across the tissues and sometimes differ compared to other species, suggesting that the tissues are entrained differently. This was consistent with the different phases of the clocks in goldfish gut and liver, and across mouse tissues that entrain to feeding at varying paces (Velarde et al. 2009; Damiola et al. 2000).; however, the synchrony seen between the gut, liver, and muscle clocks in turbot shows that the clock networks of various species function differently (Ceinos et al. 2019). The arrhythmicity observed in the retina is inconsistent with findings from Rainbow trout retina, zebrafish eye, and goldfish retina, which are all rhythmic under similar rearing conditions (López Patiño et al. 2011; Zhdanova et al. 2008; Velarde et al. 2009). This finding can be attributed to the high variability observed across the animals from the Credit River, or to the long-day rearing (16:8 LD) that causes loss of rhythmicity in Atlantic salmon brain clocks compared to a short day of 8:16 LD (Davie et al. 2009). The first possibility would be addressed by studying the YIAL fish population which is a genetically more homogenous group; the brain was harvested from these animals instead of the retina, so studying it would give a closer view into the master clock. A group of fish could

be reared under a shorter day (14:10 LD, similar to the photoperiod at the southern end of the Chinook salmon distribution) to test whether the retina would become rhythmic under such conditions.

A special property seen here in the intestine is the synchronous peaking of the positive and negative arms of the clock, which was detected throughout the three data chapters of this thesis. This is not normal of the clock, since the positive regulators are typically the drivers of expression of the negative regulators as seen in the schematic in Figure 15; however, these findings are similar to those observed in skeletal muscle of zebrafish, Chinese perch and Crucian carp (Amaral and Johnston 2012; Wu et al. 2016, 2018). Future experiments are needed to determine whether the clock is still functional, which would be the case if the proteins are manufactured at different times, as seen in mouse liver Cry and Per protein expression lagging behind their RNA, whereas Bmal and Clock RNA and protein peak simultaneously (Lee et al. 2001). This would allow for the possibility of Bmal and Clock transcription to drive the expression of Per and Cry which should peak later. An alternative explanation is that different paralogs of *Bmal1* and *Clock1b* may be performing the activation of *Cry* and *Per*, and hence those paralogs would be on different phases. Such a scenario is possible given that Chinook salmon potentially has many duplicates of each circadian gene given that its genome is tetraploid, and the duplicates of each gene have sub-specialized functions such that *Bmal1* and *Clock1b* serve a function different from activating *Per* and *Cry*. A similar situation is seen in zebrafish muscle, where some paralogs of the *Cry* gene peak synchronously with the positive regulators whereas other paralogs peak later (Amaral and Johnston 2012). This points to the possibility that some of the *Cry* paralogs are driven by genes other than *Bmal*

and *Clock*, such as *Rev-erb* and *Ror*. In the Taqman™ intestine results, signs of normal anti-phasic peaks among positive and negative regulators are seen for the first time in Chinook salmon, where *Cry1* lags behind *Bmal1* under LL and evening feeding. This reveals an influence of rearing conditions on the behaviour of the clock, since synchronous peaking of *Clock1b* and *Cry1* still happens under LD with evening feeding, and of *Bmal1* and *Cry1* under LL with morning feeding. Overall, the anomaly observed in the Chinook salmon intestinal clock remains a mystery and further study is required to reveal whether the clock is truly dependent on protein rhythms alone instead of RNA rhythms, whether the various paralogs of each clock component contribute to clock function differently, or whether certain rearing conditions cause such disruptions in the normal anti-phasic mechanism of the clock.

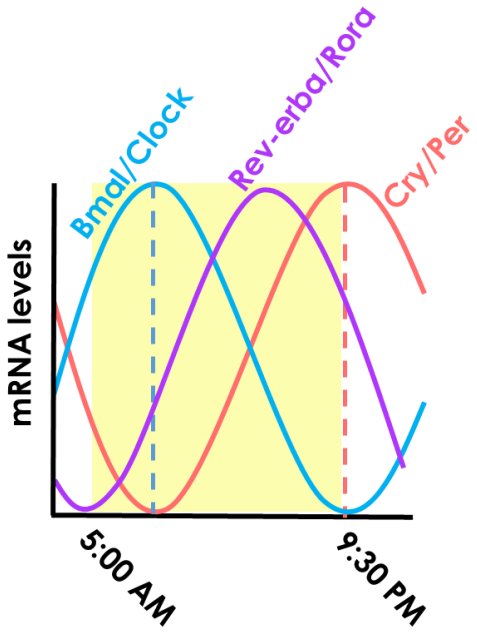

Figure 15: Schematic representing the phase relationships between different components of the circadian clock. Normally, Bmal and Clock peak in advance of the other components and drive their expression.

The liver and intestinal clocks show stronger entrainment to photoperiod than to feeding, whereas the skeletal muscle clock is less reliant on photoperiod and entrains to feeding. Rearing under normal photoperiod rather than constant light, and feeding throughout the day rather than only once, were found to be better for maintaining normal clock function at the transcript level. This is no surprise, as these fish would normally be experiencing natural LD cycles at this latitude, and they would be feeding throughout the day as they encounter prey in order to grow in size and prepare for either ocean migration or winter in the streams. Therefore, their clocks have adapted to this photoperiod length and food intake pattern. It may be best to match these rearing conditions in aquaculture to achieve the best growth rates.

The intestine was further studied to reveal rhythmicity in various intestinal processes like food intake, digestion, inflammation, and tissue regeneration. Rhythmicity was detected in most of the genes from these classes, but variability exists in the rhythmicity under the various rearing conditions (Figure 16). The highest number of rhythmic genes among appetite and digestion genes was found under LD and feeding throughout the day (Figure 17). This finding is consistent with these two rearing conditions being the most optimal for clock function as well (Figure 13). Under the most optimal rearing condition, rhythmicity was detected in one of the inflammatory genes (*Tnf*) and in one of the regeneration genes (*Myc*), implicating the clock in regulating these pathways. Finally, there are indications of both nocturnal and diurnal feeding patterns based on the peaking time of various appetite and digestive genes, but more information is needed to determine whether Chinook salmon are diurnal or nocturnal eaters.

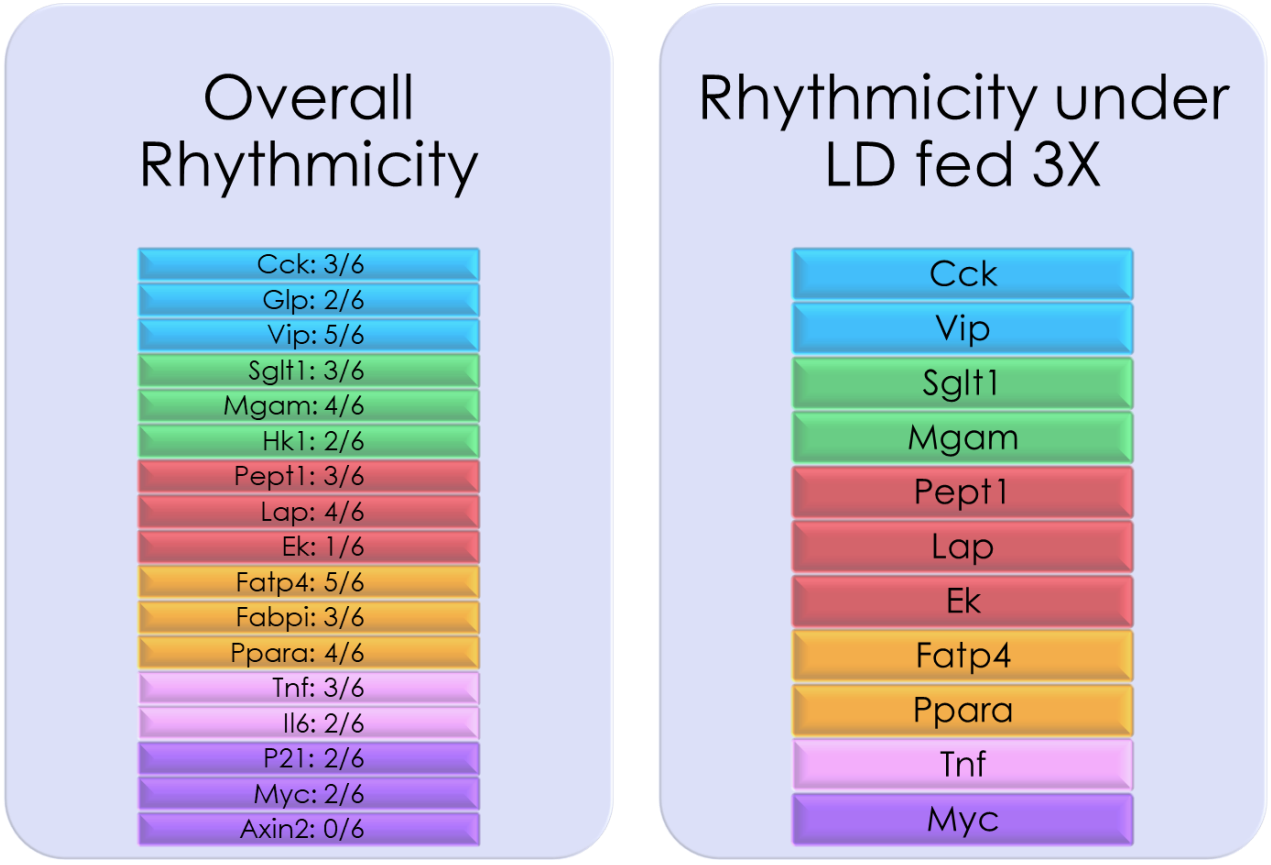

Figure 16: Rhythmicity occurs variably in genes involved in various intestinal processes, depending on the rearing conditions. Most of these genes are rhythmic under the best rearing condition for the clock, LD rearing coupled with feeding throughout the day.

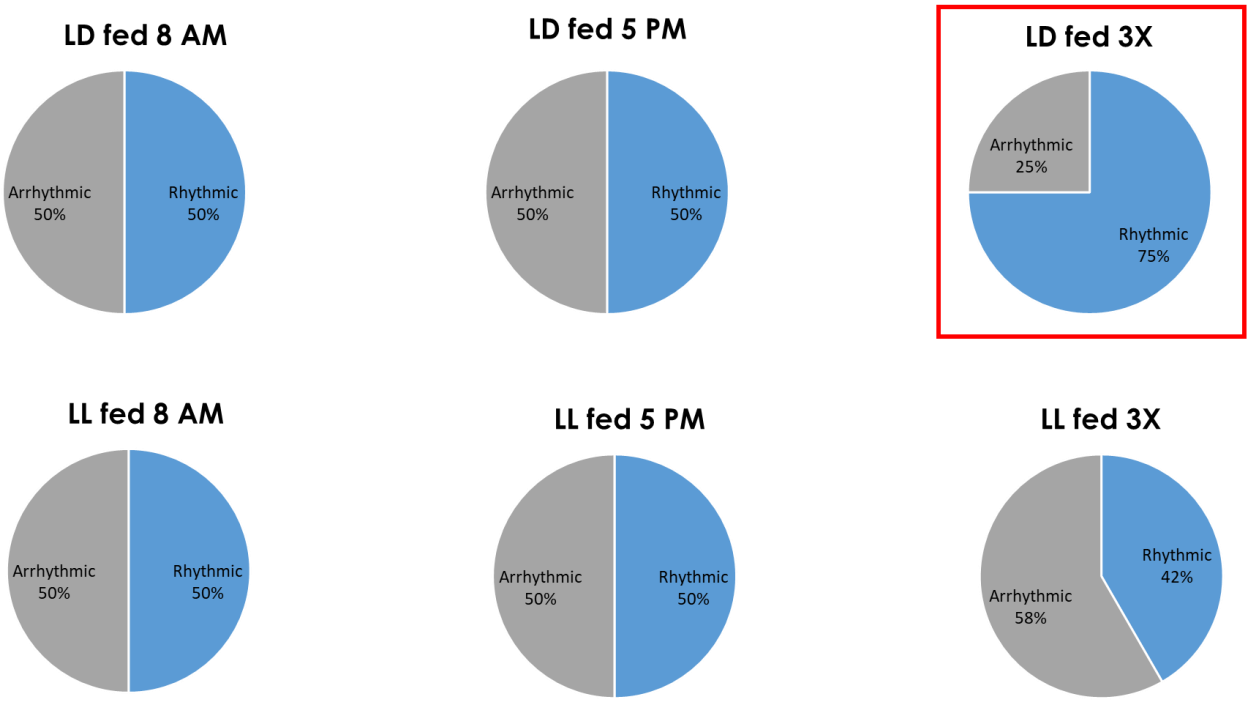

Figure 17: Pie charts showing the percentage of rhythmic genes from the appetite and digestion classes in the intestine. The blue shaded areas represent the percentage of rhythmic genes, whereas the grey areas represent the percentage of arrhythmic genes. The LD rearing conditions coupled with feeding throughout the day result in the percentage of rhythmic genes in appetite and digestion.

Future Directives

It remains very important to verify that the clock in Chinook salmon is free-running, as this has not been done yet. This means testing if the clock is able to continue oscillating in the absence of entraining cues. It would have been beneficial to have a fourth group raised under constant light and starved. This would eliminate the possibility that the rhythms seen are simply a transient response to light or feeding and not an autonomous

clock that is self-sustained in the absence of cues. In goldfish hindgut and gilthead seabream liver, short-term absence of both entrainment cues is not detrimental to clock rhythmicity, and I suspect the same to be true in Chinook salmon (Nisembaum et al. 2012; Vera et al. 2013). One limitation of this study is that feeding behaviour and food content in the gut were not assessed. In the future, taking these two factors into account may facilitate stronger conclusions about the role of feeding on entraining the genes in this study. Another expansion of this project would be to isolate the SCN and see if it displays shifts according to feeding time to decipher whether the central clock in this animal is entrained to feeding, or only to light. This is also important because the retina does not display rhythmic clock function, and it is critical to test the brain and confirm the presence of a master clock. Furthermore, because it is best in other animals to match food supply time to the natural feeding time of a species, monitoring fish feeding behaviour during experiments would help inform better feeding practices (Kotani and Fushimi 2011). Also, assaying clock expression at the protein level is needed to ascertain whether anti-phasic relationships between the positive and negative clock regulators are maintained at the protein level in the cases where synchronous peaking of is happening at the mRNA level. Finally, uncovering the truth behind the contribution of the polyQ domain to migration timing and reproduction is critical to the knowledge of circadian clock output in Chinook salmon. Experiments that measure the polyQ length and test the response to photoperiod specifically in terms of migration and reproduction are key for this understanding.

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