Clock Genes Localize to Quantitative Trait Loci for Stage-Specific Growth in Juvenile Coho Salmon, *Oncorhynchus kisutch*

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In most organisms, an internal circadian clock coordinates the expression of biological rhythms and enables individuals to anticipate and respond to the seasonally changing environment. There is remarkable conservation of function in the molecular machinery underlying this circadian clock across taxa with 4 canonical proteins interacting to form an autoregulatory feedback loop: CLOCK, CRYPTOCHROME, PERIOD, and BMAL. We mapped duplicated copies of *Clock* and *Cryptochrome* in coho salmon (*Oncorhynchus kisutch*) to determine if these genes localize to quantitative trait loci (QTL) for hatch timing, weight, length, and growth rate measured throughout the juvenile life-history stage. We found that *Cryptochrome2b* mapped to a QTL region for growth (measured at 304 days post-hatching) on linkage group OKI06. The percentage of variation (PEV) explained by this QTL was 15.2%. *Cryptochrome2b* was also associated with a marginally nonsignificant QTL for length (measured at 395 days post-hatching). *OtsClock1b* mapped to a QTL region for growth rate (PEV 10.1%) and length (PEV 10.5%) on linkage group OKI24 (measured at 479 days post-hatching). Neither gene localized to QTL for hatch timing or weight. Our findings indicate that the growth rate and length QTL associated with *OtsClock1b* and *Cryptochrome2b* are development stage-specific and may result from temporally differentiated gene expression patterns.

**Key words: circadian rhythms, Clock, Cryptochrome, growth, QTL, smoltification**

Circadian rhythms underlie diverse life functions ranging from cellular activities to seasonal behavior (Schwassmann 1988). These rhythms are the external expression of an endogenous clock that is entrained by photoperiod and thus enables organisms to anticipate and respond to cyclically changing environments. The molecular mechanism underlying circadian rhythms has been well characterized in several model systems (i.e., *Drosophila*, mouse, and zebrafish) and relies on the opposing effects of transcriptional activators and repressors in generating a feedback loop. The 4 canonical circadian rhythm proteins involved in this process are: CLOCK, BMAL, PERIOD (PER), and CRYPTOCHROME (CRY). Although there is remarkable conservation of function across taxa, major gene and genome duplication events have generated diversity in copy number. For instance, mammals have 2 copies of *Clock* and *Bmal*, 4 *Period* genes, and 2 *Cryptochrome* genes (Reppert and Weaver 2002), whereas zebrafish have 3 copies of *Clock* and *Bmal*, 4 *Period*, and 6 *Cryptochrome* genes (Hirayama et al. 2005).

Circadian regulation is of particular importance to anadromous fishes, such as Pacific salmon (*Oncorhynchus* spp.), which must coordinate physiological and behavioral adaptations with daily and seasonal cues for migrations between fresh and salt water. Two major developmental conversions that characterize the salmon life cycle: smoltification, which is the set of morphological, physiological, and behavioral changes associated with migration of juvenile fish from the freshwater to the marine environment (Hoar 1976), and sexual differentiation and maturation, a long protracted process that starts soon after the egg is fertilized and proceeds intermittently until the individual is capable of spawning (Thorpe 2007). Different species of salmon and different populations within a species may vary in the seasonal timing of both the onset and completion of these processes. This temporal variability has resulted in remarkable life-history diversity among salmonid fishes.

The timing of smoltification is especially critical for anadromous salmon because migrating fish typically experience mortality rates greater than 95% during the first few months of marine residency (Pearcy 1992). This process,
which requires rapid growth and major metabolic changes, is likely a gated event in an annual cycle; if a particular size or physiological threshold is not reached during a seasonal “decision window,” smoltification may be delayed (Duston and Saunders 1990; Arnesen et al. 2003). Given that the circadian rhythm gene network is believed to play an important role in energy balance (Turek et al. 2004), component genes are likely candidates influencing the timing of smoltification.

Previous studies have demonstrated that circadian rhythm genes likely play a key role in regulating the timing of adult migration (O’Malley et al. 2007; O’Malley and Banks 2008) and spawning (Leder et al. 2006). There is some indication that these genes may also influence the timing of juvenile outmigration as the Clock gene maps to a region of the genome associated with smoltification-related traits in rainbow trout, Oncorhynchus mykiss (Nichols et al. 2008).

Size and growth rate are important factors in the timing of smoltification and maturation. Larger, faster growing juveniles have been shown to migrate to the ocean at an earlier age and earlier in the season (Beckman and Larsen 1998). Coordination of the timing of saltwater entry with the completion of the physiological process of smolting also affects growth after migration (Maxime 2002). Smolts that enter salt water too early experience a decrease in growth and a disruption of other circadian rhythms such as routine oxygen consumption (Maxime 2002). As adults, growth rates at seasonally determined critical periods determine the age at which fish return to their natal streams to spawn (Shearer and Swanson 2000; Thorpe 2007).

Given the importance of both circadian regulation and growth to the transition between life-history stages in Pacific salmon, this study aims to evaluate a possible association between genes involved in these traits. One way to achieve this goal in an unsequenced genome is to identify regions of linkage that contain circadian rhythm genes were analyzed by interval mapping. Associations between these QTL and between previously characterized circadian rhythm genes can then be examined.

Here, we mapped duplicated copies of the circadian rhythm genes, Clock and Cryptochrome, to determine if these genes localize to the same region as QTL for hatch timing, weight, length, and growth rate measured throughout the juvenile life-history stage of coho salmon, O. kisutch (McClelland and Naish 2010).

Materials and Methods

Candidate Genes

Four circadian rhythm genes, OtsClock1a (GenBank accession no. DQ78082), OtsClock1b (GenBank accession no. DQ780894), Cryptochrome2b (GenBank accession no. GU826146), and Cryptochrome3 (GenBank accession no. GU826148), were amplified in coho salmon using polymerase chain reaction (PCR) primers previously designed for Chinook salmon (O. tshawytscha). Oligonucleotide primers and thermal cycling conditions used to amplify the polyglutamine domain of OtsClock1a and OtsClock1b are described in O’Malley and Banks (2008). The primers used to amplify microsatellites located in the noncoding region of each of the 2 Cryptochrome genes are listed in Table 1. Cryptochrome2b was amplified using the same protocol as for OtsClock1b, whereas Cryptochrome3 was amplified using the following touchdown PCR profile: one initial denaturing cycle at 3 min at 95 °C, followed by 2 cycles of 30 s at 95 °C, 20 s at 62 °C annealing temperature, and 30 s at 72 °C. In subsequent cycles, the annealing temperature was decreased by 2°C until 54 °C was reached, followed by 27 more cycles of 30 s at 95 °C, 20 s at 54 °C, 30 s at 72 °C, and a final extension of 10 min at 72 °C. PCR products were visualized on an Applied Biosystems 3100 DNA Analyzer and analyzed as length polymorphisms using GeneScan (Version 3.5.1).

Linkage Mapping

Circadian rhythm genes were added to the existing coho salmon linkage map (McClelland and Naish 2008) using the program LINKMFE v2.0 (Danzmann and Gharbi 2001). Males and females were treated separately due to differences in recombination rates between the sexes. A logarithm of odds (LOD) score of 3.0 was set as the threshold for clustering markers into linkage groups. Marker order was established using MAPORD, and distances between markers were calculated using MAPDIS (both available as part of the LINKMFE package). The program MAPCHART (Voorrips 2002) was used to create graphical representations of the linkage groups containing the newly mapped loci.

QTL Analysis

QTL analysis was used to determine possible associations between the circadian rhythm genes and several growth-related traits including hatch timing, weight, length, and growth rate. These traits have previously been mapped to several coho salmon linkage groups by McClelland and Naish (2010). Because markers and QTL in individuals used to create outbred crosses can be in different linkage phases, QTL analysis was performed with MultiQTL v2.5 (MultiQTL Ltd, Haifa, Israel) using a simple interval model (Lander and Botstein 1989). Data were analyzed using a single trait backcross model with the default settings that assume equal variance and no covariance between QTL and no epistasis. This backcross model was used to examine associations between either the alleles from the dam or the alleles from the sire and the phenotypes. Markers on the linkage groups that contained circadian rhythm genes were analyzed by interval analysis. Significance levels were estimated using the permutation test of Churchill and Doerge (1994) with 10 000 permutations of the trait data and an experimentwise false detection rate of 10%. P values less than 0.05 from the interval analysis permutation test were considered
indicative of a significant QTL effect. Unlinked circadian rhythm genes were analyzed using the marker option of MultiQTL, and significance was determined by permutation testing as above. Because $P$ values for single marker analyses are less conservative than for interval mapping (O’Malley et al. 2003), $P$ values of 0.01 were considered indicative of significant QTL effect. QTL which explained greater than 25% of the variance (PEV) in the trait value were considered to be major QTL (Bradshaw et al. 1998). The substitution effect ($|d|$), the phenotypic difference between progeny with alternate alleles, was also estimated. The substitution effect reflects the additive component of variance.

**Results**

**Candidate Gene Mapping**

Two of the 4 circadian rhythm genes, OtSClock1b and Cryptochrome2b, were polymorphic in the mapping parents and were included in the linkage analysis of the existing coho salmon linkage map (McClelland and Naish 2008) (Figure 1). OtSClock1a and Cryptochrome3 were monomorphic in the mapping parents and were therefore uninformative for linkage analysis.

Linkage analysis revealed that OtSClock1b maps to linkage group OKI24 and was polymorphic in both sexes. OtSClock1b is positioned 2.6 cM from the microsatellite marker Omm1211 in the female and shows zero recombination with Omy1212 in the male. Cryptochrome2b maps to linkage group OKI06 in both sexes. Recombination distances also vary between the sexes. Omm1300 had a zero recombination distance with Cryptochrome2b in the male, whereas these 2 markers are 13.2 cM apart in the female.

**QTL Analysis**

OtSClock1b mapped to a QTL region for growth rate and length, measured at 479 days post-hatching and after smolt metamorphosis, on the male OKI24 linkage group (Figure 1). The PEV for growth rate and length QTL were 10.1% and 10.5%, respectively (Table 1).

Cryptochrome2b mapped to a QTL region for growth rate, measured at 304 days post-hatching as presmolts, on the male OKI06 linkage group (Figure 1). The PEV for this QTL was 15.2% (Table 2). There was also evidence for a marginally nonsignificant QTL for length associated with Cryptochrome2b measured during the presmolt life-history stage (395 days post-hatching). The $P$ value after 10 000 permutations was 0.038 (LOD 1.13). However, after a genome wide correction for multiple tests, this result was no longer significant.

OtSClock1b and Cryptochrome2b did not localize to previously detected QTL regions associated with hatch timing and weight in juvenile coho salmon.

**Discussion**

We found that 2 circadian rhythm genes, Cryptochrome2b and OtSClock1b, localize to QTL regions for growth rate and length on coho salmon linkage groups OKI06 and OKI24, respectively. The QTL effects, however, appear to be developmental stage-specific as they were only detected at specific time intervals (i.e., days post-hatching). For example, Cryptochrome2b colocated with QTL for growth rate and length at 304 days post-hatching, whereas OtSClock1b colocated with QTL for growth rate and length at 479 days post-hatching.

### Table 1 Oligonucleotide primers used to amplify Cryptochrome genes in coho salmon, *Oncorhynchus kisutch*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’-3’)</th>
<th>Microsatellite repeat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cryptochrome 2b</td>
<td>GGGAGAATCAGAAGAGATACAT (TGT)3(TG)8 impure(CA)22(TA)7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TAACATACAGACATGTCAGACAG</td>
<td></td>
</tr>
<tr>
<td>Cryptochrome 3</td>
<td>CTGTATCTGCTGGTGGTGG</td>
<td>(AC)36 impure</td>
</tr>
<tr>
<td></td>
<td>TGAGCCTCTATGGATCAATAG</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 1.** Map position of growth related QTL and genes involved in circadian rhythm in coho salmon, *Oncorhynchus kisutch*. Bars denote marker intervals for QTL. Linkage group names follow the nomenclature of McClelland and Naish (2008).
The interval between these 2 time periods brackets the major physiological transformation during smoltification, which generally concludes with individuals outmigrating between April and early June when they are approximately 18 months old (Quinn 2005). The growth rate QTL linked to Cryptochrome2b was detected in presmolt individuals, whereas the growth rate and length QTL linked to OtsClock1b were identified in individuals that had undergone the smoltification process. These findings suggest that Cryptochrome2b and OtsClock1b may show temporally differentiated gene expression patterns throughout the development of juvenile coho salmon. This notion is consistent with the roles of Clock and Cryptochrome as opposing drivers in the circadian autoregulatory feedback loop. CLOCK, and a second positive element, BMAL, heterodimerize and initiate transcription of target genes, including Cryptochrome and Period. Negative feedback is achieved by PERIOD: CRYPTOCHROME heterodimers that translocate back to the nucleus to repress their own transcription by acting on the CLOCK:BMAL complex. As a result, rhythmic transcription of Clock and Cryptochrome is variable throughout a circadian phase in a wide-range organisms (reviewed in Ko and Takahashi 2006), including Atlantic salmon (Davie et al. 2009). Although developmental stage-specific expression of circadian rhythm genes has not been investigated in any salmonid species, Curran et al. (2008) showed that circadian rhythm genes are not co-expressed in the same tissue during early development in Xenopus laevis. Other studies on the ontogeny of circadian rhythms demonstrated that the Clock transcript is developmentally regulated in mice (Morse et al. 2003).

The amount of variance in trait value explained by the growth rate and length QTL on linkage group OKI24 was approximately equivalent at 10%, whereas the growth rate QTL on linkage group OKI06 was 15.2%. These findings indicate that while Cryptochrome2b and OtsClock1b may contribute to the phenotypic variation in growth and length of juvenile coho salmon, additional genes likely underlie the genetic architecture of these 2 quantitative traits. Because both Clock and Cryptochrome regulate the transcription of hundreds of clock-controlled genes (CCGs), we would expect to detect additional QTL associated with these downstream targets. In accordance, McClelland and Naish (2010) identified a second growth rate and length QTL measured at 479 days post-hatching. These QTL have nearly equivalent PEV values as those associated with OtsClock1b and measured at the same time period. Similarly, a second QTL for growth measured at 304 days post-hatching had a PEV 0.9% greater than that associated with Cryptochrome2b.

Microarray screens in mice have identified several hundred transcripts that cycle in abundance during different circadian phases. These CCGs encode proteins involved in various aspects cell physiology and metabolism (reviewed in Hardin 2004). Therefore, as transcription regulators, we would expect Clock and Cryptochrome to have pleiotropic effects on related traits such as growth rate and length as was revealed for OtsClock1b.

The QTL associated with OtsClock1b and Cryptochrome2b were only detected in the male parent. This result may be due to the fact that there is more power to detect QTL of moderate effect (PEV = 0.10) in regions where linkage distances are small (Roff 2007; Hu and Xu 2008). Because the male map is condensed relative to the female map, the ability to detect QTL is much greater in the male. Thus, we cannot exclude the possibility that QTL linked to these 2 circadian rhythm genes would be located in the female if a higher density linkage map were available.

Comparative mapping provides insight into genome evolution and helps to identify conserved and divergent regions among closely related species. By comparing the microsatellite linkage map from coho salmon to that for rainbow trout, McClelland and Naish (2008) identified 20 homologous linkage group pairs between these 2 species including the pair of OKI24 and RT24. Not only does OtsClock1b map to RT24 but previous studies also show that QTL related to growth and development localize to this region in rainbow trout as well (O’Malley et al. 2003; Nichols et al. 2007; Haidle et al. 2008). However, fine scale mapping is required to determine if OtsClock1b exhibits pleiotropic effects for life-history traits or if there is a major gene complex contributing to differences in growth and reproduction in both species of Pacific salmon.

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**Table 2** Coho salmon, *Oncorhyncus kisutch*, linkage groups, and markers to which QTL mapped

<table>
<thead>
<tr>
<th>Trait</th>
<th>Linkage group</th>
<th>Markers</th>
<th>LOD</th>
<th>Effect, m</th>
<th>Substitution effect,</th>
<th>P</th>
<th>PEV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth rate day 304–326</td>
<td>OKI06</td>
<td>Cry2b/Omm1300 Omm5026</td>
<td>1.10</td>
<td>1.61</td>
<td>1.48</td>
<td>0.040</td>
<td>0.152</td>
</tr>
<tr>
<td>Growth rate day 479–526</td>
<td>OKI24</td>
<td>Omm1293/ii Clk1b/Omy1212</td>
<td>1.27</td>
<td>2.60</td>
<td>-1.19</td>
<td>0.023</td>
<td>0.101</td>
</tr>
<tr>
<td>Length day 479</td>
<td>OKI24</td>
<td>Omm1293/ii Clk1b/Omy1212</td>
<td>1.33</td>
<td>276.22</td>
<td>-43.17</td>
<td>0.024</td>
<td>0.105</td>
</tr>
</tbody>
</table>

The trait mapped, linkage group, maximum LOD score (LOD), effect (m) of the allele present on the trait, allelic substitution effect (|d|) at the QTL position, P values for QTL (P), and the proportion of variance explained by the QTL marker (PEV) are shown. Where 2 markers are listed for a linkage group the maximum LOD score for that QTL was detected in the interval between those markers.
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References


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