Clock polymorphism in Pacific salmon: evidence for variable selection along a latitudinal gradient

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Seasonal timing of life-history events is often under strong natural selection. The *Clock* gene is a central component of an endogenous circadian clock that senses changes in photoperiod (day length) and mediates seasonal behaviours. Among Pacific salmonids (*Oncorhynchus* spp.), seasonal timing of migration and breeding is influenced by photoperiod. To expand a study of 42 North American Chinook salmon (*Oncorhynchus tshawytscha*) populations, we tested whether duplicated *Clock* genes contribute to population differences in reproductive timing. Specifically, we examined geographical variation along a similar latitudinal gradient in the polyglutamine domain (PolyQ) of *OtsClock1a* and *OtsClock1b* among 53 populations of three species: chum (*Oncorhynchus keta*), coho (*Oncorhynchus kisutch*) and pink salmon (*Oncorhynchus gorbuscha*). We found evidence for variable selection on *OtsClock1b* that corresponds to latitudinal variation in reproductive timing among these species. We evaluated the contribution of day length and a freshwater migration index to *OtsClock1b* PolyQ domain variation using regression trees and found that day length at spawning explains much of the variation in *OtsClock1b* allele frequency among chum and Chinook, but not coho and pink salmon populations. Our findings suggest that *OtsClock1b* mediates seasonal adaptation and influences geographical variation in reproductive timing in some of these highly migratory species.

Keywords: circadian clock; cline; day length; migration; polyglutamine domain; reproduction

1. INTRODUCTION

The seasonal timing of life-history events is often under strong natural selection, requiring organisms to integrate and respond to multiple environmental signals. Fitness depends on forecasting the optimal timing of season-specific activities, such as migration and reproduction, to exploit favourable conditions.

Photoperiod is a predictable environmental cue that organisms use to respond to seasonally varying conditions. The daily molecular oscillator, known as the circadian clock, senses changes in the photoperiod and mediates a diverse number of photoperiodic responses such as flowering time in plants (Yano *et al.* 2000) and hormone secretion in mammals (Goldman 2001). The molecular mechanism underlying this circadian oscillator has been characterized in several species and relies on the opposite effects of transcriptional activators and repressors in generating a negative feedback loop. One of the critical activators of the loop is the transcription factor CLOCK (Lowrey & Takahashi 2004). An essential domain of this protein is the carboxyl-terminal polyglutamine repeat motif (PolyQ) since it regulates the transcription-activating potential of CLOCK (Darlington *et al.* 1998). Experimental studies in mice discovered that

expansion or contraction in the number of glutamine repeats in this region directly affects the corresponding gene product and influences the circadian phenotype (Vitaterna *et al.* 1994).

Geographical surveys of *Clock* gene variation in birds and fish have found evidence for a latitudinal cline in the PolyQ repeat length that is probably maintained by selection (blue tit, Johnsen *et al.* 2007; Chinook salmon, O'Malley & Banks 2008a). Such clines indicate that environmental factors correlated with latitude (i.e. day length, temperature) might shape variation in the *Clock* gene (Kyriacou *et al.* 2008). O'Malley & Banks (2008a) hypothesized that since *Clock* plays a central role in the light input pathway entraining the circadian timing mechanism (DeBruyne *et al.* 2006), clinal variation in the Chinook salmon (*Oncorhynchus tshawytscha*) PolyQ domain may reflect an adaptation to seasonally changing day length.

Pacific salmon (*Oncorhynchus* spp.) show considerable diversity in their temporal patterns of migration and breeding within and among species. Photoperiod is regarded as a major environmental cue influencing migration to sea, initiation of gonadogenesis and migration back to freshwater for spawning (Clarke *et al.* 1994; Quinn & Adams 1996). These events are probably gated such that if a particular size or physiological threshold is not reached during a seasonal 'decision window,' these processes are delayed (Arnesen *et al.* 2003). Experimental studies have demonstrated that photoperiod manipulation can induce or delay the timing of migration and breeding (e.g. Saunders & Henderson 1970; Beacham & Murray 1988); however,

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the genetic pathway underlying these physiological responses remains unknown.

Here, we expand on a previous study in Chinook salmon *O. tshawytscha* (O'Malley & Banks 2008b) to test for evidence of a latitudinal cline in the *Clock* PolyQ domain in three additional Pacific salmon species in North America: chum (*Oncorhynchus keta*), coho (*Oncorhynchus kisutch*) and pink salmon (*Oncorhynchus gorbuscha*). While all four of these closely related species have overlapping geographical ranges, patterns of migration and reproductive timing are distinct among species along this latitudinal gradient (table 1). We predict that if clinal variation in *Clock* PolyQ length reflects an adaptation to seasonally changing day length, influencing the timing of migration and reproduction, there will be a robust latitudinal cline in the chum salmon *Clock* gene, a weak cline in the pink salmon *Clock*, and no distinct geographical pattern in coho salmon *Clock* gene variation. To test for evidence of selection, we compare variation in *Clock* to a set of allozyme loci for chum, coho and pink salmon. To identify potential ecological factors influencing geographical patterns in *Clock* in each of the four species, we use univariate regression trees (URTs; Breiman *et al.* 1984) to partition populations into groups for which the clinal variation in *Clock* among them best corresponds to these factors. For chum, coho and pink salmon, we considered two factors for each population: day length on the date of peak spawning and a freshwater migration index constructed from distance, to and elevation at, spawning location. For Chinook salmon, we also included a third factor, peak run timing (RT), to account for the four seasonal freshwater entry 'classes' recognized for this species (Healey 1991).

2. MATERIAL AND METHODS

(a) The salmon *Clock* gene study system

We sampled 756 individuals from 53 Pacific salmon populations representing three species: chum, coho and pink salmon (table 2). Sample information for the 42 Chinook salmon populations (14 spring run, 15 summer run, 12 autumn run and 1 winter run based on migration run type) is described in O'Malley & Banks (2008a). Spawning locations for all 95 populations are shown in figure 1.

(b) Characterization of the *Clock* PolyQ domains

We extracted genomic DNA from fin clips using the DNeasy Tissue Kit (Qiagen) and used two different sets of previously designed oligonucleotide primers to amplify the *OtsClock1a* and *OtsClock1b* PolyQ domains (O'Malley *et al.* 2007; O'Malley & Banks 2008a,b). The fluorescently labelled *OtsClock1a* sense primer was.

5'-GGTTCCTAATGTAGTTCCTGTGCTT-3' and 5'-GATTTCTCACCTGGACACTGGGCT-3' the anti-sense. For *OtsClock1b*, the fluorescently labelled sense primer was.

5'-CCTGTGTTTGTCTCCAACAGCA-3' and the anti-sense primer was 5'-CTGTCACTGCGAAATTACAGTCCT-3'. DNA was amplified in 5 µl reactions using two touchdown PCR profiles: one initial denaturing cycle of 3 min at 94°C, followed by one cycle of 1 min at 94°C, 1 min at 62°C annealing temperature and 1 min 30 s at 72°C. In subsequent cycles, the annealing temperature was

decreased by 2°C until 52°C was reached for *OtsClock1a* and 56°C for *OtsClock1b*, followed by 29 more cycles of 1 min at 94°C, 1 min at 52°C/56°C, 1 min 30 s at 72°C and a final extension of 10 min at 72°C. We electrophoresed PCR products on an Applied Biosystems 3100 DNA Analyzer and scored these products as DNA fragment length polymorphisms using GeneScan (v. 3.5.1) and Genotyper (v. 3.6NT). We standardized allele sizes to correspond to those reported in O'Malley & Banks (2008a).

(c) Statistical analysis of *Clock* gene variation related to ecological and life-history factors

We examined associations between latitude and both mean allele length (MAL) and the frequencies of the two most common alleles (MCAs) using linear regression. O'Malley & Banks (2008a) defined MAL as the sum of allele lengths of individuals in a population sample divided by the sample size. Johnsen *et al.* (2007) hypothesized that since the CLOCK protein dimerizes with the BMAL protein before binding to target control elements in the genome, the PolyQ alleles are probably codominant at the molecular level, making MAL a relevant summary statistic for population characterization.

To determine if the frequency distributions of the two *OtsClock* MCAs differed significantly from neutral expectations for each species, we compared the latitudinal regression fits (allele frequency on latitude) of the two MCAs with a distribution of regression fits for the set of polymorphic allozyme loci. For chum salmon, we calculated the regression fit of allele frequency on latitude for 21 allozyme alleles in 45 populations ranging from approximately 46–67° N (allele frequency data provided by C. Kondzela, NOAA Fisheries, Juneau, Alaska). For coho salmon, we calculated the regression fit of allele frequency on latitude for 12 polymorphic allozyme alleles in 28 populations ranging from approximately 49–64° N (Teel *et al.* 2003). For pink salmon, we calculated the regression fit of allele frequency on latitude for 14 allozyme alleles in 39 even-year pink salmon populations (approx. 54–61° N) as well as for 13 alleles in 37 odd-year pink salmon populations (approx. 52–65° N), pooling these data to generate a single distribution (allele frequency data provided by S. Wildes, NOAA Fisheries, Juneau, Alaska). Significant deviation from the expected distribution, determined by box plots, provides evidence for selection acting on *Clock*. We compared our findings with those previously reported for Chinook salmon (O'Malley & Banks 2008a).

To identify potential explanatory factors influencing patterns in *Clock*, we used URT analysis, a non-parametric analogue of multiple regression that, unlike most forms of gradient analysis, does not assume an underlying linear relationship between population differentiation and ecological distance. We used the mvpart package in R (R Development Core Team 2009), based on De'ath (2002), to perform separate URT analyses of variation in the frequency of the MCA (MCA.1) for chum, coho and pink salmon, based on two explanatory factors: day length on the date of peak spawning (DL) and an index of freshwater migration constructed with the Pythagorean theorem from freshwater migration distance to, and elevation at, the spawning grounds (MD). For Chinook salmon, we used three explanatory factors in the URT analyses: DL, MD and the date of peak RT (electronic supplementary material, appendix A and table 1).

Table 1. Summary of key life-history characteristics, typical of North American chum, coho, pink and Chinook salmon populations, that could be influenced by variation at the *Clock* gene. This information was used to support the hypotheses tested with the univariate regression tree (URT) analyses, and was taken from Groot & Margolis (1991), Weitkamp *et al.* (1995), Hard *et al.* (1996); Johnson *et al.* (1997); Myers *et al.* (1998) and Quinn (2005).

species	freshwater residency (yr)	marine residency (yr)	age at spawning (yr)	geographical variation in age at spawning	geographical variation in spawn timing	predicted latitudinal cline in <i>Clock</i>
chum salmon	<1	3–5	3–5	increases with latitude	advances with increasing latitude	strong
coho salmon	1–3	1.5	2–4	weak increase with latitude	no pattern	none
pink salmon	<1	2	2	none	weak advance with increasing latitude	weak
Chinook salmon	<1–2	1–6	1–8	increases with latitude	advances with increasing latitude	strong

First, we standardized MCA.1 by subtracting for each trait each population's value from the mean among populations and dividing by the standard deviation among populations. Next, we used URT analysis to recursively partition populations within each species by splitting them into two groups, based on variation in MCA.1, at every possible value of each explanatory factor, in a way that minimized the variation in *Clock* within each group. The variance at each tree node is the total sum of squares of MCA.1 about the node mean, and each split minimizes this value within the nodes formed by the split (and maximizes the between-node sum of squares). This procedure was continued until an over-large tree was grown, which was then pruned to a smaller size using cross-validation, with the final tree having the smallest predicted mean square error (De'ath 2002). Trees were pruned so that any split that did not decrease overall lack of fit (as measured by total variance explained) by 0.01 was discarded. We identified the best-fit pruned tree (within 1 s.e.; Breiman *et al.* 1984) by the relative error (Error), which is the total variance among the terminal tree leaves divided by the variance of the undivided data; $1 - \text{Error}$ is equivalent to the total variance explained by the pruned tree. The cross-validated relative error provides a more robust measure of the predictive value of the tree ($1 - \text{CV Error}$), which varies from close to zero for a poor predictor to one for a perfect predictor (De'ath 2002).

3. RESULTS

(a) Conservation of the *OtsClock1a* PolyQ domain

We found no evidence for length polymorphism in the *OtsClock1a* PolyQ domain among populations of chum, coho or pink salmon. A single 199 base pair (bp) allele was identified in coho, while a single 196 bp allele was identified in both chum and pink salmon.

(b) *OtsClock1b* length variation within and among Pacific salmon species

We identified four length variants of the *OtsClock1b* PolyQ domain among 18 chum salmon populations. The two MCAs, 335 and 341 bp, had frequencies of 0.519 and 0.476, respectively (table 2). Among the 19 coho salmon populations examined, we identified six

OtsClock1b PolyQ length variants. The two MCA, 335 and 338 bp, had frequencies of 0.761 and 0.174, respectively (table 2). We identified 10 length variants of the pink salmon *OtsClock1b* PolyQ domain among 16 populations. The MCA, 452 bp, was present in all populations with an overall frequency of 0.923, while the second MCA was present in all but three populations with a low frequency of 0.049 (table 2). Similar to chum and coho salmon, the most frequent of the eight *OtsClock1b* PolyQ alleles identified in Chinook salmon was 335 bp (0.737), whereas the second MCA was 359 bp (0.225) (O'Malley & Banks 2008a).

(c) Latitudinal distribution of *OtsClock1b* PolyQ alleles compared with allozymes

We found a significant association between chum salmon *OtsClock1b* PolyQ variation and latitude. Among the 18 populations examined, MAL decreased with latitude ($r^2 = 0.914$, $p = 0.0001$, regression coefficient $\beta = -0.158$; figure 2a). Allele 335 bp increased in frequency at higher latitudes ($r^2 = 0.890$, $p = 0.0001$, $\beta = 0.026$), while allele 341 bp increased in frequency at lower latitudes ($r^2 = 0.907$, $p = 0.0001$, $\beta = -0.026$; figure 2a). We compared the latitudinal regression fits for the two *OtsClock1b* PolyQ alleles (335 and 341 bp) with those for 21 allozyme alleles (r^2 range: 0.005–0.662; critical $r^2_{0.05} = 0.622$); the r^2 s for alleles 335 and 341 bp (0.890 and 0.907, respectively) were both highly significant ($p < 0.001$) outliers (figure 3).

For coho salmon, we found no significant association between MAL or the two most common *OtsClock1b* PolyQ variants and latitude among 19 populations using linear regression (MAL: $r^2 = 0.082$, $p = 0.234$; 335 bp: $r^2 = 0.016$, $p = 0.60$; 338 bp: $r^2 = 0.048$, $p = 0.366$; figure 2b). Latitudinal regression fits for the two *OtsClock1b* PolyQ alleles (335 bp: $r^2 = 0.016$ and 338 bp: $r^2 = 0.048$) did not show significant ($p > 0.50$) departures from neutral expectations when compared with those for 12 allozyme alleles (r^2 = range: 0.002–0.694; critical $r^2_{0.05} = 0.594$; figure 3).

For the 16 pink salmon populations, we found a significant association between allele frequency and latitude but not between MAL and latitude (MAL:

Table 2. Length variation in the *OtsClock1b* PolyQ domain (base pairs) among chum, coho, and pink salmon populations. Year sampled, source, number of alleles (*n*), latitude/longitude coordinates, allele frequency and mean allele length (MAL) is listed for each spawning population. n.a., not applicable.

population	year	ref.	<i>n</i>	latitude	longitude	329	332	335	341	MAL
<i>chum salmon</i>										
Porcupine River	2007	T Beacham; Department of Fisheries and Oceans, Canada	54	66.9905	-143.1377	0.04	0	0.83	0.13	335.55
Snake River	2004	J Olsen; U.S. Fish and Wildlife Service	56	64.7001	-165.3995	0	0	0.77	0.23	336.39
Little Susitna River	1989	C Kondzela; NOAA, Alaska Fisheries Science Center	52	61.2968	-150.2832	0	0	0.65	0.35	337.07
Herman Creek	2006	C Kondzela; NOAA, Alaska Fisheries Science Center	56	59.4161	-136.0923	0	0	0.59	0.41	337.46
Port Dick Creek	2000	J Olsen; U.S. Fish and Wildlife Service	58	59.3091	-151.3320	0	0	0.64	0.36	337.17
Spasski River	1991	C Kondzela; NOAA, Alaska Fisheries Science Center	60	58.0898	-135.2899	0	0	0.70	0.30	336.80
Saltery River	1991	J Olsen; U.S. Fish and Wildlife Service	34	57.9017	-136.2688	0	0	0.71	0.29	336.76
Fish Creek	2006	C Kondzela; NOAA, Alaska Fisheries Science Center	56	55.9958	-130.0947	0	0	0.57	0.43	337.57
Joshua Green River	1995	J Olsen; U.S. Fish and Wildlife Service	54	55.3897	-162.4860	0	0	0.69	0.31	336.88
Nass River	2005	T Beacham; Department of Fisheries and Oceans, Canada	48	54.9842	-129.8942	0	0	0.56	0.44	337.62
Mace River	1989	C Kondzela; NOAA, Alaska Fisheries Science Center	56	53.6101	-132.8095	0	0	0.50	0.50	338.00
Squawkum Creek	2005	T Beacham; Department of Fisheries and Oceans, Canada	58	49.2392	-122.0024	0	0	0.38	0.62	338.72
Grays River	n.a.	O Johnson; NOAA, Northwest Fisheries Science Center	42	46.3653	-123.5553	0	0.05	0.19	0.76	339.42
Necanicum River	2006	O Johnson; NOAA, Northwest Fisheries Science Center	36	45.9029	-123.8489	0	0	0.36	0.64	338.83
Foley Creek	2004	O Johnson; NOAA, Northwest Fisheries Science Center	42	45.8137	-123.7751	0	0	0.28	0.72	339.28
Hamilton Creek	1996	O Johnson; NOAA, Northwest Fisheries Science Center	34	45.6311	-121.9864	0	0	0.38	0.62	338.70
Ives Island	n.a.	O Johnson; NOAA, Northwest Fisheries Science Center	44	45.6266	-121.9974	0	0	0.32	0.68	339.09
Yaquina River	2004	O Johnson; NOAA, Northwest Fisheries Science Center	46	44.6193	-124.0311	0	0	0.22	0.78	339.69

population	year	ref.	n	latitude	longitude	335	338	341	353	356	362	MAL
<i>coho salmon</i>												
Snake River	2006	Olsen et al. (2003) <i>Conserv. Genet.</i> 4 , 557–569	62	64.7000	–165.3995	0.82	0.18	0	0	0	0	335.53
Tuluksak River	2004	Olsen et al. (2003) <i>Conserv. Genet.</i> 4 , 557–569	30	61.1022	–160.9605	0.90	0	0.07	0	0	0.03	336.40
Skilak River	1999	Olsen et al. (2003) <i>Conserv. Genet.</i> 4 , 557–569	38	60.4039	–150.1236	0.89	0	0	0	0.03	0.08	337.68
Alagnak River	1997	Olsen et al. (2003) <i>Conserv. Genet.</i> 4 , 557–569	36	59.0006	–156.8621	0.89	0.08	0.03	0	0	0	335.41
Buskin River	1998	Olsen et al. (2003) <i>Conserv. Genet.</i> 4 , 557–569	20	57.7500	–152.4786	0.70	0	0.10	0.20	0	0	339.20
Reflection Lake	1997	Olsen et al. (2003) <i>Conserv. Genet.</i> 4 , 557–569	32	56.0484	–131.5980	0.53	0.44	0.03	0	0	0	336.50
Russell Creek	2000	Olsen et al. (2003) <i>Conserv. Genet.</i> 4 , 557–569	40	55.1799	–162.6897	0.95	0.05	0	0	0	0	335.15
Chiliwack River Hatchery	1997	Van Doornik et al. (2007) <i>N. Am. J. Fish. Manag.</i> 27 , 220–237	42	49.0779	–121.7082	0.72	0.21	0.07	0	0	0	336.07
Nitinat River Hatchery	1996	Van Doornik et al. (2007) <i>N. Am. J. Fish. Manag.</i> 27 , 220–237	26	48.8205	–124.6597	0.62	0.15	0.19	0	0	0.04	337.65
Skagit River Hatchery	2001	Van Doornik et al. (2007) <i>N. Am. J. Fish. Manag.</i> 27 , 220–237	28	48.3875	–122.3672	0.68	0.32	0	0	0	0	335.96
Elwha River Hatchery	2004	Van Doornik et al. (2007) <i>N. Am. J. Fish. Manag.</i> 27 , 220–237	38	48.1152	–123.5493	0.58	0.39	0.03	0	0	0	336.34
Sol Duc River Hatchery	1994	Van Doornik et al. (2007) <i>N. Am. J. Fish. Manag.</i> 27 , 220–237	30	48.0555	–124.3061	0.63	0.1	0.27	0	0	0	336.90
Big Beef Creek Hatchery	2003	Van Doornik et al. (2007) <i>N. Am. J. Fish. Manag.</i> 27 , 220–237	36	47.6523	–122.7835	0.67	0.31	0	0	0	0.02	336.67
Quinault River Hatchery	2000	Van Doornik et al. (2007) <i>N. Am. J. Fish. Manag.</i> 27 , 220–237	32	47.4611	–123.8678	0.81	0.19	0	0	0	0	335.56
Soos Creek Hatchery	1996	Van Doornik et al. (2007) <i>N. Am. J. Fish. Manag.</i> 27 , 220–237	42	47.3095	–122.1695	0.76	0.21	0.02	0	0	0	335.78
Cowlitz River Hatchery	2002	Van Doornik et al. (2007) <i>N. Am. J. Fish. Manag.</i> 27 , 220–237	28	46.5113	–122.6294	0.71	0.29	0	0	0	0	335.85
Trask River Hatchery	2001	Van Doornik et al. (2007) <i>N. Am. J. Fish. Manag.</i> 27 , 220–237	36	45.4336	–123.7257	0.75	0.11	0.14	0	0	0	336.16
Umpqua River	2000	Van Doornik et al. (2007) <i>N. Am. J. Fish. Manag.</i> 27 , 220–237	10	43.3352	–123.0014	0.90	0.10	0	0	0	0	335.30
Trinity River Hatchery	1993	Van Doornik et al. (2007) <i>N. Am. J. Fish. Manag.</i> 27 , 220–237	20	40.7197	–122.8028	1.00	0	0	0	0	0	335.00

(Continued.)

Table 2. (Continued.)

population	year	ref.	<i>n</i>	latitude	longitude	404	410	428	431	446	452	458	476	479	497	MAL
<i>pink salmon</i>																
Swanson Creek	1997	B Templin; Alaska Department of Fish and Game	38	60.8482	-148.4124	0	0	0.05	0	0	0.95	0	0	0	0	450.73
Kenai River	1994	J Olsen; U.S. Fish and Wildlife Service	10	60.4835	-151.0591	0	0	0	0	0	1.00	0	0	0	0	452.00
Herring Creek	1994	B Templin; Alaska Department of Fish and Game	86	60.4154	-147.7891	0	0	0.01	0	0	0.98	0	0.01	0	0	452.00
Constantine	1996	B Templin; Alaska Department of Fish and Game	42	60.3646	-146.6018	0	0	0	0	0	0.98	0	0.02	0	0	452.57
Totemoff Creek	1994	B Templin; Alaska Department of Fish and Game	76	60.3435	-148.0850	0	0	0.03	0	0	0.97	0	0	0	0	451.37
Gertrude Creek	1999	J Olsen; U.S. Fish and Wildlife Service	36	58.1664	-156.2126	0	0	0.03	0	0.03	0.91	0	0	0	0.03	452.42
Spasski River (odd)	1991	S Wildes; NOAA, Alaska Fisheries Science Center.	54	58.0899	-135.2899	0	0	0.06	0.09	0	0.85	0	0	0	0	448.72
Spasski River (even)	1992	S Wildes; NOAA, Alaska Fisheries Science Center	58	58.0899	-135.2899	0	0	0	0	0	0.98	0	0.02	0	0	452.41
Kizhuyak River	1989	S Wildes; NOAA, Alaska Fisheries Science Center	32	57.6974	-152.8969	0	0	0.03	0	0	0.97	0	0	0	0	451.25
Oil Creek	1999	J Olsen; U.S. Fish and Wildlife Service	24	57.6457	-155.6959	0	0	0	0	0	1.00	0	0	0	0	452.00
Fish Creek	1987	S Wildes; NOAA, Alaska Fisheries Science Center	32	55.9952	-130.0958	0	0	0.06	0	0	0.88	0.03	0.03	0	0	451.43
Bulkley River	2003	T Beacham; Department of Fisheries and Oceans, Canada.	50	55.2488	-127.6653	0.02	0	0.06	0	0	0.88	0	0.04	0	0	450.56
Atnarko River	1989	S Wildes; NOAA, Alaska Fisheries Science Center	26	52.3698	-126.1005	0.03	0.03	0.12	0	0	0.81	0	0	0	0	445.65
Indian River	2005	T Beacham; Department of Fisheries and Oceans, Canada	56	49.4643	-122.8764	0	0	0.20	0	0	0.78	0	0	0.02	0	447.76
Snohomish River (even)	1996	S Blankenship; Washington Department of Fish and Wildlife	24	48.0201	-122.2068	0	0	0.08	0	0	0.92	0	0	0	0	450.00
Snohomish River (odd)	1997	S Blankenship; Washington Department of Fish and Wildlife	22	48.0201	-122.2068	0	0	0.05	0	0	0.90	0	0	0.05	0	452.13

Figure 1. Distribution of Pacific salmon populations sampled for *Clock* gene variation. Geographical location of the 95 Pacific salmon spawning populations representing four species sampled along the west coast of North America. Yellow diamonds, Chinook salmon; green squares, chum salmon; red circles, coho salmon; blue triangles, pink salmon.

$r^2 = 0.214$, $p = 0.071$; figure 2c). Allele 428 bp increased in frequency at lower latitudes ($r^2 = 0.470$, $p = 0.003$, $\beta = -0.008$) while allele 452 bp increased in frequency at higher latitudes ($r^2 = 0.403$, $p = 0.008$, $\beta = 0.01$; figure 2c). Comparing latitudinal regression fits for these two *OtsClock1b* PolyQ alleles (428 bp $r^2 = 0.470$ and 452 bp: $r^2 = 0.403$) to those for 27 allozyme alleles (r^2 range: less than 0.001–0.634; critical $r^2_{0.05} = 0.418$) revealed that allele 428 bp was a significant outlier at the 5 per cent level and allele 452 bp at the 10 per cent level ($p < 0.10$; figure 3).

(d) Correspondence of *Clock* gene diversity to ecological and life-history factors

For the 18 chum salmon populations, we identified the best-fit URT for the frequency of the most common *OtsClock1b* allele (MCA.1; 335 bp) on day length on the date of peak spawning (DL) and the freshwater migration index (MD). The relative error ($E = 0.129$) indicates that this simple tree separated the variation among populations into two groups by day length, and populations with longer day lengths into two groups again by DL, resulting in three terminal leaves. The first split along

Figure 2. Clinal variation in the *Clock* gene in four species of Pacific salmon. Linear regressions of *OtsClock1b* PolyQ mean allele length (MAL) and the frequencies of the two most common *OtsClock1b* alleles (MCA) on latitude (° N, x-axis) for each species: (a) chum, (b) coho, (c) pink and (d) Chinook salmon. (a) Allele, filled circles, 335 bp; open triangle, 341 bp. (b) Allele, filled circles, 335 bp; open triangle, 338 bp. (c) MCA, filled circles, 428 bp; open triangle, 452 bp. (d) Allele, filled circles, 335 bp; open triangle, 359 bp.

Figure 3. Evidence for selection on the Pacific salmon *Clock* gene. Box plots of the regression fit (r^2) of allele frequency on latitude for allozyme loci and the *OtsClock1b* gene in chum, coho and pink salmon. Regression fits for two *OtsClock1b* MCA in chum (335 bp: $r^2 = 0.890$, $p = 0.0001$, $\beta = 0.026$; 341 bp: $r^2 = 0.907$, $p = 0.0001$, $\beta = -0.026$) as well as pink salmon (428 bp: $r^2 = 0.407$, $p = 0.003$, $\beta = -0.008$; 452 bp: $r^2 = 0.403$, $p = 0.008$, $\beta = 0.01$) were significant ($p < 0.05$) outliers. O'Malley & Banks (2008a) provided similar evidence for selection on the *OtsClock1b* gene for the Chinook salmon populations.

DL explained 80.1 per cent of the variance and the second split along DL, 6.9 per cent of the variance, with the overall tree explaining 87.1 per cent (1 – Error) of the total variance. The cross-validation predictive error (CV Error) indicates that the ability of this tree to assign populations to groups correctly (1 – CV Error) on the basis of DL was moderately high (0.661, s.e. = 0.125, $p < 0.05$).

For the 17 coho salmon populations, the best-fit URT for the frequency of the most common *OtsClock1b* allele (MCA.1; 335 bp) separated the variation among populations into two groups by DL, and populations with shorter day lengths into two groups by the freshwater migration index (MD), resulting in three terminal leaves. The first split along DL explained 30.7 per cent of the variance, the second split along MD explained 14.8 per cent of the variance and the overall tree explained 45.5 per cent of the total variance. However, the ability of this tree to assign populations to groups correctly on the basis of these factors was essentially zero (s.e. = 0.405, $p > 0.05$).

For the 16 pink salmon populations, the best-fit URT for the frequency of the most common *OtsClock1b* allele (MCA.1; 452 bp) separated the variation among populations into two groups by DL resulting in two terminal leaves. This tree explained 53.2 per cent of the total variance. Similar to coho salmon, however, the ability of this tree to assign populations to groups correctly on the basis of these factors was essentially zero (s.e. = 0.437, $p > 0.05$).

For the 39 Chinook salmon populations, the best-fit URT for the frequency of the most common *OtsClock1b* allele (MCA.1; 335 bp) on DL, MD and RT separated the variation among populations into two groups by DL, and populations with shorter day lengths each into two groups by MD, resulting in three terminal leaves. The first split along DL explained 41.9 per cent of the

variance, the second split along MD explained 25.8 per cent of the variance and the overall tree explained 67.8 per cent of the total variance. RT was not a significant explanatory factor in this tree. The ability of this tree to assign populations to groups correctly on the basis of these factors was low (0.284, s.e. = 0.168, $p > 0.05$).

4. DISCUSSION

(a) Clock PolyQ diversity among Pacific salmon species

We found that closely related species with overlapping geographical ranges do not show similar allelic diversity at the *OtsClock1b* gene. Rather, species that show greater diversity in the timing of reproduction (i.e. spawn timing and age at spawning) also show increased length polymorphism in the PolyQ domain of *OtsClock1b*. For instance, pink salmon that show limited differences in spawn timing and a fixed age at spawning, have one allele that constitutes approximately 92 per cent of the variation in *OtsClock1b*. In contrast, Chinook salmon, which show extensive variability in both spawn timing and age at spawning, have four PolyQ length variants that constitute approximately 99 per cent of the variation in *OtsClock1b*.

Similar to what has been described for Chinook salmon (O'Malley & Banks 2008a), we found no intra-specific length variation in the *OtsClock1a* PolyQ domain among populations of chum, coho or pink salmon. In a comparative analysis of five teleost fish genomes, Wang (2008) discovered that *Clock* duplicates have been subject to asymmetric evolutionary rates and may have been retained through the process of neo-functionalization (one copy evolves to perform a novel function) or sub-functionalization (partitioning of ancestral function between duplicates). Such functional divergence between duplicates is usually required for their long-term retention in the genome (He & Zhang 2005). Subsequent to the *Salmonidae* genome-wide duplication event between 25 and 100 Myr (Allendorf & Thorgaard 1984), it is possible that one copy of *Clock*, or a flanking gene, experienced strong purifying selection (*OtsClock1a*) while the second copy (*OtsClock1b*) or a flanking region was subject to diversifying selection or a relaxed functional constraint. Consequently, the two copies may have undergone neo- or sub-functionalization, thereby eliminating genomic redundancy and preserving duplicated *Clock* genes among Pacific salmon species. To gain insight into the molecular evolution of these duplicated *Clock* genes in Pacific salmon, future work will be directed towards sequencing the PolyQ length variants and characterizing the nucleotide variation within the repeat motif as well as in the flanking regions.

(b) Clinal variation in the Pacific salmon Clock PolyQ

As predicted, *OtsClock1b* PolyQ length polymorphism diversity does not vary uniformly with latitude among these Pacific salmon species. We found that for each species the latitudinal variation in *OtsClock1b* PolyQ length appears to correspond to the species' clinal variation in reproductive timing. For instance, the steepest clines in PolyQ allele frequencies as well as MAL were identified in chum salmon, a species that shows robust

latitudinal clines in both spawn timing and age at spawning (Johnson *et al.* 1997). The moderate latitudinal clines in pink salmon MCAs and MAL reflects a modest cline in spawn timing and lack of variation in age at spawning among populations (Hard *et al.* 1996). In contrast, we found no evidence for latitudinal variation in coho salmon MCAs or MAL, a result which parallels the lack of a distinct geographical pattern in spawn timing and age at spawning throughout this species' range (Weitkamp *et al.* 1995).

Given evidence for selection maintaining *OtsClock1b* clinal variation in three of the four salmon species, combined with the fact that *Clock* plays a central role in the light input pathway of the circadian timing mechanism (DeBruyne *et al.* 2006), we hypothesize that the observed clinal variation may reflect an adaptation to photoperiodic parameters correlated with latitude. Pittendrigh *et al.* (1991) discovered that as day length increases, the amplitude of the circadian pacemaker declines, ultimately producing a latitudinal cline in the timing of seasonal events in natural populations. Experimental studies have demonstrated that a mutation in the *Clock* PolyQ domain can reduce the amplitude of the pacemaker and increase the efficiency of entrainment of organisms to light (Vitaterna *et al.* 2006). The PolyQ length variants observed in chum, Chinook and to a lesser extent pink salmon populations may fine-tune amplitude such that selection for conservation of pacemaker function results in a latitudinal cline in *OtsClock1b* in these species.

Other ecological factors correlated with latitude, such as temperature, may contribute to the variation observed in *OtsClock1b* PolyQ domain length. For instance, there is a latitudinal cline in the threonine–glycine–encoding (Thr–Gly) repeat region of the *Drosophila melanogaster* *Period* gene (Costa *et al.* 1991) that has been shown to be a temperature compensation mechanism of the circadian clock (Sawyer *et al.* 1997). If variation in the salmon *OtsClock1b* PolyQ domain is a temperature compensation mechanism, then the process by which this operates varies interspecifically since chum, coho and Chinook salmon all share the same MCA (335 bp) yet show distinct patterns in allelic variation along a latitudinal gradient. Furthermore, if variation in PolyQ length is associated with a temperature compensation mechanism, one might not expect to observe distinct patterns in clinal variation among highly migratory animals that encounter extensive fluctuations in temperature along a latitudinal gradient.

Not only does the strength of the latitudinal cline in *OtsClock1b* PolyQ length differ among closely related species, we determined that clines in chum and Chinook salmon vary in the opposite directions. In chum salmon, the 335 bp increases in frequency with increasing latitude, whereas the 335 bp allele in Chinook salmon decreases in frequency with increasing latitude. We can only speculate as to why these trends exist in these two species. It is possible that phenotypic variation in the same life-history trait may be driven by different genetic mechanisms regulating the circadian clock. Rosenblum *et al.* (2010) recently demonstrated that even when the same gene has been shown to influence phenotypic variation in a given trait, the molecular mechanisms underlying this variation may differ between closely related species. Future studies will be directed towards trying to understand the functional

significance of the polyglutamine repeat motif in the salmon *OtsClock1b* gene.

Contrasting latitudinal trends between chum and Chinook salmon MALs could also be driven by patterns of historical contingency. Disentangling the effects of selection from population history has proven to be quite challenging (Kyriacou *et al.* 2008). The fossil record of Pacific salmon indicates that by 6 Ma all five species were extant (Smith 1992); however, the current meta-population structure was established during post glacial recolonization within the last 10 000 years (McPhail & Lindsey 1970). Information regarding these species-specific patterns of recolonization is limited, leading to uncertainty as to how the current interspecific variation in population genetic structuring evolved along the west coast of North America. Therefore, we cannot exclude the possibility that historical recolonization patterns also contribute to the observed clinal variation in *OtsClock1b* among these species of Pacific salmon.

(c) *Relating Clock gene diversity in Pacific salmon to ecology and life history*

We found a high concordance between day length on the date of peak spawning and variation in chum salmon *OtsClock1b* PolyQ allele frequency. Similarly, we detected a high concordance between day length on the date of peak spawning as well as the freshwater migration index and variation in Chinook salmon *OtsClock1b* PolyQ allele frequency. In contrast, we found no significant concordance between variation in *OtsClock1b* and the two explanatory variables for either coho or pink salmon. These findings correspond to our results from the simple linear regression analyses and provide support for the hypothesis that clinal variation in the *OtsClock1b* PolyQ domain reflects an adaptation to the seasonally changing day length and influences geographical variation in reproductive timing in some of these highly migratory species.

One notable trend in both the chum and Chinook salmon regression tree analyses is the latitude at which populations are split based on day length on the date of peak spawning. For chum salmon, the primary split occurs between 49° N and 53° N while the primary split for Chinook salmon occurs between 53° N and 54° N. It is around Vancouver Island (49° N) that the North Pacific Current (NPC) bifurcates into a subpolar branch that forms the Alaska Current and a subtropical branch that includes the California Current (Cummins & Freeland 2007). It is possible that migrating populations of chum and Chinook (as well as possibly pink) salmon are influenced by this major current system and contribute to the geographical patterns, we observe in *Clock* variation for these species.

We conclude that *OtsClock1b* gene diversity appears to play a key role in mediating seasonal adaptation in several, but not all, Pacific salmon species. While *OtsClock1b* probably influences the timing of reproduction in chum, Chinook and possibly pink salmon, there are apparently alternative ecological factors and genetic mechanisms regulating the timing of these life-history events in coho salmon. Thus, our findings suggest that caution should be taken when predicting the function of these homologous genes even among closely related species. Other

factors such as competing selection and epistatic or pleiotropic interactions, may lead to a greater reliance on other timing-related genes or gene networks.

Recent climate change has resulted in warming winter temperatures, particularly at higher latitudes, and an overall increase in the length of the growing season (Bradshaw & Holzapfel 2008). Consequently, the optimal timing of major life-history transitions such as migration and reproduction has shifted (Bradshaw & Holzapfel 2008), leading to the potential disruption of photoperiod to reliably forecast optimal temperatures for spawning, incubation and rearing in the freshwater environment. Successful avoidance of unfavourable conditions associated with climate change will probably require adaptive shifts in key life-history traits and probably involve photoperiodic response rather than evolutionary changes in thermal tolerance (Bradshaw & Holzapfel 2006). Rapid shifts in both reproductive timing and *Clock* gene variation have previously been documented for a recently introduced population of Chinook salmon (O'Malley et al. 2007), revealing the adaptive potential of this species. Similarly, climate change will require Pacific salmon to adapt to alterations in environmental conditions (i.e. decoupling of day length and optimal timing of migration and spawning time) and adjust life-history strategies.

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