EVOLUTION OF PHOTOPERIODISM IN THE THREESPINE STICKLEBACK, *GASTEROSTEUS ACULEATUS*

by

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DISSERTATION ABSTRACT

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In seasonal environments, the ability to take advantage of the favorable seasons and avoid or mitigate the effects of the unfavorable ones is essential for organismal fitness. Many polar and temperate organisms use photoperiod (length of day) to time seasonal life history events because photoperiod's regular annual cycle makes it a very reliable indicator of seasonality. This reliability allows organisms to anticipate and properly prepare for seasonal change. Although photoperiodism is widespread in polar and temperate vertebrates, little is known relative to invertebrates regarding how its use varies with environment and this method's underlying genetic and physiological basis. This dissertation is focused on demonstrating the proper methodology for the study of photoperiodism and establishing the threespine stickleback as a model of vertebrate photoperiodism.

Chapter I is an introduction to photoperiodism, how it is influenced by environment, the physiological basis of its output, and a summary of the chapters that follow. Chapter II explains an analytical method to test for causality and applies this method to data that have been interpreted as evidence that the circadian clock is causally involved in photoperiodism. Chapter III describes the photoperiodic response of threespine stickleback *Gasterosteus aculeatus* populations from two latitudes. These

results are used to inform an empirical examination of the previously described assertion that the circadian clock is causally involved in photoperiodism. Chapter IV examines the physiological basis of early photoperiodic response using the threespine stickleback as a model teleost fish. Chapter V summarizes the previous chapters, describes their significance, and suggests future research directions.

This dissertation includes both previously published and co-authored material. Supplementary Excel files demonstrating the analyses used in Chapter III are also included in this dissertation.

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This dissertation is dedicated to my grandmothers. To my maternal grandmother, Elizabeth Savage Hart, the first of my family to study biology in Oregon and a true *tierfreundin*: living here has helped me discover more of you within me. I now understand why you always smiled when the conversation turned to Oregon. And to my paternal grandmother, Ruth Hazel O'Brien: for the grace she brings to our family. Your intelligence and resolve are wonderful to watch and, hopefully, internalize.

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CHAPTER I

INTRODUCTION

The importance of photoperiodism

Our planet experiences two great environmental rhythms. The daily cycle of light and dark is caused by the rotation of the earth about its axis, and the seasonal cycle in climate is caused by the angle of the earth's axis relative to its plane of orbit. Organisms must be adapted to the effects of these rhythms to maximize evolutionary fitness.

Some prokaryotes and all eukaryotes have circadian rhythms (from the Latin circa, meaning "around" and diem meaning "day") that control daily organismal processes. Circadian rhythms are endogenous (internal, self-sustained) and set by the daily light/dark cycle (Dunlap *et al.*, 2004). Organismal behavior and development that occurs on a seasonal scale is often mediated by photoperiod, or length of day. Photoperiodism is widespread among temperate and polar organisms (Goldman *et al.*, 2004; Bradshaw & Holzapfel, 2007). Empirical results demonstrate that proper functioning of these timekeeping mechanisms is critical to organismal fitness in the context of daily (Emerson *et al.*, 2008; Yerushalmi *et al.*, 2011) and seasonal (Bradshaw *et al.*, 2004) rhythms.

Fitness in seasonal environments depends on an organism's ability to exploit the favorable season (e.g. to maximize growth and reproduction), avoid or mitigate the

effects of the unfavorable season (e.g. through dormancy and migration) and to make a timely transition between the two life history modes (Bradshaw *et al.*, 2004; Bradshaw & Holzapfel, 2007). Photoperiod is widely used for this purpose because it has a highly reliable annual cycle. At any given latitude, its cycle is exactly the same from year to year, unlike other environmental indicators of seasonality, such as temperature or rainfall. The reliability of the photoperiodic cycle means that it can be used as an anticipatory cue of seasonal change. Transitions between life history modes take time, and the ability to anticipate future conditions means that these transitions can be completed at the optimal time of year.

In shorter-lived organisms, such as invertebrates, absolute photoperiod tends to govern a go/no-go response that once initiated, runs to completion (reviewed in Bradshaw & Holzapfel, 2007). In longer-lived organisms, such as most vertebrates, photoperiodic response results from an interaction between photoperiod and a circannual rhythm (Bradshaw & Holzapfel, 2007). These are physiological rhythms endogenous to the organism that persist under constant temperature and photoperiod (Goldman *et al.*, 2004). They are set by the natural photoperiod cycle and produce an annual rhythm in photosensitivity and photorefractoriness. Long or increasing photoperiods initiate the go/no-go response during photosensitive phases. The refractory phases cause organisms to be non-responsive to photoperiods that would otherwise be stimulatory, thus allowing time to properly prepare for seasonal change (Bradshaw & Holzapfel, 2007). Short photoperiods reset the circannual rhythm (Goldman *et al.*, 2004).

Photoperiodism is widespread in Gnathostomata (the "higher" vertebrates), but its distribution in more basal vertebrate lineages is largely unknown. There is a lack of

studies in Cephalochordates, Agnathans, Chondrichthyes, as well as the basal Actinopterygii lineages. The few studies that have been conducted focused on a single life history event and none examined the effect of photoperiod on sexual maturation or reproduction (reviewed by Bradshaw & Holzapfel, 2007). At least one basal sarcopterygian, the Australian lungfish, is photoperiodic (Kemp, 1984), but this appears to be the sole study of photoperiodism in basal Sarcopterygii.

Photoperiod provides the go/no-go signal for sexual maturation in many species of teleost fishes, mammals and birds (Bradshaw & Holzapfel, 2007), although it also affects other phenotypes, including the physiological processes associated with migration and dormancy (Bromage *et al.*, 2001; Dawson *et al.*, 2002; Goldman *et al.*, 2004). Despite the prevalence of photoperiodism within vertebrates and its effects on important phenotypes, fundamental questions remain unresolved. This dissertation is focused on developing a vertebrate model that can address these questions, which range from the involvement of photoperiodism in life history, to the physiological and genetic processes that underlie its phenotypic outputs.

What is the relationship between the circadian clock and vertebrate photoperiodic time measurement?

The circadian clock is the endogenous time keeping mechanism that is the basis of daily rhythms in organismal processes ranging from gene expression to behavior. It is entrained by the daily light/dark cycle. Photoperiodic time measurement controls seasonal processes and is entrained by either the absolute photoperiod (day length) or change in photoperiod. It is intuitive to suggest that the circadian clock forms the

mechanistic basis of photoperiodic time measurement as both are entrained by properties of the daily light cycle. If true, a single mechanism would be responsible for the timing of behavior and development on both daily and seasonal scales.

This hypothesized connection between the circadian clock and photoperiodic time measurement has been long-standing (Bünning, 1936) and over time has become assumed in the literature (e.g. Hazlerigg & Loudon, 2008). The two time keeping mechanisms have a causal relationship in plants (Wilczek *et al.*, 2009; Kobayashi & Weigel, 2007) and in a laboratory strain of Syrian hamsters (Shimomura *et al.*, 1997; Lowrey *et al.*, 2000), but it remains unknown if they are causally connected in animal populations that exist in nature.

If the circadian clock is the basis of photoperiodic time measurement, we expect a correlation between photoperiodic response and genetic variation in aspects of the circadian clock, such as period or amplitude of the circadian rhythm, or duration or timing of circadian activity. To our knowledge, this potential correlation has only been studied in outbred populations of one vertebrate. Northern populations of the white-footed mouse, *Peromyscus leucopus*, may exhibit gonadal regression in response to short day lengths, while those from southern populations are unaffected and breed year round (Lynch *et al.*, 1981; Heideman *et al.*, 1999). The period of circadian rhythm does not differ under constant darkness, long days or short days among these populations (Carlson *et al.*, 1989). Selection for increased and decreased photoperiodic response in lines derived from a single population produced similar results (Majoy & Heideman, 2000). Thus, neither genetic variation in photoperiodism within or among populations of white-footed mice is correlated with circadian rhythmicity, nor does selection on photoperiodic

response produce a corresponding change in circadian rhythmicity. Although we must be cautious in extrapolating from studies of a single vertebrate, these results support independence of the circadian clock and photoperiodic time measurement.

Correlation between allelic variation in the *clock* gene, and variation in the timing of seasonal traits mediated by photoperiodic response, has been interpreted as support for a circadian clock – photoperiodic time measurement connection. The Clock protein is a basic-helix-loop-helix transcription factor with four protein domains: a DNA binding domain, two protein dimerization domains and a transactivation domain that is characterized by a carboxyl-terminal repeat motif (Darlington *et al.*, 1998). The daily circadian rhythm is set by the expression of *clock*, which autoregulates its own transcription through a negative feedback loop (Darlington *et al.*, 1998). This series of molecular interactions is well characterized in mammals. It appears to have the same function in other vertebrate taxa, including teleost fishes and birds, but this has not been directly tested (Helfer *et al.*, 2006; Vatine *et al.*, 2011). Differences in the number of glutamine repeats in the transactivation domain may affect the circadian rhythm by altering the ability of *clock* to promote transcription (Darlington *et al.*, 1998; Gekakis *et al.*, 1998; Avivi *et al.*, 2001).

Length variation of the *clock* polyglutamine transactivation domain (hereafter termed polyQ) has been proposed as the mechanism by which the circadian clock, via its hypothesized role in photoperiodic time measurement, affects seasonal timing in several species (O'Malley *et al.*, 2010). An association between polyQ domain length of *OtsClock1b*, a *clock* paralog specific to the salmonid lineage and run timing (the seasonal timing of upstream migration in freshwater) was found in two of four Pacific salmon

species examined (O'Malley *et al.*, 2010): the stronger the latitudinal cline in run time, the stronger the latitudinal cline in polyQ domain length. Comparison of *Otsclock1b* allele frequency and neutral genomic loci suggested that these clines were maintained by divergent selection (O'Malley & Banks, 2008a; O'Malley *et al.*, 2010). As migration run time in anadromous salmon is mediated through photoperiodic entrainment of the endogenous circannual rhythm (Quinn & Adams 1996; Bromage *et al.*, 2001), these data were interpreted as evidence that *OtsClock1b* mediates seasonal adaptation by affecting photoperiodic time measurements (O'Malley *et al.*, 2010).

A similar association was found in European populations of a non-migratory passerine. *Clock* polyQ length increases with latitude in the blue tit *Cyanistes caeruleus* (Johnsen *et al.*, 2007). As with the Pacific salmonids, this cline also appears to be maintained by selection (Johnsen *et al.*, 2007). A follow up study in the same populations demonstrated an association between polyQ domain length and breeding time; female blue tits with shorter alleles tended to breed earlier in the season (Liedvogel *et al.*, 2009). Because photoperiodic response controls the timing of blue tit reproductive behavior (Lambrechts *et al.*, 1997), these studies concluded *clock* affects seasonal timing through a causal role in photoperiodic time measurement (Johnsen *et al.*, 2007; Liedvogel *et al.*, 2009).

The proposed connection between allelic variation in a central circadian clock gene and photoperiodism is tantalizing because it supports the hypothesis of a causal connection between the two biological clocks, and suggests a mechanism for variation in photoperiodic response. It is premature, however, to conclude that the circadian clock is involved in photoperiodic time measurement. These studies implicitly assumed that any

effect of *clock* allelic variation on photoperiodic time measurement would be through its role in the circadian clock. This is an unreasonable assumption, as we do not know the role of the *clock* gene in the teleost circadian clock and the possibility of a pleiotropic function for the *clock* gene, independent of its role in the circadian clock, is not considered. In addition, these studies were limited by their reliance on phenological variation as a proxy for variation in photoperiodic response. Run timing in chum (*Oncorhynchus keta*) and Chinook salmon (*Oncorhynchus tshawytscha*), the two species showing the strongest associations between latitude and *OtsClock1b* polyQ, is mediated by photoperiod (Clarke *et al.*, 1994; Quinn & Adams, 1996), but proximate environmental factors, such as water temperature and flow rate can affect run timing, especially in Chinook (Crozier *et al.*, 2008). Likewise, the timing of egg laying in the blue tit is largely controlled by photoperiodic response (Lambrechts *et al.*, 1997), but it is also influenced by local climate and food availability (Westwood & Murton, 1997).

Chapter II describes a simple, but underutilized method that can be used a priori to determine if further investigation of *clock* allelic variation and run timing is appropriate. This genetic variation and phenotypic variation are assumed to have a causal (i.e. necessary) relationship with one another because of their covariation across a latitudinal gradient. If *clock* allelic variation and run timing have a causal relationship, the correlation between them should remain if the common effect of latitude is removed. If the causal relationship does not remain after this test, further consideration of the relationship is inappropriate and tests of the assumptions underlying the hypothesized connection between the circadian clock and photoperiodic time measurement should not be conducted in this context.

Chapter III examines the photoperiodic response of multiple threespine stickleback populations from two latitudes in controlled conditions independent of other environmental signals that may affect response. Allelic variation of the *clock* gene polyQ domain is also assessed in these populations. The results are discussed in the context of methodological approaches to understanding the genetic basis of seasonal timing and the benefits of assessing photoperiodic response directly for such studies.

How does geography influence vertebrate photoperiodism?

As latitude increases, length of the favorable season decreases, resulting in greater consequences for the mistiming of seasonal development or behavior at higher latitudes. Thus, reliance on photoperiod as a seasonal timer is expected to increase with latitude, while the effects of photoperiod on southern populations are more likely to be mediated by other environmental characters, such as temperature.

This trend is well supported by intraspecific comparisons along latitudinal gradients in insects (reviewed by Danilevskii, 1965). For instance, the correlation between latitude and the photoperiod that terminates diapause is $\geq .95$ in the pitcher plant mosquito *Wyeomyia smithii* (Bradshaw & Holzapfel, 2001). The data available from vertebrates is limited, but also supports this trend. As latitude increases, photoperiod has an increasing effect on metabolic traits in three species of lizards (Lashbrook & Livezey, 1970; Angilletta, 2001; Uller & Olsson, 2003). In northern populations of Scandinavian frogs, photoperiod strictly regulates the timing of reproduction, while in southern populations it interacts with temperature to regulate the timing of reproduction (Laurila *et al.*, 2001). As latitude increases, non-stimulatory photoperiods have increasing control

over gonadal regression in two species of Peromyscus mice (Lynch *et al.*, 1981; Sullivan & Lynch, 1986; Heideman *et al.*, 1999; Lowrey *et al.*, 2000), and embryonic dormancy in several mustelid species (Thom *et al.*, 2004).

Conclusions regarding the effects of latitude on the prevalence of photoperiodism in other vertebrate taxa have been limited for two reasons. First, many studies of vertebrate photoperiodism use model animals that are difficult to raise and maintain in the lab and are impractical to manipulate in experimental conditions that control for the effects of environmental signals beyond photoperiod. Instead, these studies rely upon assumed proxies of photoperiodic time measurement, such as timing of migration, seasonal quiescence, or reproduction. Although these traits are mediated by photoperiodic response, other, unmeasured environmental signals, such as temperature, nutritional availability and presence of con-specifics can affect the timing of their expression (Crozier *et al.*, 2008; Dawson, 2002). Although this type of study is often necessary for non-model organisms that may be of ecological or economic importance, the potential effects of these unmeasured environmental signals can confound interpretation of results. This has limited our ability to understand among population variation in the timing of photoperiodically mediated traits and its effects on life history and local adaptation to the environment.

A second type of study utilizes model organisms that can be studied in laboratory conditions, but only considers, or is only able to consider, individuals from a single, laboratory maintained population. This type of model has produced considerable insights into the transcriptional, hormonal and anatomical responses to photoperiod (Yasuo & Yoshimura, 2009), but the focus on single populations as representative of a species has

limited our ability to understand the genetic and physiological basis of photoperiodic response in natural animal populations, and how it may vary among populations inhabiting different environments.

Bridging the gap between these two types of vertebrate photoperiodic models a model organism with a large natural range that can be raised, maintained, and manipulated in controlled conditions to eliminate the potential effects of environmental signals besides photoperiod. In addition, it must be amenable to the hormonal and genetic techniques necessary to examine the mechanisms that underlie the phenotypic response to photoperiod. Chapter III describes the phenotyping of photoperiodic response in populations of threespine stickleback, a teleost fish found across a wide latitudinal range. These data demonstrate that the threespine stickleback meets these criteria for a model of vertebrate photoperiodic response.

How conserved is the physiological basis of vertebrate photoperiodic response?

The physiological basis of photoperiodic response appears to be conserved across vertebrates. The role of thyroid hormone (TH) in reproduction may have originated in basal chordates (Heyland *et al.*, 2005; Paris *et al.*, 2010) and its control of the photoperiodic initiation of sexual maturation is remarkably similar between mammals and birds (Yasuo & Yoshimura, 2009). Its role in teleost fishes, the most speciose vertebrate clade, is less clear. A full understanding of the role of the TH pathway across vertebrates requires a teleost model in which the physiological basis of photoperiodic response can be studied using hormonal and anatomical techniques. The threespine stickleback is an ideal model for such studies because we have shown that it has a strong

photoperiodic response, which can be assessed in isolation from other environmental factors (Chapter III) and is amenable to techniques necessary to make direct comparisons to mammal and bird models.

Initiation of sexual maturation via photoperiodic response has two basic elements: (1) reception and encoding of the photoperiod signal and (2) a neuroendocrine axis that stimulates gonadotropin production. The anatomical and hormonal basis of these elements will be described in mammals and compared to birds and teleost fishes to highlight areas of conservation, divergence, and those that require study.

Figure 1.1 is a comparative diagram of this process that complements the following sections. It illustrates the high degree of conservation in the hormonal and anatomical basis of the neuroendocrine axis in mammals and birds and the relative lack of comparable data in photoperiodic teleost fishes.

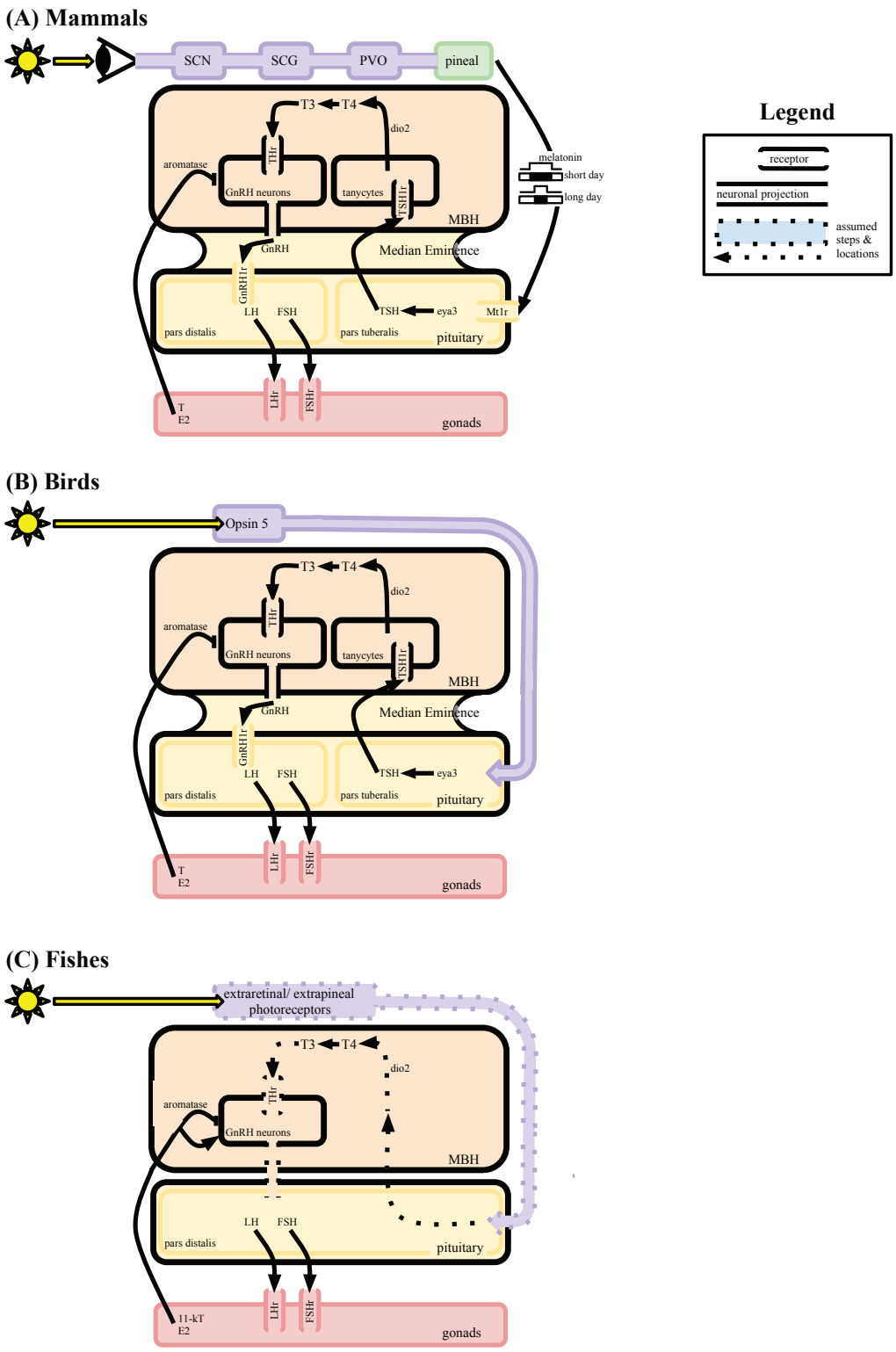


Fig. 1.1. The photoperiodic neuroendocrine axis.

Mammals

Signal reception and encoding

In mammals, the photoperiodic signal is received by the retina and neuronally communicated through the suprachiasmatic nuclei (SCN), the paraventricular nucleus (PVN), and the superior cervical ganglion (SCG) to the pineal gland (Moore *et al.*, 1995). Melatonin production from the pineal gland is inhibited by light and permitted in darkness, creating a daily rhythm where concentration peaks at night. Thus, melatonin secreted from the pineal gland encodes the photoperiodic signal and communicates it to the neuroendocrine axis that mediates sexual maturation (Cassone *et al.*, 1998).

Neuroendocrine axis

A single stimulatory photoperiod (hereafter referred to as a "long day") is sufficient to invoke hormonal response of the neuroendocrine axis. In the pars tuberalis of the anterior pituitary, a long day melatonin rhythm stimulates expression of the transcription factor Eya3 and its coactivators (Dardente *et al.*, 2010; Masumoto *et al.*, 2010). These induce expression of the thyroid stimulating hormone (TSH) β and α subunits (Hanon *et al.*, 2008; Ono *et al.*, 2008; Yasuo *et al.*, 2010). Like the gonadotropins, TSH is heterodimer consisting of protein-specific β subunit and an α subunit (GTHα) that is common to TSH and all gonadotropins.

This long day response is only induced in pars tuberalis cells expressing the melatonin receptor MT1 (Dardente *et al.*, 2003). In fact, the MT1 receptor may be the sole mechanism by which the neuroendocrine axis receives the photoperiodic signal: photoperiod mediates expression of MT1 in the pars tuberalis (Dardente *et al.*, 2003),

especially as the second melatonin receptor subtype is nonfunctional in at least one photoperiodic mammal (Weaver *et al.*, 1996).

TSH travels to the mediobasal hypothalamus (MBH). Here it stimulates production of Deiodinase 2 (*dio2*) from tanycytes lining the third ventricle (Watanabe *et al.*, 2004; Yasuo *et al.*, 2005; Yasuo *et al.*, 2007). One of three deiodinases found in bony vertebrates, the main role of *dio2* is to deiodinate thyroxine (T4) to triiodothyronine (T3). T4 is produced in the thyroid gland and transported via the hypophyseal portal system to the MBH. Here, Dio2 catalyzes the conversion of T4 to T3, which is several times more biologically active than T4.

T3 acts locally to stimulate production of gonadotropin releasing hormone (GnRH) from neurons in the MBH, probably through direct action on the neuronal dendrites. All mammals have GnRH1 and GnRH2 and some have lamprey GnRH3 (l-GnRH3) (Yahalom *et al.*, 1999; Hiney *et al.*, 2002). GnRH1 is the primary hypophysiotropic paralog in mammals as it is primarily expressed in preoptic neurons that have extensive projections to the median eminence (Dubois *et al.*, 2002). GnRH2 is primarily found in the midbrain, although its distribution can overlap with that of GnRH1 (Dubois *et al.*, 2002). It is thought to function as a neuromodulator and may play a role in regulating sexual behavior (Millar, 2005). The function of l-GnRH3 is less clear. Its distribution largely overlaps with GnRH1 and it may regulate FSH secretion in some mammals (Hiney *et al.*, 2002), although the effect of photoperiod upon it has not been studied.

GnRH is released from axon terminals in the median eminence into the pituitary portal system. The portal system transports the GnRH to the pars distalis of the pituitary

(Yasuo & Yoshimura, 2009), where it stimulates gonadotropin secretion in cells expressing the GnRHI receptor (Millar, 2005). Seasonal plasticity of GnRH1 receptor expression varies amongst photoperiodic mammals (Ciechanowska *et al.*, 2008; Townsend *et al.*, 2009). This expression pattern suggests a role in mediating photoperiodic response, but to our knowledge, this has not been studied.

The gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), are heterodimers of protein specific β subunits and GTHα. They are secreted from gonadotropic cells in the pars distalis and released into the bloodstream where they act upon peripheral targets. Their main targets are the gonads, where they stimulate production of the sex hormones. In mammals, the main steroid sex hormones are estradiol in females and testosterone in males (which is intracellularly aromatized at the site of action to estradiol). Feedback of sex hormones on gonadotropin expression is an important mediator of sexual maturation (Shupnik, 1996).

Birds

Signal reception and encoding

In birds, photoperiodic signal reception is extraretinal and does not involve melatonin or the pineal gland (Sharp, 2005). Opsin 5 is expressed in neurons located in the paraventricular organ (PVO) of the photoperiodic Japanese quail (Nakane *et al.*, 2010). These neurons project from the PVO to the median eminence. The median eminence is adjacent to the pars tuberalis of the pituitary, which is the anatomical location of the first hormonal response to a stimulatory photoperiod, which suggests a pathway for photoperiod signal transduction to the neuroendocrine axis. In addition, the

peak wavelength absorbance of Opsin 5 is within the range known to elicit gonadotropin release in quail (Nakane *et al.*, 2010), further suggesting it is the receiver of the photoperiod signal.

Although these results strongly support Opsin 5 as a mechanism for light reception, both melanopsin and vertebrate ancient opsin have similar absorbance spectra and distributions within the brain (Chaurasia *et al.*, 2005; Halford *et al.*, 2009). Given current data, the role of these opsins and that of Opsin 5 in photoperiod signal reception cannot be confirmed.

Neuroendocrine axis

Reception and communication of the photoperiodic signal differs between mammals and birds, but the neuroendocrine axis itself is broadly conserved. As in mammals, the earliest response to a long day occurs in the pars tuberalis, where TSH pulses during the first shortened night (Nakao *et al.*, 2008). As in mammals, the TSH pulse coincides with a pulse in the transcription factor *eya3* expression (Nakao *et al.*, 2008), which suggests a conserved role for its inducement of TSH expression. These pulses during the first shortened night are followed shortly by an increase in *dio2* expression around the third ventricle and a corresponding decrease in dio3 (Yasuo *et al.*, 2005; Nakao *et al.*, 2008). Increased TSH binding around the third ventricle during this initial response suggests it is driving the observed increase in *dio2* (Nakao *et al.*, 2008).

Long day responses of the thyroid hormones in birds mirror those in mammals. Long days cause an increase in T4 and T3 levels in the MBH, although their initial long day response has not been studied (Yoshimura *et al.*, 2003). Dio2 in the MBH catalyzes

the local conversion of T4 to the bioactive T3 (Yoshimura *et al.*, 2003; Yasuo *et al.*, 2005).

T3 stimulates hypophysiotropic GnRH in long day conditions. Birds contain GnRH1 and GnRH2 and, at least in some species, l-GnRH3 (Dubois *et al.*, 2002; Bentley *et al.*, 2004). GnRH1 and l-GnRH3 neurons are distributed in the preoptic area and have extensive projections to the median eminence (Dubois *et al.*, 2002), consistent with a hypophysiotropic function. GnRH2 is found in the midbrain and is thought to function primarily as a neuromodulator (Dubois *et al.*, 2002). As with mammals, the function of l-GnRH3 is largely unknown.

Under long days, GnRH nerve terminals move closer to the boundary of the median eminence, a process that is induced by T3 (Yamamura *et al.*, 2004; 2006). Presumably, T3 acts locally upon the GnRH neurons after it is catalyzed from T4 in the MBH. This change in terminal positioning could facilitate release of GnRH into the median eminence, where it can be transported to the pituitary. It is unknown if mammalian GnRH release is facilitated by the same mechanism, although this would be expected given the conservation of the photoperiodic neuroendocrine axis between the two taxa.

LH and FSH plasma levels have similar profiles over the course of a breeding season in several photoperiodic birds (Silverin *et al.*, 1999), but different mechanisms appear to regulate their release. GnRH1 acts upon the gonadotropic cells of the pars distalis to stimulate the release of LH (Sharp *et al.*, 1998), which can happen after a single long day (Nakao *et al.*, 2008). Expression of the GnRH1 receptor varies throughout the reproductive cycle in at least one bird species (Bedecarrats *et al.*, 2006)

but how this may mediate photoperiodic response is unclear. Although FSH has an important role in the stimulation of sex hormones and gonadal function, the direct mechanism of its regulation is unclear, but is unlikely to be GnRH1 in at least one photoperiodic bird (Proudman *et al.*, 2006). Lamprey-GnRH3 regulates FSH in some mammals, but its role in birds, especially in response to photoperiod is unknown (Leska *et al.*, 2007).

Birds are similar to fishes, but different from mammals in that, separate cells in the pars distalis produce FSH and LH (Proudman *et al.*, 1999; Puebla-Osorio *et al.*, 2002). The functional significance of this difference is unknown.

As in mammals, the main sex hormones in birds are testosterone and estradiol. As in mammals and fishes, sex hormone feedback on gonadotropin expression mediates sexual maturation in response to photoperiod (Dawson & Sharp, 2007). Overall, the hormonal and anatomical basis of the photoperiodic neuroendocrine axis is highly conserved between mammals and birds.

Fishes

Signal reception and encoding

In fishes, photoperiod signal reception appears to be extraretinal and extrapineal (Masuda *et al.*, 2005; Borg, 2010). This suggests that deep brain photoreceptors receive the photoperiodic signal, as in birds. Indeed, multiple photoreceptor types are distributed throughout the brain of two photoperiodic fishes: the Atlantic salmon (Philp *et al.*, 2000) and the common minnow (Álvarez-Viejo *et al.*, 2004). Unlike birds, the wavelength of light does not appear to affect photoperiodic inducement of sexual maturation

(McInernev & Evans, 1970). Although these results are based on a single species, they suggest multiple photoreceptors can receive the photoperiod signal and communicate it to the neuroendocrine axis.

The daily melatonin rhythm has been proposed as an alternative to neuronal communication of photoperiod in some fishes (Migaud *et al.*, 2010). However, methodological limitations make it difficult to distinguish between the potential effects of melatonin as a communicator of the photoperiod signal and its effects on targets downstream of reception and initial response to photoperiod (Mayer *et al.*, 1997; Bromage *et al.*, 2001; Borg, 2010). We can conclude that the melatonin signal does not affect signal reception or downstream response in at least some photoperiodic fish (Masuda *et al.*, 2005; Borg, 2010) and further work is required to distinguish between potential effects on these elements in other fishes.

Neuroendocrine axis

Of the three taxa, the least is known about the neuroendocrine axis in photoperiodic fishes. Hormonal response appears to be largely conserved, but neuroanatomy is not, with differences between the mammal, bird and fish neuroanatomy affecting how hormonal signals are transmitted between the brain and pituitary. It must be noted that anatomical regions within the hormone-secreting adenohypophysis of the pituitary are more distinct in mammals and birds relative to fishes. In mammals and birds, the adenohypophysis contains the pars tuberalis, the location of the earliest hormonal response to long days, and the pars distalis, which secretes gonadotropins in response to GnRH stimulation (Kah & Dufour, 2010). These regions are not

morphologically distinct in fishes. To avoid confusion, they will be referred to in fishes by the corresponding mammalian structure when localization of gonadotropin expression makes this possible.

The earliest long day hormonal response in mammals and birds is a TSH increase in the pars tuberalis, which stimulates Dio2 production in the MBH. There is no clear anatomical analog of the pars tuberalis in fishes (Kah & Dufour, 2010), but TSH is produced in the pars distalis of the fish pituitary (Kasper *et al.*, 2006; Cerdá-Reverter & Canosa, 2009). This distribution of TSH and the lack of a median eminence in higher teleosts (Cerdá-Reverter & Canosa, 2009) suggest that if TSH does stimulate *dio2*, it would do so through neuronal communication between the pars distalis and MBH. For now, its photoperiodic response remains unknown.

As in mammals and birds, fish Dio2 catalyzes the conversion of T4 to the bioactive T3 (Orozco & Valverde, 2005). The long day response of *dio2* and its distribution within the brain of photoperiodic fishes is unknown.

The role of TH hormone in photoperiodically induced sexual maturation in fishes is unclear. Methodology and focus (e.g. TH levels in plasma versus TH levels in the brain) often differ among studies, making it difficult to compare results and make general inferences regarding its possible role. In addition, many of these studies did not control for the potential effects of other environmental signals, including temperature and nutrient availability (Raine, 2010). Nevertheless, there are examples of T3 increasing during early sexual maturation in photoperiodic fishes (Cyr & Eales, 1996; Nordberg *et al.*, 2004). In at least one species, a change in the photoperiod regime is the specific trigger for T3 increase (Cyr *et al.*, 1988). In general, there is a correlation between

reproductive stage and TH levels in seasonally breeding fishes (Cyr & Eales, 1996), although the extent to which photoperiod or other environmental signals mediate TH levels in these fishes is unknown. T3 can stimulate GnRH neurons in fish (Parhar *et al.*, 2000), but this has not been tested in photoperiodic fishes.

To date, three GnRH paralogs have been identified in teleosts: GnRH1 and GnRH2 also occur in mammals and birds, but GnRH3 is unique to teleosts (Kah *et al.*, 2007). As in mammals and birds, GnRH1 neurons are found in the preoptic area. They share a common developmental origin with GnRH3 neurons (Kah *et al.*, 2007), which are found primarily in the ventral telencephalon (Cerdá-Reverter & Canosa, 2009) although GnRH3 distribution often overlaps with GnRH1 (Kah *et al.*, 2007). GnRH2 neurons are distributed throughout the midbrain tegmentum (Cerdá-Reverter & Canosa, 2009).

Any addition functions of the three paralogs beyond stimulation of the gonadotropins are unclear. GnRH1 is considered the hypophysiotropic paralog as it is expressed in neurons that innervate the pituitary and is capable of stimulating gonadotropin production and gonadal development (Cerdá-Reverter & Canosa, 2009). In teleosts where GnRH1 is not present, GnRH3 has been shown to be the hypophysiotropic paralog (Chen & Fernald, 2008; Cerdá-Reverter & Canosa, 2009). GnRH2 is thought to regulate sexual behavior as a neuromodulator (Cerdá-Reverter & Canosa, 2009). This is its primary role in mammals and birds, but this conclusion is based on its distribution within the brain and has not been functionally examined.

Work on GnRH expression in photoperiodic fishes is limited. In the masu salmon and rainbow trout, photoperiod treatment stimulates production of GnRH3 (Amano *et al.*, 1995; Davies *et al.*, 1999), but not of GnRH2 (Bromage *et al.*, 2001). Long days increase

expression of all GnRH paralogs in the photoperiodic pejerrey (Miranda *et al.*, 2009). In the photoperiodic grey mullet, GnRH1 increases, but GnRH3 decreases during sexual maturation (Nocillado *et al.*, 2007). These results indicate that the role of GnRH1 as the main hypophysiotropic paralog is largely conserved among mammals, birds, and fishes, but they also support functional differences among fishes during photoperiodic response.

There are two anatomical differences in gonadotropin secretion among the mammal and bird models relative to fishes that may affect response of the fish neuroendocrine axis to photoperiodic stimulation. First, stimulation of gonadotropin secretion by GnRH neurons must be via direct innervation from the hypothalamus to the pituitary because there is no median eminence in higher teleosts. The pars distalis of at least one photoperiodic fish has extensive GnRH innervation (Andersson *et al.*, 1995), but the effect of photoperiod on its expression has not been studied. Second, in mammals the gonadotropins are produced in the same cells (Childs, 2006), whereas they are produced in separate cells in fishes and birds (Puebla-Osorio *et al.*, 2002; Kanda *et al.*, 2011). The significance of this is unknown, but it may reflect differences in regulation of gonadotropin secretion.

Despite these differences and uncertainty, studies in photoperiodic fishes show that GnRH stimulates the gonadotropins, in manner that is largely similar to its actions in photoperiodic mammals and birds (Davies *et al.*, 1999; Amano *et al.*, 2001; Hellqvist *et al.*, 2006; Miranda *et al.*, 2009; Choi *et al.*, 2010). Long day exposure results in gonadotropin stimulation and these gonadotropins are transported from the pars distalis to the gonads where they induce sex hormone production (Borg, 2010).

Gonadotropins stimulate steroid hormone production from the gonad, which have feedback effects on gonadotropin secretion. The main sex hormones are estradiol and 11-ketotestosterone, although testosterone also has androgenic effects (Borg, 2010).

Section summary

Although the precise mechanisms of photoperiod signal reception and transduction to the neuroendocrine axis have not been established in birds or teleost fishes, all evidence suggests that retinal reception and melatonin communication in mammals are derived traits. Testing this hypothesis requires establishing a teleost model of photoperiodic response in which the techniques necessary to study these mechanisms are practical. Previous work using the threespine stickleback (McInernev & Evans, 1970; Borg, 2010) and data presented herein (Chapter III; Chapter IV) demonstrates that it meets this criterion.

The hormonal and anatomical basis of the photoperiodic neuroendocrine axis is highly conserved between mammals and birds. Available data regarding the function and photoperiodic responses of GnRH and the gonadotropins suggest they are conserved with respect to fishes as well, but there are several important unknowns that make this an open question. First, the functions, locations, and responses of TSH and TH in long days are unknown in fishes. Many studies on photoperiodic fishes do not control for the potential effects of environmental signals beside photoperiod, which has limited our ability to compare results among fishes or to other vertebrate taxa. Furthermore, the lack of a median eminence and differences in pituitary morphology in fishes relative to mammals

and birds have not been examined in the context of the photoperiodic neuroendocrine axis.

Such studies require a teleost fish model that can be manipulated in controlled conditions and is amenable to the techniques whose results can be directly compared to mammals and birds. Chapter III demonstrates that threespine stickleback from multiple populations have a strong photoperiodic response in controlled conditions, which allows phenotypic and physiological results to be interpreted solely in relation the influence of photoperiod. Chapter IV establishes the stickleback as a teleost model for the photoperiodic neuroendocrine axis by measuring gene expression of key hormones in the TH pathway. The findings are discussed in relation to other photoperiodic vertebrates.

Brief outline of this dissertation

Despite the prevalence of photoperiodism within vertebrates, and its obvious ecological significance, important questions regarding its variation with geography and its physiological and genetic foundations remain. These are:

- How do geography and environment affect vertebrate photoperiodism?
- Are the daily circadian clocks and the seasonal photoperiodic timer causally connected?
- Is the physiological basis of photoperiodic response conserved among vertebrates?
- What is the mechanism by which animals interpret photoperiod?
- What is the genetic basis of variation in photoperiodic response?

Progress on these questions has been limited by; (1) a reliance on the candidate gene approach and inappropriate assumptions regarding interpretation of these results, (2) models where consideration of intraspecific variation is impractical or has not been conducted, and (3) models in which we cannot attribute phenotypic changes solely to photoperiodic response and not the correlated effects of other environmental signals. This dissertation addresses these limitations by empirically demonstrating proper methodology for the study of photoperiodic time measurement and by establishing the threespine stickleback as a model of vertebrate photoperiodism.

The threespine stickleback is a small teleost fish with a diversity of phenotypes and life history forms (Bell & Foster, 1994). Long or increasing day lengths stimulate sexual maturation in both sexes (Baggerman, 1985; Yeates-Burghart *et al.*, 2009). Phenological variation has been observed between populations from different latitudes (Borg, 1982; Crivelli & Britton, 1987). This may be due to variation in photoperiodic response, but its contribution cannot be distinguished from the potential effects of other environmental signals. The gonadotropins have an annual cycle in wild-caught individuals, with plasma concentrations peaking early in the breeding season (Hellqvist *et al.*, 2006), which suggests that at least the output of the photoperiodic neuroendocrine axis is conserved among mammals, birds and the threespine stickleback.

Chapter II and part of Chapter III were motivated by a question I've been asked at several conferences: "Shouldn't you be using the circadian clock to understand photoperiodism?" This assumption is particularly widespread in teleost fish biology, where the previously described work in Pacific salmonids is widely accepted. Although this proposed connection is an intuitive solution for control of organismal action on two

time scales, we must consider it an open question because of the untested assumptions described above and the work presented herein.

Chapter II is the result of collaboration between W.E. Bradshaw, C.M. Holzapfel and myself and has been previously published (O'Brien *et al.*, 2011). We address the potential for autocorrelation to produce an assumption of a causal (i.e. necessary) relationship between two variables. Two traits are often assumed to have a causal relationship with one another because both covary with a third factor. We describe a simple method to test for autocorrelation and apply it to the previously described latitudinal clines in Pacific salmon *clock* gene alleles. The results demonstrate that phenological variation and *clock* gene allelic variation are uncorrelated, which means they cannot be causally connected. We suggest this technique as a necessary first step when studying covariation across geography or any phenomena where a causal relationship is being investigated and autocorrelation may exist.

Chapter III is the result of collaboration between L. Unruh, C. Zimmerman, W.E. Bradshaw, C.M. Holzapfel, W.E. Cresko and myself. Threespine stickleback populations from Alaska and Oregon were raised in a common environment and phenotyped for photoperiodic response. We show that all populations are photoperiodic and there is no difference in response within or between the two latitudes. This result was unexpected, as the difference between Alaska and Oregon (~ 18°) is generally large enough to demonstrate intraspecific genetic variation in photoperiodic response or proxies of photoperiodic response (reviewed in Danilevskii, 1965; Bradshaw & Holzapfel, 2007). We suggest that this constancy of response in the lab may be mediated by differences in response to increasing temperature in the wild. These results are the first steps in

establishing the threespine stickleback as a vertebrate model of photoperiodic response. By phenotyping multiple, outbred populations absent of maternal or field effects, we demonstrate the utility of the threespine stickleback for the study of photoperiodic response.

In Chapter III, we also show that there is no partitioning of variation in threespine stickleback *clock* allele length within or between the two latitudes. We discuss these results in the context of the assumption that *clock* allelic variation affects photoperiodic response through its role in the circadian clock. Our data is further evidence that the role of *clock* in seasonal timing is equivocal at best. We urge caution in the interpretation of allelic variation across geographic gradients.

Chapter IV is the result of collaboration between R. Bourdo, W.E. Bradshaw, C.M. Holzapfel, W.E. Cresko and myself. Photoperiodic response of the neuroendocrine axis that initiates sexual maturation is conserved between mammals and birds, but it is unknown if it is observed across vertebrates, including teleost fishes. To fully address this hypothesis, studies comparable in detail and technique to those performed in tetrapods must be conducted in teleost fishes. We quantified the photoperiodic response of genes coding for key hormones in the thyroid hormone pathway in three populations of threespine stickleback. These data were complemented with spatial expression analysis when anatomical location of a hormone's response in fish was unknown. Response of the neuroendocrine axis implicated the thyroid hormone pathway in the photoperiodic initiation of sexual maturation and was robust among the study populations.

This study is the first to examine early photoperiodic response of the neuroendocrine axis in a teleost fish in highly controlled experimental conditions.

Chapter III shows that the phenotypic response to photoperiod does not differ between two latitudes. Herein we demonstrate that the neuroendocrine basis of this response is also consistent. These data strongly support conservation of the photoperiodic neuroendocrine axis among mammals, birds and fishes, which suggests that results from the study of threespine stickleback photoperiodism can be applied to our understanding of vertebrate photoperiodism in general.

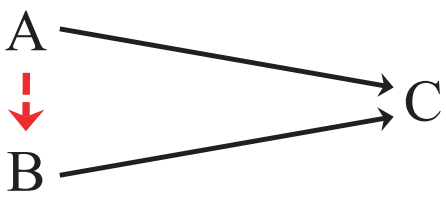
Finally, Chapter V summarizes the results from Chapters II – IV, discusses how they have contributed to the study of biological timing and our understanding of vertebrate photoperiodism, and suggest future avenues of research.

CHAPTER II

TESTING FOR CAUSALITY IN COVARYING TRAITS: GENES AND LATITUDE IN A MOLECULAR WORLD

A paper published in *Molecular Ecology*, authored by C. O'Brien, William E. Bradshaw, and Christina M. Holzapfel

With the advent of modern molecular techniques, increasing attention is being paid to non-model organisms for investigating the genetic basis of various phenotypes in physiological, ecological or geographical contexts. As genes are discovered that covary with an environmental parameter such as temperature, light or latitude, there is a natural temptation to ascribe causality to these correlations. However, correlations are only the tantalizing starting points for robust experimental designs and, in themselves provide evidence for neither causality nor an underlying functional mechanism. Herein, we use covariation of traits with latitude to illustrate the problem of confounding causation and correlation over geographic gradients. We begin with a simple diagram:



If A is correlated with C and B is correlated with C, then A will automatically be correlated with B. There follows the natural temptation to infer or conclude that A causes B, that is genetic variation in A constitutes the genetic basis of B. As an example, we consider the relationship between the circadian clock regulating daily activities of organisms and the photoperiodic timer regulating seasonal activities of organisms. This relationship has a long and contentious history (Tauber & Kyriacou, 2001; Hazlerigg & Loudon, 2008; Bradshaw & Holzapfel, 2010; Saunders, 2010; Koštál**,** 2011), a legacy of Bünning's (1936) proposition that the circadian clock formed the causal basis of photoperiodism. At the molecular level, a probabilistic cause between circadian rhythmicity and photoperiodism occurs in plants (Kobayashi & Weigel, 2007; Wilczek *et al.*, 2009) and in a long-established laboratory strain of Syrian hamsters (Shimomura *et al.*, 1997; Lowrey *et al.*, 2000). However, there are no examples where the circadian clock has been shown to be necessary, let alone sufficient for regulating photoperiodic response in natural populations of any animal. Yet, elements of the circadian clock have been shown to vary with latitude, as have phenotypes of the photoperiodic timing mechanism (Fig. 2.1). Therein lies the problem: Covariation is not proof of causation.

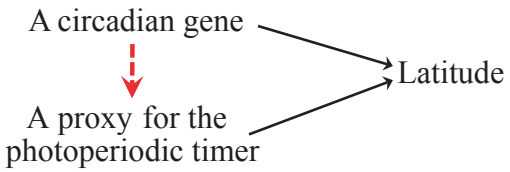


Fig. 2.1. Inference of causality between circadian rhythmicity and photoperiodism due to their common covariation with the independent variable, latitude. If allelic variation in a circadian gene is correlated with latitude and a proxy for the photoperiodic timer is correlated with latitude, the incorrect conclusion could be drawn that the circadian clock forms the causal basis of the photoperiodic timer, that is that the circadian clock is necessary for or forms the mechanistic basis of photoperiodic time measurement. In fact, the circadian clock, the photoperiodic timer, and an endless array of other variables are correlated with latitude but are not necessarily causally connected.

The seasonal timing of life-history events, which is typically orchestrated by the photoperiodic timer, is correlated with latitude in both plants (Wilczek *et al.*, 2009) and animals (Bradshaw & Holzapfel, 2007). An increasing number of circadian-related genes are now known to vary with latitude in *Neurospora* (Michael *et al.*, 2007), plants (*Arabidopsis*: Michael *et al.*, 2003; Caicedo *et al.*, 2004; Stinchcombe *et al.*, 2004; Glycine: Zhang *et al.*, 2008), *Drosophila* (Kyriacou *et al.*, 2008; Rand *et al.*, 2010), fish (O'Malley & Banks, 2008a, O'Malley *et al.*, 2010), birds (Johnsen *et al.*, 2007), and humans (Cruciani *et al.*, 2008). Given the observation that both circadian genes and photoperiodically mediated seasonal traits vary with latitude, the tendency is to conclude a causal connection between the circadian clock and the photoperiodic timer based on their latitudinal covariation.

The covariation of two traits with latitude could indeed be due to a common causal mechanism (pleiotropy), in which case an interesting relationship has been established and the question then becomes resolving the mechanistic basis of their coevolution. However, while latitude usually and appropriately serves as a composite variable, latitudinal variation represents multiple environmental factors, any one or a combination of which could be exerting parallel selective forces. The covariation of two traits with latitude could be a result of different selective forces acting on the two traits, the same selective force acting on two genetically independent traits, or a single selective force acting on one trait accompanied by genetic hitchhiking of a closely linked trait (Li, 1997; Schluter *et al.*, 2004; 2010). Examination of the relationship between variables can be made using techniques described in Sokal and Rohlf (1995): partial correlation examines the relationship between two variables, while all the other correlated variables

are held constant; path analysis incorporates simultaneously the contribution of several correlated variables. While useful, these statistics are complex, may suffer from collinearity of the independent variables (Petraitis *et al.*, 1996), are not readily accessible in many statistical packages and heretofore have not incorporated discrete variables. We are proposing a more transparent test that requires little more than a hand calculator or an Excel spreadsheet and incorporates both linear regression and analysis of variance. Below, we provide examples from flies and fish to illustrate the simplicity and usefulness of the analysis of residuals to avoid a spurious conclusion of causation when only correlation exists. When Y is regressed on X, the regression equation, $\hat{Y} = a + bX$ plots the regression line and $Y_i - \hat{Y}$ = deviations from regression (residuals). The residuals are zero correlated with *X*, that is the effect of *X* on *Y* has been factored out. If A is a causal element of B and both are correlated with latitude, then even when the common element of latitude is factored out the residuals should still be correlated; if not, their common correlation with latitude is due to linkage or independent evolution and not due to a basic underlying causal relationship between A and B. When A or B is a discrete and not a continuous variable, the residuals are computed as deviations from mean latitude for each category of *Y*. Although applicable to the covariation or association of any two traits or processes with any environmental parameter, we continue with examples from the biological timing literature. To illustrate the test, we have chosen two specific examples because of their connection with latitude, because of the large number of sample populations over a wide latitudinal range, and because the numerical data were available in the source papers. This sort of analysis was not possible for most of the papers we read because either the sample size was too small or the tabular, numerical data from which

figures were generated were not available either in the body of the text or in supplemental online material. The advent of requiring the posting of such data (Fairbairn, 2011) will make subsequent verification via independent analysis tractable.

First, in *Drosophila littoralis*, Lankinen (1986) found significant correlations between latitude and a proxy for the photoperiodic timer (critical photoperiod necessary to induce adult diapause) and between latitude and the two most fundamental properties of any circadian rhythm (the period and amplitude of its oscillation) (Fig. 2.2). Insightfully, he factored out the common effect of latitude and showed that the residuals of critical photoperiod were no longer correlated with the residuals of either period or amplitude of the circadian eclosion rhythm. Hence, he proposed that their covariation with latitude was due to linkage and not a causal relationship between them.

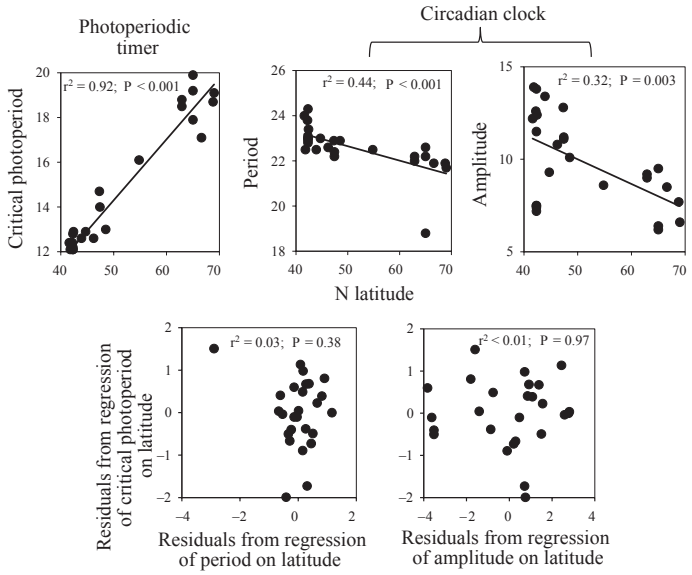


Fig. 2.2. Use of residuals to test for a causal relationship between circadian rhythmicity and photoperiodism in *Drosophila littoralis*. (Top) Latitudinal covariation in photoperiodic response (critical photoperiod) and two fundamental properties of the circadian clock, period and amplitude of the oscillation; (Bottom) lack of correlation between deviations from regression of critical photoperiod, period and amplitude on latitude. Any significant relationship between photoperiodic response and properties of the circadian clock is eliminated when the common element of latitude is factored out (plotted from Table 2 in Lankinen, 1986). Details of analyses are in Appendix 2.1.

To verify this conclusion, Lankinen and Forsman (2006) crossed two extreme populations, allowed free recombination and then imposed selection for nondiapause on short days. The hybrid lines exhibited a more 'southern' photoperiodic response and a more 'northern', circadian-based eclosion rhythm than found in any of Lankinen's original geographic strains (Fig. 2.3), that is the reverse of what would have been expected had the circadian clock been a causal component of photoperiodism. These experiments confirmed Lankinen's earlier conclusion (1986) that when the common effect of latitude was factored out, critical photoperiod was not correlated with either fundamental property of circadian rhythmicity. More generally, Lankinen and Forsman's (2006) experiments confirmed the robustness of testing for a potentially causal connection between two traits by using residuals to factor out their common, correlated element.

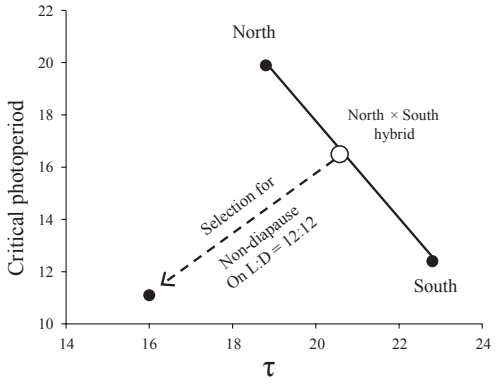


Fig. 2.3. Verification of analysis of residuals as a test for a causal relationship between photoperiodism and circadian rhythmicity in *D. littoralis* by response to selection on critical photoperiod and period (**s**) of the circadian oscillation in *D. littoralis*. A northern and a southern population were hybridized, maintained for eight generations on constant light (L:L) to allow free recombination, selected for nondiapause under short days (L:D = 12:12) for 30 generations, maintained in L:L for 10 generations, and the descendents of a full-sib pair maintained in L:L for a further six generations (plotted from data in Lankinen & Forsman, 2006).

Second, in Chinook salmon, *Oncorhynchus tshawytscha*, O'Malley and Banks (2008) found a significant correlation between latitude and their proxy for the circadian clock (length of the polyglutamine repeat in the gene *OtsClock1b*, hereafter, Poly Q) (Fig. 2.4A). They also found a significant association between latitude and their proxy for the photoperiodic timer (run time = seasonal timing of upstream migration in freshwater) (Fig. 2.4B). O'Malley and Banks (2008, p. 2813) conclude with the suggestion 'that length polymorphisms in *OtsClock1b* may be maintained by selection and reflect an adaptation to ecological factors correlated with latitude, such as the seasonally changing daylength.' After extending their correlative analyses to three more species of salmon (*Oncorhynchus*), O'Malley *et al.*, (2010, p. 3705) state more boldly that the '*Clock* gene is a central component of an endogenous circadian clock that senses changes in photoperiod (day length) and mediates seasonal behaviors.' At the heart of the conclusion is the association between latitude, Poly Q and the timing of migration and spawning. This conclusion makes at least three essential, but untested assumptions.

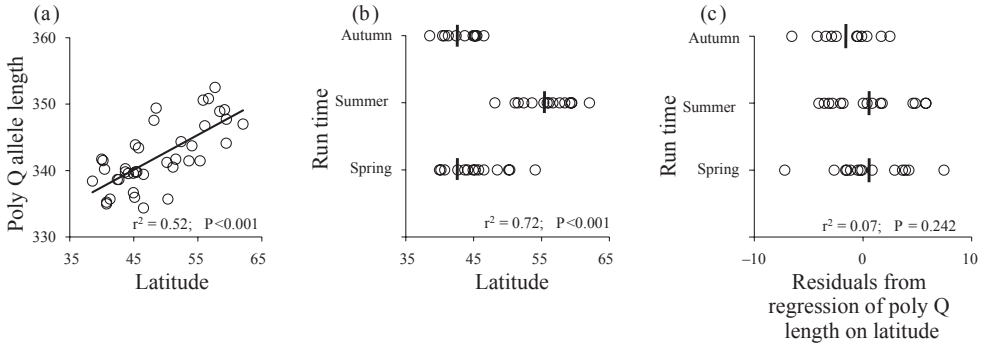


Fig. 2.4. Latitudinal covariation of mean *OtsClk1b* Poly Q domain length (Poly Q) and run (migration) time in Chinook salmon. (a) r^2 = coefficient of determination from the regression. The plot is redrawn from data from Table 3 in O'Malley & Banks (2008); n = 40, omitting the single 'W' and undefined 'F' runs as did O'Malley & Banks; (b-c) vertical lines show means; r^2 = reduction in total sum of squares from one-way ANOVA. Plots and analyses are based on the same data set as in (a). Details of analyses are provided in Appendix S2.2.

First, the conclusion requires that a single genotype (high frequency of the 335 allele and concomitant low frequency of the 359 allele) of the Chinook Poly Q domain is the primary determinant of two different run times, spring and autumn (Fig. 2.4B), even in the same river. This assumption may or may not be true. Second, the conclusion assumes that the salmon specific *OtsClock1b* plays a functional role in salmon circadian rhythmicity. There are two *Clock* paralogs in salmon: *OtsClock1a* and *OtsClock1b*, only the latter of which shows a significant correlation with latitude.

However, the assumption that *OtsClock1b* has the same functional role in salmon as its ortholog in the mammalian circadian clock (Baggs *et al.*, 2009) is untested. Third, the conclusion assumes that there is a causal relationship between the daily circadian clock and the seasonal photoperiodic timer. This assumption is at best contentious (Bradshaw & Holzapfel, 2010; Saunders, 2010; Koštál, 2011) and has not been tested in any fish. There is then a great leap from observing a correlation between latitude and only the *OtsClock1b* paralog and a correlation between latitude and run time or spawning date to concluding that the circadian clock is responsible for the evolution of photoperiodism and, hence, seasonal timing (O'Malley *et al.*, 2010). Strictly for purposes of illustration, we assume the first two of the above three assumptions to be true. We then use Lankinen's (1986) approach of analyzing residuals to test for an association between Poly Q and run time by factoring out the effect of latitude on Poly Q. In this case, Poly Q is a continuous variable and run time is a discrete variable. We therefore calculated the residuals from regression of Poly Q on latitude (Fig. 2.4A) and performed one-way ANOVA of the residuals using run time as treatments. After factoring out the effect of

latitude, run time accounted for a non-significant 7% of the residual variation in Poly Q (Fig. 2.4C). We therefore conclude that there is no basis to infer or suggest a causal relationship between them, either as a direct, independent effect of Poly Q on run time or as an indirect effect of Poly Q on the circadian clock. Further discussion of the adaptive significance of Poly Q in relation to run time is unwarranted, as is any speculation about a potential connection between the circadian clock and the seasonal photoperiodic timer. Future research might well be directed towards determining the function and adaptive significance of *Clock1b* in salmon in the context of the circadian clock itself, much as have other studies in diverse organisms (Yerushalmi & Green, 2009).

Hence, we propose that before inferring a causal relationship in similar cases of covariation of two or more traits with a third physiological or ecological independent variable, that a straightforward analysis of deviations from the common independent variable be used. Absent a significant association, no causal relationship should be inferred or suggested. Even an inference of a causal relationship would be reasonable only if all of the following were true: (i) Variation in each trait is significantly correlated with a third common element, in our case, with latitude. (ii) The significant correlation between the two traits persists after the effect of the common element is factored out. (iii) The environmental conditions used to show the correlations in (a) and (b) were in the same organism and determined under the same conditions.

Note that our test accommodates the situation where both the trait and the gene are associated with latitude in the same way. In that case, their latitudinal covariation is due to an environmental factor(s) selecting concomitantly on both the gene and the trait; no correlation between them should persist once the latitude-dependent causal

environmental factor(s) is accounted for. If the relationship between the gene and the trait is due to an underlying causal connection, then a significant correlation between them should persist independently of latitude.

Significant, positive results from analysis of residuals serve as a point of departure for future experiments but, in of themselves, do not substitute for an understanding of the functional connection between genotype and phenotype (Kingsolver & Schemske, 1991; Petraitis *et al.*, 1996; Dalziel *et al.*, 2009; Blackman 2010; Storz & Wheat, 2010). Successful connections between molecular variation and functional phenotypes have been established (but only after additional study) in both model organisms such as *Drosophila* (Schmidt *et al.*, 2008; McKechnie *et al.*, 2010; Paaby *et al.*, 2010) or *Arabidopsis* (Wilczek *et al.*, 2009) and in natural populations of non-model organisms such as the house mosquito, *Culex pipiens* (Labbé *et al.*, 2009), lizards (Rosenblum *et al.*, 2010), and organisms cited by Storz and Wheat (2010) and Dalziel *et al.*, (2009), their Appendix S1, Supporting information), including killifish, butterflies, garter snakes, deer mice, oldfield mice, threespine stickleback, and Darwin's finches.

With the advent of tractable molecular approaches in an increasing number of non-model organisms with interesting physiological or ecological backgrounds, there will be increasing impetus to ascribe an adaptive significance to molecular genetic variation. Because postglacial climate change has established many eco-climatic selection gradients across latitudes in nature, any correlation between molecular variation in SNPs, nonsynonymous substitutions or transcriptional profiles with latitude provides a tempting avenue for concluding an adaptive significance for the observed genetic variation. Instead of proposing untested suggestions or implications because of their inherent plausibility,

investigators should first examine residuals as described herein. If non-significant, further discussion or speculation of the potential adaptive significance of their covariation is not warranted. If significant, then an inferred causal connection can be used as a platform from which to seek a functional connection between genotype, phenotype and, ultimately, fitness.

Bridge

In Chapter II, we described how residuals analysis should be used as an initial test of causality when interpreting the relationship between two factors that both covary with latitude. We apply it to two examples, one of which has been interpreted as evidence that the circadian clock is causally involved in photoperiodism. Analysis of this relationship shows that there is no association once the common effect of latitude is factored out. Thus, further investigation of the circadian clock and its relationship to photoperiodism in this context is inappropriate. In Chapter III, we examine this same gene across two latitudes in the threespine stickleback *Gasterosteus aculeatus* and phenotype multiple the photoperiodic responses of multiple populations of threespine stickleback.

CHAPTER III

GEOGRAPHY OF THE CIRCADIAN GENE *CLOCK* AND PHOTOPERIODIC RESPONSE IN WESTERN NORTH AMERICAN POPULATIONS OF THE THREEPSINE STICKLEBACK

An unpublished paper submitted to the *Journal of Fish Biology,* authored by C. O'Brien, L. Unruh, C. Zimmerman, W. E. Bradshaw, C. M. Holzapfel and W. A. Cresko.

Introduction

Proper timing of seasonal events in the life histories of organisms is a key component of fitness at temperate and polar latitudes. A wide variety of animals use the length of day (photoperiodism) to anticipate and prepare in advance for future seasonal changes (Bradshaw & Holzapfel, 2007a). Over 70 years ago, Erwin Bünning (1936) proposed that the circadian clock that organizes the daily activities of organisms also formed the basis of the seasonal photoperiodic timer. Evidence for this proposition is strongest in plants (Kobayashi & Weigel, 2007; Wilczek *et al.*, 2009) and highly inbred strains of the golden hamster (Shimomura *et al.*, 1997; Lowrey *et al.*, 2000). Otherwise, the connection between the two physiological processes remains highly contentious (Hazlerigg & Loudon, 2008; Goto *et al.*, 2010; Bradshaw & Holzapfel, 2010a,b; Saunders, 2010; Koštál, 2011; Schiesari *et al.*, 2011). Historically, causal connections

between the daily circadian clock and the seasonal photoperiodic timer were inferred from parallel peculiarities of their physiological behavior to exotic light:dark cycles (vaz Nunes & Saunders, 1999; Tauber & Kyriacou, 2001; Goldman, 2001; Saunders, 2002, 2010, 2011).

With the advent of tractable molecular techniques, a common approach to examine the relationship of circadian and photoperiodic timers has been to use circadian clock genes as candidate loci and then to seek a correlation between mutations or knockdowns of those genes and variation in diapause response in photoperiodic insects (Saunders, 1990; Goto *et al.*, 2006; Stehlík *et al.*, 2008; Han & Denlinger, 2009; Ikeno *et al.*, 2010). The expression of diapause involves a neuroendocrine pathway and it is not clear whether variation in diapause response is due to the effect of the circadian clock on photoperiodism, which is the desired result by the authors, or to an individual clock gene somewhere in the neuroendocrine pathway leading to diapause independently of photoperiodism (Bradshaw & Holzapfel, 2007b; Emerson *et al.*, 2009; Bradshaw & Holzapfel, 2010a, 2010b; Schiesari *et al.*, 2011).

Three logical associations have led investigators to ask whether evolution of the photoperiodic timer, especially over latitudinal gradients, is associated with allelic variation in candidate circadian clock genes segregating in natural populations (Tauber *et al.*, 2007; Mathias *et al.*, 2007; Liedvogel *et al.*, 2009; O'Malley *et al.*, 2010). First, photoperiodism is a physiological mechanism for anticipating seasonal change and preparing in advance for that seasonal change. Second, seasonal environments change with latitude. Third, the timing of seasonal activities (phenology) changes with latitude. The speculative leap in logic is then to assume that any correlation between a circadian

clock gene and latitude or phenology implies a causal connection between the circadian clock and photoperiodism.

The canonical circadian gene *clock* has been the focus of several studies seeking to relate variation in C-terminal polyglutamine domain length within the circadian gene *clock* (PolyQ) to infer a role of the circadian clock in photoperiodism. In *Drosophila melanogaster* deletion of two of the three PolyQ domains of *clock* resulted in altered circadian behavior (Darlington *et al.*, 1998). In mice, excision of a glutamine-rich exon also resulted in altered circadian behavior (King *et al.*, 1997). These findings provided the point of departure for studies aimed at correlating variation in PolyQ with latitude (Johnsen *et al.*, 2007) or with seasonal events acting as a presumptive proxy for photoperiodism in nature (Liedvogel *et al.*, 2009; O'Malley & Banks, 2008a; O'Malley *et al.*, 2010). However, correlation does not demonstrate causation (Kingsolver & Schemske, 1991; Petraitis *et al.*, 1996; O'Brien *et al.*, 2011). In fact, none of the aforementioned studies actually determined photoperiodic response directly or sought to determine the relationship between PolyQ and photoperiodism under controlled conditions free from maternal or field effects.

Herein, we determine variation in PolyQ and in photoperiodic response as measured by sexual maturation of the threespine stickleback *Gasterosteus aculeatus* L. in northwestern North American populations from Oregon and Alaska (18° difference in latitude). *Gasterosteus aculeatus* is found from marine to freshwater habitats (Bell & Foster 1994), shows extensive population-level variation in phenology in natural populations (Borg, 1982; Crivelli & Britton, 1987), and has been shown to be photoperiodic in both wild-caught (Baggerman, 1985; Bornestaf & Borg, 2000) and

laboratory-reared populations (Yeates-Burghart *et al.*, 2009). Among wild-caught fishes from the Baltic Sea (c. 56-59°N), long days promote reproduction in the late spring and early summer (Borg, 1982; Borg & Van Veen, 1982; Borg *et al.*, 2004). In males, sexual maturation is manifest through increased bright body coloration, territoriality, nest building, courtship, and hypertrophy of the kidney to produce spiggin, the glue used for nest construction (Borg, 1982; Borg *et al.*, 2004; Mayer *et al.*, 2004). Kidney hypertrophy is therefore a reliable indicator of sexual maturity in males. In females, sexual maturation is manifest through increased ovarian mass as a consequence of oocyte maturation (Baggerman, 1972, 1985; Bornestaf *et al.*, 2001; Mayer *et al.*, 2004).

Materials and Methods

Photoperiodic response

Northern (Alaskan) stocks were established from Bear Paw Lake (61°37'N, 149°45'W) and Rabbit Slough (61°34' N, 149°15'W). Southern stocks (Oregon) were established from Cushman Slough (43°36'N, 124°2'W) and Eel Creek (43°35'N, 124°11'W). The animals used for these experiments were G_7 (AK), G_1 (Eel Creek, OR), and G_2 (Cushman Slough, OR) outbred descendants of wild-caught individuals. All collection and care of fish conformed to approved animal care protocols.

The experimental fish were produced, hatched and reared using standard protocols (Cresko *et al.*, 2004; Yeates-Burghart *et al.*, 2009). Briefly, experimental fish were reared on a 10L:14D cycle for 11 – 12 months (Alaska fish) or 11 months (Oregon fish). All fish used in the experiment were at least 50 mm standard length (SL), measured from the dorsum of the pre-maxilla to the end of the caudal peduncle. Within each stock,

fish from several parental lines were pooled and split into male-female pairs for the experiments. Experiments were run in light-tight air-cooled cabinets in climate-controlled rooms at 20°C. Aquaria were visually separated and cleaned separately to avoid the possibility of transferring visual or hormonal cues between aquaria. Fish from each population were exposed to six different photoperiod regimes, ranging from 8L:16D to 23L:1D. Fish that died were not replaced. At the end of six weeks, all surviving fish were included in the data set.

To quantify sexual maturation, the ovary-somatic index (I_O) and the kidney-somatic index (I_K) were determined. Kidneys or ovaries were dissected out and transferred to 37°C with the respective soma in a desiccator containing Drierite (www.drierite.com) until there was no decrease in mass between two successive weighings. Ovaries, kidneys and soma were weighed using a Mettler AT261 DeltaRange electronic balance (mt.com). I_O and I_K were calculated as the ratio of ovary and kidney to total body mass, respectively. I_O and I_K values were raised by 10^3 before log transformation to ensure positive values on a log scale.

Clock *polyglutamine domain*

Northern (Alaskan) collections were made from Bear Paw Lake, Rabbit Slough, Hidden Lake (60°29’N, 150°16’W), and Anchor River (59°45’N, 151°30’W). Rabbit Slough and Anchor River are populations in oceanic environments, whereas Bear Paw Lake and Hidden Lake are isolated freshwater populations. Southern (Oregon) collections were made from Eel Creek, Winchester Marsh (43°16’N, 124°19’W), Miner Creek (43°20’N, 124°22’W), and the junction of the Smith and Umpqua Rivers (43°43’N,

124°05’W). All fish were collected using unbaited minnow traps, anesthetized in MS-222 (Aquatic Eco-systems) and preserved in 200 proof ethanol. DNA was extracted from caudal fin clips using a MasterPure DNA Purification Kit (Epicentre).

The human *clock* ortholog (Ensembl ID ENSG00000134852) was BLASTed against the threespine stickleback genome (Ensembl) to find the gene *clock*. Reciprocal Best Hit (RBH) analysis was then conducted to ensure that the resulting gene was the only *clock* paralog in the stickleback genome. To do so the putative stickleback ortholog was BLASTed against the human genome. The best match that it returned was reciprocally BLASTed against the stickleback genome to ensure that its best match was stickleback *clock*. As an additional check, syntenic analysis of the genomic regions surrounding the *clock* orthologs was performed. The synteny database detects synteny between a specified genomic region (in this case, the genomic region surrounding stickleback *clock*) and regions from an outgroup genome (the human genome) using automated RBH analysis (Catchen *et al.*, 2009).

All further sequence annotation and analysis used Geneious Pro 4.7.6 software (Invitrogen). The stickleback *clock* gene was annotated by identifying exons using Ensembl’s automatic gene annotation (Curwen *et al.*, 2004), and then confirmed by comparing the translated protein against the amino acid sequence of other, annotated paralogs. The polyQ domain was apparent in the reference sequence as a region containing only glutamines and a single arginine.

To sequence the polyQ domain, we used flanking primers: a forward primer (CAGGGAGGTCAAACCCAGAC) located on exon 19 of *clock* and a reverse primer (TACTGTGGTTGGCTGCTGAC) located in the 3' UTR. These primers were designed

using NCBI Primer Design (NCBI). PCR products were amplified in an MJ Research PTC-200 (Applied Biosystems): 95˚C three minutes, 32 cycles of 95˚C 30s, 60˚C 30s, 72°C 60s, single cycle of 72°C 7 minutes. Because of a high degree of heterozygosity, PCR products were not sequenced directly, but instead were cloned into a pCR® 4-TOPO® vector (Invitrogen) and sequenced using a 3130x Genetic Analyzer (Applied Biosystems). In order to capture variation in polyQ length among alleles within individuals, multiple TOPO clones were sequenced from each individual.

Resulting sequences were translated and the polyQ domain was manually annotated in ten fish from each population. Sequences with low quality scores in the domain were discarded and re-sequenced. The number of glutamines within the polyQ domain was counted and the positions of the arginine within the polyQ domain were recorded.

Analyses

We used Microsoft Excel (Microsoft) for linear and quadratic regressions. For regressions of I_O or I_K on day length, linear regression was always significant ($P < 0.003$); in no case did the addition of a quadratic term significantly increase the reduction in total sum of squares. We therefore used linear regression for all analyses. We used JMP IN 4 (Sall *et al.*, 2005) for ANOVAs. In the latter case, we modeled latitude (AK = north vs. OR = south) and day lengths as fixed effects. Variation between populations within latitudes was incorporated into the error term.

Results

Photoperiodic response is very similar across populations at northern and southern latitudes

Sexual maturation in both males and females from both northern and southern latitudes increased with day length (Fig. 3.1). The kidney:somatic index (I_K) depended on day length (Two-way ANOVA: $F_{5,219} = 19.4$; $P < 0.001$) did not differ between northern and southern males ($F_{1,219} = 2.31$; $P = 0.130$) and there was no latitude by photoperiod interaction ($F_{5,219} = 0.43$; $P = 0.829$). The ovary:somatic index (I_O) depended upon day length ($F_{5,230} = 20.26$; $P < 0.001$) and was higher in southern than northern females ($F_{1,230} = 9.28$; $P = 0.023$) but there was no significant latitude by photoperiod interaction ($F_{5,230} = 0.91$; $P = 0.477$). These results show that while sexual maturation increased with day length at both latitudes (Fig. 3.1) photoperiodic response did not differ between northern and southern latitudes (no significant photoperiod by latitude interaction).

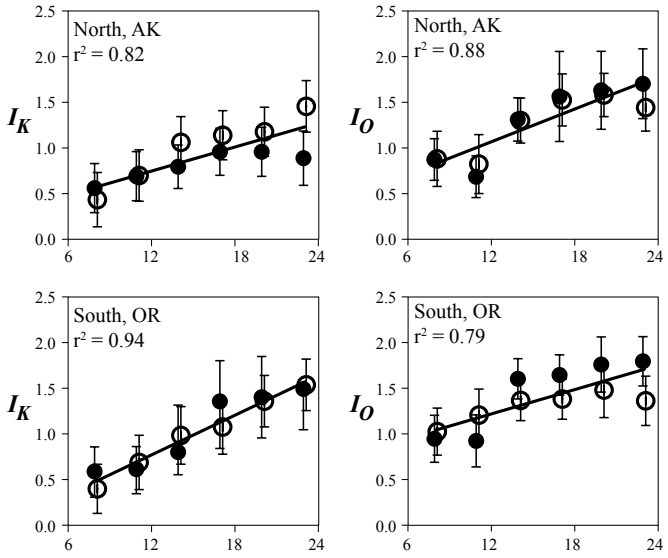


Fig. 3.1. Photoperiodic response of male and female threespine stickleback (*Gasterosteus aculeatus*) in Oregon (43.5°N) and Alaska (61.5°N) in western North America. Male response is represented by kidney:body mass ratio (I_K); female response is represented by the ovary:body mass ratio (I_O). Open circles show results from Yeates-Burghart *et al.* (2009); closed circles show results from the present study. Error bars are ± 2SE.

The polyglutamine domain of clock *varies across individual stickleback but shows no population structuring*

The BLAST search and syntenic analysis found one *H. sapiens clock* ortholog in the stickleback genome (Ensembl ID ENSGACG00000015939) (Supplementary Fig. 3.1). *Gasterosteous aculeatus clock* contains 20 exons from bp 489,361 – 499,374 on linkage group IX (Ensembl). Examination of the sequence shows that the polyQ domain is located in exon 20.

The polyQ domains (Fig. 3.2A) contained between 22 and 38 glutamine repeats and did not differ between latitudes (Nested ANOVA: $F_{1,6} = 0.74$; $P = 0.422$) or among populations within latitudes ($F_{6,72} = 1.162$; $P = 0.336$) . An arginine residue (Fig. 3.2B) occurred within each of the PolyQ domains between positions 2 and 26. Mean position of the arginine residue did not differ between latitudes ($F_{1,6} = 0.533$; $P = 0.493$) or among

populations within latitudes ($F_{6,72} = 0.907$; $P = 0.495$). These results show that there is no significant difference in either length of the PolyQ domain or position of the arginine residue within the PolyQ domain between latitudes or among populations within the northern (AK) and southern (OR) latitudes.

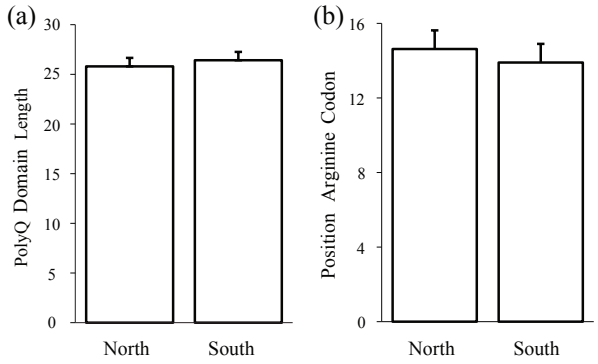


Fig. 3.2. Polyglutamine domain (PolyQ) in the *clock* gene in southern (Oregon) and northern (Alaska) populations of *G. aculeatus*. (a) Domain length in number of glutamine repeats; (b) position of the arginine codon within the polyglutamine domain. Error bars are ± 2SE.

Discussion

Stickleback have similar photoperiodic responses at northern and southern latitudes

Previously (Yeates-Burghart *et al.*, 2009), found that photoperiodic response of a single southern (Oregon) population exhibited no significant variation with photoperiod in either ovarian development or male kidney enlargement whereas a single northern (Alaska) population exhibited a strong photoperiodic response. After using replicate populations within Oregon and Alaska (Fig. 3.1), it is now clear that threespine stickleback are photoperiodic at both latitudes and do not differ in photoperiodic response between latitudes. This pattern is inconsistent with other vertebrates where photoperiodic response tends to increase with latitude and northern populations typically exhibit a

stronger photoperiodic response than southern populations (Bradshaw & Holzapfel, 2007a). In both Yeates-Burghart *et al.* (2009) and the present study all experiments were run at 20°C using laboratory-reared fishes where field and maternal effects were minimized. Experimental fishes consisted of a single male paired with a single female that were visually and chemically isolated from other experimental fish and, hence, represented independent replicates. Consequently, the similarity in their photoperiodic responses cannot be ascribed to phenotypically plastic responses to a variable environment or to visual or water-borne cues. We therefore conclude that genetically determined photoperiodic responses do not differ between Oregon and Alaskan populations separated by ~18° of latitude.

Constancy of photoperiodic response in a common laboratory environment does not necessarily translate into a constant physiological response to natural environments over a latitudinal gradient. In threespine stickleback from the field, gonadal maturation is accelerated by both increasing day lengths and warmer temperatures (Borg, 1982; Borg *et al.*, 1987; Andersson *et al.*, 1992; Hellqvist *et al.*, 2004) and cold-acclimated fishes have greater facility in adjusting to warm temperatures with increasing day lengths (Guderley *et al.*, 2001). These physiological responses to day length and temperature need to be considered in the context of the photic and thermal environments of Alaska and Oregon. We only manipulated one of these variables, photoperiod, while keeping the others constant. Although climates are colder in coastal Alaska than Oregon (U. S. Department of Commerce, 1968), spring and summer day lengths are longer and spring temperatures rise faster in Alaska than Oregon (Fig. 3.3). We therefore propose that the accelerating effects of longer day lengths and increasing temperatures in the more northern

environment may compensate for the lower average temperature in Alaska than Oregon. Hence, northern fishes would be reproductively prepared to exploit the shorter northern growing season during the brief period when summer waters are warmest. Finally, we encourage rearing animals from different localities in a common environment before using them to infer an underlying genetic basis for differences in functional phenotypes.

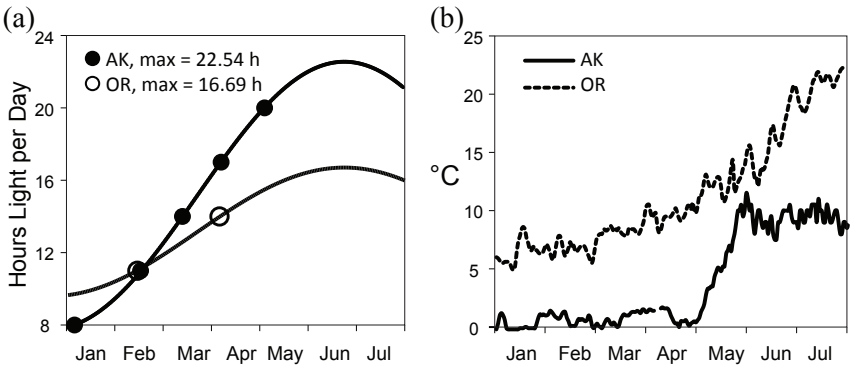


Fig. 3.3. Day length and temperature profiles during the winter and spring in Oregon (OR) and Alaska (AK). (a) Circles show the day lengths at which photoperiodic responses were determined. Note that the Oregon populations do not experience day lengths as short as eight hours or as long as 17 hours light per day. Day lengths are calculated as the time from the onset of civil twilight in the dawn until the end of civil twilight in the dusk for Florence, OR, and Seward, AK (http://www.sunrisesunset.com). (b) Water temperatures in the Rogue River near Agness, OR (42° 34.7' N, USGS 14372300), and Wasilla Creek, near Palmer, AK (61°38.5' N, USGS 15285000), based on data from 2010 and 2011 (http://waterdata.usgs.gov/usa/nwis/).

Absence of clock *polyglutamine domain length (polyQ)*

In *Drosophila melanogaster*, the Clock protein heterodimerizes with the Cycle protein to promote the transcription of period and timeless. Heterodimerization of Period and Timeless and their migration into the nucleus lead to the inhibition of their own transcription by Clock and Cycle (Darlington *et al.*, 1998). The interest in PolyQ comes from the observation that "a truncated dCLOCK protein lacking two of the three polyglutamine repeats [dCLOCK (ΔQ)] only weakly activates per and tim" (Darlington *et*

al., 1998, p. 1602). In the mouse, the *clock*$^{\Delta 19}$ mutant results in a long circadian period (Gekakis *et al.*, 1998; Jin *et al.*, 1999; Lowrey & Takahashi, 2004). King *et al.* (1997), found that "an AàT transversion at the third base position of the 5' splice donor site of intron 19" results in skipping the exon immediately upstream, i.e., exon 19. Exon 19 is in the "glutamine-rich region of the C-terminus of the predicted Clock protein (amino acids 514-564)," but not in the downstream PolyQ region (amino acids 739-837) (King *et al.*, 1997). These studies provided new and interesting insights into *clock* in the context of daily circadian timing, but they revealed nothing about any relationship between circadian rhythmicity and photoperiodism. The tractability of measuring PolyQ provided a convenient proxy for variation in the circadian clock that potentially could create functional differences in circadian rhythmicity. Unfortunately, various investigators made a logical error by seeking a causative relationship between the circadian clock and photoperiodic timer by demonstrating correlation between variation in PolyQ and latitude or phenology as assumed proxies for the photoperiodic timer.

Our findings of a lack of correlation between polyQ domain and aspects of photoperiodic response are not unique. We found no association between PolyQ and latitude in western North American populations of stickleback (Fig. 3.2). Similarly in the European blue throat Luscinia svecica there is no correlation between PolyQ and latitude from Armenia to Norway (40°30' – 70°30'N) (Johnsen *et al.*, 2007). Hence, in both species, there is no evidence of a connection between PolyQ and local or regional variation in phenology or photoperiodic response.

Photoperiodism, more than any other proximal factor, is responsible for the onset of first clutches among populations of the blue tit *Cyanistes caeruleus* and photoperiodic

response can vary between island and mainland populations at the same latitude (Lambrechts *et al.*, 1997). In a transect from Italy to Finland (36°44' – 62°37'N), Johnsen *et al.* (2007) found a significant correlation between latitude and PolyQ but only when an atypical, monomorphic, southernmost population was entered into the correlation. Johnsen *et al.* (2007) did not provide any correlation between PolyQ variation and phenological events and, in fact, made the appropriate warning (p. 4878): "Determination of the phenotypic effects of different ClkpolyQcds alleles described here would require detailed studies of both circadian and photoperiod-related behaviours of birds of differing ClkpolyQ genotypes."

Within a single site (Wytham Woods, UK; 51°47'N), Liedvogel *et al.* (2009) sought to correlate PolyQ with laying date, hatch date, and incubation duration of 950 blue tits over a two-years period. No "significant overall year*genotype interaction was found for any of the timing traits in focus (all results with $P > 0.213$)." However, when the authors continued their search for significance within the observed "non-significant" data, they found that by considering the second year in isolation, they could find a significant correlation between PolyQ and laying date and hatch date ($P = 0.047$ and $P = 0.033$, respectively, but without any table-wide adjustment for a-posteriori multiple comparisons). A follow up study on a great tit *Parus major* population at the same site found no association between PolyQ and the same measures of reproductive timing (Liedvogel & Sheldon, 2010). Hence, studies among birds over a large latitudinal range or within a single locality with a large sample size provide at best equivocal evidence for an association between *clock* polyglutamine repeat length and the timing of phenological events, much less photoperiodism.

Among teleost fishes, the molecular basis of daily circadian rhythmicity has been studied in the zebrafish *Danio rerio*. In zebrafish, the core loop of the circadian clock involves three paralogs of *clock* whose proteins form heterodimers with three paralogs of bmal that drive rhythmic expression of three paralogs of period and cryptochrome (Vatine *et al.*, 2011). No connection has been made between any core circadian rhythm genes and photoperiodically controlled seasonal life histories in zebrafish.

Salmonids as a family are photoperiodic for many seasonal life-cycle transitions, such as smolting, precocious sexual maturation, migration to sea, and the initiation of migration back to freshwater (Bromage *et al.*, 2001). Two paralogs of *clock* have been identified in Chinook salmon *Oncorhyncus tshawytscha*, *OtsClock1a* and *OtsClock1b* that arose from a tetraploidation event during divergence of salmonids from other teleost fishes (O'Malley & Banks, 2008b). No functional connection has yet been made between either of these paralogs and circadian rhythmicity in salmonids. Likewise, their functional role in photoperiodism, if any, has not been established. There is no evidence for polyglutamine length polymorphism in the *OtsClock1a* paralog among four species in the genus *Oncorhyncus*. In the *OtsClock1b* paralog, polyglutamine length is polymorphic within and among populations of Chinook, chum *O. kita*, coho *O. kisutch* and pink *O. gorbuscha* salmon (O'Malley & Banks, 2008a; O'Malley *et al.*, 2010). Mean length of the glutamine domain (PolyQ) is not significantly correlated with latitude among 19 populations of coho or 16 populations of pink salmon, but is correlated with latitude in Chinook and chum salmon (O'Malley & Banks, 2008a; O'Malley *et al.*, 2010). O'Malley *et al.* (2010) used univariate regression trees to identify correlations between the frequency of the most common polyglutamine domain length allele of *OtsClock1b* and

day length on the date of peak spawn and a freshwater migration index over a wide latitudinal range of Chinook, coho, chum and pink salmon. They found that the ability of the univariate regression tree "to assign populations to groups correctly on the basis of these factors" (day lengh and migration index) was not significant (O'Malley *et al.*, 2010, p. 3711) and significant ($P < 0.05$) only in pink salmon where length of the most common allele varied with day length on the date of peak spawn but not the freshwater migration index. They did not test for a persistent correlation between the frequency of most common *OtsClock1b* allele and latitude after their common covariation with latitude was factored out (O'Brien *et al.*, 2011).

Hence, in fishes as in birds, there is little evidence for a correlation between polyglutamine domain length and latitude or the timing of phenological events. Even if there had been a general pattern of correlation, correlation is not causation (Kingsolver & Schemske, 1991; Petraitis *et al.*, 1996; O'Brien *et al.*, 2011). In neither the birds nor the fishes was there any determination of the actual effect of PolyQ on circadian function or any actual direct measurement of photoperiodic response.

Bridge

In Chapter III, we examine the relationship between allelic variation in a circadian clock gene and latitude, which has been interpreted as evidence that the circadian clock is causally involved in photoperiodism. We show a lack of association with latitude or photoperiodic response in the threespine stickleback. These data demonstrate that caution should be employed when studying genetic variation across ecogeographic gradients and the importance of examining photoperiodism in controlled conditions. Chapter IV builds

on the phenotyping results of Chapter III by examining the physiological basis of photoperiodic response in threespine stickleback manipulated in controlled conditions.

CHAPTER IV

CONSERVATION OF THE PHOTOPERIODIC NEUROENDOCRINE AXIS AMONG VERTEBRATES: EVIDENCE FROM THE TELEOST FISH, *GASTEROSTEUS ACULEATUS*

An unpublished paper submitted to *General and Comparative Endocrinology*, authored by C. O'Brien, R. Bourdo, W. E. Bradshaw, C. M. Holzapfel, and W. A. Cresko.

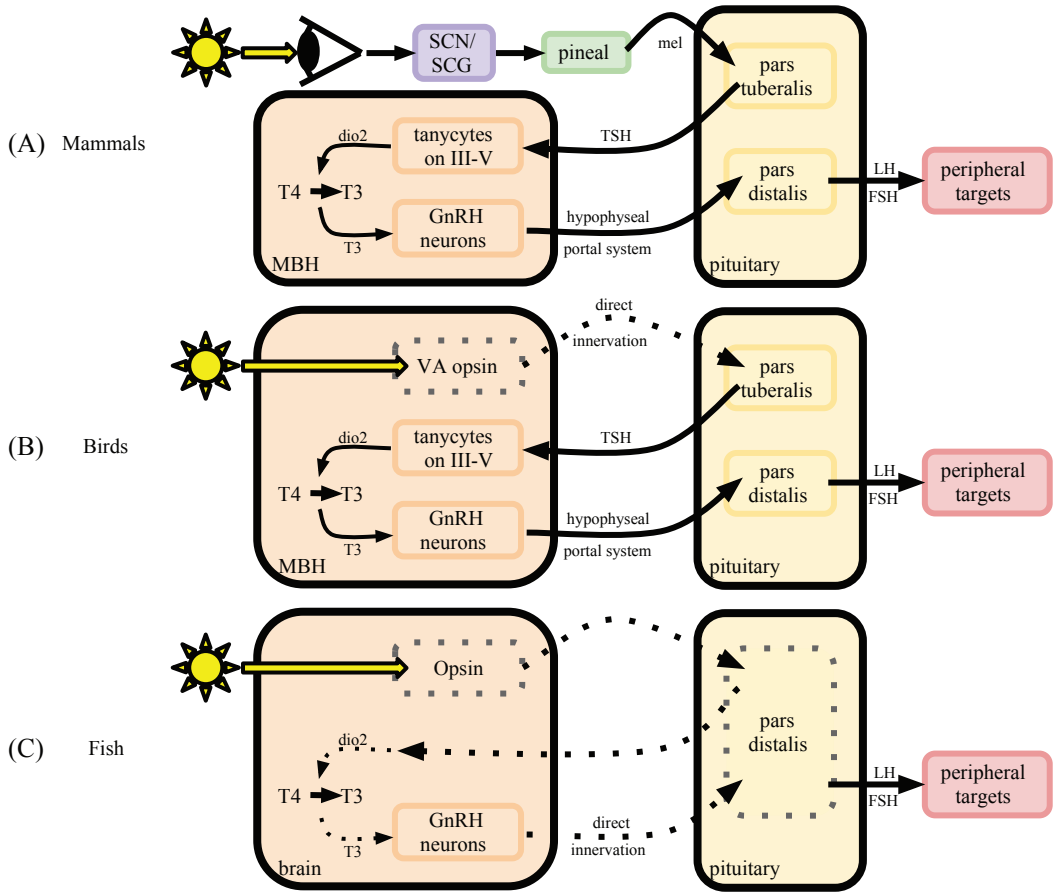
Introduction

Proper timing of life-history events is critical to fitness (Bradshaw *et al.*, 2004). Photoperiod, or length of day, has a highly reliable annual cycle that makes it an ideal environmental signal that organisms can use to anticipate and prepare for seasonal changes. The use of photoperiod for the timing of sexual maturation and reproduction is widespread among polar and temperate animals (Bradshaw *et al.*, 2007; Goldman *et al.*, 2004). The extensive use of photoperiod across diverse organisms in order to time critical life-history events underscores the importance of this environmental signal for fitness, and leads to the hypothesis that organismal systems that sense and respond to photoperiod have been molded by the action of natural selection for millennia.

Photoperiodic induction of sexual maturation in vertebrate animals begins with reception of a stimulatory photoperiod regime that leads to induction of gonadotropin

release, which in turn stimulates production of gonadal sex hormones Bradshaw *et al.*, 2010). In photoperiodic mammals and birds, the thyroid hormone (TH) pathway initiates the release of gonadotropins (Anisimova & Gascuel, 2006; Nakao *et al.*, 2008; Yasuo & Yoshimura, 2009; Yoshimura, 2010). Although the mechanisms of photoperiod signal reception and transduction differ between mammals and birds, the initial hormonal cascade and its location within the brain are conserved (Fig. 4.1).

Fig. 4.1. (next page). The photoperiodic TH pathway as inferred in fish from mammals and birds. Solid lines and borders indicate established steps and known neuroanatomical locations, respectively. Dashed lines and borders indicate suggested or inferred steps and neuroanatomical locations. *Signal reception in mammals and birds*: **(A)** In mammals, the photoperiod signal is received by the retina and neuronally communicated via the suprachiasmatic nuclei (SCN) and superior cervical ganglion (SCG) to the pineal gland (Moore, 1995). Melatonin (mel) produced by the pineal encodes the signal (Cassone, 1998). **(B)** In birds, the signal is received by extraretinal photoreceptors, most likely hypothalamic opsins (Halford *et al.*, 2009; Nakane, 2010). Communication of the signal is neuronal and does not involve melatonin (Sharp, 2005). *The neuroendocrine response in mammals and birds*: In both **(A)** mammals and **(B)** birds, the earliest hormonal response to a stimulatory photoperiod occurs in the pars tuberalis, where production of thyroid stimulating hormone beta (TSHβ) and chorionic gonadotropin alpha (CGα) increase (Hanon *et al.*, 2008; Nakao *et al.,* 2008; Yasuo *et al.*, 2010). TSHβ and CGα heterodimerize to form thyroid-stimulating hormone (TSH), which stimulates deiodinase 2 (*dio2*) production in tanycytes lining the third ventricle of the hypothalamus (III-V). Dio2 catalyzes the conversion of the thyroid hormone thyroxin (T4) to the bioactive triiodothyronine (T3) (Hanon *et al.*, 2008; Yoshimura *et al.*, 2010). T3 stimulates production of gonadotropin releasing hormone (GnRH) from neurons in the mediobasal hypothalamus (MBH). GnRH is transported via the pituitary portal system to the pars tuberalis, where it stimulates production of the gonadotropins follicle stimulating hormone (FSHβ) and luteinizing hormone (LHβ) (Yasuo & Yoshimura, 2009). These heterodimerize with CGα and are released into the bloodstream where they act upon the gonads and other peripheral targets. *Signal reception and neuroendocrine response in fish*: **(C)** In fish, photoperiodic signal reception is extraretinal (Borg, 2010; Masuda *et al.*, 2005) and may be an hypothalamic opsin (Philp *et al.*, 2000). The early responses of TSHβ and dio2 to a stimulatory photoperiod have not been studied in fish. In general, plasma T3 increases during sexual maturation in photoperiodic fishes (Biswas *et al.*, 2006; Norberg *et al.*, 2004), but its effects on GnRH in photoperiodic fishes are unknown. Photoperiodic manipulation stimulates GnRH, LH and FSH production (Amano *et al.*, 1999; Choi *et al.*, 2010; Hellqvist *et al.,* 2006; Miranda *et al.*, 2009). LH and FSH stimulate the gonads to produce sex hormones (Borg, 2010).



These findings lead to the hypothesis that involvement of TH in reproduction is conserved among vertebrates and therefore may have originated in chordates prior to the diversification of vertebrates (Heyland *et al.*, 2005; Paris *et al.*, 2010). Support for this hypothesis is limited, however, because most studies have occurred on organisms from just the tetrapod clade of vertebrates. A further test of this hypothesis requires comparable studies in other vertebrates, particularly teleost fishes, which is the most speciose vertebrate clade but for which we have precious little data regarding this hypothesis.

Photoperiodic control of sexual maturation is widespread among teleost fishes () (Bromage *et al.*, 2001; Bradshaw & Holzapfel, 2007; Borg, 2010) and the function of the

TH pathway is conserved in fish (Orozco & Valverde, 2005; Raine, 2010). However, photoperiodic control of the TH pathway remains unclear (Fig. 4.1C). Our current understanding is limited by an inability to compare studies directly due to differences in measurement techniques, the variety of species examined, and the ability to relate hormonal changes solely to photoperiodic response.

In seasonally reproducing fishes, the bioactive form of thyroid hormone, triiodothyronine (T3), tends to increase during early sexual maturation (Cyr & Eales, 1996; Norberg *et al.*, 2004; Biswas *et al.*, 2006) and, in at least rainbow trout, photoperiod is the specific trigger for this increase in T3 (Cyr *et al.*, 1988). T3 can stimulate GnRH secretion from GnRH neurons in the Nile tilapia (Parhar *et al.*, 2000), but this stimulation of GnRH has not been tested in a photoperiodic fish.

GnRH orthologs are often referred to by the species in which they were first discovered, but can also be referenced by their paralog name to facilitate comparison of their roles among species. We adopted the latter convention for our work. GnRH1 is expressed in neurons located in the preoptic area and GnRH2 neurons are found in the midbrain tegmentum. GnRH3 is unique to teleosts and is expressed in the ventral telencephalon (Chen & Fernald, 2008; Cerdá-Reverter & Canosa, 2009). GnRH1 is considered the hypophysiotropic form, (acting on the pituitary), as it is capable of stimulating gonadotropin production and gonadal development and is expressed in neurons that innervate the pituitary (Cerdá-Reverter & Canosa, 2009). In fishes where GnRH1 is not present, GnRH3 is the hypophysiotropic form (Chen & Fernald, 2008; Cerdá-Reverter & Canosa, 2009). In masu salmon exposed to a stimulatory photoperiod

regime, GnRH3 neurons increase in number (Amano *et al.*, 1999), but the response of GnRH3 in other photoperiodic fishes is unknown.

Extending the role of the TH pathway in photoperiodic induction of sexual maturation to teleosts requires a species with a strong photoperiodic response that can be manipulated in controlled conditions and that can be measured using techniques that make the results comparable to those in mammals, birds and other fishes. These criteria are met in the threespine stickleback, *Gasterosteus aculeatus*, in which we are able to isolate hormonal responses to photoperiod from other environmental variables using controlled laboratory experiments.

The threespine stickleback is a small teleost fish with a wide latitudinal and environmental range that uses photoperiod to initiate sexual maturation (Baggerman, 1985; Borg *et al.*, 2004; Hellqvist *et al.*, 2006; Yeates-Burghart *et al.*, 2009). Like birds (Dawson, 2002; Nakane *et al.*, 2010) and other fishes (Borg, 2010), reception of light related to photoperiodism is extraretinal and extrapineal (Borg *et al.*, 2004). A stimulatory photoperiod increases gonadotropin production (Hellqvist *et al.*, 2004) and wild-caught sticklebacks have an annual cycle of gonadotropin production that peaks early in the reproductive season (Hellqvist *et al.*, 2006). As in mammals and birds, androgens exert a feedback effect on gonadotropin production in both stimulatory and non-stimulatory photoperiod regimes (Borg *et al.*, 2004). In controlled photoperiod conditions in the laboratory, morphological changes of photoperiodic response can be measured Yeates-Burghart *et al.*, 2009; O'Brien *et al.*, in prep).

The goals of this study were to determine if the TH pathway is involved in the photoperiodic initiation of teleost sexual maturation and, if so, whether the dynamics of

the response of the pathway are conserved among mammals, birds and teleosts. To accomplish these goals, we quantified gene expression levels of key TH pathway genes in the brains and pituitaries of threespine stickleback during exposure to a stimulatory photoperiod regime. In the mammal and bird models, an increase in thyroid stimulating hormone (TSH) is the first known response of the photoperiodic neuroendocrine cascade (Fig. 4.1A and Fig. 4.1B). An increase in hypophysiotropic gonadotropin releasing hormone (GnRH) is the first indicator of the initiation of sexual maturation. Luteinizing hormone (LH) is one of the two gonadotropins that are secreted by the pituitary into circulation to stimulate sex hormone production (Fig. 4.1). By measuring these hormones in controlled conditions we determined the effects of photoperiod on expression of these genes independently of other environmental factors. We were then able to make direct comparisons between *G. aculeatus* and mammals and birds. In addition, evaluating multiple populations allowed us to determine the robustness of the results within a single species.

Materials and Methods

Gasterosteus aculeatus *stocks*

Two northern populations were established from Alaska, Rabbit Slough (AK1: 61°34' N, 149°15'W) and Boot Lake (AK2: 61°43'N, 149°7'W). One southern population was established from Oregon, Eel Creek (OR: 43°35'N, 124°11'W). We will refer to these populations as AK1, AK2, and OR, respectively, throughout the rest of the text.

Crosses were made via *in vitro* fertilization using established laboratory procedures, and then the fish were reared under standard laboratory conditions [Yeates-

Burghart *et al.*, 2009; stickleback.uoregon.edu/index.php/Crossing_and_Rearing_Protocols]. Experimental fish were reared to adulthood at 20°C on a non-stimulatory 10L:14D photoperiod cycle for 11 – 12 months (L:D = Light:Dark). They were at least 50 mm standard length (SL), as measured from the dorsum of the pre-maxilla to the caudal peduncle before they were subjected to any experimental treatment. All fish care and experimental procedures complied with University of Oregon IACUC-approved animal care protocols.

Experimental design

Conditions were identical to those previously used in measure the phenotypic effects of photoperiod (Yeates-Burghart *et al.*, 2009). One adult male and one adult female were paired in a single aquarium that was visually separated from other aquaria to avoid confounding visual cues. Aquaria were cleaned separately to avoid the possibility of transferring hormonal cues. The fish were fed twice a day *ad libitum*. All experiments were run in light-tight, air-cooled cabinets at 20°C. Photoperiods were programmed with Chrontrol XT electronic timers (www.chrontrol.com).

Upon being placed in the experimental aquaria, male-female pairs were given two short-day cycles of light:dark = 10:14 (hereafter: 10L:14D) before being exposed to 17L:7D stimulatory long days. This long-day regimen was chosen because it is the shortest photoperiod at which phenotypic indicators of sexual maturation in threespine stickleback plateau (Yeates-Burghart *et al.*, 2009). We used the males exclusively for all of the following experiments.

For *in-situ* mRNA hybridization of TSHβ, male stickleback from the AK2 line

were sampled six hours after dawn during a short-day regimen or six hours after dawn after exposure to a single 17L:7D long day regimen. Fish were anesthetized in MS-222 (Sigma) and the entire brain, including the pituitary, was dissected out and stored in 4% paraformaldehyde solution (Sigma Aldrich) at 4°C. The brains with pituitaries were then cryostat sectioned along the coronal plane, and placed on slides that were stored at -80° C until use.

For quantitative real-time PCR measurement of target genes, males from the three populations were sampled six hours after dawn following exposure to 0, 1, 2, 5 or 10 long days. Fish were anesthetized in MS-222 (Sigma) and the entire brain including the pituitary was dissected out and stored in Trizol (Invitrogen) at -80° C. Total RNA was extracted following a standard phenol chloroform protocol. Synthesis of cDNA was performed using random hexamers (Invitrogen) and SuperScript III (Invitrogen). Sample sizes per treatment ranged from 8 – 13 adult males (Supplementary Table 4.1).

Target gene identification

Thyroid stimulating hormone (TSH) and luteinizing hormone (LH) are both heterodimers consisting of a protein-specific β subunit and an α subunit common to TSH and LH. Therefore, we targeted the β subunits to ensure hormone specificity. First, *Homo sapiens and* zebrafish *Danio rerio* TSHβ, GnRH and LHβ orthologs were compared to the stickleback genome using BLAST to produce a set of candidate genes for further analysis.

Second, we performed phylogenetic reconstructions of the gene families to confirm the identity of the candidate genes, and in the cases of TSHβ and GnRH3, we

annotated all paralogs found in the stickleback genome. Complete amino acid sequences of orthologs from the three gene families were downloaded from the NCBI protein database. If a species contained multiple paralogs, all were included. Alignments of the three gene families were made using Muscle (Edgar, 2004). PhyML 3.0 (Guindon *et al.*, 2009) was used to estimate phylogenies and compute their likelihood scores. The parameters of the phylogenetic model were searched and optimized using M3L (code.google.com/m3l/), which implements the Broyden-Fletcher-Goldfarb-Shanno algorithm (Nocedal, 1980). The best-fitting model for each gene family was selected using the Akaike information criterion test (Akaike, 1973). Approximate likelihood ratio tests for each node were scaled using Shimodaira and Hasegawa (SH-like) support (Anisimova & Gascuel, 2006). For the phylogenetic reconstruction of all three gene families, the best model was JTT (Jones *et al.*, 1992) with a gamma-distributed set of evolutionary rates (Yang, 1996) (Supplementary Fig. 4.1).

Third, we used syntenic analysis of the target orthologs to confirm the results of the phylogenetic reconstructions. The Synteny Database uses Reciprocal Best Hit Analysis (RBH) to detect synteny between two genomes (Catchen *et al.*, 2009; Catchen *et al.*, 2011). Here, genes from a target genome and an outgroup genome are compared to one another using BLAST. Genes in the two genomes are considered orthologous if they are each other's best BLAST matches. If regions of the two genomes have a high number of orthologs, syntenic conservation due to common descent is inferred (Catchen *et al.*, 2009; Catchen *et al.*, 2011). We compared the regions around our target orthologs in the stickleback genome to the spotted green puffer fish *Tetraodon nigroviridis* and *Homo sapiens* genomes using the Synteny Database (Catchen *et al.*, 2011).

mRNA in-situ hybridization

A riboprobe complementary to stickleback TSHβ1 mRNA was synthesized using digoxigenin-labeled UTP (Roche Applied Science). It was hybridized to coronal sections of brains and pituitaries removed from males from the AK2 population to visualize the location of the TSHβ expression. The hybridization protocol was adopted from Thisse *et al.* (1993) with the following modifications: sections were not dehydrated prior to hybridization, and incubation with the anti-digoxigenin antiserum solution was performed at room temperature.

Quantitative real-time PCR (qPCR)

First, target and housekeeping gene primer sets were tested using serial dilutions of cDNA to ensure specificity and consistent amplification across a wide range of concentrations. CDNA concentrations of biological samples were quantified using a Qubit fluorometer (Invitrogen). Two hundred nanograms of cDNA were added to individual qPCR reactions. Reactions were performed in 10 µl volumes using a Kapa SYBR ® Fast kit (Kapa Biosystems). Three technical replicates were performed per gene per biological sample.

Two normalization steps created ΔΔCt values. First, Ct values for technical replicates were averaged and normalized to expression of the housekeeping gene β-actin (Ensembl ID# ENSGACG00000007836) (Hibbeler *et al.*, 2008). The resulting value was then normalized to the Day 0 population means for each individual gene.

Data were analyzed in R using a two-way population*photoperiod treatment with a

Tukey HSD correction for multiple comparisons (R Development Core Team, 2007) and Dunnett's test for comparison of treatment means with a control (Zar, 1996). Both photoperiod and population were treated as fixed effects.

Results

The genomic location and annotation of hypothesized targets of photoperiodism.

We identified two TSHβ paralogs in the stickleback genome, confirming previous results (Kitano *et al*., 2010). TSHβ1 (ENSGACG00000005276) is on linkage group XVII and TSHβ2 (ENSGACG00000009897) is on linkage group XII (Fig. 4.2). Phylogenetic reconstruction places them within their expected clades with high support (Supplementary Fig. 4.1A). TSHβ2 is nested within the teleost TSHβ1 clade with the Siberian sturgeon *Acipenser baerii*, the as an immediate outgroup to the clade containing both TSHβ paralogs (Supplementary Fig. 4.1A). This topology indicates that the TSHβ duplication resulted from the teleost-specific genome duplication, as the sturgeon lineage is known to have diverged prior to the teleost-specific genome duplication (Postlethwait *et al.*, 2004). Furthermore, there is a high number of paralogs between the genomic regions where TSHβ1 and TSHβ2 are found, indicating that they originated from a single chromosomal region (Fig. 4.2D). As TSHβ1 is the most conserved paralog among teleosts (Fig. 4.2 and Supplementary Fig. 4.1A), and we could not detect TSHβ2 expression in our biological samples, only the photoperiodic response of TSHβ1 was measured.

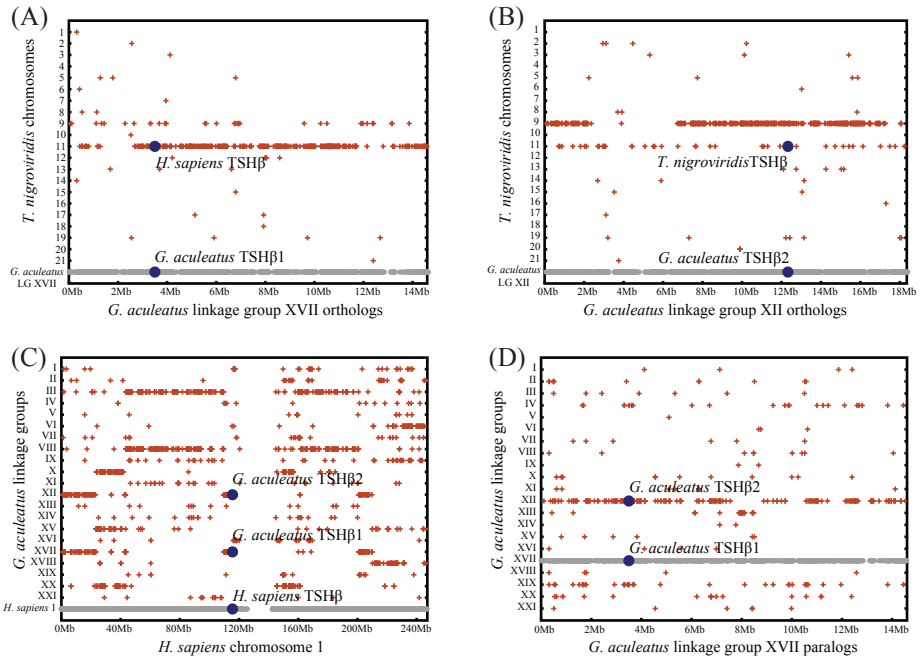


Fig. 4.2. The threespine stickleback genome contains two thyroid stimulating hormone beta subunit (TSHβ) paralogs. Genes along the x-axis and their orthologs are labeled with grey and red dots respectively. Blue circles indicate TSHβ orthologs. **(A)** Threespine stickleback TSHβ1 (ENSGACG00000005276), orthologous to the single TSHβ in the green spotted puffer fish *Tetraodon nigrovirdis* (ENSTNIG00000018284). **(B)** Threespine stickleback TSHβ2 (ENSGACG00000009897), orthologous to the single *T. nigrovirdis* TSHβ. **(C)** *H. sapiens* TSHβ (ENSG00000134200), orthologous to the two threespine stickleback TSHβ paralogs. **(D)** Syntenic relationships within the threespine stickleback genome show that linkage groups XVII and XII have a high number of paralogs. TSHβ1 (ENSGACG00000005276) and TSHβ2 (ENSGACG00000009897) are labeled.

Two GnRH paralogs (GnRH2 and GnRH3) were found in the stickleback genome, but GnRH1 is absent. GnRH2 (ENSGACG00000009021) is located on linkage group XVII and GnRH3 (ENSGACG00000009582) is on linkage group VI (Fig. 4.3). The phylogenetic reconstruction shows strong support for separation between the three GnRH paralog clades, with the stickleback GnRH paralogs placed in their expected clades (Supplementary Fig. 4.1B). GnRH3 is unique to teleosts and nested within the GnRH1 clade, confirming previous results (Kitano *et al.*, 2010). As GnRH3 is the

hypophysiotropic form in fish when GnRH1 is absent (Chen & Fernald, 2008; Cerdá-Reverter, & Canosa, 2009), the photoperiodic response of GnRH3 was measured.

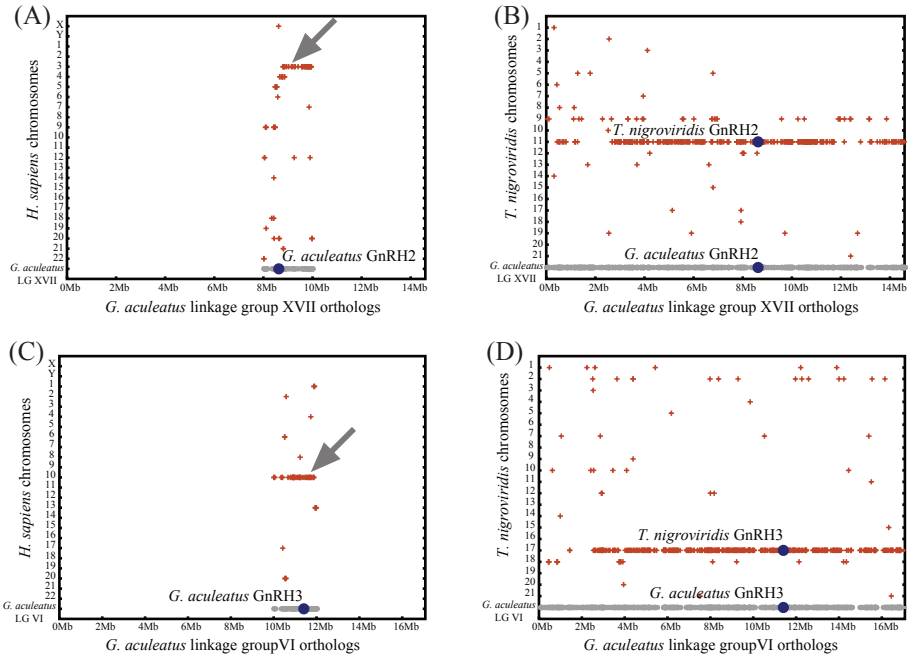


Fig. 4.3. The threespine stickleback genome contains two of the three vertebrate gonadotropin releasing hormone (GnRH) orthologs. Genes along the x-axis and their orthologs are labeled with grey and red dots respectively. Blue circles indicate GnRH orthologs. Grey arrows indicate the expected positions of missing orthologs. **(A)** Threespine stickleback GnRH2 (ENSGACG00000009021), which has no ortholog in the *H. sapiens* genome. The two megabase region surrounding threespine stickleback GnRH2 is isolated to show syntenic conservation, but the absence of an ortholog. **(B)** Threespine stickleback GnRH2 has a single ortholog in the *T. nigrovirdis* genome (ENSTNIG00000002767). **(C)** Threespine stickleback GnRH3 (ENSGACG00000009582), which has no ortholog in the *H. sapiens* genome. The two megabase region surrounding Threespine stickleback GnRH3 is isolated to show syntenic conservation, but the absence of an ortholog. **(D)** Threespine stickleback GnRH3 has a single ortholog in the T. *nigrovirdis* genome (ENSTNIG00000013337).

A single LHβ ortholog (ENSGACG00000011475) was identified in the stickleback genome, on linkage group VI (Fig. 4.4 and Supplementary Fig. 4.1C). The phylogenetic reconstruction shows strong support for separation between the teleost and tetrapod clades with the LHβ clade, with stickleback LHβ placed in the expected clades.

Interestingly, synteny of the surrounding genomic region is conserved between stickleback and the spotted green pufferfuish, *T. nigroviridis* (Fig. 4.4B), but not between stickleback and *H. sapiens* (Fig. 4.4A), that the genomic location of LHβ changed after the divergence of teleosts and tetrapods, but prior to the divergence of stickleback and *T. nigrovirdis* from their most recent common ancestor.

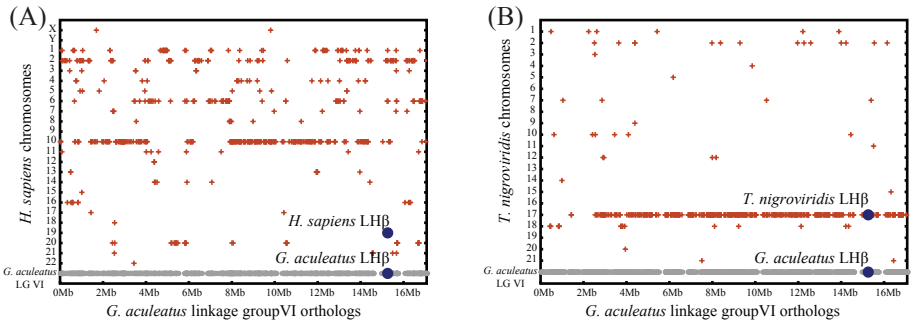


Fig. 4.4. The threespine stickleback genome contains a single luteinizing hormone beta subunit (LHβ). Genes along the x-axis and their orthologs are labeled with grey and red dots respectively. Blue circles indicate LHβ orthologs. Synteny dot plots for threespine stickleback LHβ (ENSGACG00000011475), which has a single ortholog in **(A)** *H. sapiens* (ENSG00000104826) and **(B)** *T. nigrovirdis* (ENSTNIG00000009862).

TSHβ1 expression is localized to expected regions of the brain

TSHβ1 mRNA is expressed in the pars distalis of the pituitary, as measured by visual inspection of brain section slides after *in situ* hybridization (Fig. 4.5). TSHβ1 may also be expressed around the third ventricle (III-V in Fig. 4.5), although the latter is too faint to distinguish from background staining with certainty. We expected expression to be localized to the pars distalis, as it is the region of the teleost pituitary that produces the gonadotropins (Kah & Dufour, 2010). TSHβ1 mRNA expression appeared to increase after exposure to a single long day (Fig. 4.5).

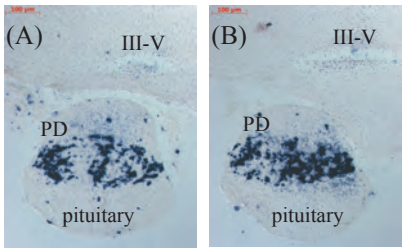


Fig. 4.5. TSHβ1 expression in the pars distalis of the pituitary labeled via mRNA *in-situ* hybridization. Exemplar coronal sections of the ventral hypothalamus and pituitary from adult male threespine stickleback exposed to (A) short days and (b) one long day. Scale bar is 100 μm. III-V: Third ventricle; PD: pars distalis.

Quantitative real-time PCR (qPCR) shows rapid response of the target genes to stimulatory photoperiods

ΔΔCt treatment means for TSHβ1, GnRH3 and LHβ are illustrated in Fig. 4.6. Results of the two-way ANOVA are reported in Table 4.1. Photoperiod has a significant effect on the expression of all three genes (for all three, $P < 0.0001$). There is a significant difference among the populations for GnRH3 ($P = 0.001$) and LHβ expression ($P = 0.015$). There is only a photoperiod*population interaction term for TSHβ1 expression ($P = 0.037$). The significant interaction term for TSHβ1 requires a closer examination of the main effect of photoperiod. As can be seen in Fig. 4.6, the general trend of the effect of photoperiod is still clear across populations, and the significant interaction term is due to a lower level of expression on day 1.

There is a significant difference among the three populations in the response of TSHβ1 to photoperiod. A single long day causes a pulse in TSHβ1 expression in AK1 and AK2, but the response of OR is not significantly different from baseline values (Figure 4.6 and Table 4.1; photoperiod*population effect: $P = 0.037$). This pulse demonstrates a significant effect of photoperiod on TSHβ1 expression (Fig. 4.6 and Table

4.1; photoperiod effect: $P << 0.0001$), although subsequent long days produce no response that is significantly different from baseline values in any of the populations (Fig. 4.6).

		Effect	DF	F-ratio	P
Gene	TSHβ1	Photoperiod	4, 131	25.52	<< .0001
		Population	2, 131	1.37	0.26
		Photoperiod * Population	8, 131	2.14	0.037
	GnRH3	Photoperiod	4, 131	21.32	<< .0001
		Population	2, 131	7.09	.0012
		Photoperiod * Population	8, 131	1.62	0.124
	LHβ	Photoperiod	4, 131	38.89	<< .0001
		Population	2, 131	4.35	0.015
		Photoperiod * Population	8, 131	1.74	0.096

Table 4.1. A two-way population*photoperiod ANOVA for TSHβ1, GnRH3, and LHβ expression in the brain and pituitary.

There is no difference among the three populations in response of GnRH3 to photoperiod (Fig. 4.6 and Table 4.1; photoperiod effect: $P = 0.124$). Long days cause a gradual decrease in GnRH3 expression in the brain and pituitary of all populations, with an eventual return to baseline values after six to ten long days (Fig. 4.6 and Table 4.1; photoperiod effect: $P << 0.0001$). This decrease is first significant after two to five long days, and the return to baseline levels occurs after five to ten long days (Fig. 4.6). There are significant differences among the populations in overall GnRH3 expression (Fig. 4.6 and Table 4.1; population effect: $P = 0.00119$).

Long days cause a gradual increase in LHβ expression in the brain and pituitary of all populations (Fig. 4.6 and Table 4.1; photoperiod effect: $P << 0.0001$). Differences in the timing of this increase among the populations are not significant (Table 4.1; photoperiod*population: $P = 0.096$). There are significant differences among the populations in overall LHβ expression (Figure 6; population effect: $P = 0.015$).

Discussion

Answers to primary questions

The primary questions addressed in this study were to ask (1) whether photoperiodic control of sexual maturation occurred via the thyroid hormone (TH) pathway in a teleost fish and, hence, whether this inductive pathway was conserved from fishes to birds and mammals, (2) whether the order of hormonal events in this pathway coincided with birds and mammals, (3) whether this order of events was robust among different populations. In the threespine stickleback, *Gasterosteus aculeatus*, the answer to all three questions is affirmative, but with variations.

Thyroid stimulating hormone

The cascade of response to gonad-stimulating long days begins with up-regulation of thyroid stimulating hormone (TSH). One day of exposure to gonad-stimulating long days elicits an increase in TSHβ1 in the pars distalis of the pituitary (Table 4.1, Fig. 4.5, and Fig. 4.6). In mammals and birds, the first short night elicits a similar response of TSHβ (Nakao *et al.*, 2008; Dardente *et al.*, 2010; Masumoto *et al.*, 2010) but in the pars tuberalis of the pituitary (Nakao *et al.*, 2008; Yasuo *et al.*, 2010). As the pars tuberalis as

a distinct region of the pituitary is found only in tetrapods (Kah & Dufour, 2010), the pulse of TSHβ1 in the stickleback pars distalis indicates that function of the tetrapod pars tuberalis is contained within the pars distalis of teleosts.

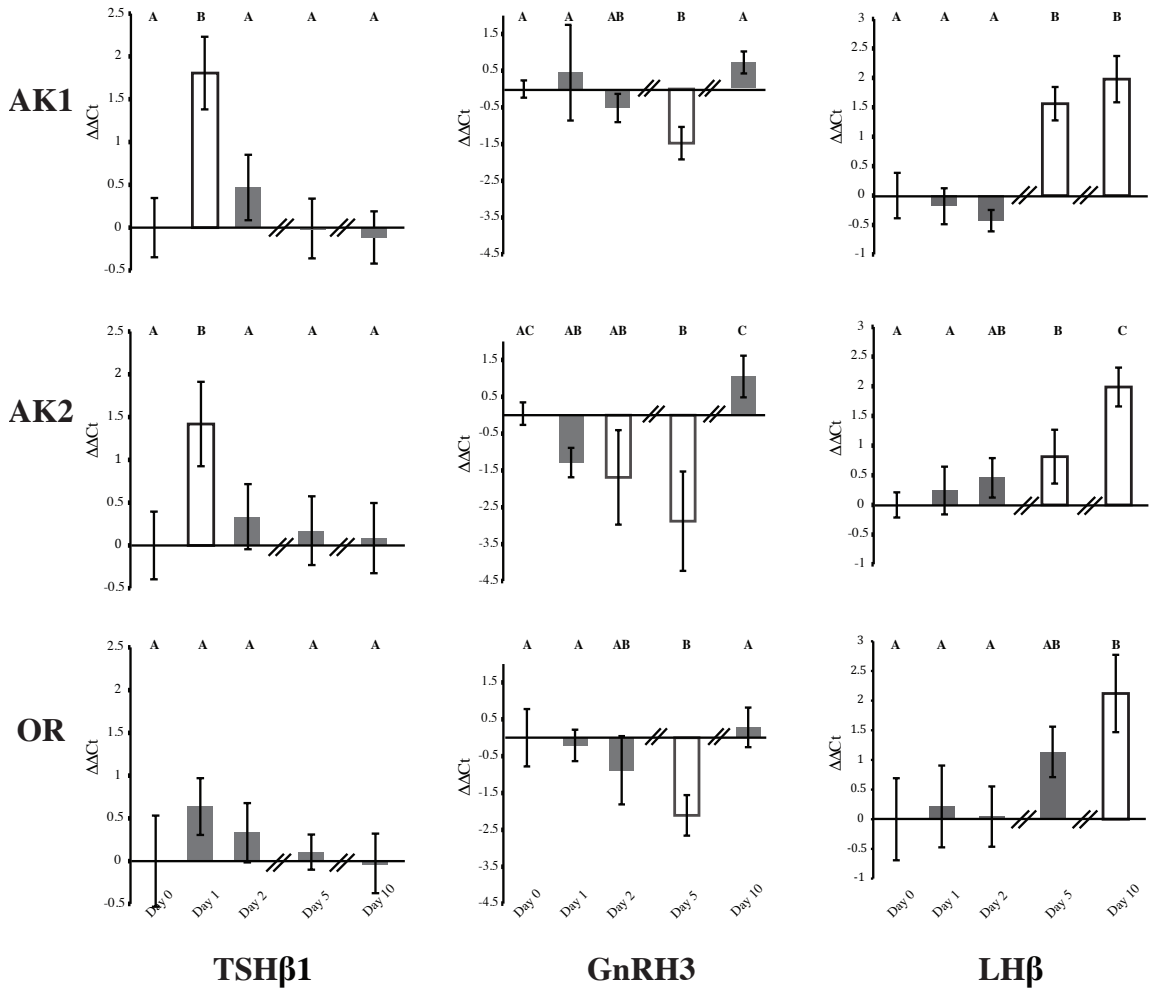


Fig. 4.6. The effect of photoperiod on the thyroid stimulating hormone pathway in the brain of adult male threespine stickleback, *Gasterosteous aculeatus*. Male sticklebacks from two populations in Alaska (AK1, AK2) and one population in Oregon (OR) were reared from hatch to adulthood on short days and then exposed to another short day (control, Day 0) or 1, 2, 5, or 10 long days. Expression of TSHβ1, GnRH3 and LHβ were quantified with qPCR and long-day treatments normalized to the short-day control. Error bars are ± 2S.E. Sample sizes are given in Supplementary Table 4.1. Time points that share a letter are not significantly different at $P < .05$ according to one-way ANOVA with a Tukey HSD correction for multiple a posteriori comparisons; open bars indicate hormone expression levels that differ significantly from the control at $P < 0.05$ according to Dunnett's test.

A previous study on the stickleback TSHβ paralogs found no difference in TSHβ1 pituitary expression between immature and sexually mature adults (Kitano *et al.*, 2010). Our findings contrast with those results (Fig. 4.6), but the studies are not directly comparable. Whereas our study compared lab-raised adult males, Kitano *et al.* (2010) compared TSHβ1 in lab-raised 8-month-old fish on a short day regimen to 12-month-old fish on a long-day regimen, potentially confounding age and time of exposure to long days. The important early pulse of TSHβ1 (Fig. 4.6) would not have been observed by Kitano *et al.* (2010).

The early pulse TSHβ1 was higher in the two more northern populations where the growing season is shorter and the winters are longer and more severe (Table 4.1 and Fig. 4.6). Stickleback in populations that are ecologically similar and geographically proximal to the northern sites in this study breed strictly from mid-May through July (Karvé *et al.*, 2008), whereas in the southern population where winters are mild (O'Brien, in prep.), some sexually mature individuals are found nearly year round (Q. Yeates-Burghart and C. O'Brien, unpublished results). Future studies might consider whether a lower threshold expression of TSHβ1 is required to initiate the cascade of events leading sexual maturation in *G. aculeatus*.

Gonadotropin releasing hormone

The cascade of response to gonad-stimulating long days in *G. aculeatus* continues with a change in the level of gonadotropin releasing hormone (GnRH), but with a decrease (Fig. 4.6) rather than an increase in expression, as is seen in photoperiodic tetrapods (Yasuo & Yoshimura, 2009). We propose three potential explanations.

First, regulation of LH may be independent of GnRH in stickleback. However, GnRH stimulates LH secretion throughout vertebrates, including photoperiodic fishes (Borg, 2010). In stickleback, the pars distalis has extensive innervation from GnRH neurons (Andersson *et al.*, 1995). As the pars distalis is the site of gonadotropin production and secretion in the vertebrate pituitary (Kah & Dufour, 2010), this innervation supports the concept of a direct control of gonadotropins by GnRH.

Second, GnRH3 may not be the actual hypophysiotropic paralog of GnRH. Although the distribution of GnRH2 and GnRH3 expression is similar to that in other teleost fishes that have lost GnRH1 (Anderson *et al.*, 1995; Okubo & Nagahama, 2008), GnRH3 and not GnRH2 is the hypophysiotropic form in other species of fish that lack GnRH1 (Chen & Fernald, 2008; Cerdá-Reverter & Canosa, 2009). Future research should consider the possibility that GnRH2 may be the hypophysiotropic form in *G. aculeatus* as well as other teleosts.

Third, the hypophysiotropic function of GnRH1 and, in the fish species where it is absent, GnRH3, is well documented, but additional functions of either paralog are much less understood (Chen & Fernald, 2008). In species where GnRH3 has assumed the hypophysiotropic function, we would expect it to retain its other functions as well. If GnRH3 expression is inhibited by long days in areas of the brain related to these other functions, but simultaneously stimulated in areas related to its hypophysiotropic function, the net expression of GnRH3 in the brain and pituitary combined could still decrease during long days. Sexual maturation in the grey mullet is regulated by photoperiod (Kuo *et al.*, 1974) and it has retained GnRH1 as the hypophysiotropic GnRH (Nocillado *et al.*, 2007). GnRH3 expression decreases in the brain of the grey mullet during sexual

maturation (Nocillado *et al.*, 2007), presumably in the context of its other, non-hypophysiotropic functions. Future research should probe the other functions of GnRH paralogs, unrelated to gonadal maturation, especially in photoperiodic fish.

Luteinizing hormone

LHβ is the third hormone to be expressed in the sequence of events leading downstream from gonadal stimulating long days (Table 4.1 and Fig. 4.6). LHβ is a gonadotropin that stimulates the sex hormones required to initiate sexual maturation in vertebrates. In the Japanese quail, a single long day stimulates LHβ release (Nakao *et al.*, 2008). A single long day also affects phenotypic indicators of sexual maturation in at the photoperiodic Siberian hamster (Finley *et al.*, 1995). In the threespine stickleback, LHβ expression rises above baseline after 5-10 long days but, given the effects of a single long day on quail and hamsters, the later expression in LHβ does not necessarily mean that 5-10 long days are necessary for LHβ expression or to commit stickleback to sexual maturation. Future research should determine the number of long days required to activate the entire TSHβ to LHβ cascade in stickleback and whether, once increased above baseline, expression of LHβ is sufficient to commit stickleback to seasonal reproductive maturation.

Conclusions

Our results strongly support a direct role for the TH pathway in the photoperiodic initiation of sexual maturation in teleosts, supporting the functional conservation of the TH pathway among photoperiodic vertebrates. Our use of lab-raised populations and

controlled experimental conditions allowed us to eliminate the potential influence of other environmental or historical factors that have limited inference in previous studies of the physiological basis of photoperiodic response in teleost fish. Although the photoperiodic responses of the populations in this study are phenotypically indistinguishable (O'Brien *et al.*, in prep), their physiological responses demonstrate the benefits of replicating studies across multiple populations. First, the differences in early TSHβ1 response between the southern and northern populations may reflect differences in seasonal reproductive patterns. Second, the initial decrease in GnRH3 was unexpected, but is robust because this decrease was consistent among these populations. Future work motivated by these GnRH3 results will illuminate functional variation in a highly conserved hormone family (Chen & Fernald, 2008). Finally, the gradual LHβ increases in all populations suggests differences between fish and birds in the timing of sexual maturation in response to photoperiod. To our knowledge, the ecological significance of the early LH release in birds has not been addressed. The results herein are motivation and a basis for such studies.

Taken together, our results further establish the threespine stickleback as a vertebrate model of photoperiodic response (Borg *et al.*, 2004) and form a foundation for future investigations into the hormonal basis of vertebrate photoperiodic response in varied seasonal environments.

Bridge

Chapter IV examines the physiological basis of early photoperiodic response using the threespine stickleback as a model teleost fish. We show that the thyroid

hormone pathway initiates sexual maturation, which strongly suggests that the hormonal and anatomical basis of photoperiodic response is conserved among vertebrates. In Chapter V, I conclude by summarizing the data presented in this dissertation, their significance, and suggest ways in which the threespine stickleback can contribute as a model of vertebrate photoperiodism

CHAPTER V

CONCLUSION

The presence of biological clocks among nearly all forms of life underscores the importance of correct time perception for dealing with periodic environmental variation. The circadian clock controls the timing of daily organismal activities. The timing of seasonal actions in polar and temperate organisms is controlled by the photoperiodic timer, which is set by photoperiod (length of day). The highly reliable annual cycle of photoperiod allows animals to anticipate and properly prepare for future environmental conditions so that these seasonal actions occur at the optimal time of year. Despite its ecological and evolutionary importance, very little is known about the genetic basis of photoperiodic interpretation in natural vertebrate populations. My dissertation research was motivated by a desire to address this question. It does so demonstrating the proper methodology for studying photoperiodism and establishing the threespine stickleback as model of vertebrate photoperiodism.

How should a complex trait like vertebrate photoperiodism be studied?

Proper study of the genetic basis of photoperiodism requires a clear understanding of the assumptions underlying interpretation of results and the necessary attributes of a model organism whose study will produce solid progress. Our work herein addresses the

effects of unreasonable assumptions on the state of the field and establishes the threespine stickleback as a model that can overcome limitations that have hindered progress.

The assumption that the circadian clock forms the basis of the photoperiodic timer in animals is widespread, but has not been demonstrated in natural populations (Bradshaw & Holzapfel, 2007). In O'Brien *et al.* (2011; Chapter II), we examine the correlation between a proxy for the circadian clock (allelic variation in a circadian clock gene) and the timing of migration in several salmonid species as a proxy for photoperiodic time measurement. This correlation has been interpreted as support for a causal connection between the circadian clock and photoperiodic time measurement (O'Malley *et al.*, 2010).

This interpretation rests on the key assumptions that the circadian clock is functionally integrated with the photoperiodic timer and that genetic variation in a circadian clock gene must affect the photoperiodic timer through its role in the circadian clock. The role of the circadian clock in seasonal timing is debated (Hazlerigg & Loudon, 2008; Goto *et al.*, 2010; Bradshaw & Holzapfel, 2010a, 2010b; Saunders, 2010; Koštál, 2011; Schiesari *et al.*, 2011), so causal relationships among all these elements must be established to support the hypothesis. We described a simple, but underemployed method that can be employed before this work is undertaken to determine if the association between the proxy of the circadian clock and migratory timing remains once their correlation due to their common covariance with latitude is removed. We found that it does not, which means their putative relationship was due to autocorrelation caused by a

common association with latitude. Thus, further investigation of a causal relationship is inappropriate.

The result of a lack of covariation between latitude and allelic variation in the candidate gene demonstrates both the value of applying this test as an initial step before examination of a correlation across an ecogeographic gradient and that caution must be employed when inferring causality between the circadian clock and photoperiodic time measurement, especially when employing proxies for each.

In Chapter III, we addressed the hypothesized association between a proxy of the circadian clock, the eponymous gene *clock*, and the response of traits mediated by photoperiod. First, we demonstrated that a stimulatory photoperiod elicits sexual maturation in male and female threespine stickleback. We developed a method and the equipment necessary to phenotype photoperiodic response in conditions that controlled for environmental variables that may affect output from the photoperiodic timer, which include temperature, nutrient availability, water quality, hormonal cues, and visual cues.

The ability to directly assess output of the photoperiodic timer means that our interpretation of the results is unhindered by the potentially confounding effects of other environmental signals. Isolating the effects of individual variables is important so as to not draw incorrect causal connections. This point is well demonstrated by considering salmonid migratory timing, which is mediated by the photoperiodic timer, but is also affected by local temperature (Crozier *et al.*, 2008) and perhaps other environmental signals. O'Malley *et al.* (2010) assumed that observed variation in migratory timing is strictly due to variation in photoperiodic response. This is not a reasonable assumption as the effects of these other environmental signals are not measured or controlled.

This assumption underlies the conclusions of O'Malley *et al.* (2010) that a correlation between variation in a circadian clock gene and migratory timing supports a role for the circadian clock in the photoperiodic timer. We tested this conclusion by examining allelic variation in the circadian clock gene among threespine stickleback populations that had been phenotyped for photoperiodic response in controlled conditions. By examining a potential correlation strictly with photoperiodic response and not phenological variation that may be affected by other environmental signals we were able to conduct a much stricter test of the hypothesized relationship. Although there is a high degree of variation in the circadian clock gene, it is not associated with populations or latitude. We conclude that there is not a causal connection between this component of the circadian clock and the photoperiodic timer. This conclusion complements that of Chapter II, by empirically demonstrating a lack of association between allelic variation and latitude in another photoperiodic teleost fish. The results of Chapters II and III show that interpretation of genetic variation along a geographic gradient should be done cautiously. In particular, suggesting that the previously observed allelic variation in a circadian clock gene is related to variation in seasonal activities mediated by photoperiodic response is imprudent at best.

Is the hormonal basis of photoperiodic response conserved among vertebrates?

Many temperate and polar vertebrates use photoperiod to mediate sexual maturation (Bromage *et al.*, 2001; Bradshaw & Holzapfel, 2007). The thyroid hormone (TH) pathway initiates this process in photoperiodic mammals and birds (Yasuo & Yoshimura, 2009), but it is unknown if the pathway's function and stimulation by

photoperiod is conserved in teleost fishes. In Chapter IV, we measure the response of key hormones in the threespine stickleback TH pathway in response to a stimulatory photoperiod. The data show that these hormones generally have the same response and neuroanatomical location in threespine stickleback as they do in mammals and birds (Chapter IV). This strongly supports conservation of the TH pathway's role among mammals, birds, fishes and perhaps all of vertebrates. The advantages of examining photoperiodic response in controlled conditions are also demonstrated, as the changes in hormone levels can be attributed solely to photoperiod, thus making the results directly comparable to those from mammal and bird models.

s**What makes a good model of vertebrate photoperiodism?**

The second way in which this dissertation addresses the main motivation of understanding the physiological and genetic basis of photoperiodism is by establishing the threespine stickleback as a vertebrate model of photoperiodic response. In general, there are two types of vertebrate models used in studies of photoperiodism. The first type comprises species that are of ecological or economic interest, but are difficult to raise, maintain, and/or manipulate in controlled conditions. Studies of these species, such as salmonids, passerines, and wild rodents, often rely upon assumed proxies of photoperiodic time measurement, such as migratory timing, metabolic dormancy, reproductive maturation or reproductive quiescence. These phenotypes may be mediated by photoperiod, but the impracticality of studying them in strictly controlled conditions means that the effects of other environmental signals upon their timing, such as temperature, nutrient availability and presence of con-specifics, are unmeasured. We are

therefore unable to attribute any observed variation in seasonal timing strictly to variation in photoperiodic response. As a result, our ability to make inferences regarding the contribution of photoperiodism to life history modes and adaptation to local environments in vertebrates is compromised.

The second category of vertebrate models include organisms that are practical to manipulate in controlled laboratory conditions and are amenable to the techniques necessary to understand the processes underlying phenotypic responses to photoperiod, such as hamsters, Soay sheep, and Japanese quail. Studies using such models have greatly advanced our understanding of the transcriptional, hormonal and anatomical basis of photoperiodism. Major findings include the earliest indicators of the transcriptional and hormonal responses to long days and the conserved nature of these responses in mammals and birds (Yasuo & Yoshimura, 2009). However, studies using this type of model have not considered or are unable to consider how intraspecific comparisons of different life histories or latitudes may inform our understanding of vertebrate photoperiodism. In addition, these results may be affected by inadvertent evolution of the study lines caused by their maintenance in small populations over many generations in laboratory conditions, which may result in evolution due to inbreeding and/or adaptation to an unnatural environment. The effects of reliance on single populations or laboratory maintained lines on our understanding of the transcriptional and physiological basis of photoperiodic response is unknown. At the least, it limits our ability to address these aspects of photoperiodism in natural vertebrate populations and, in the future, to understand the underlying basis of among population variation in vertebrate photoperiodic response.

The threespine stickleback has the advantages of both these types of models. Like the first type, it inhabits a wide latitudinal (i.e. climatic) range and has several distinct life history strategies (Bell & Foster, 1994). Like the second type, its photoperiodic response can be phenotyped in laboratory conditions that control for the potential effects of other environmental signals (Yeates-Burghart *et al.*, 2009; Chapter III) and it is amenable to the techniques necessary to understand the basis of phenotypic responses to photoperiod (Cresko *et al.*, 2007; Chapter IV). Chapters III and IV demonstrate how these advantages are employed to advance our understanding of vertebrate photoperiodism.

Chapter III measures the photoperiodic response of multiple populations from Alaska and Oregon. The severity of seasonality increases with latitude, so the consequences for the mistiming of seasonal behavior are also expected to increase. This is hypothesized to result in a greater reliance on photoperiod as a predictable indicator of seasonal change at higher latitudes (Bradshaw & Holzapfel, 2007). Although there are multiple examples of variation in invertebrate photoperiodic response and proxies of vertebrate photoperiodic response over such a latitudinal range (reviewed in Danilevskii, 1965; Bradshaw & Holzapfel, 2007), there is no difference among populations or between the two latitudes in photoperiodic response. We suggest that the lack of inter-latitudinal difference may result from differences in how the populations respond to temperature in the wild.

These data are the first study of photoperiodic response across multiple populations of a vertebrate in controlled conditions. They demonstrate that outbred lines created from multiple populations are practical to raise and maintain in a common environment and that the photoperiodic response of these lines can be measured in

conditions that control for the effects of other environmental signals. As such, they are the initial steps in establishing the threespine stickleback as a model of vertebrate photoperiodism.

How will the threespine stickleback inform our understanding of photoperiodism in relation to life history and local environment?

Threespine stickleback occur from northern Alaska (Bell & Foster, 2004) to as far south as the Baja peninsula on the west coast of North America (Sánchez-Gonzáles *et al.*, 2002). Future work regarding the relationship between the severity of seasonal change and the reliance upon a reliable, predictive cue of it should take advantage of this large latitudinal range. We expect that more southern populations than Oregon and Alaska will rely less on photoperiod and more on proximate environmental signals, such as nutrient availability and water temperature, as cues for the initiation of sexual maturation. This work would be the first to address latitudinal trends in vertebrate photoperiodism.

The threespine stickleback is also a valuable model for understanding the effects of life history on photoperiodism. Very little is known about how photoperiodism constrains, facilitates, or otherwise influences adaptation to an environment. There are several examples in vertebrates of the rapid evolution of seasonal timing after introduction to a new environment or during environmental change (Quinn & Adams, 1996; Quinn *et al.*, 2000; Réale *et al.*, 2003; Bearhop *et al.*, 2005; Møller, 2007). These traits are mediated by photoperiodism, but the relative contributions of photoperiodism, response to other environmental signals, or phenotypic plasticity to the observed changes have not been determined.

Many of the divergent life history forms produced in the threespine stickleback adaptive radiation have been used as behavioral, ecological, and, more recently, genomic models (Bell & Foster, 1994; Kingsley *et al.*, Cresko *et al.*, 2007). This background knowledge would aid the selection of populations for the phenotyping of photoperiod response and inform interpretation of results regarding the effects of life history on photoperiodism. For instance, adaptive morphological differences are maintained over small spatial scales between populations of lake and stream stickleback populations in British Columbia (Hendry *et al.*, 2002; Berner *et al.*, 2008). Lake stickleback sexually mature several weeks later than stream stickleback (A. Hendry, pers. comm.). This difference is genetic, as it maintained in laboratory conditions (A. Hendry, pers. comm.), but it is unclear if it is a result of variation in photoperiodic response or temperature response. This can be tested using the straightforward methods we demonstrated in Chapter III. If there is variation in photoperiodic response between these lake and stream populations, it suggests that such variation may quickly evolve as an isolating mechanism for the maintenance of local adaptation in the presence of maladaptive gene flow.

This example demonstrates the potential of the stickleback model to inform our understanding of how photoperiodism evolves during adaptation to an environment, its interactions with life history, and its potential as a mechanism to facilitate adaptation. Such studies will provide a foundation for understanding the genetic and physiological bases of photoperiodism.

How will the threespine stickleback inform our understanding of the genetic basis of photoperiodism?

Most laboratory studies on vertebrate photoperiodism have focused on the processes underlying photoperiodic response, at the expense of addressing the genetic basis of photoperiodic interpretation. Many studies that have addressed this question have done so using the candidate gene approach, often under the assumption that the circadian clock forms the basis of the photoperiodic timer. As is discussed in Chapter I and demonstrated in Chapters II and III, the evidence for this is equivocal at best. Actual progress will come from the forward genetic approach, which is unconstrained by selecting a priori candidates for examination.

Forward genetics is initially more difficult than the candidate gene approach, as it requires understanding a complex trait such as photoperiodism in a way that makes it amenable to genetic dissection. Recent work in mammals, birds (Nakao *et al.*, 2008; Dardente *et al.*, 2010; Masumoto *et al.*, 2010), and now a teleost fish (Chapter IV) that defined the earliest transcriptional responses to a stimulatory photoperiod has made this possible. This early response can be used as a time point around which to conduct tissue specific sampling of transcribed genes. Those that differ in expression between organisms in stimulatory and non-stimulatory photoperiods will be involved in either interpretation of photoperiod or the initial response.

These genes will be the basis of two types of follow-up studies. The first is to determine their function: where and when they are expressed, what other genes they interact with, and the phenotypic results of interfering with their expression. This work is

necessary to establish evidence of their causal involvement in photoperiodic interpretation beyond the initial correlation of differential expression with photoperiodic response.

The second type of study proceeds from the first: if a subset of these genes is functionally involved in photoperiodic interpretation, it is necessary to determine if they vary in natural populations and if this variation is associated with phenotypic variation in photoperiodism. A gene may be functionally involved in a trait without being involved in evolution of that trait. This may be due to constraints on its evolution resulting from pleiotropy, the function of its protein product, or a lack of genetic variation within it that can respond to selection. Thus, a survey of these genes in natural populations that vary in photoperiodic response is necessary to understand which are actually involved in the evolution of photoperiodism. Such work will rely on establishing phenotypic variation in photoperiodism among populations, as described in the previous section.

Conclusion: how studies of vertebrate photoperiodism will inform our understanding of evolution

Determining which genes underlie the apparent ability of the photoperiodic timer to rapidly evolve in response to a changing environment (Quinn & Adams, 1996; Quinn *et al.*, 2000; Réale *et al.*, 2003; Bearhop *et al.*, 2005; Møller, 2007) will allow us to make a connection between genotype and phenotype in a complex trait that is essential for organismal fitness in the wild. Connecting genetic variation to phenotypic variation is a central goal of evolutionary genetics (Lewontin, 1974). The modern field of evolution of development was founded in large part because of the realization that this requires

understanding the physiological and cellular processes that connect these two levels of biological organization. In this context, the study of vertebrate photoperiodism will provide valuable insight into how a trait whose downstream physiological response can be conserved across hundreds of millions of years of evolution (Yasuo & Yoshimura, 2009; Chapter IV) can also rapidly evolve in response to a changing environment. Although it is a complex trait, whose study is made more difficult by the timescale on which its output occurs, this conservation suggests that it may be a more tractable phenotype for genetic dissection than it first appears and that results from an appropriate model organism may be generalizable across vertebrates.

The importance of proper seasonal timing for organismal fitness and its prevalence in polar and temperate organisms validates our work to understand photoperiodism on multiple levels of biological organization. The threespine stickleback is an excellent model for such studies because of its wide latitudinal range, varied life history and our ability to raise and manipulate outbred populations in controlled conditions. The work presented herein establishes it as a model of vertebrate photoperiodism and forms the foundation for future studies. The results will further our understanding of the timing of organismal processes in a seasonal environment and the connection between genotype and phenotype in a widespread and ecologically relevant trait.

APPENDIX A

SUPPLEMENTARY INFORMATION: CHAPTER II

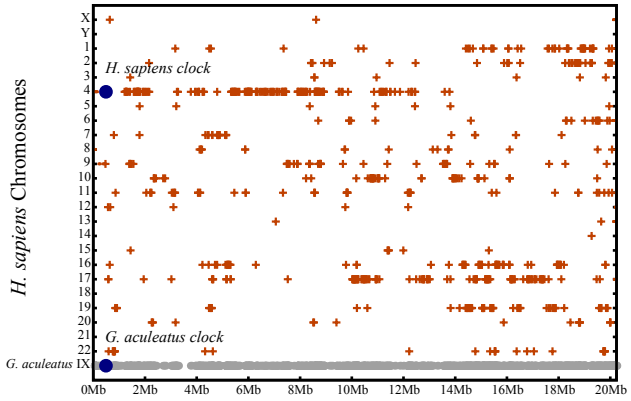
These supplementary Excel files can be downloaded separately from the dissertation.

Appendix S2.1, Regressions and calculations used to generate Fig. 2 in the main text. File title: "MEC_5133_sm_AppendixS1.xls."

Appendix S2.2. Data, regression and ANOVAs for average PolyQ length, run time, and latitude for Fig. 4 in the main text. File title: "MEC_5133_sm_AppendixS2.xls."

APPENDIX B

SUPPLEMENTARY INFORMATION: CHAPTER III



Supplementary Fig. 3.1. Syntenic analysis of the *G. aculeatus clock* gene. Gray dots indicate genes found on *G. aculeatus* linkage group IX. Red crosses indicate orthologs in the *H. sapiens* genome. *H. sapiens* (ENSG00000134852) and *G. aculeatus* (ENSGACG00000015939) *clock* homologs are indicated by blue circles.

APPENDIX C

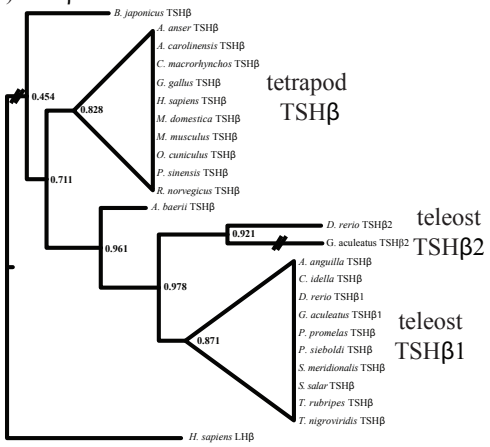
SUPPLEMENTARY INFORMATION: CHAPTER IV

		Photoperiod Treatment				
		Day 0	**Day 1**	**Day 2**	**Day 5**	**Day 10**
Population	**North 1**	10	12	14	10	10
	North 2	10	10	10	10	8
	South	8	10	8	8	8

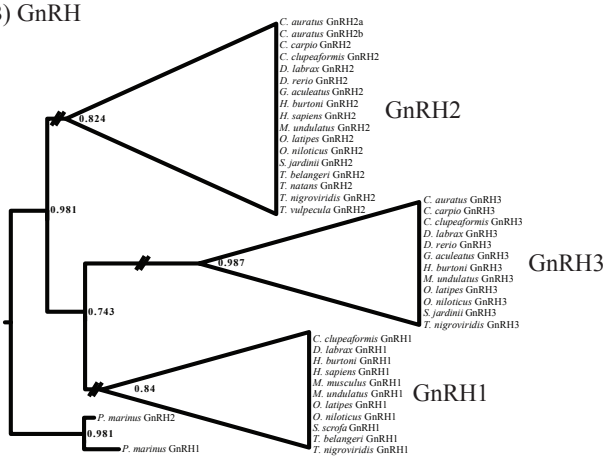
Supplementary Table 4.1. Sample sizes per treatment for quantitative PCR experiments. All fish were adult males.

Supplementary Fig. 4.1. (next page). Phylogenetic reconstructions**.** For all trees, the best model was JTT (Jones *et al.*, 1992) with a gamma-distributed set of evolutionary rates (Zang *et al.*, 1996). Although the preprohormones of the target orthologs are short (90 – 150 amino acids), and thus have poor phylogenetic signal, the *G. aculeatus* orthologs measured in this study are placed in the expected clades with high support. SH-like support scales the likelihood ratio test between 0 – 1. Nodes with values greater than .7 are considered highly supported. Branch lengths are proportional within phylogenies, except where hash marks indicate truncation. Clades with poor internal branch support are collapsed. Species within collapsed clades have been alphabetized by genus name. SH-like support values are listed at their respected nodes. **(A) TSHβ.** *Homo sapiens* LHβ was used to root the TSHβ phylogeny. There is a strong separation between tetrapod and teleost TSHβ orthologs, although incorrect placement of *B. japonicus* makes the tetrapod TSHβ paraphyletic. This is a result of the poor phylogenetic signal, as indicated by the weak node support. The threespine stickleback TSHβ1 ortholog measured in this study is within the expected clade. **(B) GnRH.** The *Petromyzon marinus* GnRH paralogs were used to root this phylogeny. There is strong support for monophyly of the three GnRH forms. As expected, GnRH3 is nested within the GnRH3 clade, as GnRH3 is unique to teleosts (Chen & Fernald, 2008). As expected, the *G. aculeatus* GnRH3 ortholog measured in this study is within this clade. **(C) LHβ.** *Homo sapiens* TSHβ was used to root the gonadotropin phylogeny. There is strong support for distinct FSHβ and LHβ clades. Within these, there is strong support for tetrapod and teleost clades, although the incorrect placement of *X. laevis* LHβ and *N. forsteri* LHβ makes the tetrapod LHβ clade paraphyletic and lowers the node support for the division between it and the teleost LHβ clade. As expected, the *G. aculeatus* LHβ ortholog measured in this present study is within the teleost LHβ clade.

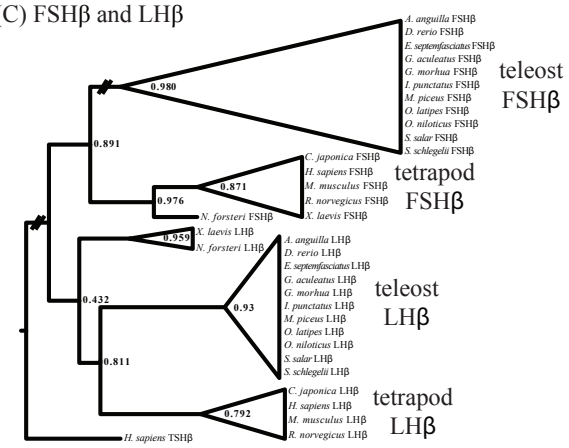
(A) TSHβ



(B) GnRH



(C) FSHβ and LHβ



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