

Published in final edited form as:

*Curr Biol.* 2010 December 7; 20(23): 2124–2130. doi:10.1016/j.cub.2010.10.050.

## Adaptive Divergence in the Thyroid Hormone Signaling Pathway in the Stickleback Radiation

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### Summary

During adaptive radiations, animals colonize diverse environments, which requires adaptation in multiple phenotypic traits [1]. Because hormones mediate the dynamic regulation of suites of phenotypic traits [2–4], evolutionary changes in hormonal signaling pathways might contribute to adaptation to new environments. Here, we report changes in the thyroid hormone signaling pathway in stream-resident ecotypes of threespine stickleback fish (*Gasterosteus aculeatus*), which have repeatedly evolved from ancestral marine ecotypes [5–8]. Stream-resident fish exhibit a lower plasma concentration of thyroid hormone and a lower metabolic rate, which is likely adaptive for permanent residency in small streams. The thyroid stimulating hormone- $\beta 2$  (*TSH $\beta 2$* ) gene exhibited significantly lower mRNA expression in pituitary glands of stream-resident sticklebacks relative to marine sticklebacks. Some of the difference in *TSH $\beta 2$*  transcript levels can be explained by *cis*-regulatory differences at the *TSH $\beta 2$*  gene locus. Consistent with these

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#### Accession Numbers

Microarray data reported herein have been deposited at the Center for Information Biology Gene Expression (<http://cibex.nig.ac.jp/index.jsp>) with the accession number CBX139.

#### Supplemental Data

Supplemental Information includes Supplemental Experimental Procedures, two figures, and one table.

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expression differences, a strong signature of divergent natural selection was found at the *TSH $\beta$ 2* genomic locus. By contrast, there were no differences between the marine and stream-resident ecotypes in mRNA levels or genomic sequence in the paralogous *TSH $\beta$ 1* gene. Our data indicate that evolutionary changes in hormonal signaling have played an important role in the postglacial adaptive radiation of sticklebacks.

### Highlights

- Low plasma concentration of thyroid hormone has evolved in stream sticklebacks.
- Lower metabolic rate of stream sticklebacks may be adaptive.
- Pituitary *TSH $\beta$ 2* mRNA level is reduced in stream sticklebacks.
- Evidence of *cis*-regulatory divergence and signatures of natural selection in *TSH $\beta$ 2*.

## Results and Discussion

### Divergence in Thyroid Hormone Concentration

Hormones mediate the regulation of diverse phenotypic traits. Therefore, hormonal divergence between ecotypes can facilitate or constrain the adaptive evolution of a suite of correlated phenotypic traits in diverse environments and is likely to underlie several life history trade-offs [2]. Although suites of morphological, behavioral, and physiological changes are common in adaptive radiations [1], little is known about the hormonal basis of these changes. The threespine stickleback (*Gasterosteus aculeatus*) provides a great model system to explore the hormonal basis for adaptive radiation. After the last glacial recession about 12,000 years ago, ancestral marine sticklebacks colonized newly formed freshwater streams and lakes in many coastal regions of the Northern hemisphere, resulting in the repeated evolution of freshwater-resident ecotypes (Figures 1A and B) [5–8]. The genetic basis of morphological traits that have evolved repeatedly during freshwater colonization has been investigated [7,9–14], but the hormonal and genetic basis for adaptive physiological traits is less well understood.

In the present study, we investigated divergence in thyroid hormone signaling pathways between marine and stream-resident sticklebacks. Marine sticklebacks have a migratory lifecycle in which adults migrate to freshwater or estuaries to spawn in the spring and summer, and juveniles migrate back to the sea in the fall, whereas stream-resident sticklebacks stay in freshwater for their entire life [5,15]. Thyroid hormone plays a key role in regulating many of the physiological and behavioral changes associated with migration in fish and birds [16–19]. For example, thyroid hormone regulates metabolic rate [20,21], swimming behavior [22,23], salinity preference [24], salinity tolerance [25], silvering [26], olfactory cellular proliferation [27], and sexual maturation [23] in fishes. Therefore, we examined whether thyroid hormone physiology has diverged between marine and stream-resident sticklebacks with contrasting migratory behaviors.

We first collected marine and stream-resident sticklebacks from multiple populations in the Pacific Northwest and Japan during the summer (Figures 1A and B) and measured plasma thyroid hormone (thyroxine; T4) concentration by radioimmunoassay (Figure 1C). Marine populations had significantly higher plasma T4 concentrations than stream-resident populations (Figure 1C; nested ANOVA,  $F_{1, 11} = 50.4$ ,  $P < 0.001$ ). Next, we made pure crosses from both a marine population and a stream-resident population that have a

parapatric distribution in the Little Campbell River (Site 1 and Site 8 in Figure 1B; [15]); these parapatric populations were chosen because these two collection sites are at the same latitude (i.e. similar seasonal photoperiodic regime). We then measured the plasma T4 concentration of laboratory-raised fish under both short (light-dark cycle LD = 8:16) and long photoperiods (LD = 16:8). The laboratory-raised marine fish had significantly higher T4 concentration than the laboratory-raised stream-resident fish under both short and long photoperiods (Figure 1D; nested ANOVA; effect of ecotype,  $F_{1,4} = 26.04$ ,  $P = 0.007$ ; effect of photoperiod,  $F_{1,4} = 0.30$ ,  $P = 0.614$ ; interaction between ecotype and photoperiod,  $F_{1,4} = 0.12$ ,  $P = 0.751$ ). Thus, the marine and stream-resident sticklebacks have genetically-based differences in T4 concentration, and the low T4 concentration in stream-resident fish does not simply result from environmental factors in fresh water, such as lower environmental levels of iodine, a precursor of thyroid hormone [28].

Animals have another form of thyroid hormone in plasma, triiodothyronine (T3). T3 is considered the more biologically active form of thyroid hormone since thyroid hormone receptors generally show a greater affinity for T3 over T4 [29]. Although T4 is the dominant thyroid hormone secreted from thyroid follicles, plasma T4 is converted to T3 in the periphery by iodothyronine deiodinase (DIO) activity [30]. We measured plasma T3 and found that the plasma T3 concentration was generally lower than the plasma T4 concentration in sticklebacks (Figure 1E). Furthermore, we found that laboratory-raised marine sticklebacks have a higher plasma T3 concentration than the laboratory-raised stream fish under the short photoperiod (ANOVA,  $F_{1,18} = 6.82$ ,  $P = 0.0177$ ). Interestingly, plasma T3 concentration did not differ between ecotypes caught at the Little Campbell River in summer (ANOVA,  $F_{1,16} = 0.022$ ,  $P = 0.883$ ), which may indicate that photoperiod influences thyroid hormone physiology. In order to further investigate divergence in thyroid hormone physiology within target tissues, we conducted a genome-wide transcriptome analysis of the gill, one of the main target tissues of thyroid hormone in teleosts. Although there was no divergence in thyroid hormone receptor mRNA levels between ecotypes, *DIO3*, which converts the active T3 to the inactive T4, was expressed at higher levels in stream-resident fish (Figure S1D). In addition, *DIO2*, which converts T4 to T3, was expressed at higher levels in marine fish than in stream-resident fish. Interestingly, an enzyme involved in iodide salvage, *iodotyrosine deiodinase (IYD)* was expressed at higher levels in marine fish than in stream-resident fish (Figure S1D); loss-of-function of *IYD* can cause hypothyroidism in humans [31]. These results suggest that stream-resident sticklebacks have diverged from marine sticklebacks not only in plasma concentration of thyroid hormone, but also in other downstream signaling pathways.

### Role of Thyroid Hormone in Metabolic Regulation

A previous study demonstrated that a marine stickleback population has a higher oxygen consumption rate (i.e. metabolic rate) than a sympatric stream-resident population [32]. Here, we found a similar difference between laboratory-raised marine and stream sticklebacks (Figure 1F) (ANOVA; effect of ecotype,  $F_{2,29} = 38.9$ ,  $P < 0.001$ ), suggesting that the divergence in oxygen consumption rate has a genetic basis. We also conducted a liver metabolome analysis and found that laboratory-raised marine sticklebacks have higher levels of most intermediates in glycolysis and the TCA cycle than laboratory-raised stream-resident sticklebacks (Figure S1E). In our genome-wide transcriptome analysis, we also found that more genes involved in oxidative phosphorylation were expressed at a higher level in laboratory-raised marine fish than in laboratory-raised stream fish (Figure 1G). These collective data suggest that the marine ecotype has a higher metabolic rate than the stream-resident ecotype.

Because thyroid hormone can regulate metabolic rate in fishes [20,21], we tested whether the differences in thyroid hormone concentration are related to differences in metabolic rate

between the ecotypes. We treated fish with T4 and a T4 synthesis inhibitor (thiourea; TU). We found that treatment with T4 increased oxygen consumption rate, while treatment with TU decreased oxygen consumption rate in both ecotypes (ANOVA; effect of treatment,  $F_{2,29} = 5.89$ ,  $P = 0.007$ ) (Figure 1F). Although the oxygen consumption rate of the stream-resident ecotype did not increase to the level of that of the marine ecotype even when exposed to T4, this is not surprising, given the fact that the stream-resident ecotypes might have a reduced ability to produce T3 in peripheral target tissues (see above). However, there are likely factors that contribute to divergence in metabolic rate between ecotypes in addition to thyroid hormone physiology.

Because thyroid hormone treatment is known to increase swimming activity in teleosts [22], some of the effects of thyroid hormone on oxygen consumption rate might be due to effects on swimming activity rather than direct effects on the basal metabolic rate. To answer this question, we observed the swimming behavior of hormone-treated fish. Fish were released into a circular tank immediately after the measurement of oxygen consumption rate, and their behavior was monitored for 20 min. T4 and TU increased and decreased, respectively, the frequency of turning behavior in both ecotypes of sticklebacks (repeated measures ANOVA; effect of treatment on turning activity,  $F_{2,27} = 7.73$ ,  $P = 0.0022$ ; effect of ecotype,  $F_{1,27} = 1.43$ ,  $P = 0.242$ ) (Figure S1C). Therefore, we added turning activity (turns/min) as a covariate and re-analyzed the effects of hormonal treatment on the oxygen consumption rate. The effect of thyroid hormone and ecotype on the oxygen consumption rate remained significant, even after including turning activity as a covariate (ANCOVA with turning activity as a covariate; effect of treatment,  $F_{2,24} = 6.42$ ,  $P = 0.006$ ; effect of ecotype,  $F_{1,26} = 34.6$ ,  $P < 10^{-5}$ ; effect of activity,  $F_{1,24} = 0.38$ ,  $P = 0.543$ ). These results suggest that thyroid hormone may be involved in the regulation of both swimming activity and basal metabolic rate.

Divergence in metabolic rate might have evolved as an adaptation to the differential abundance of nutrients and oxygen in marine and freshwater environments. Temperate marine environments generally have more abundant energy sources than freshwater environments [33]. This is the case in the Pacific Northwest of North America; primary production in a marine environment of that coastal region (eg., 1.89~3.46 gC/m<sup>2</sup>·d in Elliot Bay, Washington) [34] is higher than in small lowland streams (eg., 0.02~0.55 gC/m<sup>2</sup>·d in Kelsey Creek, Washington) [35]. In addition, dissolved oxygen is consistently high in marine environments (> 5 mg/L in the Puget Sound, Washington) [36], while it can drop below 3.0 mg/L (Evans Creek and Big Soos Creek, Washington) [37,38] or even below 0.2 mg/L (Little Campbell Stream, British Columbia, Canada) [39] in small lowland streams. Thus, greater nutrient and oxygen availability in the marine environments may allow marine stickleback to have increased metabolic rate and swimming activity, which are likely adaptive for migration. By contrast, stream-resident stickleback with similarly high levels of thyroid hormone might be predisposed to increased risks of energy deficiency or cellular hypoxia given the comparatively limited energy sources and oxygen in their habitats [40,41]. In fishes, both hypoxic stress and low food availability are known to induce low thyroid hormone levels [42,43], which suggests that the genetically-based low thyroid hormone concentration we observe in stream-resident sticklebacks may be adaptive for permanent residency in oxygen- and nutrient-limited environments.

### Divergence in *TSHβ* Expression between Marine and Resident Ecotypes

The synthesis and secretion of thyroid hormone from thyroid follicles is stimulated by a pituitary glycoprotein hormone, thyroid stimulating hormone (TSH), in many animals [44–47], although this has not yet been confirmed in sticklebacks. The functional TSH hormone is a dimer of a TSH-specific β-subunit (*TSHβ*) and an α-subunit (*GPHα*) that is shared by other pituitary glycoprotein hormones. The threespine stickleback has two *TSHβ* paralogs,

one on linkage group (LG) 17 (*TSHβ1*) and another on LG12 (*TSHβ2*) (Figure S2A). Because many paralogous genes are found on stickleback LG17 and LG12 (Figure S2B) [48], *TSHβ1* and *TSHβ2* are likely the products of the teleost-specific genome duplication [49]. Further supporting this conclusion, two clades of teleost *TSHβ* genes are found in a phylogenetic tree created using the *TSHβ* protein from multiple vertebrate species (Figure S2B).

*TSHβ1* and *TSHβ2* mRNA levels in the stickleback pituitary gland were measured by quantitative polymerase chain reaction (qPCR) in laboratory-raised crosses from the Little Campbell marine and stream-resident populations [15]. Little variation was found in pituitary *TSHβ1* mRNA levels between photoperiods or between ecotypes (Figure 2A; nested ANOVA, effect of ecotype,  $F_{1,46} = 4.03$ ,  $P = 0.051$ ; effect of photoperiod,  $F_{1,46} = 1.73$ ,  $P = 0.194$ ; interaction between ecotype and photoperiod,  $F_{1,46} = 0.49$ ,  $P = 0.489$ ). By contrast, we found significant differences in *TSHβ2* mRNA levels between photoperiods as well as between ecotypes (Figure 2A). The marine ecotype has significantly higher levels of *TSHβ2* mRNA than the stream-resident ecotype (nested ANOVA, effect of ecotype,  $F_{1,46} = 33.68$ ,  $P < 0.001$ ). In both ecotypes, transcript levels were high under the short photoperiod and dropped significantly under the long photoperiod (nested ANOVA, effect of photoperiod,  $F_{1,46} = 84.57$ ,  $P < 0.001$ ). However, the degree of change in *TSHβ2* mRNA levels was different between the marine and stream-resident ecotypes (nested ANOVA, interaction between ecotype and photoperiod,  $F_{1,46} = 8.65$ ,  $P = 0.005$ ). In the marine ecotype, mRNA levels were 8 times higher under the short photoperiod than under the long photoperiod, while in the stream-resident ecotype, mRNA levels were only about 2 times higher under the short photoperiod than under the long photoperiod. This reduction in the magnitude of photoperiodic regulation of *TSHβ2* is consistent with the fact that stream-resident fish spend their entire lives in the freshwater environment and do not perform seasonal migrations.

Although plasma T3 concentration exhibited a similar photoperiodic change (Figure 1E), plasma T4 did not. A previous study in killifish indicates that the relationship between TSH level and thyroid hormone concentration can change according to photoperiod because negative feedback of thyroid hormone on TSH is stronger under a long photoperiod than under a short photoperiod [50]. In sticklebacks, we saw a similar pattern; T4 treatment reduced *TSHβ2* expression under the long photoperiod but not under the short photoperiod (Figure S2D). Therefore, a difference in the sensitivity of the pituitary gland to negative feedback of thyroid hormone on TSH might explain the difference in photoperiodic response between T4 and *TSHβ2*. Furthermore, there is evidence in mammals that TSH might act directly on peripheral tissues to stimulate DIO2 activity and the conversion of T4 to T3 [51], which is consistent with our observation that *TSHβ2* mRNA level and plasma T3 concentration are higher during the short photoperiod than during the long photoperiod. Clearly, further studies on the relationship between *TSHβ* expression and thyroid hormone signaling are required to better understand the thyroid hormone signaling pathway in sticklebacks.

### Divergent *TSHβ2* Haplotypes between Marine and Resident Ecotypes

Because *TSHβ2* expression was different between the marine and stream-resident ecotypes, we compared the genomic sequences of the 5'-non coding regions of *TSHβ2* and *TSHβ1* between the two ecotypes. This region of the *TSHβ1* gene did not differ between ecotypes (Table S1); nested analysis of molecular variance (AMOVA) revealed that little of the genetic variance (-7.57 %) was explained by ecotype ( $P = 0.923$ ), while most was explained by populations within ecotype (61.20%) and by individuals within populations (46.37 %). By contrast, marine and stream-resident sticklebacks diverge in the 5'-non coding region of the *TSHβ2* gene. Nested AMOVA revealed that 40.83 % of the total genetic variance in the

5'-non coding region of the *TSH $\beta$ 2* gene was explained by ecotype ( $P < 0.001$ ), while 33.03 % was explained by populations within ecotype, and 26.13 % was explained by individuals within populations (Table S1). In particular, four single nucleotide polymorphisms (SNPs) exhibited striking differences in frequency between ecotypes (Figure 2B); all marine fish except a single heterozygote have one haplotype (24/25 fish), regardless of their geographical origin. In contrast, most stream-resident fish were either homozygous or heterozygous for an alternative haplotype (39/46 fish).

The phylogenetic analysis of *TSH $\beta$ 2* alleles revealed that the stream-resident alleles form a single branch except that a Japanese stream-resident population (Hiranoi Creek) had an exclusively marine haplotype (Figure S2H). We confirmed that the Japanese stream-resident population also had a lower *TSH $\beta$ 2* mRNA expression level (Figure S2E). Interestingly, a nearby Japanese stream population was also found to have a marine haplotype at an armor plate gene, despite the fact that these fish exhibit armor reduction typical of stream-resident stickleback populations [7]. Thus, the genetic basis for freshwater adaptation in Japanese stream populations from this region may differ from the North American stream populations [7].

Divergence in the *TSH $\beta$ 2* haplotype is unlikely to have resulted from the phylogenetic relatedness of each ecotype. Previous phylogenetic studies of the Pacific Northwest sticklebacks indicated that stream-resident ecotypes have been independently derived from marine sticklebacks in each region [6–8,12]. In addition, ecotype was not a significant predictor of genotype at other genes in the thyroid hormone pathway (the *TSH $\beta$ 1* locus, see above; *Pit-1* locus, nested AMOVA,  $\Phi_{CT} = -0.15$ ,  $P = 1.00$ ; *TSH receptor 2* locus, nested AMOVA,  $\Phi_{CT} = 0.029$ ,  $P = 0.197$ ) or in a microsatellite marker upstream of the *TSH $\beta$ 2* gene (Figure S2G; nested AMOVA,  $\Phi_{CT} = -0.011$ ,  $P = 0.352$ ) (Table S1).

In order to confirm that the *TSH $\beta$ 2* *cis*-regulatory region contributes to divergence in the *TSH $\beta$ 2* mRNA levels, we generated F1 hybrids heterozygous for marine and stream-resident *TSH $\beta$ 2* alleles and tested whether allele-specific expression differences can be seen in the pituitary gland of F1 hybrid fish. Differential expression of alleles in the F1 fish with an otherwise identical genetic background indicates functional *cis*-regulatory differences [12,52]. Comparison of expression levels of two different alleles in the F1 fish was conducted using Pyrosequencing technology, which can compare the relative abundance of cDNA derived from the two alleles when a SNP is present in the cDNA [52]. In all of the F1 hybrids ( $n = 10$ ), the marine allele was expressed at a higher level than the stream-resident allele (Figure 2C). The ratio of the marine allele to the stream-resident allele was significantly larger than one (mean  $\pm$  SEM =  $1.60 \pm 0.13$ , *t*-test,  $t = 4.72$ ,  $df = 9$ ,  $P = 0.0010$ ). The higher expression of the marine allele than the stream-resident allele in the same F1 hybrid fish indicates that functional *cis*-regulatory differences contribute to the divergence in *TSH $\beta$ 2* mRNA expression levels between ecotypes.

Interestingly, we found that one SNP (LGXII:12329950) of the marine haplotype is located within a consensus motif of a thyroid hormone response element (Fig. S2G), while the stream-resident allele lacks this motif due to a nucleotide substitution at this site. Furthermore, a computational analysis demonstrates that RNA secondary structure might differ between these two alleles (Fig. S2I). Although we do not know yet whether these SNPs are actually causing the *cis*-regulatory expression changes or altering RNA processing, further molecular studies on the functional divergence of the ecotype-specific alleles will provide novel insights into the genetic and endocrine basis for the repeated evolution of freshwater-resident sticklebacks.

## Conclusions

We found divergence between marine and stream-resident sticklebacks in thyroid hormone physiology. Because thyroid hormone has diverse functions related to migration, divergence in thyroid hormone physiology might contribute to adaptation not only in metabolic rate and swimming activity, but also in other phenotypic traits that are known to differ between marine and freshwater sticklebacks. For example, marine and stream-resident sticklebacks differ in salinity preference behavior [24], salinity tolerance [21], the timing of sexual maturation [15], and many morphological traits [5]. There is evidence for the regulation of both osmoregulatory ability [25] and reproduction by thyroid hormone in fishes [53]. Although few studies have so far investigated the hormonal regulation of morphology in sticklebacks, thyroid hormone and TSH have been shown to regulate skeletal development and morphogenesis in many vertebrates including fish [43,54,55]. Further studies investigating the role of thyroid hormone in the morphological, physiological and behavioral divergence between marine and freshwater-resident sticklebacks should lead to a better understanding of how genetic changes can alter suites of phenotypic traits.

Here, we found that *cis*-regulatory changes are likely important for the difference in *TSH $\beta$ 2* mRNA expression between the marine and stream-resident ecotypes, consistent with a signature of divergent natural selection at that locus. Similar to results for loci underlying plate reduction and color change in stickleback [7,12,56], we identified a single heterozygote in an ancestral marine population with an allele that is predominant in freshwater environments. These data support the idea that the pre-existing allelic variation in an ancestral population can facilitate rapid adaptation to novel environments [7,56–58]. Overall, our results suggest that divergence in hormonal signaling may play a substantial role in the evolution of multiple phenotypic traits during an adaptive radiation in animals. The threespine stickleback system provides an opportunity to further explore the molecular basis for hormonal divergence underlying multiple adaptive traits.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

We thank Ikumi Nakamura, Masakado Kawata, Manabu Kume, Jon Dickey, Felicity Jones, Frank Chan, David Kingsley, Yoshio Takei, Susumu Hyodo, Sanae Ogasawara, Shaun McCann as well as all members of the Peichel and Swanson Labs for valuable advice, discussion, and technical assistance. This work was supported by the Uehara Memorial Foundation (J.K.), the Brain Science Foundation (J.K.), JST PRESTO program (J.K.), and Grant-in-Aid for Young Scientist (B) from the Ministry of Education, Science, Sports, and Culture (J.K.), the NOAA Oceans and Human Health Initiative (S.C.L.), a Burroughs Wellcome Fund Career Award in the Biomedical Sciences (C.L.P.), and National Institutes of Health grant P50 HG02568 (C.L.P.). Sampling in Washington State was conducted under Washington Department of Fish and Wildlife permits to CLP (05-049, 06-159, 07-047). Canadian and Japanese fish were collected with the courtesy of Dolph Schluter and Manabu Kume, respectively. All experiments were reviewed and approved by the Fred Hutchinson Cancer Research Center Institutional Animal Care and Use Committees (protocol #1575).

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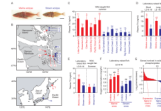
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**Figure 1. Low plasma thyroid hormone concentration and metabolic rate in stream-resident stickleback**

(A) Representative images of a marine ecotype (Duwamish River, Site 2 in Figure 1B) and a stream-resident ecotype of threespine stickleback (Big Soos Creek, Site 14 in Figure 1B). Skeletal structures are visualized with alizarin red staining. Scale bar = 10 mm.

(B) Map of collection sites in the Pacific Northwest of North America (upper panel) and the Japanese Archipelago (lower panel). Red dots indicate the collection sites of marine fish, while blue dots indicate the collection sites of stream-resident fish. Numbers indicate the sampling sites: (1) Little Campbell River marine; (2) Duwamish River marine; (3) Clam Bay marine; (4) Seabeck Bay marine; (5) Quilcene Bay marine; (6) Akkeshi Pacific Ocean marine; (7) Akkeshi Japan Sea marine; (8) Little Campbell River stream; (9) Salmon River stream; (10) Evans Creek stream; (11) Forbes Creek stream; (12) Kelsey Creek stream; (13) May Creek stream; (14) Big Soos Creek stream; (15) Allen Creek stream; (16) Hiranoi Creek stream.

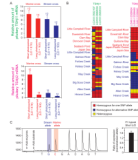
(C) Plasma T4 concentration (mean  $\pm$  SEM) of sticklebacks caught in early summer. Red bars indicate marine populations, while blue bars indicate stream-resident populations. Sample sizes for each population are shown above each bar. The number in parenthesis indicates the collection site shown in Figure 1B.

(D) Plasma T4 concentration (mean  $\pm$  SEM) of laboratory-raised sticklebacks sampled under a short photoperiod (left) or a long photoperiod (right). A grand mean ( $\pm$  SEM) of two independent families for each cross is shown in the graph. Data for each family are available in Figure S1A. Sample sizes for each group are shown above each bar.

(E) Plasma T3 concentration (mean  $\pm$  SEM) of laboratory-raised sticklebacks sampled under a short photoperiod (left) or wild fish (Little Campbell River marine and stream-resident ecotypes) caught in early summer (right). Sample sizes for each group are shown above each bar.

(F) Effect of thyroid hormone inhibitor (thiourea) and thyroid hormone (thyroxine, T4) treatment on oxygen consumption rate. Oxygen consumption rate was divided by body weight (g) and shown as rate per hour. Mean  $\pm$  SEM is shown. Sample sizes for each group are shown above each bar. Letters above the bars indicate the results of Waller-Duncan's posthoc tests after ANOVA.

(G) Expression levels of 74 genes involved in oxidative phosphorylation were compared between marine and stream-resident ecotypes by microarray. Red bars indicate the genes expressed more in marine fish than in stream-resident fish, while the blue bars indicate the genes expressed more in stream-resident fish than in marine fish. Fold change of transcript level (marine fish signal divided by the stream-resident fish signal) is shown as the logarithm to base 2, so 1 indicates that the signal intensity of the transcript is twice as high in marine fish than in stream-resident fish, while -1 indicates the signal intensity of the transcript is twice as high in stream-resident fish than in marine fish.



**Figure 2. Divergent *TSHβ2* transcript level and *cis*-regulatory sequence between marine and stream-resident sticklebacks**

(A) Photoperiodic response of total pituitary *TSHβ1* (upper panel) and *TSHβ2* (lower panel) mRNA level. A grand mean ( $\pm$  SEM) of two independent families is shown in the graph. Sample sizes for each group are shown above each bar. Expression level was determined by comparing the amplification threshold cycle to that of a serially diluted standard cDNA sample, followed by multiplication of the amount of RNA isolated from each pituitary gland.

(B) Three representative SNPs at the *TSHβ1* locus did not show any significant segregation between marine and stream-resident ecotypes (left panel). Four representative SNPs at the *TSHβ2* locus significantly differed in frequency between marine and stream-resident ecotypes (right panel). Results of genetic analyses are available in Table S1. In both panels, red squares indicate that an individual is homozygous for one SNP allele, blue squares indicate that an individual is homozygous for the alternative allele, and yellow squares indicate that an individual is heterozygous. Details of SNP information are shown in Figure S2.

(C) Pyrosequencing of pituitary cDNA in F1 hybrid fish between a Little Campbell River marine female and a Little Campbell River-resident male. The left panel indicates a representative pyrogram showing a higher peak on C than on G at a SNP site (for the SNP, see Fig. S2G). The right panel indicates the ratio (mean  $\pm$  SEM) of marine to stream-resident *TSHβ2* allelic expression (n = 10).