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Divergence in thyroid hormone concentrations between juveniles of marine and stream ecotypes of the threespine stickleback (*Gasterosteus aculeatus*)

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ABSTRACT

Background: Hormones regulate the expression of multiple phenotypic traits. Therefore, divergence in hormone concentrations may lead to evolutionary changes in the coordinated physiological and behavioural traits that comprise an organism's integrated phenotype. Adults of marine ecotypes of threespine stickleback (*Gasterosteus aculeatus*) have higher concentrations of the thyroid hormone thyroxine (T_4) than adults of stream-resident ecotypes (Kitano *et al.*, 2010). Thyroid hormones are well-established mediators of osmoregulation and migratory behaviours in fish, and the difference in T_4 concentrations indicates that changes in thyroid hormone signalling may underlie the evolutionary and ecological divergence of migratory and non-migratory ecotypes.

Questions: Is the variation in T_4 concentrations present in earlier life stages where it could contribute developmentally to differences in phenotype? Do T_4 concentrations change in marine ecotypes before seaward migration?

Organisms: A parapatric pair of marine and stream ecotypes of threespine stickleback collected from British Columbia, Canada, and a marine ecotype collected from Washington State, USA.

Methods: We collected juvenile fish of both marine and stream ecotypes on the same day in a single river to compare the whole body concentrations of T_4 using radioimmunoassay. We also sampled juvenile fish of the marine ecotype in another river at three different times to determine whether these fish exhibit temporal changes in T_4 concentrations before seaward migration.

Results: Juvenile stickleback of the marine ecotype had higher T_4 concentrations than the parapatric stream-resident juveniles. The T_4 concentrations in another marine population varied slightly across sampling times before seaward migration.

Conclusions: T_4 concentrations differ consistently between marine and stream ecotypes in both juvenile and adult life stages consonant with the hypothesis of evolutionary changes in thyroid signalling.

Keywords: endocrine, phenotypic correlation, physiology, stickleback, trade-offs.

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INTRODUCTION

Adaptation to contrasting environments is often accompanied by divergence in multiple phenotypic traits (Lande and Arnold, 1983; Schluter, 2000). When selection favours multiple phenotypic traits in combination and a certain permutation of traits conveys a fitness advantage, genetically based trait correlations can evolve resulting in an adaptive, integrated phenotype (Pigliucci, 2003). Alternatively, phenotypic correlations may exist as a non-adaptive consequence of inherent genetic and developmental constraints (Pigliucci, 2003). Given such opposing mechanisms, it has been proposed that phenotypic correlations can either facilitate adaptive evolution to divergent environments or constrain independent evolution of different phenotypic traits (Lande, 1979; Schluter, 2000; Agrawal and Stinchcombe, 2009; Kirkpatrick, 2009). Understanding the distinction between these two possibilities, therefore, requires not only investigation of the multiple phenotypic traits that have evolved in divergent environments, but also identification of the genetic and physiological mechanisms that underlie correlations between those traits, and how those mechanisms may create evolutionary trade-offs for populations occupying dissimilar environments.

Since animal migration involves many different phenotypic attributes, including morphological, physiological, and behavioural characters (Dingle, 1996), evolutionary divergence between migratory and non-migratory ecotypes requires divergence in a suite of phenotypic traits. Migratory and non-migratory ecotypes can evolve within a single species, and examples of such ecotypes are known across diverse taxa (McKeown, 1984; McDowall, 1988; Berthold, 1993; Hendry *et al.*, 2004; Roff and Fairbairn, 2007). Previous theoretical and ecological studies have demonstrated trade-offs associated with animal migration. Migration not only has advantages such as optimal foraging, avoidance of adverse environments, and selection of better spawning sites, but also has disadvantages such as high energetic costs and increased predation (McKeown, 1984; Dodson, 1997; Hendry *et al.*, 2004). The optimal trade-offs between these advantages and disadvantages may vary in different environments, which can lead to diversity in migratory behaviours.

In fish and other vertebrates, hormones have been shown to coordinate the expression of migration-related characters as organisms prepare for and undertake migration. Hormones act on a variety of target tissues to regulate the expression of multiple phenotypic traits at the organismal level and, therefore, have been proposed to be key mediators of phenotypic integration (Finch and Rose, 1995; Ketterson and Nolan, 1999; Flatt *et al.*, 2005; Zera *et al.*, 2007; McGlothlin and Ketterson, 2008; Ketterson *et al.*, 2009). Thyroid hormones mediate many physiological and behavioural functions related to fish migration, including locomotor activity (Castonguay and Cyr, 1998; Katzman and Cech, 2001; Edeline *et al.*, 2005; Kitano *et al.*, 2010), rheotactic behaviour (Edeline *et al.*, 2005; Imbert *et al.*, 2008), swimming speed (Katz and Katz, 1978), metabolic rate (Kitano *et al.*, 2010), salinity preference (Baggerman, 1957), osmoregulation (Gutz, 1970), reproduction (Costadinos *et al.*, 1994; Swapna and Senthilkumaran, 2007), growth (Power *et al.*, 2001), olfactory imprinting (Nevitt and Dittman, 1998; Lema and Nevitt, 2004), and silvering (Ura *et al.*, 1994). Therefore, studying the evolutionary changes in hormone signalling that underlie phenotypic divergence between migratory and non-migratory ecotypes may not only inform how hormones mediate the morphological, physiological, and behavioural changes necessary for migration (McKeown, 1984; McDowall, 1988; Roff and Fairbairn, 2007), but also provide new insights into the genetic changes underlying the evolution of hormonally mediated trait correlations under dissimilar environmental conditions.

The threespine stickleback (*Gasterosteus aculeatus*) provides a tractable model system for elucidating the genetic mechanisms of variation in migratory behaviour (Kitano *et al.*, 2012).

Tremendous diversification of threespine stickleback has occurred during the last few million years and resulted in the evolution of multiple phenotypically and ecologically divergent ecotypes, including sea-run migratory and non-migratory ecotypes (Wootton, 1976, 1984; Bell and Foster, 1994). Even within sea-run migratory ecotypes, some breed in estuaries, while others breed in fresh water (see Kitano *et al.*, 2012 and references therein). In this paper, we call both marine ecotypes. Marine and stream-resident ecotypes have diverged in many morphological, physiological, and behavioural traits. For example, the marine ecotypes have higher tolerance of seawater (Heuts, 1946, 1947; Gutz, 1970), higher metabolic rate (Tudorache *et al.*, 2007; Kitano *et al.*, 2010; Dalziel *et al.*, 2012a), and higher swimming endurance (Taylor and McPhail, 1986; Dalziel *et al.*, 2012b) than stream ecotypes. Interestingly, hormonal manipulations have demonstrated that thyroid hormones improve performance on most of these physiological and behavioural measures of migratory ability (Gutz, 1970; Kitano *et al.*, 2010), suggesting that thyroid hormones may play a key role in mediating the phenotypic changes necessary for migration in sticklebacks, as these hormones do in other teleost fish taxa (McCormick *et al.*, 1998). Previous studies provide evidence to support this idea, as adult marine ecotypes have higher plasma concentrations of the thyroid hormone thyroxine (T_4) than adult stream ecotypes (Kitano *et al.*, 2010). These differences in thyroid hormone concentrations appear to have a genetic basis, as laboratory-raised fish continue to show the same ecotypic differences in plasma T_4 concentrations (Kitano *et al.*, 2010).

Currently, however, we do not know whether the variation in T_4 concentrations between marine and stream ecotypes of threespine stickleback is also present in earlier life stages where it could contribute developmentally to differences in phenotype. Thyroid hormone signalling pathways can regulate skeletal development in animals (Power *et al.*, 2001; Abe *et al.*, 2003; Waung *et al.*, 2012), and developmental variation in thyroid hormones has been shown in teleost fishes to result in differences in body morphology, fin growth, and behaviour (Brown, 1997; Power *et al.*, 2001; Lema and Nevitt, 2006; Shkil *et al.*, 2012). In this study, we collected juvenile fish of a parapatric pair of marine and stream ecotypes on the same day to compare the whole body titres of T_4 using radioimmunoassay.

We also do not know whether T_4 concentrations change in fish of the marine ecotype before seaward migration. If expression of the traits important for migration has some costs, titres of these hormones may increase only before and during migration. In salmonids, for instance, plasma T_4 concentrations increase only before and/or during downstream migration (Dickhoff *et al.*, 1978; Grau *et al.*, 1981; Youngston and Simpson, 1984). To determine whether juvenile marine stickleback exhibit temporal changes in T_4 concentrations before seaward migration, we sampled juvenile fish of the marine ecotype in another river at three different time points and analysed temporal changes in T_4 concentrations.

MATERIALS AND METHODS

Juvenile fish of threespine stickleback were collected from the estuary ($n = 18$) and upstream (approximately 7 km from the sea) ($n = 14$) of the Little Campbell River in British Columbia in Canada on 20 August 2006, and from the estuary of Duwamish River in Washington State, USA on 1 August ($n = 12$), 17 August ($n = 19$), and 11 September 2006 ($n = 8$) (Fig. 1). No stickleback were caught after early September in the Little Campbell estuary (Hagen, 1967) and no stickleback were caught after 20 September in the Duwamish River estuary (J. Kitano, unpublished observation), so all fish in these sampling sites are likely marine ecotypes with seasonal migration. In addition, previous studies demonstrated that all adults

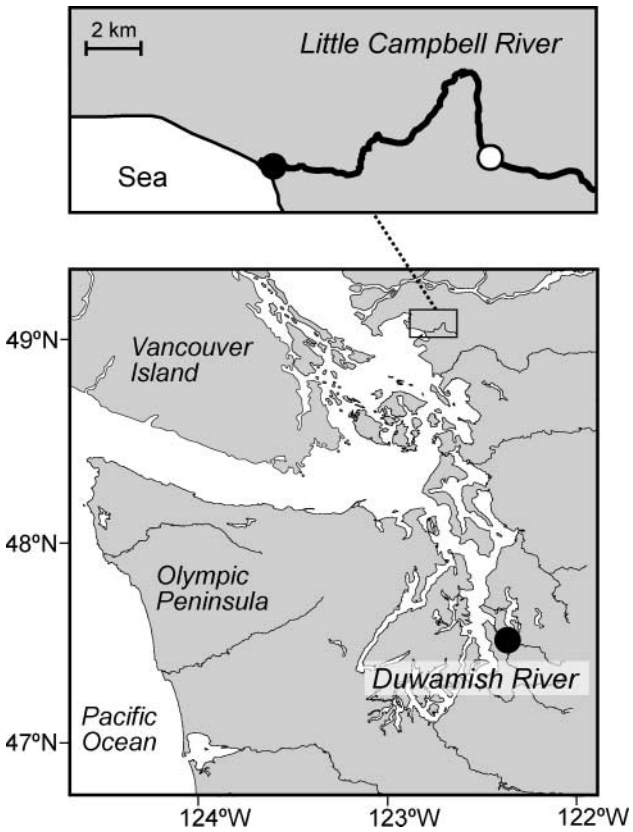

Fig. 1. Map showing the collection sites in the Little Campbell River and the Duwamish River. Solid circles indicate the sampling sites of the marine ecotype, while the open circle indicates the collection site of the stream ecotype.

caught in the Little Campbell estuary and the majority of adults caught at the Duwamish estuary were completely plated (Hagen, 1967; Kitano *et al.*, 2008): armour plate morph is often well correlated with ecotypes in threespine stickleback (Bell and Foster, 1994). In contrast, we consider that all fish caught 7 km upstream of the Little Campbell River were stream-resident ecotypes, because all fish caught at this site were low plated and several falls exist between the estuary and the sampling site (Hagen, 1967; Kitano *et al.*, 2008). Fish were collected under the permits WA06-159 and NA/SUS06-21454. The fish were caught with a hand-net, euthanized with lethal doses of MS222, frozen immediately on dry ice, and stored at −70°C until use.

Whole-body concentrations of total T_4 were measured as described previously (Lema and Nevitt, 2006). Fish were homogenized in ice-cold 100% ethanol (0.9 mL) containing 1 mM 5-propyl-2-thiouracil (PTU) to block endogenous deiodinase activity, and the homogenate was spiked with 10 μL of I^{125}-labelled T_4 (1240 counts per minute; Perkin-Elmer, Waltham, MA, USA) to permit subsequent calculation of extraction efficiency. After sonication for 20 s followed by centrifugation at 3000 *g* for 20 min at 4°C, the supernatant was removed and saved for T_4 quantification. The tissue pellet was resuspended in 0.3 mL of ice-cold

ethanol with PTU (1 mM) by vortexing and sonication for 20 s, and centrifuged (3000 *g* for 20 min at 4°C). The resulting supernatant was then combined with the previously saved supernatant. After centrifugation at 64 *g* for 5 min at 4°C, the supernatant was vacuum dried at 37°C under N_2 gas, and then reconstituted in 100 μL of ice-cold phosphate buffered saline (0.1 mM, pH 7.4) containing 1 mM PTU. For 20 μL volumes of each sample, T_4 concentrations were then quantified in duplicate using radioimmunoassay as described previously (Dickhoff *et al.*, 1982). Samples were incubated for 2 h at 37°C in 0.11 M sodium barbital buffer with anti-L-T_4 antiserum (1:5000; Accurate Chemical & Scientific Corp., Westbury, NY, USA) and I^{125}-labelled T_4 (Perkin-Elmer, Waltham, MA, USA). Sodium barbital buffer containing 20% polyethylene glycol was then added to each sample, and samples were centrifuged at 1400 *g* for 20 min at 4°C. The supernatants were removed to separate free and bound hormone, and the remaining pellets were assayed for radioactivity (Cobra II gamma counter, Packard, Downer's Grove, IL, USA). The intra-assay coefficient of variation was 11.3%. The T_4 extraction efficiency from whole-body tissues was 69.04 ± 0.01% (mean ± S.E.), and the resulting T_4 value for each sample was corrected using the respective extraction efficiency for that same sample. Standards of 1.25 to 60 $ng \cdot mL^{-1}$ were used to define the sensitivity of the T_4 assay. All data were normal log (Ln)-transformed before statistical analysis.

RESULTS

In the Little Campbell River, juvenile fish of marine and stream ecotypes collected on 20 August 2006 did not differ in body weight (ANOVA, $F_{1,30} = 2.5$, $P = 0.124$; body weight of the marine ecotype = 0.67 ± 0.02 g; body weight of the stream ecotype = 0.71 ± 0.02 g; mean ± S.E.). However, the whole-body T_4 titres were significantly higher in marine juveniles than in stream juveniles (Fig. 2; ANOVA, $F_{1,30} = 51.2$, $P < 0.001$). Even after inclusion of body weight as a covariate, the difference remained significant (ANCOVA, effect of ecotype: $F_{1,28} = 51.7$, $P < 0.001$; effect of body weight: $F_{1,28} = 1.7$, $P = 0.205$; effect of interaction: $F_{1,28} = 0.6$, $P = 0.449$).

In the Duwamish River, body weight differed significantly between sampling times (Fig. 3; ANOVA, effect of date: $F_{2,36} = 25.7$, $P < 0.001$), which might be due to sampling bias. Therefore, to compare T_4 concentrations among the sampling times, body weight was included as a covariate. T_4 concentrations did not differ significantly among the sampling times when the interaction term between date and body weight was included (ANCOVA, effect of date: $F_{2,33} = 0.50$, $P = 0.613$; effect of body weight: $F_{1,33} = 1.5$, $P = 0.228$; effect of interaction: $F_{2,33} = 0.3$, $P = 0.737$). After excluding the interaction term, however, we found significant differences among sampling dates (ANCOVA, effect of date: $F_{2,35} = 4.3$, $P = 0.021$; effect of body weight: $F_{1,35} = 1.0$, $P = 0.319$).

DISCUSSION

In the present study, we found that pre-migratory juvenile marine stickleback in the Little Campbell River had higher whole-body T_4 concentrations than the juvenile stream-resident stickleback in that same watershed, suggesting that divergence in thyroid hormone physiology already exists between marine and stream-resident ecotypes at the juvenile stage before seaward migration. Treatment of adult stickleback with exogenous T_4 has been shown to increase metabolic rate (Kitano *et al.*, 2010), basal swimming activity (Kitano *et al.*, 2010),

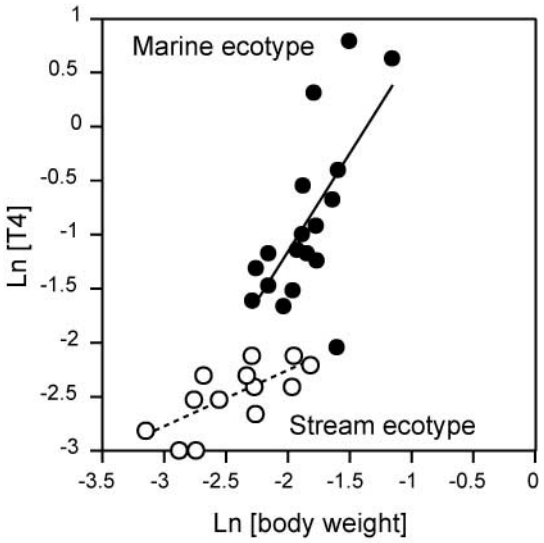

Fig. 2. Relationships between Ln-transformed body weight and Ln-transformed T_4 concentrations in the juvenile fish collected in the Little Campbell River. Solid and open circles indicate the T_4 concentrations of marine and stream ecotypes, respectively. Solid and broken lines indicate the regression lines for marine and stream ecotypes, respectively.

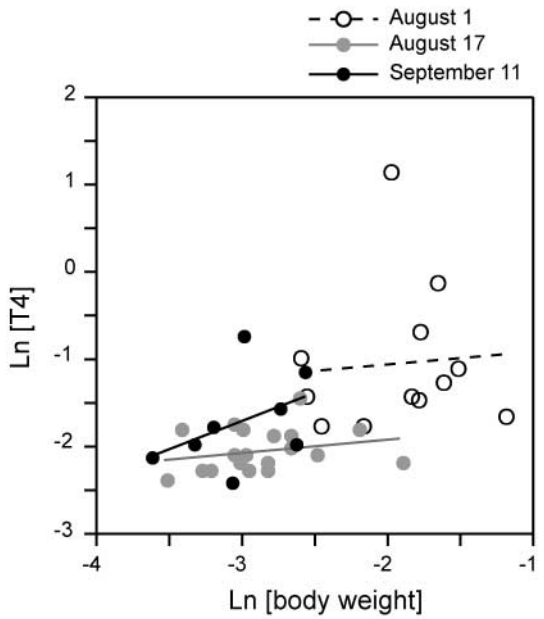

Fig. 3. Relationships between Ln-transformed body weight and Ln-transformed T_4 concentrations in the juvenile fish collected on 1 August (open circles and broken lines), 17 August (grey circles and grey lines), and 11 September (solid circles and black solid lines) in the Duwamish River.

seawater tolerance (Gutz, 1970), and seawater preference (Baggerman, 1957), indicating that the elevated thyroid hormone concentrations in juvenile marine stickleback may mediate the expression of physiological and behavioural traits that increase fitness in marine environments. The presence of higher T_4 concentrations in juvenile marine stickleback also points to a possible role for thyroid hormones as developmental mediators of phenotypic differences between ecotypes. For example, the marine and stream ecotypes have diverged in body size and body shape (Wootton, 1976, 1984; Bell and Foster, 1994; Rogers *et al.*, 2012). Thyroid hormones are involved in growth and skeletal development in fishes (Power *et al.*, 2001; Shkil *et al.*, 2012), and variation in developmental thyroid status can result in differences in body shape (Brown, 1997; Lema and Nevitt, 2006). Although our common garden experiments indicated that divergence in T_4 concentrations between adults of marine and stream ecotypes are genetically determined (Kitano *et al.*, 2010), we do not know whether the differences between juveniles of different ecotypes are determined by genetic factors, environmental factors, or a genotype × environment interaction. Studies exploring the effects of thyroid hormone treatment on juvenile stickleback and the genetic mechanisms causing the divergence in thyroid hormone concentrations should help to resolve how this divergence in thyroid hormone signalling links to the divergence in integrated physiological, behavioural, and morphological phenotype that characterizes the marine and stream ecotypes of this species.

In many fishes, including salmonid species (Dickhoff *et al.*, 1978; Grau *et al.*, 1981; Youngston and Simpson, 1984), migratory populations exhibit a surge in the production of thyroid hormone concentrations with migration. The thyroid hormone surge in salmonid species plays a key role in coordinating the physiological and behavioural changes required for freshwater individuals to undertake the downstream migration and transition to seawater, and can be triggered by environmental factors such as lunar cycles (Grau *et al.*, 1981), temperature variation (Youson *et al.*, 1994), and dissimilar or changing water currents (Youngston, 1989; Youngston and McLay, 1989). In the present study, we found a trend that the juvenile fish caught earlier in time were larger in body size and had higher T_4 concentrations. Because size thresholds for the decision to migrate have been found in salmonids (Nicieza *et al.*, 1994), our data may indicate that larger fish with higher T_4 concentrations leave the estuary first, followed by smaller individuals who delay their migration decision due to either their sub-threshold body size or sub-threshold T_4 concentrations. In addition, some fish caught at the Duwamish estuary on 17 August had similar T_4 concentrations to the stream-resident fish of the Little Campbell River (Figs. 2 and 3), suggesting that some environmental or temporal factors may influence the T_4 concentrations of marine ecotypes. However, we cannot exclude the possibility that significant effects of sampling date on body size and T_4 concentrations observed in this study are due to our sampling bias and small sample sizes, so more thorough sampling of pre-migratory fishes should be conducted as part of future studies.

Peripheral regulation of thyroid hormones may also be important. Diverse functions of thyroid hormones are mediated by the action of thyroid hormones on thyroid hormone receptors in a variety of peripheral target tissues (e.g. liver, brain, gills, gonads), and tissue specificity in thyroid hormone action is determined both by the abundance and type of receptors in a given tissue, as well as by tissue-specific expression of converting enzymes (Ishikawa and Kitano, 2012). For example, iodothyronine deiodinase enzyme types 1 and 2 convert thyroid hormones to more active forms, while iodothyronine deiodinase enzyme type 3 primarily converts thyroid hormones to less active forms (Orozco and Valverde-R, 2005; Orozco *et al.*, 2012). In other teleost fishes, the genes encoding thyroid hormone receptors and deiodinase enzymes exhibit tissue-specific patterns of transcriptional regulation (Lema *et al.*, 2009; Johnson and

Lema, 2011), suggesting that the many physiological, behavioural, and morphological traits regulated by thyroid hormones could be evolutionarily decoupled by peripheral tissue-specific changes in receptor or deiodinase enzyme expression. In salmonids, the expression of thyroid hormone receptors changes during preparation for seaward migration (Harada *et al.*, 2008). Although such peripheral regulation has not yet been examined in sticklebacks, the marine ecotype was found to exhibit both elevated type 1 deiodinase mRNA concentrations and lower concentrations of type 3 deiodinase mRNA abundance in the gills compared with stream-resident fish (Kitano *et al.*, 2010), indicating that ecotypic variation in thyroid hormone physiology may extend beyond upstream hormone production, and include downstream differences in the regulatory mechanisms for thyroid hormone action in peripheral tissues. Given that we now know that thyroid hormone status differs between marine and stream-resident ecotypes in both the juvenile and adult life stages, future work examining ecotypic variation in mechanisms of thyroid hormone action in peripheral target tissues may lend fundamental insights into how hormonally mediated traits are coupled and decoupled as integrated phenotypes evolve.

In conclusion, we found that migratory and non-migratory ecotypes within a species can differ in the levels of systemic hormone titres. Because hormones regulate the expression of many morphological, physiological, and behavioural traits, hormones can mediate the trade-offs between migratory and non-migratory ecotypes. For a better understanding of the genetic and physiological mechanisms of adaptive evolution of multiple traits, it will be essential to know whether such hormone-mediated phenotypic integration can either facilitate or constrain adaptive evolution of divergent life-history ecotypes.

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