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Comparison of freshwater tolerance during spawning migration between two sympatric Japanese marine threespine stickleback species

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ABSTRACT

Background: The colonization of empty niches can trigger phenotypic diversification. For example, the colonization of newly formed freshwater environments by marine ancestors led to phenotypic diversification in threespine sticklebacks. However, not all lineages of threespine stickleback have taken advantage of these ecological opportunities; all Japanese freshwater populations are derived from the Pacific Ocean lineage rather than the Japan Sea lineage of *Gasterosteus*.

Hypothesis: Marine ancestors of these two lineages differed in their ability to survive in freshwater environments. Japan Sea and Pacific Ocean marine sticklebacks may differ in freshwater tolerance and transcript levels of the prolactin (PRL) gene, which encodes a hormone important for freshwater osmoregulation.

Methods: We collected Japan Sea and Pacific Ocean marine sticklebacks migrating upstream near the mouth of a brackish water lake in early May and challenged them with fresh water. We then compared the survival rates, plasma sodium concentrations, and PRL expression levels of the two species.

Results: When challenged with fresh water, Japan Sea fish showed significantly higher death rates and a trend towards a greater reduction in plasma sodium concentration than the sympatric Pacific Ocean fish. Levels of PRL were consistently higher in the Pacific Ocean fish both before and after the freshwater challenge.

Keywords: hormones, osmoregulation, physiology, prolactin, stickleback.

INTRODUCTION

An ecological opportunity can trigger phenotypic diversification (Glor, 2010; Losos, 2010; Yoder *et al.*, 2010). The colonization of new habitats, appearance of new resources, or extinction of competitors can allow a lineage to adapt to a broad range of newly available ecological niches, leading to rapid speciation and/or adaptive diversification (Losos, 2010; Yoder *et al.*, 2010).

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However, not all lineages have taken advantage of these ecological opportunities. Many lineages fail to diversify even in the presence of ecological opportunities. For example, Darwin's finches and Hawaiian honeycreepers have acquired tremendous phenotypic diversity after their colonization of oceanic islands, while Galapagos mockingbirds and Hawaiian thrushes have not (Losos, 2010).

Several potential factors can prevent some taxa from utilizing ecological opportunities (Glor, 2010; Losos, 2010). Early colonizers may preclude further colonization by later colonizers; thus, extrinsic factors, such as differences in geographical accessibility, may explain variation in the exploitation of new niches. Alternatively, some taxa may lack intrinsic genetic and physiological abilities to colonize and utilize new niches, or may be less capable in this regard than others. Therefore, identification of the intrinsic constraints on exploitation of new empty niches is important to understand and predict patterns of evolutionary diversification in nature.

Repeated glacial cycles provided a number of novel empty niches in freshwater environments during the Quaternary period. Multiple marine ancestors of cnidarians, annelids, molluscs, arthropods, and teleosts have colonized these newly formed freshwater environments, resulting in rapid evolutionary diversification of these taxa (Schluter and Rambaut, 1996; Lee and Bell, 1999). Threespine sticklebacks (*Gasterosteus aculeatus*) provide a notable example of freshwater colonization followed by phenotypic diversification (Wootton, 1976, 1984; Bell and Foster, 1994). After the last glacial retreats, ancestral marine sticklebacks have colonized and adapted to a variety of newly formed freshwater environments throughout the northern hemisphere. Freshwater sticklebacks showed tremendous diversification of morphological, physiological, and behavioural traits to adapt to diverse freshwater habitats (Bell and Foster, 1994; McKinnon and Rundle, 2002; Kitano *et al.*, 2012). Because of the presence of extant marine ancestors, we can compare derived freshwater populations with their marine ancestors to investigate the ecological and genetic mechanisms underlying adaptation to freshwater environments, such as reduction of armour, trophic morphology, body shape, and hormonal signalling (Bell and Foster, 1994; Schluter, 1994; Colosimo *et al.*, 2005; Chan *et al.*, 2010; Kitano *et al.*, 2010; Wark *et al.*, 2012; Cleves *et al.*, 2014; O'Brown *et al.*, 2015).

However, not all stickleback lineages have colonized newly formed freshwater environments. In Japan, there are two genetically distinct marine *Gasterosteus* species: the Japan Sea sticklebacks (*Gasterosteus nipponicus*), which are distributed mainly in the Sea of Japan and Sea of Okhotsk (Higuchi and Goto, 1996), and Pacific Ocean populations of *G. aculeatus* (hereafter referred to as Pacific Ocean sticklebacks), which are distributed along the Pacific coast (Kitano *et al.*, 2007). These two species diverged about 1.5–2 million years ago when the Sea of Japan was isolated from the Pacific Ocean by sea-level changes (Kitano *et al.*, 2007). Previous phylogenetic analysis demonstrated that all Japanese freshwater populations, including recently introduced populations, are derived from Pacific Ocean sticklebacks, not from Japan Sea sticklebacks (Higuchi and Goto, 1996; Cassidy *et al.*, 2013; Ravinet *et al.*, 2014). Because there are many freshwater lakes and rivers near the Sea of Japan as well as the Pacific Ocean, the Japan Sea sticklebacks may have lower intrinsic ability to exploit freshwater niches than the Pacific Ocean sticklebacks.

In eastern Hokkaido, Japan, the distribution of Japan Sea sticklebacks overlaps with that of the Pacific Ocean sticklebacks (Higuchi and Goto, 1996; Kitano *et al.*, 2007). In sympatric conditions, these two sticklebacks are reproductively isolated by multiple barriers, including spatial isolation of spawning grounds (Kitano *et al.*, 2009). Most Pacific Ocean sticklebacks migrate to upstream freshwater regions for spawning, while a large portion of Japan Sea fish remain in

brackish water for breeding (Kume *et al.*, 2005, 2010). This is consistent with the idea that the Japan Sea sticklebacks may have lower intrinsic ability to exploit freshwater niches than the Pacific Ocean sticklebacks.

Environmental differences between marine and freshwater habitats include differences in osmotic pressure and ionic concentration (Lee and Bell, 1999; Willmer *et al.*, 2005; Hill *et al.*, 2008). Because the ionic concentration of fresh water is low, freshwater teleosts need to counteract the passive gain of water and loss of ions by several physiological mechanisms, such as the production of dilute urine and the active intake of ions across the gill epithelium (McCormick, 2001). We therefore hypothesized that the Japan Sea sticklebacks possess a lower osmoregulation ability in low-salinity environments than do Pacific Ocean sticklebacks, resulting in differing abilities to colonize freshwater environments.

Osmoregulatory adaptation is generally regulated by the neuroendocrine system (McCormick, 2001). In a number of euryhaline teleosts, prolactin (PRL), a multifunctional peptide hormone, plays a central role in osmoregulation in low-salinity environments (Manzon, 2002; Sakamoto and McCormick, 2006). Prolactin has been demonstrated to increase plasma ion concentrations and decrease the permeability of osmoregulatory organs, such as gills, kidneys, intestines, the urinary bladder, and skin. After freshwater exposure, PRL gene expression, synthesis, secretion, and plasma levels increased (Manzon, 2002); treatment with PRL was also shown to decrease the ion and water permeability of osmoregulatory surfaces (Manzon, 2002). Injection of PRL markedly increased the survival rate of threespine sticklebacks when transferred from salt to fresh water (Lam and Leatherland, 1969; Leatherland and Lam, 1969), changed their kidney structure (Wendelaar Bonga, 1976), and increased their preference for low salinities (Audet *et al.*, 1985). Thus, we hypothesized that different PRL levels may underlie the differing abilities of Japan Sea and Pacific Ocean sticklebacks to exploit freshwater habitats.

To test whether these two marine sticklebacks differ in osmoregulation ability, we first conducted freshwater challenge experiments in which we observed survival rate and measured changes in plasma sodium concentrations. Next, to compare the patterns of PRL levels between the two species, we quantified the expression of the PRL gene in the two stickleback species both before and after freshwater exposure.

MATERIALS AND METHODS

Fish sampling and freshwater challenge experiment

Threespine sticklebacks of the Japanese sympatric pair, Pacific Ocean fish (*G. aculeatus*) and Japan Sea fish (*G. nipponicus*), were collected with seine nets in Akkeshi Bay, Hokkaido, Japan on 11 May 2012. Details of the sampling are described elsewhere (Kume *et al.*, 2005). Adult fish were collected on the same day and from the same location to minimize potential environmental effects: mean (± SD) standard length was 86.57 ± 4.00 mm and 66.72 ± 3.51 mm for the Pacific Ocean and Japan Sea sticklebacks, respectively. To compare freshwater tolerance of Pacific Ocean and Japan Sea fish, we conducted a freshwater challenge experiment immediately after collection. Pacific Ocean and Japan Sea fish ($N = 15$ and 13, respectively) were exposed to fresh water for up to 24 hours. Fresh water used in the experiment was obtained from groundwater at the Akkeshi Waterfowl Observation Center (salinity = 0.01%). As controls, some Pacific Ocean and Japan Sea fish ($N = 15$ and 13, respectively) were maintained in the water of Akkeshi Bay (salinity = 1.40–1.44%) from which they were caught. Four or five fish of each species were held in a bucket containing

approximately 6 litres of fresh water or brackish water (three buckets for each experimental group). Except salinity, all buckets were treated in the same way: they were maintained at ambient temperature, which ranged from 0.4 to 8.0°C during the experiment. We counted the number of survivors 24 hours after freshwater transfer. Fisher's exact test was used to test the difference in the number of survivors between species using R v.3.1.0. We also collected plasma and whole brain from the survivors. For sampling, fish were killed using an overdose of buffered MS222 (300 $mg \cdot L^{-1}$). Blood samples were collected from the caudal veins using heparinized capillary tubes (Fisher Scientific, Waltham, MA, USA), as described previously (Kitano *et al.*, 2010, 2011). After centrifugation for 10 minutes at 2000 *g*, the plasma was frozen and transported to the laboratory at −20°C, and stored at −80°C until use. Each whole brain was immersed in RNAlater solution (Ambion, Austin, TX, USA) overnight at 4°C, before being stored at −80°C until RNA extraction.

Plasma sodium measurement

Plasma samples were thawed on ice, and 0.5-μL sub-samples were used for the measurement of Na^+ concentration using an atomic absorption spectrophotometer (Z-5300 Hitachi, Tokyo, Japan), as described previously (Kusakabe *et al.*, 2014). Na^+ measurements were performed in duplicate when possible. Sufficient plasma for duplicate measurements could not be obtained from three Japan Sea fish experiencing the freshwater challenge and one Japan Sea fish from the control experiment. For statistical analysis, a two-way ANOVA was conducted with species, salinity, and their interaction as explanatory variables using R v.3.0.2 (R Development Core Team, 2013).

Quantification of PRL gene expression

To assess whether expression of the PRL gene differed between the Pacific Ocean and Japan Sea fish, we analysed PRL expression levels using the quantitative real-time PCR method (qPCR). Total RNA was extracted from whole-brain samples with the AllPrep DNA/RNA Micro Kit (Qiagen, Valencia, CA, USA) (N = 12, 7, 4, and 12 for Pacific Ocean and Japan Sea fish in the freshwater challenge, and Pacific Ocean and Japan Sea fish in the control group, respectively). One microgram of total RNA was treated with DNase I (Invitrogen, Waltham, MA, USA) to eliminate genomic DNA contamination, and was then used for cDNA synthesis using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA). As an endogenous control, we conducted qPCR of a housekeeping gene encoding L13A ribosomal binding protein, which shows stable expression in the context of immune system function (Hibbeler *et al.*, 2008). Our analysis of potential reference genes encoding beta-actin, eukaryotic elongation factor I, L13A ribosomal binding protein, and ubiquitin, also showed that transcript levels of the L13A ribosomal binding protein gene was the most stable in the brain among different salinity conditions in the North American sticklebacks (A. Ishikawa, unpublished data). Therefore, the gene encoding L13A ribosomal binding protein was used as a reference gene to quantify relative expression levels of the PRL gene. Primers of PRL were designed with Primer Express 3.0 Software (Applied Biosystems). Primer sequences used were as follows: PRL forward primer, 5′-TTTGTCCACCTCTGCCAACTT; PRL reverse primer, 5′-TCT TGTTGGATATGGCGTTTTG; L13A forward primer, 5′-CACCTTGGTCAACTTGA-

ACAGTG; L13A reverse primer, 5′-TCCCTCCGCCCTACGAC. The Fast SYBR Green Master Mix (Applied Biosystems) was used for qPCR reaction, and qPCR was run on StepOnePlus™ (Applied Biosystems). Relative expression levels were calculated from standard curves drawn from serially diluted cDNA pools of all analysed fish. We analysed expression levels of the PRL gene by a two-way ANOVA with species, salinity, and their interaction as explanatory variables. Correlations between expression levels of the PRL gene and plasma sodium levels were tested by Spearman's rank correlation test using R v.3.0.2 (R Development Core Team, 2013).

RESULTS

Japan Sea fish showed higher death rates in the freshwater challenge experiments than Pacific Ocean fish. Specifically, 7 of 13 Japan Sea fish died during the freshwater challenge, while only 1 of 15 Pacific Ocean fish died (Fisher's exact test: $P = 0.011$). All fish survived in the control experiments ($N = 15$ and 13 for the Pacific Ocean and Japan Sea fish, respectively).

Freshwater exposure significantly reduced the plasma sodium level of both sticklebacks (two-way ANOVA, effect of salinity: $F = 168.323$, $P < 0.001$; effect of species: $F = 0.002$, $P = 0.965$) (Fig. 1). Japan Sea fish showed a trend towards a greater reduction in plasma sodium concentration than did Pacific Ocean fish. However, the difference was not significant (two-way ANOVA, interaction between species and salinity: $F = 2.341$, $P = 0.133$) (Fig. 1).

PRL levels were consistently higher in Pacific Ocean fish than Japan Sea fish regardless of salinity (two-way ANOVA, effect of species: $F = 20.267$, $P < 0.001$) and tended to increase in response to the freshwater challenge (two-way ANOVA, effect of salinity: $F = 3.145$, $P = 0.089$) (Fig. 2). In freshwater challenge experiments, PRL levels were positively

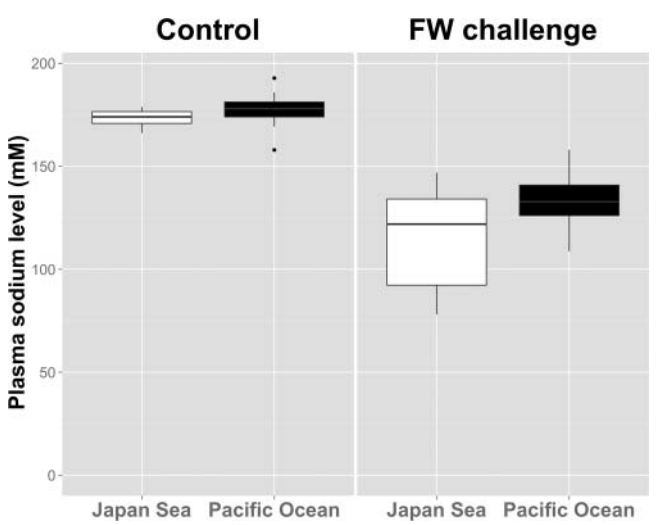

Fig. 1. Box plots of plasma sodium concentration in control and freshwater-challenged fish. White and black boxes indicate the Japan Sea and the Pacific Ocean sticklebacks, respectively.

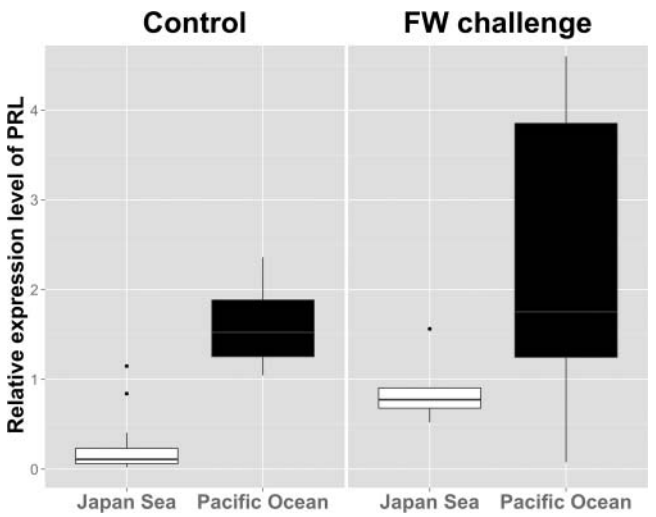

Fig. 2. Box plots of relative expression levels of PRL in the brains of control and freshwater-challenged fish. White and black boxes indicate the Japan Sea and the Pacific Ocean sticklebacks, respectively.

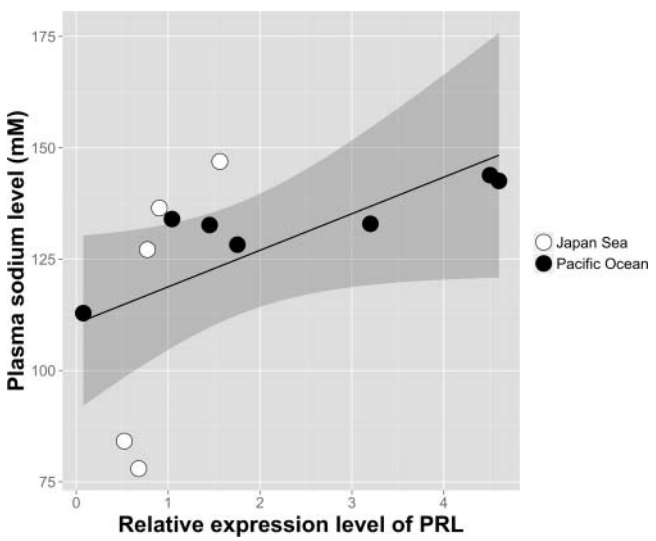

Fig. 3. Relationship between plasma sodium concentration and relative expression levels of PRL. The grey band indicates 95% confidence intervals around the fitted line. White and black circles indicate the Japan Sea and Pacific Ocean sticklebacks, respectively.

correlated with plasma sodium levels when data from both species were pooled and analysed together (Spearman's rank correlation test: $\rho = 0.74$, $P = 0.008$). The same trend was observed even when data were analysed separately for each species (Spearman's rank correlation test: $\rho = 0.71$ and 0.9, $P = 0.081$ and 0.083, for Pacific Ocean and Japan Sea fish, respectively) (Fig. 3).

DISCUSSION

In the present study, when challenged with fresh water, Japan Sea sticklebacks showed significantly higher death rates and a trend towards a greater reduction in plasma sodium concentration compared with sympatric Pacific Ocean sticklebacks. This suggests that Japan Sea sticklebacks have lower freshwater tolerance than Pacific Ocean sticklebacks, which may partially explain why there are no freshwater populations derived from the Japan Sea lineage.

Furthermore, we found significantly higher expression levels of the PRL gene in Pacific Ocean sticklebacks than in Japan Sea sticklebacks. Although PRL expression levels did increase in response to freshwater transfer in both species, expression levels were consistently higher in Pacific Ocean fish than in Japan Sea fish. Prolactin is generally important for freshwater osmoregulation in euryhaline fishes (Manzon, 2002; Sakamoto and McCormick, 2006). Injection of ovine PRL was reported to reduce freshwater mortality of a Canadian marine threespine stickleback population (Lam and Leatherland, 1969). Furthermore, our present study revealed that PRL expression levels were correlated with plasma sodium levels. Taken together, lower PRL expression levels may be one of the physiological mechanisms underlying the lower freshwater tolerance in the Japan Sea fish than in the Pacific Ocean fish during spawning migration.

Previous studies of both marine and freshwater ecotypes of *G. aculeatus* indicated that PRL activity shows seasonal variation associated with migration between salt and fresh water. Specifically, it decreases in early-winter fish (around the time of sea-run migration) and becomes maximally active in spring fish (around the time of spawning migration) (Lam and Hoar, 1967; Leatherland, 1969; Benjamin, 1974). If similar seasonal changes in PRL levels occur in Japan Sea sticklebacks, Japan Sea sticklebacks may show a much greater reduction in PRL expression levels and lower freshwater tolerance in other seasons such as early winter, which may function as an additional constraint for freshwater colonization.

Hormones generally perform multiple functions, and hormonal divergence can hence lead to changes in multiple traits (Ketterson and Nolan, 1999; Kitano *et al.*, 2014). Prolactin is a multifunctional hormone and plays important roles not only in freshwater adaptation, but also in freshwater preference behaviour (Audet *et al.*, 1985). Therefore, divergent PRL levels may contribute to the divergent choice of breeding habitats – relative upper reaches for Pacific Ocean fish and lower reaches for Japan Sea fish – possibly leading to spatial isolation between the two species (Kume *et al.*, 2005, 2010). Prolactin is also known to be involved in paternal behaviours in *G. aculeatus* (Pall *et al.*, 2004), although differences in paternal behaviours between the Japan Sea and Pacific Ocean sticklebacks have not yet been investigated. Further studies on PRL levels across multiple developmental stages and their roles in multiple functions will help to clarify the contribution of PRL levels as a constraint for freshwater colonization, reproductive isolation, and other possible behavioural differences between Japan Sea and Pacific Ocean sticklebacks.

In the present study, we only analysed freshwater tolerances of adult fish migrating to spawning grounds. Other developmental stages were not analysed. Furthermore, freshwater challenge experiments were limited to transfer from brackish water to fresh water, and freshwater acclimatization was not assessed for other salinity regimes such as gradual salinity reduction. Future studies should assess acclimatization abilities of these fish at multiple developmental stages using other salinity regimes. Because the fish analysed in these experiments were collected from the same location on the same day, environmental

effects were minimized, suggesting that the observed differences in freshwater tolerance may have a genetic basis. However, we cannot exclude the possibility of other environmental factors. For example, early exposure to different salinities is known to influence salinity tolerance at later developmental stages in Nile tilapia (Watanabe *et al.*, 1985). The spawning grounds of the Pacific Ocean fish are generally lower in salinity than those of the Japan Sea fish (Kume *et al.*, 2005, 2010), and Pacific Ocean juvenile fish remain longer in the spawning sites than Japan Sea juvenile fish (Kitamura *et al.*, 2006; Kume and Mori, 2009). Therefore, different durations of exposure to low-salinity environments at the juvenile stages could affect salinity tolerance and PRL expression levels in the adult stages. Testing with laboratory-raised fish will be essential to assess genetic contributions to the differing freshwater tolerances between the two species. Finally, we tested only one sympatric pair collected from Akkeshi Bay in the present study. Since some populations of Japan Sea sticklebacks spawn in freshwater environments (Higuchi *et al.*, 2014), further studies on other Japan Sea populations are necessary to confirm that low ability to tolerate fresh water is general across the distribution of Japan Sea sticklebacks.

In conclusion, this study demonstrates that an intrinsic difference may exist in the abilities of these two marine sticklebacks to survive in freshwater environments, and that this difference may be a result of divergent expression levels of the PRL gene. These differences may underlie their differing ability to colonize freshwater environments and/or the spatial isolation between the two lineages. Further molecular and genetic studies on the different abilities of these two lineages to survive in freshwater environments may suggest why some lineages can colonize and utilize empty niches to achieve adaptive radiation, while others cannot.

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