Assessing niche differences of sex, armour and asymmetry phenotypes using stable isotope analyses in Haida Gwaii sticklebacks

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Summary
Identifying phenotype-specific selective landscapes within populations continues to challenge evolutionary biologists in studies of adaptive variation. We explore here the potential application of carbon and nitrogen stable isotope ratios of tissues as time-integrated proxies of niche space among sex, armour and asymmetry phenotypes within an endemic population of giant threespine stickleback from Haida Gwaii, western Canada. Muscle tissues were extracted from 289 stickleback collected from Drizzle Lake, taken in transects during June 1981, June 1982, September 1982 and June 1983 and isotopic ratios of $\delta^{15}$N/$\delta^{14}$N ($\delta^{15}$N) and $\delta^{13}$C/$\delta^{12}$C ($\delta^{13}$C) determined by continuous-flow isotope ratio mass spectrometry. Among all fish, $\delta^{15}$N values, which reflect relative trophic level, ranged from 6.5‰ to 10.6‰ while $\delta^{13}$C, which reflects reliance on different carbon pathways of primary producers, ranged from $-30.5$‰ to $-27.5$‰. The sexes did not differ in $\delta^{15}$N but females were significantly enriched in $\delta^{13}$C relative to males among all samples. In each transect, lateral plate phenotypes differed in $\delta^{15}$N, with higher plate counts generally enriched in $\delta^{15}$N. $\delta^{13}$C did not vary among plate phenotypes. Approximately 50% of the adult population exhibit lateral plate asymmetries usually with one plate different between sides. Sticklebacks that were asymmetric (absolute asymmetry) did not differ from symmetric fish for either $\delta^{15}$N or $\delta^{13}$C signatures. However, this result masked a significant and consistent effect of signed asymmetries (right side − left side), with $\delta^{15}$N enrichment among right-biased compared to left-biased asymmetric. These unexpected results indicate the presence of subtle and previously unrecognized niche differences in lateral plate number and asymmetry phenotypes and are consistent with the predictions of niche-width and adaptive variation hypotheses. The results also suggest the wider application of isotopic techniques to elucidate phenotype-specific ecological and selective landscapes.

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Introduction

In spite of the central importance of the multi-dimensional niche, evolutionary biologists have not readily incorporated niche dimensions into studies of phenotypic variability and adaptive variation within populations. Part of this is due to the challenge in quantifying phenotype-specific niches over the life history of organisms. As such, niche differences within species are not well documented and their extent or importance is not well established. Initial formulation of hypotheses addressing intraspecific niche variation were advanced by Levene (1952) on multi-niche polymorphisms who modeled an equilibrium in which two genotypes would be preserved in a population if each occupied different niches. Van Valen (1965) formulated hypotheses on the relationship between niche width and morphological variance in which individuals along a phenotypic continuum exhibit niche specialization over a resource axis. In addition to early field tests on polymorphic taxa (e.g., Reimchen, 1979; Jones & Probert, 1980), more extensively studied were quantitative traits involving trophic axes in which niche diversification may be a natural extension of intraspecific competition (Grant et al., 1976; Grant & Price, 1981; Robinson & Wilson, 1994; Ackermann & Doebeli, 2004; Egas et al., 2005). Recent efforts to manipulate intraspecific competition lead to disruptive selection at higher densities (Bolnick, 2004) consistent with the importance of phenotype-specific trophic differentiation.

To provide a test of the adaptive variation hypothesis using quantitative traits, a field study was initiated in 1976 on the Haida Gwaii archipelago in British Columbia, Canada. This study has investigated the morphological variability in predator defenses within several insular lake populations of threespine stickleback (Gasterosteus aculeatus). Previous investigations in this archipelago had shown that populations differ in the average number, variance and asymmetry of their bony lateral plates in relation to predation regime among localities (Moodie, 1972; Moodie & Reimchen, 1976). Assessment of sources of mortality on stickleback demonstrated taxonomic diversity of predators and an associated diversity in foraging methods (Reimchen & Douglas, 1984; Reimchen, 1994). Some of the intrapopulation variation in lateral plates and other defense structures was predictably associated
with a spatially and temporally variable predation regime (Reimchen, 1980, 1995; Reimchen & Nosil, 2002, 2004). The cumulative evidence suggests that variable predation regimes leads to diversifying selection on the defense apparatus, which could be a common feature facilitating the maintenance of phenotype variability in these populations. While there is evidence for phenotype-specific niche space in pelvic girdle morphs (Reimchen, 1980; Reimchen & Nosil, 2001a), as well as frequency differences in plate phenotypes between littoral and limnetic regions (Reimchen, 1995), there is no direct evidence for niche differences among the bony lateral plate phenotypes.

Spatial data are useful to identify potential niche differences among individual phenotypes, but this is a limited proxy for niche. Testing the niche variation hypothesis requires quantification of phenotype niche parameters in a way that is ecologically meaningful. Stable isotope ratios $\delta^{15}N$ and $\delta^{13}C$ are increasingly used as time-integrated indicators of dietary niche, because food items typically vary in stable isotope composition and enrichment among trophic levels (fractionation) is predictable. $\delta^{15}N$ is typically used as a measure of trophic position because fractionation is relatively large (mean $+3.4\%$; Minagawa & Wada, 1984; Vander Zanden & Rasmussen, 1999; Post, 2002). Conversely, $\delta^{13}C$ varies among primary producers and undergoes little or no trophic fractionation ($<1\%$, DeNiro & Epstein, 1978; Post, 2002) and is, therefore, most commonly used to infer the relative importance of different sources of primary production in the diets of consumers. Sources of primary production in lakes that typically vary in carbon signatures include phytoplankton, benthic algae and terrestrial input. Relative dependence on these different sources of production are reflected in the stable isotope signatures of zooplankton, benthic invertebrates and fish (France, 1995; Matthews & Mazumder, 2004). We used nitrogen and carbon stable isotope analysis to test for fine-scale niche partitioning among sexes, lateral plate number and asymmetry phenotypes of stickleback from Drizzle Lake. This approach will complement empirical data that has shown slight spatial differences among sex and defense phenotypes (Reimchen, 1995). Based on the higher occurrence of male stickleback in littoral zones relative to females and the marginally higher occurrence of higher plate phenotypes in littoral habitats, we predict differences in the combination of $\delta^{15}N$ and $\delta^{13}C$ signatures; however, we cannot reliably predict the direction of the effect given
the lack of contemporary isotope data from the environment. Patterns of environmental isotope signatures are difficult to predict in this case, as the large contribution of allochthonous nutrients in dystrophic habitats (Carpenter et al., 2005), such as Drizzle Lake, may obscure the established littoral $\delta^{13}$C enrichment in autochthonous watersheds (France, 1995). We also tested for differences in isotopic signatures among lateral plate asymmetry phenotypes to evaluate the potential of habitat partitioning but we were not able to make explicit predictions.

**Study area and methods**

Drizzle Lake occurs on the *Sphagnum*-dominated lowlands on the northeastern part of Haida Gwaii off the mid-coast of British Columbia. The 100 ha lake (max depth 20 m) is dystrophic (heavily stained from tannins) and fish are generally found within 4 m of the surface (Reimchen, 1988). The lake supports an estimated 80,000 adult stickleback, 200 cutthroat trout and 16 species of avian piscivores (Reimchen & Douglas, 1984; Reimchen, 1990, 1994). Sampling occurred during 18 transects among seasons between 1980 and 1983. Sticklebacks were collected using baited minnow traps at various depths and horizontal distances from shore (detailed sampling protocols in Reimchen & Douglas, 1980; Reimchen, 1988, 1995). Each stickleback was scored for sex, standard length (SL) and lateral plate phenotype (following Reimchen, 1983), then fixed in 10% formalin and transferred to 95% ethanol following 2–4 years of storage. In order to consolidate the large number of distinct lateral plate phenotypes when number and position are taken into account, we categorized individuals based on their number of left lateral plates ($\leq 3, 4, 5, \geq 6$) and calculated total plates ($L_{\text{side}} + R_{\text{side}}$). We calculated plate asymmetries ($R_{\text{side}} - L_{\text{side}}$) and classified fish as being left-biased asymmetric ($N = 80$), symmetric ($N = 135$) or right-biased asymmetric ($N = 74$). While $R_{\text{side}} - L_{\text{side}}$ asymmetries for individual samples do not depart significantly from zero ($p > 0.84$), consistent with fluctuating asymmetry, calculations for the complete data set ($N = 10,133$) indicate a slight but significant left side directional asymmetry ($p < 0.001$).

We sub-sampled sticklebacks from October 1981, $N = 45$ (transect 9), June 1982, $N = 20$ (transect 12), September 1982, $N = 132$ (transect 14) and June 1983, $N = 92$ (transect 18) for stable isotope analysis. Most fish
were greater than 60 mm SL; however transects 12 and 14 also included sub-adults (SL 40–60 mm, \( N = 10 \) and 43, respectively). Frequency distributions of number of left lateral plates and signed lateral plate asymmetry are shown in Figure 1. We removed dorsal trunk muscle tissue posterior to the pectoral fin and above the lateral line from each individual. Tissues were dried at 60°C for 8 to 12 days, then powdered using a Wig-L-Bug grinder (Crescent Dental, Chicago, IL, USA) for 30–40 s. We weighed 1.0 mg (±0.05) of the powder for continuous-flow isotope ratio mass spectrometry analysis at the Stable Isotope Facility, University of Saskatchewan (Saskatoon, SK, Canada). Isotope signatures are presented in the conventional delta notation (\( \delta^{13}C \) and \( \delta^{15}N \)), as ratios relative to the international standards Pee Dee Belemnite for carbon and atmospheric nitrogen. Replicated samples indicate a measurement precision of ±0.35‰.

Lipid synthesis causes depletion in \( ^{13}C \) relative to dietary carbon (DeNiro & Epstein, 1978, Schlechtriem et al., 2003; Sweeting et al., 2004; Post et al., 2007) and can lead to \( \delta^{13}C \) differences that are unrelated to diet. There were negative correlations between \( \delta^{13}C \) and C:N ratio (used as a proxy for lipid content) in three of the four samples \( (r < -0.4, p < 0.01) \). This relationship is usually addressed either by extracting lipids prior to isotopic analysis or by `normalizing’ \( \delta^{13}C \) to a standard lipid content (McConnaughey & McRoy, 1979). We accounted for lipids by normalizing to C/N ratio (following McConnaughey & McRoy, 1979) and repeating all analyses using both original
and normalized δ¹³C signatures. As normalizing did not affect any significance values, we present the results of the analysis using non-normalized δ¹³C signatures. Fixation in formalin and preservation in ethanol can also affect stable isotope signatures of fish muscle tissue but as preservation techniques were consistent, effects on both isotopes should be highly uniform among all fish within each sample (Arrington & Winemiller, 2002).

We standardized isotopic signatures to remove differences among transects and ontogenetic effects. For each transect, we used linear regressions of δ¹⁵N and δ¹³C against SL, saving the standardized residuals (Z-scores) as new variables: Zδ¹⁵N and Zδ¹³C. This analysis reduced the isotopic signatures in each transect to a mean of zero, and removed the effects of size that were present in the form of positive relationships between δ¹⁵N and SL in transects 14 (p < 0.001) and 18 (p = 0.07) and positive relationships between δ¹³C and SL in transects 14 and 18 (p < 0.01). We used the standardized residuals from the regression to test (ANOVA) for remaining isotopic variance caused by differences among sexes and lateral plate phenotypes.

Results

Overall isotopic signatures were variable among individuals within and among transects (Figure 2). Within each transect, stickleback ranged about 2% for each isotope and there was an average 1% maximum difference among transects for both isotopes (δ¹⁵N, $F_{3,284} = 7.2$, $p < 0.001$, δ¹³C, $F_{3,284} = 13.8$, $p < 0.001$, SL as a covariate).

The sexes differed in signatures but only for one of the isotopes. There were no overall sex differences for Zδ¹⁵N ($F_{1,281} = 0.8$, $p > 0.4$) but for Zδ¹³C signatures, these were enriched in females relative to males in each transect (mean difference on raw data = 0.7%, $F_{1,281} = 10.9$, $p < 0.001$, Figure 3).

Lateral plate phenotypes were compared for isotopic values. We first examined number of plates on the left side of the trunk using 4 plate number categories (2–3, 4, 5, 6–7) and found in both sexes a weak and non-significant ($p > 0.5$) increase in Zδ¹⁵N signatures with increased plate numbers. Because of the frequent bilateral asymmetry in the plates, we also examined counts on the right side of the trunk. This yielded a similar but statistically stronger tendency for greater enrichment in higher plate counts ($p = 0.07$).
Figure 2. Raw data for nitrogen and carbon isotopic signatures for muscle tissues of Drizzle Lake stickleback ($N = 289$).

Figure 3. $\delta^{13}C$ isotope signatures for male and female stickleback from Drizzle Lake.
Partitioning the data among transects shows that September 1982, which had the largest sample, had a significant ($F_{3,124} = 2.7, p < 0.05$) association between increased plate counts and $Z\delta^{15}N$ enrichment on the right side but not on the left side ($F_{3,124} = 1.1, p > 0.3$). There were no trends with either sex for $Z\delta^{13}C$ and plates either on the left side or the right side. We also combined the left and right plates and examined total plates in relation to isotopic signatures (Figure 4). This yielded a highly significant ($F_{10,278} = 2.29, p < 0.004$) association between $Z\delta^{15}N$ signatures and total plate counts with trends that were concordant among years (Figure 4). Over most of the range in plates (4 thru 4), there was enrichment with increased total plate counts. The exceptions to this trend occurred at the edge of the plate distribution (where samples were small), but the lowest plate count (4) appeared to be enriched relative to most plate counts. There was a significant interaction between total plate count and sex ($p < 0.001$) and the associations were much stronger in females than in males (females $F_{10,183} = 2.2, p < 0.004$; males $F_{8,96} = 1.3, p = 0.24$).

We also assessed relationships between isotope signatures and lateral plate asymmetry. For absolute asymmetries (Rside – Lside), there were no consistent isotopic differences between symmetric and asymmetric fish for either isotope ($Z\delta^{15}N$, $F_{1,273} = 0.5, p = 0.5$; $Z\delta^{13}C$, $F_{1,273} = 1.7, p = 0.2$) and no interactions with transect ($p = 0.9$) or sex ($p = 0.18$). However, comparison of signed asymmetries (left bias versus right bias) showed $Z\delta^{15}N$ enrichment in right biased phenotypes ($F_{1,152} = 6.0, p < 0.02$) but no differences for $Z\delta^{13}C$ ($F_{1,152} = 1.2, p = 0.3$). When the directionality of the plates are partitioned for each of the common asymmetry phenotypes (3–4 vs. 4–3, i.e., 3 plates on the left and 4 plates on the right vs. 4 plates on the left and 3 plates on the right; 4–5 vs. 5–4; 5–6 vs. 6–5), the $\delta^{15}N$ enrichment occurs in each of the right biased phenotypes ($F_{1,114} = 12.9, p < 0.001$; Figure 5). Similar to the trends with total plates, the enrichment in the right biased plate phenotypes is accentuated in females ($F_{1,75} = 16.9, p < 0.001$) and not detectable in males ($F_{1,33} = 0.4, p = 0.52$).

**Discussion**

We used stable isotopes to bridge ecological and evolutionary approaches in the investigation of intrapopulation variation. An expanding literature shows
Figure 4. $Z\delta^{15}N$ residuals for total lateral plate count (left side + right side) of stickleback from Drizzle Lake.
a coupling between tissue isotopic signatures and diet, in which $\delta^{15}N$ exhibits a mean fractionation of 3.4‰ per trophic level (Minagawa & Wada, 1984; Post, 2002; Bearhop et al., 2004a). $\delta^{13}C$ undergoes only marginal fractionation (mean 0.4‰; DeNiro & Epstein, 1978), making it a more reliable indicator of dependence on different carbon sources.

It is reasonably well established that the sexes in *Gasterosteus* differ in multiple components of the niche. Males tend to be more littoral and more benthic than are females and exhibit trophic adaptations (larger gape, larger eyes) that reflect these differences in habitats (Reimchen, 1980; Reimchen & Nosil, 2001b, 2006; Caldecutt et al., 2001). Although there are no clear morphological differences between the sexes in any trophic structures in the Drizzle Lake stickleback, consistent with the similarity in $\delta^{15}N$ signatures, females are more prevalent in limnetic habitats than are males (Reimchen & Nelson, 1987). These differences are probably reflected in the $\delta^{13}C$ signatures, with females in each transect enriched relative to males. This reverse and unusual pattern of $\delta^{13}C$ enrichment in limnetic habitats could occur if the allochthonous input of nutrients that are prevalent in dystrophic lakes (Carpenter et al., 2005) differ between littoral and limnetic habitats.

The bony lateral plates on *Gasterosteus* have received considerable investigation over the last 100 years. This includes attributes such as exten-
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Isotopic variability among freshwater populations (review in Wootton, 1984), high heritability (Hagen, 1973), intrapopulation variability in frequencies (Moodie, 1972; Moodie & Reimchen, 1976; Bell & Richkind, 1981; Reimchen, 1995; Bell, 2001; Bell et al., 2004) and functional morphology of the predator-defense structure (Reimchen, 1983, 1994). Lateral plate phenotypes also can exhibit differences in behaviour which alters susceptibility to predation (Moodie, 1972; Moodie et al., 1973; Bell & Haglund, 1978; Huntingford, 1981). The cumulative studies on lateral plates suggest population-distinctive landscapes differing in modal phenotypes and the extent of stabilizing and directional selection (review in Bell & Foster, 1994).

Our current isotopic data add an additional level of complexity to lateral plate variation. Stickleback from Drizzle Lake typically vary from 3 to 6 lateral plates per side, which include plates at positions 4, 5, 6 and 7 that buttress the dorsal and pelvic spines, as well as flanking plates at positions 3 and 8 (Reimchen, 1983). These individual phenotypes, which can vary by a single plate, differ in isotopic niche space. We observed slight (0.5%) but significant increase in δ15N signatures with increased number of plates, trends that were consistent among years, suggesting that the quantitative variation in number of lateral plates within this population comprises a distribution of phenotype-specific niches. This might reflect trophic partitioning among phenotypes within any region of the lake or possibly spatial partitioning of phenotypes in different regions of the lake where nitrogen sources differ. That δ13C signatures did not co-vary among the lateral plate phenotypes suggests that lake spatial differences are less probable than trophic effects inferred from δ15N signatures, as carbon isotope signatures are general indicators of spatial effects in the diet (France, 1995).

What might account for the association between the lateral plates, which are predator-defense structures, and trophic niche? Spatial or temporal differences in foraging behaviour can alter risk of predation (Hart & Gill, 1994). The population of stickleback from Drizzle Lake incur mortality from 21 species of predators including avian piscivores such as kingfishers, loons and grebes, as well as salmonids, otters and macroinvertebrates, each with distinctive spatial and temporal foraging behaviour (Reimchen, 1994). Consequently, lateral plate phenotypes differing in isotopic niche space potentially differ in the probability of encountering any particular predator species. If higher trophic level zooplankton or macrobenthos are more available to stickleback near crepuscular periods when predatory trout are also foraging...
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(Reimchen, 1990), then this would generate a selective landscape in which higher plate number phenotypes would have an advantage given that these individuals are better protected against predatory trout (Reimchen, 1992). If there is long-term continuity of these processes, the genetic coupling between trophic and defense traits could occur (Ford, 1964).

Slight departures from bilateral symmetry have been widely investigated in multiple taxa and in the case of fluctuating asymmetry are generally thought to reflect developmental instability and subsequent fitness reduction (Palmer & Strobeck, 1986; Moller, 1997, for review). Asymmetries in the number of lateral plates occur in many freshwater stickleback populations (Moodie & Reimchen, 1976; Francis et al., 1985; Bergstrom & Reimchen, 2002). Reduced fitness of asymmetric phenotypes has been inferred based on the low frequencies of asymmetric stickleback in lakes with extensive trout predation (Moodie & Reimchen, 1976), the low incidence of asymmetries in the structural plates that buttress the dorsal and pelvic spines (Bergstrom & Reimchen, 2000) and the elevated incidence of intestinal and coelomic parasites in asymmetric relative to symmetric plate phenotypes (Reimchen & Nosil, 2001c).

Yet there are additional lines of evidence that plate asymmetries are not invariably associated with fitness reduction. Adult male stickleback from Mayer Lake, Haida Gwaii, that were asymmetric for lateral plate counts were more likely to have fry in their nests than were males that were symmetric, possibly associated with differences in aggressivity of the males (Moodie & Moodie, 1996). Plate asymmetries are common with frequencies ranging from 20% to 60% among freshwater populations on Haida Gwaii (Bergstrom & Reimchen, 2000). Relative survival of asymmetric phenotypes, assessed from age class frequencies, tends to be similar and occasionally greater than symmetrical phenotypes (Bergstrom & Reimchen, 2002). Furthermore, investigations of the armor-reduced population of stickleback at Boulton Lake, Haida Gwaii show differences in dietary niche between symmetric and asymmetric lateral plate and spine phenotypes (Reimchen & Nosil, 2001a,c).

Given this previous evidence for dietary niche differences, we predicted isotopic differences between symmetric and asymmetric stickleback. However, for absolute asymmetries, we did not detect any consistent differences in nitrogen or carbon isotopic signatures between symmetric and asymmetric fish, suggesting a similar trophic niche space for these phenotypes. Yet, our results differed for signed asymmetries. Unexpectedly, we found slight but
consistent differences (0.5‰) in nitrogen isotope signatures between the left-biased and right-biased plate asymmetries. Right-biased phenotypes were significantly higher in δ^{15}N than left-biased stickleback even when the phenotypes are matched for the same total number of plates.

Currently, we are not able to satisfactorily account for the directional asymmetry and isotope associations but there are two general interpretations of these results. The data may simply reflect relative physiological condition of the phenotypes, as condition factor is known to be associated with different isotopic signatures in other taxa (Bearhop et al., 2004b). If tenable, we would have expected differences in their condition such as relative body size and depth, emaciation or parasitism. Although we did not observe such effects, we cannot exclude such a possibility without a detailed evaluation of subtle condition factors for individual phenotypes. A second interpretation is that the data reliably reflect spatial or trophic niche differences between the left-biased and right-biased phenotypes, perhaps as a consequence of intraspecific competition. Such niche differences in asymmetric structures have been reported in the case of jaw anti-asymmetry for the left versus right scale-eating African cichlids (Takahashi & Hori, 1998). In stickleback, the plate asymmetry is not functionally tied to feeding, but rather is part of an integrated defense structure between spines and plates (Reimchen, 1983; Bergstrom & Reimchen, 2003). Isotopic differences between these plate asymmetries suggest phenotype-specific niches and hint at potential functionality to asymmetries of the defense armour. The emerging evidence for major behavioural laterality in vertebrate predator-prey interactions (Bisazza et al., 1998; Vallortiga, 2006) combined with the diversity of avian and piscine predators on stickleback raises the possibility that non-random directionality of predator approach and attack may contribute to the prevalence and directionality of plate asymmetries in stickleback.

If validated, we feel our data provide conceptual support for the niche-width and adaptive variation hypotheses that genetic and phenotype variability within populations represents an adaptation to ecological heterogeneity (Van Valen, 1965; Grant & Price, 1981; Smith & Skulason, 1996; Bolnick et al., 2003). The identification of isotopic niche differences among phenotypes differing in number and signed asymmetries of plates suggests novel opportunities for evaluating the ecological context of phenotypic variability.
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