

Isotopic trophic segregation associated with asymmetry direction in a polymorphic flatfish, *Platichthys stellatus* (Pleuronectiformes: Pleuronectidae)

CAROLYN A. BERGSTROM^{1*} and THOMAS E. REIMCHEN²

¹Department of Natural Sciences, University of Alaska Southeast, 11120 Glacier Highway, Juneau, AK 99801, USA

²Department of Biology, PO Box 3020, University of Victoria, Victoria, BC, Canada V8W 3N5

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Ecologically relevant resource polymorphisms have received increased attention over the last two decades, yet marine examples are relatively rare and the evolutionary mechanisms that maintain them are not thoroughly understood. Here, we investigated trophic segregation associated with whole-body asymmetry direction within a polymorphic marine flatfish species, the starry flounder (*Platichthys stellatus*). Stable isotope ratios ($^{15}\text{N}/^{14}\text{N}$, $^{13}\text{C}/^{12}\text{C}$) were determined for 213 fish from nine localities throughout the northeastern Pacific. Sinistral (left-eyed) flounder had slightly but significantly enriched $\delta^{15}\text{N}$ over dextral (right-eyed) flounder, suggesting that sinistral flounder were targeting higher trophic level prey. Sinistral flounder had enriched $\delta^{13}\text{C}$ in the southernmost samples only, suggesting that their prey was more marine derived than that of dextral fish in that region. A principal component composed of body depth and caudal peduncle depth was weakly associated with $\delta^{13}\text{C}$, suggesting that prey of flounder with decreased body and caudal depth were more freshwater derived. Gut contents revealed significantly greater trophic breadth of prey in sinistral flounder than dextral flounder. These data, combined with previous findings of differences between morphs in ecomorphology and prey strike behaviours in *P. stellatus*, and gut content differences in a congeneric (*Platichthys flesus*), provide evidence of trophic segregation associated with the direction of whole-body asymmetry within polymorphic flatfish species.

ADDITIONAL KEYWORDS: biased antisymmetry – niche segregation – resource polymorphism – stable isotopes – starry flounder

INTRODUCTION

In the two decades since the publication of a seminal review of resource polymorphisms in vertebrates (Smith & Skúlason, 1996), greater attention has been given to resource-based divergence within species. Examples of this are found across taxa, but surprisingly little is known of the underlying conditions and mechanisms necessary for their evolution. Intraspecific resource competition clearly plays a role in some instances (Bolnick, 2004; Martin & Pfennig, 2009; Cucherousset *et al.*, 2011; Reimchen, Steeves & Bergstrom, 2016), but given its ubiquity across taxa it fails to explain why resource polymorphisms are not more common. A combination of intraspecific competition and ecological opportunity might be necessary (Nosil &

Reimchen, 2005; Martin & Pfennig, 2010), which would explain, in part, why polymorphism is only an occasional result. Of the cases of ecologically relevant resource-based variation within species, examples involving freshwater and terrestrial species abound, particularly in fishes (Bolnick *et al.*, 2003; Ward, Webster & Hart, 2006). However, comparable examples in estuarine or marine species are markedly rare; for example, of the ~600 publications that have cited the paper by Smith & Skúlason (1996), at present fewer than two dozen have dealt with estuarine or marine species. Here, we show rare evidence of subtle trophic segregation within a marine pleuronectid flatfish species that is associated with the direction of whole-body asymmetry.

Whole-body asymmetry is a synapomorphy for all flatfish species (Pleuronectiformes; >700 species). The asymmetry is characterized post-metamorphosis by the presence of both eyes on one side of the body (the

*Corresponding author. E-mail: cabergstrom@alaska.edu

eyed side), absence of pigmentation on the other side of the body (the blind side), and spatial orientation with the blind side facing or lying on the benthos (Norman, 1934). The vast majority of these species exhibit monomorphism for asymmetry direction, whereas only seven are polymorphic for asymmetry direction and have stable and persistent frequencies of both morphs in wild populations (Munroe, 2005). As such, these polymorphic species exhibit classic examples of either discrete antisymmetry when both morphs are found in equal numbers or 'biased antisymmetry' when one morph is in the majority (Palmer, 2005). Starry flounder (*Platichthys stellatus*) (Pallas) is one of these polymorphic flatfishes, exhibiting a remarkable cline across its range, with equal frequencies in central California (antisymmetry), ~75% sinistral (left side is eyed) morphs in western Alaska (biased antisymmetry), and 100% sinistral morphs in Russia and Japan (Hubbs & Kuronuma, 1942). The direction of asymmetry is moderately heritable (Policansky, 1982; Boklage, 1984; C. A. Bergstrom, unpublished data), and the cline appears to have historical persistence (Bergstrom, 2007), yet the mechanisms that maintain it are not well understood.

Although such apparent 'mirror image' asymmetry morphs might reflect stochastic developmental origins, there is evidence of subtle but statistically significant morphological and behavioural differences between them that suggest ecological segregation. Dextral (right side is eyed) starry flounder have proportionately larger caudal peduncles, fewer gill rakers and blunter snouts than sinistral individuals from the same locality (Bergstrom, 2007), suggesting possible differences in their locomotion (Webb, 1984) and foraging mechanics (Wainwright & Richard, 1995; Link & Hoff, 1998; Amundsen, Bøhn & Vågak, 2004). In addition, newly settled dextral and sinistral juveniles differ behaviourally in their prey strike direction (Bergstrom & Palmer, 2007), which could result in different foraging efficiencies on prey that respond with directional evasion (Vallortigara & Rogers, 2005). In light of this morphological and behavioural evidence of ecological segregation, here we investigate whether the polymorphism has trophic significance by comparing gut contents and stable isotope ratios of muscle tissue between the two morphs.

Stable isotopes have emerged as a popular and effective tool in the study of trophic niche ecology (Post, 2002) and the detection of intraspecific specialization (Araújo, Bolnick & Layman, 2011; Paull, Martin & Pfennig, 2012). Stable isotope ratios reveal attributes of an individual's diet integrated over longer periods of time (weeks to months; Dalerum & Angerbjörn, 2005) than analyses of gut contents, which represent food choices at a single point in time. Isotopes of nitrogen and carbon are currently the most widely used in ecological studies, and there is broad consensus that

enrichment in $\delta^{15}\text{N}$ is associated with consumption of higher trophic level prey, whereas differences in $\delta^{13}\text{C}$ reflect shifts in the carbon source, such as the proportion of prey deriving carbon from marine vs. freshwater habitats (Fry, 2006). Isotopic signatures vary among individuals within many species of fishes (Grey, 2001; Box *et al.*, 2010; Cucherousset *et al.*, 2011; Zhao *et al.*, 2014), but only two studies have shown them to be associated with morphological asymmetry, both in threespine stickleback (Moodie, Moodie & Reimchen, 2007; Reimchen, Ingram & Hansen, 2008). A study of the polymorphic European flounder, *Platichthys flesus*, found differences in gut contents between dextral and sinistral individuals (Russo *et al.*, 2012), but the present study is the first to investigate isotopic differences between asymmetry morphs of a polymorphic flatfish.

The stable isotope data for the present study were collected from fish used in a previous morphometric analysis (Bergstrom, 2007), allowing us to investigate associations between isotopic signature, multiple anatomical variables and whole-body asymmetry direction. Our objectives were as follows: (1) to compare $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values between dextral and sinistral individuals within populations from nine sample locations throughout the North American Pacific Northwest; (2) to assess whether correlations exist within samples between $\delta^{15}\text{N}$ or $\delta^{13}\text{C}$ and multiple morphological traits to investigate possible biomechanical explanations for any trophic variation; and (3) to compare gut contents between asymmetry morphs to see if they are consistent with any differences in stable isotope signatures.

MATERIAL AND METHODS

SAMPLE COLLECTION AND ISOTOPE ANALYSIS

Samples of *P. stellatus* were collected from nine sites throughout the North Pacific by beach seine or otter trawl, from May to October from 1988 to 2004. Four samples were from Alaska (Belkofski Bay, $N = 24$; Port Moller, $N = 20$; Kodiak Island, $N = 21$; Bristol Bay, $N = 24$), three from British Columbia (Hecate Strait, $N = 37$; Denman Island, $N = 51$; Barkley Sound, $N = 4$) and two from Washington (Puget Sound, $N = 7$; Columbia River Estuary, $N = 25$), for a total of 213 fish. The Washington samples were from the University of Washington Fish Collection Museum (Puget Sound catalogue #48377; Columbia River Estuary catalogue #1988-IX:30). Alaskan samples were collected by the Alaskan Department of Fish and Game and the National Oceanic and Atmospheric Administration. The Denman Island and Hecate Strait samples were collected by the Canadian Department of Fisheries and Oceans, and the Barkley Sound

sample was collected by C.A.B. under guidelines of and approval by the Canadian Council on Animal Care. Data regarding sex was not available for most samples. Each sample was collected in a single trawl or seine set.

Flounder muscle tissue was analysed for isotopic ratios of $^{15}\text{N}/^{14}\text{N}$ ($\delta^{15}\text{N}$) and $^{13}\text{C}/^{12}\text{C}$ ($\delta^{13}\text{C}$) by continuous-flow isotope ratio mass spectrometry. Tissue for $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ analysis was taken from the flank musculature of each flounder's blind side, ventral to the lateral line and posterior to the abdominal cavity. Integument and bone tissue was avoided. Muscle tissue samples were dried for ≥ 2 weeks at 60°C and powdered with a Wig-L-Bug grinder (Crescent Dental Co.) for 30–40 s. For each fish, ~ 1 mg (± 0.05) powdered dry muscle mass was weighed and added to a $3.5\text{ mm} \times 5\text{ mm}$ tin capsule for analysis at the Stable Isotope Facility, University of Saskatchewan (Saskatoon, SK, Canada). Resulting isotope ratios are relative to the international standards Pee Dee Belemnite for carbon and atmospheric nitrogen. Replicated samples indicate that the average measurement error was approximately $\pm 0.079\text{‰}$ for $\delta^{15}\text{N}$ and $\pm 0.142\text{‰}$ for $\delta^{13}\text{C}$.

The fixative (formalin vs. alcohol) and the duration (months vs. years) of sample preservation can affect isotope ratios, although the effects appear to be small and inconsistent among tissue types and taxa (Arrington & Winemiller, 2002; Sweeting, Polunin & Jennings, 2004; Kelly, Dempson & Power, 2006). For $\delta^{15}\text{N}$, the potential difference in enrichment between ethanol and formalin ranges from 0.16‰ to 0.34‰ , and for $\delta^{13}\text{C}$ the potential difference is $\sim 2.2\text{‰}$; these represent 4 and 11% of the total range of isotopic signatures obtained in the present study for $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$, respectively (see Results). Most importantly, our main objective was to investigate isotopic differences among individuals within samples, all of which had the same fixative history and storage duration.

Isotopic differences in flounder among locations might reflect variation of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in the primary producer base, rather than differences in trophic level of the fish (Post, 2002). Given that $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values were primarily used for within-sample comparisons, food web baseline values were not needed. The assumption is that dextral and sinistral flounder at the same site are drawing from the same trophic base.

Lipid synthesis and tissue content can deplete $\delta^{13}\text{C}$, leading to differences in $\delta^{13}\text{C}$ among individuals that are unrelated to recent diet sources (DeNiro & Epstein, 1978; Schleichtrien, Focken & Becker, 2003; Sweeting *et al.*, 2004; Post *et al.*, 2007). A proxy for lipid content in individuals used in stable isotope studies is the C:N ratio of muscle tissue samples (McConnaughey & McRoy, 1979; Post *et al.*, 2007; Sardenne *et al.*, 2015), and there were negative correlations between $\delta^{13}\text{C}$ and

C:N in six of our nine samples (five were significant), as would be predicted by lipid depletion of $\delta^{13}\text{C}$. We therefore normalized $\delta^{13}\text{C}$ by saving the residuals of $\delta^{13}\text{C}$ against C:N within each sample and adding these to the raw $\delta^{13}\text{C}$ mean values (Post *et al.*, 2007; Sardenne *et al.*, 2015). All analyses involving $\delta^{13}\text{C}$ are presented for normalized values, and the significance of statistical tests was not changed with raw values.

ASYMMETRY AND MORPHOLOGICAL ASSOCIATIONS

We investigated associations between $\delta^{15}\text{N}$, $\delta^{13}\text{C}$ and multiple morphological traits, including asymmetry direction, using morphometric data from a previous study (Bergstrom, 2007). Only the subset of fish from that study (213 of 355) that had isotopic analyses completed were used to investigate morphology–isotope associations. Traits included were gill raker number, body depth, mouth length, head length, jaw length, snout length, head depth, caudal depth and caudal length, all of which were size standardized except for gill raker number (see Bergstrom, 2007). We ran a principal component analysis using a correlation matrix on these traits that produced four orthogonal components, each of which had eigenvalues of nearly one or greater and explained 10% or more of the total variance (Table 1). Principal components (PCs) 1–4 represented primarily head size, body and caudal peduncle depth, caudal peduncle length and gill raker number, respectively. Additional components with eigenvalues of ≤ 0.75 were excluded.

In order to assess the relative effect of asymmetry morph, body size and morphology as represented by the four PCs on isotopic signatures, we conducted two analyses of covariance (ANCOVAs), one each with $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ as dependents. Each ANCOVA included sample location as a random factor, asymmetry morph as a fixed factor, and standard length (SL, \ln transformed) and the four PCs as covariates. The dependence of each isotope signature on each covariate did not differ between asymmetry morphs (all two-way interaction terms involving morph and covariates were non-significant); therefore, the ANCOVAs were re-run with these interaction terms removed to test for main effects. Separately for each sample location, we also generated a correlation matrix between each isotopic signature and SL and the four PCs to assess whether the direction of correlation coefficients, regardless of their magnitude, was consistent among localities.

To compare isotope variance between asymmetry morphs instead of means, we computed composite $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ Euclidean distances from each individual fish to its locality mean (standardized to Z -scores and locality means of zero). We then analysed these mean distances in a two-way ANOVA, with sample location as a random factor and asymmetry morph as a fixed factor.

Table 1. Resulting components from a principal component analysis including component eigenvalues (Eigen.), percentage of variance explained (% Var.) and loading scores (PC1–PC4) for gill raker number and eight size-standardized morphological traits (traits from Bergstrom, 2007)

Traits	PC1 (head size)		PC2 (body, caudal depth)		PC3 (caudal length)		PC4 Gill raker number	
	Eigen.	% Var.	Eigen.	% Var.	Eigen.	% Var.	Eigen.	% Var.
	3.68	40.0	1.70	18.9	1.00	11.1	0.98	10.9
Gill raker number	0.023		0.381		0.093		0.908*	
Body depth	0.315		0.833*		0.004		-0.019	
Mouth length	0.791*		0.139		-0.102		-0.071	
Head length	0.861*		-0.276		-0.043		0.016	
Jaw length	0.907*		-0.072		-0.082		-0.027	
Snout length	0.771*		-0.126		0.141		0.041	
Head depth	0.845*		0.279		0.165		-0.098	
Caudal peduncle depth	-0.280		0.763*		0.252		-0.370	
Caudal peduncle length	0.012		-0.284		0.927*		0.011	

*Largest loadings for each principal component (PC).

In a separate analysis, we investigated differences in isotopic divergence between asymmetry morphs among the three sampled regions: Washington (Columbia Est., Puget Sound), British Columbia (Barkley Sound, Denman Is., Hecate Strait) and Alaska (Kodiak Is., Bristol Bay, Port Moller, Belkofski Bay). For each sample in each region, we computed the difference between morphs in mean $\delta^{15}\text{N}$, in mean $\delta^{13}\text{C}$ and in the composite Euclidean distances between mean $\delta^{15}\text{N}$ and mean $\delta^{13}\text{C}$. For Euclidean distances, we first *Z*-standardized $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values so that each had a mean of zero. Consequently, for the $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ bivariate plots, Euclidean values represent the absolute distances to the geometric mean, and distances between morphs comprise a measure of dual-isotope trophic differences between them for each sample. Sample means of morph isotopic differences ($\delta^{15}\text{N}$, $\delta^{13}\text{C}$ and Euclidean) were investigated for regional differences with three separate one-way ANOVAs, with 'region' as a fixed factor and each isotopic difference as a dependent.

GUT CONTENTS

Contents of stomachs were not available from specimens used for stable isotope analysis. A sample of 79 starry flounder juveniles, of which 42 were sinistral (mean SL = 15.5 cm, range 6.4–28.0 cm) and 37 dextral (mean SL = 15.4 cm, range 8.2–25.3 cm), was collected from an estuary near Juneau, AK, in June 2010 with a 50' seine net with 1/2" mesh. Contents of each stomach were removed and identified under

a dissecting microscope to the nearest taxonomic category possible. Prey were categorized into trophic groups as filter feeders, grazers or carnivores. We computed the frequency of fish that contained each individual taxonomic category and trophic group, and the average abundance of each individual taxonomic category and trophic group in the flounders' stomachs when present. Frequencies of sinistral vs. dextral fish that contained each prey taxonomic category or trophic group were compared with Fisher's exact tests because of small sample sizes of some comparisons. Abundances of taxonomic categories and trophic groups were compared between morphs using Mann–Whitney *U* tests owing to non-normality of abundance distributions. Mann–Whitney *U* tests are not informative if sample sizes are less than five, so they were not run in those cases. We also calculated the total number of combined prey taxonomic categories and prey trophic groups present in each flounder's stomach, regardless of abundance, as a proxy of diet richness. We used contingency tables and χ^2 tests to compare diet richness of all prey taxonomic categories and trophic groups between morphs.

Throughout the paper, for all analyses that involved multiple comparisons we used Benjamini–Hochberg corrections with a false discovery rate of 0.10 (Benjamini & Hochberg, 1995). These are more powerful than sequential Bonferroni corrections, which can be inappropriately conservative for ecological studies such as this one, with small to modest sample sizes and multiple variables to investigate (García, 2004).

RESULTS

SITE SAMPLE ISOTOPE MEANS

Isotopic signatures varied among site samples and among individuals within samples. Overall mean $\delta^{15}\text{N}$ of all fish in the study was 13.4‰ and ranged from means of 12.6‰ in the Hecate Strait, BC, to 15.3‰ in Bristol Bay, AK (Table 2). The $\delta^{15}\text{N}$ fractionation within any location ranged from 0.30 to 5.94‰, with an average of 2.60‰, and the range of all fish was from 9.3 to 17.9‰, representing a total fractionation of 8.6‰. Mean $\delta^{13}\text{C}$ of all fish in the study was -17.3‰ (range -29.9 to -6.1‰ among all individuals) and ranged among site sample means from -24.1‰ in the Columbia River estuary, WA, to -13.1‰ in Barkley Sound, BC (Table 2).

ASYMMETRY AND MORPHOLOGICAL ASSOCIATIONS

Sinistral flounder had slightly but significantly enriched $\delta^{15}\text{N}$ over dextral flounder (Fig. 1, Table 3), and this pattern was consistent across most samples (seven of nine; Fig. 1). None of the other morphological traits had a significant effect on $\delta^{15}\text{N}$ overall. Within locations, we found a significant negative correlation between $\delta^{15}\text{N}$ and SL in Bristol Bay after Benjamini–Hochberg false discovery rate corrections, but there was no overall effect of SL, with five samples showing negative correlations regardless of strength, and four showing positive correlations (Table 4).

Unlike $\delta^{15}\text{N}$, there was no significant effect of asymmetry morph on $\delta^{13}\text{C}$ (Fig. 1, Table 3), although both SL and PC2 (body and caudal depth) were significantly associated with $\delta^{13}\text{C}$. Within locations, there was variability in the direction of correlations between $\delta^{13}\text{C}$ and SL (Table 4), and only one (Columbia Est.) was significantly positive after correcting for multiple corrections. However, for PC2 (body and caudal depth) there was a positive correlation with $\delta^{13}\text{C}$ in eight of the nine locations (binomial test $P < 0.05$ after Benjamini–Hochberg false discovery rate corrections). Although this overall pattern was weak, it suggests the possibility of a general pattern of decreased body and caudal depth in fish using prey of greater freshwater influence. Raw $\delta^{13}\text{C}$ scores (not lipid normalized) did not change the significance or non-significance of these results.

To examine whether the asymmetry morphs differed in total isotopic (trophic) breadth within each locality, we computed composite $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ Euclidean distances from each individual fish to its locality mean (standardized to Z -scores and locality means of zero). There were no significant differences in isotopic breadth between morphs or among sample location (two-way ANOVA: side [fixed] $F_{1,212} = 0.04$; d.f. = 1; $P = 0.84$, sample [random] $F_{8,205} = 0.68$; d.f. = 8;

Table 2. Summary statistics for nine samples of *Platichthys stellatus*

Location	N [T(S,D)]	SL (cm)	Sample date	$\delta^{15}\text{N}$			$\delta^{13}\text{C}$ (normalized)				
				Mean	Range	Minimum	Maximum	Mean	Range	Minimum	Maximum
Belkofski Bay (AK)†	24 (17,7)	46.8 ± 4.9	08/2004	13.4	2.0	12.6	14.5	-16.9	1.9	-18.8	-16.9
Port Moller (AK)†	20 (15,5)	42.9 ± 5.3	06/2004	13.8	2.8	12.6	15.5	-17.3	6.4	-21.0	-14.7
Bristol Bay (AK)†	24 (13,11)	35.1 ± 5.6	05/2004	15.3	4.5	13.4	17.9	-17.4	6.0	-21.2	-15.2
Kodiak Is. (AK)†	21 (13,8)	46.8 ± 6.1	07/2004	13.0	1.8	11.9	13.7	-16.3	6.5	-18.9	-12.3
Hecate Strait (BC)†	37 (32,5)	48.2 ± 5.6	05/2004	12.6	2.1	11.8	13.9	-16.6	6.0	-20.0	-14.0
Denman Is. (BC)†	51 (27,24)	26.5 ± 3.9	10/2003	13.2	5.9	9.3	15.3	-15.8	13.7	-24.0	-10.3
Barkley Sound (BC)†	4 (2,2)	11.5 ± 3.4	02/2004	13.8	0.3	13.7	14.0	-13.2	3.2	-15.0	-11.8
Puget Sound (WA)‡	7 (3,4)	32.1 ± 1.5	10/2003	13.0	0.9	12.6	13.5	-13.3	9.4	-15.5	-6.1
Columbia Est. (WA)§	25 (14,11)	14.1 ± 3.7	08/1988	12.8	3.1	11.4	14.5	-24.1	12.0	-29.9	-17.9
Total	213 (136,77)	35.4 ± 12.9		13.4	8.6**	9.3	17.9	-17.3	23.8*	-29.9	-6.1
					2.6***						7.2***

N is the sample size; T (S,D) is total (sinistral, dextral); SL, standard length mean ± SD; Sample date is given as month/year. AK, Alaska; BC, British Columbia; WA, Washington.

†Fresh or frozen; ‡fixed and stored in 95% EtOH; §fixed in 10% formalin, stored in 75% EtOH.

*Range among sample means; **average range within samples.

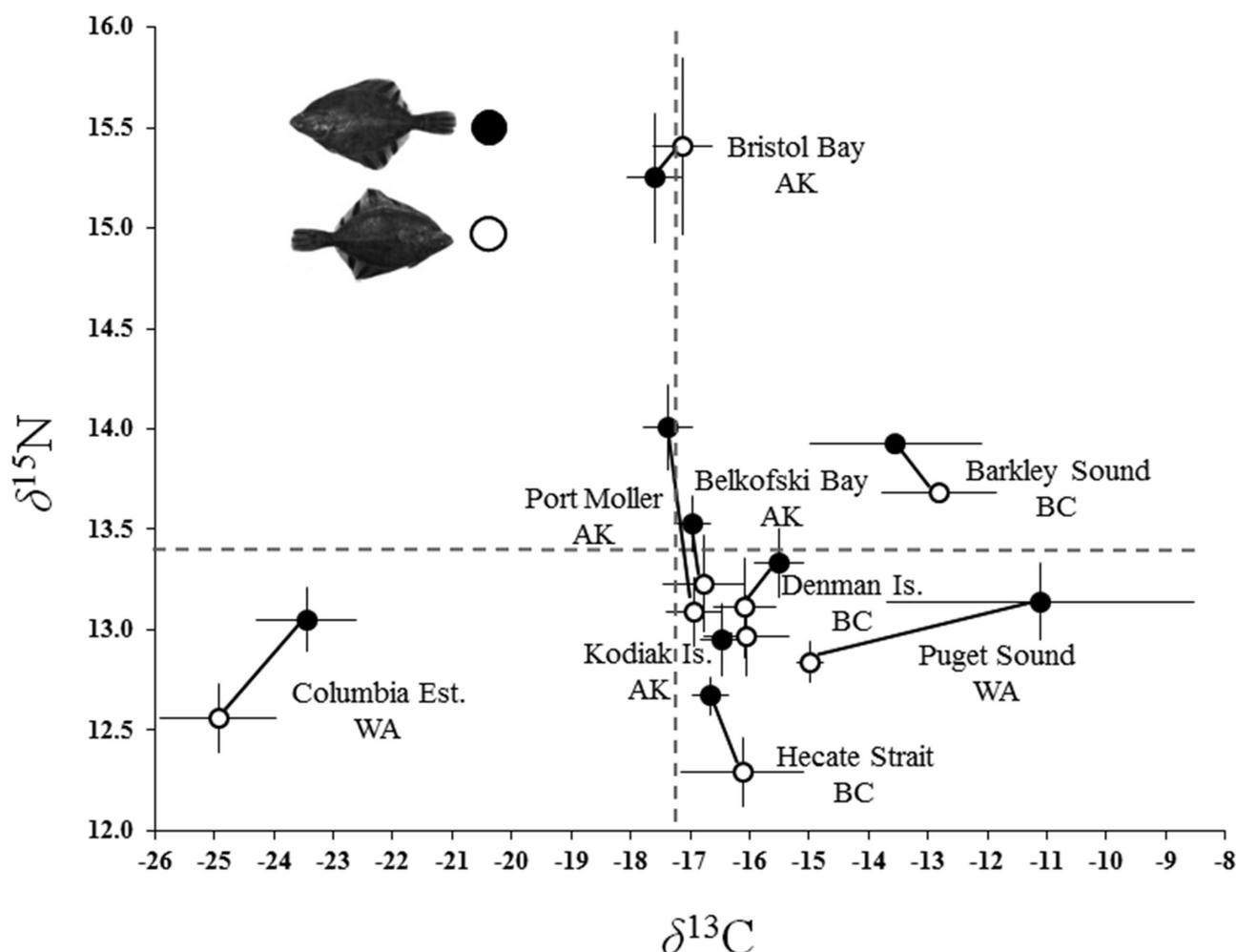


Figure 1. Mean (± 1 SE) $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ (lipid normalized) values for *Platichtys stellatus* morphs (filled circles represent sinistrals; open circles represent dextrals) from nine sample sites. AK, Alaska; BC, British Columbia; WA, Washington. Vertical and horizontal dashed lines represent overall mean $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, respectively. Diagonal continuous lines connect sinistral and dextral means from a single site and represent total isotopic Euclidean distances. Barkley Sound $\delta^{15}\text{N}$ standard errors are smaller than the marker diameter.

$P=0.70$). For both asymmetry morphs, we also compared correlation coefficients for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in order to evaluate the strength of covariation. Denman Is. and Columbia Est. had positive, significant correlations for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ for both morphs (all $r > 0.43$; all $P < 0.03$), and Belkofski Bay had a positive, significant correlation for sinistrals ($r = 0.53$; $P = 0.03$). These remained significant after Benjamini–Hochberg false discovery rate corrections for 18 multiple comparisons. All other correlations were non-significant, and there was no trend for sinistrals to have larger or smaller correlation coefficients than dextrals.

There was considerable variability in the divergence between asymmetry morphs in isotopic signatures ($\delta^{15}\text{N}$, $\delta^{13}\text{C}$ and total Euclidean distances) among sample locations (Fig. 1). When compared among geographical regions, the largest differences in $\delta^{13}\text{C}$

between morphs occurred in the southernmost samples from Washington state, compared with the British Columbian and Alaskan samples (one-way ANOVA: $F_{2,6} = 7.735$; d.f. = 2; $P = 0.022$; Fig. 2), with dextral fish exhibiting significantly more freshwater-associated signatures than sinistrals (Fig. 1). Total Euclidean distances between morphs exhibited considerable variation among samples, with the largest differences found between Puget Sound and Port Moller flounder and the smallest between Kodiak Island flounder (Fig. 1). Washington flounder had slightly greater Euclidean isotopic differences between asymmetry morphs than did Alaskan and British Columbian morphs (Fig. 2), although this was non-significant (one-way ANOVA: $F_{2,6} = 3.093$; $P = 0.119$) and probably driven by the significant regional differences in $\delta^{13}\text{C}$ divergence. There were no significant differences

among geographical regions in divergence in $\delta^{15}\text{N}$ between morphs (one-way ANOVA: $F_{2,6} = 0.095$; d.f. = 2, $P = 0.991$; Fig. 2).

Table 3. ANCOVA results of main effects on mean $\delta^{15}\text{N}$ and mean $\delta^{13}\text{C}$

Isotope	Factor	MS	d.f.	F	P
$\delta^{15}\text{N}$	Asymmetry morph	2.97	1	4.35	0.04
	Sample location	14.85	8	21.76	< 0.001
	SL	0.80	1	1.18	0.28
	PC1	0.15	1	0.21	0.65
	PC2	0.13	1	0.19	0.66
	PC3	0.43	1	0.63	0.43
	PC4	< 0.01	1	< 0.01	0.99
	Error	0.68	191		
$\delta^{13}\text{C}$	Asymmetry morph	2.01	1	0.49	0.48
	Sample location	85.09	8	20.75	< 0.001
	SL	54.22	1	13.23	< 0.001
	PC1	0.65	1	0.16	0.69
	PC2	26.63	1	6.50	0.01
	PC3	0.76	1	0.19	0.67
	PC4	3.89	1	0.95	0.33
	Error	4.10	191		

Asymmetry morph is fixed, sample location is random, SL (ln transformed) and principal components (PC1–PC4) are covariates. Two-way interaction terms involving covariates and asymmetry morph have been removed (all were non-significant).

Table 4. Correlation matrix between isotope signatures and morphology within locations, showing (r , P)

Location	Isotope	SL	PC1 (head size)	PC2 (body, caudal depth)	PC3 (caudal length)	PC4 (gill raker number)
Belkofski Bay (AK)	$\delta^{15}\text{N}$	-0.122 (0.569)	0.112 (0.602)	0.005 (0.981)	-0.138 (0.521)	-0.091 (0.672)
Port Moller (AK)		-0.186 (0.433)	0.258 (0.273)	-0.114 (0.632)	-0.260 (0.267)	0.005 (0.982)
Bristol Bay (AK)		-0.684* (0.001)	-0.026 (0.903)	0.003 (0.988)	-0.196 (0.358)	0.281 (0.184)
Kodiak Is. (AK)		-0.310 (0.172)	-0.260 (0.255)	0.176 (0.446)	0.208 (0.365)	-0.406 (0.068)
Hecate Strait (BC)		0.263 (0.121)	0.146 (0.396)	-0.307 (0.068)	0.028 (0.869)	0.043 (0.804)
Denman Is. (BC)		0.033 (0.816)	-0.215 (0.146)	0.080 (0.595)	-0.167 (0.261)	-0.069 (0.646)
Barkley Sound (BC)		0.591 (0.409)	0.399 (0.601)	-0.520 (0.480)	0.016 (0.984)	0.791 (0.209)
Puget Sound (WA)		-0.654 (0.111)	0.545 (0.342)	0.384 (0.523)	0.303 (0.621)	-0.046 (0.942)
Columbia Est. (WA)		0.395 (0.051)	0.221 (0.290)	0.461 (0.020)	-0.020 (0.924)	0.411 (0.041)
Belkofski Bay (AK)	$\delta^{13}\text{C}$	0.247 (0.244)	-0.120 (0.578)	0.100 (0.642)	-0.125 (0.561)	-0.165 (0.440)
Port Moller (AK)		0.009 (0.968)	0.199 (0.401)	0.237 (0.315)	0.058 (0.807)	-0.315 (0.176)
Bristol Bay (AK)		0.387 (0.062)	0.428 (0.037)	0.088 (0.681)	0.074 (0.733)	-0.062 (0.775)
Kodiak Is. (AK)		-0.501 (0.021)	0.094 (0.687)	-0.161 (0.485)	0.107 (0.645)	0.146 (0.528)
Hecate Strait (BC)		0.180 (0.292)	-0.047 (0.785)	0.143 (0.406)	-0.167 (0.330)	-0.122 (0.477)
Denman Is. (BC)		0.065 (0.648)	-0.200 (0.177)	0.179 (0.229)	-0.171 (0.252)	0.006 (0.967)
Barkley Sound (BC)		-0.764 (0.236)	-0.961 (0.039)	0.969 (0.031)	0.903 (0.097)	-0.522 (0.478)
Puget Sound (WA)		-0.364 (0.422)	0.315 (0.605)	0.658 (0.227)	0.535 (0.353)	-0.076 (0.903)
Columbia Est. (WA)		0.714* (0.001)	-0.038 (0.856)	0.370 (0.069)	-0.071 (0.735)	0.108 (0.607)

Morphs were pooled within each location. See Table 2 for sample sizes.

AK, Alaska; BC, British Columbia; PC1–PC4, principal components 1–4; SL, standard length; WA, Washington.

*Statistically significant after table-wide Benjamini–Hochberg false discovery rate corrections.

GUT CONTENTS

Among the 79 starry flounder juveniles from Juneau, AK, 75 (35 dextral, 40 sinistral) had prey in their stomach. We identified 11 different taxonomic categories of prey: clams, mussels, mysids, barnacles, limpets, snails, amphipods, isopods, polychaete and nemertean worms and insect larva. The most common were clams, mussels, isopods, polychaete worms and insect larva (Table 5). We placed taxonomic categories into three trophic groups: filter feeders (clams, mussels, mysids and barnacles), grazers (limpets, snails, amphipods and isopods) or carnivores (polychaete and nemertean worms). Insect larvae were not placed into a trophic group owing to uncertainty of insect type. There were no significant differences in frequency or abundance of any individual taxonomic category or trophic group between morphs (Table 5).

Individual flounder contained from zero to seven of these 11 taxonomic categories, and from zero to three trophic groups (Table 6). In order to run χ^2 tests with sufficiently large sample sizes of cells and expected values, we combined some cells (as per Fig. 3 x-axes). Although sinistral fish did not have significantly greater numbers of taxonomic categories in their stomachs than dextrals ($\chi^2 = 6.31$, d.f. = 3, $P = 0.10$; Fig. 3A), they did have two or more of the three trophic groups in their guts significantly (after Benjamini–Hochberg corrections) more often than dextral fish did ($\chi^2 = 4.33$, d.f. = 1, $P = 0.04$; Fig. 3B).

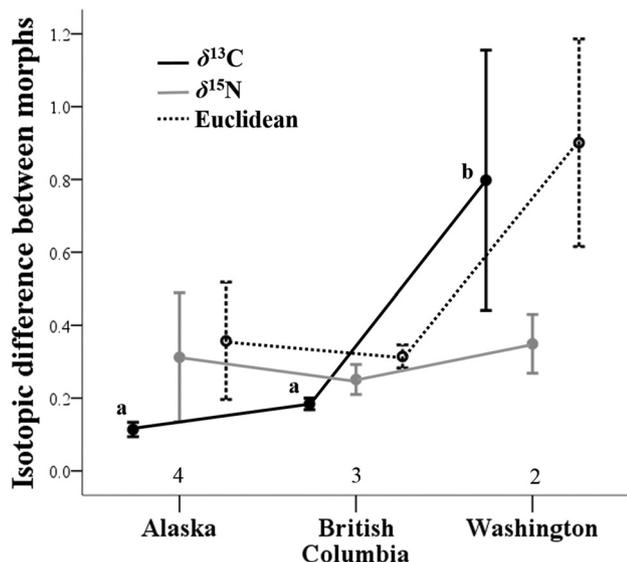


Figure 2. Absolute isotopic differences (mean \pm 1 SE) between asymmetry morphs among Alaskan, British Columbian and Washington samples. Numbers above the x-axis are the number of sample sites. For illustrative purposes only, $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ are converted to Z-scores to enable comparison on the same scale. Lower case letters next to means for $\delta^{13}\text{C}$ designate significant groupings resulting from Bonferroni *post hoc* tests.

DISCUSSION

Asymmetry direction in *P. stellatus* is associated with stable isotope signatures among multiple populations in the eastern Pacific. This, combined with the subtle but consistent anatomical differences between morphs (Bergstrom, 2007), differences between them in prey strike behaviours (Bergstrom & Palmer, 2007) and temporal persistence of the geographical cline in their relative proportion (Hubbs & Kuronuma, 1942; Bergstrom, 2007), supports the hypothesis that morphs are segregated in one or more axes of their ecological niche. One probable axis is resource use, given that the isotopic differences detected here implicate trophic level differences in their prey, and thus this might be one of very few published examples of resource polymorphism between asymmetry morphs (but see Moodie *et al.*, 2007; Reimchen *et al.*, 2008).

Platichthys stellatus exhibited considerable isotopic variability, making the evolution of resource segregation plausible. Across animal taxa, average $\delta^{15}\text{N}$ fractionation between trophic levels is usually 3–4‰ (Peterson & Fry, 1987; Post, 2002), although it can range from 2 to 5‰ (Adams & Sterner, 2000). In the present study, the total fractionation of all fish was 8.6‰. This represents what would be found across at least two trophic levels, although some of this could be attributable to variation in foodweb base $\delta^{15}\text{N}$ or

fixative history among locations. The $\delta^{15}\text{N}$ fractionation within any location ranged from 0.3 to 5.9‰, indicating that on average *P. stellatus* are occupying one to two trophic levels within a single location. Despite this trophic variability, the biomechanical explanations that cause resource segregation between asymmetry morphs remain obscure. Aside from asymmetry, there were no significant correlations between morphology and $\delta^{15}\text{N}$ isotopic signature within any of the nine samples, precluding the identification of the cause of differences in diet. Foraging success could be linked with other traits, such as behaviour, unidentified anatomical differences and/or physiological and metabolic differences. Dextral *P. stellatus* exhibit shorter snouts, fewer gill rakers and larger caudal peduncles than sinistrals, suggesting that they could be targeting larger, harder and, possibly, faster prey (Bergstrom, 2007), although these differences in performance have not been demonstrated. Given the range of isotope signatures in marine prey (see below) that are not necessarily associated with size or hardness (e.g. high trophic level polychaete worms vs. low trophic level bivalves), a proclivity for catching fast prey might not necessarily predict the resulting $\delta^{15}\text{N}$ level in muscle tissue.

Behavioural differences between morphs in particular might provide a functional link between foraging dynamics and asymmetry direction. There are a growing number of examples of correlations between morphological and behavioural asymmetries in invertebrates and fishes, some of which are associated with predator–prey outcomes (Yasugi & Hori, 2012), including examples in cephalopods (Lucky *et al.*, 2012) and other invertebrate prey (Takeuchi & Hori, 2008; Takeuchi, Tobo & Hori, 2008; Frasnelli, Vallortigara & Rogers, 2012; Tobo, Takeuchi & Hori, 2012). Predators with asymmetric attack trajectories, such as largemouth bass, have greater success in capturing freshwater gobies whose escape bias is in the opposite direction (Yasugi & Hori, 2012), potentially leading to differences in fitness between predator asymmetry morphs as a function of the frequency of left- vs. right-biased prey. Models predict that both predator–prey and intraspecific interactions can maintain different frequencies of left- vs. right-biased individuals within a species (Vallortigara & Rogers, 2005; Ghirlanda, Frasnelli & Vallortigara, 2009). Starry flounder juveniles exhibit behavioural asymmetry during foraging that is linked to their morphological asymmetry direction (Bergstrom & Palmer, 2007). Asymmetric avoidance responses in their prey, if present, could lead to selection on flounder asymmetry morphs that vary temporally and spatially with prey availability. Our present data showing isotopic differences between starry flounder asymmetry morphs support this hypothesis.

Table 5. Summary statistics for gut contents

Trophic group	Taxonomic category	Number (%) of morphs with at least one of the prey types		Fisher's exact test (<i>P</i> -value)		Abundance of prey type, when present, per morph (mean ± SD)		Mann-Whitney $U_{0.05(2)}$ (<i>P</i> -value)
		Sinistral	Dextral	Sinistral	Dextral	Sinistral	Dextral	
Filter feeders	Clam	23 (54.8)	24 (64.9)	0.49		31.1 ± 36.1	32.2 ± 39.3	$U_{23,24} = 247.5 (0.54)$
	Mussel	28 (66.7)	20 (54.1)	0.36		17.3 ± 24.3	18.4 ± 28.4	$U_{28,20} = 272.0 (0.87)$
	Mysid	8 (19.0)	2 (5.4)	0.09		13.8 ± 11.1	35.0 ± 21.2	—
	Barnacle	2 (4.8)	0 (0.0)	0.50		62.5 ± 17.7	—	—
Grazers	Any filter feeder	35 (83.3)	27 (73.0)	0.29		41.0 ± 35.3	44.8 ± 38.6	$U_{35,27} = 429.0 (0.54)$
	Limpet	4 (9.5)	0 (0.0)	0.12		10.8 ± 10.4	—	—
	Snail	5 (11.9)	4 (10.8)	1.00		23.2 ± 34.6	10.5 ± 16.4	$U_{5,4} = 10.0 (0.99)$
	Amphipod	17 (40.5)	13 (35.1)	0.65		42.1 ± 35.8	31.1 ± 31.5	$U_{17,13} = 90.0 (0.41)$
Carnivores	Isopod	25 (59.5)	20 (54.1)	0.66		25.2 ± 32.9	45.3 ± 41.9	$U_{25,20} = 166.5 (0.06)$
	Any grazer	32 (76.2)	22 (59.5)	0.15		47.1 ± 41.9	61.5 ± 39.9	$U_{32,22} = 270.0 (0.15)$
	Polychaete	21 (50.0)	15 (40.5)	0.50		5.8 ± 10.6	5.1 ± 6.4	$U_{21,15} = 155.0 (0.95)$
	Nemertean	1 (2.4)	0 (0.0)	1.00		8.0 ± 0.0	—	—
Unknown	Any carnivore	21 (50.0)	15 (40.5)	0.50		6.1 ± 10.5	5.1 ± 6.4	$U_{21,15} = 155.0 (0.95)$
	Insect larva	21 (50.0)	16 (43.2)	0.65		41.1 ± 33.6	52.8 ± 36.0	$U_{21,16} = 128.0 (0.23)$

Sinistral, *N* = 42. Dextral, *N* = 37.

Marine invertebrates, including taxa targeted by *P. stellatus* in our sample, differ considerably in their isotopic signatures from the same location (France, 1994; Bouillon *et al.*, 2002). For example, in the northwestern Atlantic large polychaete worms and benthic isopods exhibit some of the highest $\delta^{15}\text{N}$ signatures relative to euphausiids, amphiods and bivalves (Fry, 1988), a finding consistent with reports from invertebrates in the north Pacific (Dehn *et al.*, 2007) and the North Sea (Jennings *et al.*, 2002). Elevated $\delta^{15}\text{N}$ signatures in sinistral starry flounder could reflect a foraging proclivity for invertebrates of higher trophic levels, because of either greater search, pursuit and/or capture success of those prey, or associations with prey's microhabitat. In the present

Table 6. Contingency tables showing the number of sinistral and dextral *Platichthys stellatus* containing different numbers of prey taxonomic categories (A) and prey trophic groups (B) in their stomachs

Number of prey		Number of fish	
		Sinistral	Dextral
A, taxonomic categories	0	2	2
	1	1	6
	2	9	2
	3	5	12
	4	12	8
	5	7	6
	6	4	1
	7	2	0
	8–11	0	0
B, trophic groups	0	2	2
	1	7	14
	2	18	13
	3	15	8

Sinistral, $N = 42$. Dextral, $N = 37$.

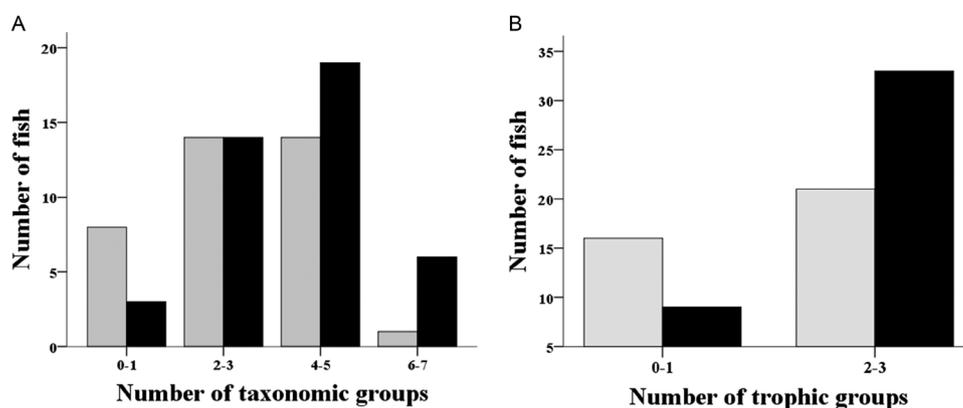


Figure 3. Gut-content prey diversity for sinistral (filled bars) and dextral (open bars) *Platichthys stellatus* morphs. A, number of prey taxonomic categories per stomach. B, number of prey trophic groups per stomach.

study, the number of trophic prey groups in stomachs of sinistral flounder was significantly greater than in those of dextral flounder (Fig. 3B). However, this was evaluated from a single sample, and there could be spatial and/or temporal variability in trophic breadth between morphs. Most *P. stellatus* samples included in our isotope analysis were larger in body size (Table 2) than those in our gut-content analysis (~15–16 cm), and it is possible that differences in diet between morphs shift among age classes, although there are no reports that document this to our knowledge. However, if this pattern is found to have general relevance it could indicate that sinistral flounder have increased opportunity to include higher trophic prey as a consequence of their greater diet breadth.

Although differences in $\delta^{15}\text{N}$ means between morphs were largely consistent across the sampled region from southern Washington to the Alaskan Aleutian Peninsula, differences in $\delta^{13}\text{C}$ were not (Fig. 1). We found overall $\delta^{13}\text{C}$ depletion in our Columbia Estuary sample compared with our other sampled sites (Fig. 1, Table 2) as predicted based on the relatively large freshwater influence there. In addition, $\delta^{13}\text{C}$ means were significantly depleted in dextral fish in samples from Washington state (Fig. 2), but not so elsewhere. Despite relatively small sample sizes, this implies the possibility that the $\delta^{13}\text{C}$ variability of the base of the food web is greater in those southern samples and/or that ecological opportunity to specialize on freshwater sources of prey might be greater there than further north. Interestingly, the congeneric European flounder (*P. flesus*) is also polymorphic and exhibits resource segregation, as left- and right-sided morphs in a sample from the Irish Sea also differ in gut contents and other subtle anatomical features (Russo *et al.*, 2012) that are comparable to differences in *P. stellatus* (e.g. head and caudal peduncle shape, eye position; Bergstrom, 2007; Bergstrom & Palmer, 2007). *Platichthys flesus* has an average incidence of the

dextral morph of ~77%, yet the dextral morph ranges from 72 to 80% and is most abundant in coastal areas of the Baltic Sea with reduced salinity (Fornbacke, Gombrii & Lundberg, 2002). This is consistent with our findings and suggests possible preference of dextral morphs for freshwater-associated prey in both *Platichthys* species.

In general, there was considerable variation among sampled locations in the divergence of isotopic signatures between morphs (Figs 1, 2). Spatial or temporal variation of the breadth of food web base isotopic signatures could translate into variation in higher trophic levels, and without quantifying $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of primary producers in these locations this cannot be ruled out. However, variation in isotopic divergence could also reflect spatial or temporal variation in the degree of ecological opportunity and intraspecific competition in higher trophic levels (Van Valen, 1965; Nosil & Reimchen, 2005; Martin & Pfennig, 2010), including those of *P. stellatus*. Even in locations with large isotopic breadth of lower trophic levels, strong interspecific competition could reduce opportunities for trophic divergence between flounder asymmetry morphs. Therefore, spatial variation in morph isotopic divergence could be attributable to a combination of food base isotopic breadth, ecological opportunity and competition. Anatomical differences between asymmetry morphs are greatest in locations where morph proportions approach 1:1, suggesting that intraspecific competition between morphs could be driving character displacement (Bergstrom, 2007). We did not detect this correlation with isotopic signatures, but this is not altogether surprising given the temporally ephemeral nature of isotopic signatures relative to morphology.

Our data showing trophic segregation between asymmetry morphs contribute to a growing body of evidence supporting the hypothesis that their persistence along a cline is attributable to adaptive divergence and spatially variable selection (Bergstrom, 2007; Bergstrom & Palmer, 2007). Selection could result if there was a spatial cline in *P. stellatus* prey availability or, possibly, a north Pacific cline in the predation landscape. The occurrence of lateralized behaviours in predator–prey interactions among taxa (Bisazza, Rogers & Vallortigara, 1998) suggests the potential for such selection. This novel example of asymmetry-associated trophic differences broadens the context in which intraspecific trophic specialization can occur (Smith & Skúlason, 1996; Araújo, Bolnick & Layman, 2011; Reimchen & Klinka, 2017). Investigation of segregation between asymmetry morphs in other genera of flatfishes will be informative with regard to the general ecological significance of asymmetry direction in Pleuronectiformes.

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