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Benthic food webs support the production of sympatric flatfish larvae in estuarine nursery habitat

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Abstract

Identifying nursery habitats is of paramount importance to define proper management and conservation strategies for flatfish species. Flatfish nursery studies usually report upon habitat occupation, but few attempted to quantify the importance of those habitats to larvae development.

The reliance of two sympatric flatfish species larvae, the European flounder *Platichthys flesus* and the common sole *Solea solea*, on the estuarine food web (benthic vs. pelagic) was determined through carbon and nitrogen stable isotope analysis. The organic matter sources supporting the production of *P. flesus* and *S. solea* larvae biomass originates chiefly in the benthic food web. However, these species have significantly different $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values which suggests that they prey on organisms that use a different mixture of sources or assimilate different components from similar OM pools (or both).

Keywords

Platichthys flesus; *Solea solea*; stable isotopes; estuary; resource partitioning

INTRODUCTION

Estuaries function as nursery areas for several flatfish species, including European flounder *Platichthys flesus* (L., 1758) (Daverat *et al.*, 2012) and common sole *Solea solea* (L., 1758) (Vinagre *et al.*, 2005), since these ecosystems provide favorable conditions for growth and survival of larvae and juveniles, and thus enhancing recruitment (Beck *et al.*, 2001).

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Nonetheless, most studies focused on the dynamics and feeding behavior of juvenile flatfish species in nursery areas (Vinagre *et al.*, 2005; Freitas *et al.*, 2009), and few have focused on their larval stages (Ramos *et al.*, 2010).

Stable isotope ratios are useful to understand the nursery function of estuarine ecosystems for fish larvae, not only because they can provide information about their basal sources of nutrition (Hoffman *et al.*, 2007), but also because they help tracking dispersal and settlement, when larvae move between habitats with isotopically distinct prey (Herzka, 2005; Hoffman, 2016). The carbon (C) and nitrogen (N) stable isotope composition ($^{13}\text{C}/^{12}\text{C}$, $^{15}\text{N}/^{14}\text{N}$) of consumers' tissues is a time-integrated signal of the available food sources in the ecosystem and that were incorporated into an organism's structural components and energy reserves (Peterson and Fry, 1987). Thus, the stable isotope ratio of a consumer reflects its diet, demonstrating an average trophic fractionation (i.e. the difference between the consumer and its diet) of +0.4 ‰ $\delta^{13}\text{C}$ and +3.2 ‰ $\delta^{15}\text{N}$ per trophic level (Vander Zanden and Rasmussen, 2001).

Thus, this study aims to determine the dependence of two sympatric flatfish larvae on estuarine habitats, by quantifying their reliance on the benthic and pelagic estuarine food webs. This was accomplished by determining the C and N stable isotope contents of the most common flatfish species in the Minho River estuary, *P. flesus* and *S. solea*, and the most likely organic matter (OM) basal sources providing nutritional support to these larvae.

MATERIAL AND METHODS

Minho estuary (NW- Iberian Peninsula) covers an area of 23 km², and it is a mesotidal system with tides ranging between 0.7 m and 3.7 m (Alves, 1996). The limit of tidal influence is about 40 km inland, and the uppermost 30 km are tidal freshwater wetlands. This estuary is partially mixed, but during periods of high floods tends to evolve towards a salt wedge estuary (Sousa *et al.*, 2005). Samples were collected bi-weekly in March 2011, during the high tides of spring tides. Flatfish larvae and OM sources (or proxies) that could support larvae production were collected at fixed stations along the first 4 km of the estuary (Fig. 1). This is the period when flatfish larvae begin their recruitment into northern Portuguese estuaries (Ramos *et al.*, 2010), and corresponds to the end of the high river discharge, during which phytoplankton availability in the estuary is potentially low (Dias *et al.*, 2016).

Flatfish larvae were collected using a modified version of a stow net and preserved in 70% ethanol. The OM sources of interest were phytoplankton, microphytobenthos (MPB), particulate OM (POM), macroalgae, and sediment OM (SOM). At each station, surface (50–100 cm below the surface) and bottom water samples (0.5 m off the bottom) were collected using a Ruttner bottle. From these samples, we measured the isotopic composition of POM (particulate organic carbon (POC) $\delta^{13}\text{C}$, particulate nitrogen (PN) $\delta^{15}\text{N}$, molar C:N), and isotopic composition of total dissolved inorganic carbon (DIC: $\delta^{13}\text{C}_{\text{DIC}}$). POM and DIC samples were processed following the methods described in Dias *et al.* (2014). The MPB samples were scraped from a pipe fixed in the estuary sediment for MPB colonization.

Drifting macroalgae were collected by hand. POM, MPB, and macroalgae samples were kept frozen (-20°C) until analysis.

Stable isotope ratios were measured using a Thermo Scientific Delta V Advantage IRMS via ConFlo IV interface (MARINNOVA). Stable isotope ratios are reported in δ notation, $\delta X = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 10^3$, where X is the C or N stable isotope, R is the ratio of heavy:light stable isotopes, and Vienna Pee Dee Belemnite and air are standards for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, respectively. The analytical error, the mean standard deviation of replicate reference material, was $\pm 0.1\text{‰}$ for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$. DIC $\delta^{13}\text{C}$ was measured using a GasBench II system interfaced to a Delta V Plus IRMS (University of California Davis, Stable Isotope Facility). The analytical error was $\pm 0.2\text{‰}$ $\delta^{13}\text{C}$.

The most likely OM sources supporting flatfish larvae production were identified using $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ bi-plots, where *P. flesus* and *S. solea* larvae $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ average values were compared with OM sources $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ average values. For OM sources, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values were pooled by station (4 stations) to estimate the average values at the estuary mouth. Phytoplankton $\delta^{13}\text{C}$ ($\delta^{13}\text{C}_{\text{phytoplankton}}$) values were estimated from DIC $\delta^{13}\text{C}$ ($\delta^{13}\text{C}_{\text{DIC}}$) values, assuming an uptake fractionation of -21‰ (i.e., $\delta^{13}\text{C}_{\text{phytoplankton}} = \delta^{13}\text{C}_{\text{DIC}} - 21\text{‰}$; Peterson and Fry, 1987). The $\delta^{15}\text{N}_{\text{phytoplankton}}$ values were those reported by Bode *et al.* (2007) for the Atlantic Iberian Peninsula Coast ($6 \pm 1.5\text{‰}$). The SOM stable isotope ratios used in the bi-plots are those reported for the Minho estuary (Dias *et al.*, 2014). To remove at most the maternal effect on the stable isotope ratios, only flatfish larvae with the yolk-sac absorbed were analyzed. For the bi-plot, *P. flesus* and *S. solea* stable isotope averages were adjusted for trophic fractionation: $+0.8\text{‰}$ $\delta^{13}\text{C}$ ($+0.4\text{‰}$ per trophic level), and $+5.9\text{‰}$ $\delta^{15}\text{N}$ ($+2.5\text{‰}$ for primary consumers and $+3.4\text{‰}$ for secondary consumers; Vander Zanden and Rasmussen, 2001). Also, flatfish larvae $\delta^{13}\text{C}$ values were corrected for lipid content based on tissue C:N ratio (Eq. 2 in Logan *et al.*, 2008), and $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values for ethanol preservation ($+0.4\text{‰}$ $\delta^{13}\text{C}$, $+0.6\text{‰}$ $\delta^{15}\text{N}$; Feuchtmayer and Grey, 2003). The relationship between the stable isotope ratios and size was tested to check if flatfish larvae were at equilibrium with their diet. To test for possible differences in the $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values between species, we used a one-way PERMANOVA. PERMANOVA tests the simultaneous response of one or more variables to one or more factors in an ANOVA experimental design on the basis of any distance measure, using permutation methods (Anderson, 2001). The statistical significance of variance ($\alpha = 0.05$) was tested using 9999 permutations of residuals within a reduced model. Statistical tests were conducted using the PRIMER software (v.6.1.6, PRIMER-E) with the permutational multivariate analysis of variance (PERMANOVA) + 1.0.1 add-on (Anderson *et al.*, 2008).

The contribution of the different OM sources to flatfish larvae biomass was quantified by a dual-isotope stable isotope mixing model, which uses Bayesian inference to solve the indeterminate equations (more than $n+1$ sources relative to n stable isotopes), producing a probability distribution that represents the likelihood a given source contributes to the consumer biomass (Stable Isotopes in R-SIAR; Parnell *et al.*, 2010). Phytoplankton (average bottom and surface), MPB, POM (average bottom and surface), macroalgae, and SOM were the OM sources used in the model. Fish larvae $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values were adjusted for trophic fractionation as previously described.

RESULTS

The OM sources were isotopically well differentiated (Fig. 2). Among sources, macroalgae and MPB presented the highest average $\delta^{13}\text{C}$ ($\pm\text{SD}$) values ($\delta^{13}\text{C}$: $-15.9 \pm 2.8\text{‰}$ and $-18.6 \pm 0.1\text{‰}$, respectively), whereas macroalgae presented the highest average $\delta^{15}\text{N}$ ($\pm\text{SD}$) values ($\delta^{15}\text{N}$: $9.5 \pm 1.3\text{‰}$), and SOM the lowest average $\delta^{15}\text{N}$ ($\pm\text{SD}$) values ($\delta^{15}\text{N}$: $1.7\text{‰} \pm 0.3\text{‰}$).

A total of 51 *P. flesus* larvae and 44 *S. solea* larvae were collected, with total length varying between 6.0–8.3 mm and 6.7–9.0 mm, respectively. Composite samples were generally required for smaller larvae (composites of 4 larvae). We found a poor relationship between size and the stable isotope values of both species ($r^2 < 0.2$; $p > 0.05$), which suggests that the flatfish were in isotopic equilibrium with their diet, and thus maternal or cross-habitat (i.e. OM sources from other habitats) influence must have been minimal (Fig. 2). *Platichthys flesus* ($\delta^{13}\text{C}$: $-19.3 \pm 0.2\text{‰}$; $\delta^{15}\text{N}$: $2.5 \pm 0.2\text{‰}$) and *S. solea* ($\delta^{13}\text{C}$: $-18.5 \pm 0.2\text{‰}$; $\delta^{15}\text{N}$: $3.3 \pm 0.3\text{‰}$) larvae stable isotope ratios, after adjusting for trophic fractionation, were intermediate between the OM sources measured, indicating reliance on multiple OM sources (Fig. 3). Nevertheless, the average stable isotope ratios of both flatfish species were close to the stable isotope ratios of MPB, suggesting a relevant contribution of this source to their biomass (Fig. 3).

The results of the dual-stable isotope mixing model (95% CI) indicate that MPB had the highest proportional contribution for both species: between 58.3% and 88.0% for *P. flesus*, and between 56.1% and 92.9% for *S. solea* (Fig 4A, 4B). However, *S. solea* had significantly higher $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values than *P. flesus* (Pseudo- $F = 78.82$; $p < 0.001$), suggesting they use a different mixture of sources. We estimate that macroalgae (^{15}N - and ^{13}C -enriched) contribution to *S. solea* varied between 1.1% and 26.2% (Fig. 4), whereas phytoplankton (^{15}N - and ^{13}C -depleted) contribution to *P. flesus*'s biomass was up to 32.0% (Fig. 4). Sediment OM proportional contribution was low in both species: up to 22% in *P. flesus*, and up to 14% in *S. solea* (Fig. 4).

DISCUSSION

Platichthys flesus and *S. solea* larvae relied principally on OM sources with benthic origin (MPB and SOM), indicating dependence on the estuarine benthic food web. One possible explanation may be the low phytoplankton availability in the POM pool in this estuary, especially during high river discharge conditions ($\sim 40\%$; Dias *et al.*, 2016). Previous studies indicate that flatfish larvae start to feed exogenously during the yolk-sac stage, and that their main prey include dinoflagellates, tintinnids, copepods, and other zooplankton (Last, 1978). However, because the European flounder (< 5.5 mm) and the common sole larvae (< 6.5 mm) we sampled had developed past the yolk-sac stage, it is likely that they were consuming more zooplankton than protistoplankton. Thus, the most likely mechanisms by which they obtain their energy from the benthic food web is indirect, i.e. they feed on prey that rely on benthic OM. In a study conducted during the same period, it was found that calanoid copepods, which are the most abundant copepods in this estuary (Vieira *et al.*, 2015), relied mostly on SOM in the brackish portion of the estuary (up to 60%; Dias *et al.*,

2016). Also, it is possible that epibenthic prey, such as polychaete, harpacticoid copepods, small bivalves and gastropods, were also consumed (Lagardère *et al.*, 1999).

Although MPB was the main OM source supporting the biomass of *P. flesus* and *S. solea* larvae in the Minho estuary, the differences in the stable isotope values indicate that they may be preying on organisms that use a different mixtures of sources or assimilate different components from identical OM pools (or both). *Platichthys flesus* presents lower $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values than *S. solea*, suggesting a greater dependence on a detritus-based food web due to the assimilation of SOM and surface terrestrial- derived POM ($\text{C:N}_{\text{POM}} > 10$; Hedges *et al.*, 1986, 1997; Dias, 2014). These results suggest that, to some extent, food source partitioning may occur. This could also favor the reduction of the potential for interspecific competition. However, we do not have enough data to support this hypothesis, because data on food selectivity is inexistent.

In contrast, sympatric juvenile *P. flesus* and *S. solea* display high trophic overlap (Vinagre *et al.*, 2005, Banaru and Harmelin-Vivien, 2009), but because they have a generalist and opportunistic feeding behavior, competition is likely reduced in highly productive estuarine ecosystems (Vinagre *et al.*, 2005). However, we note that juvenile European flounder and common sole in the Minho estuary potentially reduce resource-competition (space and food) because flounder juveniles generally occupy freshwater habitats, where it is the only flatfish species present, while common sole is restricted to the lower estuary where it shares this habitat with other flatfish species, including flounder (Freitas *et al.*, 2009).

In conclusion, the sympatric *P. flesus* and *S. solea* populations of Minho estuary are highly dependent on the benthic estuarine food web during their critical larval development.

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Fig. 1.
Location of the sampling stations in the Minho River estuary (NW-Portugal, Europe).

Fig. 2. Nitrogen and carbon stable isotope values (‰) from composite samples of larvae from *Platicthys flesus* and *Solea solea* as a function of average larvae size (mm).

Fig. 3.

Average ($\pm$ SD) $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of *Platichthys flesus* (Pf) and *Solea solea* (Ss) larvae adjusted for two trophic levels fractionation ($+0.8\text{‰}$ $\delta^{13}\text{C}$; $+5.9\text{‰}$ $\delta^{15}\text{N}$). Larvae were collected at the mouth of the Minho estuary (March 2011). Potential organic matter (OM) sources include surface (s) and bottom (b) phytoplankton (Phyto) and particulate OM (POM), microphytobenthos (MPB), macroalgae and sediment OM (SOM).

Fig. 4.

Proportional contribution of each potential organic matter (OM) source to *Platichthys flesus* and *Solea solea* biomass collected in the Minho river estuary in March 2011 based on a dual stable isotope mixing model. The OM sources include phytoplankton (Phyto), particulate organic matter (POM), macroalgae, microphytobenthos (MPB), sediment organic matter (SOM). Closed squares indicate the most likely value (mode) and lines indicate the 95% Bayesian credibility intervals.