

1 **Effect of diet on breeders and inheritance in Syngnathids: Application of**
2 **isotopic experimentally derived data to field studies**

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13 **Running page head:** Diet in Syngnathids and stable isotopes

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15 **KEY WORDS:** Stable isotopes, diet, trophic enrichment factor, inheritance,
16 Syngnathidae, prey sources, Cíes archipelago.

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19 **ABSTRACT**

20 Syngnathids are vulnerable ovoviviparous fishes with repeated brooding of males within
21 a reproductive season. The isotopic effects of diet on both breeders (pooled sexes) and
22 parents-egg transmission have been demonstrated in a few fish species but never in
23 syngnathids. Quantifying isotopic changes due to diet is necessary to assess parent-
24 newborn conversions and to estimate accurate trophic enrichment factors (TEF). We
25 assessed the isotopic ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) effects of three experimental diets on TEFs in
26 seahorse *Hippocampus guttulatus* breeders and isotopic inheritance. Our results
27 suggest that *H. guttulatus* follows an income-capital continuum pattern for “parent-egg”
28 transmission. The isotopic variability in diets for breeders and the resulting
29 experimentally derived TEFs were compared with fixed TEFs from reviews to estimate
30 their impact on the relative contribution of potential prey sources in syngnathids from
31 Cíes archipelago (Atlantic Islands National Park, NW Spain). We estimated source

1 contributions using stable isotopes mixing models (SIMMs) by combining prey sources
2 into ecologically informative groups and incorporating informative priors. We
3 demonstrate (1) that most frequently used TEFs from reviews might not be suitable to
4 all fish species, particularly to syngnathids, and (2) that dietary source variability has a
5 great effect on source contribution estimates. This study also provides for the first time
6 specific TEFs for syngnathids.

7

1. INTRODUCTION

According to their biology and ecology, syngnathids are vulnerable fishes (Ahnesjö & Craig 2011, Vincent et al. 2011). A few species are threatened, endangered or critically endangered by the IUCN Red List of Threatened Species (IUCN 2016). Others are data deficient due to the need of further information. Syngnathids are atypical ovoviviparous fish characterized by repeated mating, production of multiple batches of offspring annually, parental care and a distinctive anatomy of ovaries (Selman et al. 1991, Poortenaar et al. 2004, Planas et al. 2017). Egg production is synchronized with brooding in males (Foster & Vincent 2004, Planas et al. 2010). When mature, the eggs are transferred from females to male's brood pouches, and new cohorts of follicles will develop immediately. This cycle will extend for a variable period of the breeding season (commonly extends from spring to summer in European species), with 2-4 week inter-batch intervals.

The effects of fish diet on tissues and egg composition/quality were demonstrated decades ago, particularly for fatty acids (Sargent 1995). Even though several studies have focused on the reproduction of syngnathids (Boisseau 1967, Lin et al. 2006, Ripley & Foran 2006, Faleiro et al. 2008, Planas et al. 2010, 2012, Scobell & MacKenzie 2011, Palma et al. 2014), the effects of diet on spawning quality, nutrients assimilation, parental investment in eggs and embryos, or newborn success have been scarcely investigated (Otero-Ferrer et al. 2012, 2014, Palma et al. 2012, Saavedra et al. 2014, Uriarte et al, 2016). Egg production and embryogenesis demand a high input of energy and nutrients, more or less maternally or paternally derived depending on the species (Ripley & Foran 2006). However, the role of males would not be critical for embryo

1 survival, and the yolk is the primary energy source for developing embryos (Azzarello
2 1991, Kvarnemo et al. 2011).

3 One of the pivotal factors in fish reproduction, which is closely related to ecological
4 ones, is the availability and quality of prey sources (Hempel & Blaxter 1967, Hempel
5 1979). The nutritive constituents of an egg largely determine its quality since eggs take
6 up little nutrients once ovulated (Brooks et al. 1997). On the other hand, spawning in
7 wild fish populations may occur only when environmental conditions are adequate,
8 particularly those affecting food supply (Hempel 1979). The newborn diet can
9 compensate for some imbalances in egg composition (Pérez & Fuiman 2015), but
10 feeding conditions of parents should ensure that nutritive investment in an egg provides
11 the necessary nutrients for the fry at least until first exogenous feeding.

12 Stable isotope analysis (SIA) is a useful tool to assess the structure and dynamics of
13 food webs, to evaluate the incorporation of dietary nutrients into consumers, to
14 distinguish between populations and to track animal migration (DeNiro & Epstein 1978,
15 1981, Peterson & Fry 1987, Doucett et al. 1999, Vander Zanden & Rasmussen 2001, Post
16 2002, Schlechtriem et al. 2004, Gamboa-Delgado et al. 2008, Caut et al. 2008b, Martínez
17 del Río et al. 2009, Le Vay & Gamboa-Delgado 2011, Laiz-Carrión et al. 2019, McCormack
18 et al. 2019). SIA is practical to experimentally evaluate isotopic relationships between
19 parental and egg or newborn tissues, to estimate isotopic discrimination factors
20 (isotopic enrichment between consumer and diet) (Caut et al. 2008a, Zbinden et al.
21 2011), and to assess the investment of endogenous versus exogenous nutrients into
22 eggs/newborn (Jenkins et al. 2001, Sare et al. 2005, McMeans et al. 2009, Vaudo et al.
23 2010, Stephens et al. 2014, Kaufman et al. 2014, Tanaka et al. 2016, Uriarte et al. 2016).
24 About the later, a distinction is generally made between capital breeding (use of

1 endogenous reserves) and income breeding (use of concurrent energy intake).
2 Vertebrates commonly follow mixed capital- and income-breeding strategy, and, for
3 many species, the allocation of capital towards reproduction is a plastic reproductive
4 strategy (Williams et al. 2017), where shifts may occur with ontogeny or in relation to
5 environmental conditions (McBride et al. 2015).

6 Alternatively to other approaches, consumer diets may be reconstructed by SIA when
7 direct observations are impossible or when the sacrifice of consumers must be avoided
8 (e.g. threatened species). Consumers reflect the isotopic composition ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$)
9 of food sources plus a consistent trophic enrichment factor (TEF) at each trophic level
10 (DeNiro & Epstein 1978, 1981), theoretically ranging from 0 to 2‰ for $\delta^{13}\text{C}$ and from 2
11 to 5‰ for $\delta^{15}\text{N}$ (DeNiro & Epstein 1978, 1981, Minagawa & Wada 1984, Post 2002, Caut
12 et al. 2009). SIA is widely applied in trophic ecology to quantify the relative contributions
13 of different food sources in consumer's diet by the use of Bayesian mixing models (Wolf
14 et al. 2009, Bond & Diamond 2011). Stable isotope mixing models (SIMMs) require the
15 knowledge of accurate TEFs values. Fixed generalist TEFs have been reported from data
16 reviews including a wide range of organisms. However, erroneous models might
17 generate incorrect results, particularly in diet reconstruction and trophic relationships
18 assessment (Caut et al. 2009). As TEFs are diet-dependent (Caut et al. 2008a, 2009),
19 discrimination factors should be experimentally determined for different diets at the
20 lowest taxa level possible.

21 Understanding both the connections of diet with isotopic patterns in breeders
22 (accurate TEFs estimates), and parents-newborn transmission patterns (isotopic
23 inheritance) is critical in ecological research, particularly in trophic studies. The later
24 would be potentially interesting to determine maternal isotope composition from

1 hatchling tissues and evaluate trophic relationships and foraging strategies of breeders
2 without sampling them (Frankel et al. 2012).

3 In our study, we (1) estimated discrimination factors for seahorse *Hippocampus*
4 *guttulatus* breeders in mesocosm experiments comparing three dietary regimes
5 differing in isotopic values, (2) evaluated isotopic inheritance, and (iii) applied
6 experimentally derived TEFs to infer diet composition of syngnathids in the wild Cíes
7 (Spanish National Park). For diet reconstruction, we built a series of SIMMs using the
8 MixSIAR package (Stock & Semmens 2016), comparing TEFs from published reviews with
9 our experimentally derived TEFs.

10 By means of both laboratory experiments and field observations, we addressed the
11 following questions: (1) What is the effect of breeder's diet on isotopic discrimination
12 factors?, (2) How breeder's diets affect newborn isotopic signals?, (3) Do experimentally
13 derived TEFs perform better than fixed generalist TEFs?, and (4) What are the relative
14 contributions of potential sources to diet in wild syngnathids?

15 **2. MATERIALS AND METHODS**

16 **2.1. Fish breeding in mesocosm**

17 Adults (n=42) of the syngnathid *Hippocampus guttulatus* were reared in captivity or
18 collected in Galicia (NW Spain) from August 2016 to January 2017 with permission of the
19 Regional Government (Xunta de Galicia). Prior to the start of the experiment (mid-March
20 2017) seahorses were maintained for more than two months at the facilities of Instituto
21 de Investigaciones Marinas (CSIC) (Vigo, NW Spain) in 320 L aquaria (Planas et al. 2008)
22 in a semi-closed system. Temperature and photoperiod natural-like regimes were
23 established, fluctuating from 15 to 19°C and from 10L:14D to 16L:8D, respectively
24 (Planas et al. 2010). Water quality was checked periodically for NO₂, NO₃ and NH₄/NH₃

1 content (0 mg L^{-1}). Salinity and pH levels were 37 ± 2 (mean \pm sd) and 8.0 ± 0.2 ,
2 respectively. The fishes were fed on a diet comprising long-time enriched adult *Artemia*
3 and captured/frozen mysidaceans (see below for further details).

4 Males and females were kept separate until the start of the breeding period in mid-
5 March 2017, when all seahorses were randomly distributed (1:1 sex ratio; six pairs per
6 aquaria) in three 320 L aquaria and maintained for a whole breeding season (until early
7 October 2017). All aquaria were maintained under the same conditions except for the
8 diet offered. Average size (curved standard length) and weight of seahorses did not
9 differ across treatments (ANOVA, $p = 0.702$, $17.4 \pm 2.6 \text{ cm}$; ANOVA, $p = 0.470$, 11.5 ± 5.0
10 g).

11 Within the entire experimental breeding period, each husbandry aquaria received a
12 different diet (two daily doses):

- 13 - Diet A: Non-enriched adult *Artemia* (MC450; Iberfrost, Spain) (Planas et al. 2017).
- 14 - Diet M: Captured (*Siriella armata* and *Leptomysis* sp.) and frozen mysidaceans
15 (*Neomysis* sp.; Ocean Nutrition, USA) (1:1)
- 16 - Diet AM: Mixture (1:1) of diets A and M.

17 Small portions of dorsal fin were sampled (partial fin-clipping) in breeders both at the
18 start and at the end of the experimental period for further isotopic analysis. Fish
19 breeders were monitored regularly to check for newborn releases from male's pouches.
20 Newly released juveniles were sampled (bulk juveniles) and euthanized with Tricaine
21 MS-222 (0.1 mg L^{-1} , Sigma Aldrich), measured for curved standard length (Lourie et al.
22 2003), weighted ($\pm 0.01 \text{ mg}$) and stored at $-80 \text{ }^\circ\text{C}$ for further stable isotope analysis (SIA).

23 **2.2. Collection of syngnathids in the field**

1 Syngnathids (n= 27) were hand-caught collected by scuba diving in 2016 (Spring and
2 summer) on the Eastern coast of Cíes archipelago (Atlantic Islands National Park – PNIA,
3 NW Spain). Captured fishes were anaesthetized and fin-clipped. Fin samples were
4 transferred to screw-capped tubes containing 95% ethanol and conserved at 4°C for SIA
5 (Valladares & Planas 2012). The fishes were released at the capture site within 2-3 hours
6 after sampling.

7 Samples of epifauna (associated to vegetal cover) and mysidaceans (water column)
8 were collected in PNIA in spring, summer and autumn 2017-2018. For epifauna, a nylon
9 bag (100 µm mesh size) was placed over macroalgal canopies (*Asparagopsis armata*,
10 *Codium* sp. and *Cystoseira baccata*) and above-ground macroalgae and associated
11 epifauna inside the bag were collected. Mysidaceans were handily collected using a nylon
12 net. The samples were brought back to the laboratory, preserved at 4°C in 95% ethanol,
13 taxonomically identified, grouped into 29 OTUs (Operational Taxonomic Groups), and
14 prepared for SIA as described above for fin samples (Table S1).

15 **2.3. Stable isotopes**

16 Carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) isotopes were analyzed in fin samples,
17 experimental prey (live and frozen) for breeders, and epifauna and mysidaceans from
18 Cíes archipelago. The samples were rinsed with distilled water, dried in oven at 60°C for
19 24 h and homogenized when necessary. The analyses were carried out on tin capsules
20 containing sub-samples of 0.5 - 1 mg dry weight biomass at Servicios de Apoio á
21 Investigación (SAI) of the University of A Coruña (Spain). Fin samples were not further
22 treated for full defatting (Valladares & Planas 2012) but arithmetical corrections were
23 applied to some groups of epifauna to normalize for defatting and acidification (M.
24 Planas unpubl. data).

1 Samples were measured by continuous flow isotope ratio mass spectrometry using a
2 FlashEA1112 elemental analyser (Thermo Finnigan, Italy) coupled to a Delta Plus mass
3 spectrometer (FinniganMat, Bremen, Germany) through a ConFlo II interface. Stable
4 isotope abundances were expressed as permil (‰) in conventional delta relative to
5 VPDB (Vienna Pee Dee Belemnite) and Atmospheric Air. The precision (standard
6 deviation) for the analysis of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of the laboratory standard (acetanilide) was
7 $\pm 0.15\text{‰}$ (1-sigma, n= 10).

8 Experimentally derived trophic enrichment factors (TEFs) for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in
9 breeders were estimated as the difference between isotopic values in fin tissue at the
10 end of the breeding period (δX_f) and diet (δX_{diet}) ($\text{TEF} = \delta X_f - \delta X_{\text{diet}}$). We assumed that
11 the isotopic equilibrium of breeders with diets was reached at the end of the breeding
12 period (200 days from the start of the experiment). Our assumption was based on
13 previous data (S. Valladares unpubl. data) on seahorses *H. guttulatus* captured in the
14 wild and subsequently maintained in captivity for nearly 2 years at our facilities. In
15 addition, Thomas and Crowther (2015) pointed out that consumers are considered to
16 have achieved isotopic steady state with a new food source after approximately four to
17 five half-lives (93.8 or 96.9% isotopic replacement). Accordingly, the isotopic equilibrium
18 in seahorse breeders of 10 g weight at 20°C submitted to a diet shift would be achieved
19 in 144 days for $\delta^{13}\text{C}$ and 152 days for $\delta^{15}\text{N}$.

20 **2.4. Statistical analysis**

21 Data normality was analyzed using the Kolmogorov–Smirnov test. Differences in
22 isotopic compositions among experimental treatments (dorsal fin and newborn) were
23 assessed by Kruskal–Wallis one-way analysis of variance ($p = 0.05$). One-way analysis of
24 variance (ANOVA) and unequal N HSD post-hoc test were used in field data to compare

1 fish size, fish weight and isotopic profiles of syngnathids and prey sources ($p = 0.05$). All
2 statistical analyses were conducted in Statistica 8.0 and R 3.6.1 software packages.

3 **2.5. Bayesian Stable Isotope Mixing Models (SIMM)**

4 For SIMM analysis, we adapted the procedure of Lerner et al. (2018). A first
5 hierarchical clustering analysis (Ward's method) was applied to isotopic data of 29 OTUs
6 of epifauna and mysidaceans (Table S1) from Cíes archipelago to analyze prey group
7 similarities. Average isotopic values for each grouped source were calculated from
8 weighted values considering relative abundances in spring, summer and autumn 2017-
9 2018. The initially achieved clusters were subsequently reduced to 12 and finally to 5
10 distinct source groups: Amphipoda, copepoda, caridea, isopoda and mysidacea (Table
11 1). Grouped selected sources differed in 2.45 ‰ for $\delta^{13}\text{C}$ and 3.60 ‰ for $\delta^{15}\text{N}$
12 (corresponding to about one trophic level).

13 A Monte Carlo simulation of SI was used to calculate the probability that each
14 consumer can be explained by the source data (five selected OTUs) (Smith et al. 2013),
15 to evaluate mixing models and to determine the proportion of consumers included
16 inside the mixing polygon bound by all potential sources (Phillips & Gregg 2003, Jackson
17 et al. 2011). For that, average source isotopic signatures were adjusted for TEF values
18 (see below). The probability that a consumer's isotope value fit within the proposed
19 mixing polygon was calculated for each fish individually by a simulation of 1,500
20 iterations, providing a quantitative basis for consumer exclusion in the model. Fishes
21 with a low probability (e.g. 5%) were excluded from the subsequent mixing model (Smith
22 et al. 2013). Estimates of diet component proportions in mixing models are considerably
23 affected by isotopic discrimination factors applied and the knowledge of accurate
24 estimates of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ is critical (Bond & Diamond 2011). Given that TEFs ($\delta^{13}\text{C}$ and

1 $\delta^{15}\text{N}$) are not currently available for syngnathids, the following five TEFs were assayed
2 with mixing polygons (Table 2):

3 - Meta-analysis: Averaged ecosystem TEFs (Minagawa & Wada 1984, Post 2002).

4 - Semi-experimentally derived: *Dicentrarchus labrax* and further extrapolation to
5 fishes (Post 2002, Sweeting et al. 2007a, b).

6 - Experimentally derived from syngnathids: *Hippocampus guttulatus* fed on diets M
7 and AM, and averaged values (present study). SIMM models (Stock et al. 2018) were run
8 using the MixSIAR package (Stock & Semmens 2016) with data from Cíes archipelago,
9 including syngnathids as consumers (raw data) and selected prey groups as food sources
10 (mean \pm sd). Separate models were run for each TEF assayed. The results achieved in
11 mixing polygons with TEFs derived from diet A (not provided) showed that all consumer
12 points were located outside the 5% probability contour plot (as explained hereafter).
13 Hence, TEFs from diet A were not submitted to SIMM model analysis.

14 The models were run with Markov chain Monte Carlo (MCMC) parameters of three
15 chains of 3,000,000 iterations and a burn-in phase of 1,500,000 (extreme run). The
16 model included individuals as a random effect and one error term (process error).
17 MixSIAR analyses were run including both uninformative/generalist and informative
18 priors to increase the resolution of SIMM outputs (Moore & Semmens 2008).
19 Informative priors were incorporated using the scarce data available on gut contents in
20 the syngnathid *Syngnathus acus* in South Africa and Turkey (Bennet 1989, Taskavak et
21 al. 2010, Gurkan & Taskavak 2019). That pipefish and the seahorse *H. guttulatus* are
22 sympatric species that overlap a large extension of their breeding seasons and share
23 similar isotopic signatures and trophic levels in our study area (R. Nogueira unpubl. data,
24 A. Jiménez unpubl. data). Informative priors were adjusted for normalized dietary

1 contributions of 32.8% for amphipoda, 58.1% for copepoda, 6.8% for isopoda, 1.6%
2 caridea and 0.7% for mysidacea.

3 The models generate average estimates of source contributions. Convergence and
4 diagnostic statistics were evaluated using both Gelman–Rubin (variables < 1.05) and
5 Geweke (number of variables outside ± 1.96 in each chain) tests. Bayesian model
6 outputs are reported as mean \pm sd. Bhattacharyya's coefficients (BC) were calculated for
7 pairwise source comparisons tested (first 5000 iterations) (Bhattacharyya 1943, Rauber
8 et al. 2008). BC values range from 0 to 1 (Horn 1966). BC = 1 indicates complete similarity
9 between distributions and BC = 0 indicates complete dissimilarity. BC > 0.6 indicates
10 non-significant overlapping distributions (significant similarity) (Catry et al. 2009).

11 **2.6. Ethics**

12 Fish capture, handling and sampling were conducted in compliance with all bioethics
13 standards on animal experimentation of the Spanish Government (R.D. 1201/2005,
14 10th October) and the Regional Government Xunta de Galicia (References REGA
15 ES360570202001/15/FUN/BIOL.AN/MPO01 and REGA
16 ES360570202001/16/FUN/BIOL.AN/MPO02).

17 **3. RESULTS**

18 **3.1. Experimental study: Breeders and newborn juveniles**

19 Experimental diets A, AM and M differed by as much as 1.59 ‰ for $\delta^{13}\text{C}$ (from -22.12
20 in A to -20.53‰ in M) and 3.16‰ for $\delta^{15}\text{N}$ (from 7.67 in A to 10.83‰ in M) (Table 3).
21 Isotopic signatures in *H. guttulatus* at the onset of the breeding season did not differ
22 significantly among treatments (Kruskal-Wallis test, $H_{(2, 28)} = 2.954$, $p = 0.228$ for $\delta^{13}\text{C}$;
23 $H_{(2,28)} = 4.363$ $p = 0.113$ for $\delta^{15}\text{N}$), with average isotopic values of -16.74 ± 1.59 ‰ for
24 $\delta^{13}\text{C}$ and 12.36 ± 0.95 ‰ for $\delta^{15}\text{N}$ (Table 3). At the end of the breeding season, isotopic

1 values of dorsal fin across treatments were significantly different (Kruskal-Wallis test;
2 $H_{(2, 25)} = 14.250$, $p = 0.0008$ for $\delta^{13}\text{C}$, $H_{(2, 25)} = 16.906$, $p = 0.0002$ for $\delta^{15}\text{N}$). Isotopic values
3 in treatments AM and M were similar, differing from those in treatment A (Kruskal-
4 Wallis test, $p < 0.05$ for $\delta^{13}\text{C}$; $p < 0.01$ for $\delta^{15}\text{N}$). TEF estimates ranged from 2.50 (M) to
5 8.13 (A) for $\delta^{13}\text{C}$ and from 2.62 (A) and 4.25 (AM) for $\delta^{15}\text{N}$ (Table 2; Figure 1).

6 A total of thirteen newborn batches were released (4, 7 and 3 for A, AM and M
7 treatments, respectively) (Figure 2). Average batch sizes were 133 ± 83 , 202 ± 82 and 84
8 ± 72 newborn for A, AM and M, respectively. The first and last batches were released
9 between 72 and 229 days of the breeding season (Figure 2).

10 Isotopic signals in both first and all batches produced reflected isotopic values in
11 breeders at the end of the breeding period, with higher $\delta^{13}\text{C}$ and lower $\delta^{15}\text{N}$ signals in
12 juveniles from diet A (Table 3; Figure 2). Excluding groups A and M (too few data points),
13 $\delta^{15}\text{N}$ signatures in newborn for diet AM followed a progressive decline with time (days
14 elapsed from the onset of the breeding season). Signatures for $\delta^{13}\text{C}$ were relatively
15 constant during the whole period. As for $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ values in the last batches released
16 were similar in all treatments. Convergence of isotopic values for the three treatments
17 would occur beyond day 150. Isotopic differences between breeder's diet and last
18 newborn batches ranged from 4.43 to 6.70‰ for $\delta^{13}\text{C}$ and from 0.87 to 3.94‰ for $\delta^{15}\text{N}$
19 (Table 3).

20 **3.2. Field study: Syngnathids in Cíes Archipelago**

21 A total of 27 adult syngnathids were captured in Cíes archipelago in 2016 surveys.
22 The pipefish *Syngnathus acus* L., 1758 (13 females and 10 males) and the seahorse
23 *Hippocampus guttulatus* Cuvier, 1829 (2 males and 2 females) were the only species
24 collected. Most *S. acus* individuals were very large, being mostly (91%) associated to

1 vegetal communities. Average size (SL) and weight were 31.8 ± 10.0 cm (14.8 – 49.7 cm)
2 and 28.6 ± 21.5 g (1.3 – 67.6 g), respectively. The pipefishes captured in spring (78 %)
3 did not differ in size nor weight from those caught in summer (ANOVA, $p > 0.05$).

4 Isotopic composition (pooled sexes) for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ was $-16.10 \pm 0.41\text{‰}$ and 10.79
5 $\pm 0.71\text{‰}$ in *S. acus*, and $-16.56 \pm 0.21\text{‰}$ and $11.05 \pm 0.42\text{‰}$ in *H. guttulatus*, respectively.
6 In *S. acus*, males and females did not differ isotopically (ANOVA, $p = 0.560$ for $\delta^{13}\text{C}$;
7 $p=0.176$ for $\delta^{15}\text{N}$) but isotopic values increased with length (linear regression, $p = 0.001$
8 for $\delta^{13}\text{C}$ and $p = 0.014$ for $\delta^{15}\text{N}$). However, isotopic differences between specimens
9 within the size range analyzed (15 – 50 cm) were lower than 2.4% for $\delta^{13}\text{C}$ and 5.1% for
10 $\delta^{15}\text{N}$ (0.84‰ and 1.77‰, respectively).

11 **3.3. Field study: TEFs comparisons in mixing model polygons**

12 Due to the reduced abundance of seahorses and their isotopic similarity with *S. acus*
13 (ANOVA, $p > 0.05$), SIMMs were only carried out on pipefish specimens. Stable isotope
14 mixing model polygons (Figure 3) were constructed considering fifteen consumers (4
15 outliers not included), five prey source groups (potential prey) and five TEF values. The
16 sources were selected based on previous hierarchical cluster analysis as well as
17 ecological and functional characteristics.

18 The higher proportions of consumer (*S. acus*) points within the confidence intervals
19 of the mixing polygons were achieved using our experimentally derived TEF values.
20 Indeed, all consumer points derived from seahorses fed on diets M and from average
21 values of diets AM and M were included inside the 20% probability contour plot.
22 Conversely, as for diet A (*Artemia*), many consumers were located outside the 5%
23 probability contour plot in isopolygons constructed with fixed TEFs from published

1 reviews, particularly for values of 0.39 for $\delta^{13}\text{C}$ and 3.40 for $\delta^{15}\text{N}$ (Minagawa &
2 Wada1984, Post 2002).

3
4 **3.4. Field study: Diet reconstruction (SIMMs)**

5 There were marked differences across models (five TEFs tested) in predicted diet
6 composition (Table 4). In spite of large overlapping of source predictions, generalist
7 models A and B consistently overestimated the relative predicted contribution of
8 caridea and underestimated that of copepods and isopods. The contribution of
9 mysidacea was slight in all models, particularly with models A, B and D. Finally, the
10 predicted mean contribution of amphipoda was noteworthy for most models,
11 particularly in model C.

12 The effect of informative priors in SIMM outputs was limited (Table 4). The rank of
13 diet components resulted almost unaffected, and deviations from estimates without
14 priors were lower than 13%, affecting particularly to main diet components, particularly
15 in models C to E. Considering exclusively models that provided the best results in mixing
16 model polygons (Models C and E), SIMMs including priors predicted high diet
17 contributions of amphipods (30.6 - 53.9 %). High contributions were also predicted in
18 model E for copepoda (31.4%) and isopoda (23.5%). A simplified version of predicted
19 contributions considering significance of overlapping distributions from Bhattacharyya's
20 coefficients (Table S2) for informative priors are provided in Table 5. Accordingly, the
21 diet of *S. acus* consisted mainly of caridean shrimps (53 - 68%) and amphipods +
22 copepoda (24-31%) as predicted by generalist models A and B, whereas amphipods and
23 harpacticoid copepods (72 – 80%) would mostly contribute to bulk diet as inferred from
24 experimentally derived TEFs.

25 **4. DISCUSSION**

1 This study presents an isotopic experimental approach to investigate for the first time
2 discrimination factors in syngnathid fishes and their application to assess diet
3 reconstruction in wild populations from the Western Atlantic. TEFs were experimentally
4 derived from breeders (pooled males and females) fed on three diets comprising the
5 brine shrimp *Artemia* and/or mysidaceans. The experimentally derived TEFs were
6 submitted to SIMM (Bayesian Stable Isotope Mixing Models) analysis and compared
7 with generalist TEFs.

8 In fishes, we applied SIA to dorsal fin samples instead of muscle tissue. Compared to
9 tissues with fast isotopic turnover rates (e.g. liver), those tissues have medium-slow
10 turnover rates that provide more information over time on food sources in fishes
11 (Hobson & Welch 1992, Fry 2006). Stable isotope signatures of fin and muscle tissues
12 are correlated in many fish species (Jardine et al. 2005, 2008, Kelly et al. 2006), including
13 syngnathids (Valladares & Planas 2012), supporting the use of fins as convenient tissues
14 for SIA in food web studies. Since fin-clipping is a non-lethal tool that does not require
15 fish sacrifice, that sampling procedure is a useful method for tissue sampling in vulnerable
16 or threatened species such as syngnathids (Sanderson et al. 2009).

17 **4.1. Experimentally derived discrimination factors**

18 Dietary regimes were reflected in the corresponding isotopic signatures of breeders
19 at the end of the breeding period. Discrimination factors in seahorses fed on non-
20 enriched adult *Artemia* (Diet A) were significantly different to those from diets including
21 mysidaceans (Diets AM and M). Interestingly, standard deviations for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$
22 were considerably higher in seahorses fed on the mixed diet (*Artemia* + Mysidaceans)
23 (Figure S1), probably due to inter-individual differences in the selective choice of both
24 prey types, as shown especially in $\delta^{15}\text{N}$ signatures (Araújo et al. 2007). In spite of that,

1 the similarity of the relationship between values and discrimination factors for $\delta^{13}\text{C}$
2 (similar slopes and intercepts for $\delta^{13}\text{C}$, Figure S1) suggests similar diet assimilation across
3 treatments, in contrast to $\delta^{15}\text{N}$, which would reflect different trophic levels of the diets
4 (similar slopes but different intercepts, Figure S1).

5 The knowledge of specific diet-dependent discrimination factors is a need for
6 obtaining accurate results in interpretation of diet reconstruction studies, as pointed out
7 by Caut et al. (2008b). These authors reported a wide range of discrimination factors
8 (-8.79 to 6.1% for $\delta^{13}\text{C}$; -3.22 to 9.2% for $\delta^{15}\text{N}$) after reviewing a high number of
9 published estimates for any species. Considering our results and excluding those from
10 diet A, our discrimination factors estimates ranged from 2.50 to 4.14% for $\delta^{13}\text{C}$ and
11 from 3.91 to 4.25% for $\delta^{15}\text{N}$.

12 A common but erroneous assumption in many studies is that discrimination factors
13 are independent of diet isotope values (Caut et al. 2009). However, isotopic
14 discrimination factors vary among species, depending on diverse factors such as
15 environmental characteristics, type and quality of food source, nutritional status,
16 age/developmental stage, kind of tissue and diet isotopic ratio, among others (DeNiro
17 & Epstein 1978, 1981, Minagawa & Wada 1984, Ben-David & Schell 2001, Vanderklift &
18 Ponsard 2003, Caut et al. 2008a, 2009, Martínez del Río et al. 2009, Uriarte et al. 2016).
19 We demonstrate for the first time that breeder's diet in syngnathids has strong effects
20 on both the isotopic signals in tissues and the resulting discrimination factors. Our
21 experimentally derived estimates agree with previously published ranges of TEFs but
22 they were higher than generalist TEFs values commonly applied in ecological studies,
23 especially for $\delta^{13}\text{C}$ (Minagawa & Wada 1984, Post 2002, Sweeting et al. 2007a, b).

1 Selecting adequate experimental prey types are of pivotal importance in the
2 assessment of isotopic discrimination factors. Whenever possible, experimental
3 challenges for TEFs estimation in a given species or zoological group should consider the
4 use of prey representative of potential sources in the wild. Our study shows that
5 deviations in assessing the relative proportions of dietary resources in aquatic
6 consumers might be mostly affected by TEFs values for $\delta^{13}\text{C}$, rather than for $\delta^{15}\text{N}$. Our
7 experimental diets included two different prey. The brine shrimp *Artemia* (Crustacea
8 Branchiopoda) is a filter-feeder inhabiting inland salt waters in a wide range of salinities
9 but it is not a true marine source nor an available prey for marine syngnathids in nature.
10 Conversely, mysidaceans (Crustacea Malacostraca) are common zooplanktonic
11 predators in the marine environment. Although both prey types support growth in
12 syngnathids, the later would promote better gonad development, egg quality and
13 spawning events (Lin et al. 2007, Otero-Ferrer et al. 2012). Poor-feeding conditions can
14 lead to delayed maturation, skipped spawning, fewer spawning events per season or
15 fewer eggs produced per event (McBride et al. 2015). *Artemia* as the sole prey did not
16 seem to provide satisfactory results, resulting in few newborn batches (as for diet M)
17 and unexpectedly high TEFs for $\delta^{13}\text{C}$. Our results seem to confirm that a mixed diet (Diet
18 AM) would enhance breeding success and that it would be a reference diet for TEFs
19 assessment.

20 **4.2. Isotopic inheritance**

21 Food intake in fish generally decrease with breeding in many fish species but the
22 extent to which dietary or tissue reserves are utilized to fulfil energy and nutrients needs
23 for reproduction is unknown in syngnathids. Macromolecules are routed differentially
24 into tissues and not all species respond similarly to the use of protein and lipids over

1 embryogenesis (Ripley & Foran 2006, Wolf et al. 2009). Hence, it is feasible that egg
2 isotopic signatures differ from those in yolk-sac deprived newborn (expected higher
3 $\delta^{15}\text{N}$ and lower $\delta^{13}\text{C}$ values in the former) (Uriarte et al. 2016) and that eggs would be a
4 more reliable proxy for inheritance isotopic assessment. However, we analyzed
5 newborn instead of eggs, which are very difficult to obtain in some syngnathids (namely
6 seahorses) unless the fish be sacrificed.

7 Similarly to the findings for $\delta^{15}\text{N}$ in some marine organisms (Aberle & Malzahn 2007),
8 it seems that *H. guttulatus* newborn tended to keep “isotopic homeostasis” for $\delta^{13}\text{C}$
9 along the breeding season (likely with a minor effect of dietary $\delta^{13}\text{C}$ signatures),
10 suggesting that the biochemical composition of eggs / newborn might be selectively
11 regulated for the allocation of specific nutrients (Jardine et al. 2005), Conversely, $\delta^{15}\text{N}$
12 isotopic values of our experimental breeder’s diet were clearly reflected in first newborn
13 batches produced. However, the progressive change of isotopic signatures in bulk
14 newborn along the breeding season, the lack of a clear final isotopic steady-state (Figure
15 2), and the limited production of newborn batches in diets A and M were serious
16 constrains to assess newborn – breeder relationships. This is an interesting subject that
17 deserves further research, considering its potential applicability to marine resources
18 such as the prediction of foraging areas in wild populations when isotopic signatures of
19 breeder’s tissue and newborn are strongly correlated (Frankel et al. 2012).

20 TEFs can be experimentally estimated whenever possible by studying turnover rates
21 and patterns of isotopic change with time following a diet switch. Isotopic signatures in
22 developing larvae submitted to a diet change fit to well-known growth or time-based
23 models (Fry & Arnold 1982, Hobson & Clark 1992, Hesslein et al. 1993). However, the
24 isotopic trends displayed in our study by freshly released newborn were opposed to

1 those commonly displayed by early developing larvae, and could not be mathematically
2 modeled. Alternatively, we roughly estimate newborn – parent isotopic differences from
3 the convergence values (last batch produced for each treatment) at the end of the
4 breeding season ($-16.13 \pm 0.74\text{‰}$ for $\delta^{13}\text{C}$ and $11.88 \pm 0.37\text{‰}$ for $\delta^{15}\text{N}$). However,
5 sample size was extremely reduced in treatments A and M. It is feasible that last
6 newborn isotopic signatures will be maintained after reaching the convergence state
7 (steady-state reached). A trend towards a convergence state would reflect a progressive
8 exhaustion of parents (Svensson 1988, Ahnesjö 1992, Lyons & Dune 2003, Ripley &
9 Foran 2006). This hypothesis is supported by our results, which agree with the
10 progressive drop and final convergence observed in certain essential fatty acids as the
11 breeding season of *H. guttulatus* progresses (M. Planas unpubl. data).

12 Income (or somatic) breeders use energy gained during the reproductive season.
13 Conversely, capital breeders use energy accumulated prior to the reproductive season
14 as reproductive investment with subsequent lower quality batches being produced later
15 in the reproductive season (Stearns 1989, Stephens et al. 2009, McBride et al. 2015).
16 Many multiple-spawning fishes follow mixed capital-income strategies dropping fry
17 quality in successive clutches (McBride et al. 2016, Griffen 2018). A lag in the isotopic
18 response of newborn to breeder's diet and further progressive changes towards an
19 isotopic steady-state would imply that somatic reserves in breeders are the immediate
20 source (Fuiman & Faulk 2013). Conversely, an immediate response would be shown by
21 a small or absent lag. The delay observed in the first release of seahorse batches (6
22 weeks from the onset of the breeding period in diet AM), and the reduced number of
23 batches produced in some treatments did not allow a precise evaluation of the initial
24 impact of breeder's diet. However, the difference in isotopic signals among the first

1 batch would be the evidence of income breeding (because isotopic differences in the
2 prey were reflected in newborns). Furthermore, the decrease in the $\delta^{15}\text{N}$ of AM from
3 the first to the last batch, toward the similarity among all experimental groups, might
4 reflect the partial utilization of capital resources acquired before the experiment. Hence,
5 our results would support a capital-income continuum strategy in *H. guttulatus*.

6 An immediacy of the diet – egg or egg - newborn connection may be part of the
7 motivation for some spawning migrations (Fuiman & Faulk 2013). Seahorse and pipefish
8 may exhibit short-scale migrations, with adults occupying breeding zones during only
9 part of the year (Franzoi et al. 1993, Monteiro et al. 2001). The occupation of breeding
10 areas by adult syngnathids (*H. guttulatus*, *S. acus* and *Entelurus aequoreus*) in Cíes
11 archipelago extends from spring into summer-early autumn (R. Nogueira unpubl. data,
12 A. Jiménez unpubl. data). Such migratory pattern would result advantageous for fishes
13 as the abundance of prey sources increases significantly compared to winter periods (L.
14 Iglesias unpubl. data, S. Campos unpubl. data).

15 **4.3. Application to field collected syngnathids**

16 A large collection of sources (epifauna and nekton) was isotopically analysed from
17 Cíes archipelago but only 5 groups of combined sources were finally selected for
18 Bayesian SIMMs, resulting in output improvements but at a lesser taxonomic resolution.
19 Those combined sources agreed with those reported as main prey items in gut contents
20 of the pipefish *S. acus* from other geographic areas (Bennet 1989, Taskavak et al. 2010,
21 Gurkan & Taskavak 2019). Such reported sources were applied to SIMMs as informative
22 priors to improve models accuracy (Moore & Semmens 2008, Phillips et al. 2014).

23 Values of 0-1‰ for $\delta^{13}\text{C}$ and 3-4‰ for $\delta^{15}\text{N}$ are average TEFs reported in reviews
24 (Minagawa & Wada 1984, Post 2002, Sweeting et al. 2007a, b), being frequently used in

1 field studies. However, generalist TEFs resulted in poor fitting in our mixed polygons,
2 with a large proportion of consumer samples located outside the 95% confidence
3 contour in mixing polygons. The outliers were mostly caused by underestimations of
4 $\delta^{13}\text{C}$ discrimination factors.

5 Isotopic signatures of all analyzed sources from Cíes archipelago markedly
6 overlapped. Taxa similarity for $\delta^{13}\text{C}$ was higher than for $\delta^{15}\text{N}$ value (wider ranges). These
7 findings also applied to a lesser extent to the five combined sources selected for SIMMs,
8 particularly to mysidaceans, gammarids and carideans. The values of $\delta^{15}\text{N}$ in our
9 selected sources were similar to those reported for similar taxa from less oceanic-
10 influenced sites nearby Cíes archipelago but differed for $\delta^{13}\text{C}$ (values not corrected for
11 lipids) (Valladares et al. 2017). In any case and conversely to other studies (e.g. Lerner
12 et al. 2018), experimental conditions (and the resulting TEFs) with diet M seemed to
13 better reflect natural sources in our study site, improving SIMMs accuracy compared to
14 TEFs from reviews. Interestingly, the diet composition inferred by SIMMs for the
15 seahorse *H. guttulatus* in those nearby sites (Valladares et al. 2017) resembled our
16 results for *S. acus*. As deduced from isopolygon drawings, it is likely that SIMM models
17 accuracy be mainly due to an adequate selection of TEFs for $\delta^{13}\text{C}$.

18 SIMMs based on TEFs reviews largely over- and under-estimated the relative
19 contributions of carideans (*Hippolyte varians*) and harpacticoid copepods, respectively.
20 Models C and E provided better source discrimination, increasing notoriously the
21 contribution of amphipoda and copepods. Incorporating informative priors into SIMMs
22 increased output resolution and decreased credible intervals overlapping compared to
23 uninformative priors.

24 **4.4. Contribution of sources to bulk diet**

1 Syngnathids are secondary consumers, with specialized predatory strategies,
2 preferring macrophytes and seagrass communities (Howard & Koehn 1985, Steffe et al.
3 1989, Franzoi et al. 1993, García et al. 2005, Oliveira et al. 2007, Browne et al. 2008,
4 Varvara 2017). They ambush small prey items in complex habitats and show a variety of
5 diets and foraging behaviors across genera and locations (Manning et al. 2019),
6 depending on the species, mouth type, gender, season, habitat and prey availability
7 (Dawson 1986, Franzoi et al. 1993, Roelke & Sogard 1993, Teixeira & Musick 1995,
8 Foster & Vincent 2004, Vizzini & Mazzola 2004, Castro et al. 2008, Kuitert 2009).
9 Generally, several taxa of crustaceans and other invertebrates (including meroplankton)
10 in the water column, vegetation or among detritus on the substrate are preferred
11 (Bennet 1989, Kendrick & Hyndes 2005, Oliveira et al 2007, Kitsos et al. 2006, Taskavak
12 et al, 2010, Valladares et al. 2017, Nenciu et al. 2018, Ape et al. 2019, Gurkan & Taskavak
13 2019). However, amphipoda and copepods are among the mostly preferred prey for
14 many species (Bennet 1989, Kitsos et al. 2008, Taskavak et al. 2010, Gurkan & Taskavak
15 2019, Manning et al. 2019).

16 The analysis of overall results on prey contributions to bulk diet in *S. acus* was
17 complex and challenging due to large confidence intervals for most sources (except for
18 mysidaceans). The global scenery of percent bulk diet contribution became easier to
19 evaluate through pairwise comparisons of sources (Bhattacharyya's coefficients) (Table
20 5). According to SIMMs results including informative priors and experimentally derived
21 TEFs (Models C and E), *S. acus* from Cíes archipelago would prey mainly on amphipods
22 (54 %, model C) or amphipods + copepods (62%, model E) during spring – summer, with
23 lower contributions of caridea, isopoda and Mysidacea to bulk diet. Previously reported
24 data on gut contents in the species reported average relative contributions of 58% for

1 copepods and 33 % for amphipoda, with smaller individuals preferring copepods and
2 larger ones foraging also on decapod eggs and larvae (Bennet 1989, Taskavak et al. 2010,
3 Gurkan & Taskavak 2019, Manning et al. 2019). Accurate comparisons with reported
4 information are problematic since most data refer to gut volume percentages or prey
5 items instead of prey biomass. Another constrain is that gut content does not consider
6 prey assimilation (which is commonly unknown for most species), resulting in
7 dissimilarities with the isotopic approach, particularly for small prey (e.g. copepods)
8 (Vizzini & Mazzola 2004). In addition, prey sources may differ in gut passage time and in
9 digestion/assimilation rates (Corse et al 2015). However, our results from experimental
10 TEFs strongly agree with those from gut content analyses in wild specimens of *S. acus* in
11 other regions (Mediterranean, South Africa) (Table 5).

12 Our isotopic study suggests that *S. acus* is a specialist predator foraging preferentially
13 near the bottom on amphipods and copepods, with lower contributions of caridea,
14 isopoda and mysidaceans to bulk diet. However, seasonal changes could occur on prey
15 preferences depending on the availability and fluctuations in abundance of potential
16 resources (S. Campos unpubl. data). In *S. taenionotus*, mysidaceans are utilized only
17 when the abundances of other preferred prey decrease (Franzoi et al. 1993). Similarly,
18 *H. guttulatus* have a great ability to exploit available resources among different habitats,
19 acting as a specialist predator feeding on highly abundant prey, including nematodes
20 (Ape et al. 2019).

21 Preferences for certain prey sources rely on prey features such as diversity, size,
22 occurrence, shelter-exposure, and accessibility (mobility, escape capabilities,
23 occurrence, and vertical distribution). For example, mysidaceans are typical
24 components of suprabenthic assemblages but their abundance could vary according to

1 the habitat (Barberá et al. 2001). In Cíes archipelago, patches of mysidaceans were
2 observed mainly in the near-bottom environment, above 1 m in the water column, but
3 not strongly associated to shelter microhabitats offered by macroalgae and infauna.
4 However, amphipods or copepods abundances were especially high within or around
5 seaweed structures, being more accessible than mysidaceans to syngnathids and other
6 benthic fishes. Conversely, the pipefish *S. typhle* has a larger mouth than *S. acus*, and
7 also catches prey in the water column, consuming a significant amount of mysidaceans
8 as reflected by SIA (Vizzini & Mazzola 2004). Another important feature to consider in
9 predation is crypsis. Prey accessibility, not predator preference, can determine selection
10 of prey by predatory fishes, even at high field densities of prey (Main 1985). The
11 colouration of the caridean shrimps *Hippolyte varians* can change to effectively match
12 new backgrounds, and to provide effective visual camouflage to predators against their
13 main seaweed substrates (Green et al. 2019). Consequently, carideans might be
14 underrepresented in predator diets (Main 1987). Some fishes do not show preference
15 for mobile over immobile prey (Zhao et al. 2006) but considering that syngnathids
16 ambush their prey and the low relative contribution of isopoda to bulk diet in our study,
17 we hypothesize that mobile prey would be preferred.

18 **4.5. Issues and learnings for future studies**

19 An extensive review of the literature tested the effect of diet isotopic ratio on
20 discrimination factors and showed differences among taxonomic classes for $\delta^{13}\text{C}$, but
21 not for $\delta^{15}\text{N}$ (Caut et al. 2009). The same review pointed out that more than 60% of
22 models used a discrimination factor coming from a different species or tissues, and in
23 more than 70% of models, only one discrimination factor was used. Consumer isotope
24 fractionation is hardly to estimate in the field for some species and mixing model

1 outcomes are often highly dependent on assumptions. Ideally, simulated field trials
2 should provide accurate discrimination factor estimates to assess diet composition in
3 wild fishes. Hence, experimental trials are a convenient (the unique in most cases)
4 approach to provide more realistic discrimination factors for SIMMs.

5 Some of the most problematic challenges with the experimental approach are both
6 the adequate selection of prey sources, and the high isotopic variation between
7 experimental dietary sources, especially for $\delta^{13}\text{C}$. The feeding of marine fishes in
8 mesocosm usually rely on cultivated zooplankton (e.g. *Artemia*), which is grown on
9 microalgae and/or artificial diets. However, isotopic signatures vary largely depending
10 on several factors such as developmental stage or nutritional sources (Gamboa-Delgado
11 et al. 2008, Caut et al. 2009). For example, isotopic signatures largely differ across groups
12 and species of marine phytoplankton depending on growth phase, nutrient conditions
13 and salinity (Brutemark et al. 2009). Also, isotopic signatures in *Artemia* cysts, nauplii
14 and adults largely differ among geographical origins (Spero et al. 1992, Jomori et al.
15 2008) and nutrients availability (Aberle & Malzahn (2007). Consequently, isotopic
16 signatures of experimental prey might differ significantly from those of natural sources
17 in marine ecosystems. Ideally, isotopic signatures of experimental and natural sources
18 should be as similar as possible, which is difficult to achieve. In our study, experimental
19 diets and potential sources in nature were somehow different, particularly for $\delta^{13}\text{C}$ in
20 diets including *Artemia* (Figure S2). In spite of this, the results from isopolygons showed
21 that co-feeding on mysidaceans and lower proportions of *Artemia* provided more
22 accurate results than fixed discrimination factors from reviews, as also reported by other
23 studies (Caut et al. 2008a). Diet as a source of variation still needs assessment, and

1 understanding why discrimination factors vary as a function of the isotopic signal of the
2 diet is of pivotal importance (Caut et al. 2009).

3 Understanding of isotopic patterns in newborn fishes thorough the whole breeding
4 season is necessary to provide insight into breeders – newborn isotopic relationships
5 (i.e. isotopic inheritance). This is particularly interesting in species following a capital (or
6 capital-income) breeding strategy, in which isotopic signatures of eggs / newborn
7 display considerable changes along the breeding season. For that, the timing of sampling
8 should be precisely established in advance according to experimental aims.

9 Besides the need of further refinements in the assumptions and procedures for
10 discrimination factors estimation, other important issues to consider are the isotopic
11 similarity of potential sources and the resulting overlap (confidence) of contribution
12 estimates for dietary sources. Most studies on diet reconstruction provide relative
13 contribution estimates of dietary sources but a further assessment of overlapping
14 significance (Bhattacharyya's coefficients) for estimates are rarely considered.
15 Consequently, the accuracy of estimates might result considerably affected. In our
16 study, we failed to discriminate between some sources in the diet reconstruction. To
17 overcome such limitations, the use of a third tracer (e.g. $\delta^{34}\text{S}$) is strongly recommended
18 (Connolly et al. 2004, Soto et al. 2013).

19 For the first time, we provided specific TEFs for syngnathids, ranging from 2.5 to 4.1‰
20 for $\delta^{13}\text{C}$ and 3.9 to 4.2‰ for $\delta^{15}\text{N}$. We also demonstrated that our estimations of
21 experimentally derived TEFs applied to SIMMs were more accurate for syngnathids than
22 those obtained using fixed TEFs. On the other hand, even though *S. acus* seems to be
23 an income breeder, the present study did not allow us to rule out or confirm the
24 existence of an income-capital continuum pattern in the species. Hence, further isotopic

1 studies performed under controlled experimental conditions are needed to increase our
2 knowledge on reproductive tactics and potential factors regulating isotopic inheritance
3 and TEFs variability in syngnathids. In that regard, the effect of temperature level and
4 the use of other dietary sources would be particularly interesting.

5 **Acknowledgments**

6 This study was financially supported by the Spanish Government with projects
7 Hippoeco (Ref. CGL2015-68110-R, Ministerio de Ciencia, Innovación y Universidades and
8 Fondos FEDER) and Hippoparques (Ref. 1541S/2015, Organismo Autónomo de Parques
9 Naturales de España, Ministerio para la Transición Ecológica). Jorge Hernández-Urcera
10 was supported by a Juan de la Cierva's post-doc research grant (FJCI-2016-30990) from
11 Ministerio de Ciencia, Innovación y Universidades. We are grateful to Raquel Nogueira,
12 Laura Iglesias, Javier Cremades, Iñaki Ferreiro, David Costas, Rubén Chamorro, Cristina
13 Piñeiro, Ignacio Bárbara, and the staff and keepers of Atlantic Islands National Park for
14 technical support and invaluable assistance in the project. We also thank the Regional
15 Government Xunta de Galicia for providing capture permission of syngnathids in the
16 National Park.

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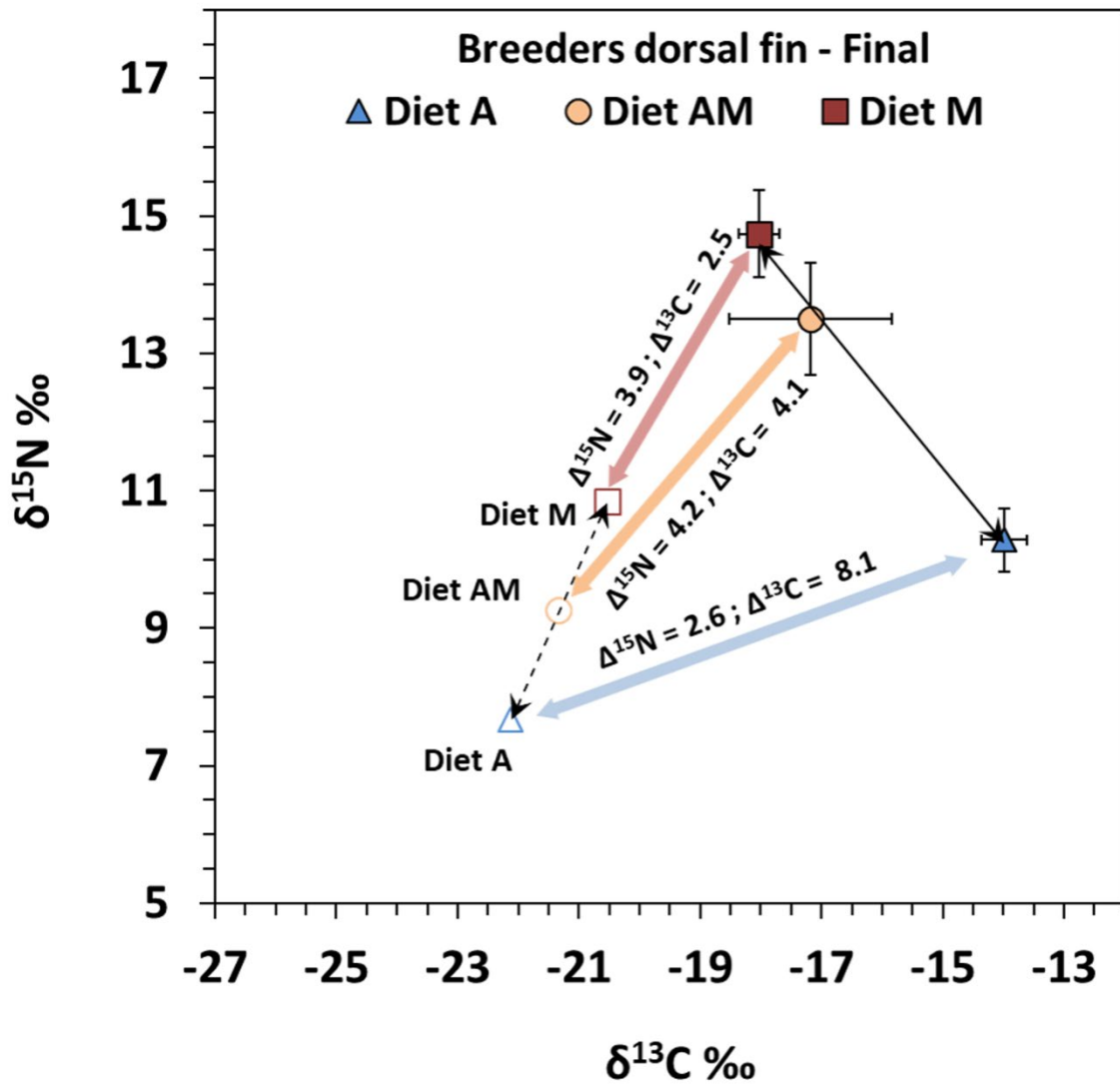
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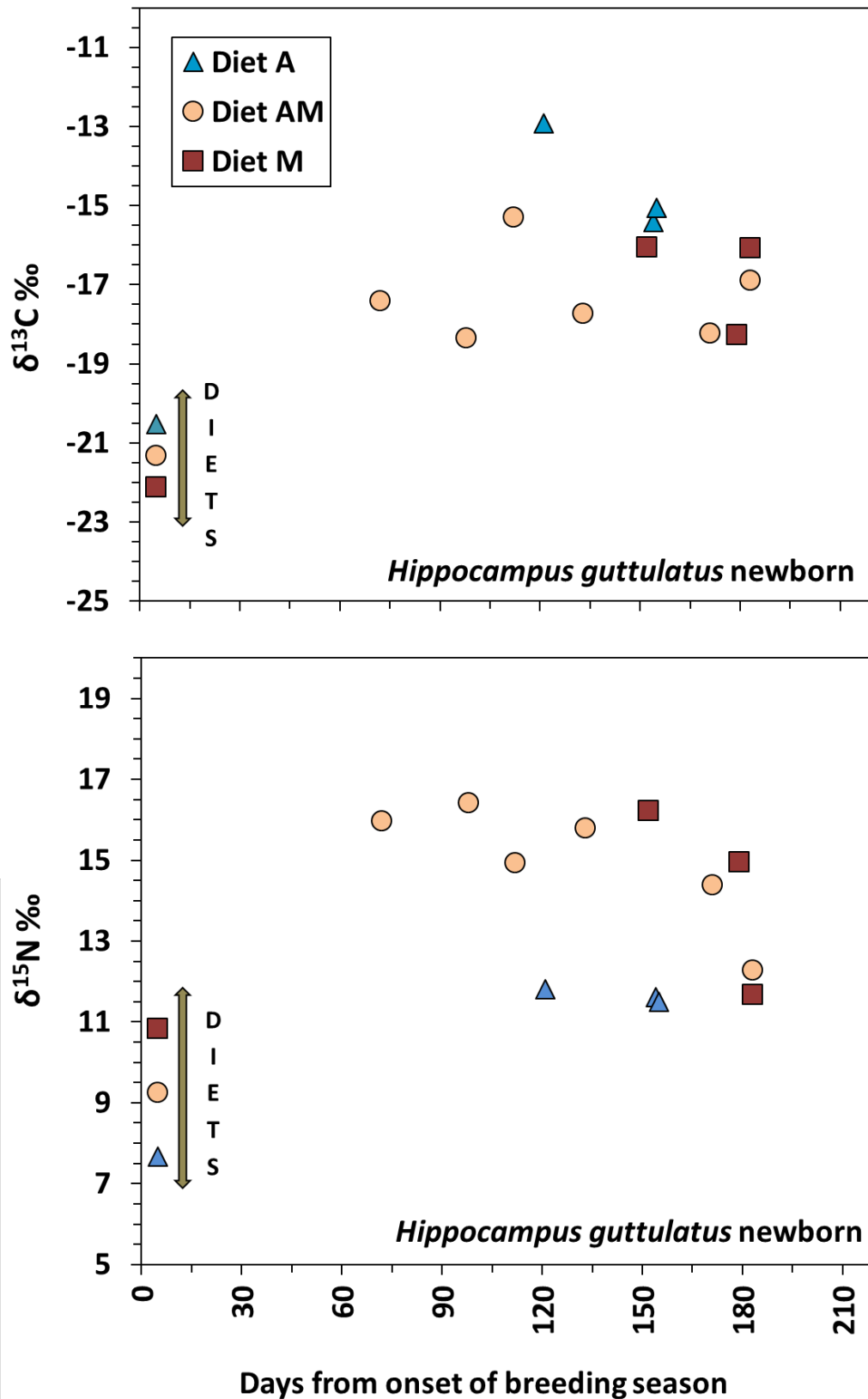
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3 Figure 1: Average isotopic signatures for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ (‰) in diets A, AM and M (open
4 symbols), and dorsal fin of *H. guttulatus* breeders at the end of the experimental period.
5 Discrimination factors are provided. See Materials and Methods section for further
6 details on diets.

7



1
 2 Figure 2: Change with time of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ (‰) signatures in newborn *H. guttulatus*
 3 along the breeding season. Breeders fed on diets A, AM and M (see Materials & Methods
 4 for further details). Isotopic signatures of experimental diets are also shown.

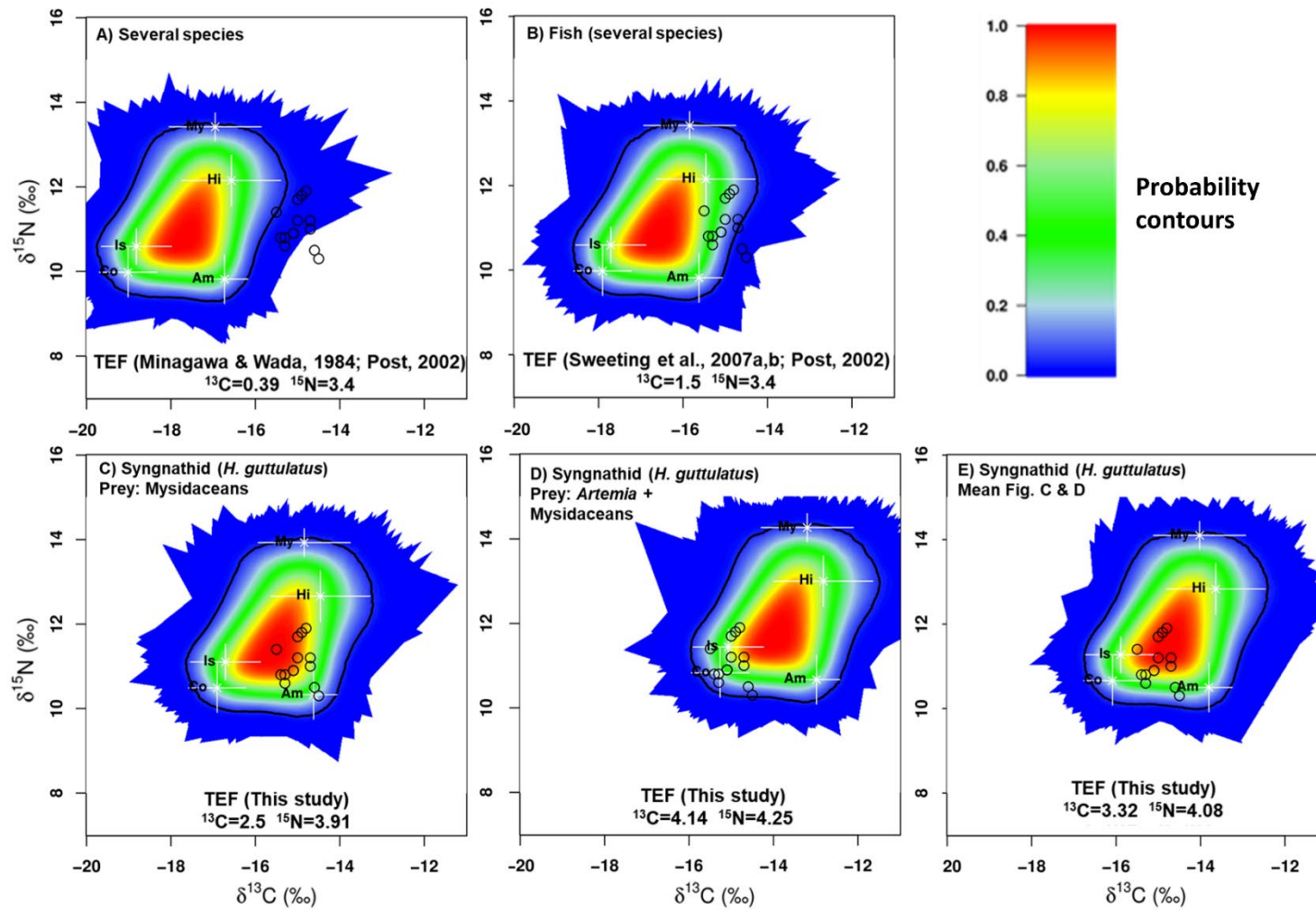


Figure 3. SIMM polygons for *Syngnathus acus* relative to five potential prey sources (white crosses) and average $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures of sources adjusted for TEF values (open circles). Coloured region represents the 95% confidence interval. Probability contours are at the 5% level. Sources: Am = Amphipoda, Co = Copepoda, Hi = Caridea (*Hippolyte varians*), Is = Isopoda and My = Mysidacea. See Table 1 for further details.

Table 1: Relative abundances (Ab – percentage of total epifauna analysed) and weighted mean values (mean ± sd) for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ (‰) in five potential sources for *S. acus* in Cíes archipelago. The weighted mean for each source was calculated considering relative abundances in spring, summer and autumn 2017-2018 (unpublished data). N.D.: Not determined (mesh bag collected).

Sources	Ab	n	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$	Species included
Amphipoda	17	308	-17.12 ± 0.25^a	6.42 ± 0.28^d	<i>Amphilocheus manudens</i> , <i>Apherusa</i> spp., <i>Caprella</i> <i>acanthifera</i> , <i>Caprella</i> <i>linearis</i> , <i>Corophium</i> spp., <i>other gammaridae</i>
Copepoda	63	18	-19.41 ± 0.38^b	6.58 ± 0.28^{cd}	<i>Harpacticoida</i>
Caridea	0.1	17	-16.96 ± 0.87^a	8.75 ± 0.30^b	<i>Hippolyte varians</i>
Isopoda	0.5	32	-19.21 ± 0.53^b	7.19 ± 0.12^c	<i>Cymodoce truncata</i> , <i>Dynamene bidentata</i>
Mysidacea	N.D.	18	-17.34 ± 0.79^a	10.02 ± 0.03^a	<i>Siriella armata</i>

Table 2. Trophic enrichment factors (TEFs) selected for SIMMs (Bayesian Stable Isotope Mixing Models). See Materials and Methods section for further details on diets M and AM.

Source	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$	Consumer species
Experimentally Derived (This study)			
Diet M (Mysidacea)	2.50	3.90	<i>Hippocampus guttulatus</i>
Diet AM (Mysidacea + <i>Artemia</i>)	4.14	4.25	<i>Hippocampus guttulatus</i>
Average Diets A and AM	3.32	4.08	<i>Hippocampus guttulatus</i>
Minagawa & Wada (1984)			
Post (2002)	0.39	3.40	Mixed groups
Post (2002)			
Sweeting et al. (2007a, b)	1.50	3.40	Fishes (<i>Dicentrarchus labrax</i>)

Table 3. Isotopic values ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in ‰) (mean \pm sd) in experimental diets, breeders (dorsal fin from males and females) and newborn juveniles of *Hippocampus guttulatus* fed on diets A (*Artemia*), AM (*Artemia* + Mysidacea) and M (Mysidacea) during the breeding season. See Materials and Methods section for further details on diets. TEF: trophic enrichment factor (breeders). Sample size (n) is given in brackets. *: Last batch for each treatment (sd not available).

	Treatment	Diet	Breeder's dorsal fin			Newborn juveniles		
			Initial	Final	TEF	Average	Final*	
$\delta^{13}\text{C}$	A	-22.12 \pm 1.01 (3)	-17.37 \pm 1.47 (7)	-13.99 \pm 1.05 (7)	8.13	-13.86 \pm 1.64 (4)	-15.42 \pm - (1)	
	AM	-21.32 \pm 0.68 (3)	-16.98 \pm 2.00 (11)	-17.18 \pm 1.64 (11)	4.14	-17.31 \pm 1.13 (6)	-16.89 \pm - (1)	
	M	-20.53 \pm 1.36 (3)	-16.04 \pm 0.87 (10)	-18.03 \pm 1.38 (10)	2.50	-16.80 \pm 1.27 (3)	-16.07 \pm - (1)	
$\delta^{15}\text{N}$	A	7.67 \pm 0.02 (3)	11.94 \pm 0.81 (7)	10.28 \pm 0.91 (7)	2.62	11.21 \pm 0.88 (4)	11.61 \pm - (1)	
	AM	9.25 \pm 0.04 (3)	12.79 \pm 1.11 (11)	13.50 \pm 0.98 (11)	4.25	14.96 \pm 1.50 (6)	12.29 \pm - (1)	
	M	10.83 \pm 0.09 (3)	12.19 \pm 0.72 (10)	14.74 \pm 0.86 (10)	3.91	14.28 \pm 2.35 (3)	11.70 \pm - (1)	

Table 4: Percent diet composition for prey sources (with and without priors) as estimated by Bayesian Stable Isotope Mixing Model (SIMM) based on dorsal fin tissue of pipefish *Syngnathus acus* individuals sampled in Cíes archipelago in spring-summer 2016. Models (TEFs for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$): A - Averaged ecosystem (Minagawa & Wada, 1984; Post, 2002); B - Semi-experimentally derived (Post, 2002; Sweeting et al., 2007a, b); Experimentally derived from Syngnathids fed on diets M (C), AM (D), and averaged values of C and D (E) (present study). Different superscript letters indicate not significant overlapping (percent diet contributions differ significantly when Bhattacharyya's coefficient < 0.6; Table S2).

Sources	Models (Trophic Enrichment Factors – TEFs)									
	A) 0.39 $\delta^{13}\text{C}$ - 3.40 $\delta^{15}\text{N}$		B) 1.5 $\delta^{13}\text{C}$ - 3.40 $\delta^{15}\text{N}$		C) 2.50 $\delta^{13}\text{C}$ - 3.91 $\delta^{15}\text{N}$		D) 4.14 $\delta^{13}\text{C}$ - 4.25 $\delta^{15}\text{N}$		E) 3.32 $\delta^{13}\text{C}$ - 4.08 $\delta^{15}\text{N}$	
	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd
Without priors										
Amphipoda	21.7	9.4 ^b	36.1	10.3 ^b	53.9	9.6 ^a	9.3	6.2 ^b	30.6	8.8 ^a
Copepoda	3.9	3.7 ^c	5.2	4.8 ^c	11.0	7.5 ^b	56.3	16.3 ^a	31.4	14.0 ^a
Caridea	68.5	8.0 ^a	47.2	10.4 ^a	14.8	8.8 ^b	4.6	3.9 ^c	8.4	6.1 ^{bc}
Isopoda	3.4	3.3 ^c	5.0	4.7 ^c	11.0	7.8 ^b	26.1	16.6 ^{ab}	23.5	14.6 ^{ab}
Mysidacea	2.5	2.3 ^c	6.5	6.1 ^c	9.3	5.9 ^b	3.7	3.2 ^c	6.1	4.3 ^c
With priors										
Amphipoda	21.0	8.7 ^b	30.3	10.5 ^b	50.0	12.1 ^a	14.7	6.8 ^b	35.5	9.6 ^a
Copepoda	9.7	5.1 ^b	13.9	6.8 ^b	22.4	8.1 ^b	68.1	15.6 ^a	44.4	12.9 ^a
Caridea	68.1	6.9 ^a	53.4	9.2 ^a	20.4	12.3 ^{bc}	0.9	2.4 ^c	5.3	7.5 ^b
Isopoda	1.1	1.8 ^c	1.7	2.7 ^c	3.2	4.8 ^d	15.9	15.6 ^b	12.2	13.2 ^b
Mysidacea	0.1	0.5 ^c	0.7	4.2 ^c	4.0	7.4 ^{cd}	0.4	1.4 ^c	2.6	4.7 ^b

Table 5: Simplified summary of table 4 showing percent diet contribution by SIMMs outputs (with or without priors for Models A to E) in the pipefish *Syngnathus acus*. Different colour densities imply significant differences (increasing order). Models (TEFs): A - Averaged ecosystem (Minagawa & Wada 1984, Post 2002); B - Semi-experimentally derived (Post 2002, Sweeting et al. 2007a, b); Experimentally derived from Syngnathids fed on diets M (C), AM (D), and averaged values of C and D (E) (present study). Previously published dietary sources inferred from gut contents analysis are also provided.

TEF models without priors	Copepoda	Amphipoda	Isopoda	Caridea	Mysidacea
A	4	22	3	68	3
B	5	36	5	47	7
C	11	54	35		
D	56	35		5	
E	62		24	14	

TEF models with priors	Copepoda	Amphipoda	Isopoda	Caridea	Mysidacea
A	31		1	68	< 1
B	24		2	53	1
C	22	50	3	20	4
D	68	31		1	
E	80		20		

Sources (Gut content)	Copepoda	Amphipoda	Isopoda	Caridea	Mysidacea
Bennet 1989	40	40	20	< 1	0
Taskavak et al. 2010	61	32	1	5	0
Gurkan & Taskavak 2019	73	27	0	0	2