*Article*

# Ecological traits and trophic plasticity in the greater pipefish *Syngnathus acus* in NW Iberian Peninsula

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**Simple Summary:** The population of the pipefish *Syngnathus acus* inhabiting Cíes Archipelago (NW Spain) was monitored in 2017-2018 for spatial and temporal changes in abundances, reproduction traits, trophic niche occupancy and dietary regimes across reproduction states through an isotopic ($\delta^{13}C$ and $\delta^{15}N$) approach. The population consisted almost exclusively of large adults, whose abundances decreased significantly from mid-autumn, after the breeding season. *S. acus* is a secondary consumer preferring amphipods but mature specimens were less selective than immature fish. The present study highlights the outstanding size of the fish and the exceptional occurrence of breeders on the studied area.

**Abstract:** The great pipefish *Syngnathus acus* is one of the most representative European syngnathid, being highly associated with seagrass and macroalgal beds. Surprisingly, the ecology of this ovoviviparous marine fish has received scanty attention. The population inhabiting three sites on Cíes Archipelago (Atlantic Islands National Park, NW Spain) was monitored in 2017-2018 for spatial and temporal changes in abundances, reproduction traits, trophic niche occupancy and dietary regimes across reproduction states through an isotopic ($\delta^{13}C$ and $\delta^{15}N$) approach. Abundances were highly variable across seasons and sites, decreasing significantly from mid-autumn. The population consisted almost exclusively of large adults that migrate by the end of the breeding season, which extended from mid-spring to summer. Operational sex ratios suggest that the species is sex-role reversed. *S. acus* is a secondary consumer (Trophic position= 3.36 ± 0.05) preferring amphipods but displaying annual and seasonal dietary plasticity. Mature fish were less selective than immatures (especially females) with a higher preference for amphipods (36-68%) in the former. The second most preferred prey were carideans, copepods or isopods, depending on the year and reproduction state. Overall, the wider trophic niches in females and immature specimens compared to males and mature fish would indicate a higher variability in both the use of prey resources and/or their origin. The present study highlights the trophic plasticity and unique features of *S. acus* population in Cíes Archipelago, especially regarding the outstanding size of the fish and the exceptional occurrence of breeders.

**Keywords:** pipefish; *Syngnathus acus*; biology; ecology; reproduction; trophic plasticity; stable isotopes; diet

## 1. Introduction

The Family Syngnathidae includes a large and diverse group of vulnerable and cryptic fishes characterized by singular morphological and biological features, a tiny mouth at the end of a tubular snout and male parental care. Pipefishes are mostly distributed in shallow waters on coasts of the temperate and tropical regions. In *Syngnathus* pipefishes, the eggs and developing embryos are enclosed within specialized brooding structures (i.e., marsupia with protective pouch flaps) located on the ventral side of the trunk or tail. The greater pipefish *Syngnathus acus* Linnaeus, 1758 is a demersal syngnathid inhabiting brackish marine areas on the Mediterranean, Aegean and Black seas and Eastern Atlantic [1]. *S. acus* is an ovoviviparous tail-brooding species that generally reproduce three times each year [2]. It is currently listed as Least Concern by IUCN Red List [3].

Despite its common occurrence in brackish seaweeds and seagrass beds, the biology and ecology of *S. acus* have been poorly investigated, and data availability is limited to a few studies [4-8, among others]. The reproduction of the species [2] and the feeding habits and prey preference [9-11] were investigated in the Aegean Sea, Jennings and van der Molen [12] estimated the trophic position in a few specimens from the Celtic Sea and the English Channel, whereas Silva et al. [13] focused on the development and early life history in ex-situ experiments.

The distribution, the habitat preference and some trophic features in syngnathids inhabiting Cíes Archipelago, on the Atlantic Islands National Park (NW Spain), have been recently assessed, focusing on three sympatric species: the long-snouted seahorse *Hippocampus guttulatus*, the snake pipefish *Entelurus aequoreus* and the great pipefish *Syngnathus acus* [14,15]. Among them, the latter was the most representative species, with a wider distribution, preferentially on macroalgal beds [16].

Available data on syngnathid populations in Cíes indicate high seasonal and spatial variability in distribution and abundances [14,15]. Such variability seemed to be governed by seasonal changes in temperature, extension and integrity of macroalgal beds, the structure of epifauna assemblages, and potential migratory events. These factors might also affect resources availability, resulting in potential dietary shifts in syngnathids, which commonly feed on small crustaceans [17] but might show some plasticity in prey selection depending on the species [18,19].

Some of the most interesting topics in marine ecology are the assessment of the trophic web and the inference of feeding regimes of targeted species. In Cíes Archipelago, the former was investigated by an isotopic approach, concluding that the three syngnathids occurring in the area were rather similar in trophic features [14]. Isotopic profiles in animals vary depending on the trophic level occupied and the dietary regime [20-26]. Stable isotope analysis (SIA) is a powerful tool to infer temporal and spatial changes in feeding regimes and migrations [27,28] or to assess the parental contribution of dietary nutrients into eggs/offspring [29-33].

To my knowledge, studies on the global assessment of *S. acus* populations are almost lacking. In the present study, the ecological traits and the variability in the feeding regime of *S. acus* inhabiting Cíes Archipelago were investigated in detail with the aim of (a) improving the knowledge on the biology, population structure and breeding features, (b) inferring temporal and spatial changes in the feeding regime using stable isotope profiles, and (c) assessing the relationship between diet and maturity state in males and females.

## 2. Materials and Methods

### *2.1. Study sites*

The study was carried out in Cíes Archipelago (42°13’N, 8°54’W) as part of the Atlantic Islands Marine National Park (NW Iberian Peninsula) (Figure 1). The Archipelago was declared Natural Park, Special Protection Area (SPA), Site of Community Importance (SCI), OSPAR area, and UNESCO World Heritage candidate [34]. Further information on Cíes Archipelago characteristics and biotopes are provided in Fernández et al. [35].

Based on previous knowledge (i.e., seaweed cover, substrate characteristics, exposure level to open water) [14-16], three subtidal sites (A, B and C) (Figure 1) were selected along the east coast of Cíes Archipelago, located on the outer area of the Ría de Vigo. The sites were positioned near the coastline (2 - 15 m depth) on rocky bottoms frequently interrupted by sandy patches, and visited in spring, summer and autumn in 2017 and 2018 (two visual censuses per site and season). Two pairs of divers conducted 36 diurnal standard underwater visual censuses (UVC) (50 min per survey; about 144 diving hours). Total areas explored per survey in sites A, B and C were 2,450, 5,625 and 2,153 m$^2$, respectively. Sighted specimens of the greater pipefish *Syngnathus acus* Linnaeus, 1758 (Family Syngnathidae) (Figure 2) were recorded and captured by the divers searching adjacent (belt transects) and separated by the maximum distance allowed for horizontal visibility (usually 2.5 m). The depth and location of fish capture were annotated.

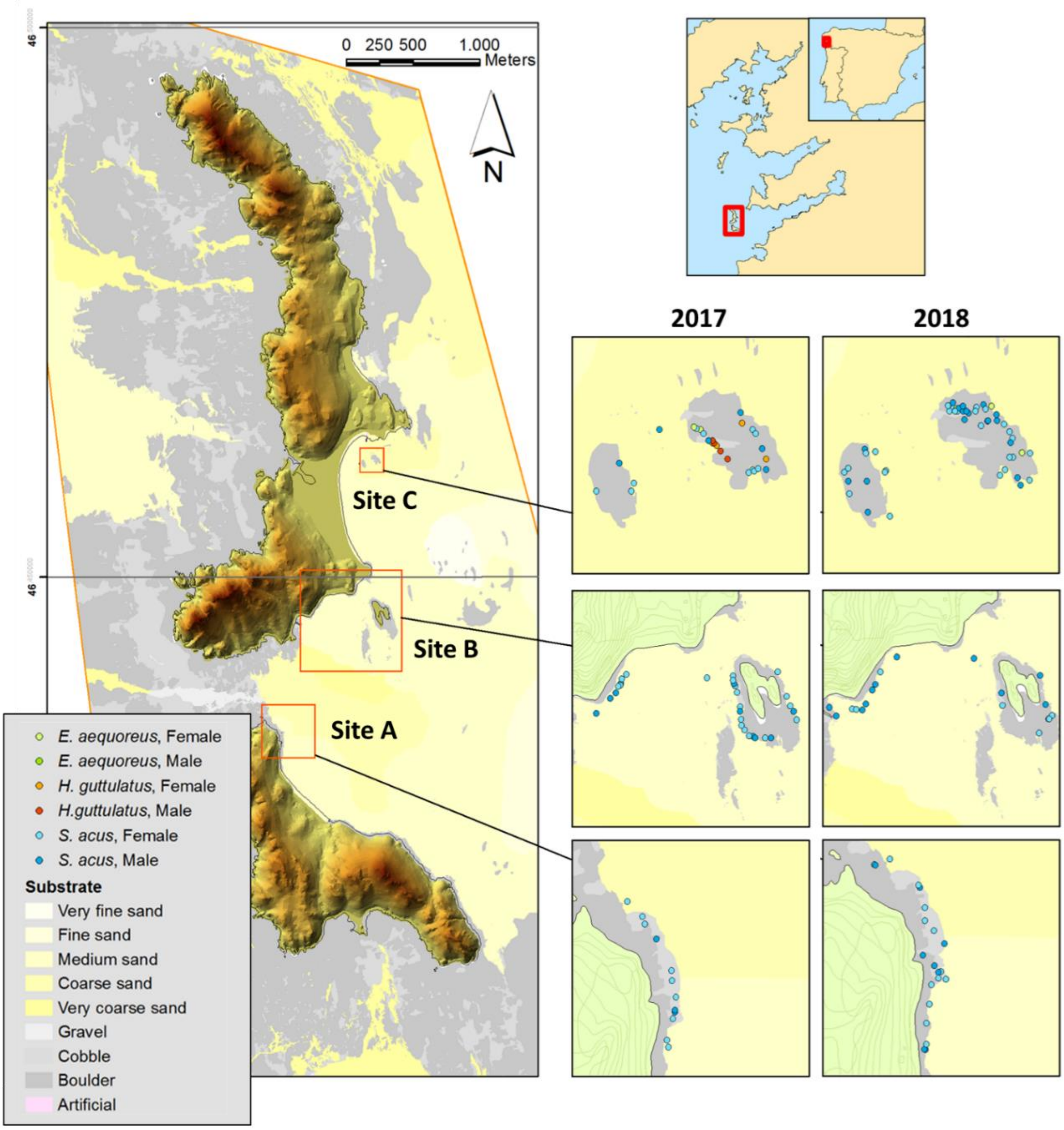

**Figure 1.** Sites (A, B and C) surveyed for *Syngnathus acus* monitoring in Cíes Archipelago (Galicia, NW Iberian Peninsula) in spring, summer and autumn (2017-2018). Substrate characteristics and syngnathids captured (*S. acus*, *Entelurus aequoreus* and *Hippocampus guttulatus)* are shown.

### *2.2. Fish collection and sampling*

*Syngnathus acus* specimens (Figure 2A) were hand-caught collected, introduced in numbered plastic bags and transferred to a support boat. Subsequently, the fish were anaesthetized with a solution of Ethyl 3-aminobenzoate methane sulfonate (MS-222; 0.1 g L-1; Sigma-Aldrich Co., USA), morphologically identified, weighted (W, g), sized for standard length (SL, cm) and marked subcutaneously using visible implant fluorescent elastomers (VIFE; Northwest Marine Technology Inc., USA) (Figure 2B). Dorsal fin samples were taken by fin-clipping [36,37], transferred to screw-capped tubes containing 95% ethanol and conserved at 4 °C for further genetic and stable isotope analysis (SIA). The presence of previous marks (recapture events), sex, reproductive condition, meristics (fin rays, body rings) and body colouration was also annotated whenever possible. All fishes were released at the capture site within 2-3 hours after sampling.

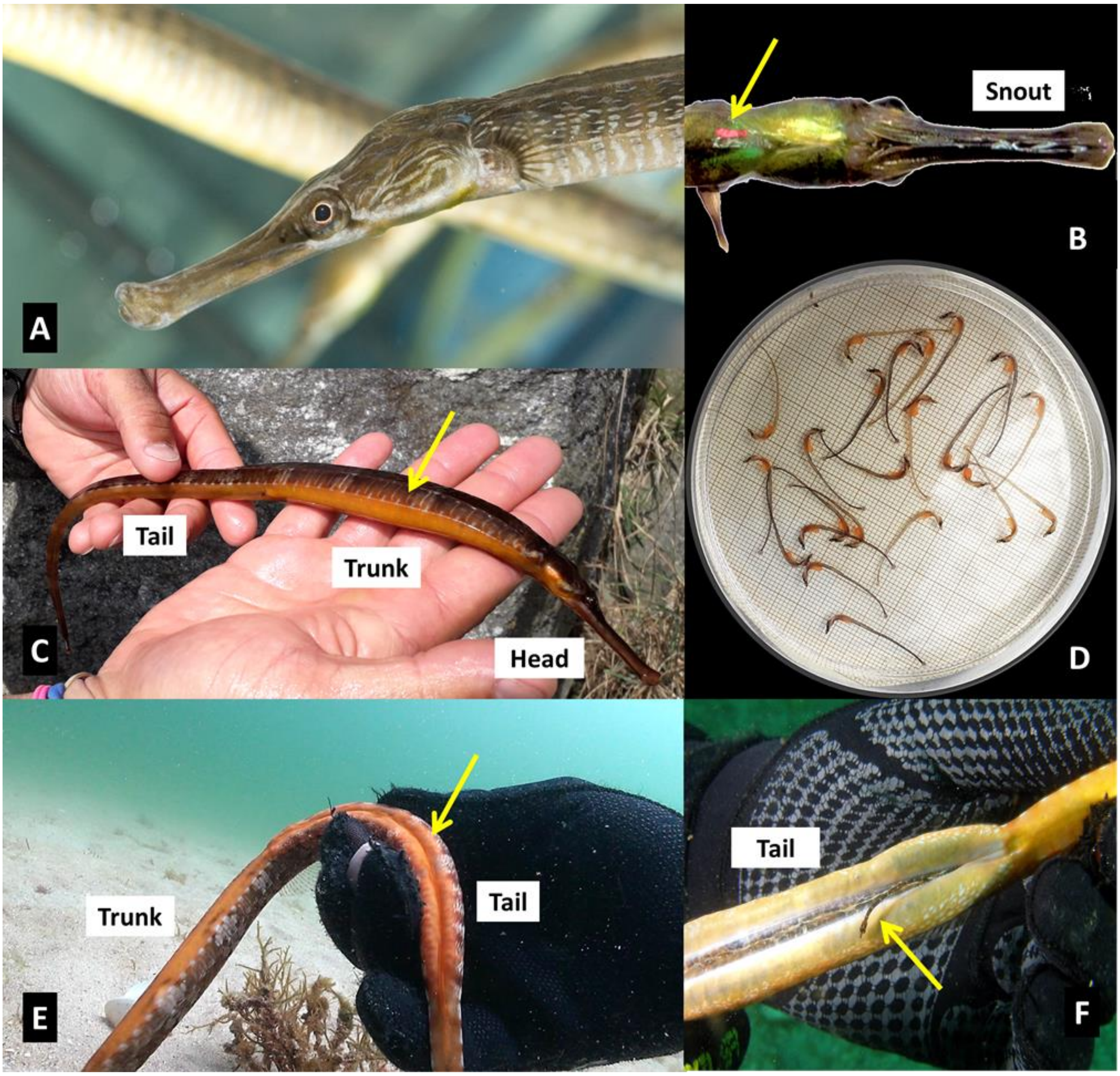

**Figure 2.** *Syngnathus acus.* A – Head morphology; B – Specimen marked with fluorescent VIFE; C – Ovigerous female showing a swollen trunk; D – Near-term embryos with small yolk-sacs; E – Pregnant male showing the enclosed breeding pouch below the tail; F - A newborn emerging from the brooding structure.

The morphological identifications were confirmed genetically [38] using the marker cytochrome b (Cytb;1149 base pairs) [39,40] in DNA extracted from dorsal fin samples.

The operational sex ratio (OSR) at a given time was calculated as the number of males available for mating as a proportion of all adults available for mating [41]. The reproductive condition was recorded considering trunk shape in females (i.e., full gonads with hydrated eggs) (Figure 2C) and pregnancy in males (i.e., fully enclosed brood pouches carrying fertilized eggs/embryos) (Figure 2D, E, F).

To minimize hampering effects in fishes, the morphological analysis and the sampling of developing eggs/larvae in brood pouches was limited to a few males. Sampling for size measurement and stable isotopes included fertilized eggs at stage B (cleavage stage; n=1 batch), embryos at stages H (eyes pigmented; n=9), I (larva/prerelease stage; reduced yolk-sac; n=5), and newly released juveniles (stage J; yolk-sacs almost fully resorbed; snout formation completed; n=4) [42]. Stage H would correspond to larvae newly emerged from the egg, but retained within the brood pouch, with jaw formation started and eyes partially pigmented [43].

The weight-length relationship in adults was investigated using the following equation:

$$W = a\ SL^{b}$$

where W is body wet weight, SL is standard length, a is an empirical coefficient, and b is the allometric exponent. For standard length (SL) measurement, the fishes were measured directly on a plate including a measurement scale.

Daily weight-specific growth rates (G; % day$^{-1)}$ were estimated from weight data in re-sighted individuals:

$$G = 100\ (e^{g} - 1)$$

where the instantaneous growth coefficient g was

$$g = (\ln W_2 - \mathrm{Ln}\ W_1) / (t_2 - t_1)$$

where $W_2$ and $W_1$ are fish wet weights (g) on days $t_2$ (recapture) and $t_1$ (first capture), respectively.

*2.3. Stable isotopes analysis (SIA)*

For $\delta^{13}C$, $\delta^{15}N$, total C and total N analyses, the samples were rinsed with distilled water, transferred to tin capsules, dried in an oven at 60 °C for 24 h and weighted (± 1 µg). The samples were analysed at SAI (University of A Coruña) by continuous-flow isotope ratio mass spectrometry using a FlashEA1112 elemental analyser (Thermo Finnigan, Italy) coupled to a Delta Plus mass spectrometer (FinniganMat, Germany) through a Conflo II interface. Isotopic values are expressed as permil (‰) in conventional delta relative to VPDB (Vienna Pee Dee Belemnite) and Atmospheric Air. As part of an analytical batch run, a set of international reference materials for $\delta^{15}N$ values (IAEA-N-1, IAEA-N-2, USGS25) and $\delta^{13}C$ values (NBS 22, IAEA-CH-6, USGS24) were analyzed. The range of C:N ratios in fin tissues (2.8 – 3.5) were within the range in reference materials (0.4 – 6.9) used. The precision (standard deviation) for the analysis of $\delta^{13}C$ and $\delta^{15}N$ of the laboratory standard (acetanilide) was ± 0.15‰ (1-sigma, n=10). Standards were run every 10 biological samples. The isotopic analysis procedure fulfils the requirements of the ISO 9001 standard. The laboratory is submitted to annual intercalibration exercises (e.g. Forensic isotope ratio mass spectrometry scheme – FIRMS, LGC Standards, UK).

Due to the low lipid content in fin samples conserved in ethanol (<5% lipids, C:N <3.56) [44,45], the normalization for lipid correction in fin samples was not necessary [37,46]. However, C:N values in epifauna indicated that lipid content was higher than 5%, and specific conversion factors constructed for lipid normalization were applied [14]. Besides, some epifaunal groups were submitted to acidification before SIA [14] by adding dilute (10%) HCl drop-by-drop, until no more release of $CO_2$ was observed [47,48].

*2.4. Data analysis*

Data and statistical analyses were conducted in R v.3.6.1 [49]. All means are reported with standard deviations. A P-value threshold of 0.05 was considered significant in all statistical analyses.

Data comparisons across groups were performed by non-parametrical tests (Kruskal-Wallis) [50]. Significant differences between groups were pairwise compared by Wilcoxon test (p. adj = Bonferroni) (Pgirmess v1.6.9 package) [51]. The analyses included comparisons for abundances, standard length, wet weight, $\delta^{13}C$ and $\delta^{15}N$ across seasons (spring, summer and autumn) and reproductive stages (ovigerous females, pregnant males, nonovigerous females and non-pregnant males).

Two-dimensional non-metric multidimensional scaling (NMDS; Euclidean distances) plots on the variation in *S. acus* abundances were constructed considering years of survey (2017 and 2018) and seasons (Sp – spring; Su – summer; Au – autumn). NMDS is based on a Bray-Curtis (BC) dissimilarity matrix [52] and it was performed with package vegan v.2.5-7 [53].

Principal Component Analyses (PCA) including fish wet weight and isotopic data as variables were performed for year, sex and reproductive states using factoMineR v2.3 [54], factoextra v1.0.7 [55] and corrplot v0.8.4 [56] packages. The data values were standardized (mean = 0; sd = 1) for clustering and PCA.

Fish trophic position (TP) was estimated from $\delta^{15}N$ values using tRophicPosition v. 0.7.7 [57], an R package incorporating a Bayesian model for the calculation of consumer TP at the population level. The bivalve *Musculus costulatus* was sampled simultaneously to fishes and the average seasonal levels used as trophic baseline (TP=2) [14,58]. Isotopic values for *M. costulatus* were -17.71‰ ± 0.07 for $\delta^{13}C$ and 5.32 ± 0.26‰ for $\delta^{15}N$. An experimentally derived TDF value (3.9 for $\delta^{15}N$) for syngnathids was applied [58]. Comparisons between groups were assessed through t-test.

Niche regions and pairwise niche overlap in fishes were assessed using $\delta^{13}C$ and $\delta^{15}N$ bi-plot as multidimensional niche indicator data. The niche region was defined as the joint probability density function of the multidimensional niche indicators at a probability alpha of 95%. Uncertainty was accounted for in a Bayesian framework. The analysis provides directional estimates of niche overlap, accounts for species-specific distributions in multivariate niche space, and produces unique and consistent bivariate projections of the multivariate niche region [59]. The packages SIBER v.2.1.4 [60] and NicheRover v.1.1.0 [61] were used to assess differences in the trophic niche features. The total convex hull areas (TA) and core trophic niche breadths were estimated using SIBER (Stable Isotope Bayesian Ellipses) while correcting for variable sample sizes (SEAc). Total trophic overlap values for 95% TA were estimated using nicheROVER, a method that is insensitive to sample size and incorporates a statistical uncertainty using Bayesian methods [59].

From the isotopic profiles in selected potential prey (amphipods, harpacticoid copepods, carideans, isopods and mysidaceans) (see [58] for isotopic data) and in the consumer *S. acus*, the dietary regimes across years and reproductive stages were estimated using Bayesian Mixing Models (SIMM) [62] using the MixSIAR package v.3.1.12 [63]. The procedure is fully described in [58]. Shortly, SIMM polygons were constructed with isotopic profiles adjusted for TDFs to determine the proportion of consumers included inside the mixing polygon bound by all potential sources [60,64,65]. TDF values applied were experimentally derived for these fishes (2.5 for $\delta^{13}C$; 3.9 for $\delta^{15}N$) [58]. Two individuals with a low probability (<5%) of being positioned inside the mixing polygon were not included in the subsequent Bayesian models [65]. The models were run with Markov chain Monte Carlo (MCMC) parameters of three chains of 1,000,000 iterations and a burn-in phase of 500,000 (very long run). The model included individuals as a random effect and one error term (process error). Convergence and diagnostic statistics were evaluated using both Gelman–Rubin (variables < 1.05) and Geweke (number of variables outside ± 1.96 in each chain) tests. Bayesian model outputs are reported as mean ± sd.

The graphics were constructed using Excel 2016, ggplot2 v3.3.0 [66] and lattice v0.20-41 [67] packages.

### *2.5. Ethics*

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

## 3. Results

### *3.1. Abundances and population characteristics*

A total of 153 *Syngnathus acus* specimens were captured (Table 1, Figure 3) (70 in 2017 and 83 in 2018). The most common meristic characteristics of pipefishes were: 62 body rings (range: 61-64), 20 trunk rings (19-20), 42 tail rings (41-44), 12 pectoral-fin rays (9-12), 38 dorsal-fin rays (37-41), 3 anal fin rays and 10 caudal-fin rays.

**Table 1.** Captures and operational sex ratios (OSR) of *Syngnathus acus* specimens (total, females and males) collected in 2017-2018 surveys on Cíes Archipelago in sites A, B and C. Recaptured specimens are not included.

| | | **2017** | | | | **2018** | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Captures** | **Total** | **A** | **B** | **C** | **Total** | **A** | **B** | **C** | **Total** |
| **Spring** | 75 | 9 | 13 | 13 | 35 | 14 | 9 | 17 | 40 |
| **Females** | 43 | 8 | 7 | 9 | 24 | 6 | 2 | 11 | 19 |
| **% Ovigerous** | 84 | 88 | 86 | 56 | 75 | 100 | 100 | 91 | 95 |
| **Males** | 32 | 1 | 6 | 4 | 11 | 8 | 7 | 6 | 21 |
| **% Pregnant** | 91 | 100 | 83 | 73 | 82 | 100 | 100 | 100 | 100 |
| **Summer** | 62 | 4 | 7 | 12 | 23 | 7 | 8 | 24 | 39 |
| **Females** | 41 | 2 | 6 | 9 | 17 | 7 | 5 | 12 | 24 |
| **% Ovigerous** | 29 | 0 | 0 | 22 | 12 | 71 | 40 | 25 | 42 |
| **Males** | 21 | 2 | 1 | 3 | 6 | 0 | 3 | 12 | 15 |
| **% Pregnant** | 76 | 50 | 100 | 100 | 83 | - | 67 | 75 | 73 |
| **Autumn** | 16 | 0 | 8 | 4 | 12 | 0 | 3 | 1 | 4 |
| **Females** | 12 | 0 | 5 | 3 | 8 | 0 | 3 | 1 | 4 |
| **% Ovigerous** | 0 | - | 0 | 0 | 0 | - | 0 | 0 | 0 |
| **Males** | 4 | 0 | 3 | 1 | 4 | 0 | 0 | 0 | 0 |
| **% Pregnant** | 0 | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Total** | 153 | 13 | 28 | 29 | 70 | 21 | 20 | 42 | 83 |
| **Females** | 96 | 10 | 18 | 21 | 49 | 13 | 10 | 24 | 47 |
| **% Ovigerous** | 50 | 70 | 33 | 33 | 41 | 85 | 40 | 54 | 60 |
| **Males** | 57 | 3 | 10 | 8 | 21 | 8 | 10 | 18 | 36 |
| **% Pregnant** | 79 | 67 | 60 | 63 | 62 | 100 | 90 | 83 | 89 |
| **OSR** | | | | | | | | | |
| **Spring** | 0.45 | 0.12 | 0.45 | 0.37 | 0.33 | 0.57 | 0.78 | 0.37 | 0.54 |
| **Summer** | 0.57 | 1.00 | 1.00 | 0.60 | 0.71 | - | 0.50 | 0.75 | 0.52 |
| **Spring + Summer** | 0.48 | 0.22 | 0.50 | 0.42 | 0.39 | 0.42 | 0.69 | 0.54 | 0.53 |

Total captures in sites A, B and C accounted for 22, 31 and 47% of total specimens, respectively, but differences were not significant due to large deviations (K-W; $X^2_{(3)}=4.99$; P=0.082) (Figure S1). Average abundance was low (12.5 fish km$^{-2}$), corresponding to 3.9, 15.2 and 18.3 fish km$^{-2}$ in sites A, B and C, respectively. The highest abundance was recorded in summer-2018 on site C (55.3 fish km$^{-2}$). Most fishes were collected in spring (49%) and to a lesser extent in summer (41%). Captures in autumn were sharply reduced (10%), especially in 2018 (2.6%). A graphical summary of abundances (nMDS plots) across years, seasons and sites are provided in Figure S2. Females (63%) outnumbered males (37%) and the captures significantly differed across seasons (Kruskal-Wallis; $X^2_{(3)}=14.7$; p<0.0001) (Figure S1).

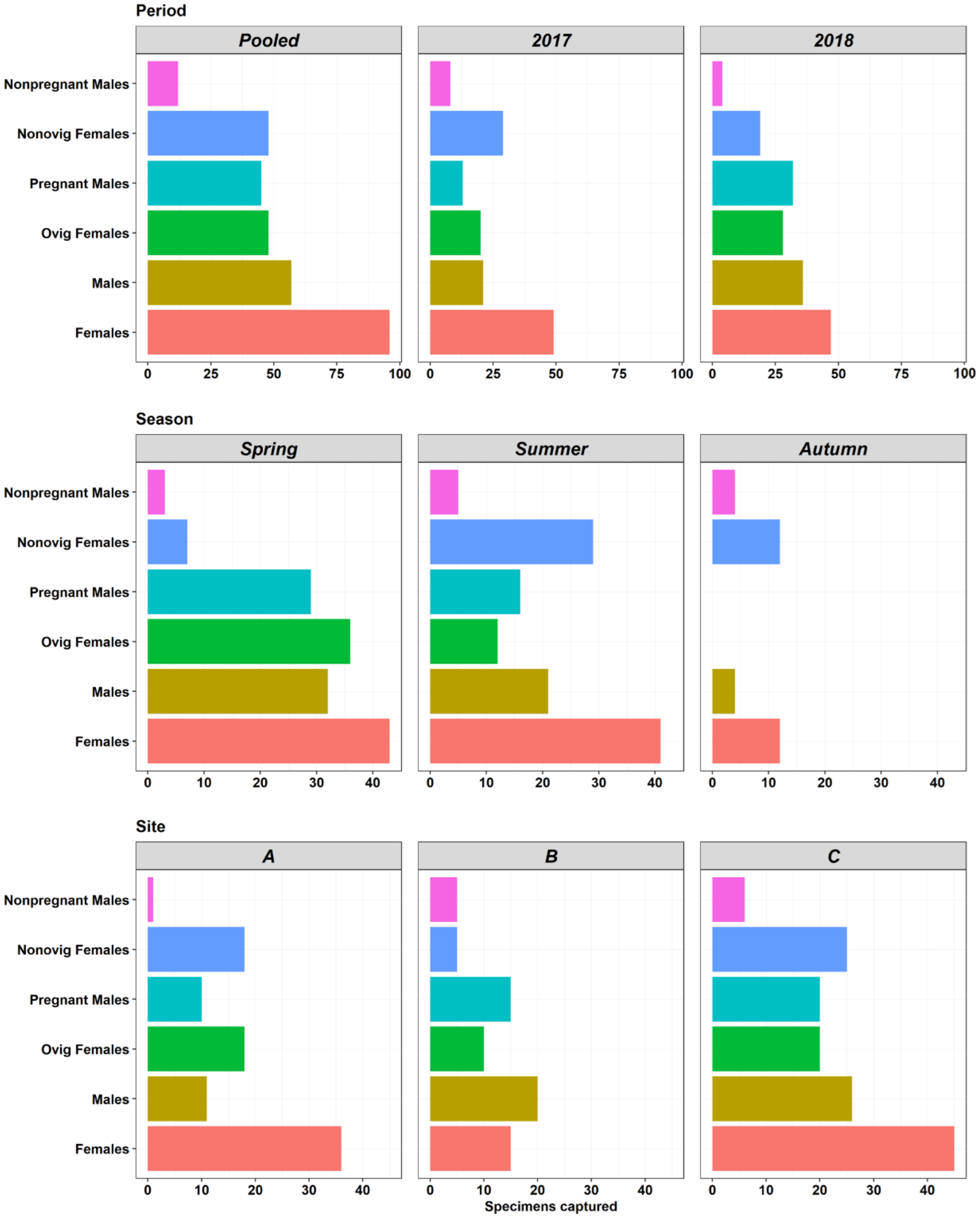

**Figure 3.** *Syngnathus acus* specimens captured in surveys carried out in spring, summer and autumn (2017 and 2018) on sites A, B and C in Cíes Archipelago.

Average size and weight values were 34.4 ± 6.8 cm SL (range: 16.4 – 49.8 cm) and 33.3 ± 19.3 g (range: 2.8 – 102.7 g), respectively (Tables 2 and S1). Adult specimens larger than 30 cm SL accounted for 78% of total captures.

Eleven *S. acus* individuals (6 females and 5 males) were re-sighted, most of them in 2018. Recaptures generally occurred on the season following the first capture but two individuals caught in 2017 were recaptured in 2018. In most cases, the first capture and further re-sighting occurred on the same site (3 in site B and 5 in site C). Two individuals initially marked in site C were recaptured in site B, at a distance of about 1 km. Another specimen previously captured in site B were re-sighted in site C. Despite the low number of individuals re-sighted, daily weight-specific growth rates (G) were roughly estimated in eight specimens, ranging from 0.08 % day$^{-1}$ (large individuals) to 0.43 % day$^{-1}$ (small individuals) (Figure S3).

**Table 2.** Standard length (SL), wet weight (WW), isotopic profiles (δ13C, δ15N) and C:N ratios in *Syngnathus acus* males and females collected in 2017-2018 in Cíes Archipelago. Different letters indicate significant differences between groups (P<0.05). Recaptured specimens were not included.

| | SL (cm) | WW (g) | $\delta^{13}$C (‰) | $\delta^{15}$N (‰) |
|---|---|---|---|---|
| **Females** | 33.7 ± 6.7 | 29.5 ± 16.6 | -15.3 ± 0.5 | 11.0 ± 0.6 |
| **Ovigerous** | 36.2 ± 5.6$^{a}$ | 34.8 ± 15.1$^{a}$ | -15.3 ± 0.4$^{a}$ | 11.3 ± 0.4$^{a}$ |
| **Nonovigerous** | 31.8 ± 6.8$^{b}$ | 24.2 ± 16.5$^{b}$ | -15.3 ± 0.6$^{a}$ | 10.7 ± 0.6$^{b}$ |
| **Males** | 35.7 ± 6.8 | 39.6 ± 21.8 | -15.5 ± 0.5 | 11.3 ± 0.5 |
| **Pregnant** | 36.3 ± 3.2$^{a}$ | 41.4 ± 20.6$^{a}$ | -15.5 ± 0.4$^{a}$ | 11.4 ± 0.3$^{a}$ |
| **Non-pregnant** | 33.2 ± 8.8$^{ab}$ | 32.6 ± 25.6$^{ab}$ | -15.5 ± 0.5$^{a}$ | 10.9 ± 0.4$^{b}$ |

Length-weight relationships for the whole population (Figure S4) indicated positive allometry (b=3.20), mostly due to the high values of coefficient b in immature individuals (b=3.32-3.35) compared to mature fishes (b=2.97-2.83).

### *3.2. Reproduction traits*

Mature females and males were mostly captured in spring and to a lesser extent in summer (Table 1, Figure 3). Opposite to spring, the relative occurrence of pregnant males in summer was higher than for ovigerous females. Average sex ratios (males:males+females) in 2017 and 2018 were 0.30 and 0.43, respectively, but differed between sites, with higher relative occurrence of females in sites A and C (Figure 3). Sex ratios in spring, summer and autumn were 0.43, 0.34 and 0.25, respectively. Operational sex ratios (OSR; Table 1) for the breeding period (spring and summer) were highly variable across sites, seasons and years but the population was slightly female-biased (0.45) in spring, the peak of the breeding season (0.33 in spring-2017; 0.54 in spring-2018). In summer, the breeding population was slightly male-biased (0.57; 0.71 in 2017; 0.52 in 2018).

Nonovigerous females (31.8 ± 6.8 cm; range: 19.5 – 45.0 cm) were significantly smaller than ovigerous females (36.2 ± 5.6 cm; range: 19.1 – 47.4 cm) and pregnant males (36.3 ± 3.2 cm; range: 24.0 - 49.8 cm) (Tables 2 and S1, Figure S5). Half of total females displayed signs of maturity, with swollen trunks and brilliant colouration.

The eggs and developing larvae were aligned inside the marsupium forming four strings that generally occupied the entire brooding structure. Morphologically, all the eggs/embryos in a male's brood pouch were frequently at the same development stage However, a few males carried more than one partial clutch or showed partially filled pouches. The eggs contained within a brooding structure were counted in one pregnant male, accounting for 166 eggs, which covered about 90% of the total available surface. The average egg diameter in stage B was 2.03 ± 0.19 mm. The lengths in pre-larvae and larvae in stages H, I and J were 2.18 ± 0.49, 2.79 ± 0.18 and 3.02 ± 0.07 cm, respectively (Table 3).

### *3.3. Isotopic profiles*

Average isotopic values for the whole population were -15.4 ± 0.5‰ for $\delta^{13}$C and 11.1 ± 0.6‰ for $\delta^{15}$N (Table S2). Females and males displayed similar $\delta^{13}$C values (-15.5 - -15.3‰) (K-W; $X^2_{(3)}$=4.22; P=0.240), but differed significantly in $\delta^{15}$N across maturity stages (10.7 – 11.4‰) (K-W; $X^2_{(3)}$=8.11; P=0.044) (Table 2, Figure S5). Average values for $\delta^{15}$N in ovigerous females and pregnant males were similar (11.3 -11.4 ‰) (P=0.757), and significantly higher than in immature specimens (10.7-10.9‰) (P<0.05).

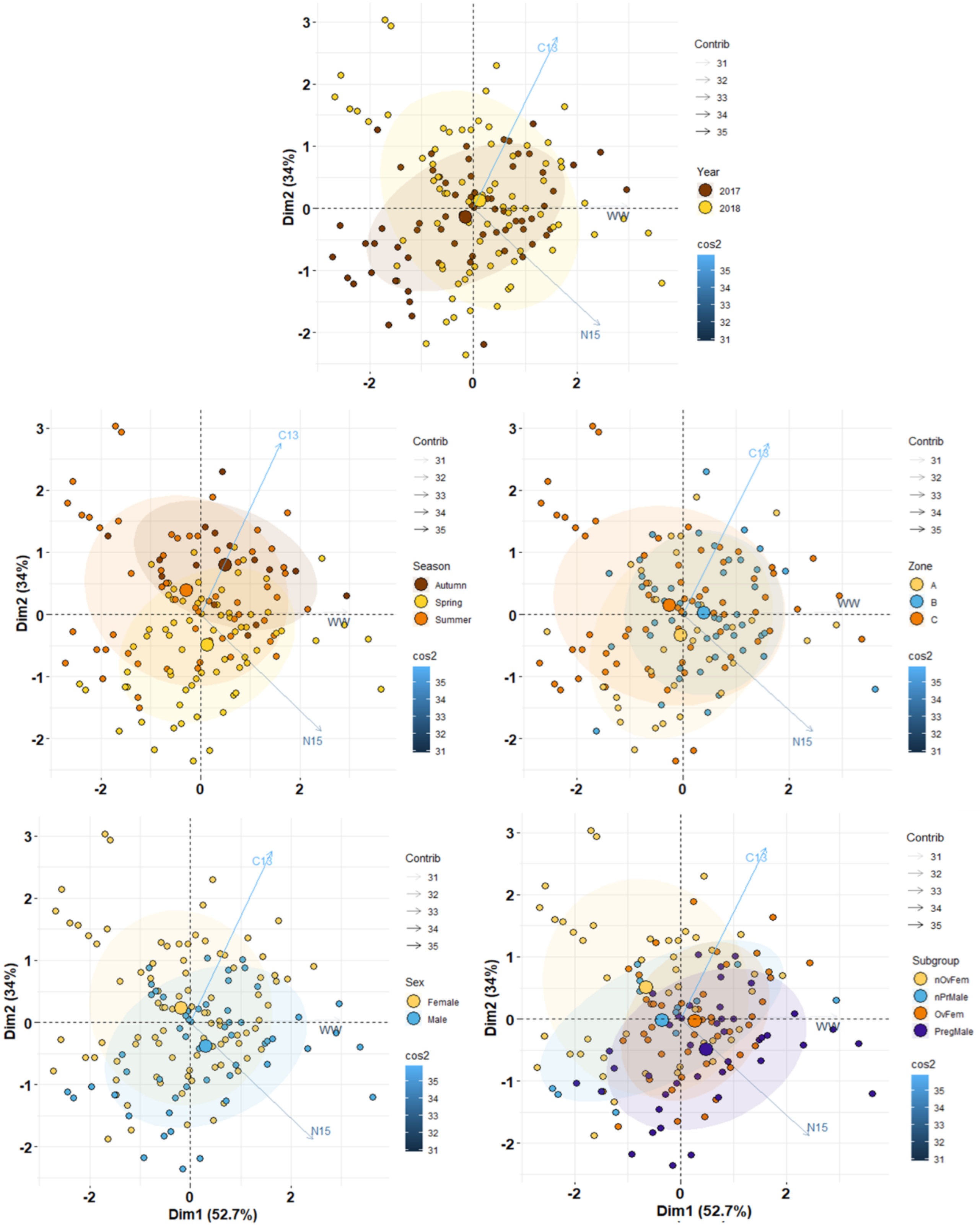

**Figure 4.** Factor score plots for the Principal Component Analysis on *Syngnathus acus* collected in spring, summer and autumn (2017 and 2018) on sites A, B and C in Cíes Archipelago. Variables: WW – Wet weight, C13 - δ*13*C (absolute values) and N15 – $\delta^{15}$N. Ellipses correspond to 95% confidence. Plots provided for year, season, site, sex and reproductive status (Nonovigerous females, non-pregnant males, ovigerous females and pregnant males).

The Principal Component Analysis (PCA) performed on weight and isotopic data revealed that the first two factors explained as much as 86.7% of total variability (Figure 4). The centroids for year and surveyed sites were very close to each other, indicating relative isotopic stability regarding those variables. However, data dispersion was higher in samples from year 2017 and site C. This finding was mainly caused by lower δ15N values in non-ovigerous females. Regarding seasonal changes, the transition from spring to autumn was characterized by a progressive $\delta^{13}$C-decrease. The PCA plots for sex and maturity state denoted higher $\delta^{15}$N values in males and an alignment of centroids following increasing $\delta^{15}$N values, from immature females to pregnant males. In contrast, the contribution of $\delta^{13}$C values on centroid positions was almost negligible.

### *3.4. Isotopic inheritance*

The isotopic profiles in embryos and newly released juveniles were rather similar, especially for $\delta^{15}$N (Table 3). Isotopic values in B-stage eggs were -16.6‰ for $\delta^{13}$C and 10.9‰ for $\delta^{15}$N. The values at that stage were slightly lower (1.5‰ for $\delta^{13}$C and 1.1‰ for $\delta^{15}$N) than in the corresponding males. Most advanced developmental stages (H to J) resulted slightly enriched compared to B-stage, ranging from -16.3‰ to -15.2‰ for $\delta^{13}$C and from 11.5‰ to 11.8‰ for $\delta^{15}$N. Furthermore, there was a direct linear relationship between the isotopic values in stages H-J and those in brooding males, so that the isotopic ratios between pre-larva/larva and males ranged from 0.97 to 1.05 for $\delta^{13}$C and from 0.99 to 1.04 for $\delta^{15}$N, and did not differ from 1 ($P=0.525$ for $\delta^{13}$C; $P=0.415$ for $\delta^{15}$N).

### *3.5. Trophic position*

The average trophic position (TP) of *S. acus* was 3.36 ± 0.05 (Table 4). TP values in males and females differed significantly (3.39 ± 0.05 and 3.33 ± 0.05, respectively) (t-test, $t=7.654$, $n=77$, $P<0.0001$), whereas mature individuals occupied a higher trophic position than immature fishes (3.41 ± 0.05 and 3.26 ± 0.05, respectively) ($t=16.488$, $n=132$, $P<0.0001$). TP values in individuals collected in summer were significantly lower than in those from spring (8.5% decrease; $t=20.365$, $n=132$, $P<0.001$) and autumn (4.9% decrease; $t=7.941$, $n=132$, $P<0.0001$) surveys. The summer decrease was not related to sex nor mature condition (3.47 ± 0.084, 3.20 ± 0.07 and 3.36 ± 0.10 for spring, summer and autumn, respectively).

**Table 3.** Standard length (SL) and isotopic values ($\delta^{13}$C and $\delta^{15}$N) in developing *Syngnathus acus* (stages B, H, I and J according to [43] collected from pregnant males in 2017-2018 on Cíes Archipelago. Isotopic values for pregnant males are given between brackets. n: number of batches analyzed (20-30 specimens per batch). *: diameter (mm).

| Stage | n | SL (cm) | $\delta^{13}$C (‰) | | $\delta^{15}$N (‰) | |
|---|---|---|---|---|---|---|
| **B** | 1 | 2.03 * | -16.6 | (-15.1) | 10.9 | (12.0) |
| **H** | 6 | 2.18 ± 0.49 | -16.3 ± 0.5 | (-15.6 ± 0.6) | 11.7 ± 0.4 | (11.6 ± 0.3) |
| **I** | 5 | 2.79 ± 0.18 | -15.2 ± 0.7 | (-15.6 ± 0.3) | 11.8 ± 0.6 | (11.8 ± 0.4) |
| **J** | 3 | 3.02 ± 0.07 | -16.1 ± 1.2 | (-16.1 ± 0.2) | 11.5 ± 0.4 | (11.1 ± 0.4) |

**Table 4.** Trophic positions (TP) in *Syngnathus acus* specimens from Cíes Archipelago in 2017-2018 surveys.

| | Pooled | n | Immature | n | Mature | n |
|---|---|---|---|---|---|---|
| **Pooled** | 3.36 ± 0.05 | 148 | 3.26 ± 0.05 | 58 | 3.41 ± 0.05 | 90 |
| **Spring** | 3.47 ± 0.08 | 71 | 3.37 ± 0.08 | 8 | 3.48 ± 0.07 | 63 |
| **Summer** | 3.20 ± 0.07 | 61 | 3.14 ± 0.07 | 34 | 3.26 ± 0.07 | 27 |
| **Autumn** | 3.36 ± 0.10 | 16 | 3.36 ± 0.10 | 16 | - - | 0 |
| **Males** | 3.39 ± 0.05 | 55 | 3.30 ± 0.05 | 12 | 3.42 ± 0.05 | 43 |
| **Spring** | 3.49 ± 0.08 | 31 | 3.34 ± 0.28 | 3 | 3.50 ± 0.08 | 28 |
| **Summer** | 3.26 ± 0.06 | 20 | 3.24 ± 0.08 | 5 | 3.26 ± 0.06 | 15 |
| **Autumn** | 3.34 ± 0.17 | 4 | 3.34 ± 0.17 | 4 | - - | 0 |
| **Females** | 3.33 ± 0.05 | 93 | 3.25 ± 0.05 | 46 | 3.39 ± 0.05 | 47 |
| **Spring** | 3.44 ± 0.07 | 40 | 3.39 ± 0.07 | 5 | 3.45 ± 0.08 | 35 |
| **Summer** | 3.17 ± 0.07 | 41 | 3.13 ± 0.07 | 29 | 3.26 ± 0.07 | 12 |
| **Autumn** | 3.36 ± 0.11 | 12 | 3.36 ± 0.11 | 12 | - - | 0 |

### *3.6. Trophic niche*

The average niche area SEAc in *S. acus* was 0.89, but an increase was noticed in 2018 (0.94) compared to 2017 (0.67) (Table 5). The niche area in summer (0.88) was larger than in spring (0.62) and autumn (0.71). In addition, males and individuals from site B occupied smaller niche areas. Differences across reproductive states were mainly due to the noticeable higher area in nonovigerous females (1.19) compared to ovigerous females (0.57) and males (0.57-0.62). The results can be visualized in detail on the isotopic bi-plots in Figure S6.

Niche overlap estimates revealed a nearly full overlap of 2017-niche by 2018-niche, and of spring-niche by autumn- and summer-niches (Table 6, Figure 5). Regarding spatial effects, the niche in individuals from site A resembled the one for site B but the probabilities that individuals from site C occupy the niches for those in sites A and B decreased to 83.7% and 73.9%, respectively. Regarding sexual condition, the trophic niche of males and mature fishes were markedly overlapped by those for females (95.8%) and immature fishes (93.8%), respectively. The probability that an immature female be included within the niches of non-pregnant males, pregnant males or ovigerous females decreased to 64.8, 73.7 and 76.6%, respectively.

**Table 5.** Estimated niche areas in *Syngnathus acus* collected in spring, summer and autumn (2017-2018) on Cíes Archipelago. TA, SEA and SEAc (SIBER package): Total area of convex hull, standard ellipse area, and corrected standard ellipse with a correction for small sample sizes, respectively.

| | | TA | SEA | SEAc |
|---|---|---|---|---|
| **Pooled** | | 5.60 | 0.88 | 0.89 |
| **Year** | | | | |
| | 2017 | 3.42 | 0.67 | 0.68 |
| | 2018 | 5.17 | 0.94 | 0.95 |
| **Season** | | | | |
| | Spring | 2.80 | 0.61 | 0.62 |
| | Summer | 3.30 | 0.86 | 0.88 |
| | Autumn | 2.03 | 0.67 | 0.71 |
| **Site** | | | | |
| | A | 2.58 | 0.83 | 0.85 |
| | B | 2.83 | 0.58 | 0.59 |
| | C | 4.55 | 1.03 | 1.05 |

| | | TA | SEA | SEAc |
|---|---|---|---|---|
| **Sex** | | | | |
| | Female | 5.23 | 0.95 | 0.96 |
| | Male | 2.39 | 0.64 | 0.66 |
| **Reproductive State** | | | | |
| | Nonovigerous female | 4.32 | 1.16 | 1.19 |
| | Non-pregnant male | 0.81 | 0.43 | 0.48 |
| | Ovigerous female | 2.66 | 0.56 | 0.57 |
| | Pregnant male | 2.16 | 0.61 | 0.62 |

### *3.7. Feeding regimes*

The results of the Bayesian mixing models on the dietary regimes in *S. acus* revealed a high contribution of amphipods (21-68%) to bulk diet in both 2017 and 2018 surveys (Table 7, Figure S7). However, two main features deserve special consideration. The first one refers to changes in the dietary regimes across years, with a substantial contribution of carideans (21-41%) in 2017 and harpacticoid copepods in 2018 (19-34%). The second interesting finding refers to changes of regimes across the four reproductive statuses recognized, with certain dissimilarities between mature and immature individuals. Accordingly, amphipods were highly consumed in nonovigerous females (46-68%) and non-pregnant males (36-63%) compared to ovigerous females (28-44%) and pregnant males (21-48%). In contrast, the contributions of copepods and isopods did not differ markedly across reproductive states, ranging from 4 to 34% in the former and from 3 to 24% in the latter. The low contribution of mysidaceans to dietary regimes was noteworthy (4-20%). However, the consumption of this carnivorous component of marine zooplankton was higher in pregnant males (10-20%).

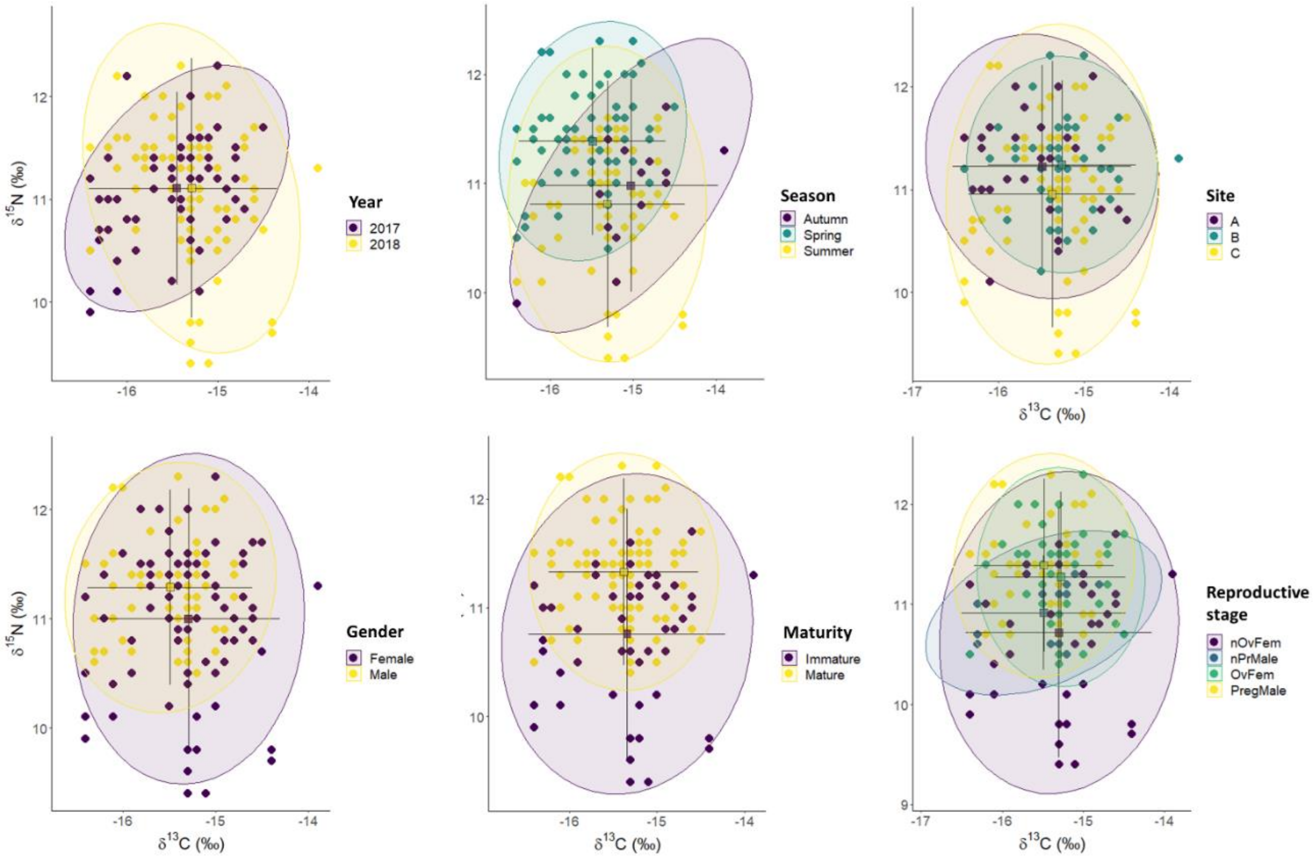

**Figure 5.** Stable isotope Bayesian ellipses showing trophic niche widths and overlaps in *Syngnathus acus* collected in spring, summer and autumn (2017-2018) in sites A, B and C on Cíes Archipelago. Ellipses with 95% credible intervals for the means are based on standard ellipses corrected for small sample sizes (SEAc; isotopic niche metrics; SIBER package). Each mark corresponds to mean isotopic values. Reproductive stages: nOvFem – Nonovigerous female; nPrM – Non-pregnant male; OvFem – Ovigerous female; PregMale – Pregnant male.

**Table 6.** Niche overlap estimates (NicheROVER package) showing posterior probabilities (alpha = 0.95) that *Syngnathus acus* individuals from rows will be found within the niches indicated by the column header. Results (%) are provided for years, seasons, sites, genders, maturity and reproductive stages in *S. acus* on Cíes Archipelago (2017-18). Seasons: Spr – Spring; Sum – Summer; Aut – Autumn. Reproductive stages (Rep Stage): nOvF – Nonovigerous female; nPrM – Non-pregnant male; OvF – Ovigerous female; PregM – Pregnant male.

| **Year** | 2017 | 2018 | **Season** | Spr | Sum | Aut | **Site** | A | B | C | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **2017** | - | 92.2 | **Spr** | - | 89.9 | 57.6 | **A** | - | 82.3 | 93.5 | |
| **2018** | 72.8 | - | **Sum** | 75.1 | - | 67.5 | **B** | 95.4 | - | 97.6 | |
| | | | **Aut** | 68.2 | 87.5 | - | **C** | 83.7 | 73.9 | - | |
| **Gender** | Female | Male | **Maturity** | Mat | Imm | | **Rep Stage** | nOvF | nPrM | OvF | PregM |
| **Female** | - | 78.7 | Mature | - | 93.8 | | **nOvF** | - | 64.8 | 76.6 | 73.7 |
| **Male** | 95.8 | - | Immature | 66.7 | - | | **nPrM** | 99.1 | - | 91.6 | 94.3 |
| | | | | | | | **OvF** | 99.4 | 77.9 | - | 97.6 |
| | | | | | | | **PregM** | 98.2 | 67.8 | 95.6 | - |

**Table 7.** Bayesian Stable Isotope Mixing Model (SIMM) (MixSIAR package) - Percent (mean ± sd) contribution of potential prey sources to *Syngnathus acus* diet. Analyses based on isotopic data from 146 specimens sampled on Cíes Archipelago in 2017-2018. Bayesian models were run (long run; chain length=300,000, burn=200,000) using experimentally derived TDF values ($\Delta^{13}$C=2.50‰; $\Delta^{15}$N=3.91‰) (see [46] for further details). Convergence and diagnostic statistics were evaluated using Gelman–Rubin and Geweke tests.

| | **Females** | | **Males** | |
|---|---|---|---|---|
| **Sources** | **Nonovigerous** | **Ovigerous** | **Non-pregnant** | **Pregnant** |
| **2017** | | | | |
| **Amphipoda** (1) | 68 ± 13 | 44 ± 10 | 63 ± 13 | 48 ± 14 |
| **Caridea** (2) | 21 ± 8 | 41 ± 13 | 22 ± 10 | 30 ± 14 |
| **Copepoda** (3) | 4 ± 6 | 6 ± 7 | 7 ± 9 | 9 ± 9 |
| **Isopoda** (4) | 3 ± 4 | 3 ± 4 | 3 ± 4 | 3 ± 5 |
| **Mysidacea** (5) | 4 ± 5 | 6 ± 8 | 5 ± 6 | 10 ± 9 |
| **2018** | | | | |
| **Amphipoda** | 46 ± 26 | 28 ± 16 | 36 ± 20 | 21 ± 12 |
| **Caridea** | 5 ± 5 | 10 ± 11 | 5 ± 7 | 8 ± 11 |
| **Copepoda** | 19 ± 9 | 29 ± 12 | 29 ± 13 | 34 ± 13 |
| **Isopoda** | 24 ± 29 | 20 ± 22 | 21 ± 24 | 17 ± 20 |
| **Mysidacea** | 6 ± 4 | 13 ± 8 | 9 ± 7 | 20 ± 10 |

(1) *Amphilochus manudens, Apherusa* spp., *Caprella acanthifera, C. linearis, Corophium* spp., and other gammaridae; (2) *Hippolyte varians*; (3) Harpacticoida; (4) *Cymodoce truncata, Dynamene bidentata*; (5) *Siriella armata*.

## 4. Discussion

### *4.1. Population characteristics*

The population of the great pipefish *Syngnathus acus* inhabiting Cíes Archipelago was irregularly distributed across sites and seasons, comprising principally very large individuals. The occurrence extended towards less sheltered and not highly exposed zones (e.g., sites A and B). A previous study revealed that the species preferred macroalgal beds on the west coast of Cíes Archipelago [15]. As for many other syngnathids, seaweed (and seagrass) coverture plays a pivotal role in the occurrence of these fishes, supplying food and shelter against predators. Environmental variables such as depth, wave's exposure and slope also govern the pattern of spatial distribution [16]

On site C, *S. acus* co-occurred sympatrically with the pipefish *Entelurus aequoreus* and the seahorse *Hippocampus guttulatus* [15]. Compared to weak swimmer syngnathids such as seahorses, the great pipefish is a fast species that swim near the substrate and that may displace to adjacent areas. Undoubtedly, the high seasonal discrepancies regarding abundances and the low recapture rates would indicate a high population turnover and displacements from/to other more distant areas, especially at the onset and end of the breeding season. Seasonal migrations to other environments have been reported in other syngnathids aiming to avoid severe autumn-winter conditions [4,68-71].

The timings of recaptures suggest that the half-life in the species is about one year, as previously suggested. [4]. *S. acus* individuals in Cíes were markedly larger than those from nearby sites [58], Mediterranean coasts [9,10,72], Black Sea [7] or southern Atlantic populations [73,74]. However, the large size in Cíes individuals agreed with those in eelgrass meadows populations from the northern Atlantic [4,6,75]. Hence, the length in *S. acus* adults seems to rely on its geographical origin [8]. Additionally, adult size also seemed to determine egg/larval size as suggested by comparing the population from Cíes with those from the Aegean Sea [2] or Ria de Aveiro in Portugal [13], whose eggs, larvae and adults were notably smaller. To my knowledge, the maximal size in newborn and adult specimens from Cíes Archipelago are new records for the species.

The weight-length relationships in immature fishes (b=3.32-3.35) agreed with the positive allometric growth reported in southern Portugal (b=3.33-3.34) [6,76], being above and below those reported for South Africa (b=3.07) [74] and Aegean Sea (b=3.71-3-73) [5,8,77], respectively. Differences in length-weight parameters across regions might be due to dissimilarities in biotic and abiotic factors [6].

The extremely low occurrence of medium-sized specimens, the lack of juveniles, the high proportion of mature individuals and the sharp reduction in autumnal abundances would indicate that Cíes Archipelago is an area where *S. acus* concentrates seasonally for reproduction purposes, as reported in *S. schlegeli* [78].

The breeding period in *S. acus* commonly extends from mid-spring to early summer [2.4], when water temperatures are warmer. Depending on the temperature, the breeding season might also extend until late summer (year 2018 in the present study). In Cíes, the extension and timing of the breeding period were also governed by water temperature. In 2018, temperatures in summer-2018 (18.1 ± 0.6 °C) were significantly higher than in summer-2017 (16.9 ± 0.2 °C), resulting in an exceptionally extended breeding season. Assuming the duration of gestation in *S. acus* is similar to that in *S. typhle* (ca. 5 weeks) [79], the recapture of pregnant males in Cíes supports the existence of three annual spawning events as in the Aegean Sea [2].

The morphology of developing embryos within the brood pouch structure was homogeneous in most broods but genetic analyses revealed that in most cases (ca. 80%) the species follows a polygyny mating system, with a maximum of three females contributing to a single brood [38]. Females usually outnumbered males as pointed out in Aegean Sea populations [2] but OSR were generally female-biased but seasonally variable. Hence, the unequal parental contribution [80,81] suggested in *S. acus* [4,82] should not be ruled out.

High variability in OSR values might rely on environmental factors (e.g., temperature) that may affect the sexes differently [78,83]. The male-biased pattern observed in advanced periods of the breeding season (i.e., summer) might be due to the temporal gap (i.e., about five weeks [79]) between last egg batch releases by females and last releases of juveniles by pregnant males. That hypothesis is in accordance with previous studies [78,84] suggesting that the pregnant males would remain sheltered on the algal bed, whereas the females might move across the patches searching for a mate or be excluded from favored habitats by intrasexual competition.

The polygyny reported in Cíes individuals is likely associated with a sex-role reversed pattern [82]. In female-biased populations, the females would compete more strongly for receptive males, whereas males would be both the more choosy sex and the limiting factor [80,85]. Sex roles in syngnathids form a continuum from conventional to reversed [86-88]. Intermediate sexual selection on females generally occurs in polygynous species, as in *S. acus* and many species with the breeding structure on the tail [89]. Sexual dimorphism in Cíes specimens seemed to be limited to an orange hue in some females. In contrast to polygynous species, female-specific ornaments as sexual signals are frequently present in polyandrous pipefishes [89,90].

### *4.2. Early development*

With the caution that the results of the present study on eggs/embryos are based on small sample size, pregnant males and advanced stages of embryogenesis were isotopically similar. Compared to eggs (stage B), the small $\delta^{15}N$-enrichment in pre-released larvae (stages H-J) suggests a fast protein turnover in developing embryos resulting in the excretion of lighter nitrogen and leading to a $^{15}N$-enrichment. Besides, selective use of some yolk reserves such as lipids and free amino acids would occur, which is a common feature in many developing fish embryos [91-94].

In most teleosts, the isotopic fluctuations in developing embryos denote a trade-off between egg nutrients provided by females and embryo metabolism supporting development and growth. However, syngnathids are fishes with male parental care in which egg yolk and embryo tissues originate from both maternal and paternal resources. The brood pouch in S. acus resembled that of *S. abaster* [95] and *S. floridae* [81], consisting of a ventral structure located below the tail and covered by two dermal flaps that form the pouch seal at the midline. During gestation, the chamber is filled with a mucous material that surrounds the eggs. Brooding males may supply nutrients, namely amino acids, proteins and carbohydrates, to embryos [96-98]. The contribution of males to $\delta^{13}C$ and $\delta^{15}N$ profiles in developing embryos is unknown, deserving further investigation that would contribute to a better interpretation of fluctuations with embryogenesis progress. Linking the isotopic profile in parents to those in eggs or offspring could also assist in providing information about paternal foraging without having to know the female/male or in inferring parental isotope profiles from hatchling tissues. Isotopic correlations between mother and offspring have been reported in turtles [28,33,99] and sharks [32,100,101]. However, available information on stable isotope discrimination values between breeders-to-eggs in teleost fishes is scanty [31,58].

### 4.3. Trophic features

The feeding regime in adult *S. acus* from Cíes agreed with other syngnathids, foraging mostly upon small crustaceans [17]. In Cíes Archipelago, the species would occupy a low trophic position among carnivorous fishes, in agreement with Jennings and van der Molen [12]. The trophic niche was highly similar to those in other co-occurring syngnathids in site C [14], with small differences across species, likely due to inter-specific dissimilarities in mouth size and shape.

In Cíes, the species preyed preferentially upon amphipods with varying proportions of other sources namely copepods, carideans and isopods. Since both sexes only occasionally come above the vegetal assemblage [4], the average contribution of suprabenthic mysidaceans to bulk diet was reduced, although more preferred by pregnant males, being less accessible to *S. acus* than epifaunal resources. The low foraging on mysidaceans and the preference for amphipods agree with the gut content in specimens from the Aegean Sea and Lake Bafa [10,11]. Hence, *S. acus* is a secondary consumer, a specialist predator foraging preferentially near the substrate in vegetated habitats [4]. Despite its tiny mouth, this fish can ingest prey much larger than expected since the prey can be cut into pieces small enough to swallow [102]

The composition of bulk diet estimates differed across seasons, sites, sexes and maturity states. Dissimilarities across seasons and length classes were also reported in populations of the Aegean Sea, with more diverse feeding in spring than in winter [9]. Overall, the individuals collected in summer from Cíes occupied lower trophic positions and wider niche areas (i.e., higher diversity in dietary resources), principally due to the impact of nonovigerous females. Another influential factor might be the seasonal variability in epifaunal assemblages, especially from summer to autumn, resulting from changes in macroalgal beds [14]. Gammaridae, Corophidae and Amphilochidae are amphipods submitted to high seasonal variability in Cíes, especially in autumn and summer.

Trophic overlap and SIMM results indicate dietary differences between immature specimens and pregnant males - ovigerous females, with a lower preference for amphipods in the former. Hence, mature fish seemed to be less selective than immatures, showing a higher contribution of other prey (i.e., Caridea and Copepoda, depending on the year) to bulk diet. Besides this, the species showed certain trophic flexibility, adapting to fluctuations in prey diversity and availability across seasons and sites, independently of gender and reproduction state. The adaptive foraging capability to changing abundances of dietary sources was in agreement with previous findings on populations from the Aegean Sea [10] and with seasonal changes reported in other syngnathids such as the pipefish *S. taenionotus* (Franzoi et al. 1993) or the seahorse H. guttulatus [19]

The wider niche area and lower trophic position in immature females were likely related to their high relative abundance in site C, an area predominantly occupied by females. Site C is a sheltered area characterized by the occurrence of vegetal communities dominated by the fucoid *Treptacantha baccata*. This macroalga provides more shelter to pipefishes and other syngnathids (e.g., Hippocampus guttulatus and Entelurus aequoreus) in Cíes Archipelago and higher relative abundances of Gammaridae and Corophidae than in Codium assemblages dominating sites A and B [14]. $\delta^{15}N$ values were lower in amphipods (6.42 ± 0.28 ‰) compared to other potential sources (6.58 - 10.02‰) included in SIMMS. Consequently, higher consumption of amphipods would explain the decrease noticed in both $\delta^{15}N$ values and trophic position of S. acus immature females.

The high isotopic similarity between males and mature females would reveal long residence periods in the same habitat. In contrast, the higher isotopic variability and the larger trophic niche in immature fishes (especially females) might reflect a higher turnover (i.e., lower residence periods), resulting in higher isotopic heterogeneity. Immature females should be more active than males, occupying wider areas within the studied zone as demonstrated in other pipefishes [103,104]. However, this hypothesis does not seem to be fully supported by the isotopic similarity in average $\delta^{13}C$ values across sex and maturity condition.

The breeding pattern, the maturation condition and the physiological processes involved in egg production and pregnancy could affect the isotopic profile in fishes. Syngnathid species differ in the use and allocation of resources for reproduction, demanding an important expenditure of energy and nutrients that can be obtained at different periods over the year [105]. The maintenance of reproductive capabilities thorough the breeding season might depend on the number of breeding events as well as other biotic and abiotic factors. Consequently, reproductive events might determine the breeding pattern followed by the species. Seahorses are synchronous batch spawners with a high number of annual breeding events such as seahorses. These fishes follow a mixed capital-income breeding strategy [106,107], in which resources are gained prior to and during the breeding period. However, the annual number of breeding events in *S. acus* and other pipefishes [108] are reduced compared to seahorses. Unfortunately, the reproduction strategy in the polygamous *S. acus* has not been properly assessed and the implications of resources availability before the breeding season on reproduction are unknown. It is feasible that *S. acus* follows a capital breeding strategy with a strong trade-off between feeding, energy storage and further allocation to reproduction. This hypothesis would be partially supported by the lower foraging on amphipods and the higher $\delta^{15}N$ values in ovigerous females and pregnant males compared to immature individuals.

## 5. Conclusions

The monitoring of *Syngnathus acus* populations in Cíes Archipelago revealed marked seasonal differences in distributions and abundances, preference for shelter sites, a breeding season highly associated with temperature and migratory events in late autumn. The population structure indicates that the studied area is an important reserve of extremely large breeders. The species should be considered a selective but flexible carnivorous fish foraging on amphipods as the main dietary contributor but adapting its regime to annual and seasonal fluctuations and resource availability. However, trophic characteristics in immature fish, especially females, differed from those in breeding specimens. The former seemed to occupy a lower trophic position, a wider niche area and a higher dietary diversity, but the factors involved in those traits need further addressing. Although the current threat and conservation status of *S. acus* is least concern, the distinctive characteristics of the population inhabiting Cíes Archipelago should deserve preferential conservation actions.

**Supplementary Materials:** The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, **Figure S1.** Abundances in *Syngnathus acus* collected in Cíes Archipelago (2017-2018) considering reproductive states (ovigerous females, pregnant males, nonovigerous females and non-pregnant males), seasons (spring, summer and autumn) and sites (A, B and C). Significances of Kruskal-Wallis test are shown. **Figure S2.** Two-dimensional non-metric multidimensional scaling (NMDS; Bray–Curtis similarities) plot for *Syngnathus acus* individuals collected in Cíes Archipelago considering period (years 2017 and 2018), seasons (spring, summer and autumn) and sites (A, B and C). The confidence limits for ellipses (95% confidence) are shown. The influence of reproductive states (ovigerous females, pregnant males, nonovigerous females and non-pregnant males) are indicated by arrows. Each mark corresponds to mean values for duplicate samples. **Figure S3.** Relationship between wet weight and daily weight-specific growth rate in *Syngnathus acus* collected in Cíes Archipelago. Only recaptured individuals are included. **Figure S4.** Length-weight relationships in mature (continuous line) and immature (dotted line) *Syngnathus acus* males and females captured in surveys carried out in spring, summer and autumn (2017 and 2018) on sites A, B and C in Cíes Archipelago. **Figure S5.** SL (cm), WW (g), $\delta^{13}C$ (‰), $\delta^{15}N$ (‰) and C:N values in *Syngnathus acus* collected in Cíes Archipelago (2017-2018) considering reproductive states (ovigerous females, pregnant males, nonovigerous females and non-pregnant males), seasons (spring, summer and autumn) and sites (A, B and C). Significances of Kruskal-Wallis test are shown. **Figure S6.** Isotopic bi-plot for reproductive stages in *Syngnathus acus* (n=146; 2017 and 2018 surveys) relative to average $\delta^{13}C$ and $\delta^{15}N$ signatures of five potential prey sources (mean ± sd; crosses) adjusted for TDF values and consumers (coloured small symbols). TDF values: 2.5 for $^{13}C$ and 3.9 for $^{15}N$ [58]. Two consumers with a low probability (<5%) were excluded on both the plot and the subsequent mixing model [65]. Sources: Am - Amphipoda, Co - Copepoda, Ca - Caridea, Is – Isopoda, and My - Mysidacea. Reproductive stages: nOvFem – Nonovigerous female; nPrM – Non-pregnant male; OvFem – Ovigerous female; PregMale – Pregnant male. **Figure S7:** Percent (mean ± sd) contribution of potential prey sources to *S. acus* diet as estimated by Bayesian Stable Isotope Mixing Model (SIMM) (MixSIAR package in R v. 3.1.12). Analyses based on isotopic data for dorsal fin tissues of 146 fishes sampled on Cíes Archipelago in 2017-2018. Bayesian models run (long run; chain length=300,000, burn=200,000) using experimentally derived TDF values ($\Delta^{13}C$=2.50‰; $\Delta^{15}N$=3.91‰) (see [58] for further details). Convergence and diagnostic statistics evaluated using Gelman–Rubin and Geweke tests. **Table S1.** Standard length (cm) and wet weight (g) in *Syngnathus acus* collected in 2017-2018 in Cíes Archipelago. Recaptured specimens are not included. **Table S2.** Isotopic profiles ($\delta^{13}C$ and $\delta^{15}N$) and C:N ratios in Syngnathus acus collected in 2017-2018 in Cíes Archipelago. Recaptured specimens are not included

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## References

1. FishBase. *Syngnathus acus*. 2022. Available online: https://www.fishbase.de/summary/Syngnathus-acus.html (accessed on 11 Jan 2022).
2. Gurkan, Ş.; Taşkavak, E.; Hossucu, B. The reproductive biology of the Great Pipefish *Syngnathus acus* (Family: Syngnathidae) in the Aegean Sea. North-West J. Zool. 2009, 5, 179–190.
3. Smith-Vaniz, W F. *Syngnathus acus*. The IUCN Red List of Threatened Species 2015: e.T198765A44933898. Available online: https://dx.doi.org/10.2305/IUCN.UK.2015-4.RLTS.T198765A44933898.en (accessed on 13 January 2022).
4. Vincent , A.C.J.; Berglund, A.; Ahnesjö, I. Reproductive ecology of five pipefish species in one eelgrass meadow. Environ. Biol. Fish 1995, 44, 347-61. https://doi.org/10.1007/BF00008250.
5. Gurkan, Ş.; Taşkavak, E.. Length-weight relationships for syngnathid fishes of the Aegean Sea, Turkey. Belg. J. Zool. 2007, 137, 2019-222.
6. Vieira, R.P.; Monteiro, P.; Ribeiro, J.; Bentes, L.; Oliveira, F.; Erzini, K.; Gonçalves, J.M.S. Length-weight relationships of six syngnathid species from Ria Formosa, SW Iberian coast. Cah Biol Mar. 2014, 55, 9-12.

7. Yildiz, T.; Uzer, U.; Karakulak, F.S. Preliminary report of a biometric analysis of greater pipefish *Syngnathus acus* Linnaeus, 1758 for the western Black Sea. Turk. J. Zool. 2015, 39, 917-24. https://doi.org/10.3906/zoo-1408-57
8. Gurkan, Ş.; Innal, D. Some morphometric features of congeneric pipefish species (*Syngnathus abaster* Risso 1826, *Syngnathus acus* Linnaeus, 1758) distributed in Lake Bafa (Turkey). Oceanol. Hydrobiol. Stud. 2018, 47, 239-247. https://doi.org/10.1515/ohs-2018-0023.
9. Taşkavak, E.; Gürkan, Ş.; Severa, T.M.; Akalına, S.; Özaydına, O. Gut contents and feeding habits of the Great Pipefish, *Syngnathus acus* Linnaeus, 1758, in Izmir Bay (Aegean Sea, Turkey). Zool. Middle East 2010, 50, 75-82. https://doi.org/10.1080/09397140.2010.10638414
10. Gurkan, Ş.; Taşkavak, E. The relationships between gut length and prey preference of three pipefish (*Syngnathus acus*, *Syngnathus typhle*, *Nerophis ophidion* Linnaeus, 1758) species distributed in Aegean Sea, Turkey. Iran. J. Fish. Sci. 2019, 18, 1093-1100. https://doi.org/10.22092/ijfs.2019.118277.
11. Gurkan, Ş.; Innal, D.; Gulle, I. Monitoring of the trophic ecology of pipefish species (*Syngnathus abaster*, *Syngnathus acus*) in an alluvial lake habitat (Lake Bafa, Turkey). Oceanol. Hydrobiol. Stud. 2021, 50, 24-32. https://doi.org/10.2478/oandhs-2021-0003.
12. Jennings, S.; van der Molen, J. Trophic levels of marine consumers from nitrogen stable isotope analysis: estimation and uncertainty, ICES J. Mar. Sci. 2015, 72, 2289-2300. https://doi.org/10.1093/icesjms/fsv120.
13. Silva, K.; Monteiro, N.M.; Almada, V.C.; Vieira, M.N. Development and early life history behaviour of aquarium reared *Syngnathus acus* (Pisces: Syngnathidae). J Mar Biol Ass UK 2006, 86, 1469-1472. https://doi.org/10.1017/S0025315406014536.
14. Piñeiro-Corbeira, C.; Iglesias, L.; Nogueira, R.; Campos, S.; Jiménez, A.; Regueira, M.; Barreiro, R.; Planas, M. Structure and trophic niches in mobile epifauna assemblages associated with seaweeds and habitats of syngnathid fishes in Cíes Archipelago (Atlantic Islands Marine National Park, NW Iberia). Front. Mar. Sci. 2021, 8, 773367. https://doi.org/10.3389/fmars.2021.773367.
15. Planas, M.; Piñeiro-Corbeira, C.; Bouza, C.; Castejón-Silvo, I.; Vera, M.; Regueira, M.; Ochoa, V.; Bárbara, I.; Terrados, J.; Chamorro, A.; Barreiro, R.; Hernández-Urcera, J.; Alejo, I.; Nombela, M.; García, M.E.; Pardo, B.G.; Peña, V.; Díaz-Tapia, P.; Cremades, J.; Morales-Nin, B. A multidisciplinary approach to identify priority areas for the monitoring of a vulnerable family of fishes in Spanish Marine National Parks. BMC Ecol. Evol. 2021, 21, 4. https://doi.org/10.1186/s12862-020-01743-z.
16. Hernández-Urcera, J.; Murillo, F.J.; Regueira, M.; Cabanellas-Reboredo, M.; Planas, M. Preferential habitats prediction in syngnathids using species distribution models. Mar. Environ. Res. 2021, 172, 105488. https://doi.org/10.1016/j.marenvres.2021.105488.
17. Manning, C.G.; Foster, S.J.;Vincent, A.C.J. A review of the diets and feeding behaviours of a family of biologically diverse marine fishes (Family Syngnathidae). Rev. Fish Biol. Fisheries 2019, 29, 197-221. https://doi.org/10.1007/s11160-019-09549-z
18. Ryer, C.H. Pipefish foraging: effects of fish size, prey size and altered habitat complexity. Mar. Ecol. Prog. Ser. 1988, 48, 37-45. https://doi.org/10.3354/meps08272.
19. Ape, F.; Corriero, G.; Mirto, S.; Pierri, C.; Lazic, T.; Gristina, M. Trophic flexibility and prey selection of the wild long-snouted seahorse *Hippocampus guttulatus* Cuvier, 1829 in three coastal habitats. Est. Coast. Shelf. Sea 2019, 224, 1-10. https://doi.org/10.1016/j.ecss.2019.04.034
20. DeNiro, M.J.; Epstein, S. Influence of diet on the distribution of carbon isotopes in animals. Geochi. Cosmochi. Act. 1978, 42, 495-506. https://doi.org/10.1016/0016-7037(78)90199-0.
21. DeNiro, M.J.; Epstein, S. Influence of diet on the distribution of nitrogen isotopes in animals. Geochi. Cosmochi. Act. 1981, 45, 341-351. https://doi.org/10.1016/0016-7037(81)90244-1
22. Hesslein, R.H.; Hallard, K.A.; Ramlal, P. Replacement of sulphur, carbon and nitrogen in tissue of growing broad whitefish (*Coregonus nasus*) in response to a change in diet traced by $\delta^{34}S$, $\delta^{13}C$ and $\delta^{15}N$. Can. J. Fish. Aquat. Sci. 1993, 50, 2071-2076. https://doi.org/10.1139/f93-230.
23. Vander Zanden, M.J.; Rasmussen, J.B. Variation in $\delta^{15}N$ and $\delta^{13}C$ trophic fractionation: Implications for aquatic food web studies. Limnol. Oceanogr. 2001, 46, 2061-2066. https://doi.org/10.4319/lo.2001.46.8.2061.
24. Gamboa-Delgado, J.; Cañavate, J.P.; Zerolo, R.; Le Vay, L. Natural carbon stable isotope ratios as indicators of the relative contribution of live and inert diets to growth in larval Senegalese sole (*Solea senegalensis*). Aquaculture 2008, 280, 190–197. https://doi.org/10.1016/j.aquaculture.2008.04.036.
25. Caut, S.; Angulo, E.; Courchamp, F. Variation in discrimination factors ($\Delta^{15}N$ and $\Delta^{13}C$): The effect of diet isotopic values and applications for diet reconstruction. J Applied Ecol. 2009, 46, 443-453. https://doi.org/10.1111/j.1365-2664.2009.01620.x.
26. Soto, D.X.; Wassenaar, L.I.; Hobson, K.A. Stable hydrogen and oxygen isotopes in aquatic food webs are tracers of diet and provenance. Funct Ecol, 2013, 27, 535-543. https://doi.org/10.1111/1365-2435.12054.
27. Hobson, K.A.; Barnett-Johnson, R.; Cerling, T. Using isoscapes to track animal migration. In *Isoscapes: Understanding movement, pattern, and process on earth through isotope mapping*; West, J.B., Bowen, G.J., Dawson, T.E., Tu, K.P., Eds.; Springer, Dordrecht, Netherlands, 2010; pp. 273-298.
28. Zbinden, J.A.; Bearhop, S.; Bradshaw, P.; Gill, B.; Margaritoulis, D.; Newton, J.; Godley, B.J. Migratory dichotomy and associated phenotypic variation in marine turtles revealed by satellite tracking and stable isotope analysis. Mar. Ecol. Prog. Ser. 2011, 421, 291-302. https://doi.org/10.3354/meps08871.

29. Doucett, R.R.; Hooper, W.; Power, G. Identification of anadromous and nonanadromous brook trout and their progeny in the Tabusintac River, New Brunswick, by means of multiple-stable-isotope analysis. Trans. Am. Fish. Soc. 1999, 128, 278-288. https://doi.org/10.1577/1548-8659(1999)128<0278:IOAANA>2.0.CO;2.
30. Sare, D.T.J.; Miller, J.S.; Longstaffe, F.J. Nitrogen- and carbon-isotope fractionation between mothers and offspring in red-backed voles (*Clethrionomys gapperi*). Can. J. Zool. 2005, 83, 712-716. https://doi.org/10.1139/z05-054.
31. Jardine, T.D; Chernoff, E.; Curry, R.A. Maternal transfer of carbon and nitrogen to progeny of sea-run and resident brook trout (*Salvelinus fontinalis*). Can. J. Fish. Aquat. Sci. 2008, 65, 2201-2210. https://doi.org/10.1139/F08-132.
32. Vaudo, J.J.; Matich, P..; Heithaus, M.R. Mother-offspring isotope fractionation in two species of placentatrophic sharks. J. Fish Biol. 2010, 77, 1724-1727. https://doi.org/10.1111/j.1095-8649.2010.02813.x.
33. Frankel, N.S.; Vander Zanden, A.B.; Reich, K.J.; Williams, K.L.; Bjorndal, K.A. Mother−offspring stable isotope discrimination in loggerhead sea turtles *Caretta caretta*. Endang. Species Res. 2012, 17, 133–138. https://doi.org/10.3354/esr00412.
34. UNESCO. World Heritage candidate. 2019, Available online: https://whc.unesco.org/en/tentativelists/6286/ (accessed on 13 Dec 2019).
35. Fernández, E.; Barañano, C.; Alejo, I.; Barreiro, R.; Bellas, J.; Besada, V.; Calviño-Cancela, M.; Cordero-Rivera, A.; González, A.; Méndez, G.; Navarro, L.; Piñeiro-Corbeira, C.; Planas, M.; Prego, R.; Saborido, F.; Sánchez, J.M.; Souza, J.; Villasante, S. *Islas Cíes: Un Ecosistema en la Frontera*. Concello de Vigo, Vigo, Spain, 2020; 240 p.
36. Planas, M.; Chamorro, A.; Quintas, P.; Vilar, A. Establishment and maintenance of threatened long-snouted seahorse, *Hippocampus guttulatus*, broodstock in captivity. Aquaculture 2008, 283, 19-28. https://doi.org/10.1016/j.aquaculture.2008.06.023.
37. Valladares, S.; Planas, M. Non-lethal dorsal fin sampling for stable isotope analysis in seahorses. Aquat. Ecol. 2012, 46, 363-370. https://doi.org/10.1007/s10452-012-9407-y.
38. Bouza, C.; Vera, M.; Pardo, B.G.; Planas, M.; Castejón-Silvo, I. Caracterización genética de signátidos en los Parques Nacionales de las Islas Atlánticas y del Archipiélago de Cabrera. In: *Proyectos de Investigación en Parques Nacionales: 2015-2018*. Amengual, J., Ed.; Organismo Autónomo de Parques Nacionales, Ministerio de Agricultura, Alimentación y Medio Ambiente, Madrid, Spain, (*in press*).
39. Wilson, A.B.; Vincent, A.C.J.; Ahnesjö, I.; Meyer, A. Male pregnancy in seahorses and pipefishes (Family Syngnathidae): Rapid diversification of paternal brood pouch morphology inferred from a molecular phylogeny. J. Hered. 2001, 92, 159-66. https://doi.org/10.1093/jhered/92.2.159.
40. Woodall, L.C.; Koldewey, H.J.; Boehm, J.T.; Shaw, W. Past and present drivers of population structure in a small coastal fish, the European long snouted seahorse *Hippocampus guttulatus*. Conserv. Genet. 2015, 16, 1139-1153. https://doi.org/10.1007/s10592-015-0728-y
41. Emlen, S.T.; Oring, L.W. Ecology, sexual selection, and the evolution of mating systems. Science 1977, 197, 215-223. https://doi.org/10.1126/science.327542.
42. Monteiro, N.M.; Almada, V.C.; Vieira, M.N. Early life history of the pipefish *Nerophis lumbriciformis* (Pisces: Syngnathidae). J. Mar. Biol. Ass. UK 2003, 83, 1179-1182. https://doi.org/10.1017/S0025315403008452h.
43. Sommer, S.; Whittingto,n C.M.; Wilson, A.B. Standardised classification of pre-release development in male-brooding pipefish, seahorses, and seadragons (Family Syngnathidae). BMC Dev. Biol. 2012, 12, 12-39. https://doi.org/10.1186/1471-213X-12-39. https://doi.org/10.1186/1471-213X-12-39.
44. Post, D.M. Using stable isotopes to estimate trophic position: models, methods, and assumptions. Ecology 2002, 83, 703-718. https://doi.org/10.1890/0012-9658(2002)083[0703:USITET]2.0.CO;2.
45. Post, D.M.; Layman, C.A.; Arrington, G.; Takimoto, J.; Quatrocchi, J.; Montaña, C.G. Getting to the fat of the matter: models, methods and assumptions for dealing with lipids in stable isotope analyses. Oecol. 2007, 152, 179-189. https://doi.org/10.1007/s00442-006-0630-x.
46. Planas, M.; Paltrinieri, A.; Dias-Carneiro, M.D.; Hernández-Urcera, J. Effects of tissue preservation on carbon and nitrogen stable isotope signatures in Syngnathid fishes and prey. Animals 2020, 10, 2301. https://doi.org/10.3390/ani10122301.
47. Jaschinski, S.; Hansen, T.; Sommer, U. Effects of acidification in multiple stable isotope analyses. Limnol. Oceanogr.: Methods 2008, 6, 12-15. http://dx.doi.org/10.4319/lom.2008.6.12.
48. Vafeiadou, A.M.; Adão, H.; De Troch, M.; Moens, T. Sample acidification effects on carbon and nitrogen stable isotope ratios of macrofauna from a *Zostera noltii* bed. Mar. Freshw. Res. 2013, 64, 741-745. http://dx.doi.org/10.1071/MF12169.
49. R Core Team. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. 2019; Available online: https://www.R-project.org (accessed on 13 Dec 2020).
50. Kassambara, A. ggpubr: 'ggplot2' based publication ready plots. 2020; Available online: https://CRAN.R-project.org/package=clinfun (accessed on 23 Feb 2020).
51. Giraudoux, P.; Antonietti, J.P.; Beale, C.; Pleydell, D.; Treglia, M. Spatial analysis and data mining for field ecologists v. 1.6.9. 2018; Available online: https://cran.r-project.org/web/packages/pgirmess/index.html (accessed on 16 Jan 2018).
52. Clarke, K.R. Non-parametric multivariate analyses of changes in community structure. Austral Eco. 1993, 18, 117-143. https://doi.org/10.1111/j.1442-9993.1993.tb00438.x.
53. Oksanen, J.; Guillaume Blanchet, F.; Friendly, M.; Kindt, R.; Legendre, P.; McGlinn, D.; Minchin, P.R.; O'Hara, R.B.; Simpson, G.L.; Solymos, P.; Stevens, M.H.H.; Szoecs, E.; Wagner, H. vegan: Community Ecology Package. R package version 2.5-7. 2020; Available online: https://CRAN.R-project.org/package=vegan (accessed on 13 Feb 2020).

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54. Husson, F.; Josse, J.; Le, S.; Mazet, J. FactoMineR: Multivariate exploratory data analysis and data mining. 2020; https://CRAN.R-project.org/package=FactoMineR (accessed on 20 March 2020).
55. Kassambara, A. factoextra: Extract and visualize the results of multivariate data analyses. 2020; Available online: https://CRAN.R-project.org/package=factoextra (accessed on 13 Feb 2020).
56. Wei, T.; Simko, V.; Levy, M.; Xie, Y.; Jin, Y.; Zemla, J. corrplot: Visualization of a correlation matrix. WWW document. 2017; Available online: https://CRAN.R-project.org/package=corrplot (accessed on 13 Dec 2020).
57. Quezada-Romegialli, C.; Jackson, A.L.; Hayden, B.; Kahilainen, K.K.; Lopes, C.; Harrod, C. tRophicPosition, an R package for the Bayesian estimation of trophic position from consumer stable isotope ratios. Methods Ecol Evol. 2018, 9, 1592-1599. https://doi.org/10.1111/2041-210X.13009.
58. Planas, M.; Chamorro, A.; Paltrinieri, A.; Campos, S.; Nedelec, K.; Hernández-Urcera, J. Effect of diet on breeders and inheritance in Syngnathids: application of isotopic experimentally derived data to field studies. Mar. Ecol. Prog. Ser. 2020, 650, 107-123. http://dx.doi.org/10.3354/meps13315.
59. Swanson, H.K.; Lysy, M.; Power, M.; Stasko, A.D.; Johnson, J.D.; Reist, J.D. A new probabilistic method for quantifying n-dimensional ecological niches and niche overlap. Ecology 2015, 96, 318-324. https://doi.org/10.1890/14-0235.1.
60. Jackson, A.L.; Inger, R.; Parnell, A.C.; Bearhop, S. Comparing isotopic niche widths among and within communities: SIBER - Stable isotope Bayesian ellipses in R. J. Anim. Ecol. 2011, 80, 595-602. https://doi.org/10.1111/j.1365-2656.2011.01806.x.
61. Lysy, M.; Stasko, A.D.; Swanson, H.K. nicheROVER: (Niche) (R)egion and Niche(Over)lap metrics for multidimensional ecological niches. R package version 1.1.0. 2014; Available online: https://CRAN.R-project.org/package=nicheROVER (accessed on 13 Dec 2020).
62. Stock, B.; Semmens, B. MixSIAR GUI user manual. 2016; Version 3. 2016; https://github.com/brianstock/MixSIAR (accesed on 13 Dec 2020). https://doi.org/10.5281/zenodo.1209993.
63. Stock, B.C.; Jackson, A.L.; Ward, E.J.; Parnell, A.C.; Phillips, D.L.; Semmens, B.X. Analyzing mixing systems using a new generation of Bayesian tracer mixing models. PeerJ 2018, 6: e5096. https://doi.org/10.7717/peerj.5096.
64. Phillips DL, Gregg JW (2003) Source partitioning using stable isotopes: coping with too many sources. Oecologia 136:261-269. https://doi.org/10.1371/journal.pone.0009672.
65. Smith, J.A.; Mazumder, D.; Suthers, I.M.; Taylor, M.D. To fit or not to fit: evaluating stable isotope mixing models using simulated mixing polygons. Methods Ecol. Evol. 2013, 4, 612-618. https://doi.org/10.1111/2041-210X.12048.
66. Wickham, H.; Chang, W.; Henry, L.; Pedersen, T.L.; Takahashi, K.; Wilke, C.; Woo, K.; Yutani, H.; Dunnington, D. ggplot2: Create elegant data visualisations using the grammar of graphics. 2020; Available online: https://CRAN.R-project.org/package=ggplot2. (accessed on 17 Sept 2019).
67. Sarkar, D.; Andrews, F.; Wright, K.; Klepeis, N.; Murrell, P. lattice: Trellis graphics for R. 2020; Available online: https://cran.r-project.org/web/packages/lattice/index.html (accessed on 17 Sept 2019).
68. Lazzari, K.; Able, K.W. Northern pipefish, *Syngnathus fuscus*, occurrences over the Mid-Atlantic Bight continental shelf: evidence of seasonal migration. Environ. Biol. Fishes 1990, 27, 177-185. https://doi.org/10.1007/BF00001671.
69. Franzoi, P.; Maccagnani, R.; Rossi, R.; Ceccherelli, V.U. Life cycles and feeding habits of *Syngnathus taenionotus* and *S. abaster* (Pisces, Syngnathidae) in of the PO River Delta (Adriatic Sea). Mar Ecol Prog Ser. 1993, 97, 71-81. https://doi.org/10.3354/meps097071.
70. Monteiro, N.M.; Almada, V.C.; Santos, A.M.; Vieira, M.N. The breeding ecology of the pipefish *Nerophis lumbriciformis* and its relation to latitude and water temperature. J. Mar. Biol. Ass. UK 2001, 81, 1031-1033. https://doi.org/10.1017/S002531540100501X.
71. Masonjones, H.D.; Rose, E.; McRae, L.B.; Dixson, D.L. An examination of the population dynamics of syngnathid fishes within Tampa Bay, Florida, USA. Curr. Zool, 2010, 56, 118–133. https://doi.org/10.1093/CZOOLO/56.1.118.
72. Valle, C.; Bayle, J.T.; Ramos, A.A. Weight-length relationships for selected fish species of the western Mediterranean Sea. J. Appl. Ichthyol. 2003, 19, 261–262. http://dx.doi.org/10.1046/j.1439-0426.2003.00492.x.
73. Day, J.H.; Blaber, S.J.M.; Wallace, H. Estuarine fishes. In *Estuarine ecology with particular reference to southern Africa*. Day, J.H., Ed.; AA Balkema, Cape Town, Soth Africa, 1981; pp. 197-221.
74. Harrison, T.D. Length-weight relationships of fishes from South African estuaries. J. Appl. Ichthyol. 2001, 17, 46-48. http://dx.doi.org/10.1046/j.1439-0426.2001.00277.x.
75. Dawson, C.E. Syngnathidae. In *Fishes of the North-eastern Atlantic and the Mediterranean*. Whitehead, P.J.P.; Bauchot, M.L.; Hereau, J.C.; Nielsen, J.; Tortonese, E., Eds.;, UNESCO, Paris, France; 1986; pp. 628-639.
76. Veiga, P.; Machado, D.; Almeida, C.; Bentes, L.; Monteiro, P.; Oliveira, F.; Ruano, M.; Erzini, K.; Gonçalves, J.M.S. Weight-length relationships for 54 species of the Arade estuary, southern Portugal. J. Appl. Ichthyol. 2009, 25, 493-496. http://dx.doi.org/10.1111/j.1439-0426.2009.01230.x.
77. Koutrakis, E.T.; Tsikliras, A.C.; Length-weight relationships of fishes from three northern Aegean estuarine systems (Greece). J Appl. Ichthyol. 2003, 19, 258-260. https://doi.org/10.1046/j.1439-0426.2003.00456.x.
78. Watanabe, S.; Watanabe, Y. Brooding season, sex ratio, and brood pouch development in the seaweed pipefish, *Syngnathus schlegeli*, in Otsuchi Bay, Japan. Ichthyol. Res. 2001, 48, 155-160. https://doi.org/10.1007/s10228-001-8130-9.
79. Ahnesjö, I. Consequences of male brood care: Weight and number of newborn in a sex-role reversed pipefish. Funct .Ecol. 1992, 6, 274-281. http://dx.doi.org/10.2307/2389517.
80. Berglund, A.; Rosenqvist, G. Selective males and ardent females in pipefishes. Behav. Ecol. Sociobiol. 1993, 32, 331-336. http://dx.doi.org/10.1007/BF00183788.

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81. Ripley, J.L.; Foran, C.M. Differential parental nutrient allocation in two congeneric pipefish species (Syngnathidae: Syngnathus spp.). J. Exp. Biol. 2006, 209, 1112-1121. https://doi.org/10.1242/jeb.02119.
82. Wilson, A.B.; Ahnesjö, I.; Vincent, A.C.J.; Meyer, A. The dynamics of male brooding, mating patterns, and sex roles in pipefishes and seahorses (family Syngnathidae). Evolution 2003, 57, 1374-1386. https://doi.org/10.1111/j.0014-3820.2003.tb00345.x.
83. Ahnesjö, I,.; Forsgren, E.; Kvarnemo, C. Variation in sexual selection in fishes. In *Fish Behaviour*. Magnhagen, C.; Braithwaite, V.A.; Forsgren, E.; Kapoor, B.G., Eds.; Science Publishers Inc., Boca Raton, USA. 2008; pp. 303-336.
84. Steffe AS, Westoby M, Bell JD (1989) Habitat selection and diet in two species of pipefish from seagrass: sex differences. Mar Ecol Prog Ser 55:23-30. http://dx.doi.org/10.3354/meps055023.
85. Rosenqvist, G. Sex role reversal in a pipefish, Mar. Behav. Physiol. 1993, 23, 219-230. https://doi.org/10.1080/10236249309378867.
86. Svensson, I. Reproductive costs in two sex-role reversed pipefish species (Syngnathidae). J. Anim. Ecol. 1988, 57, 929-942. http://dx.doi.org/10.2307/5102.
87. Vincent, A.; Ahnesjö, I.; Berglund, A.; Rosenqvist, G. Pipefishes and seahorses: are they all sex role reversed? Trends Ecol. Evol. 1992; 7, 237-241. https://doi.org/10.1016/0169-5347.
88. Berglund, A.; Rosenqvist, G. Sex role reversal in pipefish. Adv. Study Behav. 2003, 32, 131-167. https://doi.org/10.2307/3565641.
89. Rosenqvist, G.; Berglund, A. Sexual signals and mating patterns in Syngnathidae. J. Fish Biol. 2011, 78, 1647-1661 https://doi.org/10.1111/j.1095-8649.2011.02972.x.
90. Jones, A.G.; Avise, J.C. Mating systems and sexual selection in male-pregnant pipefishes and seahorses: Insights from microsatellite-based studies of maternity. J. Hered. 2001, 92, 150-158. https://doi.org/10.1093/jhered/92.2.150.
91. Finn, R.N.; Henderson, J.R.; Fyhn, H.J. Physiological energetics of developing embryos and yolk-sac larvae of Atlantic cod (*Gadus morhua*). II. Lipid metabolism and enthalpy balance. Mar. Biol. 1995, 124, 371–379. https://doi.org/10.1007/BF00363910.
92. Wiegand, M.D. Composition, accumulation and utilization of yolk lipids in teleost fish. Rev. Fish. Biol. Fish. 1996, 6, 259-286. http://dx.doi.org/10.1007/BF00122583.
93. Rainuzzo, J.R.; Reitan, K.I.; Olsen, Y. The significance of lipids at early stages of marine fish: a review. Aquaculture 1997, 155, 103-115. https://doi.org/10.1016/S0044-8486(97)00121-X.
94. Rønnestad, I.; Koven, W.; Tandler, A.; Harel, M.; Fyhn, H.J. Utilisation of yolk fuels in developing eggs and larvae of European sea bass (*Dicentrarchus labrax*). Aquaculture 1998, 162, 157-170. http://dx.doi.org/10.1016/S0044-8486(98)00203-8.
95. Carcupino, M.; Baldacci, A.; Mazzini, M.; Franzoi, P. Morphological organization of the male brood pouch epithelium of *Syngnathus abaster* Risso (Teleostea: Syngnathidae): before, during and after egg incubation. Tissue & Cell 1997, 29, 21-30. https://doi.org/10.1016/s0040-8166(97)80068-7.
96. Haresign, T.W.; Shumway, S.E. Permeability of the marsupium of the pipefish *Syngnathus fuscus* to ($^{14}$C)-alpha-amino-isobutyric acid. Comp. Biochem. Physiol. A 1981, 69, 603-604. https://doi.org/10.1016/0300-9629(81)93030-9.
97. Ripley, J.L.; Foran, C.M. Direct evidence for embryonic uptake of paternally derived nutrients in two pipefishes (Syngnathidae: *Syngnathus* spp.). J. Comp. Physiol. B 2009, 179, 325-333. https://doi.org/10.1007/s00360-008-0316-2.
98. Kvarnemo, C.; Mobley, K.B.; Partridge, C.; Jones, A.G.; Ahnesjö, I. Evidence of paternal nutrient provisioning to embryos in broad-nosed pipefish *Syngnathus typhle*. J. Fish Biol. 2011, 78, 1725-1737. http://dx.doi.org/10.1111/j.1095-8649.2011.02989.x.
99. Caut, S.; Fossette, S.; Guirlet, E.; Angulo, E.; Das, K.; Girondot, M.; Georges, J.Y. Isotope analysis reveals foraging area dichotomy for Atlantic leatherback turtles. PLoS ONE 2008, 3: e1845. http://dx.doi.org/10.1371/journal.pone.0001845.
100. McMeans, B.C.; Olin, J.A.; Benz, G.W. Stable-isotope comparisons between embryos and mothers of a placentatrophic shark species. J. Fish Biol. 2009, 75, 2464-2474. http://dx.doi.org/10.1111/j.1095-8649.2009.02402.x.
101. Carpentier, A.S.; Booth, D.T..; Arthur, K.E.; Limpus, C.J. Stable isotope relationships between mothers, eggs and hatchlings in loggerhead sea turtles *Caretta caretta*. Mar. Biol. 2015, 162, 783-797. https://doi.org/10.1007/s00227-015-2624-x.
102. de Lussanet, M.H.C.; Muller, M. The smaller your mouth, the longer your snout: predicting the snout length of *Syngnathus acus, Centriscus scutatus* and other pipette feeders. J. R. Soc. Interface 2007, 4, 561-573. https://doi.org/10.1098/rsif.2006.0201.
103. Roelke, D.L.; Sogard, S.M. Gender-based differences in habitat selection and activity level in the northern pipefish (*Syngnathus fuscus*). Copeia 1993, 2, 528-532. http://dx.doi.org/10.2307/1447155.
104. Ahnesjö, I. Behavioural temperature preference in a brooding male pipefish *Syngnathus typhle*. J. Fish Biol. 2008, 73, 1039-1045. https://doi.org/10.1111/j.1095-8649.2008.01977.x
105. Stephens, P.A.; Boyd, I.L.; McNamara, J.M.; Houston, A.I. Capital breeding and income breeding: Their meaning, measurement, and worth. Ecology 2009, 90, 2057-2067. https://doi.org/10.1890/08-1369.1.
106. Planas, M.; Olivotto, I.; González, M.J.; Laurà, R.; Zarantoniello, M. A multidisciplinary experimental study of the effects of breeders diet on newborn seahorses (*Hippocampus guttulatus*). Front Mar Sci 2020, 7, 638. https://doi.org/10.3389/fmars.2020.00638.
107. Planas, M. Carry-over effects of pre-breeding diets on seahorse (*Hippocampus reidi*) reproductive success. Aquaculture 2021, 533, 736148. https://doi.org/10.1016/j.aquaculture.2020.736148.

108. Sogabe, A.; Mohri, K.; Shoji, J. Reproductive seasonality of the seaweed pipefish *Syngnathus schlegeli* (Syngnathidae) in the Seto Inland Sea, Japan. Ichthyol. Res. 2012, 59, 223-229. https://doi.org/10.1007/s10228-012-0278-y.