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Feeding Ecology of Pipefish Species Inhabiting Mediterranean Seagrasses

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Abstract

Pipefish are a vulnerable and diverse group of ichthyofauna tightly associated with seagrass meadows, key habitats in shallow marine areas. Despite their charismatic role, the main ecological features, habitat, and diet of this group remain largely unknown. This study focuses on assessing pipefish habitat and feeding preferences, including different hosting seagrasses such as *Posidonia oceanica* and *Cymodocea nodosa* from the Balearic Islands, western Mediterranean. Four species (*Syngnathus typhle*, *S. abaster*, *Nerophis ophidion,* and *N. maculatus*) were found associated to different seagrasses. *S. typhle* and *N. maculatus* were more frequent in *P. oceanica* meadows, while *S. abaster* and *N. ophidion* in *C. nodosa*. Individuals of all species captured in *P. oceanica* were larger than those living in *C. nodosa*, suggesting a size-dependent habitat preference. Feeding preferences, however, were driven by prey availability and fish features, e.g., head/snout morphology. For the first time in the western Mediterranean, a thorough description of the diet and potential prey of this group was carried out. Epifaunal assemblages (potential prey) were dominated in both habitats by harpacticoid copepods and gammarid amphipods, and they were also the primary prey according to stomach contents of all species. These results can contribute to future pipefish conservation and management actions, such as targeting crucial habitat identification and designing culture and reintroduction protocols.

Keywords: Syngnathid; morphometry; epifauna; diet; seasonality; Balearic Islands.

Introduction

Syngnathidae is a family of fish found in temperate and tropical seas across the world, which includes seahorses, pipefishes, pipehorses, and seadragons. The name of this family is derived from Greek, “syn”, meaning “fused” or “together”, and “gnathus”, meaning “jaws”. Thus, the elongated tubular snout with a fused and toothless jaw is a particular trait of this family (Leysen *et al.*, 2011), which determines their particular feeding ecology as specialized suction feeders (Muller & Osse, 1984). The type and size of prey consumed vary depending on the species, size of the snout, and the mouth opening (Lyons & Dunne, 2004). Syngnathids mimic seagrass leaves in shape, color, and orientation, and thus, within the meadows, they remain inconspicuous to predators (Howard & Koehn, 1985; Fuller & Berglund, 1996). Their predation strategies are limited by their rigid body and mode of swimming, mainly done by fast undulations of the dorsal and pectoral fins, combined with body undulations for rapid movement (Lindsey, 1978; Ashley-ross, 2002; Neutens *et al.*, 2017). They are cryptic and sit-and-wait predators (Tipton & Bell, 1988; Franzoi *et al.*, 1993).

Among syngnathids, pipefish are the most abundant group in Mediterranean waters (Franzoi *et al.*, 2010), where 10 species have been reported, all of them included in the IUCN Red List of Threatened Species. Pipefish are a charismatic component of the ichthyofauna in vegetated coastal and estuarine lagoon habitats (Howard & Koehn, 1985; Campolmi *et al.*, 1997). In the Mediterranean Sea, they are usually associated with seagrass meadows (Vincent *et al.*, 2011) as nursery and feeding grounds (Teixeira & Musick, 1995). In the oligotrophic waters of the Mediterranean (Fourqurean *et al.*, 2007), there are seven seagrass species (Short & Coles, 2001), being *Posidonia oceanica* (Mediterranean endemism) and *Cymodocea nodosa,* the most abundant between 0.2–45 m depth (Green & Short, 2003). *P. oceanica* creates dense meadows (300 to 1000 shoots/m^2) with a compact root-rhizome mat and a high leaf stratum, achieving canopy heights of 1 m during early summer (Drew & Jupp, 1976). *C. nodosa*, in turns, may also develop high density meadows (up to 2000 shoots/m^2) (Terrados *et al.*, 2006) but the leaf stratum is lower (<50 cm), leaves are thinner with a less compact rhizome mat (Rull *et al.*, 1996). The abundance and diversity of the epifaunal communities associated to these meadows differ in species composition and abundance, related to plant characteristics such as leaf morphology or shoot density (Orth *et al.*, 1984). Mediterranean epifaunal communities are dominated by copepods and amphipods (Connolly & Butler, 1996; Sánchez-Jerez *et al.*, 1999), which also play an important role as trophic resources for fish. It has been previously reported that the feeding preferences of pipefish correspond to small crustacean conforming seagrass epifauna (Lyons & Dunne, 2004; Oliveira *et al.*, 2007; Taçkavak *et al.*, 2010). However, seagrasses are facing a rapid decline worldwide due to anthropogenic stressors, which may affect their growth and distribution and may lead to the local extinction or displacement of species associated with them (Waycott *et al.*, 2009).

In the scenario of threatened species associated with habitats in regression, it is relevant to determine the distribution and abundance and relationships between habitat and life traits. Therefore, the main objectives of this study were to assess the abundance of syngnathid species in western Mediterranean meadows and to determine their feeding habits, linking prey preferences with pipefish morphometry and with the epifaunal community associated to seagrasses inhabited by pipefish.

Materials and Methods

Study area and sampling design

The study was conducted in two seagrass habitats of the Balearic Islands, Mediterranean Sea. A comparative approach was adopted; the same procedures were used in two *Posidonia oceanica* meadows in South Mallorca (i.e., Cala Gamba, 39º32.050'N 2º42.526'E; and Port d'Andratx, 39º32.356'N 2º22.437'E) and one *Cymodocea nodosa* meadow in Cabrera Archipelago (i.e., Es Burri Bay, 39º8.604'N 2º57.524'E). Samples were taken every two months, approximately from June 2016 to December 2018. During the study, seawater temperature ranged between 14 ºC and 27 ºC. Temporal patterns were assessed considering a warm season and a cold season (T^a >20ºC and T^a <20 ºC, respectively) (Shaltout & Omstedt, 2014).

Field sampling

Pipefish were first sampled by visual censuses and second by using a small trawl net called "gambera" or "gánguil" (in Spanish) traditionally used to catch fishing bait; a light-weight epibenthic trawl with a rolling stainless steel cylinder in the bottom of the mouth that protects the *P. oceanica* and *C. nodosa* leaves from snagging and tearing while operating. The beam trawl was 3 m long and had a 0.8 m mouth aperture with a 1.2 cm^2 mesh size (Alós *et al.*, 2014). A total of 151 transects were run that varied in length between 30 and 500 meters, depending on the meteorological conditions and seafloor topography. GPS positions were taken at the beginning and end of each transect to estimate the trawled area. The transects were performed during daylight hours at a depth range of 1.7–16.5 m. Species identification was done on board (n = 105) and fish were returned to the sea, except for a subsample of fish from the total (those captured between June 2016 and April 2018). These fish were anesthetized to death with an overdose of anesthetic in solution (tricaine methanesulfonate, MS-222; concentration: 0.1–0.2 g/L), preserved in absolute ethanol, labeled and transported to the laboratory inside coolers with ice pads for length measurements in the laboratory and further procedures (e.g., stomach content, morphological features).

The epifaunal community was sampled in order to assess potential or available prey for syngnathids inhabiting seagrass meadows. Five replicated samples were randomly collected by scuba divers in each meadow and fish sampling day. Nylon mesh bags (125 μm) covering a 314 cm^2 surface of sea-bottom (20 cm diameter) were used. The bag was placed over the seagrass seafloor and leaves were removed by cutting at sediment surface level with scissors, so that all the organisms conforming to epifauna living associated with the seagrass leaves were trapped in the bag (Tuya *et al.*, 2011). Samples were processed right after collection, labeled and conserved in 70% ethanol, for further identification and analysis in the laboratory. The abundances of each taxonomic group were standardized by dividing total abundances for every 100 cm^2 of the foliar surface.

Laboratory procedures

Pipefish total length (distance from the tip of the snout to the tip of the longer lobe of the caudal fin) was measured to the lowest millimeter (n = 102 out of the total n = 105; three individuals were not photographed, and therefore not analyzed, before turning them back to the sea) with the image processing and analysis device ImageJ2 (Rueden *et al.*, 2017), and a subsample (n = 43) was used to take head morphometric measures (Fig. 1) using a precision caliper. To minimize the influence of size differences on the subsequent results, the morphometric measurements were standardized (Kupren *et al.*, 2008; Lawson *et al.*, 2013; Yildiz *et al.*, 2015). Head Length (HL) was standardized by Total Length (TL) and Snout Length (SL), Minimum Snout Height (MSH), Eye Diameter (ED), Mouth Height (HM) and Mouth Width (WM) were standardized by HL (Cakić *et al.*, 2002; Yildiz *et al.*, 2015).

For the stomach contents, after fixation of the whole individuals in 70% ethanol, necropsy was performed at the laboratory. Fishes were dissected and their digestive tracts extracted and opened. Syngnathids have a tube-shaped digestive tract, with no differentiation between stomach and intestines (Tipton & Bell, 1988), so all digestive tract was analyzed. A transverse incision was used to expose the contents from the esophagus to the anus. The food items were removed and identified at the minimum taxonomic level possible (Abel & Riedl, 1986) under a LEICA MZ16 binocular stereo microscope. Empty tracts were also recorded, and sometimes items

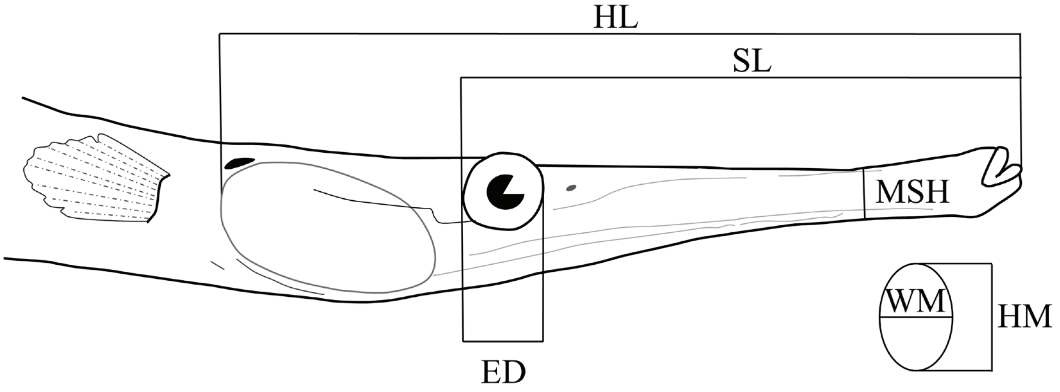

Fig. 1: Representation of the biometric parameters analyzed on pipefish heads [modified from Franzoi *et al.* (1993)]. HL, head length (distance from the tip of the snout to the operculum); SL, snout length (distance from the tip of the snout to the end of the eye); MSH, minimum snout height (minimum vertical distance in the middle of the snout); ED, eye diameter (horizontal distance of the eye); HM, mouth height (maximum height opening of the mouth); WM, mouth width (maximum width opening of the mouth). All of them were measured in mm.

were impossible to identify due to digestion. Because of the difficulty in determining the individual weights and lengths of prey items, they were just counted and then pooled into the following dietary categories: harpacticoid copepods, gammarid and caprellid amphipods, ostracods, decapods, acarus, and teleosts.

Epifaunal community samples were processed, separating leaves and the mobile organisms. Fauna was analyzed under a LEICA MZ16 binocular, categorizing individuals into broad taxonomic units to class/order level (Abel & Riedl, 1986) such as: copepods, gammarid and caprellid amphipods, ostracods, decapods, isopods, tanaidaceans, cumaceans, mysidaceans, acarus, pantopods, cnidarians, gastropods, polyplacophora, bivalves, annelids, sipunculids, chaetognaths, turbellarians, echinoderms, nematodes, and teleost. The length and width of all leaves in each sample were measured, as well as leaf and epiphyte dry weight, in order to determine foliar surface and foliar biomass as a proxy of habitat availability and to standardize organism abundances. Community diversity was also determined by the Shannon-Wiener Diversity Index (Spellerberg & Fedor, 2003).

Data analysis

Pipefish presence/absence, number of captures, and estimated abundances (average number of individuals per squared kilometer of trawled seafloor surface and their standard errors) were calculated for each transect for the different species, seasons, and habitats. The low numbers of individuals collected in many transects discouraged statistical analysis to evaluate differences in pipefish abundances among species and locations. For this reason, and due to the similar ecological characteristics of both meadows (i.e., depth, extension, and water temperature), Cala Gamba and Port d'Andratx data were merged as the *P. oceanica* habitat. Pipefish abundances were graphically evaluated and body size distributions were compared with the Mann–Whitney U test in both habitats (*P. oceanica* and *C. nodosa*) and seasons (cold and warm) for each species. Throughout the study, non-parametric analyses were used when neither the untransformed nor the logarithmic or square root transformed data met normality.

Pipefish morphometric measurements (HL, SL, MSH, ED, HM, and WM) were evaluated on a pipefish subsample through principal component analysis (PCA) in order to identify the main morphometric characters causing differences between species. A general linear model (GLM) was used to test correlations between morphometric characters and if these correlations were species-dependent. A one-way ANOVA analysis (factor: species; levels: *S. typhle*; *S. abaster*, *N. maculatus,* and *N. ophidion*) was used comparing the main character identified by PCA to test if differences between species were significant. To test differences between head morphometry characters of each species depending on the habitat and season, a one-way ANOVA was performed in those species where we had found individuals in the same habitat during both seasons or in both habitats during the same season. Factors used in this ANOVA were habitat (levels: *P. oceanica* and *C. nodosa*) and season (levels: cold and warm), depending on the species.

Feeding preferences of pipefish were assessed through four indexes: the frequency of occurrence of a specific prey (%O = [frequency of prey item/total frequency of overall prey items in this species] x100); frequency of appearance (%A = [number of stomachs containing prey i/number of stomachs containing prey] x100); prey-specific abundance (%Pi = [number of prey i in stomachs containing prey i/total number of prey in stomachs containing prey i] x100), and vacuity index (%VI = [number of empty stomachs/total number of stomachs] x100); and through Amundsen (1996) modification of the Costello (1990) graphical analysis method. A principal component analysis was used to evaluate the differences between the diets of each species. To test significant contribution of different prey types to these differences, Kruskal–Wallis tests were performed. Differences in the diet, depending on habitat and season, were tested with one-way ANOVA with the same criteria as the previous ANOVA. Factors for this analysis were habitat (levels: *P. oceanica* and *C. nodosa*) and seasons (levels: cold and warm). The Spearman correlation coefficient was used to test if morphometric characters and stomach contents (%O) were correlated.

Two analytical approaches were used to study the epifaunal community diversity: direct analysis of community composition (PERMANOVA) and the calculation of the Shannon–Wiener Index (H'). Differences in standardized abundances (i.e., abundance of each taxa/100 cm^2 of leaf) of epifaunal communities between habitats and seasons were firstly analyzed with a two-factors PERMANOVA. This approach was based on the null hypothesis of no difference in the community assemblage composition between the two habitats and across the two seasons considered. A Kruskal–Wallis test was used to evaluate which taxa had an influence on the differences found among sampling habitats and seasons. Spearman correlation coefficient was also used to evaluate the association between coincident groups of epifaunal community assemblages (standardized abundance by foliar surface) and pipefish stomach contents (%O).

All graphics were plotted using SigmaPlot 8.0.2 and ggplot2 package for RStudio (Wickham, 2016). Statistical analyses were performed using PERMANOVA+ add on PRIMER 6 (Plymouth Routines in Multivariate Ecological Research) and STATISTICA 7.1 (StatSoft Inc., Tulsa, OK, USA) software.

Results

Pipefish populations and biometrics

A total of 105 pipefish specimens of 4 different species (*Syngnathus* t*yphle*, *S. abaster, Nerophis maculatus,* and *N. ophidion*) were captured during the study (Table 1). *S. typhle* was the most abundant species in the *P. oceanica* meadows during the study period, but it was mainly captured during the warm season. Conversely, in *C. nodosa* meadow*, S. typhle* was captured in both warm and cold seasons. *S. abaster* dominated the captures throughout the year in *C. nodosa*. Regarding *N. maculatus* (N = 11) and *N. ophidion*, (N = 11), the first was more abundant in *P. oceanica* and the second in *C. nodosa,* both during the cold season.

All pipefish species showed higher TL in *P. oceanica* meadows (mean values for fish found in *P. oceanica* and *C. nodosa* respectively were: S. *typhle:* 13.3 cm and 7.9 cm; *S. abaster*: 10.3 cm and 8.8 cm; *N. maculatus*: 25.0 cm and 9.0 cm; *N. ophidion*: 19.9 cm and 14.0 cm), but significant differences were only found for *S. typhle* (U = 79.00, Z = 3.41, p <0.005) and *N. maculatus* (U = 0.00, Z = 2.56, p <0.05) (Fig. 2). No significant differences in TL were found for *S. abaster* or *N. ophidion* between habitats. Considering TL related to seasons, significant differences were only revealed for *S. abaster* in *C. nodosa* (U = 84.00, Z = -3.28, p <0.005), where pipefish captured during the cold season presented longer total lengths (mean = 10.05 cm; SE = 0.44) than in the warm season (mean = 7.57 cm; SE = 0.55).

Morphometric descriptors %HL/TL and %SL/HL were the main contributors to the morphometric variability among pipefish species (96.1% of variation explained by axis 1, eigenvalue=39.4), and both descriptors were strongly correlated between them (Pr coefficient >0.001) and with the rest of morphological descriptors measured (Pr coefficients >0.05). These correlations were not species-dependent except for %HL/TL-%ED/HL. Subsequent analyses of morphological features were then carried out only with %HL/TL. One-way ANOVA revealed statistically significant differences among the four species considered (%HL/TL F = 90.6065). *S. typhle* presented the highest %HL/TL, followed by *S. abaster, N. ophidion* and *N. maculatus,* respectively (Fig. 3). Comparisons between habitats for *S. typhle* showed no significant differences during the warm season (F = 3.710). No differences between seasons were found for *S. abaster* on *C. nodosa* (F= 2.9487).

Feeding preferences

Harpacticoid copepods and gammarid amphipods were the most frequent pipefish preys in the stomach content analyses (Table 2, Fig. 4). For *S. typhle*, the main prey was harpacticoid copepods in both habitats (%O = 76-97.7; %A

Table 1. Pipefish total number of captures (Abund) and estimated densities (Dens: number of individuals per squared kilometer) for each species captured during the study per habitat and season.

		P. oceanica		**C. nodosa**	
		Warm	**Cold**	**Warm**	**Cold**
S. typhle	Abund // Dens	20 // 6.15	0 // 0	16 // 2.36	5 // 1.11
S. abaster	Abund // Dens	0 // 0	1 // 0.28	20 // 2.95	21 // 4.65
N. maculatus	Abund // Dens	2 // 0.24	5 // 2.24	3 // 0.44	1 // 0.22
N. ophidion	Abund // Dens	1 // 0.2	0 // 0	1 // 0.14	9 // 1.99

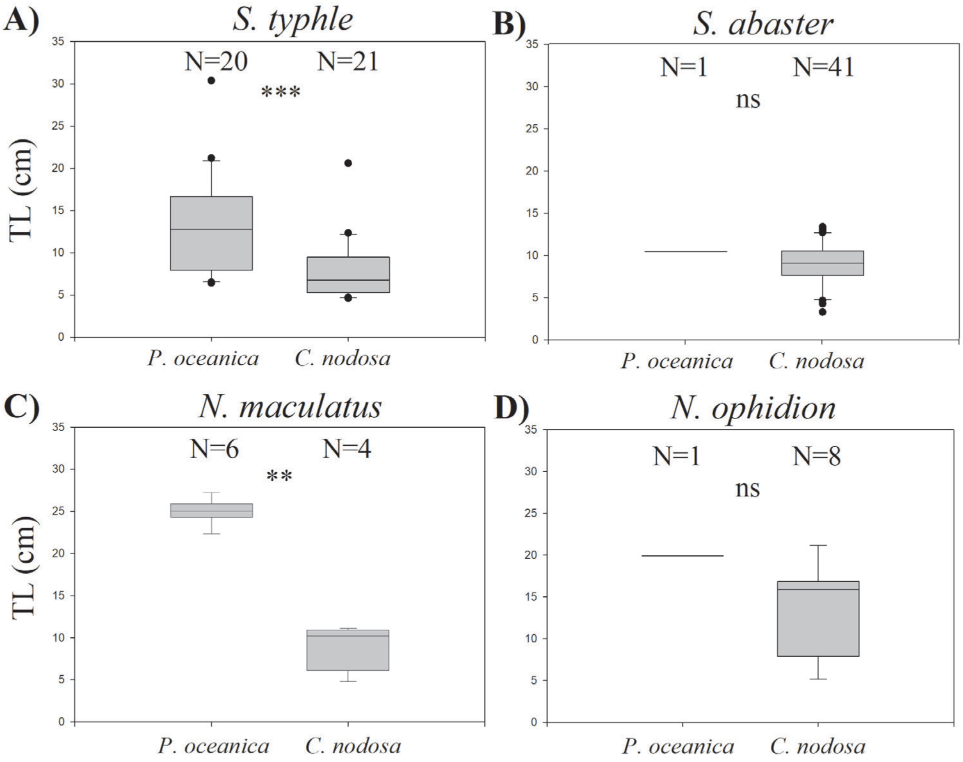

Fig. 2: Total Lengths (cm) of **a**) *S. typhle* **b**) *S. abaster* **c**) *N. maculatus* and **d**) *N. ophidion*. Boxplots show mean, confidence intervals, error bars and outliers. Sample size and significant difference between habitats according to Mann–Whitney U test are also showed: *** = p <0.005; ** = p <0.01; * = p< 0.05; ns = no significant.

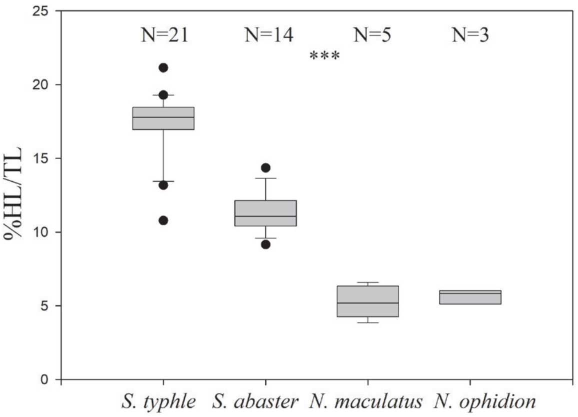

Fig. 3: Pipefish species differences on %HL/TL. Boxplot show mean, confidence intervals, error bars and outliers. Sample size and significant difference between species according to One-Way ANOVA test are also showed: *** = p <0.005; ** = p <0.01; * = p <0.05; ns = no significant.

= 66.7– 100; %Pi = 88.4–93.8), followed by teleosts and decapods (%O = 8–12; %A = 33.3; %Pi = 40–75) in *P. oceanica*, and gammarid amphipods in *C. nodosa* (%O = 6.3; %A = 62.5; %Pi = 15). The vacuity index was equal to 33.3% for *S. tyhple*. For *S. abaster*, harpacticoid copepods (%O = 37.7–60.8; %A = 80–100; %Pi = 39.4–60.8) and gammarid amphipods (%O = 33–44.9; %A = 100; %Pi = 33–44.9) were the main preys in both habitats, followed by ostracods in *C. nodosa*. No empty stomachs were found for this species. *N. maculatus* main prey were harpacticoid copepods (%O = 84.4; %A = 100; %Pi = 84.3). Secondary preys were gammarid amphipods and ostracods (%O = 9.5–5.4; %A = 100–66.7; %Pi = 9.5–6.3). On the opposite, *N. ophidion* main preys were gammarid amphipods (%O = 57.5; %A = 100; %Pi = 57.5). Secondary preys were harpacticoid copepods (%O = 42.5; %A = 100; %Pi = 42.5). *N. maculatus* and *N. ophidion* presented a 40% and 33.3% values for the vacuity index respectively. According to the Amundsen (1996) graphical analysis (Fig. 5), certain individuals of *S. typhle* in *P. oceanica* presented a clear specialization in the ingestion of decapods (%O = 12; %Pi = 75).

The main differences between pipefish diets were caused by harpacticoid copepods, gammarid amphipods, ostracods, and teleosts (Fig. 6). Significant differences between pipefish species ingestion were only found for gammarid amphipods and ostracods (H = 24.23 and H = 20.32, respectively). Differences in the diet composition between habitats were tested for *S. typhle* in the warm season and results showed no differences for fish inhabiting *P. oceanica* and *C. nodosa*. For differences between seasons, *S. abaster* individuals found in *C. nodosa* were tested and significant differences were found on the ingestion of harpacticoid copepods (F = 6.21). Pipefish morphometric characters (TL, %HL/TL and correlated ones) were negatively correlated to pipefish ingestion of harpacticoid copepods (R = –0.44), gammarid amphipods (R = –0.51) and ostracods (R = –0.41) and positively correlated to teleost ingestion (R = 0.31).

Table 2. Pipefish stomach contents including number of prey items (Abund), percentage frequency of occurrence (%O), percentage frequency of appearance (%A) and percentage prey-specific abundance (%P_i) for every habitat and season analyzed during the study.

		P. oceanica								***C. nodosa***							
		Warm				**Cold**				**Warm**				**Cold**			
		Abund	**%O**	**%A**	**%P_i**	**Abund**	**%O**	**%A**	**%P_i**	**Abund**	**%O**	**%A**	**%P_i**	**Abund**	**%O**	**%A**	**%P_i**
S. typhle	Harpacticoida	38	76	66.7	88,4	-	-	-	-	330	93.8	100	93.8	-	-	-	-
	Gammaridae	1	2	16.7	33,3	-	-	-	-	22	6.3	62.5	15	-	-	-	-
	Caprelidae	0	0	0	0	-	-	-	-	0	0	0	0	-	-	-	-
	Ostracoda	0	0	0	0	-	-	-	-	0	0	0	0	-	-	-	-
	Decapoda	6	12	33.3	75	-	-	-	-	0	0	0	0	-	-	-	-
	Acari	1	2	16.7	12.5	-	-	-	-	0	0	0	0	-	-	-	-
	Teleostei	4	8	33.3	40	-	-	-	-	0	0	0	0	-	-	-	-
S. abaster	Harpacticoida	-	-	-	-	4	57	100	57.1	188	60.8	100	60.8	26	37.7	80	39.4
	Gammaridae	-	-	-	-	3	43	100	42.9	102	33	100	33	31	44.9	100	44.9
	Caprelidae	-	-	-	-	0	0	0	0	0	0	0	0	0	0	0	0
	Ostracoda	-	-	-	-	0	0	0	0	18	5.8	75	7.6	11	15.9	80	19,6
	Decapoda	-	-	-	-	0	0	0	0	1	0.3	12.5	1,6	1	1.4	20	4
	Acari	-	-	-	-	0	0	0	0	0	0	0	0	0	0	0	0
	Teleostei	-	-	-	-	0	0	0	0	0	0	0	0	0	0	0	0
N. maculatus	Harpacticoida	-	-	-	-	124	84	100	84.4	-	-	-	-	-	-	-	-
	Gammaridae	-	-	-	-	14	9.5	100	9.5	-	-	-	-	-	-	-	-
	Caprelidae	-	-	-	-	1	0.7	33	3.2	-	-	-	-	-	-	-	-
	Ostracoda	-	-	-	-	8	5.4	67	6.3	-	-	-	-	-	-	-	-
	Decapoda	-	-	-	-	0	0	0	0	-	-	-	-	-	-	-	-
	Acari	-	-	-	-	0	0	0	0	-	-	-	-	-	-	-	-
	Teleostei	-	-	-	-	0	0	0	0	-	-	-	-	-	-	-	-
N. ophidion	Harpacticoida	0	0	0	0	-	-	-	-	-	-	-	-	17	42.5	100	42.5
	Gammaridae	0	0	0	0	-	-	-	-	-	-	-	-	23	57.5	100	57.5
	Caprelidae	0	0	0	0	-	-	-	-	-	-	-	-	0	0	0	0
	Ostracoda	0	0	0	0	-	-	-	-	-	-	-	-	0	0	0	0
	Decapoda	0	0	0	0	-	-	-	-	-	-	-	-	0	0	0	0
	Acari	0	0	0	0	-	-	-	-	-	-	-	-	0	0	0	0
	Teleostei	0	0	0	0	-	-	-	-	-	-	-	-	0	0	0	0

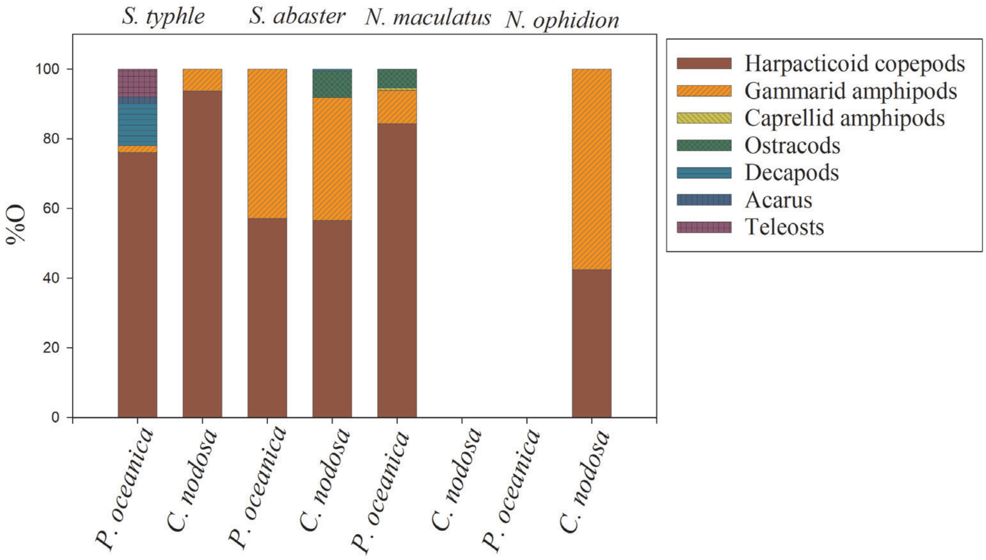

Fig. 4: Comparative stomach contents percentage frequencies of occurrence (%O) of every type of prey for every species and habitat studied.

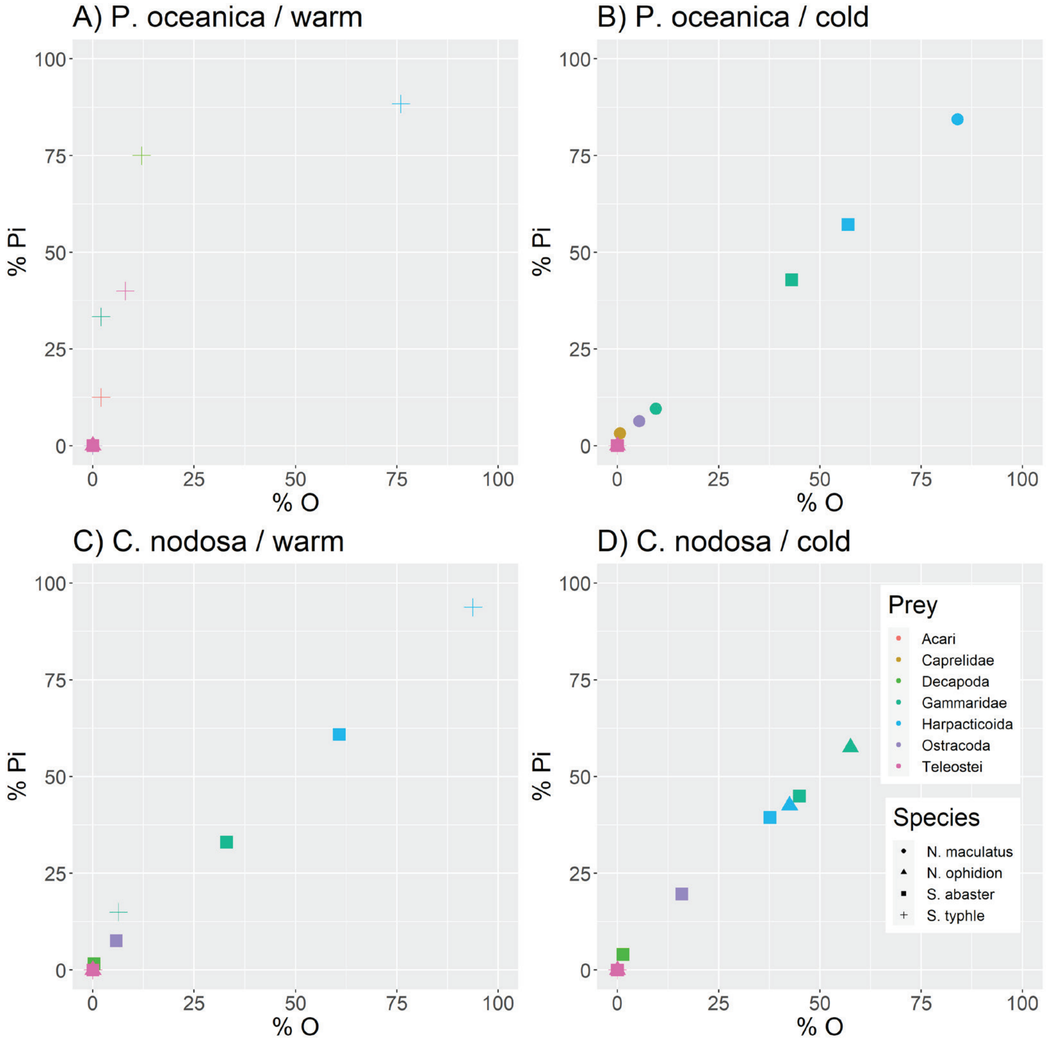

Fig. 5: Comparative feeding strategy graphical analysis according to Amundsen (1996) modification of the Costello (1990) method: a) *P. oceanica* habitat – warm season, b) *P. oceanica* habitat – cold season, c) *C. nodosa* habitat – warm season, and d) *C. nodosa* habitat – cold season. The diagonal axis from the lower left to the upper right corner measures prey importance in the diet (dominant prey in the upper and rare or unimportant prey at the lower end). The diagonal axis from the lower right to the upper left corner measures the specialization of the predator population (lower right) or the specialization of individual predators (upper left). The vertical axis represents the feeding strategy, from generalization in the lower end and specialization in the upper end.

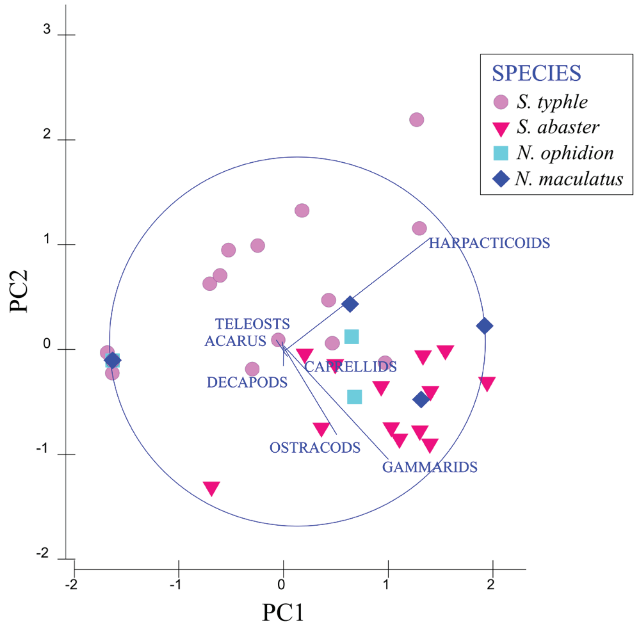

Fig. 6: PCA results for differences between pipefish species diets. The analysis explains 82.2 of cumulative variation (64.5% PC1, eigenvalue 1.49; 17.7% PC2, eigenvalue = 0.41), mainly depending on harpacticoid copepods, gammarid amphipods, ostracods, and teleosts.

Epifaunal community

Invertebrate communities from the *C. nodosa* meadow showed higher abundances (referred to 100cm^2 of foliar surface, correlated to foliar dry weight; R = 0.93) than those from *P. oceanica* (Table 3). In both habitats, standardized abundances of epifauna were higher during the cold period. These differences were statistically different (Pseudo-F = 29.862 for differences between habitats; Pseudo-F = 3.4839 for differences between seasons in each habitat). Conversely, epifaunal communities showed similar values of diversity in terms of H’ for each habitat. However, in both *P. oceanica* and *C. nodosa* meadows, diversity decreased during the cold period.

Differences between habitats were mainly due to the abundance of some taxa such as caprellid and gammarid amphipods, gastropods, or bivalves (Table 4). Concerning differences between seasons within each habitat, significant differences were found for several taxa such as harpacticoid copepods, ostracods, tanaidaceans, cumaceans or gastropods in *P. oceanica* and only for gammarid amphipods and opisthobranchs in *C. nodosa* (Table 4).

Regarding potential relationships among diet compositions of each species and presence of potential preys (epifaunal communities) on both seagrasses, the Spearman Rank R test revealed a correlation between the %O of each prey in the diets of *S. abaster* and *N. maculatus* and the composition of the epifaunal communities (standardized epifaunal abundances by foliar surface) present in *P. oceanica* (R = 0.49 and R = 0.47 respectively). Additionally, in the *C. nodosa* meadows, there was a correlation between the %O of *S. typhle, S. abaster,* and *N. ophidion* and the invertebrate community assemblage in terms of standardized epifaunal abundances by foliar surface (R = 0.49 for *S. typhle*; R = 0.46 for *S. abaster,* and R = 0.49 for *N. ophidion* (Fig. 7).

Discussion

The present study showed that seagrass meadows in the Balearic Islands harbor pipefish assemblages dominated by *S. typhle* and *S. abaster*; but *N. ophidion* and *N. maculatus* were also present in these coastal communities. The distribution range of *S. typhle* and *N. ophidion* comprehends the northeast Atlantic as well as the North, Baltic, Mediterranean, and Black Seas (Pollom, 2014, 2015). *S. abaster* and *N. maculatus* are only present in

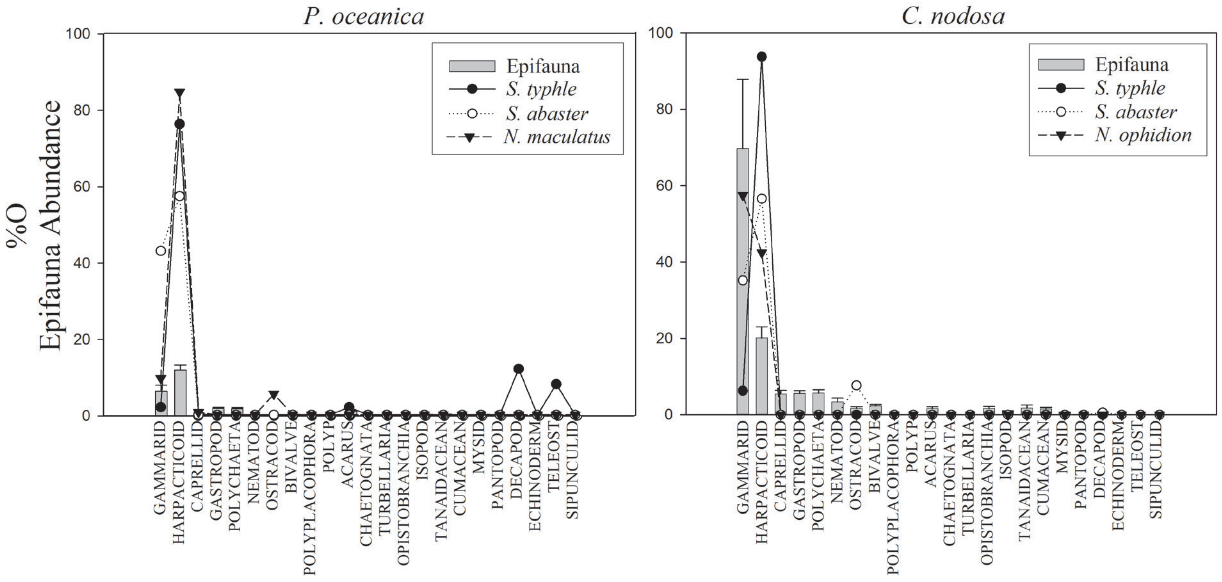

Fig. 7: Correlation between percentage frequencies of occurrence (%O) found in pipefish stomach contents and epifaunal community composition (standardized abundances by foliar surface) are shown for **a**) *P. oceanica* and **b**) *C. nodosa*. Histogram bars represent epifauna abundance and dot diagrams represent the %O of the pipefish species diets.

Table 3. Epifaunal communities' composition according to their number of individuals (Total Abund) and standardized abundances (Stand Abund) for every habitat and season considered in the study. Shannon–Wiener Index (H') is included as a proxy to communities' diversity.

	P. oceanica				***C. nodosa***			
	Warm		**Cold**		**Warm**		**Cold**	
	Total Abund	**Stand Abund**	**Total Abund**	**Stand Abund**	**Total Abund**	**Stand Abund**	**Total Abund**	**Stand Abund**
Harpacticoida	89.88	9.07	129.35	15.34	19.57	14.72	10.36	26.77
Gammaridae	19.20	2.25	76.45	11.40	16.23	12.45	65.00	141.38
Caprellidae	2.12	0.24	1.10	0.20	6.27	5.03	2.48	5.99
Ostracoda	1.24	0.14	0.00	0.00	3.90	1.82	0.68	1.47
Decapoda	0.00	0.00	0.10	0.01	0.33	0.57	0.28	0.49
Isopoda	1.24	0.16	1.00	0.15	0.60	0.75	0.32	0.88
Tanaidacea	0.84	0.13	2.25	0.36	1.80	0.85	1.00	2.88
Cumacea	0.64	0.07	0.05	0.01	0.53	0.87	1.00	2.27
Mysidacea	3.20	0.27	0.10	0.01	0.80	0.29	0.28	0.69
Acari	14.12	1.37	6.75	0.78	1.57	1.53	0.64	1.73
Pantopoda	0.00	0.00	0.10	0.01	0.13	0.06	0.08	0.07
Cnidaria	0.88	0.07	0.00	0.00	0.00	0.00	0.00	0.00
Gastropoda	28.28	2.94	2.90	0.29	11.97	6.69	1.64	4.25
Opistrobranchia	0.16	0.02	0.00	0.00	6.80	2.85	0.08	0.11
Polyplacophora	0.28	0.02	0.00	0.00	0.00	0.00	0.00	0.00
Bivalvia	2.60	0.27	0.50	0.04	3.60	2.62	0.79	2.00
Polychaeta	17.64	2.00	10.50	1.42	5.63	5.21	2.52	6.23
Sipunculidea	0.00	0.00	0.00	0.00	0.07	0.03	0.00	0.00
Chaetognatha	0.52	0.05	0.00	0.00	0.03	0.00	0.00	0.00
Turbellaria	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Echinodermata	0.12	0.01	0.25	0.04	0.07	0.01	0.00	0.00
Nematoda	1.20	0.14	0.20	0.02	2.27	2.22	2.36	4.72
Teleostei	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00
H'	1.6248		0.9702		1.9362		1.0670	

the northeast Atlantic surrounding Spain and Portugal and the Mediterranean and Black Seas (Freyhof, 2016; Wiswedel, 2016).

The low abundance of these species in the study area, as well as their protected status in coastal ecosystems, precluded obtaining more samples. However, as they are assessed at a global scale as Least Concern (LC) and Data Deficient (DD) by the IUCN Red List of Threatened Species, the results presented here are a significant contribution towards a better knowledge of syngnathids. Moreover, relatively little information is available on the abundance and food preferences of syngnathid populations in the western Mediterranean Sea. To our knowledge, no formal range-wide surveys of abundance estimates of these species have been undertaken to date, and the seasonal variability of Mediterranean pipefish is still unknown.

Seagrass species determine meadow architecture and complexity (e.g., shoot density or leaf surface), which affects habitat availability for syngnathids (Malavasi *et al.*, 2007). Due to the mimicry capacity of pipefish in these habitats, the sampling methodology had to be adapted. Firstly, visual censuses with scuba-divers along transects in the meadows resulted in very low visualizations. Thus, we decided to sample with a small trawl net traditionally employed in seagrass meadows. Because of the low natatorium speed of the syngnathids and the unselective gear, we expected to have representative samples with this

Table 4. Kruskal-Wallis results for every taxa conforming epifaunal communities and their significance on the differences between habitats and seasons. H statistic and p-value resulting on the analysis are included.

	Habitat		**Season**			
	P. oceanica vs. C. nodosa		***P. oceanica***		***C. nodosa***	
			Cold vs. Warm		***Cold vs. Warm***	
	H	**p-value**	**H**	**p-value**	**H**	**p-value**
Harpacticoida	3.0523	0.0806	4.1327	0.0421	3.5943	0.0580
Gammaridae	32.5963	0.0001	1.6951	0.1929	23.4490	0.0001
Caprellidae	28.3510	0.0001	1.4874	0.2226	0.1904	0.6623
Ostracoda	12.7705	0.0004	7.5160	0.0061	1.5067	0.2196
Decapoda	6.7347	0.0095	2.5568	0.1098	0.1664	0.6833
Isopoda	0.6527	0.4192	0.0737	0.7860	0.0025	0.9600
Tanaidacea	0.0123	0.9118	6.5741	0.0103	0.0989	0.7531
Cumacea	11.4706	0.0007	7.0222	0.0081	0.1950	0.6587
Mysidacea	0.0368	0.8479	9.1326	0.0025	0.0018	0.9659
Acari	3.0577	0.0804	2.3421	0.1259	0.0322	0.8577
Pantopoda	1.5871	0.2077	2.5568	0.1098	1.1394	0.2858
Cnidaria	8.6583	0.0033	7.5160	0.0061	0.0000	1.0000
Gastropoda	21.0979	0.0001	31.0704	0.0001	3.7745	0.0520
Opistrobranchia	10.0657	0.0015	2.5107	0.1131	8.5438	0.0035
Polyplacophora	6.3473	0.0118	5.3796	0.0204	0.0000	1.0000
Bivalvia	18.8134	0.0001	9.4063	0.0022	0.7972	0.3719
Polychaeta	7.1633	0.0074	4.6101	0.0318	0.0021	0.9634
Sipunculidea	1.0000	0.3173	0.0000	1.0000	0.8000	0.3711
Chaetognatha	3.6203	0.0571	5.3796	0.0204	0.8000	0.3711
Turbellaria	1.0000	0.3173	0.8000	0.3711	0.0000	1.0000
Echinodermata	4.6916	0.0303	0.6935	0.4050	0.8000	0.3711
Nematoda	6.9570	0.0088	6.3327	0.0119	0.0602	0.8062
Teleostei	1.0000	0.3173	0.8000	0.3711	0.0000	1.0000

method. However, regardless of the sampling methodologies, it is worth noting the low abundance of pipefish individuals found in the Balearic Islands (N = 105 on 151 fishing operations).

Due to the low sample size, it was not possible to estimate the body size distribution of the different pipefish species for the habitats and seasons studied. More sampling effort is needed to complete this information. However, some differences were found in this study for the habitat choice of each species. Our results showed that smaller specimens of *S. typhle,* as well as *S. abaster* and *N. ophidion,* preferred habitats dominated by *C. nodosa*, with short and thin leaves and sometimes associated to algal presence; while larger individuals of *S. typhle* and *N. maculatus* seemed to prefer tall canopy meadows formed by *P. oceanica*, characterized by large and broad leaves and high shoot densities. Smaller individuals of *S. abaster* were more common during the warm season in *C. nodosa*, when the plentiful vegetative growth supplies a refuge from predation for young pipefishes (Franzoi *et al.*, 1993; Riccato *et al.*, 2003). These differences in habitat choice could be explained as larger *S. typhle* and *N. maculatus* need the tall and strong leaves of *P. oceanica* as a physical support and for crypsis while entwining, exploring, and searching for prey (Malavasi *et al.*, 2007). Nevertheless, *C. nodosa* provides an important vegetated area for *S. abaster,* which are morphologically better adapted to sparse and narrow leaves (Verdiell-Cubedo *et al.*, 2007), and smaller individuals of *S. typhle, N. maculatus,* and *N. ophidion*. Differences in seagrass association between the pipefish species shown in the present study were also described by Scapin *et al.* (2018), who highlighted a more generalist behavior in habitat choice of *S. abaster*, mostly found in association to algal beds and low-density seagrass meadows, compared with *S. typhle*, which appear to be dense-seagrass specialists. *N. ophidion* was also expected to be a dense-seagrass specialist (Scapin *et al.*, 2018), but it was mainly found in *C. nodosa* during our study.

Pipefish exhibit a high degree of trophic specialization compared to other epibenthic marine teleosts (Gürkan, 2008). This specialization was thought to occur between different syngnathid species, mainly due to their head and snout morphologies (Kendrick & Hyndes, 2005). However, this study proved that apart from the morphology, the availability of prey was also important. Our results indicated that there is a relation between pipefish diet and changes in the structure of epifaunal assemblages. Divergences in the diets were found to be mainly related to the ingestion of gammarid amphipods, ostracods, and teleosts, while harpacticoid copepods were the primary prey for all pipefish. Moreover, some *S. typhle* individuals from *P. oceanica* showed an individual preference for decapods. Other authors had already stated that pipefish diets are based on small crustaceans (Teixeira & Musick, 1995; Campolmi *et al.*, 1997; Kendrick & Hyndes, 2005). Prey frequency of occurrence in stomach contents of *S. abaster* and *N. maculatus* was related to invertebrate abundances in *P. oceanica*. Additionally, *S. typhle, S. abaster,* and *N.ophidion*'s diets were related to epifaunal assemblages in *C. nodosa*. As suggested by Mattson (1990) and Oliveira *et al.* (2007), the taxonomic composition of the diet is determined by fluctuations of potential prey abundance in the environment. The availability and vulnerability of the prey species influence the consumption rates and contribution to the diet (Franzoi *et al.*, 1993). This may be the reason why harpacticoid copepods and gammarid amphipods were the most preferred prey by pipefish in both habitats despite their morphological adaptations since they clearly dominate the invertebrate assemblages in the studied seagrass meadows.

Besides epifaunal assemblage composition, the ability to catch bigger and faster prey depends on the volume of water that can be inhaled and the length of the snout (Muller & Osse, 1984), which depends on the head size of the individual. As preys are swallowed whole, the dimensions and maximum opening of the mouth determines the maximum size of prey that can be ingested (Oliveira *et al.*, 2007). Head size and morphologic variations between pipefish species found during the study may explain differences in the selection of secondary prey among species. As shown by morphometric measures taken in this study, *S. typhle* has a long and flat snout that is more than half the length of the head and a large mouth opening comparing to the other species. *S. abaster* has a cylindrical snout that is more or less half the length of the head and a small mouth opening, as well as *N. maculatus* and *N. ophidion* whose snout is cylindrical and less than half the length of the head and whose mouth opening is the smaller of all. Moreover, the opening of the mouth causes the expansion of the lateral walls of the snout, forming a tube with an increase in volume (Oliveira *et al.*, 2007). Small pipefish individuals or species with smaller mouth openings and shorter snouts are highly selective, consuming a narrow range of prey (i.e., harpacticoid copepods, gammarid amphipods, and ostracods). Conversely, larger individuals or species with larger mouth openings are less selective while capturing their prey, consuming a wider type and size range of prey, including faster swimming prey (i.e., decapods and teleost) (Kendrick & Hyndes, 2005). As found in this study, larger specimens of *S. typhle* had the ability to predate on decapods or juvenile teleosts in addition to harpacticoid copepods and gammarid amphipods. In fact, one of the largest pipefish found, whose total length was 16.5 cm, had eaten two juvenile *Symphodus ocellatus* (teleost) of 1.4 and 2.6 cm length. This result agreed with Bell's report (1983) of small fish being important in larger *S. typhle* specimen's diet. Conversely, *N. maculatus* and *N. ophidion*'s head and snout morphology only enables them, both large and small individuals, to forage on smaller prey (i.e., gammarid and caprellid amphipods or ostracods). *S. abaster*'s diet consisted of either small (i.e., gammarid amphipods or ostracods) or big prey (i.e., decapods) apart from the common prey. Species present in the same habitat, if showing differences in head morphometry, would use different trophic resources and feeding strategies, and thus their foraging niches do not concur. While *S. abaster* feeds in submerged vegetation, *S. typhle*, *N. maculatus,* and *N. ophidion* also catch prey in the water column (Vizzini & Mazzola, 2004). Relatively high vacuity indexes compared to previous studies in syngnathids (Taçkavak *et al.*, 2010) were found for *S. typhle, N. maculatus,* and *N. ophidion* (33.3%, 40%, and 33.3% respectively).

Pipefish body size has also been proved to be an important factor in their feeding ecology, as it determines the rank of prey that can be ingested. Furthermore, ontogenic changes on the feeding habits, type, and proportion of prey consumed as well as in the size of prey of *S. typhle* have been previously reported (Oliveira *et al.*, 2007). As individuals grow, the changes in prey consumed indicate a progressive substitution of gammarid amphipods for shrimps and little fish with the corresponding increase in trophic level. Hereby, more studies analyzing the relationship between pipefish body size and age are needed in the Mediterranean populations, as several studies have met with little success in attempting to use otolith increment width to age members of the Syngnathidae family (Parkinson *et al.*, 2012).

Despite all the above, studying the feeding ecology of these species by stomach contents analysis provides evidence of food preferences and foraging habits, but in many cases, it provides little information on food actually assimilated. Food items that are quickly digested, as gelatinous zooplankton, are generally underestimated compared to those that remain longer in the stomach, such as animals with a chitin cover. Carbon and nitrogen stable isotope ratios have been used in fish as a complementary approach to prey actually assimilated by consumers (Vizzini & Mazzola, 2004). It is therefore crucial to investigate the stable isotope ratios of pipefish and potential prey treated in this study to establish the relationship between prey ingested and prey assimilated.

The present study sheds light on western Mediterranean pipefish distribution, habitat, and their associated trophic sources. Habitat and feeding preferences have been proved to be dependent on a wide range of factors (species, body size, head, and snout morphometry and epifaunal assemblages present on seagrasses). *P. oceanica* habitat is preferred by individuals of larger species (*S. typhle* and *N. maculatus*) than those living in *C. nodosa* (*S. abaster* and *N.ophidion*). Feeding preferences were not driven to body size but to prey availability and head/snout morphology. Epifaunal assemblages (potential prey) were dominated in both habitats by harpacticoid copepods and gammarid amphipods and they were also the primary prey according to stomach contents of all species. These results, along with the low pipefish densities found, make this area worthy of specific protection initiatives. Conservation measures should be adopted by regional or national institutions in order to protect the diversity of species and natural resources present in the habitats where these species live. Future studies should focus on investigating a wider spatial extension of pipefish distribution in order to provide effective tools towards their population management and conservation.

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