POPULATION DYNAMICS AND FEEDING ECOLOGY OF SYNGNATHIDS INHABITING MEDITERRANEAN SEAGRASSES

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ABSTRACT

Syngnathids are an emblematic, vulnerable and diverse group of the ichthyofauna associated to vegetated coastal and estuarine habitats (Campolmi *et al.*, 1996). Seagrass meadows, where pipefish aspect and behavior makes them mimetic, provide shelter and food, and seem to be preferred habitats (Kendrick & Hyndes, 2005). Syngnathid population dynamics and feeding habits are still poorly known, especially in Mediterranean coastal waters (Vizzini and Mazzola, 2004). In order to understand syngnathid population trends and feeding ecology, pipefish assemblages from *Posidonia oceanica* and *Cymodocea nodosa* meadows in the Balearic Islands were studied. Pipefish were fished with an artisanal epibenthic trawl in both habitats, during warm and cold season. Associated epifauna (i.e. potential preys) was also sampled by scuba divers. Syngnathids were identified and measured (n=73). Head morphometry, stomach contents (n=43) and sexual maturity (n=22) were also studied. Epifauna samples were identified to main taxon. A total of 4 pipefish species were found: *Syngnathus typhle, S. abaster*, *Nerophis ophidion* and *N. maculatus*. Dominant species were *S. abaster* and *S. typhle* in *C. nodosa* and *P. oceanica* meadows respectively. Individuals captured in *P. oceanica* were significantly larger in size than those living in *C. nodosa*. Main prey items observed in stomach contents analyses were amphipods and copepods, which also were the most abundant taxa in epifaunal samples in both habitats, along with gastropods and polychaetes in *P. oceanica*. While standardized abundance of invertebrates was higher in *C. nodosa*, diversity of epifaunal communities was similar in both types of habitat. Although observed prey items were in accordance with epifaunal communities, variations were detected among pipefish species, sizes and habitats. Additionally, large individuals and some species (i.e. *S. tyhple*) have wider snouts and mouth openings, which allow them to catch and ingest larger preys such as decapods and even small teleost juveniles.

Key words: Syngnathid, seagrass, feeding habits, population, Mediterranean.

INTRODUCTION

Syngnathidae is a family of fish found in temperate and tropical seas across the world, which includes the seahorses, the pipefishes, the pipehorses, and seadragons (Figure 1). The name of this family is derived from Greek, “syn”, meaning "fused" or "together", and “gnathus”, meaning "jaws". This fused and toothless jaw trait is also something the entire family has in common, showing a particular feeding ecology (Leysen *et al.*, 2011). They are considered secondary consumers with two specialized predatory strategies: sit and wait and/or slow search behavior (Tipton & Bell, 1988; Franzoi *et al.*, 1993). They are characterized by an elongate tubular snout and could be considered as specialized suction feeders (Muller & Osse, 1984). The type and size of prey consumed by syngnathids varies depending on the size of the snout and the mouth opening, so they have obvious limitations while feeding (Lyons & Dunne, 2004).

Figure 1. Fam. Syngnathidae: a) Seahorse *Hippocampus guttulatus* b) Pipefish *Syngnathus tyhple* c) Pipehorse *Idiotropiscis lumnitzeri* d) Seadragon *Phycodurus eques*.

Syngnathids have some particular characteristics that collaborate to its vulnerability. First, male parental care (Vincent *et al.*, 1995) where females pass the eggs from the oviduct into the ventral brood pouch developed by male, where the male inseminates them (Franzoi *et al.*, 1993). However, some differences among genus can be found. For instance, within pipefish, the male brood pouch is typical in genus *Syngnathus*, while some other genus such as *Nerophis* have a simple ventral region where eggs are loosely attached without any protecting plates or covering membranes (Dawson, 1986; Monteiro *et al.*, 2005). After fecundation, males carry and care the embryos throughout the gestation period until hatchling of the independent young (Berglund *et al.*, 1986).

Vizzini & Mazzola (2004) reported that this reproductive behavior means a greater cost for males compared to females. Second, the investment in embryo protection structures that are among the most developed in fishes and the long embryonic development that lasts for one month (afterwards fully formed juveniles are released) (Monteiro *et al*., 2003; Silva *et al.*, 2006). Behavior of newborn juveniles differ depending on the genus. While *Syngnathus* juveniles present a benthonic preference, in *Nerophis* they display a pelagic life phase (Monteiro *et al*., 2003; Silva *et al.*, 2006). Third, the low number of eggs per couple and the couple fidelity habits. In this scenario juvenile survivorship is essential for species endurance, however juvenile mortality is commonly high due to predation, low mobility and habitat limitations. Recolonization of new areas will be typically very slow.

Among syngnathids, pipefish are the most abundant group found in Mediterranean waters (Franzoi *et al.*, 2010) (Figure 2), where 11 species have been reported according to the IUCN Red List of Threatened Species: *Nerophis ophidion, N. maculatus, Syngnathus abaster, S. acus, S. typhle, S. phlegon, S. rostellatus, S. schmidti, S. tanaeionotus, S. tenuirostris* and *Minyichthys sentus*. They are usually associated to seagrass meadows (Pita *et al.*, 2002) and their feeding ecology is poorly known (Lyons & Dunne, 2004; Vizzini & Mazzola, 2004).

Pipefish are an important component of the icthyofauna in vegetated coastal and estuarine lagoon habitats (Howard & Koehn, 1985; Campolmi *et al.*, 1996). They mimic thin seagrass leaves in shape, color and orientation so they are protected from predators (Howard & Koehn, 1985; Fuller & Berglund, 1996). These highly specialized fishes are characterized by limited mobility due to the small size of their fins and the occurrence on their bodies of semi-rigid dermal plates that restrict flexibility. They select the habitats that best enable them to remain inconspicuous to predators, which points out to an effect of meadow density on pipefish habitat preference (Kendrick & Hyndes, 2005).

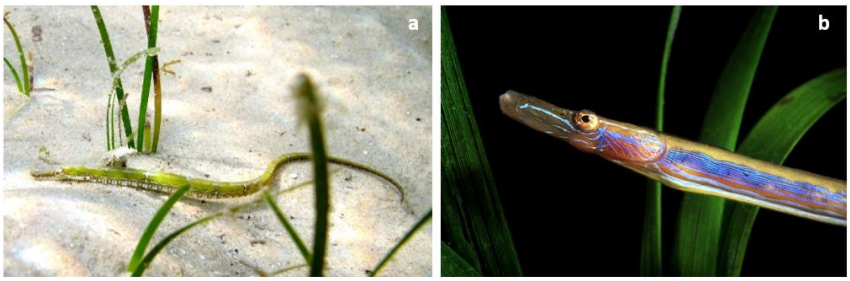

Figure 2. Mediterranean pipefish species: a) *Syngnathus abaster* b) *Nerophis ophidion*.

Distribution and abundance of seagrasses modify the spatial distribution and abundance of fauna due to combined factors such as the reduction of predation and the habitat selection for behavioral and ecological preferences of species (Sánchez-Jerez et al., 1999). It may influence pipefish life history, as they use these habitats as nursery and feeding grounds (Teixeira & Musick, 1995).

The very clear and nutrient-poor (oligotrophic) waters of the Mediterranean (Fourqurean *et al.*, 2007) host seven seagrass species (Short & Coles, 2001), being *Posidonia oceanica* (endemic) and *Cymodocea nodosa* the most abundant (Olsen *et al.*, 2012). Meadows distribution depends on the seagrass species, which have a contrasting meadow structure: *P. oceanica* meadows cover the 40% of the Mediterranean bottoms between 0-40 m. This species creates dense meadows (300 to 1000 shoots/m^2) with a compact root-rhizome mat and a high leaf stratum, achieving longest canopy height of 1 m during early summer (Drew & Jupp, 1976). Conversely, *C. nodosa* develop lax meadows (100 to 450 shoots/m^2) with a low leaf stratum and less compact rhizome mat (Rull *et al.*, 1996).

The heterogeneous habitats build by Mediterranean seagrasses have a variable influence on the abundance and diversity of epifaunal communities, conformed by groups of animals with different life forms and ecological characteristics living above the sea-bottom, among the plant leaves and stems (Short & Coles, 2001). Seagrass provide a substrate for feeding and attachment, and species composition and abundance is related to plant characteristics such as leaf morphology or shoot density (Orth *et al.*, 1984), and 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. 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 to them: fish and invertebrates (Olsen *et al.*, 2012).

It has been previously reported that feeding preferences of pipefish corresponds to small crustaceans conforming seagrass´s epifauna. However, there is a lack of information to the moment in Mediterranean waters (Franzoi *et al.*, 1993; Teixeira & Musick, 1995; Campolmi *et al.*, 1996; Carcupino *et al.*, 1997; Kendrick & Hyndes, 2005).

OBJECTIVES

The main objectives of this study were to evaluate the status of syngnathids populations in the Western Mediterranean and to expand our knowledge on syngnathids feeding habits and life cycles by:

a) Assessing the distribution and abundances of Mediterranean pipefish species in two different habitats and seasons.
b) Examining pipefish feeding habits by stomach contents and exploring its relationship with morphometric features.
c) Evaluating potential preys of pipefish examining epifaunal compositions within preferred habitats.
d) Exploring the evaluation of sexual maturation stages of pipefish through macro and microscopic descriptions.

MATERIALS AND METHODS

STUDY AREA AND SAMPLING DESIGN

This study was carried out in two 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 and Port d'Andratx) and one *Cymodocea nodosa* meadow in Cabrera (i.e. Es Burri Bay) (Figure 3). Cala Gamba is located in the inner part of Palma Bay (Mallorca), (Figure 3a), while Port d'Andratx sampling is in the exit of a natural harbor, both sites are under medium-low anthropogenic impact, mainly due to the presence of commercial and recreational harbors, urban wastes and tourist use (Figure 3b). Conversely, Es Burri is located in the Cabrera's Archipelago National Park and characterized by well-preserved seagrass as a result of the protection from fisheries, touristic pressure and any other anthropogenic impact (Figure 3c).

Two different sampling methods were used to collect pipefish and epifaunal communities, which were sampled every two months approximately from May 2017 to April 2018, so temporal variations can be analyzed. Seawater temperature ranged between 27ºC in the warm season and 14ºC in the cold season. Temperatures below 20ºC were considered as cold season, while temperatures higher than that were considered warm season (Table 1).

Figure 3. Map of the three sampled sites in the Balearic Islands with some of the transects sampled in red. a) Cala Gamba (Mallorca) b) Port D'Andratx (Mallorca) c) Es Burri (Cabrera). Google Earth.

Table 1. Sampling design followed in the present study.

HABITAT	SEASON	SITE	DATE	SEAWATER TEMPERATURE
C. nodosa	Warm	Es burri	June 2017	23ºC
			August 2017	27ºC
			October 2017	24ºC
	Cold		May 2017	19ºC
			November 2017	19ºC
			February 2018	14ºC
P. oceanica	Warm	Port D'Andratx & Cala Gamba	July 2017	26ºC
			October/November 2017	22ºC
	Cold		January 2018	15ºC
			March/April 2018	14ºC

PIPEFISH SAMPLING AND BIOMETRIES

Pipefish were sampled using a small trawl net called 'gambera' or 'gánguil' (traditionally used to catch fishing bait), a light-weight epibenthic trawl with an incorporated 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 0.8 m mouth aperture with 1.2 cm^2 mesh size (Figure 4) (Catalán *et al.*, 2014).

A total of 127 transects were run that variated on distance between 30 and 500 meters long, depending on the meteorological and orographic conditions. GPS positions were taken at the beginning and end of each transect, and were performed during daylight hours at a depth range of 1.7-16.5 m. Species identification was done on board (see ANNEX 1) after being slaughtered as soon as possible with an overdose of anesthetic in solution (tricaine methanesulfonate - MS-222; concentration: 0.1-0.2 g/L). Fishes were

preserved in absolute ethanol, labelled and transported to the laboratory inside coolers with ice pads to take pictures for later length measurements in the laboratory and further procedures (e.g. stomach content, morphological features),. Pipefish average densities and their standard error associated were calculated for each transect for the different species, seasons and habitats.

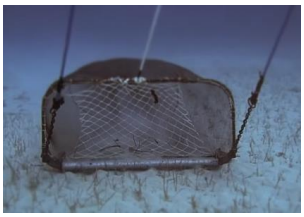

Figure 4. Pipefish sampling with the trawl net.

Once in the lab, pipefish total length was measured to the lowest millimeter with the image processing and analysis device ImageJ2 (Rueden *et al.*, 2017), and head morphometric measures were also taken using a precision caliper (Figure 5; Table 2). To minimize the influence of size differences on the subsequent results, the morphometric measurements were standardized. Head length was expressed as %TL and the other 5 were expressed as %HL (Cakić *et al.*, 2002; Yildiz & Karakulak, 2015).

Table 2. Description of the biometric characters analyzed.

Morphometric character	Acronym	Measurement	Description
Total Length	TL	cm	Distance from the tip of the snout to the tip of the longer lobe of the caudal fin
Head Length	HL	mm	Distance from the tip of the snout to the operculum
Snout Length	SL	mm	Distance from the tip of the snout to the end of the eye
Minimum Snout Height	MSH	mm	Minimum vertical distance in the middle of the snout
Eye Diameter	ED	mm	Horizontal distance of the eye
Mouth Height	HM	mm	Maximum height opening of the mouth
Mouth Width	WM	mm	Maximum width opening of the mouth

Samples from the caudal muscular tissue of every fish were taken in aseptic conditions and sent to specialized laboratories for further genetic (i.e. Servicio de Genética para la Acuicultura y la Conservación de Recursos de la Universidade de Santiago de Compostela) and stable isotopes analysis (i.e. Servizo de Apoio á Investigación de la Universidade da Coruña). These analysis are still in process.

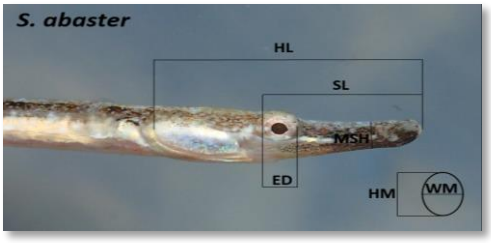

Figure 5. Head morphometric measures. HL: Head Length; SL: Snout Length; ED: Eye Diameter; MSH: Minimum Snout Height; HM: Height Mouth; WM: Wide Mouth.

PIPEFISH DISECCTIONS AND FEEDING HABITS

Additionally, pipefish stomach contents were analyzed as a proxy to feeding habits and preferences. After fixation of the whole individuals in 70% ethanol, necropsy was performed at laboratory. Fishes were dissected and their digestive tracts extracted and opened. Signathids 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 were impossible to identify some items 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 dietary categories (see Table 4).

During the dissection, gonads were removed from the pipefish to determine sex and sexual stages by macroscopic and microscopic techniques. Due to the insufficient sample size to carry out statistical analysis (N=22), these results are included as supplementary material in the ANNEX 2.

EPIFAUNAL COMMUNITY ASSESSMENT

In order to assess potential or available prey for syngnathids inhabiting seagrass meadows, organisms conforming epifaunal community (crustaceans, mollusks, etc.) were sampled. Five replicates of epifaunal community 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 (Figure 6). The bag was placed over seagrass sea-floor and leaves were removed by cutting at sediment surface level with scissors, so that all the organisms conforming epifauna living in the seagrass leaves were trapped in the bag (Tuya *et al*., 2011). Samples were fixed right after being on board,

labeled and conserved in 70% ethanol, for further identification and analysis in the laboratory.

Figure 6. Epifauna sampling in a *C. nodosa* meadow.

Once in the lab, epifaunal community samples were processed separating leaves and the mobile organisms. Fauna was analyzed under a LEICA MZ16 stereo microscope, categorizing individuals into broad taxonomic units to class/order level (Abel & Riedl, 1986) such as copepods, gammarid amphipods, caprellid amphipods, gastropods, isopods, mysids, etc. (see Table 4) and separating them in different tubes (Total Abundance, TA). Epifaunal samples were then dried (48h at 60ºC) and sent for further stable isotopes analysis (i.e. Servizo de Apoio á Investigación de la Universidade da Coruña, Galicia) still in process. Length and width of all leaves in each sample were measured to determine foliar surface as a proxy of habitat availability or complexity and standardization of organism abundances. Relative abundances of each taxa were calculated by the standardization of total abundances for every 100cm^2 of foliar surface. Community diversity was also determined with Shannon-Wiener Diversity Index.

Table 4. Identified taxons in epifaunal communities and pipefish stomach contents.

Taxa	Stomach Contents	Epifauna	Taxa	Stomach Contents	Epifauna
CRUSTACEA			PYCNOGONIDA		
Copepoda			Pantopoda		X
Harpacticoidea	X	X	CNIDARIA		X
Calanoidea		X	GASTROPODA		X
Amphipoda			Opisthobranchia		X
Gammaridae	X	X	POLYPLACOPHORA		X
Caprellidae	X	X	BIVALVIA		X
Ostracoda	X	X	ANELIDA		
Decapoda	X	X	Polychaeta		X
Isopoda		X	CHAETOGNATHA		X
Tanaidacea		X	TURBELLARIA		X
Cumacea		X	ECHINODERMATA		X
Mysidacea		X	NEMATODA		X
ARACHNIDA			TELEOSTEI	X	X
Acari	X	X			

DATA ANALYSIS

Low pipefish abundances discourage statistical analysis of species distribution. However, presence/absence and average densities (number of individuals/trawled seafloor surface) of different pipefish species collected were graphically evaluated in both habitats (*P. oceanica* and *C. nodosa*), as well as size distribution (tested with Mann-Whitney U). Cala Gamba and Port d'Andratx were both considered and merged as *P. oceanica* habitat for the analysis.

Pipefish biometrics were evaluated through Principal Component Analysis (PCA) and Percentage Similarities (SIMPER) in order to determinate the main differences between species. After testing normality of samples, a one-way ANOVA was used to test if the differences between species were statistically significant. Morphometric characters were corroborated to be correlated with the Pearson coefficient. Differences between habitats and season for each group of species were tested with a two factor permutational multivariate ANOVA (PERMANOVA). Factors were: habitat, with two levels: *P. oceanica* and *C. nodosa*; and season, with four levels: cold x *P. oceanica*, warm x *P. oceanica*, cold x *C. nodosa* and warm x *C. nodosa*.

In order to characterize feeding preferences of sampled pipefish individuals, contribution of different prey items registered from the stomach contents were calculated according to total abundance of different preys (TA); frequency of occurrence (%O=[(frequency of food 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); and vacuity index (%VI) [(number of empty stomachs/total number of stomachs)x100]. PCA and SIMPER were used to evaluate the differences between the diets of each species. To test significant contribution of each prey to these differences, Kruskal-Wallis test were performed. Differences on the diet depending on habitat and season were tested with PERMANOVA (factors habitat and season). The Spearman correlation coefficient was used to test if morphometric characters and stomach contents (%O) were correlated.

For epifaunal community assemblage, differences between samples depending on the relative abundances of different taxa were analyzed with PERMANOVA (factors habitat and season). 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. PCA and SIMPER were also used in order to determinate which taxa had a major influence on these differences and to calculate the contribution of each taxa to the dissimilarity between sampling time and habitats. Data was tested for normality and fourth root transformed. Samples were still not normal, so the Kruskal-Wallis test was used to test which were the taxons determining significant differences. Spearman correlation coefficient was used to evaluate relations between epifaunal community assemblages and pipefish stomach contents.

All graphics were plotted using SigmaPlot Version 8.0.2 and statistical analyses were performed using PRIMER 6&PERMANOVA+ and STATISTICA software.

RESULTS

PIPEFISH POPULATIONS AND BIOMETRICS

A total of 73 pipefish specimens of four different species (*Syngnathus* ty*phle*, *S. abaster, Nerophis maculatus* and *N. ophidion*) were captured during the study (Table 5; Figure 5). *S. typhle* was the most abundant pipefish specie in *P. oceanica* meadows during the study period, but it was captured only during warm season (Table 5; Figure 5a). Conversely, in *C. nodosa, S. typhle* was captured in both warm and cold seasons (Table 5; Figure 5b). *S. abaster* dominated the captures in terms of abundance throughout the year in *C.* nodosa was (Table 5; Figures 5a,b). Regarding *N. maculatus*, it was more abundant in *P. oceanica* than in *C. nodosa* meadows, where 7 and 2 individuals were captured respectively during both sampling seasons (Table 5; Figures 5a,b). The species *N. ophidion* was the least abundant in this study, where 2 individuals were captured in *C. nodosa* during the cold season and 1 individual in *P. oceanica* during warm season (Table 5; Figures 5a,b).

Highest pipefish densities (i.e. total abundance per squared kilometer) in *P. oceanica* during the warm period corresponded to *S. typhle*, and *N. maculatus* dominated the cold season (Table 5; Figure 5c). In *C. nodosa*, the species *S. abaster* presented the highest densities during both seasons, followed by *S. typhle* in the warm season as well as *S. typhle* and *N. ophidion* in the cold season (Table 5; Figure 5d).

Table 5. Pipefish Total Abundances and Densities (TA/1000m^2) for each habitat and season sampled.

		P. oceanica		*C. nodosa*	
		WARM	COLD	WARM	COLD
S. typhle	ABUNDANCE	20	0	10	5
	SE	0.24	0.00	0.25	0.12
	DENSITY	6.15	0.00	2.08	1.47
	SE	0.00	0.00	0.00	0.00
S. abaster	ABUNDANCE	0	1	12	13
	SE	0.00	0.06	0.27	0.13
	DENSITY	0.00	0.28	3.00	3.22
	SE	0.00	0.00	0.00	0.00
N. maculatus	ABUNDANCE	2	5	1	1
	SE	0.36	0.17	0.05	0.04
	DENSITY	0.24	2.24	0.22	0.19
	SE	0.00	0.00	0.00	0.00
N. ophidion	ABUNDANCE	1	0	0	2
	SE	0.06	0.00	0.00	0.05
	DENSITY	0.20	0.00	0.00	1.45
	SE	0.00	0.00	0.00	0.00

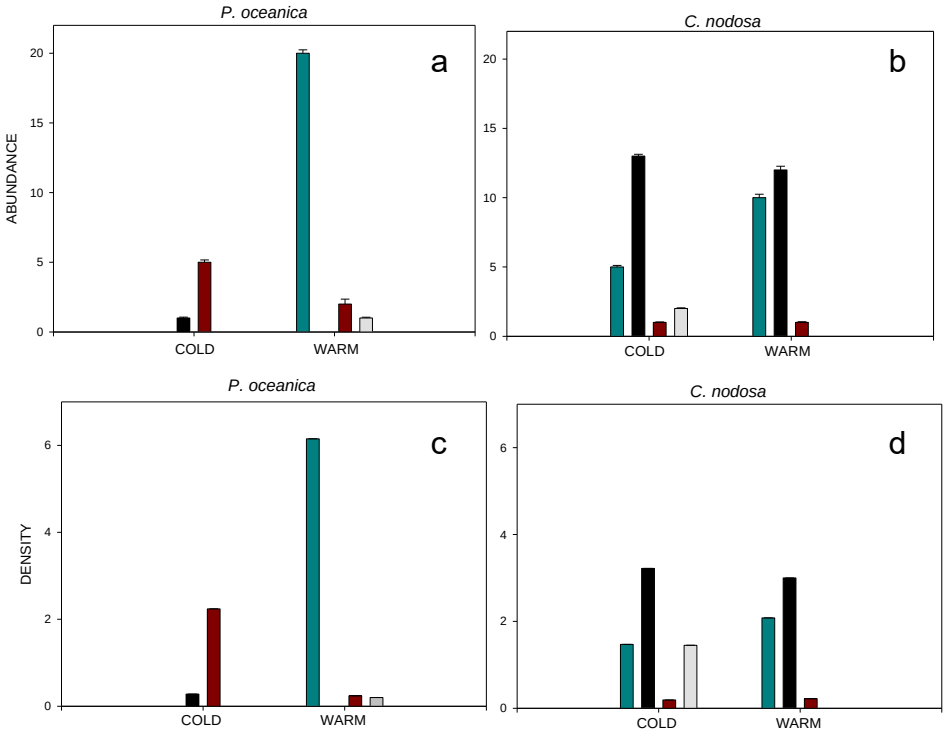

Figure 5. Pipefish Total Abundances and Densities (TA/1000m^2) for each habitat and season.

Low abundances and densities discourage statistical analysis to evaluate differences in pipefish distribution among habitats or/and seasons. For the same reason *N. maculatus* and *N. ophidion* were aggregated by genus *(Nerophis* sp.*)* on subsequent analysis.

Pipefish total length for each group of species, habitat and season were analyzed (Figure 6). Mann-Whitney U test revealed significant differences in total length for *S. typhle* (p=0.0001) and *Nerophis* sp. (p=0.0066) between *P. oceanica* and *C. nodosa*

(Table 6). Total length was higher in *P. oceanica* captures for both groups of species (Figure 6, Table 7). No differences in size were found for *S. abaster* between habitats ($p>0.05$). However, significant differences between season were only revealed for *S. abaster* ($p=0.0193$) in *C. nodosa* (Table 6), where pipefish captured during the cold season presented longer sizes (Figure 6).

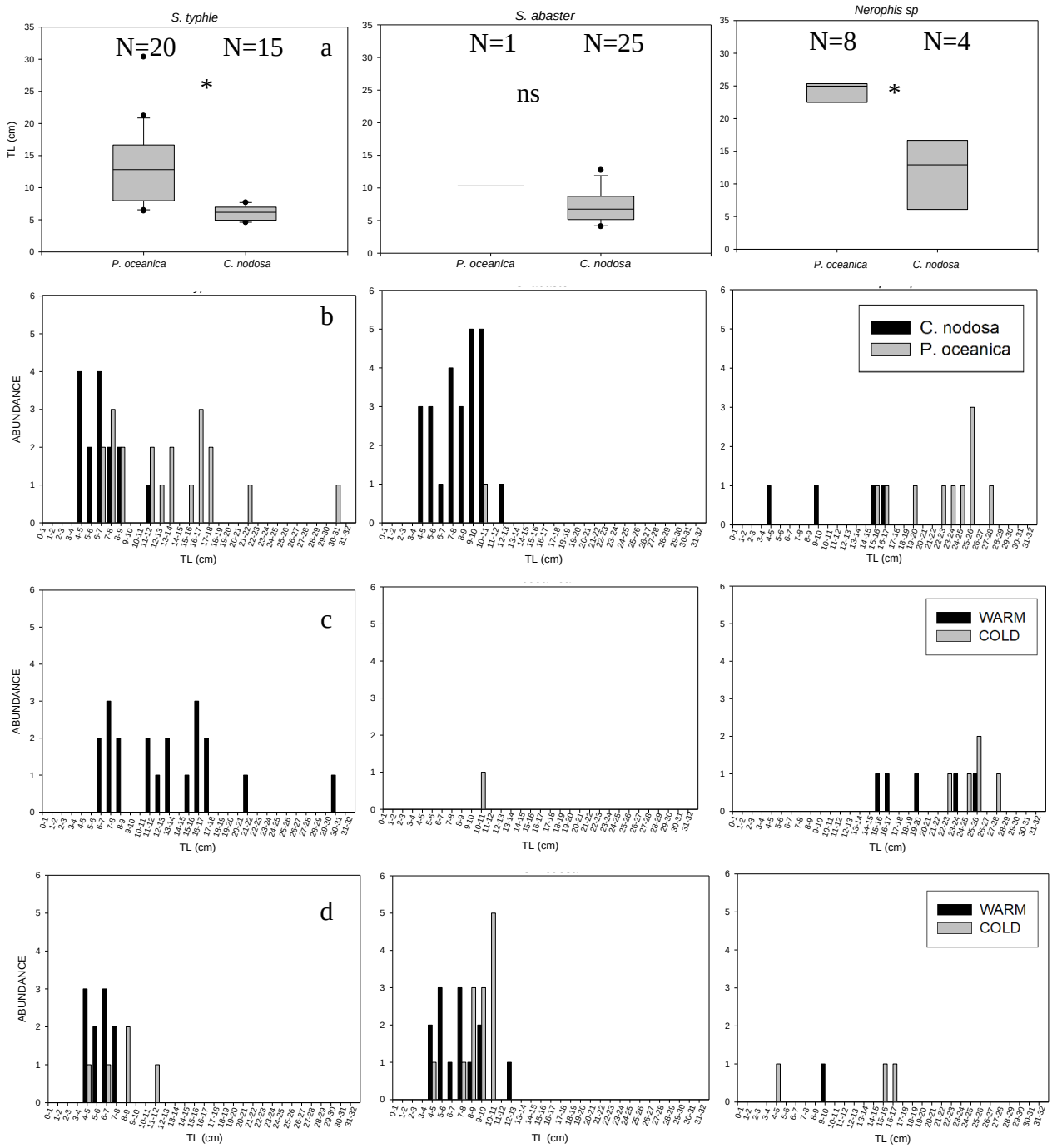

Figure 6. Pipefish Total Lengths: a) TL found for *S. typhle*, *S. abaster* and *Nerophis* sp. Boxplots shows mean, confidence intervals, error bars and outliers. Sample size and significant difference in pipefish size between habitats are also showed: $p<0.05$*; ns=no significant. b) Abundance of pipefish of *S. typhle*, *S. abaster* and *Nerophis* sp. for their TL in 1 cm intervals for each habitat. c) Abundance of pipefish for their TL in 1 cm intervals in *P. oceanica*. d) Abundance of pipefish for their TL in 1 cm intervals in *C.nodosa*.

Table 6. Mann-Whitney U Test results for differences between the size of different pipefish groups depending on the habitat and season.

SPECIES	HABITAT	M-W U TEST p	SEASON	M-W U TEST p
S. typhle	*C. nodosa*	0.0001	COLD vs. WARM	0.1416
	P. oceanica		COLD vs. WARM	No-test
S. abaster	*C. nodosa*	1.0000	COLD vs. WARM	0.0193
	P. oceanica		COLD vs. WARM	No-test
Nerophis sp	*C. nodosa*	0.0066	COLD vs. WARM	1.0000
	P. oceanica		COLD vs. WARM	0.1797

Mean TL were higher in *P. oceanica* than in *C. nodosa* for all pipefish groups (*S. typhle:* 16.23-5.99 cm; *S. abaster*: 10.31-8.26 cm; *Nerophis* sp.: 24.13-16.39 cm). Similarly, %HL/TL and %SL/TL present higher mean values in *P. oceanica* except in *Nerophis* sp. (Table 7). %HL/TL and %SL/HL are the main contributors to the variability among groups (Table 8,9, Figure 7) and both descriptors are strongly correlated with the rest of morphological relations measured ($p<0.05$; Table 10). Subsequent analyses of morphological features were then carried out only with %HL/TL and %SL/HL. One-way ANOVA revealed statistically significant differences among the groups of species depending on %HL/TL (p=0.0001) and %SL/HL (p=0.0001) (Table 11). *S.typhle* presented the higher %HL/TL and %SL/HL, followed by *S. abaster* and *Nerophis* sp. respectively (Figure 8). The PERMANOVA analysis revealed significant differences for the head morphometry of *S. typhle* (p=0.001) and *Nerophis* sp. individuals (p=0.026) inhabiting *P. oceanica* or *C. nodosa*.

Table 7. Mean, minimum and maximum values and standard error for all biometric characters studied for *S. typhle*, *S. abaster* and *Nerophis* sp. on each habitat.

Morphometric characters	*S. typhle*					
	P. oceanica			*C. nodosa*		
	MEAN	MIN-MAX	SE	MEAN	MIN-MAX	SE
TL (cm)	16.23	8.77-30.37	1.48	5.99	4.60-7.70	0.43
%HL/TL	18.04	14.47-21.14	0.45	16.22	10.77-18.20	0.96
%SL/HL	12.34	8.85-15.19	0.53	11.85	6.63-13.07	0.75
%ED/HL	1.77	1.37-2.28	0.07	2.08	1.64-2.60	0.10
%MSH/HL	2.44	1.94-2.81	0.08	1.61	1.21-2.00	0.10
%HM/HL	2.36	1.70-2.86	0.11	1.12	0.92-1.35	0.05
%WM/HL	2.09	1.53-2.55	0.10	0.85	0.66-1.01	0.05

Morphometric characters	*S. abaster*					
	P. oceanica			*C. nodosa*		
	MEAN	MIN-MAX	SE	MEAN	MIN-MAX	SE
TL (cm)	10.31	10.31	0.00	8.26	4.46-12.74	0.73
%HL/TL	12.46	12.46	0.00	11.22	9.14-14.34	0.37
%SL/HL	7.76	7.76	0.00	7.32	6.34-10.65	0.36
%ED/HL	2.09	2.09	0.00	1.92	1.47-2.39	0.08
%MSH/HL	1.55	1.55	0.00	1.36	1.05-1.97	0.06
%HM/HL	0.92	0.92	0.00	0.91	0.70-1.23	0.04
%WM/HL	0.78	0.78	0.00	0.67	0.50-0.89	0.04

Morphometric characters	*Nerophis sp*					
	P. oceanica			*C. nodosa*		
	MEAN	MIN-MAX	SE	MEAN	MIN-MAX	SE
TL (cm)	24.13	19.90-27.19	1.06	16.39	15.83-16.95	0.56
%HL/TL	5.39	3.85-6.58	0.42	5.47	5.11-5.84	0.36
%SL/HL	3.00	2.81-3.21	0.06	3.53	3.41-3.66	0.12
%ED/HL	0.60	0.40-0.78	0.07	0.99	0.97-1.01	0.02
%MSH/HL	0.50	0.31-0.66	0.06	1.29	1.26-1.33	0.04
%HM/HL	0.37	0.32-0.40	0.01	0.63	0.62-0.63	0.01
%WM/HL	0.31	0.25-0.36	0.02	0.50	0.47-0.54	0.04

Table 8. PCA results for pipefish groups of species depending on morphometric characters.

	Eigenvalues	%Variation	Cumulative %Variation	Eigenvector %HL/TL	Eigenvector %SL/HL
PC1	39.4	96.1	96.1	-0.782	-0.599
PC2	0.963	2.3	98.4	-0.506	0.756

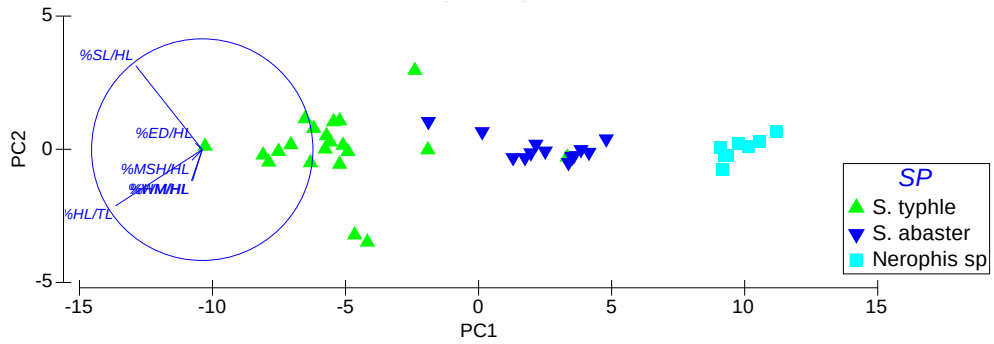

Figure 7. PCA results for pipefish groups of species depending on morphometric characters.

Table 9. SIMPER results for pipefish depending on morphometric characters. Contribution on %.

	Average dissimilarity%	Contribution %HL/TL	Contribution %SL/HL
S. typhle x *S. abaster*	23.01	43.53	34.99
S. typhle x *Nerophis sp*	54.63	45.42	34.32
S. abaster x *Nerophis sp*	37.27	45.85	32.64

Table 10. Correlation matrix for morphometric characters.

	%HL/TL	%SL/HL	%ED/HL	%MSH/HL	%HM/HL	%WM/HL
%HL/TL	1.00	0.95	0.70	0.83	0.80	0.77
%SL/HL	0.95	1.00	0.69	0.82	0.73	0.70
%ED/HL	0.70	0.69	1.00	0.50	0.35	0.29
%MSH/HL	0.83	0.82	0.50	1.00	0.90	0.89
%HM/HL	0.80	0.73	0.35	0.90	1.00	0.99
%WM/HL	0.77	0.70	0.29	0.89	0.99	1.00

Table 11. One-way ANOVA results for the factor species depending on morphometric characters.

Effect	Degr. Of Freedom	%HL/TL p	%SL/HL p
Intercept	1	0.0001	0.0001
sp	2	0.0001	0.0001

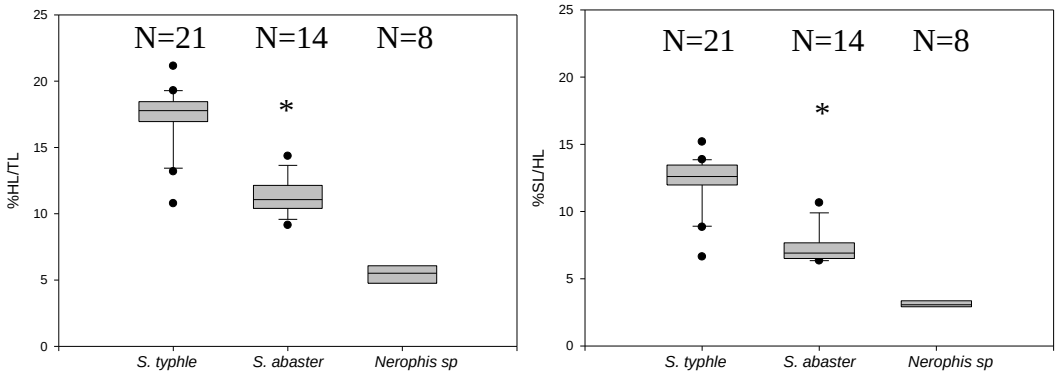

Figure 8. %HL/TL and %SL/HL for *S. typhle*, *S. abaster* and *Nerophis* sp. Boxplots shows mean, confidence intervals, error bars and outliers. Sample size and significant difference in pipefish morphometric characters are also showed: p<0.05*.

FEEDING PREFERENCES

Harpacticoid copepods and gammarid amphipods were the most frequent pipefish preys in the stomach content analyses (Table 12, Figures 9a,b). For *S. typhle*, the main prey was harpacticoid copepods in both sea grass meadows (%O=76-97.7%; %A: 66.7-100%), followed by teleostei and decapods (%O=8-12; %A: 33.3) in *P. oceanica*, and gammarid amphipods in *C. nodosa* (%O=6.3; %A: 62.5) (Table 12; Figures 9a,b). Vacuity index was equal to 33.3% for *S. tyhple*. For *S. abaster*, harpacticoid copepods (%O=37.7-60.8; %A=80-100) and gammarid amphipods (%O=33-44.9; %A=100) were the main preys in both habitats, followed by ostracods in *C. nodosa*. (Table 12; Figures 9a,b). No empty stomachs were found for this species. *Nerophis* sp. mainly forages on harpacticoid copepods in *P. oceanica* (%O=84.4; %A=100) and gammarid amphipods in *C. nodosa* (%O=57.5; %A=100). Secondary preys are gammarid amphipods and ostracods in *P. oceanica* (%O=5.4-9.5; %A=66.7-100) and harpacticoid copepods in *C. nodosa* (%O=42.5; %A=100) (Table 12; Figures 9a,b). *Nerophis* sp. presented a 37.5% value for the vacuity index.

Table 12. Pipefish stomach contents. TA: Total Abundance. %O: % Frequency of occurrence. %A: % Frequency of appearance.

	S. typhle											
	P. oceanica						*C. nodosa*					
	WARM			COLD			WARM			COLD		
	TA	%O	%A	TA	%O	%A	TA	%O	%A	TA	%O	%A
CRUSTACEA												
Copepoda												
Harpacticoida	38	76.0	66.7	-	-	-	330	93.8	100.0	-	-	-
Amphipoda												
Gammaridae	1	2.0	16.7	-	-	-	22	6.3	62.5	-	-	-
Caprelidae	0	0.0	0.0	-	-	-	0	0.0	0.0	-	-	-
Ostracoda	0	0.0	0.0	-	-	-	0	0.0	0.0	-	-	-
Decapoda	6	12.0	33.3	-	-	-	0	0.0	0.0	-	-	-
ARACHNIDA												
Acari	1	2.0	16.7	-	-	-	0	0.0	0.0	-	-	-
TELEOSTEI	4	8.0	33.3	-	-	-	0	0.0	0.0	-	-	-

	S. abaster											
	P. oceanica						*C. nodosa*					
	WARM			COLD			WARM			COLD		
	TA	%O	%A	TA	%O	%A	TA	%O	%A	TA	%O	%A
CRUSTACEA												
Copepoda												
Harpacticoida	-	-	-	4	57.1	100.0	188	60.8	100.0	26	37.7	80.0
Amphipoda												
Gammaridae	-	-	-	3	42.9	100.0	102	33.0	100.0	31	44.9	100.0
Caprelidae	-	-	-	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0
Ostracoda	-	-	-	0	0.0	0.0	18	5.8	75.0	11	15.9	80.0
Decapoda	-	-	-	0	0.0	0.0	1	0.3	12.5	1	1.4	20.0
ARACHNIDA												
Acari	-	-	-	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0
TELEOSTEI	-	-	-	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0

	Nerophis sp											
	P. oceanica						*C. nodosa*					
	WARM			COLD			WARM			COLD		
	TA	%O	%A	TA	%O	%A	TA	%O	%A	TA	%O	%A
CRUSTACEA												
Copepoda												
Harpacticoida	0	0.0	0.0	124	84.4	100.0	-	-	-	17	42.5	100.0
Amphipoda												
Gammaridae	0	0.0	0.0	14	9.5	100.0	-	-	-	23	57.5	100.0
Caprelidae	0	0.0	0.0	1	0.7	33.3	-	-	-	0	0.0	0.0
Ostracoda	0	0.0	0.0	8	5.4	66.7	-	-	-	0	0.0	0.0
Decapoda	0	0.0	0.0	0	0.0	0.0	-	-	-	0	0.0	0.0
ARACHNIDA												
Acari	0	0.0	0.0	0	0.0	0.0	-	-	-	0	0.0	0.0
TELEOSTEI	0	0.0	0.0	0	0.0	0.0	-	-	-	0	0.0	0.0

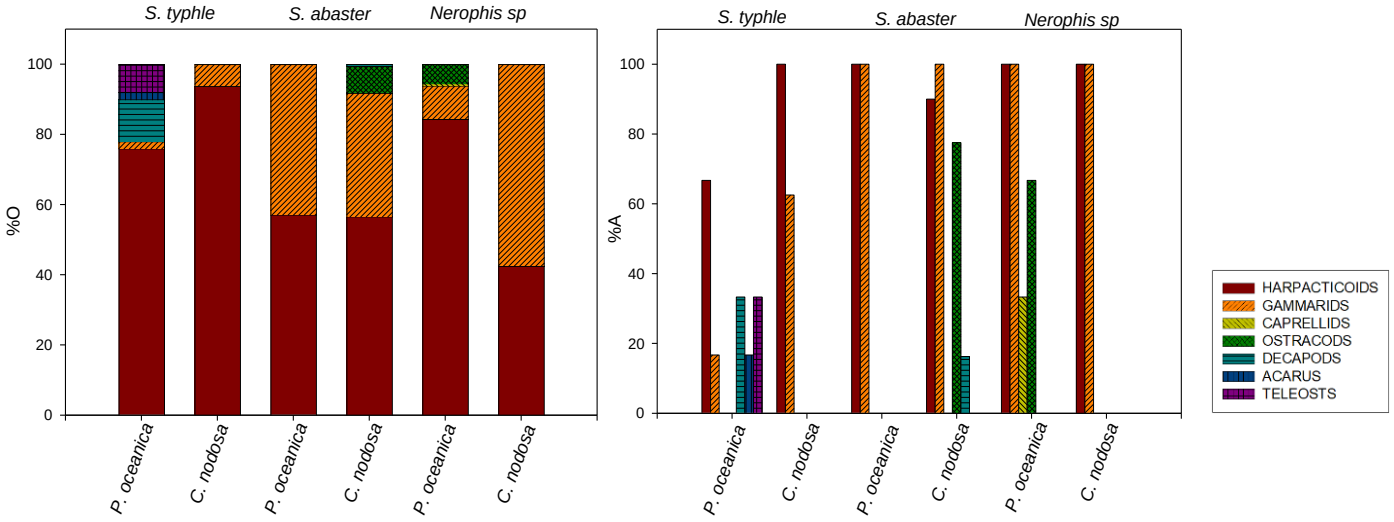

Figure 9. Pipefish stomach contents: a) %O; b) %A.

The main differences between pipefish diets were caused by gammarid amphipods, ostracods and teleosts according to PCA (Figure 10; Table 13) and SIMPER Analysis (Table 14). Similarly, Kruskal-Wallis test revealed significant differences between the different groups of species ingestion on gammarid amphipods and ostracods (Table 15).

Table 13. PCA results for pipefish groups of species depending on pipefish stomachs.

	Eigenvalues	%Variation	Cumulative %Variation	Eigenvector GAMMARIDS	Eigenvector OSTRACODS	Eigenvector TELEOSTS
PC1	1.49	64.5	64.5	0.557	0.282	-0.042
PC2	0.41	17.7	82.2	-0.617	-0.475	0.063

Table 14. SIMPER results for pipefish groups of species depending on stomach contents. Contribution on %.

	Average dissimilarity%	Contribution GAMMARIDS	Contribution OSTRACODS	Contribution DECAPODS	Contribution TELEOSTS
S. typhle x *S. abaster*	37.08	38.17	32.33	0.00	0.00
S. typhle x *Nerophis sp*	100.00	5.56	0.00	22.22	22.05
S. abaster x *Nerophis sp*	38.86	27.11	28.08	0.00	0.00

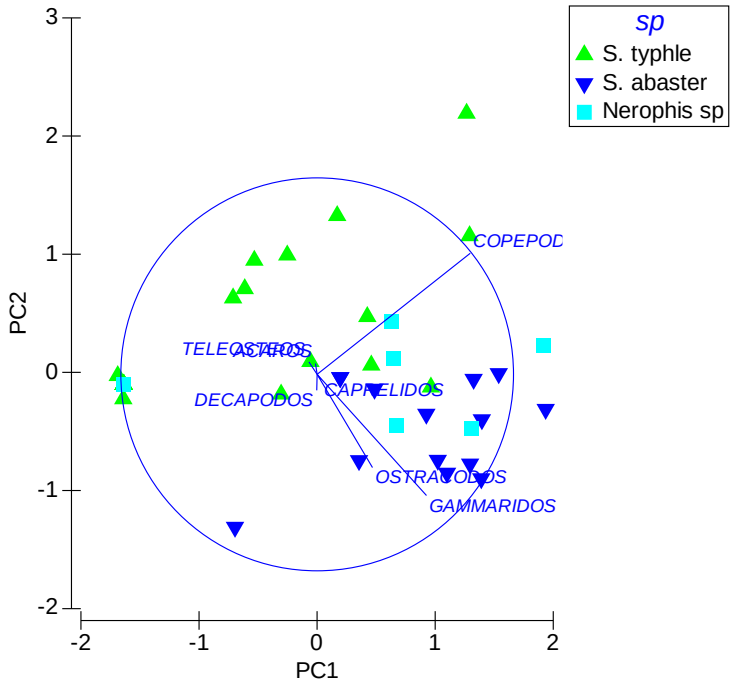

Figure 10. PCA results for pipefish groups of species depending on stomach contents.

Table 15. Kruskal-Wallis p-value for stomach contents comparing pipefish groups of species.

	p-value
Harpacticoids	0.129
Gammarids	0.001
Caprellids	0.112
Ostracods	0.001
Acarus	0.592
Decapods	0.561
Teleosts	0.342

Pipefish snout (MSH) and mouth (HM, WM) opening sizes were negatively correlated to their ingestion of harpacticoid copepods, gammarid amphipods and ostracods and positively correlated to teleost ingestion (Table 16).

Table 16. R correlation coefficient for stomach contents and morphometry.

	Harpacticoid copepods	Gammarid amphipods	Ostracods	Teleosts	Decapods	Acarus	Caprellids
TL (cm)	-0.43	-0.33	-0.22	0.13	0.05	0.07	0.21
%HL/TL	-0.22	-0.51	-0.39	0.33	0.09	0.24	-0.19
%SL/HL	-0.13	-0.50	-0.38	0.31	0.01	0.24	-0.20
%ED/HL	0.25	0.03	0.04	-0.11	0.05	-0.05	-0.21
%MSH/TL	-0.34	-0.49	-0.45	0.31	0.09	0.22	-0.20
%HM/TL	-0.44	-0.51	-0.41	0.31	0.04	0.21	-0.22
%WM/TL	-0.39	-0.47	-0.41	0.31	0.08	0.21	-0.22

EPIFAUNAL COMMUNITY

Two analytical approaches were used to study the epifaunal community diversity: direct analysis on community composition (PERMANOVA) and Shannon-Wiener Index (H'), which synthetize and aggregate this information. Invertebrate communities from the *C. nodosa* meadow showed higher standardized abundances (referred to 100cm^2 of foliar surface) than those from *P. oceanica* (Figure 11). In both habitats, relative abundances of epifauna were higher during the cold period. These differences were revealed to be statistically different with a PERMANOVA analysis (p=0.001 for differences between habitats and p=0.011 for differences between season in each habitat) (Table 17). 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 (Figure 12).

Table 17. Epifauna Total Abundance (TA); Relative Abundance (RA=TA/100cm^2 foliar Surface); and Diversity (H').

	Epifaunal communities							
	P. oceanica				*C. nodosa*			
	WARM		COLD		WARM		COLD	
	TA	RA	TA	RA	TA	RA	TA	RA
CRUSTACEA								
Copepoda	89.88	13.40	129.35	14.60	8.50	13.44	6.10	14.37
Amphipoda								
Gammaridae	19.20	30.80	76.45	56.90	13.80	16.60	27.67	6.12
Caprellidae	2.12	3.30	1.10	2.50	3.00	0.36	1.47	0.08
Ostracoda	1.23	0.36	0.20	0.40	0.40	0.00	0.20	0.00
Decapoda	0.00	0.54	0.10	0.00	0.27	0.01	0.07	0.01
Isopoda	1.24	1.16	1.00	0.31	0.80	0.24	0.33	0.17
Tanaidacea	0.84	1.25	2.25	0.02	0.07	0.69	0.60	0.20
Cumacea	0.64	1.52	0.05	0.89	0.40	0.06	0.80	0.01
Mysidacea	3.20	0.21	0.10	0.54	0.33	0.00	0.20	0.03
ARACHNIDA								
Acari	14.12	0.61	6.75	1.80	0.47	1.20	0.33	0.70
PYCNOGONIDA								
Pantopoda	0.00	0.04	0.10	0.00	0.07	0.02	-	-
CNIDARIA	0.88	0.00	0.00	0.10	0.00	0.00	0.00	0.00
GASTROPODA	28.28	4.20	2.90	3.70	6.80	1.75	1.33	0.72
Opistobranchia	0.16	0.00	0.00	0.02	0.00	0.02	-	-
POLYPACOPHORA	0.28	0.00	0.00	0.03	-	-	0.00	0.01
BIVALVIA	1.61	1.90	0.30	0.80	1.10	0.08	0.70	0.10
ANELIDA								
Polychaeta	17.64	6.60	10.50	2.40	3.93	3.41	2.00	1.14
CHAETOGNATHA	0.52	0.10	0.00	0.06	0.07	0.00	0.00	0.02
TURBELLARIA	0.04	0.00	0.00	0.01	-	-	-	-
ECHINODERMATA	0.12	0.00	0.25	0.00	0.07	0.06	0.00	0.04
NEMATODA	1.20	1.90	0.20	0.15	0.87	0.08	0.33	0.02
TELEOSTEI	0.04	0.00	-	-	-	-	0.00	0.00
H'	1.50781781		0.944756562		1.604885717		1.142545764	

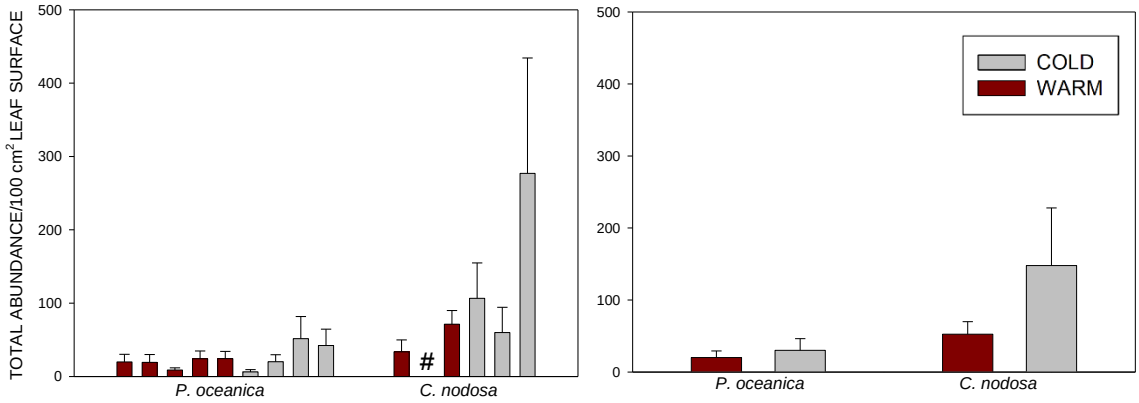

Figure 11. Invertebrate communities Total Abundance/100 cm^2 Leaf Surface. #: lack of leaf surface data.

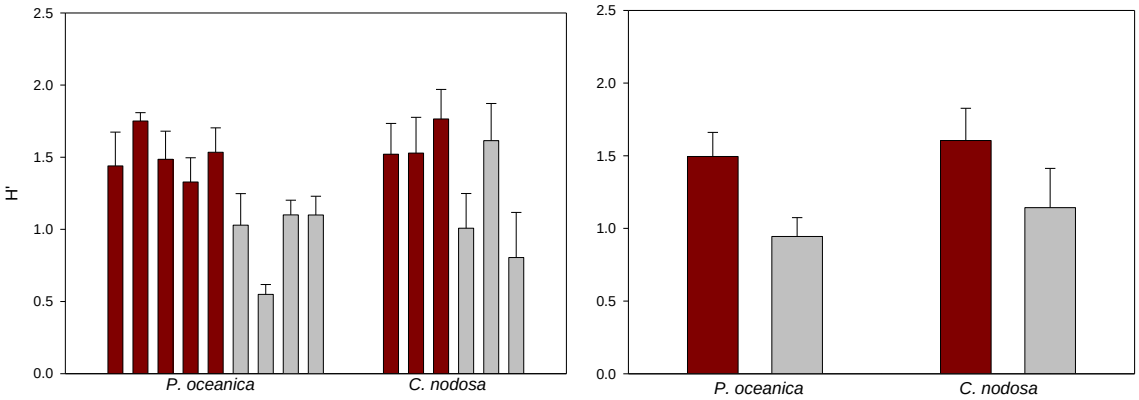

Figure 12. Diversity (Shannon-Wiener Index) of epifaunal communities.

The differences between habitats and seasons were mainly due to the relative abundance of caprellid and gammarid amphipods, gastropods and polychaetes, as it was revealed by PCA (Figure 13; Table 18) and SIMPER analysis (Table 19). Kruskal-Wallis analysis between habitats revealed significant differences for both types of amphipods and for gastropods (Table 20). Regarding differences between seasons within each habitat, significant differences were found for harpacticoid copepods and gastropods in *P. oceanica* and gammarid amphipods in *C. nodosa* (Table 20).

Table 18. PCA results for epifaunal communities depending on habitat and season.

	Eigenvalues	%Variation	Cumulative %Variation	Eigenvector GAMMARIDS	Eigenvector CAPRELLIDS	Eigenvector GASTROPODS	Eigenvector POLYCHAETES
PC1	1.01	26.6	26.6	-0.83	-0.42	0.04	-0.03
PC2	0.616	16.2	42.8	-0.13	-0.01	0.49	0.49

Table 19. SIMPER results for epifaunal communities depending on habitat and season. Contribution on %.

	Average dissimilarity%	Contribution GAMMARIDS	Contribution CAPRELLIDS	Contribution GASTROPODS	Contribution POLYCHAETES
P. oceanica x *C. nodosa*	43.86	15.07	10.02	6.98	8.58
P. oceanica Warm x Cold	34.22	11.02	7.26	10.73	5.22
C. nodosa Warm x Cold	39.33	11.60	10.82	8.14	11.46

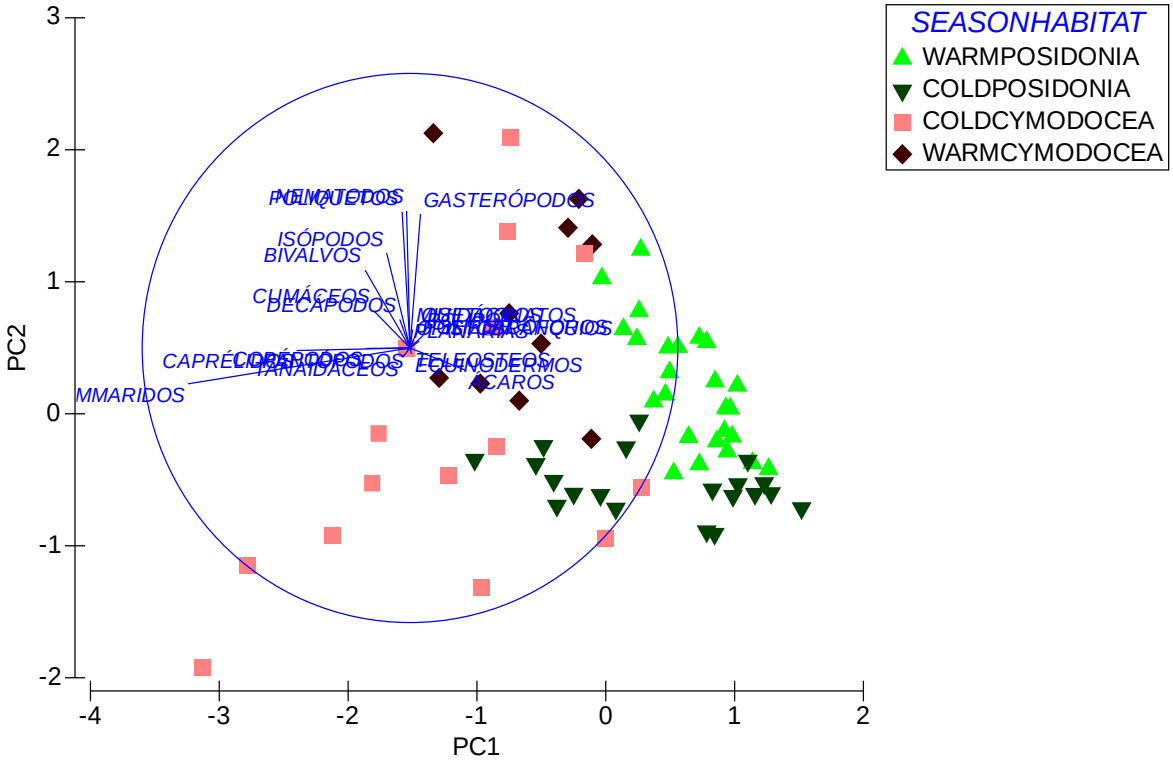

Figure 13. PCA distribution of epifauna samples.

Table 20. Kruskal-Wallis test results for epifaunal communities of different hábitats and seasons.

	P.oceanica x *C. nodosa* p-value	*P. oceanica* Cold x Warm p-value	*C. nodosa* Cold x Warm p-value
Harpacticoids	0.5199	0.0421	0.202
Gammarids	0.0001	0.1929	0.0019
Caprellids	0.0094	0.2226	0.6466
Gastropods	0.0104	0.0001	0.373
Poychaetes	0.0598	0.0318	0.1448

Regarding potential relationships among diet compositions of each group of species and presence of potential preys (epifaunal communities) on both seagrasses, Spearman Rank R test revealed a correlation between the %O of each prey in the diets of *S. abaster* and *Nerophis* sp. and the composition of the epifaunal communities present in *P. oceanica* (Table 21; Figure 14). Additionally, in the *C. nodosa* meadows, there was a correlation between the three pipefish groups and the invertebrate community assemblage (Table 21; Figure 14).

Table 21. R correlation coefficient for diets and habitats.

	S. typhle	*S. abaster*	*Nerophis sp*
P. oceanica	0.13	0.51	0.56
C. nodosa	0.51	0.50	0.52

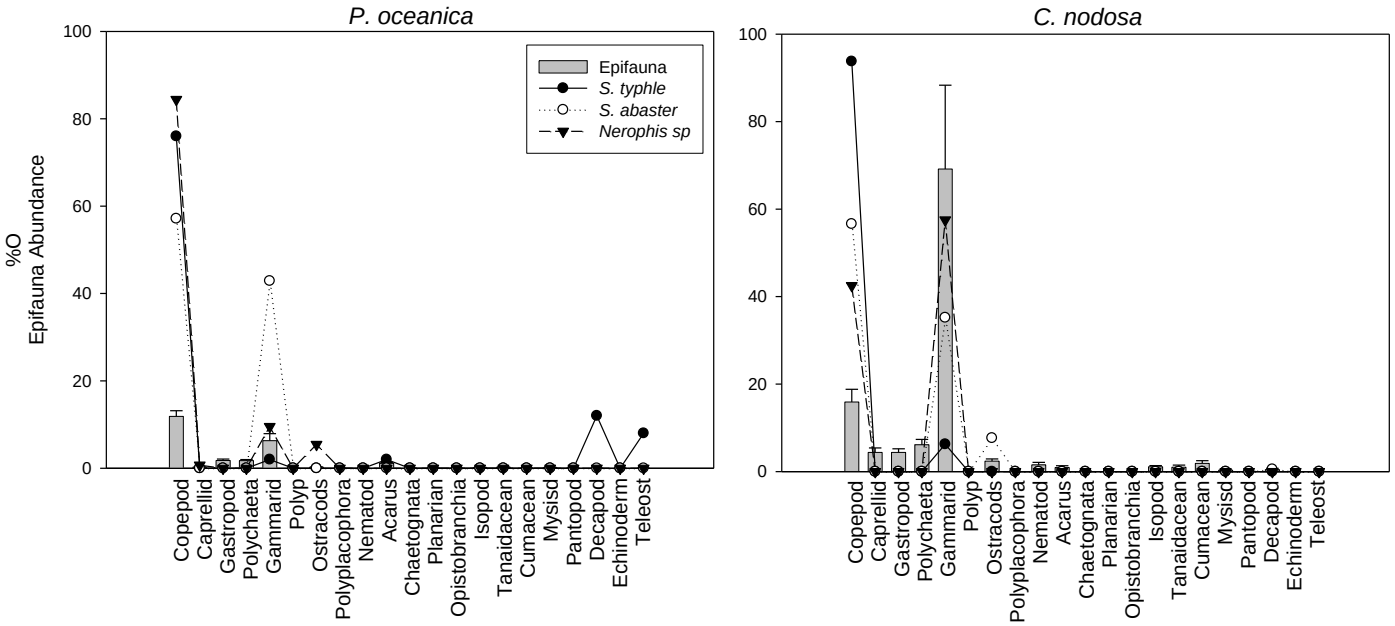

Figure 14. %O of different preys in pipefish diets and Relative Abundances of epifaunal invertebrates.

DISCUSSION

Relatively little information is available on the population trends of syngnathid populations in the Western Mediterranean Sea. To our knowledge, no formal range-wide surveys or field population estimates of these species have been undertaken to the moment and trends or seasonal variability in densities of Mediterranean pipefish are still unknown. The present study showed that seagrass meadows in the Balearic Islands support pipefish assemblages dominated by *S. typhle* and *S. abaster*, but *N. ophidion* and *N. maculatus* were also important in these communities. Three of these species are assessed at global scale as Least Concern (LC) by the IUCN Red List of Threatened Species, including *S. typhle* (Pollom, 2014), *S. abaster* (Freyhof, 2016) and *N. ophidion* (Pollom, 2015), and one is Data Deficient (DD), *N. maculatus* (Wiswedel, 2016).

The coexistence of several pipefish species in the same seagrass meadow is determined by different morphological adaptations between species, which are the most important factor determining separation in the ecological niche between them (Vizzini & Mazzola, 2004). In this study, some differences were found in the habitat choice of each group of species. As seagrass species determine some characteristics of meadow architecture such as leaf and shoot densities or leaf surface that could affect the availability of suitable habitats for syngnathids (Malavasi *et al.*, 2007), larger pipefish individuals were expected to be found preferably on *P. oceanica*, characterized by larger

and broader leaves and higher shoot densities rather than in *C. nodosa*, with shorter and thinner leaves and sometimes associated to algal presence.

Due to our low sample size it was not possible to estimate precisely the pipefish distribution depending on body size for the different habitats and seasons. More sampling effort is needed to complete this information, as total length seems to be an important factor in pipefish population dynamics. However, our results showed that smaller specimens of *S. typhle* and *Nerophis* sp. as well as almost every individual of *S. abaster* preferred habitats dominated by *C. nodosa*, while larger individuals of *S. typhle* and *Nerophis* sp. seemed to prefer tall canopy meadows formed by *P. oceanica.* A strong relationship exists between *S. typhle* and *Nerophis* sp. populations, as reported by Scapin *et al.* (2018). 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 on habitat choice could be explained as larger *S. typhle* and *Nerophis* sp. 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 smaller individuals of *S. typhle* and *Nerophis* sp. and for *S. abaster,* which are morphologically better adapted to sparse and narrow leaves (Verdiell-Cubedo *et al.,* 2007). Differences in seagrass association between *S. typhle, Nerophis* sp. and *S. abaster* showed 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 lax seagrass meadows, compared with *S. typhle* and *N. ophidion*, which appear to be dense seagrass specialist.

Moreover, pipefish exhibit a high degree of trophic specialization compared to other epibenthic marine teleosts (Gürkan, 2008). This specialization occurs between different syngnathid species due to their head and snout morphologies (Kendrick & Hydnes, 2005). Differences in the diets were found to be mainly related to the ingestion of gammarid amphipods, ostracods and teleosts, while harpacticoid copepods are the primary prey for all pipefish. Other authors had already stated by stomach contents analysis that pipefish diets are based on small crustaceans (Teixeira & Musick, 1995; Campolmi *et al.*, 1996; Kendrick & Hyndes, 2005). 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 of the individual growth of individuals. As prey are swallowed whole, the dimensions and maximum opening of the mouth determine the maximum size of prey that can be ingested (Oliveira *et al.,* 2007). Morphologic variations between pipefish groups of species and sizes found in this study may explain differences in the selection of secondary prey, although primary preys are always harpacticoid copepods. 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 *Nerophis* sp., whose snout is cylindrical and less than half the length of the head and whose mouth opening is the smaller of all. Besides, the opening of the mouth causes the expansion of the lateral walls of the snout forming a tube with increase in volume (Oliveira *et al.,* 2007).

Small pipefish individuals or species with smaller mouth openings are highly selective consuming a narrow range of prey (i.e. harpacticoid copepods, gammarid amphipods and ostracods) because of their limitations on mouth opening and short snout. 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).

Larger specimens of *S. typhle* had the ability to predate on bigger prey (i.e. decapods or juvenile teleosts) apart than harpacticoid copepods and gammarid amphipods. In fact, one of the largest pipefish found on this study, 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 to Bell's report (1983) of small fish being important on larger *S.typhle* specimen's diet. 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 correspondent increase in trophic level. Conversely, *Nerophis* sp.'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 on either small (i.e. gammarid amphipods or ostracods) or big prey (i.e. decapods) apart from the common prey.

Pipefish body size has been proved to be an important factor in their feeding ecology, as it determines the rank of prey that can be ingested. Hereby, more studies analyzing the relation 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).

Differences on head morphology and feeding strategies are needed because pipefish share the same habitats. In this sense, species that are present in the same habitat would however use different trophic resources (i.e. preys) and feeding strategies, and thus their foraging niches do not concur. While *S. abaster* feeds in submerged vegetation, *S. typhle* and *Nerophis* sp. also catch prey in the water column (Vizzini & Mazzola, 2004). Relatively high vacuity indexes compared to previous studies in syngnathids (Taçkavat *et al.,* 2010) were found for *S. typhle* and *Nerophis* sp. (33.3 and 37.5% respectively).

Divergences in the diet has sometimes been thought to be only related to the pipefish ability to catch a wider range of prey and not to fluctuations in prey abundance (Oliveira *et al.,* 2007). However, results in this study probed that apart from the morphology, the availability of prey is also important. Epifaunal communities were more abundant in *P. oceanica*, but when this abundance was related to foliar surface availability results showed a higher invertebrate's relative abundance in *C. nodosa* meadows. These differences were mainly caused by the presence of gammarid amphipods and gastropods. Furthermore, this abundance was higher during the cold season for both habitats, being copepods the responsible for this difference in *P. oceanica* and gammarid amphipods in *C. nodosa*. Nevertheless, epifaunal communities' diversity was similar in both habitats but higher during the warm season.

Results of this study indicated that there is a relation of the pipefish diet with the changes in the structure of the epifaunal assemblages. Prey frequency of occurrence in stomach contents of *S. abaster* and *Nerophis* sp. was related to invertebrate abundances in both habitats. Additionally, *S. typhle*'s diet was related to epifaunal assemblages in *C. nodosa*. As suggested by Mattson (1990), the taxonomic composition of the diet is determined by the composition of the potential prey in the environment. The availability and vulnerability of the prey species influences the consumption rates and contribution to the diet (Franzoi *et al.,* 1993). This may be the reason why harpacticoid copepods and gammarid amphipods are 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.

Some other habitats apart from seagrass meadows, such as oyster reefs, could possibly have an important role in supporting pipefish assemblages (Scapin *et al.*, 2018). Future studies should focus on investigating a wider spatial extension of pipefish distribution in order to provide a better understanding on habitat characteristics affecting pipefish distribution and provide effective tools towards their population management and conservation. Moreover, studying the feeding ecology of these species by stomach contents analysis provides evidences of food preferences and foraging habits but in many cases 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, 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 urgent to investigate the stable isotopes ratios of pipefish and potential prey treated in this study to establish the relation between prey ingested and prey assimilated.

CONCLUSIONS

The main conclusions obtained in this study are:

1. Pipefish communities in the Western Mediterranean are dominated by *S. typhle* in *P. oceanica* meadows and *S. abaster* in *C. nodosa* meadows.
2. Habitat choice of the different species depends on their morphological adaptations and the meadow architecture.
3. The habitat preference of *S. typhle* and *Nerophis* sp. (*P. oceanica*) might be conditioned to their body size and non-conditioned by season. Conversely, the presence and abundances of *S. abaster* on *C. nodosa* might be independent on their body size but dependent on the season.
4. Feeding preferences of pipefish depend on body size and head and snout morphology. The maximum snout dimension and mouth opening allows *S. typhle* to feed on bigger and faster prey than *S. abaster* and *Nerophis* sp.
5. Regardless the pipefish species, harpacticoid copepods and gammarid amphipods are considered primary prey, while ostracods, decapods and teleosts secondary prey and caprellid amphipods and acarus occasional prey.

6. Ingested prey also depends on prey availability in the seagrass meadows they inhabit, where harpacticoid copepods and gammarid amphipods are also the dominant component of invertebrate assemblages in both studied habitats.

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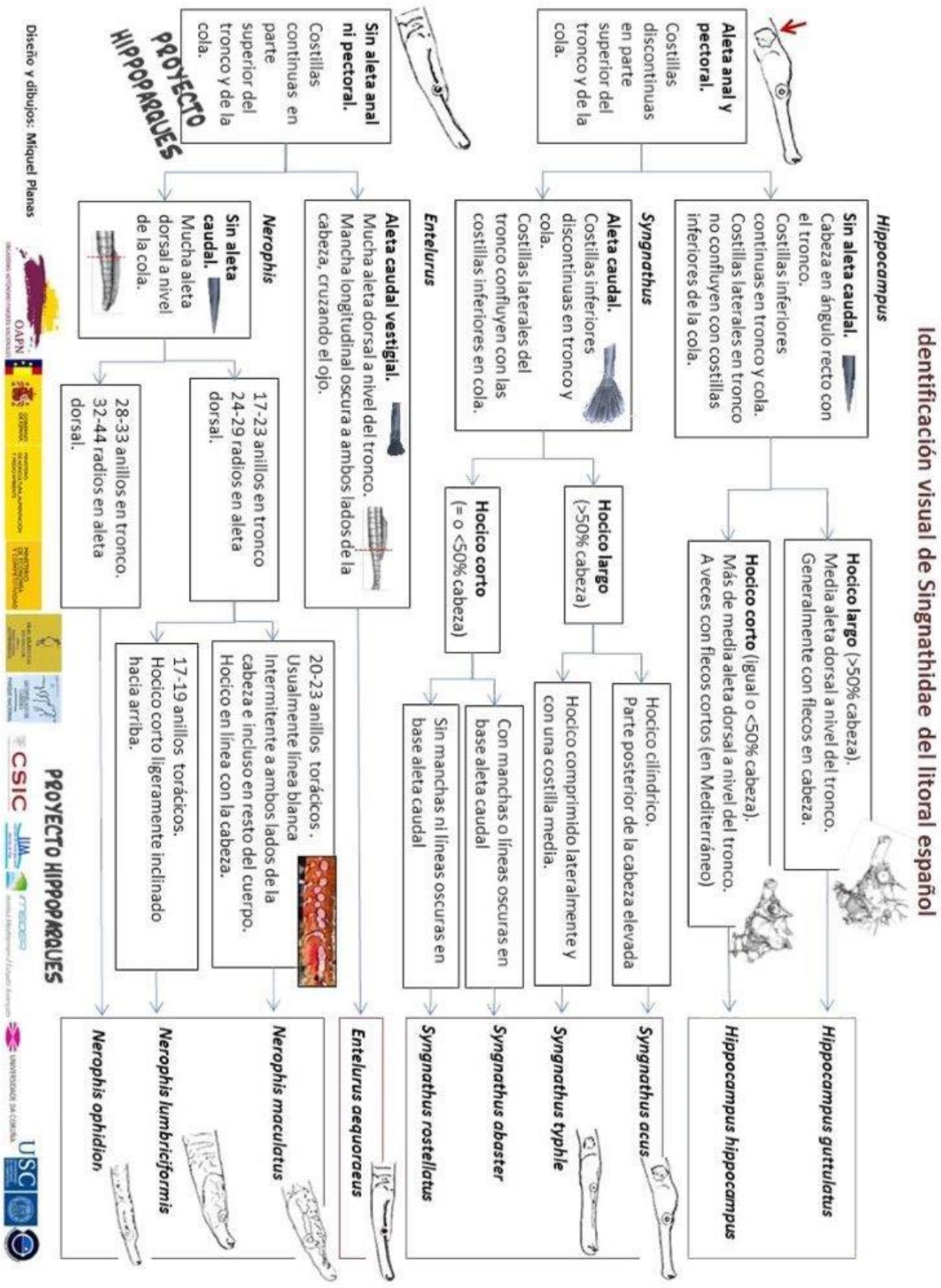
ANNEX 1:

Syngnathids identification guide used in `` Hippoparques Project ´´.



ANNEX 2:

The evaluation of fish basic life history traits, such as breeding season or fecundity, is fundamental to understand responses of different species to environmental changes (overfishing, fisheries management, etc.) (Winemiller & Rose, 1992). The objective of this part of the study was to identify and describe the ovary and testes developmental stages along the annual reproductive cycle in four different species of syngnathids.

After fixation of the whole individuals in 70% ethanol or 10% formaldehyde v/v, necropsy was performed at laboratory and gonads were extracted. In immature or resting individuals, the gonads were small, thread-like structures attached to the swim-bladder wall and coated by the dorsal mesentery. As they were difficult to dissect, the entire swimbladder wall was removed, preserved and sectioned transversally in order to observe the gonad structure.

Gonad samples were dehydrated in alcohol and embedded in paraffin wax for routine histological purposes at LIMIA (Marine Investigation and Aquaculture Laboratory; Dirección General de Pesca y Medio Marino, Govern Illes Balears). Samples were processed for their embedding with the automatic inclusive STP120 Myr. Gonads are introduced into plastic 'cassettes', washed in tap water in order to remove formaline remnants, dehydrated through ethanol series, and embedded in paraffin wax. Block making was performed with the block forming unit AP300-1 Myr. Blocks were sectioned using disposable razor blades using a rotation microtome HM330 Microm. The embedded gonads were sectioned transversely at 3-4 μm thickness, and stained with Mayer's haematoxylin and eosin 1% (Luna, 1968). Histological sections obtained were observed under a LEICA DMRA2 microscope and pictures were taken with the coupled LEICA DFC425 C camera.

Sex were established macroscopically by morphological differential trends between individuals: presence or absence of eggs (free and located along the ventral side of the male's body in genus *Nerophis*) or the male's structure to protect the eggs (complete developed brood pouch in genus *Syngnathus*) (Kornienko, 2001). Sexual stage was determined visually when possible, by the presence or absence of ripe and developing oocytes, coloration and length of the gonads, despite estimates of the stage of ovarian development based on macroscopic inspection of the gonads has proven to be imprecise and may lead to biased inferences (Murua *et al*., 2003). When gonads were too small, sex

and maturity could not be determined by this method. Then, sex and gonad development were determined based on microscopic observations.

According to Kornienko (2001), ovaries of female syngnathids are cilindrical, with no ovigerous plates, and developing follicles are located between the outer wall of the ovary and the inner coelomin epithelium, so that they go through subsequent developmental stages: from oogonia to mature oocytes. Testes of male syngnathids are paired bag-shaped glands. Gametes in the testis wall are mixed with somatic cells and no true cyst structure is found.

For the classification, a scale divided in 4 stages depending on macro and microscopic characteristics of the gonads was used following Brito & Bazzoli's work (2003) (Table 1a,b). Ovary reproductive phases were determined based on the occurrence of young oocytes (YO), pre-vitellogenic oocytes (PV) atretic vitellogenic oocytes (VO) and spawning oocytes (SO), as well as postovulatory follicles (PF). Male gonad stages were based on male germ cell development and the presence of spermatogonia (SG), spermatogenic cells (SC) or spermatozoa (SZ).

Table 3. Brito & Bazzoli's (2003) classification of fish gonads by macro and microscopic characteristics: a) Ovaries b) Testes

	OVARIES	TESTES
STAGE I	Translucent and with only presence of young (YO) and pre-vitellogenic oocytes (PV)	Whitish and poorly developed, with presence of spermatogonia (SG)
STAGE II	Yellowish and with presence of YO, PV and cortical alveoli oocytes (CA)	White and with spermatogenic cells (SC) and small quantity of spermatozoa (SZ) in the lumen of seminiferous tubules
STAGE III	Yellow and with YO, PV, CA and vitellogenic oocytes (VO)	White and with large quantity of spermatozoa in the lumen of seminiferous tubules (LU)
STAGE IV	Yellow, maximum length and with spawning oocytes (SO) visible with the naked eye in addition to YO, PV and VO. Sometimes postovulatory follicles are fund (PF)	White, maximum length and with seminiferous tubules with open lumen

In addition to these macro and microscopic characteristics, we have included brooding males into STAGE IV. According to this criterion and due to low pipefish abundances found on the study area, we still haven't found some of the developmental gonad stages previously described for all the species (Table 2).

Table 4. Sexual maturation stages found on pipefish from the Balearic Islands.

	S. typhle		*S. abaster*		*N. ophidion*		*N. maculatus*	
	FEMALE	**MALE**	**FEMALE**	**MALE**	**FEMALE**	**MALE**	**FEMALE**	**MALE**
STAGE I	Fig 1.a	Fig 2.a	Fig 3.a					
STAGE II						Fig 4.a		
STAGE III	Fig 1.b		Fig 3.b					
STAGE IV	Fig 1.c	Fig 2.b	Fig 3.c Fig 3.d				Fig 5.a	Fig 6.a

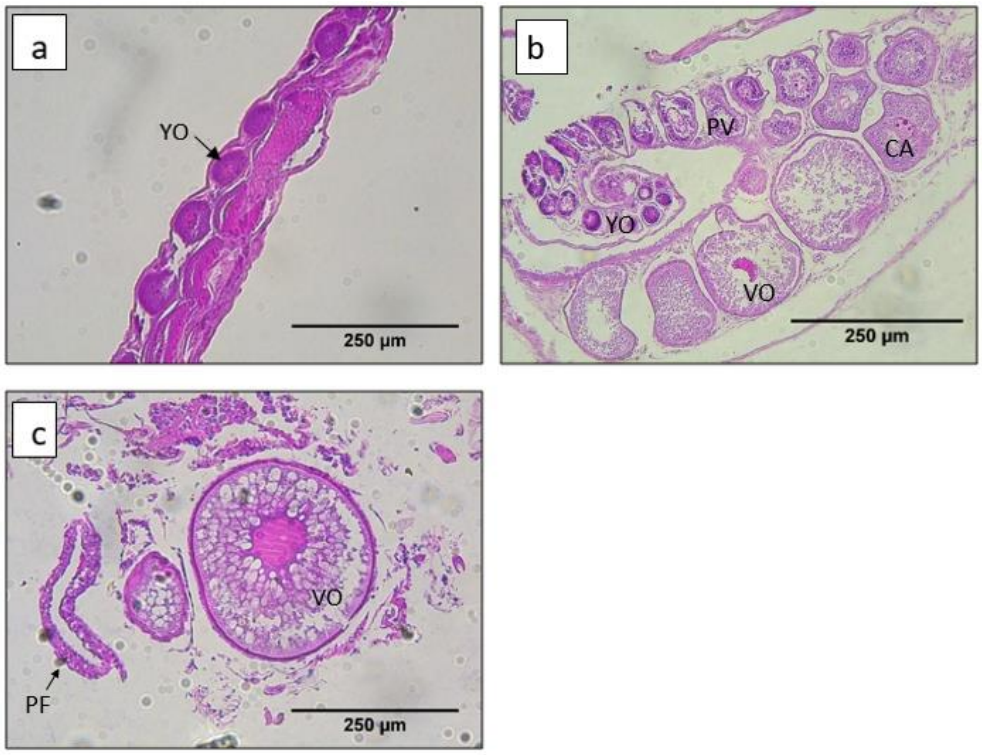


Figure 1. Female *S. typhle* reproductive stages: a) STAGE I b) STAGE III c) STAGE IV. YO: young oocytes. PV: pre-vitellogenic oocytes. CA: cortical alveoli oocytes. VO: vitellogenic oocytes. PF: postovulatory follicles.

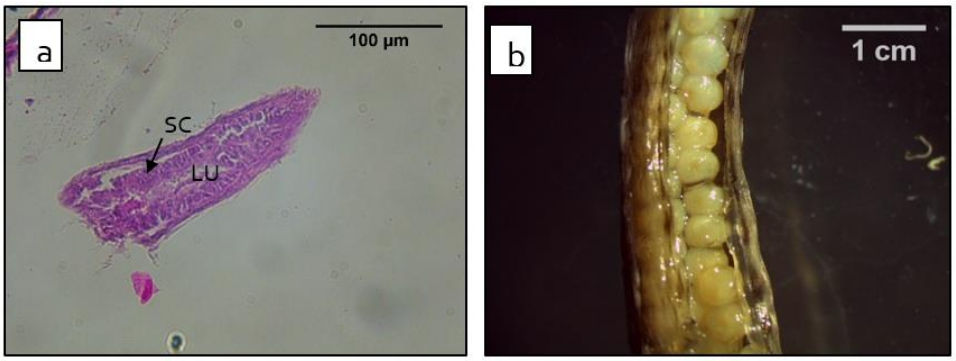


Figure 2. Male *S. typhle* reproductive stages: a) STAGE I b) STAGE IV (macroscopic picture, classified by the presence of brooding structure and eggs). SC: spermatogenic cells. LU: lumen of seminiferous tubules.

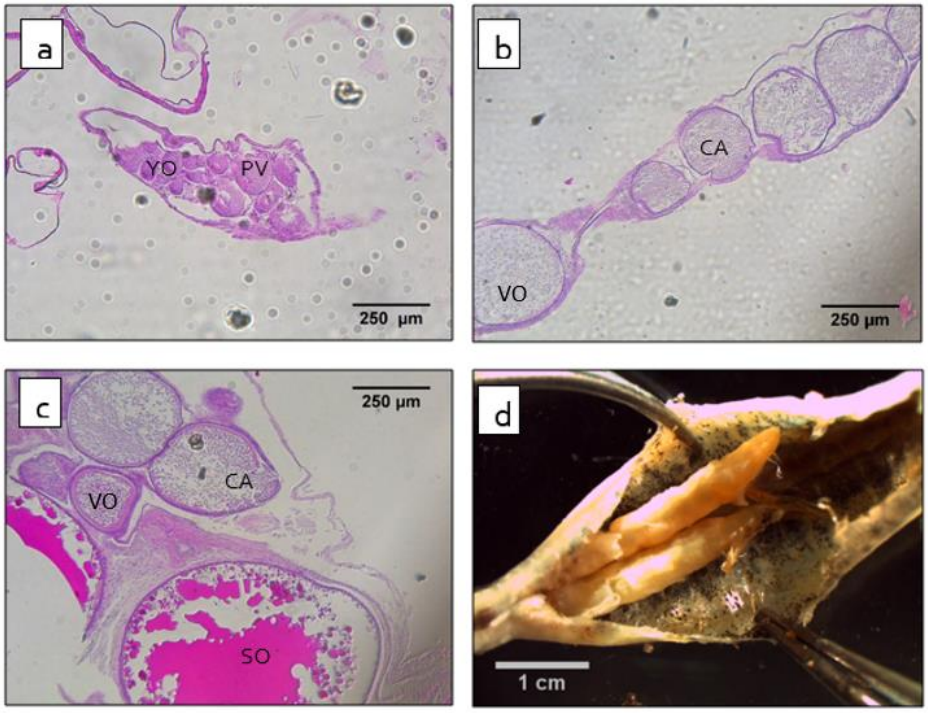


Figure 3. Female *S. abaster* reproductive stages: a) STAGE I b) STAGE III c) STAGE IV d) STAGE IV (macroscopic picture, classified by coloration and length of the gonad). YO: young oocytes. PV: pre-vitellogenic oocytes. CA: cortical alveoli oocytes. VO: vitellogenic oocytes. SO: spawning oocytes.

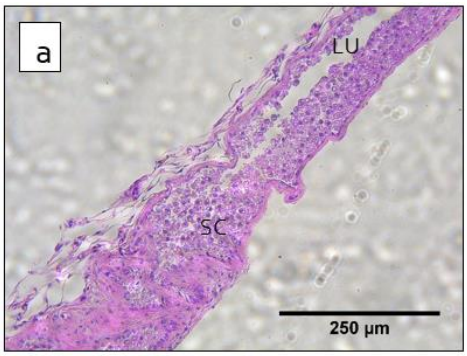


Figure 4. Male *N. ophidion* reproductive stage: a) STAGE II. SC: spermatogenic cells. LU: lumen of seminiferous tubules.

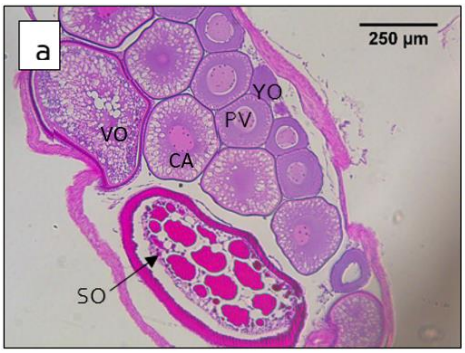


Figure 5. Female *N. maculatus* reproductive stage: a) STAGE IV. YO: young oocytes. PV: pre-vitellogenic oocytes. CA: cortical alveoli oocytes. VO: vitellogenic oocytes. SO: spawning oocytes.

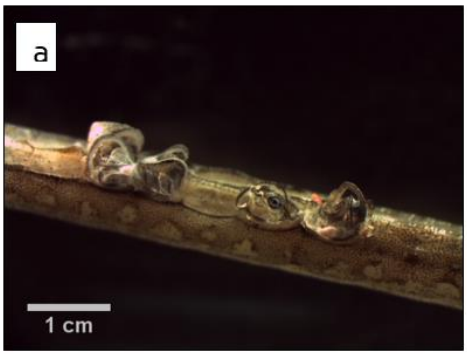


Figure 6. Male *N. maculatus* reproductive stage: a) STAGE IV (macroscopic picture classified by the presence of brooding structure and eggs).