Variation in multiple traits of vegetative and reproductive seagrass tissues influences plant–herbivore interactions

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Abstract Plant–herbivore interactions have strong ecological and evolutionary consequences, but have been traditionally overlooked in marine higher plants. Despite recent advances in seagrass ecology that highlight the importance of herbivory, the mechanisms that regulate the feeding behaviour of seagrass consumers remain largely unknown. Herbivores have been shown to reduce the sexual reproductive success of seagrasses through direct consumption of inflorescences and seeds, but we know little about intraspecific variation in susceptibility to grazing of different seagrass tissues. We contrasted the relative palatability of reproductive and vegetative tissues of the temperate seagrass *Posidonia oceanica* in the field, and we assessed the feeding preferences among these tissues of the main consumers of the plant, the fish *Sarpa salpa* and the urchin *Paracentrotus lividus*. Moreover, we identified the plant traits that explained the observed feeding behaviour. We provide strong evidence for herbivore selectivity among seagrass tissues. In the field, 70–90% of inflorescences were damaged by herbivores compared to 3–60% of leaves of similar age. In feeding assays, the urchin *P. lividus* showed over a twofold preference for reproductive tissue at various stages of development.

By contrast, we detected no feeding activity on either leaves or inflorescences from the fish *S. salpa*, which is known to migrate to deeper waters soon after flowering starts and during the period of fruit maturation. Despite being the preferred food of urchins, inflorescences were chemically defended, had higher levels of phenolics and lower nutrient and calorific content than leaves. We experimentally demonstrated that leaf structural defences are the primary factor in determining urchin feeding preferences. Removal of plant structure results in a drastic shift in urchin selectivity towards the most nutritious and less chemically defended leaf tissue, indicating that multiple mechanisms of defence to herbivory may coexist in seagrasses.

Keywords Chemical defence · Feeding preference · Plant–herbivore interactions · Plant toughness · Phenolics

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Introduction

Herbivores can control the composition, distribution and abundance of plant species (Lubchenco and Gaines 1981; Fritz and Simms 1992; Prusak et al. 2005) and strongly influence ecosystem organization and functioning. Plants can minimise the impact of consumption by reducing herbivore preference or performance, diminishing the negative effects of consumption on plant fitness and/or escaping spatially or temporally (Lubchenco and Gaines 1981; Boege and Marquis 2005). Such defensive mechanisms are not mutually exclusive and comprise diverse structural, morphological and chemical adaptations that can be regulated in

response to consumers (Pennings and Paul 1992; Hay et al. 1994; Karban and Baldwin 1997; Orians 2005). These dynamic traits vary throughout plant ontogeny (Boege and Marquis 2005), and across space and time (e.g., Poore 1994; Wright et al. 2000; Brenes-Arguedas and Coley 2005). Such variation creates a complex feeding ground from which herbivores must select their prey (Simpson and Raubenheimer 1999; Behmer et al. 2002). Although most herbivores select for food with high nutritional content or low levels of defences (Alm et al. 2002; Goecker et al. 2005; Moore and Foley 2005; Stolter et al. 2005), we know little about how these multiple traits interact and influence feeding preferences (Hay et al. 1994).

Seagrasses are clonal flowering plants that mainly inhabit shallow marine soft-sediment habitats worldwide (Williams and Heck 2001). Despite the relevance of plant–herbivore interactions in terrestrial plants and seaweeds (Duffy and Hay 1990; Coley and Barone 1996), little is known about seagrass–consumer interactions. Herbivory was previously considered to have only a minimal impact on these marine plants due to the alleged unpalatability of their tissues (Thayer et al. 1984; Valiela 1995) and the loss of major seagrass consumers such as waterfowl, sirenians, fish and sea turtles (Jackson et al. 2001). However, recent studies indicate that herbivory has been greatly underestimated and present-day direct grazing on seagrasses can be substantial (see review by Valentine and Duffy 2005). To date, studies on seagrass herbivory have mainly focused on consumption of vegetative tissues (see review by Heck and Valentine 2006). However, there is increasing evidence that herbivores can also reduce the reproductive success of seagrasses through direct consumption of inflorescences (Holbrook et al. 2000; Piazzi et al. 2000; Balestri and Cinelli 2003) and through post-dispersal seed predation (Fishman and Orth 1996; Holbrook et al. 2000; Nakaoka 2002; Orth et al. 2002).

Despite the capacity of herbivores to decrease the fitness of seagrasses, we know little about their defensive traits and how these change amongst tissues and throughout their ontogeny. The main reported defence mechanism in seagrasses is the presence of phenolics as chemical deterrents (Zapata and McMillan 1979; McMillan 1984), which decrease in concentration with tissue age (Cariello and Zanetti 1979; Ravn et al. 1994; Agostini et al. 1998). Seagrasses also have a high C/N ratio and a high content of cellulose (Lawrence 1975; Hemminga and Duarte 2000), which lower their nutritional value and may deter herbivores. Additionally, seagrasses tolerate moderate levels of herbivory through compensatory growth or shoot recruitment

(Tomasko and Dawes 1989; Valentine et al. 1997, 2000; Moran and Bjorndal 2005). Since the consequences of herbivory on seagrasses will vary depending on whether vegetative or reproductive tissues are consumed, the allocation of defences between these plant parts may vary and will be shaped by resource allocation constrains (Bryant et al. 1991; Stamp 2003; Weiner 2004). Optimal defence theory (ODT) predicts that tissues that contribute more to plant fitness or are more prone to predation should be better defended (McKey 1979; Rhoades 1979). Since fitness ultimately depends on reproductive success, it is often reasoned that organisms should be under strong selection to allocate resources to the protection of these reproductive parts against potential consumers. Many seaweeds, vascular plants and lichens have indeed been shown to allocate more defences to reproductive than to vegetative or somatic parts (e.g., Steinberg 1984; Hyvarinen et al. 2000; Ohnmeiss and Baldwin 2000; but see Pavia et al. 2002). This variability often has a strong influence on herbivore preference for particular plant parts (Zangerl and Bazzaz 1992; Van Alstyne et al. 2001; Taylor et al. 2002).

In this study we investigated the susceptibility to herbivory of reproductive and vegetative tissues of the temperate seagrass *Posidonia oceanica*. This species is endemic to the Mediterranean Sea, where it forms extensive meadows that are considered key and endangered ecosystems (Boudouresque et al. 1989; Marbà et al. 1996). Flowering in *P. oceanica* is patchy and highly variable (Balestri 2004). In the past 2 decades, there has been a notable increase in the reports of both flowering and seedling recruitment in this species (Diaz-Almela et al. 2006). Recent evidence suggests that sexual reproduction may play a more important role in the long-term population dynamics of *P. oceanica* than previously thought, although its shoot flowering probability is quite low compared to other seagrasses (Diaz-Almela et al. 2006). We quantified the natural impact of consumers on reproductive and vegetative tissues of *Posidonia oceanica* in the field and measured the nutritional, structural and chemical traits in those tissues. We used a series of experiments to test the feeding preferences of the urchin *Paracentrotus lividus* and the sparid fish *Sarpa salpa*, the main consumers of this plant. Furthermore, we established the role of nutrients, structural components and secondary chemistry in determining the observed feeding preferences. More specifically, we asked: (1) does susceptibility to herbivory differ between sexual and somatic tissues in *P. oceanica*? (2) What are the feeding preferences between reproductive and vegetative tissues of the main consumers of the plant (*P. lividus* and

S. salpa)? (3) Do herbivore feeding preferences change throughout reproductive tissue development from inflorescence to fruit formation? (4) What is the importance of within-plant variation in multiple plant traits in determining the observed feeding preferences?

Materials and methods

Study organisms

Posidonia oceanica (L.) Delile is the dominant seagrass in the Mediterranean Sea. It is a monoecious species that produces hermaphroditic flowers containing male and female organs formed in a series of spikes on a long peduncle. Flowering in *P. oceanica* varies highly temporally and spatially, but with no clear pattern (Balestri 2004). The reproductive season typically starts in September–October in shallow meadows, and fruit maturation takes approximately 4–6 months (Buia and Mazzella 1991). Inflorescences are rare along the NE Spanish Mediterranean coast, but we benefited from an exceptional flowering event in the autumn 2003 that allowed us to pursue this study. The two major macroherbivores in the Mediterranean and the main consumers of *P. oceanica* are the edible sea urchin *P. lividus* Lamarck and the sparid fish *S. salpa* Linnaeus (Verlaque 1987, 1990). Grazing on *P. oceanica* by both herbivores varies greatly temporally and bathymetrically. Both are mostly present in shallow meadows, and their maximum activity takes place in summer (Tomas et al. 2005). The urchin *P. lividus* stays in the meadow throughout the year, while the fish descends to deeper waters (40–45 m) for spawning and shelter from autumn to the beginning of summer (Verlaque 1990; Francour 1997).

Incidence of herbivory

We first quantified the incidence of herbivory on reproductive and vegetative tissues of *Posidonia oceanica*. Reproductive shoots were collected in early December 2003 (*n*=28–50) from three shallow meadows (3–8 m) located along the north Catalan coast (NE of Spain; NW Mediterranean Sea): Port-Lligat (42°17′30″N; 3°17′16″E), Montjoi (42°15′36″N; 3°13′49″E) and Fenals (41°41′23″N; 2°49′43″E). Presence or absence of herbivore bite marks was recorded on (1) inflorescences, and (2) the youngest and second youngest leaves of each shoot (hereafter "leaf 1" and "leaf 2", respectively). Bite marks were quantified on these leaves because they are roughly the same age as the inflorescences (leaf 1 slightly younger and leaf 2

slightly older; Romero 1985) and because they were always flanking the inflorescence, i.e., they had been equally exposed to herbivores. Even though the bite marks from the fish *S. salpa* and from the urchin *P. lividus* can be readily identified in leaves (Boudouresque and Meinesz 1982), we were not able to differentiate bites by the two herbivores in inflorescences due to the intricate floral structure, and consequently we only discriminated between intact and grazed tissues. We were able to identify instances where grazers consumed most of the reproductive structures, since inflorescences are attached to the base of the shoot through a peduncle, and the basal 1–2 cm of this structure is protected from the consumers by the other leaves and the sheaths, and remained always intact. We used log-linear models to analyse the incidence of bite marks (presence or absence) in relation to tissue type (leaf 1, leaf 2 and inflorescence) and site (Port-Lligat, Montjoi, and Fenals). We considered "bite marks" as a response variable, and consequently all models include the interaction between tissue type and site (Taulman et al. 1998).

Analyses of plant traits

Secondly, we quantified structural, nutritional, chemical and calorific traits in reproductive and vegetative tissues of *Posidonia oceanica* to determine whether within-plant variation in those traits relates to herbivore preference. Flowering shoots bearing young inflorescences were collected in Port-Lligat, Montjoi and Fenals and transported to the laboratory on aerated seawater tanks. Inflorescence and leaf tissues from each shoot were separated and frozen, freeze-dried and ground into a fine powder using a Fritsch Planetary Micro Mill Pulverisette. Nitrogen and carbon content, organic matter, soluble protein, total phenolic content, calorific value and tissue toughness were quantified independently in leaves and inflorescences from five flowering shoots from each location unless otherwise specified.

Nitrogen and carbon content were analysed using a Carlo-Erba autoanalyser. Organic matter was measured as the ash-free dry weight remaining after burning 30 mg of the sample for 4 h at 500°C (Pillans et al. 2004). Soluble protein concentration was measured using the Bradford assay as modified by Cronin and Hay (1996). We used a modified Folin–Ciocalteu assay to quantify phenolic content (Bolser et al. 1998). Calorific value was measured on Port-Lligat samples only (*n*=6). An IKA C5000 bomb calorimeter was used to burn 2 g of powdered tissue for each replicate. Due to the large amount of material needed for this particular

analysis, each replicate consisted of a pooled collection of five shoots or five inflorescences. Tissue toughness was measured on thawed flowering shoots from Port-Lligat ($n=5$) using a TA.XT2i Texture Analyser (Stable Microsystems), which calculates the force required to rupture the plant tissue by a pointed needle. Data obtained are only used for comparative purposes between tissue types, since absolute toughness of fresh and thawed material might differ. Toughness was measured on leaves 1 and 2, and on bracts within each inflorescence. Leaf and inflorescence length were also measured in 12 randomly collected inflorescences from one of the sites (Fenals) to determine whether reproductive and vegetative tissues were equally accessible to herbivores.

Plant traits were measured from inflorescences and leaves from the same flowering shoot ($n=5$). We used a split-plot three-factor partially nested ANOVA to analyse nitrogen and carbon content, organic matter, soluble protein and the total phenolic content. Site (three locations) and tissue type (reproductive and vegetative) were treated as fixed factors, and shoot was treated as a random factor and nested in site. Because we had no estimate of within-shoot variance (as only one inflorescence and one leaf were measured per shoot), the model does not include an interaction term of shoot (site) × tissue. To prevent an inflated type-I error in our multiple univariate approach, we kept an overall α of 0.05 using Bonferroni adjustments. We used paired t tests to analyse data on calorific value since all reproductive and vegetative tissues came from a single location (Port-Lligat). We used a two-factor mixed model ANOVA to analyse data on toughness from a single site (Port-Lligat), with tissue type as a fixed factor and shoot as a random factor. Where appropriate, transformations of the data were made to satisfy the parametric test assumptions of normality and heterogeneity of variance.

Herbivore consumption and feeding preferences

Thirdly, we used a number of field and laboratory feeding experiments to quantify consumption and tissue preference of the fish *S. salpa* and the sea urchin *P. lividus* during the flowering event. We also ran a number of feeding assays to evaluate the role of multiple plant traits in determining herbivore feeding preferences.

For the fish *Sarpa salpa*, tethered leaves and inflorescences of *Posidonia oceanica* from the same flowering shoot were offered in pairs ($n=20$). Each replicate pair consisted of one peg with tethered leaves and another peg with tethered inflorescences (ca. 1.5 g FW of each). Plant tissues were attached to metal pegs by a

cable tie, leaving 10–11 cm of both tissue types above the sediment. To standardise replicates and eliminate existing herbivore marks, we cut all leaves at the apex and used only inflorescences without obvious herbivore marks. The experiment was set up in sandy patches at least 5 m away from seagrass meadows and rocky reefs to target the herbivore fish *S. salpa* and prevent consumption by the sea urchin *P. lividus*. Replica pairs were at least 3 m apart from each other. Also, five control replicate pairs were individually protected from herbivores with 1 cm plastic mesh cages. Consumption was calculated as $[(H_0 \times C_f/C_0) - H_f]$, where H_0 and H_f were pre-assay and post-assay wet masses of tissue exposed to the herbivore and C_0 and C_f were pre-assay and post-assay wet masses of controls (Sotka and Hay 2002; Taylor et al. 2003). We ran two 12-day independent tethering experiments in December and January in Fenals and in the Medes Islands Marine Reserve (42°2′50″N; 3°13′18″E), where *S. salpa* densities are remarkably high (Macpherson et al. 2002; Tomas et al. 2005).

For *Paracentrotus lividus*, we ran two laboratory experiments to determine the urchin feeding preferences. For each experiment, urchins were collected from shallow seagrass meadows (Port-Lligat or Fenals) and transported in aerated seawater tanks to the Aquarium of Barcelona facilities, where experiments took place immediately. Even though *Posidonia oceanica* produces hermaphroditic flowers, at the beginning of the reproductive season each inflorescence was characterised by the dominance of either male or female organs. We carried out a preliminary study to determine whether *P. lividus* showed any preferences between the two types of organs by offering individual urchins a choice between an inflorescence bearing well-developed male organs versus an inflorescence bearing female organs. We found no differences between the consumption of male and female reproductive structures (mean ± standard error: 0.48±0.25 and 0.45±0.19 g wet biomass consumed, respectively; $P=0.94$; $t=0.079$; $n=4$). We therefore indistinctively used inflorescences bearing male or female organs in subsequent assays.

Two independent paired feeding choice assays were run to determine urchin feeding preference for leaf or inflorescence tissues at different stages of development. In the first assay (December), young inflorescences without fruits were offered against leaves. In the second assay (March), inflorescences with well-developed fruits were offered against leaves. In each paired experiment, individual urchins were offered a choice between ca. 1.5 g of fresh leaves and inflorescences from the same flowering shoot ($n=20$). Inflorescences

and leaves were stapled flat side by side onto a 2-mm plastic mesh fixed to the bottom of each cage with plastic ties, thus making the two tissues equally available. Plant samples were pad-dried of excess water and weighed at the beginning and end of each experiment. Pairs of inflorescences and leaves were placed in cages with no urchins as a control to account for changes in plant mass unrelated to consumption. Experiments ended when roughly half of one treatment had been consumed. Replicates where the sea urchin failed to feed were discarded. Consumption was calculated as described above for the fish *S. salpa*.

All data were checked for normality and homogeneity of variances, and where necessary data were transformed to meet parametric test assumptions. In those bioassays where these assumptions were met, differences in consumption in choice experiments were analysed using paired *t* tests. In those instances where bioassay results had a non-normal distribution, we used a non-parametric Wilcoxon signed-ranks paired test to compare feeding between the control and treatment foods.

Role of plant traits in determining herbivore preferences

Lastly, we experimentally tested the role of structural and chemical components in determining the feeding preferences of *P. lividus*. To test whether differences in structural properties were responsible for the feeding preferences observed with fresh tissue, we freeze-dried and ground the plant parts to a fine powder, and we subsequently reconstituted the samples into rectangles of agar-based artifical diets. This process does not affect most chemical or nutritional properties of the plants, and thus allows us to specifically test the effects of morphology/structure (Hay and Fenical 1998). We added 1 g of plant powder to a solution of 1.2 g agar in 25-ml distilled water. This mixture was poured into an acrylic mould (1×5 cm) beneath which lay a thin plastic 2-mm window screen mesh. When the mixture solidified, it adhered to the mesh, forming a thin rectangle that was offered to each urchin in inflorescence-leaf pairs attached to the bottom of each cage. This experiment was similar to the fresh tissue feeding choice experiments explained above (paired replicates, *n*=20), except that we now offered a choice between agar items of identical shape and size with either inflorescence or leaf tissues. Consumption was measured as the number of mesh squares completely cleared of food. Control cages were set up with agar strips and no urchins. The shape of the strips and the number of squares per strip in the control cages remained unchanged throughout the experiment. The assay ended when roughly half of one of the options was eaten.

To assess the role of plant chemistry in determining urchin feeding behaviour, we first extracted plant metabolites from a pooled sample of leaves and inflorescences with an equal proportion of material from Port-Lligat, Montjoi, and Fenals. We extracted 6 g of freeze-dried material with 100 ml of water:methanol (1:1) for 3 h, as these solvents have previously been used successfully to extract phenolic compounds from this species (Cuny et al. 1995). The solvent was collected, and we repeated the extraction twice more. We sonicated the samples during 15 min at the beginning of each extraction. We combined the three extractions and dried the solvent using rotary evaporation to obtain a crude extract. We weighed the crude extract and calculated the percent yield in inflorescences and leaves as the mass of crude extract over the mass of freeze-dried material ×100.

We tested the activity of these extracts by incorporating them into an agar-base food as before and maintaining the naturally occurring concentrations of extract in inflorescences and leaves (per dry mass) but including 1 g of finely-ground dried tissue of the green alga *Ulva* sp. (Becerret et al. 2001). *Posidonia oceanica* extracts were added at the last stage of the reconstitution process to ensure that the secondary metabolites were not damaged by the high temperatures. We used *Ulva* because it is a highly palatable alga that is readily eaten by *P. lividus* (Boudouresque and Verlaque 2001). Controls were identical to treatments, but did not contain plant extracts. Control and treatment foods solidified in mould strips that were offered to individual urchins, as described previously (paired replicates, *n*=20). Differences in consumption were analysed using paired *t* tests when parametric test assumptions were met. Otherwise, data were analysed using the non-parametric Wilcoxon signed-ranks paired test.

Results

Incidence of herbivory

Regardless of location, the number of bite marks was found to be dependent on the type of tissue (log-linear model, significant "bite marks by tissue" interaction, χ^2=126.63, *df*=2, *P*<0.001; non-significant "bite marks by site by tissue" interaction, χ^2=8.32, *df*=4, *P*=0.081; non-significant "bite marks × site" interaction, χ^2=4.13, *df*=2, *P*=0.127), regardless of location. Between 74 and 86% of inflorescences were damaged by herbivores,

compared to 3–18% of leaf 1 and 14–56% of leaf 2 (Fig. 1).

Analyses of plant traits

There was great within-individual variation in the quality traits of *Posidonia oceanica*, as indicated by the number of significant differences in structural, nutritional and chemical characteristics between vegetative and reproductive tissues (Fig. 2; Table 1). Leaf tissues had significantly higher calorific content (13.77± 0.28 kJ/g DW; all data given as means + standard error) than inflorescence tissues (10.49±0.74 kJ/g DW; paired *t* test, *t*=−6.399, *df*=5, *P*=0.001). Both leaf 1 (0.74±0.06 N) and leaf 2 (0.68±0.05) were tougher than floral bracts (0.37±0.05); two-factor mixed model ANOVA, *F*=13.352, *df*=2, *P*=0.003). Protein content in leaves was higher than in flowers and showed no differences between sites (Fig. 2; Table 1). Carbon/nitrogen (C/N) ratios were higher in inflorescences than in leaves and also varied across sites (Fig. 2; Table 1) with significantly higher C/N ratios in Fenals than in Montjoi and Port-Lligat (Tukey HSD test, *P*<0.001 after Bonferroni adjustment for both comparisons).

Nitrogen, carbon, phenolic and organic matter differences between reproductive and vegetative tissues varied depending on the site (Table 1). Both the carbon and nitrogen contents in leaves were higher than in inflorescences in Montjoi and Fenals, but not in Port-Lligat (Fig. 2). Organic matter content in leaves was higher than in inflorescences in all three sites. Inflorescences from Fenals had the lowest nitrogen, carbon and organic matter content (Tukey HSD tests, *P*<0.01 after Bonferroni adjustment for any comparison). Inflorescences had higher phenolic content than

leaves in Port-Lligat and Montjoi, but not in Fenals (Fig. 2), and inflorescences from Port-Lligat had the highest concentration of phenolics (Tukey HSD test, *P*<0.001 after Bonferroni adjustment for any comparison). Organic matter varied with tissue type and site (Fig. 2; Table 1), with higher values in leaves than in flowers and with the highest values in Port-Lligat and the lowest in Fenals (Tukey HSD test, *P*<0.03 after Bonferroni adjustment for any comparison).

Herbivore consumption and feeding preferences

We found no herbivore marks by the sparid fish *S. salpa* in either leaf or inflorescence tissue in any of the two assays performed, and we observed no *S. salpa* individuals during the experiments. Having successfully used the tethering technique in the same two locations the previous summer, we discarded the possibility of a methodological problem. We concluded that the fish did not exert any trophic pressure on either tissue at the time of the experiments, and discontinued further experimentation with this species.

When given a choice, the sea urchin *P. lividus* consumed more than double the amount of inflorescence than leaf tissue (Fig. 3) whether the choice was between leaves and young inflorescences (Fig. 3a; paired *t* test *t*=−3.651; *df*=14; *P*=0.003) or between leaves and inflorescences with well-developed fruits (Fig. 3b; paired *t* test *t*=−4.836; *df*=11; *P*=0.001).

Role of plant traits in determining herbivore preferences

Both structural traits and chemical components significantly influenced *P. lividus* feeding choices (Fig. 3). When we offered *P. lividus* a choice between ground reproductive and vegetative tissues embedded in agar, the sea urchin consumed twofold more leaf than inflorescence tissues (Fig. 3c; Wilcoxon signed-ranks test *T*=159; *P*=0.001; *n*=18). When we offered *P. lividus* a choice between artificial diets containing either leaf or inflorescence crude extracts, we observed a 17-fold preference for the leaf-extract artificial food (Fig. 3d; Wilcoxon signed-ranks test *T*=78; *P*=0.002; *n*=12).

Discussion

We provide strong evidence for herbivore selectivity among seagrass reproductive and vegetative tissues. Inflorescences of *Posidonia oceanica* were found to suffer strikingly higher rates of herbivory than leaves, both in shallow meadows in the field and in aquaria
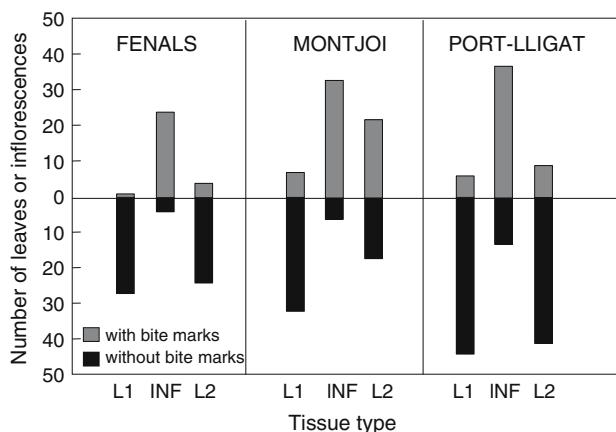
Fig. 1 Number of leaves (L1 and L2) and inflorescences (INF) damaged by herbivores in each of the three locations: Port-Lligat (*n*=50), Fenals (*n*=28) and Montjoi (*n*=39)
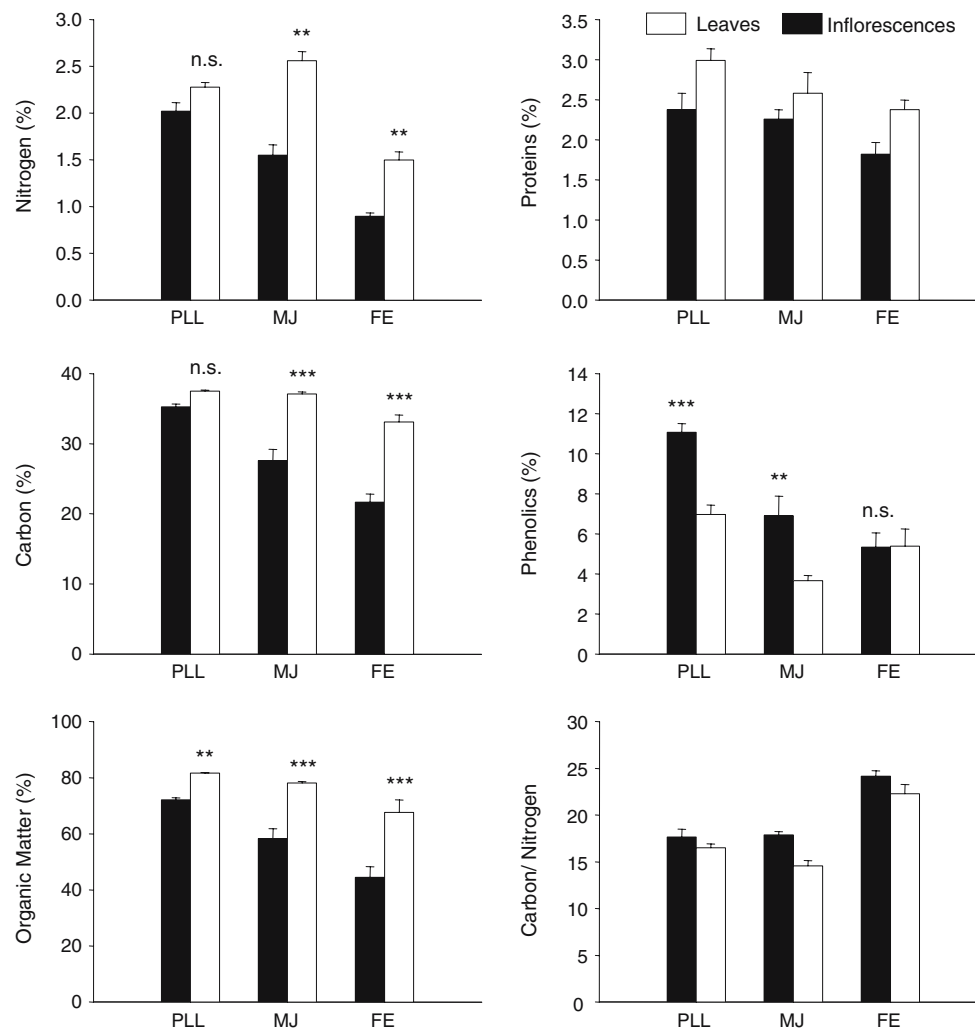
Fig. 2 Inflorescence and leaf nutritional traits from Port-Lligat (PLL), Montjoi (MJ) and Fenals (FE). Data are means (±SE). Sample size *n*=5. In those variables where there was a significant site-by-tissue interaction, differences between tissue types per site are represented by *asterisks*. *$P<0.05$; **$P<0.01$; ***$P<0.001$. Probability values generated by Tukey HSD multiple comparisons

conditions, despite the fact that leaves are more nutritious and have lower levels of chemical defences than the reproductive structures. Leaf toughness appears to operate as a structural defence that deters herbivores and emerges as the primary factor in determining herbivore feeding preferences. Interestingly, we observed a reversal of herbivore feeding preferences in the absence of structural defences. When morphology and texture were experimentally homogenised, the herbivores shifted their preference towards the least chemically defended and more nutritious tissue, indicating that multiple mechanisms of defences to herbivory might coexist in seagrasses.

The high percentage of inflorescences we found damaged by herbivores closely mirrors the results of Balestri and Cinelli (2003) and Piazzi et al. (2000), both of whom found herbivore marks in over 80% of developing

inflorescences. Moreover, our field observations indicate that inflorescences are specifically targeted by herbivores over vegetative tissue of equivalent age. Given the crucial role of sexual reproduction in the conservation of seagrasses through colonisation of bare areas (Duarte and Sand-Jensen 1990; Olesen et al. 2004) and in maintaining and increasing genetic diversity in existing populations, it is important to understand those processes such as herbivory that can compromise seagrass reproductive success.

Of the two main consumers of *Posidonia oceanica*, the sea urchin *P. lividus* showed a clear and consistent feeding preference not only for young inflorescences early on, but also for later-stage flowers bearing mature fruits, and emerges as a facultative florivore. The fish *S. salpa* could also be feeding on flowers at the beginning of the reproductive period (September and

Table 1 Results of the three-factor partially-nested ANOVA assessing the differences between tissue types (leaves and inflorescences) and sites (Port-Lligat, Montjoi; Fenals)

Source of variation	Nitrogen				Proteins			Carbon		
	df	MS	F	P	MS	F	P	MS	F	P
Site	2	2.749	63.711	**0.000**	0.863	6.417	0.078	203.280	37.848	**0.000**
Shoot (site)	12	0.043	1.649	1.194	0.135	0.812	3.828	5.371	1.654	1.188
Tissue	1	2.920	111.590	**0.000**	1.865	11.258	**0.036**	446.988	137.629	**0.000**
Site × tissue	2	0.352	13.468	**0.006**	0.060	0.359	4.230	58.899	18.128	**0.001**
Error	12	0.026			0.166			3.248		

Source of variation	Phenolics				Organic matter			Carbon/nitrogen		
	df	MS	F	P	MS	F	P	MS	F	P
Site	2	45.630	16.776	**0.002**	0.125	16.907	**0.000**	145.559	46.851	**0.000**
Shoot (site)	12	2.720	3.172	0.168	0.007	6.602	**0.006**	3.107	2.186	0.570
Tissue	1	44.289	51.657	**0.000**	0.268	240.216	**0.000**	33.127	23.311	**0.002**
Site × tissue	2	12.115	14.131	**0.006**	0.011	10.080	**0.018**	3.048	2.145	0.960
Error	12	0.857			0.001			1.421		

Organic matter data were arcsine converted to meet parametric test assumptions

All values are Bonferroni adjusted. Significant values at *P*<0.05 are in bold

October), but we did not detect any *S. salpa* feeding activity on either leaves or inflorescences during the time of our experiments (December–January). *S. salpa* migrates to deeper waters during the autumn and winter (October–March) (Francour 1997; Tomas et al. 2005), and thus the presence of reproductive structures in the field from September until March only marginally overlaps with fish feeding activity in seagrass meadows. The fish *S. salpa* is known to exert a much higher herbivory pressure on shallow *P. oceanica* meadows than *P. lividus*, with annual fish consumption values of 40% APP compared to annual urchin consumption values of 17% APP (Prado et al. 2006). If the fish were present during the flowering months, it seems likely that inflorescence herbivory rates would be even higher, since Piazzi et al. (2000) showed that *S. salpa* readily consumes reproductive structures under aquaria conditions. Thus, inflorescences seem to largely escape predation from *S. salpa*, the plant's most voracious herbivore, through phenological escape. Likewise, many terrestrial plants avoid pre-dispersal seed consumption by flowering or fruiting when herbivores are at low abundance (Schemske 1984; Evans et al. 1989; Pilson 2000). Incidentally, *Posidonia oceanica* is unusual amongst temperate seagrasses in that it flowers in the autumn whilst most others, including all congeneric species, flower in the spring (Hemminga and Duarte 2000). The mechanisms behind this shift in flowering season from spring to autumn are unknown, but as a result of fruit maturation taking place during the colder winter months, the reproductive structures have fewer chances of being eaten.

Paracentrotus lividus preferred feeding on inflorescences instead of leaves despite their lower calorific value and lower concentrations of nitrogen, organic matter and carbon. Moreover, reproductive tissues were also more chemically defended against urchin herbivory than leaves, as indicated by a strong preference for agar-based foods containing leaf crude extracts over those with inflorescence extracts. Phenolic concentration was also significantly higher in inflorescences than in leaves in two of the three sites studied. Phenolic compounds are secondary metabolites known for their defensive properties in both terrestrial and aquatic plants (Feeny 1976; Swain 1977; Harborne 1983; Hay and Fenical 1988) and have been shown to inhibit amphipod grazing on the seagrass *Zostera marina* (Harrison 1982). Greater allocation of phenolic compounds to reproductive parts than to leaves is a strategy used by the kelp *Alaria marginata*, where reproductive fronds have higher phenolics and lower consumption rates than blades (Steinberg 1984). However, in the present study *P. lividus* consistently preferred the more chemically defended inflorescences both in the field and in laboratory experiments with fresh plants, showing that secondary chemistry is not the primary factor influencing urchin feeding behaviour.

Our results indicate that plant structure is the most important factor in determining the feeding preferences of the urchin *P. lividus*. Floral bracts were less tough than leaves, and when plant structure was destroyed, the urchin feeding preference radically changed and leaves were significantly preferred over
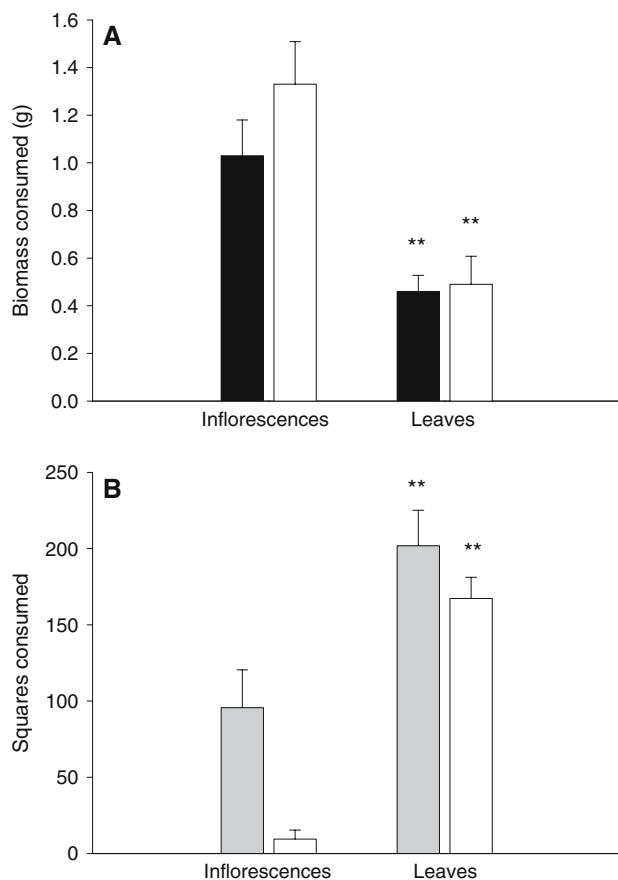
Fig. 3 Results from *Paracentrotus lividus* feeding bioassays with fresh plants and agar-based foods. **a** Paired feeding preference assays with fresh seagrass tissues. In the first experiment (*in black*), young inflorescences were offered alongside leaves in a shoot of equivalent biomass (*n*=15). In the second experiment (*in white*), older inflorescences bearing fruits were offered alongside leaves in a shoot of equivalent biomass (*n*=12). **b** Feeding preference assays using agar-based artificial diets of identical shape and size. In the first experiment (*in grey*) each urchin was offered a choice of either leaf or inflorescence freeze-dried ground tissue (*n*=18). In the second experiment (*in white*) urchins were offered artificial diets containing *Ulva* spp. ground tissues with either inflorescence or leaf crude extract. * *P*<0.05; ** *P*<0.01; ***P*<0.001

inflorescences. Structural factors that increase the difficulty in detaching and masticating plant material directly limit the herbivore feeding rate, and a feeding preference for both vascular plants and algae that are less tough and easier to process has been described in several studies (Coley 1983; Pennings and Paul 1992; Sagers and Coley 1995; Pennings et al. 1998; Lucas et al. 2000; Cronin et al. 2002). However, if the sea urchin feeding preferences were based solely on morphological or structural plant traits such as toughness, we would have expected *P. lividus* to consume the two types of tissues indistinctly when these factors were made equal. Conversely, the urchin drastically reversed its feeding preference towards the more

highly nutritious and less chemically defended leaf diet when structural defences were removed. Similarly, Bolser et al. (1998) and Cronin et al. (2002) found that the crayfish *Procambarus clarkii* prefers those freshwater macrophyte species that are not defended structurally, even if they are of low nutritional value, whereas the most highly nutritious plants are preferred when offered in an agar-based diet lacking structural defences. Likewise, in our study structural traits were the most important factor determining the urchin's feeding choices and appear to have been masking a secondary preference of the urchin for the less chemically defended and more nutritious parts of the plant.

Given the widely accepted assumption that reproductive tissues have a higher fitness value than vegetative tissues, it may seem paradoxical that seagrass leaves are more effectively defended against sea urchin herbivory than inflorescences. However, the relative estimation of the fitness of plant parts is often based on general reasoning rather than empirical evidence (Steinberg 1984; Zangerl and Bazzaz 1992; Van Alstyne et al. 1999; Hyvarinen et al. 2000). In many studies where the fitness value of different plant tissues has been measured objectively, survival of long-lived perennial terrestrial plants and algae has been found to be the most important component of population growth, while a reduction in fertility (e.g., through loss of reproductive tissue) is comparatively less important (Ang and Dewreede 1990; Silvertown et al. 1993; Engel et al. 2001; Pavia et al. 2002). *Posidonia oceanica* is also a long-lived perennial plant, with individual leaves living up to 300 days, the longest life-span known in seagrasses (Romero 1985, Hemminga and Duarte 2000), with individual shoots living more than 30 years (Marbà et al. 1996), and meadows persisting for millennia (Mateo et al. 1997). Accordingly, vegetative tissues of *Posidonia oceanica* may contribute more to the fitness of the plant than inflorescences, thus explaining the differences in defences against herbivory reported in our study.

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