Vol. 458: 89–102, 2012
doi: 10.3354/meps09757

MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser

Leaf-fracture properties correlated with nutritional traits in nine Australian seagrass species: implications for susceptibility to herbivory

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ABSTRACT: Seagrasses are exposed to the constant risk of structural damage due to abiotic factors, such as waves and currents, and biotic factors, e.g. herbivory. Leaf mechanical resistance is therefore essential in protecting plants from structural failure and may also have ecological consequences. For example, mechanical traits of seagrass leaves may play an important role in plant–herbivore interactions and food-preferences of herbivores in these ecosystems, as widely reported for terrestrial plants. However, little is known about leaf mechanical resistance against structural damage in seagrasses and how it varies with other traits such as their nutritional value. We analysed the correlation between fracture properties relevant to herbivory and the nutritional value of seagrass leaves, testing the general assumption that species that invest heavily in mechanical resistance (toughening of the leaves) will present low nitrogen and high carbon and fibre contents. Direct measurements of leaf traits were conducted on 9 seagrass species from south-western Australia: (1) leaf-fracture properties from shearing and tearing tests, (2) nutritional values (carbon to nitrogen ratio and fibre content) and (3) morphological and structural traits (specific leaf area and leaf thickness). Results showed that leaf-fracture properties in seagrasses were tightly correlated to their C:N ratio, which reflects their nutritional value, thus supporting the general assumption that C investment is inversely correlated to N content. This close correlation suggested that patterns of seagrass consumption may be influenced not only by the C:N ratio but also by the leaf-fracture properties. Among co-existing seagrasses, we found a continuous spectrum of mechanical and nutritional traits across species, which provides fundamental information about species assembly, herbivore behaviour and ecosystem functions.

KEY WORDS: Biomechanics · Herbivory · Nitrogen · Nutritional trait · Leaf toughness · Mechanical resistance

INTRODUCTION

Seagrasses, flowering plants adapted to marine life, are exposed to constant risk of structural damage due to abiotic factors, such as waves and currents, and biotic factors, i.e. herbivory. Mechanical resistance is therefore essential in protecting plants from structural failure (Read & Stokes 2006 and references therein). Leaf mechanical resistance can be assessed by their fracture properties such as toughness and

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strength, which are traits describing how and under what conditions the leaf will break (reviewed by Aranwela et al. 1999). In plants, high investment in cell wall material and fibres is the basis of leaf toughening and increases the leaf mechanical resistance (Lucas et al. 2000), modifying structural traits such as specific leaf area and leaf dry matter content. Cell wall material and fibres may also have non-mechanical effects, such as nutrient dilution and digestibility reduction (reviewed by Read & Stokes 2006). Features determining leaf physical integrity such as leaf-fracture properties, structural traits and fibre content may thus have ecological consequences (Read & Stokes 2006). For instance, mechanical resistance by leaf toughening have been commonly reported as an effective anti-herbivore defence in terrestrial ecology because it limits the ability of the herbivores to shear or tear the leaves for their intake (Grime et al. 1996, Wright & Vincent 1996, Pennings et al. 1998, Lucas et al. 2000, Díaz et al. 2001, Sanson et al. 2001, Siska et al. 2002, Cingolani et al. 2005, Sanson 2006).

Recent publications on traits mediating feeding choices of seagrass consumers suggest that structural traits of seagrass leaves (related to leaf mechanical resistance) may play an important role in regulating the plant–herbivore interactions in seagrass ecosystems (Prado & Heck 2011, Vergés et al. 2011). Other leaf traits, such as the synthesis of chemical feeding deterrents (Zapata & McMillan 1979, McMillan et al. 1980, Vergés et al. 2007a) or nitrogen content (Vergés et al. 2007b, Prado et al. 2010, Prado & Heck 2011), have also been reported to influence food selection by seagrass consumers, since their variation may reduce plant quality as food, making leaves less palatable by conferring low attractiveness. Additionally, fibre content also influences herbivore feeding selectivity, since fibrous tissues are difficult to break down mechanically and digest (Klumpp & Nichols 1983, Lanyon & Sanson 2006). In general, seagrass living tissues are characterised by their poor nutritional quality and low digestibility, which led to the conclusion that the direct consumption of these plants was insignificant or minimal (Birch 1975, Phillips & McRoy 1980, Klumpp & Nichols 1983, Thayer et al. 1984). However, a compilation of recent studies reveals that herbivory on seagrasses has been greatly underestimated and may currently be a significant process in seagrass ecosystems (Valentine & Heck 1999, Valentine & Duffy 2006). Yet, very little is known about leaf mechanical resistance against structural damage in seagrasses and how it varies with other leaf traits. This study focuses on the leaf-fracture properties of seagrass leaves and how they correlate to nutritional value (fibre and nitrogen content) and structural traits. We tested the general assumption that species investing heavily in mechanical resistance of leaves will have lower nitrogen and higher carbon and fibre contents, thus influencing biomechanical characteristics, which are likely to have ecological consequences. Specifically, we aimed to: (1) quantify mechanical, nutritional and structural traits of 9 coexisting seagrass species, and (2) examine the associations between these traits that may be contributing various ecological strategies of these seagrass species. Finally, we discuss the potential consequences of our findings for the susceptibility of these seagrass species to herbivory.

MATERIALS AND METHODS

Location and plant selection

South-western Australia was selected as the study area for its large number of co-occurring seagrass species (14 species, Carruthers et al. 2007). The study was carried out in mid-November 2008 on 9 seagrass species centred around the geographical area of Perth, including 5 sites along ca. 50 km of the coastline (Table 1). These species included a wide range of growth strategies, life histories and morphologies (Cambridge 1999) to cover as much variation in the leaf traits as possible: 3 species with a cluster of leaves arising from a stem (*Amphibolis antarctica* [Labill.] Sonder et Aschers, *A. griffithii* [J.M. Black] den Hartog, *Thalassodendron pachyrhizum* den Hartog), 4 species with strap-like leaves (*Posidonia australis* Hook. f., *P. coriacea* Kuo & Cambridge, *P. sinuosa* Cambridge & Kuo, and *Heterozostera nigricaulis* Kuo), 1 species with oval leaves (*Halophila ovalis* [R. Brown] Hook. f.) and 1 species with terete leaves (*Syringodium isoetifolium* [Aschers.] Dandy). In addition, there were species from sheltered (e.g. *P. sinuosa*, *H. ovalis*) as well as exposed locations (*P. coriacea, T. pachyrhizum*); short-lived (*H. ovalis* and *S. isoetifolium*) and long-lived species (*Posidonia* spp.); 2 species occurring widely in the tropics (*H. ovalis* and *S. isoetifolium*) and 7 warm temperate species endemic to the southern half of Australia.

Entire specimens (leaves with rhizomes and roots) were collected from sub-tidal sites from 1 to 10 m deep. Plant material was carefully manipulated after collection to minimise changes in turgence and physical integrity. For transportation to the laboratory of the School of Plant Biology (University of Western Australia, Perth), seagrass shoots were laid out horizontally, wrapped completely in moist tissue and

Table 1. Nine seagrass species investigated and their collection sites, all in the vicinity of Perth, Western Australia

Species	Code	Family	Location	Coordinates	
				S	E
Amphibolis antarctica (Labill.) Sonder et Aschers	Aa	Cymodoceae	Garden Island and Cottlesloe	32°14′36.46″ 32°0′10.62″	115°40′41.93″ 115°45′1.12″
Amphibolis griffithii (J.M. Black) den Hartog	Ag	Cymodoceae	Cottesloe	32°0′10.62″	115°45′1.12″
Halophila ovalis (R. Brown) Hook. f.	Ho	Hydrocharitaceae	Cockburn Sound and Swan River	32°15′53.54″ 31°58′54.28″	115°41′52.65″ 115°49′17.79″
Heterozostera nigricaulis Kuo	Hn	Zosteraceae	Cockburn Sound (outer part)	32°15′53.54″	115°41′52.65″
Posidonia australis Hook. f.	Pa	Posidoniaceae	Cockburn Sound	32°15′21.01″	115°42′29.04″
Posidonia coriacea Kuo & Cambridge	Pc	Posidoniaceae	Lal Bank	31°48′33.0″	115°43′00.4″
Posidonia sinuosa Cambridge & Kuo	Ps	Posidoniaceae	Cockburn Sound	32°15′21.01″	115°42′29.04″
Syringodium isoetifolium (Ascher.) Dandy	Si	Cymodoceae	Cockburn Sound (outer part)	32°15′53.54″	115°41′52.65″
Thalassodendron pachyrhizum den Hartog	Tp	Cymodoceae	Garden Island	32°14′36.46″	115°40′41.93″

placed in plastic bags sealed to avoid desiccation, under cool and dark conditions in an ice chest. The following day, the plant material was transported under the same conditions by airplane to the laboratory of the Department of Biological Sciences (Macquarie University, Sydney), where it was immediately submerged in tanks with aerated natural seawater at 20°C in a climate-controlled chamber.

Structural traits

Measurements of leaf traits were taken in healthy and fully-developed but not senescent leaves from at least 5 shoots per species and location. We cut a leaf fragment of ~5 cm length and measured the fresh weight (FW; g) and projected area (PA; mm^2) using image analysis software (ImageJ) after scanning the fragment. Subsequently, we determined blade width (Wd; mm) and thickness (Th; mm) or diameter (D; mm) with digital callipers and a thickness gauge to calculate the blade cross-sectional area (CA; mm^2), assuming the cross-section had either a rectangular or round shape. After measuring the mechanical properties (see below), these leaf fragments were freeze-dried and used to measure their dry weight (DW; g) with a precision balance. The whole fragment volume (V; mm^3) of each sample was estimated considering the leaf fragment length (L; mm) and the CA. Specific leaf area (SLA; m^2 kg^{-1} DW) was then calculated as the leaf fragment area divided by its correspondent DW, and leaf dry matter content (LDMC; %) as the percentage of DW in FW.

Mechanical traits

Leaf-fracture properties were evaluated by 2 tests (tearing and shearing tests) and expressed at 2 levels: (1) whole-leaf mechanical traits, that is, total quantity of force or work needed to tear or shear a single leaf blade, which depends on the leaf size and its mechanical properties at the material level; (2) material mechanical traits, normally called 'material properties', which are inherent properties to the material, normalized by leaf volume or leaf CA. Regarding the ecological significance of these traits, whole-leaf mechanical traits indicate the work or force in absolute terms to shear or tear a single leaf blade by, for example, a herbivore, whereas material properties show invested work or force for an amount of material ingested, giving an idea of the cost-efficiency in the feeding process.

The leaf mechanical properties were measured within 4 d of sampling and the specimens tested in the sequence that they were collected so that the time of storage was homogeneous among species. Prior to the measurements, leaves were lightly and carefully cleaned of epiphytes and debris. The first outermost fully-developed leaf of the plant shoot was selected (normally the second youngest leaf), and a portion of the blade leaf (4 to 5 cm above the ligule, not including the leaf sheath) was cut for testing. These portions were the same used for the measurements of the structural traits described in the previous section.

Shearing test measures the force required for foliar breakage (Wright & Vincent 1996, Aranwela et al. 1999). We used a purpose-built shearing machine with a constant cutting angle (20°) and speed (see Wright & Cannon 2001 for more details). Although this test was not intended to mimic any specific herbivore-induced stress, it provides an indication of physical resistance (Wright & Cannon 2001) and it has been identified as 1 of the best ways to measure leaf toughness (Sanson et al. 2001). The test was conducted in 2 directions, transverse and longitudinal, across the leaf (n = 5 to 11 for each species and test) except for *Syringodium isoetifolium*, where only transversal testing was possible due to its terete morphology. During the test, a force to displacement curve was monitored, and the absolute work-to-shear (W_{SA}; mJ) needed to cut the specimen was calculated as the area under this curve (Fig. 1). This amount of work was expressed per unit fracture area (CA), to calculate the specific work-to-shear (W_{SS}; J m^{-2}; Read & Sanson 2003). As the whole leaf was cut transversally during the test, the measured W_{SA} and W_{SS} reflect a gross measure of all of the components of the leaf, i.e. the work done to shear the lamina, including the leaf veins and midribs, if present (Sanson et al. 2001; our Fig. 1).

The tensile (tearing) test was carried out on another set of leaf portions similarly selected as described for the shearing test. The mechanical properties in tension were measured with an Instron testing machine (model 5542) and the BlueHill® software (v. 2.18) with a 500 N-load cell and pneumatic action grips (model 2712). The tests were conducted to the long axis of the leaf, including the midrib in the case of *Halophila ovalis*. The leaves were clamped individually into the grips of the tensometer with the mountings from 10 to 135 mm apart (depending on the species size), with the exact distance measured to the closest 1 mm. When necessary, the inner part of the grips was covered with rubber to avoid slippage of the leaf. Once clamped in the grips, the leaf specimens were stretched at a constant velocity of 10 mm min^{-1}, while the displacement (δ, mm) and the load force (F, N) were recorded every 0.1 s until they broke, when the maximum force (absolute force-to-tear, F_{TA}, N) was recorded. From the force-displacement curve and the size traits, we obtained the following mechanical properties: (1) F_{TA} (N), the maximum force that the specimen can bear before breaking; (2) specific force-to-tear (F_{TS}, N mm^{-2}), the maximum force per unit of CA needed for the specimen to break, which is equivalent to the property known as 'tensile strength' or 'breaking stress' in engineering (Gere & Goodno 2009); (c) absolute work-to-tear (W_{TA}, mJ), i.e. the work needed to break the leaf, calculated as the area under the force-displacement curve; (d) specific work-to-tear (W_{TS},

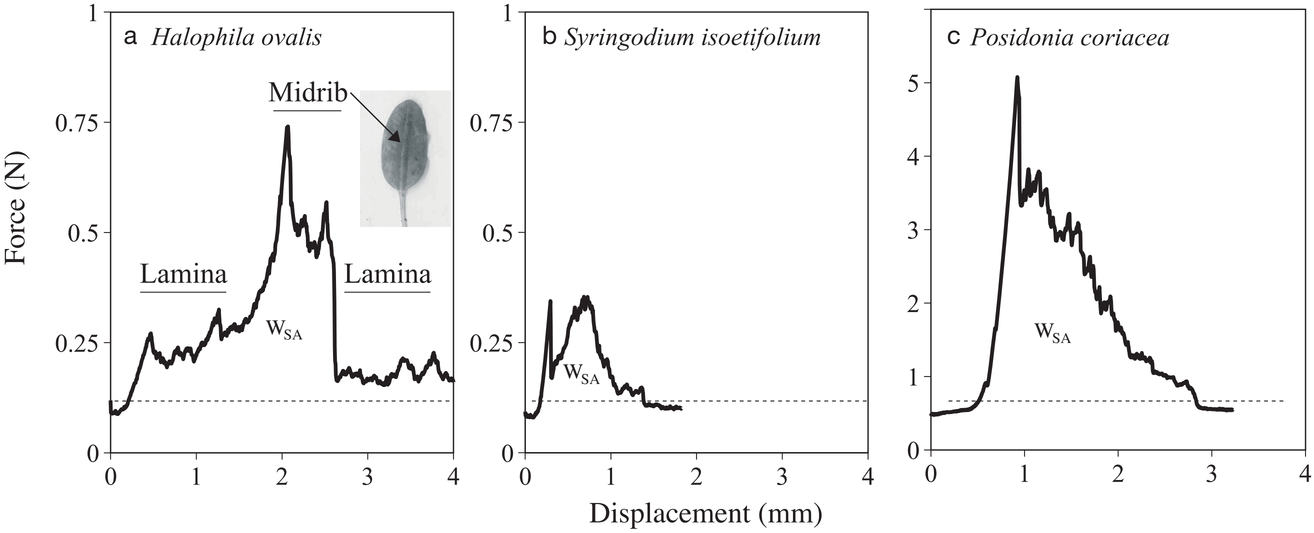

Fig. 1. *Halophila ovalis*, *Syringodium isoetifolium* and *Posidonia coriacea*. Representative force-displacement curves from a shearing test for 3 different species. The displacement (*x*-axis) is the distance travelled by the horizontal blade. The full area under the curve is used to estimate absolute work-to-shear (W_{SA}; mJ). The shearing test can be used to capture an image of the variation in fracture properties along a leaf transect (Aranwela et al. 1999). (a) *H. ovalis* leaf, showing the force applied to cut transversally the lamina or leaf blade (with cross-veins) and the increase when the cutting blade of the machine reaches the midrib (more energy needed to cut). (b) In *S. isoetifolium*, the force decreases when the cutting blade is reaching the aerenchyma in the leaf centre. (c) In *P. coriacea*, the highest force across the leaf is reached when the cutting blade first touches the outer part of the leaf; note that the *y*-axis scale is 6-fold higher than scales used for panels (a) and (b). Dotted line: baseline of the cutting device, i.e. the force without cutting any sample

J m^{-3}) is the W_{TA} normalized by the tissue volume (V), which is also called ‘tensile toughness’. Tests were made on 5 to 15 replicates per species, excluding the specimens that slipped during the test or broke close to or at the grips.

Nutritional traits

After measuring the leaf-fracture properties, the epiphyte-free portions of leaves from each species were freeze-dried and pulverized in a ball-grinder. Subsamples of dried biomass were then used for the determination of total C and N content (% DW) using a Perkin-Elmer 2400 elemental analyser. C:N ratio was calculated based on dry biomass. A second subsample of the dried biomass was used to determine the neutral detergent fibre content (NDF; method modified from Van Soest et al. 1991). Samples of 20 to 30 mg of dry biomass were heated to boiling (100°C) in 2 ml of neutral detergent for 1 h, followed by centrifuging (2500 × *g*, 5 min). The pellet was kept in the tube, washed and centrifuged (2500 × *g*, 5 min) with distilled water (×2), ethanol (×2) and acetone (×1). The final pellet, free of non-cell wall components and chlorophyll, was dried overnight in an oven at 60°C and weighed again. The amount of fibre for each sample was obtained by the difference in mass and expressed as fibre percentage of dry biomass (% or g of fibre per 100 g of dry biomass). The NDF content is referred to as ‘fibre content’ hereafter. For each species, 3 to 6 replications were made.

Data and statistical analysis

The interspecific variability in traits was assessed by the coefficient of variation (CV). Non-parametric 1-way analysis of variance (Kruskal-Wallis test), followed by non-parametric multiple comparison test (Behrens-Fisher test for all pairs), was used to compare leaf traits among species since data did not satisfy analysis of variance assumptions (Shapiro-Wilk test for normality and Levene's test for homoscedasticity) even after transformations. We constructed a correlation matrix with Spearman's coefficient to all pairs of traits. A principal component analysis (PCA) was conducted with a selection of a suite of key variables in each trait group to observe the relationship of species in relation to these variables. A critical level of 0.05 was used for all hypothesis tests. Statistical analyses were computed with R 2.11.1 (R Development Core Team 2010).

RESULTS

Comparison across species

Leaf traits varied significantly among species (Tables 2 & 3), and inter-species variability tended to be higher in the mechanical traits than in the nutritional traits (Tables 3 & 4). In this inter-specific comparison, the 2 cosmopolitan species *Syringodium isoetifolium* and *Halophila ovalis* had the lowest C and NDF and the highest N contents in their leaves, thus the lowest C:N ratio (<17; Table 2, Fig. 2). In structural traits, *H. ovalis* had the highest SLA,

Table 2. Nutritional and structural leaf traits. Values are means ± SD of replicates (n = 3 for nutritional traits and n = 5 to 15 for structural leaf traits). C: leaf carbon content, N: leaf nitrogen content, C:N ratio (dry weight, DW-based), NDF: leaf neutral detergent fibre content, LDMC: leaf dry matter content, SLA: specific leaf area, Th: leaf thickness, CV: coefficient of variation, p: statistical significance associated with Kruskal-Wallis test for testing equality of medians among species

Species	Nutritional traits				Structural traits		
	C (% DW)	N (% DW)	C:N ratio	NDF (% DW)	LDMC (%)	SLA (m^2 kg^{-1} DW)	Th (mm)
Amphibolis antarctica	32.0 ± 1.5	1.7 ± 0.2	21.7 ± 1.6	43.5 ± 2.6	23.8 ± 2.3	19.1 ± 2.6	0.16 ± 0.01
Amphibolis griffithii	30.6 ± 1.1	1.9 ± 0.3	19.2 ± 2.3	47.8 ± 0.8	27.0 ± 4.4	16.1 ± 2.6	0.21 ± 0.02
Halophila ovalis	28.9 ± 0.8	2.2 ± 0.2	15.6 ± 1.9	43.9 ± 0.5	23.5 ± 10.8	36.5 ± 6.4	0.09 ± 0.01
Heterozostera nigricaulis	32.1 ± 0.4	1.8 ± 0.1	20.8 ± 1.2	55.2 ± 1.4	33.2 ± 15.6	28.6 ± 13.2	0.17 ± 0.03
Posidonia australis	33.0 ± 0.5	1.2 ± 0.1	31.4 ± 2.3	57.2 ± 1.2	22.7 ± 1.7	14.2 ± 2.0	0.35 ± 0.06
Posidonia coriacea	32.3 ± 2.0	0.8 ± 0.3	52.3 ± 15.3	58.4 ± 0.8	17.2 ± 0.6	8.2 ± 1.3	1.03 ± 0.16
Posidonia sinuosa	34.5 ± 0.4	1.3 ± 0.3	30.7 ± 5.7	59.5 ± 1.4	23.9 ± 5.4	18.9 ± 4.0	0.25 ± 0.03
Syringodium isoetifolium	26.5 ± 0.5	2.0 ± 0.1	15.4 ± 0.6	38.1 ± 1.8	12.8 ± 0.5	8.5 ± 0.7	1.21 ± 0.22
Thalassodendron pachyrhizum	31.2 ± 0.7	1.3 ± 0.1	28.0 ± 2.3	49.4 ± 1.1	24.4 ± 1.7	22.2 ± 2.3	0.19 ± 0.04
CV inter-species (%)	8	28	44	15	25	48	102
p	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Table 3. Mechanical leaf traits. Values are means ± SD of replicates. Whole-leaf mechanical traits: F_{TA}, absolute force-to-tear; W_{TA}, absolute work-to-tear; W_{SA}, absolute work-to-shear (T: transversal, L: longitudinal). Material mechanical traits: F_{TS}, specific force-to-tear; W_{TS}, specific work-to-tear; W_{SS}, specific work-to-shear. CV: coefficient of variation inter-species; p: statistical significance associated with Kruskal-Wallis test for testing equality of medians among species

Species	Whole-leaf mechanical traits				Material mechanical traits			
	F_{TA}(N)	W_{TA}(mJ)	W_{SA}-L (mJ)	W_{SA}-T (mJ)	F_{TS} (N mm^{-2})	W_{TS} (MJ m^{-3})	W_{SS}-L (J m^{-2})	W_{SS}-T (J m^{-2})
Amphibolis antarctica	9.96 ± 1.34	9.48 ± 2.31	0.26 ± 0.09	0.73 ± 0.20	8.32 ± 1.54	0.630 ± 0.445	196.5 ± 68.9	587.8 ± 149.7
Amphibolis griffithii	12.35 ± 1.97	12.45 ± 3.45	0.28 ± 0.11	0.51 ± 0.15	9.22 ± 0.78	0.668 ± 0.216	141.8 ± 52.6	376.7 ± 68.9
Halophila ovalis	4.20 ± 0.49	2.10 ± 0.97	0.15 ± 0.05	0.34 ± 0.13	4.36 ± 1.36	0.266 ± 0.204	176.3 ± 42.7	299.1 ± 113.6
Heterozostera nigricaulis	3.21 ± 0.26	3.38 ± 0.41	0.15 ± 0.04	0.18 ± 0.02	9.66 ± 1.75	0.179 ± 0.023	103.8 ± 37.1	407.7 ± 43.7
Posidonia australis	48.85 ± 11.36	72.01 ± 22.32	0.67 ± 0.23	1.60 ± 0.25	9.48 ± 2.36	0.522 ± 0.303	172.3 ± 36.4	394.3 ± 55.7
Posidonia coriacea	29.49 ± 6.19	87.88 ± 36.95	3.41 ± 0.87	3.73 ± 0.45	4.45 ± 0.59	0.270 ± 0.097	286.8 ± 61.4	583.9 ± 93.7
Posidonia sinuosa	31.57 ± 7.92	35.61 ± 21.71	0.38 ± 0.17	1.35 ± 0.35	16.18 ± 4.33	0.756 ± 0.512	155.7 ± 64.1	724.2 ± 128.1
Syringodium isoetifolium	2.35 ± 0.36	5.50 ± 2.02	–	0.14 ± 0.07	0.69 ± 0.39	0.134 ± 0.083	–	49.0 ± 13.7
Thalassodendron pachyrhizum	17.84 ± 6.51	44.90 ± 25.74	0.32 ± 0.10	1.25 ± 0.29	7.45 ± 2.58	0.594 ± 0.295	182.8 ± 50.5	492.5 ± 65.7
CV (%)	89	105	157	103	56	52	30	45
p	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.01	<0.001

Table 4. Pairwise Spearman's correlation coefficient among mechanical, nutritional and structural traits. Correlations have been done between averaged values for each species (n = 9, except for correlations including W_S-L and W_{SS}-L, where n = 8 as no data are available for *Syringodium isoetifolium*). The corresponding statistical significance are given by asterisks (*p < 0.05, **p < 0.01, ***p < 0.001). Nutritional leaf traits as in Table 2. Whole-leaf and material mechanical traits as in Table 3. Correlations underlined are due to calculations of the traits. Leaf traits used for principal component analysis are in **bold**

	Nutritional traits				Structural traits			Whole-leaf mechanical traits				Material mechanical traits			
	C	N	C:N	**NDF**	LDMC	**SLA**	**Th**	**F_{TA}**	**W_{TA}**	**W_{SA}-T**	W_{SA}-L	F_{TS}	W_{TS}	W_{SS}-T	W_{SS}-L
C	–	−0.77*	0.87**	0.88**	0.12	−0.18	0.18	0.77*	0.59*	0.75*	0.65	0.75**	0.39	0.75*	−0.05
N		–	−0.97***	−0.72*	0.12	0.43	−0.38	−0.80**	−0.89**	−0.90***	−0.89**	−0.32	−0.25	−0.65	−0.50
C:N			–	0.82**	−0.08	−0.35	0.28	0.87**	0.85**	0.95***	0.91**	0.42	0.34	0.72*	0.43
NDF				–	0.17	−0.18	0.27	0.77*	0.61*	0.73*	0.71*	0.63	0.31	0.58	−0.14
LDMC					–	0.62	−0.57	−0.05	−0.22	−0.18	−0.56	0.63	0.42	0.25	−0.71*
SLA						–	−0.92***	−0.33	−0.63	−0.40	−0.87**	0.16	0.07	0.02	−0.24
Th							–	0.28	0.57	0.28	0.93***	−0.12	−0.19	−0.10	0.10
F_{TA}								–	0.85**	0.93***	0.92**	0.46	0.60	0.53	0.21
W_{TA}									–	0.90***	0.97***	0.18	0.35	0.47	0.40
W_{SA}-T										–	0.97***	0.28	0.47	0.63	0.55
W_{SA}-L											–	0.07	0.25	0.40	0.38
F_{TS}												–	0.54*	0.53*	−0.71*
W_{TS}													–	0.58	0.06
W_{SS}-T														–	0.33
W_{SS}-L															–

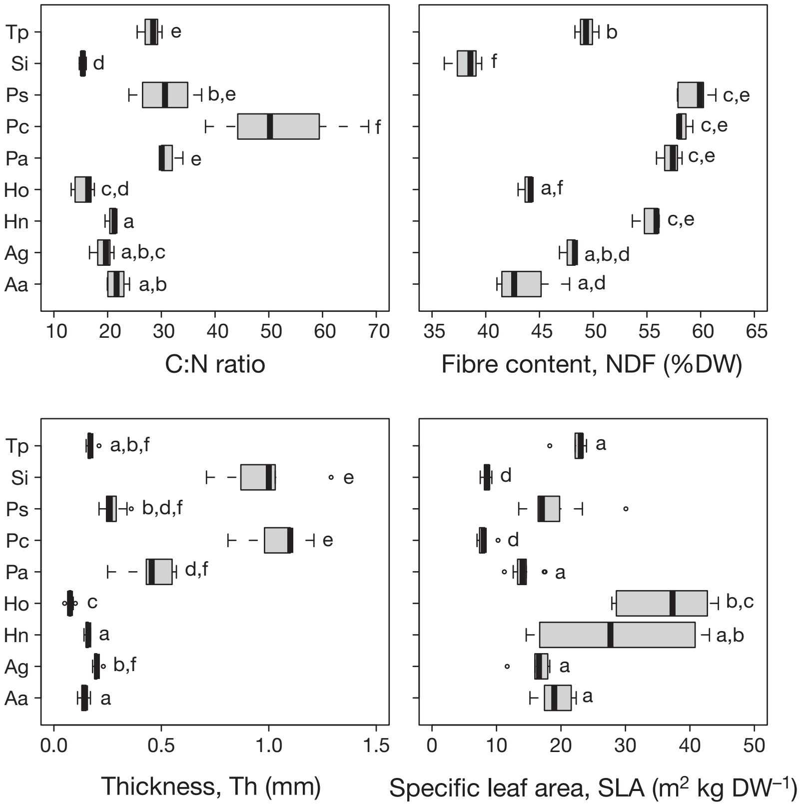

Fig. 2. Differences across species for selected nutritional and structural traits. Lettering indicates species with significantly different values for the variables at $p < 0.05$ (non-parametric multiple comparisons). Mid-line is the median, box limits are the 25th and 75th quartiles, whiskers show the 1.5 interquartile range, and points are the observations out of this range. NDF: neutral detergent fibre content. Species on the *y*-axis: Tp, *Thalassodendron pachyrhizum*; Si, *Syringodium isoetifolium*; Ps, *Posidonia sinuosa*; Pc, *P. coriacea*; Pa, *P. australis*; Ho, *Halophila ovalis*; Hn, *Heterozostera nigricaulis*; Ag, *Amphibolis antarctica*; Aa, *A. griffithii*

whereas *S. isoetifolium*, with a fleshy, cylinder-like leaf, had the lowest SLA and LDMC (Table 2, Fig. 2). In mechanical traits, *S. isoetifolium* and *H. ovalis* presented the lowest values for most of the whole-leaf and material properties (Table 2). In contrast, the 3 species of the genus *Posidonia*, and especially *P. coriacea*, reached the lowest N content and the highest C and fibre content values in their foliar tissue (Table 2, Fig. 2). This genus also showed the highest values in all of the whole-leaf mechanical traits (Table 3, Fig. 3); for instance, the absolute force needed to tear a *P. coriacea* leaf was 35 N on average, 25 times higher than the force needed for *S. isoetifolium*. The other species with a similar morphology consisting of a cluster of leaves arising directly from a vertical stem (*Amphibolis* spp., *Heterozostera nigricaulis* and *Thalassodendron pachyrhizum*) had nutritional values in between the *Posidonia* spp. (high C:N ratio, high NDF) and the 2 smaller, more ephemeral species, *H. ovalis* and *S. isoetifolium*, (C:N ratio <17, low NDF; Fig. 2), with a similar pattern observed for mechanical traits (Fig. 3). The comparison of transverse versus longitudinal toughness across the species indicated that 2 to 4 times more work was required to fracture leaves transversely than longitudinally, except for *P. coriacea*, where a similar force was needed to cut the leaf in both directions (Table 3). In longitudinal section, almost all species grouped together, except for the *Posidonia* spp. group (Fig. 3).

Leaf trait intercorrelation

We obtained a correlation matrix with all pairwise comparisons among the leaf traits (Table 4). Overall, we observed strong correlations among nutritional traits, among whole-leaf mechanical traits and between nutritional traits and whole-leaf mechanical traits (Table 4). Among the nutritional variables, the fibre content (NDF) was positively correlated to C content and negatively to N across the species (Table 4). As a result, NDF was positively correlated to the C:N ratio. The whole-leaf mechanical traits such as F_{TA}, W_{TA} and W_{SA} were positively correlated with the C:N ratio and NDF (Table 4). These correlations mean that the leaves that need a high amount of work or force to be cut or torn present a higher proportion of fibres and C, and a lower proportion of N. Additionally, we found stronger correlations among whole-leaf mechanical traits, such as W_{TA}, F_{TA} and W_{SA}, than among material properties (Table 4).

After analysing the correlations between all pairs of traits, we selected key variables in each group of traits including: Th and SLA; C, N and fibre contents (NDF); and 3 whole-leaf mechanical properties (F_{TA}, W_{TA} and absolute work-to-shear in the transversal direction W_{SA}-T) for PCA. We did not include shearing test data in the longitudinal direction, as they were not measured in *Syringodium isoetifolium.* We retained 2 components explaining 87% of the variance. The leading component (PC1, 61.6% of variance) was negatively correlated with N and posi-

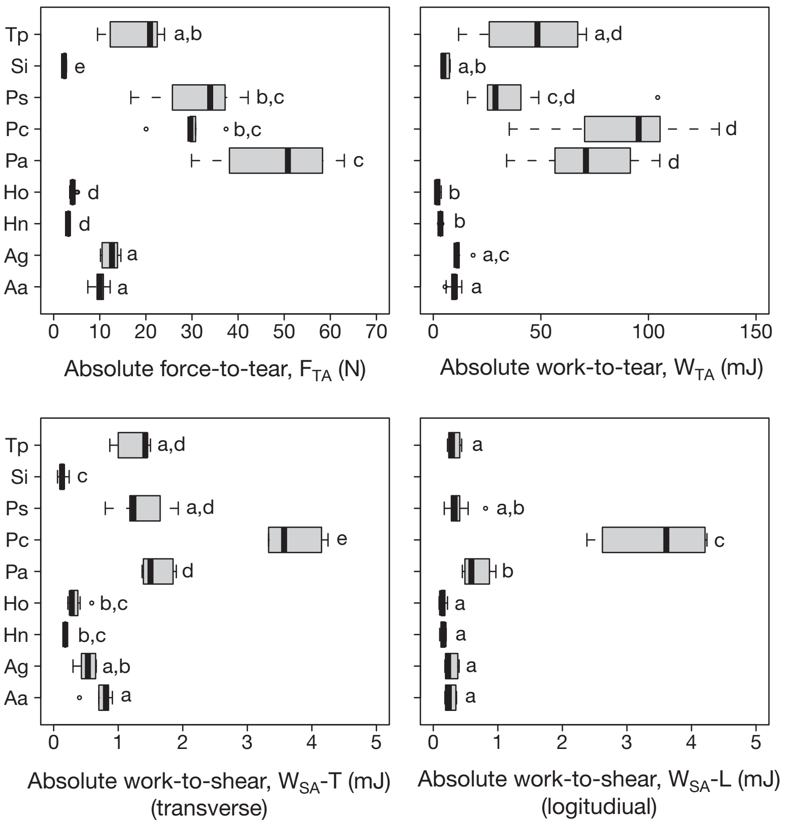

Fig. 3. Differences across species for whole-leaf mechanical leaf traits. Lettering indicates species with significantly different values for the variables at $p < 0.05$ (non-parametric multiple comparisons). Box plots and abbreviations as in Fig. 2

tively with the whole-leaf mechanical properties, whereas PC2 (25.9% of variance) was negatively associated with C, NDF and SLA and positively with blade thickness (Fig. 4). NDF presented similar loadings in both components. *S. isoetifolium* and *Halophila ovalis* displayed the lowest scores for PC1, whereas *Posidonia* spp. exhibited the highest (Fig. 4). *Amphibolis* spp., *Heterozostera nigricaulis* and *Thalassodendron pachyrhizum* (all species with a similar morphology, a cluster of leaves arising from a vertical stem), were situated in an intermediate position. In reference to PC2, in which thickness, SLA and C were strongly associated, *P. coriacea* and *S. isoetifolium* had a similar score due to their analogous thickness and SLA (Fig. 4).

DISCUSSION

Our results showed that leaf-fracture properties and nutritional traits were negatively and tightly inter-correlated in seagrasses, indicating that species with high mechanical resistance (that is, more force or work is needed to shear or tear the leaves) tend to exhibit a low leaf nutritional value (high C:N, high fibre content). Similar correlations have been described for terrestrial plants, suggesting that there has been a parallel selection among species for a combination of certain traits both in terrestrial and aquatic environments (Pérez-Harguindeguy et al. 2003, Read & Stokes 2006, Read et al. 2009, Molinari & Knight 2010). The correlations reflect a mixture of direct and indirect causal relationships between leaf traits (Wright et al. 2004), as C is the main building element of supporting tissue. Therefore, a high fraction of C is used for the structural carbohydrates forming cell walls and fibre bundles, explaining the correlation between C and leaf reinforcement. Accordingly, high C:N ratios (i.e. poor nutritional value) are found in species with tough tissues, since the primary source of leaf toughening in plants is the composite cell wall and fibres (Lucas et al. 2000).

Mechanical resistance and fibre content are leaf traits that confer protection against a range of mechanical damage, both abiotic and biotic (reviewed by Read & Stokes 2006). The leaf mechanical resistance may be important in the adaptation to abiotic factors such as water motion, and it may be the result of the plant life-history strategy (persistence

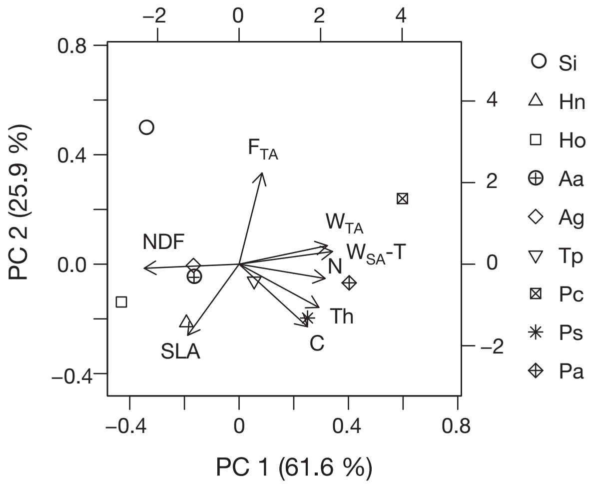

Fig. 4. Principal component analysis with the projection of the 8 variables (arrows) and 9 species (each represented by a different symbol). Abbreviations as in Figs. 2 & 3

versus productivity). For instance, *Syringodium isoetifolium* and *Halophila ovalis* often experience low mechanical stress as they live in sheltered places or form the understorey beneath other seagrasses (Cambridge & Lambers 1998), so their leaves resist low mechanical loads and have a relatively low amount of fibres, in comparison with other seagrasses. In contrast, *Posidonia* spp. have large amounts of foliar fibres which provide mechanical support to withstand the mechanical forces from strong water movement.

The described correlations of leaf traits may have implications in other ecological aspects. For instance, both types of traits, mechanical and nutritional, have been described as factors underlying the potential palatability of seagrass leaves and the food preference of their consumers. Some studies actually found a positive correlation between seagrass leaf N content and preference by herbivores (e.g. Bjorndal 1980, Zieman et al. 1984, McGlathery 1995, Preen 1995, Goecker et al. 2005), although other studies showed negative correlations (Mariani & Alcoverro 1999, Valentine & Heck 2001, Kirsch et al. 2002, Vergés et al. 2007b, White et al. 2011). Preferences of seagrass consumers may thus be influenced by factors other than N, such as fibre content or leaf-fracture properties, which may be more relevant. Vergés et al. (2007a, 2011) found that sea urchins preferentially consumed the most nutritious but less chemically defended leaf tissues only when structural defences were removed in artificial diet experiments. Along the same lines, Prado & Heck (2011) suggested that structural plant features are the most important factors driving discrimination between seagrass species by omnivorous fish, whereas strict herbivores make feeding decisions influenced by nutritional characteristics. These observations suggest that both mechanical and nutritional traits may determine the food-preferences of seagrass consumers.

Among the 9 coexisting seagrass species, we found a continuous spectrum of mechanical and nutritional leaf traits. According to this continuum, we could expect a preference by seagrass consumers toward the species with high nutritional value and low mechanical resistance. Previous studies reported herbivory preference for pioneering over climax species (Lanyon 1991, Preen 1995, Aragones 1996, Mariani & Alcoverro 1999). In our study, results showed that the small, cosmopolitan and fast-growing species *Syringodium isoetifolium* and *Halophila ovalis* (Kirkman 1985, Cambridge & Lambers 1998, Cambridge 1999) may be more susceptible to herbivory because of their higher N and lower C and fibre contents, as well as having 'tender' tissues (i.e. low mechanical resistance). In fact, there is an extensive literature on the grazing of the cosmopolitan species (*H. ovalis*, *Halodule uninervis* and *S. isoetifolium*) by dugongs (Lanyon et al.1989, Preen 1995, Nakaoka & Aioi 1999) and green turtles (Bjorndal 1980, Kuiper-Linley et al. 2007) in tropical seagrass meadows. These are fast-growing species that do not invest in leaf toughening as much as the large, long-lived *Posidonia* spp. (Cambridge & Lambers 1998, Cambridge 1999) because this would be a trade-off to photosynthesis investment in growth. These long-lived species might be less susceptible to herbivory given their structural reinforcement as a mechanism of persistence, which is expressed as high C content, low N content and tough leaves. *Amphibolis* spp., *Heterozostera nigricaulis* and *Thalassodendron pachyrhizum* (all species with a similar morphology, a cluster of leaves arising from a vertical stem) were situated in an intermediate position between the short- and long-lived species.

We conducted a literature survey on studies of seagrass consumers to assess their preferences for the 9 seagrass species included in our study (Table 5). Information is lacking about herbivory pressure on half of the species (e.g. *Thalassodendron pachyrhizum*, *Heterozostera nigricaulis*, *Halophila ovalis* and *Syringodium isoetifolium*). Low levels of direct grazing were reported for leaves of *Posidonia* spp. and *Amphibolis* spp., with periphyton, epiphytes and ageing leaves with high epiphyte or epifauna loads being the preferred food sources in Western and Eastern Australian seagrass beds (Table 5). We found a few studies reporting a preference for *A. griffithii* and *P.sinuosa* over *A. antarctica* and *P. coriacea* (for herbivorous fishes; MacArthur & Hyndes 2007, White et al. 2011) and young tissues of *P. australis* over *P. sinuosa* (for isopods; Brearley & Walker 1995, Brearley et al. 2008). In fact, *Posidonia* spp. appear to contribute very little directly to the secondary production of higher trophic levels in Western Australian meadows (Table 5).

Halophila ovalis and *Syringodium isoetifolium* were the only 2 species in our study characterised by a C:N ratio <17, which is considered necessary in animal nutrition (Russel-Hunter 1970), and presenting weak or 'soft' tissues, i.e. with a low mechanical resistance to herbivore attack. In contrast, we found that the majority of the species, specially *Posidonia* spp., presented a high C:N ratio, presumably explained by their fibre-reinforced or tough leaves. Valentine & Duffy (2006, p. 469) suggested that 'it is unlikely that high C:N ratios (>17) by themselves are

Table 5. Literature information on the leaf consumption in the seagrass species included in the study. Geographical areas include both Western and Eastern Australia

Seagrass species	Geographical area	Consumers	Type and extent of consumption	Preferences observed	Source
Studies based on *in situ* observations (measurements on gut content of potential seagrass consumers)					
Posidonia spp., *Heterozostera tasmanica*, *Zostera muelleri*, *Amphibolis antarctica*	South coast of Australia, from Thomsons Bay to Jervis Bay (30 localities)	Gut contents of 106 species were studied and only *Meuschenia freycineti* consumed seagrass in substantial quantities	0 to 59.2 mg m^{-2} d^{-1}	Seagrass was possibly an incidental item ingested for its associated sessile invertebrates	Edgar & Shaw (1995)
Posidonia australis	Port Hacking, New South Wales, eastern Australia	39 species of fish community. Those consuming seagrasses and algae (7): *Girella tricuspidata*, *Petroscirtes lupus*, *Monacanthus chinensis*, *Meuschenia freycineti*, *M. trachylepis*, *Arothron hispidus*, *Torquigener pleurogramma*	Seven species consumed algae and seagrasses (ca. 10% of the overall community diet). 29 to 63% gut content of monocanthid fish	Authors suggest that fish grazed on fauna-encrusted *Posidonia* blades and on their attached epiphytic algae	Burchmore et al. (1984)
Posidonia australis	New South Wales, eastern Australia	Omnivorous fish: *Monacanthus chinensis*, *Meuschenia freycineti*, *M. trachylepis*	Up to 65% of gut content in *M. freycineti*	Preference towards pieces with encrusted bryozoans and polychaetes	Bell et al. (1978)
Posidonia australis, *Amphibolis antarctica*	Cliff Head, Western Australia	Crab: *Portunus pelagicus*	Low volume of seagrass in gut content (6.1 to 23.2%)	Opportunistic omnivores with preferences for slow-moving animal prey	Edgar (1990)
Posidonia sinuosa, *P. coriacea*, *Amphibolis griffithii*	Fremantle, south-western Australia	Fish: *Odax acrophilus*, *Haletta semifasciata*	0.3 to 17.1% volume in gut content	*A. griffithii* and *P. sinuosa*, but no *P. coriacea*	MacArthur & Hyndes (2007)
Heterozostera tasmanica (Zosteraceae)	Duck Point, Corner Inlet, south-eastern Australia	Fish (sea garfish): *Hyporhamphus melanochir*	35 mg seagrass d^{-1} g^{-1} DW (body weight)	Not specified	Klumpp & Nichols (1983)
Studies based on *in situ* observations (measurements on seagrass biomass consumption)					
Posidonia australis	Pittwater, Botany Bay, Port Hacking, south-eastern Australia	Monocanthid fish: *Meuschenia freycineti*, *M. trachylepis*	Direct consumption. Low level of grazing although can result in high localised removal rates in certain areas in winter 0 to 2.4 mg DW m^{-2} d^{-1}	Not specified	Wressnig & Booth (2008)
Posidonia australis, *P. sinuosa*	Rottnest Island, south-western Australia	Isopods: *Lynseia* spp.	Direct consumption by burrowing beneath the epidermis and consuming the mesophyll and cells of the vascular bundles	Preference for *P. australis* leaves over *P. sinuosa* Preference for ageing leaves	Brearley & Walker (1995)

Table 5 (continued)

Seagrass species	Geographical area	Consumers	Type and extent of consumption	Preferences observed	Source
Amphibolis griffithii, *A. antarctica*, *Posidonia* spp., *Thalassodendron pachyrhizum*	Western Australia coastline (along 1000 km)	Isopod: *Limnoria agrostica*	Direct consumption of new tissues within the sheath of *A. griffithii*	Isopods preferentially attacked young tissues, the most recently apical clusters and tissues within the sheath area	Brearley et al. (2008)
Posidonia sinuosa, *P. coriacea*, *Amphibolis griffithii*, *A. antarctica*	Geographe Bay, south-western Australia	8 potential grazing fish species: *Odax acrophilus*, *O. cyanomelas*, *Girella zebra*, *Siphonognathus beddomei*, *Meuschenia hippocrepis*, *Scobynichthys granulatus*, *Polyspina piosae*, *Torquigener pleurogramma*	Direct consumption by biting leaves. Extremely low level of seagrass grazing (0.00065 % of the biomass in the bay)	No preference towards leaves with high nitrogen content. Preference for *P. sinuosa* and *A. griffithii* over *A. antarctica*	White et al. (2011)
Posidonia sinuosa	Cockburn Sound, Western Australia	Sea urchin: *Temnopleuris michaelsenii*	Not quantified	Not specified	Cambridge et al. (1986)
Halophila ovalis	Western Australia	Black swan: *Cygnus atratus*	23 % of seagrass production	Not specified	Eklöf et al. (2009)
Posidonia sinuosa	Marmion Lagoon off Perth, Western Australia	Gastropods: *Thalotia conica* Amphipod: *Tethygeneia* spp., *Ampithoe* spp., *Hyale* spp.	Not quantified	Grazers did not eat *Posidonia* leaves, but ate their epiphytes and periphyton	Jernakoff & Nielsen (1997, 1998)
Studies based on multiple-choice experiments					
Posidonia australis	Botany Bay, Port Hacking, south-eastern Australia	Monocanthid fish: *Meuschenia freycineti*, *M. trachylepis*	Direct consumption	Preference toward seagrass leaves with high epiphyte loads (old leaves). Young, soft, nutrient-rich seagrass blades were consumed less than mature blades	Wressnig & Booth (2007)
Posidonia australis	Shoalwater Bay and Boyinaboat Reef, south-western Australia	Gastropods: *Pyrene bidentata*, *Cantharidus lepidus*	Low level of grazing on seagrass leaves (0.00–0.24 mg ind.$^{-1}$ d^{-1})	Preference towards kelp and periphyton and red algae and avoidance of seagrass consumption	Doropoulos et al. (2009)

Table 5 (continued)

Seagrass species	Geographical area	Consumers	Type and extent of consumption	Preferences observed	Source
Studies based on stable isotope techniques					
Amphibolis griffithii	Success Bank, Western Australia	Several fish species: *Cochleocepts* sp., *Notolabrus parilus*, *Acanthaluteres vittiger*, *Scobinichthys granulatus*, *Odax acroptilus*, *Siphonognathus radiatus*, *Leviprora inops*, *Pelates sexlineatus*, *Pelsartia humeralis*	Not quantified	Macroalgae were identified as the main contributor of carbon to the trophic structure, whereas seagrass is the main contributor to seston and sedimentary organic matter, and very little of it is incorporated into the trophic level	Smit et al. (2005)
Posidonia sinuosa	Fremantle, Western Australia	Gastropods: *Cantharidus lepidus*, *Thalotia conica*	Not quantified	Authors suggest that these gastropods consumed significant amount of *P. sinuosa* leaves	Smit et al. (2006)
Posidonia australis	South-eastern Australia	Monocanthid fish: *Meuschenia freycineti*, *M. trachylepis*	Not quantified	Epifauna and epiphytes of *P. australis* were the main food source of the 2 grazers	Wressnig (2006)

responsible for the relative low grazing on seaasses'. Our results suggested that not only the C:N composition but also the mechanical resistance, which was correlated with this ratio, may explain the low seagrass leaf palatability, as seagrasses generally exceed this C:N ratio threshold.

The comparison of transverse versus longitudinal toughness across the species indicated that 2 to 4 times more work was required to fracture leaves transversely than longitudinally. The differences are due to fibre orientation in the leaf, since many veins lie longitudinally in monocot species (Onoda et al. 2011). To our knowledge, this is the first study to compare longitudinal and traversal toughness for a range of species. Ecologically, the high transverse toughness may influence the feeding behaviour of herbivores. For example, ingesting, grazing or detaching leaf blades by pulling them along the longitudinal axis may be easier in terms of energy consumption than doing so in the transverse axis. The longitudinal orientation of fibres may also explain the feeding behaviour of isopod miners in *Posidonia* spp. and *Amphibolis griffithii*, which burrow beneath the epidermis consuming the mesophyll and forming linear mines along the longitudinal axis of the leaf lamina (Brearley & Walker 1995).

In summary, we quantified mechanical resistance and nutritional value in a pool of 9 seagrass species. We confirmed the general assumption of the correlation between high C:N ratio and high investment in structural reinforcement, and leaf toughening. Among co-existing seagrasses, we found a continuous spectrum of mechanical and nutritional traits across species, which will be useful as fundamental information of species assembly, herbivore behaviour and ecosystem functions. Further research on experimental feeding trials and multiple-choice experiments are required for more comprehensive understanding of the role of mechanical traits on animal–seagrass interactions.

Acknowledgements. We thank I. Wright for help with the shearing machine, as well as M. Westoby for use of the facilities at the Department of Biological Sciences (Macquarie University, Sydney) and for comments on the preliminary results. Also thanks to G. and D. Kendrick, J. Ooi, R. Hovey and J. Statton (The University of Western Australia, Perth) for help with field work, and J. Kuo for receiving us. We appreciate the constructive criticism given by 3 anonymous reviewers on the early version of the manuscript. Funds were provided by the projects Imachydro (CTM2008-00012/MAR) and Sea-Live (CTM2011-24482) from the Spanish Ministry of Science and Innovation, and by the Junta de Andalucía Excellence project PAMBIO (P08-RNM-03783). C.B.S. holds a contract within the project Imachydro. This is CEIMAR Journal publication no. 9.

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Editorial responsibility: Hans Heinrich Janssen, Oldendorf/Luhe, Germany

Submitted: April 28, 2011; Accepted: April 16, 2012
Proofs received from author(s): June 26, 2012