Feeding preferences of amphipod crustaceans *Ampithoe ramondi* and *Gammarella fucicola* for *Posidonia oceanica* seeds and leaves

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Summary: The functional importance of herbivory in seagrass beds is highly variable among systems. In Mediterranean seagrass meadows, macroherbivores, such as the fish *Sarpa salpa* and the sea urchin *Paracentrotus lividus,* have received most research attention, so published evidence highlights their importance in seagrass consumption. The role of small crustaceans in seagrass consumption remains less studied in the region. Herbivory on *Posidonia oceanica* seeds has not previously been reported. In turn, crustacean herbivory on *P. oceanica* leaves is broadly recognized, although the species feeding on the seagrass are mostly unknown (except for *Idotea baltica*). This work evaluates *P. oceanica* consumption by two species of amphipod crustaceans commonly found in seagrass meadows. *Ampithoe ramondi* and *Gammarella fucicola* actively feed on *P. oceanica* leaves and seeds. Both species preferred seeds to leaves only when the seed coat was damaged. This study provides the first direct evidence of consumption of *P. oceanica* seeds by the two named amphipod crustaceans, and confirms that they also consume leaves of this seagrass species.

Keywords: herbivory; mechanical traits; nutritional quality; invertebrate food choice; crustacean; gammarid.

Preferencia alimentaria de los anfípodos *Ampithoe ramondi* and *Gammarella fucicola* sobre hojas y semillas de *Posidonia oceanica*

Resumen: La herbivoría tiene una importancia funcional muy variable entre los sistemas de praderas de angiospermas marinas. En las praderas mediterráneas, el papel de los macroherbívoros, como el espárido *Sarpa salpa* y el erizo marino *Paracentrotus lividus,* ha concentrado buena parte de la atención científica y, en consecuencia, la evidencia y bibliografía científica enfatizan su importancia como consumidores de angiospermas marinas. Los trabajos de investigación sobre el papel de pequeños crustáceos como consumidores de angiospermas marinas en la región mediterránea es todavía escasa. La herbivoría sobre semillas de *Posidonia oceanica* no se había reportado hasta la fecha. En cambio, el consumo de hojas de *P. oceanica* por crustáceos sí está ampliamente aceptado, aunque las especies responsables de este consumo son en su mayoría desconocidas (con la excepción de *Idotea baltica)*. Este trabajo evalúa el consumo de semillas y hojas de *P. oceanica* por dos especies de anfípodos gammáridos frecuentes en las praderas de angiospermas marinas mediterráneas y su preferencia alimentaria entre ambos tejidos. Nuestros resultados indican que *Ampithoe ramondi* y *Gammarella fucicola* consumen activamente tanto las hojas como las semillas *P. oceanica*. Ambas especies prefirieron consumir las semillas de *P. oceanica* a las hojas, pero sólo cuando la cubierta exterior de la semilla estaba dañada. Este estudio es la primera evidencia de consumo directo de semillas de *P. oceanica* por anfípodos y confirma que las dos especies estudiadas consumen hojas.

Palabras clave: herbivoría; propiedades mecánicas; calidad nutricional; selección alimentaria; invertebrados; gammáridos.

INTRODUCTION

Herbivores play a functional role in benthic marine ecosystems by channelling primary production to higher trophic levels (Poore et al. 2012, Hillebrand 2009, Gruner et al. 2008). Current seagrass herbivores are dominated by waterfowl, fish, urchins and small invertebrates, which have replaced large vertebrate herbivores (e.g. dugongs and manatees, turtles) (Thayer et al. 1984, Heck and Valentine 2006, Valentine and Duffy 2006).

Invertebrate abundance associated with seagrass meadows may be three times greater than that of other highly productive ecosystems such as coral reefs (Nakamura and Sano 2005). The invertebrate communities associated with seagrass meadows have a crucial importance in the cycling of carbon, controlling epiphyte biomass (Jaschinski et al. 2009, Jernakoff and Nielsen 1997), sustaining higher trophic levels (Edgar and Shaw 1995a) and enabling, for example, the achievement of higher fish densities compared with adjacent environments (Edgar and Shaw 1995b).

Crustaceans are one of the most abundant invertebrate taxonomic group in epifaunal seagrass communities and, among crustaceans, amphipods are one of the dominant groups (Barnes 2017, Sturaro et al. 2015, Moore and Hovel 2010, Sanchez-Jerez et al. 1999). The amphipods associated with seagrass systems are considered to be dominated by detritus or/and epiphyte feeders (Valentine and Duffy 2006). Apart from sea urchins, the direct consumption of seagrass leaves by invertebrates is considered accidental and is generally associated with grazing on epiphytes (but see Rueda et al. 2009). Invertebrate herbivores' preference for epiphytic algae rather than seagrass (Michel et al. 2014) is frequently explained by the presence of chemical defence compounds in seagrass tissues and/or their lower nutrient content compared with macroalgae (Cruz-Rivera and Hay 2000, 2003). Several amphipod species, particularly from the family Ampithoidae and the genus Gammarus (e.g. *Gammarus mucronatus, G. locusta, G. oceanicus* and *Ampithoe longimana*) show a specific level of tolerance to algal (e.g. *Dyctiota, Gracilaria* and *Ulva*) chemical defences (Andersson et al. 2009, Duffy and Hay 1994).

Seagrass leaves may constitute an abundant food source. Seagrass seeds, which are nutritionally richer than leaves because they are rich in concentrated storage components such as starch and protein, could be a valuable food source for herbivores (Delefosse et al. 2016, Uchida et al. 2014, Dall et al. 1992). Seagrass seed consumption has been confirmed in North Atlantic meadows (Fishman and Orth 1996), Australian meadows (Orth et al. 2002, 2006, 2007, Wassenberg 1990) and Japanese meadows (Nakaoka 2002). Between 18% and 75% of the sampled seeds from five *Posidonia australis* meadows showed herbivore damage (Orth et al. 2002). Wassenberg (1990) revealed that seeds of *Zostera capricorni* are an important component of the diet of juvenile stages of the decapod crustacean *Penaeus esculentus* during the period of seed production. Field experiments have shown *Zostera marina* and *Zostera caulescens* seeds and spathes as a trophic resource for the decapod *Callinectes sapidus* and the tanaid *Zeuxo* sp. (Nakaoka 2002, Fishman and Orth 1996). Seed-tethering experiments have also evidenced the direct consumption of seagrass seeds by crustaceans, sometimes with high percentages of damaged seeds (>50% for *Halophila ovalis* and *Posidonia sinuosa*, and >60% for *Posidonia australis* and *Amphibolis antartica*) (Orth et al. 2006, 2007). Seemingly, laboratory assays have demonstrated the direct consumption of both seeds and seedlings of *Z. marina* by crustaceans when an alternative food source is not available (Wigand and Coolidge Churchill 1988), as well as inflorescence consumption by non-native amphipod *Ampithoe valida* (Reynolds et al. 2012). However, seagrass seed consumption either by fishes, sea urchins or small invertebrates remains unreported in Mediterranean meadows.

The dominant Mediterranean seagrass species, *Posidonia oceanica*, flowers irregularly, both spatially and temporally, and consequently seeds represent an eventual and ephemeral resource for herbivores (Díaz-Almela et al. 2006). Nutritionally, free sugars and starch are the main carbohydrates stored in *P. oceanica* seeds and represent between 2% and 10% (free sugars) and 4% and 30% (starch) of seed dry weight (DW) (Hernán et al. 2017, Celdrán and Marín 2013). Regarding nutrient content, *P. oceanica* seeds exceed both adult and seedling leaves (Balestri et al. 2009), but despite their comparatively low nutritional value, leaves represent an abundant and permanent potential trophic resource for the invertebrate community. *P. oceanica* leaves have a lower nutrient content (as % of DW) and a higher C/N ratio than leaf epiphytes or algae (Prado et al. 2010, Lepoint et al. 2007).

Vergés et al. (2007, 2011) studied the macroherbivore (*Paracentrotus lividus*) feeding preferences for different *P. oceanica* tissues and found that the inflorescences were preferred to leaves. The authors found no differences in the concentration of chemical defence compounds or in the nutritional value of different parts of the plant and suggested that this preference was driven by plant structural traits. Similar drivers (e.g. structural traits and nutrient content) could also affect amphipod preference to consume epiphytes rather than *Posidonia* leaves or litter fragments (i.e. *Apherusa chiereghinii, Aora spinicornis* and *Gammarus aequicauda*) or rizhomes (*Dexamine spiniventris*) (Michel et al. 2014). There is little published evidence of direct consumption of *P. oceanica* tissues by amphipod crustaceans or by other herbivores (but see Guidetti 2000, Peirano et al. 2001).

Here we assess whether *P. oceanica* seeds and leaves represent a trophic resource for two amphipod species commonly found in Mediterranean seagrass meadows and whether these amphipods show any feeding preference for leaves or seeds. Consumption and food choice experiments were performed in microcosms with the amphipods *Ampithoe ramondi* Audouin, 1826 and *Gammarella fucicola* Leach, 1814, two species commonly found in *P. oceanica* meadows (Bellan-Santini et al. 1982). *A. ramondi* and *G. fucicola* show a broad distribution across the Mediterranean,

Atlantic, Red Sea and Indian Ocean. Both species are described as mainly algae and detritus feeders (Michel et al. 2015, Zakhama-Sraieb et al. 2011, Lepoint et al. 2006). First, we performed consumption tests to determine whether *A. ramondi* and *G. fucicola* could feed on *P. oceanica* leaves and seeds. To this end, non-epiphytized leaves and seeds with or without a damaged coat were offered to amphipods. Next, we performed food choice experiments to determine whether amphipods preferred epiphytized versus non-epiphytized leaves and whether they preferred seeds (richer in stored resources) to leaves. We distinguished between seeds with an undamaged coat ("sealed seeds") and a damaged coat ("open seeds") to determine whether the seed coat protection was an intrinsic seed trait affecting amphipod food choice. We analysed nitrogen and phosphorus concentration in leaves and seeds and determined the mechanical resistance to puncture (a proxy of resistance to herbivory) of the same organs to enrich the discussion about food choices.

MATERIALS AND METHODS

Collection and identification

Drifting, naturally-produced *Posidonia oceanica* fragments, including leaves, rhizomes and roots and associated fauna, were collected at Alcúdia Bay (39.826292°N 3.177788°E) in June 2014 and housed in the laboratory inside a 4000 L tank (4 m long × 1 m wide × 1 m high) with continuous seawater input (84 L per hour) and recirculation. Tank temperature was kept below 22°C and day/night natural cycle was simulated with daylight fluorescent lights (280.0-0 lux). Light intensity and temperature were recorded using a data logger (Onset Hobo). A second collection of *P. oceanica* leaves and seedlings for the consumption and feeding choice tests was performed during summer 2015 and housed in a second tank of similar conditions to the one described above.

During summer 2015, amphipods associated with *P. oceanica* were collected in the first tank, fixed in ethanol 95% and transported to the laboratory for taxonomic identification. They were identified using a stereomicroscope (Leica MZ16 with integrated camera EC3) with the animals submerged in lactic acid. Four species were recorded in the samples: *Ampithoe ramondi, Gammarella fucicola, Liljeborgia dellavallei* and *Microdeutopus stationis*. Hereafter, the identifications were done on living amphipods and animal manipulation was reduced to the minimum to avoid stress or damage. Due to the low number of available individuals of *L. dellavallei* and *M. stationis*, consumption and preference tests were performed with *A. ramondi* and *G. fucicola* only.

Consumption tests

Herbivory on *P. oceanica* leaves and seeds was tested using containers made of transparent acrylic pipe of 4.8 cm internal diameter and 10 cm length. The top and bottom of each container were closed with a 0.5 mm nylon mesh to allow water exchange with the main tank and prevent amphipods from going out/in. The leaf portions and seeds used in the tests were measured (length and width) before the assays.

A number (between 5 and 12) of similar-sized amphipods of the same species were placed into each container together with one piece of leaf or seed. The consumption test endpoint was established after 6 days but the tests ended when detectable consumption occurred (with a minimum duration of 21 hours). Three feeding materials were offered to the amphipods separately: a portion of the second youngest leaf of a non-epiphytized *P. oceanica* shoot (gently scraped with a razor blade), a *P. oceanica* seed cut from a seedling at shoot base (as a proxy of a naturally damaged seed having holes in the seed coat, hereafter "open seed") and a similar *P. oceanica* seed with the cut section sealed with 100% bee wax ("sealed seed"), as a proxy of a seed with an undamaged coat. Seed coats are usually covered by a hydrophobic waxy cuticle to prevent water exchange with the environment (Freeman 2008), so beeswax was used to innocuously cover the section plane formed after cutting the seed from the seedling and to avoid amphipod access to internal seed tissues through the scar . A total of 43 tests were performed, 20 with *A. ramondi* (open and sealed seeds n=16; leaves n=4), 23 with *G. fucicola* (open and sealed seeds n=14; leaves n=9).

Preference tests

The same acrylic containers described above were placed in the tank with one amphipod and one of the following choice options: epiphytized leaf versus non-epiphytized leaf; non-epiphytized leaf versus open seed; and non-epiphytized leaf versus sealed seed. For each combination, between 10 and 20 trials were performed. Trials in which both or none of the offered materials were eaten were excluded. The number of valid replicates analysed for each combination of choice options and species were the following for *A. ramondi* and *G. fucicola*, respectively: epiphytized leaf/non-epiphytized leaf, n=10 and n=8; open seed/non-epiphytized leaf, n=10 and n=10; sealed seed/non-epiphytized leaf, n=11 and n=14. Preference tests lasted until the first consumption mark appeared (19-143 hours) or the animal died. Consumption marks were detected with a stereomicroscope (Zeiss Stemi DV4) and the most representative ones were captured using a Leica MZ16 with integrated EC3 camera.

Nutrient concentration analysis

At the end of the preference assays, the leaves and seeds were placed individually in plastic bags and stored frozen at –20°C until processing. In the laboratory, the leaves and seeds were dried out (60°C, 48 h) and ground to powder with a stainless steel ball mill (MM200 RETSCH, Haan, Germany). An aliquot of the ground material was used to determine total nitrogen content using a Heraeus CHN-o-rapid elemental analyser and phosphorous content following the protocol

described by Fourqurean et al. (1992) with certified standard beech leaves (CRM No. 100). Nitrogen and phosphorous content in leaves and seeds are expressed as the % of DW.

Tissue mechanical property tests

During the summer of 2016, eight *P. oceanica* seeds and shoots were collected to perform mechanical resistance tests. We tested second the youngest leaves in shoots, seeds with intact coat and seeds without coat (emulating open seeds in the treatments). To avoid differences in thickness between tested leaves, basal and apical portions of each leaf were not used. Seed slices 2 mm thick were used in tests. A Zwick Z100 mechanical testing machine was employed to perform punching tests, which measure the force (N mm^{-2}) required to punch a hole through the leaf lamina, a proxy of mechanical resistance to herbivory (Ibanez et al. 2013, Aranwela et al. 1999). The punch and die method was adapted from Onoda et al. (2008).

Statistical analyses

A chi-squared test was used to assess differences in amphipod feeding frequency depending on the type of food offered (i.e. epiphytized leaf, non-epiphytized leaf, open seed or sealed seed). The null hypothesis assumes independence of amphipod consumption pressure (frequency of bites) from food type. The expected frequencies under the null hypothesis were compared with the observed frequency of bites. Analysis of variance was performed to assess differences in mechanical resistance between leaf, coated seed and uncoated seed. A t-test was performed to evaluate leaf and seed nutritional features. A chi-squared test was done following Sokal and Rohlf (1981). One-way ANOVA and a t-test were performed with the Statistica 7.1 data analysis software system, StatSoft Inc.

RESULTS

Ampithoe ramondi and *Gammarella fucicola* were able to feed on *Posidonia oceanica* leaves and seeds (either open or sealed). All the leaves offered to *A. ramondi* were attacked (100%), whereas 80% and 60% of open and sealed seeds were bitten, respectively. *G. fucicola* bit all the open seeds offered and 60% of the sealed seeds; it fed on 67% of the leaves offered. *A. ramondi* and *G. fucicola* started scraping the seed coat and bored through, forming irregular holes. The marks on the leaves displayed a dogtooth pattern (Fig. 1) on
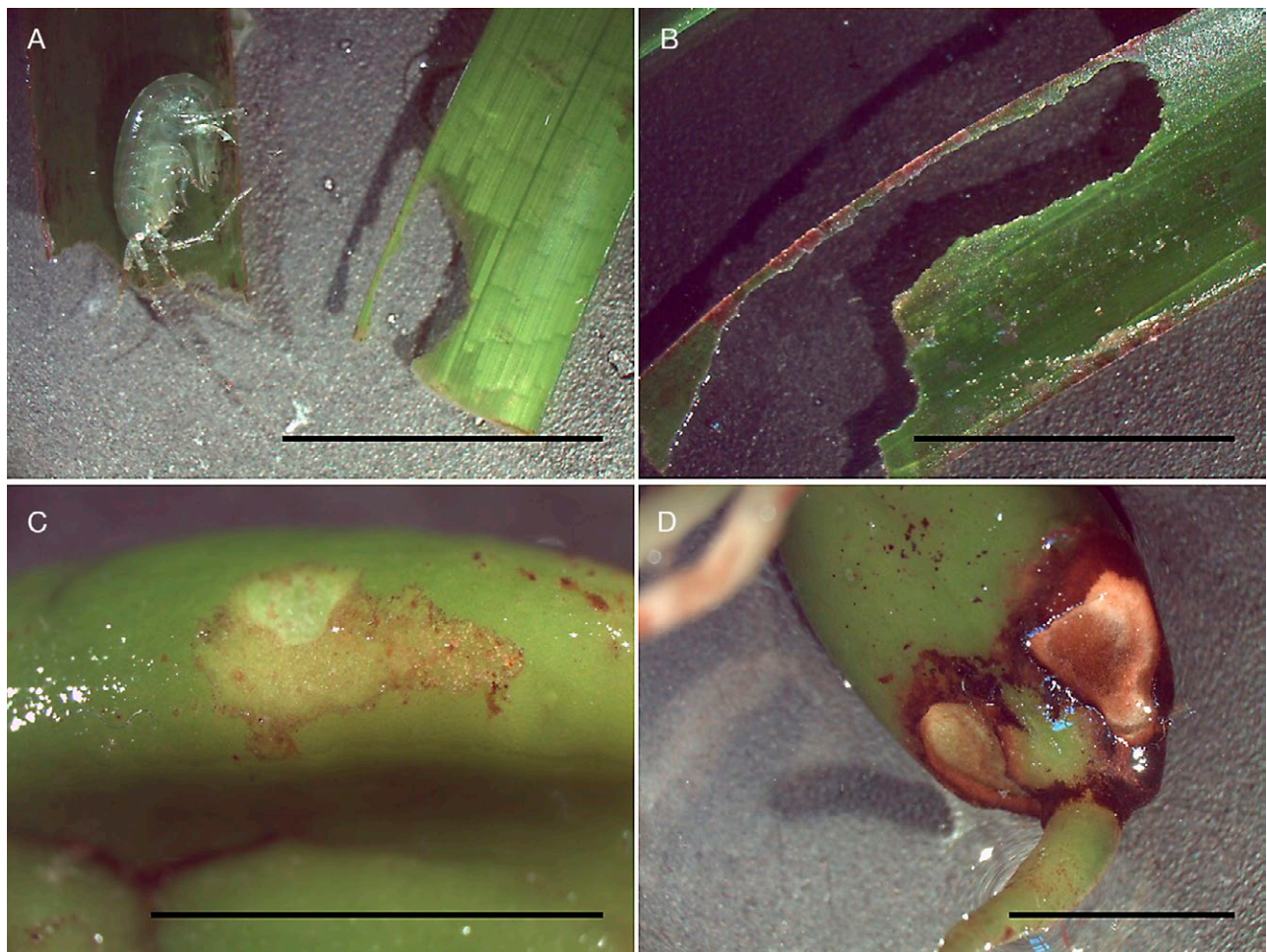

Fig. 1. – Dogtooth bite pattern on leaves and irregular holes on seeds produced by *Gammarella fucicola* and *Ampithoe ramondi*. Scale bars show 1.0 cm and 0.5 cm for leaf (A, B) and seed (C, D) photos respectively. A specimen of *A. ramondi* is also shown in photo A.
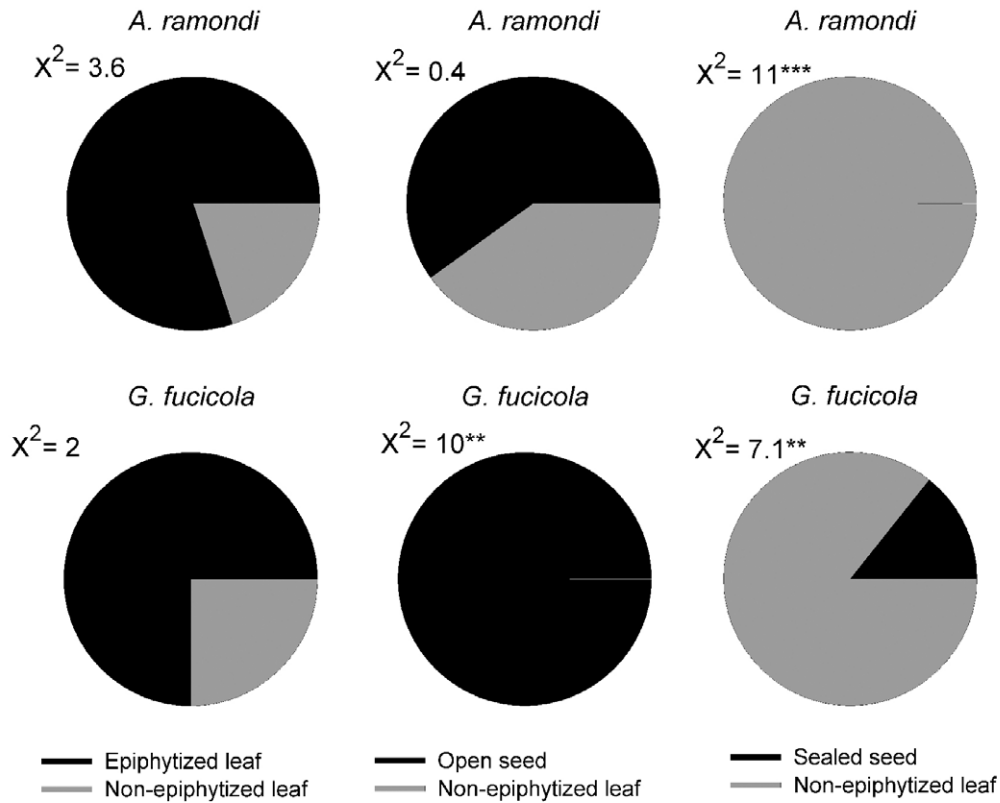
Fig. 2. – Frequency of bites of *Gammarella fucicola* and *Ampithoe ramondi* on leaves, open seeds and sealed seeds. Chi-squared statistic and statistical significance is shown: ** p<0.01, *** p<0.001.
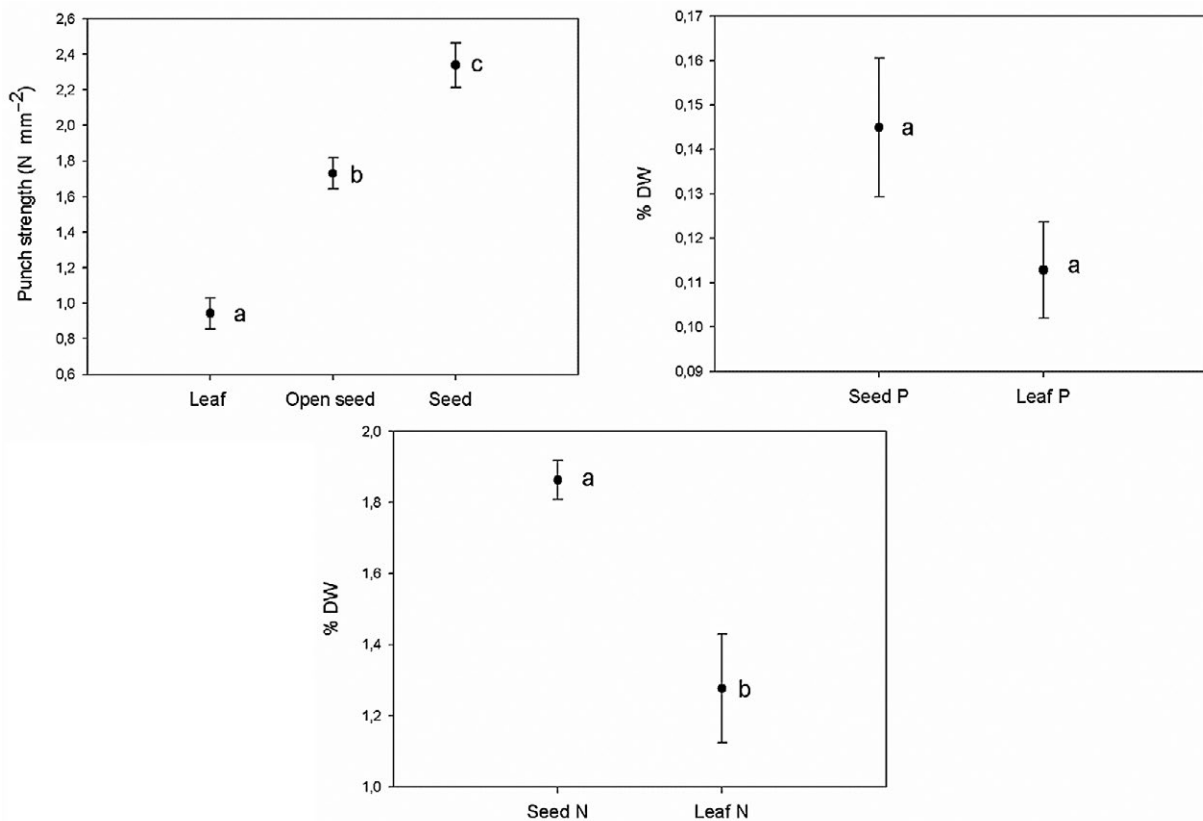

Fig. 3. – Mechanical resistance and nutrient content of leaves, open seeds and seed with coat. Error bars represent standard error. Punch strength (N mm^{-2}) for leaves, open seeds and seeds with   coat. Nutrient content (% DW) of seeds and leaves. Differences between groups are indicated by different letters.

the leaf margin. Visual differences between marks produced by the two species were unnoticeable using a stereomicroscope (Zeiss Stemi DV4).

Feeding choice test

The two species showed a similar pattern with respect to feeding preferences. Open seeds were preferred over non-epiphytized leaves, but this choice reversed when sealed seeds were offered. Apparently, both species preferred epiphytized leaves over non-epiphytized leaves, although the chi-squared statistic was not significant at this point, probably because of the low number of replicates (Fig. 2).

Mechanical and nutritional traits

The leaves showed a lower mechanical resistance to herbivory (mean±SE: 0.94 ± 0.086 N mm^{-2}) than open seeds (1.88 ± 0.171 N mm^{-2}), which were pierced more easily than seeds with a coat (2.21 ± 0.168 N mm^{-2}) (ANOVA: $F=51.7016$; $p<0.0001$) (Fig. 3). Nitrogen content was higher (t-value=3.6078, $p<0.01$) in seeds ($1.86\pm0.055\%$ N) than in leaves ($1.14\pm0.066\%$ N). There were no differences (t-value=1.6468, $p>0.5$) in phosphorus content between leaves and seeds) (Fig. 3).

DISCUSSION

We identified two potential consumers of *Posidonia oceanica* leaves and seeds in the field: the gammarid amphipods *Ampithoe ramondi* and *Gammarella fucicola*. Both species preferred nutritionally poorer leaves to the richer seed tissue when the seed coat was intact. However, this choice pattern reversed when the seed coat was damaged, suggesting that the coat protects the seed against invertebrate herbivory. Seed protection against herbivory assures carbon and nutrient supply, which are essential for seedling survival and the success of recruitment. Plants can prevent seed herbivory through chemical (i.e. secondary metabolites) (e.g. Rhoades and Cates 1976, Veldman et al. 2007) or structural defences (e.g. coat strength) (Davis et al. 2008, Rodgerson 1998). The coat, as the outermost protective tissue of seeds, is the first line of defence against pathogens and herbivores (Freeman 2008). Our results suggest that the mechanical defence associated with the presence of a coat on *P. oceanica* seeds effectively discourages *A. ramondi* and *G. fucicola* herbivory. Apart from this study, the understanding of *P. oceanica* seed mechanical defence is still poor. Seed coat chemical defences have not been evaluated in this work, but they might also drive herbivore preference. In turn, *P. oceanica* leaves display the strongest mechanical defences known among seagrasses; they show a substantially higher proportion of fibre than terrestrial herbaceous plants (De los Santos et al. 2016, Onoda et al. 2011), which seems to deter macroherbivores (Vergés et al. 2007, 2011). *P. oceanica* seed phenolic content (about 6% of seed DW) (Hernán et al. 2016, 2017) exceeds the phenolic concentration found for seagrass species with higher seed production (e.g.

Reynolds et al. 2012). Given the high amount of seed structural reserve storage, *P. oceanica* has a high theoretical reproductive effort capacity (Cabaço and Santos 2012). *P. oceanica* seed production is low compared with other seagrass species (Díaz-Almela et al. 2006, Conacher et al. 1994b, Silberhorn et al. 1983), and a strong chemical defence would be essential for seed protection, seedling recruitment and, thus, for the maintenance and persistence of the meadows (Kendrick et al. 2012). Since *P. oceanica* seeds have no dormancy, a positive effect of seed scarring by amphipods is not expected (Conacher et al. 1994a, Loques et al. 1990). Our results show that a seed coat deters the attack by small invertebrates, likely enhancing seedling survival.

Previous studies have assessed the influence of nutritional quality of algae and the presence of chemical defence compounds on the feeding choice and ingestion rate of marine invertebrate herbivores (Vergés et al. 2007, 2011, Cruz-Rivera and Hay 2000, Duffy and Hay 1994), but similar studies on seagrass are still scarce. In addition, plant tissue toughness has been widely recognized as the main constrictor of invertebrate herbivory in terrestrial systems, well above plant nitrogen content (Caldwell et al. 2016, Ibanez et al. 2013). The importance of mechanical characteristics of seagrass compared with its nutritional quality in determining food choice in small marine invertebrates had not been recognized until now. The importance of mechanical traits and fibre content in the food choice of large marine invertebrates (i.e. sea urchins) had been previously acknowledged in algae (Cruz-Rivera and Friedlander 2011) and seagrasses (Jiménez-Ramos et al. 2017), and our results suggest that similar food choice mechanisms may also operate for amphipods.

Posidonia oceanica leaves and seeds are a complementary food source for certain species, especially in healthy meadows where the amphipod community is richer and denser (Zakhama-Sraieb et al. 2006). Our work shows that small amphipods (e.g. *A. ramondi* and *G. fucicola*) may use *P. oceanica* epiphytized leaves as a trophic resource and eventually benefit from seeds, especially when the coat protection is damaged. Seed availability and their higher nutritional value compared with leaves (Hernán et al. 2016, 2017) would also drive the amphipod food preference for seeds. The preference of *A. ramondi* and *G. fucicola* for epiphytized leaves rather than non-epiphytized leaves is in accordance with the algae and detritus feeding behaviour considered for both species elsewhere (Michel et al. 2014, Navarro-Barranco et al. 2013, Lepoint et al. 2006). However, the relative importance of the different food sources in their diet varies among studies; even crustacean rests have been found in the gut content of *G. fucicola* (Michel et al. 2014), suggesting an opportunist and generalist feeding behaviour. Changes in available trophic resources, nutritional quality, quantity, and palatability will have stronger effects on the food choice and consumption rate of opportunistic consumers than on specialists.

The role of small herbivores as drivers of ecological processes in Mediterranean meadows, such as in seed-based seagrass recruitment or the percentage of

seagrass organic matter transferred to higher trophic levels, remains elusive; mesocosm or tethering field study approaches should be performed to address it. Results of studies on Western Australian (Orth et al. 2002) and North Pacific (Nakaoka 2002) meadows suggest that seed ingestion by small invertebrates may be a significant factor in seed-based recruitment failure (percentage of damaged seeds: 34%-53% in *Posidonia australis*, 14% in *Zostera marina* and 27% in *Zostera caulescens*). A field assessment of the importance of amphipod herbivore pressure on *P. oceanica* tissues remains to be carried out.

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