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### **Trade-offs in macroalgal chemical defences battle of the sexes,invaders and consumers**

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Trade-offs in macroalgal chemical defences:  
Battle of the sexes, invaders and consumers

Martyn Kurr

Supervised by Andrew Davies & Jan Hiddink

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For Dad

## Summary

Macroalgae are useful as model organisms because their simple physiology and modular growth allows their investments into growth, defence, and reproduction to be quantified. *Ascophyllum nodosum* is a dioecious perennial which responds clearly to environmental stressors, and *Sargassum muticum* is an invasive pseudo-annual which grows in discrete populations of known time-since-invasion. Two chapters investigate the occurrence of sexual dimorphism in *A. nodosum* under stress, and incorporate surveys, chemical analyses, and feeding trials to demonstrate that females invest more into sexual reproduction at sites where stressors impact juvenile mortality, and compensate by reducing investment into defence, leaving them more vulnerable to grazers. At sites where stressors impact mainly on adult mortality and performance with lower influence on juvenile mortality, limited sexual dimorphism is observed. Similar results have been demonstrated in terrestrial plants, but none have quantified responses to stress in such detail. The second two chapters test the Enemy Release Hypothesis, the Evolution of Increased Competitive Ability hypotheses, and the Biotic Resistance hypothesis using surveys, chemical analyses, and feeding trials with *S. muticum* and its associated grazers from populations established for different lengths of time, over 40 years. Grazers from older *S. muticum* were more likely to feed upon it in the laboratory, but surveys of chemical defences did not reflect any increased pressure over time in the field. Instead, *S. muticum* increased its defensive investment in the presence of a greater diversity of grazers, irrespective of the length of time those grazers had been exposed to it. Therefore although grazers can learn to consume *S. muticum*, they are still unlikely to do so in the field, such that many species must be present before at least some begin to impart top-down pressure upon it. Collectively these investigations demonstrate the high value of using macroalgae in wider ecology.

Chapter One  
General Introduction

*Overview*

This thesis is focussed on using macroalgae as model organisms to test ecological theories, previously explored almost entirely in vascular plants. Although these theories are borne from research into higher plants, none are specific to them and testing their predictions in a relatively-unexplored context is vital to furthering our understanding of ecology. Macroalgae, as photoautotrophs, are subject to the same fundamental pressures as all primary producers, requiring space, light, and nutrients. They experience attack from herbivores, and have therefore evolved defences and other strategies to compensate for top-down pressure. Morphologically and physiologically however, macroalgae are simpler than vascular plants. This makes them useful as model organisms, because expenditure into growth, defence, and reproduction are often easier to quantify. Furthermore, their intertidal ecology is composed of far fewer biotic and abiotic variables than experienced by terrestrial plants. This makes quantifying ‘stress’ much simpler than for example, in a woodland, or on a prairie, where both above and below-ground factors must be considered relative to very large species diversities and complex interactions with seasonality. Although present on rocky shores such interactions are minimal in intricacy, by comparison, and this fact has been exploited by ecologists for decades.

The research in this thesis falls into two facets, with two chapters covering each. The first section concerns sexual dimorphism in the perennial, native and long-lived *Ascophyllum nodosum* with specific interest into the stressors which drive and modify differences between the genders. The first experimental chapter (chapter two) is an investigation into the presence of sexual dimorphism in *A. nodosum*, and its modification by stress. The second chapter (chapter three) is an investigation into the different stressors acting on *A. nodosum*, with the aim of determining which drive sexual dimorphism, and which do not. The second section concerns the invasive ecology of *Sargassum muticum*, a fast-growing, pseudo-annual currently expanding its range northwards around the West coast of Britain, with a specific focus on comparing populations established for different lengths of time-since-invasion. The first chapter (chapter four) is a large observational survey of populations of *S. muticum* around the UK, and their respective mesoherbivore populations, which allows the

simultaneous testing of the Enemy Release Hypothesis, the Evolution of Increased Competitive Ability hypothesis, and the concept of Biotic Resistance. The second chapter in this section (chapter five) utilises a series of feeding trials with *S. muticum* and herbivores of *S. muticum* taken from several differently-aged populations of the algae, with a view to testing the preferences and palatability of the herbivores and algae, respectively. Both of these sections focus on the interaction between mesoherbivores (or ‘grazers’) and the model alga used, and are therefore constructed around the principal chemical defence in brown algae; phlorotannins. The introduction which follows is composed of four sections; the first concerns sexual dimorphism in photoautotrophs, the second is an introduction to the invasive stratagem, the third is an introduction to chemical defences in macroalgae, and the fourth is broad comparison between *A. nodosum* and *S. muticum* which serves primarily to paint a picture of the considerable differences in their life-history.

### *1. Dioecy and Sexual Dimorphism in Photoautotrophs*

Dioecy and sexual dimorphism often co-occur in the literature on photoautotrophs, and with good reason. In any species with distinct genders there will be some degree of sexual dimorphism, even if this is only in the reproductive characters (Barrett & Hough 2012). Sexual dimorphism is not solely confined to dioecious species however (Ashman *et al* 2004, Delph 2005), and appreciating this fact is crucial to understanding the origin of dioecy. In a monoecious population, all individuals produce gametes transporting an X-chromosome (e.g. oogonia, ovules) and gametes transporting a Y chromosome (e.g. spermatozoa, antheridia) and they therefore reproduce as both males and females. However, the proportion of reproductive biomass assigned to each ‘reproductive function’ is not constrained to a 1:1 ratio, and neither is the fitness gained from reproducing as either role – the ‘functional gender’ of the individual. These ratios also differ between individuals, between populations, and through time (Delph & Wolf 2004). Crucially, the requirements of each reproductive function are always different, irrespective of species. For example, sperm (in whatever form) usually requires more nitrogen to produce, whereas the manufacture of seeds and fruits in internally-fertilised species (such as angiosperms) requires more water (Meagher 1984, Sánchez Vilas & Pannell 2010). Therefore environmental pressures of many kinds impact differently upon an individual’s capacity to reproduce as a male or female (Barrett & Hough 2012). This simple statement has consistently been the explanation for both sexual

dimorphism and the evolution of dioecy in plants (e.g. Pannell & Barrett 1998, Hesse & Pannell 2011a, b).

Dioecy has evolved many times in nearly half of plant families (Renner & Ricklefs 1995). It is well-established that dioecy evolves from monoecy, through transitional reproductive demographics collectively called sub-dioecy (Pannell & Barrett 1998, Delph & Wolf 2004). In sub-dioecious populations, some individuals reproduce as both genders, and some individuals reproduce as one gender only. In any one population, these individuals can be of either gender, all be male (androdioecy), or – as is the most common – all be female (gynodioecy). In these scenarios, sex-ratio can be highly variable, as can the functional gender of the remaining hermaphrodites. These transitional demographics can be stable if individuals are plastic in their reproductive function. For example, if males retain the physiological capacity to produce fruit and do so when conditions permit, complete dioecy need never evolve because the trade-off between reproductive function and survivability is reduced (e.g. Sánchez Vilas & Pannell 2014). If, however, mono-sexual (i.e. complete males or females) gain a consistent advantage, their frequency within the population will rise. In this scenario, the functional gender of remaining hermaphrodites becomes skewed towards the opposing function in order to keep sex-ratios functionally around 1:1, thereby maximising the probability of successful reproduction. When the all hermaphrodites have gained 100% of their fitness from reproducing solely as one gender, complete dioecy has evolved (Delph and Wolf 2004). The crucial point here is that some advantage must be conferred by reproducing solely as one gender, and this rare because – intrinsically speaking – considerable reproductive assurance is sacrificed by reproducing as one gender only. The probability of finding a mate in a dioecious population is half that in a monoecious population with functionally 1:1 sex-ratios, and this probability can often be lower because selfing is impossible (Heilbut *et al* 2001).

Sexual selection (attractiveness) in plants is believed to be far less important in the majority of cases, than by viability (survival) or reproductive (fecundity) selection (Kohorn 1995; Moore & Pannell 2011; but see Delph & Herlihy 2012). Selection for optimal gender-specific traits is common in purely dioecious species (Obeso 2002), although selection-pressures for these optima can be opposing. Traits which promote survivorship may be the same as those which afford high fecundity in one gender, but not in the other (Delph & Herlihy 2012). Therefore the manifestation of ‘male-like males’ or ‘female-like males’ for example, can depend on environmental conditions (Yu *et al* 2011). Dimorphism in leaf and flower morphology is common, as is dimorphism in growth-rate, competitive ability and defence

(e.g. Cepeda-Cornejo & Dirzo 2010, Midgley 2010, Sánchez Vilas *et al* 2010). More efficient resource allocation, the ability to specialise in micro-niches, and the reduction of the potential for in-breeding depressions have been postulated as possible causes for the evolution of dioecy (Eppley & Pannell 2007). The idea that top-down pressures from herbivores may force dioecy to evolve, rather than that it only evolves through some conferred advantage at the individual-level, has also been posited (Ashman 2002).

Once dioecy has evolved it confers advantages at the population-level, under certain conditions. Long-lived, mass-reproducing, densely aggregated species living in relatively permanent habitats are more likely to be dioecious (Renner & Ricklefs 1995) and this has been postulated to be because dioecy functions well when intraspecific competition for resources are more important than the availability or reliability of resources *per se* (Dorken & Barrett 2003, 2004). Possible reasons for this are that outcrossed individuals grow faster and are competitively superior on average to selfed progeny (e.g. Ashman 1992), and that niche-partitioning allows better use of an available habitat (Álvarez-Cansino *et al* 2010). This latter theory is well supported by empirical evidence and highlights the fact that dioecy and sexual dimorphism are intertwined in the evolution, ecology, and life-history of photoautotrophs. Sexual dimorphism causes dioecy, and dioecy permits further sexual dimorphism.

## 2. An Overview of Invasive Ecology

Biological dispersal is ubiquitous in all life (Hanski & Gilpin, 1997), but the ease with which different species colonise new ranges differs markedly, even between congeners (Lockwood & Somero 2011). Species which move or expand ranges readily, often becoming established or even dominant within a few generations, are termed ‘invasive’. Whilst this remains the strictest definition of the term, ‘invasive’ is often used synonymously with ‘biological pollutant’, this referring specifically to a species whose colonisation is deleterious to an area (Occhipinti-Ambrogi 2007).

Some plant groups such as invasive shrubs, occupy niches necessitating hardiness and therefore favour K-selection (Sutherland 2004). However, most invasives are *r*-selected, with rapid onset of sexual maturity and high fecundity (Bownes & McQuaid 2006, Troost 2010). This is conducive to rapid colonisation and evolution, at the expense of individual resilience and competitive ability (McMahon 2002). The Argentine ant, *Linepithema humile*, was first reported in New Zealand in 1990, and rapidly became prolific (Harris 2002). However, 40% of these populations collapsed after 14 years of initial establishment, probably due to limited



genetic diversity yielding low resistance to disease (Cooling *et al* 2011). Thus, whilst evidence suggests that rapid evolution is afforded to many invasives (Lee 2002, Cano *et al* 2009), genetic bottlenecks can still cripple an invasive population long after successful establishment and proliferation.

Limited individual resilience results in short life-spans and ephemeral life-history characteristics. Many invasive animals live for less than 10% of native counterparts (McMahon 2002) and invasive photoautotrophs are typically annuals, either partially or completely dying-off in winter months (McCourt 1984; Schlaepfer *et al* 2009). Though more vulnerable to disease and predation than K-selected equivalents (Bishop & Peterson 2006), selected invasives can find spatial refuge from both by expanding and altering their ranges, in accord with the Enemy Release Hypothesis (ERH) (Keane & Crawley 2002). By expanding into a previously unsettled range, an invasive presents a novel food-source to the local faunal assemblage which, even in the case of generalist foragers, may reject such items (Williamson & Fitter 1996, Keane & Crawley 2002, Cacabelos *et al* 2010a, Rossi *et al* 2010).

If, however, an abundant native consumer accepts an invasive as a food source before it can become established, a ‘biotic resistance’ to the invasion may inhibit colonization altogether. Grazer preference-trials in a variety of species including vertebrates and invertebrates from marine, terrestrial, and freshwater-aquatic environments show almost always that more non-native biomass is consumed (Parker & Hay 2005). This lends weight to the idea that the concepts behind the ERH work both ways, with the non-indigenous species being at a disadvantage through lack of defensive mechanisms against local consumers; the ‘Increased Susceptibility Hypothesis’ (Hokkanen & Pimentel 1989; Colautti *et al*, 2004). These two hypotheses are not mutually exclusive, and each may explain the fate of an invasion. Unfortunately, the ERH has received far more investigative effort to date, no doubt because failed invasions will be almost always unnoticed or ignored due to lack of significant ecological (and financial) consequences.

The invasive stratagem is not a guarantee of success (Bax *et al*, 2003). In most cases, the invasive will not succeed in colonizing a new area, and to maximise the options available for settlement without lengthy evolutionary adaptation – albeit accelerated – invasives must be physiologically flexible (Claridge & Franklin 2002, Piola and Johnston 2009, Troost 2010, Lockwood & Somero 2011). Invasives often show greater plasticity of morphology than native counterparts, allowing a better response to environmental changes, and a competitive advantage over the native species (Hoonkoop *et al* 2003). For example, the highly invasive alga *Codium fragile* fragments under levels of sheer-stress close to the limits of holdfast

retention. By allowing disposable vegetative tissue to be lost, surface area is lowered and so the force exerted by water movement is lessened (Bégin & Scheibling, 2003, D'Amours & Scheibling 2007). The lost tissue can also aid in dispersal, as the fragments have the capacity to regenerate complete clonal individuals, and this – combined with broad physiological tolerance – has afforded pronounced invasive success (Trowbridge 1996, 1998).

Above all other aspects of invasive ecology, propagule dispersal is the most important (Allendorf & Lundquist 2003). At the start of any invasion there will be a genetic bottleneck, and unless this is overcome the new population will falter after a few generations, as with the Argentine ant. This paradox of invasive ecology can be solved by additions to the new population, via propagules, from established ones. The number of individuals contributing propagules to the new population, internally and from extraneous sources, multiplied by the frequency of these events is termed 'propagule pressure' (Allendorf & Lundquist 2003). Rapid onset of sexual maturity – a fundamental staple of the *r*-selected stratagem – increases the frequency of dispersal events, whilst high fecundity increases the number of individuals surviving to maturity, and hence the number of contributors in the next event. As such the establishment phase of an invasion can be described logarithmically, akin to the exponential growth in bacterial colonisation (Monod 1949).

In Lough Hyne, Ireland, the invasive barnacle *Austrominius modestus* is replacing native species such as *Semibalanus balanoides*, the former taking around 8 weeks to reach sexual maturity, in contrast to the native which takes 1-2 years (Lawson *et al* 2004, White 2008). Furthermore, *S. balanoides* produces one brood per year of around 1500-3500 eggs, whereas *E. modestus* produces one brood every 2 weeks of around 180 eggs, totalling >4600 per year (Barnes & Barnes 1968). Whilst these totals are not particularly different, that *E. modestus* produces a steady supply year-round promotes maximum contribution to propagule pressure by each individual; even if a particular barnacle only lives for 6 months after settlement, it will have produced some 2000 eggs, compared to 0 by an equivalent *S. balanoides*.

*E. modestus* originates from Australia. *C. fragile* which is abundant in Europe, originated near Japan as did the oyster *Crossostrea gigas*, both of which are also common in North American waters (Trowbridge & Todd 2001, Lawson *et al* 2004, Troost 2010). None of these species could have colonised Europe or North America using natural vectors, such as ocean currents. In fact many of the most ecologically significant invasions to date are by species far beyond scope of possible individual movement (Bax *et al* 2003). Vectors for these transmissions can be classified as either deliberate or accidental, and both are of course,

anthropogenic. Deliberate introductions include those for ornamental or game-sport purposes, or more recently as scientific test-animals and desirable ecosystem engineers such as soil-stabilising shrubs. But by far the most common motive for deliberate introductions are for food-culture and domestication. These species are selected specifically for the same invasive characteristics discussed herein, which also make them ideal as hardy, fast-growing, rapidly-reproducing livestock, and ironically make them near-impossible to irradiate once they escape and become pests (IUCN 2011).

In the marine environment, accidental introductions are more commonplace and none more so than those associated with hull-fouling and ballast-water. Organisms have been ‘hitch-hiking’ since the first days of sea-travel, and some 150 species would be present on a typical Eighteenth century ship (Carlton 1999). Today an estimated 10,000 species are transported globally in ballast water (although most die) and it is of little surprise that a new species becomes established once every 32-85 weeks in large ports (Carlton 1999, Hewitt 2003). Harbours, and large estuaries which bear major transport hubs on their banks, are frequently the first rung on the ladder of invasion. Coastal, intertidal, and estuarine environments are abiotically variable and hence promote competitive advantages to ecologically and physiologically flexible organisms (Willmer *et al* 2005). This natural stochasticity is exacerbated by anthropogenic impacts, further promoting invasion by selecting against natives which have evolved locally (Bax *et al* 2002, Piola and Johnston 2009, West *et al* 2009). Brackish waters also have lower species diversity than fully marine or fresh waters, making invasion simpler by the presence of empty niches, and propagule pressure is maintained by regular shipping activity (Wolff 1999).

Once established, invasive species can have both positive and negative effects on local communities (Wallentinus & Nyberg 2007). Many modify the habitat by increasing heterogeneity and complexity, which in turn enhances biodiversity (Schmidt & Scheibling 2007, Bruschetti *et al* 2009). Most, however, negatively impact biodiversity and current scientific consensus paints invasive species as one of the fundamental problems of climate change (Molnar *et al* 2008), particularly in aquatic ecosystems (Sorte *et al* 2013). Mitigations for the prevention and control of invasions, are growing ever more prominent in the literature, as are the consequences of successful invasions (Funk 2015). It is clear that the invasive stratagem is a complex one, and its success depends on a variety of human, biotic, and abiotic interactions.

### 3. *The Role of Chemical Defence in Marine Macroalgal Ecology*

Though macroalgae differ from true plants physiologically, both share similar ecological traits with familiar pressures and limitations, including biotic pressure from herbivores (Overnell 1976, Stromgren 1977, 1986, De Ruyter Van Steveninck & Breeman 1987, Miller & Pearse 1991, Shurin *et al* 2002, Jormalainen & Honkanen 2007).

Actual grazing upon macroalgae is limited, with usually only around 10% of production directly ingested from living algae, though this value is highly variable (Pomeroy 1980, Mann 1982). The majority of this grazing pressure comes from the mesoherbivore assemblages which comprise of gastropods and small (<2.5cm) crustacea (Brawley 1992; Kraufvelin & Salovius 2004, Riera *et al* 2009), although in some areas fish and urchins are also important consumers (Van Alstyne and Paul 1990, Thomsen *et al.* 2006). Damage to algal fronds during grazing, particularly by voracious feeders, or those which graze close to the holdfast, such as limpets, can weaken an individual alga and cause loss of tissue post-attack (Davies *et al* 2007). As such, grazing by these species can cause loss of established canopies, or preclude reestablishment of a canopy after a disturbance event. Herbivores reduce the abundance of primary producers by an average of 68%, and macroalgae are the most vulnerable to grazing disturbance (Poore *et al* 2012). Whether lost through disturbance, grazing, or senescence, the remaining 90% of macroalgal production enters the detrital food-web. Here it is again food for many gastropods and scavenging crustacea, as well as annelids and terrestrial species should the material be washed ashore (Rodil *et al* 2008, Riera *et al* 2009).

When large canopy-forming macroalgae do develop stands, water is slowed and surface area is increased dramatically. Both the algal surfaces and interstitial water play host to a huge variety of organisms, although not all are herbivores, and of these not all graze directly on macroalgae (Viejo 1999, Kraufvelin & Salovius 2004, Cacabelos 2010a,b). Spatial and three-dimensional heterogeneity is increased, causing nutrient and sediment retention, with knock-on effects for recruitment, biodiversity, primary and secondary productivity, and coastal processes such as erosion and sediment flow (Steneck *et al* 2002).

The dynamic environments inhabited and created by macroalgae require a suite of specialisations to survive. Macroalgal adaptations do manifest as morphological traits, such as specific frond structures to survive high water-flow (Friedland & Denny 1995), or toughened thalli to resist grazing and physical damage from rocky substrata (Viejo & Arrontes 1992). But far more pronounced and diverse are the specialised biochemicals which

macroalgae produce *de novo*. Many of these chemicals serve multiple functions, such as DMSP (Dimethylsulfoniopropionate) and its cleavage products, which are found within algae, plants, invertebrates, and fish (Van Alstyne 2007). DMSP serves as both a primary and secondary metabolite in algae, and is functional as both an osmolyte (Kirst 1996), and a cryoprotectant (Karsten *et al* 1996), and its cleavage products have roles as antioxidants (Sunda *et al* 2002), antibiotics (Seiburth 1960) and it is believed, sulphur excretion (Stefels 2000).

Additionally, DMSP is the latent form of an activated herbivore defence (Van Alstyne 2007). Chemical defences can be grouped into 3 categories; constitutive, induced, and activated. Constitutive defences are always present within the tissues, and vary little with time. As such, the tissues are always defended, but to maintain such protection is expensive in terms of energy and nutrient delegation, especially if required nutrients are limited. As would be expected therefore, constitutive defences are reserved for the most important tissues (Van Alstyne *et al* 1999).

Induced defences are those not present in the tissues until herbivory, or the threat of herbivory, occurs. At this point, production of some secondary metabolic compound begins, so as to decrease the palatability of the tissues under attack. The precipitating cues differ between species, as do the responses, but in general;

- some level of inducible defence is present in most macroalgae, actual herbivory is more likely to elicit a response than damage from physical abrasion or frond tear; the differentiating factor most likely being herbivore mucus or some other substance released during grazing (Coleman *et al* 2007, Pavia & Toth 2008).
- waterborne cues from nearby grazing on conspecifics or even the mere presence of grazers has been shown to induce defence (Pavia & Toth 2008).
- The type of grazing threat results in different responses (Pavia & Toth 2008). Highly mobile grazers such as isopods, or voracious feeders such as large fish, will tend not to elicit induced responses, most likely because such herbivores will have ceased to feed before chemical production reaches biologically significant levels, whereas less mobile herbivores, such as gastropods, will still be present.

As such, induced defences are less expensive when herbivory is infrequent or idiosyncratic, but ineffective against fast consumers, or during the initial stages of attack (Sotka & Whalen 2007).

Activated chemical defences are those whose precursor-molecule is present constitutively, often serving other functions, and at the onset of herbivory is produced via alteration – through rearrangement, addition, or cleavage – of the constitutive molecule to become defensive (Jormalainen & Honkanen 2007). The latter is the case for DMSP, which is metabolised to DMS and acrylic acid by a dedicated enzyme, DMSP-ase, when an alga is under herbivore attack (Van Alstyne *et al* 2001). DMS and acrylic acid are toxic to both herbivores and algal cells, and by producing them quickly when needed, algae avoid the problems of constitutive defence and storage, but without the marked lag-time of induced defences (Van Alstyne 2007). Note, however, that unless the precursor molecule has additional functions which require its presence at levels biologically significant upon cleavage, and that the products of its metabolism are sufficient to deter herbivory, activated defences are not as effective as the more specific constitutive and induced defences.

All three defensive strategies have advantages and disadvantages, and many algae, particularly Phaeophyta, possess all three. The trade-off becomes evident when considering that species' ecological niche. Attempts to describe this compromise in terms of defence theories such as the Optimal Defence Model (ODM) and the Carbon Nutrient Balance Model (CNBM) have yielded mixed results, with the more phytocentric ecological perspectives of the ODM currently having greater evidential support, suggesting that an alga's defensive stratagem is determined more by top-down (i.e. grazer) pressure, and less by bottom-up (i.e. nutrient availability) constraints (Pavia *et al* 1999; Van Alstyne *et al* 1999).

Phlorotannins are polymers of benzene (polyphenolics), and are therefore composed predominantly of carbon and hydrogen, two typically non-limiting micronutrients. Phlorotannins are, however, often 10-100kDa in mass, and can be up to 650kDa (Boettcher & Targett 1993, Targett & Arnold 2001). This makes them comparatively cheap in terms of nutrient-delegation, but expensive in terms of energy expenditure, and therefore their production is compromising to other physiological processes (Pavia *et al*, 1999). Found only in the Phaeophyta amongst algae, phlorotannins, like DMSP, serve many functions to justify their cost. These include UV absorption, as well as free-radical foraging roles, making them effective sunscreens (Ragan & Glombitza 1986, Pavia *et al* 1997), and they have been shown to retard microbial activity of both bacteria and fungi (Seiburth & Conover 1965).

A role in wound-healing was suggested by a single investigation with light-microscopy (Lüder & Clayton 2004) and whilst widely-cited, the conclusions of the researchers were conjecture based on known chemical properties of phlorotannins and observation of phlorotannin accumulation about wound-sites. An actual wound-healing mechanism was never demonstrated, nor was its abundance experimentally manipulated, to confirm any suggestion made into a wound-healing function. Phlorotannins are known, however, to chelate heavy metals, thereby reducing their toxicity (Ragan *et al* 1979), though this is not the case for copper ions (Toth & Pavia 2000).

The most abundant evidence for phlorotannin function is for roles in herbivore defence by reducing palatability, lowering grazer fitness, and retarding digestive efficiency (Ragan & Glombitza 1986, Pavia & Toth 2000, Jormalainen *et al* 2005, Toth *et al* 2005, Wikström *et al* 2006, Pavia & Toth 2008, Shibata *et al* 2014). Whilst not all herbivores are deterred (Pavia *et al* 1997, Jormalainen *et al* 2005), phlorotannins are nevertheless highly important in brown algal ecology, accounting for 2-20% dry weight of a typical individual (Ragan & Glombitza 1986, Amsler & Fairhead 2006). This amount varies between species and conspecifics, but also within an individual, and over time. Phlorotannins are costly to produce (Pavia *et al* 1999) but not to store since they are non-toxic to algal tissues, stable (Gin-Nae *et al* 2007) and borne in specialised structures (physodes) (Ragan & Jensen 1978), making them ideal for both constitutive and inducible chemical defence. Phlorotannins vary with season and may be lower during reproductive stages, probably due to energy constraints (Ragan and Jensen 1978). Phlorotannins are often to be used as a proxy for biotic stress but are also a useful indicator of relative tissue fitness contribution (Pavia *et al* 2012). Investigations into the latter have revealed that the phaeophyta distribute constitutive defences in accordance with the ODM, with the more valuable tissues being the more heavily defended (Pavia & Toth 2008). Though variable between species, the most valuable tissues are usually structural, such as the primary thallus and holdfast. Apical tissue is of less importance, even if it is meristematic. Abundance also varies markedly at smaller temporal and spatial scales as production is induced through direct grazing, waterborne, and even airborne cues, although presence and degree of response is highly context and species specific (Pavia & Toth 2008, Pavia *et al* 2012).

Although much is still left to learn about chemical defences and phlorotannins in algae, they evidently play a crucial role in the ecology of the Phaeophyta, and provide a useful quantifiable proxy for grazing pressure, once the effects of UV-induction are properly considered.

#### 4. Physiological and Ecological Comparison of *Ascophyllum nodosum*, and *Sargassum muticum*

Some 10-40% of accidentally introduced marine species are macroalgae (Schaffelke *et al*, 2006) and one of the most prolific is *Sargassum muticum*, which was introduced to both North America and Europe by the shipment of *Crossastrea gigas* from Japan, for aquaculture during the 1940's (Plouguerne 2006). It has since spread across both the Pacific and Atlantic coasts of North America, the Mediterranean, and throughout the Atlantic coasts of Western Europe (Davison 2009). It forms dense canopies of branching thalli which play host to intertidal and subtidal fauna and epiphytes on sheltered shores (Cacabelos *et al* 2010a, b).

*Ascophyllum nodosum* also forms dense stands, and these too are important habitats in the sheltered intertidal zone. *Ascophyllum nodosum* however, is indigenous to Europe and North America (Chock & Mathieson 1976). Morphologically the two species are very different; both display apical branching and attain typical lengths of 1-3 metres, although *S. muticum* is feathery and delicate whereas *A. nodosum* is leathery with broader thalli and fronds (Cousens 1985, Davison 2009). Being fucales of the Phaeophyta, both produce phlorotannins to compensate for biotic and abiotic pressures (Toth & Pavia 2000, Plouguerne *et al* 2006).

##### 4.1 Habitat

*S. muticum* favours sheltered shores, where it forms belts on the low to mid-eulittoral, preferably 2-4m below chart datum, but as shallow as 1m above chart-datum and as deep as 6m below it depending on light and competition for space, similar to many other fucales (Fernández *et al* 1990, Thomsen 2006). *Sargassum muticum* will, however, grow on more exposed shores, albeit with lower performance (Fernández *et al*, 1990; Harries *et al*, 2007).

*Ascophyllum nodosum* grows only on sheltered shores, where it is limited to a band 2-3m above chart-datum (Åberg 1996). So specific is it, that *A. nodosum* is actually used as a bioindicator for low exposure (Connan *et al* 2006).

At the most sheltered sites, *S. muticum* growth becomes retarded by high epiphyte load (Baer & Stengel 2010), a problem experienced less frequently by the more specialised *A. nodosum* which undergoes continual sloughing of the epidermis (Filion-Myalebust & Norton 1981), although this adaptation is not always effective (Mathieson & Guo 1992). Both require rocky substrate for attachment, but *S. muticum* will grow on far less stable boulders than will



*A. nodosum*, and large individuals can be observed travelling with such stones in strong currents (Baardseth 1970, Haries *et al* 2007).

Table 1- Comparison of the defining characteristics of *Ascophyllum nodosum* and *Sargassum muticum* (DM = Dry Mass)

Trait	<i>Ascophyllum nodosum</i>	<i>Sargassum muticum</i>
Tidal Height tolerance	+2 m - +3 m <sub>1</sub>	+1 m - +6 m <sub>2,3</sub>
Typical daily growth	2 mm <sub>4</sub>	25 mm <sub>5,6</sub>
Life-history, max age	Perennial, 40+ years	Pseudo-annual, unclear
Typical length, growth-type	~1m (0.5 m - 3 m), dichotomous branching <sub>7</sub>	~1m (0.5 - 4 m), Branchings <sub>8</sub>
Reproduction, investment by mass	Dioecious, annual 2 week gamete release, mature at ~5 years, 50% DM <sub>1</sub>	Monoecious (self-fertile), monthly gamete release for 3-5 months, 50% DM <sub>9</sub>
Fertilization	External	Internal
Germlings, effective dispersal	Small zygotes, no rhizoids, 3-5 m <sub>10</sub>	Large zygotes, rhizoids, 3-5 m <sub>11</sub>
Dispersal	Germlings only <sub>10</sub>	Germlings, detached reproductive branches, adults dragged attached to small stones <sub>8,11</sub>
Physical tolerances	5-25°C, 15-30 PSU <sub>12,13</sub>	5-25°C, 15-30 PSU <sub>14</sub>
Phlorotannin abundance	12% DM (variable depending on tissue type) <sub>15</sub>	1.5-3% DM <sub>16</sub>

Upper 0.5 m  
of frond



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1 Åberg 1996 2 Fernández *et al* 1990 3 Thomsen 2006 4 Stromgren 1981 5 Arenas *et al* 1995  
6 Wernberg *et al* 2001 7 Cousens 1985 8 Davison 2009 9 Norton & Deysher 1989 10 Cervin  
& Åberg 1997 11 Dudgeon *et al* 2001 12 Stromgren 1981 13 Stromgren 1986 14 Norton  
1977 15 Ragan & Jensen 1978 16 White 2010

## 4.2 Growth

*Sargassum muticum* grows significantly faster than most fucales. The rhizoids for example, grow three times faster than those of *Fucus distichus* (Norton 1977). Vegetative growth is apical, and typically in the region of 20-30mm per day (Arenas *et al* 1995; Wernberg *et al* 2001), but in optimum conditions can average 40mm per day, although only during its growing-season between April and June (in temperate climates), decreasing to a more commonplace 4mm per day in July before growth terminates in August for the reproductive season. More fertile individuals grow slower overall, particularly close to reproduction (Norton 1977). As such, an individual measuring a few centimetres can attain a length of many metres within 2-3 months if conditions permit (Davison 2009).

*Ascophyllum nodosum*, on the other hand, grows far slower than a typical fucale. A germling might only grow 2mm in its first year, and only a further 15mm in its second. Adults are genetically differentiated into ‘fast’ and ‘slow’ growers, with the former typically growing 15cm per year, and the latter perhaps only 5cm (Stromgren 1981), equating to around 2mm per day for an ‘average’ individual. Like *S. muticum*, growth is apical (Toth & Pavia, 2000).

## 4.3 Life-Cycle

*Sargassum muticum* is a pseudo-annual, with a distinct spring growing period, followed by a phase of reproduction, and then an autumn die-off, similar to an annual plant. Over winter, however, the primary axis, or sometimes only the holdfast, remains (Critchley 1983, Engelen 2009, Baer & Stengel 2010). As such, *S. muticum* stands are ephemeral, and do not function as ‘ecosystem engineers’ (Schmidt & Scheibling 2007) for the whole year. At more exposed sites, *S. muticum* stands can vary markedly in density between years, sometimes not re-growing at all (Plouguerne *et al* 2006, *Pers. Obs.*). The autumn die-off provides a huge nutrient influx to the detrital pool, the effects of which have been likened to eutrophication events (Pederson *et al* 2005). The perennial holdfast is crucial to its invasive success of *S. muticum*, ensuring retention of primary space for the following year’s growth (Engelen & Santos 2009, White 2010).

*Ascophyllum nodosum* only loses the fruiting bodies (receptacles) after its summer reproductive season. The thalli are perennial and therefore the crucial habitat they provide remains throughout the winter (Sundene 1973). Input to the detrital pool also varies

throughout the year, but at orders of magnitude less, and far less abruptly than, that from *S. muticum* (Riera *et al* 2009).

#### 4.4 Morphology

Both species display a degree of morphological plasticity common to macroalgae, although the responses are somewhat different and the flexibility greater in *S. muticum*. *Sargassum muticum* grows shorter and bushier on more exposed shores, resulting in the ‘squat’ profile typical of intertidal organisms growing on such coasts (Trussel 1997), whilst the weight of sheltered and exposed forms remains similar (Andrew & Viejo 1998, Baer & Stengel 2010). In sheltered localities or when conspecific density is high, *S. muticum* grows longer, with less lateral-branching. These heteromorphs of *S. muticum* do not result from genetic differentiation, as has been shown to be the case for its congener, *Sargassum cymosum* (De Paula & De Oliveira 1982) but rather through the genuine phenotypic plasticity shown in other intertidal species (e.g. Etter 1988).

*Ascophyllum nodosum* grows faster on more exposed shores, and produces many reproductive secondary laterals, whereas growth is slower on sheltered shores and laterals are vegetative until the individual becomes competitive in the upper canopy, at which point laterals become reproductive. These different strategies are not responses to the wave-stress, but are instead the result of intraspecific competition for light (Cousens, 1985).

#### 4.5 Reproduction & dispersal

As is typical for the fucales, both species invest some 50% of their annual production into reproductive output (Norton & Deysher 1989, Åberg 1996). *Sargassum muticum* is monocious and self-fertile (Deysher & Norton 1982), with 10-25% of conceptacles being male, whereas *A. nodosum* is dioecious and is therefore not capable of self-fertilisation. A typical female *A. nodosum* will produce around 60 million eggs, compared to only 30 million by an average *S. muticum* individual, resulting in similar output *per-capita*; assuming a 50% female proportion in a population of *A. nodosum* (Norton & Deysher 1989, Åberg & Pavia 1997).

*Sargassum muticum* becomes mature in its 1<sup>st</sup> year, and reproduces July through September, though this can be May through September in warmer climes (Norton 1977, Plouguerne *et al* 2006). During the reproductive season, bursts of propagules are released

every 2 weeks, synchronised with the spring tides (Fletcher 1980, Norton 1981); thereby maintaining propagule pressure throughout this time. *Ascophyllum nodosum*, on the other hand, takes 5 years to become mature, and releases all of its propagules in a two week period in April, a significantly shorter reproductive season than most fucales (Åberg & Pavia 1997). Fertilisation is external, and extremely inefficient (Baardseth 1970).

The zygotes of *S. muticum* are unusual in that they remain adhered upon the conceptacle for 1-3 days after release, during which time they develop rhizoids and grow to around 150µm; far larger than the 10-100µm typical of fucales (Norton 1981, Dudgeon *et al* 2001). Essentially miniature adults, these germlings sink faster than most macroalgal propagules, but even in calm waters, turbulence and not gravity is the principle force behind sedimentation of particles so small (Norton 1992), and therefore *S. muticum* loses nothing by this adaptation, another K-selected trait which may account for its success. Germlings have been shown to drift for up to 1km, although recruitment is negligible after 3-5m; typical values for a fucale (Deysher & Norton, 1982; Dudgeon *et al*, 2001). The germlings lose the ability to settle after around one month, but have been shown to grow perfectly well without attachment, though whether such individuals survive in the field is unknown. Secondary thalli can detach at any time, bear vesicles for buoyancy and grow as if held-fast. If these possess receptacles, a single thallus can drift many thousands of kilometres, only to release self-fertilised germlings and commence colonisation on a distant shore, as is believed to have been the case in California (Deysher & Norton, 1982).

*Ascophyllum nodosum* propagules are released as zygotes only, and form rhizoids after settlement, as is usual for macroalgae. The ability to settle is lost after only 10 days (but see Cervin & Åberg 1997), and recruitment is negligible after 4m. Remarkably however, over 50% of propagules disperse over 6m (Dudgeon *et al* 2001). This suggests extreme post-settlement mortality, as would be expected for such slow-growing germlings, and it has been suggested that grazing by gastropods, dislodgement and damage by waves, as well as overgrowth by diatoms and biofilm are responsible (Baardseth, 1970; Lazo *et al* 1994, Åberg & Pavia, 1997; Cervin & Åberg, 1997). Thalli can detach, as with *S. muticum*, and have been shown to drift for thousands of kilometres due to buoyant vesicles (John 1974). Because self-fertilisation is impossible however, this is not a viable dispersive mechanism for *A. nodosum*.

#### 4.6 Ecology

Both species are euryhaline and eurythermal as would be expected from intertidal organisms, with ranges of 15-30 PSU and 5-25°C being applicable for growth (Norton 1977, Stromgren 1981, Stromgren 1986). *Sargassum muticum* is the more ‘warm-adapted’, and displays an exponential increase of growth with raising temperature and an optimum of 25°C (Norton 1977), compared to a linear response in *A. nodosum* and an optimum of 14-18°C (Stromgren 1981). *Sargassum muticum* is also tolerant of temperatures in excess of 25°C, levels which cause mortality in *A. nodosum*. Whilst *A. nodosum* performance is undoubtedly influenced by the physical regime (Stromgren 1986), *S. muticum*’s responses are the more pronounced. Overall biomass production is far lower in spatially and temporally heterogeneous environments such as coasts of Scotland (Harries 2007) and Ireland (Baer & Stengel 2010), and it cannot attain the marked dominance it does in more homogenous locales such as Limfjorden, Denmark (Thomsen 2006).

Grazing upon both species is limited. Both field observations and laboratory trials show a lower palatability for each than is typical for both fucales, and macroalgae in general (Pavia *et al* 1999; Thomsen 2006; Sarà *et al* 2007; Riera *et al* 2009; Cacabelos *et al* 2010a; Engelen *et al* 2011). Using stable isotopic analysis, Golléty *et al* (2010), indicated that no one organism specialises on *A. nodosum*, and its relative contribution to the diets of all grazers was lowest in winter. This is perhaps not surprising, since phlorotannin levels are at their highest in *A. nodosum* at this time (Ragan & Jensen 1978).

*Ascophyllum nodosum*’s investment into phlorotannin production is markedly higher than that of *S. muticum*, and many other fucales. Typical values reported for *Ascophyllum* are ~12% by dry weight (Ragan & Jensen 1978), compared to ~1.5% for *S. muticum*. These levels fluctuate in both species with season and within individuals, and each varies by up to 100% of typical cited values across the literature, with levels greater than 3% reported for *S. muticum* (Gorham 1984), and less than 1% reported for both (Targett 1992, White 2010). Limited evidence suggests that *S. muticum* produces more phlorotannins inside its native range, in accordance with the Enemy Release Hypothesis (White, 2010). *Sargassum muticum* is innocuous and scarce in its native range, rarely exceeding 45cm (Rueness 1989; Hirata *et al* 2003), but freed from grazer pressure, genotypes are selected for which prioritise growth and reproduction over defence, in accordance with an extension of the ERH; the Evolution of Increased Competitive Ability hypothesis (EICA, Blossey & Notzold 1995).

### *Concluding remarks*

Because phlorotannins are clearly so important to the ecology of *A. nodosum* but are also highly plastic in their abundance, they present an ideal fulcrum to investigate sexual dimorphism in this species. Likewise the posits of Biotic Resistance theory, the ERH, and the EICA all hinge around herbivory and an invasive photoautotroph's reaction to it, and therefore phlorotannin abundance can also be used to test the predictions of these theories. Approaching these avenues of research by quantifying defensive investment is the crux of this thesis, and has yielded a series of interesting findings. Both species of algae are very important in their respective communities, and each provides a habitat for a diverse community of organisms. Therefore the research in this thesis is of value to rocky-shore ecologists, but each investigation is taken from a general perspective and both species are utilised primarily as model organisms. More importantly then, the findings herein are of interest to all wider ecologists with an interest in chemical ecology, dioecious reproduction, sexual dimorphism, and invasive ecology.

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## Chapter Two

### Stress intensifies sexual dimorphism in a long-lived alga

#### *Abstract*

In plants and algae, dioecy is common in dense populations where intraspecific competition is important. This competition can be alleviated by sexual dimorphism in physiology, morphology, and ecology between the genders, allowing niche-separation. Sexual dimorphism arises because stress acts on each gender differently, owing to different costs of reproducing as a male or female. As such, stress increases sexual dimorphism in many plants. However, specifically-designed, holistic investigations into stress-effects on sexual dimorphism, with quantified stressors and responses, are lacking. Little research has focussed on sexual dimorphism in seaweeds, although they present a useful avenue to investigate sexual dimorphism with stress. The macroalga *Ascophyllum nodosum* is a modular organism, with somatic and sexual structures that are easily differentiated. It responds predictably to stress, and its defensive allocations are easily quantifiable. In this study, three populations under different levels of quantified and ecologically significant wave-stress and herbivory were monitored for one full year, to test the hypothesis that stress would increase sexual dimorphism, and impact on competitive ability. Defensive chemical assays were combined with field-observations and herbivore feeding-trials to test for sexual dimorphism in size, mortality, reproduction, defence, and susceptibility to herbivory throughout a full annual-cycle at all three sites. Greater stress resulted in more pronounced sexual dimorphism. Females invested more into reproduction when stress from wave-exposure and herbivory was high, but males did not. In the months post-reproduction, females subsequently allocated less to defence. Although reduced defence led to increased susceptibility to herbivory for females, size and mortality were not affected. These results confirm the assumptions of previous studies on sexual dimorphism, with respect to stress and herbivory, and the ecological impacts of dioecy in this species are discussed. This is the first study, of plants or algae, to quantitatively demonstrate that stress increases sexual dimorphism in defence, leading to gender-biased herbivory. This is also one of few studies to show sexual dimorphism in algae, and the first study to quantify sexual dimorphism over a whole annual cycle. These results advance our knowledge of the selection-pressures and circumstances that promote sexual dimorphism in plants and algae.

## *Introduction*

Dioecy is a common reproductive strategy in animals but less so in plants and algae. Only an estimated 25% of angiosperm species occur as separate males and females (Bawa 1980), and of these only around 6% are solely dioecious (Heilbuth, Ilves & Otto 2001). However, the strategy is widespread globally, occurring in over half of all plant families (Renner & Ricklefs 1995). Being dioecious reduces the chances of reproduction because there are fewer mates available, and self-fertilisation is impossible (Heilbuth, Ilves & Otto 2001; Hesse & Pannell 2011a). Therefore for dioecy to evolve, there must be considerable selective pressure (Delph 2005), and even then the strategy only proves advantageous under stringent conditions, such as where intraspecific competition is strong, and habitats are relatively permanent (Dorken & Barrett 2003). The selection pressures that promote dioecy are diverse, and the strategy has probably evolved independently many times (Renner & Ricklefs 1995). Dioecy is more common in dense populations of perennial, long-lived species, where reproductive assurance is high because of the close proximity to mates, provided that sex ratios are around 1:1 (Delph & Wolf 2004). Under these conditions, inbreeding depressions can occur in monocious populations, particularly if individuals are large and reproduce *en masse* (see Pannell & Barrett 1998; Dorken & Barrett 2003). Whilst self-fertilisation can have considerable advantages for populating new or disturbed habitats (Pannell & Barrett 1998), outcrossed individuals have been shown to be competitively superior to selfed progeny (Eppley & Pannell 2007; Pannell 2010).

Intraspecific competition in dioecious populations can be alleviated by sexual dimorphism in physiology, morphology, and ultimately ecology. Some degree of niche-separation between genders is very common, although not ubiquitous, in dioecious populations and permits a greater use of the available space in a habitat, known as 'microhabitat partitioning' (Eppley & Pannell 2007; Álvarez-Cansino *et al.* 2010; Hesse & Pannell 2011a). Sexual dimorphism can occur in sub-dioecious (hermaphrodites and males/females) and even monocious (all hermaphrodites) populations of plant, and individuals that prioritised one reproductive function (production either sperm or eggs) in a previously hermaphroditic species, is the most probable route for the evolution of dioecy (Pannell & Barrett 1998).

Dioecy evolves through sexual dimorphism because of the differential costs associated with reproducing as a male or female (Meagher 1984). Unsurprisingly, sexual dimorphism most commonly manifests in sexual and reproductive traits (Delph 2005), but sexual dimorphism

in size, growth-rate, photosynthetic ability, defence, and nutrient uptake have all been reported, and are quite common in dioecious plants (Delph & Herlihy 2012, and references therein). Some of these non-sexual traits do directly influence reproductive success however, such that 'somatic' and 'reproductive' investments cannot be clearly separated. For example, female trees typically invest more heavily into reproduction because they produce both fruits and flowers, which leaves fewer resources for growth, meaning male trees are usually larger than female trees (Obeso 2002). However, investment into growth positively benefits fecundity in males because they are able to support more flowers, and in wind-pollinated species growing taller improves reproductive success by ensuring pollen travels further (Hesse & Pannell 2011a).

According to the resource allocation hypothesis, an individual's resource expenditure can be grouped into growth, maintenance (e.g. defence) and reproduction (Coley, Bryant & Chapin 1985), and allocating resources into reproduction must come at the expense of growth and/or defence, and vice-versa. Therefore because it elicits responses in growth or defence, herbivory, like any pressure, can impact upon males and females differently, and has been postulated as a possible selective pressure for the evolution of dioecy in some plants (Ashman 2002; Cornelissen & Stiling 2005). It has been shown in numerous species of perennial shrub, palm, tree, and some herbs that males grow faster, and larger than females, which in turn have higher levels of defensive compounds and lower susceptibility to herbivory (Barrett & Hough 2012). It is clear that even when sexual dimorphism manifests in non-sexual traits, it is always linked with reproductive effort (Cornelissen & Stiling 2005).

There remain numerous questions about the origins and ecological impacts of dioecy in plants and algae, and an important avenue of investigation concerns the degree to which sexual dimorphism might be modified by biotic and abiotic stress (Boecklen & Hoffman 1993; Ashman 2002; Cornelissen & Stiling 2005). Since stress impacts differently on the genders, sexual dimorphism should increase along a stress gradient (Shine 1989), and a number of studies have confirmed this when comparing populations in stressful environments to those more benign (Barrett & Hough 2012). However, these studies have been terrestrial only, defence (when assessed) has frequently been inferred rather than directly quantified, and reported stress responses were sometimes ambiguous. This latter consideration is of importance because plants respond to many environmental variables (Murphy & Doust

2004), and researchers have often defined habitat characteristics based solely on one variable such as nutrient or water availability, making it unclear if other factors were important.

This study aimed to investigate sexual dimorphism in a common marine macroalga, *Ascophyllum nodosum*, in order to address the question of how stress impacts upon sexual dimorphism in seaweeds, and how this then impacts upon competitive ability. Very little data exists on sexual dimorphism in seaweeds (but see Vergés, Paul & Steinberg 2008), but research into their ecology has revealed analogies between them and higher plants (discussed by Cousens 1985). *Ascophyllum nodosum* is dioecious, perennial and long-lived (up to 30-40 years), and grows in dense, monospecific stands which result in intense competition for space and light (Åberg 1992), making its ecology concomitant with many dioecious plants (Dorken & Barrett 2003; Hesse & Pannell 2011b). Intraspecific competition means growing large is of paramount importance for individual success, and its dichotomous branching morphology also means larger individuals are proportionally more fecund (Cousens 1985; Åberg 1996). Because of this, and apparently in contrast to many terrestrial plant species, *A. nodosum*'s vegetative tissues are more important to its fitness than its sexual tissues (Pavia, Toth & Åberg 2002). These it produces in vast abundances, often equalling the mass of its vegetative tissues during the reproductive season (Åberg 1996). As such, sexual dimorphism is unlikely to manifest in differences in size, because if one gender became rare or under-fecund, the persistence of the whole population would be at risk (the 'Allee' effect, discussed by Hesse & Pannell 2011b).

Taking stress to be 'any external pressure which reduces growth and reproduction below the genotype's potential' (Osmond *et al.* 1987; Gehring & Monson 1994), three populations of *A. nodosum* which were exposed to different levels of wave-exposure (abiotic stress) and herbivory (biotic stress), were selected. Being a sheltered-shore species, *A. nodosum* is negatively impacted by strong wave-action (see Cousens 1985). Also the success of its germlings (fertilised zygotes) is highly dependent on them settling within the established stand, where even then grazing by gastropods can result in high post-settlement mortality (Åberg & Pavia 1997; Cervin & Åberg 1997; Dudgeon *et al.* 2001). Defensive investment positively correlates with levels of herbivory, and the production of defensive compounds has negative impacts on growth (Pavia, Toth & Åberg 2002; Toth, Karlsson & Pavia 2007). Herbivory also causes frond-breakage, and therefore has substantial negative impacts on

individual survivability and reproductive output (Cousens 1985; Åberg 1996; Pavia & Toth 2000; Davies, Johnson & Maggs 2007).

By monitoring investment into the three allocations of growth, defence, and reproduction for a full annual cycle in each of the three populations, four hypotheses were tested. 1) Females will invest more into reproduction than males as stress increases. 2) Sexual dimorphism will be greater as stress increases. 3) Sexual dimorphism will manifest in defence, but not growth or mortality. 4) Sexual dimorphism in defence will leave females more susceptible to herbivory. Testing these hypotheses advances our knowledge of the selection-pressures and circumstances that promote sexual dimorphism, and the possible ecological consequences of dioecy in this species are discussed with respect to competitive ability.

## *Materials and Methods*

### *Study organisms*

*Ascophyllum nodosum* is a large (1-3m long), common, mid-intertidal macroalga found on temperate sheltered rocky shore habitats (Olsen *et al.* 2010). It is a useful species to investigate stress-mediated effects because it has a narrow tolerance to wave exposure (Jenkins *et al.* 2008) and shows clear responses to herbivory (Toth, Karlsson & Pavia 2007). Therefore it is simple to locate well-established populations of *A. nodosum* close to one another, each experiencing near-identical temperature, light, and salinity regimens, but under different ecologically-significant levels of wave-stress and herbivory, which differ greatly at small spatial scales (<1 km) between intertidal habitats (Cousens 1985; Toth, Karlsson & Pavia 2007).

*Ascophyllum nodosum* grows with a dichotomous branching morphology, displays apical meristematic growth and produces a new vesicle (air-bladder) once per year at the tip of each vegetative lateral shoot in the years after maturity (~5 years) (Cousens 1985). Laterals are seldom broader than 10mm or thicker than 3mm and so frequently break, particularly under high degrees of wave exposure and herbivory, but still survive and continue to grow provided the holdfast remains attached (Cousens 1985; Toth, Karlsson & Pavia 2007). Receptacles (reproductive structures) are produced in pairs from pits on the vegetative shoots, and individual size is directly related to fecundity. Receptacle size positively correlates with individual size, but numbers of gamete-producing structures within the receptacles (conceptacles) does not differ between individuals (Åberg 1996; Åberg & Pavia 1997).

Timing of receptacle production differs depending on latitude but typically begins in mid-summer. Receptacles are shed after releasing the externally-fertilised gametes during a 2-week period at the end of the following spring (Cousens 1985; Pavia, Toth & Åberg 2002; Dudgeon & Petraitis 2005).

*Ascophyllum nodosum* produces phlorotannins as defensive compounds in response to herbivory and UV exposure (Pavia, Carr & Åberg 1999; Pavia, Toth & Åberg 1999). These compounds are inducible by direct herbivory from slow-moving gastropods, and by waterborne cues from nearby conspecifics. However, they are not induced by more motile crustacean herbivores, or by artificial clipping (simulated herbivory) (Pavia & Toth 2000). Phlorotannins are expensive to produce (Pavia & Toth 1999) and therefore are generated only as needed. Their abundance is independent of available nitrogen (Toth, Karlsson & Pavia 2007).

*Littorina obtusata* (Linnaeus 1758) is a common consumer of *A. nodosum* found throughout Western Europe (Watson & Norton 1987; Hayward & Ryland 2006) which occurs in high abundances on sheltered shores. This species shows a feeding preference towards algal tissues containing lower levels of phlorotannins, making it a good indicator for biologically relevant differences in defensive investment (Pavia & Toth 2000; Pavia, Toth & Åberg 2002). In this study, it is used as a model herbivore of *A. nodosum* in laboratory-based feeding trials, allowing an estimation of susceptibility to herbivory (Pavia & Toth 2000).

#### *Stress gradient and site selection*

The study was undertaken on the Isle of Anglesey, North Wales, an area central in the latitudinal distribution of *A. nodosum*. The region is temperate, with an average sea surface temperature of ~13°C, and a tidal-range of approximately 6m. Three sites known to contain established stands of *A. nodosum*, with three different levels of wave exposure stress, were selected by estimating potential wave energy from the distance of open water at 10° compass intervals emanating from the sites (Davies & Johnson 2006). Longer distances lead to the generation of greater wave height by winds and therefore have a significant impact on the species composition of a site (Lindgarth & Gamfeldt 2005). The three sites selected were, 1) Ynys Faelog near Menai Bridge (53.226793°N, 4.157054°W) (hereafter Menai), is a southern-facing rocky shore in the Menai Strait, and represents the low-stress study site. 2) Cemlyn Bay near Cemaes (53.414750°N, 4.511564°W) (hereafter Cemlyn) is a north-easterly

facing bay on the north coast of Anglesey, and represents the medium-stress study site. 3) Bull Bay, near Amlwch (53.422543°N, 4.368959°W) (hereafter Bull) is an easterly-facing inlet on Anglesey's northern shore, and represents the high-stress study site (Figure S1).

Each site was initially surveyed to validate the exposure model and establish the degree of biotic and abiotic stress. Firstly, intensity of herbivory was estimated from the mean abundance of gastropod herbivores known to consume *A. nodosum* (*Littorina littorea*, *Littorina fabalis*, *Littorina obtusata* and *Patella* sp.) on each shore, estimated from ten 0.25 m<sup>2</sup> quadrats thrown haphazardly within the *A. nodosum* population (Toth, Karlsson & Pavia 2007). Abundances of total herbivores observed were different by orders of magnitude between sites, and are unlikely to have changed significantly throughout the year (see Pavia & Toth 2007). *Littorina fabalis* was included in surveys because although it has been previously shown to consume algal films (Viejo & Åberg 2003), it does also consume macroalgae including *Sargassum muticum*, *Fucus serratus*, and *Ascophyllum nodosum* (Kurr, unpublished data). Secondly, the modelled exposure estimates were verified by placing gypsum (CaSO<sub>4</sub>) clod cards (Thompson & Glenn 1994) in the general area of the *A. nodosum* population on each of the identified shores for 12 tidal cycles in August, taking care to ensure that the clods were not touched by the surrounding algal stands (n = 14 at each site). Clod dissolution correlates with mean flow conditions, and is an established method that has proven to demonstrate differences in wave exposure levels between sites (Jonsson *et al.* 2006). Thirdly, the background sediment composition of each site was established by taking three replicate sediment samples from each site within the boundaries of the *A. nodosum* stand. Samples were washed, dried, and mechanically sieved into  $\phi$  components (a logarithmic scale of particle size) using sieves with mesh sizes at quarter  $\phi$  intervals (see Blott & Pye 2001). Sediments finer than 63  $\mu$ m were analysed using a Malvern Mastersizer laser particle-sizer. All sediment analysis was conducted using the Gradistat software (version 4, Blott & Pye 2001). More exposed sites will generally contain larger sediment particles (i.e. sand, gravel) compared to sheltered sites (i.e. mud, silt).

#### *Sampling and analysis protocols*

Individual *A. nodosum* were differentiated as per Åberg (1989). Genders were determined by the colour of the conceptacles (sites of gamete production with receptacles), males being orange and females being green (Dudgeon *et al.* 2001). This method was validated by light-microscopy and found to be applicable from December to April. Dry Masses were estimated

by measuring the length (l) and circumference (c) of each individual to the nearest half centimetre, and dry-mass (DM) was estimated using the formula  $DM = 0.057 (1c^2)^{0.7900}$  for spring (when receptacles are full), and  $DM = 0.034 (1c^2)^{0.8605}$  for autumn (when receptacles are small) (Åberg 1990).

Energy-content of algal tissues, both somatic (for stress response) and reproductive, was estimated by ascertaining living-biomass (Ash-Free Dry Mass per unit Dry Mass); the percentage of organic compounds in the tissues (Littler & Littler 1985, Lamare & Wing 2001). Samples were cleared of epiphytes, washed thoroughly with distilled water, freeze-dried to constant mass, homogenised, and a known quantity (~1000mg) was weighed to the nearest milligram. This was ashed-off at 450 °C for 3.5 hours, and reweighed (Holme & McIntyre 1984). Organic carbon is used extensively in algal metabolism (Neilson & Lewin 1974), including growth, defence and reproduction, making its abundance a useful measure of condition and tissue investment (Holme & McIntyre 1984; Littler & Littler 1985; Lamare & Wing 2001).

Phlorotannin abundance was estimated using a modified version of the Folin-Ciocalteu method (Van Alstyne 1995). Initially, *A. nodosum* tissues were washed thoroughly with distilled water and cleared of epiphytes. The samples were then freeze-dried to constant mass, ground until homogenous, and 0.2 g subsampled for phlorotannin extraction. 60 % aqueous acetone was used as a solvent, and phlorotannins were extracted under constant agitation in the dark for 1 hour. The algal pulp was separated from the supernatant by centrifugation (5300 rpm for 10 minutes) and the acetone removed using *in-vacuo* cold-distillation (80 kPa, 38 °C) until only the 40 % aqueous fraction remained (typically ~2 hours). This was then diluted, filtered so as to remove precipitated lipophilic compounds (Pavia & Toth 2000), and a 1 ml subsample was diluted in 11 ml of water. To this extract, 1 ml of Folin-Ciocalteu's phenol reagent (Sigma-Aldrich F9252) was added, followed immediately by 12 ml of 1M aqueous sodium carbonate decahydrate solution (Sigma-Aldrich 71360) to act as a buffer. The resultant solution was incubated in the dark for 30 minutes and analysed by spectrophotometry at 760 nm, using phloroglucinol (1,3,5-trihydroxybenzene, Sigma-Aldrich P3502) as a standard (Van Alstyne 1995). 12 samples were selected from each month to be analysed in triplicate, so as to ensure accuracy. These were never more than 0.1 standard deviations from their respective means. Defensive investment was expressed as the percentage of algal dry mass composed of phlorotannins. Relative defensive investment of



receptacles was estimated by expressing receptacle phlorotannin abundance as a fraction of vegetative phlorotannin abundance.

#### *Response of *Ascophyllum nodosum* to stress*

Measurements of size and living-biomass were made on the *A. nodosum* populations at each site, in order to assess the degree to which the alga was responding to the observed levels of wave exposure and herbivory. Algal DM was estimated *in situ*, and the vegetative meristems of 240 individuals sampled per site (120 male, 120 female,  $n = 720$ ) for living-biomass estimation. The sex-ratio between male and female *A. nodosum* at each site was determined by a random, non-destructive survey of 100 individuals at each site in April. A sex-ratio significantly different to 1:1 can be an indicator of greater mortality in one gender (Viejo *et al* 2011).

#### *Investment into growth and chemical defence*

To investigate sexual dimorphism in *A. nodosum*, a year-round survey was undertaken at each of the three sites. Because genders cannot be differentiated between May and November, 100 male and 100 female individuals were selected haphazardly across each site, and tagged in April to allow sampling to continue throughout the year. From December to November, 10 male and 10 female *A. nodosum* individuals were sampled in the first week of each month at each site ( $n = 720$ ). Dry masses were estimated *in situ*, and five to six undamaged meristematic apices were pulled manually from each individual. All manipulation was done by tearing the tissues by hand in order to simulate physical damage and therefore limit artificial phlorotannin induction (Pavia *et al.* 1997). Each month between December and April, receptacles (~100 per individual) were removed by hand from the same reproductive laterals from which apices were sampled, so as to avoid bias from potential intra-individual differences in phlorotannin abundance ( $n = 300$  individuals) (Cousens 1985). The phlorotannin abundance and living biomass of both somatic and reproductive tissues were then determined.

#### *Investment into reproduction*

*Ascophyllum nodosum* produces a large quantity of receptacles, and enumerating them for 720 individuals across the observed period was not possible. However, abundance and size of receptacles differs between individual *A. nodosum* (Åberg 1996), and could therefore differ between males and females. To test this, 15 male and 15 female *A. nodosum* were collected

from each of the three sites across a three day period in March, when receptacles were full ( $n = 90$ ). Dry-mass of the individuals was ascertained *in situ*, and samples were bagged whole, taking care not to damage the fronds or cause loss of receptacles, for return to the laboratory. The receptacles were then removed by hand, and counted. A subsample of exactly 100 receptacles (sampled at random to avoid selection bias for larger ones) from each individual was taken. These were then freeze-dried to constant mass and weighed to the nearest milligram, to allow for an estimation of the total dry-mass of receptacles produced by each individual. An estimation of the relative reproductive investment (RRI) for each individual was then calculated by the formula:

$$RRI = \left( \frac{ab}{c} \right) 100$$

Where  $a$  is the estimated dry-mass of all receptacles produced by the alga,  $b$  is the mean living-biomass in receptacles from that alga's respective site and gender (ascertained from the receptacles of individuals collected for the annual survey,  $n = 300$  individuals,  $\sim 100$  receptacles each), and  $c$  is that individual alga's total estimated DM.

This proportional index estimates the living biomass allocated to reproduction, per unit DM of alga, averaged over five months. It proves useful here because it does not correlate to individual size (Regression analysis  $n = 90$ ,  $R^2 < 0.001$   $p = 0.618$ ), unlike other estimations of reproductive effort (e.g. Åberg 1996).

#### *Role of stress and gender on herbivore preference*

To determine susceptibility to herbivory, feeding trials were undertaken across a five-month period between May and October (hereafter 'summer trials'), and a three-month period between January and March (hereafter 'winter trials'). Due to space limitations, trials were undertaken as a series of blocks, which were separated by between one and three weeks throughout the 'summer' and 'winter' periods. Eight blocks of trials were undertaken in summer ( $n = 48$ ), and eight blocks of trials were undertaken in winter ( $n = 48$ ). Different *A. nodosum* individuals were selected for each block giving a total of 96 individuals used (48 in summer and 48 in winter). Each block was of the same design, one male and one female *A. nodosum* was selected from each of the three sites on one day ( $n = 6$ ) and dry-mass was estimated. Samples were maintained in ambient flow-through seawater overnight before each trial began. From each individual, nine 500 mg blotted wet-mass ( $\pm 50$  mg) pieces of meristematic apical tissue were sampled, cleared of epiphytes and weighed to the nearest

milligram (Pavia, Toth & Åberg 1999; Pavia & Toth, 2000; Pavia, Toth & Åberg 2002). Each piece came from a different apical shoot to prevent certain pieces being more damaged than others. All manipulation was done by tearing the tissues by hand in order to simulate physical damage and therefore limit artificial phlorotannin induction (Pavia *et al.* 1997). Most shoots contained an annual airbladder, and this was punctured to make all pieces negatively buoyant, thereby avoiding encounter rate bias. Of the nine pieces, three were used as controls to correct for autogenic changes in mass, three were used in no-choice feeding trials, and three were used in choice feeding trials. An additional three to four apical shoots were also collected for phlorotannin analysis to confirm the expected sexual dimorphism in defence.

All *L. obtusata* were collected seven days prior to experimentation from each of the study sites, and starved to ensure an even level of hunger in all individuals. Each *L. obtusata* was used in one trial only with *A. nodosum* from its site of origin, and no *L. obtusata* collected died during starvation or experimentation ( $n = 1728$ ). All trials were undertaken for 10 days in outdoor aquaria, supplied with ambient flow-through seawater ( $\sim 16$  °C summer,  $\sim 9$  °C winter, 34 psu,  $\sim 30$  seconds flushing time). Each individual aquarium (270 ml volume) was positioned partially submerged in a water bath to limit fluctuations in temperature, which was itself under a roof to prevent rainwater contaminating the system and to provide shade. No individual aquaria were connected to any other to ensure independence, and to remove any potential for chemical signalling between treatments. Photosynthetically relevant levels of incident radiation (400-700 nm) under the roof were approximately 25% ambient (measured with an MDS-MkV/L Adventec photometer on a sunny day at noon, in July). For no-choice feeding trials, one piece of algae was presented to three *L. obtusata*. For choice feeding-trials, each piece was paired with one from the opposite gender from that respective shore, and presented to six *L. obtusata* collected from within that *A. nodosum* stand. Coloured string was tied around each piece of algae in the choice trials to discern male from female. At the end of the trial all pieces were then blotted dry and reweighed to the nearest milligram.

### *Statistical analysis*

Differences in clod dissolutions, grazer densities, and abundances of vegetative living-biomasses between sites were assessed with one-way analysis of variance (ANOVA), and means compared with Tukey's HSD procedures. Sex-ratios were tested for significant deviation from 1:1 using chi-square analyses. The effects of site, gender, and time on dry mass, phlorotannin concentration and living-biomass in *A. nodosum* tissues were assessed

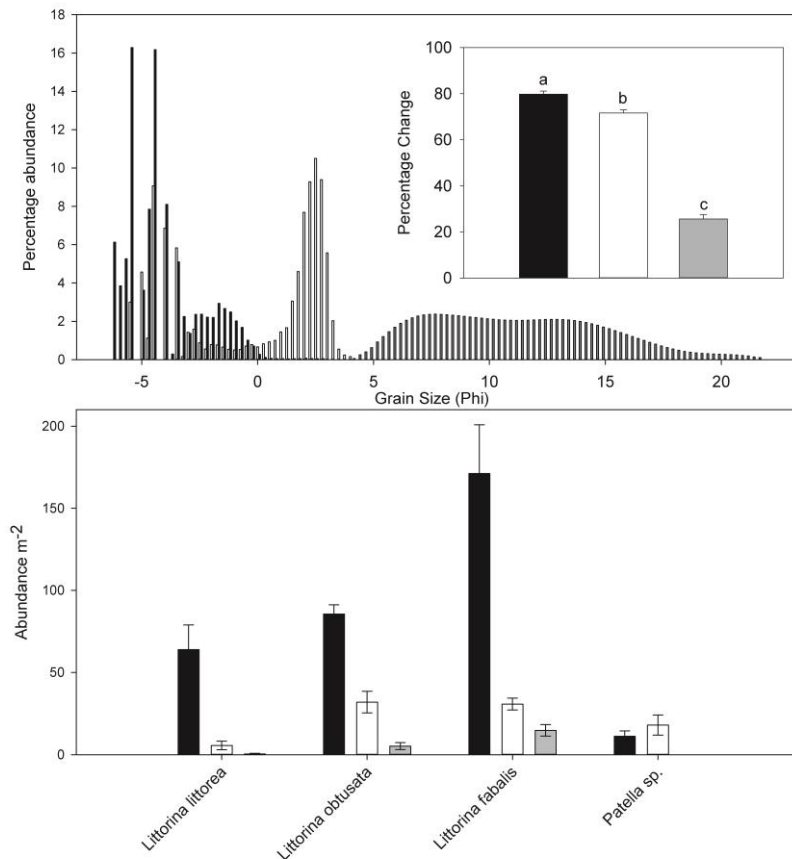
using 3-way ANOVA, with all factors as fixed and orthogonal. Means were compared using Tukey's HSD post-hoc procedures. Differences between feeding-rates by *L. obtusata* were assessed using one-way ANOVA. Due to the length of time over which feeding trials were undertaken (5 months for summer trials, 3 months for winter trials), phlorotannin abundances were markedly different between individuals used in the first and those used in the last blocks. To compensate, differences in phlorotannin abundance between tissues used in feeding trials were assessed using paired T-tests. Levene's tests were used to ensure all data conformed to the assumptions of homogeneity of variance, prior to analysis. In the event of non-conformity, data were either transformed or Welch's ANOVA was used and means compared with Game's Howell post-hoc tests (Domingues, Martinelli & Ehleringer 2006).

## Results

### *Stress gradient and site selection*

All biotic and abiotic metrics demonstrated the presence of a clear gradient of stress in the form of wave energy and herbivory from the selected sites (Menai < Cemlyn < Bull) (Fig. 1 and Fig. S1). At Menai, the average modelled fetch distance was 342 m on mainly south facing bearings. In contrast, both Cemlyn and Bull had open bearings mainly facing north to east, with an average fetch of 17 km and 48 km respectively (Fig. S1). These differences were also reflected with significantly more gypsum clod dissolution at Bull over Cemlyn, and Cemlyn over Menai (One-way ANOVA,  $F_{2,39} = 163.7$ ,  $SS = 10.928$ ,  $p < 0.001$ , Fig. 1a). Sediment composition was also clearly different at each site, with Menai classified as poorly sorted fine silt, Cemlyn mostly poorly sorted very coarse sand, and Bull as poorly sorted coarse gravel (Fig. 1a)

Herbivore density was significantly different between sites (ANOVA,  $F_{2,27} = 90.97$ ,  $p < 0.001$ ), with Menai hosting the fewest number of herbivores ( $20 \text{ m}^{-2}$ , S.E.  $\approx 4$ ), followed by Cemlyn ( $80 \text{ m}^{-2}$ , S.E.  $\approx 15$ ) and Bull ( $>300 \text{ m}^{-2}$ , S.E.  $\approx 26$ ) (Fig. 1b). *Littorina obtusata* abundance differed the most between sites although *Littorina fabalis* was the most abundant herbivore. *Littorina littorea* were found at Menai and Cemlyn, but were abundant at Bull only. *Patella vulgata* was absent from Menai, very rare at Bull, but slightly more common at Cemlyn, although its distribution was patchy and it was often absent from quadrats (Fig. 1b).



**Fig. 1.** a) Pooled grain-size distributions from 3 sediment grabs taken from each of the 3 study sites. Inset: gypsum clod mass-losses from 44 replicates deployed simultaneously for 12 tides in September 2014, b) Mean herbivore abundances from 10 quadrats placed haphazardly within *Ascophyllum nodosum* stands. Error bars show  $\pm 1$  S.E, groupings on a) inset are from Tukey's HSD analysis of a one-way ANOVA. On all plots, grey bars show data from Menai Bridge, white bars from Cemlyn Bay and black bars from Bull Bay.

#### *Response of Ascophyllum nodosum to stress*

*Ascophyllum nodosum* was in poorer condition at sites of higher stress. Both individual dry masses (Table 1) and living-biomass (One-way ANOVA,  $F_{2,717} = 117.99$ ,  $SS = 13873.5$ ,  $p < 0.001$ , Tukey's HSD, Bull < Cemlyn < Menai, Fig. S3) decreased along the stress-gradient. At Menai, large *A. nodosum* individuals, typically ~1.5 m (S.E. 0.075 m), but as much as 3 m in length were widespread, whereas those at Cemlyn grew to ~1.2 m (S.E. 0.034 m) on average, and at Bull, most individuals were ~0.7 m (S.E. 0.021 m) in length and seldom grew longer than 1m. Importantly, there was no significant difference in the sex ratio at each site (Menai:  $n = 100$ ,  $\chi^2 = 0.64$ ,  $p = 0.424$ , Cemlyn:  $n = 100$ ,  $\chi^2 = 1.44$ ,  $p = 0.230$ , Bull:  $n = 100$ ,  $\chi^2 = 2.56$ ,  $p = 0.110$ ) suggesting no sexual dimorphism in mortality.

*Investment into growth and defence*

Male and female *A. nodosum* had similar dry-masses at all three sites throughout the year, indicating that no sexual dimorphism existed in size (Table 1). Investment into receptacles during the spring meant individuals were larger in DM during this time of the year although a significant interaction term between month and site suggests asymmetry in the timing and/or magnitude of receptacle production in different populations. Menai individuals developed receptacles earlier than their more stressed conspecifics, particularly those from Bull. A significant interaction term for gender and site shows that although males and females were of similar sizes within populations, their relative sizes between populations did differ, with Menai males being as small as Cemlyn males, and Bull females being as large as Cemlyn females (Table 1).

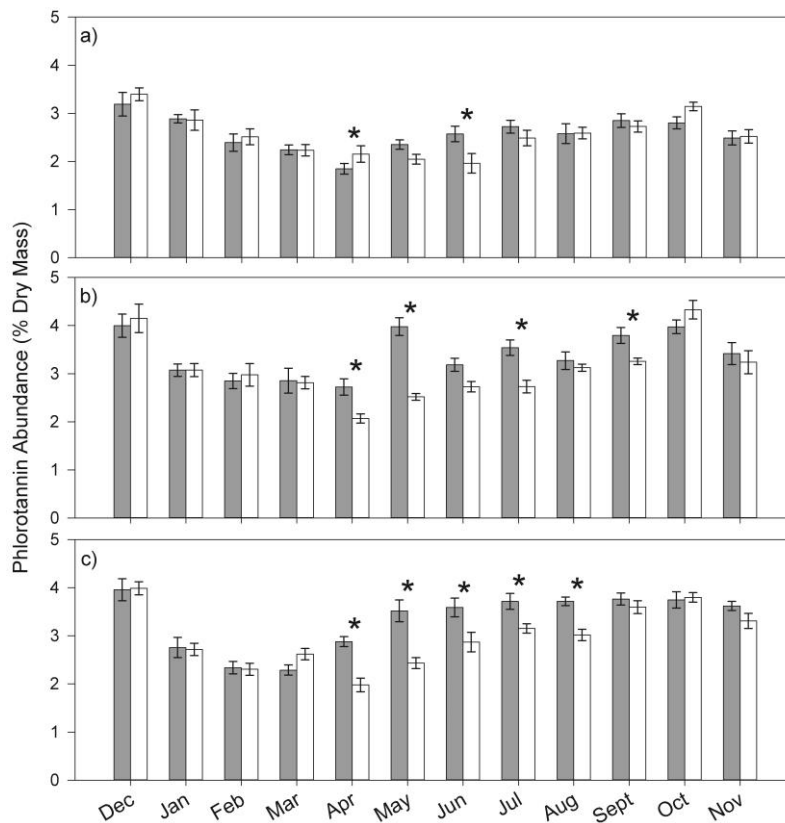
**Table 1.** 3-way ANOVA for dry-masses of *Ascophyllum nodosum* individuals collected from Menai Bridge (M), Cemlyn Bay (C), and Bull bay (B) across a 12 month period from December to November

	DF	MS	F	P
Site	2	601268	26.84	< <b>0.001</b>
Gender	1	9060	0.40	= 0.525
Month	11	308814	13.79	< <b>0.001</b>
Site x Gender	2	77626	3.47	= <b>0.032</b>
Site x Month	22	104335	4.66	< <b>0.001</b>
Gender x Month	11	7482	0.33	= 0.978
Site x Gender x Month	22	10.999	0.49	= 0.976
Residual	648	22402		

Tukey's HSD post-hoc analyses for significant effects;

**Site x Gender-** MF (A) MM (AB) CM (BC) CF (CD) BF (CD) BM (D)

**Site x Month-** Dec M (A) Jan M (A) Mar M (AB) Feb C (BC) Mar C (BCD) Jan C (BCD) Dec C (BCD) Apr M (BCD) Feb M (BCD) Apr C (BCD) Mar B (CD) Jan B (CD) Nov M (CD) Nov B (CD) Jun M (CD) Apr B (CD) Oct C (CD) Sep C (CD) Oct M (CD) Nov C (CD) Jul M (CD) Dec B (CD) Sep B (CD) Jun C (CD) Sep M (CD) Feb B (CD) Jul C (CD) May C (CD) May M (CD) Oct B (CD) May B (CD) Aug M (CD) Aug B (CD) Jul B (CD) Aug C (D) Jun B (D)



**Fig. 2.** Phlorotannin abundance (% dry mass) in the meristematic tissues of male (grey bars) and female (white bars) *Ascophyllum nodosum* collected from Menai Bridge (a), Cemlyn Bay (b), and Bull Bay (c) on the Isle of Anglesey across a 12-month period from December to November. Each bar represents 10 individuals ( $n = 720$ ), error bars show  $\pm 1$  S.E. \* indicates periods when differences between males and females were significant based on Tukey's HSD multiple comparisons tests ( $p < 0.05$ ).

Sexual dimorphism existed in phlorotannin abundance over the summer, with males being better defended than females. However, sexual dimorphism was largely absent from Menai, and was stronger at Bull than at Cemlyn (Fig. 2, Table 2a). No sexual dimorphism in phlorotannin abundance existed at any site over the winter (October-March) (Fig. 2, Table 2b). *Ascophyllum nodosum* from all three sites demonstrated a sinusoidal pattern of phlorotannin production, being lowest at the end of spring and highest in winter (Fig. 2), although the timing of production and reduction was different between each site (Fig. 2, Table 2). Phlorotannin production at Bull showed the greatest variation throughout the year, with production in January, February, and March being amongst the lowest seen at any site, and production around October and December being amongst the highest.

**Table 2.** 3-way ANOVA for Phlorotannin concentration (% of dry-mass) of meristematic tissues from *Ascophyllum nodosum* collected from Menai Bridge, Cemlyn Bay, and Bull bay across: a) the 6 month period from April to September (summer), b) the 6 month period from October to March (Winter)

a)				
	DF	MS	F	P
Site	2	21.2986	102.04	< <b>0.001</b>
Gender	1	23.2890	111.57	< <b>0.001</b>
Month	5	7.6598	36.70	< <b>0.001</b>
Site x Gender	2	2.7559	13.20	= <b>0.001</b>
Site x Month	10	0.4256	2.04	= <b>0.029</b>
Gender x Month	5	0.9562	4.58	< <b>0.001</b>
Site x Gender x Month	10	0.4976	2.38	= <b>0.010</b>
Residual	324	0.2087		

b)				
	DF	MS	F	P
Site	2	13.7099	46.92	< <b>0.001</b>
Gender	1	0.3447	1.18	= 0.278
Month	5	17.0954	58.51	< <b>0.001</b>
Site x Gender	2	0.0854	0.29	= 0.747
Site x Month	10	1.3646	4.67	< <b>0.001</b>
Gender x Month	5	0.2821	0.97	= 0.439
Site x Gender x Month	10	0.1013	0.35	= 0.967
Residual	324	4201.9206		

Tukey's HSD post-hoc for significant effects;

**Site x Month-** C Oct (A) C Dec (A) B Dec (AB) B Oct (ABC) B Nov (BCD) C Nov (CDE) M Dec (CDE) C Jan (DEF) M Oct (DEFG) C Feb (DEFGH) M Jan (DEFGH) C Mar (EFGH) B Jan (EFGHI) M Nov (FGHI) M Feb (GHI) B Mar (GHI) B Feb (HI) M Mar (I)

#### *Investment into reproduction and defence of receptacles*

Sexual dimorphism was present in the living-biomass of reproductive tissue. There was significantly less living biomass in male reproductive tissues ( $\bar{x} = 49.60\%$ , S.E. = 0.35) than in female reproductive tissues ( $\bar{x} = 54.86\%$ , S.E. = 0.56) in *A. nodosum* from all three sites. As with levels of vegetative living biomass the levels of reproductive living biomass correlated with site stress, Menai showing the greatest content and Bull showing the least (Table 3a).



**Table 3.** a) 3-way ANOVA for living biomass in the reproductive tissues of *Ascophyllum nodosum* from Menai Bridge, Cemlyn Bay, and Bull Bay, b) 2-way ANOVA for relative reproductive indices of *Ascophyllum nodosum* from Cemlyn and Bull Bays, c) 3-way ANOVA for investment into sexual defence (phlorotannin abundance in receptacles) normalised for investment into vegetative defence (phlorotannin abundance in meristems) for *Ascophyllum nodosum* from Menai Bridge, Cemlyn Bay, and Bull Bay

a)

	DF	MS	F	P
Site	2	623.21	29.35	< <b>0.001</b>
Gender	1	2070.88	97.54	< <b>0.001</b>
Month	4	338.49	15.94	< <b>0.001</b>
Site x Gender	2	94.28	4.44	= <b>0.013</b>
Site x Month	8	77.09	3.63	< <b>0.001</b>
Gender x Month	4	148.32	6.99	< <b>0.001</b>
Site x Gender x Month	8	18.88	0.89	= 0.526
Residual	299	11953.46		

Tukey's HSD post-hoc for significant effects;

**Site x Gender-** MF (A) CF (B) BF (BC) MM (BC) CM (C) BM (D)

**Site x Month-** M Dec (A) C Dec (A) M Jan (AB) C Jan (ABC) M Apr (ABC)

M Feb (ABCD) M Mar (BCDE) B Jan (BCDEF) B Dec (CDEF) C Feb (CDEF)

B Mar (CDEF) B Apr (DEF) B Feb (EF) C Apr (EF) Cem Mar (F)

**Gender x Month-** F Dec (A) F Jan (AB) F Feb (BC) F Apr (CD) M Dec (CD) F Mar (CD)

M Jan (D) M Apr (D) M Mar (D) M Feb (D)

b)

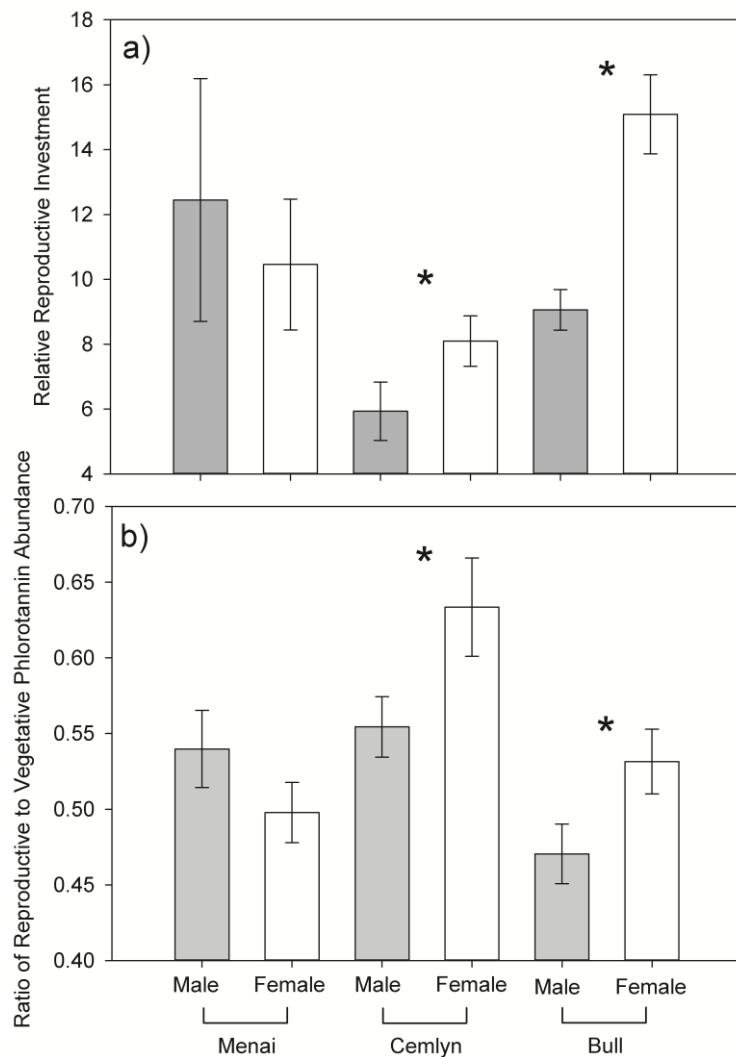
	DF	MS	F	P
Site	1	383.83	22.95	< <b>0.001</b>
Gender	1	251.31	13.03	< <b>0.001</b>
Site x Gender	1	55.91	0.22	= <b>0.038</b>
Residual	59	1380.69		

c)

	DF	MS	F	P
Site	2	0.24348	11.96	< <b>0.001</b>
Gender	1	0.08021	3.94	= <b>0.048</b>
Month	4	0.34899	17.15	< <b>0.001</b>
Site x Gender	1	0.10665	5.24	= <b>0.006</b>
Site x Month	4	0.07883	3.87	< <b>0.001</b>
Gender x Month	4	0.09598	4.72	= <b>0.001</b>
Site x Gender x Month	4	0.03508	1.72	= 0.093
Residual	270	0.02035		

Male and female *A. nodosum* from Menai displayed similar levels of relative reproductive investment (RRI). However, at both Cemlyn and Bull, female RRI was higher than that of

males, and the disparity in reproductive investment was significantly greater at Bull than Cemlyn (Fig. 3a and Table 3b)



**Fig. 3a)** Estimated relative reproductive investment (RRI) of male (grey bars) and female (white bars) *Ascophyllum nodosum* collected from 3 sites on the Isle of Anglesey in March; calculated as ash free dry mass of reproductive material per unit dry mass of total algal mass. Each bar represents 15 individuals ( $n = 90$ ). **b)** The ratio of phlorotannin in the reproductive tissues, to phlorotannin in the vegetative tissues, of of male (grey bars) and female (white bars) *Ascophyllum nodosum* collected from 3 sites on the Isle of Anglesey from December to April. Each bar represents 50 individuals ( $n = 300$ ). Error bars show  $\pm 1$  S.E. \* indicates where differences between males and females were significant, based on Tukey's Post Hoc ( $p < 0.05$ ).

There was no significant difference between raw phlorotannin concentrations in male and female reproductive tissues in *A. nodosum* from any site or at any time (3-way ANOVA; Gender  $p = 0.190$ , Site x Gender  $p = 0.195$ , Gender x Month  $p = 0.346$ , Site x Gender x Month  $p = 0.102$ ). However because of the significant decrease in vegetative phlorotannin levels in females from Cemlyn and Bull during April, when normalised for levels of vegetative defence, female reproductive tissues were relatively better-defended than male reproductive tissues in *A. nodosum* from these two sites (Table 3c, Figure 3b). This manifestation of sexual dimorphism did not differ in magnitude between Cemlyn and Bull.

#### *Role of stress and gender on herbivore preference*

*Littorina obtusata* showed differences in preference between males and females from Cemlyn and Bull, but only in the summer (Fig 4). Menai *L. obtusata* exhibited no preference for feeding upon either male or female tissue in summer (One-way ANOVA,  $F_{3,81} = 0.1$ ,  $SS = 7249$ ,  $p = 0.959$ , Fig. 4a) or winter (One-way ANOVA,  $F_{3,86} = 1.01$ ,  $SS = 29620$ ,  $p = 0.392$ , Fig. 4b) feeding trials, and there was no significant difference between summer (male  $\bar{x} = 2.65$  % DW, S.E. = 0.18; female  $\bar{x} = 2.58$  % DW, S.E. = 0.17; Paired T-test,  $T_7 = 0.59$ ,  $p = 0.574$ ) or winter (male  $\bar{x} = 2.75$  % DW, S.E. = 0.1; female  $\bar{x} = 2.62$  % DW, S.E. = 0.06, Paired T-test,  $T_7 = 0.82$ ,  $p = 0.438$ ) phlorotannin levels in *A. nodosum* used during these trials.

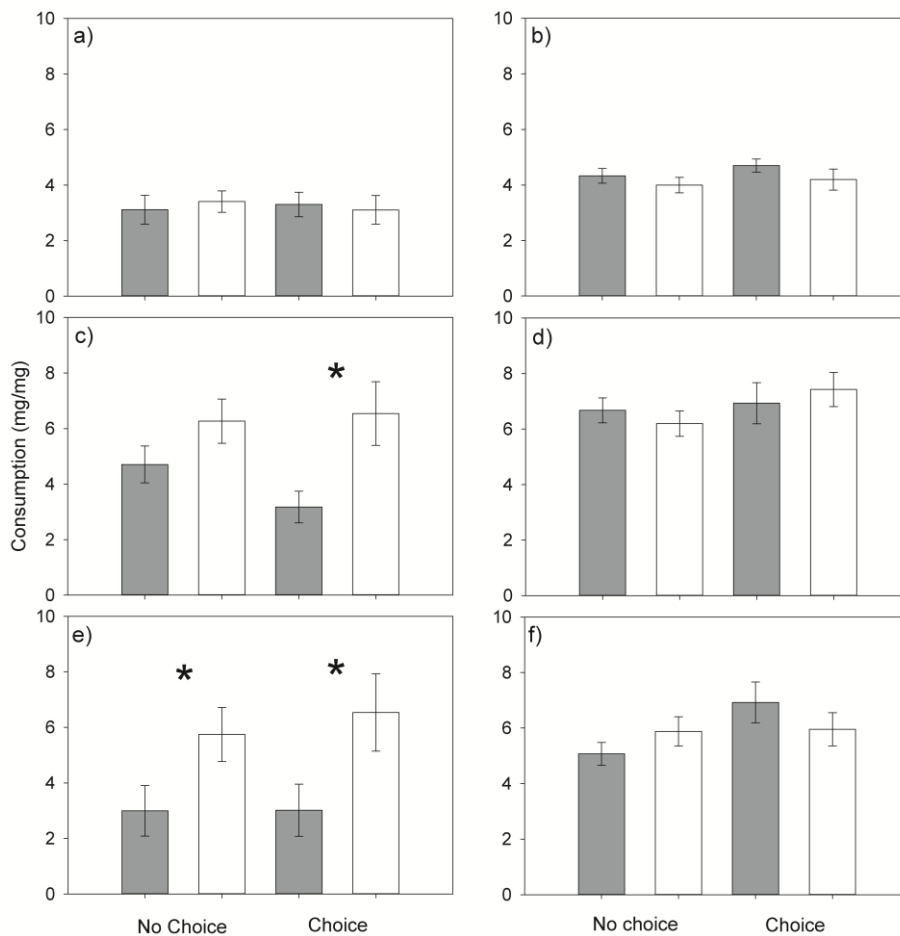
When presented with the option to feed on either male or female *A. nodosum* in summer choice trials, Cemlyn *L. obtusata* preferred female tissue (One-way ANOVA,  $F_{1,47} = 6.86$ ,  $SS = 114013$ ,  $p = 0.012$ , Fig. 4c). However, when presented with no such choice, male and female tissues were consumed to similar degrees (One-way ANOVA,  $F_{1,47} = 2.27$ ,  $SS = 24497$ ,  $p = 0.139$ , Fig. 4c). Phlorotannins were more abundant in male tissues used in these summer trials (male  $\bar{x} = 3.96$  % DW, S.E. = 0.13; female  $\bar{x} = 3.35$  % DW, S.E. = 0.27 Paired T-test,  $T_7 = 3.31$ ,  $p = 0.013$ ). Conversely, during the winter no preference for either gender was observed in either choice or no-choice trials (One-way ANOVA,  $F_{3,92} = 0.78$ ,  $SS = 15828$ ,  $p = 0.507$ , Fig. 4d), and phlorotannin levels between the two genders were similar (male  $\bar{x} = 3.93$  % DW, S.E. = 0.20; female  $\bar{x} = 4.26$  % DW, S.E. = 0.36, Paired T-test,  $T_7 = 0.78$ ,  $p = 0.460$ ) in the *A. nodosum* tissues presented to the grazers.

During summer feeding trials, Bull *L. obtusata* consumed female tissue preferentially to male tissue irrespective of treatment (One-way ANOVA,  $F_{3,92} = 2.93$ ,  $SS = 204300$ ,  $p = 0.038$ , Fig.

4e), and male tissues ( $\bar{x} = 4.00$  % DW phlorotannin, S.E = 0.23) used in these trials were more heavily defended than those female ( $\bar{x} = 3.41$  % DW phlorotannin, S.E = 0.24) (Paired T-test,  $T_7 = 2.40$ ,  $p = 0.048$ ). Consistent with both Menai and Cemlyn trials, no preference for either gender was observed during the winter (One-way ANOVA,  $F_{3,92} = 1.7$ ,  $SS = 34577$ ,  $p = 0.172$ , Fig. 4f), and the phlorotannin abundances within the tissues used of both sexes were similar during this time (male  $\bar{x} = 3.67$  % DW, S.E = 0.17; female  $\bar{x} = 3.80$  % DW, S.E = 0.12; Paired T-test,  $T_7 = 0.94$ ,  $p = 0.377$ ). Observed sexual dimorphism in phlorotannin abundance was similar between Cemlyn and Bull A. *nodosum* used in feeding trials (Table 4). There was no effect of block on the consumption of tissue in summer (One-way ANOVA,  $F_{7,137} = 1.94$ ,  $SS = 252347$ ,  $p = 0.068$ ) or winter (One-way ANOVA,  $F_{7,133} = 1.88$ ,  $SS = 235767$ ,  $p = 0.077$ ).

**Table 4.** 2-way ANOVA for phlorotannin concentration (% dry-mass) from *Ascophyllum nodosum* tissues used in Cemlyn and Bull Bay summer feeding trials

	DF	MS	F	P
Site	1	0.0192	0.05	= 0.832
Gender	1	2.8814	6.84	= <b>0.014</b>
Site x Gender	1	0.0003	0.00	= 0.978
Residual	31	14.6986		



**Fig. 4.** Consumption (change in algal mass corrected for autogenic changes in mass) per milligram of grazer of male (grey bars) and female (white bars) meristematic *Ascophyllum nodosum* tissues from (a) Menai Bridge in summer, (b) Menai Bridge in winter, (c) Cemlyn Bay in summer, (d) Cemlyn Bay in winter, (e) Bull Bay in summer, (f) Bull Bay in winter, by *Littorina obtusata* (3 grazers per 500 mg  $\pm$  50 mg) in 10-day feeding trials. Each bar represents 24 replicates from eight algal individuals ( $n = 192$ ), error bars show  $\pm 1$  S.E. \* indicates when differences in grazing rate were significant, based on One-way Analysis of Variance ( $p < 0.05$ )

### Discussion

This study demonstrates that the size and condition of *Ascophyllum nodosum* was clearly different between the three sites tested, and there was an obvious correlation between these responses and observed stress. This is in accordance with previous work on *A. nodosum* populations under different magnitudes of wave exposure (Cousens 1985) and herbivory

(Toth, Karlsson & Pavia 2007). Although these stressors did not cause differences in the size or mortality of males and females, sexual dimorphism in reproductive effort was significantly different between the three sites, being absent at the low-stress site, present at the intermediate-stress site, and very strong at the high-stress site. This disparity in reproductive effort was because of higher investment by females compared to males, in accordance with many plant species (Delph 1999; Obeso 2002). Due to this, females at the intermediate and high-stress sites were lesser defended than their male conspecifics in the months after reproduction, and this disparity was greater at the high-stress site than at the intermediate-stress site. The lower defences in female vegetative tissues from the intermediate and high-stress sites in the months post-gamete release, left them more susceptible to herbivory than males. Herbivores from the high-stress site, but not the intermediate-stress site, consumed lower abundances of male tissue even when they had no other food available, despite defences being similar between males from these two sites.

To our knowledge this is the first study of plants or algae, to quantitatively demonstrate that stress increases sexual dimorphism in defence, leading to gender-biased herbivory. These results are in accordance with a well-accepted prediction of the resource allocation hypothesis which predicts that expenditure in reproduction must be balanced by austerity in growth or defence (Coley, Bryant & Chapin 1985), and confirms the qualitative conclusions of previous research in the terrestrial environment assessing sexual dimorphism with stress (Boecklen & Hoffman 1993; Ashman 2002; Cornelissen & Stiling 2005; Barrett & Hough 2012).

Typically in plants, females invest more heavily into reproduction than males, though these results show this is not always the case in *A. nodosum*. Although living biomass was always higher in female reproductive tissues, suggesting a greater energetic cost associated with their production (Lamare & Wing 2001), males produced more receptacles than females at the low-stress site. As such, relative investment into reproduction was equal at the low-stress site, but females did invest more at the higher-stressed sites. Two previous findings combine to explain this pattern. Firstly, strong herbivore pressure and wave activity reduces germling survival rates, leading to increased importance of fecundity at the local scale (e.g. Lazo, Markham & Chapman 1994; Åberg & Pavia 1997; Dudgeon *et al.* 2001). Waves are known to dislodge germlings and although they can survive for up to 90 days in the dark and reattach (Cervin & Åberg 1997), they are far more likely to be removed from the stands entirely when wave-action is strong, presumably resulting in selection for individuals which prioritise

reproduction. Secondly, the reproductive structures of male and female *A. nodosum* are structurally similar, differing only in the nature of the gametes produced in the conceptacles, numbers of which do not differ between receptacles (Åberg and Pavia 1997). The ratio of sperm to eggs is not known for *A. nodosum* but in three closely-related species (*Fucus vesiculosus*, *F. spiralis*, and *F. serratus*) ratios are known to be between 40 and 400 to 1 (Vernet & Harper 1980). As seen in many plants therefore (Hesse & Pannell 2011b), spermatozoa are unlikely to be limited, and selection for higher reproductive investment acts more on females at stressful sites, resulting in sexual dimorphism.

Assuming equal resource availability, an increase in expenditure for one trait must be balanced by a reduction in expenditure elsewhere (Coley, Bryant & Chapin 1985). In plants this often manifests in disparities in size or morphology, but this is not always the case (Barrett & Hough 2012). As expected considering its ecology, both male and female *A. nodosum* were similar sizes at all three sites. Instead, the females at the higher-stressed sites sacrificed defence in their vegetative tissues to compensate for their increased expenditure on reproduction, although this only occurred after gamete release and was absent for the rest of the year. Annual patterns of resource allocation are known to display sexual dimorphism in many plants, owing to the differential costs of reproduction (e.g. Montesinos, García-Fayos & Verdú 2012; Cipollini *et al.* 2013).

Both genders reduced vegetative defensive investment during the reproductive period and increased production over the summer, in accordance with previous annual surveys of phlorotannin production (Ragan & Jensen 1978). This was probably to compensate for increased UV exposure (Pavia, Carr & Åberg 1999; Pavia, Toth & Åberg 1999), and possibly herbivory, which may be more intense owing to the higher temperatures during summer. Males were able to increase their phlorotannin production immediately, suggesting that resources were readily available in the tissues to do so. Females lagged behind males, gradually increasing their phlorotannin production over several months. Crucially the males at the highest-stressed site were consistently better defended over a full five months, whereas males from the intermediate-stressed site demonstrated more variable phlorotannin abundance, possibly due to variation in site-specific herbivore pressure inducing phlorotannin production, as opposed to UV exposure which would have been near identical between sites. Alternatively, increased shading because of the thicker canopy at the intermediately stressed site may have been responsible, but interindividual variability amongst males would have

likely increased within months, and no such effect was observed. Phlorotannin abundances take around two to four weeks to increase after induction (Pavia & Toth 2000), making the time between surveys (four weeks) relevant for detecting such variability. Interestingly at both the intermediate and high-stress site, females did not reduce phlorotannin production in their reproductive structures, resulting in them being relatively better defended than those of males. Pavia, Toth and Åberg (2002) report that *A. nodosum* concentrate defences in tissues with the greatest fitness value, and this further suggests that female receptacles are comparatively more important than those of males at the intermediate and high stress sites.

Unsurprisingly, the lower phlorotannin abundance in female vegetative tissues in the summer months resulted in greater preference for them by *Littorina obtusata*. Although phlorotannin abundances were similar between the two higher-stressed sites, *L. obtusata* from the high-stress site displayed a more consistent preference for female tissue than those from the intermediate-stress site, electing to feed less on male tissue even when offered no choice. This could only have been behavioural in nature, since these same herbivores were quite capable of consuming male tissue during winter trials when phlorotannin levels were greater than in summer. It is possible that these individuals expected lesser-defended material to be close-by and were conditioned to reject well-defended tissue. This conditioning would not be beneficial at the intermediate-stress site, where sexual dimorphism in defence was less consistent over the summer. Although limited in number, other investigations have provided evidence that sex-biased herbivory may become more pronounced under differing nutrient-regimens (Elmqvist & Gardfjell 1988) or in years of fluctuating precipitation (Boecklen, Price & Mopper 1990). Unfortunately, these studies were correlative only and no measurement of plant defensive-performance or internal state was taken. Nor is it apparent if the sexual-dimorphism observed changed in magnitude throughout the annual cycle of these species (Cornelissen & Stiling 2005).

Despite the increased susceptibility to herbivory, the ecological impacts of sexual dimorphism in *A. nodosum* appear to be minimal. Size and mortality were unaffected between sites, suggesting that females remained equally competitive to males irrespective of stress, despite their reproductive handicap. There are three non-mutually exclusive theories that may explain this pattern. Firstly, females may have higher rates of nutrient uptake or better photosynthetic ability. Although this has been reported in higher-plants, differences in leaf, flower, or root morphology have been responsible (e.g. Gehring & Monson 1994; Delph



& Meagher 1995; Hesse & Pannell 2011a). Therefore, unless differences exist at the cellular-level, this ability seems unlikely in *A. nodosum*. Secondly, gender-specific microhabitat partitioning (Álvarez-Cansino *et al.* 2010; Hesse & Pannell 2011a) may exist in *A. nodosum*. Inter-specific and intra-specific microhabitat partitioning, other than that caused by intertidal zonation, has been shown in seaweeds previously, and enhances total cover by algal stands (Taylor & Hay 1984, Stachowicz *et al.* 2008). It is quite possible that microhabitat partitioning by females allows them to compensate for their defensive handicap in the field, since densities of gastropod herbivores can vary at very small (<1m) spatial scales (Forrest, Chapman & Underwood 2001). Thirdly, sexual dimorphism could simply be too localised within the tissues to significantly impact upon *A. nodosum*'s ecology. This study examined phlorotannin abundance in apical tissue only, but in the field, herbivorous gastropods are known to concentrate feeding on the holdfasts, which provide a better site for attachment of the foot (Watson & Norton 1987). Since intra-individual variation in phlorotannin abundance exists (Pavia, Toth & Åberg 2002), it is possible that sexual dimorphism in defence does not manifest in the lower regions of the fronds. However, since phlorotannins are expensive to generate (Pavia & Toth 1999) males would not have produced more in the apical tissues unless there was some advantage in doing so. Microhabitat partitioning is therefore the most probable mechanism explaining the competitive similarities between genders in *A. nodosum*.

*Ascophyllum nodosum* prefers sheltered habitats, although it is abundant and successful at both higher-stressed sites studied. Whilst this investigation did not replicate site stressors, the populations selected represent typical habitats for this species, and similar demographics have been reported between sites of similar wave exposure (Cousens 1985). Therefore it is likely that some degree of sexual dimorphism manifests in many populations of *A. nodosum*. However, the design of the present study did not permit distinction between the stressors of wave exposure and herbivory, and so determining which is the more important was not possible from these results. Growing in dense, gregarious stands is vital to the success of this species, and positively benefits survival of germlings (Dudgeon & Petraitis 2005). Being dioecious should facilitate this strategy (Pannell & Barrett 1998; Dorken & Barrett 2003; Eppley & Pannell 2007; Pannell 2010), but without further investigation into the photosynthetic ability, capacity for nutrient uptake, intra-individual variability in phlorotannin concentration, and most importantly microhabitat partitioning between genders, the wider ecological impacts of sexual dimorphism are unclear for *A. nodosum*. What is evident from these findings is that abiotic stress drove females to invest more heavily into

reproduction than males, resulting in trade-offs in defensive investment but not in growth. This study therefore demonstrates a clear and positive relationship between stress and sexual dimorphism in this species.

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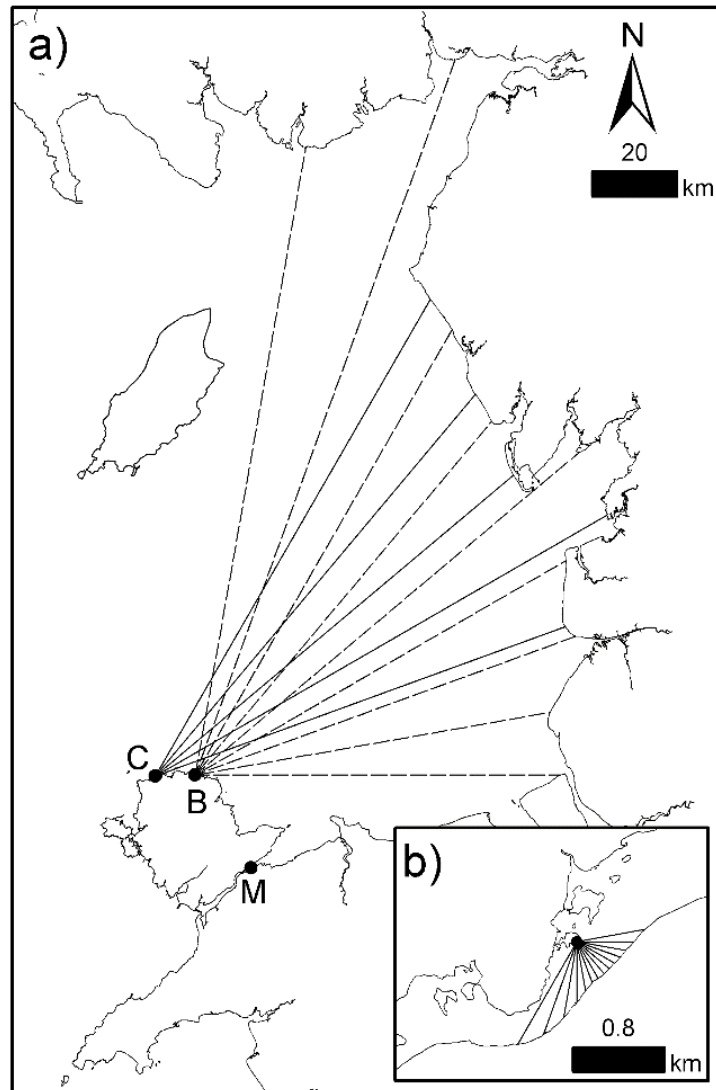
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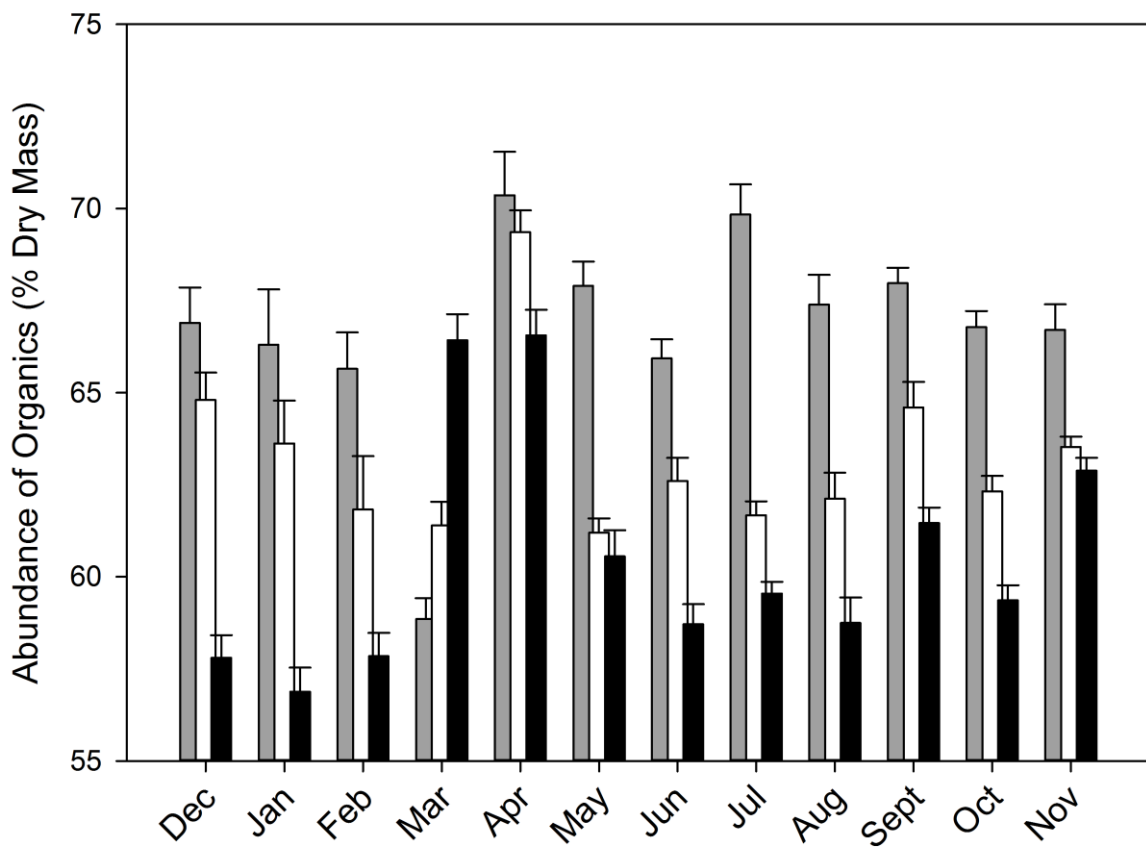
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Appendix



**Fig. 1.** Modelled wave fetch distances, a) Menai Bridge (M), Cemlyn Bay (C) and Bull Bay (B). Cemlyn Bay is represented as solid lines and Bull Bay as dashed lines (fetch from Menai Bridge is not visible). b) Inset around the Menai site, and shows the limited fetch as solid lines.



**Fig. 2.** Living biomass (abundance of organic compounds, expressed as a percentage of dry mass), in the meristematic tissues of *Ascophyllum nodosum* collected from Menai Bridge (grey bars), Cemlyn Bay (white bars), and Bull Bay (black bars) on the isle of Anglesey across a 12 month period from December to November. Each bar represents 20 individuals ( $n = 720$ ), error bars show  $\pm 1$  S.E.

## Chapter Three

### Certain stressors amplify sexual dimorphism in the marine alga *Ascophyllum nodosum* (L.)

Le Jolis

#### *Abstract*

Sex differences in a number of physiological variables have been reported in algae, although in contrast to terrestrial plants, little is known about sexual dimorphism and its causes in seaweeds. The alga *Ascophyllum nodosum* exhibits sexual dimorphism at sites where wave-action and herbivory are stronger, because females increase their investment into reproduction at the expense of chemical defence. Females may do this at stressed sites because of high germling and juvenile mortality, or because their smaller individual sizes have negative impacts for fecundity. This study aims to determine which is the case by comparing *A. nodosum* in an area where stressors (wave-action and herbivory) impacted upon both adult performance and juvenile mortality, to one where only adult performance was impacted (by ice-damage). Seven populations of the algae are compared at both the regional (> 1000 km) and local scales (< 50 km), to assess the presence of sexual dimorphism in algal size, sex-ratio, and the chemical defences and tissue condition of both vegetative and reproductive tissues. Taking a multi-scale approach is a useful way to determine which abiotic variables are driving biological patterns, because variability in the latter mirrors variability in the former. Sexual dimorphism was more common at both the regional and local scale when herbivory and wave-exposure were high. Other factors caused differences in physiology at both scales, but did not drive sexual dimorphism. Furthermore, sexual dimorphism was consistent in the defence of reproductive tissues at all sites, suggesting it was not driven by stress at all. Therefore, sexual dimorphism in *A. nodosum* is caused by some stressors, not caused by others, and is present in some fashion regardless of stress. This is the first study to directly quantify sexual dimorphism at different spatial scales, in populations of either plants or algae. As such it reveals novel insights into the driving forces behind it in *A. nodosum*.

#### *Introduction*

Limited data exists on sexual dimorphism in seaweeds, but sex differences in mortality (Engel *et al* 2001), production of metabolites (Payo *et al* 2011), threshold size for reproduction (Viejo *et al* 2011), and susceptibility to herbivory (Verges *et al* 2008; Chapter 1) have been reported. In accordance with many terrestrial species, the females of some

seaweed species put more energy into reproduction (Vernet & Harper 1980; Chapter 1), although sexual dimorphism in reproductive effort is not always present (Viejo *et al* 2011; Chapter 1). Sexual dimorphism arises in plants and algae because stressors act on males and females differently, owing to gender-specific costs associated with reproduction (Delph & Herlihy 2012). Stress therefore increases sexual dimorphism in many plants (Barrett & Hough 2012), although only one investigation has so far examined the manifestation of sexual dimorphism with stress in a marine alga (Chapter 1).

In accordance with results on many higher-plant species, sexual dimorphism positively correlates with stress in *Ascophyllum nodosum*, with females investing more into reproduction and therefore being lesser-defended than males at sites of high wave-action and herbivory (Chapter 1). Because eggs are limited compared to sperm in fucoids (Vernet & Harper 1980), and female reproductive tissue is both more valuable and often more expensive to produce (Chapter 1), there are two non-mutually exclusive hypotheses that explain the pattern of sexual dimorphism in this species. Firstly, wave-stress and herbivory reduce the survivability of young algae (Lazo *et al* 1994; Dudgeon *et al.* 2001; Åberg & Pavia 1997). Germling mortality in areas impacted by even moderate wave-action can be 85-99 % (Vadas *et al* 1990), juveniles can experience 50-85 % mortality in wave-exposed populations (Cousens 1985), and 99.99 % of recruits can be consumed in their first year by herbivores (Lazo *et al* 1994). Therefore females may be selected for which prioritise reproduction, since increasing fecundity at the local scale requires more eggs but not more sperm, since sperm outnumber eggs by orders of magnitude in fucoid seaweeds (Vernet & Harper 1980). Expenditure in reproduction comes at the cost of defence, and so sexual dimorphism manifests at these highly stressed sites (Chapter 1). Secondly, wave action and herbivory cause substantial frond damage, often resulting in the loss of the upper half of the frond (Cousens 1985, Toth *et al* 2007). As fecundity correlates with individual size in *A. nodosum* (Åberg 1996), the loss of receptacles (fruiting-bodies) and the lack of space upon which to bear them may be more problematic to females, which produce more expensive and better-defended fruiting-bodies (Chapter 1). Each reproductive structure lost is consequently more detrimental to female fitness (Pavia *et al.* 2002, Moore & Pannell 2011, Barrett & Hough 2012). Therefore, sexual dimorphism may be the result of recruit mortality, detriments to adult performance, or both, in *A. nodosum*.

Determining the cause of sexual dimorphism in *A. nodosum* therefore requires an assessment of populations under stressors that impact adult performance, whilst causing minimal juvenile and germling mortality. This investigation aims to compare the degree of sexual dimorphism in *A. nodosum* from a region predominantly stressed by wave-action and herbivory (Anglesey, North Wales), to one predominantly stressed by ice-damage (Tjärnö, Western Sweden) at two different spatial scales, the regional (> 1000km) and local (< 50km). Taking a multi-scale approach is a useful way to determine which abiotic variables are driving biological patterns, because variability in the latter will mirror variability in the former (discussed by Levin 1992). For example, if a physical parameter varies between regions, but not within regions, biological factors driven by this parameter will only be detectable at the regional scale. Likewise, if a physical parameter varies between local sites in one region but not others, biological factors driven by it will only vary within that region.

Ice-damage presents a very different stress to wave-action or herbivory, being more stochastic and absent entirely for much of the time. The degree of wave-action upon a site does of course change through time, and storms can dislodge considerable amounts of standing biomass (Ugarte 2011). Likewise, grazer-stress on algae can increase with temperature over summer (Cubit 1984). Both, however, are consistent pressures across the year when compared with ice-damage, which is entirely confined to winter in sub-arctic/northern-temperate regions, and in many localities such as western Sweden, is absent for years at a time (Åberg 1992a,b). The germlings of *A. nodosum* settle over the summer and grow into juveniles by their first winter (Dudgeon & Petraitis 2005). Germlings are therefore highly unlikely to be directly impacted by ice-damage, even if the sea freezes in their first year. In contrast, juvenile (established recruits) mortality has been estimated to be 60-80 % during ice-years in *A. nodosum* populations near Tjärnö, depending on severity (Åberg 1992b). However, in years without ice this figure drops to 10 % (Åberg 1992b), and because the sea remains ice-free in 75 % of years, juvenile mortality has been estimated to be only 24 % in this region (Åberg 1992a). Because of comparatively low-levels of wave-action and herbivory at Tjärnö therefore, germling and juvenile mortality of *A. nodosum* is likely to be far less than that on Anglesey.

Ice-damage still impacts upon adult performance, however. Non-fatal cropping due to ice-damage usually affects the upper 50 % of a frond (Mathieson *et al* 1982), and is therefore similar to the impacts of wave-action (Cousens 1985) and herbivory (Toth *et al* 2007) which

also result in *A. nodosum* fronds being around 50 % smaller. Because of *A. nodosum*'s slow growth, the impacts of disturbance events can persist for many years (Jenkins et al. 2004), meaning that even in Tjärnö where the sea freezes only one in every four years, *A. nodosum* individuals remain smaller because of the recurrent disturbance (Åberg, 1992b). As fecundity correlates with individual size in *A. nodosum* (Åberg 1996), individuals experiencing greater ice-damage must invest more into reproduction to compensate for their smaller sizes (Åberg 1992a). Complete losses of stands in Western Sweden, with its small tidal-range and sheltered geography, are far less common than in areas where tides and waves combine to buckle and drag ice floes across the benthos (McCook & Chapman 1997). Consequently in this area, adult performance is impacted to a similar degree by ice-damage than by wave-action and herbivory elsewhere. This leads to the hypothesis that if germling and juvenile mortality drives sexual dimorphism, it will be more common on Anglesey. However, if detriments to adult performance are the cause, sexual dimorphism will be equally common in both regions. To test this, four populations near Tjärnö and three on Anglesey were sampled for algal size, sex-ratio, and the living biomass content and phlorotannin abundance of both vegetative and reproductive tissues in mid-summer, late-summer, autumn, and the following spring. Sampling populations of *A. nodosum* separated at different spatial scales reveals much about the environmental variables driving disparities between them (*sensu* Åberg & Pavia 1997) and assessing sexual dimorphism in this regard is a novel approach in research both in algae and higher plants.

## *Materials and Methods*

### *Study Organism*

*Ascophyllum nodosum* is an abundant and often dominant furoid, which grows on the mid-intertidal of sheltered rocky shores in temperate latitudes (Olsen et al 2010). It displays apical, dichotomous, branching growth, reaching sizes of up to 3m in some habitats, but is more typically around 1m in length. Every year after it matures (at around 2-5 years of age), it produces a buoyant vesicle at the tip of its apical meristems (Cousens 1985). Receptacles (fruiting bodies) are produced in pairs along the length of its fronds between mid-summer and the following spring, when externally-fertilised gametes are released over a two-week period towards the end of April (Cousens 1985; Pavia *et al* 2002; Dudgeon & Petraitis 2005). The dry mass of the whole alga can double once the receptacles are ripe (Åberg 1996; Pavia *et al* 2002). Fecundity positively correlates with individual size (Åberg 1996), and because of the dense nature of established populations, intraspecific competition for light is strong (Cousens

1985). Although fronds can break due to wave-action, herbivory, and ice-damage, they continue to grow provided that the holdfast remains attached to the substrate (Toth & Pavia 2006).

Like other brown algae, *A. nodosum* produces phlorotannins to retard herbivory and limit damage by UV radiation (Pavia *et al* 1997). Phlorotannins can be induced by light, gastropod herbivory, and by waterborne cues from nearby *A. nodosum* under attack. However, they are not produced in response to crustacean herbivores, or by simulated herbivory (Pavia & Toth 2000). Phlorotannins are expensive to generate and there is a trade-off between their production and both growth and reproduction (Pavia *et al* 1999, Chapter 1). Therefore they are produced only as required, and their abundance is independent of growth-limiting nutrients, such as nitrogen (Toth *et al* 2007).

#### *Site selection*

The study was undertaken at seven locations bearing established *A. nodosum* populations. Four of these were in the archipelago west of the Sven Lovén Centre for Marine Sciences, Tjärnö in south-western Sweden, and three were on the Isle of Anglesey in North Wales. Western Sweden experiences very small tides (~0.3 m) and has an average salinity of approximately 25 (ranging from 15-30), owing to large freshwater input into the Skagerrak Strait (Åberg 1992b). The four Swedish locations were S1) 58.893467°N, 11.123100°E. S2) 58.895333°N, 11.124367°E. S3) 58.851933°N, 11.134983°E. S4) 58.856894°N, 11.139860°E. Anglesey, by contrast, experiences large (6m) tides and has a salinity more typical of open seawater (~34). The Anglesey locations were Menai (near Menai Bridge); 53.226793°N, 4.157054°W. Cemlyn (in Cemlyn Bay); 53.414750°N, 4.511564°W. Bull (in Bull Bay); 53.422543°N, 4.368959°W. Potential wave energy was estimated from the distance of open water at 10° compass intervals around each site (Davies & Johnson 2006), using high-resolution coastline data from Google Earth. Greater fetch allows larger waves to develop, and is a useful quantitative indicator of 'wave exposure' (Lindegarth & Gamfeldt 2005).

Herbivore abundance at each site was estimated at the date of first sampling, by haphazardly placing ten quadrats within the *A. nodosum* stand and carefully collecting all gastropods by hand (Toth *et al* 2007). Three known herbivores of *A. nodosum* were found to be present at all sites (*Littorina littorea*, *Littorina fabalis*, *Littorina obtusata*) and *Patella vulgata* was also



present at Cemlyn and Bull on Anglesey. *Littorina fabalis* was included in surveys because although it has been previously shown to consume algal films (Viejo & Åberg 2003), it does also consume macroalgae including *Sargassum muticum*, *Fucus serratus*, and *A. nodosum* (Kurr, unpublished data).

### *Sampling protocols*

Sampling procedures differed slightly between regions due to the logistics of travelling to Sweden from Anglesey, where this research was conducted. In June, 24 genet per Tjämnö site (n = 96, identified as per Åberg 1989) were selected haphazardly and tagged with cable-ties around the holdfast, since determining their gender at this time was not possible. Only individuals with at least four vesicles present on the fronds were included, because those younger than this are likely to be immature (Pavia *et al.* 1999). Length and circumference were measured to the nearest half-centimetre, allowing an estimation of dry mass (Åberg 1990). Apical meristems (4-5) were torn by hand from each genet to minimise phlorotannin induction (Pavia *et al.* 1997), returned to the laboratory, and frozen within 12 hours of sampling. These same individuals were then resampled in August, October, and the following April, but because of mortality throughout the experiment, sample sizes decreased from 96 to 82. Only individuals that survived the entire experiment were included in analysis, leaving sample sizes of 22 at Site 1, 19 at Site 2, 23 at Site 3, and 18 at Site 4.

In April, the conceptacles (sites of gamete production) were visible within the receptacles, and this allowed differentiation of genders by a combination of light-microscopy (Vernet & Harper 1980) and by the colour of the conceptacles (Dudgeon *et al.* 2001). Receptacles (~100 per genet) were collected from the same reproductive laterals as that month's vegetative samples, to avoid bias by intra-individual variation in tissue condition (Cousens 1985). Because genders were not known at the time of initial tagging, the numbers of males and females ultimately shown to be tagged in April, provided an estimate of sex-ratio in Tjämnö.

Sampling on Anglesey differed due to greater access, 10 males and 10 females per month were sampled from each site exactly as per Tjämnö algae, except that genets were different in each month due to an excess of males and females being haphazardly tagged during the previous spring. Unlike in Tjämnö, equal numbers of each gender had been specifically tagged and therefore a separate survey was needed to assess sex-ratio as a means to estimate sexual dimorphism in mortality (Viejo *et al.* 2011). This was undertaken non-destructively in April.

100 individuals were selected haphazardly at each site, and their genders differentiated (Dudgeon *et al.* 2001).

#### *Phlorotannin analysis and energy content*

To assess the degree of sexual dimorphism in defence and energy content, both vegetative material and the reproductive material collected in April were cleared of epiphytes, washed with distilled water, freeze-dried to constant mass, and ground until homogenous. A known amount (200 mg) was then assessed for phlorotannin abundance using a modified variation of the Folin-Ciocalteu method (Van Alstyne 1995). A further subsample was assessed for energy content by estimating the living-biomass (ash-free dry mass per unit dry mass) of the tissues (Littler & Littler 1985). A known quantity (~1000mg) was weighed to the nearest milligram, ashed-off at 450 °C for 3.5 hours, and reweighed to the nearest milligram (Holme & McIntyre 1984). This quantification of the organic fraction of the tissues is a useful measure of condition and energetic cost of tissue-production, since organic carbon is used extensively in algal metabolism (Neilson & Lewin 1974), and AFDM per unit DM correlates almost exactly with energy content in algae (Lamere & Wing 2001).

#### *Statistical analysis*

Sex-ratio of *A. nodosum* at each site was tested for significant difference from 1:1 with a one-proportion binomial analysis. Prior to analysis of means, all data were Levene's-tested for equal variance. Differences in herbivore abundances between sites were tested by one-way ANOVA if variances were homogenous, and Welch's ANOVA if they were not. Differences in phlorotannin and living-biomass abundance were assessed with 3-way repeated measures nested ANOVA's, with site nested in region. Differences in whole-algal dry mass, receptacle phlorotannin abundance, and receptacle living-biomass were analysed with two-way nested ANOVA's, with gender and region as fixed orthogonal factors, and site nested in region. All means were compared post-hoc with Tukey's HSD tests.

#### *Results*

##### *Stress*

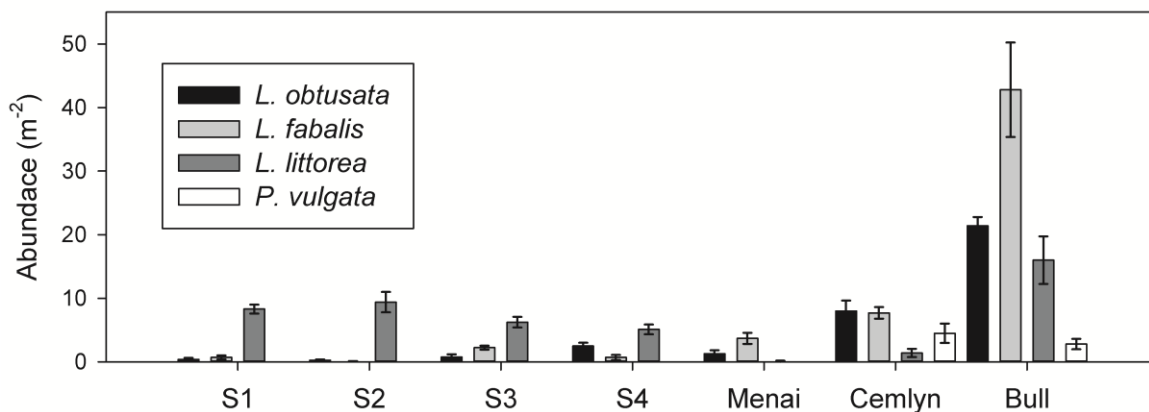
Tjärnö sites were similar in their fetch (Table 1), and all four sites had similar total abundances of grazers (Figure 1), although there were differences in species composition between sites. S4 hosted the most *L. obtusata*, whereas S3 had more *L. fabalis*. *Littorina*

*littorea* were the most common grazer, but abundances were similar between sites (Figure 1). In contrast, Anglesey showed large differences in both fetch and grazer density, with both being lowest at Menai and highest at Bull (Table 1, Figure 1). Both the average fetch and total grazer abundances were similar between Menai and all four Tjärnö sites (Table 1, Figure 1). *Patella vulgata* were higher at Cemlyn than Bull, but their abundances were small by comparison to *L. fabalis* and *L. obtusata* which were extremely abundant at Bull (Figure 1).

**Table 1.** Descriptive statistics for the fetch of four Swedish sites (S1-S4) and three Welsh sites (Menai, Cemlyn, Bull) bearing established stands of *Ascophyllum nodosum*

	Mean	S.E.	Sum	Maximum
S1	193.6	44.6	6969.7	1022.0
S2	221.8	51.0	7985.3	1002.0
S3	144.5	62.7	5200.9	1680.0
S4	296.0	81.7	10654.9	1500.0
Menai	209.4	47.8	7539.6	1042.1
Cemlyn	16724	6894	602076	130977
Bull	29449	8941	1060178	181133

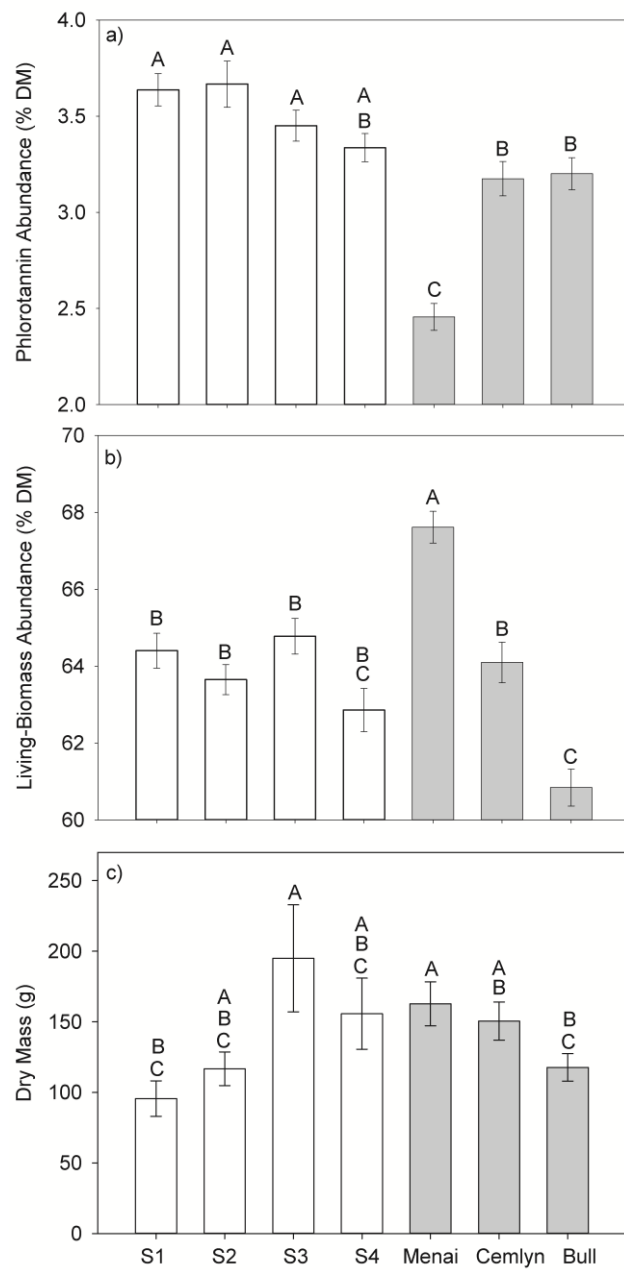
Overall, Anglesey was significantly more exposed to wave-stress, with an average fetch distance of 15.4 km (S.E. 3.9 km) compared to 0.2 km (S.E. 0.03 km) at Tjärnö. Abundances of grazers were also higher on Anglesey, with an average of 146 individuals m<sup>-2</sup> (S.E. 26.7), compared to 9 m<sup>-2</sup> (S.E. 0.64) at Tjärnö. Both *Littorina obtusata* and *Littorina fabalis* were common on Anglesey ( $\approx 10$  m<sup>-2</sup>, S.E. 1.71, and  $\approx 18$  m<sup>-2</sup>, S.E. 4.06 respectively) but both were rare at Tjärnö ( $< 1$  m<sup>-2</sup>, S.E. 0.19), whereas *Littorina littorea* were equally common in both regions (Anglesey  $\approx 7$  m<sup>-2</sup>, S.E. 1.81, Tjärnö  $\approx 6$  m<sup>-2</sup>, S.E. 0.63). The limpet *Patella vulgata* was present on Anglesey, albeit rarely ( $\approx 2$  m<sup>-2</sup>, S.E. 0.66), but absent entirely from Tjärnö (Figure 1). *Ascophyllum nodosum* from both regions had similar total dry-masses (Two-way ANOVA SS = 1572, F<sub>1,310</sub> = 0.11,  $p = 0.737$ ).



**Figure 1.** Abundances of the known gastropod herbivores of *Ascophyllum nodosum* in each of seven established stands of the alga. S1, S2, S3, and S4 are near Tjärnö in Western Sweden, Menai, Cemlyn, and Bull are on Anglesey in North Wales. Error bars show +/- 1 S.E

### Responses to Stress

There were significant differences in the mean phlorotannin abundance between sites (Table 2, Figure 2a), although these did not relate to grazer abundance (regression analysis phlorotannin abundance v grazer abundance;  $p = 0.134$ ). There were also significant differences in living biomass abundance between sites (Table 3, Figure 2b), which showed a weak negative relationship with grazer abundance (regression analysis; gradient = -0.01, intercept = 64.84, SS = 920,  $F = 46.92$ ,  $p < 0.001$ ). On Anglesey, both dry mass and living-biomass content negatively associated with higher stress (wave-exposure and grazer abundance), but there were also differences in both dry mass and living-biomass abundance between Tjärnö populations despite stressors being broadly similar between these sites. S3 *A. nodosum* were larger than those from S1 and S2 (Figure 2c), and living-biomass was higher in *A. nodosum* from S1 and S3 than that from S4 (Figure 2b), although these differences were variable over time (Table 3). Dry-masses of *A. nodosum* from S3 and Menai were greater than those from S1 and Bull (two-way ANOVA term for site nested in region, SS = 194753,  $F_{5,310} = 2.81$ ,  $p = 0.017$ ), but algae from all other sites were of a similar size (Figure 2c).



**Figure 2.** Abundance of a) phlorotannin (percentage dry mass), and b) living-biomass (percentage dry mass) in the vegetative tissues of *Ascophyllum nodosum*, and the total drymasses of those same algae (n = 569) from seven established populations, collected in June, August, October, and April. S1, S2, S3, and S4 are near Tjärnö in Western Sweden (white bars), Menai, Cemlyn, and Bull are on Anglesey in North Wales (grey bars). Error bars show +/- 1 S.E, groupings based on tukey's post-hoc analysis

**Table 2.** Three-way repeated-measures nested ANOVA for the phlorotannin abundance in *Ascophyllum nodosum* meristematic tissue samples ( $n = 570$ ), from four established populations near Tjärnö in Western Sweden, and three established populations on Anglesey in North Wales, sampled in April, June, August, and October.

	DF	MS	F	P
Month	3	37.78	112.94	< <b>0.001</b>
Gender	1	1.62	4.86	= <b>0.028</b>
Region	1	43.38	129.66	< <b>0.001</b>
Month x Gender	3	1.42	4.25	= <b>0.006</b>
Month x Region	3	5.11	15.28	< <b>0.001</b>
Gender x Region	1	3.04	9.08	= <b>0.003</b>
Site	5	6.61	19.77	< <b>0.001</b>
Month x Gender x Region	3	1.18	3.54	= <b>0.015</b>
Month x Site (Region)	15	1.10	3.28	< <b>0.001</b>
Gender x Site (Region)	5	0.98	2.92	= <b>0.013</b>
Month x Gender x Site (Region)	15	0.31	0.93	= 0.531
Residual	514	0.33		

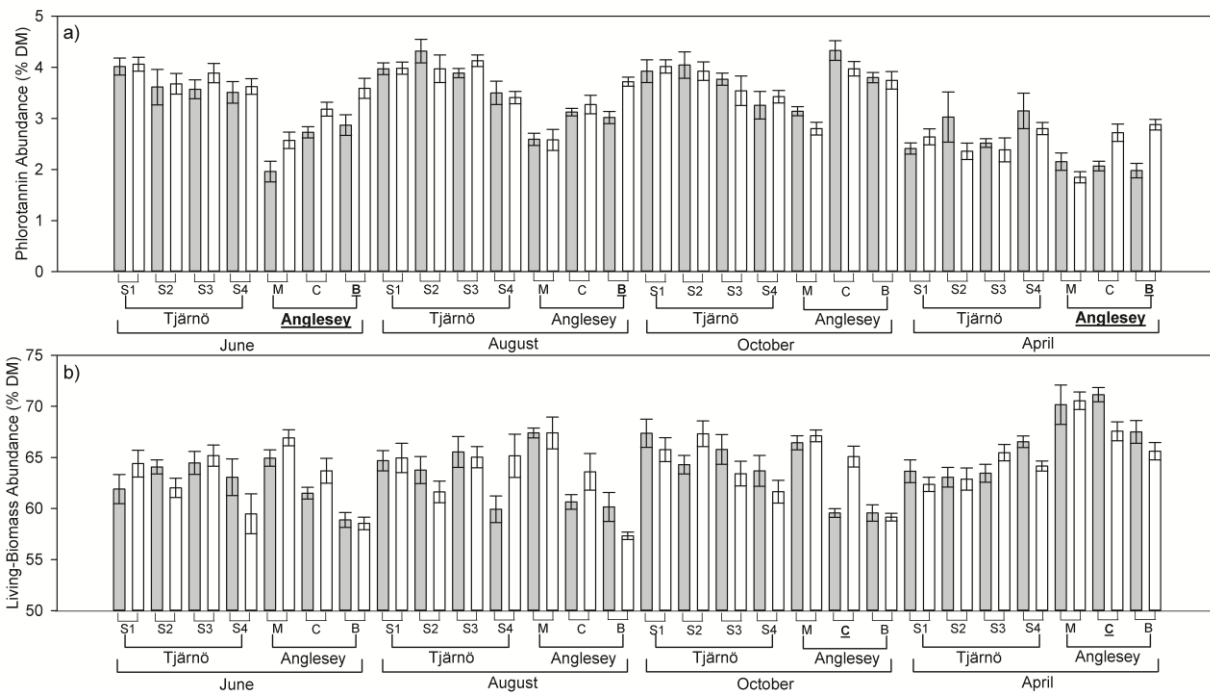
**Table 3.** Three-way repeated-measures nested ANOVA for the living-biomass abundance in *Ascophyllum nodosum* meristematic tissue samples ( $n = 569$ ), from four established populations near Tjärnö in Western Sweden, and three established populations on Anglesey in North Wales, sampled in April, June, August, and October.

	DF	MS	F	P
Month	3	339.82	24.54	< <b>0.001</b>
Gender	1	0.436	0.03	= 0.859
Region	1	8.14	0.59	= 0.444
Month x Gender	3	19.52	1.41	= 0.239
Month x Region	3	321.73	23.23	< <b>0.001</b>
Gender x Region	1	14.86	1.07	= 0.301
Site	5	396.85	28.65	< <b>0.001</b>
Month x Gender x Region	3	30.81	2.22	= 0.084
Month x Site (Region)	15	32.49	2.35	= <b>0.003</b>
Gender x Site (Region)	5	21.59	1.56	= 0.170
Month x Gender x Site (Region)	15	35.03	2.53	= <b>0.001</b>
Residual	513	13.85		

#### *Sexual dimorphism in vegetative tissues*

Sexual dimorphism in phlorotannin abundance was significant in the vegetative tissues of *A. nodosum* at Bull only, in June, August, and April (Table 2, Figure 3a). Sexual dimorphism was not evident in size or sex-ratio at any site, and although Cemlyn females had higher

abundances of living-biomass than males in April, and lower abundances in October, no other incidences of sexual dimorphism occurred in living-biomass abundance (Table 3, figure 3b). By region there was no sexual dimorphism in living-biomass abundance of *A. nodosum* at any time, although that of Anglesey *A. nodosum* was higher than that from Tjärnö in April, but lower in October (Table 3, figure 3b).



**Figure 3.** a) Abundance of phlorotannin (% DM) and b) Abundance of living-biomass (% DM) in the vegetative tissues of male (white bars) and female (grey bars) *Ascophyllum nodosum* ( $n = 569$ ) collected from seven established populations. S1, S2, S3, and S4 are near Tjärnö in Western Sweden, Menai, Cemlyn, and Bull are on Anglesey in North Wales. Error bars show  $\pm 1$  S.E., **highlighted text** indicates where genders were significantly different (based on Tukey's post-hoc analysis)

With all sites pooled together, male *A. nodosum* ( $n = 273$ ) had more phlorotannin in their vegetative tissues ( $\bar{x} = 3.32\%$  DM, S.E 0.05) than female *A. nodosum* ( $n = 297$ ,  $\bar{x} = 3.24\%$  DM, S.E 0.05), although by region this was significant on Anglesey only, and by month in June and April only (Table 2, Figure 3a). Phlorotannin abundance increased in both regions over summer, and was higher in Tjärnö *A. nodosum* than that from Anglesey in April, June,

and August, although in October *A. nodosum* from both regions bore similar abundances (Figure 3).

*Sexual dimorphism in reproductive tissues*

Female *A. nodosum* defended their receptacles more than males and this was consistent across all seven sites, despite there being between-site differences in overall abundance (Table 4).

**Table 4.** Two-way nested ANOVA for phlorotannin abundance in the reproductive tissues of *Ascophyllum nodosum* ( $n = 137$ ), sampled from four established populations near Tjärnö in western Sweden, and three established populations on Anglesey in North Wales, in April.

	DF	MS	F	P
Gender	1	0.63	9.51	= <b>0.003</b>
Region	1	1.62	24.32	< <b>0.001</b>
Gender x Region	1	0.005	0.07	= 0.787
Site (Region)	5	0.64	9.56	< <b>0.001</b>
Gender x Site (Region)	5	0.13	2.01	= 0.082
Residual	123	0.06		

There was no sexual dimorphism in the living-biomass abundance in receptacles, and *A. nodosum* from all sites showed similar total abundances, except for Menai *A. nodosum* which showed ~6% more living-biomass abundance in its receptacles, than that from other sites (Table 5).

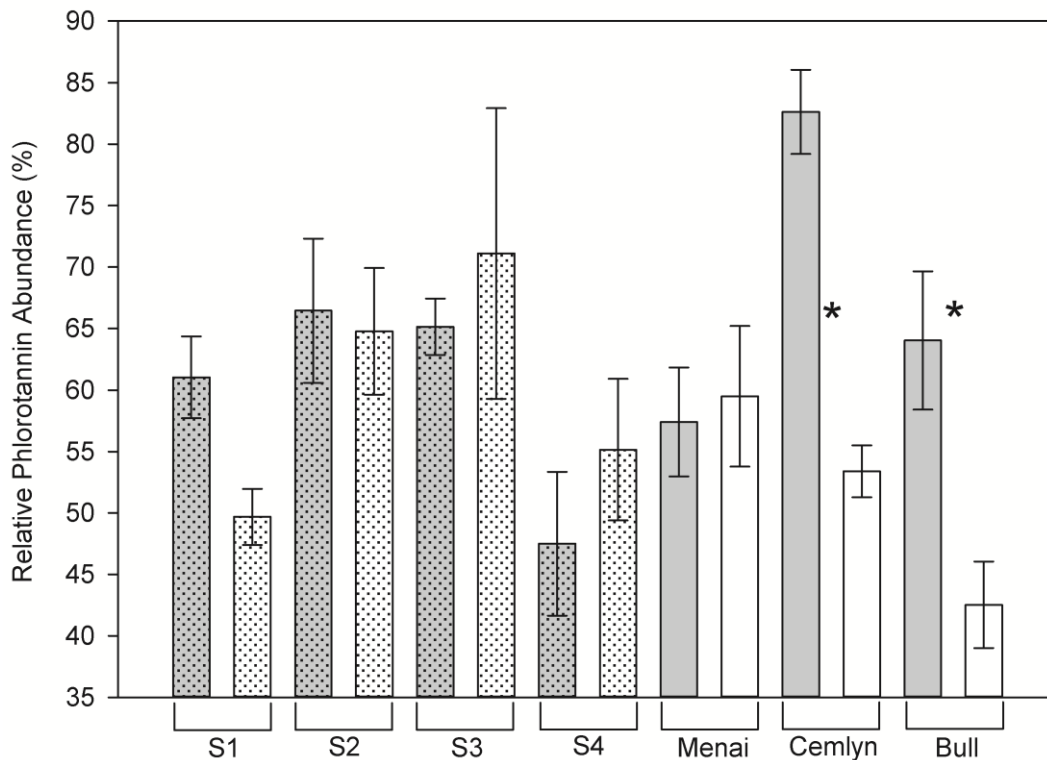
**Table 5.** Two-way nested ANOVA for living-biomass abundance in the reproductive tissues of *Ascophyllum nodosum* ( $n = 137$ ), sampled from four established populations near Tjärnö in western Sweden, and three established populations on Anglesey in North Wales, in April.

	DF	MS	F	P
Gender	1	39.77	1.97	= 0.163
Region	1	308.51	15.31	< <b>0.001</b>
Gender x Region	1	31.74	1.58	= 0.212
Site (Region)	5	83.15	4.13	= <b>0.002</b>
Gender x Site (Region)	5	13.04	0.65	= 0.664
Residual	123	20.15		

*Ascophyllum nodosum* from both Cemlyn and Bull displayed sexual dimorphism in receptacle phlorotannin abundance relative to vegetative phlorotannin abundance (Table 6, Figure 4). Females from both sites invested ~20% more phlorotannin into their receptacles



relative to their vegetative tissues than males, whereas this difference (non-significant) was ~4% elsewhere (Figure 4). The degree of sexual dimorphism in relative receptacle defence was not significantly different between Cemlyn and Bull.



**Figure 4.** Relative phlorotannin abundance of the receptacles of female (grey bars) and male (white bars) *Ascophyllum nodosum* from seven established populations, sampled in April. S1, S2, S3, and S4 are near Tjärnö in Western Sweden (speckled bars), Menai, Cemlyn, and Bull are on Anglesey in North Wales (plain bars). Abundances in the receptacles are expressed as a percentage of abundance in the vegetative tissues, error bars show +/- 1 S.E, \* indicates where significant differences exist between the genders (determined by Tukey's post-hoc analysis)

When pooled, Tjärnö *A. nodosum* defended their receptacles more than Anglesey *A. nodosum* (Table 4). Also, when considered relative to vegetative defence, females defended receptacles more, with receptacle phlorotannin abundance being 64% that of vegetative phlorotannin abundance, whilst males invested only 56% as much phlorotannin in their receptacles as in their vegetative tissues (Table 6). This sexual dimorphism was similar between regions, and

both groups of *A. nodosum* invested comparable amounts phlorotannin into their receptacles relative to that in their vegetative tissues ( $\approx 60\%$ ). There was no sexual dimorphism in the living-biomass content of receptacles in *A. nodosum* from either region, although living-biomass abundance was higher in Anglesey *A. nodosum* than that from Tjärnö (Table 5).

**Table 6.** Two-way nested ANOVA for receptacle phlorotannin abundance expressed as a fraction of vegetative phlorotannin abundance for *Ascophyllum nodosum* ( $n = 137$ ), sampled from four established populations near Tjärnö in western Sweden, and three established populations on Anglesey in North Wales, in April.

	DF	MS	F	P
Gender	1	0.21	10.15	= <b>0.002</b>
Region	1	< 0.001	0.01	= 0.938
Gender x Region	1	0.21	10.51	= <b>0.002</b>
Site (Region)	5	0.11	5.43	< <b>0.001</b>
Gender x Site (Region)	5	0.07	3.66	= <b>0.004</b>
Residual	123	0.02		

### Discussion

Sexual dimorphism was more apparent in *Ascophyllum nodosum* on Anglesey than in those from near Tjärnö. Male *A. nodosum* were better defended than females on Anglesey as a whole for two months, and specifically at the Bull site for three months, whereas no sexual dimorphism in vegetative defence ever occurred in Tjärnö algae, at any site. Females from both populations defended their receptacles more than males, both when raw phlorotannin abundances were compared, and when they were considered relative to phlorotannin in the vegetative tissues. However, sexual dimorphism in the relative defence of receptacles manifested significantly in Cemlyn and Bull only. Sexual dimorphism in living-biomass also manifested twice at Cemlyn, with both males and females displaying a better condition than the opposite gender for one month.

Differences between population demographics of *A. nodosum* are apparent at some scales but not others (Åberg & Pavia 1997). For example, abundances of juveniles differ considerably at spatial-scales less than 1.5m but not over larger scales (Åberg & Pavia 1997). This is to be expected, since recruitment is impacted by micro-scale heterogeneity in shelter and abundance of grazers, which differ at small spatial-scales (Forrest *et al* 2001; Dudgeon & Petraitis 2005). Abundances of adults however, differ only at large spatial-scales (hundreds of kilometres, Åberg & Pavia 1997), suggesting abiotic factors such as light levels, ice-damage,

and available nutrients are more important to adults. This is the first study to assess sexual dimorphism in algae at different spatial-scales, and therefore reveals interesting insights into the potential driving-forces behind it.

Differences in sexual dimorphism in *A. nodosum* occurred at the regional scale (> 1000km) but were more prevalent at the local scale (< 50km), suggesting that multiple stressors can be responsible for sexual dimorphism, but critically that not all are. As discussed previously, wave-action and herbivory appear to drive sexual dimorphism in both vegetative defence, and relative defence of receptacles which occurred only at the most wave- and grazer-stressed sites (Chapter 1). Menai had similar wave-stress and herbivory to the Tjärnö sites, and displayed similar degrees of sexual dimorphism. Ice-damage however, appears not to drive sexual dimorphism at all. Despite there being a considerable sea-ice event prior to April sampling (Gunnar Cervin, *pers. comm.*), vegetative defence never differed between the genders at Tjärnö sites, and sexual dimorphism in reproductive tissues was similar to that found on Anglesey. Therefore it is likely that sexual dimorphism occurs because of high germling and juvenile mortality selecting for higher reproductive investment in females, and not because of direct stress on adults. Phlorotannin levels were considerably higher in *A. nodosum* from Tjärnö, despite grazers being fewer in number there, and differences occurred between Tjärnö sites and Menai despite abundances of herbivores being similar between them. This contrasts with previous local-scale investigations into phlorotannin abundance in *A. nodosum*, which showed that grazer abundances were the primary reason for between-site differences (Toth *et al* 2007). Although salinity was lower in Tjärnö, low salinity actually causes a reduction in phlorotannin production (Connan & Stengel 2011), and so the cause of this variation is unclear. Whichever physical or biological factor/s drove this difference in phlorotannin content however, it did not cause sexual dimorphism.

In contrast, sexual dimorphism in reproductive defence (phlorotannin abundance in reproductive tissues) occurred ubiquitously, suggesting that 1) This manifestation of sexual dimorphism is independent of stress entirely, and further suggesting that 2) female reproductive tissues are more valuable than male reproductive tissues, because they are better-defended (Pavia *et al.*, 2002). This is unsurprising since antheridia (sperm) to oogonia (egg) ratios can be between 40 and 400 to one in fucoids (Vernet & Harper 1980), although living-biomass abundance and by extension, energy content (Lamere & Wing 2001), did not differ between male and female tissues. However it is worth noting that receptacles were

collected in April, and sexual dimorphism in living-biomass abundance has previously been shown to manifest in December to March only in *A. nodosum* (Chapter 1).

### *Conclusions*

These findings further our knowledge of sexual dimorphism in algae by demonstrating that not all stressors cause it to manifest. Populations experiencing stressors which impact upon germling and juvenile mortality (wave-action and herbivory) demonstrate sexual-dimorphism more often than those which experience stressors impacting primarily on adult performance and less on recruit mortality (i.e. ice-damage). Some stressors other than herbivory may still necessitate a defensive response by the algae through the production of secondary metabolites (phlorotannins), which impact negatively upon primary metabolism. However, these stressors do not act differently upon the genders, and therefore do not cause sexual dimorphism. This is despite the fact that females invest more heavily into reproduction than males, if only because they produce more expensive defences in their reproductive structures. This study highlights the value of a multi-scale approach to the research of sexual dimorphism in both plants and algae.

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## Chapter Four

### Biotic resistance to an invasive alga is dependent on native herbivore diversity, not time-since-invasion

#### *Abstract*

Three widely cited theories explaining biological invasions are the Enemy Release Hypothesis (ERH), the Evolution of Increased Competitive Ability hypothesis (EICA), and the Biotic Resistance Hypothesis. Evidence suggests that a lag-phase in top-down-pressure exists during the initial stages of a biological invasion, and the ERH and EICA may apply in this period, before herbivore-mediated Biotic Resistance becomes more important when the invasive is abundant. The predictions of these models are tested with the invasive marine macroalga, *Sargassum muticum*, using chemical analyses of defence and algal condition, as well as surveys of its associated mesoherbivore community from seven populations with known time-since-invasion. Contrary to the predictions of the ERH and EICA, older populations did not host greater abundances or diversities of herbivores, and levels of chemical defence did not relate to herbivore abundance or population age. However, with one exception, *S. muticum* from older populations were of lower condition than those younger. This suggests that Biotic Resistance increases on *S. muticum* the longer it has been established in an area. Levels of chemical defence strongly correlated with mesoherbivore diversity, suggesting that herbivore-mediated Biotic Resistance is dependent on more than mesoherbivore abundance alone. Since different fractions of a mesoherbivore assemblage impart distinct pressures on their host algae, it follows that the greater the species diversity the greater the probability that species are present which impart biotic resistance. These findings add a new dimension to the classic idea that community diversity positively effects resilience to invaders.

#### *Introduction*

Invasive species constitute one of the largest threats to biodiversity and ecosystem functioning on the planet. Human activities in the marine environment have left less than 15% of the world's ocean waters free from invasive species, and alongside crustaceans and molluscs, algal species are amongst the most prolific and damaging non-native groups (Molnar et al. 2008). However, research into invasive terrestrial plants and their ecological impact is far more mature by comparison, and has led to the development of a number of

widely-cited theories describing the processes underlying invasions by photoautotrophs. These include the Enemy Release Hypothesis (ERH) (Keane and Crawley 2002), its phytocentric extension the Evolution of Increased Competitive Ability hypothesis (EICA) (Blossey and Nötzold 1995), and the model of Biotic Resistance (Elton 1958).

The ERH posits that invasive species are removed from a considerable fraction of top-down pressure because local consumers, particularly specialists, are unable or unwilling to feed upon them (Keane and Crawley 2002). The EICA is specific to plants and algae, and predicts that in an invasive population released from top-down pressure, phenotypes will be selected for that prioritise growth and reproduction over defence. This affords the invasive an advantage over native plants, and allows it to become competitively dominant (Blossey and Nötzold 1995). The Biotic Resistance Hypothesis (BRH) contrasts with the ERH and EICA, predicting that local communities are inherently resilient to the addition of new species. By the same logic as the ERH, the BRH is predicted to manifest because invasive species have not evolved specific defences to compensate for pressures exerted within their new environments (Elton 1958).

Attempts to test the validity of the ERH and EICA in both terrestrial and marine environments have yielded evidence in support of (e.g. Jogesh et al. 2008, Lankau 2007, Abhilasha and Joshi 2009) and in opposition to (e.g. Agrawal and Kotanen 2003, Van Kleunen and Schmid 2003, Leger and Forister 2005, Huang et al. 2010, Lind and Parker 2010) these theories, whilst other studies suggest that some species conform only partially to their predictions (e.g. Joshi and Vrieling 2005, Hull-Sanders et al. 2007, Cano et al. 2009). Evidence for the BRH is abundant and convincing, but even invasives with similar ecologies can experience very different levels of this pressure depending on the nature of their new habitat, and their time-since-invasion (Maron and Vilá 2001, Levine et al. 2004, Eschtruth and Battles 2009).

Biotic Resistance comprises interspecific competition, local species diversity, pathogenesis, and herbivory, and each may change in importance as the invasive arrives, establishes, proliferates, and impacts upon the native community (Levine et al. 2004). The herbivory component of biotic resistance is an important determinant on the progress of an invasion, and native herbivores often readily consume invasives, even preferring them to native plants (Parker and Hay 2005). Large native generalists are the most important fraction of the faunal assemblage responsible, and the abundance of these herbivores is generally more important than their diversity (Maron and Vilá 2001, Parker et al. 2006). However, herbivory only becomes an important component of biotic resistance once an invasive has

begun to proliferate, thereby serving to regulate the invasion, rather than preventing it altogether (Maron and Vilá 2001, Levine et al. 2004). As they first establish, invasive species may therefore 'slip through the net' of native herbivory, and only experience significant top-down pressure once they are abundant (e.g. Poore 2004). This is to be expected since optimally foraging animals are more likely to feed upon common sources of food, and theoretically a lag-phase should occur after introduction, when an invasive is temporarily released from significant top-down pressure (Maron and Vilá 2001, Theoharides and Dukes 2007). Rather than being entirely contrasting theories therefore, the sum of current evidence suggests a chain of events linking the ERH, EICA and the herbivory component of the BRH together. The invasive is initially free from native herbivory (ERH), giving it a competitive advantage over native plants (EICA), which allows it to proliferate, thereby attracting the attention of generalist native herbivores (herbivore-mediated Biotic Resistance). Very few studies have specifically tested time-since-invasion when assessing the ERH, EICA, and BRH, and most simply compare invasive populations in their native range, to those in their invaded range.

This study aims to further our understanding of the way in which the ERH, the EICA, and herbivore-mediated Biotic Resistance may manifest at different phases of the invasion of the marine macroalga, *Sargassum muticum*, in the British Isles. The spread of this species provides an excellent opportunity to investigate invasive ecology for three reasons. Firstly, *S. muticum* is not consumed by large herbivores such as fish and urchins, unlike tropical *Sargassum* species, and instead attracts a diverse range of mesoherbivores (small crustaceans and gastropods) (Van Alstyne and Paul 1990, Thomsen et al. 2006, Strong et al. 2009). In contrast to large herbivores which can eat whole plants and algae at once, even closely-related mesoherbivores can impart very different top-down pressures, because of their broad range of feeding-modes and foraging preferences (e.g. Pavia et al. 1999a, Karez et al. 2000, Pavia and Toth 2000, Lankau 2007). Individuals of even highly polyphagous mesoherbivore species display strong host-plant specificity and feeding preferences, possibly because of their relative lack of mobility compared to large generalist herbivores which can quickly graze across an area far larger than the plants they eat (Sotka 2005). As such, many mesoherbivores are particularly unlikely to feed upon a plant or alga unless it is common in their local habitat (e.g. Arrontes 1990, Bell and Sotka 2012, Mattila et al. 2014). Therefore the ERH and EICA could apply to the initial stages of a *S. muticum* invasion, but once it becomes prolific, mesoherbivores are more likely to consume it and thereby impart Biotic Resistance.

Secondly, the responses of *S. muticum* to herbivory are easily quantified by measuring its investment into defence. As a brown alga, *S. muticum* produces polyphenolic compounds, phlorotannins, as a defence against herbivory (Van Alstyne and Paul 1990, Pavia and Toth 2008) both in its annual fronds, and in its perennial holdfast. Although not universally deterrent against all consumers, phlorotannins retard feeding by a broad suite of mesoherbivores and many brown algae have been shown to alter their production in response to levels of herbivory, as opposed to abiotic conditions such as nutrient levels (Pavia and Toth 2008). Phlorotannins therefore make a useful proxy for responses to top-down pressure, particularly because they are expensive to produce and are therefore made only when necessary (Pavia and Toth 1999). Finally, *S. muticum* prefers sheltered shores, growing in discrete stands in bays, inlets, and harbours which are frequented by sailors, fishermen, and other members of the public. Because it is well known beyond the academic community as a pest organism, a detailed chronology of its 50-year invasion across Western Europe exists (Davison 2009).

By sampling *S. muticum* individuals from seven populations of known time-since-invasion, three hypotheses are tested. 1) More recently established populations of *S. muticum* will host less-abundant and less diverse mesoherbivore assemblages, in accordance with the ERH. 2) Because individuals from these more recently established populations are under less pressure, they will have more living biomass and fewer chemical defences present in their tissues, in accordance with the EICA. 3) Biotic Resistance to *S. muticum* will be dependent not only on mesoherbivore abundance, but also on mesoherbivore diversity because of their wide-variety of feeding-modes and preferences.

Furthermore, this study provides the opportunity to investigate the relationship between phlorotannin and energy content in this species. Phlorotannins are known to be expensive to produce in other furoids (Pavia and Toth 2008), and so the hypothesis is tested that phlorotannin production will be negatively related to living biomass abundance.

## *Materials and Methods*

### *Sample Sites*

Seven populations of *S. muticum* were surveyed from locations around the British Isles. Each of these were selected based on time-since-invasion, estimated from Critchley et al. (1983), and Davison (2009) (Figure 1). Site morphologies were broadly similar and the meso-scale positions of *S. muticum* stands were alike, since *S. muticum*'s tolerance of exposure is

minimal (Davison 2009). Populations were all shallow subtidal (0.5m-1.3m above chart datum), and in the lee of rock formations or sand-bars. *Sargassum muticum* stands were always amongst or nearby to common native algae such as *Fucus serratus*, *Ascophyllum nodosum*, *F. vesiculosus*, *F. spiralis*, and usually stands of *Laminaria digitata*, *L. hyperborea* and/or *Sacharina latissima* were within 50m of the sample site. Locations were named after the time-since-invasion by *S. muticum* (Figure 1).

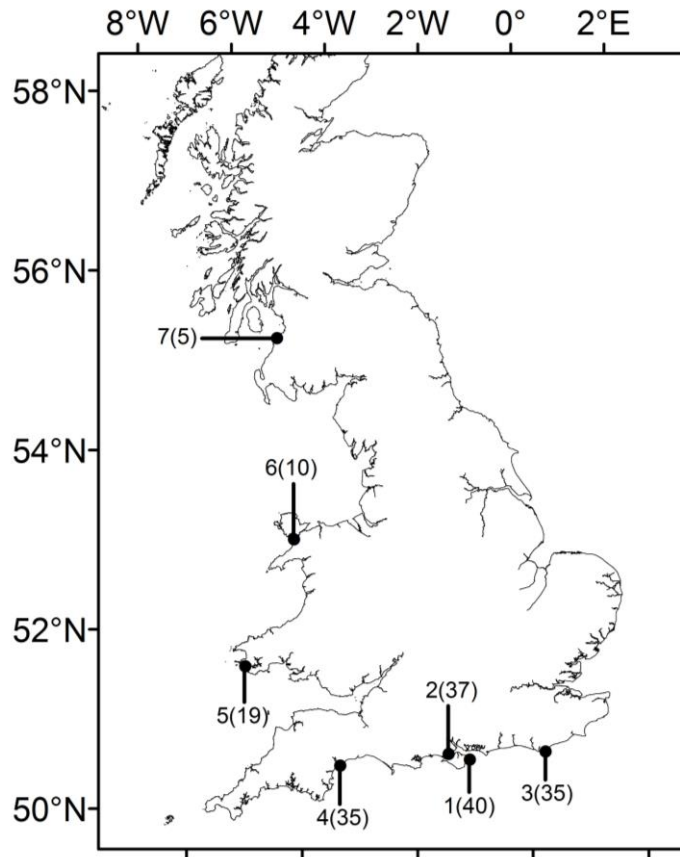


Figure 1: Locations of *Sargassum muticum* populations sampled for algal material and mesoherbivores. Sites are named by the time-since-invasion of *S. muticum* (years in parentheses). Scale-bar represents 100km. Site 1 (40): Bembridge Ledge (50.680466°N, 1.072554°W). Site 2 (37): Lepe (50.783981°N, 1.355657°W). Site 3 (35): Eastbourne (50.750541°N, 0.270442°E). Site 4 (35): Sandy Bay, near Exmouth (50.607876°N, 3.363291°W). Site 5 (19): West Angle Bay, near Milford Haven (51.688676°N, 5.110854°W). Site 6 (10): The northern shore of the Foryd estuary near Caernarfon (53.131581°N, 4.304016°W). Site 7 (5): Culzean Bay (55.377332°N, 4.771487°W).

Most of these locations are within 1 degree of latitude, although the Foryd Estuary, 6(10), and Culzean bay, 7(5), are 3° and 5° further north respectively than the most southern site of Exmouth. They therefore differ slightly in terms of average temperature and incident UV levels. Sea temperatures at Exmouth were around 2°C higher than at Culzean Bay, for example, and total solar irradiance differed by approximately 200kWh/m<sup>2</sup> annually (Šúri et al. 2007). The UVR fraction is known to account for around 4% of total solar irradiance depending on humidity and cloud levels, giving an approximate difference of around 1.2% UVR between Exmouth and Culzean Bay (Escobedo et al. 2009).

Temperature is not known to influence phlorotannin abundance, although it may increase the feeding rate of mesoherbivores (Cubit 1984). However, UVR radiation has been shown to induce phlorotannin production in brown algae during laboratory trials (Pavia et al. 1998). Assuming a similar induction in *S. muticum* to that reported for *A. nodosum*, a 1.2% increase in UVR levels would equate to a 0.72% higher phlorotannin production at the southernmost site compared to the northernmost, a negligible difference considering that herbivory can increase phlorotannin abundance by over 70% (Pavia and Toth 2000).

#### *Phlorotannin Abundance and Tissue Condition*

Twenty-four *S. muticum* individuals were selected at random from each site in mid-summer (July), and vegetative tissues were removed from the upper 20cm of the frond so as to ensure only fresh annual growth was sampled. The epiphyte loading differed between fronds, but rarely between individuals. Care was taken to select fronds free from epiphytes from any given individual, since removal was impossible without damaging the fronds. Separately, the holdfasts (discerned as per Norton 1977) of 20 further individuals were sampled in the same fashion, although the tougher nature of these tissues allowed those bearing epiphytes to be sampled, and these were removed by hand in the laboratory. Samples were kept refrigerated at 5°C in a mobile chiller during transit to avoid degeneration.

The tissues were initially washed thoroughly with distilled water, freeze-dried to constant mass, ground until homogenous, and analysed for phlorotannin abundance. 60% aqueous acetone was used as a solvent, and phlorotannins were extracted over 1 hour under constant agitation in the dark. The algal pulp was separated from the supernatant by centrifugation (5300rpm for 10 minutes) and the acetone removed from this using *in-vacuo* cold-distillation (80 kPa, 38°C) until only the 40% aqueous fraction remained (typically ~2 hours). This was then diluted, filtered so as to remove precipitated lipophilic compounds (Pavia and Toth 2000), and analysed by photometry using a variation of the Folin-Ciocalteu

method, using phloroglucinol (1,3,5- trihydroxybenzene, Sigma-Aldrich P3502) as a standard (Pavia and Toth 2000). Six samples were selected from each site to be analysed in triplicate, so as to ensure accuracy. These were never more than 0.1 standard deviations from their respective means.

A known quantity (~1000mg) of dried, homogenised frond-material was subsampled to ascertain ash-free dry-mass (AFDM) of the tissues per unit dry-mass, as a measurement of living-biomass and therefore relative energetic content at the time of sampling (Holme and McIntyre 1984). Samples were weighed to the nearest milligram, ashed-off at 450°C for 3.5 hours, and reweighed to provide an estimate the percentage of organic compounds (living-biomass) in the tissues by weight (Littler and Littler 1985). Organic compounds are used extensively in algal metabolism, and so provide a useful indicator of tissue condition (Neilson and Lewin 1974). Holdfasts were not analysed for living-biomass because many of those collected, particularly from younger populations, comprised only of very small quantities of material, which was expended during phlorotannin analysis.

#### *Mesoherbivore Survey*

From each of the sites, 20 *S. muticum* individuals were selected at random in mid-summer (July) and sampled for associated fauna by placing a bag over the whole alga *in situ* at low tide ( $n = 140$ ). The entire individual, including the holdfast, was removed and returned to the laboratory before being frozen at -20°C to fix any trapped animals. These were then separated from the fronds by washing the alga over a 1mm sieve, and the length of each *S. muticum* was measured to the nearest 5 mm.

Animals were identified to species level, and those known to feed on algal tissue were weighed to the nearest 0.01 mg, and measured to the nearest 0.1mm shell/body length. Variation in size and mass of herbivore conspecifics between and within sites was minimal, and so average individual masses for each species were pooled and converted to dry-mass as per Ricciardi and Bourget (1998). This allowed for the calculation of mesoherbivore dry-mass per meter of *S. muticum* length. Ideally, dry mass of algae or at the very least an estimation of ‘algal volume’ (see Åberg 1990) is preferred. However, almost all *S. muticum* individuals bore at least some fronds host to epiphytes, which made such measurements impossible. The principal epiphyte was *Ectocarpus* sp. and removing this filamentous alga without causing substantial damage to the feathery *S. muticum* fronds was not possible, particularly since this material had been frozen and defrosted. Only species known to be herbivores of macrophytes were included, although *Lacuna vincta* were excluded from



analysis because they were juveniles, and would therefore have been feeding on sediment (Fretter and Manly 1977).

Five different species diversity indices were calculated, since drawing conclusions from one alone is inadvisable (Boyle et al., 1990). Simpson's diversity Index (1-D) measures the probability that two randomly selected individuals will belong to the same species. Margalef's diversity index (natural log) shows the number of species present, controlled for sampling effort. Pielou's evenness index (natural log) quantifies how similar the abundance of each species are to one another. The Shannon-Weiner Index (natural log) is the ratio of species number to their relative importance within the sample. The Brillouin's index (natural log) is functionally similar to the Shannon-Weiner index, but accounts for non-random sampling, possibly caused in this case by the differences in motility between species, which could have resulted in fewer highly-motile crustacea being sampled.

#### *Statistical analyses*

All data was tested for normality and homogeneity of variances by Anderson-Darling and Levene's tests respectively. In the event of non-conformity, data were either transformed, or non-parametric tests were used to compare means. Data for the abundance of organic compounds in the fronds, and between-site phlorotannin abundances in the fronds and holdfasts of *S. muticum* were approximately normal, but did not conform to the assumption of homogeneity of variance, and means were therefore compared with a Welch's ANOVA with a Games-Howell post-hoc analysis. The frond lengths, mesoherbivore abundances, and species diversity indices for associated mesoherbivores were compared with one-way Analyses of Variance (ANOVA), and means were compared with Tukey's HSD post-hoc analyses. The relationships between living-biomass abundances in the fronds and phlorotannin abundances in both the fronds and holdfast were tested for using a Pearson Product-Moment Correlation Coefficient and the interaction between this relationship and site was assessed using a two-way ANOVA. The relationships between mesoherbivore abundance and frond length, species diversity indices with both phlorotannin abundances in the fronds and holdfasts, and living-biomass abundances in the fronds, were assessed using regression analysis with mesoherbivore abundance, and species diversity as the independent variables.

## Results

### *Phlorotannin Abundance and Tissue Condition*

There was no relationship between time-since-invasion of *Sargassum muticum* population and the phlorotannin abundance in either the fronds or the holdfasts (Figure 2a). There were, however, significant differences in phlorotannin abundance between sites in both the holdfasts and in the fronds (Welch's ANOVA,  $F_{13,110} = 43.82$ ,  $R^2 = 46.93\%$ ,  $p < 0.001$ ), Figure 2a). All pooled there was significantly more phlorotannin present in the holdfasts (4.62% DM, S.E. = 0.13) of *S. muticum* than in the fronds (3.96% DM, S.E. = 0.07) (Welch's ANOVA,  $F_{1,211} = 12.49$ ,  $p = 0.001$ ), although this was significant at site 5 (19) only, and the trend was reversed at site 3 (35) (Figure 2a). There was no relationship between phlorotannin abundance in the holdfasts, and phlorotannin abundance in the fronds (Pearson's correlation,  $p = 0.295$ ).

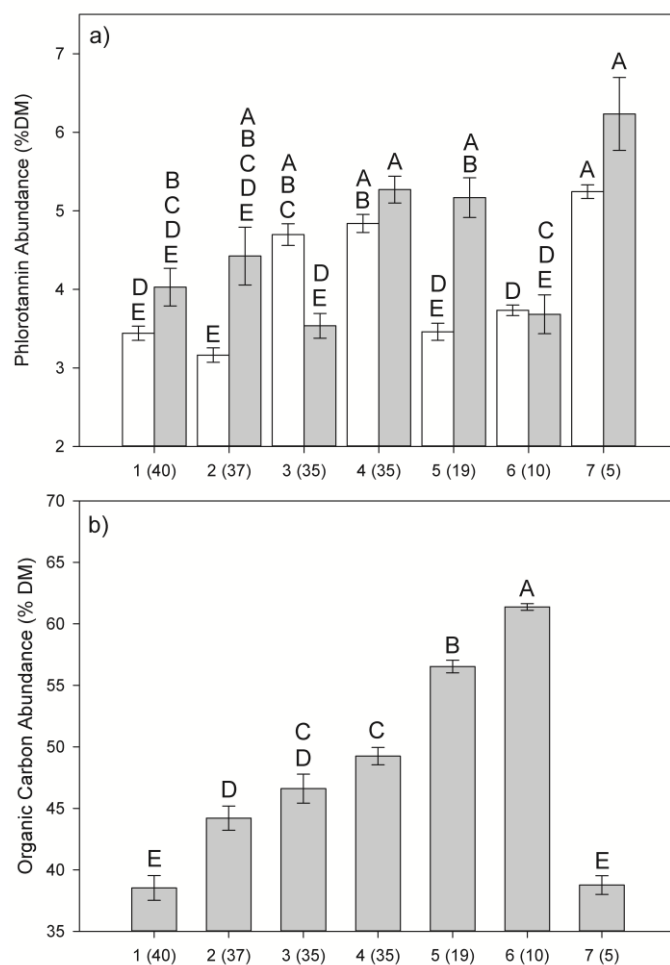


Figure 2: a) Phlorotannin abundance (% DM) in the upper fronds of *Sargassum muticum* (white bars) ( $n = 168$ ) and holdfasts of *Sargassum muticum* (grey bars) ( $n = 140$ ) collected from seven populations around the British Isles. Groupings are based on the result of a Games-Howell test performed post-hoc on a one-way Welch's ANOVA. b) Living-biomass abundance (% DM) in the upper fronds of *Sargassum muticum* ( $n = 168$ ) collected from seven populations around the British Isles, groupings are based on the result of a Games-Howell test performed post-hoc on a one-way Welch's ANOVA ( $F_{6,68.56} = 241.27$ ,  $R^2 = 80.24\%$ ,  $p < 0.001$ ). Sites are named by the time-since-invasion of *S. muticum* (years in parentheses), error bars show  $\pm 1$  S.E.

Living-biomass abundance in the frond tissues of *S. muticum* displayed a negative trend with time-since-invasion, showing a regular, linear pattern across all sites except at site 7 (5), which bore similar abundances to the oldest population, 1 (40) (Figure 2b). There was

no relationship between living-biomass in the fronds, and phlorotannin abundance in the holdfasts. There was, however, a weak but significant negative correlation between the living-biomass in the fronds of *S. muticum*, and the phlorotannin abundance in those same tissues (Pearson Correlation, d.f. = 167,  $r = -0.219$ ,  $p = 0.004$ , Figure 3). This relationship was independent of site (2-way ANOVA interaction term,  $SS = 0.829$ ,  $F_{6, 154} = 0.58$ ,  $p = 0.742$ ), and was only detectable when all data were pooled. Lengths of *S. muticum* fronds differed between sites (One-way ANOVA,  $SS = 77874$ ,  $F_{6,133} = 16.19$ ,  $p < 0.001$ , Tukey's HSD,  $6(10) > 7(5) = 1(40) = 3(35) > 5(19) = 4(35) = 2(37)$ ) although there were no relationships between time-since-invasion, phlorotannin abundance in the fronds or holdfasts, or living-biomass abundance in the fronds.

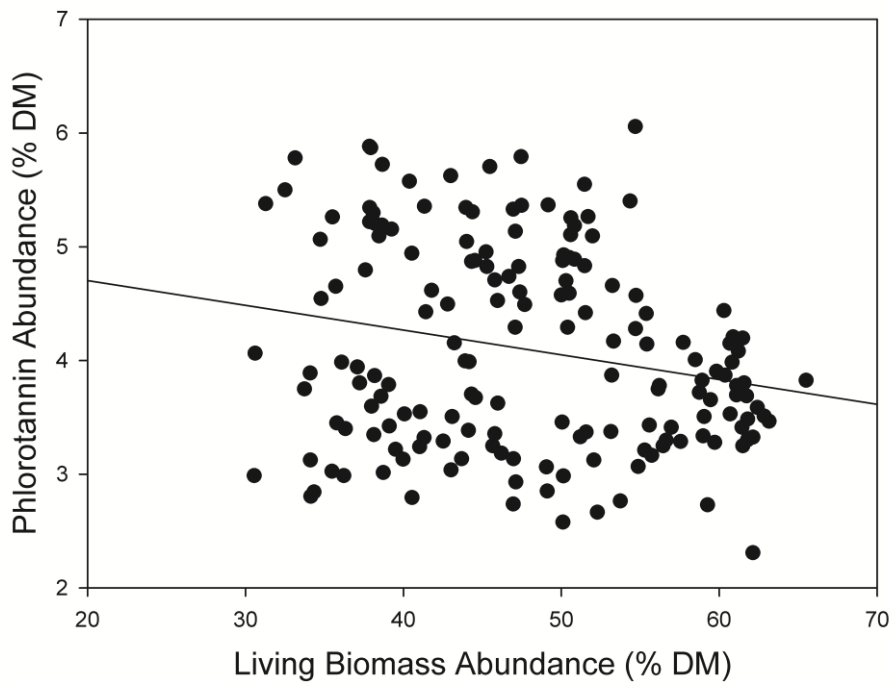


Figure 3: The relationship between the abundance of living-biomass (% DM) and the abundance of phlorotannins (% DM) in the fronds of *S. muticum* ( $n = 168$ ) collected from throughout the British Isles.

#### *Mesoherbivore Survey*

A large variety of animals were found on *S. muticum* fronds from all sites, including multiple species of caprellids, *Palaemon*, fish, and juvenile mussels (*Mytilus* sp.) (ESM Table 1). Holdfasts were often epiphytised by other seaweeds, including *Ulva* sp., *Chondrus*

*crispus*, and *Fucus serratus* recruits. Anemones, bryozoans, and tunicates were also common on these perennial structures, whereas fronds were epiphytised by *Ectocarpus* sp. almost exclusively. Nearly all individuals bore at least some fronds host to extensive amounts of *Ectocarpus* sp., and whilst quantifying it was not logistically viable, general observations did not indicate any variability between or within sites. Seven species of adult mesoherbivore were found on *S. muticum*, and their total biomass (herbivore DM) correlated linearly with *S. muticum* length (l) (Regression analysis,  $r^2 = 7.51\%$ ,  $p = 0.001$ ). Abundances differed greatly between sites (Figure 4), and only individuals from population 1(40) consistently hosted every mesoherbivore found elsewhere. Only *Gammarus locusta* was found in all populations (Figure 4d), and abundances did not differ between them (One-way ANOVA,  $SS = 149.8$ ,  $F_{6, 133} = 1.62$ ,  $p = 0.146$ ). Within-species sizes of individuals did not differ between sites, and their average lengths were often smaller than that reported to be typical for their species (Table 1).

Table 1: Comparison of typical adult body lengths for mesoherbivores found on *Sargassum muticum*. Typical lengths are taken from Haywood and Ryland (1995), and where applicable male and female lengths have been averaged. Hayward PJ, Ryland JS (1995). Handbook of the marine fauna of North-West Europe. Oxford University Press, Oxford.

Species	Typical Adult Length (mm)	Mean Length on <i>Sargassum muticum</i> (mm) and S.E
<i>Littorina fabalis</i>	<11	5 (0.02)
<i>Idotea granulosa</i>	5-17	7 (0.10)
<i>Idotea balthica</i>	10-25	8 (0.30)
<i>Gammarus locusta</i>	33	4 (0.06)
<i>Rissoa parva</i>	<5	3 (0.01)
<i>Ampithoe rubricata</i>	20	4 (0.04)
<i>Dynamene bidentata</i>	<7	5 (0.05)

There was no relationship between the biomass of mesoherbivores on *S. muticum* fronds (estimated AFDM of mesoherbivores per unit length), and the mean phlorotannin abundance of those tissues from that population (Table 2a). In fact *S. muticum* from all populations except the oldest, 1 (40), hosted similar biomasses of mesoherbivores. This disparity was due entirely to the dense abundances of *Littorina fabalis* on these fronds, which averaged ~150

individuals  $m^{-1}$  (Figure 4a). No species diversity index displayed any relationship with population age, however all but one of the five tested indices positively correlated with known phlorotannin abundances from the *S. muticum* populations (Table 2b), with Simpson's Diversity Index showing the clearest relationship (Figure 5a). Only Margelef's Species Richness Index displayed no relationship, although this was approaching significance ( $p = 0.051$ , Table 1). Conversely, mesoherbivore-diversity did not correlate with abundances of phlorotannin in the holdfasts of *S. muticum*. The abundances of living-biomass in the fronds of *S. muticum* showed generally negative trends with species diversity (Figure 5b), but residuals displayed high degrees of variability and relationships were not significant.

Table 2 a) Regression analysis statistics for the relationships between five species diversity indices, for mesoherbivores found on the fronds of *Sargassum muticum* ( $n = 140$ ) from seven sites around the British Isles, and the time-since-invasion of *S. muticum* from those same sites. b) Regression analysis statistics for the relationships between five species diversity indices, for mesoherbivores found on the fronds of *Sargassum muticum* ( $n = 140$ ) from seven sites around the British Isles, and phlorotannin abundance from the upper 20cm of *S. muticum* ( $n = 168$ ) frond material from those same sites

a)	Gradient	Intercept	MS	$r^2$	F	p
<b>Margelef's SR</b>	0.001	0.460	0.6232	2.37	3.26	= 0.073
<b>Pielou's Evenness</b>	0.001	0.520	0.0802	1.09	1.29	= 0.257
<b>Shannon-Weiner</b>	0.003	0.407	0.2383	0.35	1.48	= 0.225
<b>Simpson's Index</b>	0.000	0.277	0.0014	0.00	0.02	= 0.879
<b>Brillouin's Index</b>	0.002	0.340	0.2041	1.38	1.90	= 0.171

b)	Gradient	Intercept	MS	$r^2$	F	p
<b>Margelef's SR</b>	0.746	0.327	0.4353	56.46	6.48	= 0.051
<b>Pielou's Evenness</b>	0.368	0.200	0.16193	80.07	20.09	= <b>0.007</b>
<b>Shannon-Weiner</b>	0.890	0.338	0.4632	63.57	8.73	= <b>0.032</b>
<b>Simpson's Index</b>	0.541	0.201	0.16474	70.81	12.13	= <b>0.018</b>
<b>Brillouin's Index</b>	0.687	0.270	0.2961	59.69	7.40	= <b>0.042</b>

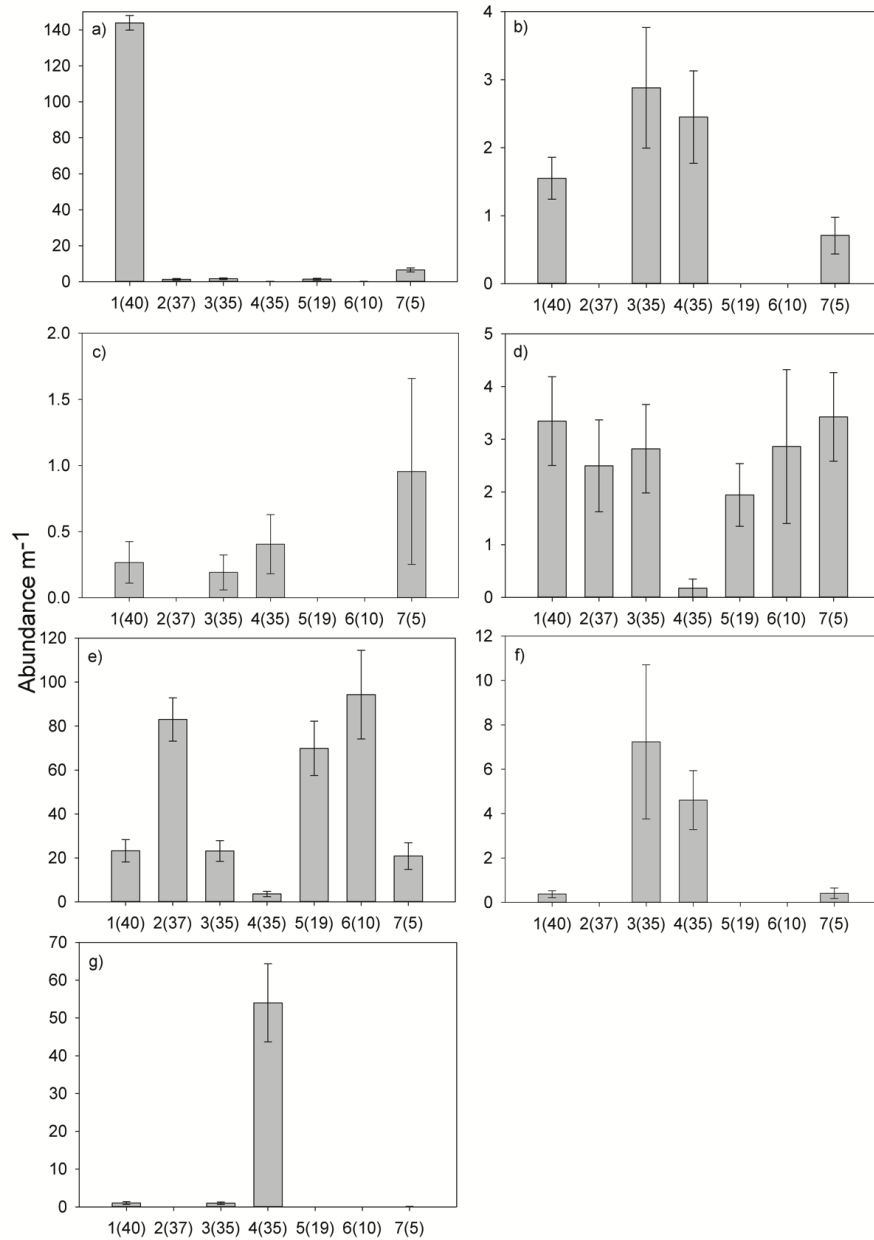


Figure 4: The abundance of adult mesoherbivores (>1mm) per meter of frond length, collected from whole *Sargassum muticum* individuals (n = 140) from seven sites around the British Isles, a) *Littorina fabalis*, b) *Idotea granulosa*, c) *Idotea balthica*, d) *Gammarus locusta*, e) *Rissoa parva*, f) *Ampithoe rubricata*, g) *Dynamene bidentata*. Sites are named after their time-since-invasion of *S. muticum* (years in parentheses).

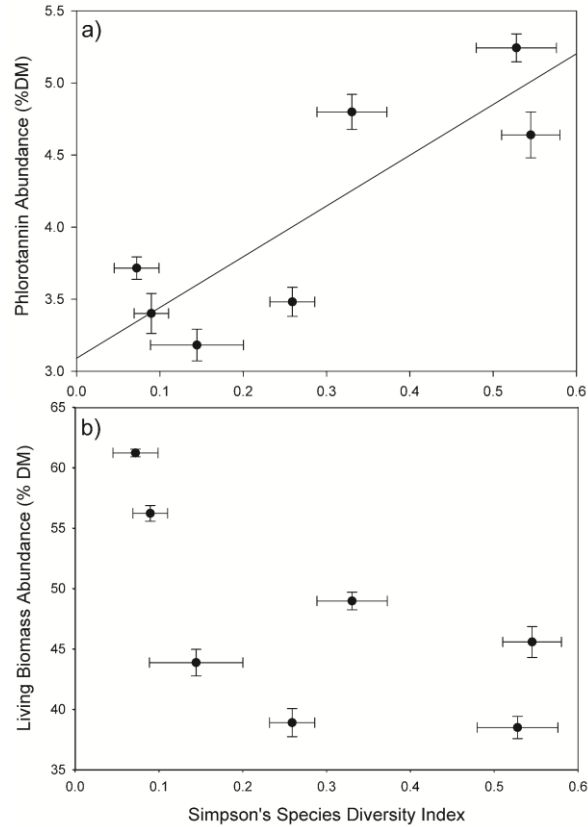


Figure 5: a) The relationship between the Simpson's species diversity index for the mesoherbivores collected from the fronds of *Sargassum muticum* ( $n = 140$ ) growing in seven different populations around the British Isles, and the mean phlorotannin abundance (% DM) in the fronds of *S. muticum* ( $n = 168$ ) from those sites. b) The relationship between the Simpson's species diversity index for the mesoherbivores collected from the fronds of *Sargassum muticum* ( $n = 140$ ) growing in seven different populations around the British Isles, and the mean living-biomass abundance (% DM) in the fronds of *S. muticum* ( $n = 168$ ) from those sites. Error bars show  $\pm 1$  S.E.

### Discussion

These findings provide little evidence to suggest that the Enemy Release Hypothesis (ERH) currently applies to the *Sargassum muticum* invasion within the British Isles, since herbivore abundance and diversity did not correlate with time-since-invasion. These findings are in accordance with previous research comparing the fauna associated with *S. muticum* to that associated with local macroalgae, which found minimal differences between species composition between the two (Engelen et al. 2013, and references therein), indicating a lack



of enemy release on *S. muticum*. These results should be interpreted with caution however; although no comparison was made with local native macroalgae in this study, those animal individuals found were generally smaller than would be expected, and may therefore exert less top-down pressure than their larger conspecifics. Individuals of both epifaunal (Viejo 1999) and infaunal (Strong et al. 2006) species associated with *S. muticum* canopies have been found to be smaller than those outside the canopy. Furthermore, epiphytes, which were abundant on *S. muticum* throughout the UK, are known to be the principal food for many species of mesoherbivore found on *S. muticum* (Viejo 1999), and the abundance of mesoherbivores positively correlates with that of epiphytes when between-site differences in epiphyte load exist (Cacabelos et al. 2010a). Although *S. muticum* host similar faunal diversities to natives therefore, it is unclear if actual top-down pressure is similar between it and native algae.

Levels of chemical defence in *S. muticum* also showed no relationship with time-since-invasion and the only population which bore host to a significantly higher mesoherbivore abundance did not possess the highest levels of defence. This contradicts both the Evolution of Increased Competitive Ability hypothesis (EICA) and previous research on intraspecific levels of chemical defence in brown algae, which has shown higher defences are produced in the presence of more mesoherbivores (Toth et al. 2007, Pavia and Toth 2008). This further suggests that many of the animals found were not feeding directly on the fronds of *S. muticum*, in accordance with laboratory-based feeding experiments that show a general aversion towards *S. muticum* by mesoherbivores (Monteiro et al. 2009, Cacabelos et al. 2010b, Engelen et al. 2011, but see Strong et al. 2009).

In accordance with the EICA however, *S. muticum* individuals were generally in poorer condition in older populations, having less living-biomass in their tissues. This could be an indication that individual performance is adversely affected by growing in a community that over several decades has become acclimatised to the presence of *S. muticum*. This would suggest that that the manifestation of Biotic Resistance to *S. muticum* takes many generations, although whether this was the result of increased herbivory and is therefore in accordance with the predictions of the ERH and EICA, is unclear. This would be difficult to conclude, since living-biomass did not correlate with herbivore abundance or diversity, and the other components of Biotic Resistance, such as interspecific competition or pathogens, were not assessed (Levine et al. 2004).

As a pseudo-annual with a large investment into annual reproduction (Norton and Deysher 1989), *S. muticum* is most vulnerable to specialist co-evolved herbivores, and is

expected therefore to confirm to the posits of the ERH and EICA (Maron and Vilá 2001). Both help to explain its success beyond its native range of Japan, where it is far less abundant (Davison 2009) and less aggressive in its proliferation (Tokuda et al. 1994). These data suggest, however, that herbivore-pressure on *S. muticum* has remained consistent over a 35-year period in Britain, such that chemical defences in older populations are no greater than those in younger ones. Gorham and Lewey (1984) also sampled from Bembridge Ledge ('site 1 (40)' herein) and reported similar phlorotannin abundances (~4% DM) from mid-summer *S. muticum* fronds, from a population that would have been approximately five years old at that time (Critchley et al. 1983). This suggests that top-down pressure has not increased at this location in the 35 years separating these studies. Therefore either the effects of the ERH and EICA are not relevant at this timescale, or that they do not apply in this instance, rather than that site-specific differences between populations are masking the effect of the ERH and EICA. Previous work into the time taken for species diversity of mesoherbivores to reach their maximum in introduced crops, suggests timescales of less than 200 years (Strong et al. 1977). In this instance, either levels of top-down pressure have reached their maximum in less than five, or will take more than 35 years to do so.

These results do demonstrate however, that phlorotannin abundance is highly variable in *S. muticum*, and this plasticity as well as the capacity for production of large quantities of these defences may explain its continued success, in contrast to the EICA, but in accordance with findings on other invasives (Van Kleunen and Schmid 2003, Leger and Forister 2005, Huang et al. 2010, Lind and Parker 2010). Phlorotannins are known to be energetically expensive to produce and are composed of large quantities of organic carbon (Pavia and Toth 2008). These results indeed suggest that individuals which prioritise defence do so at the expense of organic resources, which could be otherwise used for other biological processes (Neilson and Lewin 1974, Coley et al. 1985). Crucially there were fewer such resources present in individuals from older populations, and yet phlorotannin abundances were still at their highest in two of these. The youngest population disrupted the trend of living-biomass with population age, but phlorotannin abundances in these individuals were amongst the highest observed. As such, chemical defences appear to be important to the ecology of *S. muticum*, and it will produce them even when resource availability is low.

Overall, abundances of phlorotannins in the holdfasts were higher than in the fronds, although differences were rarely significant at site-level. Curiously however, the abundances of phlorotannin in the holdfasts did not correlate to the abundances in the fronds, the abundance of organics in the fronds, the age of the population, nor herbivore density or

diversity. The holdfasts are typically the most important tissues for macroalgae (Tugwell and Branch 1992, Pavia et al. 2002), and although free-floating fronds are an important dispersal mechanism for *S. muticum* (Deyscher and Norton 1982), detachment of the entire individual is clearly undesirable and defending these tissues against herbivory is therefore of crucial importance. Evidently the holdfasts, being the only perennial part of *S. muticum* (Norton 1977), possess a somewhat different chemical ecology to the annual fronds, the loss of which are probably of less importance.

Despite there being considerable differences in phlorotannin abundance between populations, all but the individuals in the very oldest population hosted similar biomasses of mesoherbivores. *Sargassum muticum* individuals from populations that hosted the greatest diversity of mesoherbivores however, invested significantly more into chemical defence. This suggests that top-down pressure, and therefore the herbivory component of Biotic Resistance, was dependent on the variety of herbivore attacks. An abundance of research suggests that ‘species diversity’ (of controphics) leads to more stable communities better able to resist invasive plants and algae (Price and Pärtel 2013, but see Eschtruth and Battles 2009), including *S. muticum* (White and Shurin 2007). These researchers typically conclude that some mechanism of intraspecific competition leads to obligate niche segregation of the invasive population, and in certain manipulative experiments (e.g. Naeem et al. 2000) this can only be the case. However these studies typically consider ‘herbivory’ and ‘species diversity’ to be independent components of biotic resistance (Maron and Vilá 2001, Levine et al. 2004), and many do not test for or even attempt to control for herbivory in field experiments, despite herbivore diversity often positively correlating with the diversity of primary consumers (Duffy et al. 2003).

These approaches may be acceptable when considering pressure from large herbivores in terrestrial environments, which may be of low species diversity. However, marine mesoherbivore assemblages can be remarkably diverse, and different components within these assemblages may exert different pressures on a plant or alga, owing to their wide variety of feeding-modes (see Pavia et al. 1999a, Karez et al. 2000, Pavia and Toth 2000, Lankau 2007). Therefore it is to be expected that there be a positive relationship between mesoherbivore diversity and top-down pressure on macroalgae, since the presence of a greater number of herbivore species leads to a greater probability that the alga is under biologically-significant attack by at least some individuals, and must therefore compensate with defences. Indeed a greater diversity of herbivores results in the evolution of a broader suite of chemical defences in specialist algae (Hay et al. 1987), and artificially increased

herbivore diversities negatively impact the functioning of monospecific eelgrass beds (Duffy et al. 2003). It has been speculated previously that herbivore diversity may impede the success of invasives (Agrawal and Kotanen 2003), although to our knowledge no other study has attempted to measure top-down pressure in this fashion with respect to one.

Mesoherbivore abundance did not differ significantly between six of the seven populations sampled, and so it was not possible to determine whether abundance is more or less important than diversity in this instance. Likewise due to logistical constraints, this study did not quantitatively survey the native algal community near to the *S. muticum* populations investigated, nor the mesoherbivores associated with this native assemblage. As such it was not possible to conclude that the diversity of the native algae lead to an increase in the diversity of the mesoherbivore assemblage, and therefore that the ‘species diversity’ and ‘herbivory’ components of biotic resistance were in fact interlinked. However, considering that photoautotroph and herbivore diversities often covary (Duffy et al. 2003), and that mesoherbivore species associated with native algae are generally the same as those associated with *S. muticum* (Engelen et al. 2013), this avenue of investigation warrants further effort.

### *Conclusions*

No lag-phase in enemy-release could be detected in the 40 year span of the invasion of *S. muticum* around the British Isles. Contrary to the predictions of the Enemy Release Hypothesis, top-down pressure, be it determined through direct abundance measurements, diversity of herbivore species, or production of chemical defences does not correlate with the length of time *Sargassum muticum* has been established at a location. Individuals from older populations were, however, in poorer condition, having lower quantities of living-biomass per unit mass. This could be due to increased Biotic Resistance over time, although is unlikely to be herbivore-mediated.

Chemical defences are costly to produce, and yet appear to be very important to the success of *S. muticum* in its invasive range, in contrast to the predictions of the Evolution of Increased Competitive Ability Hypothesis. It will produce them irrespective of the available pool of organic compounds, and is more likely to prioritise defence of the highly important perennial holdfast, as opposed to the less important annual fronds. The factors determining the levels of chemical defence in the holdfasts are, however, unknown.

The defences in the annual fronds positively correlated with mesoherbivore diversity. Since different components of mesoherbivore assemblages exert distinct top-down pressures, be they differences in feeding-rate, feeding-mode, or feeding-preferences for certain tissue-

types, the greater the number of species present the greater the chances that at least one is feeding in a way which warrants the production of phlorotannins. These results suggest that 'herbivory' as a component of Biotic Resistance, is not determined solely by mesoherbivore abundance. This is an important consideration for the interpretation of research that attempts to quantify the various components of Biotic Resistance using observations, as opposed to field-manipulations or laboratory trials.

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Appendix: Epiphytic and epifaunal species present on *Sargassum muticum*

<u>Site</u>	<u>Alga</u>	<u>Bryozoa</u>	<u>Chordata</u>	<u>Cnidaria</u>	<u>Crustacea</u>	<u>Mollusca</u>
<u>1 (40)- Bembridge Ledge</u>	<i>Chondrus crispus</i> <i>Cladophora rupestris</i> <i>Ectocarpus</i> sp. <i>Fucus serratus</i> <i>Palmaria palmata</i> <i>Polysiphonia</i> sp. <i>Ulva lactuca</i> <i>Ulva intestinalis</i>	<i>Membranipora membranacea</i>	<i>Asciidiella scabra</i>	<i>Actinia equina</i>	<i>Ampithoe rubricata</i> <i>Dynamene bidentata</i> <i>Caprellidae</i> sp. <i>Gammarus locusta</i> <i>Idotea balthica</i> <i>Idotea granulosa</i> <i>Palaemon</i> sp. <i>Semibalanus balanoides</i>	<i>Gibbula cineraria</i> <i>Gibbula umbilicalis</i> <i>Lacuna vineta</i> <i>Littorina fabalis</i> <i>Littorina littorea</i> (eggs) <i>Mytilus edulis</i> <i>Rissoa parva</i>
<u>2 (37)- Lepe</u>	<i>Chondrus crispus</i> <i>Ectocarpus</i> sp. <i>Palmaria palmata</i> <i>Polysiphonia</i> sp. <i>Ulva lactuca</i>	<i>Membranipora membranacea</i>			<i>Gammarus locusta</i> <i>Palaemon</i> sp	<i>Littorina fabalis</i> <i>Rissoa parva</i>
<u>3 (35)- Eastbourne</u>	<i>Chondrus crispus</i> <i>Cladophora rupestris</i> <i>Ectocarpus</i> sp. <i>Fucus serratus</i> <i>Palmaria palmata</i> <i>Polysiphonia</i> sp. <i>Ulva lactuca</i> <i>Ulva intestinalis</i>	<i>Membranipora membranacea</i>	<i>Asciidiella scabra</i> <i>Sprattus sprattus</i>	<i>Actinia equina</i>	<i>Ampithoe rubricata</i> <i>Dynamene bidentata</i> <i>Gammarus locusta</i> <i>Idotea balthica</i> <i>Idotea granulosa</i> <i>Palaemon</i> sp. <i>Semibalanus balanoides</i>	<i>Gibbula cineraria</i> <i>Gibbula umbilicalis</i> <i>Lacuna vineta</i> <i>Littorina fabalis</i> <i>Littorina littorea</i> (eggs) <i>Mytilus edulis</i> <i>Rissoa parva</i> <i>Sepia officinalis</i> (eggs)
<u>4 (35)- Exmouth</u>	<i>Ulva intestinalis</i> <i>Ectocarpus</i> sp. <i>Cladophora rupestris</i>				<i>Ampithoe rubricata</i> <i>Dynamene bidentata</i> <i>Gammarus locusta</i> <i>Idotea balthica</i> <i>Idotea granulosa</i> <i>Semibalanus balanoides</i>	<i>Gibbula cineraria</i> <i>Littorina fabalis</i> <i>Mytilus edulis</i> <i>Rissoa parva</i>

**5 (19)-**  
**Milford Haven**

*Cladophora rupestris*  
*Ectocarpus sp*  
*Ulva lactuca*

*Gammarus locusta*

*Gibbula cineraria*  
*Gibbula umbilicalis*  
*Littorina fabalis*  
*Rissoa parva*

**6 (10)-**  
**Caernarfon**

*Ectocarpus sp.*  
*Palmaria palmata*  
*Polysiphonia sp.*

*Membranipora*  
*membranacea*

*Asciidiella scabra*

*Gammarus locusta*  
*Palaemon sp.*

*Littorina fabalis*  
*Rissoa parva*

**7 (5)-**  
**Culzean Bay**

*Chondrus crispus*  
*Cladophora rupestris*  
*Ectocarpus sp.*  
*Fucus serratus*  
*Palmaria palmata*  
*Polysiphonia sp.*  
*Ulva lactuca*  
*Ulva intestinalis*

*Membranipora*  
*membranacea*

*Asciidiella scabra*  
*Sprattus sprattus*

*Actinia equina*

*Ampithoe rubricata*  
*Dynamene bidentata*  
*Caprellidae sp.*  
*Gammarus locusta*  
*Idotea balthica*  
*Idotea granulosa*  
*Palaemon sp.*  
*Semibalanus*  
*balanoides*

*Aplysia punctata*  
*Gibbula cineraria*  
*Gibbula umbilicalis*  
*Lacuna vincta*  
*Littorina fabalis*  
*Littorina littorea*  
(eggs)  
*Mytilus edulis*  
*Rissoa parva*

## Chapter Five

### Time-Since-Invasion Increases Mesoherbivore Feeding Preference For The Invasive Alga, *Sargassum muticum* (Yendo) Fensholt

#### *Abstract*

Non-native algae can have substantial negative impacts in their invaded ranges. One widely-cited mechanism that attempts to explain how invasive plants and algae are often able to spread quickly, and even become dominant in their invaded ranges, is the Enemy Release Hypothesis. Although the ERH has been criticised in review, the predictions of the hypothesis are still valid in many circumstances. This study assessed the feeding-preferences of two species of gastropod herbivore from populations exposed to the invasive alga *Sargassum muticum* for different lengths of time. Feeding-trials, consisting of both choice and no-choice, showed that the herbivores from older stands of *S. muticum* were more likely to feed upon it than those taken from younger stands. These findings provide evidence in support of the ERH, by suggesting that top-down pressure from gastropods increases the longer *S. muticum* has been present. These findings are in accordance with the results of other feeding-trials with *S. muticum*, but in contrast to research that utilises observations of herbivore abundance and diversity to assess top-down pressure. The former tend to validate the ERH, and the latter typically reject it. The potential causes of this disparity are discussed, as are the importance of palatability, herbivore species, and time-since-invasion when considering research into the ERH. This study takes an important, yet neglected, approach to the study of invasive ecology, and highlights the value of multi-faceted experiments when testing the predictions of theories such as the ERH.

#### *1. Introduction*

The introduction of non-native marine algae can have substantial negative impacts upon native communities in their invaded ranges (Molnar et al., 2008). When an invasive plant or alga begins to proliferate in a new range, it presents a novel food-source to native consumers. Although specialists may avoid the invasive, generalist consumers can be attracted to such species and even consume them preferentially to natives (Parker and Hay, 2005; Parker et al., 2006). However, this is not always the case and in many instances both plants (e.g. Jogesh et al., 2008) and algae (e.g. Davis et al., 2005) have been shown to be avoided by generalist

consumers. This may account for their invasibility, as described by the Enemy Release Hypothesis (ERH) (Keane and Crawley, 2002).

*Sargassum muticum* is a highly invasive marine alga which is not controlled by large herbivores such as fish and urchins (Britton-Simmons, 2004; Thomsen et al., 2006). Although it attracts a range of mesoherbivores (Strong et al., 2009), many of these still prefer to feed upon native algae or the epiphytes of *S. muticum* (Britton-Simmons, 2004; Cacabelos et al., 2010a; Critchley et al., 1986; Engelen et al., 2011; Monteiro et al., 2009; Norton and Benson, 1983; Rossi et al., 2010; Viejo, 1999). Because of their size, mesoherbivores are less mobile than large herbivores, and individuals or localised populations of generalist species can display strong host-plant specificity, even when the species as a whole does not (Bell and Sotka, 2012; Mattila et al., 2014; Vesakoski et al., 2009). This is particularly evident in those that are slow-moving or brood their young (Sotka, 2005). This may be because a mesoherbivore's host plant is both its food and habitat, and some algae provide better protection from predators (Watanabe, 1984; Jormalainen et al., 2001). As such it can be hypothesised that mesoherbivores, particularly those less mobile, will take many generations to develop feeding preferences for a species such as *S. muticum*, or even to accept it as a food-source at all.

Optimally-foraging animals are expected to prefer readily available sources of food, and in cases where host specificity is strong, an invasive may therefore escape local herbivores when it is first introduced to a new range (Maron and Vilá, 2001). Once an invasive proliferates however, encounter-rate with local consumers will increase and these may then accept it as a food source. Therefore it is likely that the longer such a species has been present in an environment, the greater the propensity of local consumers to feed upon it. This has been shown to take as little as 20 years in beetles that consume the leaves of invasive trees (Auerbach and Simberloff, 1988), and data on introduced crops shows that species diversity can be as rich as that found in native controphics after less than 200 years (Strong et al., 1977). However, little is known about the lengths of time it might take marine mesoherbivores to accept a species such as *S. muticum*. Also – even amongst the far more abundant terrestrial literature – few studies that specifically incorporate time-since-invasion as a variable utilise behavioural experiments. Most opt instead for observational surveys of abundance or diversity to infer consumer choice (sensu Maron and Vilá, 2007).

This study aims to enhance our understanding of the ERH by investigating the feeding preferences of slow-moving mesoherbivores, taken from stands of *S. muticum* that have been established for different lengths of time. *Sargassum muticum* is an ideal species with which to



investigate mesoherbivore responses with time because it is well known beyond the scientific community as a pest. It prefers sheltered habitats and so is quickly reported when it spreads to harbours and inlets frequented by fishermen and sailors. Therefore a detailed and reliable chronology of its spread around western-Europe and the British Isles exists (Davison, 2009). As a brown alga, it produces quantifiable polyphenolic chemicals as a defence against herbivory (Pavia and Toth, 2000; Van Alstyne and Paul, 1990). Although not deterrent against all species, these phlorotannins retard herbivory by a broad range of mesoherbivores, particularly slow-moving generalist gastropods (Pavia and Toth, 2008). Brown algae have been shown to increase phlorotannin production in response to increased herbivory, making themselves less-palatable (Pavia and Toth, 2008; Van Alstyne and Paul, 1990), although investigations into invasive plants have shown variable responses in the production of chemical defences in their invaded ranges (i.e. increases: Caño et al., 2009; decreases: Willis et al., 1999; no recorded change, but still lowered palatability in herbivore performance trials: Hull-Sanders et al., 2007). Using laboratory-based feeding experiments with *S. muticum* and two species of common generalist gastropod mesoherbivores, this study tests two hypotheses. Firstly, generalist gastropod mesoherbivores are more likely to accept *S. muticum* as a food source the longer it has been present in their local habitat. Secondly, the palatability of *S. muticum* will be lower in long-established populations, compared to those recently established, because *S. muticum* will be better defended as a result of more top-down pressure. Testing these hypotheses aims to further our knowledge of invasive ecology and time-since-invasion effects, specifically with respect to the ERH.

## 2. Materials and Methods

### 2.1 Study organisms

*Sargassum muticum* bears all of the hallmarks of a classic invasive marine species, being temperate, pseudo-annual, fast-growing and *r*-selected, with broad physiological tolerances and a propensity for high dispersal rates and rapid growth in areas of strong anthropogenic influences, such as harbours (Andrew and Viejo, 1998; Arenas et al., 1995; Claridge and Franklin, 2002; Critchley, 1986; Engelen and Santos, 2009; Norton, 1977). High levels of propagule pressure have allowed it to overcome genetic bottlenecks, and the story of *S. muticum*'s spread and proliferation starkly illustrates the possibilities of a successful invasive stratagem (Allendorf and Lundquist, 2003; Davison, 2009). *Sargassum muticum* was first

sighted in the British Isles in 1973, on the eastern coast of the Isle of Wight, and has subsequently spread as far east as Kent, and as far north-west as the Firth of Clyde.

*Ascophyllum nodosum* and *Fucus serratus* were utilised as control organisms, as they are common native intertidal algal species often found on sheltered shores where they adhere to hard substrata in the mid to lower intertidal, and are therefore often sympatric with *S. muticum* (Boaden et al., 1975; Dudgeon and Petraitis, 2005). Both are consumed by the selected grazers, *Littorina obtusata* and *Littorina fabalis*, which are common generalist consumers of seaweeds, found throughout Western Europe (Hayward and Ryland, 2006; Watson and Norton, 1987). They occur in high abundances on sheltered shores, both intertidally and subtidally. Both species show an aversion towards algal tissues containing high levels of phlorotannins, making them good indicators for biologically relevant differences in algal defensive investment (Pavia et al., 2002; Pavia and Toth, 2000).

## 2.2 Study sites

Four *Sargassum muticum* populations were sampled for material to be used in feeding trials (Figure 1). The three oldest populations grow on the upper sub-tidal of moderately exposed sandy shores, in lagoons formed in the lee of a rock formations. The youngest population differed slightly in that it grows in the lee of a sand-bar formed by the fluvial outflow of the Foryd Estuary in the Menai Strait. Salinity and temperature differences at time-of-sampling were minimal (salinity within 1 practical salinity scale, and temperature within 2°C), and all populations grew amongst or very near to common native algae such as *Fucus serratus*, *Ascophyllum nodosum* and *Fucus vesiculosus*. The northernmost and southernmost sites differ by 3° of latitude, and UV exposure causes induction of phlorotannins (Pavia et al., 1998). However UV-R levels (which account for a small percentage of total solar irradiance) differ by only 0.72% between Bembridge and Caernarfon (estimated from data in Escobedo et al., 2010; Šúri et al., 2008). If *S. muticum* responds to UV in the same way as other fucoids, this would equate to a ≈0.4% difference in phlorotannin abundance between the northernmost and southernmost sites (Pavia et al., 1997), negligible considering that herbivory can induce phlorotannin production by 70% (Pavia and Toth, 2000).

Locations are named herein by the length of time-since-invasion by *S. muticum* (Davison, 2009), as follows; ‘40YR’: Bembridge Ledge, on the eastern coast of the Isle of Wight. ‘35YR’: Eastbourne on the south eastern coast of England. ‘19YR’: West Angle Bay, near Milford Haven in south Wales. ‘10YR’: The northern shore of the Foryd estuary near Caernarfon in North Wales (Figure 1).

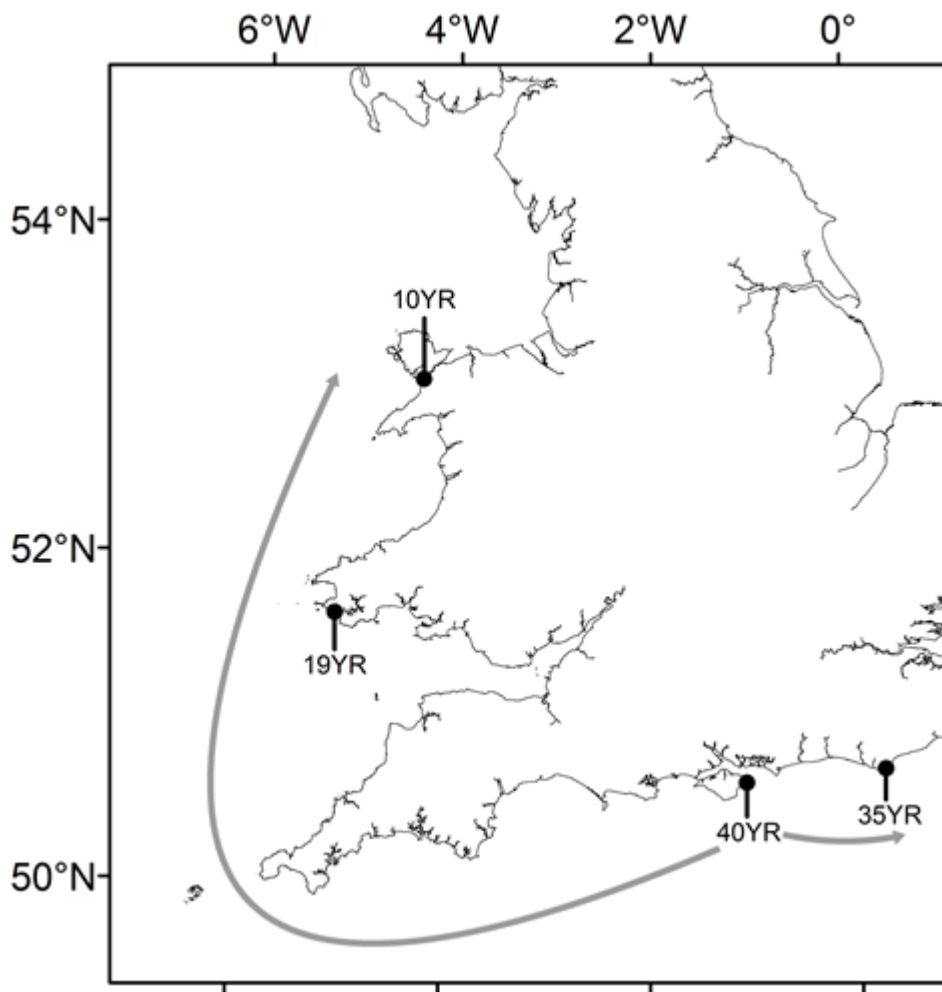


Figure 1: Locations of *Sargassum muticum* populations sampled for algal material and mesoherbivores. Sites are named by the time-since-invasion of *S. muticum*. Grey lines represent general invasion path from first observed occurrence. Site 40YR: Bembridge Ledge (50.680466°N, 1.072554°W). Site 35YR: Eastbourne (50.750541°N, 0.270442°E). Site 19YR: West Angle Bay, near Milford Haven (51.688676°N, 5.110854°W). Site 10YR: The northern shore of the Foryd estuary near Caernarfon (53.131581°N, 4.304016°W).

### 2.3 Feeding Trials

Several experiments were conducted: (1) *S. muticum* from stands of different ages were presented to *L. obtusata* from one site (to assess the palatability of the algae). (2) *L. obtusata* from *S. muticum* stands of different ages were presented with *S. muticum* from one site (to assess the willingness-to-feed of the snails). (3) *L. fabalis* from an ‘old’ and a ‘young’ *S. muticum* stand were presented with *S. muticum*, and the common native alga *Ascophyllum nodosum* from both sites in a suite of choice and no-choice trials to simultaneously assess

palatability of the algae, and both the willingness-to-feed and feeding preferences of the snails.

*A. nodosum* is present at 40YR, 35YR, and 10YR, but is not found close to the *S. muticum* population at 19YR, and therefore *Fucus serratus* was selected as the native control algae for experiments 1 and 2. As such, herbivores used in these trials were collected from *F. serratus* only, at all 3 study sites. Likewise, *Littorina obtusata* was common at 35YR, 19YR, and 10YR, but is almost entirely absent from 40YR. However, the *S. muticum* from 40YR was found to be woodier and darker in colouration than that from other sites, and this allowed it to be differentiated from that from other sites, thereby permitting choice trials to be undertaken. Experiment 3 therefore used 40YR as the 'old' site, and *Littorina fabalis* as the herbivore, which is common at both 40YR and 10YR. *Ascophyllum nodosum* was selected as the native alga, since this was the preferred native host-alga of *L. fabalis* at both sites. All algae were collected within one week of being used in trials, and maintained in ambient (~19°C, salinity 34) seawater within the same outdoor aquaria used for feeding trials. Algae were removed by pulling the holdfast from the substrata, epiphytes were cleared from the fronds, and all individuals were returned to the laboratory within cool-boxes, inside of one day. From each alga used, 500mg (+/- 50mg) blotted-wet-weight clippings were taken from the apical region (one clipping per apex, Pavia et al. 1999) and autogenic changes in algal mass were corrected for by taking control clippings and maintaining these in parallel to the experimental trials. Additionally, ~5 clippings were taken for phlorotannin analysis in palatability trials, and for the estimation of wet-mass to dry-mass conversion ratios in choice trials (explained further below). Handling of algae was kept to a minimum, and great care was taken during blotting of *S. muticum* fronds to avoid dislodging vesicles or causing other damage.

All animals used were collected one week prior to feeding-trials and maintained as above, without food to ensure even levels of hunger. Because starved herbivores can display compensatory feeding for the first two days (Cronin and Hay, 1996) change in algal masses were only recorded after the full seven-day trial period. At the end of the trials, change in algal mass was corrected for average autogenic changes in the controls (identical treatments without herbivores) (Monteiro et al., 2009).

### 2.3.1 The palatability of *S. muticum* to naïve grazers

To determine whether *S. muticum*'s palatability was different depending on time-since-invasion, six algal individuals were collected at random from the 35YR, 19YR, and 10YR populations (n = 18). These were offered to *Littorina obtusata* (three per clipping) collected

from Bull Bay in North Wales (53.422543°N, 4.368959°W) in a no-choice trial. Although the no-choice technique has been criticised for not producing ‘true’ feeding responses (Roa, 1992), no-choice trials were used for two reasons. Firstly, *S. muticum* fragments as it fed upon, making it impossible to differentiate between algae from most sites at the end of the trials. Secondly, the technique still holds value when determining the ‘willingness-to-feed’ of a particular herbivore on a plant or alga (sensu Jogesh et al., 2008), or ‘palatability’ of a particular plant or alga (sensu Toth et al., 2007), as opposed to purely ‘preference’ of consumers. *Sargassum muticum* does not grow at Bull Bay, nor the surrounding coastline, and so these individuals were extremely likely to be naïve to this food-source. This was done to avoid biasing the study by using mesoherbivores with a history of *S. muticum* consumption, thereby ensuring differences in feeding responses were a product of the algae’s condition, and not that of the consumer. All trials were run in triplicate (n = 54).

### 2.3.2 Willingness of exposed grazers to feed upon *S. muticum*

To determine whether *L. obtusata* had a greater appetite for *S. muticum* when their population has been exposed to it for longer, 30 individuals were collected at random from the 35YR, 19YR, and 10YR populations (n = 90). One *S. muticum* individual and one *Fucus serratus* individual were collected from the 10YR site, it being the most accessible from the laboratory. One individual was used for each species to keep phlorotannin abundances, algal condition, and any other variables which may influence palatability, as constant as possible between treatments. Half of the *L. obtusata* were provided with one clipping of *S. muticum*, and the other half were provided with one clipping of *F. serratus* to gauge for population-specific differences in feeding-rates on a typical sympatric native alga. *Littorina obtusata* from these three sites differed considerably in size, and so the AFDM of each individual (n = 90) was determined at the end of the trials. Individual feeding rates were then controlled for animal size.

Each alga from the palatability trials (Sections 2.3.1 and 2.3.2 above), were assessed for phlorotannin abundance. Samples from the fronds were washed in distilled water, frozen at -20°C, and then freeze-dried to constant weight. These were ground until homogenous, and 0.2 g subsampled for chemical assay. 60 % aqueous acetone was used to extract phlorotannins over 1 hour under constant agitation, in the dark. The algal pulp was separated by centrifugation (5300 rpm for 10 minutes) and the acetone removed using *in-vacuo* cold-distillation (80 kPa, 38 °C). Lipophilic compounds were filtered from this extract (Pavia and Toth, 2000) and 40% Folin-Ciocalteu’s phenol reagent (Sigma-Aldrich F9252) was used in

conjunction with 1M aqueous sodium carbonate decahydrate solution (Sigma-Aldrich 71360) to act as a buffer. The resultant solution was incubated in the dark for 30 minutes and analysed by spectrophotometry at 760 nM, using phloroglucinol (1,3,5-trihydroxybenzene, Sigma-Aldrich P3502) as a standard (Van Alstyne, 1995). Replicates were run in triplicate. Differences in phlorotannin abundance for *S. muticum* used in the palatability trials were assessed with a one-way analysis of variance (ANOVA). Change in algal mass was calculated by averaging the increase in weight observed in the control clippings for each individual alga, and adding this value to the loss in weight observed in the experimental clippings. A one-way ANOVA was used to analyse the ‘palatability’ consumption, and a two-way ANOVA used to analyse the ‘willingness-to-feed’ consumption with ‘site’ and ‘species’ as fixed orthogonal factors.

### 2.3.3 The willingness to consume *S. muticum* and a native alga by *Littorina fabalis*

To compare willingness-to-feed on *Sargassum muticum* and a native sympatric alga by mesoherbivores (*Littorina fabalis*) from a site invaded by *S. muticum* 40 years prior, to those from a site invaded 10 years prior, three *S. muticum* and three *A. nodosum* individuals were collected at random from 40YR and 10YR sites (n = 6 of each species). *Littorina fabalis* were collected at random from *Fucus serratus*, *Fucus vesiculosus*, *Fucus spiralis*, and from the substrata nearby to the *S. muticum* and *A. nodosum* stands at these locations. One clipping was provided to three randomly-assigned *L. fabalis* in each of the following treatments: (1) 40YR *L. fabalis* with 40YR *S. muticum*, (2) 40YR *L. fabalis* with 40YR *A. nodosum*, (3) 40YR *L. fabalis* with 10YR *S. muticum*, (4) 40YR *L. fabalis* with 10YR *A. nodosum*, (5) 10YR *L. fabalis* with 40YR *S. muticum*, (6) 10YR *L. fabalis* with 40YR *A. nodosum*, (7) 10YR *L. fabalis* with 10YR *S. muticum*, (8) 10YR *L. fabalis* with 10YR *A. nodosum*. All trials were run in triplicate (n = 72). *Littorina fabalis* were of similar sizes from both sites and therefore it was not deemed necessary to correct for animal dry mass.

### 2.3.4 *L. fabalis* preference for *S. muticum* over native alga

To compare preferences of 40YR and 10YR *Littorina fabalis* when offered *Sargassum muticum* and *Ascophyllum nodosum* from their native site, and when offered 40YR *S. muticum* and 10YR *S. muticum*, each of the *S. muticum* individuals was randomly paired with another *S. muticum* individual from the other site, and one *A. nodosum* individual from its local site. One clipping of each was then offered to six *L. fabalis* individuals in the

following four treatments: (1) 40YR *L. fabalis* with 40YR *S. muticum* & 40YR *A. nodosum*, (2) 40YR *L. fabalis* with 40YR *S. muticum* & 10YR *S. muticum*, (3) 10YR *L. fabalis* with 10YR *S. muticum* & 10YR *A. nodosum*, (4) 10YR *L. fabalis* with 40YR *S. muticum* & 10 YR *S. muticum*. All trials were run in triplicate ( $n = 36$ ). *Littorina fabalis* were of similar sizes from both sites and therefore it was not deemed necessary to correct for AFDM. This experiment could not be run fully-orthogonally (e.g. including ‘40YR *L. fabalis* with 40YR *S. muticum* & 10YR *A. nodosum*’ or ‘10YR *L. fabalis* with 40YR *S. muticum* & 10YR *A. nodosum*’) because both space in the outdoor aquarium was limited, and some of the individuals used – particularly the *A. nodosum* – did not bear sufficient apical material to conduct every variation of treatments.

Changes in algal mass from the no-choice experiment (Section 2.3.3) were analysed using a three-way ANOVA with ‘familiarity’ (i.e. algal origin in relation to herbivore origin), ‘species’ (of algae), and ‘site’ (from which the herbivores were taken) as fixed orthogonal factors. Change in algal mass from the choice experiments (Section 2.3.4) were converted into changes in dry-mass, based on wet-mass to dry-mass conversion ratios calculated for each individual alga used. Estimated changes in dry-mass are then independent of one another and were therefore analysed parametrically with two two-way ANOVAs (Pavia et al., 2002). The first with ‘site’ and ‘species’, as fixed orthogonal factors, and the second with ‘*S. muticum* site’ and ‘*L. fabalis* site’ as fixed orthogonal factors.

All data were Levene’s-tested to ensure conformity to the assumption of homogeneity of variance, Anderson-Darling-tested to ensure data were approximately normal, and means were compared using Tukey’s HSD post-hoc tests.

### 3. Results

#### 3.1 The palatability of *S. muticum* to naïve grazers

*Sargassum muticum* from 35YR contained the highest abundance of phlorotannins (5.0% DW, S.E. = 0.15), and that from 19YR (3.6% DW, S.E. = 0.23) and 10YR (3.8%, S.E. = 0.14) bore similar concentrations (ANOVA  $F_{2,15} = 18.66$ ,  $SS = 7.07$   $p < 0.001$ , Tukey’s HSD; 35YR > 19YR = 10YR). However, naïve *Littorina obtusata* taken from Bull Bay did not respond to these differences, consuming similar levels of *S. muticum* tissue irrespective of its origins (ANOVA,  $F_{2,51} = 0.15$ ,  $SS = 5706$ ,  $p = 0.861$ , Figure 2a).

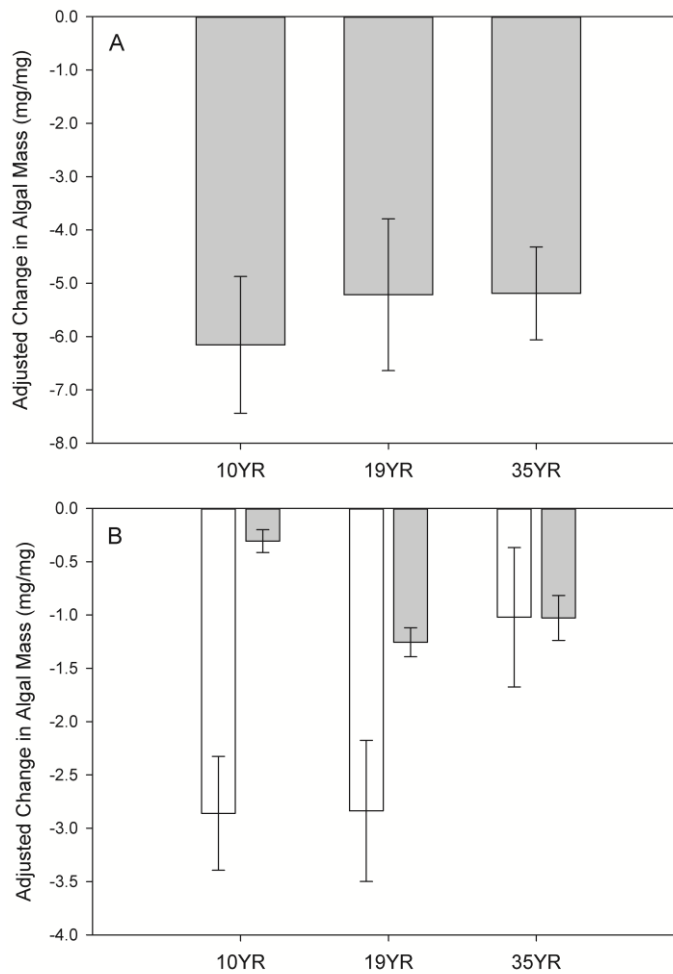


Figure 2: a) Change in algal mass (mg) after seven days, of *Sargassum muticum* fronds collected from populations established for different lengths of time (named by reported time-since-invasion), by *Littorina obtusata* naïve to *S. muticum*. b) Change in algal mass per unit ash-free-dry-mass of herbivore (mg<sup>-1</sup>) of *Fucus serratus* (white bars) and *Sargassum muticum* (grey bars) after seven days in the presence of *Littorina obtusata* collected from *S. muticum* populations established for different lengths of time (named by reported time-since-invasion). All data are adjusted for autogenic changes in algal mass. Error bars show +/- one S.E.

### 3.2 Willingness of exposed grazers to feed upon *S. muticum*

There were significant differences between the willingness-to-feed on *S. muticum* by *L. obtusata* from all three sites (Table 1, Figure 2b). Although *L. obtusata* consumed similar amounts of *Fucus serratus* per mg of animal dry mass regardless of site (ANOVA,  $F_{2,42} = 2.9$ ,  $SS = 33.43$ ,  $p = 0.066$ ), consumption of *S. muticum* differed. *Littorina obtusata* from 10YR



consumed less *S. muticum* than those from 19YR, which consumed similar amounts to those from 35YR (ANOVA,  $F_{2,42} = 9.95$ ,  $SS = 7.369$ ,  $p < 0.001$ ). However, consumption of *F. serratus* and *S. muticum* by 35YR *L. obtusata* was similar, indicating that these herbivores were prepared to consume the invasive as readily as a native, in contrast to those from 19YR and 10YR, which demonstrated a greater willingness-to-feed on the native *F. serratus*. Overall, consumption of algal material per unit dry mass of herbivore was similar between the three sites (ANOVA,  $F_{2,87} = 2.10$ ,  $SS = 15.73$ ,  $p = 0.128$ ).

Table 1: 2-way ANOVA statistics for the feeding preferences of *Littorina obtusata* collected from three sites, each bearing *Sargassum muticum* for different lengths of time (10, 19, and 36 years), in no-choice feeding trials on *Sargassum muticum* and *Fucus serratus* collected from those sites.

	<b>DF</b>	<b>MS</b>	<b>F</b>	<b>P</b>
Site	2	7.87	2.57	= 0.083
Species	1	42.61	13.90	< <b>0.001</b>
Site x Species	2	12.53	4.09	= <b>0.020</b>
Residual	84	3.065		

### 3.3 The willingness-to-feed on *S. muticum* and a native alga, by *L. fabalis*

There were significant differences between the willingness-to-feed upon both *S. muticum* and *Ascophyllum nodosum* from both sites, by both populations of *L. fabalis*. *Littorina fabalis* from both populations consumed roughly equal amounts of the native *A. nodosum* in the no-choice feeding trials, and both preferred the 10YR *A. nodosum* (Figure 3). 40YR *L. fabalis* consumed more *S. muticum* from both the old and the young population, than did 10YR *L. fabalis* (Figure 3). Interestingly, 40YR *S. muticum* was the more palatable for both populations of *L. fabalis*. There was a significant interaction between familiarity of material and herbivore population, because 40YR *L. fabalis* consumed more algal material from their local site than did 10YR *L. fabalis*, but this was an artefact of the palatability of 40YR *S. muticum* and 40YR *L. fabalis*' strong preference for it (Table 2).

Table 2: 3-way ANOVA statistics for the feeding preferences of *Littorina fabalis* collected from two sites, one bearing *Sargassum muticum* for 40 years, and one for 10 years, in no-choice feeding trials on *Sargassum muticum* and *Ascophyllum nodosum* collected from those sites.

	<b>DF</b>	<b>MS</b>	<b>F</b>	<b>P</b>
Familiarity	1	2952	0.34	= 0.564
Species	1	48724	5.56	= <b>0.021</b>
Site	1	37037	4.22	= <b>0.044</b>
Familiarity x Species	1	12827	1.46	= 0.231
Familiarity x Site	1	54066	6.17	= <b>0.016</b>
Species x Site	1	53737	6.13	= <b>0.016</b>
Familiarity x Species x Site	1	458084	52.25	< <b>0.001</b>
Residual	64	8767		

#### 3.4 *L. fabalis* preference for *S. muticum* over a native alga

There were significant differences in the preferences of *L. fabalis* from 10YR and 40YR when provided the choice between the native *A. nodosum* and the invasive *S. muticum*. When given the choice to feed on either *S. muticum* or *A. nodosum*, and on *S. muticum* from both sites, 40YR *L. fabalis* consumed more algal material overall than did 10YR *L. fabalis* (Figure 4, Table 3a, b). *Littorina fabalis* from 40YR showed a strong preference for *S. muticum* over *A. nodosum* (Figure 4a), but no preference for either species was observed in 10YR *L. fabalis* (Figure 4 b, Table 3a).

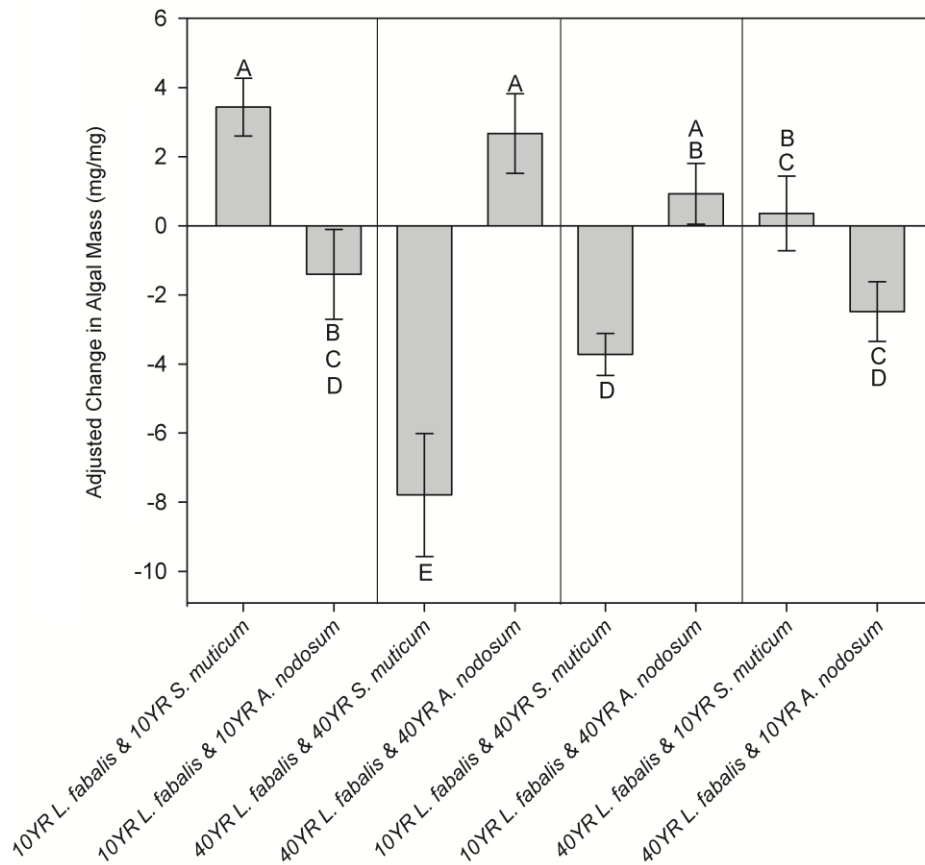


Figure 3: Change in algal mass (mg) of *Sargassum muticum* and *Ascophyllum nodosum* fronds collected from populations established for different lengths of time (named by reported time-since-invasion of *S. muticum*), after seven days of exposure to *Littorina fabalis* collected from those same sites, in no-choice feeding trials. All data are adjusted for autogenic changes in algal mass. Error bars show +/- one S.E. Letter groupings are based on Tukey's HSD post-hoc comparison of ANOVA.

10YR *Littorina fabalis* in fact reduced its feeding-rate in the presence of *S. muticum*, such that very little or no feeding upon 10YR *A. nodosum* was observed in these treatments (Figure 4b) in contrast to no-choice trials (Figure 3). In accordance however with the no-choice trials, *L. fabalis* from both populations showed a preference for 40YR *S. muticum* over 10YR *S. muticum*, and 40YR *L. fabalis* consumed more of it than 10YR *L. fabalis* (Figure 4 b & c). However there was no significant effect of site on this preference, both sets of *L. fabalis* finding 40YR *S. muticum* equally preferable over 10YR *S. muticum* (Table 3b).

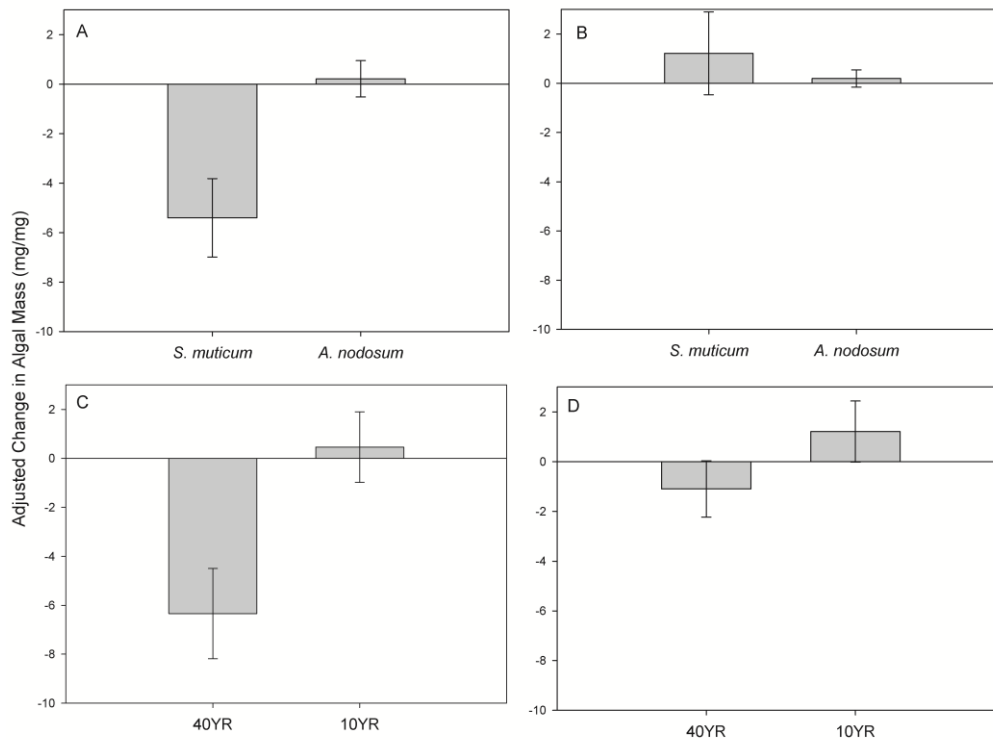


Figure 4: Change in algal mass (mg) of *Sargassum muticum* and *Ascophyllum nodosum* collected from sites bearing *S. muticum* populations of different ages (named by reported time-since-invasion of *S. muticum*), after seven days of exposure to *Littorina fabalis* collected from the same sites, in choice feeding trials. a) 40YR *L. fabalis* fed 40YR algae, b) 10YR *L. fabalis* fed 10YR algae, c) 40YR *L. fabalis* fed on *S. muticum* from both sites, d) 10YR *L. fabalis* fed on *S. muticum* from both sites. All data are adjusted for autogenic changes in algal mass. Error bars show +/- one S.E.

Table 3: a) 2-way ANOVA statistics for the feeding preferences of *Littorina fabalis* collected from two sites, one bearing *Sargassum muticum* for 40 years, and one for 10 years, in choice feeding trials on *Sargassum muticum* and *Ascophyllum nodosum* collected from those sites. b) 2-way ANOVA statistics for the feeding preferences of *Littorina fabalis* collected from two sites, one bearing *Sargassum muticum* for 40 years, and one for 10 years, in choice feeding trials on *Sargassum muticum* collected from both sites.

a)

	<b>DF</b>	<b>MS</b>	<b>F</b>	<b>P</b>
Site	1	76729	7.26	= <b>0.011</b>
Species	1	37249	3.52	= 0.070
Site x Species	1	77841	7.36	= <b>0.011</b>
Residual	32	10570		

b)

	<b>DF</b>	<b>MS</b>	<b>F</b>	<b>P</b>
<i>S. muticum</i> site	1	146306	10.04	= <b>0.003</b>
<i>L. fabalis</i> site	1	63420	4.35	= <b>0.045</b>
<i>S. muticum</i> site x <i>L. fabalis</i> site	1	7112	0.60	= 0.128
Residual	32	14569		

#### 4. Discussion

There were considerable differences in both the willingness-to-feed upon *Sargassum muticum*, and the preferences for it over native algae, by mesoherbivores from populations exposed to it for different lengths of time. The greater the time-since-invasion, the greater the acceptance of *S. muticum* by herbivores from those sites. Chemical defences in *S. muticum* also correlated with time-since-invasion in one experiment (but see Chapter 4), although palatability tested by naïve herbivores did not, indicating that some exposure to an alga is required to develop preferences. Palatability was, in fact, higher in the older of the two populations sampled in a different experiment, where grazers did have some experience of the invasive. Interestingly, although herbivores from an ‘old’ *S. muticum* population consumed more of it than those from a ‘young’ population, both groups of herbivores demonstrated similar behaviours in choice. Each preferred both *S. muticum* from the ‘old’ site, and *Ascophyllum nodosum* from the ‘young’ site. This suggests that despite the differences in acceptance of *S. muticum* as a food-source, the capacity to detect food of

different quality remained consistent. In other words, herbivores from the ‘old’ site had not simply become less selective.

It is unclear whether the increased preference for *S. muticum* by grazers is the result of exposure to it over a long time, or whether increased abundance of the alga in ‘older’ populations simply leads to greater encounter-rate. However, since *S. muticum* abundance at any site is dependent on time, the two factors of repeated exposure through time and repeated exposure through space are interlinked. The longer *S. muticum* has been present, the more the local population of grazers will encounter it, and therefore the more they will eat it. These results provide evidence in support of the Enemy Release Hypothesis (ERH), which posits that non-natives experience lower herbivore pressures in their introduced ranges because local consumers are unfamiliar with them (Keane and Crawley, 2002). The ERH is a popular and widely cited explanation for the invasibility of many photoautotrophs, but it is now apparent that the hypothesis frequently – and perhaps more often than not – fails verification (Parker et al., 2006; Parker and Hay, 2005). Remarkably few studies have specifically tested the ERH with respect to time-since-invasion, and fewer still incorporate feeding-trials to directly assess preferences or willingness-to-feed of consumers. During the 1970’s, a number of studies directly assessed what was known as the “stability-time” hypothesis. This theory predicts that younger habitats, such as stands of non-native plants, should host lower faunal diversities. Little evidence was found to support it (discussed by Strong et al., 1977), however, these studies were largely focused on deliberately-introduced, and highly-palatable, crops such as sugar-cane, cultivated in near-monospecific fields. Time-since-introduction was in the order of centuries, and the fauna surveyed were highly-mobile flying insects (Strong et al., 1977). It is perhaps unsurprising, therefore, that such experiments found no evidence for a theory that bears many similarities to the ERH.

Evidence for the ERH in *S. muticum* can be largely divided into those studies which assessed herbivore abundance and diversity (e.g. Cacabelos et al., 2010a; Engelen et al., 2013; Norton and Benson, 1983; Strong et al., 2009; Viejo, 1999; Withers et al., 1975) and those like this one, which assessed feeding-rates by herbivores (e.g. Cacabelos et al., 2010b; Critchley et al., 1986; Engelen et al., 2011; Monteiro et al., 2009; Norton and Benson, 1983; Strong et al., 2009). Typically, observational studies on faunal abundance and diversity showed similar patterns between *S. muticum* and sympatric native algae, and therefore rejected the ERH. However, the results of feeding-trials usually show an aversion towards *S. muticum* or a preference for natives in feeding-trials, in support of the ERH. Although different to previous feeding-trials because of the incorporation of time-since-invasion, this

study also supports the ERH and shows different herbivore feeding preferences for natives over *S. muticum*. These apparently contradictory sets of conclusions could be the result of animals moving into *S. muticum* stands during the day for protection against visual predators, but then returning to native alga to feed during the night (Buschmann, 1990). However, the idea that branching morphology of host-alga has positive effects on survivability in this regard, has been challenged (Holmlund et al., 1990). Alternatively, those herbivores present on *S. muticum* could be feeding mainly on epiphytic material, and even detritus, which is abundant on *S. muticum*. Epiphytes and detritus have been shown to account for the majority of the diets of mesoherbivores collected from *S. muticum* fronds (Viejo, 1999), and the abundance of herbivores also positively correlates to epiphyte load in *S. muticum* stands (Cacabelos et al., 2010b).

Few other studies have found preferences for *S. muticum* over native algae, or even lack of preference for natives when paired with it (e.g. Engelen et al., 2011). However, Strong et al. (2009) demonstrated that the amphipod *Dexamine spinosa* exhibited very large preferences for *S. muticum* in Strangford Loch. The amphipod fed directly upon *S. muticum*'s fronds even when they were epiphytised, showing neither the preference nor the aversion towards epiphytes seen in other species of crustacean (Karez, 2000). *Sargassum muticum* was present in Strangford Loch for eight years prior to these feeding-trials, although it was probably abundant for less than five (Davison, 2009). These preferences therefore developed extraordinarily quickly, especially considering the time-since-invasion for *S. muticum* populations sampled in other studies which reported lack-of-preference for natives, such as the 23 years in the study by Cacabelos et al. (2010a), or the 35 years in the present study. The findings by Strong et al. (2009) may be due to the herbivore studied, as swimming crustaceans are more mobile than benthic gastropods and their feeding-modes differ, making it easier for them to feed on thinner algal fronds (Pavia and Toth, 2000) such as those of *S. muticum*. It is interesting that the gastropods in Monteiro et al. (2009), as well as those from the 'younger' stands in this study, exhibited preference for natives, whereas the crustaceans (both amphipods and isopods) in Engelen et al. (2011) did not. These studies therefore arrive at different conclusions with regards the ERH, possibly because of the capabilities of the herbivores used.

Engelen et al. (2013) did not specifically test time-since-invasion, but noted that the older *S. muticum* populations sampled had greater faunal diversity with respect to sympatric native algae. Had the experiment been undertaken at the older sites only, the ERH would also have appeared not to apply. Likewise, although not specifically tested, Monteiro et al. (2009)

discuss that the feeding-preferences for natives over *S. muticum* in the herbivores they assayed, did not differ with time. Three sites were used, and their estimated time-since-invasions were 22, 12, and 7 years. However, the results presented herein suggest modifications of gastropod preferences do not begin until somewhere between 19 and 35 years after invasion. Therefore when drawing conclusions from both field observations and feeding-trials with a view to testing the ERH, a number of important factors must be taken into consideration. These are the general palatability of the non-native (ideally assessed with data from its native range), the herbivores included (their relative motility, feeding-modes, and diet), and crucially, the time-since-invasion at the site or sites being sampled.

It is curious that despite differences in phlorotannin concentration in the *S. muticum* sampled, palatability appeared unaffected in the first experiment. The younger two populations bore similar abundances of phlorotannins, but the older population had around 1.5% more phlorotannin by dry mass. *Littorina obtusata* have previously been shown to respond to differences in phlorotannin of around 1% DM when feeding on *A. nodosum* (Pavia et al., 2002). Additionally, the no-choice trials in Experiment 2 (and those by Jogesh et al., 2008 and Toth et al., 2007) indicate that feeding-rates can be substantially different between treatments in such trials, in spite of the known effects of compensatory-feeding in no-choice experiments (Roa, 1992).

The lack of differences in feeding response could have arisen because the animals used were naïve to *S. muticum*. Three species of freshwater apple snail from the genus *Pomacea* have been shown to display large preferences for novel food sources, consuming around ten times as much material compared to familiar foods (Morrison and Hay, 2011). Likewise the terrestrial slugs *Arion subfuscus* and *Agriolimax caruanae* (*sensu Deroceras invadens*) initially preferred novel food sources, despite them containing toxins. These preferences had then ceased in a second feeding-trial with the newly-experienced individuals (Whelan, 1982). Crucially, the naïve *L. obtusata* in the palatability trials consumed around twice as much *S. muticum* material, per individual, than the experienced *L. obtusata* in the willingness-to-feed trials. This despite them being of similar size to those from the ‘oldest’ site, and substantially smaller than those from the ‘youngest’ site in those trials. Although naïve animals were specifically selected to avoid bias in recent foraging history, this may have been an error in design, although it does reveal that biotic resistance in the very early stages of an invasion may be exceptionally high (Parker and Hay, 2005), albeit temporary if the invasive proves unpalatable (When, 1982). That there were differences in phlorotannin abundance however, does suggest differences in herbivory between the sites sampled (e.g.



Toth et al., 2007). An assessment of herbivory with respect to phlorotannin abundance between *S. muticum* populations of different ages, would reveal a great deal about the purpose and ecological significance of phlorotannins in this species.

The results presented herein represent a preliminary assessment of grazing preference with time-since-invasion. Sampling a greater number of both sites and mesoherbivores, would permit a more concrete understanding of the behavioural shifts undertaken by these consumers in response to invasions. The logistical constraints of including time-since-invasion are notably restrictive, and even equipped with the chronology of *S. muticum*'s spread, selecting sites similar to one another proved to be difficult. Even those sampled differed in terms of species composition, such that allowances had to be made in the design of experiments. Additionally, caution must be applied to interpreting the broader ecological significance of these trials. As discussed previously (Monteiro et al., 2009), mesoherbivore feeding-behaviours in the laboratory are not necessarily reflected in the field. Equally, presence of herbivores on algae in the field, does not necessarily indicate direct herbivory on the tissues of the macrophyte (Viejo, 1999). It is worth noting that *S. muticum* is vastly more abundant at both of the 'older' sites sampled, and although grazing marks are visible upon the fronds, they grow to similar lengths observed elsewhere (Kurr, *unpublished data*). However, these findings still cast new light onto the processes underlying invasions by unpalatable photoautotrophs, and highlight the need to consider time-since-invasion during the design and interpretation of research into the ERH.

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## Chapter Six

### General Discussion

The research in this thesis demonstrates the value in incorporating chemical assays, physical and biological observational surveys, and feeding-trials to address important questions in ecology. Chapters Two and Three address the question, ‘does stress cause sexual dimorphism on *A. nodosum*?’, with Chapter Two taking an intensive look at three sites, and Chapter Three taking a broader view of seven sites. Each complements the other and both reveal different aspects of the reproductive ecology of *A. nodosum* that the other cannot. Chapter Two illustrates that sexual dimorphism occurred in *Ascophyllum nodosum* because of an increased reproductive burden on females at sites where germling and juvenile mortality were probably higher because of wave-stress and herbivory. Chapter Three strengthened this argument by demonstrating that stressors which impact on adults only do not cause sexual dimorphism – even if they are biologically significant – although some sexual dimorphism occurs irrespective of stress.

Chapters Four and Five arrive at slightly different conclusions. Both ask the question ‘does time-since-invasion change the ecology of *S. muticum*?’, and whilst Chapter Four found little evidence to suggest it did whilst taking a broad multi-faceted observational approach, Chapter Five showed the opposite through small-scale manipulative experiments. Both still complement each other by finding that which the other could not, although their combined results are harder to explain. Chapter Four showed that time-since-invasion has no impact on the chemical defences of *Sargassum muticum*, because the abundance and diversity of mesoherbivores remain constant across a 35 year time-frame. Diversity, however, correlated with increased chemical defences, suggesting that top-down pressure changed as a function of species-richness. Chapter Five complicates the findings of Chapter Four, by demonstrating that time-since-invasion influences the feeding rates and preferences of grazers, with those from populations exposed to *S. muticum* for longer showing greater propensity to eat it.

#### *Sexual dimorphism in Ascophyllum nodosum*

The findings in Chapters Two and Three are primarily important for two reasons. Firstly, this is one of few studies to specifically test for the modification of sexual dimorphism with stress. Although other studies have found increasing degrees of sexual dimorphism in



populations of plant growing in difficult conditions (e.g. Lloyd & Webb 1977, Elmqvist & Gardfjell 1988, Popp & Reinartz 1988, Dawson & Bliss 1989, Boecklen *et al* 1990, Dawson & Ehleringer 1993, Boecklen & Hoffman 1993, Kohorn 1995, Dudley 2006), none quantified stress, and investments into growth, defence, and reproduction, and used feeding-trials simultaneously to elicit such a comprehensive view. Likewise, no study so far has assessed sexual dimorphism at multiple scales to reveal which stressors cause sexual dimorphism to be expressed. The holistic approaches in this thesis are readily applicable to studies of many species of plant and algal species and would reveal much about the driving-forces behind sexual dimorphism in both.

Secondly, very few studies have found sexual dimorphism in the somatic traits of seaweeds, and even fewer have specifically tested for it (Engel *et al* 2001, Verges *et al* 2008, Payo *et al* 2011, Viejo *et al* 2011, Lipinska *et al* 2015). Macroalgae possess a huge variety of sexual strategies (Frenkel *et al* 2014), and like plants, many algal genera contain dioecious species (Bold & Wynne 1985). Since sexual dimorphism in growth, defence and ecology are borne of differences in the requirements for each reproductive role (Barrett & Hough 2012), it might be expected that sexual dimorphism be extensive in macroalgae. It is unusual therefore that so few studies report sexual dimorphism in algae when it is so abundant in plants. There are two factors which may explain this disparity in plant and algal research.

Firstly, as organisms plants are far more complicated than algae. Angiosperms, which have been the focus of most studies on sexual dimorphism in primary producers, have a wide-range of interconnected tissues from various below-ground structures bearing mutualistic fungi (Brundrett 2002), to an array of above-ground structures; leaves, branches, flowers, and ramets, all of which can display sexual dimorphism (Cepeda-Cornejo & Dirzo 2010, Midgley 2010, Sánchez Vilas *et al* 2010, Hesse & Pannell 2011, Delph & Herlihy 2012). Sexual dimorphism in reproductive characters is pronounced in angiosperms because females produce expensive fruits which require energy and water (Harris & Pannell 2008). Macroalgae on the other hand comprise of at most four tissue types, the holdfast, the stipe, the frond, and reproductive tissues, and in many cases these tissues lack distinct separation (Bold & Wynne 1985). Likewise the sexual characters between males and females are often very alike, with both sexes producing similar fruiting bodies (Vernet & Harper 1980, Dudgeon *et al* 2001, but see Liu *et al* 2012) that are often inexpensive to produce, not least because of their ability to photosynthesise (Guillemin *et al* 2013). Therefore, both the reasons and the capacity for algae to manifest sexual dimorphism is probably limited, particularly since many somatic structures can be genetically constrained from differentiating

to avoid their functions being compromised (Spigler *et al* 2010, Yu *et al*, 2011). Crucially, sexual dimorphism never manifested in growth in *A. nodosum*, and this unsurprising because being large is important to each individual (as discussed in Chapter Two). Secondly, although considerable effort has been expended in studying seaweeds, compared to research on plants which have been both 1) readily accessible, and 2) more important as resources, research into macroalgae is immature. In this thesis, sexual dimorphism was detected in *A. nodosum* because of the considerable amount of sampling effort invested both in time and space. Sample sizes were orders of magnitude larger than any previous research into phlorotannin abundance, and were dispersed over many months, a tactic seldom seen even in plant studies (Cornelissen & Stiling 2005). In effect, demonstrating sexual dimorphism proved to be difficult and even then it was largely confined to two of seven sites, and only manifested for a brief period of time. Therefore although sexual dimorphism may indeed be common in seaweeds, it might be so subtle as to have largely gone unnoticed.

#### *The Invasive Ecology of Sargassum muticum*

The findings in Chapters Four and Five are less conclusive than those in Two and Three, and are of value largely because they reveal interesting avenues of research, rather than that they demonstrate clear patterns. As discussed in Chapter Five, each reaches different conclusions when testing the Enemy Release Hypothesis (Keane & Crawley 2002), which predicts that invasives in new habitats are released from top-down pressure. This theory hinges on the idea that consumers ignore invasive species because 1) co-evolved specialists are absent and 2) generalists prefer native plants. Evidence against this latter view is abundant (Parker & Hay 2005), although the ERH is still investigated and evidence continues to mount both for (e.g. Schaffner *et al* 2011) and against it (e.g. Lombardero *et al* 2012). The reason for this contradiction is discussed below, but in essence the ERH functions in some circumstances but not others, and the specific reasons for this probably differ on a near case-by-case basis.

Generalist consumers come in as many varieties as invasive plants, and whether or not the former will consume the latter depends on many variables. There are three factors to consider when a herbivore selects a food: 1) It must actually recognise the material as a food, 2) It must be mechanically capable of handling the tissue (i.e. it possesses appropriate mouthparts to render the plant's cells), and 3) It must be physiologically capable of digesting the material (i.e. it possesses the appropriate digestive apparatus or gut-fauna, to result in a net-positive energy flux upon consuming the plant's cells). In the case of an invasive food-

source, it is more likely that these three conditions will be met if the invasive is similar to native food sources (Lombardero *et al* 2012, Saul & Jeschke 2015). This is probably why, in Chapter Four, phlorotannin abundance correlated to species diversity – the more species present, the greater the probability that some mesoherbivores were already consuming similar species to *S. muticum* and therefore the transition onto eating the invasive would have been easier.

These ‘transitions’ may occur through a number of non-mutually-exclusive routes. Firstly, shifts in either population or individual-level behaviour over generations may be responsible. Generalist consumers exert different top-down pressures to specialist consumers (Lankau 2007). Within these generalist species are individuals predisposed to risk-prone behaviour and this fraction of the community is the most likely to accept a novel food-source (Wilson *et al* 1994; Wolf *et al* 2007). Should capitalising on a novel food-source offer benefits to fitness, this fraction of the community will grow in size and competitive ability (Dingemanse & Réale 2005, Smith & Blumstein 2008), and in such a scenario an herbivore-mediated biotic resistance may develop (Maron & Vilá 2001, Levine *et al* 2004, Eschtruth & Battles 2009). However, feeding choices are not always preferential or even facultative and the dynamic between mesoherbivore behaviour and invasive-proliferation is poorly understood. Risk-prone individuals may be so because of competitive inferiority (McKillup & McKillup 1994), or because they are juveniles and therefore more likely to prioritise shelter over food-quality, which may result in unusual choices of host plants or algae (Gosselin & Chia 1995, Jormalainen *et al* 2001). In these instances, feeding preferences for an invasive will be obligate, and therefore unpredictable (Vandendriessche *et al* 2006).

Furthermore, microbes in the guts of marine invertebrates have been shown to increase digestive efficiency (Bärlocher 1982), provide additional outputs from digestion (Odintsov 1981; Wainwright & Mann 1982), and provide resistance to toxins (Zachary *et al* 1983), including polyphenolics (Tugwell & Branch 1991). Since many common algal anti-herbivore defences also possess anti-microbial functions (Pavia & Toth 2008), it is likely that the mutualism between herbivore and gut-flora has considerable influence over algal-herbivore interactions. Gut microbes of mesoherbivores can vary both within and between populations, being more abundant in some individuals, or absent altogether in others (Atlas *et al* 1982, Mattila *et al* 2014). These variations in microbial consortia occur when such microbial species are ‘transient’, being capable of functioning extraneously from their hosts, and their possession requires that the mesoherbivore ingest them from the environment (Harris 1993), which during the proliferation of an invasive will be changing. It is unclear

how long it might take a herbivore to garner the correct suite of microbes to functionally digest an invasive. Some species may already possess the correct consortia, others may acquire one over time, and others may never acquire one at all (Sullam *et al* 2012).

Therefore finding complete enemy release or biotic resistance in an invasive may almost be as unusual as finding sexual dimorphism in algae. Chapter Five demonstrated that littorinids from populations exposed to *S. muticum* for less time will impart less top-down pressure than those from populations exposed to it for longer, and this may have been because of behaviour, microbiology, or both. However, this pattern may not be observed for all species, and some species may be more likely to demonstrate this pattern than others. Very few littorinids were actually found on *S. muticum* in Chapter Four, with the exception of the very oldest site. In fact, only one species was found in every stand of *S. muticum* (*Gammarus locusta*) and this was rarely in abundance. The only other organism found associated with every *S. muticum* population was the epiphytic *Ectocarpus sp.*, and it is probably that this was the primary food of the *G. locusta* found (Costa & Costa 1999, Viejo 1999, Cacabelos *et al* 2010, but see Karez *et al* 2000). Therefore even with their increased propensity to consume *S. muticum*, top-down pressure by littorinids is probably minimal at most sites, and the same can be said for many other species. Strong *et al* (2009) demonstrated a considerable preference for *S. muticum* by an amphipod (*Dexamine spinosa*) but was quick to point out that abundance of *S. muticum* in their study site appeared unaffected. The same can be said of the oldest *S. muticum* population in Chapter Four, which hosted over one hundred (albeit very small) *L. fabalis* per individual, which were visibly causing frond damage. However, abundance of *S. muticum* at this site was still far greater than at any other location. A pure study of abundance at either site would conclude that ERH is in effect because of *S. muticum*'s rampant spread, whilst a study of feeding rates would show evidence for Biotic Resistance.

Therefore as discussed in Chapter Five, the precise methodology used to investigate invasive ecology appears to hold considerable sway over the results, because invasive ecology is particularly complex and multi-faceted (Johnson & Chapman 2007). For one experiment in Chapter Five, palatability of algae was tested using naïve herbivores. These were selected specifically to avoid biasing the results by collecting individuals which had never before encountered *S. muticum*, but which then consumed large quantities of the entirely novel food, irrespective of phlorotannin abundance (*sensu* Whelan 1982, Morrison & Hay 2011). Therefore even using the same species, with the same basic methodology, and in the same aquaria, provided entirely different results to the other experiments. It is

unsurprising therefore that, considering the variety of invasive species, herbivores, and methodologies used in invasive research, contrasting results are regularly found with regards the ERH, and other theories on invasive ecology.

As such, invasions cannot be studied in the same regard as other aspects of biology and ecology, with ‘model species’ representing all invasives and their herbivores (discussed previously by Parker *et al* 2006). Instead, genuinely holistic, highly-intensive approaches which consider herbivore behaviour, herbivore physiology, and that of the invasive are required. The outcome of an invasion depends on the co-similarity between its accumulated eco-evolutionary experience, and that of its enemies and competitors (Cox & Lima 2006, Saul *et al* 2013, Saul & Jeschke 2015). In other words, if the new niche filled by an invasive is bordered by species which lack the tools or ability to impart *significant* pressure upon it (e.g. through competition or herbivory) it will spread (i.e. ERH), and *vice-versa* (i.e. Biotic Resistance). Which scenario manifests will vary on a case-by-case basis, as will the underlying mechanisms responsible. Furthermore, the results in Chapters Four and Five suggest that the niche occupied by an invasive also changes through time. Although phlorotannins did not relate to time-since-invasion, with one exception living-biomass showed a clear pattern for lower condition with greater population age. Whether this was a result of internal shifts in the algae (e.g. senescence) or external pressures from the biotic environment (e.g. herbivory, competition, pathogens) may be unclear, but considering the negative relationships between phlorotannin and living-biomass in *S. muticum*, and both tissue types studied in *A. nodosum*, this suggests some physiological correlates may be associated with time-since-invasion.

It is incredibly difficult to correctly assay the changing ecology of an invasion, to factor in all variables responsible for the stress experienced by an invasive and appreciate how these might change in the future. Furthermore, findings on one species or habitat may not apply to even similar invasive scenarios, making it very difficult to develop practical models which may apply to all invasions. This, however, must be the next step in invasive ecology if we are to further understanding of our rapidly changing biome and the threat invasive species present.

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