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Time-since-invasion increases native mesoherbivore feeding rates on the invasive alga, *Sargassum muticum* (Yendo) Fensholt

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Abstract:	Invasive 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. This study assessed the feeding behaviours of two species of gastropod herbivore from populations exposed to the invasive alga <i>Sargassum muticum</i> for different lengths of time. Feeding-trials, consisting of both choice and no-choice, showed that the herbivores from older stands (35–40 years established) of <i>S. muticum</i> were more likely to feed upon it than those taken from younger (10–19 years established) stands. These findings provide evidence in support of the ERH, by showing that herbivores consumed less <i>S. muticum</i> if they were not experienced with it. These findings are in accordance with the results of other feeding-trials with <i>S. muticum</i> , 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.

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1 TSI INCREASES FEEDING RATES ON S. MUTICUM

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3 **Time-since-invasion increases native mesoherbivore feeding rates on the invasive alga,**

4 ***Sargassum muticum* (Yendo) Fensholt**

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17 *Abstract*

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35

36 *Key Words:* Behaviour, Enemy Release Hypothesis, Feeding trials, Herbivory, Invasive
37 species, Non-native species

38

39 1. INTRODUCTION

40

41 The introduction of invasive marine algae can have substantial negative impacts upon native
42 communities in their newly established range (Williams & Smith, 2007; Thomsen *et al.*,
43 2009). When an invasive plant or alga begins to proliferate in a new range, it presents a novel
44 food-source to native consumers. Although specialists may avoid the invasive organism,
45 generalist consumers can be attracted to such species and even prefer to consume them over
46 native species (Parker & Hay, 2005; Parker *et al.*, 2006). However, this is not always the case
47 and in many instances both plants (e.g. Jogesh *et al.*, 2008) and algae (e.g. Davis *et al.*, 2005)
48 have been shown to be avoided by generalist consumers. Herbivore preference may therefore
49 account for the invasibility of some species, a scenario described by the Enemy Release
50 Hypothesis (ERH) (Keane & Crawley, 2002).

51 *Sargassum muticum* Yendo (Fensholt) is a highly invasive marine alga which is not
52 controlled by large herbivores such as fish and urchins (Britton-Simmons, 2004; Thomsen *et*
53 *al.*, 2006; Engelen *et al.*, 2015). Although it attracts a range of mesoherbivores (Strong *et al.*,
54 2009), many of these still prefer to feed upon native algae or the epiphytes on *S. muticum*
55 (Norton & Benson, 1983; Critchley *et al.*, 1986; Viejo, 1999; Britton-Simmons, 2004;
56 Monteiro *et al.*, 2009; Cacabelos *et al.*, 2010a; Rossi *et al.*, 2010; Engelen *et al.*, 2011).
57 Because of their size, mesoherbivores are less mobile than large herbivores, and individuals
58 or localised populations can display strong host-plant specificity, even when the species as a
59 whole does not (Vesakoski *et al.*, 2009; Bell & Sotka, 2012; Mattila *et al.*, 2014). Specificity
60 is particularly evident in species that are slow-moving or brood their young (Sotka, 2005),
61 and may be because the host alga of a mesoherbivore is both its food and habitat, and some
62 algal species can provide better protection from predators (Watanabe, 1984; Jormalainen *et*
63 *al.*, 2001). As such it can be hypothesised that mesoherbivores, particularly species that are

64 less mobile, will establish a feeding preference for an invasive species such as *S. muticum*
65 when exposed to it, but the time scales involved in such development is unclear.

66 Optimally-foraging animals are expected to prefer readily available sources of food,
67 and in cases where host specificity is strong, an invasive population may therefore escape
68 local herbivores when it is first introduced to a new range (Maron & Vilá, 2001). However,
69 once an invasive species proliferates, encounter-rates with local consumers will increase and
70 these may then accept it as a food source. Therefore, it is likely that the longer such a species
71 has been present in an environment, the greater the propensity of local consumers to feed on
72 it will be (Schultheis *et al.* 2015). This has been shown to take as little as 20 years in beetles
73 that consume the leaves of invasive trees (Auerbach & Simberloff, 1988), and data on
74 introduced crops shows that species diversity can be as rich as that found in native
75 controphics after less than 200 years (Strong *et al.*, 1977). However, little is known about the
76 lengths of time it might take marine mesoherbivores to consume a species such as *S.*
77 *muticum*, and few studies that specifically incorporate time-since-invasion as a variable
78 utilise behavioural experiments. Most opt instead for observational surveys of abundance or
79 diversity to infer consumer choice (*sensu* Maron & Vilá, 2007). Time-since-invasion is a vital
80 and understudied element of invasive ecology, since both the invader and the local community
81 change the longer an invasive population has been established (Strayer *et al.*, 2006).

82 This study aimed to enhance our understanding of the ERH by investigating the
83 feeding behaviours of slow-moving mesoherbivore grazers, taken from stands of *S. muticum*
84 that have been established for different lengths of time. The Space-for-Time Substitution
85 methodology is well established in invasive ecology and climate change studies, and although
86 it is not fool-proof it provides a convenient alternative to the time-for-time approach which is
87 often not viable (Pickett, 1989; Thomaz *et al.*, 2012; Blois *et al.*, 2013). *Sargassum muticum*
88 is an ideal species with which to investigate mesoherbivore responses with this approach,

89 because it is well known beyond the scientific community as a pest. It is large, conspicuous,
90 intertidal, and prefers sheltered habitats and so is quickly reported when it spreads to
91 harbours and inlets frequented by fishermen and sailors. As such and in contrast to many
92 other marine invasions, a detailed and reliable chronology of its spread around western-
93 Europe and the British Isles exists (Davison, 2009). As a brown alga, *S. muticum* produces
94 quantifiable polyphenolic chemicals as a defence against herbivory (Van Alstyne & Paul,
95 1990; Pavia & Toth, 2000). Although not deterrent against all species, these phlorotannins
96 impede herbivory by a broad range of mesoherbivores, particularly slow-moving generalist
97 gastropod grazers (Pavia & Toth, 2008). Brown algae have been shown to increase
98 phlorotannin production in response to increased herbivory, making themselves less-palatable
99 (Van Alstyne & Paul, 1990; Pavia & Toth, 2008), although investigations into invasive plants
100 have shown variable responses in the production of chemical defences in their invaded ranges
101 (i.e. increases: Caño *et al.*, 2009; decreases: Willis *et al.*, 1999; no recorded change, but still
102 lowered palatability (i.e. the attractiveness of the food in the absence of others) in herbivore
103 performance trials: Hull-Sanders *et al.*, 2007). Using laboratory-based feeding experiments
104 with *S. muticum* and two species of common generalist gastropod grazers, this study tested
105 two hypotheses. Firstly, generalist gastropods are more likely to accept *S. muticum* as a food
106 source the longer it has been present in their local habitat. Secondly, the palatability of *S.*
107 *muticum* will be lower in long-established populations, compared to those recently
108 established. Testing these hypotheses aimed to further our knowledge of invasive ecology
109 and time-since-invasion effects, specifically with respects to the ERH.

110

111

112 2. MATERIALS AND METHODS

113

114 **2.1 Study organisms**

115 *Sargassum muticum* was used as a model invader because it bears all of the hallmarks of a
116 classic invasive marine species, being temperate, pseudo-annual, fast-growing and *r*-selected,
117 with broad physiological tolerances and a propensity for high dispersal rates and rapid growth
118 in areas of strong anthropogenic influences, such as harbours (Norton, 1977; Critchley *et al.*,
119 1986; Arenas *et al.*, 1995; Andrew & Viejo, 1998; Claridge & Franklin, 2002; Engelen &
120 Santos, 2009). *Sargassum muticum* was first sighted in the British Isles in 1973, on the
121 eastern coast of the Isle of Wight, and has subsequently spread as far east as Kent, and as far
122 north-west as the Firth of Clyde.

123 *Ascophyllum nodosum* (L.) Le Jolis and *Fucus serratus* (L.) were used as control
124 organisms to assess typical feeding rates from snails of the different populations. Both are
125 common native intertidal algal species, which are closely related to *S. muticum*. Because the
126 three species are all fucoids, they bear a similar chemical composition (Davis *et al.* 2003).
127 Both control species are also found on sheltered shores where they adhere to hard substrata in
128 the mid to lower intertidal (Boaden *et al.*, 1975; Dudgeon & Petraitis, 2005), and are
129 therefore often sympatric with *S. muticum*. Both are consumed by the selected grazers,
130 *Littorina obtusata* (L.) and *Littorina fabalis* (Turton), which are common grazers on
131 seaweeds throughout Western Europe (Watson & Norton, 1987; Hayward & Ryland, 2006)
132 and can be found on *Fucus spiralis*, *Fucus vesiculosus*, *A. nodosum*, *F. serratus*, *S. muticum*,
133 *Halidrys siliquosa*, *Ulva lactuca*, and *Ulva intestinalis* in high abundances on sheltered
134 shores, both in the intertidal and shallow subtidal (Kurr, unpublished data). Both species
135 show an aversion towards algal tissues containing high levels of phlorotannins, making them

136 viable indicators for ecologically relevant differences in algal defensive investment (Pavia &
137 Toth, 2000; Pavia *et al.*, 2002).

138

139 2.2 Study sites

140 Four *S. muticum* populations were sampled for material to be used in feeding trials (Figure 1).

141 All populations grew on the upper sub-tidal of moderately exposed sandy shores, in lagoons

142 formed in the lee of a rock formations or sand-bars. Salinity and temperature differences at

143 time-of-sampling were minimal (salinity within 1 unit, and temperature within 2 °C), and all

144 populations were amongst or very near to common native algae such as *F. serratus*, *A.*

145 *nodosum* and/or *F. vesiculosus*. The northernmost and southernmost sites differed by 3° of

146 latitude, and whilst UV exposure causes induction of phlorotannins (Pavia *et al.*, 1998), UV-

147 R levels (which account for a small percentage of total solar irradiance) differ by only 0.72%

148 (estimated from Šúri *et al.*, 2007; Escobedo *et al.*, 2009). If *S. muticum* responds to UV in the

149 same way as other fucoids, this would equate to an approximate difference of 0.4% in

150 phlorotannin abundance between the northernmost and southernmost sites (Pavia *et al.*,

151 1998), a negligible amount, considering that herbivory can induce phlorotannin production by

152 70% (Pavia & Toth, 2000).

153 For clarity, locations have been named by the length of time since the first

154 observation of *S. muticum* (Davison, 2009), as follows; ‘40YR’: Bembridge Ledge, on the

155 eastern coast of the Isle of Wight. ‘35YR’: Eastbourne on the south eastern coast of England.

156 ‘19YR’: West Angle Bay, near Milford Haven in south Wales. ‘10YR’: The northern shore of

157 the Foryd estuary near Caernarfon in North Wales (Figure 1). It can be assumed that the first

158 observation equates to time-since-invasion in *S. muticum* populations as it expresses limited

159 DNA polymorphism within UK and European populations, suggesting that it has spread from

160 a single point of invasion (Hallas, 2012; Le Cam, 2015). Communities impacted by an

161 invasive population with a time-since-invasion of 10 years or less, are expected to experience
162 considerably different effects to those exposed for 30 years or more, making the timescale
163 investigated here valid for detecting shifts in behaviour (see Strayer *et al.*, 2006).

164

165 **2.3 Feeding trials**

166 Four experiments were conducted: (1) *Sargassum muticum* from stands of different ages were
167 presented to *L. obtusata* collected from a site where *S. muticum* has not been recorded, to
168 assess the palatability of the algae (i.e. the attractiveness of the food in the absence of other
169 foods). (2) *Littorina obtusata* from *S. muticum* stands of different ages were presented with *S.*
170 *muticum* and *F. serratus* from one site, to assess the willingness-to-feed of the snails (i.e. how
171 eager the animals are to feed in the material in the absence of others). Finally, *L. fabalis* from
172 an 'old' and a 'young' *S. muticum* stand were presented with *S. muticum*, and the common
173 native alga *A. nodosum* from both sites in (3) no-choice trials to assess the willingness-to-
174 feed of grazers, and with *S. muticum* from the 'young' and 'old' stands in (4) choice trials to
175 determine feeding preferences of the snails (i.e. which material will be selected when given a
176 choice).

177 For each experiment, a standardised collection protocol was utilised. Firstly, all algae
178 were collected within one week of being used in trials, and maintained in ambient (~19°C,
179 salinity 34) seawater within the same outdoor aquaria used for feeding trials. Algae were
180 removed by pulling the holdfast from the substrata, epiphytes were cleared from the fronds,
181 and all individuals were returned to the laboratory within cool-boxes, inside of one day.

182 From each alga used, 500mg (+/- 50mg) blotted-wet-weight clippings were taken from the
183 apical region (one clipping per apex) and autogenic changes in algal mass were corrected for
184 by taking control clippings and maintaining these in parallel to the experimental trials.

185 Additionally, ~5 clippings were taken for phlorotannin analysis in palatability trials.

186 Handling of algae was kept to a minimum, and great care was taken during blotting of *S.*
187 *muticum* fronds to avoid dislodging vesicles or causing other damage.

188 Secondly, all animals used in the trials were collected one week prior to experiments
189 and maintained as above, without food to ensure even levels of hunger. Because starved
190 herbivores can display compensatory feeding for the first two days (Cronin & Hay, 1996)
191 change in algal masses were only recorded after seven days. Change in algal mass was
192 corrected for snail ash-free dry mass and compared with autogenic changes in the controls
193 (identical treatments without grazers) (Monteiro *et al.*, 2009; Forslund *et al.*, 2010). All trials
194 were conducted in separate 250 ml aquaria with an individual water-line providing a
195 flushing-time of around 30 s.

196

197 **2.3.1 The palatability of *S. muticum* to naïve grazers**

198 To determine whether *S. muticum*'s palatability was different depending on time-since-
199 invasion, six algal individuals were collected at random from each of the 35YR, 19YR, and
200 10YR populations (n = 18). To capture individual *S. muticum* variation, three clippings from
201 each individual were provided to three *L. obtusata* (n = 54) collected from Bull Bay in North
202 Wales (53.422543°N, 4.368959°W) in a no-choice trial. *Sargassum muticum* does not grow
203 at Bull Bay, nor on the adjacent coastline, and so these individuals were extremely likely to
204 be naïve to this food-source. This was done to avoid biasing the study by using
205 mesoherbivores with a history of *S. muticum* consumption, thereby ensuring differences in
206 feeding responses were a product of the algal condition, and not that of the consumer.

207 Although the no-choice technique has been criticised for not producing 'true' feeding
208 responses (Roa, 1992), no-choice trials were used for two reasons. Firstly, *S. muticum*
209 fragments as it is fed upon, making it impossible to differentiate between algae from most
210 sites at the end of the trials. Secondly, the technique still holds value when determining the

211 ‘willingness-to-feed’ of a particular herbivore on a plant or alga (i.e. how much material are
212 herbivores willing to consume; sensu Jogesh *et al.*, 2008), or ‘palatability’ of a particular
213 plant or alga (i.e. how easy the material is to consume; sensu Toth *et al.*, 2007), as opposed to
214 purely ‘preference’ of consumers, which requires that a choice be offered. No-choice trials
215 can therefore be useful in predicting the results of direct interaction between grazers and
216 hosts (Pearse *et al.*, 2013). Data were analysed in a One-way ANOVA, with site as a fixed
217 factor, the response variable was the mean of the three clippings from each individual alga to
218 provide a better estimate of the palatability of an individual *S. muticum* and to avoid non-
219 independence arising from using clippings from individual alga in the analysis (n = 18).

220 Phlorotannin abundances in each of the six *S. muticum* individuals were determined.
221 Samples from the upper frond were washed in distilled water, frozen at -20°C, and then
222 freeze-dried to constant weight. These were ground until homogenous, and 0.2 g subsampled
223 for chemical assay. 60 % aqueous acetone was used to extract phlorotannins over 1 hour
224 under constant agitation, in the dark. The algal pulp was separated by centrifugation (5300
225 rpm for 10 minutes) and the acetone removed using *in-vacuo* cold-distillation (80 kPa, 38
226 °C). Lipophilic compounds were filtered from this extract (Pavia & Toth, 2000) and 40%
227 Folin-Ciocalteu’s phenol reagent (Sigma-Aldrich F9252) was used in conjunction with 1M
228 aqueous sodium carbonate decahydrate solution (Sigma-Aldrich 71360) to act as a buffer.
229 The resultant solution was incubated in the dark for 30 minutes and analysed by
230 spectrophotometry at 760 nM, using phloroglucinol (1,3,5-trihydroxybenzene, Sigma-Aldrich
231 P3502) as a standard (Van Alstyne, 1995). Replicates were run in triplicate and phlorotannin
232 abundances compared with a one-way analysis of variance (ANOVA) with ‘site’ as a fixed
233 factor. Differences in means were compared using Tukey’s *post-hoc* tests.

234

235 **2.3.2 Willingness of grazers experienced with *S. muticum* to feed upon it**

236 To determine whether *L. obtusata* consumed more *S. muticum* when their population had
237 been exposed to it for longer, 30 individuals were collected at random from each of the
238 35YR, 19YR, and 10YR sites (n = 90). One *S. muticum* individual and one *F. serratus*
239 individual were collected from the 10YR site. Apex material from one large individual was
240 used for each algal species to keep phlorotannin abundances, algal condition, and any other
241 variables that may influence palatability, as constant as possible between treatments, given
242 that the focus in this experiment was on the grazers. Fifteen *L. obtusata* individuals were
243 provided with one clipping of the *S. muticum* individual each, and the other fifteen were
244 provided with one clipping of the *F. serratus* individual each to gauge for population-specific
245 differences in feeding-rates on a typical sympatric native alga. Differences in ‘willingness-to-
246 feed’ did not conform to the assumption of homogeneity of variance because the variability in
247 *F. serratus* consumption was greater than that for *S. muticum* consumption. Therefore, means
248 were compared using a Kruskal-Wallis test, and paired Mann-Whitney U tests for *post-hoc*
249 analysis.

250

251 **2.3.3 The willingness to consume *S. muticum* and a native alga by *L. fabalis***

252 To compare willingness-to-feed on *S. muticum* and a native sympatric alga by
253 mesoherbivores (*L. fabalis*) from a site invaded by *S. muticum* 40 years prior, to grazers from
254 a site invaded 10 years prior, three *S. muticum* and three *Ascophyllum nodosum* individuals
255 were collected at random from 40YR and 10YR sites (n = 6 of each species). *Littorina fabalis*
256 were collected at random from adjacent stands of fucoids, and from the substrata nearby to
257 the *S. muticum* and *A. nodosum* stands at these locations. One clipping of each alga was
258 provided to three randomly-assigned *L. fabalis* from each site, and all trials were run in
259 triplicate (n = 72). Changes in algal mass were analysed using a three-way nested-ANOVA
260 with ‘algal species’, ‘algal origin, and ‘grazer origin’ as fixed orthogonal factors, and

261 'individual' (alga) nested in the interaction between 'algal species' and 'algal origin'.

262 Differences in means were compared using Tukey's *post-hoc* tests.

263

264 **2.3.4 *L. fabalis* preference for *S. muticum* from sites with different time-since-invasion**

265

266 Clippings from the same algal individuals used for the willingness to feed experiment

267 (Experiment 3) were used to compare the preference of *L. fabalis* collected from the 40YR

268 and 10YR sites when offered *S. muticum* from their site of origin, against algae collected

269 from the other site. Each treatment was allocated to six *L. fabalis* individuals in the following

270 randomly assigned treatments: (1) 40YR *L. fabalis* with 40YR *S. muticum* & 10YR *S.*

271 *muticum*, and (2) 10YR *L. fabalis* with 40YR *S. muticum* & 10 YR *S muticum*. All trials were

272 run in triplicate (n = 18). To capture autogenic changes in algal mass each experimental

273 aquaria (i.e. with grazers) was paired with a control aquaria containing the same algal

274 combination but no grazers. Following Forslund *et al.* (2010), a paired *t* test approach was

275 used to compare the differences in algal mass-change between clippings in both the

276 experimental and control aquaria, whereby, a significant result indicates that one clipping has

277 changed mass more than the other in the presence of grazers.

278 3. RESULTS

279

280 **3.1 The palatability of *S. muticum* to naïve grazers**

281 *Sargassum muticum* from 35YR contained the highest abundance of phlorotannins
282 (5.0% DW, S.E. = 0.15), and that from 19YR (3.6% DW, S.E. = 0.23) and 10YR (3.8%, S.E.
283 = 0.14) bore similar concentrations (ANOVA $F_{2,15} = 18.66$, $SS = 7.07$, $p < 0.001$, Tukey's
284 HSD; 35YR > 19YR = 10YR). However, the consumption of *S. muticum* tissue from
285 different sites was not significantly different (ANOVA $F_{2,15} = 0.0741$, $SS = 1902$, $p = 0.929$;
286 Figure 2a), suggesting that naïve *L. obtusata* taken from Bull Bay did not respond to
287 differences in phlorotannins and/or any differing palatability of *S. muticum* from sites with
288 different time-since-invasion.

289

290 **3.2 Willingness of grazers experienced with *S. muticum* to feed upon it**

291 There were significant differences between the willingness-to-feed on the *S. muticum*
292 individual by *L. obtusata* from all three sites (Kruskal-Wallis, $H = 28.69$, $p < 0.001$; Figure
293 2b). *Littorina obtusata* consumed broadly similar amounts of the *F. serratus* individual per
294 mg of animal dry mass regardless of site, although those from 19YR consumed more *F.*
295 *serratus* than those from 35YR. Consumption of the *S. muticum* individual relative to the *F.*
296 *serratus* individual showed an incremental increase with time-since-invasion. The 10YR
297 grazers consumed less of the *S. muticum* individual than those from the other populations,
298 and more of the *F. serratus* individual. The 19YR grazers consumed as much of the *S.*
299 *muticum* as those from 35YR, but consumed more of the *F. serratus* than the *S. muticum*. The
300 35YR grazers consumed as much of the *S. muticum* as they did the *F. serratus* individual,
301 indicating that they were equally willing to feed on the invasive and native algae.

302

303 **3.3 The willingness-to-feed on *S. muticum* and a native alga, by *L. fabalis***

304 Willingness to feed differed between species and grazer origin (Table 1, Figure 3). 40YR *S.*
305 *muticum* was the most readily consumed by both groups of grazers, however the 40YR
306 grazers consumed more of the invasive overall. Additionally, whilst the 10YR grazers were
307 willing to consume 10YR *A. nodosum* in similar quantities to 40YR *S. muticum*, 40YR
308 grazers consumed more of the invasive. No differences in consumption of the native *A.*
309 *nodosum* were detected between grazer populations, both consuming more of that from
310 the 10YR site.

311

312 **3.4 *L. fabalis* preference for *S. muticum* from sites with different time-since-invasion**

313 The experienced grazers demonstrated a clear preference for *S. muticum* from one location,
314 but these preferences were not evident in the more naïve group. 40YR *L. fabalis* preferred
315 40YR *S. muticum* to 10YR *S. muticum* ($t_9 = 2.44$, $p = 0.041$, Figure 4a), whereas no
316 preferences were observed for *S. muticum* from either site by 10YR grazers ($t_9 = 0.87$, $p =$
317 0.411, Figure 4b).

318

319 4. DISCUSSION

320 There were considerable differences in the acceptance and selection of *Sargassum muticum*
321 as a food source, by mesoherbivores from populations exposed to it for different lengths of
322 time. Overall, the greater the time-since-invasion, the greater the feeding rates on *S. muticum*
323 by grazers from those sites (Experiments 2, 3 and 4). Chemical defences in *S. muticum* were
324 highest at the site with greatest time-since-invasion (Experiment 1), although palatability,
325 when tested by naïve grazers, did not differ (Experiment 1). Likewise, more experienced
326 grazers demonstrated a clear preference for *S. muticum* from one site when offered a choice,
327 whereas naïve conspecifics consumed indiscriminately (Experiment 4). This suggests that
328 some exposure to an alga may be required to develop subjectivities based on chemical
329 defence or condition (Experiment 1 and 4).

330 It is unclear whether the increased acceptance of *S. muticum* as a food source is the
331 result of exposure over decadal timescales, or differences in encounter rate because of greater
332 proliferation of the alga in older populations. However this point is probably moot, since the
333 abundance of a non-native species in an introduced range is also dependent on time (Bennett
334 *et al.* 2013; Byers *et al.*, 2014; Vicente *et al.*, 2014) Therefore the longer *S. muticum* has been
335 present, the more the local population of grazers will encounter it, and therefore the more
336 likely they will be to consume it. These results provide evidence in support of the Enemy
337 Release Hypothesis (ERH), which posits that non-native species experience lower herbivore
338 pressures in their introduced ranges because local consumers are unfamiliar with them
339 (Keane & Crawley, 2002). The ERH is a popular and widely cited explanation for the
340 invasibility of many photoautotrophs, but it is now apparent that the hypothesis typically fails
341 verification (Parker & Hay, 2005; Parker *et al.*, 2006). Only a few studies have specifically
342 tested the ERH with respect to time-since-invasion (Strayer *et al.* 2006, Heger and Jeschke

343 2014, Schultheis *et al.* 2015), and fewer still incorporate feeding-trials to directly assess
344 preferences or willingness-to-feed of consumers.

345 Despite our findings, the question of whether or not the ERH applies to *S. muticum*
346 remains unclear since top-down control by specialist grazers in its native range has never
347 been demonstrated. Certainly the impacts of any increase in grazer preference have not been
348 sufficient to limit the spread of this species around the UK. Evidence for the ERH in *S.*
349 *muticum* can be divided into those studies that assessed grazer abundance and diversity (e.g.
350 Withers *et al.*, 1975; Norton & Benson, 1983; Viejo, 1999; Wernberg *et al.*, 2004; Strong *et*
351 *al.*, 2009; Cacabelos *et al.*, 2010a; Engelen *et al.*, 2013) and those like this the current study
352 that assessed feeding by grazers (e.g. Norton & Benson, 1983; Critchley *et al.*, 1986;
353 Pedersen *et al.*, 2005; Monteiro *et al.*, 2009; Strong *et al.*, 2009; Cacabelos *et al.*, 2010b;
354 Engelen *et al.*, 2011). Typically, observational studies on faunal abundance and diversity
355 demonstrated similar patterns between *S. muticum* and sympatric native algae, and therefore
356 show evidence against the ERH (e.g. Cacabelos *et al.*, 2010a; Engelen *et al.*, 2013) However,
357 the results of feeding-trials usually show an aversion towards *S. muticum* or a preference for
358 native species in feeding-trials, in support of the ERH (e.g. Monteiro *et al.* 2009, Cacabelos *et*
359 *al.* 2010b). Although different to previous feeding-trials because of the incorporation of time-
360 since-invasion, the present study also supports the ERH. These apparently contradictory
361 conclusions may be the result of animals moving into *S. muticum* stands during the day for
362 protection against visual predators, but then returning to native alga to feed during the night
363 (Buschmann, 1990; Machado *et al.*, 2015). Alternatively, grazers on *S. muticum* could be
364 feeding mainly on epiphytic material and detritus in the field (Viejo, 1999; Cacabelos *et al.*,
365 2010b), and may therefore avoid *S. muticum* under laboratory conditions due to experimental
366 removal of epiphytes or the selection of individuals that are relatively epiphyte free.

367 However, a small number of previous studies have found preferences for *S. muticum*
368 over native algae (e.g. Pedersen *et al.*, 2005), or a lack of preference for native algae when
369 paired with it (e.g. Engelen *et al.*, 2011). Strong *et al.* (2009) demonstrated that the amphipod
370 *Dexamine spinosa* from Strangford Lough in Northern Ireland, exhibited a clear preference
371 for *S. muticum* in feeding trials. The amphipod fed directly upon *S. muticum*'s fronds even
372 when they were epiphytised, showing neither the preference for, nor the aversion towards,
373 epiphytes seen in other species of crustacean (Karez *et al.*, 2000). *Sargassum muticum* was
374 present in Strangford Lough for eight years prior to these feeding-trials, although it was
375 probably abundant for less than five (Davison, 2009). These preferences therefore developed
376 quickly, far less than the 23 years Cacabelos *et al.* (2010a) show it took for grazers to adapt to
377 *S. muticum*, or the 19–35 years in this study. The findings by Strong *et al.* (2009) may be due
378 to the grazer studied, as swimming crustaceans are more mobile than benthic gastropods and
379 their feeding-modes differ, making it easier for them to feed on thinner algal fronds (Pavia &
380 Toth, 2000) such as those of *S. muticum*. In addition, the gastropods in Monteiro *et al.* (2009)
381 exhibited preference for native algae, whereas the crustaceans (both amphipods and isopods)
382 in Engelen *et al.* (2011) did not. These studies therefore arrive at different conclusions for the
383 ERH, possibly because of the capabilities of the grazers used.

384 Engelen *et al.* (2013) did not specifically test time-since-invasion in field
385 observations, but noted that the older *S. muticum* populations sampled had greater faunal
386 diversity with respect to sympatric native algae. Had the experiment been undertaken at the
387 older sites only, the ERH would also have appeared not to apply. Likewise, Monteiro *et al.*
388 (2009) discuss that the feeding-preferences for native algae over *S. muticum* in the grazers
389 they assayed, did not differ with time-since-invasions of 22, 12, and 7 years. However, the
390 results presented here suggest modifications of gastropod preferences do not begin until
391 somewhere between 19 and 35 years after invasion. Therefore, when drawing conclusions

392 from both field observations and feeding-trials with a view to testing the ERH, the nature of
393 the grazers included (their relative mobility, feeding-modes, and diet), and the time-since-
394 invasion at the site or sites being sampled (Strayer *et al.*, 2006; Schultheis *et al.*, 2015) must
395 be taken into consideration.

396 It is curious that despite differences in phlorotannin concentration in the *S. muticum*
397 sampled, palatability appeared unaffected in the first experiment. The younger two
398 populations bore similar abundances of phlorotannins, but the older population had around
399 1.5% more phlorotannin by dry mass. *Littorina obtusata* responds to differences in
400 phlorotannin of around 1% DM when feeding on *A. nodosum* (Pavia *et al.*, 2002). The lack of
401 differences in feeding response could have arisen because the animals used were entirely
402 naïve to *S. muticum*, since gastropods may display high rates of indiscriminate feeding on
403 novel foods (e.g. Whelan, 1982; Morrison & Hay, 2011). The naïve *L. obtusata* in the
404 palatability trials (experiment 1) consumed much more *S. muticum* material than the
405 experienced *L. obtusata* in the willingness-to-feed trials (experiment 2). These findings
406 suggest that biotic resistance in the very early stages of an invasion may be exceptionally
407 high (Parker & Hay, 2005), albeit temporary if the invasive species proves unpalatable (see
408 Whelan 1982).

409 These results represent a preliminary assessment of grazing preference with time-
410 since-invasion. Sampling a greater number of both sites and grazers would permit a more
411 concrete understanding of the potential behavioural shifts undertaken by these consumers, in
412 response to invasions. The logistical constraints of including time-since-invasion are notably
413 restrictive. Even equipped with the chronology of *S. muticum*'s spread, selecting sites similar
414 to one another proved to be difficult since. Sites differed in terms of species composition and
415 compromises had to be made in the design of experiments. Caution must also be applied in
416 interpreting the broader ecological significance of these trials, since mesoherbivore feeding-

417 behaviours in the laboratory are not necessarily reflected in the field (Monteiro *et al.*, 2009),
418 and presence of grazers on algae in the field does not necessarily indicate direct herbivory on
419 the tissues of the macrophyte (Viejo, 1999; Pearse *et al.* 2013). It is worth noting that *S.*
420 *muticum* is vastly more abundant at both of the ‘older’ sites sampled (35 and 40), and
421 although grazing marks are visible upon the fronds, they grew to similar thallus lengths as
422 observed from other sites elsewhere (Kurr, *unpublished data*). However, these findings do
423 suggest that native marine mesoherbivores have the potential to alter their behaviour in the
424 presence of non-native species, developing an ability to feed on novel foods over time. This
425 adds to the growing body of literature (e.g. Dostál *et al.*, 2013; Harvey *et al.*, 2013; Byers *et*
426 *al.*, 2015; Schultheis *et al.*, 2015, and references therein) which shows that time-since-
427 invasion is a fundamental component of invasive ecology.

428

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436

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715

716 *Figures*

717 **Fig 1.** Locations of *Sargassum muticum* populations sampled for algal material and
718 mesoherbivores. Sites are named by the time-since-invasion of *S. muticum*. Grey lines
719 represent general invasion path from first observed occurrence. Site 40YR: Bembridge Ledge
720 (50.680466°N, 1.072554°W). Site 35YR: Eastbourne (50.750541°N, 0.270442°E). Site
721 19YR: West Angle Bay, near Milford Haven (51.688676°N, 5.110854°W). Site 10YR: The
722 northern shore of the Foryd estuary near Caernarfon (53.131581°N, 4.304016°W).

723

724 **Fig 2.** Algal consumption by native herbivores: A) Change in algal mass per unit ash-free-
725 dry-mass of herbivore (mg/mg) after seven days of *Sargassum muticum* fronds collected from
726 populations established for different lengths of time (named by reported time-since-invasion),
727 by *Littorina obtusata* naïve to *S. muticum*. B) Change in algal mass per unit ash-free-dry-
728 mass of herbivore (mg/mg) of *Fucus serratus* (white bars) and *Sargassum muticum* (grey
729 bars) after seven days in the presence of *Littorina obtusata* collected from *S. muticum*
730 populations established for different lengths of time (named by reported time-since-invasion).
731 Letters indicate groupings in consumption by each algal species and site, and * differences
732 between algal species within a site based on Mann-Whitney U tests. All data are adjusted for
733 autogenic changes in algal mass and error bars show +/- 1 S.E.

734

735 **Fig. 3.** Change in algal mass (m g/mg) of *Sargassum muticum* and *Ascophyllum nodosum*
736 fronds collected from populations established for different lengths of time (named by
737 reported time-since-invasion of *S. muticum*), after seven days of exposure to *Littorina fabalis*
738 collected from those same sites in no-choice feeding trials. All data are adjusted for autogenic
739 changes in algal mass. *Post hoc* analysis is presented in Table 1, and error bars show +/- 1
740 S.E.

741

742 **Fig. 4.** Change in algal mass of *Sargassum muticum* collected from sites bearing *S. muticum*
743 populations of different ages (named by reported time-since-invasion of *S. muticum*), after
744 seven days of exposure to *Littorina fabalis* collected from the same sites in choice feeding
745 trials (grey bars), and autogenic controls that did not contain grazers (white bars). A) 40YR *L.*
746 *fabalis* and B) 10YR *L. fabalis*, each treatment contained *S. muticum* from both sites. Error
747 bars show +/- 1 S.E.
748

For Review Only

749 **Table 1.** Three-way Nested ANOVA for the change in mass of algal individuals exposed to
 750 *Littorina fabalis* collected from two sites, one bearing *Sargassum muticum* for 40 years, and
 751 one for 10 years, in no-choice feeding trials on *Sargassum muticum* and
 752 *Ascophyllum nodosum*.

753

	DF	MS	F	P
Algal species	1	48724	8.01	0.007
Algal origin	1	54066	8.89	0.004
Grazer origin	1	37037	6.09	0.017
Algal species*Algal origin	1	458084	75.32	< 0.001
Algal species*Grazer origin	1	53737	8.84	0.005
Algal origin*Grazer origin	1	2952	0.49	0.489
Individual (Algal species*Algal origin)	1	23747	3.90	0.001
Algal species*Algal origin*Grazer origin	1	12827	2.11	0.153
Individual (Algal species*Algal origin)*Grazer origin	8	9899	1.63	0.142
Error	68	6082		

754 Tukey's *post-hoc* analysis:755 **Algal Species*Algal Origin**; 10YR *S. muticum* (A), 40YR *A. nodosum* (A), 10YR *A. nodosum* (B), 40YR *S.*
756 *muticum* (C)757 **Algal Species*Grazer Origin**; *A. nodosum* and 40YR grazers (A), *S. muticum* and 10YR Grazers (A), *A.*
758 *nodosum* and 10YR Grazers (A), *S. muticum* and 40YR Grazers (B)759 **Algal Origin*Grazer Origin**; 10YR Algae with 10YR Grazers (A), 10YR Algae with 40YR Grazers (AB),
760 40YR Algae with 10YR Grazers (AB), 40YR Algae with 40YR Grazers (B)

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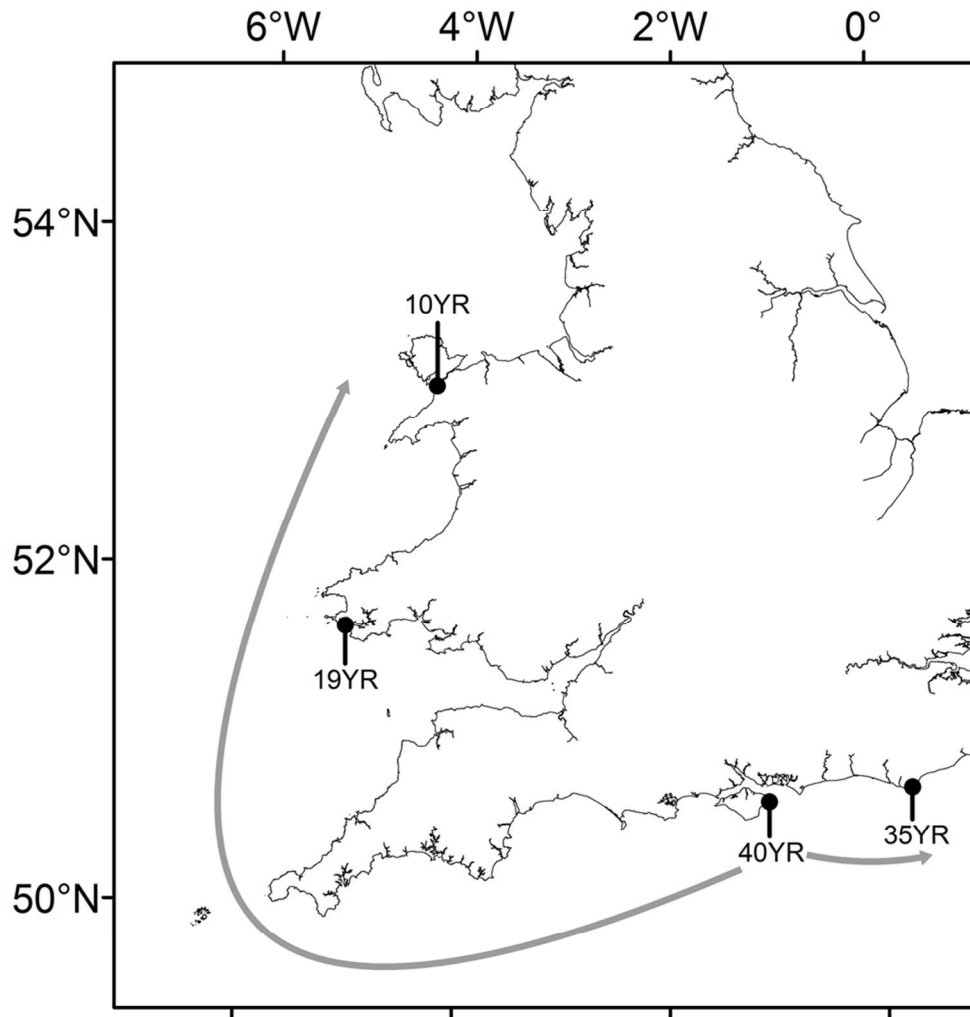


Fig 1. Locations of *Sargassum muticum* populations sampled for algal material and mesoherbivores. Sites are named by the time-since-invasion of *S. muticum*. Grey lines represent general invasion path from first observed occurrence. Site 40YR: Bembridge Ledge (50.680466°N, 1.072554°W). Site 35YR: Eastbourne (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).

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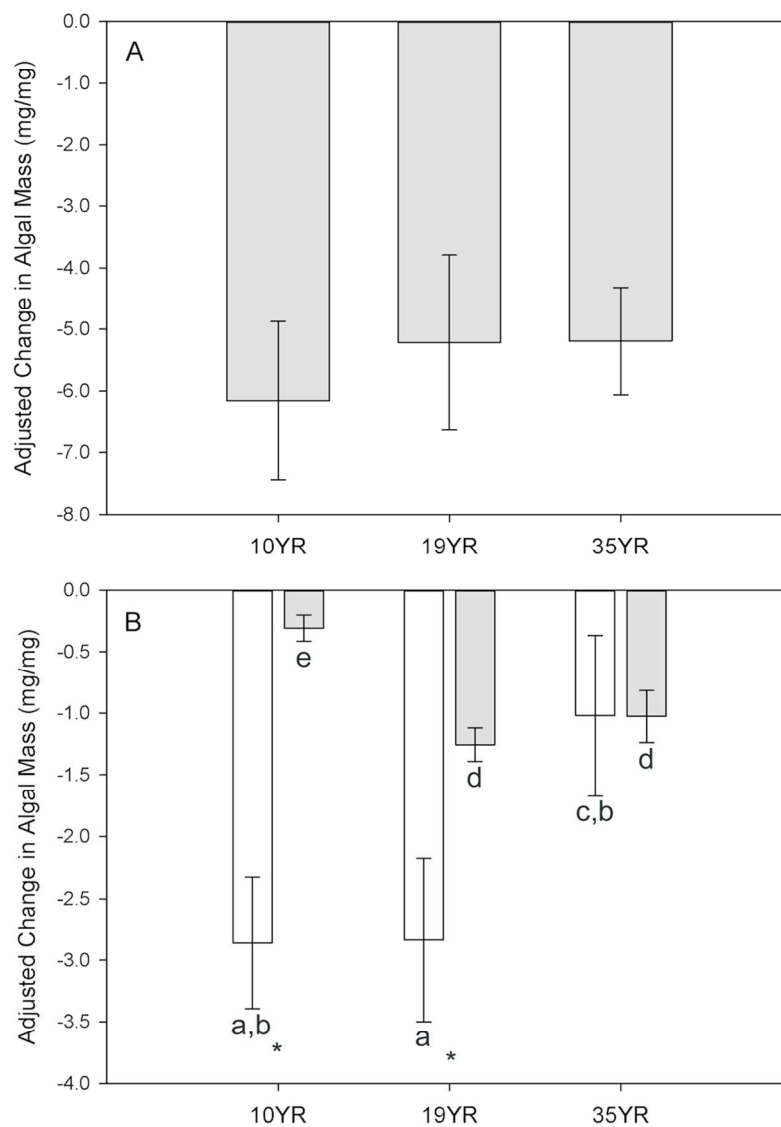


Fig 2. Algal consumption by native herbivores: A) Change in algal mass per unit ash-free-dry-mass of herbivore (mg/mg) after seven days of *Sargassum muticum* fronds collected from populations established for different lengths of time (named by reported time-since-invasion), by *Littorina obtusata* naïve to *S. muticum*. B) Change in algal mass per unit ash-free-dry-mass of herbivore (mg/mg) 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). Letters indicate groupings in consumption by each algal species and site, and * differences between algal species within a site based on Mann-Whitney U tests. All data are adjusted for autogenic changes in algal mass and error bars show ± 1 S.E.

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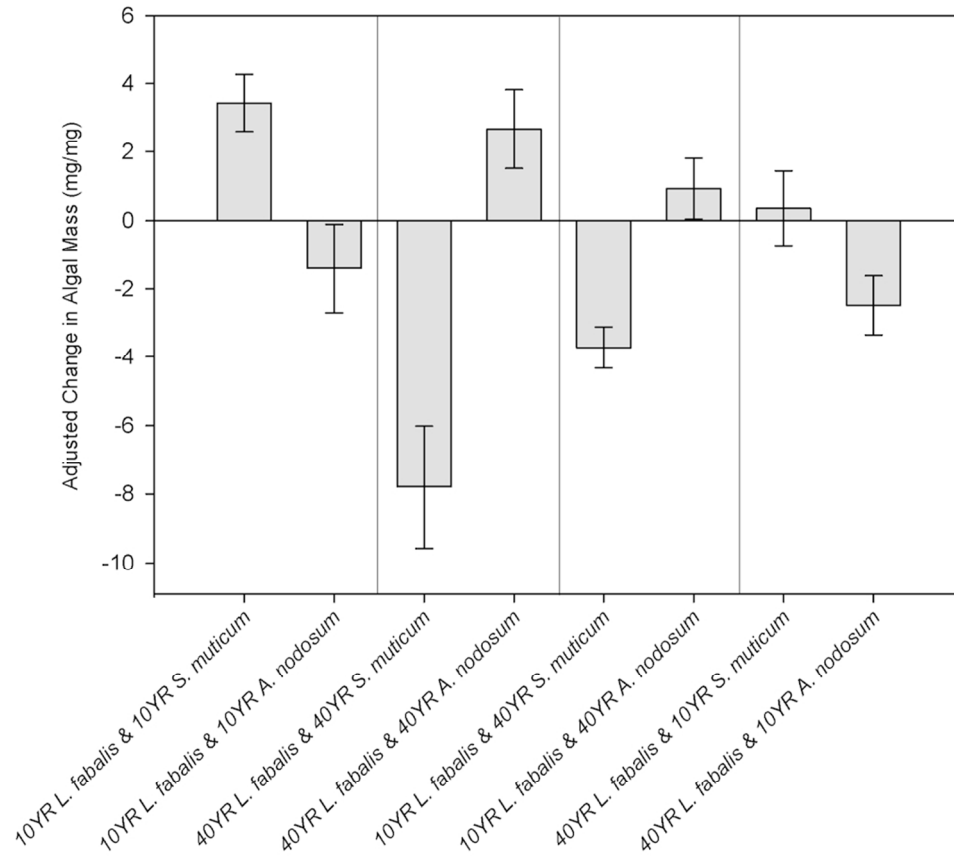


Fig. 3. Change in algal mass (mg/mg) of *Sargassum muticum* and *Ascophyllum nodosum* fronds collected from populations established for different lengths of time (named by reported time-since-invasion of *S. muticum*), after seven days of exposure to *Littorina fabalis* collected from those same sites in no-choice feeding trials. All data are adjusted for autogenic changes in algal mass. Post hoc analysis is presented in Table 1, and error bars show ± 1 S.E.

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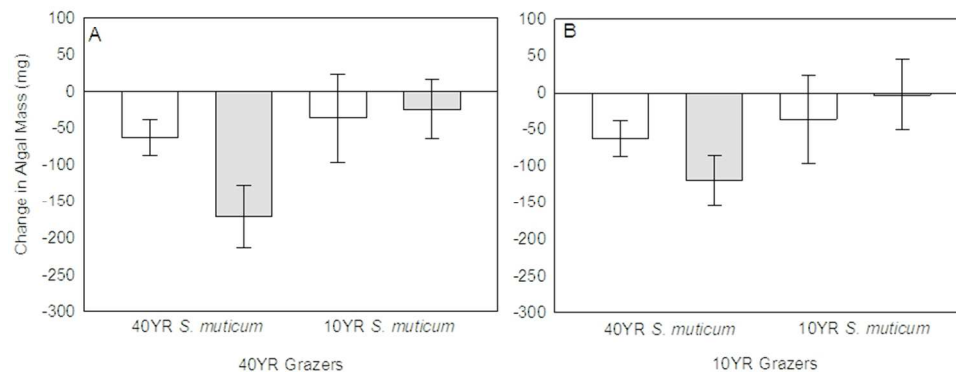


Fig. 4. Change in algal mass of *Sargassum muticum* collected from sites bearing *S. muticum* populations of different ages (named by reported time-since-invasion of *S. muticum*), after seven days of exposure to *Littorina fabalis* collected from the same sites in choice feeding trials (grey bars), and autogenic controls that did not contain grazers (white bars). A) 40YR *L. fabalis* and B) 10YR *L. fabalis*, each treatment contained *S. muticum* from both sites. Error bars show ± 1 S.E.

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