Small herbivores and abiotic heterogeneity promote trait variation of a saltmarsh plant in local communities

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Intraspecific trait variation (ITV) enables plants to respond to global changes. However, causes for ITV, especially from biotic components such as herbivory, are not well understood. We explored whether small vertebrate herbivores (hares and geese) impact ITV of a dominant clonal grass (*Elytrigia atherica*) in local communities. Moreover, we looked at the relative importance of their direct (e.g. selective grazing) and indirect effects (altering genotypic richness/diversity and abiotic environment) on ITV. We used exclosures at two successional stages in a Dutch saltmarsh, where grazing pressure at the early successional stage was ca. 1.5 times higher than that of the intermediate successional stage . We measured key functional traits of *E. atherica* including height, aboveground biomass, flowering (flower or not), specific leaf area, and leaf dry matter content in local communities (1 m ×1m plots) inside and outside the exclosures. We determined genotypic richness and diversity of each plant using molecular markers. We further measured abiotic variations in topography and clay thickness (a proxy for soil total nitrogen). Structural equation models revealed that small herbivores significantly promoted ITV in height and flowering at the early successional stage, while they marginally promoted ITV in height at the intermediate successional stage. Moreover, the direct effects of herbivores played a major role in promoting ITV. Small herbivores decreased genotypic diversity at the intermediate successional stage, but genotypic richness and diversity did not impact ITV. Small herbivores did not alter topographic variation and variation in clay thickness, but these variations increased ITV in all traits at the early successional stage. Small herbivores may not only impact trait means in plants as studies have shown but also their ITV.

1 **Small herbivores and abiotic heterogeneity promote trait variation of a saltmarsh plant in**

2 **local communities**

3 **running title:** small herbivores and trait variation

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10 **Abstract**

11 Intraspecific trait variation (ITV) enables plants to respond to global changes. However, causes

12 for ITV, especially from biotic components such as herbivory, are not well understood. We

13 explored whether small vertebrate herbivores (hares and geese) impact ITV of a dominant clonal

14 grass (*Elytrigia atherica*) in local communities. Moreover, we looked at the relative importance

15 of their direct (e.g. selective grazing) and indirect effects (altering genotypic richness/diversity

16 and abiotic environment) on ITV. We used exclosures at two successional stages in a Dutch

17 saltmarsh, where grazing pressure at the early successional stage was ca. 1.5 times higher than

18 that of the intermediate successional stage. We measured key functional traits of *E. atherica*

19 including height, aboveground biomass, flowering (flower or not), specific leaf area, and leaf dry

20 matter content in local communities (1 m ×1m plots) inside and outside the exclosures. We

21 determined genotypic richness and diversity of each plant using molecular markers. We further

22 measured abiotic variations in topography and clay thickness (a proxy for soil total nitrogen).

23 Structural equation models revealed that small herbivores significantly promoted ITV in height

24 and flowering at the early successional stage, while they marginally promoted ITV in height at

25 the intermediate successional stage. Moreover, the direct effects of herbivores played a major

26 role in promoting ITV. Small herbivores decreased genotypic diversity at the intermediate

27 successional stage, but genotypic richness and diversity did not impact ITV. Small herbivores

28 did not alter topographic variation and variation in clay thickness, but these variations increased

29 ITV in all traits at the early successional stage. Small herbivores may not only impact trait means

30 in plants as studies have shown but also their ITV.

31   **Keywords:** trait variation; plant-herbivore interaction; grazing; abiotic; genetic diversity;

32 saltmarsh

33 **Introduction**

34 Studies show that intraspecific trait variation (i.e. variability in traits of conspecific individuals;

35 hereafter ITV) can enable plant species to respond to global changes (Westerband, Funk &

36 Barton, 2021), impact community composition and structure (Whitlock, Grime & Burke, 2010),

37 and govern ecosystem processes (Boege & Dirzo, 2004; Crutsinger et al., 2006; Crutsinger,

38 Souza & Sanders, 2008; Lecerf & Chauvet, 2008). The importance of ITV has been increasingly

39 realized over the years (Violle et al., 2012; Siefert et al., 2015; Funk et al., 2017; Westneat et al.,

40 2019; Westerband, Funk & Barton, 2021), therefore, exploring the causes for ITV is of prime

41 importance, particularly under the current rapid global changes (Westerband, Funk & Barton,

42 2021). Several components including genotypic richness/diversity and phenotypic plasticity

43 triggered by biotic and abiotic environments can drive ITV (Valladares, Gianoli & Gomez,

44 2007). However, biotic components, particularly herbivory, received much less attention than

45 genotypic richness/diversity and abiotic environments (Valladares, Gianoli & Gomez, 2007).

46

47 Whilst herbivores are one of the major drivers for plant trait differentiation globally (Díaz et al.,

48 2007; He & Silliman, 2016), the majority of the studies that document the effects of herbivores

49 on plant traits focus on trait means without considering ITV (e.g. Bullock et al., 2001; Louault et

50 al., 2005; Kahmen & Poschlod, 2008). Limited studies suggest that herbivores may also impact

51 ITV (e.g. Jessen et al., 2020). However, these studies mainly focus on large herbivores. Large

52 and small herbivores are relative. Here we refer to small herbivores as vertebrate herbivores with

53 body mass ranging from 1 to 10 kg such as hares and geese. Small herbivores sometimes can

54 have stronger impacts on plant communities than large ones especially when their abundance is

55 high (Olofsson et al., 2004). Additionally, small herbivores are usually more selective with

56 forage plants (Olff & Ritchie, 1998). Therefore, small herbivores may also impact ITV, however,

57 we currently lack empirical evidence. As small herbivore populations are changing dramatically

58 due to human actions (Smith, Jennings & Harris, 2005; Menu, Gauthier & Reed, 2002), it is

59 important to understand whether and how small herbivores impact ITV.

60 Small herbivores may impact ITV via multiple non-exclusive processes. First, small herbivores

61 can promote ITV through selective grazing. Studies show that large herbivores generally prefer

62 young plants that are often more nutritious (e.g. higher protein and lower lignin and cellulose)

63 (Augustine & McNaughton, 1998). Thus, larger herbivores decrease plant height by grazing

64 them down (Díaz et al., 2007; Evju et al., 2009). Similarly, small herbivores may prefer young

65 plants and decrease plant height, but because small herbivores consume less and they are more

66 selective with forage plants, some young plants may escape from small herbivores via

67 association with unpalatable species (Van Der Wal et al., 2000; Kuijper & Bakker, 2008). Thus,

68 small herbivores may promote variation in plant height. Second, small herbivores may impact

69 ITV indirectly through altering genotypic richness and diversity. Increased genotypic richness

70 and diversity can increase ITV (Evans et al., 2016). Studies looking at the effects of large

71 herbivores on genotypic diversity show that large herbivores can increase genotypic diversity by

72 promoting seed transportation (Rico & Wagner, 2016) or decrease it by promoting clonal growth

73 (Kleijn & Steinger, 2002). Small herbivores can have similar effects to large ones on many

74 processes such as seed dispersal (Bakker & Olff, 2003). Therefore, small herbivores can either

75 increase or decrease genotypic richness and diversity. Third, small herbivores may promote ITV

76 indirectly through altering abiotic heterogeneity (e.g. topographic variation and variation in soil

77 nutrients) via grazing the vegetation at particular spatial scales, trampling, and localized

78   deposition of droppings (Adler, Raff & Lauenroth, 2001). Abiotic heterogeneity usually

79   promotes ITV (Westerband, Funk & Barton, 2021). However, whether small herbivores impact

80   ITV via these processes and the relative importance of them in shaping ITV remains

81   underexplored.

82

83   To test these hypotheses, we used long-term (22-year) exclosure experiment at the early and

84   intermediate successional stages of a Dutch saltmarsh. In the eastern part of the saltmarsh of the

85   island of Schiermonnikoog, hares and geese are the abundant herbivores while large herbivores

86   are absent (Kuijper & Bakker, 2005; Schrama et al., 2015; Chen et al., 2019). A well-calibrated

87   successional gradient is present here (Olff et al., 1997). Hares and geese are more abundant at the

88   early successional stages but their abundance decreases at intermediate and late successional

89   stages due to less preferred late successional plants such as *Elytrigia atherica* (synonym *Elymus*

90   *athericus*) increase in dominance (Kuijper & Bakker, 2005; Schrama et al., 2015; Chen et al.,

91   2019). *Elytrigia atherica* mainly reproduces clonally, but sexual reproduction may occur

92   occasionally via windows of opportunity and their seeds are mainly dispersed by tidal water, thus

93   genotypic richness is high in this grass (Bockelmann et al., 2003; Change et al., 2005; Chen,

94   2020). Topographic variation and variation in clay thickness are important factors for the growth

95   and expansion of *E. atherica* in saltmarshes (Olff et al., 1997; Nolte et al., 2019). Thus, the long-

96   term exclosure experiment along the successional gradient in this saltmarsh provides ideal

97   conditions to look at the effects of small herbivores on ITV in plants.

98

99   In this study, we look at the effects of hares and geese on ITV of *E. atherica* in local

100 communities (1 m ×1m plots). This is because ITV is usually strongly driven by processes

101 operating at small spatial scales (Westerband, Funk & Barton, 2021). We measured key

102 functional traits of this dominant grass including height, aboveground biomass, flowering

103 (flower or not), specific leaf area, and leaf dry matter content within local communities inside

104 and outside the exclosures. Using structural equation models, we test whether hares and geese

105 impact ITV of *E. atherica*, and the relative importance of their direct effects (e.g. selective

106 grazing) and indirect effects (via altering genotypic richness/diversity and abiotic variables) on

107 ITV. We expect that hares and geese would increase ITV in all traits measured and their effects

108 would be more apparent at the early successional stage (relative to the intermediate one) where

109 grazing pressure was higher. Although adult plants of *E. atherica* are less preferred, hares and

110 geese considerably graze on its seedling/young plants (Kuijper, Nijhoff & Bakker, 2004;

111 Fokkema et al., 2016). However, short seedlings/young plants may escape from grazing by

112 associating with non-preferred plants such as *Artemisia maritima*. Hares and geese may increase

113 topographic variation via trampling (Wijnen, Wal & Bakker, 1999; Elschot et al., 2015), they

114 may also impact sediment accumulation (measured by clay thickness) by trampling and altering

115 vegetation structure (Boorman, Garbutt & Barratt, 1998). However, because hares and geese play

116 a less important role than tidal water in seed dispersal (Chang et al., 2005), it is unlikely that they

117 can increase genotypic richness and diversity by dispersing seeds. Instead, hares and geese may

118 decrease genotypic richness and diversity by promoting clonal spread of *E. atherica* (Van Der

119 Graaf, Stahl & Bakker, 2005). Therefore, we also expect that hares and geese may increase ITV

120 through selective grazing and promoting topographic variation and variation in clay thickness but

121 may decrease ITV by decreasing genotypic richness and diversity.

122

123 **Materials and methods**

124 *Study site*

125 A natural successional gradient is present in the back-barrier saltmarsh of the island of

126 Schiermonnikoog (53°30' N, 6°10' E), the Netherlands. Because this island expands eastward,

127 thus the eastern part of the island is younger relative to the western part (Olff et al., 1997). The

128 western part of the saltmarsh is enclosed for cattle grazing, while the eastern part of it is grazed

129 by wild small herbivores such as spring staging geese, year-round present hares and rabbits.

130 Hares and geese are the most abundant herbivores (Van de Koppel et al., 1996; Van Der Wal,

131 Kunst & Drent, 1998; Wal et al., 2000; Kuijper & Bakker, 2005; Schrama et al., 2015). Rabbits

132 are very rare. Kuijper and Bakker (2005) found that biomass removed by rabbits in 2000 was

133 6.33% and 0.16% of the total biomass removed by small herbivores at the early and intermediate

134 successional stage, respectively.

135

136 We used long-term hare and goose exclosures that were initiated in 1994 (details in Chen et al.,

137 2019). We selected exclosures located at the early and intermediate successional stages, which

138 are approximately 2.5 km apart (Fig. 1A). Vegetation succession has undergone around 30 and

139 60 years at the early and intermediate successional stage, respectively. Age of the vegetation

140 succession was counted from the year of vegetation establishment to 2016. The year of

141 vegetation establishment was determined by checking aerial photographs (Olff et al., 1997).

142 Grazing pressure from hares and geese was ca. 1.5 times higher at the early successional stage

143 than the intermediate successional stage based on year-round dropping count in 2000 and 2016

144 (see Table S1 for more details; Kuijper & Bakker, 2005; Chen et al., 2019; Chen et al., 2019a).

145

146 Hare and goose exclosures (one per each successional stage) were located in a similar elevation

147 (early stage: 1.42 ± 0.004; intermediate stage:1.44 ± 0.004; mean ± 1se; N=24; m+NAP; Normal

148 Amsterdam Water Level). Exclosures (8 m × 12 m and 6 m × 8 m at the early and intermediate

149 successional stages) were made by chicken mesh (mesh width 25 mm) supported by wooden

150 posts every 3.5 m to exclude hare grazing inside the exclosures. Exclosures were around 1 m in

151 height, ropes were suspended on top of the wooden posts to stop geese flying into the exclosures.

152 At the beginning of the exclosure experiment (1995), *E. atherica* rarely occurred (< 2.5 %;

153 percent cover) inside and outside the exclosures at these two stages. Vegetation composition

154 differed in the grazed areas and inside the exclosures in 2016 (Fig.1; also see Table S2 for

155 species composition for the three most abundant species). Specifically, *Artemisia maritima* was

156 the most dominant plant in the grazed area at these two stages, while *E. atherica* was the most

157 dominant plant inside the exclosures.

158

159 *Experimental design*

160 To make our results spatially comparable, we sampled within ca. 6 m × 8 m inside and outside

161 the exclosures at both successional stages in June 2017. We chose this size because this is the

162 largest area we can sample inside the exclosures at the intermediate successional stage. The

163 distance between the area and the exclosure measured ca. 80 m. We randomly set up 7 plots (1m

164 × 1m) inside these two grazed areas and exclosures (Fig. 1). The distance between these plots

165   ranged from 0.5 m to 9 m. We divided each plot into 25 grids (0.2 m × 0.2 m). Within each grid,

166   we collected one individual stem of *E. atherica* (with roots), usually in the middle of the grid

167   (Fig. 1). *Elytrigia atherica* did not occur everywhere, particularly not in the grazed area, thus

168   sample size per plot varied from 9 to 25.

169

170   *Trait measurement*

171   We measured traits for individual stems of *E. atherica* in the field. We sampled individual stems

172   without obvious grazing marks. We cut individual stems at the ground level and measured

173   maximum height (cm) from the base to the highest point. We measured the maximum leaf width

174   and length of the first fully grown leaf, usually the third leaf from the top. We also recorded

175   whether individual stems flowered or not. After these measurements, each stem was stored in a

176   paper bag, sealed, labeled, and then put in a self-sealing plastic bag to reduce water loss in

177   tissues. At the end of each day, samples were brought to the lab, we measured fresh biomass of

178   the individual stems and fully-grown leaves. We also measured their dried biomass (g) after

179   oven-dry (70 °C) to constant mass (ca. 3 days). We calculated specific leaf area (mm^2 mg^{-1}) as

180   (leaf width ×leaf length) / dried leaf biomass. We calculated leaf dry matter content (mg g^{-1}) as

181   dried leaf biomass / fresh leaf biomass. Specific leaf area and leaf dry matter content were not

182   measured in the standard way (Pérez-Harguindeguy et al., 2016), and caution should be taken

183   when comparing our data with other studies. Due to that reason, in the main text, we focus on

184   height, aboveground biomass, and flowering, we present the results for specific leaf area and leaf

185   dry matter content in the online supporting file.

186

187   *Genotyping and genotype richness and diversity*

188   We used oven-dried leaf samples (ca. 2 mg per individual stem) for DNA extraction. We first

189   shredded leaf samples into smaller pieces using tissuelyser. We then extracted DNA from each

190   leaf sample using the CTAB method (Doyle & Doyle, 1987) and stored DNA samples at -20 °C

191   before PCR. We amplified DNA using PCR with fluorescence-labeled primers. We used five

192   microsatellite markers (ECGA89, WMS6, WMS44, WMS2, and ECGA89) originally designed

193   for the other Poaceae species, *Elymus caninus* (Sun, Salomon & Bothmer, 1998) and *Triticum*

194   *aestivum* (Röder et al., 1998). These five markers were used for genotyping *E. atherica* in this

195   system (Bockelmann et al., 2003; Chen 2020). The PCR products from primer ECGA89, WMS6,

196   and WMS44 were pooled together, while the PCR products from WMS2 and ECGA89 were

197   pooled together. Additionally, 1 μL rROX was added in each sample as the internal size standard

198   (Gene ScanTM–350 ROXTM, Applied Biosystem). We visualized the pooled PCR products

199   using the 3730 DNA analyzer and scored the microsatellite peak patterns (height > 100)

200   manually using GeneMapper. In total, we successfully genotyped 579 individual stems of *E.*

201   *atherica*, but 2 were excluded for further analyses as some trait data measured in the field for

202   these samples were missing.

203

204   *Abiotic variables*

205   We measured clay thickness and topographic variation for each plot (3 replicates). We measured

206   clay thickness using a 2 cm Ø soil corer with tick marks as a proxy for soil total nitrogen (Olff et

207   al., 1997). Previous studies show that clay thickness is strongly positively correlated with soil

208   total nitrogen in this system (i.e. total nitrogen (g) = 186 + 43 clay thickness (cm); r = 0.9,

209   p<0.0001; Olff et al., 1997). Also, clay thickness is used as a proxy of soil fertility (e.g. Schrama

210   et al., 2017). We measured topography using Trimble R8 (precision for elevation ca. 1 cm).

211

212   **Data analysis**

213

214   *Calculating trait means, ITV, topographic variation, and variation in clay thickness*

215   We calculated means for each trait by averaging trait values over 9 -25 individual stems in each

216   plot. We calculated ITV in each trait each plot as the standard deviation /mean. Topographic

217   variation and variation in clay thickness were calculated as standard deviation /mean (over 3

218   samples) of elevation and clay thickness, respectively.

219

220   *Calculating genotypic richness, genotypic diversity, and genetic differentiation*

221   To calculate genotype richness and diversity per plot, we first calculated pairwise genetic

222   distance using Dice dissimilarity from the R package ade4 (Dray & Dufour, 2015) based on the

223   presence/absence matrix of 42 allele bands from those five markers. We then assigned genotypes

224   based on dice dissimilarity, using the function "assignClones" from the R package polysat

225   (Lindsay, Clark & Clark, 2018). We calculated genotypic richness as the number of unique

226   genotypes detected divided by the number of individual stems genotyped for each plot.

227   Genotypic diversity – taking into account the abundance of different genotypes – was calculated

228   using the function "genotypeDiversity" with the index of "Shannon" from the package polysat

229   (Lindsay, Clark & Clark, 2018). Because small herbivores may select for some particular

230   genotypes that are more resistant or tolerant to herbivore grazing (Kotanen & Bergelson, 2000),

231   which may also impact traits. We explored genetic differentiation using principal coordinates

232   analysis (PCoA) from the R package ade4 (Dray & Dufour, 2015). We looked at genetic

233   differentiation at the treatment level, that is, grazed and ungrazed across plots at each

234   successional stage, result can be found in Fig. S1.

235

236   *Effects of small herbivores on trait means and ITV*

237   Although our focus is on ITV, to compare whether the effects of small herbivores on ITV are

238   stronger than trait means, we also looked at the effects of small herbivores on trait means. We

239   use analysis of variance, function "lm", to look at the effects of small herbivores on means and

240   ITV of each trait at the early and intermediate successional stage, separately. We checked model

241   assumptions by visually inspecting residual plots for homogeneity and quantile-quantile plots for

242   normality. In all cases, models fitted the data reasonably well.

243

244   *Direct and indirect effects of small herbivores on ITV*

245   We estimated the direct, indirect effects through altering genotypic richness and diversity,

246   indirect effects through altering abiotic variables, and total effects (sum of direct and indirect

247   effects) of small herbivores on ITV. We estimated these effects in each trait at each successional

248   stage based on the standardized path coefficients using structural equation models from the R

249   package "lavaan" (Rosseel, 2012). Grazing (0: ungrazed, 1: grazed), genotypic richness,

250   genotypic diversity, topographic variation, and variation in clay thickness were included in the

251   models. Note that genotypic diversity was not included for ITV at the early successional stage, as

252   it was significantly correlated with genotypic richness (variance inflation factor > 4). See online

253   supporting text for an example of a structural equation model for ITV in height at the

254   intermediate successional stage and calculation of the direct and indirect effects. Effects are

255   significant when $p \leq 0.05$, while marginally significant when $p > 0.05$ and p < 0.1. Data analysis

256   was performed in R3.5.3 (R Core Team 2020).

257

258   **Results**

259   *Effects of small herbivores on trait means and ITV*

260   At the early successional stage, small herbivores significantly decreased means in height,

261   aboveground biomass, and flowering of *E. atherica*. Also, small herbivores significantly

262   increased ITV in flowering and marginally increased ITV in height, but had no effects on ITV in

263   aboveground biomass. At the intermediate successional stage, small herbivores significantly

264   decreased means in height, aboveground biomass, and flowering. However, small herbivores had

265   no effects on ITV in all traits (Fig. 2; see Table S3 for test statistics). Small herbivores did not

266   impact mean and ITV in specific leaf area and leaf dry matter content at both successional stages

267   except that small herbivores decreased mean in specific leaf area at the intermediate successional

268   stage (Table S3).

269

270   *Direct and indirect effects of small herbivores on ITV*

271   The structural equation model reveals that overall (summing up the direct and indirect effects),

272   small herbivores tended to promote ITV in height and flowering, but not in other traits at the

273   early successional stage. Overall, small herbivores tended to promote ITV in height, but not in

274   other traits at the intermediate stage. Moreover, these positive overall effects of small herbivores

275   on ITV in height and flowering were mainly attributable to their direct effects. Although the

276   overall effects of small herbivores on ITV in aboveground biomass and specific leaf area were

277   not significant, small herbivores also directly significantly impacted ITV in these two traits at the

278 early successional stage (Fig 3; Fig. S2). See Table 1 for the standardized path coefficient

279 estimated from SEM for the direct, indirect, and total effects of small herbivores on ITV.

280

281 At the early successional stage, small herbivores did not have significant effects on genotypic

282 richness and diversity, but genotypic richness decreased ITV in flowering. At the intermediate

283 successional stage, small herbivores did not have significant effects on genotypic richness, but

284 significantly reduced genotypic diversity. However, genotypic richness and diversity did not

285 impact ITV in any traits at the intermediate successional stages (Fig. 3; Fig. S2).

286

287 Small herbivores did not impact topographic variation and variation in clay thickness at both

288 successional stages. However, these variations significantly increased ITV in all traits at the

289 early successional stage except that the effects of topographic variation on ITV in flowering were

290 not significant. The effects of these variations on ITV were less apparent at the intermediate

291 successional stage except that variation in clay thickness marginally decreased ITV in height

292 (Fig. 3; Fig. S2).

293

294 **Discussion**

295

296 In this study, using long-term exclosures, in combination with field observations and genetic

297 analyses, we explored ITV of a dominant clonal grass (*Elytrigia atherica*) and the direct and

298 indirect effects of small herbivores on ITV. We found that, at the early successional stage, small

299 herbivores promoted ITV in height and flowering of this grass. At the intermediate successional

300 stage, small herbivores marginally promoted ITV in height. Moreover, small herbivores

301   promoted ITV mainly through direct effects (e.g. selective grazing) but not through altering

302   genotypic richness/diversity or topographic variation and variation in clay thickness.

303

304   *Effects of small herbivores on trait means and ITV*

305   Our results suggest that small herbivores could impact both means and variations in key

306   functional traits of *E. atherica*, but their effects differed in different traits. At the early

307   successional stage, we found that small herbivores significantly decreased means in three traits

308   measured (height, aboveground biomass, and flowering) and they significantly promoted ITV in

309   two traits (height and flowering). At the intermediate successional stage, small herbivores

310   significantly decreased means in four traits (height, aboveground biomass, flowering, and

311   specific leaf area) and they marginally promoted ITV in one trait (height; Fig. 2; Fig. S2). This

312   suggests that trait means and variations may be driven by different processes. Moreover, we only

313   observed ITV in height and flowering may be because these two traits have higher plasticity than

314   other traits, and traits with a higher level of plasticity also show higher ITV (Givnish, 2002).

315   Furthermore, small herbivores had similar effects on trait means at both successional stages, but

316   had stronger impacts on ITV at the early successional stage. This may be because the effects of

317   small herbivores on trait means and variations were mediated by community composition in the

318   field. At the early successional stage, the preferred plant, *F. rubra*, was more abundant in the

319   grazed area (ca. 3 times relative to that of the intermediate successional stage), thus small

320   herbivores (although more abundant) may graze more on *F. rubra* rather than *E. atherica*. At the

321   intermediate successional stage, the abundance of *F. rubra* deceased, while the abundance of *E.*

322   *atherica* increased in the grazed area (ca. 5 times relative to that of the early successional stage),

323   thus small herbivores (less abundant) may also substantially grazed on less preferred *E. atherica*.

324   Therefore, overall, the effects of small herbivores on trait means were similar at these two stages.

325   Meanwhile, non-preferred species (*A. maritima*) was more abundant in the grazed area at the

326   early successional stage (ca. 2 times relative to that of the intermediate successional stage; see

327   Table S2 for detailed percent cover of these species). It may facilitate young *E. atherica* escape

328   from herbivore grazing. Therefore, ITV was more apparent at the early successional stage than

329   the intermediate one. Previous studies in this system have shown that hares and geese are the

330   important drivers for plant community composition and structure along this successional gradient

331   (Olff et al., 1997; Kuijper & Bakker, 2005; Chen et al., 2019). Here, extending the current

332   knowledge, we show that small herbivores may also impact trait means and variations, which in

333   turn could impact community composition and structure (Whitlock, Grime & Burke, 2010).

334

335   *Direct and indirect effects of small herbivores on ITV*

336   As we hypothesized, the direct effects of small herbivores through selective grazing increased

337   ITV. This is not only true for height and flowering but also for aboveground biomass and

338   specific leaf area, despite the overall effects of small herbivores on aboveground biomass and

339   specific leaf area were not significant. As explained in the previous paragraph, the direct effects

340   of small herbivores may be mediated by community composition especially the proportion of

341   preferred and non-preferred plants. Herz et al. (2017) found that local neighborhood diversity

342   can explain a large amount of ITV in German meadows and pastures, possibly through increased

343   plant-plant interactions (e.g. competition). In this study, plant diversity was higher in the grazed

344   than the ungrazed plots at both successional stages after 22-year grazing (Table S2). Thus, higher

345   plant diversity may also contribute to increased ITV in height under grazing at both successional

346   stages. Future studies/experiments looking at the effects of herbivores on ITV in plant

347   communities with the same composition are needed to fully separate the effects of selective

348   grazing from the effects of neighboring plants.

349

350   We found that small herbivores did not impact genotype richness and diversity of *E. atherica* at

351   the early successional stage but decreased genotypic diversity at the intermediate successional

352   stage. A previous study in the west part of this saltmarsh found that cattle grazing does not

353   impact genotype richness and diversity of this grass, but cattle grazing alters its morphological

354   traits in the field such as decreasing height and leaf width (Veeneklaas et al., 2011). Here, we

355   found that genotypic richness and diversity generally had no significant effects on ITV.

356   Microsatellite markers are commonly used in studying plant genetics (Jarne & Lagoda, 1996;

357   Vieira et al., 2016), but because they are selectively neutral, they may not control gene

358   expression for the traits measured here. More generally speaking, the genetic basis of most traits

359   in many wild plants is unknown. Thus, it is difficult to know in advance which genes or genomic

360   regions to consider, which further hinders inferring a causal relationship between genetic

361   variation and phenotypic variation (Merilä & Hendry, 2014). A low correlation between

362   variation in morphological traits and DNA markers was also reported before (Kolliker et al.,

363   1998). Additionally, we found genetic differentiation of *E. atherica* in the grazed and ungrazed

364   areas at the early successional stage, but not at the intermediate successional stage (Fig. S1),

365   however, more data (presumably from more exclosures) are needed to consolidate this result.

366   Thus, the observed trait changes (at both successional stages) may not be associated with genetic

367   changes in *E. atherica*.

368

369   In contrast, we found no effects of small herbivores on topographic variation and variation in

370 clay thickness within plots. These abiotic variations may be primarily induced by flooding and

371 inundation, which may overrule the effects of small herbivores in salt marshes. However,

372 variations in these two abiotic variables had substantial positive impacts on ITV particularly at

373 the early successional stage. Abiotic environment may be more heterogenous at the early

374 successional stage because this stage has more frequent sediment deposition than the

375 intermediate successional stage (Schrama, Berg & Olff, 2012). A previous study conducted in

376 the western part of this saltmarsh also found that topographic variation at the small spatial scales

377 (0.1 m² and 10 m²) is positively correlated with species richness and cattle grazing additionally

378 increased species richness (Ruifrok et al., 2014). Thus, abiotic variations, even at very small

379 spatial scales, may play an important role in promoting ITV and altering other plant community

380 properties.

381

382 *Long-term exclosures*

383 Although we used 22-year old herbivore exclosures, our data cannot answer whether duration of

384 herbivore grazing impacts ITV, as we only collected data for one year. To our knowledge, no

385 studies have compared the effects of short- and long-term grazing on ITV. Didiano et al. (2014)

386 found that tolerance to rabbit grazing decreased as the age of the exclosures increased in *F.*

387 *rubra*, the most abundant plant in Silwood Park, England. Therefore, grazing duration may also

388 impact ITV.

389

390 **Conclusion**

391 Our results suggest that small herbivores may not only impact means but also ITV in some key

392 functional traits of a dominant grass (*E.atherica*). Additionally, small herbivores impacted ITV

393 mainly through selective grazing but not through altering genotypic richness/diversity and

394 abiotic variations. However, topographic variation and variation in clay thickness may contribute

395 to ITV. Small herbivore populations are changing rapidly due to human actions. For instance,

396 populations of European brown hares have declined dramatically due to land-use changes

397 (Smith, Jennings & Harris, 2005), while populations of geese are rapidly increasing globally

398 (Menu, Gauthier & Reed, 2002). These changes in small herbivore populations could thus impact

399 their effects on ITV in plant species, which may have consequences for plants to respond to

400 increasing environmental changes.

401

402 **Declarations**

403

404 **Funding**: Not applicable

405 **Conflicts of interest/Competing interests**: The authors declare no conflict of interest.

406 **Availability of data and material (data transparency)**: Data can be found

407 https://figshare.com/s/d7e2cee03c89cf0a9d6a. Date will be made public once the manuscript is

408 accepted. Figures are not copyrighted.

409   **Authors' contributions**: QC conceived and designed the experiments, performed the

410   experiments, analyzed the data, and wrote the manuscript. All authors contributed to revisions

411   and gave final approval for publication.

412

413   **Acknowledgments**

414   We thank Iris Bontekoe, Erica Zuidersma for helping collect samples in the field. We thank

415   Marco van der Velde, Jan Veldsink, and Yvonne Verkuil for their help with genotyping in the

416   lab. We thank J. F. Scheepens for his constructive comments on the earlier versions of this

417   manuscript. We thank Juan Alberti and Oliver Carroll for their constructive suggestions for

418   improving the structure and readability of this manuscript. We thank three anonymous reviewers

419   and the associate editor for their constructive comments and suggestions. We thank

420   Natuurmonumenten for offering us the opportunity to work in the saltmarsh of the island of

421   Schiermonnikoog. QC is funded by CSC (China Scholarship Council).

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611

Figure 1

Sampling location and scheme

Location for the 22-year exclosures at the early and intermediate successional stage in the saltmarsh of the island of Schiermonnikoog. Pictures of the exclosures at the early and intermediate successional stages are shown. Sampling plots (1× 1 m) within an exclosure and sampling scheme for individuals of *Elytrigia atherica* within a 1× 1 m plot are shown. We followed the same sampling scheme for each plot. *Elytrigia atherica* did not always occur within each grid, thus sample size varied for each plot. Sampling plots and scheme were similar outside the exclosures (in the grazed area) at both successional stages. Number of hares and geese indicate the abundance of small herbivores such that the early successional stage had higher grazing pressure (indicated by two hares and two geese) relative to the intermediate successional stage (indicated by one hare and one goose). Note, only the map of the saltmarsh, but not exclosures and sampling plots, is projected according to its actual size.

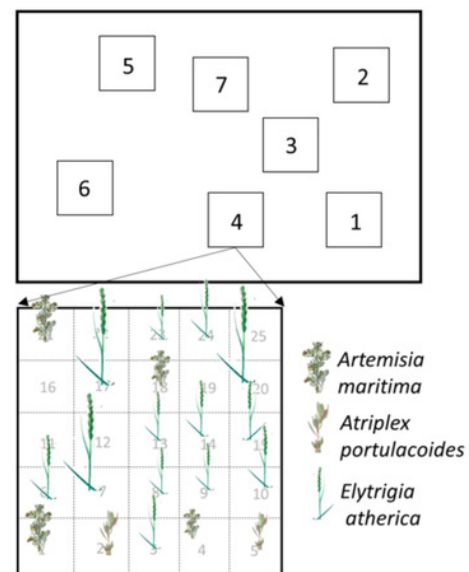
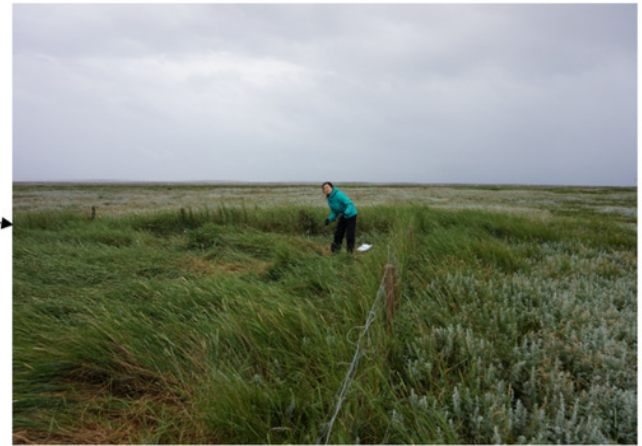



Figure 2

Means and variations in functional traits of individual stems of *Elytrigia atherica* in the grazed and ungrazed plots at the early and intermediate stages.

Traits include height (cm), aboveground biomass (g), flower frequency. Results for specific leaf area (cm^2 g^{-1}) and leaf dry matter content (g g^{-1}) are not shown because small herbivores generally did not impact mean and ITV in these two traits at both successional stages. Dots are the means, error bars show 1 se. Asterisks indicate significant levels: * P<0.1; ** P<0.05; *** P<0.001. See Table S3 for test statistics.

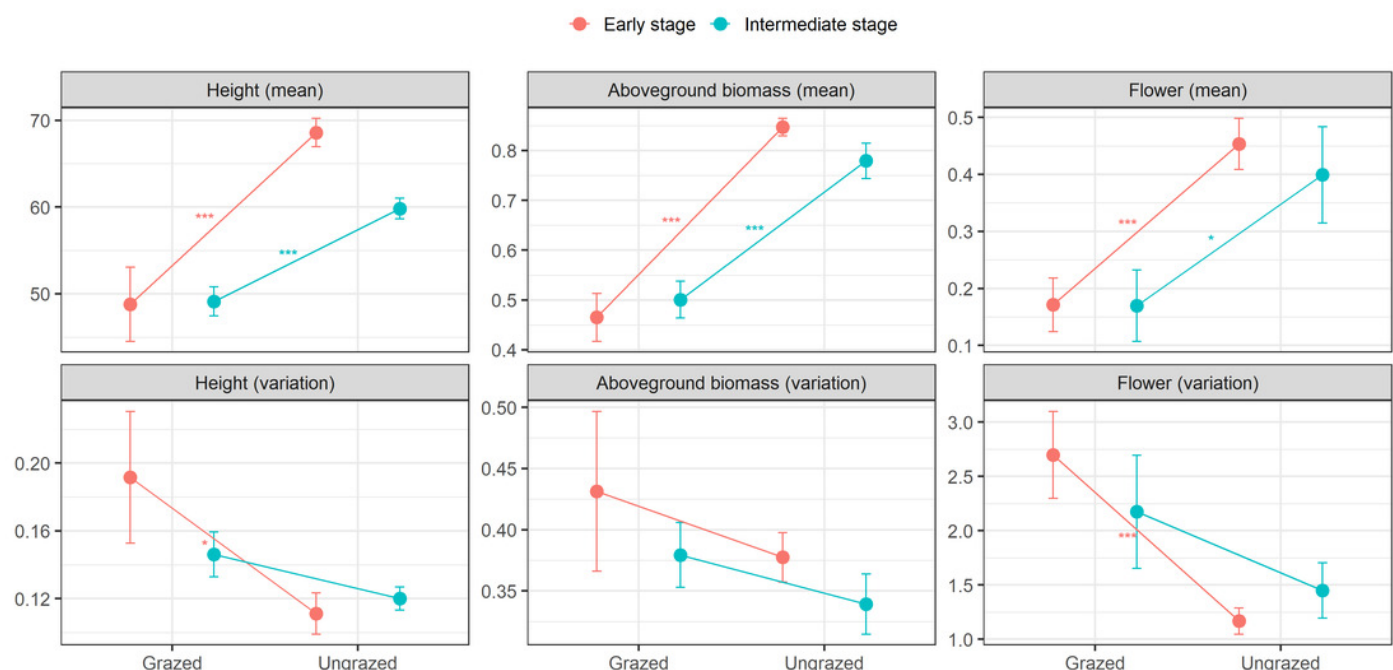
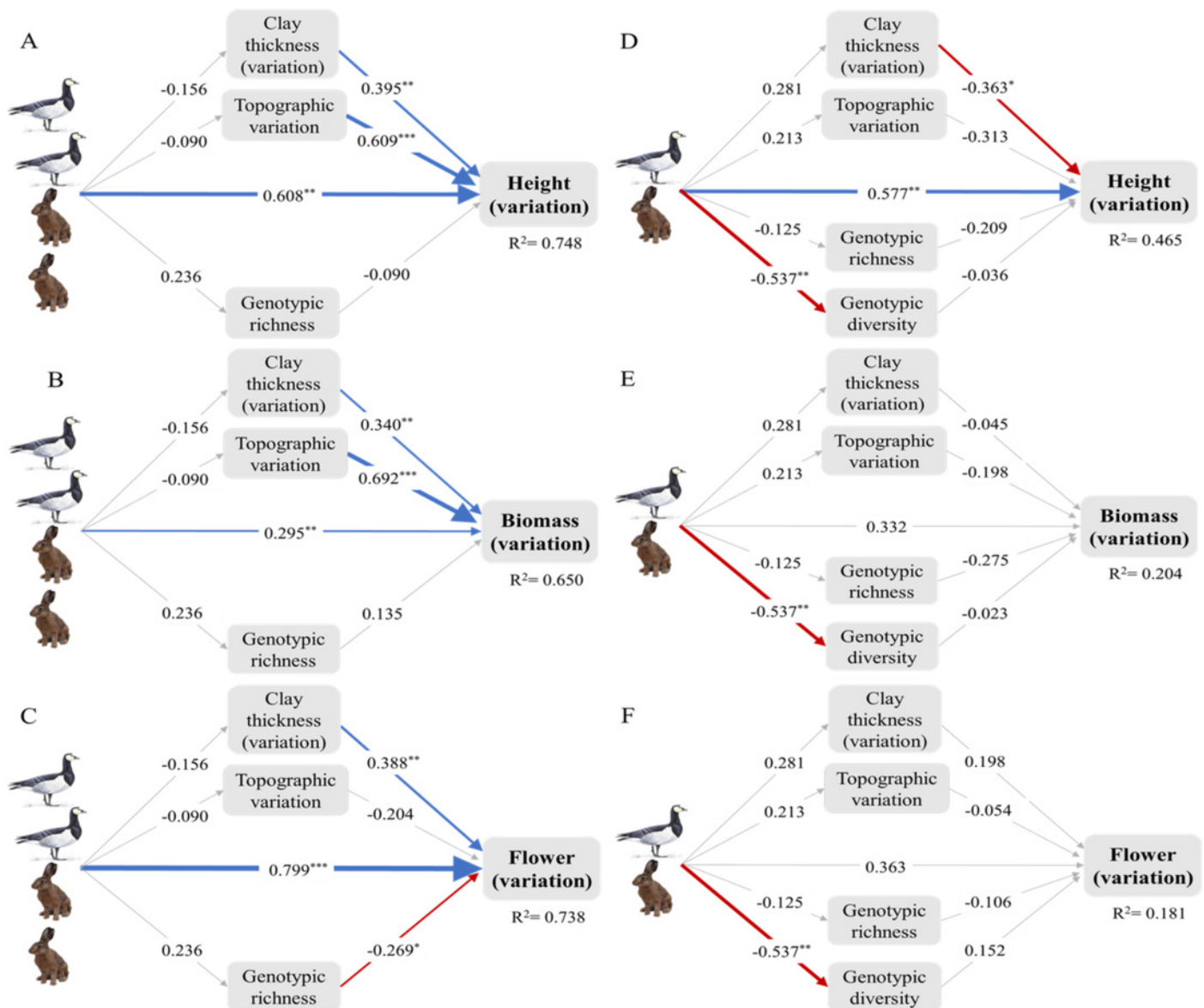



Figure 3

Intraspecific trait variation (ITV) of the dominant grass *Elytrigia atherica* and the direct and indirect effects of small herbivores on ITV in local communities at the early (A-C) and intermediate stage (D-F).

The direct effects, indirect effects through genotypes, indirect effects through abiotic variations, and total effects of small herbivores on ITV in each trait are summarized in Table 1. Model fit the data well (for all models at the early successional stage: χ^2 = 4.409, df = 3, N = 14, p > 0.05; for all models at the intermediate successional stage: χ^2 = 6.559, df = 6, N = 14, p > 0.05). Variance explained for clay thickness (variation), topographic variation, and genotypic richness for models at the early successional stage are 0.024, 0.008, and 0.056, respectively. Variance explained for clay thickness (variation), topographic variation, genotypic richness, and genotype diversity for models at the intermediate successional stage are 0.079, 0.045, 0.016, and 0.289, respectively. Number of hares and geese indicate the abundance of small herbivores such that the early successional stage had higher grazing pressure (indicated by two hares and two geese) relative to the intermediate stage (indicated by one hare and one goose). Boxes are measured variables. Arrows denote unidirectional relationships among variables. Blue arrows are significant positive relationships, red arrows are significant negative relationships, and grey arrows show non-significant relationships. The width of the arrows indicates the strength of the pathways. The values on the arrows denote standardized path coefficients. Asterisks indicate significant paths: * p < 0.1; ** p < 0.05; *** p < 0.001. See Fig. S2 for the direct and indirect effects of small herbivores on ITV in specific leaf area at the both successional stages. Note that leaf dry matter content was not evaluated in structural equation models due to limited sample size (see online supporting text).

Direct and indirect effects of hares and geese on intraspecific trait variation (ITV) of *Elytrigia atherica* at the early and intermediate successional stages.

See online supporting text for an example of how these data were calculated and how significance was determined using lavaan SEM. Asterisks indicate significant effects: * $p < 0.1$; ** $p < 0.05$; *** $p < 0.001$.

Successional stages	Traits	Direct effects	Indirect effects through genotypes	Indirect effects through abiotic variations	Total effects
Early	Height	0.608***	-0.021	-0.117	0.47**
	Biomass	0.295**	0.032	-0.116	0.211
	Flowering	0.799***	-0.063	-0.042	0.694***
Intermediate	Height	0.577**	0.045	-0.169	0.454*
	Biomass	0.332	0.022	-0.055	0.299
	Flowering	0.363	-0.068	0.044	0.339