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Impact assessment of *Leptoglossus occidentalis* in *Pinus pinea*: Integrating population density and seed loss

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30

31 **Keywords:** Western conifer seed bug, Stone pine, Mediterranean region, forest management, invasive
32 insect

33

34 **1. Introduction**

35

36 The North American Western conifer seed bug, *Leptoglossus occidentalis* Heidemann 1910 (Hemiptera:
37 Coreidae) was first introduced in Europe through Italy in 1999. The species is now distributed throughout
38 the entire continent as a result of its high dispersal ability and multiple independent introductions, both
39 natural and human mediated (Lesieur et al. 2014, 2019; Farinha et al. 2018b). The seed bug has also been
40 observed in Asia and North Africa (Zhu 2010; Ahn et al. 2013; Jamâa et al. 2013; Gapon 2015) and more
41 recently in South America and South Africa (Faúndez and Rocca 2017; Kun and Masciocchi 2019; van
42 der Heyden 2019; van der Heyden and Faúndez 2020).

43 This hemipteran species mainly feeds on reproductive conifer structures, using its sucking mouthparts. It
44 may feed on all cone development stages, from conelets to ripening cones with mature seeds (Koerber
45 1963). Occasionally, it may feed on xylem for hydration (Farinha et al. 2018b). In Europe, the species has
46 been observed feeding on several European pine species, namely on Maritime pine *Pinus pinaster* Ait.
47 (Sousa and Naves 2011), Scots pine *Pinus sylvestris* L. (Lesieur et al. 2014), Aleppo pine *Pinus halepensis*
48 Mill. (Kment and Baňar 2008; Maltese et al. 2009; Petrakis 2011; Hizal 2012), Black pine *Pinus nigra* J.
49 F. Arnold (Tescari 2004; Maltese et al. 2009; Hizal 2012; Lesieur et al. 2014) and Stone pine *Pinus pinea*
50 L. (Feducci et al. 2009; Maltese et al. 2009; Sousa and Naves 2011; Hizal 2012; Bracalini et al. 2013).

51 The Stone pine is an emblematic tree species of the Mediterranean Basin, with significant ecological and
52 landscape relevance for Mediterranean ecosystems. Its edible seeds are highly priced being sold, on the
53 market, at values up to 100€/kg (Mutke et al. 2012; Farinha et al. 2018c). The market demand for these
54 seeds is quite high, having even led producers to invest in management programs using artificial watering
55 and fertilization (fertirrigation) to increase production (Farinha et al. 2018a). Damage to Stone pine seeds,
56 cause particular concern due to their economic value (EUFORGEN 2009; Mutke et al. 2012). Stone pine
57 plantations have increased greatly in the last decades throughout the Mediterranean (Mutke et al. 2014).
58 In Portugal alone, the Stone pine area increased 61% between 1995 and 2015 (ICNF 2015). The Iberian
59 Peninsula gathers approximately 70% of the global distribution range of the species. If Italy and Turkey

N - natural exposed cones; E - cones inside insect exclusion bags; FR - fertilized and irrigated trees; C - control trees

60 are added, then the four countries account for 94% of Stone pine stands worldwide (Mutke and Calama
61 2016). Both cone and seed yield losses have been reported in Italy, Turkey, Spain, Portugal and Lebanon,
62 (Mutke et al. 2017). To note that the complete reproductive cycle of Stone pine cones takes place over
63 three years, resulting in a long exposition to damage. The spring conelets (female strobili) are wind
64 pollinated a few weeks after emergence, but fertilization only takes place during spring/summer of the
65 third year of cone maturation, when edible seeds are formed (Pimpão 2014). Conelet mortality high rates
66 as well as the presence of an abnormal numbers of both empty and small undeveloped seeds have been
67 recorded at increasing levels since the beginning of the century (Mutke et al. 2017). The two phenomena
68 together were named as Dry Cone Syndrome (DCS). The invasive western conifer seed bug has been
69 pointed out by many as the possible main cause of DCS (Bracalini et al. 2013; Mutke et al. 2014).
70 Damage to seeds in early developmental stages would cause seed and conelet abortion, whereas damage
71 on mature cones would cause damaged seeds. Still, the piercing sucking mouthparts of the seed bug leave
72 no visible traces on cones or kernels, which makes it difficult to irrefutably assign damages to the insect's
73 feeding activity. Meanwhile, other causes were suggested for this recent phenomenon of increased DCS.
74 Climatic conditions and, especially spring and summer drought intensification in the Mediterranean due
75 to climate changes has been frequently suggested (Mutke et al. 2017; Farinha et al. 2018a). In fact, studies
76 that point to *L. occidentalis* as a cause for DCS also point to the climate as acting together (Farinha et al.
77 2018a; Calama et al. 2020). In addition, the impact of emergent pest species, primarily the native cone
78 worm borer *Dioryctria mendacella* (Lepidoptera: Tortricidae) has also been discussed (Calama et al.
79 2017). Yet, in contrast with the seed bug, the activity of the cone worm borer is easy to identify, leaving
80 galleries and excrements on attacked conelets and cones. Most likely, the severe cone and seed losses
81 recorded are caused by multiple and interactive factors (Farinha et al 2018a; Calama et al 2020). In fact,
82 in a previous study using insect exclusion bags, it was demonstrated that the damage by the seed bug was
83 probably not the only cause of empty seeds, as it was observed also in protected branches (Farinha et al.
84 2018a). However, cones were protected only during one summer season, which does not allow
85 concluding on the damages done on previous years. Also, in this previous study, fertirrigated trees
86 appeared to be more susceptible to seed damage, which could be caused by *L. occidentalis*.
87 The difficulties associated with the quantification of the impact of this insect also hinder the decision on
88 how and when to act regarding pest control. In some countries, such as Turkey and Portugal, chemical
89 control has been applied in field conditions. In Portugal, an authorized commercial product, TEPPEKI,

90 based on a pyridine organic compound (flonicamid) is now authorized and available in the market
91 (DGAV 2019). However, without being able to clearly identify the damage caused by the seed bug,
92 producers may be applying chemical control treatments blindly, without knowing if they are actually
93 needed or cost-effective to control *L. occidentalis*. Furthermore, the detection of the seed bug in the field
94 is only possible through direct observation, as there are no traps available at the moment for this insect
95 pest. However, field observations of the seed bug are nearly impossible in natural non-grafted Stone pine
96 stands where trees are over 10 meters high.

97 This study presents the first attempt to estimate the impact of *L. occidentalis* on seed yield through a
98 combined field methodology that correlates the damage on seeds with the presence and abundance of the
99 bug at the site level on a long-time scale (5 years). By combining these two variables, and comparing
100 exposed and protected cones in exclusion trials, we aim to clarify to what extent the insect is responsible
101 for the damage to Stone pine seeds, as reported in the last decade in the Mediterranean region. Other
102 studies have used exclusion methods (Mutke et al. 2017; Ponce 2017; Farinha et al. 2018a) but none of
103 them were accompanied by field monitoring of *L. occidentalis* populations, leaving uncertainties on the
104 actual causes of damage. Additionally, we want to understand the impact of stand management by
105 watering and fertilization on the susceptibility to the conifer seed bug. In a previous study, by using bags
106 with caged bugs in branches with cones, we observed that both seed damage and cone mortality tended to
107 be higher on fertirrigated trees (Farinha et al. 2018a). This hypothesis is in line with the well-documented
108 observations that some insect feeding guilds, such as sapsuckers, may benefit from host plant watering
109 and nitrogen availability, which increases the nutritional value of the host plant (Jactel et al. 2009). In this
110 work, we wanted to confirm this hypothesis for *L. occidentalis*. The outcome is particular relevant for
111 landowners interested in using fertirrigation to increase productivity in orchard plantations.

112

113 **2. Material and Methods**

114

115 **2.1 Experimental field sites**

116

117 The study was conducted in two sites, hereafter called A and B, located in the center of Portugal. Both
118 plantations consisted of grafted Stone pine trees. Climate is similar on both sites - Mediterranean with a
119 cold and moist winter and dry and hot summers, with annual precipitation around 600 mm and a mean

N - natural exposed cones; E - cones inside insect exclusion bags; FR - fertilized and irrigated trees; C - control trees

120 annual temperature of 17 °C. The understory of both plantations comprises a mixture of annual grasses,
121 legumes and forbs. The soil is sandy-loam with a low organic matter (<0.5%).

122 Study site A is located in Coruche (39°06'25.7"N, 8°21'40.6"W), in a pure *P. pinea* stand planted in 2007
123 and grafted at the age of four. Within the site, an area of 3 ha was selected and four randomized blocks
124 each including a pair of plots of two different tree management treatments were installed during the
125 summer of 2014. Each plot comprehends 15 to 21 trees spaced by 3 x 8 m. The tree management
126 treatments were Control (C) and a regime consisting of fertilized + irrigation, hereafter called
127 Fertirrigation (FR). Trees were fertirrigated through a dripping irrigation system with diluted fertilizer (40
128 kg N ha⁻¹) during the growing season (March to October), from 2014 to 2017. This irrigation regime
129 corresponded to an additional water input to the system of approximately 270 mm ha⁻¹ year⁻¹. Samples of
130 ripening cones were collected always at the beginning of the cone harvesting campaign in December over
131 a period of 5 years, from 2015 to 2019.

132

133 Site B is located in Vendas Novas (38°44'34.9"N, 8°31'59.0"W), in a Stone pine plantation installed in
134 2010, with a 7 x 3.5m line spacing and grafted at the age of four. In an area of 3 ha, two blocks were
135 selected, each containing 2 plots, one for each of the two management treatments (Control, C and
136 Fertirrigation, FR). Trees were fertirrigated through a dripping irrigation system since plantation (2010)
137 with 100 kg/ha of NPK injected in the irrigation system. During the time of the experiment, an average
138 100 mm per year was added daily from March to October. The production of last year cones started in
139 2018. Thus, for this site we only have samples of cones collected in December in the years 2018 and
140 2019.

141

142 **2.2 Temporal viable seed yield variation in experimental field trials**

143

144 A field experimental trial enclosing cones in insect exclusion bags was set up at both sites, A and B. We
145 started by randomly select 10 trees of each tree management treatment (C and FR) among all productive
146 trees from each site. The 3rd year cones of each sample tree were counted, labeled and divided into two
147 sets: half were covered with an individual insect-exclusion bag and the other half were left exposed. The
148 number of sampled cones per tree varied between trees and sites, depending on the number of cones
149 available.

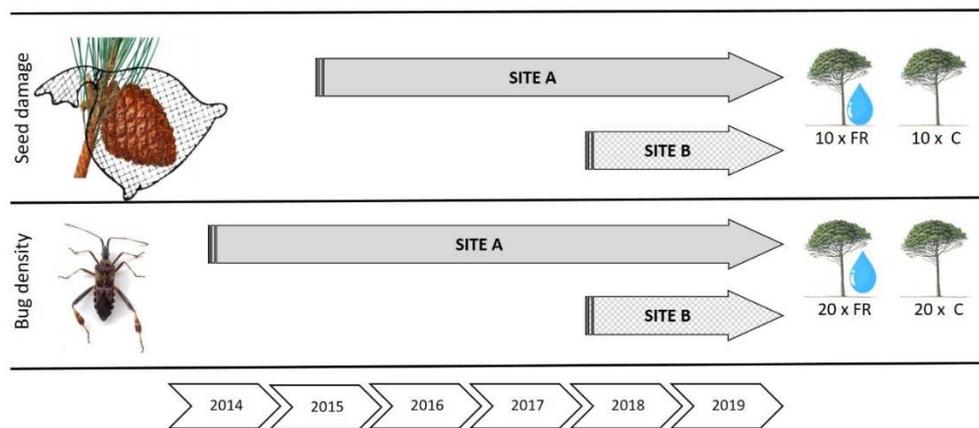
N - natural exposed cones; E - cones inside insect exclusion bags; FR - fertilized and irrigated trees; C - control trees

150 Insect exclusion bags were made out of 0.5 mm moldable metal mesh. The bags were built with enough
 151 space between the cone and the mesh, thus preventing insects from feeding from the outside. Each year,
 152 bags were set up in April, before the emergence of the overwintering adult bugs, which occurs in early-
 153 mid May in the studied region. The insect is active throughout the summer and until mid-autumn. It stops
 154 feeding and seeks a winter refuge for overwintering by end of October/ early November. Bags were
 155 removed in December and all labeled cones were harvested (Fig. 1).

156 Overall, we had two exclusion treatments: cones within insect-exclusion bags (E) and cones exposed
 157 naturally (N), and two tree management treatments: cones on control trees (C) and cones on fertirrigated
 158 trees (FR).

159 The following variables were measured for each harvested cone individually: cone fresh weight (g), cone
 160 dry weight until scales opening (55°C in oven until the scales open, which usually takes 5 to 10 days) (g)
 161 and number of pine seeds in shell. All pine seeds were opened manually and the shells separated from the
 162 kernels. The kernels were weighed and separated into four categories: i) intact kernel with no damages
 163 (which we call hereafter sound seeds); ii) kernel partially damaged/with a different color/serous
 164 appearance (named as “damage type I”); iii) completely shrunken and dry embryo with no endosperm
 165 (named as “damage type II”); and, iv) empty shell without embryo (named as “damage type III”). This
 166 classification follows Farinha et al. (2018a).

167



168

169

170 **Fig.1** Field trials. The top diagram concerns the two field trials methodology: first row corresponds to the
 171 assessment of seed damage in ripening cones by comparing cones enclosed in insect exclusion bags (E)
 172 with cones naturally exposed (N) (see Methods 2.2). The bottom row corresponds to the prospection of *L.*

N - natural exposed cones; E - cones inside insect exclusion bags; FR - fertilized and irrigated trees; C - control trees

173 *occidentalis* population density in the field (see Methods 2.3). Both trials were conducted in plots with
174 irrigated and fertilized pines (FR) and in plots with control pines (C).

175

176

177 **2.3 *Leptoglossus occidentalis* population abundance in natural field conditions**

178

179 The presence and abundance of *L. occidentalis* was assessed monthly from May to November every year
180 by direct observation of branches and cones in each site. Observations on site A were conducted from
181 May 2014 until November 2019 and on site B from May 2018 to November 2019. Insect observations on
182 site B started in 2018 due to the absence of cones up until that year. In both sites, 20 trees from each tree
183 management treatment (C and FR) were randomly selected each year, among all producing trees (Fig.1).
184 The methodology consisted on careful observation of the whole tree canopy for at least 5 minutes,
185 completing a full turn of the canopy. The direct observation of the branches and cones from the ground or
186 using a small ladder was possible in both sites due to the small size of the grafted trees (2-5 m high) and
187 the easy access to the canopy. The development stage of identified insects (nymph or adult) was recorded
188 but no collections were made in order to avoid perturbation on the records for the following months.

189

190 **2.4 Climatic data**

191

192 Daily records of meteorological data were obtained from local meteorological stations installed near the
193 sites. Air humidity and temperature (CS215; Campbell Scientific, Inc., Logan, UT, US) were collected
194 continuously in 30-min time intervals (CR10X; Campbell Scientific, Inc., Logan, UT, US). Due to a local
195 station failure at site A during the summer of 2016, missing data for average temperatures, maximum
196 temperatures and relative humidity were modeled from site B climatic data, through linear models
197 generated from data from both sites corresponding to the years of 2015 and 2017. Hemipteran insects are
198 quite sensible to air temperature and in particular to Vapor Pressure Deficit (VPD) affecting their activity
199 and even their distribution (de la Vega and Schilman 2017). To evaluate climatic data that could influence
200 summer survival, the number of days with maximum temperatures over 34°C and 36°C was recorded on a
201 monthly basis for the whole duration of the study. Vapor Pressure Deficit (VPD) was calculated using
202 maximum daily temperatures and average daily relative humidity for the period of insect's activity. We

203 considered 25 hPa threshold for the insect activity, in accordance with what was observed for other
204 Hemiptera species (de la Vega and Schilman 2017).

205

206 **2.5 Statistical analysis**

207

208 To evaluate the effects of the insect-exclusion bags (E vs N) and FR treatment (FR vs C) with the
209 correspondent control conditions we used the ratio between the number of intact kernels over the total
210 number of pine seeds per cone (*viable seed yield*).

211 Treatment comparisons were performed using generalized linear models (GLM), with a binomial
212 distribution. The response variable was the viable seed yield. Seeds with any type of damage were
213 considered as nonviable. Cones belonging to the same tree were considered as repeated measures in the
214 models, controlling the subject effect.

215 Two models were used with different objectives. In site A, with a long-term data set of 5 years, we were
216 interested in analyzing the effect of the year on the exclusion treatment. Therefore, a model was built for
217 all study years considering two fixed factors (exclusion treatment and year) and their interaction. When
218 significant differences were found, pairwise differences were further explored using Tukey tests. For this
219 location, we also analyzed separately the response variables: type I damage and type II + type III damage
220 pooled together using the methodology outlined above. Type II and III have been considered together to
221 avoid misclassifications in the laboratory as the damage is sometimes difficult to distinguish, especially
222 when the kernel is very shriveled or stuck to the shell.

223 For both site A and B, we further used generalized linear mixed model to analyze the fixed effects of tree
224 management, exclusion treatment and their interaction. Year was considered a random effect. For site A
225 we used data for the first three study years only, when tree management treatments were in place, since
226 tree treatments were discontinued after 2017.

227 Temporal trends in *L. occidentalis* populations were analyzed using the average number of insects per
228 tree (including all life stages), for each year and site. This average corresponds to the mean number of
229 insects observed per sampled tree during the month with more observations, which corresponds to the
230 peak of their activity (June-July).

231 The percentage of seed loss (per cone) due to the activity of *L. occidentalis*, was calculated using the
232 following equation:

N - natural exposed cones; E - cones inside insect exclusion bags; FR - fertilized and irrigated trees; C - control trees

233

234
$$\text{Seed loss (\%)} = \frac{\text{Average Viable Seed Yield (E)} - \text{Average Viable Seed Yield (N)}}{\text{Average Viable Seed Yield (E)}} \times 100$$

235

236 In which Average Viable Seed Yield (E) is the average proportion of sound seeds on cones inside insect
237 exclusion bags, and Average Viable Seed Yield (N) is the average proportion of sound seeds on naturally
238 exposed (unprotected) cones. We also analyzed possible correlations and linear relationships through
239 linear regression models between the average number of insects per tree and seed loss. Taking into
240 account that cones are susceptible to *L. occidentalis* during the entire reproduction cycle, the relationship
241 was analyzed between seed loss in a current year t and the average number of insects in the current N_t and
242 previous year N_{t-1} . Data is presented as average \pm SE throughout the text.

243

244 3. RESULTS

245

246 3.1 Variation of viable seed yield in experimental field trials

247

248 3.1.1 Temporal trends of viable seed yield

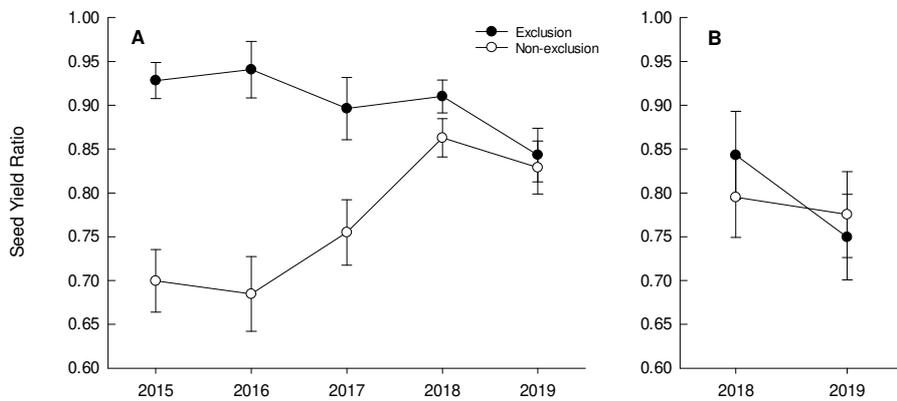
249

250 During the 5 years of the study, 560 cones were collected (354 in site A and 206 in site B) and a
251 total of 44 133 pine seeds were processed. Overall, we found 80 ± 1 pine seeds in shell per cone, of which
252 on average 67 ± 1 were sound (Table 1 Supplementary material).

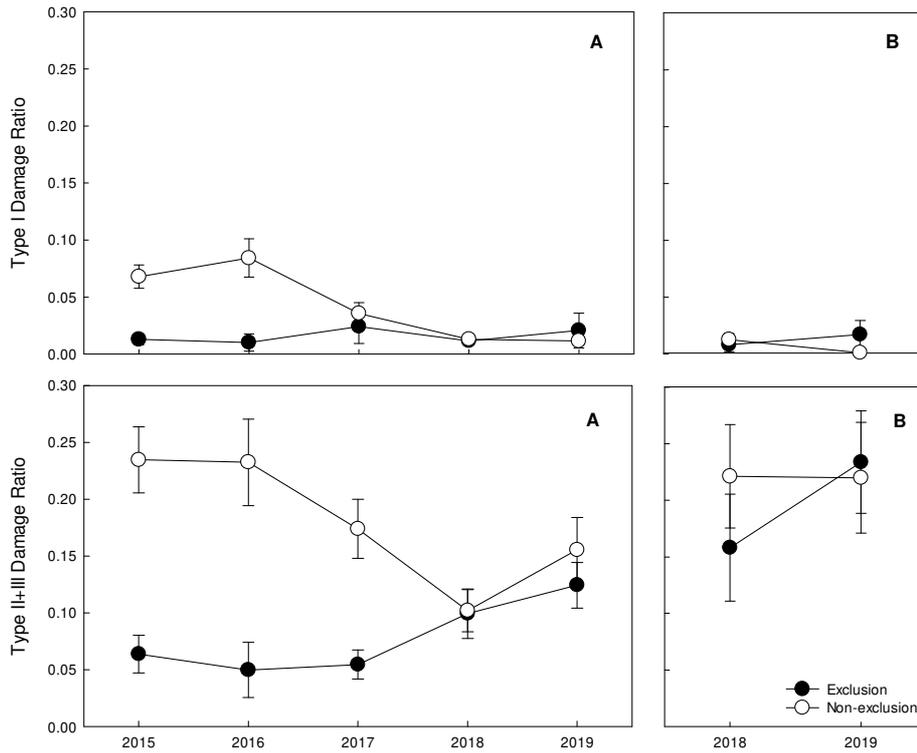
253 In site A, significant differences between exclusion treatments were concentrated in the first three study
254 years (2015: $p < 0.001$, 2016: $p < 0.001$ and 2017: $p = 0.008$), with viable seed yield of cones inside
255 insect-exclusion bags significantly higher than exposed ones (Fig. 2). During the last two years of the
256 study, no significant differences between viable seed yield of different treatments were found. Cones
257 enclosed in insect-exclusion bags have relatively constant viable seed yields, not different statistically
258 over the study period, being only slightly lower in 2019. On the contrary, viable seed yield of cones
259 without insect-exclusion bags presented a greater variation over time increasing nearly 20% from the
260 beginning to the end of the study.

261 When damage types were analyzed separately in site A (Fig. 3), we observe that type I damage was in
262 general less prevalent than more severe kernel damages (type II+III). The temporal pattern is similar in
N - natural exposed cones; E - cones inside insect exclusion bags; FR - fertilized and irrigated trees; C - control trees

263 both cases, with differences during the first study years, which gradually disappear (Fig. 3). For type I
 264 damage, there are significant differences between treatments in 2015 ($p < 0.001$) and 2016 ($p < 0.001$),
 265 and for type II+III damage, there are significant differences in 2015 ($p < 0.001$), 2016 ($p = 0.003$) and
 266 2017 ($p < 0.001$). During the first two years, both damage types contribute to the differences found
 267 between insect exclusion treatments, whereas in 2017 type II+III alone is responsible for them. From
 268 2018 to 2019, we observe an increase in type II+III damage for both insect exclusion treatments,
 269 associated with the slight decrease in viable seed yield during this period.
 270



271
 272 **Fig. 2** Temporal trends in viable seed yield (proportion of intact kernels on the total number of seeds per
 273 cone, average ratio \pm SE) according to exclusion treatments: *Exclusion* (E) - cones inside insect exclusion
 274 bags (E) and *Non-exclusion* (N) - cones naturally exposed. Error bars represent standard error. (A – site
 275 A, B – site B)
 276



277

278 **Fig. 3** Temporal trends in type I (top) and type II + III (bottom) damage ratio (average ratio \pm SE) in
 279 kernels per cone according to insect exclusion treatments. Error bars represent standard error. (A – site
 280 A, B – site B)

281

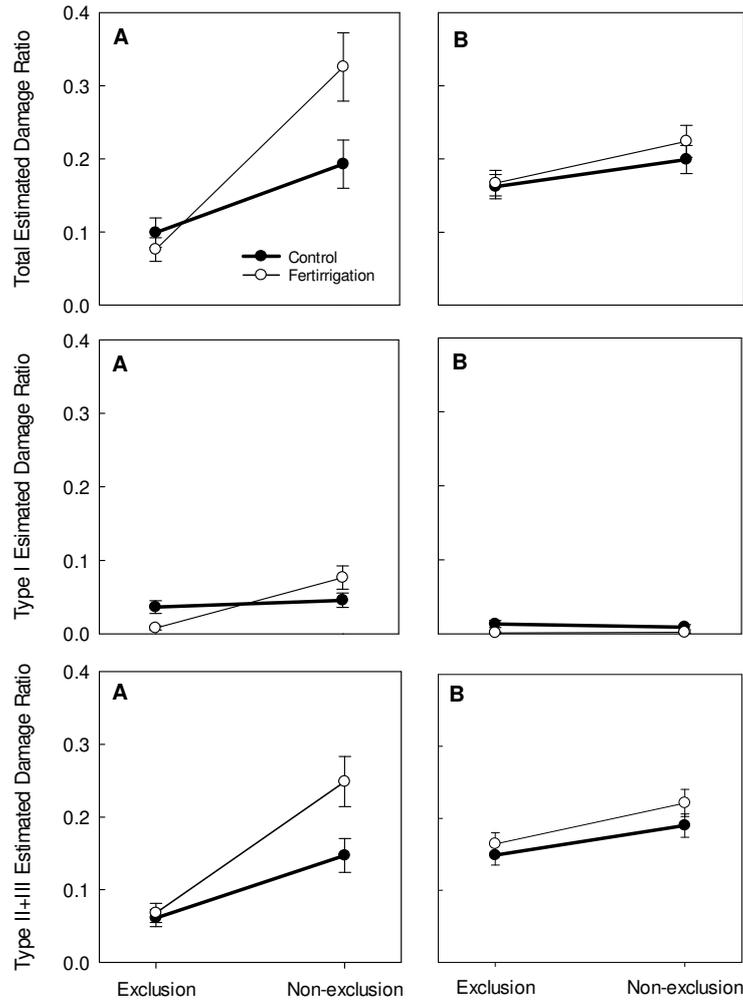
282 3.1.2 Effect of tree management on viable seed yield

283

284 When the tree management treatment (C, FR) is analyzed for seed damage, in site A significant
 285 interaction between treatment types (exclusion treatment, management treatment) is observed for all
 286 damage types (total damage - $p < 0.001$, type I damage - $p < 0.001$, type II+III damage – $p=0.001$) (Fig.
 287 4). Both in the control C and FR plots, insect exclusion cones had higher yields than non-exclusion ones
 288 ($p < 0.001$). In cones within insect exclusion bags, total damage was higher in control plots in comparison
 289 with FR plots ($p = 0.035$), while in non-exclusion cones the opposite was observed with FR cones
 290 showing a much higher damage ratio than control ones ($p < 0.001$). Considering type I damage, only FR
 291 cones showed significant higher damaged seeds on non-protected cones ($p < 0.001$). For type II+III
 292 damages, results are similar to those of total damage, but here with no significant difference between C
 293 and FR on the insect exclusion cones.

294

N - natural exposed cones; E - cones inside insect exclusion bags; FR - fertilized and irrigated trees; C - control trees



295

296 **Fig. 4** Damage ratio (estimated damage ratio \pm SE) according to both insect exclusion and tree
 297 management treatments. Error bars represent standard error. (A – site A, B – site B)

298

299 In site B, tree management treatment showed an effect for all damage types (total damage - $p = 0.034$,
 300 type I - $p < 0.001$, type II+III – $p < 0.001$). Higher damage was observed for FR cones when compared to
 301 control cones (total damage – $p=0.038$, type II+III – $p=0.001$). Insect exclusion treatment also had an
 302 effect for total damage ($p < 0.001$) and damage type II+III ($p < 0.001$), with higher damage for
 303 unprotected cones when compared with exclusion cones (total damage – $p < 0.001$, type II+III – $p <$
 304 0.001). For type I damage, despite no differences in exclusion versus exposed cones, C cones were less
 305 affected than FR ones ($p=0.006$). In site B, the models detect no significant interactions between factors
 306 for any damage type.

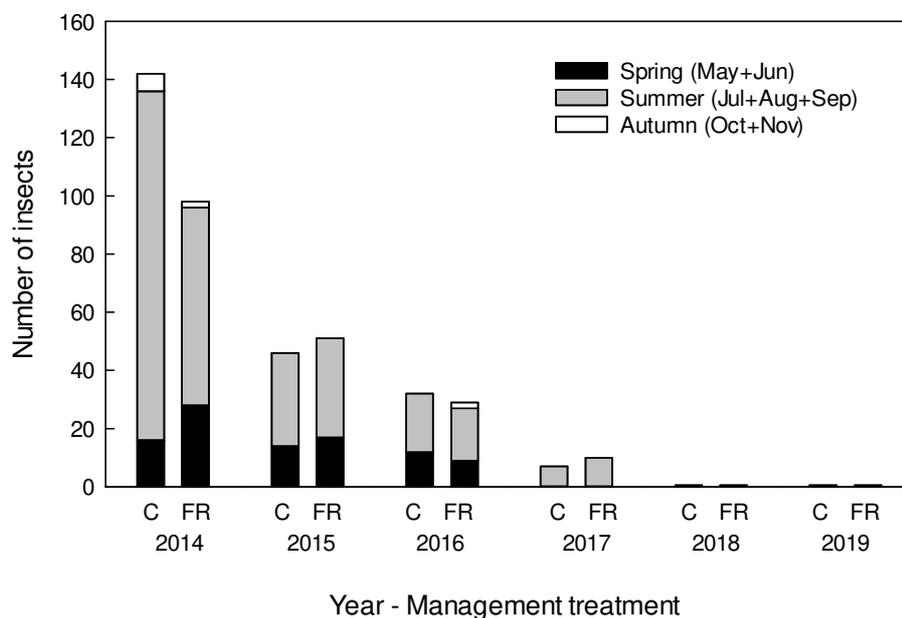
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N - natural exposed cones; E - cones inside insect exclusion bags; FR - fertilized and irrigated trees; C - control trees

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3.2. *Leptoglossus occidentalis* population abundance in natural field conditions

At site A, individuals of the local population of *L. occidentalis*, were observed in the first four years of the study but no individuals were detected in the following two years (Fig. 5). The month with the highest number of insects observed was July for all years except 2014, for which it was June. There were no differences in the number of insects observed between FR and control plots ($p = 0.620$). No insects were observed at site B for the whole duration of the study.



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Fig. 5 Total number of insects observed per year, season and tree treatment in Site A. C - control trees FR – fertirrigated trees (this treatment was applied from 2014 to 2017)

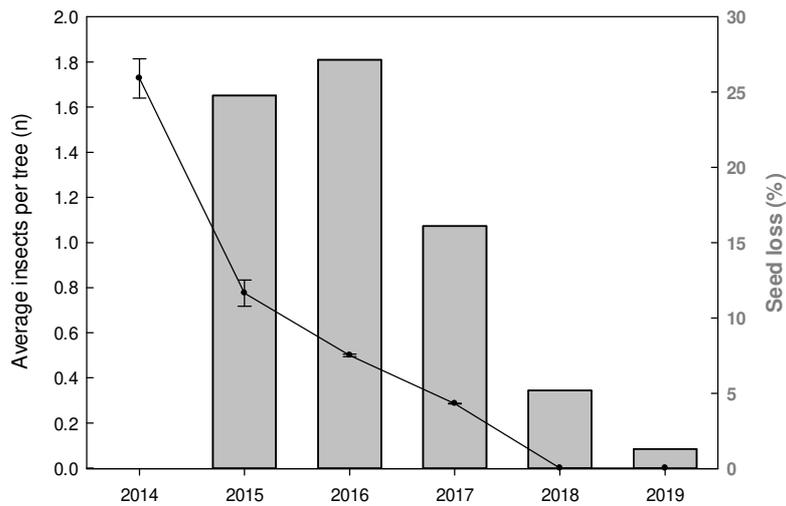
During the 6-year period, we observed a steady decrease of population numbers over time (Fig. 5). From 2014 to 2017, the period in which the insect was observed in the field, the population declined exponentially (equation $N_t = N_0 e^{\lambda t}$, growth rate $\lambda = -0.584$, $r^2 = 0.98$). A sharp drop in the average number of individuals observed per tree occurred from 2014 to 2015, with the loss of over 60% of the population (Fig. 6). From then on, a steady decrease was observed. In 2018 and 2019, no insects could be detected. Since this insect has a gregarious behavior with a patchy distribution in the field, we also calculate the percentage of trees with insects in the sample tree pool. Here too we noticed a decreasing pattern throughout the study period. Taking per year the month with the highest number of observations, in 2014

N - natural exposed cones; E - cones inside insect exclusion bags; FR - fertilized and irrigated trees; C - control trees

328 we were able to observe insects in more than half of the trees inspected (55%). In 2015, the number
329 dropped to 33%. In 2016, it continued to decline to only a quarter of the trees with insects, reaching a
330 minimum of one-fifth (20%) in 2017.

331 When we analyze temporal patterns of seed loss at site A we observe a correspondence between years
332 with significant insect exclusion treatment differences and years with high seed loss (over 15%). These
333 correspond to the years in which *L. occidentalis* was still present on site (up to 2017, Fig. 6). We also
334 observe a decrease in seed loss that follows the population decline very closely over time (Fig. 6). From
335 2018, when the population density of the insect decreased making it difficult to detect it in the field, seed
336 loss decreased also to only about 5% in 2018 and under 2% in 2019. The correlation between seed loss in
337 the current year and the average number of insects observed in the current and previous year was
338 significant ($r^2=0.95$, $p=0.02$).

339



340

341 **Fig. 6** Temporal trends in *L. occidentalis* population numbers (average number \pm SE) in site A. The
342 average corresponds to the mean number of insects observed per sampled tree during the month with
343 more observations each year. Grey bars represent percentage seed loss per cone.

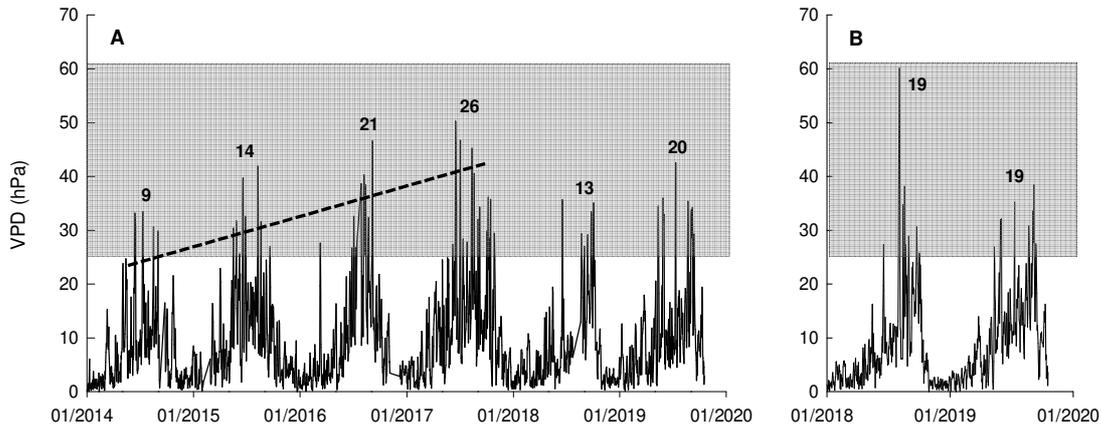
344

345 3.3 Climatic data

346

347 Average monthly temperatures during summer were similar between sites. In both sites the year of 2017
348 was exceptionally hot and dry with the period of insect activity (from April to November) showing

349 maximum records of extreme daily temperatures and vapor pressure deficits. In site A, we observed an
 350 increasing trend of the number of extremely hot days from 2014 onwards culminating in 2017 with
 351 maximum values but decreasing thereafter in 2018 and 2019 (Fig. 1 Supplementary material). Days with
 352 VPD over 25 hPa peaked also in 2017, with a record of 26 days (Fig. 7). Site B appears to show more
 353 extreme conditions for insect activity and survival during the studied period, especially in 2018.



354

355 **Fig. 7** Daily calculated values of VPD (hPa). Numbers represent the total number of days were calculated
 356 VPD was higher than 25 hPa. Grey shades represent the VPD threshold above 25 hPa. The dashed line
 357 shows the increasing trend between 2014 and 2017.

358

359 4. Discussion

360

361 By analyzing, both the densities of the insect populations observed in the field and the seed damage later
 362 obtained in harvested cones we were able to correlate the two variables and thus clearly quantify the
 363 bug's impact in seed loss per cone.

364 A recent and extensive study led by Calama et al. (2020) reported a severe decline in seed yield of *P.*
 365 *pinea* in Spain. The authors compared historical data (1996-2005) and recent data (2012-2015) of cone
 366 and seed production and highlighted the bug as the main cause of the observed decline in seed yield. Seed
 367 damage as large as 50% was observed in some sites for the year of 2015 (Calama et al. 2020). We
 368 obtained a similar result for 2015, the only year in which both studies can be compared. In fact, the year
 369 of 2015 together with 2016 were the most damaging years in our time series but a recovery trend on
 370 viable seed yield was observed after 2016 with seed losses below 2% three years after, in 2019. Our
 371 results contradict an overall decline in viable seed yield of Stone pine as suggested in some studies

372 (Mutke et al. 2014; Calama et al. 2020) but rather a variable pattern that needs clarification as to its
373 determinants. A year of low cone production might result in high yield loss due to a concentration of
374 insects feeding on a small number of cones available. The year of 2015 was the one with the lowest cone
375 production in site A, as the trees were still young (8 years old) on its early years of cone production after
376 grafting. This may have contributed to the high seed damage observed in that year. Still, from 2016 to
377 2018 cone production was more or less constant (Table 1) whereas seed damage steeply decreased (Fig.
378 6). This decrease can only be justified by the decline of the bug population.

379 Seeds from cones naturally exposed to biotic agents presented a variable percentage of viable seed yield
380 over the five years of the study (varying from 70% to 86%). On the contrary, seeds from cones inside
381 insect exclusion bags had a steady and higher viable seed yield average (92%) considering only the long
382 time series of site A. This result clearly shows that the variations observed must be related with biotic
383 agent(s) causes and should be independent of meteorological conditions or tree physiological status that
384 would equally affect the two types of cones. Other field studies using insect exclusion bags and
385 comparing viable seed yield from protected and unprotected cones also reported a similar viable seed
386 yield average (94%) in cones protected with nets (Elvira-Recuenco et al. 2016; Farinha et al. 2018a).
387 However, in none of these studies were the results of the viable seed yield correlated with the population
388 density of *L. occidentalis*. By analysing both variables we found a strong positive correlation between
389 them ($r=0.98$) with high natural insect densities corresponding to high seed loss. Furthermore, the insect's
390 near-absence in the field in the last two years (2018 and 2019) would thus explain the lower seed losses
391 per cone observed – only 2% (contrasting with 25% on the first years) as well as the striking similarity in
392 seed losses between protected and unprotected cones. These results provide strong evidence for the
393 impact of *L. occidentalis* on seed loss in Stone pine. They also support previous studies that hypothesized
394 this new invasive bug as the main responsible for cone and seed losses in the Mediterranean region
395 reported in the last decade (e.g. Bracalini et al. 2013; Mutke et al. 2014; Elvira-Recuenco et al. 2016;
396 Calama et al, 2020).

397 When analysing the type of seed damage, the results corroborate again a clear impact of the insect. Both
398 damage Type I and Type II + III are much higher in unprotected cones than in protected ones in the first
399 two years when the insect density was also very high (2015 and 2016). Interestingly, in 2017, when the
400 population of the insect greatly decreases, the Type I damage also decreases (to only 2.5%) with no
401 differences between unprotected and protected cones. It should be noted that this type of damage reflects

402 the feeding by the insect on the already fully developed seed of last year cones (ripening cones) (Farinha
403 et al. 2018a, b). The low level of the insect population in 2017 may then explain the low damage Type I
404 and the consequent lack of difference between protected and unprotected cones in this year. In contrast, in
405 this same year, the Type II + III damage was greater on unprotected cones. Again, this may be linked to
406 the different impact of the insect if it feeds on young or mature cones. This type of damage (Type II + III)
407 is observed when the insect feeds on young cones as seen in other Pine species (Schowalter and Sexton
408 1990; Bates et al. 2001). Therefore, it may reflect the presence of the insect in the previous year when it
409 was still abundant.

410 In the last two years (2018 and 2019), no differences between protected and unprotected cones were
411 observed in site A in any damage category which is in line with the near absence of the insect in the field.
412 This near-absence in the field means that the population is below detectability level by direct observation
413 and not that it has disappeared completely. The significant percentage of seeds with damage Type II + III
414 on naturally exposed cones on site B in 2018 could be the result of damage caused by the bug on previous
415 year or other undetermined causes. Nevertheless, site B corroborates the trend observed in site A
416 regarding the low density of the insect in the field in 2018 and 2019. Geographically, the two study sites
417 are located 50 km apart and have similar climate and soil characteristics. Observations elsewhere in the
418 country that encompass several regions in central and South of Portugal (Santarém, Setúbal, Évora) have
419 also revealed low insect population levels in 2018 and 2019 leading to believe that external factors may
420 be influencing population dynamics on a large scale and that the insect population fluctuations do not
421 occur locally.

422

423 We further investigated viable seed yield and the presence of the insect in trees growing in a better water
424 and nutritional environment (fertirrigated plots) compared with trees growing in natural conditions. Trees
425 from fertirrigated plots presented a consistently lower viable seed yield in unprotected cones in both sites
426 although we have not found an apparent preference in insect distribution for trees with a particular
427 management treatment. It is important to note that the patchy distribution of this insect makes it difficult
428 to carry out an unbiased sampling of its actual distribution that only a census could address. Previous
429 studies have showed this higher damage to seeds in fertirrigated trees when compared to control ones
430 (Farinha et al. 2018a). It is possible that these trees are more attractive, both nutritionally and visually
431 (larger canopies), to the seed bug *L. occidentalis*. Similar preference was shown for other plant sucking

432 insects (Moran and Whitham 1988; Kimberling et al. 1990; Price 1991) as well as other seed and cone
433 pests (Mitchell 2006). It is also likely that well irrigated and fertilized trees support higher survival of the
434 seed bug nymphs which are more demanding in humidity than adults (Farinha et al. 2018a). Forest
435 managers should therefore balance the management costs of fertirrigation, addressing the potential
436 increase in cone production but a decrease in viable seed yield when the bug is present in high densities.
437 However, there is a relevant limitation to this. Effective and timely monitoring tools for the bug are still
438 lacking which is a challenge for the scientific community. In this study, the bug monitoring was only
439 possible using grafted pine orchards where the cones are at eye level. In the absence of traps, the
440 implementation of a network of grafted seed orchards to monitor the insect populations should be
441 articulated between researchers and producers and taken as a priority as it would benefit both. Producers
442 would have more information to support their pest management decisions. In particular, the chemical
443 control could then be carried out in an appropriate and non-blinded extent avoiding negative
444 environmental impact and unnecessary costs. Due to its patchy distribution, the number of trees (instead
445 of the number of bugs) where at least one insect is spotted is a practical tool for grafted seed orchards and
446 could serve as a proxy for the density of the bug in the field. In a practical way, we could observe that in
447 a sample of a cluster of grafted pines (n=20), when the bug was spotted in one-third to one-quarter of the
448 trees the loss in seed per cone was around 25%. Whereas if spotted in 20% of the trees the damage drops
449 to 15%. These results are the first quantitative indicators of the damage that a given insect population
450 density (estimated as described in the methods section) can cause on ripening cones of Stone pine. Yet, it
451 will still be necessary to replicate the experiment in more locations and further calibrate the results with
452 the annual cone production (resources availability for the insect) to get an estimate for the economic
453 injury level of *L. occidentalis*. It is relevant to note that in this study we evaluate the impact of the insect
454 only on the seed yield. Our analysis does not take into consideration the impact of this bug on the
455 mortality of young cones and thus, in the annual production of cones. Yet, we recognize, this can be a
456 further negative impact of the seed bug (Bates et al. 2002; Mutke et al. 2017; Farinha et al. 2018a). In
457 addition, attacks on last year of cone development may cause a larger number of inviable seeds (small
458 size), thus reducing the number of pine nuts per cone (e.g. Elvira-Recuenco et al. 2016; Farinha et al.
459 2018a). However, in contrast to seed damage type I and II, which are an evident sign of the activity of *L.*
460 *occidentalis* (Farinha et al., 2018b), it is not possible to decouple inviable seeds caused by the seed bug
461 from tree physiological factors and negative effects of abiotic factors on fertilization.

462 Alongside the absence of traps, another challenge is the population dynamics of this insect. It was
463 described as chaotic because its determinants are not known (Lesieur et al, 2014). Resource availability
464 can strongly influence insect population dynamics as observed for many seed feeding insects (e.g. Van
465 Klinken and Flack 2008; Johnson et al. 2016). This may be particularly important for insects feeding on
466 host species with masting cycles. In years when there is a counter-harvest, the number of insects decrease
467 with the opposite happening in years of high cone production (bumper years). In our study, pines were
468 still very young and time period not long enough to identify years of harvest and counter-harvest and its
469 putative effect on the population dynamics of the seed bug. Moreover, the local insect population was not
470 uniquely affected by the cone production of the experimental site, considering that the site is surrounded
471 by pine stands with mature trees in full cone production, which could act as reservoir. The population
472 dynamics of this insect is still an intriguing subject, which requires specific studies and longer time series
473 of cone production, climate and insect abundance for its understanding.

474 Climate has also been widely pointed out as a key factor in the dynamics of various insects (e.g. Bale et
475 al. 2002; Stange and Ayres 2010; Kiritani 2013; Pureswaran et al. 2018) but little is known about how it
476 affects *L. occidentalis* dynamics. During the 5 years of this study, the year of 2017 was exceptionally dry,
477 with maximum VPD values among the highest of the 5 years' time series. This year the insect population
478 density in the field declined abruptly. We associate it with consecutive days with extreme air dryness
479 during the period of insect activity imposing an environmental stress that may have reduce the number of
480 individuals considerably. In fact, an increasing trend of harsh summers was observed between 2014 and
481 2017. Although there are evidences that higher temperature often promotes the abundance of insects
482 (Wallner 1987), heat waves may exceed maximum biological thresholds decreasing fecundity and
483 survival (Musolin et al. 2009). Without further information however, we cannot safely conclude that this
484 was due solely to climate conditions. Future scenarios of climate change in the Mediterranean region
485 predict an increase in frequency of heat waves (Giorgi and Lionello 2008). More studies relating climate
486 with population dynamics, especially the impact of meteorological conditions on *L. occidentalis*
487 populations during summer are required. Insects' distribution models must integrate their resistance to
488 extremely high temperature events, as it may become a key controlling factor of insects' survival in some
489 regions of the globe (Rouault et al. 2006).

490

491 **5. Conclusions**

N - natural exposed cones; E - cones inside insect exclusion bags; FR - fertilized and irrigated trees; C - control trees

492

493 This work represents the first field study that integrates, at site level, both the variation in stone pine
494 viable seed yield and the population density of a key biotic agent of Stone pine, the invasive bug *L.*
495 *occidentalis*. This combined approach provided supported estimates on the impact of the insect on stone
496 pine seeds, which until then had been rather speculative. Results revealed that variation in viable seed
497 yield is strongly correlated with variation in bug densities. Where, as expected, greater damage
498 corresponds to higher bug densities. We could thus set out a basis for the estimation of an economic
499 injury level threshold for *L. occidentalis*. This threshold would later help producers to decide at which
500 value of the bug field density an investment in pest control becomes justified.

501 To note that, scientific efforts to find tools, such as pheromone-based traps or other attracting methods
502 which would allow for the monitoring of this insect species must continue.

503

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505

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512 **Authors' contributions** AF, MB conceived the manuscript. AF and AC conducted the field experiments.
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515

516 **7. References**

517

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