Flower color preferences of insects and cattle: effects on *Gentiana lutea* reproductive success

Mar Sobral, María Losada, Tania Veiga, Javier Guitián, José Guitián, Pablo Guitián

Angiosperms diversification was primarily driven by pollinator agents, but non-pollinator agents also promoted floral evolution. Gentiana lutea shows pollinator driven flower color variation in NW Spain. We test whether insect herbivores and livestock, which frequently feed in G.lutea, play a role in G. lutea flower color variation, by answering the following questions: i) Do insect herbivores and grazing livestock show flower color preferences when feeding on G. lutea? ii) Do mutualists (pollinators) and antagonists (seed predators, insect herbivores and livestock) jointly affect G. lutea reproductive success? Insect herbivores fed more often on yellow flowering individuals but they did not affect seed production whereas livestock affected seed production but did not show clear color preferences. Our data indicate that flower color variation of G. lutea is not affected by insect herbivores or grazing livestock.

1 **Flower color preferences of insects and livestock: effects on *Gentiana lutea***

2 **reproductive succes**s

3

4 Mar Sobral[1], María Losada[1], TaniaVeiga[2], Javier Guitián[2], José Guitián[1], Pablo Guitián[2]

5 [1] Departamento de Bioloxía Celular e Ecoloxía/Facultade de Bioloxía, Universidade de Santiago

6 de Compostela, 15782 Santiago de Compostela, A Coruña, Spain.

7 [2] Departamento de Botánica/Facultade de Bioloxía, Universidade de Santiago de Compostela,

8 15782 Santiago de Compostela, A Coruña, Spain.

9

10 Corresponding Author:

11 Mar Sobral[1]

12 Departamento de Bioloxía Celular e Ecoloxía/Facultade de Bioloxía, Universidade de Santiago

13 de Compostela, 15782 Santiago de Compostela, A Coruña, Spain.

14 e-mail: sobral.bernal.mar@gmail.com

15

16

17

18

19

20 **Abstract**

21 Angiosperms diversification was primarily driven by pollinator agents, but non-pollinator agents

22 also promoted floral evolution. *Gentiana lutea* shows pollinator driven flower color variation in

23 NW Spain. We test whether insect herbivores and livestock, which frequently feed in *G.lutea*,

24 play a role in *G. lutea* flower color variation, by answering the following questions: *i*) Do insect

25 herbivores and grazing livestock show flower color preferences when feeding on *G. lutea*? *ii*) Do

26 mutualists (pollinators) and antagonists (seed predators, insect herbivores and livestock) jointly

27 affect *G. lutea* reproductive success? Insect herbivores fed more often on yellow flowering

28 individuals but they did not affect seed production whereas livestock affected seed production

29 but did not show clear color preferences. Our data indicate that flower color variation of *G. lutea*

30 is not affected by insect herbivores or grazing livestock.

31

32

33

34

35

36

37

38

39

40

41

42

Introduction

Despite pollinators being considered the principal driver of floral diversification and speciation (Bradshaw & Schemske, 2003; Herrera, Castellanos & Medrano, 2006; Whittall & Hodges, 2007; Van der Niet, Peakall & Johnson, 2014), non-pollinator agents also interact with plants and promote floral evolution (Strauss & Whittall, 2006; Andersson, 2008). This is the case with antagonist animals, such as herbivores, which damage plants as a result of resources extraction (Strauss & Whittall, 2006; Whittall & Carlson, 2009), affecting fitness and, therefore, potentially causing natural selection on plant traits (Wise & Hébert, 2010; Agrawal et al., 2012; de Jager & Ellis, 2014).

Plant reproductive success can be shaped by the balance between mutualistic and antagonistic interactions which may maintain floral trait variation (Herrera et al., 2002; Lavergne, Debussche & Thompson, 2005; McCall & Irwin, 2006; Bartkowska & Johnston, 2012). Herbivory can have a negative effect on plant fitness in a greater or lesser extent depending on pollinator presence or absence (Herrera, 2000; Herrera et al., 2002); and the positive effect exerted by pollinators may depend on herbivore presence (Gómez, 2003; Gómez, 2005a). Thus, phenotypic variation of a floral trait, such as flower color, can result from the balance between the positive selection pressures exerted by pollinators and seed dispersers, and the negative selection pressures exerted by herbivores or seed predators (Herrera, 2000; Herrera et al., 2002; Irwin et al., 2003; Asikainen & Mutikainen, 2005; Frey, 2004; Strauss, Irwin & Lambrix, 2004; Andersson, 2008; Bartkowska & Johnston, 2012). Selective pressures exerted by abiotic factors (Galen, 2000; Warren & Mackenzie, 2001; Streisfeld & Kohn, 2007) and historical processes or genetic drift (Mitchell-

64   Olds, Willis & Goldstein, 2007) may also influence phenotypic variation.

65   Invertebrate herbivory (mainly insects and gastropods) negatively affects plant fitness causing

66   natural selection on plant traits, including floral traits, such as flower color (Fineblum &

67   Rausher, 1997; Frey, 2004; Whittall & Carlson, 2009; Bartkowska & Johnston, 2012).

68   Additionally, vertebrate herbivory (typically represented by large mammal herbivores) affect

69   plant fitness and community composition (Herrera, 1984; Agustine & McNaughton, 1998;

70   Knight, 2003; Gómez, 2005b; Allsup, 2014). Interaction with mammals can also have a positive

71   effect for the plants. Reproductive success of many flowering plant species relies on browsing

72   ungulates activity, since they play an essential role if ungulates act as seed dispersal agents

73   within plant communities (for example, Herrera, 1984; Herrera, 2000). Thus, the effect of

74   mammal herbivory can be either positive, if they act as seed dispersal agents, or negative if they

75   act as herbivores. Many plant communities interact not only with wild fauna but also with

76   domesticated mammals. Domesticated ungulates such as cattle, horses and sheep often feed on

77   wild plant populations (for example during transhumance practices) potentially exerting natural

78   selection on them.

79   The type and strength of selection exerted on plant attributes depend on herbivore preferences

80   during foraging (Agustine & McNaughton, 1998; Asikainen & Mutikainen, 2005; Agrawal, Lau

81   & Hambäck, 2006). Livestock could discriminate between flower color morphs (Phillips et al.,

82   2001), and thus might be able to show color preferences. But, even if livestock could not detect

83   flower color differences, it still may differentially feed on a particular color morph if it shows

84   preferences for any trait correlated with flower color (see Lande and Arnold 1983). As it could

85   be the case for some herbivory defenses which are known to be correlated with floral pigments

86   (Simms and Bucher 1996).

87   The effect of livestock (negative or positive) is expected to be stronger than the exerted by insect

88   herbivores or pollinators –due to the amount of damage or seed dispersal that large vertebrates

89   are able to exert. Thus, it is necessary to take their effect into account in order to have a holistic

90   view of the biotic forces exerting selective pressures on traits such as floral color (Johnson,

91   Campbell & Barrett, 2015).

92   Despite it has been long recognized that both, pollinators and herbivores play an important role

93   on plant evolution (Ehrlich & Raven, 1964), their effects are usually studied independently and

94   under different scopes (Johnson, Campbell & Barrett, 2015). Moreover, the effects of grazing

95   mammals on flower traits have been disregarded (but, see Juenger & Bergelson, 1997; Gómez,

96   2003; Gómez et al., 2009; Ågren, 2013), and the potential effects of domesticated mammal

97   herbivores on floral traits are unknown. Here we explore for the first time the simultaneous effect

98   of wild and domesticated animals on plant traits, particularly regarding their potential effect on

99   flower color variation.

100   *Gentiana lutea* flower color varies continuously from orange to yellow within and among

101   populations in NW Spain (Sobral et al., 2015) –where livestock interacts with plant communities

102   (see Blanco-Fontao, Quevedo & Obeso 2011) and commonly feed on *G. lutea*. This corolla color

103   variation has a genetic basis (Zhu et al., 2002; Zhu et al., 2003) and is not related to abiotic

104   factors such as radiation, altitude, temperature or rainfall (Veiga et al., 2015a). Two flower color

105   varieties are described (Laínz, 1982; Renobales, 2003) and a partial hybridization barrier exists

106   between the yellow *G. lutea aurantiaca* and the orange *G. lutea lutea* (Losada et al., 2015). *G.*

107   *lutea* is strongly dependent on pollinators which, together with seed predators, show flower color

108   preferences causing selection on flower color (Losada et al., 2015, Veiga et al., 2015b).

109   Here we test if insect herbivores   mainly adults belonging to Orthopthera and Coleoptera

110   orders, and larvae from different insect groups   and large mammal domesticated herbivores

111   (local extensive livestock, mainly cows and horses) affect flower color variation in *Gentiana*

112   *lutea* –while having into account the effect of pollinators and seed predators. For this purpose,

113   the following questions were formulated: *i*) Do insect herbivores and livestock show flower color

114   preferences when feeding on *G. lutea*? *ii*) Do mutualists (pollinators) and antagonists (seed

115   predators, insec and livestock) jointly affect *G. lutea* reproductive success?

116   **Materials & Methods**

117   **Study Area**

118   Our study area covered the distribution of *Gentiana lutea* in the Cantabrian Mountains, NW

119   Spain (see Fig. 1). In 2010, we visited 8 populations (Cebreiro, Ancares, Leitariegos, Torrestío,

120   Ventana, San Isidro, Señales and San Glorio) and 12 in 2011 (Queixa, San Mamede, Loureses

121   and Pontón were new). All studied populations were haphazardly selected along a 230 km

122   longitudinal gradient from the San Mamede population (42º 12´ N, 7º 30' W; at the western

123   limit) to the San Glorio population (43º 04´ N, 4º 45' W; at the eastern limit), and localized at

124   high altitudes from 1,100 m to 1,700 m a.s.l., on grassy pastures and hillsides used extensively

125   by local livestock. For this research, we received a field permit from the Environmental

126   Territorial Service of León, Territorial Delegation of Government of Spain, Regional

127   Government of Castilla and León (ID:12_LE_325_RNA_PuebladeLilio_INV; Reference:

128   06.01.013.016/ROT/abp; File number: AEN/LE/103/12).

129   **Plant species**

130   *Gentiana lutea* (*Gentianaceae*) is a rhizomatous perennial herb distributed throughout central

131   and southern European mountains, living at montane and sub-alpine levels (approximately from

132   700 to 2,000 m a.s.l.) and mainly associated with livestock grazing grasslands (Hesse, Rees &

133   Müller-Schärer, 2007; Anchisi et al., 2010). This is a long-lived geophyte, which usually

134   develops one unbranched stout stem (rarely two or three) measuring up to 190 cm tall; and shows

135   a basal rosette formed from lanceolate-elliptic leaves measuring 190-350 × 55-150 mm

136   (Renobales, 2012). Fertile stems bloom in summer (June-July), and show several tens of bisexual

137   and actinomorphic flowers grouped in pseudo-whorls. Flowers present a bicarpellate ovary fixed

138   over a split calix, a stigma with two lamellae and (4-8) petals fused on the basis. Corollas have

139   an open structure, which facilitates pollinator access (mainly insects belonging to Hymenoptera

140   and Diptera orders) to flower nectaries. Corolla color varies from orange to yellow along the *G.*

141   *lutea* distribution range in the Cantabrian Mountains, Spain (Sobral et al., 2015). *G. lutea* fruits

142   are capsules, which hold a great number of elliptic, flattened and winged seeds, measuring 2.5-

143   4.5 mm, which ripen in summer (Renobales, 2012). Wind is the main seed dispersal agent

144   (Struwe & Albert, 2002). *G. lutea* may be considered a toxic species, because it contains

145   relatively high levels of herbivory deterrents (Smit, Ouden & Müller-Schärer, 2006; Hesse, Rees

146   & Müller-Schärer, 2007).

147   **Field procedures**

148   *Plant traits measurement*

149   In July 2010, during blooming, we measured flower color on ten randomly chosen flowers per

150   plant. In 2011, we measured color on only three flowers per plant because we found that the

151   coefficient of variation for flower color within plants was asymptotic, reaching a plateau after 3

152   flowers. Each petal was measured three times. Final color spectrum data for individual plants

153   came from the mean of these three measurements per petal for 10 petals belonging to 10 different

154   flowers in 2010 (30 floral color data per plant in 2010) and three measurements for each of three

155   petals belonging to 3 different flower in 2011 (nine color data per plant in 2011). We measured

156   floral color in a total of 2,711 flowers belonging to 504 plants across 12 *G. lutea* populations.

157   With the aim of taking into account indirect selection on correlated plant traits (see Veiga et al.,

158   2015b), we also measured the stalk height (the height of the stalk from the base of the plant to

159   the top, in cm) and the leaf length (the length of the longest basal leaf from the insertion to the

160   tip, in mm) in each plant.

161   Flower color was measured by means of a spectrometer (USB2000+; Ocean Optics, Inc.,

162   Dunedin, FL) and the petal color spectra were processed using the SpectraSuite® software

163   (Ocean Optics, Inc., Dunedin, FL, USA). No differences among populations and between color

164   morphs (orange or yellow colors, discernible by human eye) were found in the UV light range in

165   a previous study (see Veiga et al., 2015b). Thus, flower color variation was described by means

166   of the CIELab Colorimetric System (CIE, 2004). This colorimetric system is based on the visible

167   light range of the electromagnetic spectrum and allows for a transformation of the measured

168   reflectance spectrum into three variables, which describe the flower color variation: *L* (brightness

169   of color, from black to white), *a* (red color variation, from green to red) and *b* (yellow color

170   variation, from blue to yellow; see Veiga et al., 2015b; for more information on the flower color

171   measurements).

172   These three flower color variables (*L*, *a*, and *b*) were reduced by principal component analysis

173   (PCA). The first principal component (PC1) explained 63% of variance of a, *b* and *L*; thus, PC1

174   was used as the flower color variable in the statistical analyses. Correlations between the original

175   color variables and PC1 show that low scores in PC1 indicate orange colors and high scores

176   indicate yellow colors.

177   *Insect herbivory*

178   A total of 162 individual plants were randomly selected before blossom started (June) in each of

179   8 populations studied in 2010 (Cebreiro, Ancares, Leitariegos, Torrestío, Ventana, San Isidro,

180   Señales and San Glorio). The insect herbivory percentage was quantified visually for all leaves

181   of each plant during flowering (July). For this, we used the scale designed by Dirzo &

182   Domínguez (1995), which establishes six categories: the first category includes leaves without

183   damage; the second, leaves with 1-6% of damage; the third, leaves with 6-12% of damage; the

184   fourth, 12-25 % of damage; the fifth, 25-50% and the sixth, 50-100 % of damage. With

185   frequencies of leaves in each category of damage, we calculated the Index of Herbivory per

186   plant: $IH = (\Sigma ni * i) / N$; where n is the number of leaves in a category, i is the category number

187   and N is the number of leaves per plant.

188   *Livestock herbivory*

189   Livestock consume parts of the plants including leaves, flowers or fruits and parts of the stalk.

190   First, we tested whether the effect of livestock was a negative herbivory effect or, on the other

191   hand, whether livestock could be considered a *G. lutea* seed disperser. We examined livestock

192   herbivory during the fruits ripening season (August) establishing two categories: without

193   evidence of livestock herbivory (0 or absence) and with evidence of livestock herbivory (1 or

194   presence) in 183 plants chosen in 8 populations in 2010 and 288 plants chosen across 12

195   populations in 2011.

196   Livestock was observed feeding on *G.lutea* at the time that it was bearing fruits. During that time

197   (August 2010 and 2011), we collected 63 livestock fecal samples in all 12 *G. lutea* populations

198   and examined them (40 belonging to cows, 20 to horses and, 3 to sheep). We manually inspected

199   the fecal samples in the lab (3 grams per sample) searching for seed presence within livestock

200   pellets. *G. lutea* seeds found within livestock fecal samples were later examined for

201   germinability. Seeds were distributed on filter paper in petri plates. The state of germination and

202   wetting of the plates were examined on alternate days; the filter paper was removed every 2-3

203   days to reduce fungal infection. Germination trials lasted for 60 days. Note that we had

204   previously found that after 45 days, 25% of viable *G. lutea´s* seeds were germinated (Losada et

205   al., 2015).

206   *Effect of mutualist and antagonist interactions on seed production*

207   When fruits were not yet opened, total number of fruits was counted per plant. Afterwards, 20

208   fruits were haphazardly collected per individual. Number of viable seeds was counted in each

209   fruit sampled (over 150,000 seeds were counted). *G. lutea* plants set on average 79 fruits, each

210   with a mean of 63 seeds (Sobral et al., 2015). Thus, each plant sets approximately 5,000 seeds on

211   average; hence total number of seeds set per plant was impractical to count. Total seed number

212   per plant was estimated by multiplying the number of fruits per plant times the average number

213   of viable seeds per fruit. In this study, total seed number (reproductive output) was used as a

214   proxy for plant fitness.

215   With the aim of understanding the effect that insect and livestock herbivory have on the *G. lutea*

216   reproductive output, previously published data on pollination success and seed predation of the

217   same marked plants and reproductive seasons (Sobral et al., 2015) were used to incorporate the

218   effects of these ecological interactions into the models. Note that these models used data from 8

219   populations studied in 2010 (Cebreiro, Ancares, Leitariegos, Torrestío, Ventana, San Isidro,

220   Señales and San Glorio) because we did not collect data on insect herbivory in 2011. See Sobral

221   et al. (2015) for methods to recording pollination and seed predation.

222   **Statistical analyses**

223   Analyses were performed using the SPSS for Windows, version 20.0 (IBM Corp., 2011). Error

224   distribution, link function and model´s structure were chosen by means of the AICc criterion

225   starting with saturated models (Burnham & Anderson, 2002).

226   *Insect preferences for flower color*

227   In order to study the insect preferences for flower color, we analyzed the data from 104 plants

228   from the 8 populations studied in 2010. We used a generalized linear model (GzLM) in which

229   the explanatory variables were population, flower color, stalk height, leaf length, the *flower color*

230   ** stalk height* interaction and the *flower color * leaf length* interaction; and the per plant

231   percentage of herbivory was the response variable (Table 1). Note that the herbivory index was

232   transformed into a per-plant percentage of herbivory before analysis and was fitted to a Poisson

233   distribution with a log link function. Population was included as a fixed factor into the models

234   since they were selected following a longitudinal gradient. Additionally, the same model

235   (without the population effect) for each of the studied populations was performed (see Appendix

236   1).

237   *Livestock preferences for flower color*

238   Some populations present livestock but others do not. Populations differ in the average corolla

239   color (Sobral et al. 2015), therefore, the flower color preferences across the studied range could

240   be merely reflecting the arbitrary livestock presence on different colored populations. Therefore,

241   we analyzed the effect of livestock independently for each population. We analyzed the livestock

242   herbivory within populations (419 plants, between 17 and 64 plants per population), using a

243   generalized linear model (GzLM) equivalent to that used in the case of insect herbivores. The

244   explanatory variables were population, flower color, stalk height, leaf length, the *flower color **

245   *stalk height* interaction and the *flower color * leaf length* interaction (see Appendix 2). Livestock

246   herbivory was fitted to a Binomial distribution with a logit link function.

247   *Effect of mutualist and antagonist interactions on seed production*

248   We used a generalized linear model (GzLM) to analyze the effect of the ecological interactions

249   (pollination, seed predation, insect herbivory and cattle herbivory) that may affect total seed

250   number (the response variable), used as a proxy for plant fitness. Cattle herbivory (absence = 0;

251   presence = 1), pollinator visitation rate (Nº visits per minute), escape from seed predation (%

252   fruits not affected by seed predators) and insect herbivory (absence = 0; presence = 1) were the

253   explanatory variables. We also included the population effect into the model (Table 2). Total

254   seed set was fitted to a Poisson distribution with a log link function; and error distribution, link

255   function and model´s structure were chosen by means of the AICc criterion (Burnham &

256   Anderson, 2002). We used data from 94 plants belonging to 8 populations studied in 2010

257   (Cebreiro, Ancares, Leitariegos, Torrestío, Ventana, San Isidro, Señales and San Glorio) to

258   perform this model.

Results

260   *Insect and livestock preferences for flower color*

261   Insect herbivores showed preferences for flower color when feeding on *Gentiana lutea* (Table 1).

262   Overall, these insects preferred to feed upon yellow-flowering individuals (Fig. 2) and this

263   flower color preference depended on stalk height, as the significant interaction between these

264   two plant traits suggests. Insects prefer yellow-flowering individuals and, among these, shorter

265   individuals were more herbivorized than longer ones (Table 1; Fig. 3). The relationship between

266   the intensity of herbivory and the interaction between flower color and other plant traits also

267   happens within some of the populations (see Cebreiro, Torrestío and Ventana populations,

268   Appendix 1).

269   Livestock did not show preferences for flower color within each studied population; although,

270   the probability of livestock herbivory marginally depended on the interaction between flower

271 color and leave length in a population (Cebreiro population; see Appendix 2). Note than

272 significant effects were also not found if the analyses were split by year.

273 *Effect of mutualists and antagonists on G. lutea reproductive success*

274 After the examination of 63 livestock fecal samples (40 cow samples, 20 horse samples and 3

275 sheep samples), we found 13 seeds in 6 different fecal samples (4 cow samples and 2 horse

276 samples) from 4 different populations. None of these seeds germinated after the 60 days trial,

277 suggesting that seeds did not survive the digestive track of livestock (a previous study showed

278 25% germination of *G. lutea* after 45 days, see Losada et al. 2015). Thus, we rule out the seed

279 disperser role of livestock and consider that the interaction with livestock has a negative effect

280 on *G. lutea* reproductive success.

281 Pollinator visitation rate positively affected plant reproductive output and livestock herbivory

282 decreased seed production. Insect herbivory and seed predation did not affect seed production

283 (Table 2). The most important effect on the *G. lutea* reproductive output turned out to be

284 livestock herbivory, plants which did not suffer livestock herbivory set an average of 5,005 (+/-

285 416) seeds whereas plants eaten by livestock set an average of 1,269 (+/- 356 seeds). Livestock

286 herbivory affected *G. lutea* reproductive success 2.7 times more than pollinators (see effect sizes,

287 β values; Table 2).

Discussion

289 Insect herbivores preferred to feed on yellow-flowering individuals but livestock did not show

290 flower color preferences along the *Gentiana lutea* range studied (Table 1; Fig. 2; Appendix 2). In

291 some *G. lutea* populations pollinators and seed predators also prefer the yellow morphs (see

292 Veiga et al. 2015 b) but the color preferences vary between populations (see Sobral et al. 2015).

293 Pollinators might visit the more herbivorized individuals in some locations whereas, on other

294   locations, pollinator and herbivores might prefer different corolla colors. This is likely to depend

295   on the pollinator and herbivore community composition as well as on the range of color variation

296   in particular populations.

297   Floral pigmentation in *G. lutea* varies among individuals depending on carotenoids concentration

298   (Zhu et al., 2002). These pigments, which regulate color expression from orange to yellow, are

299   involved in the synthesis of volatile compounds considered to be defenses against insect

300   herbivory (Lakshminarayan, 2013). Alternatively, anthocyanines (which participate in red color

301   expression) may play a dual role, both attracting pollinators and alerting herbivores of a high

302   content of chemical secondary compounds that confer toxicity or, at least make plant tissues

303   difficult to metabolize (Lev-Yadun & Gould, 2009). The fact that insect herbivores preferred to

304   feed upon yellow-flowering individuals might suggest that yellowish corolla color pigments are

305   related to lower amounts of chemical deterrents than orange pigmentation.

306   Livestock herbivory negatively affected the reproductive success of *G. lutea*, whereas pollination

307   had a positive effect on *G. lutea*'s seed output. We found the effect of livestock herbivory on

308   seed production to be stronger than the effect of pollinators (see β values, Table 2). The few

309   studies dealing with the joint effect of herbivores and pollinators on floral traits show that

310   selection by herbivores is often (in 70% of the cases) as strong (or stronger) than selection

311   exerted by pollinators on flowers' characteristics –herbivores potentially affect floral traits as

312   much as pollinators do (see Johnson, Campbell & Barrett, 2015). Flower color variation in other

313   species is maintained by the balance of selective pressures exerted by mutualisms and

314   antagonisms; for example in *Raphanus sativus* (McCall et al., 2013), *Iris lutescens* (Wang et al.,

315   2013), *Ursinia calenduliflora* (de Jager & Ellis, 2014) and *Geranium thunbergii* (Tsuchimatsu,

316   Yoshitake & Ito, 2014). But, we cannot confirm that either insects or livestock play a role on

317 *G.lutea*'s flower color variation. This is because we found effects of livestock on seed production

318 but not color preferences, and insect herbivores showed color preferences but did not affect seed

319 production. However, insect herbivory also depended on correlations among vegetative traits

320 (such as stalk height or leaf length) and flower color (Table 1; Appendix 1). Thus, if leaf length

321 or stalk height are related to seed production, insects could still be playing an indirect role on the

322 maintenance of flower color variation trough pleiotropic effects among correlated plant traits

323 (Fineblum & Rausher, 1997; Herrera et al., 2002; Strauss, Irwin & Lambrix, 2004; Narbona et

324 al., 2014; Johnson, Campbell & Barrett, 2015).

325 Livestock and insect herbivores do not play a role shaping flower color variation in *G. lutea*. But,

326 pollinators show color preferences and affect *G. lutea*'s fitness. Thus, flower color variation in

327 *G. lutea* might be originated, or at least reinforced, by the selective pressures exerted by

328 pollinators but it is unrelated to insect herbivores and livestock. Flower color variation in

329 polymorphic species may originate from selection by animals which could favor isolation

330 between different color morphs and cause sympatric diversification. But, other reasons could

331 additionally explain flower color variation; for example, the geographic isolation produced by

332 the Quaternary climatic changes has been identified as the main cause of divergence in several

333 mountain plant species (e.g. Martín-Bravo et al., 2010; Alarcón et al., 2012; Blanco-Pastor

334 &Vargas, 2013; Fernández-Mazuecos et al.,2013).

335 Domesticated animals feeding on *G. lutea* do not show preferences for flower color. Despite it,

336 we argue for the importance of considering the effect of domesticated animals on plant

337 conservation and evolution. Livestock shape plant communities and ecosystems trough their

338 interaction with particular species (for example López-Sánchez et al. 2014 and López-Sánchez et

339 al. 2015). Human activities are known to have many effects on biodiversity (for example, Dirzo

340 et al. 2014). We might have overlooked a potential indirect effect that human could play on

341 diversity conservation and evolution –trough the interactions of domesticated mammals with

342 plant communities. Considering livestock effects on plant communities could better our nature

343 understanding and management.

Acknowledgments

References

348 **Agrawal AA, Lau JA, Hambäck PA. 2006.** Community heterogeneity and the evolution of

349 interactions between plants and insect herbivores. *The Quarterly Review of Biology* **81** (4): 349

350 376.

351 **Agrawal AA, Hastings AP, Johnson MTJ, Maron JL, Salminen J-P. 2012.** Insect herbivores

352 drive real-time ecological and evolutionary change in plant populations. *Science* **338**: 113 116.

353 DOI: 10.1126/science.1225977.

354 **Ågren J, Hellstrom F, Torang P, Ehrlén J. 2013.** Mutualists and antagonists drive among-

355 population variation in selection and evolution of floral display in a perennial herb. *Proceedings*

356 *of the National Academy of Sciences of the United States of America* (PNAS) **110**: 18202-18207.

357 **Agustine DJ, McNaughton SJ. 1998.** Ungulate effects on the functional species composition of

358 plant communities: herbivore selectivity and plant tolerance. *Journal of Wildlife Management* **62**

359 (4):1165 1183. DOI: 10.2307/3801981.

360 **Alarcón M, Vargas P, Sáez L, Molero J, Aldasoro JJ. 2012.** Genetic diversity of mountain

361 plants: Two migration episodes of Mediterranean *Erodium* (Geraniaceae). *Molecular*

362 *Phylogenetics and Evolution* **63** (3): 866–876. DOI:10.1016/j.ympev.2012.02.031.

363 **Allsup CM. 2014.** Overcompensation of *Ipomopsis aggregata* following ungulate herbivory:

364 getting to the root of it. D. Phil. Thesis, University of Illinois at Urbana-Champaign, EE.UU.

365 **Anchisi E, Bernini A, Piaggi E, Polani F. 2010.** *Genziane d'Europa* [Gentians of Europe].

366 Pavia: Verba & Scripta s.a.s., Italy. (In Italian)

367 **Andersson S. 2008.** Pollinator and non-pollinator selection on ray morphology in

368 *Leucanthemum vulgare* (oxeye daisy, Asteraceae). *American Journal of Botany* **95** (9): 1072

369 1078. DOI: 10.3732/ajb.0800087Am.

370 **Asikainen E, Mutikainen P. 2005.** Preferences of pollinators and herbivores in gynodioecious

371 *Geranium sylvaticum*. *Annals of Botany* **95** (5): 879 886. DOI: 10.1093/aob/mci094.

372 **Bartkowska MP, Johnston MO. 2012.** Pollinators cause stronger selection than herbivores on

373 floral traits in *Lobelia cardinalis* (Lobeliaceae). *New Phytologist* **193** (4): 1039–1048. DOI:

374 10.1111/j.1469-8137.2011.04013.x.

375 **Blanco-Fontao B, Quevedo M, Obeso JR. 2011.** Abandonment of traditional uses in mountain

376 areas: typological thinking versus hard data in the Cantabrian Mountains (NW

377 Spain). *Biodiversity and Conservation* 20(5): 1133-1140.

378 **Blanco-Pastor JL, Vargas P. 2013.** Autecological traits determined two evolutionary strategies

379 in Mediterranean plants during the Quaternary: low differentiation and range expansion versus

380 geographical speciation in *Linaria*. *Molecular Ecology* **22** (22): 5651–5668. DOI:

381 10.1111/mec.12518.

382 **Bradshaw HD, Schemske DW. 2003.** Allele substitution at a flower colour locus produces a

383 pollinator shift in monkeyflowers. *Nature* **426**: 176–178. DOI: 10.1038/nature02106.

384 **Burnham KP, Anderson DR. 2002.** *Model selection and multi-model inference: a practical*

385 *information-theoretic approach*, 2nd edn. New York: Springer-Verlag.

386 **CIE P. 2004.** *15: 2004. Colorimetry*. Vienna: CIE Central Bureau, Austria.

387 **De Jager ML, Ellis AG. 2014.** Floral polymorphism and the fitness implications of attracting

388 pollinating and florivorous insects. *Annals of Botany* **113** (2): 213 222. DOI:

389 10.1093/aob/mct189.

390 **Dirzo R, Domínguez C. 1995.** Plant-herbivore interactions in Mesoamerican tropical dry

391 forests. In: Bullock S, Mooney HA, Medina IE, eds. *Tropical Dry Forests*. New York:

392 Cambridge University Press, 304 323.

393 **Dirzo R, Young HS, Galetti M, Ceballos G, Isaac NJ, Collen B, 2014.** Defaunation in the

394 Anthropocene. *Science*, *345*(6195), pp.401-406.

395 **Ehrlich PR, Raven PH. 1964.** Butterflies and plants: a study in coevolution. *Evolution* **18** (4):

396 586 608. DOI: 10.2307/2406212.

397 **Fernández-Mazuecos M, Blanco-Pastor JL, Gómez JM, Vargas P. 2013.** Corolla

398 morphology influences diversification rates in bifid toadflaxes (*Linaria* sect. *Versicolores*).

399 *Annals of Botany* **112**: 1705–1722. DOI: 10.1093/aob/mct214.

400 **Fineblum WL, Rausher MD. 1997.** Do floral pigmentation genes also influence resistance to

401 enemies? The W locus in *Ipomoea purpurea*. *Ecology* **78** (6): 1646–1654.

402 **Frey FM. 2004.** Opposing natural selection from herbivores and pathogens may maintain floral-

403 color variation in *Claytonia virginica* (Portulacaceae). *Evolution* **58** (11): 2426 2437.

404 **Galen C. 2000.** High and dry: drought stress, sex-allocation trade-offs, and selection on flower

405 size in the alpine wildflower *Polemonium viscosum* (Polemoniaceae). *The American Naturalist*

406 **156**: 72 83.

407 **Gómez JM. 2003.** Herbivory reduces the strength of pollinator-mediated selection in the

408 mediterranean herb *Erysimum mediohispanicum*: consequences for plant specialization. *The*

409   *American Naturalist* **162** (2): 242 256.

410   **Gómez JM. 2005a.** Non-additive effects of herbivores and pollinators on *Erysimum*

411   *mediohispanicum* (Cruciferae) fitness. *Oecologia* **143**: 412–418. DOI: 10.1007/s00442-004-

412   1809-7.

413   **Gómez JM. 2005b.** Long-term effects of ungulates on performance, abundance, and spatial

414   distribution of two montane herbs. *Ecological Monographs* **75** (2): 231–258.

415   **Gómez J, Perfectti F, Bosch J, Camacho J. 2009.** A geographic selection mosaic in a

416   generalized plant-pollinator-herbivore system. *Ecological Monographs* **79**: 245-263.

417   **Herrera CM. 1984.** Seed dispersal and fitness determinants in wild rose: combined effects of

418   hawthorn, birds, mice, and browsing ungulates. *Oecologia* **63**: 386 393.

419   **Herrera CM. 2000.** Measuring the effects of pollinators and herbivores: evidence for non-

420   additivity in a perennial herb. *Ecology* **81** (8): 2170 2176.

421   **Herrera CM, Medrano M, Rey PJ, Sánchez-Lafuente AM, García MB, Guitián J,**

422   **Manzaneda AJ. 2002.** Interaction of pollinators and herbivores on plant fitness suggests a

423   pathway for correlated evolution of mutualism- and antagonism-related traits. *Proceedings of the*

424   *National Academy of Sciences of the United States of America* (PNAS) **99** (26): 16823 16828.

425   DOI: 0.1073/pnas.252362799.

426   **Herrera CM, Castellanos MC, Medrano M. 2006.** Geographical context of floral evolution:

427   towards an improved research programme in floral diversification. In: Harder LD, Barret SCH,

428   eds. *Ecology and evolution of flowers*, Oxford: Oxford University Press, 278 294.

429   **Hesse E, Rees M, Müller-Schärer H. 2007.** Seed bank persistence of clonal weeds in

430   contrasting habitats: implications for control. *Plant Ecology* **190**: 233–243. DOI:

431   10.1007/s11258-006-9203-7.

432   **IBM Corp. Released. 2011.** *IBM SPSS Statistics for Windows, Version 20.0.* Armonk, NY: IBM

433   Corp.

434   **Irwin RE, Strauss SY, Storz S, Emerson A, Guibert G. 2003.** The role of herbivores in the

435   maintenance of a flower color polymorphism in wild radish. *Ecology* **84** (7): 1733 1743.

436   **Johnson MTJ, Campbell SA, Barrett SCH. 2015.** Evolutionary interactions between plant

437   reproduction and defense against herbivores. *Annual Review of Ecology, Evolution, and*

438   *Systematics* **46**:191 213. DOI: 10.1146/annurev-ecolsys-112414-054215.

439   **Juenger T, Bergelson J. 1997.** Pollen and resource limitation of compensation to herbivory in

440   scarlet gilia, *Ipomopsis aggregata*. *Ecology* **78**: 1684 1695.

441   **Knight TM. 2003.** Effects of herbivory and its timing across populations of *Trillium*

442   *grandiflorum* (Liliaceae). *American Journal of Botany* **90** (8): 1207–1214. DOI:

443   10.3732/ajb.90.8.1207.

444   **Lande R, Arnold SJ. 1983**. "The measurement of selection on correlated

445   characters." *Evolution* (1983): 1210-1226.

446   **Laínz M. 1982.** *Mis contribuciones al conocimiento de la flora de Asturias* [My contributions to

447   the knowledge of the Asturian flora]. Oviedo: Diputación Provincial de Asturias, Instituto de

448   Estudios Asturianos (CSIC). (In Spanish)

449   **Lakshminarayan S. 2013.** Role of carotenoid cleavage dioxygenases in volatile emissions and

450   insect resistance in *Arabidopsis*. D. Phil. Thesis, University of Western Ontario, Canada.

451   **Lavergne S, Debussche M, Thompson JD. 2005.** Limitations on reproductive success in

452   endemic *Aquilegia viscosa* (Ranunculaceae) relative to its widespread congener *Aquilegia*

453   *vulgaris*: the interplay of herbivory and pollination. *Oecologia* **142** (2): 212 220. DOI:

454   10.1007/s00442-004-1721-1.

455   **Lev-Yadun S, Gould KS. 2009.** Role of anthocyanins in plant defence. In: Gould KS, Davies

456   KM, Winefield C, eds. *Anthocyanins: biosynthesis, functions and applications.* New York:

457   Springer, 21 48.

458   **López-Sánchez A, Schroeder J, Roig S, Sobral M, Dirzo R. 2014.** Effects of Cattle

459   Management on Oak Regeneration in Northern Californian Mediterranean Oak Woodlands.

460   PLoS ONE 9(8): e105472.

461   **López-Sánchez A, Perea R, Dirzo R, Roig S. 2015.** Livestock vs. wild ungulate management

462   in the conservation of Mediterranean dehesas: Implications for oak regeneration. Forest ecology

463   and management 362:99-106.

464   **Losada M, Veiga T, Guitián JA, Guitián J, Guitián P, Sobral M. 2015.** Is there a

465   hybridization barrier between *Gentiana lutea* color morphs? *PeerJ*

466   **3**:e1308.https://doi.org/10.7717/peerj.1308.

467   **Martín-Bravo S, Valcárcel V, Vargas P, Luceño M. 2010.** Geographical speciation related to

468   Pleistocene range shifts in the Western Mediterranean Mountains (*Reseda* sect. *Glaucoreseda*,

469   *Resedaceae*). *Taxon* **59** (2): 466–482.

470   **McCall AC, Irwin RE. 2006.** Florivory: the intersection of pollination and herbivory. *Ecology*

471   *Letters* **9** (12): 1351–1365. DOI: 10.1111/j.1461-0248.2006.00975.x.

472   **McCall AC, Murphy SJ, Venner C, Brown M. 2013.** Florivores prefer white versus pink petal

473   color morphs in wild radish, *Raphanus sativus*. *Oecologia* **172**: 189–195. DOI: 10.1007/s00442-

474   012-2480-z.

475   **Mitchell-Olds T, Willis JH, Goldstein DB. 2007.** Which evolutionary processes influence

476   natural genetic variation for phenotypic traits? *Nature Reviews Genetics* **8**: 845 856. DOI:

477   10.1038/nrg2207.

478   **Narbona E, Buide ML, Casimiro-Soriguer I, del Valle JC. 2014.** Polimorfismos de color

479   floral: causas e implicaciones evolutivas [Flower color polymorphisms: evolutionary causes and

480   implications]. *Ecosistemas* **23** (3): 36 47. DOI: 10.7818/ECOS.2014.23-3.06. (In Spanish).

481   **Phillips CJC, Lomas CA. 2001.** The perception of color by cattle and its influence on

482   behavior. *Journal of dairy science* **84** (4): 807 813. DOI: 10.3168/jds.S0022-0302(01)74537-7.

483   **Renobales G. 2003.** Notas acerca del tratamiento de las Gentianaceae para flora Ibérica [Notes

484   about the treatment of the Gentianaceae for Iberian flora]. *Anales del Jardín Botánico de Madrid*

485   **60** (2): 461–469. (In Spanish)

486   **Renobales G. 2012.** *Gentiana lutea* L. In: Castroviejo S, Talavera S, Andrés C, Arista M,

487   Fernández Piedra MP, Gallego MJ, Ortiz PL, Romero Zarco C, Salgueiro FJ, Silvestre S,

488   Quintanar A, eds. *Flora Ibérica Vol. XI. Plantas vasculares de la Península Ibérica e Islas*

489   *Baleares, Gentianaceae-Boraginaceae* [Iberian flora Vol. XI. Vascular plants of Iberian

490   Peninsule and Balearic Islands, Gentianaceae-Boraginaceae]. Madrid: Real Jardín Botánico

491   (CSIC), 10 13. (In Spanish).

492   **Simms EL, Bucher .1996.** Pleiotropic effects of flower-color intensity on herbivore

493   performance on Ipomoea purpurea. *Evolution* (1996): 957-963.

494   **Smit C, Ouden JD, Müller-Schärer H. 2006.** Unpalatable plants facilitate tree sapling survival

495   in wooded pastures. *Journal of Applied Ecology* **43** (2): 305–312. DOI: 10.1111/j.1365-

496   2664.2006.01147.x.

497   **Sobral M, Veiga T, Domínguez P, Guitián JA, Guitián P, Guitián J. 2015.** Selective

498   pressures explain differences in flower color among *Gentiana lutea* populations. *PLoS ONE* **10**

499   (7): e0132522. DOI:10.1371/journal.pone.0132522.

500   **Strauss SY, Irwin RE, Lambrix VM. 2004.** Optimal defence theory and flower petal colour

501   predict variation in the secondary chemistry of wild radish. *Journal of Ecology* **92**: 132 141.

502   DOI: 10.1111/j.1365-2745.2004.00843.x.

503   **Strauss SY, Whittall JW. 2006.** Non-pollinator agents of selection on floral traits. In: Harder

504   LD, Barrett SCH, eds. *Ecology and evolution of flowers*. New York: Oxford University Press,

505   120–138.

506   **Streisfeld MA, Kohn JR. 2007.** Environment and pollinator-mediated selection on parapatric

507   floral races of *Mimulus aurantiacus*. *Journal of Evolutionary Biology* **20**: 122–132. DOI:

508   10.1111/j.1420-9101.2006.01216.x.

509   **Struwe L, Albert VA. 2002.** *Gentianaceae: systematics and natural history*. Cambridge:

510   Cambridge University Press.

511   **Tsuchimatsu T, Yoshitake H, Ito M. 2014.** Herbivore pressure by weevils associated with

512   flower color polymorphism in *Geranium thunbergii* (Geraniaceae). *Journal of Plant Research*

513   **127** (2): 265 73. DOI: 10.1007/s10265-013-0598-7.

514   **Van der Niet T, Peakall R, Johnson SD. 2014.** Pollinator-driven ecological speciation in

515   plants: new evidence and future perspectives. *Annals of Botany* **113** (2): 199–211. DOI:

516   10.1093/aob/mct290.

517   **Veiga T, Guitián JA, Guitián P, Guitián JM, Munilla I, Sobral M. 2015a.** Flower color

518   variation in the montane plant *Gentiana lutea* L. (Gentianaceae) is unrelated to abiotic factors.

519   *Plant Ecology & Diversity*. DOI:10.1080/17550874.2015.1074626.

520   **Veiga T, Guitián JA, Guitián P, Guitián JM, Sobral M. 2015b.** Are pollinators and seed

521   predators selective agents on flower color in *Gentiana lutea*? *Evolutionary Ecology* **29** (3): 451–

522   464. DOI: 10.1007/s10682-014-9751-6.

523   **Wang H, Conchou L, Bessière JM, Cazals G, Schatz B, Imbert E. 2013.** Flower color

524   polymorphism in *Iris lutescens* (Iridaceae): Biochemical analyses in light of plant–insect

525   interactions. *Phytochemistry* **94**: 123–134. DOI: 10.1016/j.phytochem.2013.05.007.

526   **Warren J, Mackenzie S. 2001.** Why are all colour combinations not equally represented as

527   flower-colour polymorphisms? *New Phytologist* **151**: 237–241. DOI: 10.1046/j.1469-

528   8137.2001.00159.x.

529   **Wise MJ, Hébert JB. 2010.** Herbivores affect natural selection for floral-sex ratio in a field

530   population of horsenettle, *Solanum carolinense*. *Ecology* **91**(4): 937 943.

531   **Whittall JB, Hodges SA. 2007.** Pollinator shifts drive increasingly long nectar spurs in

532   columbine flowers. *Nature* **447**: 706–709. DOI: 10.1038/nature05857.

533   **Whittall J, Carlson M. 2009.** Plant defense: a pre-adaptation for pollinator shifts. *New*

534   *Phytologist* **182**: 5–8. DOI: 10.1111/j.1469-8137.2009.02796.x.

535   **Zhu C, Yamamura S, Koiwa H, Nishihara M, Sandmann G. 2002.** cDNA cloning and

536   expression of carotenogenic genes during flower development in *Gentiana lutea*. *Plant*

537   *Molecular Biology* **48** (3): 277–285. DOI: 10.1023/A: 1013383120392.

538   **Zhu C, Yamamura S, Nishihara M, Koiwa H, Sandmann G. 2003.** cDNAs for the synthesis

539   of cyclic carotenoids in petals of *Gentiana lutea* and their regulation during flower development.

540   *Biochimica et Biophysica Acta* (BBA) **1625** (3): 305–308. DOI: 10.1016/S0167-4781(03)00017-

541   4.

542

543

544

545

546

547

548

549

550

551

552

553

554

555

556

557 **Table 1: GzLM fitted to analyze insect herbivory (percentage of herbivory per plant) and**

558 **its relationship with flower color and other correlated plant traits, such as stalk height and**

559 **leaf length.** N = 104 individuals. The statistically significant effects are marked in bold (P <

560 0.05). Factor codes: LL, leaf length (mm); SH, stalk height (cm).

Dependent variable	Factor	Wald Chi-Square	d.f.	*P*
Insect herbivory	**Flower color**	3.876	1	**0.049**
	LL	1.646	1	0.200
	SH	1.013	1	0.314
	LL * Flower color	0.498	1	0.481
	SH * Flower color	4.887	1	**0.027**
	Population	12.592	7	0.083

561

562

563

564

565

566

567

568

569

570

571

572

573

574

575 **Table 2: GzLM fitted to analyze *G. lutea* reproductive output (total seed number) and its**

576 **relationship with the cattle herbivory (0/1), the pollinator visitation rate (Nº visits/minute),**

577 **the escape from seed predation (% fruits not affected by seed predators), insect herbivory**

578 **(0/1) and the population effect.** N = 94 individuals. The statistically significant effects are

579 marked in bold (P < 0.05).

Dependent variable	Factor	*B*	Wald Chi-Square	d.f.	*P*
Total seed number	**Cattle Herbivory**	**-0.520**	11.396	1	**0.001**
	Pollinator visitation rate	**0.193**	7.418	1	**0.006**
	Escape seed predation	0.156	1.572	1	0.210
	Insect Herbivory	0.058	0.570	1	0.450
	Population		19.929	7	**0.006**

580

581

582

583

584

585

586

587

588
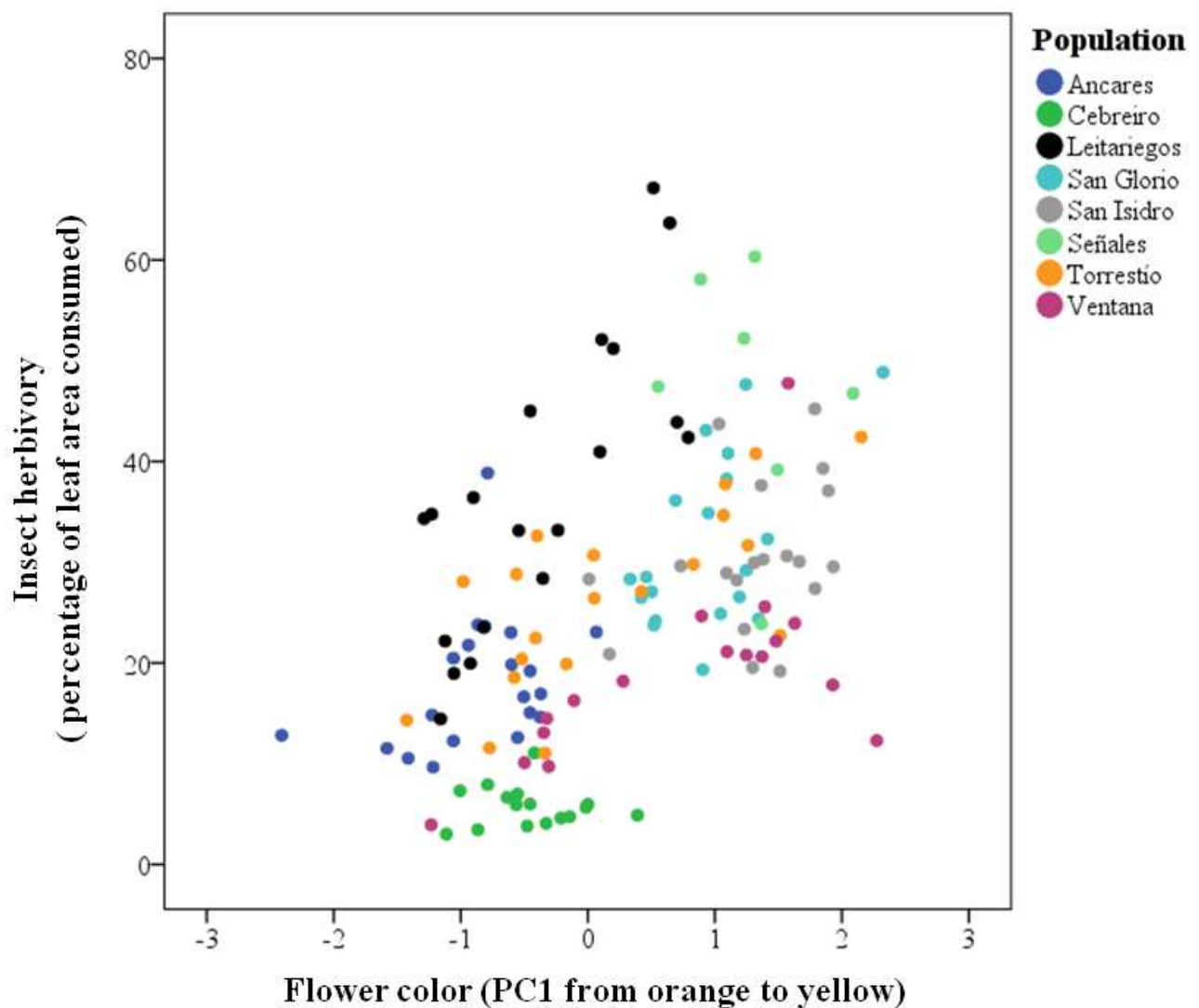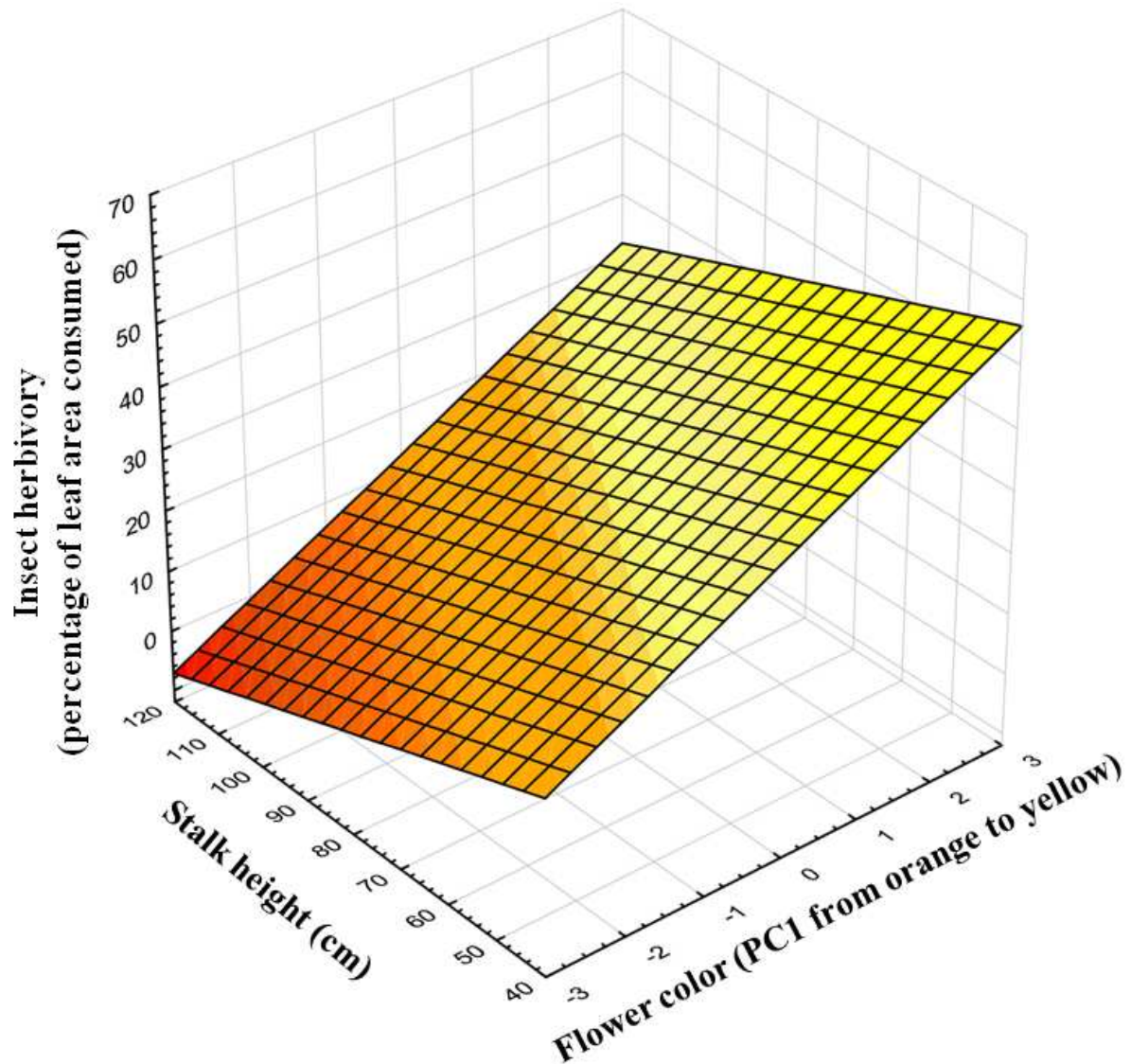
589

590

591

592

593

594

595   **Figure 1: Location of the *Gentiana lutea* populations sampled.** The shaded region indicates

596   the distribution of *G. lutea* in the Cantabrian Mountains, NW Spain. Black dots represent the 12

597   studied populations (from W to E): San Mamede, Queixa, Loureses, O Cebreiro, Os Ancares,

598   Leitariegos, Torrestío, Ventana, San Isidro, Señales, Pontón and San Glorio.

599

600

601

602

603

604

605

606

607

608

609
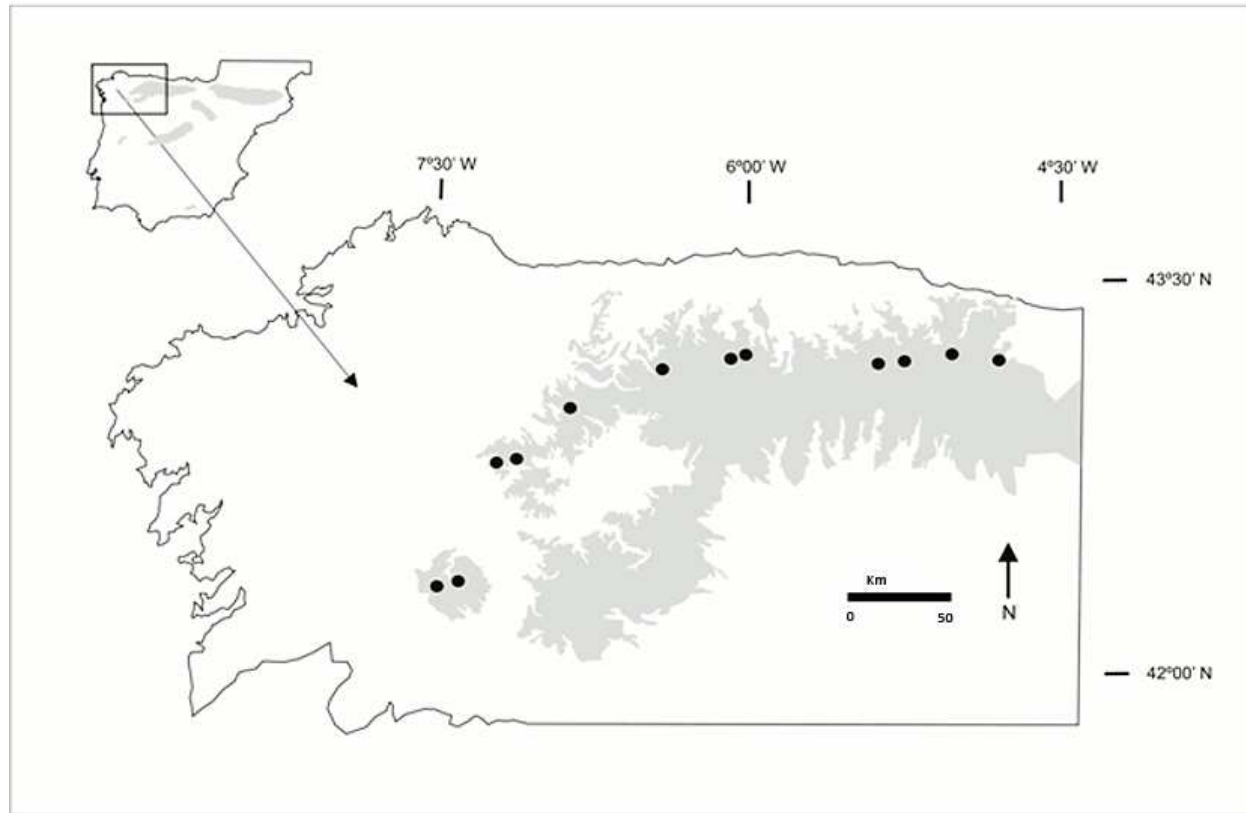
610 **Figure 2: Predicted values of insect herbivory (percentage of eaten leaf area per plant) in**

611 **relation to _G. lutea_ flower color (PC1).** N= 104 individuals, from 8 populations studied in

612   2010.

613

614

615

616

617

618

619

620

621

622

623

624

625

626

627

628

629

630

631

632

633

634   **Figure 3: Predicted values of insect herbivory (percentage of eaten leaf area per plant) in**

635   **relation to flower color (PC1) and correlated stalk height (cm).** N= 104 individuals, from 8

636   populations studied in 2010.

637

638

639

640

641

642

643

644

645

646

647

648

649

650

651

652

653

654

655

Appendix 2. Cattle herbivory results and its relationship with flower color, leaf length, and also the quadratic effect of flower color, for each marked individual from each studied population in 2010 and 2011.

Appendix 1. Insect herbivory (Herbivory Index) and its relationship with flower color and other correlated plant traits, for each of studied populations in 2010. N = individuals. In bold effects with $P < 0.05$. Factor codes: LL, leaf length (mm).