

Biodiversity and Conservation

Identifying population thresholds for flowering plant reproductive success: the marsh gentian (*Gentiana pneumonanthe*) as a flagship species of humid meadows and heathland

--Manuscript Draft--

Manuscript Number:	BIOC-D-17-00267R3	
Full Title:	Identifying population thresholds for flowering plant reproductive success: the marsh gentian (<i>Gentiana pneumonanthe</i>) as a flagship species of humid meadows and heathland	
Article Type:	Original Research	
Keywords:	Demographics; dispersal; habitat fragmentation; Ovule fertilization; Plant conservation; Pollination; Pollen limitation	
Corresponding Author:	Simon Pierce, PhD Universita degli Studi di Milano Milan, ITALY	
Corresponding Author Secondary Information:		
Corresponding Author's Institution:	Universita degli Studi di Milano	
Corresponding Author's Secondary Institution:		
First Author:	Simon Pierce, PhD	
First Author Secondary Information:		
Order of Authors:	Simon Pierce, PhD	
	Alberto Spada, PhD	
	Elisabetta Caporali, PhD	
	Filippa Puglisi, MSc	
	Andrea Panzeri, MSc	
	Alessandra Luzzaro, PhD	
	Simona Cislighi, MSc	
	Lia Mantegazza, MSc	
	Elisa Cardarelli, PhD	
	Massimo Labra, PhD	
	Andrea Galimberti, PhD	
	Roberta Maria Ceriani, PhD	
Order of Authors Secondary Information:		
Funding Information:	Fondazione Cariplo (2014-1631)	Dr. Simon Pierce
Abstract:	<p>The threshold below which population declines impact the effectiveness of plant reproduction is essential for the identification of populations that can no longer spontaneously recover following habitat management or restoration, below the minimum viable population (MVP) size. We hypothesized that risk of reproductive limitation can be evaluated from combined analysis of pollen activity, ovule fertilization and germination in the context of population demographics and fragmentation. The marsh gentian (<i>Gentiana pneumonanthe</i>), a rare emblematic species of European heathland and fen, was investigated at the southern limit of its range in eighteen populations encompassing one to several hundred thousand individuals, spanning</p>	

	<p>small fragments to extensive well-preserved areas. An index of habitat fragmentation was determined from GIS; field surveys determined the ratio of juvenile to reproductive age states; fluorescence microscopy of pistils determined, for each population, the proportion of flowers exhibiting active pollen tube growth. Analysis of seed lots determined the ovule fertilization rate and seed germination capacity. Some of the small populations occupying restricted habitat fragments showed high rates of pollination (100%) and 'normal' age state demographics. However, reproductive characters all exhibited exponential rise to maximum relationships with population size, indicating clear tipping points (for pollination, at a threshold of 7 reproductive adults, and for ovule fertilization rate and germination at 42 reproductive adults). Thus although small populations may set seed, exhibit a 'normal' age state structure, and may appear viable, reproductive effectiveness declines when population size falls below 42 generative individuals and <7 is an indicator of strong limitation. Although many remnant populations of <i>G. pneumonanthe</i> are in the order of 50-150 individuals these should be not be considered as MVPs; they are on the brink of calamity.</p>
<p>Response to Reviewers:</p>	<p>We have moved the photographic figures to the online supplementary materials, and recreated the main results figure as suggested. We have removed the species authority (L.) as requested, and we have substituted 'reproductive failure' with phrases such as 'reproductive limitation' and 'decline in reproductive success' in accordance with Reviewer 4's comments. These small changes to the text affect mainly the Abstract but also the opening paragraph of the Introduction and the end of the Discussion.</p> <p>We thank you for continuing to have faith in this manuscript!</p> <p>Yours sincerely,</p> <p>Simon Pierce on behalf of all authors</p>

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ORIGINAL PAPER

Identifying population thresholds for flowering plant reproductive success: the marsh gentian (*Gentiana pneumonanthe*) as a flagship species of humid meadows and heathland

Simon Pierce¹, Alberto Spada¹, Elisabetta Caporali^{2,3}, Filippa Puglisi⁴, Andrea Panzeri^{1,7}, Alessandra Luzzaro¹, Simona Cislaghi⁵, Lia Mantegazza⁴, Elisa Cardarelli⁶, Massimo Labra⁵, Andrea Galimberti⁵ and Roberta M. Ceriani²

¹ Department of Agricultural and Environmental Sciences (DiSAA), University of Milan, Via G. Celoria 2, I-20133 Milan, Italy

² Department of Biosciences, University of Milan, Via G. Celoria 26, I-20133 Milan, Italy

³ Orto Botanico Città Studi (City of Studies Botanic Garden), University of Milan, Via C. Valvassori Peroni 12, I-20133 Milan, Italy

⁴ Istituto di Istruzione Superiore Luigi Castiglioni, Via Garibaldi 115, I-20812 Limbiate (MB), Italy

⁵ Department of Biotechnology and Biosciences, Università degli Studi di Milano-Bicocca, Piazza della Scienza 2, I-20126 Milan, Italy

⁶ Department of Earth and Environmental Sciences, Università di Pavia, Via Ferrata 9, 27100 Pavia, Italy.

⁷ The Native Flora Centre of the Lombardy Region (Centro Flora Autoctona; CFA), c/o Consorzio Parco Monte Barro, via Bertarelli 11, I-23851 Galbiate (LC), Italy

Corresponding author: Simon Pierce, simon.pierce@unimi.it

1 **Abstract** The threshold below which population declines impact the effectiveness of plant
2 reproduction is essential for the identification of populations that can no longer spontaneously
3 recover following habitat management or restoration, below the minimum viable population
4 (MVP) size. We hypothesized that risk of reproductive limitation can be evaluated from
5 combined analysis of pollen activity, ovule fertilization and germination in the context of
6 population demographics and fragmentation. The marsh gentian (*Gentiana pneumonanthe*), a
7 rare emblematic species of European heathland and fen, was investigated at the southern limit
8 of its range in eighteen populations encompassing one to several hundred thousand
9 individuals, spanning small fragments to extensive well-preserved areas. An index of habitat
10 fragmentation was determined from GIS; field surveys determined the ratio of juvenile to
11 reproductive age states; fluorescence microscopy of pistils determined, for each population,
12 the proportion of flowers exhibiting active pollen tube growth. Analysis of seed lots
13 determined the ovule fertilization rate and seed germination capacity. Some of the small
14 populations occupying restricted habitat fragments showed high rates of pollination (100%)
15 and ‘normal’ age state demographics. However, reproductive characters all exhibited
16 exponential rise to maximum relationships with population size, indicating clear tipping
17 points (for pollination, at a threshold of 7 reproductive adults, and for ovule fertilization rate
18 and germination at 42 reproductive adults). Thus although small populations may set seed,
19 exhibit a ‘normal’ age state structure, and may appear viable, reproductive effectiveness
20 declines when population size falls below 42 generative individuals and <7 is an indicator of
21 strong limitation. Although many remnant populations of *G. pneumonanthe* are in the order of
22 50-150 individuals these should be not be considered as MVPs; they are on the brink of
23 calamity.

24

25 **Keywords** Demographics, Dispersal, Habitat fragmentation, Ovule fertilization, Plant
26 conservation, Pollination, Pollen limitation

27

28 **Introduction**

29

30 Habitat fragmentation and declining habitat availability and connectivity induce restricted
31 gene flow within metapopulations, leading to ‘Allee effects’ (Allee 1931; 1938), and are
32 particularly important for the persistence and conservation of rarer species (Matsumura and
33 Wahitani 2000; Goverde et al. 2002; Pierce et al. 2006). Pollen and seeds are the main vectors
34 of spermatophyte gene flow (Ellstrand 1992) and one of the potential impacts of plant
35 population fragmentation is pollen limitation and subsequent decline in seed production and
36 germination capacity (den Nijs and Oostermeijer 1997; Aguilar et al. 2006): a key aspect of
37 pollen limitation being not only pollen quantity but quality and ability of pollen grains to
38 germinate (Aizen and Harder 2007). Thus aside from the genetic effects of inbreeding
39 depression, one of the main potential impacts of habitat decline is a decline in reproductive
40 success. While identification of reproductive limitations may be relatively straightforward on
41 a case-by-case basis, identifying the general point at which population restriction starts to
42 impact reproductive success for a given species is a delicate problem that requires integration
43 of various aspects of plant biology and ecology. Identification of these thresholds should
44 provide additional information to aid the evaluation of minimum viable population (MVP)
45 sizes for restocking, reintroduction and other conservation activities, as part of wider
46 Population Viability Analysis (PVA; e.g. Menges 2000; Oostermeijer 2000, 2003).

47 The marsh gentian (*Gentiana pneumonanthe*: Gentianaceae) provides an example of a
48 ‘flagship species’ (Volis et al. 2005) for the conservation of European humid meadows and
49 lowland heaths. The importance of microsite availability for seedling establishment and
50 population regeneration for the local persistence of *G. pneumonanthe* is well known
51 (Oostermeijer et al. 1994a), as is the threat of habitat abandonment, succession, competition
52 with tall species and the importance of active habitat management to create gaps for seedling
53 recruitment (Oostermeijer et al. 1996; Kostrakiewicz-Gierałt 2013, 2014). Indeed, the species
54 effectively occupies part of a succession in which reproduction and seedling recruitment
55 become increasingly restricted as succession progresses (Oostermeijer et al. 1996). Seedlings
56 exhibit high mortality rates, but established adults show low mortality and no programmed
57 senescence, although each individual has an idiosyncratic reproductive capacity (Rose et al.
58 1989). Management techniques such as burning, mowing or sod cutting are all disturbances
59 that have varying impacts on seedling recruitment, the most effective being sod cutting

60 (Křenová and Lepš 1996; Oostermeijer et al. 1998). The genetic effects of inbreeding in small
61 populations are also well known for this species: indeed, small populations tend to be less
62 genetically variable and more isolated in terms of gene flow between populations (Raijmann
63 et al. 1994).

64 However, it is less clear if demographically ‘regressive’ or ‘senile’ populations
65 (characterized by limited recruitment; Oostermeijer et al. 1994a; Hegland et al. 2001; Brys et
66 al. 2003) are associated with pollination limitation, and whether this constitutes a problem for
67 the completion of the life cycle and persistence of small groups of plants (in addition to the
68 known impact of successional vegetation development on recruitment opportunities).
69 Knowledge of reproductive limitations is a crucial element for understanding whether
70 population recovery can spontaneously follow habitat management or restoration.

71 *Gentiana pneumonanthe* is self-compatible, but has been shown experimentally to exhibit
72 limited autogamy (spontaneous pollination of a flower with its own pollen) due to
73 protandrous flower development (a separate male phase preceding a female phase) and
74 herkogamy (physical separation of anthers and stigmas; Petanidou et al. 1991): autogamy may
75 vary between 0.2 to 25% depending on circumstances (Petanidou et al. 2001). *Gentiana*
76 *pneumonanthe* exhibits nastic corolla movement in response to low temperatures (i.e. flowers
77 close for the night or during cloudy weather at temperatures below 16°C; Kozuharova 2004)
78 which could press the stigma lobes close to the anthers, and has been suggested as a
79 mechanism for self-pollination, but could be an adaptation to protect the fertile parts of the
80 flower from chilling and prevent nectar dilution by dew (Kozuharova and Anchev, 2006), or
81 both. Anemophily has also been investigated and is extremely unlikely (Petanidou et al.
82 1995). It is clear that very small populations can exhibit pollen limitation, with concomitant
83 effects on seed quality and production (Petanidou et al. 1991). However, it would be useful to
84 know at what point these effects start during the decline of populations, as an additional
85 parameter when attempting to determine MVP sizes. Comparison of ovule to seed numbers
86 per fruit across populations suggests that pollination declines towards smaller population
87 sizes, particularly where populations occupy *Molinia caerulea*-dominated fen habitat where
88 less heath (*Calluna vulgaris*) is available to support pollinator populations and thus facilitate
89 *G. pneumonanthe* (Oostermeijer et al. 1998; see also Oostermeijer et al. 2000). The effects of
90 habitat fragmentation and demographic senility are recognized as crucial to the persistence of
91 *G. pneumonanthe* populations (Oostermeijer et al. 1994a; Volis et al. 2005), but possible
92 relationships between lack of pollination success and population senility (regressive age-state

93 structure) have yet to be explicitly tested. Additionally, a view of reproduction at sites at the
94 southern boundary of the species range is currently lacking. Effects of reproductive failure on
95 demographics could be determined by examining a large number of populations across
96 numerous sites and circumstances. Specifically, we ask: at what point do demographic
97 senility, habitat fragmentation and population size become associated with limitation of
98 pollination, ovule fertilization and seed germination capacity?

99 Measurement of the potential for pollination within plant populations is typically
100 performed by observation of flower visitation by insects to determine the plant-pollinator
101 network, and is ideally complemented by measurement of pollen receipt, or the deposition of
102 pollen on the stigmatic surfaces of the flower (Primack and Silander 1975; Engel and Irwin
103 2003). This is particularly informative if pollen viability and activity (germination and pollen
104 tube growth; Derksen et al. 1995) can also be confirmed *in situ*. The growth of the pollen tube
105 and in particular the presence of specific structural compounds in the tube wall allows
106 observation of pollen activity and thus can be used to confirm the viability and efficacy of
107 pollen for flowers collected in the wild. Specifically, the cell wall of the pollen tube is
108 impregnated with the polysaccharide callose, which limits lateral expansion as the pressurized
109 tube grows and avoids inflation of the cell like a balloon (Chebli and Geitmann 2007). As the
110 pollen tube extends, callose-impregnated septa ('plugs') and the action of the cytoskeleton are
111 used to keep gametes at the tip of the tube. Due to this specific structural role of callose it is
112 not found in the tissues of the stigma and underlying style and with the aid of the appropriate
113 stain can thus be used to distinguish pollen tubes from surrounding host tissues. Here we were
114 not interested in the mechanism of pollen tube growth *per se* (physiological and
115 morphological aspects of which are reviewed by Holdaway-Clarke and Hepler 2003; Chebli
116 and Geitmann 2007), but in assessing the extent to which pollination, ovule fertilization and
117 seed production occur within and between populations, in particular in relation to population
118 demographics and the extent of local habitat fragmentation.

119 When considered together, observations of the presence of pollen grains, pollen tube
120 growth, penetration of the ovule, ovule development, seed production and germination
121 capacity can provide a high degree of confidence in the effectiveness of reproduction. During
122 fertilization, pollen tube arrival at the ovule initiates ovule and ovary/fruit development, but
123 each ovule requires a viable pollen grain for fertilization and if insufficient grains are
124 deposited this will be evident as a large proportion of ovules that start development but do not
125 contain a zygote, or embryo. Thus measuring the proportion of seeds that contain embryos, or

126 not, is a direct indicator of fertilization rate, which may be particularly low in small
127 populations of rare species (e.g. Pierce et al. 2010).

128 We hypothesized that populations of *G. pneumonanthe* exhibiting senile demographics (i.e.
129 with a small proportion of juveniles; Oostermeijer et al. 1994a) occupying highly fragmented
130 habitats exhibit pollen limitation and subsequent limitation of ovule fertilization and seed
131 germinability, and that thresholds of population size and demographics can be identified to
132 help guide conservation efforts.

133

134 **Materials and methods**

135

136 **Study species**

137

138 *Gentiana pneumonanthe* is a perennial scapose hemicryptophyte (i.e. with buds at/just below
139 soil level), with the perennating organ being a rhizome: during winter the aerial parts senesce
140 and several fresh stems may be produced each spring, meaning that despite its herbaceous
141 habit it is essentially deciduous (Simmonds 1946). This allows a polycarpic life history in
142 which flowering can be repeated for decades. The erect stem is thin, but gains some support
143 from surrounding plants, most notably *Molinia caerulea*, and linear-lanceolate cauline leaves
144 are longer and thinner than the rounder basal leaves and exhibit sufficient stiffness that they
145 may help *G. pneumonanthe* to maintain its position amongst the upright leaves and stems of
146 *M. caerulea* (when cultivated in isolation, stems of *G. pneumonanthe* are too weak for the
147 plant to stand completely upright, resulting in a trailing habit; S.Pierce, personal
148 observations). This allows large individuals of *G. pneumonanthe* to position an inflorescence
149 at up to ~1.5 m (typically 10 to 50 cm) despite the relatively ephemeral nature of the stems.
150 Flowering takes place from late spring (June), through the summer until October. Fruits
151 contain between 300 and 1000 seeds (Raijmann et al. 1994) which are tiny gravity-dispersed
152 dust seeds that do not appear to persist in soil, and thus the seed bank is transient
153 (Oostermeijer et al. 1992). *G. pneumonanthe* is the exclusive larval host plant of the critically
154 endangered butterfly *Maculinea alcon* (Vanden Broeck et al. 2017), and as such plays a key
155 role in the ecology of a range of plant and insect species, including ants (*Myrmica* spp.; which
156 are also essentially parasitized by *M. alcon*) and the clumps of *Molinia caerulea* in which the
157 ants nest.

158 *Gentiana pneumonanthe* is distributed widely in western Europe, from southern
159 Scandinavia and Portugal to Russia, and is found at the southern limit of its range in northern
160 Italy, but the species is restricted to humid meadows (EU Habitats Directive code: 6410
161 ‘*Molinia* meadows on calcareous, peaty or clayey-silt-laden soils (*Molinion caeruleae*’) and
162 heathland (broadly classifiable as habitat 4030 ‘European dry heaths’, although
163 *G. pneumonanthe* occupies humid areas; see Cerabolini et al. 2017). The dependence of these
164 habitats on traditional management regimes involving sod cutting and fire means that they are
165 at risk of land-use change and concomitant ecological succession processes.

166

167 **Study area**

168

169 Study sites are listed in Supplementary Table S1 and encompassed lowland heathland
170 dominated by *Calluna vulgaris* (L.) Hull and *Molinia caerulea* (L.) Moench (EU Habitats
171 Directive code 4030: European dry heaths) and *Molinia* meadows on clayey soils (EU 6410:
172 *Molinion caeruleae*). Sites of particular conservation interest included natural parks in the
173 hinterland of the major conurbation of Milan, including the Groane Regional Park (Parco
174 Regionale delle Groane) and the Briantea Heathland Park (Parco Brughiera Briantea), which
175 include remnants of an historically extensive lowland heathland. A preliminary study,
176 including database records for the occurrence of organisms in the Lombardy region
177 (www.biodiversita.lombardia.it/ossnat_2016/PUBBLICO_flora_elenco_taxa_grid) and on-
178 site inspections, determined that *G. pneumonanthe* is now locally extinct in many historic
179 sites, including many where the species was observed during a survey of heathland vegetation
180 as late as 2008 (Brusa 2008; Fig. 1).

181 Habitat area and perimeter were determined from global positioning system track data
182 collected in the field during the summer of 2015 using a Garmin eTrex Summit GPS handset,
183 with track data imported into Quantum GIS (QGIS 2.16 Nødebo; www.qgis.org/en/site) for
184 the creation of polygons from which area and perimeter measurements were obtained. The
185 ‘shape index’ (SI) of McGarigal and Marks (1995) was used to define the relationship
186 between habitat area and perimeter as detailed in equation 1:

187

188

189

$$SI = \frac{P}{S} \times 100 \quad [1.]$$

190

191 where P denotes habitat perimeter (in m), S denotes the habitat area (m²), and higher values
192 represent larger, relatively irregular (un-circular) fragments.

193

194 **Demography**

195

196 For the definition of demographic stages, Oostermeijer et al. (1994a, 1996) identified six age
197 state classes for *G. pneumonanthe*: 1). seeds, 2). seedlings, 3). juveniles, 4). vegetative adults,
198 5). generative adults (i.e. in an evident reproductive phase), 6). dormants (winter survival as a
199 rhizome; see also Kostrakiewicz-Gieralt 2013). In practice, during surveys in the summer of
200 2015, the presence of seeds and dormants was not considered *in situ* and seedlings and
201 juveniles were considered together, following Volis et al. (2005). Juveniles were defined as
202 individuals with elliptical cauline leaves but with persistent cotyledons, vegetative adults were
203 robust, tall individuals with lanceolate rather than elliptical cauline leaves that were not in bud
204 or in flower, and reproductive adults were those with one or more flower or bud (Fig. 1B-F).
205 The ratio between the number of young plants (Y: seedlings + juveniles) and the number of
206 reproductive or generative adults (G) was used to calculate the age state ratio of Oostermeijer
207 et al. (1994a; R_O) as an indicator of demographic state:

208

209

$$210 \quad R_O = Y / G \quad [2.]$$

211

212

213 According to Oostermeijer et al. (1994a) a range of values between 0 to 0.029 indicates a
214 ‘regressive’ or ‘senile’ population in which juveniles are either absent or very weakly
215 represented, 0.03 to 2 indicates a ‘normal’ population, and 2 and 100 indicates an ‘invasive’
216 population with a preponderance of juveniles. The number of vegetative adults was not used
217 by Oostermeijer et al. (1994a), but was used here for the calculation of other parameters, such
218 as the total number of individuals and the local density of *G. pneumonanthe*.

219 The number of individuals of each age state at each site was determined from three 9 m²
220 quadrats (i.e. R_O values represent a mean with *n* = 3), with each square centimetre of every
221 quadrat checked manually to soil level for seedlings and other age states. Quadrat size was
222 chosen based on the need to represent the population at the scale of both adults and seedlings.
223 Oostermeijer et al. (1994a) used quadrats of between 4 and 16 m² based on local population

224 density, and an initial study found an intermediate quadrat size of 9 m² to be manageable and
225 capable of representing all age states and was applied universally across sites to allow
226 standardization. Local population size (in terms of both total population size and number of
227 reproductive adults) was directly counted where possible, but for the largest populations the
228 data from quadrats was used to calculate a density value that, in combination with GIS
229 measurements of site area, was used to estimate local population size (e.g. one of the habitats,
230 at Soave (site 14), had an area of 75.1 hectares and was impossible to survey in its entirety,
231 although spot-checks revealed the presence of *G. pneumonanthe* apparently throughout).

232

233 **Pollination success within populations**

234

235 ‘Pollination success rate’ was defined as the percentage of individuals within the population
236 that exhibited growth and development of pollen tubes within pistil tissues. For each
237 population, flowers were collected in the female phase of development (Petanidou et al. 2001)
238 in the 2015 field season: generally, 10 flowers per population at the correct developmental
239 stage were found, but sample size ranged from 5 to 15 flowers depending on the amount of
240 available material in nature. Each flower represented a separate individual plant. Flowers
241 were excised at the base and placed in a solution of formalin-acetic-80% alcohol (1:1:8; FAA)
242 at ~4 °C (in the field, in a cool-bag over icepacks, in the laboratory in a refrigerator). After
243 24 h samples were transferred to 70% ethanol which was replaced with fresh 70% ethanol
244 after a further 24 h, followed by longer-term storage at 4 °C.

245 Pollen tubes were stained and observed following Martin (1959): the calyx and corolla of
246 each flower were excised and tissues were cleared in 8 N sodium hydroxide solution followed
247 by staining with a 0.1% solution of water-soluble aniline blue dye dissolved in 0.1 N, K₃PO₄.
248 The pistil of *Gentiana* spp. is formed by two carpels, and these were separated longitudinally
249 along the line where they joined, and then both carpels were arranged side-by-side on a
250 microscope slide and observed whole under a conventional fluorescence microscope. Samples
251 were illuminated with ultraviolet light (wavelength 356 nm) to stimulate fluorescence of
252 aniline associated with pollen tube callose. The presence or absence of pollen on the stigmatic
253 surface was recorded, and the presence and disposition of pollen tubes within style and carpel
254 wall tissues and in the vicinity of ovules was noted for each sample.

255

256

257 **Seed production and *in vitro* germination success**

258

259 Seed was collected in autumn 2015 from ten fruits (where possible) from each population
260 using 20 ml capacity polypropylene bottles and stored for two months in the drying room of a
261 seed bank maintained at 15% relative humidity and ambient temperature (~22°C). Seed mass
262 was measured on a microbalance with a precision of 0.01 mg (model New Classic MS;
263 Mettler-Toledo, Novate Milanese, Italy). For each population seed lots were divided into 6
264 sub-lots of 50 seeds each, which were weighed and the value divided by 50 to calculate the
265 mean seed mass per sub-lot. These values were used to calculate the mean single seed weight
266 per population.

267 For germination, *Gentiana pneumonanthe* is known to germinate on water agar (Godefroid
268 et al. 2010) or on damp filter paper in the light (Oostermeijer et al. 1994b). However, the
269 germination experiment did not aim simply to compare germination rates between
270 populations, but also to produce seedlings and plants for future restocking activities as part of
271 a wider conservation project: it was deemed necessary to provide nutrients for further growth
272 and plant production in a controlled, sterile *in vitro* system. Murashige and Skoog's (1962)
273 basal medium (from here on referred to as MS) has been used for a range of *Gentiana* species
274 (Morgan, 2004; Vinterhalter et al. 2012; Kaushal et al. 2014) including *G. pneumonanthe*
275 (Bach and Pawłowksa 2003). For this motive a half-strength MS medium was used with 15 g
276 L⁻¹ sucrose and 6 g L⁻¹ agar and modified by the addition of inositol (50 mg L⁻¹), thymine (0.5
277 mg L⁻¹) and indole-3-butyric acid (IBA; 0.01 mg L⁻¹). The pH of the medium was adjusted to
278 5.8 using 0.1N NaOH or HCL immediately prior to autoclaving at 0.1 MPa and 121°C for 20
279 minutes. Medium was then poured into 6cm-diameter Petri dishes; the use of Petri dishes,
280 rather than flasks, allowed the use of a stereomicroscope to count germination and determine
281 the presence or absence of embryos within seeds (see Pierce et al. 2015).

282 For seed sowing, 40 mg sub-samples of seed (*G. pneumonanthe* seeds are extremely small
283 and samples of 'dust seed' were managed as a powder) were transferred to 1.5 ml Eppendorf
284 tubes and surface sterilised using Wilson's (1915) surface sterilization method. Specifically,
285 seeds were immersed in domestic bleach (i.e. a 5% (v/v) sodium hypochlorite solution,
286 equivalent to 3% active chlorine) containing 0.1% Tween surfactant as a wetting agent, for 3
287 minutes, followed by six rinses in sterilized distilled water in a sterile environment. Previous
288 attempts confirmed that relatively dilute bleach solutions were not effective at surface
289 sterilization for this species (see Panzeri 2015). Seeds were sown using a sterilized stainless

290 steel spatula on the agar medium contained in the Petri dishes and subsequently sealed using
291 laboratory film (Parafilm). A minimum of twenty replicate Petri dishes per population were
292 prepared.

293 Following sowing, Petri dishes were placed in a growth chamber (Snijders Economic
294 Deluxe; Thermo-Lab, Codogno (LO), Italy) with a photoperiod of 12 hours, a measured light
295 intensity of $300 \mu\text{mol Q m}^{-1} \text{s}^{-1}$, and a day/night temperature regime of 20/10°C. Petri dishes
296 were removed weekly and checked qualitatively for the presence of germinated seeds, and the
297 position of Petri dishes within the growth chamber was then re-randomised to minimise the
298 possible effects of local temperature and light variation. Final germination percentage was
299 quantified for each treatment when no further germination was observed, at approximately
300 two months after sowing.

301 Stereomicroscopic examination of Petri dishes involved counting the number of seeds
302 consisting of only external integuments (representing unfertilized ovules) and the number of
303 seeds containing internal integuments surrounding a visible embryo (intact seeds).
304 ‘Fertilization rate’ was calculated as the percentage of total seeds that were intact and thus
305 represent successfully fertilized ovules. Germination rate was defined as the percentage of
306 intact seeds for which cotyledons and emerging rhizoid-like trichoblasts were visible after two
307 months.

308

309 **Results**

310

311 **Observations of pollination**

312

313 Pollen grains were evident under UV light as yellow globular structures with fluorescent
314 pollen tubes visible both on the stigmatic surface and penetrating and growing within the style
315 (Fig. S1). Relatively well-developed (recurved) styles were observed with stigmas covered in
316 germinated pollen grains (Fig. S1A). Relatively young styles that were still straight and
317 evidently at the start of development either did not exhibit pollen grains (Fig. S1B) or pollen
318 grains were visible only on the tip, often with pollen tubes visible within the tissues of the
319 style (Fig. S1C-E).

320 Pollen tubes were observed to descend from the stigmatic surface, through the style tissues
321 in two strands that eventually penetrating the carpel walls and followed the edge of the

322 carpels (the edge that later develops to form the long edge of the dehisced fruit; Fig. S2).
323 Ovules were observed to be inserted in 4-5 parallel rows along this line. From these pollen
324 tube bundles individual pollen tubes were observed to abruptly change course in the vicinity
325 of an ovule, with single pollen tubes each entering a single ovule (Fig. S2A). Ovules closest
326 to the distal (apical) end of the ovary where fertilized first, with a gradient of decreasing
327 fertilization evident moving proximally along the ovary (Fig. S2B). Examples were evident of
328 both successful penetration of ovules by pollen tubes (Fig. S2C) and a complete absence of
329 pollen tubes in the carpel walls (Fig. S2D).

330

331 **Pollination and reproductive success across sites**

332

333 Of the site parameters (habitat/demographic measures) population size was found to be the
334 most strongly significantly correlated with pollination success (i.e. the percentage of flowers
335 sampled from the population confirmed to have active pollen growth within pistil tissues –
336 which was in turn correlated with fertilization rate and germination capacity; Table 1).
337 Population size was also strongly correlated with fertilization, seed mass and germination
338 capacity (Table 1). Habitat area and shape index (the latter essentially being the opposite of
339 ‘fragmentation’) also correlated positively with fertilization rate and germination capacity
340 (Table 1), and shape index also correlated positively with seed mass. Thus larger habitat
341 fragments supported larger populations with more effective fertilization, seed filling and
342 germination. Demographic or age state ratio was correlated positively with habitat area, but
343 not with reproductive characteristics such as pollination success, fertilization, seed mass or
344 germination capacity (Table 1).

345 Pollination success rate was highly variable between populations, ranging from 0 to 100%
346 (Fig. 2A). Even some of the smallest populations (e.g. Sites 4, 8 and 9) exhibited 100%
347 pollination success (Fig. 2A), whereas some of the largest populations exhibited only
348 moderate pollination success: e.g. 50% for a large population occupying a recently burned
349 area at Rovesenda (Site 18; Fig. 2A). In general, pollination success rate was significantly
350 correlated with population size ($R^2 = 0.578$, $F = 10.282$, $p = 0.0015$) following an exponential
351 rise to maximum relationship with a tipping point at 7 reproductive individuals, above which
352 pollination rate was consistently high and below which pollination success declined towards
353 the origin (Fig. 2A).

354 The mean fertilization rate also exhibited a statistically significant rise to maximum
355 relationship with population size, with a tipping point at 42 reproductive adults below which
356 fertilization declined to the origin ($R^2 = 0.776$, $F = 11.286$, $p = 0.0004$; Fig. 2B). Similarly,
357 germination capacity was consistently around 20% for populations of more than 42
358 reproductive adults, but below this tipping point declined in the smallest populations
359 following a similar statistically significant relationship ($R^2 = 0.543$, $F = 3.913$, $p = 0.0429$;
360 Fig. 2C).

361

362 **Discussion**

363 The results demonstrate that highly variable rates of pollination success experienced by small
364 populations of *G. pneumonanthe* occupying restricted habitat fragments are not directly
365 correlated with the degree of ‘senility’ or age-state characteristics of populations (i.e. senility
366 does not occur because of pollen limitation). This is in agreement with the observation that
367 recruitment may effectively become disconnected from annual reproductive effort because
368 long-lived individuals persist over decades as the vegetation ‘successes’ around them,
369 restricting the gaps required for seedling establishment (Oostermeijer et al. 1996). In the
370 present study, several sites with small populations represented remnants of historic heathland
371 that are now so transformed that they essentially represent a woodland understorey composed
372 mainly of *M. caerulea* hosting a few reproductive adults of *G. pneumonanthe* (e.g. site 4;
373 Table S1). Both recruitment (seedlings) and pollination were absent at these sites and this is a
374 clear sign of local extinction debt (i.e. a time-lag between the effective extinction and when
375 the last individual actually dies). However, for some small populations the extinction debt
376 was less obvious: these exhibited ‘normal’ age state distributions and appeared to be in
377 demographic good health in appropriate habitat (e.g. the 16 plants at ‘Pineta’; Site 8) but
378 reproduction was not as effective as larger populations. Indeed, pollination success exhibited
379 a sharp and significant decline below 7 flowering individuals, and a decline in ovule
380 fertilization rate and germination capacity was evident in populations of less than 42
381 reproductive adults. This suggests that for small populations pollination was sporadic (despite
382 the fact that autogamy is possible for this species it is apparently not a reliable mechanism)
383 and pollen availability was insufficient to support high fertilization rates – a clear threshold
384 for pollen limitation. While this generally confirms a similar suggestion by Oostermeijer et al.
385 (1998) based on ovule/seed set counts across populations, we can add that our direct

386 observation of pollination revealed that the relationship is not linear (the closest fit being an
387 exponential rise to maximum relationship; Fig. 2) and thus a tipping point is evident which
388 represents a threshold for severe reproductive limitation.

389 A population size of approximately 42 flowering individuals is an important general
390 threshold as many populations in managed habitat fragments (at least in northern Italy) are of
391 this order of magnitude in size, suggesting that any further declines in habitat availability,
392 pollen availability or increased fragmentation will almost certainly prove disastrous. In the
393 present study only certain aspects of the biology and ecology of the species were investigated,
394 and this population size threshold should not be interpreted as a minimum viable population
395 size *per se*: rather it is the limit below which reproductive limitations become severe. Where a
396 more extensive range of factors are accounted for the minimum viable population size is
397 estimated to be much higher: in the order of 300 to 400 (Oostermeijer et al. 2003), although
398 stochastic environmental effects may alter this estimate (Oostermeijer 2000) and a safer
399 minimum viable population size is likely to be in the order of a thousand individuals (JGB
400 Oostermeijer, personal communication). The majority of populations in the study area are
401 thus well below the minimum viable population size, but hope springs from the fact that
402 reproduction for many populations remains above the thresholds for severe limitation.

403 A further complication of restricted population size and habitat fragmentation is that of
404 inbreeding, evident for populations in the Netherlands from study of polymorphic isozyme
405 loci (Oostermeijer 1994b; Raijmann et al. 1994). For the populations in the present study,
406 investigation of possible genetic effects of habitat fragmentation including inbreeding
407 depression was beyond the scope of the analysis presented here, but genome ezRADseq-based
408 investigation of material collected from all populations during the study is currently underway
409 and results will be presented separately in the context of the ecological factors and vegetation
410 characters predominating at each site. While traditional studies of genetic variability use
411 neutral genetic markers that are not necessarily pertinent to the ecology of the species, study
412 of genome-scale variability between individuals will be able to determine specific differences
413 across hundreds of alleles that reflect ecological adaptation. Indeed, a future aim will be to
414 understand which populations can provide genetically-compatible material to restore or
415 reinforce neighbouring or even distant populations without inducing out-breeding depression,
416 particularly with regard to the possibility that 'heathland' and 'fen' ecotypes of the species
417 might exist.

418 Population restoration should ideal be based on encouraging seedling recruitment by
419 ‘resetting’ the ecological succession using traditional management techniques such as sod
420 cutting (Oostermeijer et al. 1998). This type of disturbance provides microsites for
421 establishment whilst suppressing competition by surrounding vegetation. Other options
422 include the addition of seed to sites following management or the reinforcement of
423 populations using plants produced *ex situ* from seed, particularly where there is an urgent
424 requirement to ‘boost’ a population from the threshold for severe reproductive limitation to
425 the minimum viable reproductive size. The seeds that germinated during the present study
426 produced several thousand plants that are currently being cultivated and reintroduced to
427 reinforce parent populations, particularly those at greatest risk of reproductive limitation. This
428 forms part of an integrated project including habitat management by the various regional
429 parks and bodies involved with the project, and pupils of a local agricultural college learnt *in*
430 *vitro* techniques, produced plants and helped with the practical work of mowing habitat and
431 restocking wild populations (Figs. S3-S6). An ultimate aim will be to re-establish lost historic
432 populations using appropriate ecotypes, guided by genetic analysis of the relatedness of extant
433 populations. Notably, the natural parks directly involved in the current project have
434 demonstrated a commitment to the management of their heathland sites and the ideal goal is
435 not simply that of habitat maintenance, but of habitat expansion. Populations of rare perennial
436 species can spontaneously expand in response to appropriate management regimes (Endels et
437 al. 2007) when reproduction is not limited, and this is our hope for *G. pneumonanthe* in
438 northern Italy.

439 In conclusion, even very small populations of *G. pneumonanthe* can exhibit pollination,
440 ovule fertilization and seed set, and are not necessarily senile or regressive. However,
441 populations of less than seven flowering (reproductive or generative) individuals exhibit a
442 statistically significant decline in pollination success, probably representing a shift from out-
443 crossing to highly variable and unreliable autogamy. A threshold of 42 reproductive adults is
444 important for other aspects of reproduction (ovule fertilization, seed germination), above
445 which reproduction is not limited by issues of population size and habitat fragmentation. As
446 suggested by Oostermeijer et al. (2003) minimum viable population sizes are likely to be in
447 the order of several hundred flowering individuals or more. To this we can add that local
448 groups of around 50 flowering adults may appear demographically ‘normal’ and capable of
449 out-crossing and reproduction, but should nonetheless be considered to be on the cusp of out-
450 crossing failure. Whereas the minimum viable population size can be used as an indicator of

451 successful conservation, a threshold of ~50 flowering plants represents an indicator of direct
452 conservation need.

453

454 **Acknowledgements** This study was supported by a grant from the Cariplo Foundation as part of the
455 project “*FraGenziane: Fragmented Gentiana pneumonanthe populations, habitats and associated*
456 *fauna in local ecological network*”, grant number 2014-1631: project leader, Parco Monte Barro. The
457 regional parks of Parco Regionale delle Groane and Consorzio Parco Brughiera Briantea allowed
458 access to their territories and actively facilitated the project, and we particularly thank Luca Frezzini
459 and the ecological guards of the Groane Park (particularly Paolo Ventura and Antonella Pezzotta) and
460 Daniele Piazza of Consorzio Parco Brughiera Briantea. We thank Dr. Maria Costanza Scarpini
461 (Istituto L. Castiglioni) and students of the school for their enthusiasm whilst actively facilitating
462 investigation of seed germination and plant production. We thank Guido Brusa for sharing his
463 experience and for help with finding populations. Prof. Giuseppe Bogliani coordinated project
464 activities for the University of Pavia, and Silvia Stefanelli and Arianna Musacchio provided
465 entomological assistance in the field. We thank Letizia Manzoni for help with the analysis of
466 autogamy. Prof. Maurizio Cornalba (University of Pavia) shared his expertise for the determination of
467 Apoidea. We thank the regional governments of Lombardy and Piedmont for permission to collect
468 samples of plant material. Comments from three anonymous reviewers, one of whom left strong hints
469 as to their identity, greatly improved the manuscript and in doing so improved the chances of survival
470 of *G. pneumonanthe* in northern Italy.

471

472 **Author contribution statement** SP and RMC conceived and designed the study, SP, EC (Caporali),
473 AS and AL performed the pollination analyses, SP, FP, LM, AL, AP and SC collected flowers and
474 seed, characterized seed lots and performed *in vitro* germination tests for the study populations. ML
475 and AG supervised population sampling, advised on statistics and revised the text. EC (Cardarelli)
476 performed the analysis of pollinator visitation. SP performed statistical tests, produced graphics and
477 wrote the manuscript, and all authors were involved in manuscript correction.

478

479 **Supplementary material**

480 Data analysed during this study are available as supplementary online material in the form of
481 a spreadsheet file.

482

483

484

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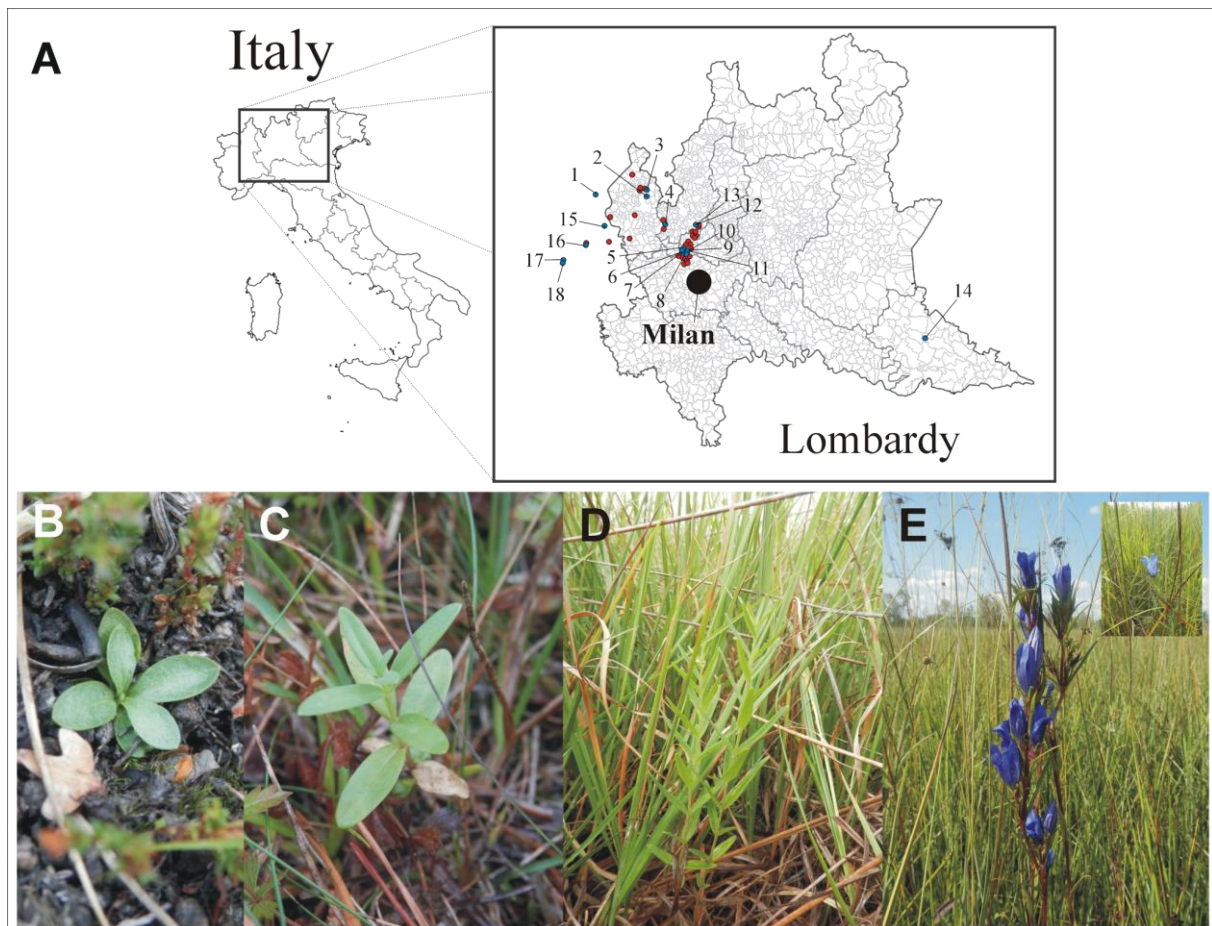
603 **Table 1.** Pearson's correlation coefficients (r) between population/habitat characters and reproductive
 604 characteristics of *Gentiana pneumonanthe* populations in northern Italy. Enboldened values represent significant
 605 correlations: the critical value of r is 0.456 (at the $p \leq 0.05$ level and with $d.f.=17$). R_0 = Oostermeijer et al.'s
 606 (1994a) age state ratio of young plants (seedlings + juveniles) to reproductive adult individuals.

607

Variables	Population size	Demographic ratio (R_0)	Habitat area	Shape index	Pollination success	Fertilization rate	Germination capacity	Seed mass
Population size	1	0.394	0.752	0.680	0.616	0.715	0.587	0.583
Demographic ratio (R_0)	0.394	1	0.460	0.457	0.190	0.248	0.113	0.227
Habitat area	0.752	0.460	1	0.977	0.206	0.528	0.488	0.424
Shape index	0.680	0.457	0.977	1	0.170	0.522	0.531	0.431
Pollination success	0.616	0.190	0.206	0.170	1	0.600	0.506	0.350
Fertilization rate	0.715	0.248	0.528	0.522	0.600	1	0.756	0.877
Germination capacity	0.587	0.113	0.488	0.531	0.506	0.756	1	0.535
Seed mass	0.583	0.227	0.424	0.431	0.350	0.877	0.535	1

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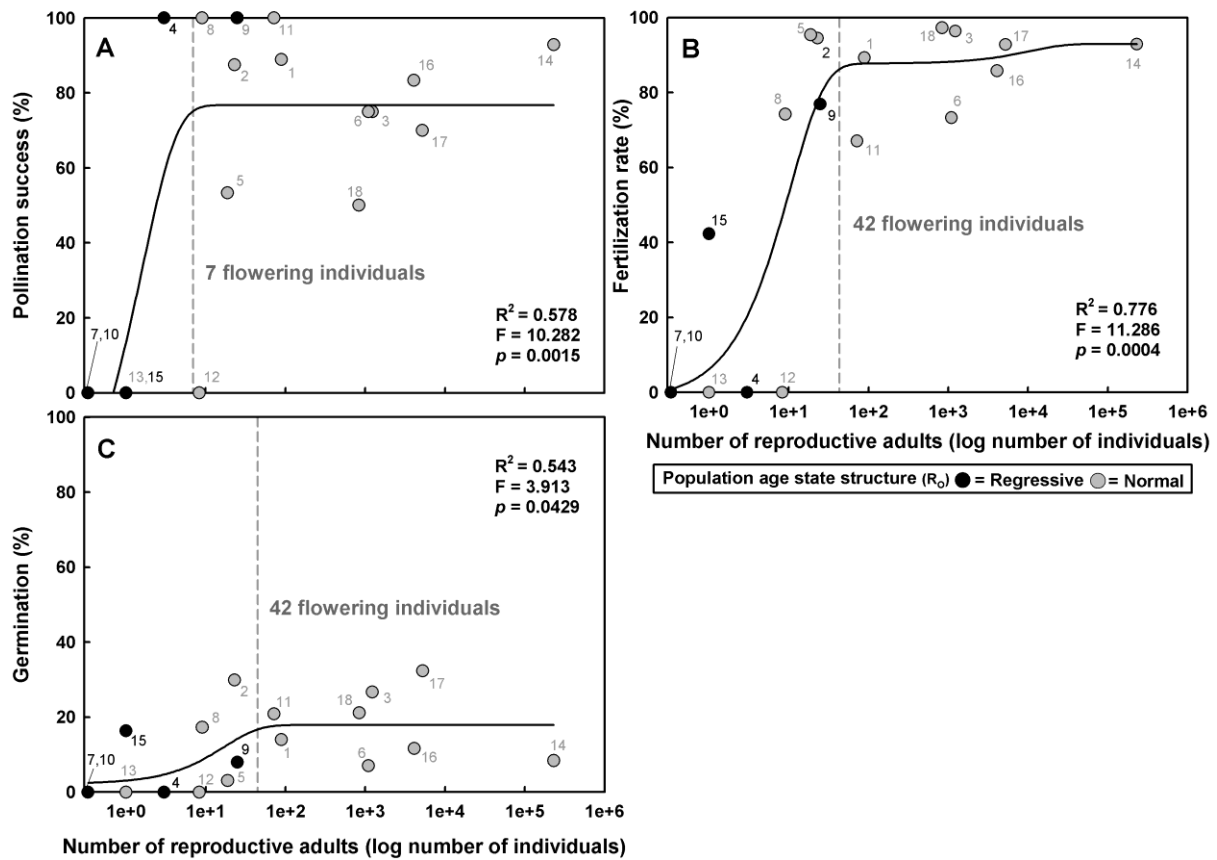


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612 **Fig. 1** *Gentiana pneumonanthe* populations investigated during the study and an overview of
 613 age state categories. A). location of study sites in northern Italy (mainly in the Lombardy
 614 region, but some sites in neighbouring Piedmont). Blue points represent sites from which
 615 flower and seed material of *G. pneumonanthe* was collected and vegetation surveys carried
 616 out (site labels and names are listed in Supplementary Table S1). Red points represent historic
 617 records or recent observations for which site inspections confirmed local extinction (site
 618 labels not reported). Age state categories include: B). seedling, C). juvenile, D). vegetative
 619 adult, E). reproductive/generative adult with multiple or (inset) single flowers. (Photos: Simon
 620 Pierce).

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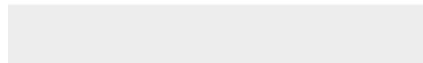


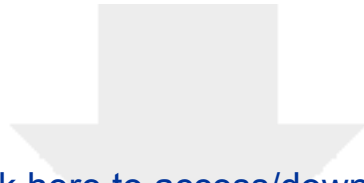
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623 **Fig. 2** The relationship between reproductive success (A, pollination success; B, fertilization
 624 rate; C, germination capacity *in vitro*) and population size (log axis) for a wide range of
 625 contrasting populations of *Gentiana pneumonanthe* from northern Italy (code numbers
 626 represent populations listed in Table S1). Non-linear regression was fitted as an exponential
 627 rise to maximum (double, 5 parameter) followed by ANOVA. Population age state structure
 628 represents the ratio of young plants (seedlings + juveniles) to reproductive adult individuals,
 629 following the age states delimited by Oostermeijer et al. (1994a).



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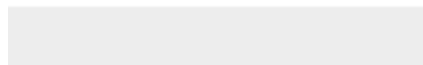




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