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

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Abstract:	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 (Gentiana pneumonanthe), 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

	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 G. pneumonanthe are in the order of 50-150 individuals these should be not be considered as MVPs; they are on the brink of calamity.
Response to Reviewers:	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. We thank you for continuing to have faith in this manuscript! Yours sincerely, Simon Pierce on behalf of all authors

ORIGINAL PAPER

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

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Abstract 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 (*Gentiana pneumonanthe*), 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 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 *G. pneumonanthe* are in the order of 50-150 individuals these should be not be considered as MVPs; they are on the brink of calamity.

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

Introduction

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

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

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

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

Gentiana pneumonanthe is self-compatible, but has been shown experimentally to exhibit limited autogamy (spontaneous pollination of a flower with its own pollen) due to protandrous flower development (a separate male phase preceding a female phase) and herkogamy (physical separation of anthers and stigmas; Petanidou et al. 1991): autogamy may vary between 0.2 to 25% depending on circumstances (Petanidou et al. 2001). *Gentiana pneumonanthe* exhibits nastic corolla movement in response to low temperatures (i.e. flowers close for the night or during cloudy weather at temperatures below 16°C; Kozuharova 2004) which could press the stigmas lobes close to the anthers, and has been suggested as a mechanism for self-pollination, but could be an adaptation to protect the fertile parts of the flower from chilling and prevent nectar dilution by dew (Kozuharova and Anchev, 2006), or both. Anemophily has also been investigated and is extremely unlikely (Petanidou et al. 1995). It is clear that very small populations can exhibit pollen limitation, with concomitant effects on seed quality and production (Petanidou et al. 1991). However, it would be useful to know at what point these effects start during the decline of populations, as an additional parameter when attempting to determine MVP sizes. Comparison of ovule to seed numbers per fruit across populations suggests that pollination declines towards smaller population sizes, particularly where populations occupy *Molinia caerulea*-dominated fen habitat where less heath (*Calluna vulgaris*) is available to support pollinator populations and thus facilitate *G. pneumonanthe* (Oostermeijer et al. 1998; see also Oostermeijer et al. 2000). The effects of habitat fragmentation and demographic senility are recognized as crucial to the persistence of *G. pneumonanthe* populations (Oostermeijer et al. 1994a; Volis et al. 2005), but possible relationships between lack of pollination success and population senility (regressive age-state structure) have yet to be explicitly tested. Additionally, a view of reproduction at sites at the southern boundary of the species range is currently lacking. Effects of reproductive failure on demographics could be determined by examining a large number of populations across numerous sites and circumstances. Specifically, we ask: at what point do demographic senility, habitat fragmentation and population size become associated with limitation of pollination, ovule fertilization and seed germination capacity?

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

When considered together, observations of the presence of pollen grains, pollen tube growth, penetration of the ovule, ovule development, seed production and germination capacity can provide a high degree of confidence in the effectiveness of reproduction. During fertilization, pollen tube arrival at the ovule initiates ovule and ovary/fruit development, but each ovule requires a viable pollen grain for fertilization and if insufficient grains are deposited this will be evident as a large proportion of ovules that start development but do not contain a zygote, or embryo. Thus measuring the proportion of seeds that contain embryos, or not, is a direct indicator of fertilization rate, which may be particularly low in small populations of rare species (e.g. Pierce et al. 2010).

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

Materials and methods

Study species

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

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

Study area

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

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

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

where P denotes habitat perimeter (in m), S denotes the habitat area (m^2), and higher values represent larger, relatively irregular (un-circular) fragments.

Demography

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

$$R_O = Y / G \qquad [2.]$$

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

The number of individuals of each age state at each site was determined from three 9 m^2 quadrats (i.e. R_O values represent a mean with $n = 3$), with each square centimetre of every quadrat checked manually to soil level for seedlings and other age states. Quadrat size was chosen based on the need to represent the population at the scale of both adults and seedlings. Oostermeijer et al. (1994a) used quadrats of between 4 and 16 m^2 based on local population density, and an initial study found an intermediate quadrat size of 9 m^2 to be manageable and capable of representing all age states and was applied universally across sites to allow standardization. Local population size (in terms of both total population size and number of reproductive adults) was directly counted where possible, but for the largest populations the data from quadrats was used to calculate a density value that, in combination with GIS measurements of site area, was used to estimate local population size (e.g. one of the habitats, at Soave (site 14), had an area of 75.1 hectares and was impossible to survey in its entirety, although spot-checks revealed the presence of *G. pneumonanthe* apparently throughout).

Pollination success within populations

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

Pollen tubes were stained and observed following Martin (1959): the calyx and corolla of each flower were excised and tissues were cleared in 8 N sodium hydroxide solution followed by staining with a 0.1% solution of water-soluble aniline blue dye dissolved in 0.1 N, K_3PO_4. The pistil of *Gentiana* spp. is formed by two carpels, and these were separated longitudinally along the line where they joined, and then both carpels were arranged side-by-side on a microscope slide and observed whole under a conventional fluorescence microscope. Samples were illuminated with ultraviolet light (wavelength 356 nm) to stimulate fluorescence of aniline associated with pollen tube callose. The presence or absence of pollen on the stigmatic surface was recorded, and the presence and disposition of pollen tubes within style and carpel wall tissues and in the vicinity of ovules was noted for each sample.

Seed production and *in vitro* germination success

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

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

For seed sowing, 40 mg sub-samples of seed (*G. pneumonanthe* seeds are extremely small and samples of 'dust seed' were managed as a powder) were transferred to 1.5 ml Eppendorf tubes and surface sterilised using Wilson's (1915) surface sterilization method. Specifically, seeds were immersed in domestic bleach (i.e. a 5% (v/v) sodium hypochorite solution, equivalent to 3% active chlorine) containing 0.1% Tween surfactant as a wetting agent, for 3 minutes, followed by six rinses in sterilized distilled water in a sterile environment. Previous attempts confirmed that relatively dilute bleach solutions were not effective at surface sterilization for this species (see Panzeri 2015). Seeds were sown using a sterilized stainless steel spatula on the agar medium contained in the Petri dishes and subsequently sealed using laboratory film (Parafilm). A minimum of twenty replicate Petri dishes per population were prepared.

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

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

Results

Observations of pollination

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

Pollen tubes were observed to descend from the stigmatic surface, through the style tissues in two strands that eventually penetrating the carpel walls and followed the edge of the carpels (the edge that later develops to form the long edge of the dehisced fruit; Fig. S2). Ovules were observed to be inserted in 4-5 parallel rows along this line. From these pollen tube bundles individual pollen tubes were observed to abruptly change course in the vicinity of an ovule, with single pollen tubes each entering a single ovule (Fig. S2A). Ovules closest to the distal (apical) end of the ovary where fertilized first, with a gradient of decreasing fertilization evident moving proximally along the ovary (Fig. S2B). Examples were evident of both successful penetration of ovules by pollen tubes (Fig. S2C) and a complete absence of pollen tubes in the carpel walls (Fig. S2D).

Pollination and reproductive success across sites

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

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

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

Discussion

The results demonstrate that highly variable rates of pollination success experienced by small populations of *G. pneumonanthe* occupying restricted habitat fragments are not directly correlated with the degree of 'senility' or age-state characteristics of populations (i.e. senility does not occur because of pollen limitation). This is in agreement with the observation that recruitment may effectively become disconnected from annual reproductive effort because long-lived individuals persist over decades as the vegetation 'successes' around them, restricting the gaps required for seedling establishment (Oostermeijer et al. 1996). In the present study, several sites with small populations represented remnants of historic heathland that are now so transformed that they essentially represent a woodland understorey composed mainly of *M. caerulea* hosting a few reproductive adults of *G. pneumonanthe* (e.g. site 4; Table S1). Both recruitment (seedlings) and pollination were absent at these sites and this is a clear sign of local extinction debt (i.e. a time-lag between the effective extinction and when the last individual actually dies). However, for some small populations the extinction debt was less obvious: these exhibited 'normal' age state distributions and appeared to be in demographic good health in appropriate habitat (e.g. the 16 plants at 'Pineta'; Site 8) but reproduction was not as effective as larger populations. Indeed, pollination success exhibited a sharp and significant decline below 7 flowering individuals, and a decline in ovule fertilization rate and germination capacity was evident in populations of less than 42 reproductive adults. This suggests that for small populations pollination was sporadic (despite the fact that autogamy is possible for this species it is apparently not a reliable mechanism) and pollen availability was insufficient to support high fertilization rates – a clear threshold for pollen limitation. While this generally confirms a similar suggestion by Oostermeijer et al. (1998) based on ovule/seed set counts across populations, we can add that our direct observation of pollination revealed that the relationship is not linear (the closest fit being an exponential rise to maximum relationship; Fig. 2) and thus a tipping point is evident which represents a threshold for severe reproductive limitation.

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

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

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

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

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

Supplementary material

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

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

Variables	Population size	Demographic ratio (R_O)	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_O)	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**

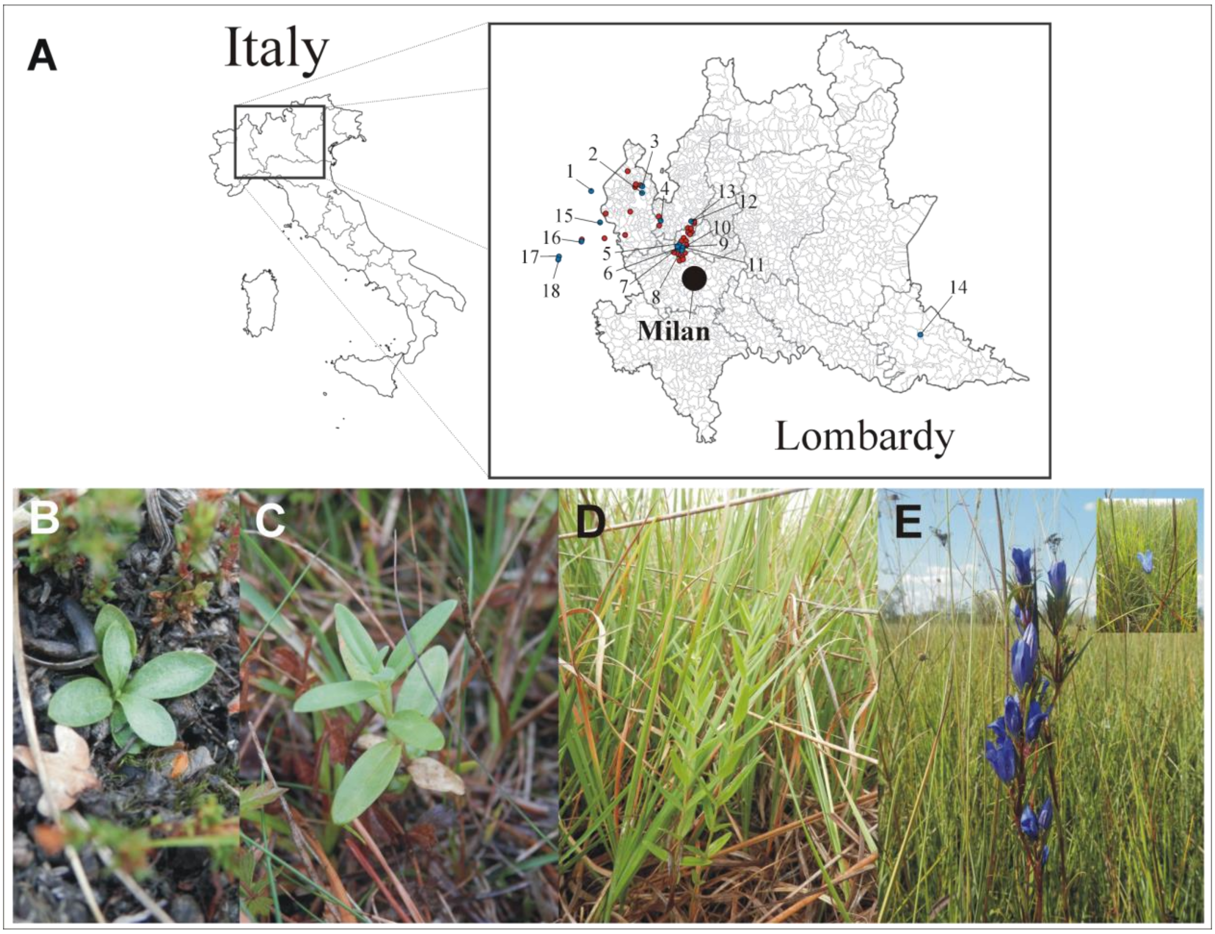

Fig. 1 *Gentiana pneumonanthe* populations investigated during the study and an overview of age state categories. A). location of study sites in northern Italy (mainly in the Lombardy region, but some sites in neighbouring Piedmont). Blue points represent sites from which flower and seed material of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

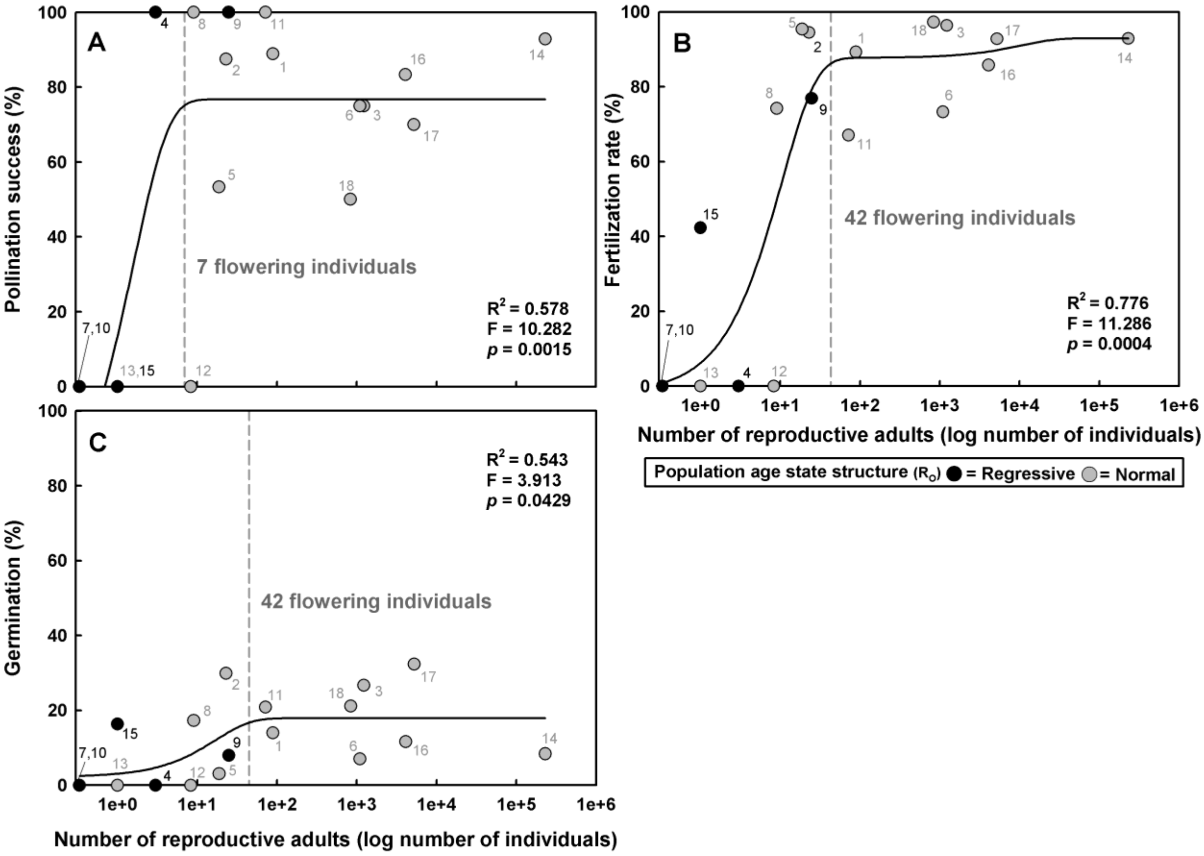

Fig. 2 The relationship between reproductive success (A, pollination success; B, fertilization rate; C, germination capacity *in vitro*) and population size (log axis) for a wide range of contrasting populations of *Gentiana pneumonanthe* from northern Italy (code numbers represent populations listed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

Attachment to Manuscript

Pierce - Pollinator observation sub-study.pdf