# Connectivity, habitat heterogeneity, and population persistence in *Ranunculus nodiflorus*, an endangered species in France

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## Summary

• Here, we explore the role of habitat spatial structure in the maintenance of metapopulations of *Ranunculus nodiflorus*. This rare species grows in puddles that can be connected occasionally by flooded corridors.
• We monitored five locations in the Fontainebleau forest, France, since 2002 and recorded the presence of corridors among puddles and evaluated their impact on puddle demography and plant fitness.
• We showed that connections increased population size, by increasing both the number of puddles occupied by the species and the density of individuals within puddles, but seemed to have no direct influence on plant fitness. We found no evidence of a large persistent soil seed bank.
• Natural corridors are likely to decrease the extinction probability of the populations, most probably by allowing recolonization of empty puddles after extinctions. Therefore, the preservation of corridors appears crucial for the conservation of *R. nodiflorus* in its natural habitat.

## Introduction

Habitat spatial structure is an essential component affecting the dynamics and persistence of species living in fragmented landscapes (Legendre & Fortin, 1989). Metapopulation models have been developed to account for such structure in populations that are discontinuous because of patchy and heterogeneous habitat (Hanski & Simberloff, 1997). These models show that metapopulation dynamics depend on the demography of each subunit (patch) and on the migration among patches (Levin, 1969). Hence, habitat fragmentation, which increases habitat structure, may strongly affect the persistence of a metapopulation over time, by (1) decreasing habitat size, therefore population size and (2) increasing isolation via decreased migration rates among patches (Young *et al.*, 1996; Thomas *et al.*, 2001). Several theoretical approaches

have confirmed this influence of habitat fragmentation by demonstrating that metapopulation persistence is governed by the number of available connected patches. From these results, Hanski *et al.* (1996) and Hanski (1997) developed the concepts of Minimum Viable Metapopulation (MVM) and Minimum Amount of Suitable Habitat (MASH). However, very few field studies have assessed how the viability of a metapopulation is actually affected by (spatial or functional) connectivity (Grasman & HilleRisLambers, 1997) or habitat characteristics (Wahlberg *et al.*, 2002; Murphy & Lovett-Doust, 2004).

Among the factors influencing metapopulation viability, migration is of central interest in conservation biology because (1) it can easily be artificially increased and (2) increasing migration is likely to decrease the extinction probability of the whole metapopulation. Migration among patches decreases

demographic and environmental stochasticity by increasing the effective size of a patch and by enabling (re)colonization of empty patches (Brown & Kodric-Brown, 1977; Fahrig & Merriam, 1985). Migration also favours the maintenance of higher levels of genetic variability via increased local effective population sizes and decreased genetic drift (Mills & Allendorf, 1996). Hence, restoring or increasing migration among small populations can improve their mean fitness, which is otherwise expected to be low because of strong genetic drift leading to loss of genetic variability and fixation or accumulation of deleterious alleles (Barrett & Kohn, 1991; Luijten *et al.*, 2000; Higgins & Lynch, 2001). Finally, migration decreases kinship among individuals within a patch and lowers biparental inbreeding depression (Richards, 2000).

To favour migration among populations in fragmented landscapes, many conservation programs promote the use of biological corridors (Simberloff *et al.*, 1992; Haddad & Baum, 1999). Corridors are spatial structures allowing movements of individuals (plants or animals) among the habitat patches. Most studies exploring the impact of corridors on population persistence concern animal populations (Saccheri *et al.*, 1998; Aars & Ims, 1999; Haddad, 1999). These studies show that corridors increase the mean fitness of the populations (Boudjemadi *et al.*, 1999) and prevent metapopulation extinctions (Mech & Hallett, 2001) by enhancing migration among patches. By contrast, the impact of corridors or connections on plant population viability has been little investigated. Tewksbury *et al.* (2002) recently demonstrated that corridors facilitating animal movements could also promote seed dispersal; other studies investigated the role of water as a vector of seed migration among patches (Johansson *et al.*, 1996; Kirchner *et al.*, 2003). However, the effect of corridors and increased migration on the persistence of these plant metapopulations was not tested.

In plant populations, migration can also occur through time, via dormant seeds remaining in the soil for several generations. Hence, soil seed banks are another factor that may influence metapopulation dynamics and persistence. Seed banks are known to decrease demographic stochasticity and genetic drift in small populations. In populations with limited dispersal ability, this opportunity for 'temporal migration' may lessen the genetic depreciation (Levin, 1990; Kalisz *et al.*, 1997) and decrease the extinction probability.

In this study, we address the role of corridors in the maintenance of a metapopulation by studying how corridors influence population size and individual fitness in small populations of the rare and endangered species *Ranunculus nodiflorus*. This small *Ranunculaceae* lives in puddles that occur on sandstone formations in the Fontainebleau Forest, France (Arnal, 1996). The habitat is therefore naturally fragmented and strongly constrains the size of the populations. Water levels are highly variable, so that puddles can be transitorily connected, after heavy rainfall, through natural water flows considered as flooding corridors.

By collecting data on the number and size of puddles, the number of empty puddles, and the connectivity among puddles, we were able to explore the influence of spatial and environmental characteristics of the landscape on the demography of the species and, for a single year, on the events of recolonization of empty puddles. We focus here on the influence of corridors on the reproductive success of individuals and on metapopulation demography but we also searched for a possible seed bank to explain their maintenance. We estimated population viability by analysing, in every puddle, vegetative and reproductive traits presumably correlated with individual fitness. Finally, we propose management measures that could favour the maintenance of the species in its natural habitat in the Parisian region.

## Materials and methods

### Description of the metapopulation

**Study species** *Ranunculus nodiflorus* L. (*Ranunculaceae*) is a rare and endangered annual plant living in wet zones in Spain, Portugal and France. It has undergone a strong decline during the last century caused by the drainage of wetlands and the regression of grazing (Danton & Baffray, 1995) and it appears on the French Red List of threatened species (Olivier *et al.*, 1995). The species has very strict habitat requirements, growing only in puddles with thick soil and highly variable water levels.

It displays small (5 mm) odourless yellow flowers on which insects have never been observed during field work (F. Noel, pers obs). According to Kirchner *et al.* (2003), it reproduces mainly by selfing. Plants produce small oval achenes (2 mm long) with a tiny tail. These achenes contain up to 14 seeds and are able to float (Kirchner *et al.*, 2003). Some of the seeds germinate in autumn and others in spring (F. Noel, pers obs, and see below). Flowering and seed production occur between April and May. The plants die rapidly after the last achene is ripe. As with all annual species, *R. nodiflorus* is unable to reproduce vegetatively.

**Study area** The study was carried out in the Fontainebleau forest, 50 km south of Paris, in five sites where *R. nodiflorus* had previously been observed (Fig. 1): Coquibus (site 1), Meun (site 2), Couleuvreux (site 3), De Oliviera (site 4) and Belle-Croix (site 5). Each site is a 'platière', characterized by a sandstone ground and the occurrence of temporary puddles during rainy periods. Water levels, which depend chiefly on the rainfall intensity, are highly variable and puddles can transitorily be connected by water corridors. Two puddles were considered as connected when flooding corridors running between them were observed at least once during the study. We visited the study sites frequently enough (once a month) to be confident that all (potential) corridors were identified. Other vegetation (mostly heather, *Calluna vulgaris*) is rather
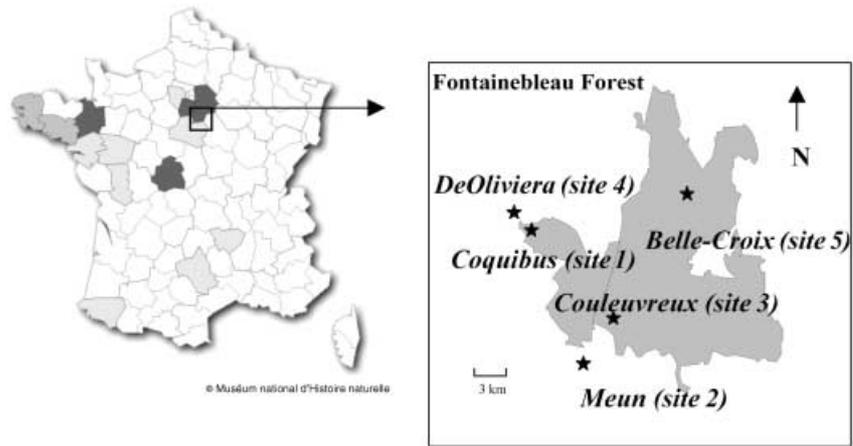
**Fig. 1** Study area in the Fontainebleau Forest (south-east of Paris, France) and map of the five study sites.

low, but birch trees (*Betula pendula*) and pines (*Pinus sylvestris*) tend to invade the sites between clearings managed by the French National Forest Office (ONF). The total surface of each 'platière' is *c.* 148 m² in site 1, 525 m² in the site 2, 4 m² in site 3, 0.5 m² in site 4 and 32 m² in site 5. The two most distant sites (2 and 5) are located 17.5 km apart (Fig. 1).

### Demographic data

The five sites were monitored during two consecutive seasons (from October to June): 2002–03 and 2003–04.

In 2002–03, we recorded *c.* 200 puddles in the five sites. At that time, *R. nodiflorus* was observed in 61 puddles over the five sites (see Fig. 2 for details of site 1 and 2). A puddle is defined either by the presence of water or by the presence of *R. nodiflorus* at the time of the study. Hence, we likely underestimated the number of 'empty' puddles (i.e. puddles not containing *R. nodiflorus*), because we do not account for 'dry' (no water) empty puddles that may be watered during particularly wet years.

A permanent quadrat (30 cm × 30 cm) was placed in each of the 61 occupied puddles: 28 in site 1, 28 in site 2, one in site 3, one in site 4 and three in site 5. From autumn 2002 to spring 2003, the number of individuals ($N_{ind}$) within each quadrat was monitored monthly. We also recorded the plant phenology, as juvenile (J) or adult bearing flowers and/or fruits (A) and the maximum plant density observed during the study period in each quadrat (referred to as 'plant density'). We define a network as a single isolated puddle (occupied, or not, by the species) or as a set of puddles with each at least one potential connection to another puddle (i.e. a potential water corridor, Fig. 2). Thus, in the terminology of metapopulation dynamics, a puddle corresponds to a patch and a network is a metapopulation.

In 2003–04, we explored the five sites to record newly colonized puddles (in which additional quadrats were placed). We also recorded extinction events in the puddles that were occupied in 2002–03.

### Environmental data

For each quadrat that was monitored in 2002–03, we also recorded environmental variables monthly: water depth (WDepth), presence (connect) and number of potential corridors converging to the puddle (Ncorr), sunlight intensity (Sun = high, low and medium) describing canopy cover, ground vegetation cover (Veget = high, low and medium density), and evidence of disturbance such as animal or human tracks (Tracks = 0 or 1). Soil depth was measured in spring 2004 and soil samples were collected near each quadrat; we evaluated the texture (sandy, sandy–organic, organic–sandy and organic) and measured the colour (by picture analysis with the software PIXIE 2.0, http://www.nattyware.com/pixie.html). The water pH was also measured with a portable pH meter (once during the study). Finally, daily records of rainfall and monthly records of temperature in the Fontainebleau forest were obtained from the French meteorological institute (Météo France).

In addition to quadrat observation, we performed a detailed mapping of site 1 in February 2004 (when water levels were high), using a high-precision tacheometer system (1 mm accuracy), to gain precision on the local distribution of the species. Other sites were not considered due to time constraints. Every puddle, within the site 1, was mapped (*n* = 151) and the presence or absence of *R. nodiflorus* was recorded. We measured the length and width of each puddle, its water depth (WDepth) and pH (in wet puddles only). We also recorded the connectivity (connected or isolated), the number of connections (Ncorr) and, for connected puddles, the status of the neighbouring puddles (connected to occupied or connected to empty). When possible, we counted the total number of individuals. For large populations (*n* > 200), we counted individuals in a fraction of the puddle and extrapolated to the whole surface. We then calculated the surface of the puddles and the population density (number of individuals per m²). This measurement of population density is more informative than density measured in the quadrats, because it includes the whole population within each puddle.
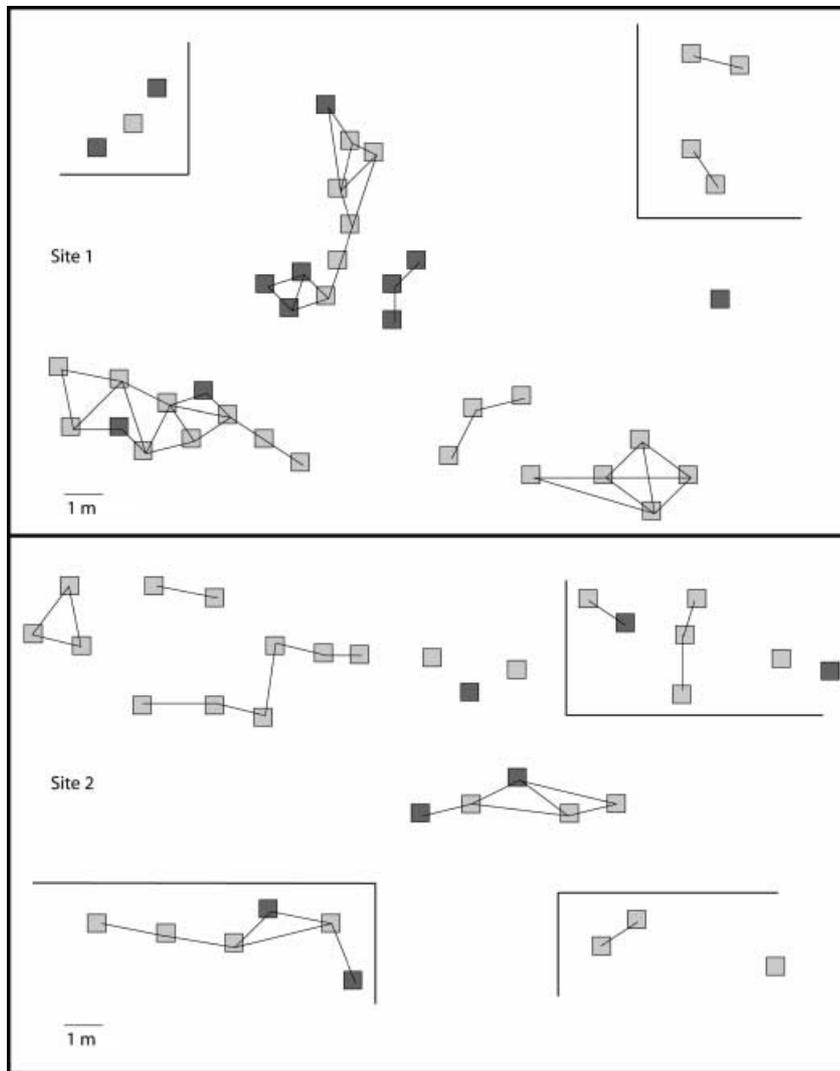
**Fig. 2** Schematic representation of the experimental field design (*in situ*) and the different networks for sites 1 and 2. Grey squares delimit quadrats placed in 2002–03 and dark squares delimit the additional quadrats placed in 2003–04. Black lines represent the temporary water corridors. Sites 3, 4 and 5 are not represented here because they contain only isolated quadrats (1, 1 and 3 quadrats, respectively). Real distances are not respected for clarity of the figure.

## Vegetative and reproductive data

To evaluate the fitness components of plants, we measured vegetative and reproductive traits of four plants per quadrat during the 2003 flowering season (measures were performed on individuals from the 2002–03 quadrats). We systematically examined the four plants located at each corner of a quadrat. Measures were performed on May 28 and May 30 on adult plants. For each plant, we measured the maximum height (Hind), the stem diameter at the soil level (Diam), the number of leaves (Nleav), the number of buds (Nbud), the number of flowers (Nflw), the number of achenes (Nak), and the number of seeds per achene (Ns/a). We also calculated the mean number of seeds per achene (s/a) and per individual (s/I). We collected one achene per quadrat on average and the resulting seeds were used to assess puddle quality, as described later.

## Germination tests

To study the ability of *R. nodiflorus* to form a soil seed bank, we performed two germination tests in experimental garden.
1 In May 2003, 375 seeds were collected directly on 47 plants from 3 sites. They were bulked and sown in June 2003 in pots with compost (25% of Fontainebleau sand and 75% of mould) and placed in an experimental garden. The germination percentage was monitored for 5 wk. The experimentation stopped after 5 wk because of a problem with watering.
2 In April 2004, after flowering but before seed production, soil was sampled in 57 of the 2002–03 quadrats (= all quadrats but four, where soil had been intensively turned over by wild boars). The soil samples were collected with a cylindrical sampler (diameter 8.5 cm) and covered the whole soil depth (a few centimetres). They were placed in an incubator with a 12 h day/12 h night photoperiod, 10°C night and 15°C day

and turned over each week. Our aim was to detect a possible soil seed bank by monitoring the number of seeds that were produced before 2003, did not germinate in 2004, but were still viable. These soil samples were monitored for 17 wk.

We also performed *in situ* germination tests in site 1, to assess the quality of puddles where *R. nodiflorus* was not observed between 2001 and 2004 ('empty puddles'). We measured water pH in four empty puddles, i.e. two puddles in each of two empty networks. In each puddle, we sowed 50 seeds (a pool of seeds collected from the same site the year before) and mapped them with a high-precision tacheometer (1 mm accuracy) geo-referenced with a global positioning system (GPS).

### Life cycle of the species

The timing of germination influences population persistence and is likely governed by joint genetic and environmental effects. To characterize the environmental variability within and among sites and specify the life cycle of the species, we studied the spatio-temporal patterns of seedling emergence in *R. nodiflorus*. For that purpose, we calculated coefficients of aggregation, CA, on the 2002–03 demographic data that were recorded monthly. This coefficient is defined as $CA = \sigma^2/X$, where $\sigma^2$ and X are the variance and mean of the number of seedlings per quadrat, respectively (Apparicio, 2000; González-Astorga & Núñez-Farfán, 2000). A CA of 1 indicates random distribution across space or time; larger values indicate aggregation. We calculated one CA per date for the spatial analysis, by computing the mean and variance over all quadrats at a given date, and one CA per quadrat for the temporal analysis, by computing the mean and variance over all dates in a given quadrat. Deviations of the CA from 1 were tested by means of a *t*-test.

### Statistical analysis

Statistical analyses were performed with JMP.5.0.1.2 (ANOVA, Wilcoxon test, Kruskal–Wallis test and correlations; SAS Institute, Cary, NC, USA, 2003) or with the freeware R 1.8.0 (mixed-model, R, a language and environment, 2003). To study correlations, we generally used Pearson's correlation, except when data were not normally distributed, in which case nonparametric statistics, such as Spearman's correlation coefficient, are more appropriate.

**Population occupancy and population size** To explain the presence/absence data of the species on site 1 (February 2004 data), we tested several correlation between environmental variables (pH or puddle size) or connectivity and the presence/absence data. We also tested whether connectivity was correlated with any of the following environmental variables; water depth, water pH or puddle size. Finally, within occupied puddles, we tested the effect of connections or number of connections on log-transformed plant density using a

mixed model analysis of covariance with puddle size and water depth as covariates:

$$Y_{ijk} = \mu + connect_i + Puddle_j + \beta(size_{ijk} - \bar{s}) + \gamma(Wdepth_{ijk} - \bar{W}) + R_{ijk} \qquad \text{Eqn 1}$$

$$Y_{ijk} = \mu + Ncorr_i + Puddle_j + \beta(size_{ijk} - \bar{s}) + \gamma(Wdepth_{ijk} - \bar{W}) + R_{ijk} \qquad \text{Eqn 2}$$

($Y$ is the population density (number of plants per m$^2$); $\mu$ is the mean population density over all puddles; *connect* is the connection status of a puddle (connected or isolated; $i = 1, 2$); *Ncorr* is the number of connections converging to the puddles ($i = 0, 1$ or $2$) in the February 2004 record; *Puddle* is an individual puddle effect ($k = 1 \ldots 41$); *size* is the puddle size (m$^2$), with mean $\bar{s}$; *WDepth* is the water depth, with mean $\bar{W}$; $\beta$ and $\gamma$ are constants; $R$ is an error term). *Puddle* was regarded here as random because the experimentation was limited to a subsample of all puddles (site 1).

This analysis to detect the effect of connections on population dynamics was conducted on population density rather than total population size, because the latter is primarily constrained by puddle size. Nevertheless, we separately tested the correlation between the total number of individuals and puddle size, both for all occupied puddles and within connected or isolated puddles.

**Spatial structure of morphological traits** To analyse the spatial structure of morphological traits (measured in 2003), we tested the effects of site, quadrat and network using a nested ANOVA. All traits were log-transformed to meet normality requirements. The model was:

$$Y_{ijkl} = \mu + Site_i + Net(Site)_{ij} + Quad(Net(Site))_{ijk} + R_{ijkl}$$

($Y$ is the log-transformed value of a morphological trait (Nleav, Nflw, Nbud, Nak, Ddiam, Hind, or s/I); $\mu$ is the mean value of the trait; *Site* is the site effect ($i = 1 \ldots 5$; 5 sites under study); *Net* is the network effect ($j = 1 \ldots 12$; up to 12 networks per site); *Quad* is the quadrat effect ($k = 1 \ldots 9$; up to nine quadrats per network); $R$ is an error term). All effects and their interactions were considered random. The site effect was tested using the network mean square as the residual and the network effect was tested using the quadrat mean square as the residual.

**Influence of connections on plant fitness** We evaluated the impact of connections among puddles on individual fitness (2002–03 data) using an analysis of covariance with the model:

$$Y_{ijk} = \mu + connect_i + Site_j + \alpha(Hind_{ijk} - \bar{H}) + R_{ijk}.$$

($Y$ is the value of individual fitness traits (s/I, Nak); Hind is a covariate, corresponding to plant height; $\alpha$ is a constant; other terms have already been defined).
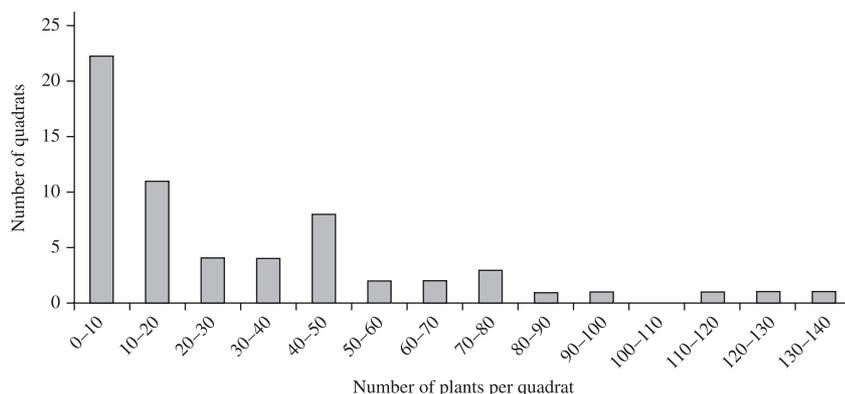
**Fig. 3** Distribution of *Ranunculus nodiflorus* flowering plant density in the quadrats.

**Table 1** Environmental variability over all sites and within sites containing more than one quadrat (sites 1, 2 and 3; 2002–03 data)

| Variables | All sites (n = 61) Mean | SD | Site 1 (n = 28) Mean | SD | Site 2 (n = 28) Mean | SD | Site 5 (n = 3) Mean | SD |
|---|---|---|---|---|---|---|---|---|
| Maximum water level (cm) | 3.12 | 3.18 | 2.16 | 3.12 | 4.56 | 3.62 | 1.93 | 0.67 |
| Total water level (cm) | 4.22 | 4.98 | 2.65 | 5.01 | 6.66 | 5.52 | 1.93 | 0.80 |
| Average immersion time (d) | 20.29 | 19.85 | 13.19 | 22.51 | 25.71 | 11.17 | 23.33 | 10.29 |
|  | Low | Medium | High | Low | Medium | High | Low | Medium | High |
| Vegetation coverage | 36.8 | 51.5 | 11.7 | 40 | 48 | 12 | 36 | 52 | 12 | 0 | 0 | 100 |
| Sunlight intensity | 5.6 | 17.9 | 76.5 | 4 | 24 | 72 | 4 | 24 | 72 | 0 | 33 | 67 |
|  | Yes | No | ND | Yes | No | ND | Yes | No | ND | Yes | No | ND |
| Tracks | 54.6 | 30.6 | 14.8 | 60 | 24 | 16 | 48 | 28 | 24 | 100 | 0 | 0 |

For each site, the number of quadrats is indicated in parentheses. Total water level is the sum of water levels observed over the year and average immersion time is the mean number of days when puddles are submerged. For vegetation coverage, sunlight intensity, and tracks, values are percentages of the total number of quadrats.

## Results

In 2002–03, we monitored the populations of *R. nodiflorus* monthly using 61 permanent quadrats. The number of corridors potentially connecting the quadrats to one another varied between zero (completely isolated quadrats) and four. Quadrats were thus grouped into 24 occupied networks (see Fig. 2 for details of site 1 and 2). The five sites consisted of 1–12 networks, containing 1–9 quadrats and ranging from 0.5 m$^2$ to 72 m$^2$. Over all sites, 7.5% of quadrats were not connected, 31% had only one connection, 40% had two, 7.5% had three and 14% had four connections.

### Distribution of plants and environmental effects

**Heterogeneous distribution of plants** The species is not uniformly distributed among puddles in the sites. The number of individuals was highly variable among quadrats (Student test on the spatial coefficient of aggregation, $t = 3.16$, $P = 0.008$), with total number of individuals per quadrat varying from 2 to 197 seedlings and 138 flowering plants. The distribution of density of *R. nodiflorus* was L-shaped, with a majority of quadrats exhibiting low densities (< 20 plants per quadrat) and a few quadrats with high densities (> 100 plants) (Fig. 3). Plant density was higher, on average, in the largest sites (sites 1 and 2) (one-way ANOVA, $F = 4.31$, $P = 0.0047$).

Table 1 presents the mean and standard deviation, or the distribution, of environmental variables over all sites and within sites 1, 2 and 5. In site 1 only ('Coquibus'), we explored all the relationships between the environmental variables (including connectivity) and the presence/absence or maximum density of the species in the puddles. We detail these correlations later.

**Influence of pH on plant distribution** *Ranunculus nodiflorus* seemed to be unable to germinate and/or grow in too acidic puddles (pH < 4.5), as shown by a strong positive relationship between pH and the occupancy status of a puddle in site 1 (logistic fit, $P < 0.0001$). The average pH of occupied puddles was 5.85 (ranging from 4.5 to 7.2, with a standard deviation of 0.75) and the average pH of empty puddles was 4.8 (ranging from 3.5 to 7.3, with a standard deviation of 0.74); the difference is highly significant (one-way Wilcoxon test, $\chi^2 = 32.07$, $P < 0.0001$). The pH requirement of *R. nodiflorus* is partly confirmed by a germination test: none of the 200

**Table 2** Characteristics of puddles in site 1 (Coquibus, February 2004 data)

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Occupied puddles | 43 | Unknown pH | 18 | Isolated | 5 | | |
| | | | | Connected | 13 | | |
| | | pH < 4.5 | 0 | | | | |
| | | pH ≥ 4.5 | 25 | Isolated | 3 | | |
| | | | | Connected | 22 | Connected to occupied | 19 |
| | | | | | | Connected to empty | 3 |
| Empty puddles | 108 | pH ≥ 4.5 | 59 | Connected | 29 | Connected to empty | 26 |
| | | | | | | Connected to occupied | 3 |
| | | | | Isolated | 30 | | |
| | | pH < 4.5 | 44 | Connected | 33 | | |
| | | | | Isolated | 11 | | |
| | | Unknown pH | 5 | Connected | 3 | | |
| | | | | Isolated | 2 | | |

The water pH is unknown when puddles are dry. Because 'dry' puddles are difficult to detect when they do not contain *Ranunculus nodiflorus*, the number of dry empty puddles is likely underestimated.

**Table 3** Analysis of covariance on *Ranunculus nodiflorus* plant density in site 1 (February 2004 data)

| A. Effect of connectivity on plant density Effects | *F* | *P* > *F* | B. Effect of number of connections on plant density Effects | *F* | *P* > *F* |
|---|---|---|---|---|---|
| Connect | 13.2 | **0.0008** | Ncorr | 6.69 | **0.0034** |
| Puddle surface | 0.58 | 0.45 | Puddle surface | 0.57 | 0.45 |
| Water depth | 0.03 | 0.86 | Water depth | 0.02 | 0.89 |

Traits and effects: Connect, connection status (connected or isolated); Ncorr, number of connections converging to the puddles (0, 1 or 2). In bold: significant effects (*P* value < 0.05).

seeds sown in the empty puddles of empty networks, with pH ranging from 4 to 4.5, germinated. Because *R. nodiflorus* is a protected species, we were not allowed to perform this kind of experiment as a control in occupied puddles or in empty puddles with a presumably favourable pH, that were all located next to occupied puddles. Empty puddles were classified into 'suitable' (pH > 4.5) or 'unsuitable' (pH < 4.5) for *R. nodiflorus* growth. In the following text we only consider suitable puddles.

**Influence of connectivity on plant distribution**  Table 2 presents the occupancy and connectivity status of puddles at site 1 (February 2004 data). Empty puddles were more numerous than occupied ones. Puddles with unknown pH correspond to puddles that were dry when water pH was measured. These puddles are not considered in the following because their connection status was less readily estimated (for example, when puddles are frequently dry, possible connections with other dry empty puddles are less detectable and network description is more difficult). Among puddles of know pH, isolated puddles were generally empty and networks tended to be either completely empty or (almost) completely occupied. The percentage of empty puddles is higher among isolated puddles than among connected ones ($\chi^2$ = 11.609, $P$ = 0.0007). Among the 25 wet occupied puddles, three (12%) were isolated in February 2004; the others were parts of networks.

Among suitable puddles, the plants were more likely found in connected puddles. In networks containing plants, all puddles but three were occupied. In addition, the density of *R. nodiflorus* was significantly larger in connected puddles than in isolated ones (ANCOVA on plant density, significant connection effect, $F_{1,37}$ = 13.2, $P$ = 0.0008) (Table 3). Finally, we detected a strong positive correlation between total number of individuals and puddle size (linear fit, $r$ = 0.28, $P$ = 0.0002). Note, however, that this correlation is only significant in connected puddles (linear fit, $r$ = 0.52, $P$ < 0.0001 in connected puddles, $r$ = 0.012, $P$ = 0.78 in isolated puddles).

In contrast, the number of connections converging to a given puddle had no influence on plant density. In an analysis of covariance, the number of connections had a globally significant effect on plant density ($F_{1,36}$ = 6.69, $P$ = 0.0034, Table 3), but only the difference between zero and one connection was significant ($t$ = 3.39, $P$ = 0.0017), not the difference between one and two connections ($t$ = 3.17, $P$ = 0.53). Hence, in terms of plant density, it seems more important for a population to belong to a network than to be connected to a large number of puddles.

This effect of connectivity on plant density and distribution could not be attributable to a correlation between connectivity and some of the environmental factors considered in this study. Because connectivity occurs via water corridors, a
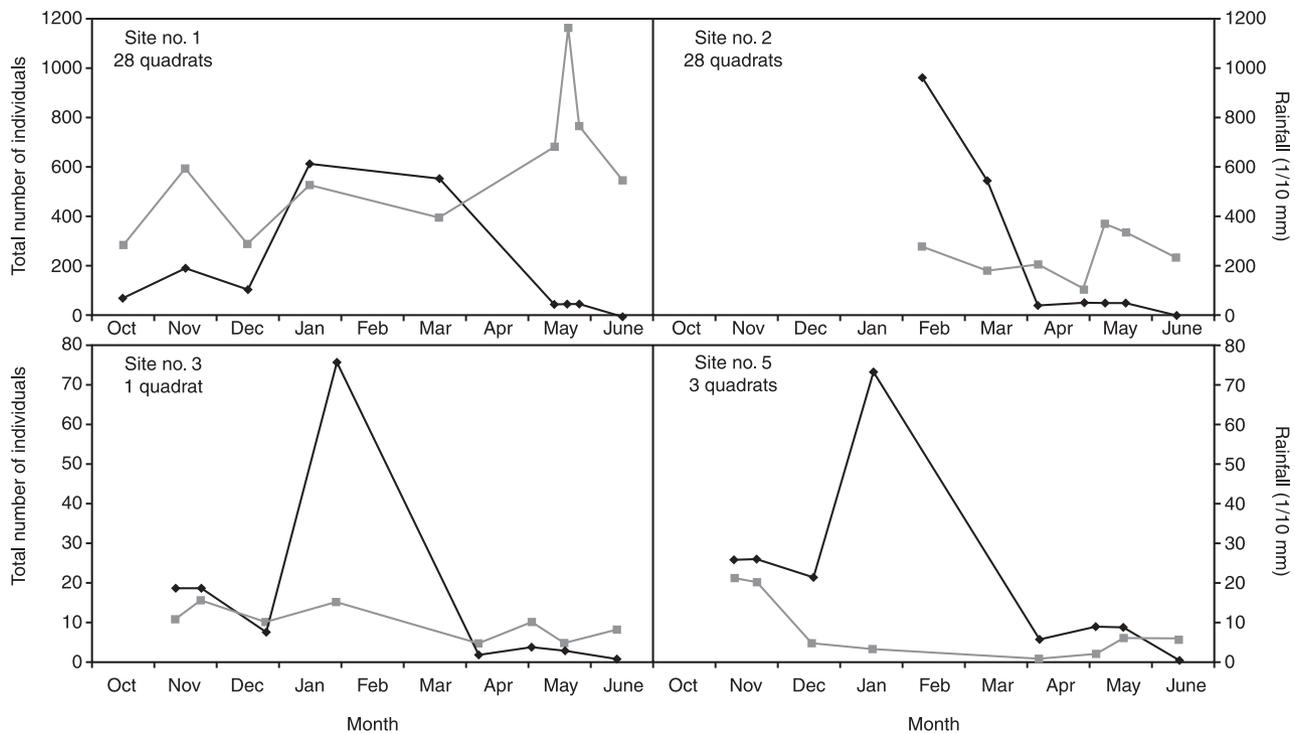
**Fig. 4** Dynamics of rainfall (black line) and *Ranunculus nodiflorus* population size (grey line) in each site. Note that the scale of the *y*-axis varies across sites.

correlation is expected between connectivity and water depth, so that higher plant densities could in fact be due to high water levels, not to higher connectivity levels. However, no such correlation between water depth and connectivity was found (One-way test, $\chi^2 = 0.11$, $P = 0.74$) and water depth had no significant effect on plant density (one-way Anova, $F = 0.48$, $P = 0.49$). Similarly, water pH had no significant effect on plant density within occupied puddles (one-way ANOVA, $F = 0.007$, $P = 0.93$) and was not correlated with connection status: the pH of isolated puddles was not significantly different from that of connected puddles (one-way Wilcoxon test, $\chi^2 = 0.5$, $P = 0.47$). In addition, within networks, the water pH of puddles connected to empty puddles was not significantly different from that of puddles connected to occupied puddles (One-way Wilcoxon test, $\chi^2 = 0.003$, $P = 0.95$) (See Table 2). Finally, puddle size had no significant effect on plant density and was not significantly different between occupied and empty puddles ($\chi^2 = 2.19$, $P = 0.13$) or between connected and isolated puddles ($\chi^2 = 0.59$, $P = 0.44$).

Between 2003 and 2004, the average population size within quadrats increased in all sites (one-way Anova, $F = 14.77$, $P = 0.0002$). The number of occupied puddles also increased in sites 1 and 2 (from 28 to 40 and from 28 to 34 puddles, respectively). Thus, we placed 12 additional quadrats in site 1 (9/29 (= 31%) in connected puddles and 3/30 (= 10%) in isolated puddles) and seven additional quadrats in site 2 (5/12 (= 42%) in connected puddles, 2/15 (= 13%) in isolated puddles) (see Fig. 2). In site 1, we also recorded the colonization

of a new network (three new connected puddles apparently not connected to others). Hence, although the differences are not significant, there is a tendency for a higher rate of recolonization in connected than in isolated puddles. Among the total number of quadrats placed in 2003, we observed a single extinction: in site 2, one isolated quadrat occupied in 2002–03 was found empty in 2004.

**Other environmental factors** Analyses on all sites pooled together (2002–03 data) showed no significant relationship between other environmental variables (soil depth, Sun, Veget, Tracks, soil texture) and the log-transformed maximum density of plants in the quadrats.

### Demographic data and life cycle of the species

In 2002–03, we observed a significant temporal variation in the number of individuals per quadrat (Student test on the temporal coefficient of aggregation: $t = 3.36$, $P = 0.001$). Figure 4, reporting the dynamics of the number of plants from October 2002 to June 2003, shows two distinct germination periods, which is consistent with this temporal aggregation. The first period of germinations occurred in autumn and winter 2002; the resulting plants remained at the seedling stage until spring, when a second period of germinations occurred. Between March and May 2003, the seedlings grew, flowered and produced achenes. In site 1, the maximum population size occurred in May whereas at sites 3 and 5, it

**Table 4** Analysis of variance on morphological traits of *Ranunculus nodiflorus* measured in the five study sites, in all 61 quadrats (2003 data)

| Variables | Effects Site | | Network (site) | | Quadrat (network, site) | |
|---|---|---|---|---|---|---|
| | F | P > F | F | P > F | F | P > F |
| Nleav | 0.17 | 0.9507 | 1.48 | 0. 1710 | 1.94 | **0.0042** |
| Nflw | 0.27 | 0.8916 | 1.05 | 0.4367 | 1.95 | **0.0073** |
| Nak | 3.11 | **0.0472** | 1.41 | 0.2052 | 1.33 | 0.1525 |
| Hind | 9.04 | **0.0006** | 1.54 | 0.1484 | 3.03 | **< 0.0001** |
| Diam | 0.55 | 0.6578 | 1.14 | 0.3630 | 2.65 | **< 0.0001** |
| s/I | 3.27 | **0.0407** | 1.61 | 0.1412 | 1.11 | 0.3549 |

Traits: Nleav, number of leaves per plant; Nflw, number of flowers per plant; Nak, number of achenes per plant; Hind, plant height; Diam, stem diameter; s/I, number of seeds per plant. In bold: significant effects (*P* value < 0.05).

**Table 5** Analysis of covariance on fitness-related traits of Ranunculus nodiflorus over all sites (2002–03 data)

| Effects | Number of achenes per plant | | Number of seeds per plant | |
|---|---|---|---|---|
| | F | P > F | F | P > F |
| Hind | 54.44 | **< 0.0001** | 13.97 | **0.0003** |
| Site | 3.37 | **0.011** | 0.62 | 0.59 |
| Connect | 0.19 | 0.65 | 1.69 | 0.19 |

Traits and effects: Hind, plant height; Connect, connection status (connected or isolated). In bold: significant effects (*P* value < 0.05).

occurred in November. For sites 2 and 4, we were unable to perform this analysis because of missing data.

Figure 4 also reports rainfall during the same period. Over the whole period, there was a significant positive correlation between the number of individuals and millimetres of rainfall (mm rainfall) for all sites. Within seasons, this correlation was significant in autumn–winter only (Spearman correlation coefficient, $r = 0.52$, $P = 0.0023$ for all sites in one year; $r = 0.73$, $P = 0.0018$ in autumn–winter (September–March); and $r = 0.44$, $P = 0.072$ in spring (April–June)). Moreover, no correlation was observed between the number of individuals and temperature (Spearman correlation coefficient, $r = 0.05$, $P = 0.77$ for all sites on one year; $r = -0.01$, $P = 0.95$ in autumn; and $r = 0.33$, $P = 0.17$ in spring). These results suggest that germinations were favoured by rainfall in autumn, whereas spring germinations tended to occur when the puddles are drying.

### Variability of vegetative and reproductive components of the plants

We analysed the soil characteristics next to 18 puddles in site 1 and tested their impact on the reproductive and vegetative performances of the plants. We found a significant negative relationship (linear fit, $r = -0.43$, $P = 0.015$) between pH and the number of achenes produced by a plant (the number of seeds per achene was not affected by pH). We also observed a positive relationship between soil depth and the number of seeds per achene (Spearman correlation coefficient = 0.56,

$P = 0.034$). No relationship was found between pH and soil depth (linear fit, $r = 0.016$, $P = 0.67$), so that the fitness of *R. nodiflorus* was optimized in acidic puddles with a good soil depth (within the range of pH suitable for the species).

A large fraction of the variation in vegetative and reproductive traits was attributable to differences among quadrats or among sites, as indicated by significant site and/or quadrat effects for most traits (Nleav, Nflw, Nak, Ddiam, Hind, and s/I, Table 4). Surprisingly, we did not observe any significant differences in vegetative and reproductive traits among networks. Finally, the fitness of individuals, measured as seed or achene production, did not depend on connectivity (ANCOVA on reproductive traits), with similar values of total number of seeds and total number of achenes (Ns/I, Nak) in connected and isolated puddles (Table 5).

### Germination tests and seed bank

Of the 375 seeds sown in June 2003, 250 (67%) had germinated in July. The experiment was stopped prematurely after 5 wk, because of an accidental lack of water. However, we can be sure that an appreciable fraction of the seeds are able to germinate just after being produced if they encounter favourable conditions and do not appear to be subject to any dormancy. By contrast, germinations were recorded for 17 wk in the 57 soil samples collected in spring 2004 before seed production. We observed only 13 germinations of *R. nodiflorus,* occurring in eight samples. This suggests that there were few viable seeds left in the soil samples, most probably

because the majority of seeds had germinated earlier. We therefore conclude that this species has little opportunity to build a persistent seed bank in the soil.

## Discussion

We have described the dynamics of a metapopulation of a small and threatened plant in the Parisian region and identified some of the major factors influencing these dynamics; such factors are likely to play a key role in the persistence of the species in its natural environment. We specifically evaluated the role of the water corridors as connections among populations, but we also investigated the effects of other environmental variables. Habitat quality can explain the absence or presence of the species in the temporary puddles (Dupré & Ehrlen, 2002). Our results highlight three main factors affecting the dynamics of *R. nodiflorus* populations: (1) the presence of corridors connecting puddles, influencing spatial migration; (2) the chemical properties of the puddles (soil and water), controlling their suitability for *R. nodiflorus*; and (3) the dispersal of the species in time (seed bank and variation in germination time). We discuss these three factors further in the following text.

### Corridors, connectivity and population dynamics

**Connectivity increases population density** We have shown that *R. nodiflorus* was frequently present in networks and rarely observed in isolated puddles. In addition, the vast majority of networks were either entirely empty or full (except for three puddles that were empty among the occupied puddles of a network). We also demonstrated that, in occupied puddles, plant density was higher in connected than in isolated puddles. In addition, the number of individuals within a puddle is positively correlated with puddle surface within networks, whereas no such relationship is observed in isolated puddles. The number of connected puddles colonized between 2003 and 2004 was greater than the colonization of isolated ones. Thus, the presence (not the number) of corridors seems to enhance the probability of puddles to be filled with plants, by promoting seed dispersal, as described below. The relationship between connectivity and plant density could not be attributed to any of the environmental factors considered in this study (water levels, water pH, soil depth, etc.), so that connectivity seems to be the main factor controlling plant density.

The presence of corridors allowed larger populations than in isolated puddles. Thus, connectivity could act positively on the persistence of populations by limiting extinctions and favouring (re)colonizations. Experimental results on *R. nodiflorus* showed a percentage of colonization for connected puddles (34%) greater than for isolated ones (11%) and a single extinction occurred in an isolated puddle. Thus, the first results collected on the short-term appear to support this hypothesis, but were not significant. Additional observa-

tions are required to confirm the role of corridors on the dynamics of extinction and recolonization of this metapopulation, notably because the signs of a metapopulation collapse are sometimes counterintuitive (Thrall *et al.*, 1998).

Previous experiments have shown that *R. nodiflorus* seeds are able to float for a period of a few weeks (3–4 wk, Kirchner *et al.*, 2003). In addition, seeds are produced in early summer, leaving ample time for dispersal before germination, which occur in autumn or in spring. Therefore, between June and March of the following year, when rainfall raises water levels in puddles, small streams of water connect the puddles and disperse seeds. The direction of the flow may depend on the relative elevation of the puddles but also on the direction of the wind because most sites are relatively flat. Two major hypothesis may explain the impact of corridors on population density: (1) corridors can reduce extinction probabilities by bringing new seeds yearly in connected puddles, therefore limiting demographic stochasticity or decreasing inbreeding depression (see later), whereas isolated puddles probably experience repeated bottleneck events; (2) alternatively, within a network, habitat quality may drive the persistence of populations in favourable puddles, acting as source populations, and corridors allow migration towards sink puddles experiencing lower population sizes or frequent extinctions. These two mechanisms are not mutually exclusive and may operate simultaneously in our metapopulations.

Regarding the role of inbreeding depression or fixation of deleterious mutations in population density, we argue below that seed production of plants is not different in isolated and connected puddles. However, in isolated puddles, genetic drift could have led to the loss of favourable genes affecting seed viability and/or germination ability. Thus, the reproductive success of individuals may be reduced in isolated puddles. This hypothesis could have been tested by examining the percentage of germination of seeds from isolated and connected puddles. Unfortunately, the quantity of seeds was too small to allow such a study.

**Connectivity, migration and spatial structure** Despite the potential role of connections and migration within networks, the analysis of the spatial structure of vegetative and reproductive traits of the plants did not reveal any significant structure at the network level, and variation in traits was mostly explained by variation among quadrats and/or among sites. Spatial structure within a metapopulation is due to genetic and/or environment effects. Using a limited number of isozymes, Kirchner *et al.* (2003) demonstrated that puddles are not genetically differentiated within networks, whereas genetic differentiation among networks is marginally significant. These conflicting observations between neutral genetic markers and potentially selected quantitative traits could be attributable to strong local environmental effects at the quadrat (puddle) level, resulting in either small-scale local adaptation or phenotypic plasticity unaffected, or little affected,

by migration. Substantial environmental variation was in fact observed among quadrats for most variables (Table 1), whereas some variable (e.g. soil composition or insolation) did not exhibit significant differences among networks or among sites, which suggests small-scale environmental variation. To evaluate the respective roles of genetic and environmental effects on the spatial structure of quantitative traits in the metapopulations of *R. nodiflorus*, we intend to use microsatellite markers (to confirm that genetic exchanges do occur within networks and to assess the role of corridors in the migration process) and a common garden experiment is under way to formally show if differences among quadrats have a genetic basis and/or correspond to phenotypic plasticity only.

**Connectivity does not increase mean fitness**  In the analysis of achene and seed production, two traits likely to be associated with plant fitness, showed that populations growing in isolated puddles did not suffer from decreased fitness compared with populations growing in connected puddles. We originally thought that isolated populations of *R. nodiflorus* would suffer from the fixation of deleterious alleles by genetic drift or a stronger inbreeding depression. However, our results did not highlight any alteration of plants in isolated puddles. The presumably highly selfing breeding system of the species could cause a regular and efficient purge of strongly deleterious alleles. Alternatively, the fixation of slightly deleterious alleles, which are not efficiently purged by selfing, could be undetectable on the traits we measured but could be expressed, for example, in the quality of the seeds (see later). The time-scale of our study was probably too small to provide much information on the impact of connectivity on individual fitness. To obtain more accurate evaluations of population viability, long-term studies of size variation, relative fitness (Heschel & Paige, 1995) or joint genetics and demography (Richards *et al.*, 2003) are required.

## Habitat suitability and occupancy

**Influence of pH on habitat suitability**  Analysing the differences between empty puddles and puddles occupied by the species allowed us to identify some components of the biotope of *R. nodiflorus*. Empty puddles had a significantly lower pH than puddles where the species was found. *Ranunculus nodiflorus* therefore appears to have a strict habitat requirement in terms of water pH, which must exceed 4.5 for seed germination to occur. The difference of pH among puddles is likely caused by the presence of calcareous stones brought in when the road running across the 'platière' was built. These stones were dropped in puddles close to the road, which thus exhibit a higher pH. Puddles more distant from the road have a more acidic pH, unless they benefit from a flow of calcareous water (or presence of calcareous nodules). *R. nodiflorus* does not occupy all suitable (pH > 4.5) puddles: 59 of 102 were empty in 2002–03. However, we have detected an increase

in population size between 2003 and 2004. The number of occupied puddles increased; newly occupied puddles were generally connected to already occupied puddles. In site 1, we also observed the colonization of one empty network (three newly occupied puddles). Thus, the species seems to be able to extend to empty suitable puddles, especially to empty connected puddles.

**Extinction threshold**  In a fragmented population, habitat suitability and habitat occupancy allow estimation of the probability of extinction. Eriksson & Kiviniemi (1999) define the 'quasi-equilibrium extinction threshold' of a fragmented population as $h'_c = 1 - s/h$ ($h$ is the fraction of suitable patches and $s/h$ the occupied fraction of suitable patches) if $h/h'_c < 1$, the population is threatened. This definition assumes that a species may persist for a while under unfavourable conditions, via dormant seeds for example, and thus incorporates a delay before extinction when the fraction of suitable habitats decreases. It was therefore more appropriate for our studied metapopulation than the classical extinction threshold ($h_c$), based on equilibrium between colonization and extinction rates.

The data on *R. nodiflorus* yielded $h = 84/128 = 0.65$, $s/h = 25/84 = 0.30$, so that the population is just below the threshold ($h/h'_c = 0.93$) and, according to the authors, does not have a high risk of extinction. This prediction however, strongly depends on our ability to discriminate between empty suitable and unsuitable puddles, and the extinction risk might be higher if some puddles have been incorrectly classified as suitable. In addition, we have likely underestimated the number of 'empty' puddles (i.e. puddles not containing *R. nodiflorus*), because we do not account for 'dry' (no water), empty puddles that may be watered on particularly wet years. This may lead to an overestimation of $h$ and to an additional underestimation of the risk of extinction for the species.

The process of extinction (caused by environmental and demographic stochasticities), although reduced within a network thanks to corridors, may still occur at a low rate. If this rate of extinction were higher than the presumably low rate of recolonization via migration between 'isolated' puddles, the total population (over all sites) would experience a slow decline eventually leading to extinction. Our data do not cover a sufficiently long period to detect such decline, but the present 'empty' networks could have been 'occupied' in the past. Observation of a single year revealed that colonization of empty networks is possible (Fig. 2), but only a thorough monitoring over several decades could confirm a decline or expansion of the metapopulations.

## Temporal dispersal and seed bank

**The soil seed bank of *R. nodiflorus***  Few viable seeds were detected in the soil after the spring episode of germination, which suggests that most viable seeds germinate in the year

following their production. We conclude that *R. nodiflorus* has not developed a major soil seed bank strategy. Note however, that the presence of a soil seed bank may depend on the climatic conditions and can fluctuate over years (Leck, 1996; Gutiérrez & Maserve, 2003). Therefore, it is possible that *R. nodiflorus* usually forms a soil seed bank but that, by chance, environmental conditions favoured the germination of all seeds in 2003. This hypothesis is consistent with the observed increased population sizes in the puddles of 2004.

In annual species, the absence of a seed bank may greatly increase extinction rates (Pavone & Reader, 1982; Levin, 1990; Kalisz *et al.*, 1997; Baskin & Baskin, 1998), especially in patches with small population size such as those observed here, because of increased demographic and environmental stochasticities. However, the production of seeds that germinate simultaneously may enhance the ability of *R. nodiflorus* to compete with other species. This strategy is observed in perennial plants with vegetative and sexual reproduction or in animal species (Winkler & Stocklin, 2002; Arendt & Wilson, 1997). Moreover, autumn germinations that survive the winter can be more vigorous and have a greater fitness than the spring germinations (Arthur *et al.*, 1973).

**The germination dynamics of *R. nodiflorus*** The population dynamics of *R. nodiflorus* are complex, and seem to be connected to climatic variation. First, our results suggest that they partly depend on water conditions and their temporal variation in autumn and spring (whereas results suggest no relation with temperature). We cannot exclude that others factors such as sunlight intensity, vegetation coverage or trampling, which may be correlated with variations in water levels, may also be responsible for the observed dynamics of germination. *Ranunculus nodiflorus* germinates during two periods: in autumn when puddles are filling up and in spring when puddles are drying. Therefore, germination of *R. nodiflorus* seems to require an alternation of wet and dry periods. This requirement may explain why individuals mainly grow on the edge of puddles where variations of water level are larger. Our observations showed that most germination occurred in the drying puddles where the water level had been particularly high, although this observation might simply indicate a better survival or seed set of plants in the preceding generation.

We did not follow seedlings individually, so that we could not establish whether autumn germinations died before spring and were replaced by spring germinations or survived and reproduce. Nevertheless, the population size never dropped to zero between autumn and spring (except in site 5, Fig. 4), which tended to demonstrate that autumn seedlings were able to resist the winter conditions and to continue their life cycle in spring. Regardless of their germination date, all plants flowered during a short period in April–May and produced seeds in May–June

The existence of two periods of germination suggested that the seeds respond to microsite differences or that there are two kinds of seeds in the population: (1) seeds germinating immediately after production, when the biotope is wet and (2) seeds germinating after a cold period (winter), when the biotope is drying. To assess these two hypotheses, experiments are being conducted under common conditions, in the garden of the Conservatoire Botanique at the Museum. However, these two kinds of 'bet-hedging' strategy (Schaffer, 1974) provide the population with opportunities to go successfully through the critical stage of germination. If seedlings are destroyed during winter or if bad climatic conditions prevent seed germination in spring, populations are nevertheless maintained.

## Conclusion

In summary, the presence of *R. nodiflorus* strongly depends on the environmental quality of puddles but large proportions of isolated empty puddles also suggest an additional role of connectivity. Isolated populations do not seem to suffer genetic depreciation, so that differences between isolated and connected puddles are more likely attributable to a higher demographic stochasticity and to smaller probabilities of recolonization after extinction. Differences between connected and isolated puddles are probably exacerbated by the absence of a consistent soil seed bank. Therefore, in view of the positive impact of natural corridors on the population demography, their maintenance appears to be a crucial condition for conservation of the species in Fontainebleau forest. Efficient protection of the sites requires careful management to prevent invasion of corridors by woody vegetation. In addition, population recovery might be greatly facilitated by occasional artificial migration from large populations to empty networks or isolated puddles (i.e. by sowing seeds in the empty puddles) and/or by manipulating the chemical characteristics of the unsuitable (pH < 4.5) puddles.

## Acknowledgements

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