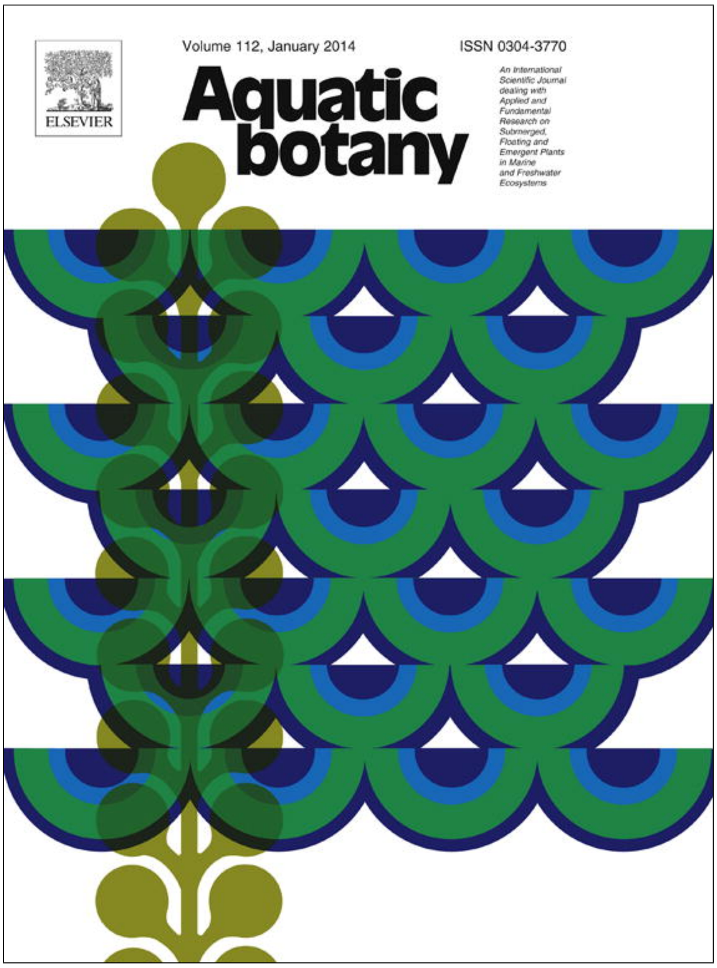
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Effects of hydrology on recruitment of *Pilularia minuta* Durieu (Marsileaceae), an endangered plant of Mediterranean temporary pools

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ABSTRACT

Hydrological requirements for germination and development of *P. minuta* were assessed through a 5-year field survey in Garâa Sejenane, Tunisia, and an experiment under controlled conditions. The spatial distribution of *P. minuta* was recorded in the field annually, while the experiment tested the effects of water levels and flooding dates on germination (the emergence of new individuals) and development. Water level was found to be the major factor affecting the germination and the development of *P. minuta* with flooding date as a secondary limiting factor. For germination to occur, the sediment must be completely waterlogged. Water depths of 5–10 cm are optimal for plant development. In addition, the plant needs a minimal flooding period of seven weeks to develop. Late-spring precipitation appears to be more critical than earlier rains.

1. Introduction

Acidic Mediterranean temporary pools are rich in species (Grillas et al., 2004) that make significant contributions to regional biodiversity while also helping to identify biodiversity hotspots (Blondel and Aronson, 1999; Médail and Quézel, 1999). The ephemeral nature, small size and shallow depth of Mediterranean temporary pools together with the extreme specialization of their plant communities could well make them highly susceptible to anthropogenic degradation, which is probably an important cause of their rapid, continual decline (*e.g.*, Rhazi et al., 2001, 2006; Dimitriou et al., 2006; Zacharias et al., 2007). The seasonal alternation of inundated and dry phases is an important ecological constraint for the flora of temporary wetlands (Santamaría et al., 1996; Grillas et al., 2004; Deil, 2005), most of which are annual, with the exception of the perennial amphibious species of genus *Isoetes*, whose bulbs are resistant to drought and whose underwater photosynthesis is enhanced by the Crassulacean acid metabolism (Pedersen et al., 2011).

In the Mediterranean Basin, hydroclimatic conditions are associated with significant inter-annual variability in rainfall (Bolle, 2003). The general effects of hydrology on the biology of Mediterranean wetland plants are well established (Webb et al., 2012). Flooding date, which combines the effects of several variables – temperature, solar irradiation, length of day – determines the germination rate (Arts and Van der Heijden, 1990; Bliss and Zedler, 1998; Warwick and Brock, 2003). Water depth and the duration of flooding influence the competition between aquatic and amphibious species, as the latter suffer from excessive water (Crosslé and Brock, 2002). Flooding frequency seems to affect neither richness nor biomass, even if the duration of individual flooding events may be important for segregating plant communities (Bornette et al.,

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1994; Casanova and Brock, 2000; Nicol et al., 2003; Drinkard et al., 2011). Lastly, the date and duration of drying appear to be critical for the seed production of certain species – for example, *Elatine brochonii* Clavaud (Rhazi et al., 2007) and *Littorella uniflora* (L.) Asch. (Arts and Van der Heijden, 1990) – but not for others such as *Ranunculus peltatus* Schrank (Volder et al., 1997). Such species-specific differences in adaptation (Rea and Ganf, 1994) could well enhance the community's resilience to hydrological disturbances.

In this paper, we present a study of the hydrological requirements of one of the rarest species of Mediterranean temporary pools (*Pilularia minuta* Durieu, Marsileaceae) whose largest populations are in Garâa Sejenane (northwest Tunisia; Daoud-Bouattour et al., 2009). For the long-term conservation of this species, we investigated how hydrology affects the biology of *P. minuta* using a combination of a field survey and an *ex situ* experiment.

P. minuta is a small, amphibious pteridophyte that only grows in Mediterranean temporary pools (Grillas et al., 2004). Usually considered to be perennial (rhizomatous geophyte or radicant hydrophyte; Prelli, 2001; Grillas et al., 2004), *P. minuta* is, in fact, an annual plant with an underground stem that dies together with the leaves during the dry period (Johnson, 1933; Ferchichi-Ben Jamaa, 2010). As an annual pteridophyte, this species combines short-lived individuals with the survival of spores during the dry phase and delayed fertilization of female gametes by motile antherozoids, triggered by flooding.

We hypothesized that late rainfall events (March–May), occurring during sporocarp maturation, are determinant for the development of *P. minuta* and verified this in the field. We tested the influence of water depth and hydroperiod experimentally on both germination and development. We hypothesized that moderate water depth and early flooding date were likely to control the success of fertilization and would enhance both the recruitment and development of *P. minuta*.

2. Materials and methods

2.1. Studied species

P. minuta Durieu (small pillwort, Marsileaceae) grows exclusively on non-calcareous substrates and is a pioneer light-demanding species with low competitive ability that benefits from disturbance-induced vegetation opening (Daoud-Bouattour et al., 2009). The very short life cycle of this amphibious species begins in very damp or flooded conditions in late February–early March. At this point, it develops creeping, filiform stems buried slightly below the surface, and 1–3 cm-long erect, slender green leaves which remain green at the beginning of the dry phase (April) but dry up and disappear quickly after May–June (Grillas et al., 2004). Sporocarps, the drought-resistant reproductive organs, form at each node in the inundated ground when the plant is submerged, after leaves have developed and are fully mature when drying out. A complete reproductive cycle of *P. minuta* requires a new flooding (Supplementary material 1). In the present paper, the term *germination* refers to the emergence of a new individual (sporophyte) identified by the appearance of the first leaf.

2.2. Field survey

The pool that we studied, Marchar2, is located in the Mogods Hills of northwest Tunisia (37°05′07″ N, 09°12′25″ E; 100 m a.s.l.; Fig. 1), at the eastern edge of Garâa Sejenane. Sheep, cattle, donkeys and mules graze here all year round. The marshy Garâa Sejenane plain of sandy-silty acidic soil includes a mosaic of cultivated/grazed fields, scattered marshes and temporary pools. The climate is Mediterranean humid with irregular rainfall (average of 880 mm year^{-1}) that is greatest during the winter months (Kassab, 1979). As measured in February 2010, the pool's maximal surface reaches an area of 620 m^2 with a maximum depth of 27 cm. The water pH, measured at diverse periods, was comprised between 6 and 7. The vegetation forms three concentric belts (Grillas et al., 2004). *P. minuta* covers about 100 m^2 in the pool's central and intermediate belts, together with *Callitriche brutia* Petagna, *Coronopus squamatus* (Forssk.) Asch., *Eleocharis palustris* (L.) Roem. & Schult., *Eryngium pusillum* L., *Illecebrum verticillatum* L., *Isoetes velata* A. Braun, *Lythrum borysthenicum* (Schrank) Litv., *L. hyssopifolia* L., *L. tribracteatum* Salzm. ex Spreng., *Myosotis sicula* Guss., and *Ranunculus baudotii* Godr. The peripheral belt is dominated by *Crassula tillaea* Lest.-Garl., *Isoetes histrix* Bory, *Ranunculus sardous* Crantz., *Rumex bucephalophorus* L. and *Sagina apetala* Ard.

Over a period of five years (2007–2011) we mapped the *P. minuta* population in late April–early May, at the end of the wet phase, recording the presence or absence of the species in a grid covering the entire pool, divided in 1934 cells of 0.25 m^2 (hereafter referred to as 'quadrats'). The grid was constituted of strings tightened between pegs that we repositioned annually in precisely the same place. Water depth was measured in each quadrat in February 2010 to reconstruct pool topography (Fig. 1). The Sejenane weather station provided precipitation data (*Source*: Bureau de l'Inventaire et des Ressources Hydrauliques, BIRH, Tunisia).

2.3. Experiment under controlled conditions

From December 15 2008 (W0) to June 1 2009 (W24), we conducted an experiment to identify the optimal hydrological conditions for the germination and development of *P. minuta*. We tested the effects of four flooding dates (December 15 (W0), January 15 (W5), February 15 (W10), March 15 (W14)) and five water levels (soil saturations of 70% and 100%, and water depths of 1 cm, 5 cm and 10 cm), and crossed these two factors with four replicates per combination of factors in a randomized design of containers (Supplementary material 2).

To avoid both disturbing the development of *P. minuta* in Maachar2 pool and impoverishing other populations of this rare species, we collected two kinds of surface sediments during the dry phase (July 2008): (1) 80 kg of sediments randomly collected at a depth of 5 cm near Maachar2 pool in an area without *P. minuta*, and sterilized for 48 h at 150 °C to remove any diaspore; (2) 5 kg of sediments collected at a depth of 2 cm in another pool near Maachar2, with dense populations of *P. minuta*. The presence of *P. minuta* sporocarps within these 5 kg of sediments was verified under binocular microscope after the sediment had been homogenized in 10 random samples of 1 cm^3 each (3–9 sporocarps per cm^3).

All sediments were conserved completely dry until the experimental flooding. The experiment was carried out *ex situ*, on the terrace of a villa at Tunis, under transparent shelter to prevent any rainwater being added. In December 2008, 84 plastic containers were sown with ~1443 cm^3 (thickness: 3 cm) of sterilized substrate covered with 100 cm^3 (thickness: 5 mm) of sediment containing *P. minuta* sporocarps. The containers were flooded on four dates (December 15 (W0), January 15 (W5), February 15 (W10), and March 15 (W14)) to different levels: 70% and 100% saturation, and 1 cm, 5 cm, and 10 cm deep, as per the protocol illustrated in Supplementary material 2. Four procedural control containers were filled exclusively with sterilized sediments and flooded on December15 (W0) to a depth of 5 cm.

During the course of the experiment, rainwater was regularly added to keep water levels constant. Containers with soil saturations of 70% and 100% were adjusted by weight. The arrangement

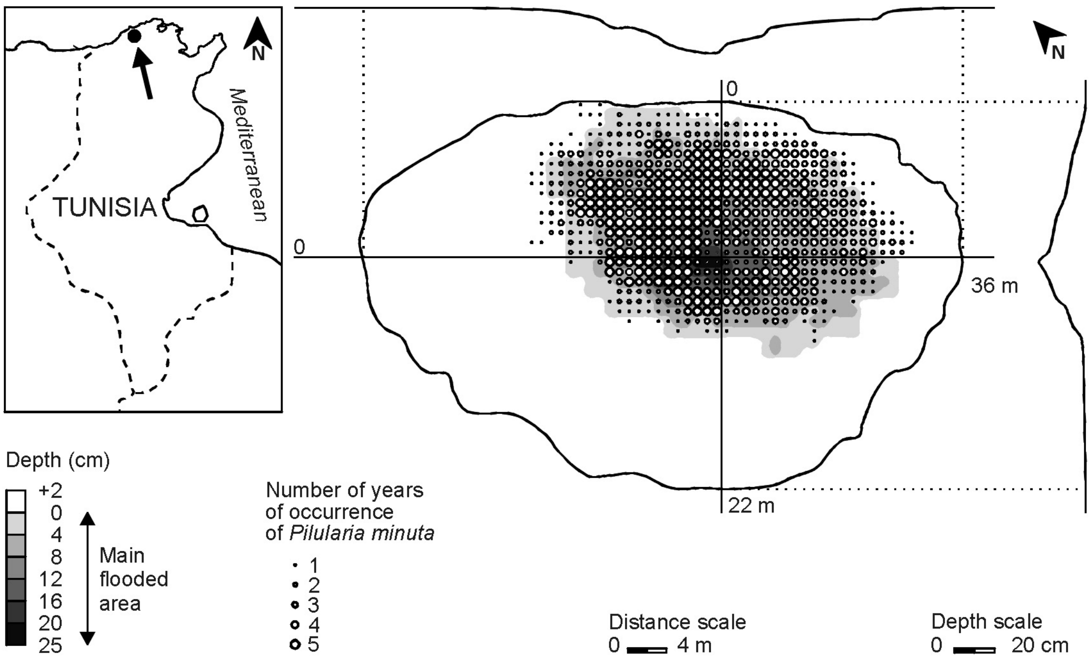

Fig. 1. *Pilularia minuta* occurrences in Maachar2 pool. Gray areas correspond to the main flooded part of the pond; white area corresponds to the part flooded only during rainy episodes, which are considered here to be outside the pool.

of the containers was re-randomized monthly to ensure homogenous light, temperature and wind conditions for the duration of the experiment. Germinations of other species were systematically and carefully extracted to avoid disturbing the development of *P. minuta* seedlings. Watering stopped on May 15 (W21), the approximate date on which Maachar2 usually dries out.

P. minuta germination (the appearance of the first leaf) and further development were monitored weekly for a period of 6 months from December 15 2008 (W0) to June 1 2009 (W24). Five randomly selected leaves per container were measured with a precision of 1 mm, marked and monitored weekly during the experiment.

2.4. *Data analysis*

2.4.1. *Field survey*

On the basis of annual field surveys, we were able to calculate the mean percentage of quadrats of each depth where *P. minuta* was found during at least one of the five years of the study, and the proportion of quadrats of each depth both where *P. minuta* never developed and where it developed every year during the five years of the study. To assess the influence of water depth on *P. minuta* development of, we tested the relationship between these proportions and quadrat depths using simple non-linear regressions. To assess the influence of rainfall quantity, we tested the relationship between the total number of quadrats where *P. minuta* was found each year and the cumulative precipitation (1) between September, December, January, February and March (*i.e.* before the pools' putative flooding date), and (2) between December, January, February, March and May (*i.e.* during the pool's putative flooding period). To test the influence of rainfall regularity, we also tested the relationship between the total number of quadrats where *P. minuta* was found each year and (1) the number of rainless days, and (2) the variation of daily precipitation between December and May. These relationships were tested using linear regression.

2.4.2. *Experiment under controlled conditions*

First, we analyzed the influence of hydrological factors (flooding date and water level) over time, and thus considered time as a factor. Since measurements were made on the same sampling units (the containers) over time, we tested differences in the number and size of leaves using repeated three-way ANOVAs (Doncaster and Davey, 2010). We used the number of leaves per container per date and the average size of the five leaves per container and per date as response variables (subjects), time (24 weeks) as within-subject factor, and flooding date and water level as between-subject factors. We log-transformed the number of leaves data to approach normal distribution and homogeneity of variance. Finally, we tested the relationship between leaf number (log-transformed) and leaf size (log-transformed) with linear regression.

Second, we analyzed the influence of the hydrological factors across the monitoring dates but did not consider time as a factor. We tested differences in the maximal leaf number per container,

Table 1
Number of quadrats where *Pilularia minuta* was found in the Maachar2 pool at the end of the wet season (late April–early May) for each year studied, and meteorological information for the corresponding growth period (September–May). Temperature was registered at the Bizerte meteorological station (*Source*: Weather Underground; http://www.wunderground.com); precipitation was measured at the Sejenane meteorological station (*Source*: Bureau de l'Inventaire et des Ressources Hydrauliques, BIRH, Tunisia). A total of 1934 quadrats were monitored in Maachar2 pool.

	2006–07	2007–08	2008–09	2009–10	2010–11
Date of field survey	22.V.2007	7.V.2008	7.V.2009	23.IV.2010	6.V.2011
Number of quadrats with *P. minuta*	515 (27%)	413 (21%)	539 (28%)	430 (22%)	212 (11%)
Minimum temperature (°C)	14	11	12	13	10
Maximum temperature (°C)	39	37	40	37	38
Mean temperature (°C)	21	20	21	21	20
Total precipitation (mm)	837	787	1046	931	864

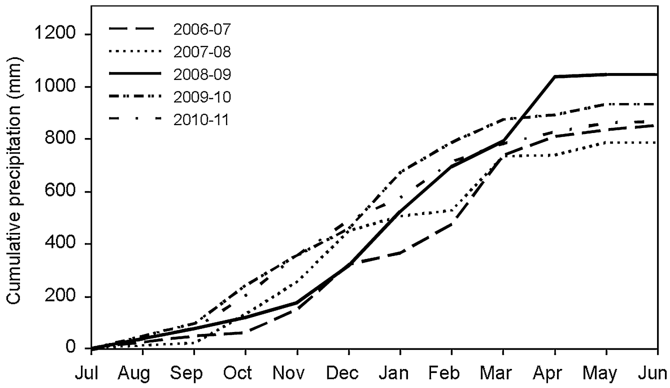

Fig. 2. Cumulative precipitation at Sejenane meteorological station during the five years of the study.

Source: Bureau de l'Inventaire et des Ressources Hydrauliques, Tunisia.

the first germination and the rate at which leaves appeared and grew, using two-way ANOVAs. The first germination time was defined for each container as the number of weeks between the flooding date and the observation of the first leaf. For containers where no leaves were observed, the first germination time was fixed at 24 weeks, *i.e.* the duration of the experiment. The leaf appearance rate per container refers to the mean number of leaves appearing weekly, calculated for each container during the first five weeks after the first leaf is observed. Leaf growth rate refers to the mean increase in leaf size weekly, calculated for each container during the first five weeks of growth. We used flooding date and water level as fixed factors.

ANOVAs were performed with Statistica (version 6.0, Statsoft, France). We always performed factorial ANOVA and therefore analyzed the primary factors and their interactions. When the overall difference tested with ANOVA was significant, we used Tukey's *post hoc* tests. We graphically verified normality and homoscedacity assumptions. All error measures stated in the text are standard error (SE).

3. Results

3.1. Field survey

Cumulative precipitation indicates that the five years of the study were rather wet, and 2008–2009 and 2009–2010 were wetter than the average 880 mm year^{-1} (Fig. 2). The mean number of quadrats per monitored year with *P. minuta* in Maachar2 was 422 ± 58, with a maximum of 539 in 2008–2009 and a minimum of 212 in 2010–2011 (Table 1). The mapped occurrence of *P. minuta* resembled the mapped pool depths (Fig. 1): the mean percentage of quadrats where *P. minuta* occurred during at least one year is significantly correlated with quadrat depth. The maximal depth is 10 cm (Fig. 3a) while beyond 10 cm, there is a negative relationship between the mean percentages of quadrats with *P. minuta* and water depths. The percentage of quadrats where *P. minuta* never appeared during the study is significantly correlated with their depth (Fig. 3b). Below 5 cm, the proportion of quadrats in which *P. minuta* never appeared increases exponentially with lower quadrat depth. The percentage of quadrats where *P. minuta* appears every year is also correlated with quadrat depth (Fig. 3c). The relationship between these two variables can be described by a polynomial model of order 2 that predicts that a maximum proportion of quadrats in which *P. minuta* appears yearly is reached for a depth of 10 cm.

We found no relationship between cumulative precipitation and *P. minuta* coverage. The number of rainless days between December and May was negatively correlated with *P. minuta* coverage ($R^2 = 0.76$, $p = 0.055$) and the variance of precipitation for rainy days was positively correlated with coverage ($R^2 = 0.76$, $p = 0.052$) (Supplementary material 3). While we cannot test it, the three best years for *P. minuta* development all included

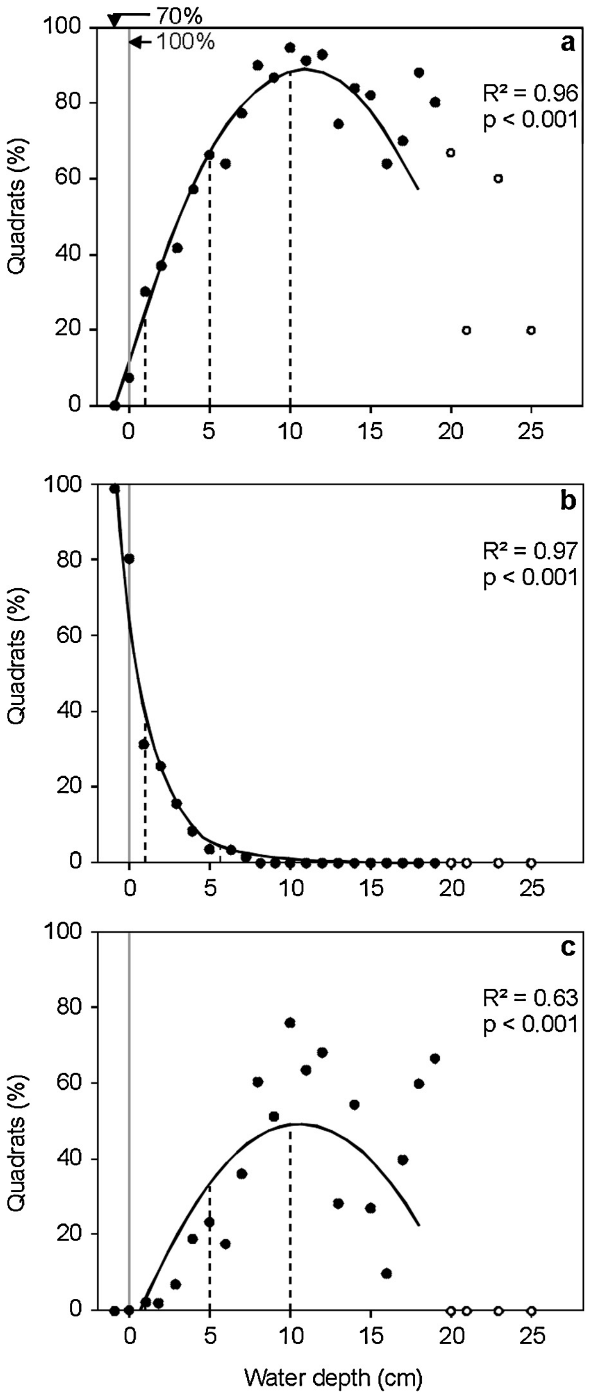

Fig. 3. Regression models between quadrat depth and *Pilularia minuta* occurrences. (a) Mean percentage of quadrats at each depth where *P. minuta* was found during at least one of the five years of the study. (b) Percentage of quadrats at each depth where *P. minuta* was never found during the five years of the study. (c) Percentage of quadrats at each depth where *P. minuta* was found every year during the five years of the study. Gray lines show the limits of pool's main flooded area, dashed lines indicate water levels selected for the experiment, with 70% and 100% of saturation placed as an indication at depths of −1 and 0 cm, respectively. White dots represent fewer than five measurements and are not considered in the regression calculation. Coefficient of determination (R^2) and significance (p) of non-linear regression are indicated.

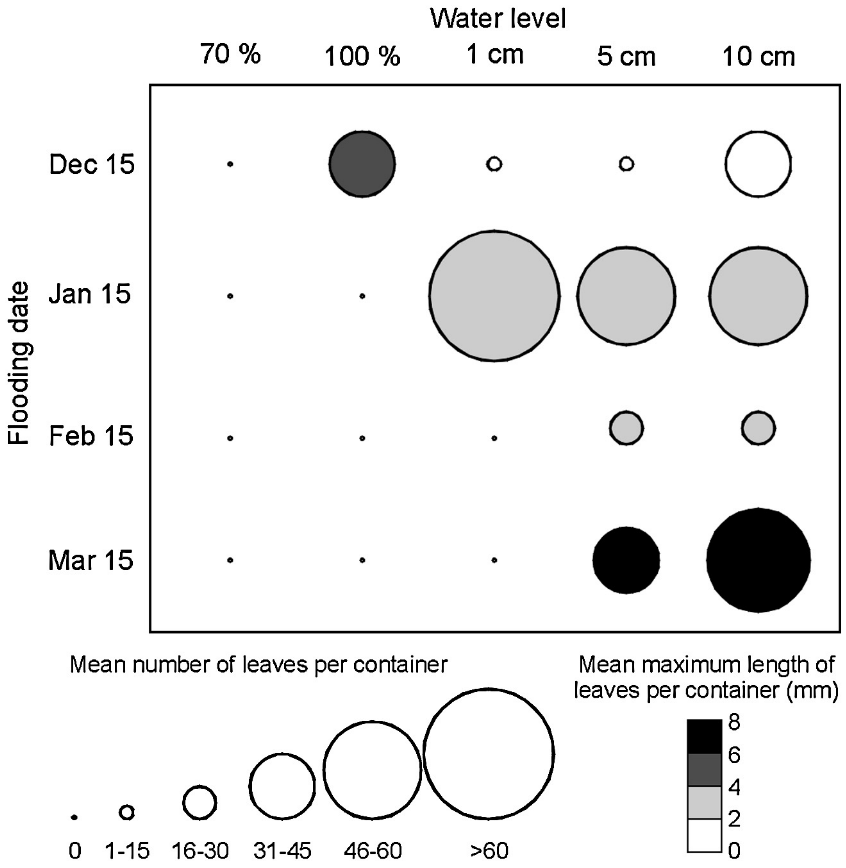

Fig. 4. Mean number and maximum size of *Pilularia minuta* leaves per container by water level and flooding date.

important rainy periods during the second part of the growing season (February–March 2006–2007, February–March 2007–2008, March–April 2008–2009; Fig. 2).

3.2. Experiment under controlled conditions

P. minuta germination appears strongly controlled by water level (Fig. 4): (1) no germination occurred at 70% saturation; (2) germination occurred only for early flooding dates where saturation was 100% and at a depth of 1 cm; (3) germination was observed under 5 and 10 cm flooding depth, irrespective of the flooding date. Independently of time and flooding date, water level had a significant effect on the number of leaves per container whereas the flooding date had no effect (Table 2a). The interactions between time, flooding date and water level had a significant effect on the number of leaves per container (Table 2a; Supplementary material 4). The first germination occurred more rapidly after a late flooding (Fig. 5). Similar results were obtained for changes in average leaf size over time (Table 2b; Supplementary material 5, 6). We found a significant positive linear relationship between leaf number and leaf size (Table 2c; leaf size = 0.8 + 0.3 × leaf number; $R^2 = 0.48$).

The ANOVAs show that water level had a significant effect on the maximum number of leaves per container (Fig. 6a), on first germination (Fig. 6b), on the rate of leaf appearance (Fig. 6c), on the maximum leaf size per container (Fig. 6d), and on the rate of leaf growth (Fig. 6e). For inundations to 5 and 10 cm, the maximum number of leaves, leaf growth rate and maximum size per container were significantly higher (Fig. 6a, d and e) while germination time was significantly shorter and leaf appearance rate

Table 2
Results of the repeated two-way ANOVAs on the number (a) and size (b) of *Pilularia minuta* leaves per container, and results of the linear regression between the number and the size of *P. minuta* leaves (c).

	DF	SS (%)	*p*
a. ANOVA on leaf number			
Flooding date	3	5	ns
Water level	4	21	0.01
Flooding date × water level	12	13	ns
Residual error	60	61	
Total	79	100	
Time	17	14	<0.001
Time × flooding date	51	12	<0.001
Time × water level	68	23	<0.001
Time × flooding date × water level	204	15	<0.001
Residual error	1020	36	
Total	1360	100	
b. ANOVA on leaf size			
Flooding date	3	3	ns
Water level	4	23	0.001
Flooding date × water level	12	12	ns
Residual error	60	62	
Total	79	100	
Time	17	13	<0.001
Time × flooding date	51	12	<0.001
Time × water level	68	20	<0.001
Time × flooding date × water level	204	15	<0.001
Residual error	1020	40	
Total	1360	100	
Regression	1	50	<0.001
Residual error	265	50	
Total	266	100	

ns: non significant.

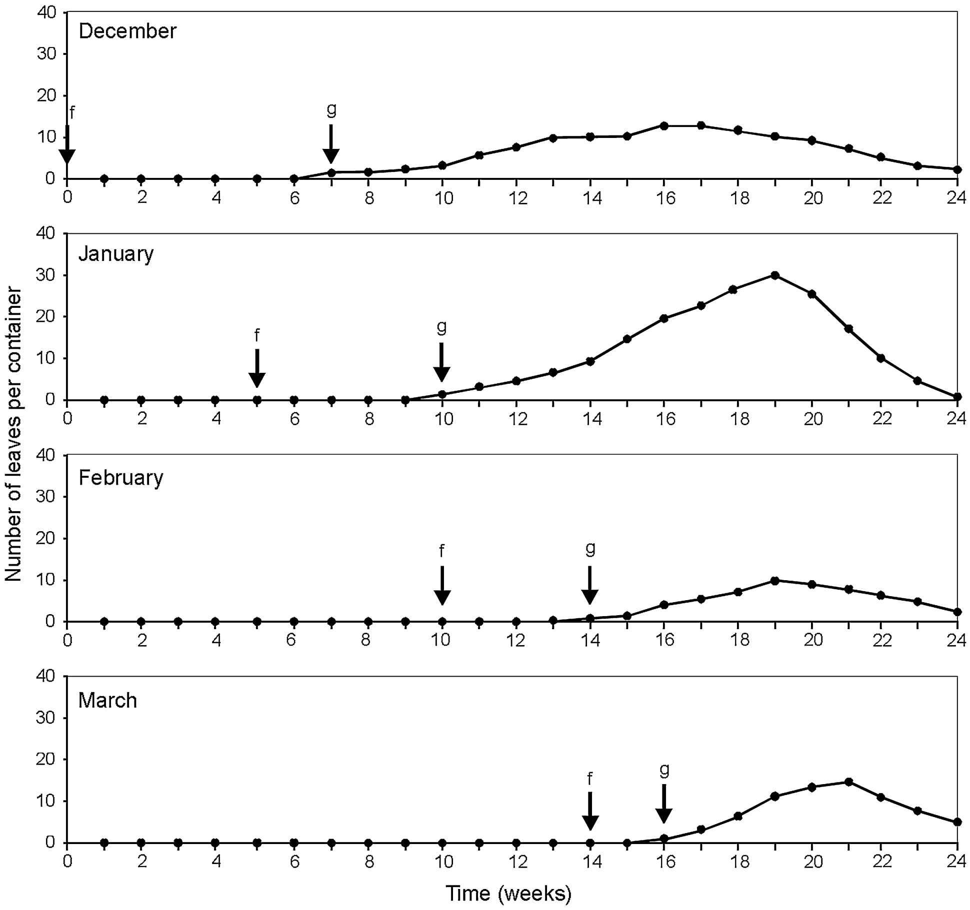

Fig. 5. Number of *Pilularia minuta* leaves per container by flooding date and during the 24 weeks of the experiment. f: date of the flooding; g: date of the first germination. Corresponding results of repeated ANOVA are $F = 6.5$, $p < 0.0001$.

significantly faster (Fig. 6b and c). Flooding date had a significant effect only on leaf growth rate (Fig. 6f), which rose with postponement of the flooding (Fig. 6f).

4. Discussion

4.1. Influence of precipitation on in situ P. minuta abundance

In situ development of the *P. minuta* population was found to show considerable year-to-year variation albeit less marked in the current wetland compared with the semi-arid Benslimane region in Morocco where *P. minuta* occurred only during the four wettest of the 10 years of temporary pools monitoring (Rhazi et al., 2009). This variability is a well-known life-history trait of annual amphibious species of Mediterranean temporary pools (Grillas et al., 2004; Deil, 2005). Comparison between *P. minuta* abundance and regional precipitation data did not show any clear influence of rainfall quantity. Cumulative precipitation was probably not an adequate parameter in the region that we studied, where the amounts of rainfall certainly exceed the needs of the plant. Precipitation variation and rainless days, as indicators of rainfall regularity, significantly influence the abundance of the species, and late rainfall events (February to April) seem to enhance the development of *P. minuta*. The observed *in situ* variability could then result from subtle inter-annual variation in water regime, including the frequency, duration, timing and predictability of flooding and drought periods (Casanova and Brock, 2000; Warwick and Brock, 2003), combined perhaps with temperature variation (Berjak et al., 1994; Santamaría and Hootsmans, 1998).

4.2. Influence of water depth on P. minuta germination and development

The experiment revealed the major influence of the water regime and, more particularly, of water level: (1) complete substrate saturation is necessary for *P. minuta* to germinate, (2) a water level of 5 cm suffices for *P. minuta* to germinate and develop independently of the flooding date, and (3) a water level of 10 cm seems optimal. In the field, at low water depths, competition with others species could affect the development of the heliophilous *P. minuta* (Daoud-Bouattour et al., 2009). The field survey also indicates that water depths greater than 10 cm limit the development of *P. minuta*, perhaps because of limited light (Rørslett and Johansen, 1995; Bornette and Puijalon, 2011) or competition with aquatic and helophytic species (Bliss and Zedler, 1998). Less abundant *P. minuta* in the pool center (maximal water depths) could also be due to the fact that the substrate dries more quickly in the center, where there is no underground water supply, than on the periphery, and, is therefore likely to prevent the sporocarps from maturing (Volder et al., 1997).

While dormancy-breakage cues are largely unknown for wetland plants (Baskin and Baskin, 1998), our results suggest that seeds and spores have mechanisms to detect water depth, perhaps through light, pressure, temperature and/or O_2 content (Keeley,

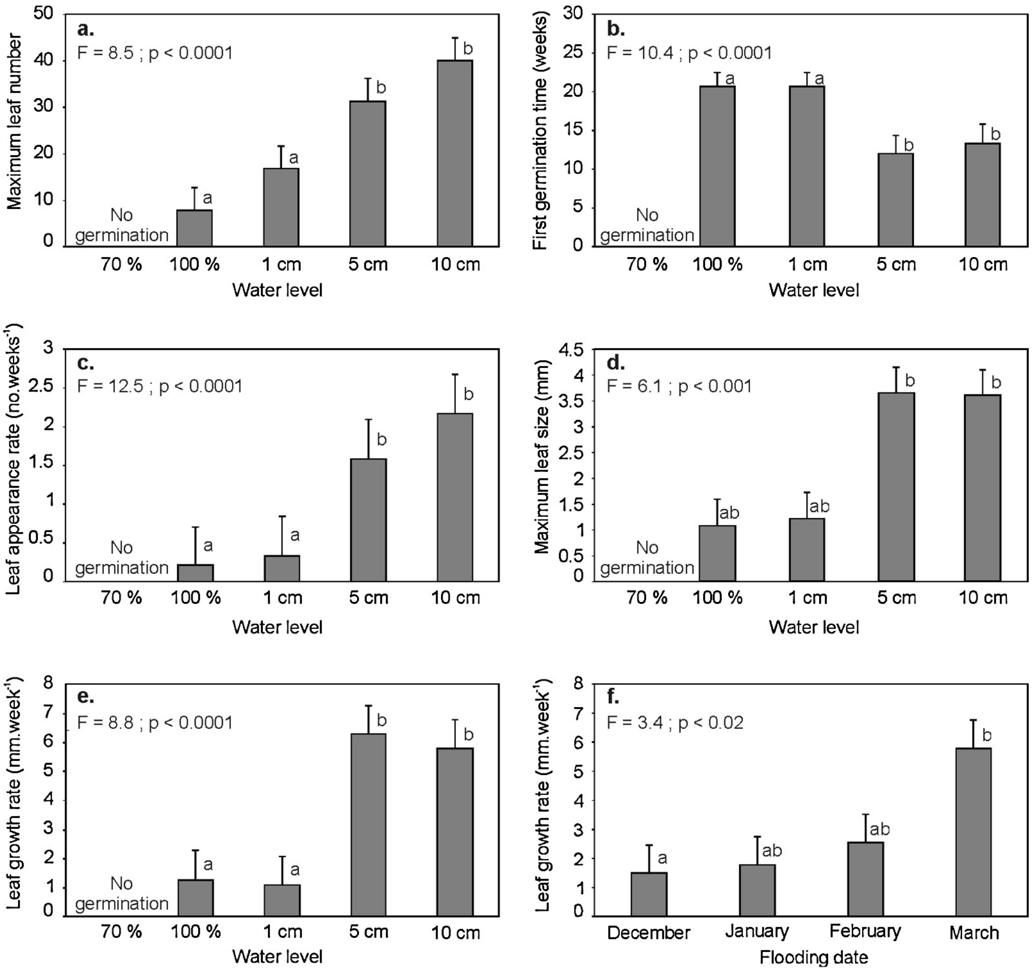

Fig. 6. Development parameters of *Pilularia minuta* by water level and flooding date. Mean ± SE per container. Results of two-way ANOVA are indicated. Different letters indicate significant differences.

1988; Grillas et al., 2004; Deil, 2005; Bornette and Puijalon, 2011). The germination time, which is longer for water levels below 1 cm, suggests that *P. minuta* sporocarps and spores also detect the water level.

4.3. Influence of flooding date on P. minuta germination and development

Our experiment suggests that flooding date only influences the germination and development *P. minuta* at low water levels. When flooding occurs later in the season, water levels must be higher, which ensures the stability of hydrological conditions when temperatures rise and evaporation increases. Although flooding dates do not influence the maximal extent of the populations, they do determine both leaf growth and germination rates, which are higher when flooding is late. Unlike other marsh species that develop more when the flooding lasts longer (Mony et al., 2010), *P. minuta* individuals and populations can compensate for their short growth period by developing more quickly. This may also be enhanced by the rise in water temperature in the spring, which is known to influence the timing and speed of reproductive mechanisms (Santamaría and Hootsmans, 1998; Schneider and Pryer, 2002) and to affect growth dynamics (Santamaría et al., 1996; Grillas et al., 2004; Deil, 2005). Our experiment indicates that *P. minuta* requires a minimum of two weeks of flooding for germination and seven weeks (with flooding in March) for maximal population development.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquabot.2013.08.001.

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