

Phenology of macrophytes in coastal environments: *Utricularia australis* (R. Br.) and *Stuckenia pectinata* (L.) Börner in an interdunal pond within the Albufera de València Natural Park

Maria A. Rodrigo and Sara Calero

Integrative Ecology lab. Ecology, Ethology and Evolution (e³) group. Cavanilles Institute for Biodiversity and Evolutionary Biology, University of Valencia, Catedrático José Beltrán 2. E-46980-Paterna, València, Spain.

* Corresponding author: maria.a.rodrigo@uv.es

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ABSTRACT

Phenology of macrophytes in coastal environments: *Utricularia australis* (R. Br.) and *Stuckenia pectinata* (L.) Börner in an interdunal pond within the Albufera de València Natural Park

Macrophytes are key elements for biodiversity in Mediterranean ponds and coastal lagoons. To predict the long-term persistence of aquatic plant populations in a changing environment is essential to understand their phenology, i.e. the timing of ecological processes coupled with environmental factors. *Utricularia australis* and *Stuckenia pectinata* (= *Potamogeton pectinatus*) populations growing in a brackish interdunal pond within the Albufera de València Natural Park were monitored over three years. We describe their life-cycles under shallow conditions and relate them to accumulated heat and radiation. *U. australis* overwintered as turions; turion germination happened in February at 125-210 growing degree-days (GDD), at a daily mean temperature (DMT) of 10-11 °C, and with 150-220 hours of accumulated sunshine (h Sun). The flowering period started in June (2450 GDD, 26 °C DMT, 1365 h Sun), but only in 2014 and in the sunniest areas of the pond. The turion formation started in early August (3800-4000 GDD, 20-28 °C DMT, 1900-2180 h Sun). A reduction in *U. australis* biomass and coverage was observed throughout time. *S. pectinata* exhibited a perennial population restricted to the pond deeper basins. It initiated its life-cycle in late February-early March, mainly by re-sprouting from old stems and roots, vigorously producing new shoots with green leaves (400-440 GDD, 11-12 °C DMT, 300-380 h Sun). In early May, the production of the first spikes was detected (840-1045 GDD, 17-18 °C DMT, 820-900 h Sun). By mid-June, fertilization was complete and drupelet growth began (1430-1800 GDD, 20-23 °C DMT, 1190-1230 h Sun). Increased salinity and competition with charophytes developed in the pond, negatively affected the fertility and abundance dynamics of *S. pectinata*. *U. australis* performance declined with the reduction in free-water surface due to the expansion of helophytes, mainly cattails. The *S. pectinata* life-cycle in this pond fits in with the latitudinal pattern of a prolonged life-cycle with a delayed reproduction for southern populations. The restoration of degraded or destroyed aquatic ecosystems is a turning point for local populations to recover, but some management of the restored waterbodies is required to guarantee the preservation of peculiar and endangered species. *In situ* continuous monitoring of water temperature, and other relevant variables for submerged vegetation (such as light availability and salinity), is needed to have a more precise image of the environmental factor values required to pass from one phenological event to another, and for a better comparison among populations from different origins.

Key words: Aquatic angiosperms, Mediterranean ecosystems, carnivorous plant, life-cycles, growing degree-days (GDD)

RESUMEN

Fenología de macrófitos en ambientes costeros: *Utricularia australis* (R. Br.) y *Stuckenia pectinata* (L.) Börner en una laguna interdunar del Parc Natural de l'Albufera de València

Los macrófitos son elementos clave para la biodiversidad de lagunas del Mediterráneo. Para predecir la persistencia a largo plazo de las poblaciones de plantas acuáticas en un ambiente cambiante es esencial entender su fenología, i.e. el ritmo de los procesos ecológicos relacionados con los factores ambientales. Hemos estudiado durante tres años una población de *Utricu-*

laría australis y una de *Stuckenia pectinata* (=Potamogeton pectinatus) en una laguna interdunal salobre del Parc Natural de l'Albufera de València. Describimos sus ciclos vitales en condiciones someras y los relacionamos con el calor y la radiación acumuladas. *U. australis* pasó el invierno en forma de turiones; su germinación sucedió en febrero con 125-210 grados-día (GD), a una temperatura media diaria (TMD) de 10-11 °C y con 150-220 horas acumuladas de radiación solar (h Sol). La floración comenzó en junio (2450 GD/26 °C TMD/1365 h Sol), únicamente en 2014 y en las zonas más soleadas. La formación de turiones comenzó en agosto (3800-4000 GD/20-28 °C TMD/1900-2180 h Sol). Observamos una reducción en la biomasa y cobertura de *U. australis* a lo largo del tiempo. *S. pectinata* presentó una población perenne restringida a las cubetas más profundas de la laguna. Inició su ciclo vital a finales de febrero-principios de marzo, a partir de los rebrotes de los viejos tallos sumergidos (400-440 GD/11-12 °C TMD/300-380 h Sol). A comienzos de mayo detectamos las primeras flores (840-1045 GD/17-18 °C TMD/820-900 h Sol). Sobre mediados de junio la fertilización se completó y comenzó el crecimiento de los frutos (1430-1800 GD/20-23 °C TMD/1190-1230 h Sol). El aumento de salinidad y la competencia con los carófitos de la laguna afectó negativamente la fertilidad y la dinámica de *S. pectinata*. El crecimiento de *U. australis* cayó con la reducción de la superficie de agua libre por expansión de los helófitos. El ciclo de vida de *S. pectinata* en esta laguna se corresponde con el patrón latitudinal que atribuye ciclos de vida prolongados con reproducción tardía a las poblaciones de baja latitud. La restauración de ecosistemas acuáticos (destruidos-degradados) es fundamental para la recuperación de poblaciones locales, si bien es necesaria la gestión de estos ecosistemas para garantizar la preservación de especies peculiares y amenazadas. Se requiere el seguimiento in situ y continuo de la temperatura del agua y otras variables (disponibilidad luminosa, salinidad) para obtener una imagen precisa de los valores de los factores ambientales requeridos para pasar de una fenofase a otra, para así comparar entre poblaciones de origen diferente.

Palabras clave: Angiospermas acuáticas, ecosistemas mediterráneos, planta carnívora, ciclos de vida, grados-día

INTRODUCTION

Ponds are the most widespread aquatic habitat, and collectively dominate the total area of continental standing waters in Europe; this being especially true for Mediterranean countries (Miracle *et al.*, 2010). Ponds also play many other valuable roles such as enhancing biodiversity, mitigating diffuse pollution or regulating temperature and humidity. Macrophytes are key elements for biodiversity in ponds and coastal lagoons of the Mediterranean (Chappuis *et al.*, 2012), one of the world's major biodiversity hot spots (Myers *et al.*, 2000), and they are of key interest for management and conservation purposes (Chappuis *et al.*, 2014).

Most aquatic angiosperms operate mixed reproductive strategies involving clonal and sexual reproduction, hence they disperse by both vegetative propagules and seeds (Philbrick & Les, 1996; Eckert *et al.*, 2016). This is the case of widely distributed species such as *Utricularia australis* R. Br. and *Stuckenia pectinata* (L.) Börner (=Potamogeton pectinatus L.). However, some shallow permanent waterbodies will turn into temporary ones under the drying situation predicted for the Mediterranean region (IPCC, 2014). Submerged macrophytes will be exposed to desiccation, and although plant fragments from

some species are partially tolerant to it (Barnes *et al.*, 2013), the production of drought-resistant propagules will determine the persistence of these communities (Eckert *et al.*, 2016). Understanding the reproductive strategies, particularly the timing (phenology) of sexual reproduction, and the life-history patterns, including the types of propagules and their time of formation and germination/sprouting, is key for the conservation of submerged macrophytes and the ecosystems they inhabit.

Utricularia australis is a free-floating carnivorous species of aquatic bladderwort (Taylor, 1989). Its rootless shoots take up all necessary nutrients, either from the water or from captured prey (aquatic crustaceans, mites, nematodes, rotifers and protozoa) inside its traps or bladders. It usually grows in standing, nutrient-poor and humic waters. Its main life-history traits include extensive clonal reproduction, high dispersal ability of vegetative propagules (shoot fragments and turions), and rare-to-sporadic sexual reproduction (Adamec, 2011). *U. australis* is distributed in a vast geographic range in temperate and tropical regions, except North and South America (Taylor, 1989). There is no evidence that the species is threatened in any major parts of its range, and therefore it is listed by the IUCN as

“Least Concern” (Champion, 2014). Conversely, its status in the Mediterranean region is not as good as would be desired. In the Spanish territory, few littoral aquatic ecosystems have survived centuries of desiccation and destruction, and even fewer are free from pollution and eutrophication problems. For example, desiccation and drainage as well as water extraction and regulation, are the main causes of macrophyte loss in Catalan stagnant waters (Chappuis *et al.*, 2011). Thus, *U. australis* is catalogued as “in danger of extinction” in Andalusia, the Basque Country and the Valencian Autonomous Community, while it is considered as “vulnerable” in Asturias, Catalonia and Madrid (Anthos, 2017).

Stuckenia pectinata, commonly known as sago pondweed or fennel pondweed, is a perennial submerged macrophyte which grows abundantly within a wide distribution area (Kantrud, 1990). Some authors have proposed a latitude-correlated switch in its life-cycle strategy (Pilon *et al.*, 2002), although, in fact, little is known about *S. pectinata*'s reproductive ecology (Ganie *et al.*, 2016). The species employs several clonal (tubers, rhizomes, nodal plantlets, plant fragments) and sexual (fruits) modes of reproduction (Van Wijk, 1989). This highly flexible reproductive strategy contributes to the rapid spread of the species across different habitats.

Over three years, one population of *U. australis* and one of *S. pectinata* growing in a brackish shallow pond within the Albufera de València Natural Park (Spain) were monitored. The main objectives of this study are (i) to describe the different phenophases (i.e. the observable stages in the annual life cycle of an organism) of these two species in shallow coastal Mediterranean ecosystems, in coexistence with others macrophytes (charophytes and helophytes), under habitat interannual variation, and (ii) to explore how their phenological patterns respond or not to abiotic environmental variables. For the first time for these species, combined data of water daily mean temperature, accumulated heat and accumulated hours of sunshine for the precise time of the different phenological events are provided. The study also delves into the understanding of the sexual reproductive phenology of the species, by comparing our data from a lower-latitude

ecosystem to the available information from other latitudes. Since the two-studied species are distributed world-wide, they constitute an excellent tool to analyse the phenological responses to environmental factors that vary with latitude.

MATERIAL AND METHODS

Study area

The Albufera de València Natural Park is located on the east coast of Spain (Miracle & Sahuquillo, 2002). It includes one of the largest coastal lagoons in the Iberian Peninsula, separated from the Mediterranean Sea by a 14 km long and 1 km wide sand bar (Fig. 1A). This area, called *Devesa del Saler*, included several dune ridges, with a developed pine forest in the inner stable one, and a set of oligohaline shallow ponds. During the 1970s, an urbanization plan destroyed the seashore dune front and the dune slacks. Later, the area was protected (becoming a Natural Park in 1986; Natura 2000 network), and the regional authorities executed several restoration projects to reconstruct the former foredunes, their vegetation, and both temporary and permanent waterbodies. One of these ecosystems, the Llacuna Nova del Canyar (LNC, hereafter; Fig. 1) was created in 2007 as a shallow permanent pond with an approximate area of 5900 m². Most of the pond has a water level between 0.5 and 1 m, except for some deeper basins (1.5 m maximum depth).

Stuckenia pectinata (L.) Börner (= *Potamogeton pectinatus* L.) has been present in the pond since its reconstruction thanks to the work of the Natural Park managers, who planted some specimens from nearby waterbodies (J.M. Benavent, pers. comm.). *Utricularia australis* R. Br. was first detected in the LNC in October 2013 (Calero *et al.*, 2015). Both species formed the submerged vegetation community of the pond together with dense beds of *Chara hispida*, which occupied the shores and the deeper parts, and *Chara aspera* and *Nitella hyalina*, mainly located on the shores (Calero *et al.*, 2015, 2017). The pond held a high density of helophytic vegetation (Fig 1B; genera *Typha*, *Phragmites*, *Juncus* and *Scirpus*).

The LNC is an oligo-haline pond with a high concentration of chloride and sodium which

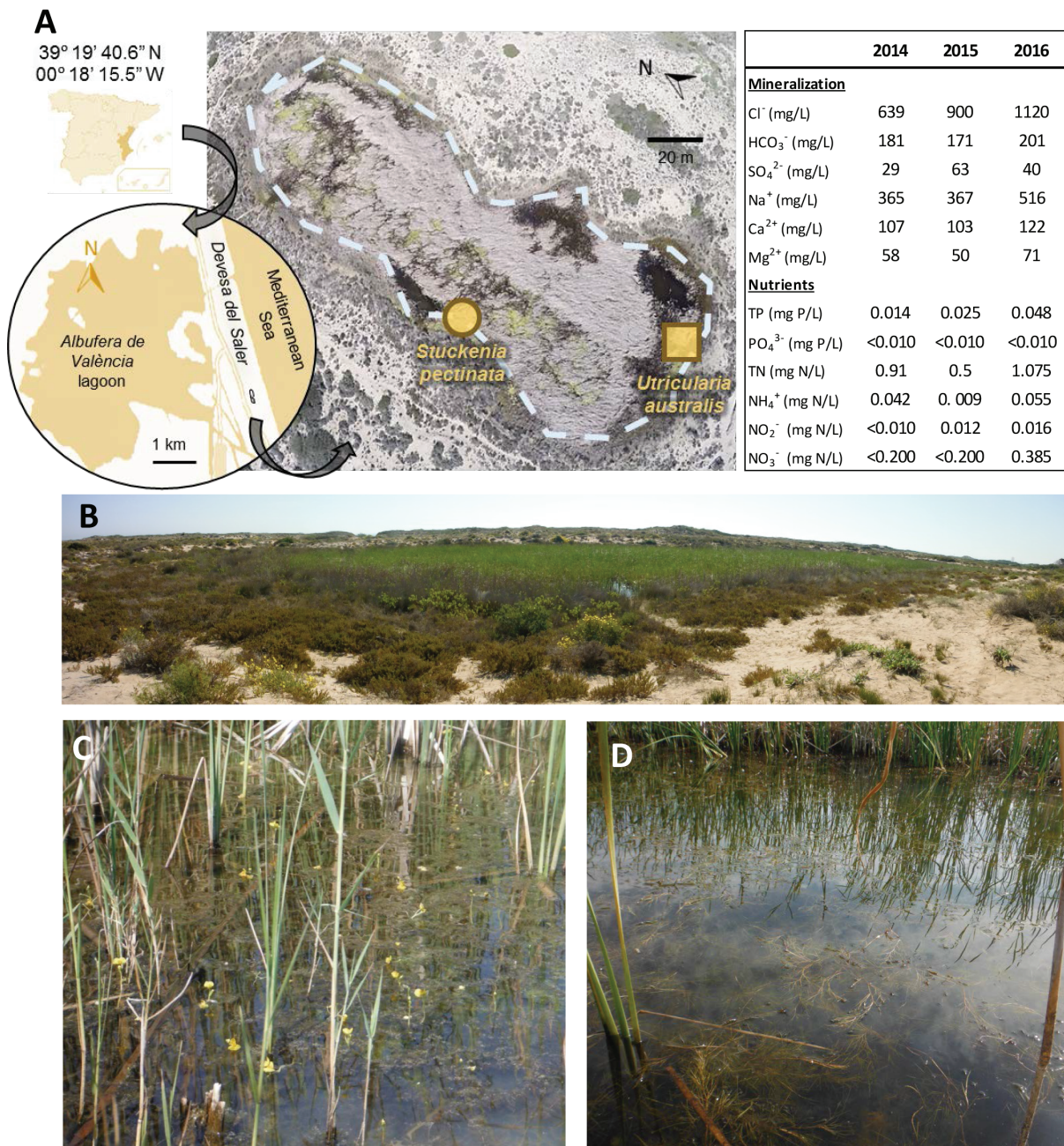


Figure 1. A: Location of the LNC within the coastal dune system of the Albufera de València Natural Park, and location of the sampling sites for *Utricularia australis* and *Stuckenia pectinata*. Chemical features of the LNC in 2014-2016. Analyses were performed in January 2014, and March 2015 and 2016. B: General view of the studied pond in spring 2015. C: Water surface of the shore of the interdunal pond covered by *U. australis* specimens, some of them flowered. D: The *S. pectinata* meadow in the interdunal pond. A: Localización de la LNC en el sistema dunar costero del Parc Natural de l'Albufera de València, y ubicación de las zonas de muestreo de *Utricularia australis* y *Stuckenia pectinata*. Características químicas de la LNC en 2014-2016. Los análisis se realizaron en enero 2014, y en marzo 2015 y 2016. B: Vista general de la laguna estudiada en primavera de 2015. C: Superficie del agua en la orilla de la laguna interdunar cubierta por especímenes de *U. australis*, algunos de ellos en flor. D: Pradera de *S. pectinata* en la laguna.

almost doubled from 2014 to 2016 (Fig. 1A). The water is nitrogen-poor; soluble reactive phosphorus concentrations were always lower than 0.01 mgP/L, and TP concentrations increased throughout time but remained below 0.05 mgP/L.

Collection of macrophytes and phenological data

The macrophyte samples were taken monthly, from January 2014 to December 2016, by sampling in the last week of each month, and on approximately the same day for the three years. During spring and summer, the sampling was bi-weekly for better detection of all phenophases throughout the expected time of the highest fertility.

Since *S. pectinata* distribution was mainly restricted to one of the deeper basins of the pond (Fig. 1D), this species was sampled only at this site where it coexisted with *Chara hispida*. The sampling was performed from the shore with the assistance of a hook. *U. australis* had a scattered

distribution throughout the whole pond, but it was collected by hand at one shallow spot (< 50 cm deep) located in the southern part of the pond (Fig. 1C). The sampling effort was intended to be the same at each date and site (for *S. pectinata* site, the hook was thrown three times into the centre of the basin and all the specimens of both *S. pectinata* and *C. hispida* were collected; for *U. australis* site, specimens were randomly collected). For *S. pectinata*, 25-30 specimens by sampling date were collected on average. For *U. australis*, 40, 20 and 15 specimens were sampled, on average, by sampling date in 2014, 2015 and 2016, respectively (due to the reduction of its biomass; see Results). Fresh macrophyte material was placed in plastic bags and transported in a portable cooler to the Integrative Ecology Lab (ICBiBE, University of Valencia).

All plants were observed with a binocular loupe (Olympus SZ61, 67.5x maximum magnification) to accurately count and monitor the aspect and development stage of sexual and vegetative structures (flowers and fruits; also bladders and

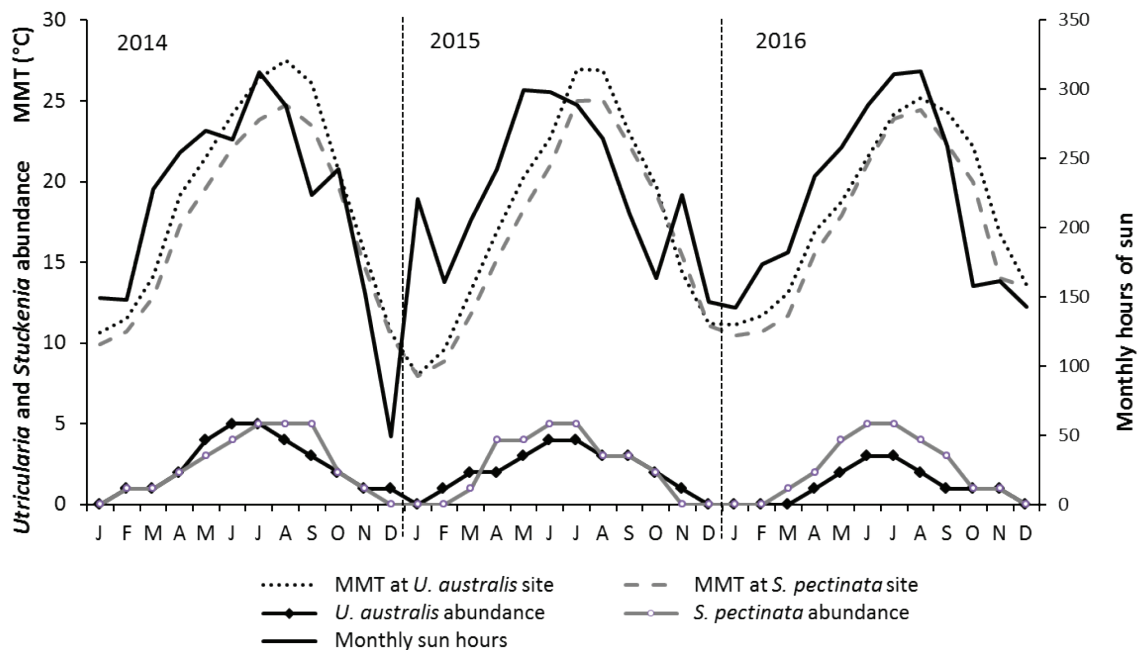


Figure 2. Dynamics of accumulated hours of sun per month, monthly mean temperature (MMT) at the two sites where the *U. australis* and *S. pectinata* were sampled and semi-quantitative (from 0 to 5) abundances of the two macrophyte species. *Dinámica del número de horas de sol por mes, temperatura media mensual en las zona de muestreo de U. australis y S. pectinata y abundancia semi-quantitativa (de 0 a 5) de las dos especies de macrófitos.*

turions for *U. australis*) in each specimen. The general aspect of *U. australis* was based mainly on the leaf colour, and four categories were considered: intense green, yellow-green, brown-green and senescent specimens. The phenological events considered were germination of turions, vegetative growth (abundance increase), flowering, formation of turions and senescence for *U. australis*. We distinguished two different phenological states for turions (Fig. S1, see Supplementary information available at <http://www.limnetica.net/en/limnetica>): (i) developing turions, already having a spherical dark green appearance, but strongly connected to the mother shoot (intense-green, yellow-green and brown-green specimens), and (ii) developed turions, with a darker brownish colour, easily detachable with forceps from the mother shoot (brown-green and senescent specimens). Two categories of bladder colour were considered: (i) purple, which is the colour of the anthocyanin from the plant tissues in an acidic medium, i.e. when the prey have not yet been digested, and (ii) dark blue-black, which indicates a basic medium due to the digestion of the animal proteins. The general aspect of *S. pectinata* was also based on the leaf colour and three categories were considered: green, brown and senescent specimens. For this species the phenological events considered were sprouting, vegetative growth, flowering and fruiting periods, as well as senescence.

Qualitative-semiquantitative estimation of macrophyte biomass and coverage

Due to the restrictions imposed by the environmental authorities concerning the collection of large samples of macrophytes from this protected pond (particularly for *U. australis*), biomass and distribution estimates are only qualitative/semi-quantitative. These estimations are derived from in situ observations and photographs which were taken at the *S. pectinata* and *U. australis* sampling sites each sampling date. Moreover, the whole pond was checked to get an approximate idea of *U. australis* coverage and flowering, and additional photographs were taken. With this information, 6 categories of qualitative abundance/coverage were established (0: No speci-

mens, 1: Very few specimens (< 10 % coverage), 2: Few specimens (10-40 %), 3: Medium abundance (40-70 %), 4: Abundant (70-90 %), 5: Highly abundant (> 90 %); (Fig. S2, see Supplementary information available at <http://www.limnetica.net/en/limnetica>). The photographs were also used to estimate the coverage of emergent vegetation in the *U. australis* site (Fig. S3, see Supplementary information available at <http://www.limnetica.net/en/limnetica>). The quantity of charophytes that appeared in the samplings with the hook when *S. pectinata* was sampled was used to roughly estimate the amount of charophytes in relation to *S. pectinata* in this basin.

Measurement of limnological variables

Two probes provided with data loggers (Onset HOBO; U20-001 model) were set up (submerged) in a shallow (*Utricularia* site) and a deeper (*Stuckenia* site) location in the pond to measure underwater temperature every half an hour. After subsequent data processing, daily mean temperature (DMT), monthly mean temperature (MMT) and growing degree-days (GDD) were calculated for the whole study period. GDD is a broadly applied parameter (Trudgill *et al.*, 2005) to measure the accumulation of heat throughout the year (from a starting point or biofix and above a baseline temperature) that allows a species to develop from one phenophase to the next in its life cycle. GDD were calculated using the HOBOWare Pro Growing Degree Days Assistant. We used the Actual Temperature Method (no cut-off), which uses the logging interval of the temperature data, 30 minutes in our case, to perform a numerical integration. We considered 4 °C as baseline temperature. The method described uses the area between the curve and this low threshold to compute the GDD. We used the 1st January as the starting date (biofix). Further information concerning the methodology can be found in Calero *et al.* (2017). Accumulated hours of sun by day (h Sun) were obtained from the European Climate Assessment and Dataset (Valencia station, number 237 –located 18 km from the pond–; Klein Tank *et al.*, 2002). Monthly hours of sun (MHS) were calculated to understand the synergistic roles of temperature and light in macrophyte phenology.

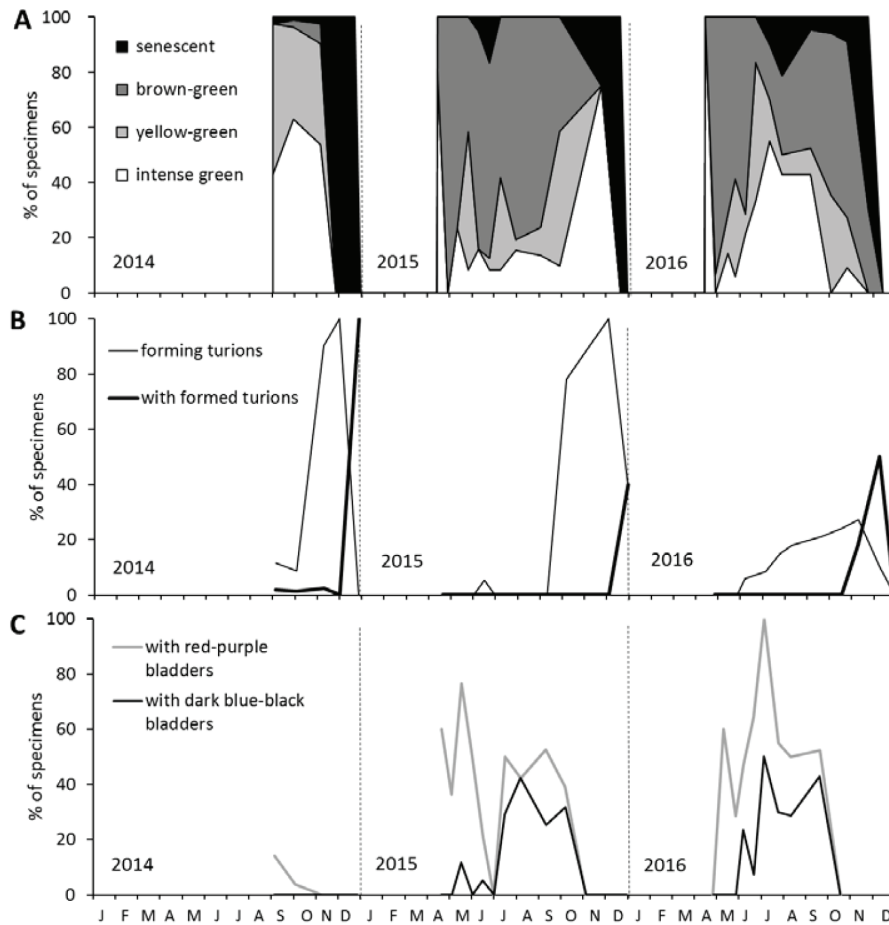


Figure 3. Phenology of some features of *U. australis* represented as the percentage of specimens showing the indicated characteristic throughout the study period. A: Changes in the colour of the plants (data registered from September 2014). B: Formation of turions. C: Presence of purple and dark blue-black bladders. *Fenología de algunas características de U. australis representadas por el porcentaje de especímenes que muestran las características indicadas a lo largo del periodo de estudio. A: cambios en el color de las plantas. B: Formación de turiones. C: Presencia de utriculos púrpura y azul oscuro-negros.*

Salinity, pH and dissolved oxygen were measured monthly with portable field equipment (Multiline F/Set-3, WTW) at both sites. Water samples were taken monthly to measure sestonic chlorophyll *a* (according to Calero *et al.*, 2015), and annually to analyse total nitrogen (TN) and total phosphorus (TP) concentrations, as well as the solute composition of the water. The IAMA laboratory (UPV) and the *Laboratorios Tecnológicos de Levante* performed these latter analyses using standard methods (APHA, AWWA, WEF, 2012).

Statistical analysis

One-way ANOVA tests for repeated measures were used to compare the means of the monthly number of hours of sun between 2014, 2015 and 2016, as well as for monthly mean temperatures in each macrophyte site. The normality of the distribution of the analysed variables and the homoscedasticity of samples were tested by the Kolmogorov–Smirnov and Levene tests, respectively. The salinity trend was analysed by means of linear regression. Two months (October 2015 and December 2016) with heavy rains were not

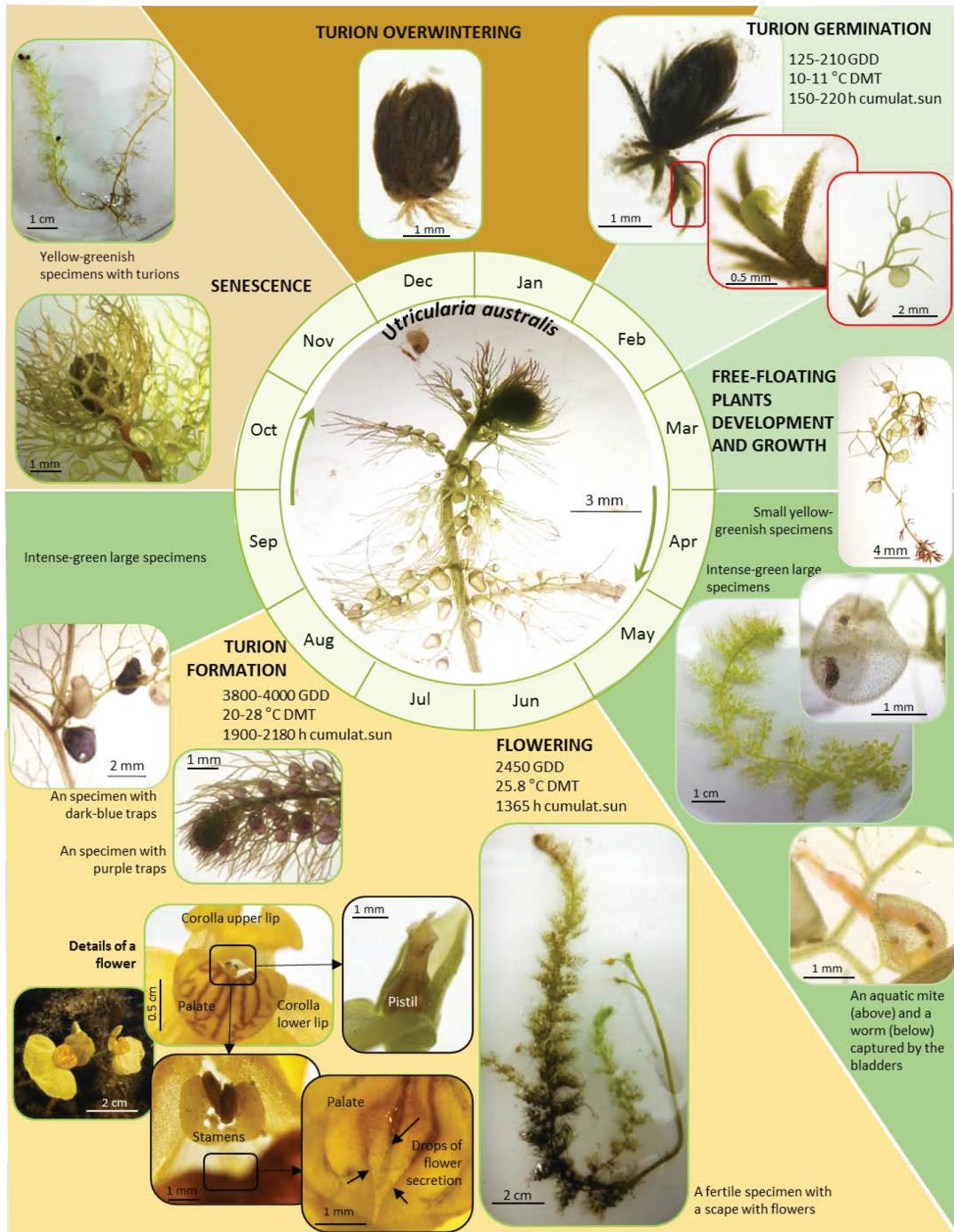


Figure 4. The life-cycle of the *Utricularia australis* population in the LNC. The different events are distributed throughout the months of the year in a circular diagram. Accumulated growing degree-days (GDD), water daily mean temperature (DMT) and accumulated sunshine (hours of sun) for the most important phenological events are shown. *El ciclo de vida de la población de Utricularia australis en la LNC. Los diferentes eventos están distribuidos a lo largo de los meses del año en un diagrama circular. Se muestran los grados-día acumulados, la temperatura del agua media diaria (DMT) y las horas acumuladas de radiación solar (horas de sol) para los eventos fenológicos más relevantes.*

considered in the analyses. To describe the relationship between macrophyte features (abundance and phenological events) and environmental data, stepwise multiple linear regression models were performed to test said relationships. Statistical significance was considered when $p < 0.05$. The IBM SPSS 19.0 statistical package was used to perform all statistical analyses.

RESULTS

Utricularia australis life-cycle

After the first detection in the pond of some specimens of *U. australis*, forming turions, in October–November 2013, no specimens were observed over the next two months. By February 2014, some free-floating specimens appeared

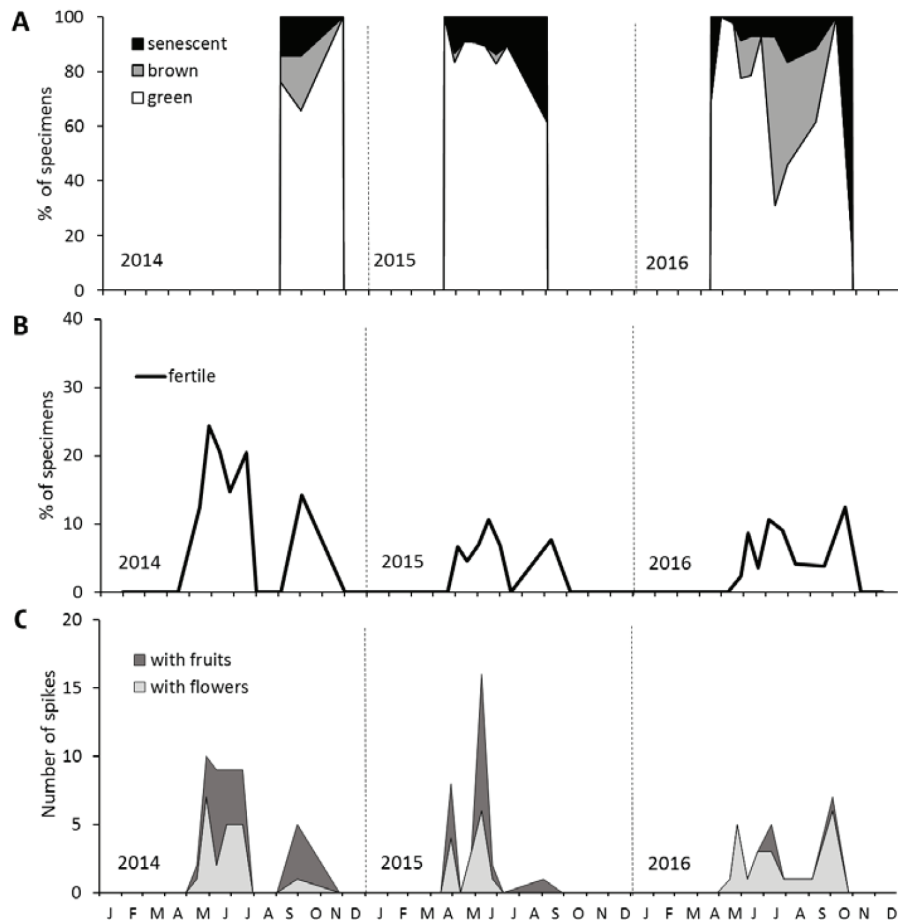


Figure 5. Phenology of some features of *S. pectinata* represented as the percentage of specimens showing the indicated characteristic throughout the study period. A: Changes in the colour of the plants (data registered from September 2014). “Green specimens” include specimens with bright green stem and leaves, but also others with brown stem and green leaves. “Brown specimens” include old plants with new sprouting leaves. B: Percentage of specimens bearing flowers and/or fruits. C: Number of spikes bearing flowers (being formed and already formed) and spikes bearing fruits in any state. *Fenología de algunas características de S. pectinata representadas como el porcentaje de especímenes que muestran la característica indicada a lo largo del periodo de estudio. A: Cambios en el color de las plantas (datos obtenidos a partir de septiembre 2014). “Especímenes verdes” incluye aquellos especímenes con tallos y hojas verdes brillantes pero algunos también con tallos oscuros y hojas verdes. “Especímenes marrones” incluye plantas viejas con nuevas hojas. B: Porcentaje de especímenes portadores de flores y/o frutos. C: Número de tallos portadores de flores (que se están formando o ya formadas) y tallos portadores de frutos en cualquier estado.*

(Fig. 2). The maximum population abundance occurred between June and July. The specimen colour changed from yellow-green, intense green to brown-green at the end of the season (Fig. 3A). While a high biomass of free-floating plants covered most of the water surface of the pond, flowering was restricted to its southern part, except for some isolated specimens that flowered on the eastern shore. The flowering period started in June 2014, lasting until August (Fig. 4; Table S1, Supplementary information available at <http://www.limnetica.net/en/limnetica>). By then, the first specimens developing turions on the tip of the shoot appeared, as well as others with developed turions (Fig. 3B). The formation of turions was observed until November, when 100 % of specimens were senescent. In 2015, the abundance peaked again between June and July. The formation of turions started one month later than in the previous year. In 2016, there was a clear reduction in abundance (Fig. 2); the first specimens were not detected until April, and some shoots already began to form turions in May. No flowering occurred in 2015 or 2016.

The plants exhibited a large quantity of bladders, on many occasions containing small animals which had been captured such as aquatic mites and worms (Fig. 4). In 2014, 14 % of the specimens possessed purple bladders at the beginning of September (Fig. 3C). This percentage largely increased in 2015 and 2016 up to 70-100 %. Dark blue-black bladders were also observed during these years in up to 40-50 % of specimens at the end of June-beginning of July.

In terms of accumulated heat and radiation (Fig. 4; Table S1, Supplementary information), the turions germinated at 125-210 GDD, 10-11 °C DMT and 150-220 h Sun. The flowering period started at 2450 GDD, 25.8 °C DMT and 1365 h Sun. The turion formation started at 3800-4000 GDD, between 20 and 27.8 °C DMT and 1900-2180 h Sun.

***Stuckenia pectinata* life-cycle**

In 2014, the overwintering old stems of *S. pectinata* started to vigorously produce new green leaves by the end of February, reached the maximum biomass in July-September and then rapidly decreased in

October, when the shoots moved into the senescence stage (Fig. 2, 5A). In 2015 and 2016, shoots started to grow one month later, in March; the maximum biomass, lower than the first year, was achieved earlier. The percentage of senescent plants particularly increased in 2016 (Fig. 5A).

The reproductive period started at the end of April-beginning of May in the three years, with the highest percentage of fertile specimens in 2014, up to 25 %. In 2015 and 2016, the percentage of fertile shoots was similar (6-7 %; Fig. 5B). In 2016, the proportion of fertile shoots with fruits was very scarce (Fig. 5C). The fructification peak took place in mid-June in 2014 and 2015.

In terms of accumulated heat and radiation (Fig. 6; Table S1, Supplementary information), the re-sprouting from old stems in late February-early March took place at 400-440 GDD, 11-12 °C DMT and 300-380 h Sun. In early May, the production of the first spikes was detected at 840-1045 GDD, 820-900 h Sun, and when the DMT was 17-18 °C. By mid-June, fertilization was complete and drupelet growth began. At this time, the accumulated heat was 1430-1800 GDD and there was a DMT of 20-23 °C and 1190-1230 h Sun.

Limnological variables

The monthly mean temperatures (MMT) (Fig. 2) between the three studied years were not statistically different in any of the macrophyte sites (repANOVA $F_{2,33} = 3.3$; $p = 0.060$ for the *Utricularia* site; repANOVA $F_{2,33} = 1.9$; $p = 0.170$ for the *Stuckenia* site). Nor was there interannual variability in the mean values of monthly hours of sun (MHS) (Fig. 2) (repANOVA $F_{2,33} = 0.44$; $p = 0.650$). Water salinity significantly increased over the three-year period (Fig. 7; $R^2 = 0.70$, $p < 0.001$ for the *Stuckenia* site; $R^2 = 0.24$, $p = 0.003$ for the *Utricularia* site). The trend of increased salinity was more remarkable in the *Stuckenia* site, where salinity changed from mean values of 1.7 g/L in 2014 to 2.5 g/L in 2016. The sestonic chlorophyll concentrations did not show any summer peak, and were below 20 µg/L most of the time in the *Utricularia* site (Fig. 7A). In the *Stuckenia* site, however, there was a constant pattern over the three year period: very low concentrations of chlorophyll during the cold months, but peaks in

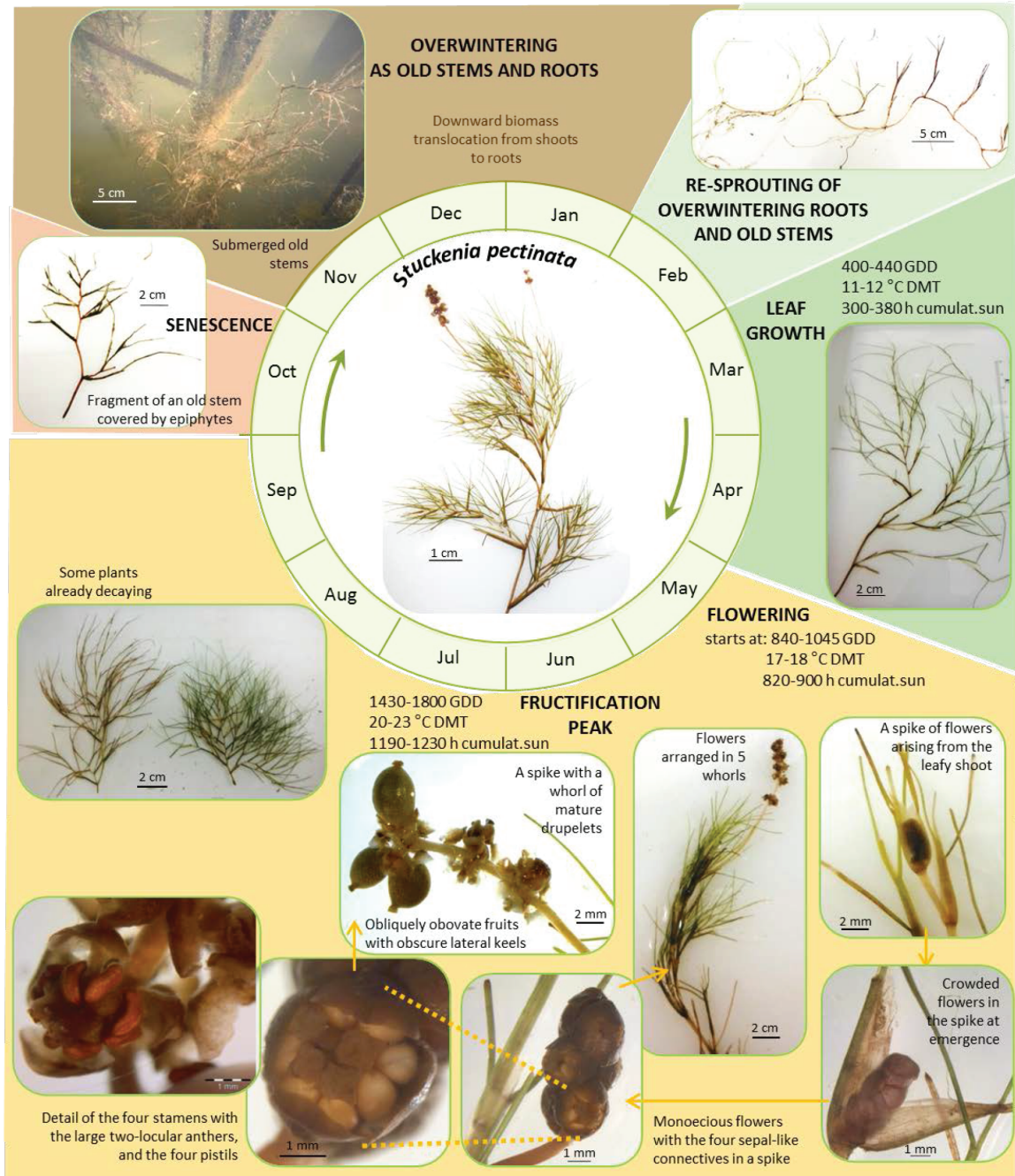


Figure 6. The life-cycle of the *Stuckenia pectinata* population in the LNC. The different events are distributed throughout the months of the year in a circular diagram. Accumulated growing degree-days (GDD), water daily mean temperature (DMT) and accumulated sunshine (hours of sun) for the most important phenological events are shown. *El ciclo de vida de la población de Stuckenia pectinata en la LNC. Los diferentes eventos están distribuidos a lo largo de los meses en un diagrama circular. Se muestran también los grados-día acumulados, la temperatura del agua media diaria (DMT) y las horas acumuladas de radiación solar (horas de sol) para los eventos fenológicos más relevantes.*

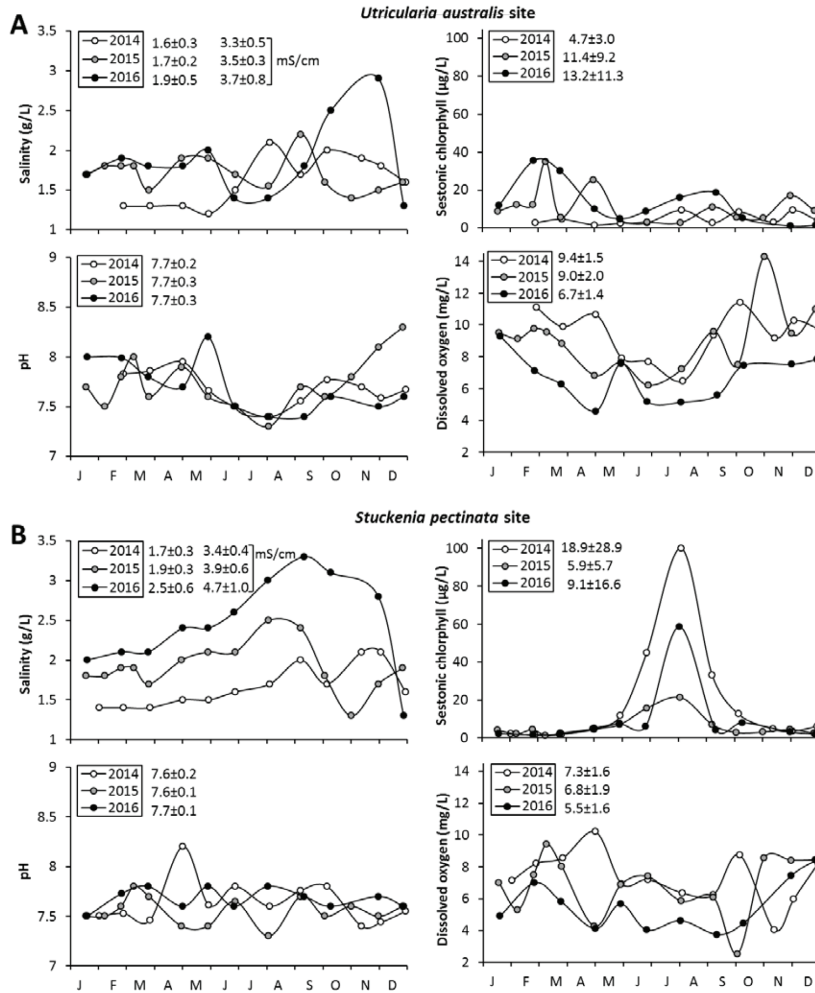


Figure 7. Fluctuations of limnological variables in the three consecutive years in the LNC. A: *Utricularia australis* site; B: *Stuckenia pectinata* site. Annual means±standard deviations are shown for each variable and each site. In the salinity graph, the annual mean (±SD) values for conductivity also appear. *Fluctuaciones de las variables limnológicas en los tres años consecutivos en la LNC. A: En la zona de Utricularia australis; B: En la zona de Stuckenia pectinata. Se muestran las medias anuales±desviaciones típicas para cada variable y en cada lugar. En la gráfica de salinidad, también aparecen las medias anuales (±DT) de la conductividad.*

the summer; the highest concentrations being found in 2014 (Fig. 7B). Water pH was very similar in both sites, with annual means of 7.6-7.7. Mean dissolved oxygen concentrations were quite variable at both sites, but always higher in the *Utricularia* site (Fig. 7A and 7B).

Multiple regression analyses of macrophyte features and the abiotic environment

The abundance of both *U. australis* and *S. pectinata* was only related to MMT and MHS (Table

1). Water salinity, pH and dissolved oxygen did not show any significant relationship. Turion formation in *U. australis* was not significantly related to either temperature or salinity. However, there was a significant negative relationship with MHS. The fertility of *S. pectinata* was significantly related to MMT and negatively related to water salinity (Table 1).

DISCUSSION

The life-cycle of *Utricularia australis* in the pond

After the arrival of *U. australis* in the LNC in autumn 2013, it overwintered by forming turions (Calero *et al.*, 2015), typical of the species (Taylor, 1989). When the turions began to germinate, the abundance of free-floating plants rapidly increased across the pond in spring 2014, despite being a nutrient-poor habitat. This situation was possible due to several ecophysiological adaptations that this plant exhibits, such as a very high net photosynthetic rate, its carnivorous activity, an efficient nutrient re-utilization from senescent shoots, and a very high affinity for mineral nutrient uptake from water (Adamec, 2011). The carnivorous activity of this *U. australis* population was proven not only by the presence of animals inside the traps, but also by the change in the colouring of the bladders, which varied throughout the annual cycle from purple to dark-blue, which is indicative of the digestion of the animal proteins (Cirujano *et al.*, 2014).

Even though sexual reproduction is quite uncommon for *U. australis* (Aguilella *et al.*, 2009; Araki & Kadono, 2003) this species flowered in the LNC during the summer of 2014, as we had already reported in Calero *et al.* (2015). The flowering species were very large and ramified, equivalent to the lower size threshold for sexual reproduction described by Adamec (2011), 40-70 cm in length depending on the system. The population from the LNC invested in sexual and vegetative reproduction at the same time. In August 2014, still in the flowering period, the specimens started to form turions; this pattern of starting with turion formation when the temperature was still high was repeated over the next two years. As shown by the negative relationship between the percentage of specimens forming turions and the monthly hours of sun, which explains more than 50 % of the variance in turion production, it seems that this process is more influenced by the length of the day. Our observations support the idea that solar radiation is a key factor for the development of *Utricularia* species (Englund *et al.*, 2003). The presence of turions, more than sexual reproduction, has

allowed the persistence of this species for at least three consecutive years in the pond.

The sites where the population from the LNC flowered (the sampling site in the southern part of the pond as well as some spots in the eastern part) received high exposure to the sun due to the scarce growth of cattails. During 2015-2016, there was no repetition of either flowering or such massive vegetative growth, showing how the same population of *U. australis* may flower, or not, depending on the interannual variations of the habitat. We consider that the species found the optimal habitat for the allocation of resources in generative reproduction (Adamec, 2011; Englund *et al.*, 2003) in this pond during 2014, but not afterwards. Since we did not find any significant relationship between *U. australis* and water salinity, and taking into account that this species is considered to be a eurytopic species with a very wide ecological tolerance to water chemistry (Adamec, 2011), it seems that the increase in salinity experienced by the pond is not the reason for the observed decrease in biomass and coverage. Charophyte meadows, possible competitors of *U. australis*, did not grow significantly in the sampled site. However, the cattail surface greatly expanded from 2014 to 2016 (see Fig. S3, Supplementary information), particularly in the shallow areas of the shores, considerably reducing the solar radiation reaching the water surface as well as the spatial habitat for *U. australis* development. We consider that the decrease in *U. australis* biomass and coverage, and the lack of further flowering, is closely related to this reduction in the free-water surface throughout the pond.

The life-cycle of *Stuckenia pectinata* in the pond

The *S. pectinata* population from the LNC showed a perennial life-cycle which initiated in late February, mainly by rapid outgrowth of old plants that vigorously produced new shoots with green leaves. *S. pectinata* has been described to employ habitat-specific reproductive strategies (Van Wijk, 1989; Wan *et al.*, 2016). Sexual fruits, tubers and nodal plantlets are the most important reproductive propagules in standing waters (Ganie *et al.*, 2016). In the LNC, the main observed remains of *S. pectinata* during the cold

season were old stems, these being the primary mode of vegetative reproduction and playing a key role in the annual regeneration. *S. pectinata* from the LNC also invested in sexual reproduction through the formation of flowers and achenes. However, the allocation of resources to sexual reproduction was not too high, since less than 25 % of the specimens in 2014, and less than 10 % in 2015 and 2016, were fertile. Other studies reported how *S. pectinata* can flower

abundantly, but the significance of seed production for yearly survival is considered to be very limited (Van Wijk, 1989). In the LNC, the high density of charophytes (Calero *et al.*, in preparation) may have hindered the germination of seeds, the sprouting of tubers, and even the growth of small plantlets from the pond bottom.

S. pectinata has been described to grow luxuriantly in many aquatic habitats (Eid *et al.*, 2013). Its growth in the LNC, though, is quite limited

Table 1. Results of the stepwise multiple linear regression analyses performed on the abundance of the two macrophytes species, and on turion formation in *U. australis* and *S. pectinata* fertility taking into account the abiotic environmental variables. *: significant; **: highly significant. *Resultados de los análisis de regresión múltiple realizados sobre la abundancia de las dos especies de macrófitos y sobre la formación de turiones en U. australis y la fertilidad en S. pectinata usando las variables abióticas ambientales.* *: significativo; **: altamente significativo.

	Coeff.	R ²	Multiple ²	p
<i>Utricularia australis</i>				
Abundance				
Monthly Mean Temp.	0.15	0.66	0.661	0.001**
Monthly Hours of Sun	0.01	0.52	0.694	0.042*
Water Salinity	0.26	0.03	0.696	0.564
Water pH	-0.16	0.15	0.696	0.836
Water Dissol. O ₂	0.12	0.07	0.715	0.171
Turion formation				
Monthly Hours of Sun	-0.50	0.51	0.508	0.005**
Monthly Mean Temp.	-0.65	0.28	0.523	0.730
Water Salinity	-20.00	0.00	0.552	0.303
<i>Stuckenia pectinata</i>				
Abundance				
Monthly Mean Temp.	0.19	0.74	0.737	<0.001**
Monthly Hours of Sun	0.01	0.69	0.819	0.001**
Water Salinity	-0.28	0.12	0.821	0.497
Water pH	-0.10	0.01	0.822	0.928
Water Dissol. O ₂	-0.05	0.15	0.823	0.650
Fertility				
Monthly Mean Temp.	1.64	0.43	0.428	0.001**
Water salinity	-5.73	0.002	0.540	0.025*
Monthly Hours of Sun	-0.04	0.22	0.570	0.273

and its presence is restricted to the deeper basins. In one such basin, the cover and biomass of *S. pectinata* was lower in 2015 and 2016 than in 2014. Although the species has a high tolerance to water conductivity changes (Menéndez & Sánchez, 1998), fertility decreased over the three years, along with a gradual increase in salinity; higher salinity values also negatively affected *C. hispida* reproduction in a nearby pond (Calero *et al.*, 2018). Thus, we consider that the salinity increase observed in the LNC, particularly in this site, together with the massive coverage increase in charophytes (Calero *et al.*, in preparation) were the main causes of the *S. pectinata* reduction in performance throughout time. Hidding *et al.* (2010) reported how *Chara*'s ability to act as a nutrient sink may contribute, through macronutrient-mediated competition, to the decline of *S. pectinata* in shallow lakes. In fact, in the present study, *S. pectinata* reached its maximum biomass after the other aquatic plants (*Typha domingensis* and *C. hispida*) which have their peak growth in summer. This can have a significant survival value to avoid competition for light and nutrients (Eid *et al.*, 2013).

Latitudinal variation of life-cycles

Several studies have reported latitudinal variation in the life-cycle of aquatic species, particularly in marine macro-algae, seagrasses and charophytes (Peters & Breeman, 1993; Calero *et al.*, 2017). However, information about latitudinal responses in freshwater angiosperms is not so abundant. For *U. australis*, there is an extensive bibliography concerning different aspects of its biology, but almost no information is available regarding its phenology (Thor, 1988); this fact prevents comparisons with our data. For *S. pectinata*, conversely, several studies focus on the phenology, biomass production and reproductive allocation at different locations. Based on information from Western Europe, Pilon *et al.* (2002) proposed a latitude-correlated switch in the *S. pectinata* life-cycle strategy: while northern populations show a short life-cycle, with an early and high investment in tuber biomass, low latitude populations have a prolonged life-cycle with delayed reproduction, an increased total

plant biomass, but an earlier biomass peak. The *S. pectinata* life-cycle in the southern LCN fits in with this pattern; although the biomass peak was observed later than in other southern European sites, there was interannual variability among the three years under consideration. The differences in *S. pectinata*'s phenology between studies in Spanish lagoons (Menéndez & Comín, 1989; Menéndez *et al.*, 2002; Obrador *et al.*, 2007; Prado *et al.*, 2013, etc.), and in many other sites (Van Wijk, 1988, 1989; Pilon *et al.*, 2002, etc.) may be due to intrinsic (i.e. genetic) and extrinsic factors such as local abiotic factors (temperature, solar irradiance, length of growing season, day length, water light extinction, water depth, nutrient condition, etc.) and local biotic factors (competition and/or herbivory). Santamaría (2002) found, regarding irradiance, that latitudinal variation was less important than local and seasonal differences. Thus, we need simultaneous studies across latitudes with special emphasis on standardizing the samplings and analysing important variables such as GDD, cumulative sunshine, etc., to draw geographical patterns.

On the conservation of macrophytes

The case of *U. australis* is a good example of the situation of many aquatic plants in Spain. Although initially the species do not appear to have difficulties regarding their preservation, since they reproduce asexually and have a rapid development, the loss of habitats has endangered their populations. In the Albufera de València Natural Park, *U. australis* was formerly cited in several aquatic ecosystems, but the species was not found in any cited locality during the survey performed in 1995, nor in the surveys carried out from 1999 (Aguilella *et al.*, 2009). Thus, the discovery of the population in the LNC (Calero *et al.*, 2015) represented the reappearance of the species after an absence of more than 20 years in the Park. During the samplings, we observed that waterfowl, mainly ducks and herons, regularly visited the ponds. Thus, the establishment of the population likely occurred by means of waterfowl dispersion (a fragment on the legs, plumage and/or guts; Figuerola *et al.*, 2005). This new population of *U. australis* proves that the restora-

tion of destroyed or degraded aquatic ecosystems is a turning point for local populations to recover, particularly for those endangered species in the Iberian Peninsula. In the studied waterbody, some management was necessary to reduce the extension of emergent vegetation, which seems to be the main cause of the regression of *U. australis*. Local authorities mechanically removed part of the helophytic biomass in November 2016, however, the emergent vegetation re-occupied most of the water surface by the next year (pers. observ.; October 2017). The removal of cattails to recover large populations of *U. australis* in this coastal pond is expensive and not sustainable in the long term. Therefore, maintaining small populations in different ponds, ensuring connectivity, appears to be the best option to recover populations of *U. australis* in the Natural Park.

S. pectinata reproduced mainly asexually in the LNC, but allocated some resources to flowering and fruit formation. This investment in sexual reproduction could assure the population's resistance to desiccation, a predicted trend for the ponds and coastal lagoons of the Mediterranean region (IPCC, 2014), as well as the genetic variability needed for a more likely adaptation of the species facing a changing environment. In this context, we highlight the need to continuously monitor water temperature and other relevant variables in situ, such as light availability and water salinity, in the long-term to better understand the environmental factors determining the reproduction of submerged vegetation, and its timing.

As addressed here, exploring or inferring relationships between biotic and abiotic variables in the short-term helps to disentangle which environmental factors to follow in long-term monitoring programs for macrophyte populations. Thanks to accurate knowledge of the conditions (the values of parameters such as accumulated degree-days and daily/monthly mean temperatures) that are required to pass from one phenological event to another, we will be able to compare among populations from different origins and across biogeographic ranges. All this information will eventually enable the prediction of the long-term persistence of species facing desiccation.

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