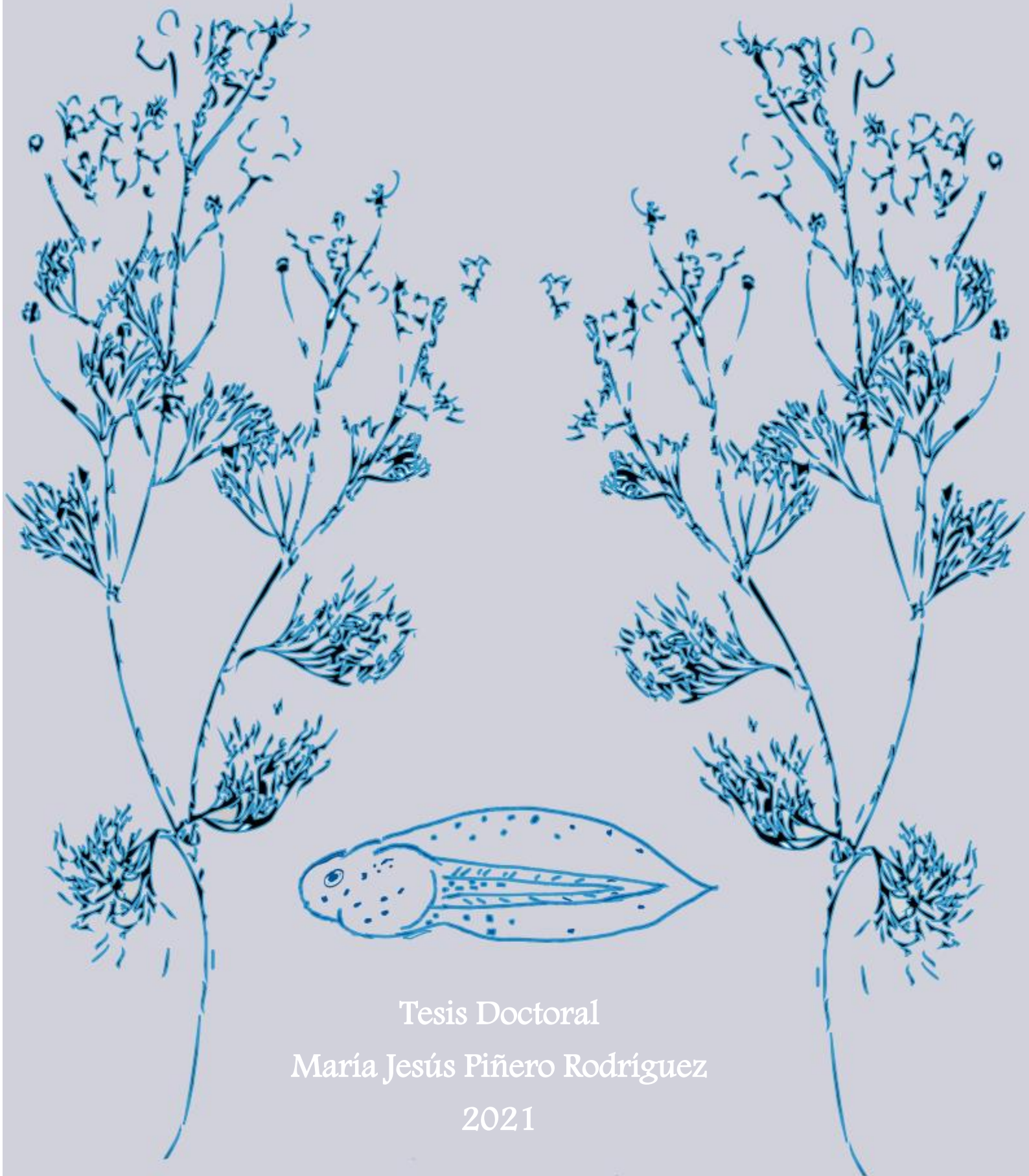


Interacciones ecológicas entre larvas de anfibios y macrófitos acuáticos en el Parque Nacional de Doñana



Tesis Doctoral
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INTERACCIONES ECOLÓGICAS ENTRE LARVAS DE ANFIBIOS Y MACRÓFITOS ACUÁTICOS EN EL PARQUE NACIONAL DE DOÑANA

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Resumen:

A pesar del incremento del conocimiento científico sobre la herpetofauna, mucho queda aún por descubrir en cuanto a su ecología básica. Así, aún no comprendemos de manera profunda cuáles son todas sus funciones ecosistémicas, ni las repercusiones que puede ocasionar la disminución o la pérdida de sus poblaciones. Con los trabajos desarrollados en esta tesis queremos profundizar en el conocimiento de las interacciones entre las larvas de anuros y los macrófitos acuáticos: comprendiendo el papel que ejercen las larvas de anuros como herbívoros; conociendo cómo responden a este estrés los macrófitos acuáticos; analizando cómo las especies vegetales invasoras pueden afectar a las larvas de los anfibios; y conociendo los movimientos espaciales de las larvas de anfibios, ya que con ello podemos detectar las conexiones espaciales y la magnitud de las interacciones con los diferentes componentes del ecosistema. Para conseguir las respuestas a todas estas cuestiones hemos llevado a cabo diferentes estudios entre los años 2015 y 2017, tanto en condiciones experimentales en mesocosmos como directamente en las lagunas temporales del Parque Nacional de Doñana. Como resultados más notables podemos destacar que hemos observado cómo las larvas de *Pelobates cultripes* desarrollan un importante papel como herbívoros en las lagunas temporales, reduciendo la biomasa de los macrófitos acuáticos, y con ello su éxito reproductivo, y disminuyendo la complejidad estructural de los hábitats acuáticos. Hemos comprobado también cómo las larvas de *P. cultripes* alcanzan altas densidades incluso en lagunas de gran extensión, y además son capaces de recorrer grandes distancias en un corto espacio de tiempo, sugiriendo que su impacto es generalizado en toda la laguna. Por otro lado, destacamos que la especie invasora *Azolla filiculoides* ocasiona un efecto en cascada en los medios acuáticos que invade, alterando y modificando todos los niveles del ecosistema al concurrir en una fuerte competencia con los macrófitos autóctonos, a los que ocasiona la disminución de su biomasa o su eliminación completa. Asimismo, el deterioro que sufre el medio acuático afecta negativamente a la condición corporal de los metamórficos de *Hyla meridionalis* e incluso incrementa la mortalidad de *Pelobates cultripes*. En general, *A. filiculoides* disminuye la calidad de los nutrientes a lo largo de la cadena trófica. Por todo ello, podemos concluir que las interacciones entre las larvas de anfibios y los macrófitos acuáticos están relacionadas por múltiples vías de conexión, tanto de forma directa como indirecta, lo cual hasta ahora había sido escasamente documentado, necesitándose un mayor conocimiento para esclarecer estos nexos de conexión y con ello contribuir a la conservación de la dinámica de los medios acuáticos temporales.

Introducción general:

Las lagunas temporales son cuerpos de agua relativamente pequeños y someros que se caracterizan por sufrir fases de desecación recurrentes, tanto de manera cíclica como aperiódica (Ramsar, 2002; D.D.Williams, 2006). Esta definición de lagunas temporales engloba un amplísimo abanico de cuerpos de agua, desde charcas efímeras o praderas inundadas a lagunas de gran extensión que pueden tener tanto largos como cortos periodos de inundación (o *hidroperiodo*). En general, son cuerpos de agua pequeños y someros (menores de 10 ha), y con una flora y fauna muy especializada, ya que se componen de especies acuáticas adaptadas a resistir periodos secos (Williams, 2006; Grillas et al., 2004).

El área de la superficie inundada no es necesariamente un indicador fiable del hidroperiodo de una laguna temporal, sino que éste está determinado además de por la topografía del terreno y las precipitaciones, por la composición de los suelos y la dinámica de las aguas subterráneas. De este modo, grandes extensiones inundables pero cuyo único aporte sea la precipitación directa pueden ser más efímeras que lagunas de un tamaño mucho más reducido pero localizadas en zona de afloramiento de un acuífero. En cuanto a los flujos de salida de agua de estos sistemas temporales, se deben sobre todo a evaporación directa e indirectamente a la transpiración de las plantas, aunque el agua puede perderse también por percolación a través de sustratos permeables como arenas o karst (Grillas et al., 2004). La calidad y composición química del agua en las lagunas temporales, además de la cantidad, se deriva de la resultante entre la precipitación, la escorrentía, la conexión con las aguas subterráneas y los procesos de desecación (Serrano et al., 2006). El tipo de sustrato sobre el que se asientan las lagunas determina también las características físico-químicas del agua, lo que a su vez condiciona la composición de las comunidades biológicas que pueden albergar. Así, lagunas situadas sobre sustratos rocosos o arenosos normalmente tienen aguas claras, mientras que aquellas sobre terrenos con un menor tamaño de partícula, como arcillas o limos, causan una mayor turbidez. Del mismo modo, lagunas que se asientan sobre arenas silíceas suelen tener mayor acidez, y en cubetas con una elevada concentración de materia orgánica disuelta de origen vegetal pueden presentar aguas fuertemente coloreadas por los polifenoles derivados, como sucede en el Parque Nacional de Doñana (Serrano, 1992).

Las lagunas temporales son sistemas extremadamente dinámicos y heterogéneos, pudiendo constituir desde un pobre pastizal de hierbas anuales que resisten temporalmente la inundación, a lagunas donde la producción primaria corresponde a una gran riqueza de macrófitos y algas. Esta heterogeneidad tiene como consecuencia que las lagunas temporales

alberguen una alta biodiversidad (Williams, 1997; Williams, 2004; Florencio et al., 2009; Jeffries, 2011; Florencio et al., 2020), compuesta además por especies singulares, sometida a la peculiaridad de enfrentarse a sucesiones ecológicas secundarias recurrentes (Williams, 1997; Boix et al., 2003). En particular, las lagunas temporales de las áreas Mediterráneas presentan ciclos de inundación con una enorme variabilidad interanual, con periodos de sequía intercalados por años de abundantes o escasas precipitaciones. Esta heterogeneidad de los ciclos de inundación hace que las características de las lagunas, desde su tamaño a su composición química varíen enormemente espacial y temporalmente, variando también la composición de sus comunidades y por tanto la intensidad de las interacciones ecológicas que se dan dentro de ellas (Wilbur, 1997; Gómez-Rodríguez et al., 2010; Florencio et al., 2011).

En consecuencia, los organismos que habitan estos ambientes tan fluctuantes han evolucionado adaptaciones que les permiten subsistir a pesar de la estocasticidad ambiental (Williams, 1985). Estas adaptaciones comprenden desde bancos de semillas de macrófitos (Tuckett et al., 2010; Carta, 2016; Metzner et al., 2017), a acinetos de algas y huevos o quistes de resistencia del zooplancton (Holzinger & Karsten, 2013; Hairston et al., 2000; Brendonck & De Meester, 2003; Cáceres & Tessier, 2004) o la capacidad de ajustar el desarrollo larvario a las condiciones imperantes (Newman, 1992; Gomez-Mestre et al., 2013). Uno de los grupos taxonómicos y ecológicos fundamentales en las lagunas temporales, y que cobran un valor único en las lagunas Mediterráneas, son las comunidades de plantas acuáticas, que a menudo presentan una alta diversidad de especies y contienen una gran proporción de especies amenazadas (Grillas et al., 2004). Las comunidades de macrófitos acuáticos comprenden tanto aquellas especies que completan su ciclo en el medio acuático como aquellas que sólo desarrollan parte del mismo en el agua, y además de plantas vasculares, se componen de varios géneros de briófitos y algas carófitas (García-Murillo et al., 2010). Las comunidades de macrófitos en particular son un elemento fundamental de las lagunas temporales porque constituyen el grupo principal de productores primarios y confieren complejidad estructural al sistema. Los macrófitos pueden ser emergentes, estar totalmente sumergidos o con parte de sus estructuras flotantes o aéreas, o totalmente flotantes (Cronk & Fenessy, 2001). Presentan adaptaciones estructurales específicas para la vida en el agua, como tallos endebles con finas cutículas y escaso xilema en las especies sumergidas, o la heterofilia, con hojas estrechas o lineares sumergidas, combinadas con otras ensanchadas cuando alcanzan la superficie (Schulthorpe, 1967; Cronk & Fenessy 2001).

Las lagunas temporales albergan también una enorme diversidad de comunidades de invertebrados, que dependen de estos cuerpos de agua para su reproducción. Al igual que en

el caso de las comunidades de plantas, algunas especies de invertebrados solamente pasan en las lagunas temporales parte de su ciclo biológico (p.e. dípteros y odonatos), mientras que otros completan su ciclo vital en ellas, como los grandes branquiópodos y muchas especies de coleópteros y heterópteros. La ausencia de peces en la mayor parte de las lagunas temporales favorece la presencia de crustáceos zooplanctónicos, incluyendo notostráceos, cladóceros, anostráceos y conchostráceos (Alonso, 1996; Díaz-Paniagua et al., 2010; Fahd et al., 2009). La composición y abundancia de las comunidades de invertebrados en las lagunas temporales está determinado por el hidropериодо, la profundidad, la superficie, la concentración de oxígeno disuelto y la riqueza de macrófitos (della Bella et al., 2005; Florencio et al., 2009).

En cuanto a los vertebrados, encontramos varios grupos taxonómicos ligados a las lagunas temporales. Muchas especies de aves están ligadas a los medios acuáticos temporales y cumplen una función importante en la dispersión de semillas y propágulos (Figuerola & Green, 2002, 2003). Algunas especies migratorias de anátidas también usan estos medios como lugares de invernada o de descanso durante su migración (Grillas & Roché, 1997). También encontramos especies de reptiles, como culebras o galápagos, con un importante papel como depredadores o consumidores primarios en lagunas temporales (Bodie & Semlitsch, 2000; Pérez-Santigosa et al., 2011). No obstante, el grupo de vertebrados más íntimamente ligado a las lagunas temporales son los anfibios, para quienes son hábitats de reproducción claves (Griffiths, 1997), y en los cuales llegan a representar una amplia proporción de la biomasa total (Gibbons et al., 2006).

La mayoría de las especies de anfibios retienen aún el ciclo de vida bifásico ancestral, con una fase larvaria acuática que metamorfosea en un juvenil terrestre. La explotación de un medio tan heterogéneo en su duración y calidad como son las lagunas temporales ha favorecido la evolución de mecanismos por los que las larvas de anfibios pueden ajustar sus trayectorias de crecimiento y desarrollo para maximizar las oportunidades mientras las condiciones locales son adecuadas. Esta adecuación de la fase larvaria a las condiciones del medio ha evolucionado apoyándose en dos peculiaridades de las larvas de anfibios. Por un lado, son organismos capaces de percibir con gran detalle información ambiental que les permite evaluar las fluctuaciones en el medio. Pueden, por ejemplo percibir visualmente la presencia de depredadores de cierto tamaño como peces o cangrejos (Stauffer et al., 1993; Melotto et al., 2020), pero también percibir las pistas químicas de depredadores más pequeños y menos conspicuos como las larvas de odonatos o de coleópteros (Kiesecker et al., 1996; Gomez-Mestre & Díaz-Paniagua 2011). Del mismo modo, son capaces de advertir la disminución de la columna de agua y el aumento de la densidad de conoespecíficos como

indicadores de la desecación de la laguna (Newman 1989, 1992). Por otro lado, las larvas de anfibios tienen la capacidad de desacoplar su crecimiento y desarrollo en función de la percepción de la calidad ambiental, deteniendo el desarrollo y maximizando el crecimiento en condiciones benignas, o deteniendo el crecimiento y precipitando una metamorfosis temprana cuando las condiciones empeoran (Wilbur & Collins 1973; Gomez-Mestre et al., 2010; Touchon et al., 2015).

Los anfibios, y en especial sus larvas, cumplen múltiples funciones en los medios acuáticos temporales, llegando a afectar las propiedades físico-químicas del agua y a determinar la estructura y dinámica de las comunidades acuáticas (Bucket et al. 2012; Arribas et al., 2014). En concreto, las larvas de anfibios tienen un gran impacto sobre la producción primaria, los ciclos de nutrientes, la descomposición de hojarasca, la dinámica poblacional de invertebrados (Wilbur, 1997; Whiles et al., 2006; Regester, Whiles & Lips, 2008; Costa & Vonesh, 2013; Arribas et al., 2014). Las larvas de muchas especies de anuros son fundamentalmente consumidores primarios, pudiendo ingerir grandes cantidades de fitoplancton y de macrófitos (Díaz-Paniagua, 1985; Arribas et al., 2014; Arribas et al., 2015), aunque también pueden favorecer el crecimiento de macrófitos presuntamente al consumir el perifiton adherido a los tallos y ramas sumergidas (Arribas et al., 2014), e incluso la dispersión de sus semillas (Arribas, 2016). Algunas especies con larvas de gran tamaño podrían condicionar los patrones de crecimiento y la reproducción de las plantas acuáticas. En el primer capítulo de esta tesis nos aproximamos a esta cuestión analizando el efecto de las larvas de sapo de espuelas (*Pelobates cultripes*) sobre el crecimiento y la reproducción de macrófitos sumergidos comunes en el Parque Nacional de Doñana (Capítulo 1), examinando esta interacción renacuajo-planta también en el contexto de la variación del hidropereodo.

Por otro lado, las larvas de anfibios también pueden ser consumidores secundarios que condicionan las comunidades de zooplancton (Arribas et al., 2014; Escoriza et al., 2020). Gran parte del conocimiento obtenido sobre las interacciones ecológicas de las larvas de anfibios se ha generado mediante aproximaciones experimentales, tanto en laboratorio como en mesocosmos. Estas aproximaciones son muy eficaces a la hora de examinar hipótesis, pero en cambio limitan en cierta medida el realismo de las condiciones manipuladas, en el tiempo y en el espacio. Uno de los aspectos esenciales de la biología de cualquier organismo, y del que menos conocemos en el caso de las larvas de anfibios es el de sus patrones de movimiento. Los patrones de movimiento de cualquier especie determinan el grado de heterogeneidad ambiental a la que se expone, así como su probabilidad de encuentro con conespecíficos, depredadores, parásitos, etc. En esta tesis abordamos por primera vez un estudio en campo de

los patrones de movimiento de larvas de anuros en lagunas temporales, como un primer paso para contextualizar en el medio natural el conocimiento que vamos obteniendo del papel de las larvas de anfibios en los medios acuáticos (Capítulo 2).

Por todo lo mencionado anteriormente, las lagunas temporales, y dentro de estas, las lagunas temporales Mediterráneas en particular, son sistemas de un enorme valor ecológico. Por desgracia, son sistemas bastante vulnerables y sujetos a un alto nivel de amenaza. Las mayores amenazas a las lagunas temporales son su destrucción directa mediante desecación o sobreexplotación de los acuíferos que puedan alimentarlas, pero también su exposición a contaminantes como pesticidas o herbicidas, y su colonización por especies exóticas invasoras (Gallardo et al., 2016). Aunque el propio dinamismo y corto hidropериodo de estos sistemas puede dificultar el establecimiento a largo plazo de especies invasoras acuáticas (Díaz-Paniagua et al., 2014), el impacto de especies como el cangrejo rojo americano (*Procambarus clarkii*), la gambusia (*Gambusia holbrooki*), o el corixido *Trichocorixa verticalis* son muy marcados y ampliamente estudiados. De las especies invasoras con un mayor impacto en los medios acuáticos se han descrito ampliamente algunas plantas como el carrizo (*Phragmites australis*) o el espartillo (*Spartina alterniflora*) (Gallardo et al., 2014), o el jacinto de agua (A.E. Williams 2006). Una especie invasora con un gran efecto potencial sobre las lagunas temporales pero cuyo efecto sobre los ecosistemas acuáticos es menos conocido es el del helecho flotante *Azolla filliculoides*, del que se ha descrito ampliamente su expansión en las marismas de Doñana (García Murillo et al., 2007; Aragonés et al., 2009; Fernández-Zamudio et al., 2013; Espinar et al., 2015). Los dos últimos capítulos de esta tesis se centran en estudiar el impacto de esta especie invasora sobre las comunidades de las lagunas temporales, con especial énfasis en los macrófitos y las larvas de anfibios (Capítulo 3), así como en estudiar las posibles respuestas de algunos de estos organismos al helecho invasor (Capítulo 4).

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1. Los renacuajos como herbívoros en las lagunas temporales (Herbivory by spadefoot toad tadpoles and reduced water level affect the abundance and life-history of submerged plants in temporary ponds)

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En revisión en *Inland Waters*.

Herbivory by spadefoot toad tadpoles and reduced water level affect the abundance and life-history of submerged plants in temporary ponds:

Abstract

In temporary ponds, submerged plants have adapted to recurrent periods of drought. These ponds often lack the impact of large herbivores, but can harbor large numbers of herbivorous tadpoles. Tadpoles of the Iberian spadefoot toad (*Pelobates cultripes*) may reach large body sizes and consume large quantities of macrophytes during their long larval period, which they can shorten when faced with risk of pond drying. We conducted a mesocosms experiment to analyze the interplay between hydroperiod and impact of herbivorous tadpoles on the abundance and life history of three species of submerged plants common to temporary ponds. We observed differences in growth among plant species resulting in lags in their timing of maximum cover, probably related to interspecific competitive interactions. Some plants responded to pond drying with a remarkable increase in the production of flowers. Tadpoles greatly reduced the plant cover, although their impact varied across plant species. Differential plant consumption by these large tadpoles can substantially modify the internal structure and complexity of temporary ponds, and they can also interfere with the plants' responses to shortened hydroperiod.

keywords: Aquatic macrophytes, amphibians, pond hydroperiod, plant phenology

Introduction

Submerged plants are important components of freshwater ecosystems, where they can modify the physical and chemical environment (see e.g. Carpenter and Lodge 1986; Lodge 1991) and also provide structural complexity that serves as refuge, food or support to other aquatic organisms (Cronk and Fennessy 2001). Herbivores may considerably alter aquatic habitat structure (Lodge et al. 1998; Bakker et al. 2016), causing greater impact the more biomass they can consume. Many aquatic invertebrates feed mainly on periphyton or phytoplankton, whereas others, as gastropods and large crustaceans, graze submerged macrophytes (Carpenter and Lodge 1986). Among vertebrates, waterfowls and many fish are important herbivores, grazing high quantities of aquatic plants that may considerably affect the structure of these habitats. Also, aquatic turtles include an important fraction of aquatic vegetation in their omnivorous diet (Lodge et al. 1998; Pérez-Santigosa et al. 2011). In the case of anuran amphibians, although postmetamorphic stages rarely consume plants, tadpoles are commonly herbivorous (Altig et al. 2007; Montaña et al. 2018). Taking into account the proportion of aquatic macrophytes consumed by vertebrates, Lodge et al. (1998) considered that this group could cause a higher impact on vascular plants than any other taxonomic group, even though the impact of amphibians was not considered in this study.

Tadpoles may reach high abundances in their habitats (Gibbons et al. 2006; Pinero-Rodriguez et al. 2020) in which they have an important role, contributing to nutrient cycling, altering the trophic web, and even causing bioturbation by modifying the distribution of sediments (Costa and Vonesh 2013; Cortés-Gómez et al. 2015, Kohl et al. 2019). They can potentially cause an important decrease of their food resources, including submerged plant biomass (Arribas et al. 2014). Tadpoles are often primary consumers, or even omnivorous, for which algae, phytoplankton or periphyton are the most important components of their diet. Nevertheless, they can shift their diet in response to the availability of resources, or as a result from competition and predation risk (Altig et al. 2007; Montaña et al. 2018, Arribas et al 2018). Tadpole diets of some Iberian species have been described in detail (Díaz-Paniagua 1985; 1987). They mainly feed on algae and detritus, and also include a high proportion of macrophytes in their diet (Díaz-Paniagua 1985). An isotopic analysis revealed that submerged macrophytes were the most assimilated food source in some of these species (Arribas et al. 2015). The macrophyte contribution to the tadpoles' diet may differ considerably among species and also in relation to the body size of tadpoles. Anurans with small tadpoles

can favor the growth of aquatic plants by grazing the periphyton attached to their submerged stems (Arribas et al. 2014). In contrast, large tadpoles, as those of the western spadefoot toad, *Pelobates cultripes*, can cause a great impact on aquatic media, largely decreasing or even exhausting the macrophyte biomass (Arribas et al. 2014; 2015).

Temporary wetlands have a singular flora and fauna, including aquatic species which can resist the dry phase of these media (Grillas et al. 2004; Williams 2006). Pond drying is therefore the main risk for the inhabitants of these wetlands, and they have evolved different strategies to cope with the dry periods. Many amphibian species, for instance, develop as larvae in temporary ponds, and are also adapted to the fluctuations in hydroperiod by either having a short developmental time or the ability to perceive the risk of pond drying and accelerate their development accordingly (Richter-Boix et al. 2006). In Mediterranean temporary ponds, the emergence of aquatic organisms is synchronized with the beginning of the wet cycle, as detected for zooplankton (Florencio et al. 2020) and macrophyte communities (Fernández-Zamudio et al. 2017). Mediterranean temporary ponds are particularly rich in macrophytes (Grillas et al. 2004; Rhazi et al. 2006; Pinto-Cruz et al. 2012; Bagella and Caria 2012; Díaz-Paniagua et al. 2015), and the emergence of seedlings is conditioned by the timing of pond inundation, independently of its occurrence in Autumn, Winter or Spring (Fernández-Zamudio et al. 2017). The life cycle of most plants in temporary ponds is annual, decaying when the water temperature increases and the ponds are close to drying out. In the subsequent aquatic phase, the plant assemblage is re-established by means of a persistent seed bank (Bonis et al. 1995; Brock et al. 2003; Aponte et al. 2010). However, how plants respond to pond drying, and the extent to which amphibian larvae can interfere with that response, are largely unknown.

In our study area, some amphibians start breeding immediately after pond inundation (Díaz-Paniagua 1992). In particular, the western spadefoot toad, *Pelobates cultripes* lays eggs immediately after pond inundation, and extends the larval phase until the ponds are about to dry out. This is the anuran with the largest tadpole body size in all of the Iberian Peninsula. The diet of spadefoot toad tadpoles is omnivorous, although they are predominantly herbivorous feeding on algae and macrophytes, they also commonly ingest detritus and invertebrates (Díaz-Paniagua 1985, Arribas et al 2015, Escoriza et al. 2016), exploiting primary production in the ponds and consuming such

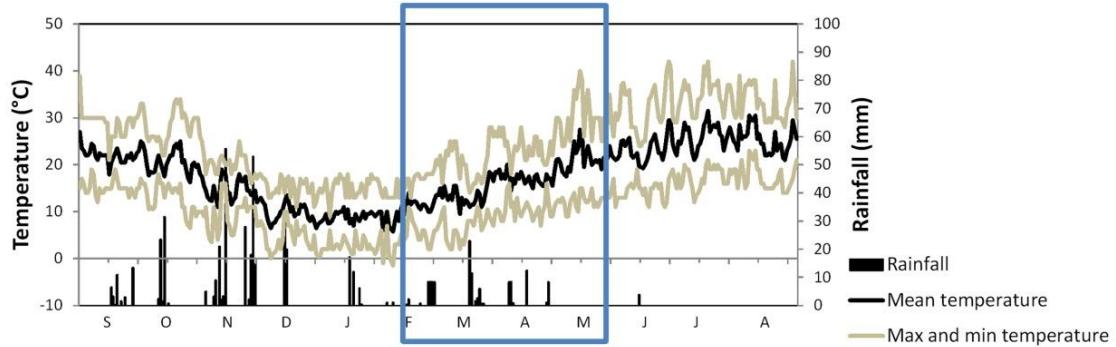
large quantities of macrophyte biomass that can modify the structure of these aquatic habitats (Arribas et al. 2014; 2015).

Here we study the interplay between the impact of large herbivorous tadpoles and risk of pond drying on submerged plants, to assess the potential interference of herbivory on the plant responses to pond drying. For this purpose, we carried out a mesocosms experiment simulating the annual wet cycle of temporary and permanent ponds, while manipulating the presence/absence of spadefoot toad tadpoles. We predicted that i) tadpoles would differentially reduce the biomass of the plant species included in the experiment; ii) that plants would respond to pond drying advancing their reproductive cycle, and iii) that tadpole herbivory would interfere the plants' responses to pond drying.

Material and Methods

We carried out a mesocosms experiment at the Doñana Biological Reserve (37°00'N, 6°38'W), within Doñana National Park. This area is located in southwestern Spain, close to the Atlantic coast. Temporary ponds are very abundant in this area, being the main breeding site for seven of the 11 amphibian species present in the park. After a summer dry phase, the wet phase of the ponds starts when the accumulated rains raises the groundwater table over the bottom of the basin, which may occur either in autumn, winter or spring. At the end of the spring, or during the summer, the rise of temperatures and evaporation cause pond drying (a detailed description of the inundation cycle of this pond network is given in Díaz-Paniagua et al. 2010; 2015). The variation of temperature and rainfall of the area during the annual cycle in which the experiment was carried out is shown in Fig. 1.

Figure 1. Variation of temperature and daily rainfall in Doñana Biological Reserve and the extension of the experimental period within the hydrological cycle (Sept 1st 2014 to August 31st 2015) of the study year. The experimental period during which tadpoles of *Pelobates cultripes* were included in the mesocosms is framed in blue.



We established mesocosms using 40 round 500L tanks volume (96 cm high and 120 cm in diameter). Each tank received 50 L of sand plus a homogenized mixture of 4 L of pond sediment from several nearby ponds to provide them with propagules (seeds, spores or zooplankton cysts) enabling plants and zooplankton to grow naturally in the tanks, upon inundation. The tanks filled naturally with rainfall in the autumn, and we added additional well water until the water level reached the 400 L mark, over the thick layer of substrate. We left the mesocosms undisturbed until February 2015, when we first visually inspected the abundance of plants of different species in the tanks. The vegetation of the tanks were mainly composed of three macrophyte species: *Callitriche obtusangula*, *Myriophyllum alterniflorum* and *Ranunculus peltatus*. These are common macrophytes in Doñana temporary ponds, where they reach can high density, providing spatial complexity to the water column (Fernández-Zamudio et al. 2016). The presence of other species was scarce and very occasional (*Elatine macropoda*, or some charophytes). We removed or transplanted plants among tanks until all of them had a similar proportion of the three most abundant submerged macrophytes. All the tanks received transplanted plants, in order to homogenize the effect of this perturbation.

We used a 2x2 factorial design, considering the effect of two factors: hydroperiod, and herbivory. The effect of hydroperiod was tested with two levels, simulating either permanent ponds with a constant high water level (400 L, PERM), or temporary ponds where we gradually decreased the water level (TEMP). To analyze the

effect of herbivory, we manipulated the presence/absence of herbivorous tadpoles. In mesocosms assigned to the herbivory treatment (Pcul), we introduced two tadpoles of *P. cultripes*, which are big-sized and macrophyte grazers (Arribas et al. 2014), whereas tadpoles were absent from the remaining tanks (No Pcul). We only included two tadpoles based on our previous experience, as a higher number of tadpoles could have exhausted macrophytes too fast and it would not allow us to measure a gradual variation of plant biomass throughout the experimental period. Each treatment was replicated 10 times: PERM-NoPcul, PERM-Pcul, TEMP-NoPcul, TEMP-Pcul.

The experiment began when we introduced the tadpoles, February 26th 2015. Average tadpole body mass was 0.21 g [0.02 SD], and total length was 29.39 mm [2.41 SD]. We kept tadpoles in the tanks until April 14th, at which time they had reached an average body mass of 15.43 g [4.46 SD] g and total length of 111.11 mm [13.17 SD]. To keep the target water level in each treatment, we checked the tanks weekly. In permanent tanks, we added water as needed to maintain the 400L level. In tanks simulating temporary ponds, 15 days after tadpole introduction, we removed 25L water weekly until they reached the 250L mark on April 15th, and maintained this reduced level constant until June 5th 2015, when we terminated the experiment.

We estimated the plant cover over the water surface of the tanks by counting the number of cells containing each plant species in a sampling quadrat with 36 cells of 10x10 cm. These data were recorded thrice: on February 25th (initial data), March 26th and April 10th..

We obtained phenological data of *M. alterniflorum* and *R. peltatus*, recording the day when we observed the first flower of each species per tank, and estimating the length of the period from the first to the last flower observation. We could not get similar data for *C. obtusangula* due to the difficulty of detection of its submerged flowers, and also because many of these plants scarcely produced flowers due to the early consumption of the stems by tadpoles. We recorded plant phenological data weekly, from March 7th to May 22nd (13 weeks in total). We also quantified the number of cells with flowers, using a 12x12 cells quadrat, with a cell size of 5x5cm. Also, in April, we marked three individuals of each plant species per tank, and monitored them weekly until the end of the experiment. We could only monitor individuals of *M. alterniflorum* (since April 8th) and *R. peltatus* (since April 2nd), because of the early consumption of *C. obtusangula* in the tanks. We counted the number of the monitored

plants surviving (still present in the tank) each week, to estimate survival curves through the remaining experimental period.

Statistical analyses

To compare plant cover among treatments through the three sampling times we used generalized linear mixed models (GLMM) with binomial error distribution, considering hydroperiod and herbivory as fixed factors, and tanks as random factor to control the variation of the cover in a same tank in the three different sampling times. The response variable was a vector including, for each tank and month, the values of the number of cells containing each macrophyte species and the number of cells which did not contain it. A similar analyses was performed to compare the number of cells with flowers through the experiment. We used GLMM with binomial error distribution, in which the response variable was a vector (number of cells with flowers, number of cells with no flowers), the fixed factors were herbivory and hydroperiod, and tank (weeks) was the random factor. We also included the initial plant cover as covariable to control for different plant densities among tanks. To analyze the time to the first flower production, we fit a generalized model with negative binomial error distribution, using data of the first flower in each cell of the quadrat per tank as variable response, and hydroperiod and herbivory as predictors, and initial plant cover as covariable. The length of the flowering period fitted a generalized linear model with Gaussian error distribution and the same predictor variables, after checking for the normality assumptions required.

We analyzed the effect on plant survival taking into account the survival of individually monitored plants at the end of the experiment (data from April 30th data for *R. peltatus* and from May 22nd for *M. alterniflorum*). We used a GLMM with binomial error distribution, using tank as random factor, and herbivory and hydroperiod as fixed factors. The response variable was a vector including for each monitored plant, if it was still present and surviving (1) or if it had been completely consumed and did not survive (0). We used R version 3.6.3 (R Core Team 2020) for all analyses.

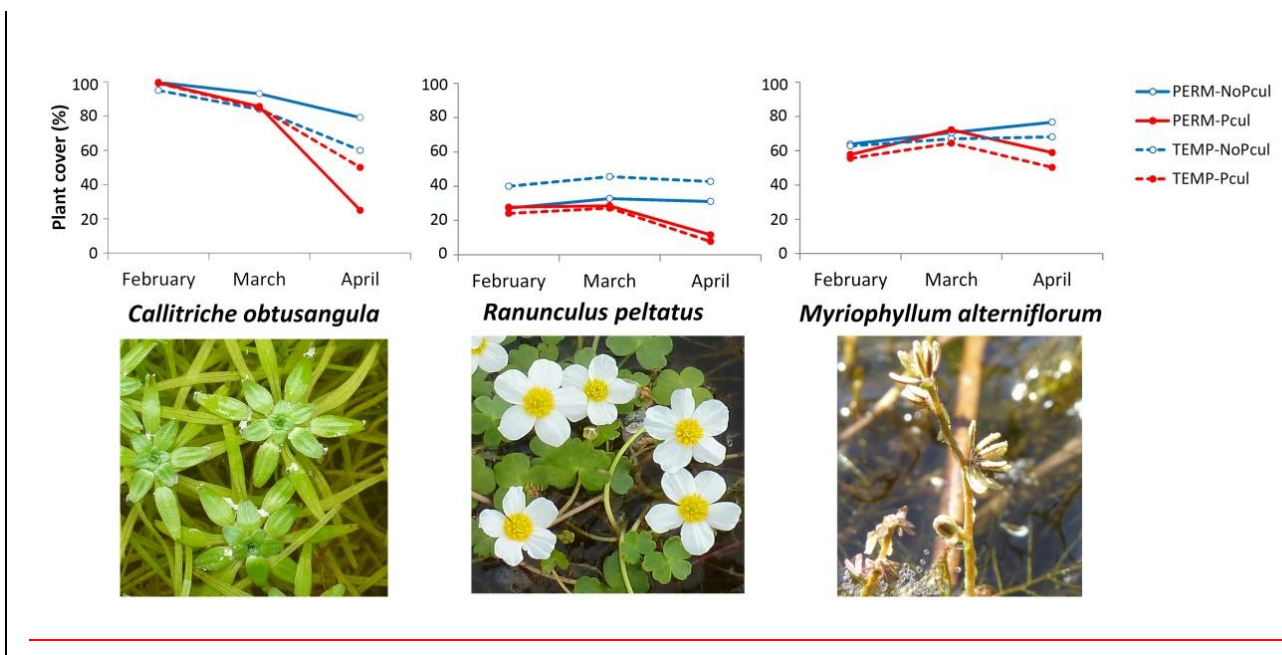
Results

Effect of tadpole herbivory and pond drying on plant cover

At the beginning of the experiment, the species with the highest plant cover was *C. obtusangula*, present on average in 99% of the water surface across all tanks, whereas the cover of *M. alterniflorum* was 60% and that of *R. peltatus*, 30%. We observed a

gradual reduction in the cover of *C. obtusangula* in March and April in all tanks, significantly affected by the tadpole herbivory ($\chi^2 = 7.403$, $p = 0.006$) and by the interaction of herbivory and hydroperiod ($\chi^2 = 4.3944$, $p = 0.036$). The presence of tadpoles caused the highest reduction in cover in tanks with temporary hydroperiod, with a 54% reduction in the cover of *C. obtusangula*. The other two species still showed a slow increase in their cover in March. However in April, *M. alterniflorum* showed only a low decrease in cover in the presence of herbivores, and a slight increase in the absence of herbivores, not exhibiting significant effects of the treatments (Fig 2, Table 1A).

Figure 2. Differences among the average plant cover occupied by each of the three submerged macrophyte species in the presence or absence of herbivorous spadefoot toad tadpoles, and in constant high or decreased water levels.



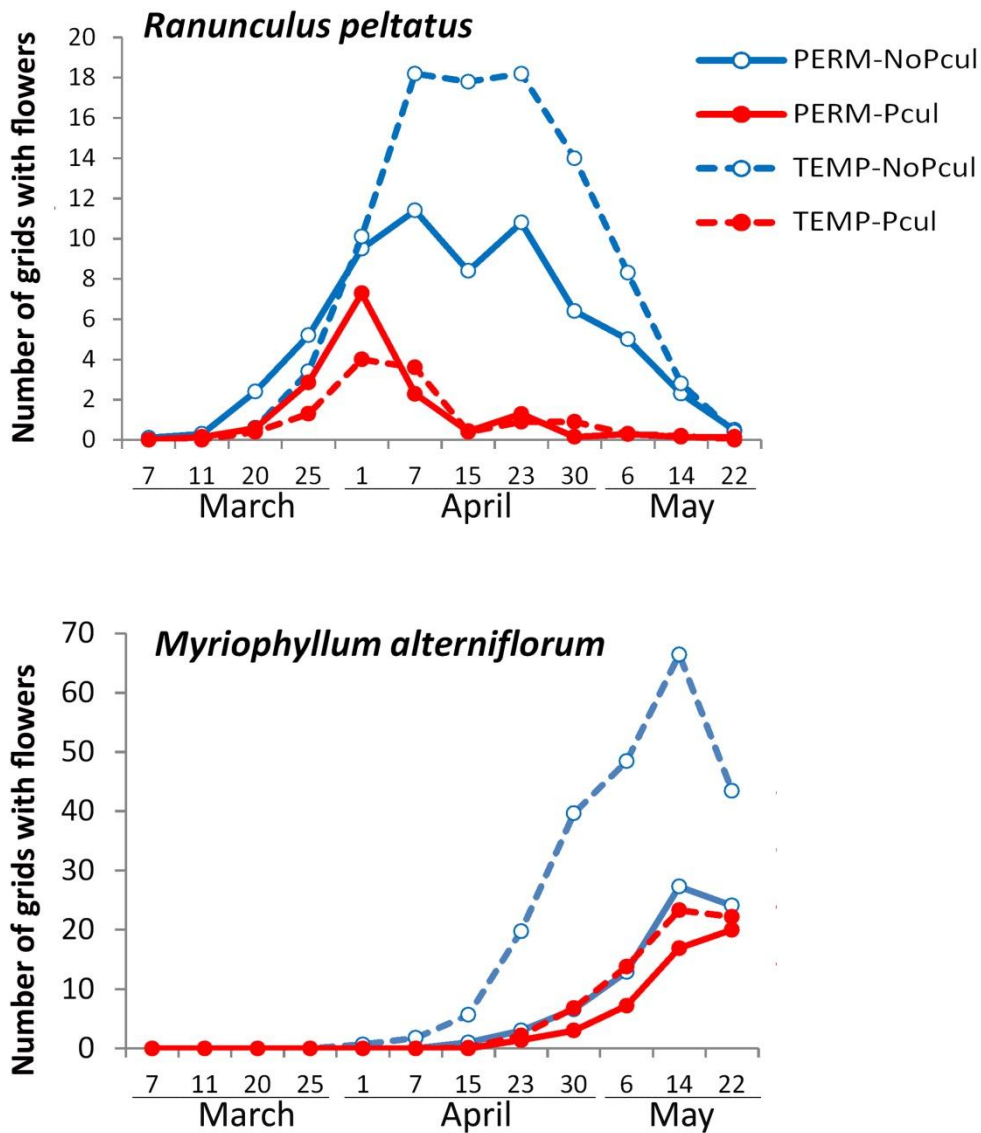
Phenology of plant reproduction

Number of flowers

Throughout the experimental period, the presence of tadpoles significantly decreased the number of flowers of *R. peltatus* ($\chi^2 = 29.26$, $p < 0.0005$), whereas hydroperiod did not affect it. In the case of *M. alterniflorum*, tadpoles also reduced the number of flowers produced ($\chi^2 = 5.94$, $p = 0.015$), but pond drying produced an increase in flower

production ($\chi^2 = 4.08$, $p = 0.026$). The interaction of both factors was not significant (Fig. 3, Table 1B).

Figure 3. Variation in the average number of grid cells containing flowers of *M. alterniflorum* and *R. peltatus* in mesocosms in the presence/absence of herbivorous tadpoles and either constant high or decreased water levels.



Effects of herbivory and pond drying on the flowering period

Ranunculus peltatus began producing flowers on March 7th in tanks with permanent hydroperiod and in the absence of herbivorous tadpoles. On March 20th, we already

detected flowers in all treatments, reaching the maximum number of flowers between April 1st and 23rd (Fig. 3). However, neither herbivory nor hydroperiod significantly affected the date of the first flower blooming in *R. peltatus*.

Myriophyllum alterniflorum flowered later than *R. peltatus*, starting on April 1st in tanks with temporary hydroperiod, which had a gradual and slow increase in flower production. Flowering peaked in mid May in all treatments. Contrary to *R. peltatus*, hydroperiod ($\chi^2 = 9.86$, $p = 0.002$) and herbivory ($\chi^2 = 5.68$, $p = 0.017$) significantly affected the beginning of the flowering period in *M. alterniflorum*. The earliest flowers appeared in the tanks with the highest flower abundance, which occurred in temporary hydroperiod tanks with no tadpoles (Fig. 3, Table 1C). The interaction of both factors was marginally non-significant ($\chi^2 = 2.93$, $p = 0.087$).

Herbivory significantly affected the length of the flowering period in *R. peltatus* ($\chi^2 = 13.06$, $p = 0.0003$), as flower production finished in the tanks with tadpoles 4-5 weeks earlier than in the other treatments (Fig. 3, Table 1D). We did not record the complete flowering period of *M. alterniflorum*, as we still detected flowers during the last week of our experimental period, in spite of the small number of individuals still remaining in the tanks.

Individual monitoring of plants

Survival

Herbivory strongly determined the survivorship of *R. peltatus* individuals ($\chi^2 = 24.80$, $p < 0.0005$), whereas hydroperiod and the interaction of both factors did not (Table 1D). In the tanks with herbivorous tadpoles, plant survival decreased from the first week. By the end of the experiment, only 6.7% of *R. peltatus* plants survived in the presence of tadpoles, whereas survivorship was 73.3 % in tanks without them (Fig 4, Table 1D).

For *M. alterniflorum*, the lowest plant survival was observed in the tanks containing tadpoles and also experiencing pond drying (*ca.* 37%), and tadpole herbivory had little impact on plant survival when at constantly high water level (Fig. 4, Table 1D). By the end of the experiment, both hydroperiod and herbivory did not showed significant effects on plant survival (Table 1D).

Figure 4. Surviving plants individually monitored for *Ranunculus peltatus*, and *Myriophyllum alterniflorum* plants in each week during the experimental period.

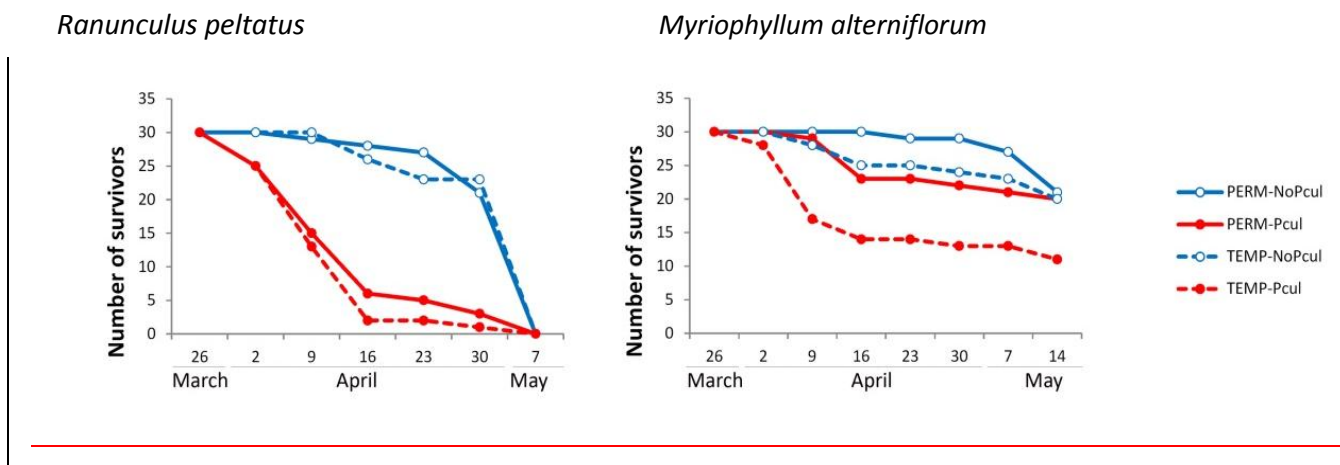


Table 1. Statistical results for A) Generalized linear mixed models, with binomial error distribution, on plant cover of the three macrophyte species as variable response, using the presence of tadpoles (Herbivory) and hydroperiod as fixed factors and tank as random factor; B) Generalized linear mixed models, with binomial error distribution, on the flower production (number of cells with flowers) through the experiment with the same fixed and random factors as in the previous models; C) Generalized linear model, with negative binomial error distribution, on the time to the observation of the first flower of *Myriophyllum alterniflorum* and *Ranunculus peltatus*, using herbivory and hydroperiod as fixed factors; D) Generalized linear model, with Gaussian error distribution, on the length of the flowering period of *M. alterniflorum* and *R. peltatus* using the same fixed factors as in the previous analyses; E) Generalized linear mixed models on survival of individually monitored plants, using the same fixed and random factors as in A and B.

A) Plant Cover. Variable response: Vector (Number of cells with macrophyte species, number of cells with no macrophyte specues)

Macrophyte species		df	χ^2	p	Model
<i>C. obtusangula</i>	Hydroperiod	1	0.5499	0.458	GLMM binomial
	Herbivory	1	7.4026	0.007	
	Hydoperiod:Herbivory	1	4.3944	0.036	
	Residuals	115			
<i>M. alterniflorum</i>	Hydroperiod	1	0.183	0.669	
	Herbivory	1	1.602	0.206	
	Residuals	116			
<i>R. peltatus</i>	Hydroperiod		0.068	0.795	
	Herbivory		4.511	0.0337	
	Residuals	116			

B) Flower production. Variable response: vector (Number of cells with flowers, Number of cells with no flowers)

Macrophyte species		df	χ^2	p	Model
<i>M. alterniflorum</i>	Hydroperiod	1	4.082	0.026	GLMM binomial
	Herbivory	1	5.938	0.015	
	Residuals	307			
<i>R. peltatus</i>	Hydroperiod	1	1.149	0.284	
	Herbivory	1	29.257	<0.0005	
	Residuals	439			

C) Variable response: Time to first flower

Macrophyte species		df	χ^2	p	Model
<i>M. alterniflorum</i>	Hydroperiod	1	9,309	0.0023	GLM Negative binomial
	Herbivory	1	5.361	0.021	
	Residuals	1	2.935	0.087	
		35			
<i>R. peltatus</i>	Hydroperiod	1	1.423	0.233	GLM Negative binomial
	Herbivory	1	2.718	0.1	
	Residuals	33			

D) Variable response: Length of the flowering period

Macrophyte species		df	F	p	Model
<i>M. alterniflorum</i>	Hydroperiod	1	8.441	0.0064	GLM Gaussian
	Herbivory	1	4.917	0.033	
	Residuals	34			
<i>R. peltatus</i>	Hydroperiod	1	0.004	0.948	GLM Gaussian
	Herbivory	1	13.059	0.001	
	Residuals	32			

E) Plant survival. Variable response: vector with data on survival(1) or not (0) of each monitored plant

Macrophyte species		df	χ^2	p	Model
<i>M. alterniflorum</i>	Hydroperiod	1	2.586	0.108	GLMM binomial
	Herbivory	1	2.112	0.146	
	Residuals	116			
<i>R. peltatus</i>	Hydroperiod	1	0.0012	0.972	
	Herbivory	1	24.801	<0.0005	
	Residuals	116			

Discussion

The three macrophyte species included in this study commonly form dense and complex masses in Mediterranean temporary ponds (Fernández-Zamudio et al. 2016). We observed broad changes in the composition of the plant assemblage in our experimental setup caused by the herbivorous impact of spadefoot toad tadpoles and also due to pond drying. In tanks with permanent high water level, *C. obtusangula* was the most abundant species in the initial phases of the experiment, but experienced a steep gradual reduction while the other two macrophytes increased in cover. These reverse trends indicate that *C. obtusangula* may be affected by competition from the other two species, either for light or nutrients. Interspecific competition may be important in the structure of aquatic macrophytes (Grace 1991), and the morphological characteristics of some species may make them more advantageous than others when they are competing for common resources (McCreary 1991). For instance, species with floating leaves may be

directly exposed to sunlight while shading macrophytes. Thus, in dense and extensive masses of aquatic vegetation, some species are only predominant if they have earlier and faster growth than the other macrophytes that will predominate later in the season (Larson 2007). In our study, *C. obtusangula* was the smallest species with thinner and fewer stems. However, it was also the earliest species, and prevailed while the other species were still small sized and not very abundant.

Herbivory by tadpoles had a strong impact on the cover and volume of the three submerged macrophyte species. The most affected was *C. obtusangula*, especially in tanks with a permanent hydroperiod, where tadpoles reduced its cover to less than 25%. Tadpoles showed a feeding preference towards *C. obtusangula* in relation to the other two macrophytes, possibly associated with the higher initial abundance of this species which then resulted in its main food resource. This plant may also be easier for tadpoles to ingest, due to its thin stems and its simple and poorly branched structure. This may be particularly important when tadpoles are still small, and they cannot cut or shred the thicker or harder stems or leaves of other species. The effect of herbivorous tadpoles was higher in permanent than in temporary tanks, probably because in a larger volume of water, *C. obtusangula* reached higher biomass than in the gradually decreasing volume of temporary tanks. Pond drying constrained the growth of this plant to the extent that the cover and volume reached by *C. obtusangula* in tanks with decreasing water level was similar to those observed in permanent tanks but exposed to tadpole herbivory.

In contrast, the least consumed plant species that tadpoles consumed the least was *M. alterniflorum*, which was also the latest plant to grow and to produce flowers. In previous studies, we have detected that this macrophyte is less consumed by tadpoles (Arribas et al. 2014) and can even have lethal effects on them when it is the only food available (Cabrera-Guzmán et al. 2020). Other *Myriophyllum* species contain allelopathic compounds (Leu et al. 2002), and it is therefore possible that *M. alterniflorum* also contains them, which would work as a defense mechanism to avoid or reduce its consumption by herbivores, including tadpoles. These effects may also explain why tadpoles fed on this plant mainly at the end of the experiment, when the biomass of *R. peltatus* had been reduced to a few survivors that did no longer produce flowers. Our experiment demonstrates that *P. cultripes* tadpoles have a strong herbivorous impact on submerged macrophytes and therefore may alter the structure of aquatic ecosystems. However, this conclusion should not be generalized to other tadpole

species. Indeed, small tadpoles, even if also herbivorous, can favor the growth of macrophytes by removing periphyton growing over their stems or leaves (Arribas et al. 2014). In particular, the negative effect of the *P. cultripes* herbivory is mainly due to the high volume of food they need to ingest at the late phases of their larval period, when they reach a large body size. The observed herbivory is perfectly realistic given that the natural abundance and density of tadpoles may be much greater than the one used in our mesocosms (7 tadpoles/m²; Pinero-Rodriguez et al. 2020).

Hydroperiod also affected *M. alterniflorum*, although only by the end of the experiment did we detect a reduction in cover for this species due to reduced water level. Growth of submerged macrophytes is constrained by the light and nutrient availability, so the higher plant density, consequence of the reduced volume of water, limits access to these resources (Cronk and Fennessy 2001). In temporary ponds, the extent and volume of the ponds is variable and gradually decreases from spring to summer. While the height of the water column decreases, the mass of macrophytes is more and more dense, reducing the availability of light to individual plants. By these late phases of the inundated period of the ponds, other factors are stressing, such as higher temperature or lower oxygen content (Williams 2006), thus favoring the deterioration of the plants and accelerating their senescence and death.

Phenological changes

Herbivory is detrimental to plants and plants may respond increasing growth, flower and fruit production, or elevating their photosynthetic rate to compensate the loss of parts of the plant consumed (see Belsky 1986). These compensatory effects have been described mainly for terrestrial species, whereas for aquatic species, studies about the pressure of herbivory mainly refer to changes in the distribution, diversity and abundance of macrophytes in the ponds (Sheldon 1987; Wood et al. 2016). In this study we detected shifts in plant phenology in response to herbivory and pond drying. Thus, *R. peltatus* shortened its flowering period when exposed to herbivorous tadpoles, but maintained its flower production despite its shorter life-span. In response to pond drying, however, *R. peltatus* markedly augmented its flower production, hence increasing the probability of successful plant reproduction when at risk of pond drying, improving its resilience by securing persistence in future inundation cycles.

In contrast, *M. alterniflorum* showed similar flowering period across treatments. In our experiment, *M. alterniflorum* produced flowers when ambient temperature was

above 30 °C, whereas *R. peltatus* flowered when in the range between 20-30 °C. The beginning of the flowering period and flower production in *M. alterniflorum* occurred earlier in tanks with decreased water levels in the absence of tadpoles. Such phenological shift could constitute an adaptive plastic response to pond drying, coincidentally occurring in the species with delayed growth and maturation and consequently more prone to face such risk. This effect could alternatively be related to the higher temperature reached in tanks with lower water volume, although we lack precise temperature data to confirm this, and it would warrant experimental testing.

In general, tadpole herbivory did not affect the flower production per plant of *M. alterniflorum*, although it did impact plant survivorship. Due to its delayed growth, *M. alterniflorum* is commonly exposed to herbivory from spadefoot toad tadpoles that have been growing already for a long time and have therefore attained large sizes. Tadpole herbivory may therefore have played a role in the association between delayed phenology of this macrophyte species and its production of allelopathic compounds.

It is important to consider the temporal variation in macrophyte abundance in relation to the reproductive phenology and growth patterns of potential herbivores (Lodge 1998). In our study, we detected different peaks of abundance among the three species. *Callitriche obtusangula* was the earliest growing species, and the most affected by herbivory. *Ranunculus peltatus* was intermediate, and maintained a high flower production in a shorter flowering period to secure a successful reproduction under the pressure of herbivory. *Myriophyllum alterniflorum* was the latest species and was the least affected by herbivory, which could be associated with its capability to produce allelopathic compounds.

The herbivory exerted by *P. cultripes* tadpoles on submerged macrophytes may be as great as the effect of large herbivores, since they can remove all plant biomass in the mesocosms in a short period of time (Arribas et al. 2014). In aquatic ecosystems, plant removal involves important changes in the physical and chemical characteristics of the water that affect the composition of the aquatic communities, by increasing phytoplankton abundance and also altering nutrient fluxes and reducing the habitat complexity with consequent reduction of shelter and food resources for other species (Scheffer 2001). Our study reveals that large tadpoles may consume such large amounts of plant biomass that they can be considered among the main herbivores in temporary ponds, modifying the life history of the submerged macrophytes and the spatial complexity of the water column.

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2. Movilidad, distancias máximas recorridas y selección de hábitat de las larvas de sapo de espuelas en las lagunas temporales. (*Movement patterns, maximum distances traveled and microhabitat selection of spadefoot toad tadpoles in temporary ponds*)

Movement patterns, maximum distances traveled and microhabitat selection of spadefoot toad tadpoles in temporary ponds

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Abstract. Understanding the movement of individuals across natural habitats is important to determine fundamental aspects of their ecology, from dispersal patterns and habitat selection to interactions with predators, pathogens or conspecifics. Amphibian larvae are key to aquatic systems, playing fundamental roles as both consumers and prey, affecting nutrient cycling and even physico-chemical properties of the water bodies they occupy. Nevertheless, despite an extensive body of literature on most other aspects of their ecology, we know very little about spatial movements of amphibian larvae in their natural habitats, and the little information we have is restricted to lotic systems. Here we use a mark-and-recapture procedure using elastomers to determine movement patterns and microhabitat selection of western spadefoot toad tadpoles (*Pelobates cultripes*) in two large ponds in southwestern Spain. We observed maximum distances moved by these tadpoles of >67 m and >245 m, depending on the pond. These distances coincided with the maximum cross distance in each pond, suggesting that they could potentially move even farther in larger wetlands. Mean distances moved were 29.7 and 105 m in each pond. We sampled at two, four and nine days after release of tagged individuals, and we observed a stable, not expanding, movement pattern across sampling dates, suggesting that tadpoles resumed normal activity immediately after release. Tadpoles showed preference for deeper and more densely vegetated areas of the ponds, especially associated with plant species such as *Ranunculus peltatus* and *Potamogeton pectinatus*.

Keywords: habitat use, home range, *Pelobates cultripes*, spatial ecology, tadpole movement, temporary ponds.

Introduction

The study of the movement of individuals in natural populations is vital to understand many fundamental aspects of the relationship between organisms and the environment they inhabit (Nathan et al., 2008; Clobert et al., 2009). Understanding how organisms move across the landscape in terms of frequency of moved distances, diel patterns and dependence of movement upon sex or phenotypic features such as body condition are key in contextualizing population interconnectivity and expansion, spread of emerging diseases, predator-prey dynamics, propagule dispersal, biological invasions or resource use (Clobert et al., 2001; Fèvre et al., 2006; Knowlton and Graham, 2010).

Movement ecology is unevenly developed across taxa, with vertebrates among the most thoroughly studied species. Nevertheless,

within vertebrates, movement ecology and demography of amphibians are still largely missing components of our otherwise extensive knowledge on the group (Pittman et al., 2014; Conde et al., 2019). This is a major gap of knowledge since amphibians are the most threatened group of vertebrates worldwide (Stuart et al., 2004; Wake and Vredenburg, 2008; Hoffmann et al., 2010). Amphibians are key ecological agents in both aquatic and terrestrial ecosystems, in addition to natural vectors of energy between the two (Regester et al., 2006, 2008). Yet, despite their ecological importance and the critical need to improve their conservation, our knowledge on amphibian movement is limited (Pittman et al., 2014). Nevertheless, information about terrestrial movement of juvenile and adult amphibians has been compiled over the past two decades using a combination of mark-and-recapture and molecular techniques (Cayuela et al., 2020). Amphibians typically show low dispersal distances (most species showing postmetamorphic or breeding dispersal

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of less than 1 km) and high degree of breeding site fidelity or philopatry (Gamble et al., 2007; Cayuela et al., 2020), although different strategies of disperser vs. philopatric individuals may coexist within populations (Denoël et al., 2018). Recent reviews on movement ecology (Pittman et al., 2014) and dispersal (Cayuela et al., 2020) of amphibians have summarized our current knowledge on the topic and identified areas where more effort is needed, both in terms of taxonomic breadth and mechanistic understanding of amphibian movement. However, it is remarkable that even such reviews that vindicate the need to further study amphibian movement fail to identify a major gap in our knowledge: we do not know how amphibian larvae move in lentic systems. The literature on amphibian movement focuses almost exclusively on the terrestrial juvenile and adult stages (Pittman et al., 2014; Cayuela et al., 2020), with the exception of tadpole transportation on land on the back of their parents (Pašukonis et al., 2019). This is in sharp contrast with the abundant literature on most other aspects of the ecology of amphibian larvae, from how they adjust their growth and development according to local conditions, to their effects on the structure and dynamics of aquatic communities, primary production, and nutrient cycling (Wilbur, 1997; Whiles et al., 2006; Costa and Vonesh, 2013; Arribas et al., 2014).

Our limited knowledge about movement patterns of amphibian larvae refers exclusively to lotic environments over relatively short distances. Thus, in rivers and streams, amphibian larvae are known to drift downstream, with steeper stream gradients and stronger currents increasing the proportion of larvae drifting (Thiesmeier and Schuhmacher, 1990). Downstream drifting is reduced when streams flow through well conserved forests and/or contain suitable hiding places (Thiesmeier and Schuhmacher, 1990; Wahbe and Bunnell, 2001; Chelgren and Adams, 2017). For instance, *Ascaplus truei* tadpoles drifted downstream between 0.30 and 3.76 m a day, moving longer distances

when they were in patches of streams under unmanaged forest canopy than when they were in sections flowing through open fields resulting from clearcuts (Wahbe and Bunnell, 2001). Individual characteristics have also been shown to affect the likelihood of drifting, as larvae in early developmental stages are more likely to drift (Thiesmeier and Schuhmacher, 1990; Chelgren and Adams, 2017), and so are hungry larvae compared to satiated ones (Thiesmeier and Schuhmacher, 1990).

However, no information about movement patterns is available for amphibian larvae inhabiting ponds or marshes, and such information would be highly relevant to understand multiple aspects of the ecology of amphibian larvae. For instance, it is well established that tadpoles vary their activity rate according to local conditions, increasing their activity as they feel hungry, and decreasing it with increased risk of predation, (e.g., Horat and Semlitsch, 1994; Van Buskirk and Arioli, 2002; Touchon et al., 2013). Amphibian larvae also modulate their activity to thermoregulate, and in response to pathogen infection (Lefcort and Blaustein, 1995). Nevertheless, we do not yet know how changes in activity rate translate into actual movement patterns or total distance moved by amphibian larvae in natural environments, which will determine rates of predator encounter, and differential vulnerability against predators with different strategies (e.g., ambush vs. active hunting). Assessing patterns of larval movement in natural systems will also improve our understanding of competitive interactions within amphibian guilds, trophic niche partitioning, exposure to pollutants (salinity, pesticides, herbicides), effect of canopy and light heterogeneity, thermoregulation, parasite load, importance for seed dispersal or propensity to school. Here we present the first characterization of the movement patterns of *Pelobates cultripipes* tadpoles (Western spadefoot toad) in two ponds in southwestern Spain, aimed at determining how much these tadpoles move, over which timeframe, and whether they show microhabitat preferences.

Material and methods

We have selected the larvae of the Western spadefoot toads as model system to study larval movement patterns in lentic habitats because *P. cultripes* are the largest tadpoles in the Iberian peninsula and have a very important ecological role in Mediterranean aquatic systems, where they can be found at high densities, accounting for a large fraction of the ponds' biomass. Spadefoot toad tadpoles are mostly herbivores that consume large quantities of macrophytes (which represent up to 70% of their diet), and algae, although they also include detritus and small crustaceans (Díaz-Paniagua, 1985; Arribas et al., 2015). These tadpoles exert a big competitive effect on other amphibian larvae, and condition both the physico-chemical properties of the water and the zooplanktonic guild of the ponds they inhabit (Arribas et al., 2014, 2015). Their large size allowed an easier manipulation during elastomer tagging and higher odds of survival after manipulation.

To study the movement of *P. cultripes* tadpoles we selected two seasonal ponds within the Doñana National Park, on the right bank of the Guadalquivir river mouth in southwestern Spain. We selected these two ponds because they are representative of the ponds where *P. cultripes* commonly breeds within the National Park, and because they had a sufficient extension to detect longer displacements if these indeed occurred. The climate in this area is Mediterranean subhumid with Atlantic influence, characterized by hot, dry summers and by rainfall occurring mainly in autumn or winter, from November to March (mean annual precipitation of $544.6 \text{ mm} \pm 211.3 \text{ mm}$; Díaz-Paniagua et al., 2010). The park comprises more than 54 000 ha of marshes, shrubland, pinewoods and dunes, and contains a pond network including more than 3000 temporary ponds (Díaz-Paniagua et al., 2015). These ponds are usually flooded in the fall or in winter and last until June or July depending on rainfall, showing large interannual variation in hydroperiod (Gómez-Rodríguez et al., 2009) and are common breeding habitats for eight amphibian species (Díaz-Paniagua et al., 2005). For this study we chose two large bodies of water that we knew harbored large spadefoot toad populations and studied tadpole movement in them between late April and early May 2017. One of the ponds, locally known as Laguna del Zahillo (hereafter Zahillo; $-6.5066, 36.9868$), had a regular basin with homogeneous depth. During our sampling period we estimated that the inundated area at Zahillo extended over a surface of 7909.79 m^2 , 114 m long and 96 m wide, with an average depth of 26.3 cm. Aquatic vegetation was homogeneously distributed in this pond, with *Myriophyllum alterniflorum*, and *Ranunculus peltatus* predominating the macrophyte guild. The other pond, Laguna de Las Pajas (hereafter Las Pajas; $-6.4709, 36.9795$), had a more irregular shape, and the inundated area extended over $34\,861.64 \text{ m}^2$, with a maximum length of 286 m and a maximum width of 246 m, and an average depth of 38.6 cm. We georeferenced the perimeter of each pond using a handheld GPS (Juno SB, Trimble, CA, USA) and estimated the inundated surface using QGIS (QGIS Development Team, 2019).

Visible implant elastomer (VIE) tadpole tagging

We selected and georeferenced in each pond three points rather distant from each other for initial tadpole capture. At dusk we set up between 3 and 5 fyke nets around each point of each pond and left them open overnight (ca. 12h). The fyke nets used have a semicircular aperture ($68 \times 34 \text{ cm}$) and are built with 5 mm mesh. They have a conical structure with an approximate total length of 100 cm, including three internal inverted funnels to prevent animals from escaping. They also have an external wing of net (length ca. 125 cm) that intercepts animals and directs them towards the trap (Florencio et al., 2012).

Early the following morning we collected all amphibians trapped in the nets and haphazardly assigned a tagging color to each trapping area. We arranged the collected amphibian larvae in several 50 l containers in the shade near the shore while we processed the spadefoot toad tadpoles, simultaneously identifying, counting and releasing individuals from other species. Within a few hours we tagged all spadefoot toad tadpoles with elastomers (VIE; Northwest Marine Technologies, Shaw Island, Washington, USA), each group of tadpoles tagged with the color assigned to their specific place of capture within each pond. VIE tagging presents a high level of efficacy, with elastomer retention rates of 100 % after 20 days in larval salamanders (Grant, 2008) and after a three-month period in hydrid tadpoles (Bainbridge et al., 2015). We placed the tags sub-epidermally dorso-laterally near the insertion of the tail fin, as placement of VIE in that part of the body does not seem to cause mortality or differential growth of tagged animals (Iannella et al., 2017). Gloves were used throughout the tagging procedure and renewed frequently. Tadpoles were held carefully over a plastic tray covered with filter paper moist with pond water to prevent desiccation. The majority of tagged *P. cultripes* tadpoles were large, with an average total length of 76.8 mm (± 11.4 [SE]) for a subsample of 60 individuals from Zahillo and $82.8 \pm 7.0 \text{ mm}$ for a sample of 58 individuals from Las Pajas. To obtain these measurements, tadpoles were photographed and digitally measured using ImageJ software (version 1.45 s, National Institutes of Health, USA). We avoided tagging larvae at developmental stages 38 Gosner and later (Gosner, 1960) as they would have been too close to metamorphosis likely altering their movement behavior, and also because long hindlimbs could have represented a drag to long distance movement (Chelgren and Adams, 2017). Upon tagging, tadpoles were released back precisely into the point where the net was originally placed.

Recapture and movement estimation

The following day after tagging and releasing the animals we designed a homogeneous array of regularly spaced fyke nets in each of the ponds, with 30 nets placed at Zahillo and 35 nets in Las Pajas. Nets were held in place with steel re-bars labeled and georeferenced that stayed in place throughout the study. Each net represented a potential sampling site for tagged tadpoles, and at each one we recorded water depth, macrophyte species present and macrophyte density (categorized as high, medium, low). At each position, we

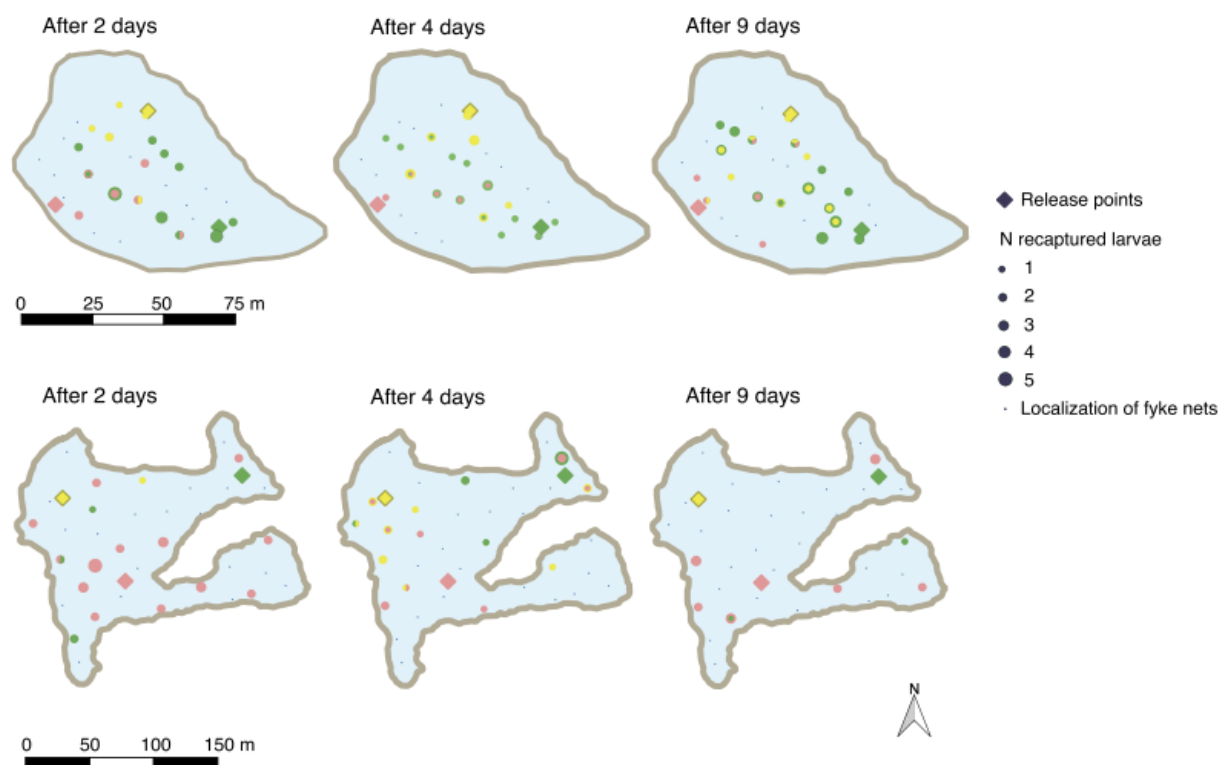


Figure 1. Maps of each pond representing the releasing points and the position of the nets for tadpole captures, indicating higher point size for higher number of tadpoles captured. Different colors are used for different releasing points and their corresponding captured tadpoles.

alternated the orientation of the aperture of the net to avoid a systematic bias regarding directionality of tadpoles' trajectory. Given the spatial distribution of the nets in each of the ponds, the average distance between adjacent nets was 8.2 m for Zahillo and 25.4 m for Las Pajas. We conducted three samplings after the release of the tagged tadpoles in each pond: after two days, four days, and nine days. At each sampling time, we set up the fyke nets at dusk and checked them at dawn, recording the total number of tadpoles captured and the number of tagged tadpoles in them, releasing them immediately. We observed most of the tadpoles released from the nets sprinting away over a short distance and then resuming foraging on macrophytes. Upon checking the nets at each sampling time, we removed the nets until the next sampling but left the metal rods on site to ensure the sampling points were identical across samplings. Using the fraction of tagged animals recaptured in the first sampling, we applied Chapman's method to estimate population density in each pond using the package *FSA* (Ogle et al., 2019) in *R* statistical package (R Core Team, 2018). We used *QGIS* to determine distances between the points where tadpoles were released after tagging and each of the recapture fyke nets, proxies for minimum distances moved. We also tested for microhabitat selection as a function of depth, distance to shore, vegetation density, vegetal species richness and abundance of the macrophyte species present using non-metric multidimensional scaling with the function *metaMDS* from package *vegan* in *R*. The results of the first two NMDS dimensions were then related to the number of larvae captured in each point using the function *envfit*.

Results

We tagged a total of 825 *P. cultripes* tadpoles at Zahillo, with 172, 287, and 366 individuals captured in each of the three initial capture sites (fig. 1A). At Las Pajas, we tagged 1420 tadpoles with 315, 479, and 626 individuals captured, respectively, at each of the three initial capture sites (fig. 1B). At Zahillo, we recorded a total of 8481 tadpole captures over the course of the study, 115 of which (1.36%) were recaptures of tagged individuals. At Las Pajas we recorded a total of 8727 spadefoot toad tadpole captures with only 66 recaptures (0.76%). The recapture rates in each pond across the different sampling times of the study is shown in table 1. Given the number of tagged individuals released, the number of them recaptured, and the total number of tadpoles captured in each pond, we estimate population sizes of 57 239 tadpoles for Zahillo (95% confidence intervals: 41 657-80 791) and 201 042 tadpoles (95% confidence intervals: 136 912-306 893) for Las Pajas, which given the size of the ponds at the time of the study resulted

Table 1. Number of *Pelobates cultripes* captured in each pond at each sampling time: 2, 4 or 9 days post-release of tagged individuals, indicating the fraction of tagged individuals (i.e., recaptures).

Pond	1st sampling (2 days)		2nd sampling (4 days)		3rd sampling (9 days)	
	Total	Recaptures	Total	Recaptures	Total	Recaptures
Zahillo	2494	36	2812	36	3175	43
Las Pajas	3360	24	3559	31	1808	11

Table 2. Minimum, median, mean and maximum distances moved (in m) by *Pelobates cultripes* tadpoles in two temporary ponds at three sampling periods post tagging and release.

Pond		Distance moved (m)		
		2 days later	4 days later	9 days later
Zahillo	Minimum	2.2	2.2	2.2
	Median	21.3	27.6	27.8
	Mean	25	28.4	29.7
	Maximum	60.6	67	67.9
Las Pajas	Minimum	28	10.4	42.2
	Median	57.25	54.6	60.3
	Mean	79.3	70	105.5
	Maximum	206.9	170	245.6

in average larval densities of 7.2 tadpoles/m² for Zahillo and 5.8/m² for Las Pajas.

The minimum observed distance moved was 2.2 m in Zahillo and 10.4 m in Las Pajas, corresponding to the closest net to the releasing point in each pond. At Zahillo we observed a mean movement of 27.7 m and a maximum distance of 67.9 m. At the larger Las Pajas, mean distance averaged over sampling times was 57.4 m, with maximum distance covered of 245.6 m, which represented the maximum possible distance within the pond. Distances recorded were rather constant over the different sampling times in both ponds, with greater variance in the larger pond (table 2). A large fraction of the tadpoles remained within relatively short distances from the release location, but many tadpoles also moved considerable distances even within the first 48h (fig. 2).

In terms of microhabitat selection, the two ponds offered different scenarios. Zahillo had a regular oval shape, with very little variation in depth, and with a dense cover of macrophytes, hence offering a very homogeneous

habitat for tadpoles (fig. 3A). We consequently found no relationship between number of spadefoot toad tadpoles and NMDS factors in that pond ($R^2 = 0.017$, $P = 0.851$; fig. 3). Conversely, the larger Las Pajas was more heterogeneous, irregularly shaped, with a more complex bathymetry and a patchier distribution of macrophytes. Results from the NMDS analysis were significantly related to the abundance of *P. cultripes* tadpoles in that pond, which were more abundant in deeper sites, far from the shore, with rich and dense vegetation predominated by *R. peltatus* and *Potamogeton pectinatus*. Tadpole abundance was in turn negatively associated with the presence of *M. alterniflorum*, the macroalga *Chara connivens*, and helophytes ($R^2 = 0.310$, $P = 0.009$; fig. 3).

Discussion

Here we estimated movement of pond-dwelling spadefoot toad tadpoles using color tagging, release of marked individuals at specific locations, and recapturing them at various time points using regularly spaced fyke nets. To the best of our knowledge, this is the first time tadpole movement is quantified in lentic aquatic systems. Interestingly, the distances recorded did not conform to a stepping-stone pattern in which tagged tadpoles gradually move away from the release point. Instead, within 48 h released tadpoles had already moved across the whole area. A large fraction of tadpoles remained within 40 m of the release place in Zahillo and within 100 m in Las Pajas, whereas a few individuals would swim up to the maximum linear distances pos-

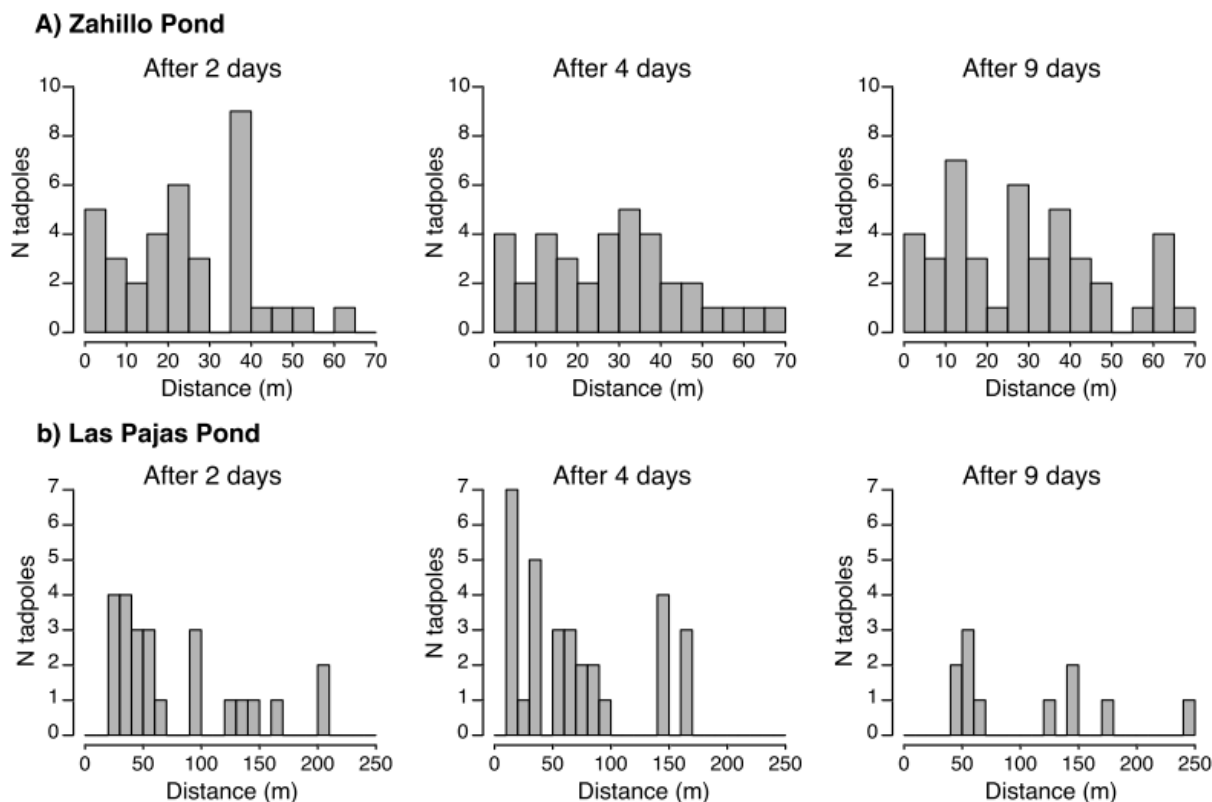


Figure 2. Number of *P. cultripes* tadpoles recaptured at different distances from their initial releasing point, after two, four or nine days.

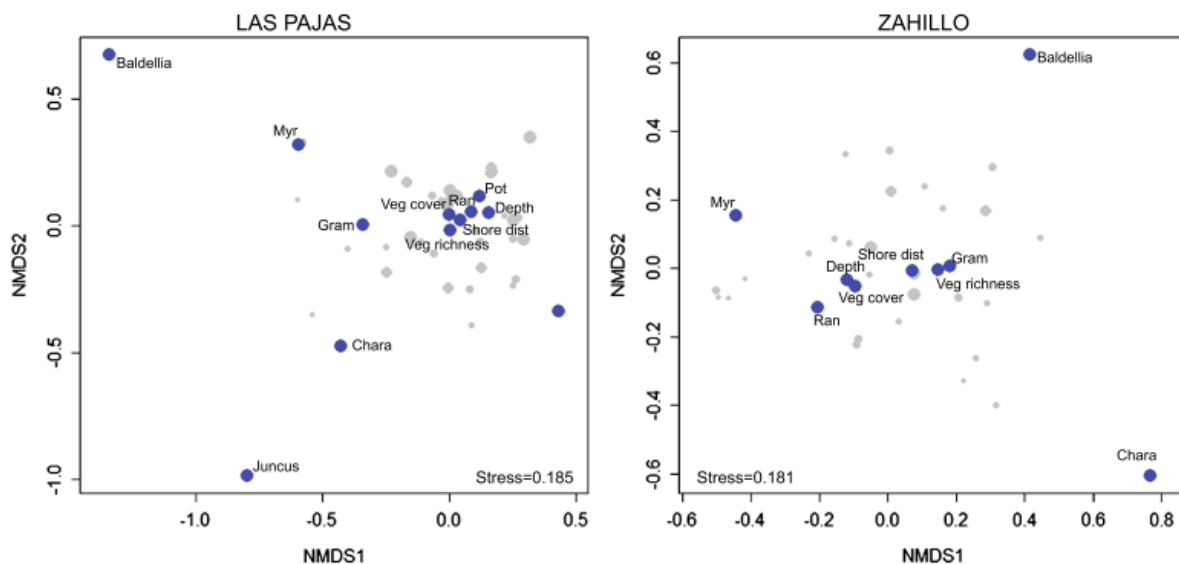


Figure 3. Representation of the NMDS scores of the environmental variables characterizing each capture point in the two study ponds. The abundance of tadpoles captured in each net is shown in relation to the point size. (Gram = Gramineans, *Baldellia* = *B. ranunculoides*, *Myr* = *M. alterniflorum*, *Pot* = *P. pectinatus*, *Ran* = *R. peltatus*).

sible in each pond. Tadpoles therefore showed a consistent and broad movement pattern that was independent of time over the timescale studied.

Spadefoot toad tadpoles in these ponds moved over considerable distances, even more so when we realize that the distances here estimated are very conservative, and should be

considered minimal distances moved. First, we estimated movement as the linear distance between the release point and the traps where tadpoles were recaptured, but tadpoles do not move in a straight line, so the actual distance moved is greater than the one observed. Also, in the absence of individual markings, some tadpoles that were apparently staying close to the release point could have moved farther and returned. However, the low recapture rate is somewhat discouraging for use of individual marking technology such as pit-tagging. Also, the fact that tadpoles could move across such large ponds in less than 48h indicates that they have an even greater moving potential.

Previous reports on movement of amphibian larvae refer exclusively to stream dwellers, urodeles in most cases. Stream larval salamanders have been shown to move both upstream and downstream between 20-50 m (Lowe, 2003; Cecala et al., 2009; Honeycutt et al., 2016; Chelgren and Adams, 2017). Tadpoles moved well above that range of distances, especially in the largest pond studied, and without the contribution of water current, as in lotic systems. Moreover, our observed events of longer distances traveled by *P. cultripipes* tadpoles (between 100 and 200 m) are congruent with occasional long distances moved in streams (Lowe, 2003), except that in our system tadpoles covered those distances within very short time spans (48 h or less). Moreover, the longest distances moved coincided with the maximum possibilities of the inundated areas of the ponds, suggesting that pond extension was constraining the maximum distance moved by these tadpoles, which could therefore be even greater in larger wetlands.

Regarding anuran tadpoles, little is known about their movement patterns in streams. Under experimental conditions, *Rana catesbeiana* (*Lithobates catesbeianum*) tadpoles were shown not just to drift downstream, but to actively swim away from the current and into areas where the flow was reduced (Schmidt et al., 2011). Tadpoles of the coastal tailed frog,

Ascaphus truei, showed very timid incursions upstream but drifted over 180 m downstream (Chelgren and Adams, 2017). Both bullfrog and tailed frog tadpoles were shown to move in a biased or directed random walk fashion (Huang et al., 2002). Absence of skeletal elements in tadpoles' tails improve their maneuverability, and their viscoelastic fins form a light and flexible structure that yet provides considerable thrust power (Hoff and Wassersug, 2015). In terms of propeller efficiency and sprint speed, tadpoles are capable of performing as well as teleost fish of comparable sizes (Wassersug, 1989).

Tadpole morphology is directly associated with swimming performance and speed (Arendt, 2010), and different environmental factors, both biotic and abiotic, can induce changes in morphology and behavior that result in different activity levels and affect locomotion performance (Touchon and Warkentin, 2011; Van Buskirk, 2017). Being capable of sustained thrust is likely to be more important for stream dwelling tadpoles to prevent drifting downstream, than for pond dwelling tadpoles that do not have to swim against current. Turbidity in ponds is likely to be high reducing visibility, except if dense communities of macrophytes help maintaining clear waters, in which case macrophytes themselves would add structural complexity. Either way, tadpoles are more likely to prime low swimming speed and enhanced maneuverability rather than sustained high speed (Wassersug, 1989). In view of these general features of tadpole locomotion, and taking into account that most of the tadpoles were not far from the releasing points, we speculate that tadpoles in our study ponds presented slow swimming, so that long distances would have been moved by individuals sustaining constant swimming, possibly alternated with bursts of faster swimming. The high vegetation density in the ponds studied provided tadpoles with high resource availability for feeding. Therefore, they were not forced to search for food in areas farther apart, and

great tadpole abundances were found associated with macrophyte density and macrophyte species richness in both ponds.

The distances moved by tadpoles in our study are also highly relevant because of the high local abundance of the species. Even if only a small fraction of tadpoles move over long distances, the high abundance of tadpoles of this species warrants numerous overall events of long distance movements. We estimated larval densities in our study ponds well within the range of densities reported for this species, for which there have been reports of up to 100-120 larvae per m² (Petit and Delabie, 1951; Cei and Crespo, 1971). Given the estimated population size of spadefoot toad tadpoles in these ponds (between 50 000 and >200 000) and the ample range of individual larval weights (4.56-16.14 g per tadpole, depending on stage and larval density), the total biomass that tadpoles of this species can account for is huge, in the order of thousands of kg to several tons. Amphibian larvae are hubs in aquatic ecological networks, as they are both prey and predators to multiple taxa, as well as primary consumers and detritivores (Gibbons et al., 2006; Barzaghi et al., 2017; Arribas et al., 2018). They can account for large fractions of wetlands' biomass and here we show that they can move over considerable distances and show habitat preference or not depending on the conditions of the water body.

In conclusion, we found that spadefoot toad tadpoles moved long distances (>250 m), and over a relatively short time span (<48 h), as indicated by the lack of a marked stepping stone process. The study of spatial ecology of amphibian larvae will complement our knowledge on predator-prey dynamics, impact of invasive species, disease dynamics, or resource use.

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3. Efecto del helecho acuático invasor sobre la calidad del agua, vegetación acuática y las larvas de anfibios en ambientes mediterráneos (*The invasive aquatic fern *Azolla filiculoides* negatively impacts water quality, aquatic vegetation and amphibian larvae in Mediterranean environments*)



ORIGINAL PAPER

The invasive aquatic fern *Azolla filiculoides* negatively impacts water quality, aquatic vegetation and amphibian larvae in Mediterranean environments

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Abstract Among invasive species, floating plants have a great impact on aquatic ecosystems, forming dense mats that cause a great alteration of the physical and chemical water features. We experimentally assessed the effect of *Azolla filiculoides* mats on Mediterranean temporary waters using mesocosms with sediments from an invaded marsh and amphibian larvae from surrounding wetlands. *Azolla filiculoides* formed a dense mat over the water surface in the experimental tanks, which decreased pH and oxygen concentration, and increased nutrients, nitrogen and phosphorus compounds in the water compared to mesocosms lacking the exotic fern. Macrophyte abundance and richness were reduced under the *Azolla* mat. Invaded mesocosms also had higher phytoplankton abundance and different zooplankton composition, which was mainly characterized by higher abundance of juvenile copepods. Regarding amphibian development, tadpoles requiring a long developmental period,

the western spadefoot toad, *Pelobates cultripes*, had significantly lower survival in the presence of *A. filiculoides* (4.8% compared to 60% survival in fern-free mesocosms). For tadpoles of the Mediterranean tree frog, *Hyla meridionalis*, which develop faster, we did not detect effects on survival, but on body size and physical condition of metamorphs. The formation of *A. filiculoides* mats produced a deterioration of the trophic web, as it caused most sources to be depleted in ^{13}C and ^{15}N . Primary consumers, tadpoles and zooplankton, were also ^{13}C depleted, although the diet of tadpoles from *Azolla* mesocosms was mainly composed of the invasive fern. We therefore conclude that *A. filiculoides* has a negative impact on physical and chemical water features, reduces the richness and biomass of the submerged macrophyte guild and reduces survival and body condition of tadpoles. Overall, *A. filiculoides* causes substantial deterioration of the food web of the aquatic ecosystem.

Keywords Invasive species · Floating plants · Amphibians · Isotopes · Aquatic macrophytes · Food web effects · Temporary wetlands

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Introduction

Aquatic plants exert an important influence on the biotic and abiotic factors characterizing wetlands.

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They can alter chemical and physical processes, affecting oxygen content, pH, nutrient cycling, turbidity, and light penetration in the water column (Cronk and Fennessy 2001). They also provide complexity to microhabitats, and give support, food, and refuge to other species, determining the composition of aquatic communities (Cronk and Fennessy 2001). The establishment and proliferation of a single invasive plant species can produce a strong transformation of the original aquatic ecosystem, affecting its structure and function (Strayer 2010; Schultz and Dibble 2012). This alteration of the aquatic environment may result in an impoverishment of the food web, negatively affecting native coexisting organisms, and reducing the richness and biodiversity of invaded ecosystems. In particular, free floating plants tend to form dense mats over the water surface which attenuate light penetration, severely reducing the abundance of submerged plants (Scheffer et al. 2003). For example, the invasive water hyacinth *Eichornia crassipes* alters the structure of aquatic microhabitats by forming dense mats that reduce light and oxygen leading to a decrease in plankton primary production (Williams 2006), and reduces the diversity of aquatic invertebrates, hence also reducing the diversity of fish diet (Toft et al. 2003). Similar effects are produced by *Salvinia natans*, which outcompetes other macrophytes, mainly due to their shading effect but also through warming of the top layer of the water column and limiting the temperature increase below the mat (Netten et al. 2010). Invasive aquatic plants also cause negative effects on amphibians, whose larvae are often the main group of vertebrates in temporary waters. Nevertheless, the impact of invasive macrophytes on amphibians has been poorly documented and the few existing studies are mainly related to terrestrial plants or helophytes (see review in Bucciarelli et al. 2014). Among the effects of invasive aquatic plants reported, the mosquito fern, *Azolla filiculoides*, has been shown to cause a decline in animal diversity, including tadpoles, fish, and aquatic invertebrates in Zimbabwean streams (Gratwicke and Marshal 2001), *Myriophyllum spicatum* leachates reduced tadpole body mass (Curtis and Bidart 2017) and *Lythrum salicaria* reduced developmental rate and survival of tadpoles (Brown et al. 2006).

Although *A. filiculoides* has spread worldwide as an invasive species, its effects on the biodiversity of invaded ecosystems have only been addressed in two

previous studies (Janes et al. 1996; Gratwicke and Marshal 2001). Here, we analyze experimentally the effect of the mosquito fern, *Azolla filiculoides*, on the abiotic features (pH, electrical conductivity, turbidity, oxygen and nutrient content of the water), primary producers, and primary consumers, such as zooplankton and tadpoles in Mediterranean temporary freshwater ecosystems. This fern is native to warm temperate and subtropical America and is now considered cosmopolitan. It was introduced in Europe in 1880, initially in France, from where it was spread invader to other European countries, mainly those of the Atlantic-Mediterranean regions (Hussner 2012). It is considered one of the most dangerous invasive aquatic plants and the second most widely spread in Europe (Hussner 2012). The species was first found in the Iberian Peninsula around 1920 in the mouth of Sado River in Portugal and was associated with rice fields (García-Murillo et al. 2007). This is a floating heterosporous fern with a short branched rhizome (2–5 cm) covered by small overlapping leaves and filiform roots. It may rapidly propagate vegetatively through rhizome fragmentation or sexually by producing spores (García-Murillo et al. 2007). In the absence of environmental constraints, a given individual may multiply its biomass 100 times in one year by shoot fragmentation (Fernández-Zamudio and García-Murillo 2013). The life span of individuals is much longer (over 200 days) than common native floating macrophytes, and their propagules have high survival, over 98% (Fernández Zamudio and García-Murillo 2013). In favorable still waters, *A. filiculoides* forms dense floating mats turning color from greenish to reddish (Online Resource 1), a transition that in southern Spain has been described to take place from February to June (Florencio et al. 2015). The species of the genus *Azolla* lives in symbiosis with *Anabaena azollae*, a nitrogen (N) fixating cyanobacteria, which allows the fern to grow under low N availability, hence phosphorus (P) becomes its main limiting nutrient (Carrapiço 2010; Peeters et al. 2016). An excess of this nutrient may cause *Azolla* blooms, as detected in Portugal in 1993 and 1995 (Carrapiço et al. 1998), and also later in Spain (García-Murillo et al. 2007). In southern Spain, its most suitable habitats are characterized by low salinity and medium to high P levels, while the cyclical droughts of Mediterranean temporary waters reduce the production of viable spores,

limiting its expansion (Fernández-Zamudio et al. 2010).

In this study, based on an experimental approach using mesocosms, we hypothesized that the development of the *Azolla* mats affects the physical and chemical features of the water, and also the composition of aquatic plant and animal communities. We aimed to evaluate the changes associated with *A. filiculoides* in the freshwater ecosystems invaded, including survivorship and development of amphibians, a threatened group of vertebrates. Tadpoles are primary consumers that present wide differences across species in their ecological requirements. Many species live in fluctuating environments and show a high degree of developmental plasticity, which allows them to accelerate development when they find adverse environmental conditions (Alford 1999; Gomez-Mestre et al. 2010). Some species have short larval periods and the larvae can escape from their aquatic habitats by completing metamorphosis with a small body size. In contrast, other species, with larger size at metamorphosis require a longer larval period and larvae are forced to withstand the deterioration of their aquatic habitats until they reach the minimum body size at which they can trigger metamorphosis (Wilbur and Collins 1973; Wells 2007). In this study, we assessed the effect of *A. filiculoides* on two amphibians that differ in their larval requirements by carrying out two different experiments in subsequent years. In the first experiment, approximating an annual inundation cycle (October to July), we used a species with short larval period: the Mediterranean tree frog, *Hyla meridionalis*. Tadpoles of this species can complete their development in 2.5–3 months, reaching a metamorphic body size of 12–16 mm (Díaz-Paniagua 1986). In the second experiment, we used a species with a longer larval development: the Western spadefoot toad, *Pelobates cultripes*. Tadpoles of this species develop for 5–8 months and reach large body sizes at metamorphosis, around 35–40 mm (Díaz-Paniagua et al. 2005). Previous laboratory experiments have demonstrated that *P. cultripes* tadpoles may accelerate development, in response to adverse environments, once they have reached a specific developmental stage (stage 35 Gosner, Gosner 1960), but not during earlier stages of development (Kulkarni et al. 2011).

In addition to the monitoring amphibians and physical and chemical variables of the water, we also

recorded macrophyte richness twice in the first experiment. We recorded analogous variables in the second experiment, but finished it at an intermediate phase of the annual inundation cycle, coinciding with the peak of the vegetation growth. This allowed us to record data on macrophytes (richness and biomass) before they decayed or were consumed by *P. cultripes*, a species that consumes macrophytes in large quantities (Arribas et al. 2015). Finally, to obtain a qualitative idea of whether *Azolla* mats caused a deterioration of the invaded habitat, we also analyzed the nutrient flow across the food web using stable isotopes.

Methods

We performed both experiments in outdoor mesocosms located in Doñana National Park (36° 59' N, 6° 27' W), in southwestern Spain. This is a protected wetland, especially preserved for the conservation of aquatic species, such as waterbirds, amphibians and macrophytes (García-Novo and Marin 2006). An extensive marsh covering about 50,000 km², and more than 3000 temporary ponds constitute the main aquatic habitats of the park, most of them being seasonal, including a dry summer phase. *Azolla filiculoides* was first detected in some points of the marsh in 2000 (García-Murillo et al. 2007), reaching a maximum cover of around 1700 ha seven years later (Espinosa et al. 2015).

We used 500 L round tanks (upper diameter 120 cm) as mesocosms. As substrate, we used 100 L of sand and 40 L of sediments from a nearby pond in which dense covers of *A. filiculoides* mats had been observed over the previous 14 years. Pond sediment served also as inoculum as it harboured seeds from native macrophytes and *A. filiculoides* spores, as well as resting eggs and propagules for zooplankton and phytoplankton.

Experiment 1 Effects of *Azolla filiculoides* on the physical and chemical water features, primary producers, zooplankton assemblages and development of *Hyla meridionalis* tadpoles.

Experimental procedure and tadpole data

The tanks were naturally filled with rainfall in October 2015. We then filled them up with well water until all reached 300 L. We monitored the water level weekly until March, refilling water when necessary to keep a constant 300 L volume, and observing native plant growth and the presence of *A. filiculoides*. From March, we did not control the water level anymore, thereafter being only altered by natural causes (evaporation and/or rainfall). We considered two different treatments in relation to *A. filiculoides*. We randomly selected 11 tanks for the treatment with *A. filiculoides* (hereafter AZOLLA), and 9 tanks without *A. filiculoides* (hereafter NO AZOLLA). In AZOLLA tanks, we allowed all plants to grow, including the exotic fern. In NO AZOLLA tanks, we weekly removed all *A. filiculoides* detected, leaving only native macrophytes. In March, we introduced 10 *H. meridionalis* tadpoles in each tank. They had been previously captured by dipnetting in a neighbouring temporary pond. All tadpoles were mixed and randomly assigned to the experimental tanks. Tadpoles had an average total length of 10.12 (\pm 1.25) mm and were in 25 Gosner developmental stage (after Gosner 1960) when introduced in the tanks. The tanks were covered with window screening to prevent *H. meridionalis* metamorphs from escaping and the accidental introduction of other vertebrates or flying insects. We observed the first *H. meridionalis* metamorph 67 days after the introduction of tadpoles in the tanks, and from then on we checked for metamorphs every third day. Upon tail resorption, we measured body mass on a digital scale (\pm 0.01 g) and snout to vent length (SVL) using scaled graph paper (\pm 0.5 mm). The ratio SVL/body mass was considered as a measure of physical condition. We estimated time to metamorphosis as the number of days elapsed since the day tadpoles were introduced to the tanks until the day when each reabsorbed the tail. We found a higher number of metamorphs than we expected on the first day we checked for them, suggesting that we had probably overlooked metamorphs prior to that day. Because juveniles continue to lose weight for some time after metamorphosis and their weight is therefore not directly comparable with that of newly metamorphosed individuals, we only included in our analyses data from metamorphs found from the 67th day onwards. For survival, we estimated the proportion of

tadpoles emerged as metamorphs in each tank, and only considered data from those tanks in which we found metamorphs from the first checking day.

Physical and chemical water features

We recorded physical and chemical variables of the water on two occasions: the first was taken two days before we introduced the tadpoles (March), and the second took place two months later (May). We recorded pH and dissolved oxygen content using a multiprobe 340iWTW; electric conductivity with a HANNA HI9373 probe; and turbidity with a HANNA HI 93703. We also obtained two 0.5 L water samples from each tank and frozen them until further analyses. One of those samples was filtered with Whatman GF/C 47-mm glass microfibre filters and then frozen until their ammonium, nitrite, nitrate and phosphate content was determined using a multichannel Seal Analytical Autoanalyzer (Model 3HR). The other water sample per tank was directly frozen until assayed and was used to determine total N and total P.

Primary producers

To quantify the abundance of phytoplankton, we estimated the chlorophyll-a content in the filters, following the trichromatic method (Holm-Hansen and Riemann 1978). With the same method, we estimated chlorophyll-a content in periphyton samples, obtained from suspended glass slides placed in the middle of the water column of the tanks in March, and protected from tadpole grazing by placing them in a 2 mm mesh wire cages. These slides were recovered two months later. They were then submerged in acetone for 24 h in dark and filtered through a GF/C filter which was used for estimation of chlorophyll-a content.

We also estimated the macrophyte cover in the tanks using a 60 \times 60 cm square quadrat, with 5 \times 5 cm cells, placed in the center of the tank over the water surface, once in March and once in May. We recorded the number of cells with the presence of each plant species. We considered the experiment finished on the date the last *H. meridionalis* metamorph was detected, 14th June.

Zooplankton

Additionally, in March, we filtered a 1-L water sample from each tank through a 100 μm mesh size net to collect a zooplankton sample. We preserved the filtered zooplankton in 70% ethanol for later quantification in the laboratory. We counted the number of planktonic crustaceans belonging to each of the main groups (calanoid and cyclopoid copepods, daphnid and chydorid cladocerans, and ostracods) and rotifers, differentiating adults from juvenile forms of copepods.

Experiment 2 Effects of *Azolla filiculoides* on aquatic plant biomass, survival of *Pelobates cultripes* tadpoles, and nutrient cycling.

Experimental procedure, tadpole introduction and plant biomass data

In October 2016, we used 32 tanks to quantify the abundance of macrophytes in relation to the presence or absence of *A. filiculoides*, and in relation to the presence or absence of tadpoles of the Western spadefoot toad, *P. cultripes*. We thus followed a 2×2 factorial design crossing the presence/absence of *Azolla* and spadefoot toad tadpoles, with 8 tanks randomly assigned to each treatment.

The tanks began to fill with rains in autumn 2016, and then the plants started to grow. Because we did not get a natural development of a dense and thick mat of *A. filiculoides* in the tanks by February 2017, we collected individuals of the invasive fern from the nearby invaded marsh pond, and placed them into 16 of the 32 tanks (AZOLLA tanks), until they covered 100% of the water surface. By this time, native aquatic plants had also grown in all the tanks. We then filled the tanks with well water to approximately 350 L and visited them periodically to remove the small amounts of *A. filiculoides* observed in NO AZOLLA tanks. On 20th March 2017, we observed a plague of aphids (*Ropalosiphon nymphaea*) that initially deteriorated most of the *A. filiculoides* plants. Then, we had to reintroduce *A. filiculoides* plants from the close marsh pond to restore *A. filiculoides* cover to 100%. We recorded the same physical and chemical variables described for Experiment 1 in all tanks, confirming that AZOLLA and NO AZOLLA tanks in Experiment 2 showed similar differences in physical and chemical

water features to those described for Experiment 1 (Online Resource 2), despite our manipulation and replacement of *A. filiculoides* mats.

We collected eggs from several clutches of *P. cultripes* from a natural pond of the study area. Eggs were first kept in the laboratory until hatching and then brought to a large tank with natural plants until the beginning of the experiment. On 20th March 2017, we introduced six *P. cultripes* tadpoles in half of the tanks (PCUL tanks). Tadpoles had an average (\pm SE) SVL length of 5.1 (\pm 0.11) mm and a total length of 12.7 (\pm 1.9) mm, all of them in Gosner stage 25. We left them to grow in the tanks until 24th April 2017, when we finished the experiment. We then removed the *A. filiculoides* mat and harvested all other natural plants grown in the tanks. We weighed them after removing the excess water with a manual centrifuge, obtaining the fresh biomass of each species per tank.

Survival and diet of spadefoot toad tadpoles

We also analyzed the effect of *A. filiculoides* on tadpole survival. We captured all tadpoles present in the tanks and estimated the number of survivors per tank. We euthanized them using a buffered lethal solution of MS-222 and collected the terminal half of the tail (including the crest and muscle) of two tadpoles from each tank with survivors (one tadpole from two AZOLLA tanks which only had one survivor) and dried them at a constant 35 $^{\circ}\text{C}$ for stable isotopic analyses. We preserved the remaining part of these tadpoles, as well as the other tadpoles, in ethanol 70%. To analyze the diet of tadpoles, we extracted the intestine of one tadpole of three different AZOLLA tanks and three NO AZOLLA tanks. We selected 4 pieces (around 15–20 mm length) of the intestine and extracted their content on a slide, which we first examined under the microscope at 4x magnification, and then under 10x. We counted the presence of items belonging to each food category on each examined 10x microscopic field, finally estimating the percentage of occurrence over the total number of fields examined. Food categories considered were: chlorophyceans, bacillariophyceans, zooplankton, insects, *A. filiculoides*, angiosperms, charophytes, and detritus. The sum of the percentages estimated for different food sources exceeded 100% because different sources commonly coincided in a same field.

Stable isotope analyses

We collected samples for stable isotope analyses of *A. filiculoides*, submerged macrophytes, filamentous algae, periphyton (on slides hanging in the tanks), filtered zooplankton and detritus from the bottom of the tanks at the end of the experiment. The samples were dried in the oven at 35 °C to constant dry weight and ground to a fine and homogeneous powder with mortar and pestle. The determination of ^{13}C and ^{15}N content in the samples was carried out at the Stable Isotope Laboratory at Doñana Biological Station. The samples were combusted at 1020 °C using a continuous flow isotope-ratio mass spectrometry system using Flash HT Plus elemental analyzer coupled to a Delta-V Advantage isotope ratio mass spectrometer via a CONFLO IV interface (Thermo Fisher Scientific, Bremen, Germany). Stable isotope ratios are expressed in the standard δ -notation (‰) relative to Vienna Pee Dee Belemnite ($\delta^{13}\text{C}$) and atmospheric N ($\delta^{15}\text{N}$), using the equation $\delta^{13}\text{C}$ or $\delta^{15}\text{N} = ((R_{\text{sample}}/R_{\text{standard}}) - 1) \times 1000$, where R is $^{13}\text{C}/^{12}\text{C}$ or $^{15}\text{N}/^{14}\text{N}$. Replicate assays of laboratory standards routinely inserted within the sampling sequence, and previously calibrated with international standards, indicated analytical measurement errors of $\pm 0.1\text{‰}$ and $\pm 0.2\text{‰}$ for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, respectively.

At the end of the experiment, we detected the occurrence of one exotic crayfish (*Procambarus clarkii*) in one of the tanks assigned to the AZOLLA + PCUL treatment, and we consequently excluded data of tadpole survival and plant biomass from this tank in further analyses. Nevertheless, due to the low number of tadpoles surviving in AZOLLA tanks, we used the surviving tadpoles for isotopic and diet analyses.

Statistical analyses

We used R software, version 3.2.5 (R Core Team 2018), for statistical analyses. We applied generalized linear models (GLM) with Gaussian error distribution to assess how physical and chemical variables, plant biomass, and diversity were affected by the presence or absence of *A. filiculoides* in the first experiment, and also by the presence of *P. cultripes* tadpoles in the second experiment. We checked for normality of the residuals of all these models, requiring the square root transformation for the plant biomass variables. We

estimated the significance of the tests with *lmtest* package and permutation test (in *lmPerm* package) when parametric assumptions were not met. Differences in the isotopic signatures of the sources in relation to the presence or absence of *A. filiculoides* were analyzed using Kruskal–Wallis test. To analyze differences among treatments in macrophyte cover (number of cells containing the plants) in Experiment 1 and plant richness in Experiment 2, we used generalized linear models with a Poisson error distribution (quasipoisson when overdispersion was detected). To compare tadpole survival, we used generalized linear models with binomial error distribution (or quasibinomial when there was overdispersion), in which the response variable was a two-column vector binding the number of survivors and the number of non-survivors per tank.

To assess whether tadpole body mass, SVL, and physical condition were affected by the presence or absence of *A. filiculoides*, we used generalized linear models with mixed effects (GLMM), using tank as a random factor. GLMM models were fitted with *lme4* package. Using the *Vegan* package, we performed a multivariate non-metric multidimensional scaling (NMDS) analysis for zooplankton composition, based on a Bray–Curtis dissimilarity matrix, which was related to the physical and chemical variables of the tanks using the function *envfit*. Then, we applied a permutational multivariate analysis of variance (Adonis, also included in the *Vegan* package) to test for significance of the relationships between the zooplankton data and the physical and chemical variables.

Results

Experiment 1 Effects of *Azolla filiculoides* on the physical and chemical features, primary producers, zooplankton assemblages and development of *Hyla meridionalis* tadpoles.

Physical and chemical composition of water

In March, AZOLLA tanks had significantly lower pH and dissolved oxygen content than NO AZOLLA tanks. We did not detect differences in either electrical conductivity or turbidity (Table 1). AZOLLA tanks also reached higher contents in nitrogen compounds than NO AZOLLA tanks, although the effect was

Table 1 Mean and standard error of the physical and chemical variables and chlorophyll-a content in NO AZOLLA and AZOLLA tanks at two different times (March and May) of the experimental period

Variables	March				May			
	NO AZOLLA	AZOLLA	$F_{1,18}$	P	NO AZOLLA	AZOLLA	$F_{1,18}$	P
pH	9.3 (0.2)	8.1 (0.1)	31.877	< 0.001	8.1(0.2)	7.3 (0.03)	28.24*	< 0.001
Oxygen (mg/L)	4.9 (0.2)	2.8(0.2)	44.25*	< 0.001	4.1 (0.3)	1.7 (0.1)	57.54*	< 0.001
Electrical conductivity ($\mu\text{S}/\text{cm}$)	741.4 (31.8)	792.5(37.8)	0.973	0.337	748.4 (39.2)	778.1 (34.1)	0.3282	0.574
Turbidity (NTU)	4.3 (1.5)	5.7(2.6)	0.203	0.658	4.4 (0.5)	7.2 (0.03)	1.105*	0.307
Ammonium ($\mu\text{g}/\text{L}$)	18.7 (4.6)	38.2(8.5)	3.496	0.078	24.2 (1.8)	287.7 (133.2)	3.169*	0.092
Phosphate ($\mu\text{g}/\text{L}$)	72.6 (24.5)	43.9(6.7)	1.414	0.25	463.0 (90.6)	672.3 (83.2)	2.882	0.107
Nitrite ($\mu\text{g}/\text{L}$)	1.8 (0.3)	2.5(0.2)	4.347	0.052	2.0 (0.1)	13.2 (5.8)	2.981*	0.101
Nitrate ($\mu\text{g}/\text{L}$)	0.001 (0.0)	1.0(2.6)	3.748*	0.069	1.6 (0.2)	84.2 (29.5)	6.37*	0.021
Total P ($\mu\text{g}/\text{L}$)	173.2 (36.7)	115.4(16.5)	2.148*	0.16	572.4 (91.9)	804.1 (88.4)	3.191	0.091
Total N ($\mu\text{g}/\text{L}$)	1371.8 (169.9)	1474.2(82.4)	0.375	0.548	1857.7 (154.0)	2386.5 (282.2)	2.38	0.14
Phytoplankton chlorophyll-a ($\mu\text{g}/\text{L}$)	1.339 (3.6)	9.027 (2.9)	5.614	0.029	3.733 (1.1)	10.693 (3.8)	2.549	0.128
Periphyton chlorophyll-a ($\mu\text{g}/\text{L}$)	–	–	–	–	0.338 (0.1)	0.210 (0.1)	0.529	0.476

F and P indicate the results and significance of generalized linear models fitted to normal distribution of errors (except when an asterisk indicates that the significance was tested with permutation analyses). Bold text enhances significant results

*LmPerm

marginally non-significant. In that first sampling, we observed no differences in total N, and total P content between experimental treatments.

Similar differences in pH and dissolved oxygen still persisted among the two treatments two months later (May), when most of the vegetation cover in AZOLLA tanks was composed of dead ferns. Higher nutrient contents persisted in AZOLLA tanks, and the differences were larger than in March (Table 1). Total P in AZOLLA tanks was on average 40% higher than in NO AZOLLA tanks, whereas total N was 30% higher in AZOLLA tanks (Table 1). Substantial variation was observed within treatments, and only nitrate significantly differed between treatments, with ammonium and total P content showing only marginal non-significance ($P < 0.10$, Table 1).

Phytoplankton and periphyton

AZOLLA tanks had higher chlorophyll-a content than NO AZOLLA tanks ($F_{1,18} = 5.61$, $P < 0.029$) in

March. The same trend persisted in May, but the differences between treatments attenuated and no significant differences were found ($F_{1,18} = 2.55$, $P = 0.128$). Content of chlorophyll-a in periphyton samples, only measured in May, did not differ between treatments ($F_{1,18} = 0.53$, $P = 0.476$) (Table 1).

Native macrophytes

In March (five months after tanks were filled), *A. filiculoides* covered on average 97% (± 5.6) of the water surface in AZOLLA tanks, whereas it remained absent from NO AZOLLA tanks. Native macrophytes were predominantly represented by the submerged species *Ranunculus peltatus* and *Callitriche truncata* (Table 2). Most of the macrophytes were negatively influenced by *A. filiculoides*. In March, *R. peltatus* reached 100% cover in NO AZOLLA tanks, but only 12.1% in AZOLLA tanks ($F_{1,18} = 61.87$, $P < 0.005$). *Callitriche truncata* extended over 56.3% of cover in NO AZOLLA, and 53% in AZOLLA tanks

Table 2 Mean percentage of live macrophyte cover (\pm SE) and of the number of tanks in which each species was observed on the water surface in March (when AZOLLA tanks had reached maximum *A. filiculoides* cover), and May (when *A. filiculoides* plants were dying) in relation to the presence or absence of *A. filiculoides* in Experiment 1

Sampling month	Species	NO AZOLLA		AZOLLA	
		% Cover	N tanks (%)	% Cover	N tanks (%)
March	<i>R. peltatus</i>	100	100	12.1 (\pm 4.8)	36.3
	<i>C. truncata</i>	56.3 (\pm 11.6)	100	53.3 (\pm 11.3)	100
	<i>R. peltatus</i> aerial morphs	5.4 (\pm 2.1)	77.8	18.9 (\pm 3.5)	100
	<i>C. truncata</i> aerial morphs	7.3 (\pm 2.7)	55.6	10.9 (\pm 3.9)	63.6
	<i>A. filiculoides</i>	–	–	Total: 97.8 (\pm 1.3) Dead plants: 5.43 (\pm 1.3)	100
May	<i>R. peltatus</i>	17.8 (\pm 5.1)	70	0	0
	<i>C. truncata</i>	42.8 (\pm 11.4)	90	1.7 (\pm 0.9)	25
	<i>R. peltatus</i> aerial morphs	0	0	0	0
	<i>C. truncata</i> aerial morphs	0	0	0	0
	<i>A. filiculoides</i>	–	–	Total: 73.0 (\pm 21.0) Dead: 39.0 (\pm 10.0)	72.7 (all dead in 55.5)

For *A. filiculoides*, the percentage of total cover and dead plants cover are indicated, as well as the percentage of tanks with only dead plants

($F_{1,18} = 1.05$, $P = 0.294$). In May, the *A. filiculoides* mat persisted in eight AZOLLA tanks (72.7% of them), but the ferns were dead on the surface of five tanks (45.5%). Among native plants, *R. peltatus* were absent from AZOLLA tanks and only covered 17.8% of the surface of NO AZOLLA tanks ($F_{1,18} = 39.18$, $P < 0.005$). *Callitriche truncata*, which had resisted *Azolla* earlier in the season, showed only 1.7% cover in AZOLLA tanks whereas in NO AZOLLA tanks still covered 42.8% of the surface ($F_{1,18} = 29.67$, $P < 0.005$) (Table 2).

Zooplankton

The presence of *A. filiculoides* modified the zooplankton composition. The ordinal representation of the first two NMDS dimensions grouped NO AZOLLA tanks in relation to the higher abundance of cladocerans and ostracods, and AZOLLA tanks in relation to higher copepod abundances (Fig. 1a). The zooplankton composition of the tanks significantly differed in relation to the presence or absence of *Azolla* ($F_{1,17} = 6.72$, $P = 0.001$), and to total N content ($F_{1,17} = 4.51$, $P = 0.012$), chlorophyll-a ($F_{1,17} = 4.20$, $P = 0.004$) and conductivity ($F_{1,17} = 3.34$, $P = 0.027$). Juvenile copepods were the most abundant group in AZOLLA

tanks, while cladocerans and ostracods predominated in NO AZOLLA tanks (Fig. 1b).

Emergence, body length and mass of Mediterranean tree frog metamorphs

The proportion of *H. meridionalis* tadpoles emerged (survivors) from NO AZOLLA tanks was 61.7% (\pm 0.83), whereas only 44.4% (\pm 0.71) emerged from AZOLLA tanks ($F_{1,23} = 2.39$, $P = 0.147$). Individuals emerged until 11th June in NO AZOLLA tanks and until 15th June in AZOLLA tanks.

Metamorphs from AZOLLA tanks were smaller in snout-to-vent length (mean \pm SE: 14.1 \pm 0.3 mm in AZOLLA; 15.5 \pm 0.9 mm in NO AZOLLA; $\chi^2 = 4.7293$, $P = 0.030$), and in body mass (mean \pm SE: 0.23 \pm 0.02 g in AZOLLA; 0.30 \pm 0.07 g in NO AZOLLA; $\chi^2 = 5.90$, $P = 0.015$), and experienced poorer physical condition ($\chi^2 = 5.05$, $P = 0.025$; Fig. 2). Tadpoles were in better conditions when they had longer time to metamorphosis ($\chi^2 = 6.36$, $P = 0.011$), and this tendency was marginally affected by the presence of *A. filiculoides* ($\chi^2 = 3.39$, $P = 0.066$), being negative in AZOLLA tanks whereas positive in NO AZOLLA tanks (Fig. 2).

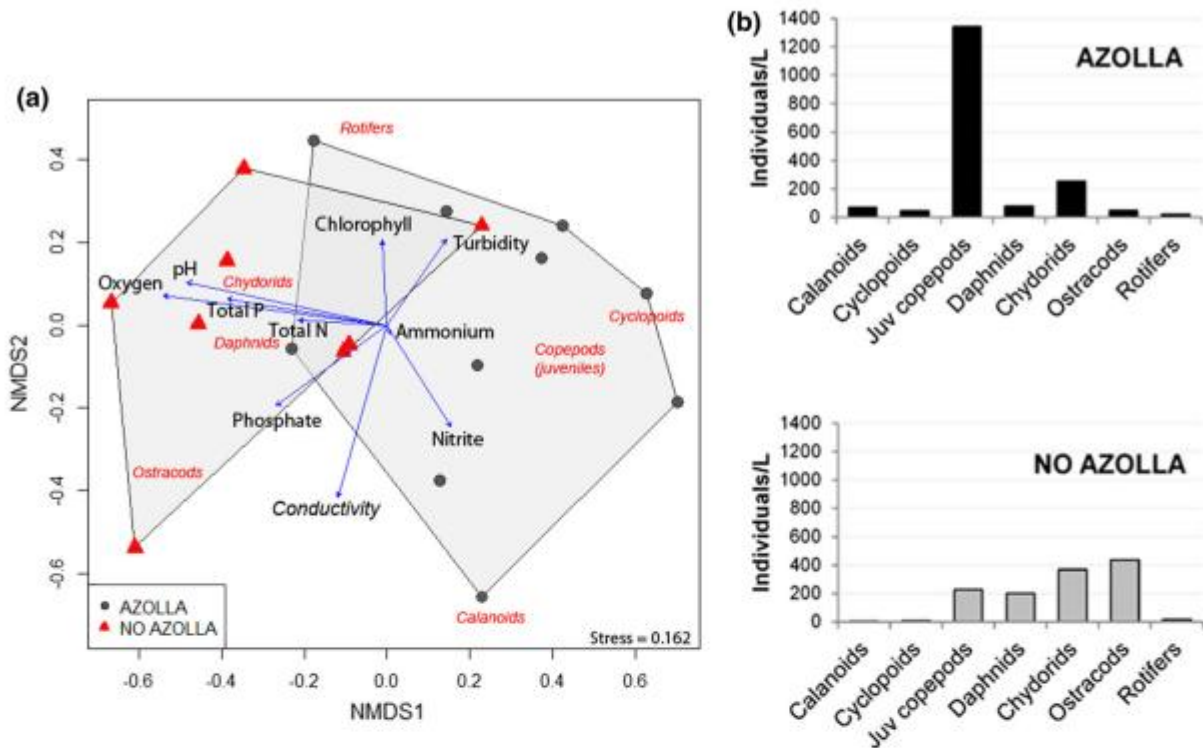


Fig. 1 a Non-metric multidimensional scaling ordination of the zooplankton groups (italic red letters) in AZOLLA and NO AZOLLA tanks, also including the vectors representing the

influence of physical and chemical variables (in black letters) to the ordination, **b** mean number of individuals of different groups of zooplankton counted in AZOLLA and NO AZOLLA tanks

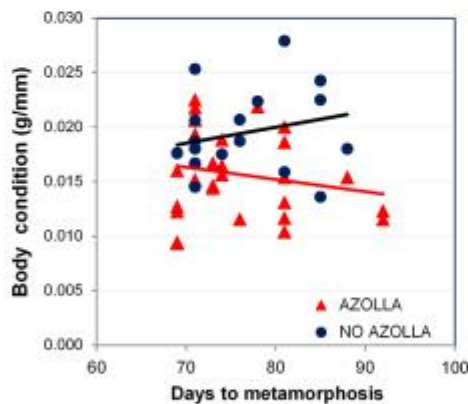


Fig. 2 Physical condition (Body mass/Snout-to-vent length) of *Hyla meridionalis* metamorphs in relation to the day of emergence (only included data of individuals metamorphosed after the day 67th)

Experiment 2 Effects of *Azolla filiculoides* on aquatic plant biomass, *Pelobates cultripis* tadpoles, and nutrient cycling.

Native macrophytes

Nine macrophyte species were found in the tanks at the end of the experiment (Table 3). The presence of *A. filiculoides*, but not of *P. cultripis* tadpoles, affected plant species diversity and richness. NO AZOLLA tanks had significantly higher diversity than AZOLLA ones (Fig. 3a; AZOLLA: $F_{1,24} = 45.44$, $P < 0.001$, PCUL: $F_{1,24} = 0.51$, $P = 0.482$). Species richness had similar results (Fig. 3b; AZOLLA: $\chi^2_{1,28} = 30.79$, $P < 0.001$; PCUL: $(\chi^2_{1,28} = 0.61$, $P = 0.433)$). In the absence of *A. filiculoides*, tanks had on average 4.1 (± 0.8) plant species, four times the number of species found on average in AZOLLA tanks (0.9 ± 0.21 species).

Plant biomass was significantly affected by the presence of *A. filiculoides* and *P. cultripis* tadpoles (Fig. 4; AZOLLA: $F_{1,28} = 143.70$, $P < 0.001$; PCUL: $F_{1,28} = 4.70$, $P = 0.030$, after square root transformation). NO AZOLLA tanks had high plant biomass (mean total biomass = 782.0 ± 82.0 g), whereas AZOLLA tanks had mean total biomass of

Table 3 Mean biomass (g) and standard error (in parentheses) of each plant species recorded and number of tanks in which they were present in relation to the presence or absence of *Azolla filiculoides* in Experiment 2

	NO AZOLLA		AZOLLA	
	Mean + SE	n tanks	Mean + SE	n tanks
<i>Chara connivens</i>	306.7 (± 64.4)	16	19.3 (± 8.9)	8
<i>Nitella translucens</i>	0	0	0.2 (± 0.2)	1
<i>Callitriche truncata</i>	149.9 (± 48.0)	15	0.5 (± 0.5)	4
<i>Myriophyllum alterniflorum</i>	22.3 (± 12.5)	3	1.9 (± 2.0)	1
<i>Ranunculus peltatus</i>	262.0 (± 66.0)	15	0.02 (± 0.02)	2
<i>Elatine alsinastrum</i>	18.4 (± 11.6)	13	0	0
<i>Baldellia ranunculoides</i>	5.2 (± 3.8)	2	0	0
<i>Eleocharis multicaulis</i>	0.4 (± 0.4)	1	0.11 (± 0.11)	1
<i>Lemna minor</i>	–	1	–	2
Total plants	782.0 (± 84.7)	16	27.1 (± 11.8)	16

For *Lemna minor*, only the presence was recorded

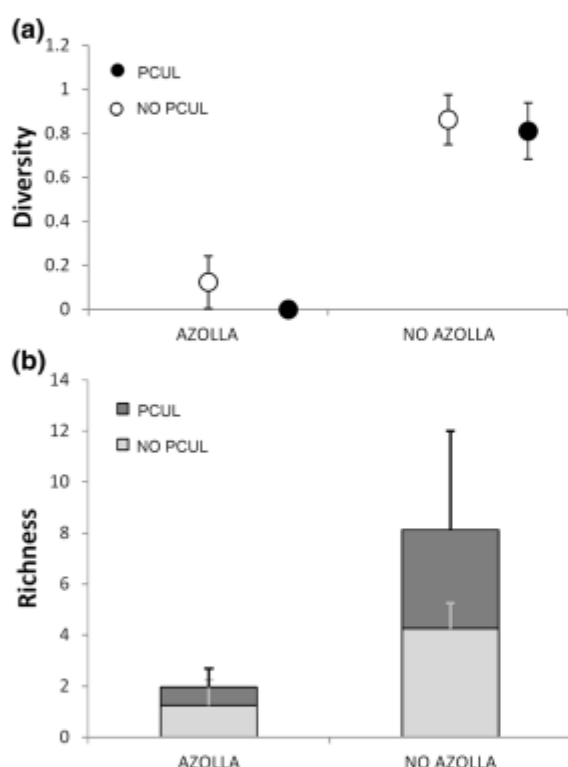


Fig. 3 Mean and standard error of: **a** the number of submerged plant species, and **b** species diversity (Shannon index) in tanks from the AZOLLA and NO AZOLLA treatments

27.1 ± 11.8 g, including four tanks completely lacking plants by the end of the experiment. Biomass of phanerogams was scarce in AZOLLA tanks, as it was significantly affected by the presence of *A. filiculoides*, but not by *P. cultripis* (log transformed phanerogam biomass: AZOLLA: $F_{1,28} = 207.39$, $P < 0.001$,

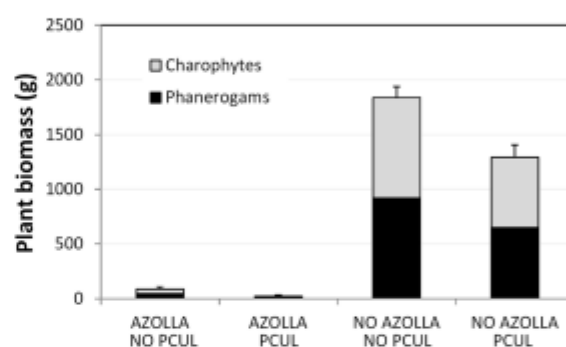


Fig. 4 Mean and standard error of the total plant biomass recorded in the four treatments combining the presence or absence of *Azolla filiculoides* and the presence or absence of *Pelobates cultripis* tadpoles. Mean biomass of phanerogams and charophytes are differentiated

PCUL: $F_{1,28} = 1.55$, $P = 0.214$), resulting in 10 (69%) AZOLLA tanks with no phanerogams. Charophytes were present in 100% of NO AZOLLA tanks, but only in six AZOLLA tanks (40%), and their biomass was significantly reduced by the presence of *A. filiculoides* (log transformed, $F_{1,28} = 48.55$, $P < 0.001$) and of *P. cultripis* (log transformed: $F_{1,28} = 4.31$, $P = 0.047$). We observed oogonia and/or antheridia in charophytes in 14 out of the 16 NO AZOLLA tanks but in none of the AZOLLA tanks. The interaction of both factors was not significant for any of the plant biomass variables.

Survival of spadefoot toad tadpoles

One month after tadpoles were introduced in the tanks, we found a significant effect of *A. filiculoides* on tadpole survival ($F_{1,13} = 14.46$, $P = 0.002$). On

average, 60% (\pm 58.3) of tadpoles survived in NO AZOLLA tanks, whereas only 4.8% (\pm 3.1) survived when *A. filiculoides* mats covered the tanks. There were no survivors in five AZOLLA tanks and in one NO AZOLLA tank. All tadpoles living under the *Azolla* mat (even those coexisting with crayfish) had a dark grey color, while those from NO AZOLLA tanks had a pale greenish color (Online Resource 3).

Diet of spadefoot toad tadpoles and isotopic values of food sources

Tadpoles significantly differed in their $\delta^{15}\text{N}$ signature between treatments and reached higher values in NO AZOLLA than in AZOLLA tanks. Similarly, most of the sources included in their diet had the same trend, with lower $\delta^{15}\text{N}$ values in AZOLLA tanks, except for the aquatic plants, which had similar or even higher values in AZOLLA tanks. In contrast, tadpoles did not differ in $\delta^{13}\text{C}$ isotopic signature, and there was a general trend in the sources to reach lower $\delta^{13}\text{C}$ values in AZOLLA tanks than in the absence of *A. filiculoides*, which were significantly different for zooplankton and detritus (Fig. 5).

The intestine of *P. cultripes* tadpoles living in NO AZOLLA tanks mainly contained detritus (85.4%),

Table 4 Percentages of presence of each food resource in the total number of microscopic fields analyzed (10 \times magnification) from the intestines of six *Pelobates cultripes* tadpoles grown in three AZOLLA tanks and three NO AZOLLA tanks

	AZOLLA	NO AZOLLA
Chlorophyceans	18.08	24.12
Bacillariophyceans	19.18	29.46
Zooplankton	15.99	35.43
Insects	15.16	3.81
<i>Azolla filiculoides</i>	75.17	0
Angiosperms	14.35	68.04
Charophytes	0	7.88
Detritus	46.79	42.71

Legends to the figures

phanerogams (62.5%, only *C. truncata*), zooplankton (66.7%) bacillariophyceans (diatoms) (54.7%) and a small frequency of the macroalga *Chara connivens* (1.6%). Those from the AZOLLA tanks had a more diverse diet (Table 4). The resources found at the highest frequencies were *A. filiculoides* (67%, appearing fronds, roots, male and female sporocarps, and spores) and detritus (53.5%). They also fed on zooplankton, filamentous algae and diatoms, invertebrates (mainly aphids) and phanerogams (10–28%). In these tadpoles, the phanerogams *R. peltatus* and *C. truncata* were present in the intestine, but the highest frequency corresponded to *Lemna minor*, abundant in one of the analyzed intestines (Table 4).

Discussion

The dense mat formed by *A. filiculoides* on the water surface altered the physical and chemical conditions of the water and, consequently, all the aquatic organisms living underneath it. Floating plants, either native or invasive, form mats on the water surface as a strategy to outcompete other aquatic plants. Other studies (Scheffer et al. 2003; de Tezanos Pinto and O'Farrell 2014) have previously demonstrated that the development of a dense mat over the water surface reduces light penetration into the water column, which limits the photosynthetic capacity of submerged plants. It also hampers direct gas exchange with the atmosphere, resulting in low dissolved oxygen in the water (Janes

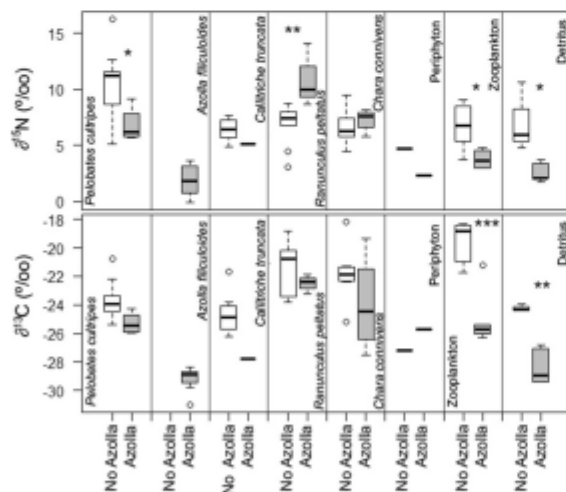


Fig. 5 Boxplot (median, interquartile range, and total range) of the $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ signatures of *Pelobates cultripes*, aquatic plants, periphyton, zooplankton and detritus recorded in AZOLLA and NO AZOLLA tanks. Significant differences are indicated with asterisks (* $P < 0.01$; ** $P < 0.001$; *** $P < 0.005$). Only submerged parts of *Ranunculus peltatus* were included

et al. 1996; Peeters et al. 2016). Therefore, an important consequence of *A. filiculoides* spread observed in our mesocosms was the debilitation and ultimate disappearance of most submerged plants. *Azolla filiculoides* plants usually grow faster and form mats thicker than other floating native plants, such as *Lemna gibba* and *L. minor* (Peeters et al. 2016). The faster mat formation probably increases the negative impact on native aquatic plants.

Most of the submerged plants growing under the *Azolla* mat ended up dying without seed production, except those species that were able to develop aerial forms extending across the thick mat. These species may plastically develop terrestrial morphotypes under non-flooded conditions. In our study, we observed aerial forms growing over the *A. filiculoides* mat of *R. peltatus* and *C. truncata*. The morphotype of *R. peltatus* over the *A. filiculoides* mat has been described in detail in a previous study as an exaptation to resist the habitat perturbation caused by *A. filiculoides* (Pinero-Rodríguez et al. 2018). When *R. peltatus* grew in co-occurrence with *A. filiculoides*, the species reduced its cover, density, and vegetative period, producing fewer flowers and consequently fewer seeds (Pinero-Rodríguez et al. 2018).

Only charophytes were able to withstand the environmental alterations caused by the *A. filiculoides* mat, although they also experienced a considerable reduction in biomass and showed a delay in sexual propagule production. The higher resistance of charophytes may be explained by their morphological adaptations to low light intensity, producing longer shoots and taller plants at low light conditions (Blindow et al. 2003). Also, charophytes have lower minimum light requirements and are consequently able to colonize deeper areas than angiosperms (Chambers and Kalf 1985; Middelboer and Markager 1997).

We observed a decrease in pH and dissolved oxygen concentration under the *Azolla* mat, as previously described by Janes et al. (1996). These authors also described an increase in carbon dioxide (CO₂) which could benefit photosynthesis, while the decreased oxygen concentrations reduce respiration. Photosynthesis and respiration are the main processes affecting pH (Bronmark and Hansson 2005). The consumption of CO₂ during photosynthesis would increase pH, but when the *Azolla* mat reduces light penetration, it also reduces the photosynthetic capacity

of submerged plants, contributing to decreased pH, while CO₂ would remain higher in relation to NO AZOLLA tanks. In addition, the lower number of plants photosynthesizing in AZOLLA tanks would also explain the lower consumption of CO₂ and their lower pH.

We have observed a notable increase in nutrients over time in all our mesocosms, but mainly so in AZOLLA tanks. The disproportionate 15-fold increase of nutrients in AZOLLA tanks may be due to the negative effect of the *Azolla* mat on other aquatic plants, which died and decomposed, hence contributing to increase the quantity of organic matter deposited in the ponds. In addition, *A. filiculoides* has the ability to store phosphorus (Peeters et al. 2016) and lives in symbiosis with the cyanobacteria *Anabaena azollae* that fixes nitrogen (Peters and Meek 1989). Thus, the species has an advantage over other aquatic plants in environments where nitrogen or phosphorus are limiting. When the plants of the thick mat die, decompose and fall to the pond basin, they are also contributing with an excess of nutrients to the organic matter deposited in the pond bottom.

The higher concentration of chlorophyll-a revealed that phytoplankton was more abundant in AZOLLA tanks than in NO AZOLLA tanks. This was a direct consequence of the eutrophication caused by the presence of *A. filiculoides*. The lower oxygen availability causes phosphate release from sediments to the water column, making this nutrient available for phytoplankton (Bronmark and Hansson 2005). In our study, we only detected higher abundance of phytoplankton in AZOLLA tanks, but not increased abundance of periphyton, probably due to the absence of aquatic plants required as substrate for the development of these algae.

All the changes observed in the environment dominated by *A. filiculoides* also affected the animals living underneath the mat. The effect of *A. filiculoides* on animal communities has been only described in streams invaded by this exotic fern in Zimbabwe, where a decrease in invertebrates, and in tadpole and fish abundance and diversity were detected (Gratwicke and Marshal 2001). The absence of egg strings on a common reproductive habitat of natterjack toads, *Epidalea calamita*, in Spain was also reported as an effect of the expansion of *A. filiculoides* (Galán 2012). In our study, the zooplankton community varied significantly. The alteration of the environmental

conditions in AZOLLA tanks resulted in impoverishment zooplankton diversity, favoring the parthenogenetic cycles of copepods, which increased their juvenile forms. The increase in phytoplankton should have benefitted filter-feeder species, such as small cladocerans, but also nauplius and copepodites, which consequently also favored a higher abundance of adult copepods.

The effect on tadpoles of two different species of anurans revealed different results for each species, which may be due to differences in the length of their larval period and the relative importance of macrophytes in their diet. For *H. meridionalis* we did not find differences in mortality in relation to the presence or absence of *A. filiculoides*. These tadpoles can reach the minimum body size required to complete their development within a short period. They are herbivorous, including in their diet a high proportion of detritus, algae and aquatic plants (Díaz-Paniagua 1985; Arribas et al. 2015). In our experiment, the impoverishment of the aquatic environment mostly affected aquatic plants, which died and decomposed to be transformed in organic matter deposited as detritus among sediments. The reduced body size and physical condition of tadpoles from AZOLLA tanks revealed the poorer nutritive value of the remaining available food. Lower body condition of the emerging metamorphs could be due to the deterioration of food sources or to a dietary shift towards greater ingestion of detritus. In fact, *A. filiculoides* did not have an effect on survival, but on the size and condition of the metamorphs. This effect was accentuated in tadpoles metamorphosing later, which probably were forced to extend their larval period in order to reach the adequate size to complete their development, while their habitat was becoming more and more inhospitable. In contrast, in NO AZOLLA tanks, where aquatic plants were a common and abundant resource, metamorphs reached larger body sizes and better conditions when they had longer developmental periods.

In the second experiment, tadpoles of *P. cultripes* (characterized by a long larval period) were kept in the mesocosms during an early phase of their developmental period. After only one month in the mesocosms, surviving tadpoles experienced a sixfold increase in their SVL under the *Azolla* mat and a sevenfold increase in NO AZOLLA tanks. For this species, the most significant impact of *A. filiculoides* was tadpole mortality. The main components of the

diet of large *P. cultripes* tadpoles are aquatic macrophytes (Arribas et al 2015). The absence of submerged plants in AZOLLA tanks probably affected the development of most of the tadpoles. While the main component of the diet of tadpoles in NO AZOLLA tanks were phanerogams, zooplankton, bacillariophycean algae and detritus, survivors in AZOLLA tanks mainly fed *A. filiculoides* and detritus, with lower consumption of other food resources. They compensated for the absence of phanerogams by consuming *A. filiculoides*, which had lower nutrient content, and probably lower digestibility than the aquatic plants commonly fed by these tadpoles. While the stems and leaves of submerged macrophytes are soft and flexible, usually lacking lignin (Cronk and Fennessy 2001), floating plants have a hard cuticle covered by wax and containing suberine and/or lignine (Borisjuk et al 2018), which probably can not be shredded by small tadpoles. The high mortality detected in most AZOLLA tanks was probably related to the absence of phanerogams, as small tadpoles were still not able to feed on the harder *Azolla* fronds, after depleting the organic matter of sediments.

Our isotopic analyses confirmed a deterioration of the food sources under the *A. filiculoides* mat. Submerged plants show a wide spatial and temporal variation in isotopic composition (Boon and Bunn 1987), as they depend on the inorganic C and N availability in the aquatic environment (Finlay and Kendall 2007). Except for periphyton, which was not an abundant resource, our analyses revealed that submerged aquatic macrophytes and detritus in AZOLLA tanks were depleted in $\delta^{13}\text{C}$. Consequently, primary consumers as zooplankton and tadpoles in these tanks were also $\delta^{13}\text{C}$ depleted. Although the fronds and roots of *A. filiculoides* were an alternative food resource for tadpoles, this plant showed a low $\delta^{13}\text{C}$ signature, providing lower carbon and energy than other plants to primary consumers.

Except for *C. truncata*, which was very scarce and deteriorated at the end of the experiment, other macrophytes like *C. connivens* did not differ in their $\delta^{15}\text{N}$ values in relation to the presence of *A. filiculoides*, which is in accordance with the high availability of N compounds in AZOLLA tanks. In contrast, tadpoles (which are primary consumers) had lower $\delta^{15}\text{N}$ values in the presence of the *Azolla* mat, as a consequence of the scarcity of macrophytes in AZOLLA tanks, which forced them to shift their diet

towards sources with lower $\delta^{15}\text{N}$ values, mainly *A. filiculoides* and detritus.

In summary, the invasion by *A. filiculoides* has negative consequences for the animal and plant communities of aquatic ecosystems. When *A. filiculoides* forms a dense mat over the water surface, it reduces light penetration and oxygen exchange in the water column, causing a steep reduction in macrophyte biomass and consequently their photosynthetic activity. The decay of dead plant biomass caused eutrophication of the water and impoverishment of the basal organisms of the trophic web, also reducing the trophic position of primary consumers, like tadpoles, compromising their body condition at metamorphosis or even causing a steep decline in their survival.

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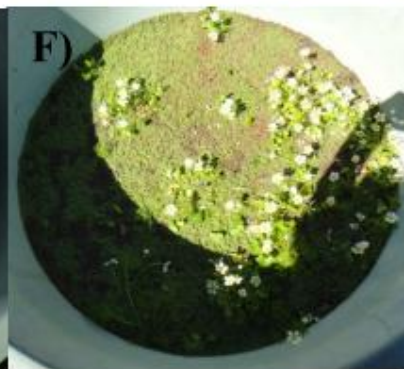
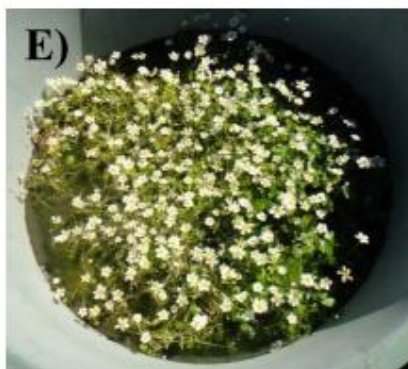
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Supplementary material:

Online Resource 1

A) Aerial view of the border of the marshes invaded by *Azolla filiculoides* in Doñana National Park; B) a closer view of *Azolla* mat on the surface of a pond; C and D) Details of the borders of the marsh invaded by *A. filiculoides*; E) NO AZOLLA tank in February 2016; F) AZOLLA tank in February 2016; G) AZOLLA tank in May 2016.



Online Resource 2

Mean and Standard error of physical and chemical variables and chlorophyll-a content in relation to the presence or absence of *Azolla filiculoides*. The results of the models analyzing the effect of *Azolla filiculoides* presence (AZOLLA) or *Pelobates cultripes* presence (PCUL) are also indicated.

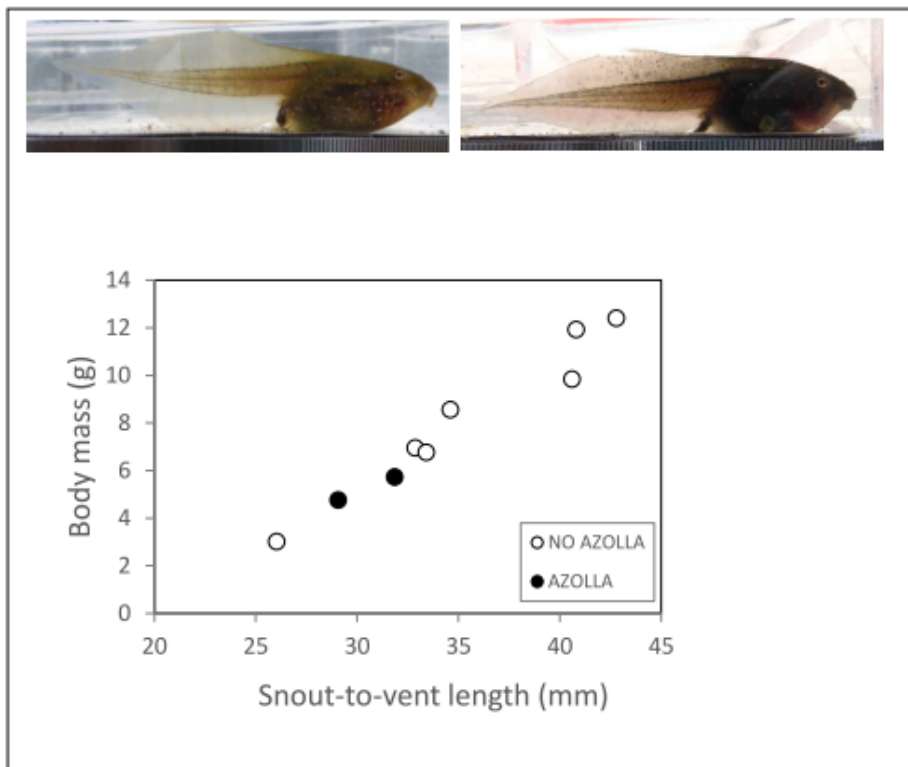
	AZOLLA	NO AZOLLA	AZOLLA	PCUL
	mean+SE	mean+SE	F,p	F,p
Oxygen(mg/L) ⁽¹⁾	3.23(0.67)	9.76 (0.91)	33.2539, <0.0005	3.2613, 0.081
E Conductivity (μ S/cm)	925 (20.9)	782.8 (42.2)	9.2043, 0.005	1.1674, 0.289
pH ⁽²⁾	7.9 (0.06)	8.36 (0.1)	15.1179, <0.0005	1.6978, 0.203
Turbidity (NTU) ⁽²⁾	14.0 (0.25)	14.1 (0.26)	0.0690, 0.795	0.2794, 0.601
Ammonium (μ g/L)	626.2 (107.5)	35.3 (14.7)	28.7807, <0.0005	0.0847, 0.773
Phosphates (μ g/L)	813.3 (98.2)	228.4 (64.5)	37.4495, <0.0005	0.0098, 0.922
Nitrites (μ g/L)	120.5 (10.3)	7.7 (2.8)	107.9040, <0.0005	0.0008, 0.978
Nitrates (μ g/L)	672.3 (108.5)	12.22 (5.4)	122.0266, <0.0005	0.1359, 0.715
Total P(μ g/L)	940.4 (114.7)	317.3 (78.9)	35.7721, <0.0005	0.1765, 0.677
Total N(μ g/L)	2994.4 (142.4)	1623.2(178.1)	34.9820, <0.0005	0.0362, 0.850
Chlorophyll-a (μ g/L)	3.4 (1.0)	1.9 (0.26)	3.4085, 0.075	1.4974, 0.231
Periphyton Chlorophyll-a (μ g/L)	0.6 (0.1)	3.9 (0.9)	12.7743, 0.001	0.4478, 0.509

⁽¹⁾This variable was log-transformed for GLM comparisons

⁽²⁾Lmperm was used for comparisons because these variables did not fit normal error distributions

Online resource 3

Mean snout to vent length vs mean body mass of *Pelobates cultripes* tadpoles from mesocosms in the presence or absence of *Azolla filiculoides*. Tadpoles were weighed (± 0.01 g) and photographed on a millimetric scale for a posterior estimation of their SVL using Image 2.0 software. Above: Green phenotype of *P. cultripes* tadpoles grown in NO AZOLLA tanks (left) and dark phenotype of *P. cultripes* grown in AZOLLA tanks (right).



4. Desarrollo de un morfotipo emergente en *Ranunculus peltatus*, en respuesta al efecto de sombra producido por el helecho exótico *Azolla filiculoides*. (*Ranunculus peltatus develops an emergent morphotype in response to shading by the invasive Azolla filiculoides*)



Short communication

Ranunculus peltatus develops an emergent morphotype in response to shading by the invasive *Azolla filiculoides*

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ABSTRACT

The invasive aquatic fern *Azolla filiculoides* forms dense mats that shade submerged macrophytes. Here we show that submerged plants of *Ranunculus peltatus* switch to a different morphotype when shaded by *Azolla filiculoides* mats. We used outdoor mesocosms to assess the cover of submerged plants in tanks with presence or absence of the exotic fern. In tanks with *A. filiculoides* mat covering the water surface, the submerged stems of *R. peltatus* elongated to surpass the dense mat, while their submerged capillary leaves were deteriorated and lost. Once above the *Azolla* cover, *Ranunculus* developed an aerial part of the plant of reduced height with erect aerial laminar and thick capillary leaves, which subsequently produced flowers and fruits. The plants with this aerial amphibian morphotype survived for shorter spans and produced fewer flowers than the aquatic morphotypes growing without *A. filiculoides* mats. We conclude that the morphological plasticity that characterizes *R. peltatus* allows this species to subsist the adverse transformation of the aquatic environment by the development of *Azolla* mats.

1. Introduction

Ranunculus peltatus Schrank is a common submerged aquatic plant, widespread in Europe and found in all Mediterranean countries. This species, as others of the subgenus *Batrachium*, usually lives in temporary aquatic habitats, developing aquatic forms, but growing as terrestrial plants in wet soils (Cook, 1963). Many species of this group are heterophyllous and exhibit an extreme degree of phenotypic plasticity (Cook, 1966). Heterophylly, the ability to produce different leaf types, is one type of phenotypic plasticity commonly observed among macrophytes (van der Valk, 2012). This strategy allows aquatic plants to survive under both dry and submerged conditions, as an adaptation to fluctuating water levels (Cronk and Fennessy, 2001).

Ranunculus peltatus is perennial in permanent waters, but annual in temporary waters. This species shows wide morphological plasticity, with different lengths of their internodes and branches and different reproductive strategies in relation to the quality and disturbance of the environment (Garbey et al., 2004). Plants with aquatic forms may even shift to terrestrial forms when they are taken out of the water and placed in a terrestrial environment, where they can thus survive and produce flowers and fruits (Volder et al., 1997). The aquatic

morphotype is characterized by long erected stems with submerged capillary leaves (Fig. 1E) as well as floating laminar leaves on the water surface (Fig. 1C) and adventitious roots in the nodes of submerged stems (Garbey et al., 2004). The terrestrial morphotype, developing under drought conditions, consist of prostrate stems of short height (Volder et al., 1997), with aerial and capillary leaves (Fig. 1D).

A common threat to aquatic plants is the introduction of exotic invasive plants that compete with native species or modify their habitats, reducing their survival and compromising their reproduction (Strayer, 2010; Hussner, 2012). *Azolla filiculoides* is a free-floating aquatic fern native to tropical and temperate America that has been widely introduced across the globe (Brinkhuis et al., 2006). Its first presence in Spain was reported at the beginning of the 20th century (Bolòs and Masclans, 1955) and is now widely spread throughout the country, with the greatest abundance in the southwest (Cirujano Bracamonte et al., 2014). *Azolla filiculoides* may form thick and dense floating mats that cause a negative effect on submerged plants and algae as well as on animal populations (Hussner, 2010).

Here we describe how aquatic form of *R. peltatus* plants are able to modify their morphology to survive in places where dense *A. filiculoides* mats cover the water surface, shading the water column.

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Fig. 1. Aerial forms of *Ranunculus peltatus* growing over *Azolla filiculoides* mat (A and B). The common aquatic form (C) and terrestrial form (D) of *R. peltatus*, submerged aquatic stems and capillary leaves in common aquatic plants (E), and long submerged stems without leaves in *R. peltatus* individuals grown in the presence of *A. filiculoides* mats (F and G).

2. Material and methods

We simulated the inundation cycle of temporary aquatic habitats in Doñana National Park (SW Spain) using *in situ* mesocosms. In this area *A. filiculoides* forms thick mats at the border of an extensive marsh, where it was first detected in 2001 (Fernández-Zamudio et al., 2006). Our mesocosms consisted of 23 plastic round tanks (volume = 500 L, upper diameter = 120 cm), located outdoors in Doñana National Park under natural light and temperature variations. We put 100 L of sand in the bottom of the tanks and added 40 L of sediment from the nearby marsh where *A. filiculoides* is abundant. The tanks were naturally filled during rainfall in October 2015 and were topped up with well water, until all the tanks reached a volume of 300 L. We then allowed natural

vegetation to grow over the next four months, from the seeds in the sediment. *Ranunculus peltatus*, *Callitriche truncata* Guss and *Chara con-nivens* P.Salzmann ex A. Braun were the most common plant species growing in the tanks. We randomly assigned each tank to either presence ($n = 12$ AZOLLA tanks) or absence ($n = 11$ CONTROL tanks) of *A. filiculoides*. Any *A. filiculoides* starting to grow in CONTROL tanks were manually removed on a weekly basis for the entirety of the experiment, whereas they were left to grow undisturbed in the AZOLLA tanks. In March and May, we measured pH and electrical conductivity (using multi 340i WTW probe) to monitor the water quality in the tanks. CONTROL tanks averaged pH of $9.3 (\pm 0.17 \text{ SE})$ and $8.1 (\pm 0.17 \text{ SE})$, respectively for March and May, with a conductivity of $738.2 (\pm 30.2 \text{ SE})$ and $747.9 (\pm 35.1 \text{ SE}) \mu\text{S}/\text{cm}$. The AZOLLA tanks had pH

averages of $8.1 (\pm 0.09 \text{ SE})$ and $7.3 (\pm 0.03 \text{ SE})$ and conductivity averaged at $808.0 (\pm 37.9 \text{ SE}) \mu\text{S/cm}$ and $786.8 (\pm 32.3 \text{ SE}) \mu\text{S/cm}$ in March and May respectively.

In February, March, April and May, we took photographs of the water surface of all tanks in order to quantify the presence of *A. filiculoides* and the emergent parts of *R. peltatus*, estimating the area covered by live and/or dead plants of these two species (*A. filiculoides* and *R. peltatus*) and also counted the number of flowers of *R. peltatus*. In addition, the photographs taken in March, when the maximum abundance of *R. peltatus* was reached in AZOLLA tanks, were also used to quantify the abundance of different types of emergent leaves of *R. peltatus*, namely the aerial capillary leaves, aerial laminar leaves and floating laminar leaves observed on the surface. To estimate the density of this plant in the tanks, we superimposed an image of a 12×12 grid to each photograph (a central rectangle with the vertices touching the edges of the circular perimeter of each tank) and counted the number of grids in which *R. peltatus* was present. We did not quantify the presence of underwater plants since they were not visible in the AZOLLA tanks.

2.1. Statistical analyses

We used generalized linear models (GLM) to compare how macrophytes differed between treatments, using R version 3.1.0 (R Core Development Team, 2014). We compared the number of flowers, number of floating laminar leaves, number of aerial laminar leaves, and number of aerial capillary leaves of *R. peltatus* between treatments fitting models with a Poisson error distribution. However, all these models were overdispersed. We therefore refitted models for *R. peltatus* density, number of aerial and floating laminar leaves and number of aerial capillary leaves to assume a negative binomial (GLM-nb) error distribution, whereas we used a quasipoisson error distribution (GLM-quasipoisson) for analyzing the number of flowers.

3. Results and discussion

In the AZOLLA tanks, we detected *R. peltatus* plants with a particular morphotype when the fern cover formed a dense mat on the water surface (Fig. 1A, B). This morphotype included an aerial part of the plant emerging over the *A. filiculoides* mats similar to the terrestrial forms that this species develops in dry conditions (Volder et al., 1997): short erected stems, aerial laminar leaves and thick capillary aerial leaves that do not touch the water. These plants had submerged stems, extending from the substrate to the water surface, and did not exhibit submerged capillary leaves, only short adventitious roots under the nodes (Fig. 1F, G).

The cover of *A. filiculoides* in the experimental tanks changed over time. By February, *A. filiculoides* had developed thick mats over the water surface in 75% of AZOLLA tanks, covering more than 75% of the water surface in each tank. All tanks had dense mats in March and April, but about 20–30% of the *A. filiculoides* cover was composed of dying plants. In May, three of the AZOLLA tanks had no *A. filiculoides* anymore, and while the others ($n = 9$) still had dense covers, seven of them had only dead individuals. In June, *A. filiculoides* was absent from the water surface in all tanks (Fig. 2A).

We observed *R. peltatus* plants reaching the water surface of the tanks from February to May in CONTROL tanks, but only from February until April in AZOLLA tanks. Cover of *R. peltatus* was lower in AZOLLA than in CONTROL tanks throughout the experiment (Fig. 2B). We observed *R. peltatus* stems with aquatic capillary leaves emerging through the *Azolla* layer. Once they surpassed the mat they developed short erect and thicker forms, as well as erect laminar leaves and flowers. The *R. peltatus* plants with this morphotype started to decline in April, when half of the plants were dying. In May the few remaining *R. peltatus* plants were clearly decaying. Throughout all monthly samples, the number of *R. peltatus* flowers was notably higher in the CONTROL than in the AZOLLA tanks (Fig. 2B).

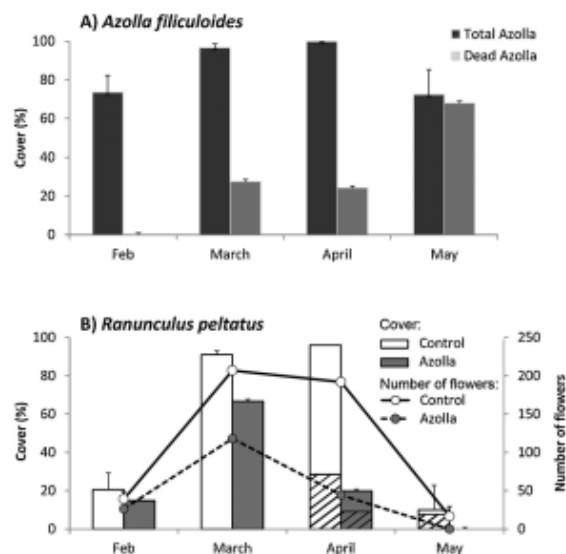


Fig. 2. A) Variation in plant cover (mean + SE) of *Azolla filiculoides*; and B) Variation in plant cover and number of flowers of *R. peltatus* in the experimental tanks from February to May. Hatched areas indicate the proportion of dead *R. peltatus* plants.

In March, the AZOLLA tanks showed the highest densities of *R. peltatus* (more than 50% of the grids with presence of the species), but densities were still significantly lower than in CONTROL tanks (GLM-nb: $z = 2.294$, $P = 0.022$; average number of grids (\pm SE) with *R. peltatus* presence = 96.1 ± 8.86 in AZOLLA tanks vs. 131.1 ± 8.59 grids in CONTROL tanks). AZOLLA tanks had an average of 118 ± 21.0 (SE) flowers, significantly differing from CONTROL tanks (GLM-quasipoisson: $t = 2.286$, $P = 0.033$), which had approximately doubled the number of flowers (207 ± 34.0 flowers). At that point in the year, all AZOLLA tanks contained the aerial morphotype of *R. peltatus*. The plants in CONTROL tanks had mostly laminar leaves floating on the water surface, whereas almost all laminar leaves in AZOLLA tanks were erected and emerging over the mats. The number of floating laminar leaves therefore also differed between treatments (GLM-nb, $z = 4.587$, $P < 0.0005$: average number of floating laminar leaves (\pm SE) in AZOLLA tanks = 6.5 ± 5.1 , in CONTROL tanks = 191.5 ± 50.1). However, the number of laminar leaves emerging from the water surface (aerial laminar leaves) did not differ among treatments, as they were also common in CONTROL tanks when the density of *R. peltatus* was high (average \pm SE: in AZOLLA tanks = 163.7 ± 28.8 , in CONTROL tanks = 86.3 ± 39.2). In CONTROL tanks there were no aerial capillary leaves, whereas AZOLLA tanks had on average 26.6 ± 6.3 grids with aerial capillary leaves (GLM-nb: $z = -5.358$, $P < 0.0005$).

The development of a thick mat of *A. filiculoides* usually forms a barrier for other submerged aquatic plants that reduces light and oxygen availability in the water bodies (Janes et al., 1996). The shading effect produced by floating plants like *A. filiculoides* commonly affect the development and growth of submerged macrophytes, which can survive by adopting different strategies, such as the rapid elongation of stems or tolerance to low light intensity (Lu et al., 2013). *Ranunculus peltatus* is a very plastic plant that may resist adverse conditions by transforming its structure from an aquatic to a terrestrial form (Cook, 1963; Volder et al., 1997; Garbey et al., 2004). Such a transition was evidenced in this species in response to drought conditions (Volder et al., 1997), and suggests that this plant is not only adapted to immersion but also to desiccation (Lumbreras et al., 2009). In the aquatic form, photosynthesis is mainly performed by submerged capillary leaves, using water carbon dioxide (CO_2) or bicarbonate (HCO_3^-), whereas laminar leaves have mainly a buoyancy function for the

flowering stalks (Nielsen and Sand-Jensen, 1993). In terrestrial forms, the formation of aerial laminar and capillary leaves allows the plant to resist desiccation and to photosynthesize in the aerial environment (Nielsen and Sand-Jensen, 1993).

In our study, under the deterioration of the aquatic environment by *A. filiculoides*, mostly as a consequence of reduced light penetration and possibly oxygen concentration, *R. peltatus* plants lost their submerged capillary leaves, probably associated to the inability to perform their photosynthetic function. However, they were able to grow through the thick *A. filiculoides* mat and develop new emerging aerial structures that enabled them to not only persist for several months, but also to produce flowers and seeds. Under these conditions, *R. peltatus* plants combined both aquatic and terrestrial forms, developing aerial leaves with thick cuticles to resist desiccation and maintaining their submerged stems rooted to the ground, revealing the high capacity of this species to overcome disturbances. The amphibious morphotype found in this study suggests that the plasticity of *R. peltatus* in response to the event of a drying environment can act as an exaptation (*sensu* Gould and Vrba, 1982) to respond to the detrimental effect of invasive mat-forming plants. *Ranunculus peltatus* was not the only submerged species exhibiting forms with aerial leaves, but we also observed them in *Callitriche truncata* Guss. (Fig. 1b), a common aquatic plant in our area that usually does not reach the water surface. However, it did not reach as high a cover in AZOLLA tanks as *R. peltatus*.

In our experiment, the main transformation of the aquatic environment caused by *A. filiculoides* was reduced light penetration, which forced *R. peltatus* to have to emerge over the fern mat. Other possible limiting factors such as oxygen availability may have also been circumvented with the development of the aerial morphotype. We observed the occurrence of aerial structures in *R. peltatus* in response to shading by *A. filiculoides*, not only in the experimental tanks, but also in natural areas invaded by *A. filiculoides* in the nearby marsh (Supplementary Fig. 1).

We conclude that the morphological plasticity of *R. peltatus* allows this species to survive and reproduce in a wide range of contrasting environments. Although the *A. filiculoides* mats shaded the water column and was adverse for the submerged part of the plants, the development of an aerial part over the mat made their photosynthetic functions and the production of fruits and seeds possible, just as their terrestrial morphotypes do in dry years.

Author contributions

MJP and CDP recorded and analyzed the experimental data and all authors equally contributed to discussing and writing the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.aquabot.2018.09.007>.

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Supplementary material:



Supplementary Figure 1: Aerial forms of *Ranunculus peltatus*, growing among *Azolla filiculoides* in Doñana marsh.

Discusión general:

Los anfibios son un grupo de especies enormemente heterogéneo, a lo que se une el hecho de que la fase larvaria se realice en condiciones completamente diferentes de la que requieren los adultos. En concreto, la mayoría de las especies de anuros desarrollan la fase larvaria en el medio acuático, donde en muchas ocasiones alcanzan altas densidades, por lo que pueden causar un impacto importante sobre la estructura de las comunidades acuáticas. Algunos estudios recientes sobre la estructura de las comunidades acuáticas han destacado el papel que las larvas de anfibios ejercen sobre las redes tróficas. Muchas especies de anfibios son consumidores primarios, u omnívoros en los que predomina la materia vegetal como alimento (Altig *et al.*, 2007), contribuyendo al flujo de nutrientes, alterando las cadenas tróficas, la distribución de los sedimentos e incluso la estructura espacial del hábitat (Arribas *et al.*, 2014; Cortés-Gómez *et al.*, 2015; Kloh *et al.*, 2019). La mayoría de los anuros usan medios acuáticos temporales para reproducirse (Wells, 2007), muchos de ellos de escasas dimensiones, pero de gran productividad y compleja estructura (Grillas *et al.*, 2004; Williams, 2006). En ellos, los anfibios, y en particular sus larvas, son frecuentemente los únicos vertebrados, llegando a ejercer un gran impacto sobre la estructura de estos hábitats al alcanzar altas densidades. Esta tesis se centra principalmente en el análisis de las relaciones de las larvas de anfibios con los macrófitos que predominan en las lagunas temporales mediterráneas, cuyas interacciones han sido escasamente abordadas previamente.

Los renacuajos, y en concreto los que adquieren gran tamaño corporal como los del sapo de espuelas, llegan a tener un gran impacto sobre las plantas acuáticas, por lo que pueden considerarse entre los herbívoros más importantes de las lagunas temporales mediterráneas. Nuestro trabajo experimental demuestra que los renacuajos de *P. cultripes* pueden reducir considerablemente la biomasa de los macrófitos, alterando con ello la compleja estructura espacial de los ambientes acuáticos, y consecuentemente afectando también a otros organismos que utilizan a las plantas como soporte, refugio o alimento. Nuestro estudio, además, demuestra que las larvas de anfibios pueden tener también un efecto importante en medios acuáticos permanentes, aunque el efecto se magnifica en los medios temporales, especialmente en las especies de macrófitos más tempranas, contribuyendo probablemente al rápido crecimiento de los renacuajos.

Los macrófitos sumergidos propios de medios temporales están adaptados a soportar las amplias variaciones que soportan estos medios (ver por ejemplo Bliss & Zedler, 1998; Cronk & Fennesi, 2002; Williams, 2006; Fernández-Zamudio *et al.*, 2017), especialmente en cuanto a

la impredecibilidad del periodo de inundación. En nuestro estudio, también detectamos que las plantas son capaces de responder a la herbivoría ejercida por los renacuajos, lo cual ofrece un resultado novedoso que resalta el importante papel que las larvas de anfibios pueden ejercer sobre la dinámica de las comunidades de los medios acuáticos temporales. Por una parte, plantas como *Ranunculus peltatus* tienen una respuesta ante la temporalidad y ante la presión de herbivoría, manteniendo una alta producción de flores, a pesar de que acortan su periodo de floración, con lo que garantizan su éxito reproductivo y sus probabilidades de desarrollo en futuros ciclos hidrológicos. Pero no todas las especies de macrófitos presentan esta respuesta, ya que no se ha observado sobre la otra especie estudiada, *Myriophyllum alterniflorum*, probablemente porque esta especie parece haber desarrollado defensas químicas ante los herbívoros (Cabrera-Guzmán *et al.*, 2020). Alternativamente, también hay que considerar que *M. alterniflorum*, que no parece responder a la herbivoría ejercida por los renacuajos, tiene una reproducción considerablemente más tardía que otras especies de macrófitos, coincidiendo con los periodos en los que los renacuajos alcanzan mayor tamaño y por tanto pueden ejercer mayor impacto sobre las plantas. Por ello, también podría ser posible que la producción de compuestos alelopáticos como defensa ante la herbivoría hubiera evolucionado en respuesta a la presión de herbivoría de las larvas de anuros.

El efecto de la herbivoría por parte de las larvas de *P. cultripes* se detectó con tan solo dos renacuajos en un volumen de aproximadamente 400 L de agua, mientras que en las lagunas temporales las densidades de renacuajos pueden alcanzar valores mucho más altos, multiplicando enormemente el impacto esperado sobre los macrófitos sumergidos. En esta tesis se ha hecho también un esfuerzo por cuantificar la densidad que alcanzan los renacuajos en sus hábitats acuáticos, y su movilidad. Estos resultados son también novedosos, ya que no se ha analizado anteriormente en otros estudios, probablemente por las dificultades que presentan estos organismos para su seguimiento en libertad. Por una parte, hemos conseguido comprobar que alcanzan altísimas abundancias, como fueron más de 57000 individuos en una laguna de unos 8000 m² (7.2 renacuajos/m²) o más de 200000 en una laguna de unos 34000 m² (5.9 renacuajos/m²). Ello refuerza la importancia que tienen como herbívoros, pues el impacto observado experimentalmente adquiere su verdadera magnitud al considerar las altas densidades en que se presentan en el medio natural.

También hemos demostrado la gran movilidad que pueden tener los renacuajos a lo largo en tan solo dos días. Sin embargo, esta movilidad se circunscribe a la extensión del área inundada de la laguna, y aunque muchos de los hábitats reproductivos de los anfibios son de escasas dimensiones, también pueden reproducirse en lagunas temporales someras y

extensas. Nuestro estudio demuestra que las larvas del sapo de espuelas pueden recorrer, de un extremo a otro, lagunas de un tamaño considerable en un periodo de tiempo relativamente corto. Sumando esta movilidad a la gran cantidad de renacuajos que pueden contener las lagunas, se puede considerar que constituyen un medio de dispersión de los organismos o propágulos que puedan transportar en su tracto intestinal. En general, la mayoría de las larvas de anuros consumen materia vegetal (Altig *et al.*, 2007, Montaña *et al.*, 2010). La especie estudiada, *Pelobates cultripes*, es gran consumidora de macrófitos (Díaz-Paniagua *et al.*, 1985; Arribas *et al.*, 2015), incluyendo en su dieta tanto partes vegetativas de las plantas, como también semillas, que pueden posteriormente germinar (Arribas, 2016). En realidad, los renacuajos de esta especie se pueden considerar omnívoros, pues consumen también materia animal (Escoriza *et al.*, 2017), y, sobre todo, una alta proporción de los detritos acumulados en el fondo de sus hábitats (Díaz-Paniagua, 1985; Arribas *et al.*, 2015). Entre estos detritos se encuentran, además de restos de macrófitos en descomposición y perifiton, propágulos de plantas y de invertebrados como crustáceos zooplanctónicos. Todo este tipo de material puede ser potencialmente transportado en el interior del intestino de los renacuajos (endozoocoria), siendo intensamente dispersado por todo el fondo de las lagunas en las que se desarrollan las larvas. Los renacuajos realizan, tanto una intensa movilización de nutrientes, como la redistribución de plantas y zooplancton por toda la laguna, homogeneizando la distribución de los propágulos por toda la cubeta inundada. En una laguna de escasas dimensiones, este transporte puede parecer de escasa importancia. Sin embargo, las lagunas temporales son enormemente variables de un año a otro, pudiendo quedar gran parte de la zona inundada de la laguna fuera de la que se inundará el año siguiente, con lo que los propágulos caídos en las zonas inundadas un año, podrían no tener éxito de emergencia en el siguiente ciclo. Esto es particularmente importante en el caso de las plantas acuáticas, que mantienen una zonación característica en las lagunas que delimita el área donde se concentran las semillas que caen al fondo (Aponte *et al.*, 2010; Grillas *et al.*, 1993). El papel de los renacuajos en estos ecosistemas acuáticos, repartiendo propágulos por toda el área inundada de una laguna, puede incrementar el éxito reproductivo de muchas especies en el ciclo hidrológico siguiente, contribuyendo de manera importante a la composición, dinámica y estructura de las comunidades de los medios acuáticos temporales. Asimismo, la redistribución de las semillas por toda el área favorecería el desarrollo de una amplia y densa capa de macrófitos que incrementaría la disponibilidad de alimento para la cohorte de renacuajos del siguiente ciclo hidrológico.

Actualmente, una de las principales amenazas para la conservación de los medios acuáticos es la introducción de especies exóticas (Strayer, 2010). Entre ellas, encontramos especies de depredadores o competidores que directamente afectan a la diversidad de especies de animales (Weiss, 2012), o también macrófitos con alta capacidad de crecimiento y dispersión que pueden desplazar a las plantas nativas (Strayer, 2010; Schultz & Dibble 2012). Ambos tipos de especies invasoras alteran considerablemente la estructura y calidad de los ecosistemas acuáticos, produciendo cambios importantes a los que las especies nativas deben adaptarse para conseguir mantenerse en los hábitats invadidos. En este estudio hemos abordado los cambios que produce la invasión de una planta flotante en los ecosistemas de Doñana. Se trata de un helecho flotante, *Azolla filiculoides*, cuya expansión no solo afecta a otros macrófitos sino también a las comunidades de animales. A pesar de ser actualmente una de las principales especies acuáticas invasoras en Europa (Heusser, 2012), sus efectos sobre los ecosistemas acuáticos no habían sido estudiados en detalle anteriormente, sino que solo se había descrito su efecto sobre comunidades de animales acuáticos en arroyos de Zimbabwe (Gratwicke & Marshall 2001) y experimentalmente sobre dos especies de macrófitos (Janes *et al.*, 1996). En esta tesis se ha estudiado, experimentalmente bajo condiciones semi naturales, los cambios que este helecho produce en el medio acuático, tanto en cuanto a las características fisicoquímicas del agua, como a las comunidades acuáticas. En concreto, se ha analizado su impacto sobre los macrófitos acuáticos y sobre sus principales consumidores en el interior de las lagunas temporales, las larvas de anfibios, aportándose una información novedosa sobre la alteración de las redes tróficas al ser afectadas por esta especie invasora. Además de estos efectos, el diseño experimental que contempla tanto la presencia como la ausencia de la especie invasora, nos ha permitido también profundizar en las características de las comunidades acuáticas, en este caso de las marismas de Doñana, y analizar sus redes tróficas, donde las larvas de anuros tienen un importante papel como consumidores primarios, para los que las plantas acuáticas son un recurso de gran importancia.

El efecto más importante que produce *A. filiculoides* se debe a la formación de una densa cobertura de plantas sobre la superficie del agua que bloquea el acceso de la luz a la columna de agua. Esto reduce enormemente la capacidad fotosintética de las plantas, que tienden a elongar sus tallos y hojas, y acaban finalmente muriendo. Sin embargo, nuestros experimentos han ofrecido un resultado muy novedoso, detectando que algunos macrófitos son capaces de resistir estas adversas condiciones mediante el desarrollo de formas aéreas sobre la capa de *A. filiculoides*. Esto nos ha permitido realizar la descripción de un morfotipo de *Ranunculus peltatus* que permite a esta planta acuática sobrevivir y reproducirse con éxito

(Pinero-Rodríguez *et al.*, 2018), a pesar de las adversas condiciones del medio invadido por el helecho invasor. *Ranunculus peltatus* es una planta que presenta una gran plasticidad morfológica (Cook 1966), y en ella se han descrito morfotipos acuáticos, que son los que habitualmente se desarrollan en el interior de las lagunas, y también morfotipos terrestres, que son los que adquieren las plantas cuando se localizan en las zonas que se van secando alrededor de las lagunas o en las cubetas húmedas de las lagunas en años en que no llegan a inundarse (Cook, 1966; Garbey *et al.*, 2004). La planta posee dos tipos de hojas: unas filiformes o capilares, con las que puede captar los nutrientes directamente del medio acuático, que son las que predominan en las formas acuáticas, y otras laminares, que flotan sobre la superficie del agua, captando directamente la luz. Cuando se produce la invasión por *A. filiculoides*, *R. peltatus* sufre las consecuencias de la falta de luz y reducción de nutrientes, y cuando sus tallos alcanzan la superficie del agua, crece sobre la densa mata de *A. filiculoides* adquiriendo una morfología similar a la de las plantas terrestres. Esta forma consiste en tallos engrosados y elevados y favorece la formación de hojas laminares, a la vez que se forman también algunas capilares más engrosadas y fuertes que las de las formas acuáticas. A la vez, en el interior del agua, mantienen tallos elongados que las anclan al suelo, donde a través de las raíces pueden captar nutrientes. El desarrollo de este morfotipo permite a esta especie resistir la invasión de *A. filiculoides*, e incluso producir flores, frutos y semillas que permitan su subsistencia en ciclos futuros. El filtrado de la luz por la capa de helechos afecta negativamente a la fotosíntesis y la respiración de las plantas, que mueren y forman al descomponerse una densa capa de detritos en el fondo y la eutrofización del medio al aumentar la cantidad de nutrientes en la columna de agua. En general, la invasión de *A. filiculoides* deteriora la calidad del agua, reduciendo su pH y la concentración de oxígeno, y disminuye la heterogeneidad del ambiente al eliminar gran parte de los macrófitos, lo que consecuentemente afecta también a las comunidades animales, como se observa con los crustáceos zooplanctónicos. Estudios previos habían determinado que también reduce la diversidad de invertebrados acuáticos, de larvas de anfibios y de peces (Gratwicke y Marshall 2001). Con nuestros experimentos, hemos tenido la oportunidad de analizar en detalle cómo afecta a las larvas de anuros.

Un resultado importante es que la modificación del hábitat que produce la invasión de *A. filiculoides* no tiene el mismo efecto sobre distintas especies de anfibios. Una de las características de las larvas de anfibios es su capacidad de flexibilizar su desarrollo en función de la disponibilidad de recursos que les ofrecen sus hábitats (Alford, 1999; Gomez-Mestre *et al.*, 2010). Existe una amplia heterogeneidad en las características y requerimientos del desarrollo larvario entre las especies de anfibios, y en este estudio se eligieron dos especies

bien diferenciadas. La primera de ellas, *Hyla meridionalis*, es una especie que completa su desarrollo metamórfico con un tamaño corporal relativamente pequeño, lo que permite que las larvas se puedan desarrollar en 2,5-3 meses. La segunda especie, *Pelobates cultripes*, metamorfosea con mayor tamaño corporal, y es una de las especies que alcanza mayor tamaño larvario entre las especies ibéricas. Los renacuajos tienen que alcanzar un tamaño mínimo larvario antes de que puedan acelerar la metamorfosis (Kulkarni *et al.*, 2011) por lo que están obligados a permanecer en el medio acuático hasta alcanzarlo, y su periodo larvario suele durar entre 5 y 8 meses (Díaz-Paniagua *et al.*, 2005). En la primera especie no detectamos efectos sobre la supervivencia de las larvas, sino que las que se desarrollaban bajo la capa de *Azolla* tardaron más en completar el desarrollo y lo hicieron con menor tamaño y peor condición física que las larvas desarrolladas en un medio sin el helecho invasor. Los renacuajos de esta especie no consiguieron acelerar el desarrollo para escapar de las adversas condiciones del medio, sino que las peores condiciones dificultaron su crecimiento y retrasaron su desarrollo, probablemente reflejo de la menor disponibilidad o calidad del alimento. En el caso de *P. cultripes*, el efecto de la invasión fue la mortalidad de las larvas, a pesar de que los renacuajos fueron capaces de diversificar su dieta, consumiendo incluso las plantas de *A. filiculoides*. Los macrófitos son un componente importante de las dietas de las larvas de esta especie, y les permite alcanzar un gran tamaño corporal durante el primer tercio de su desarrollo. La reducción o ausencia de macrófitos tuvo consecuencias letales en estos renacuajos que al verse obligados a permanecer en un medio empobrecido, no consiguieron completar la metamorfosis. Un análisis isotópico de las fuentes alimenticias de los renacuajos, confirmó el deterioro de toda la red trófica en presencia del helecho invasor. En ella, la mayoría de los recursos redujeron la proporción de carbono y nitrógeno, forzando a los renacuajos a utilizar las plantas de *Azolla* como alimento, que a su vez aportan menor cantidad de nutrientes y son más difíciles de digerir que las otras plantas.

En resumen, esta tesis aporta una información novedosa sobre la ecología de las lagunas temporales, en las que los principales vertebrados son las larvas de anfibios, que además alcanzan alta abundancia y ejercen un gran impacto sobre sus principales recursos tróficos. Tanto las larvas de anfibios (Díaz Paniagua, 1992), como los macrófitos acuáticos (Fernández Zamudio *et al.*, 2017), están adaptados a la temporalidad de estos hábitats, flexibilizando su periodo de crecimiento en función del inicio de la inundación y de la duración de las lagunas. Hemos podido avanzar en los capítulos de esta tesis en nuestro conocimiento acerca de cómo interaccionan anfibios y macrófitos, tanto con efectos negativos sobre las plantas, ejercidos por la herbivoría, como positivos, al considerar el incremento del éxito de

reproducción en ciclos de inundación siguientes, ocasionado por la dispersión de los propágulos por todo el fondo de las lagunas. Asimismo, ante la invasión de plantas exóticas, tanto las plantas nativas, como los renacuajos revelan la posibilidad de desarrollar una respuesta alternativa que les permite subsistir en las deterioradas condiciones de las lagunas, aunque esta respuesta no resulta posible en la especie de anfibio que requiere un largo periodo de crecimiento en el medio deteriorado.

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Conclusiones:

1. Los renacuajos de gran tamaño, como las larvas del sapo de espuelas, *Pelobates cultripes*, pueden ocasionar un consumo de biomasa vegetal tan elevado que pueden ser considerados entre los principales herbívoros de las lagunas temporales, ya que son capaces de modificar la historia de vida de los macrófitos sumergidos y la complejidad espacial del hábitat.
2. La formación de capas densas de la especie invasora *Azolla filiculoides* sobre la superficie del agua ocasiona consecuencias negativas para las comunidades acuáticas. Los efectos ocasionados sobre las comunidades vegetales repercuten en una menor presencia o incluso su eliminación.
3. La formación de capas densas de la especie invasora *Azolla filiculoides* también ocasiona efectos negativos en las comunidades de larvas de anfibios que pueden ver comprometida su condición corporal o incluso incrementar su mortalidad.
4. La alta plasticidad morfológica de *Ranunculus peltatus* le permite persistir frente a la especie invasora *Azolla filiculoides*, ya que consigue desarrollar una parte aérea, sobrepasando la densa capa de helechos flotantes, para realizar las funciones fotosintéticas, y la producción de frutos y semillas.
5. Encontramos que los renacuajos de *Pelobates cultripes* pueden desplazarse largas distancias (> 250 m) en un lapso de tiempo relativamente corto (<48 h), estos aportes en la ecología espacial de las larvas de anfibios complementan nuestro conocimiento sobre la dimensión espacial en que se producen las interacciones con los diferentes elementos del ecosistema, como la dinámica depredador-presa, el impacto de las especies invasoras, la dinámica de las enfermedades o el uso de los recursos.

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