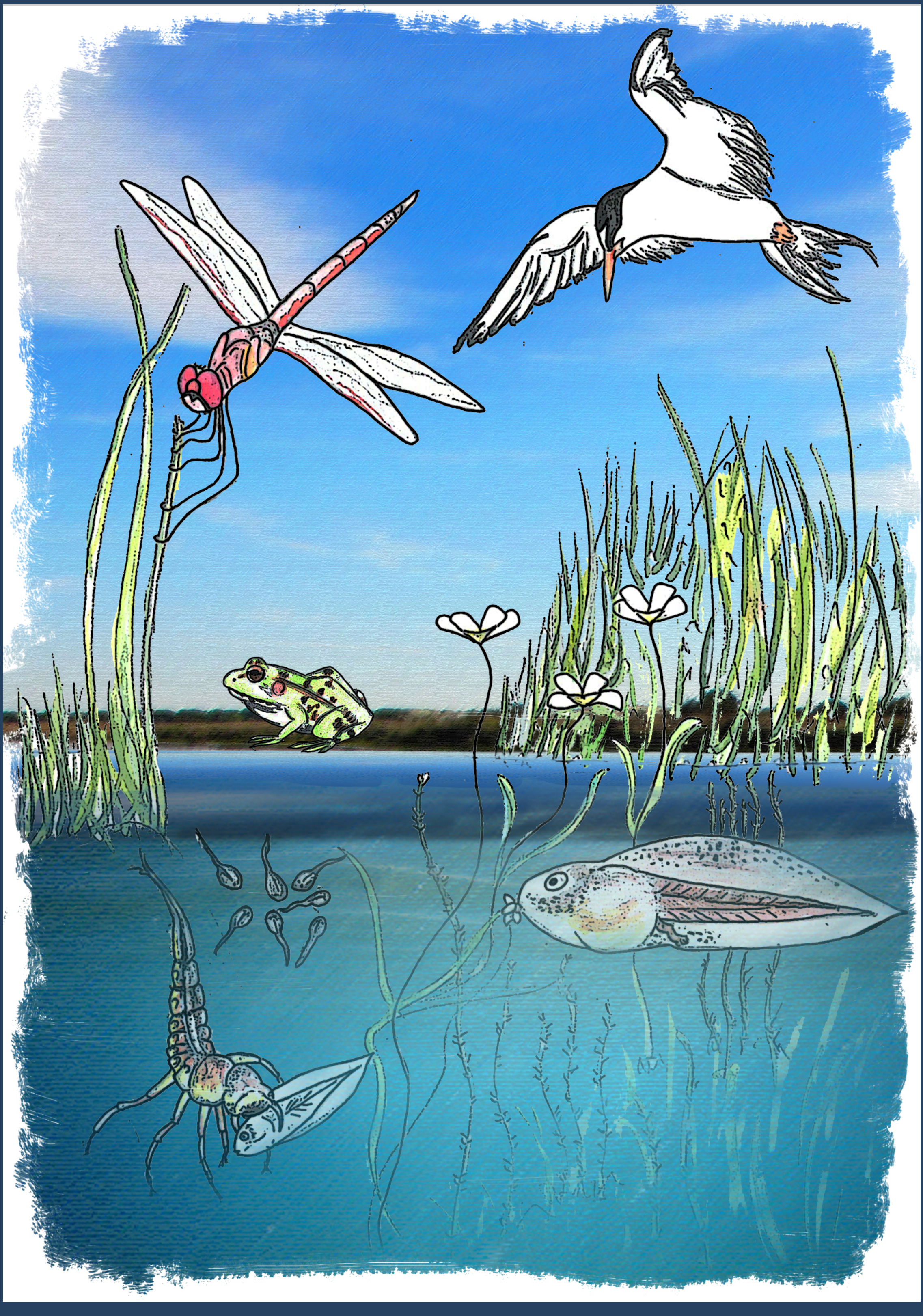
El papel funcional de las larvas de anfibios en los ecosistemas acuáticos temporales



Mª Rosa Arribas Ramos
Tesis Doctoral

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EL PAPEL FUNCIONAL DE LAS LARVAS DE ANFIBIOS EN LOS ECOSISTEMAS ACUÁTICOS TEMPORALES

Tesis Doctoral

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Estación Biológica de Doñana, CSIC

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Universidad Pablo de Olavide
Facultad de Ciencias Experimentales
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Doctorado en Estudios Medioambientales

EL PAPEL FUNCIONAL DE LAS LARVAS DE ANFIBIOS EN LOS ECOSISTEMAS ACUÁTICOS TEMPORALES

Memoria presentada por la Licenciada en Biología
M. Rosa Arribas Ramos para optar al título de Doctora
por la Universidad Pablo de Olavide

Fdo. M. Rosa Arribas Ramos

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CERTIFICAN:

Que M. Rosa Arribas Ramos, Licenciada en Biología por la Universidad Autónoma de Madrid, ha realizado bajo su dirección la presente memoria de Tesis Doctoral, titulada "*El papel funcional de las larvas de anfibios en los ecosistemas acuáticos temporales*" y que a su juicio reúne los méritos suficientes para optar al grado de Doctor por la Universidad Pablo de Olavide.

Y para que así conste, firman el presente documento en Sevilla a 16 de septiembre de 2015.

Firma Director: Dr. Ivan Gomez-Mestre

Firma Directora: Dra. Carmen Díaz-Paniagua

Firma Tutora : Dra. Antonia Jiménez Rodríguez
Universidad Pablo de Olavide

A mi familia,

A mis amigos,

Y a Grego,

por su apoyo incondicional

ÍNDICE

RESUMEN

La estructura y dinámica de las comunidades ecológicas están determinadas y reguladas por una serie de procesos clave que influyen en la distribución y la abundancia de las especies (p. ej. selección, deriva, especiación y dispersión), y por interacciones ecológicas como la competencia y la depredación. La importancia relativa de la competencia y la depredación dependerá en gran medida del grupo local de especies, el tipo de depredadores y el nivel de segregación de nicho. Los anfibios constituyen un sistema idóneo para abordar estas cuestiones, ya que son especies clave en las redes tróficas, con un papel importante en diversos procesos ecológicos. Además, el efecto de las larvas de anfibios en los sistemas acuáticos, así como su nicho trófico, dependen de sus complejas interacciones con competidores y depredadores, ya que responden a ellos alterando su morfología y comportamiento. Ante una situación generalizada de dramáticos declives en las poblaciones de anfibios a nivel global, es fundamental caracterizar el papel funcional de los anfibios en los sistemas naturales para poder comprender las posibles consecuencias de su declive y extinción local. El principal objetivo de la presente tesis es conocer el potencial regulador de las larvas de anfibios según las condiciones ambientales, centrándonos en investigar su papel sobre otros niveles tróficos y sus interacciones con la vegetación acuática, así como comparar sus efectos en sistemas mediterráneos y sistemas tropicales.

Gracias a experimentos en mesocosmos, que simulan las características ambientales en condiciones controladas, evalué el efecto de una comunidad de larvas de anfibios del Parque Nacional de Doñana sobre diversos parámetros físico-químicos y biológicos característicos del medio. Se detectó un importante efecto de las larvas de anfibios sobre estos parámetros, mayoritariamente cuando se encontraban en alta densidad, causando una marcada reducción de la biomasa de plantas, aumentando la turbidez del agua y los nutrientes tales como el amonio. Las larvas de anfibios afectaron asimismo la abundancia de algas, inferida indirectamente estimando la concentración de clorofila del agua, y redujeron la diversidad de zooplancton. Sin embargo, se observó que estos efectos no eran consecuencia general de todas las especies de anfibios, sino que se debían fundamentalmente a las larvas del sapo de espuelas (*Pelobates cultripes*). Las larvas de esta especie son desproporcionalmente grandes comparadas con las larvas de las otras especies con las que coexiste, y consume una gran cantidad de biomasa de macrófitos. Esta especie por sí sola alteró la composición del zooplancton, que en presencia de las larvas de *P. cultripes* se caracterizó por una mayor proporción relativa de copépodos respecto a cladóceros. A este sesgo en la comunidad de zooplancton pueden contribuir también las especies de urodelos

(tritones y salamandras), pues son eficientes carnívoros que pueden alterar considerablemente la composición de las comunidades de zooplancton.

Además de la densidad, también se manipuló experimentalmente la presencia de depredadores nativos e invasores, que se introdujeron libres en el medio o mantenidos en jaulas para distinguir los efectos indirectos no derivados de su depredación. Los efectos directos del depredador invasor, el cangrejo rojo americano, fueron los más perjudiciales, tanto en la reducción exacerbada de la supervivencia de anfibios como en las características fisicoquímicas del medio (p. ej. gran aumento de la turbidez y los nutrientes o reducción casi total de la biomasa de plantas).

Mediante el uso de isótopos estables se evaluó la plasticidad trófica en la comunidad de larvas de anfibios derivada de los cambios que se habían detectado en las condiciones ambientales. Observamos variaciones en los valores isotópicos de carbono (δ^{13}C) y nitrógeno (δ^{15}N) entre especies y según los tratamientos a los que fueron expuestos. La alta densidad de larvas, la presencia de *P. cultripes* y el cangrejo rojo americano produjeron una disminución en el δ^{13}C de los anfibios, relacionado con un aumento en su dieta de recursos con menor δ^{13}C, como detritos o zooplancton. Por tanto, la alimentación oportunista de los anfibios está ampliamente condicionada por la competencia, mientras que los efectos indirectos de los depredadores son leves en el sistema templado estudiado. Sin embargo, en sistemas neotropicales se observa que los depredadores tienen un mayor papel en la estructura de las comunidades de anfibios, siendo responsables de una alteración de la posición trófica relativa de las diferentes especies estudiadas en la zona central de Panamá, así como de cambios en su crecimiento y en su supervivencia. Asimismo, la composición de la comunidad tiene también un gran efecto en el nicho trófico de las larvas de anfibios ya que distinguimos cambios en el δ^{15}N de algunas especies.

Dado que el conjunto de nuestros resultados ponen de manifiesto que las larvas de anfibios anuros de sistemas acuáticos temporales son importantes consumidores primarios, no sólo de fitoplancton o perifiton, sino también de plantas vasculares acuáticas, con esta tesis se pretende además averiguar las preferencias de diferentes larvas de anfibios por varias especies de macrófitos y carófitos. El sapo de espuelas es el mayor consumidor de macrófitos, mostrando especial preferencia por los del género *Callitriche*. La herbivoría por parte de larvas de anfibios puede tener un importante papel en estos ecosistemas, afectando a la composición de las comunidades de plantas, su fenología reproductora o la dispersión de sus semillas. De hecho, el papel de las larvas de anfibios en la dispersión directa o indirecta –por depredadores de anfibios- de semillas de plantas acuáticas ha sido ignorado hasta el momento, por lo que nuestros datos son las primeras observaciones realizadas en este sentido. Además de las semillas ingeridas directamente de las plantas, los renacuajos pueden consumir también las presentes en el banco de semillas del sedimento junto a los detritos, que es otro componente habitual de su dieta. Se observó que entre un 9 y un 32% de los renacuajos, dependiendo de la especie, transportaban semillas o esporas de la vegetación de las lagunas. De ellos, *P. cultripes* fue la especie con mayor número de semillas. La especie de planta preferida para el consumo (*Callitriche*) fue también de la que encontramos más semillas en las heces de los renacuajos y su porcentaje de germinación fue entre el 40 y el 88 % en los renacuajos de las diferentes especies.

Los resultados de la presente tesis revelan el importante papel que ejercen las larvas de anfibios sobre la compleja estructura de la comunidad de los sistemas acuáticos. Por ello es esencial ampliar nuestro conocimiento respecto a su papel funcional, con el objetivo de anticipar y entender las consecuencias de sus acusados y previsibles declives poblacionales.

SUMMARY

The structure and dynamics of ecological communities is determined and regulated by several key processes that influence in the species distribution and abundance (e.g., selection, drift, speciation, and dispersal), and by ecological interactions as competition and predation. The relative importance of competition and predation will depend largely on the local assemblage of species, the type of predators, or the degree of niche segregation. Amphibians constitute an ideal system to address these questions, as they are key species in the trophic webs with a profound role in several ecological processes. Moreover, the effect of amphibian larvae in the aquatic systems and their trophic niche largely depend on their complex ecological interactions with competitors and predators, as they respond to them altering their morphology and behavior. As amphibians continue showing generalized dramatic and global population declines, it is essential to fully characterize the functional role of amphibians in the natural systems to understand the possible consequences of their local declines and extinction. The main objective of this thesis is to know the regulatory potential of amphibian larvae according to the environmental conditions, focusing on the research about their role over other trophic levels, and their interactions with the aquatic vegetation, comparing their effects in Mediterranean and Neotropical systems.

By means of experiments with mesocosms, which simulate the environmental conditions in controlled conditions, I evaluated the effect of a guild of amphibian larvae from Doñana National Park on several biological and physicochemical parameters of the system. We detected an important effect of amphibian larvae on these parameters, mostly when at high larval density, causing a marked reduction of plants biomass, increasing water turbidity and several nutrients like the ammonia. Amphibian larvae also affected algae abundance, indirectly inferred by estimating chlorophyll concentration in the water, and they reduced zooplankton diversity. However, these changes were not produced as a consequence of all amphibian species, but they were caused mainly to the larvae of the western spadefoot toad (*Pelobates cultripes*). The larvae of this species are disproportionally larger than the other amphibian larvae of the area, and they consume a large amount of macrophyte biomass. This species by itself altered the zooplankton composition, as the presence of tadpoles of *P. cultripes* increased the relative proportion of copepods in respect to cladocerans. Urodeles (newts and salamanders) can also contribute to this bias in the zooplankton community, as they are efficient carnivorous that can considerably alter the zooplankton community composition.

Apart from density, we also experimentally manipulated the presence of native and invasive predators, which were introduced either freely roaming or caged in the mesocosms to distinguish their non-consumptive predator effects. The direct consumptive effects of the invasive predator, the red swamp crayfish, were very harmful in the great survival reduction of amphibians as well as in the physicochemical characteristics of the system (e.g., marked increase in water turbidity and nutrients, pronounced reduction of the plants biomass).

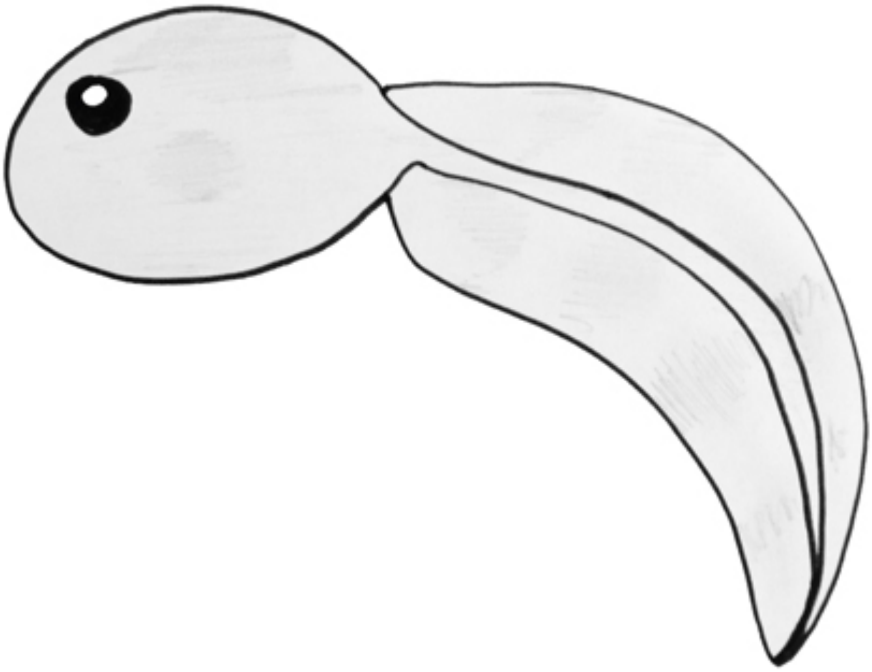
Using stable isotopes, we evaluated the trophic plasticity of the amphibian larvae community derived of the changes in the environmental conditions detected in the former experiment. We observed variations in the stable isotopic values for carbon (δ^{13}C) and nitrogen (δ^{15}N) among species and according to the treatments the amphibians were exposed to. High larval density, presence of *P. cultripes* and presence of red swamp crayfish produced a decrease in the δ^{13}C of amphibians, related to an increase in carbon-depleted resources in their diet such as detritus or zooplankton. Therefore, the opportunistic feeding of amphibians is widely conditioned by competition, whereas the indirect predator effects are negligible in this temperate system. However, in neotropical systems predators have a greater structuring role in the amphibian community, altering the relative trophic position of the different studied species in central Panama, and changing their growth rate and survival. Furthermore, the community composition also has a great effect in the trophic niche of the amphibian larvae as we distinguished changed in the δ^{15}N of some of the amphibian species.

Since our results bring to light that anuran larvae of temporary aquatic systems are important primary consumers, and not only of phytoplankton or periphyton but also of aquatic macrophytes, this thesis also try to figure out the feeding preferences of different amphibian larvae regarding several species of macrophytes and charophytes. The western spadefoot toad is the greatest macrophyte consumer, showing special preference for those from the genus *Callitriche*. Herbivory by amphibian larvae can have an important role in these ecosystems, affecting the community composition of plants, their reproductive phenology or their seed dispersal. In fact, the role of amphibian larvae in the direct or indirect –through seed predators- seed dispersal of aquatic plants has been ignored until the present study, and therefore our data are the first observations concerning this. Apart from the seeds directly ingested from the plants, tadpoles can also consume the seeds buried in the seed bank while feeding on detritus, as it is a common resource for tadpoles. We observed that between 9 and 32% of the tadpoles, depending on the species, transported seeds or spores from the pond vegetation. Among them, *P. cultripes* was the species with greatest number of seeds. The preferred plant in

the feeding experiment (*Callitriche*) was also the one from which we found in greatest number in the tadpole feces, whose germination rate was between 40 and 88% depending on the tadpole species.

The results of this thesis reveal the important role of amphibian larvae on the complex community structure of aquatic systems. Thus, it is essential to widen our knowledge in respect to their functional role aiming to anticipate and understand the consequences of their pronounced and predictable population declines.

INTRODUCCIÓN GENERAL



So still the Tadpole cleaves the watery vale
With balanced fins, and undulating tail;
New lungs and limbs proclaim his second birth,
Breathe the dry air, and bound upon the earth.

Erasmus Darwin, *The Temple of Nature* (1803)

INTRODUCCIÓN GENERAL

Ecología de comunidades

La composición de las comunidades está regulada por diversos procesos ecológicos y evolutivos, y la importancia relativa de un factor u otro depende en gran medida de la escala geográfica y temporal considerada. A una escala macroecológica, son determinantes en la composición de las comunidades la biogeografía, las variaciones climáticas y la historia evolutiva de los distintos grupos de organismos. Así, la estructura y la dinámica de las comunidades ecológicas están últimamente determinadas por cuatro procesos básicos: selección, deriva, especiación y dispersión (Vellend 2010, Kozak and Wiens 2012). Las especies tienen tendencia a mantener sus rasgos ecológicos similares a lo largo del tiempo. Esta conservación del nicho puede tener un gran efecto en la determinación de patrones respecto a la riqueza de las especies y la composición de las comunidades a nivel global (Wiens and Graham 2005, Wiens et al. 2010). A nivel más local, no obstante, la composición de las comunidades depende en mayor medida de las variaciones microclimáticas y de las interacciones ecológicas que se establecen en el seno de la comunidad, como las relaciones de competencia, depredación o parasitismo.

Sin embargo, sabemos también que la plasticidad fenotípica es ubicua en los organismos, y que por tanto éstos modifican su comportamiento, su morfología, su fisiología y sus historias de vida en función de las condiciones ambientales. De hecho, muchos rasgos ecológicos clave presentan una gran labilidad evolutiva (Ackerly 2003, Knouft et al. 2006), del mismo modo que la plasticidad adaptativa evoluciona rápidamente entre taxones próximamente emparentados (Pigliucci and Schmitt 1999, Gomez-Mestre et al. 2008). Intuitivamente, la idea de la conservación de nicho y la capacidad plástica de las especies parecen en gran medida discordantes o incompatibles (i.e. los rasgos ecológicos de las especies permanecen inmutables o varían rápidamente en respuesta a estímulos ambientales), pero en realidad son conceptos fáciles de conciliar. Por ejemplo, la plasticidad puede reducir el impacto de interacciones ecológicas como la competencia y la depredación e incrementar así la probabilidad de coexistencia entre especies en un área geográfica determinada (Miner et al. 2005) sin necesidad de una divergencia adaptativa de sus nichos y por tanto prolongando la conservación de nicho.

Dependiendo de las condiciones locales, los organismos pueden alterar rápidamente su nicho y su fenotipo de forma adaptativa (West-Eberhard 2005, Levin 2009, Ashton et al. 2010), lo que puede tener consecuencias sobre toda la estructura de la comunidad. Desde la década de 1960 numerosos autores han estudiado experimentalmente factores potenciales determinantes de la estructura de las comunidades (p. ej. Paine 1966, Hall et al. 1970) y sus implicaciones en las redes tróficas o en la diversidad de especies (Levins 1968). Los factores bióticos más importantes que determinan el funcionamiento de las comunidades a escala local son la **competencia** (interacciones dentro de un nivel trófico) y la **depredación** (interacciones entre diferentes niveles tróficos) (p. ej. Connell 1975, Holt and Polis 1997, Sih et al. 1998). La depredación influye enormemente en la composición de especies, la supervivencia y por tanto la densidad de las poblaciones o en la estructuración de los tamaños. Además, afecta a las interacciones competitivas, llegando incluso a revertirlas (p. ej. Morin 1983, Wilbur et al. 1983, Gonzalez et al. 2011). Por otro lado, la alta densidad de individuos puede asimismo conllevar una baja supervivencia (p. ej. Loman 2004) debido a la competencia por recursos o por hábitats de mejor calidad o libres de depredadores (p. ej. Szabo 2002). Los depredadores pueden influir positivamente en el crecimiento de sus presas al reducir sus densidades antes de que los recursos disponibles sean más escasos que la demanda de la población (p. ej. Wilbur et al. 1983, Brodin and Johansson 2002).

Además del consumo directo de las presas por parte de los depredadores, éstos a menudo inducen **alteraciones de ciertos rasgos en las presas** (p. ej., en el comportamiento, morfología, o en historias de vida) que pueden reducir o evitar el riesgo de depredación (Lima and Dill 1990, Lima 1998, Sih et al. 1998, Railsback and Harvey 2013). Estas modificaciones normalmente tienen un coste para los supervivientes, como un crecimiento reducido o un menor éxito reproductivo, debido principalmente a la menor tasa de adquisición de recursos (Relyea 2002a). Los **efectos indirectos** (los que no derivan del consumo por parte del depredador) pueden variar con la densidad de las presas o con factores ambientales como el nivel de nutrientes o la temperatura (Werner and Peacor 2003). Además, este impacto puede implicar efectos indirectos en otros organismos de la comunidad de diferentes niveles tróficos. Estos efectos cascada por parte del depredador son más acusados en ecosistemas acuáticos que en terrestres (Shurin et al. 2002).

Ecología trófica

Para entender la dinámica y estructura de las comunidades es importante conocer las relaciones tróficas que se dan en dicha comunidad (Pimm 1982, Morlon et al. 2014). Las redes tróficas representan de manera simplificada la magnitud de las interacciones tróficas (consumidor-recurso) entre especies o individuos de un ecosistema (Cohen

1977, Post 2002). La longitud de la red trófica, es decir, el número de eslabones o transferencias tróficas desde los recursos basales hasta los depredadores superiores, es un factor importante en la regulación de la estructura de la comunidad y el funcionamiento del ecosistema (Hairston et al. 1960, Duffy et al. 2005). Las interacciones tróficas son mecanismos esenciales en la organización de las comunidades (Wilbur 1987, Polis and Strong 1996, Chase 2000). Estas interacciones son variables en el espacio y en el tiempo (Paine 1980, Warren 1989) y están influenciadas tanto por las variaciones ambientales (Winemiller 1990, Winemiller and Polis 1996, Sabo et al. 2009, 2010) como por el movimiento de nutrientes y energía entre hábitats (Polis et al. 1997, Nowlin et al. 2007). Sin embargo, el **estado trófico de los organismos no es estático** sino que depende del contexto ecológico en el que se encuentren, ya que como hemos visto anteriormente, tanto el propio fenotipo de los organismos interactuantes como la intensidad de la interacción pueden variar sustancialmente **según las condiciones ambientales**. Además, muchos animales son omnívoros, lo que implica que consumen recursos de varios niveles tróficos y por lo tanto no pueden ser asignados a un único nivel de la red trófica (Cousins 1987, Phillips and Gregg 2003). Es esencial por tanto identificar los procesos ecológicos que determinan la estructura trófica para ser capaces de entender las causas y consecuencias de la diversidad biológica a escala local. Los **isótopos estables**, especialmente los de carbono y nitrógeno, constituyen una herramienta de importancia creciente y muy potente para estudiar las relaciones tróficas tanto en ecosistemas acuáticos como terrestres, así como las variaciones temporales o espaciales en la dieta (McCutchan Jr et al. 2012; **Caja 1**).

CAJA 1: El uso de los isótopos estables en la caracterización de la estructura trófica

El estudio de la composición de la dieta de los animales puede realizarse mediante métodos tradicionales, tales como el análisis del contenido estomacal u observaciones comportamentales, y también mediante el análisis de isótopos estables. Los métodos tradicionales son muy útiles pero solamente representan una información instantánea en cuanto a búsqueda de comida e ingesta, requieren un gran tamaño muestral y además son normalmente laboriosos en cuanto a tiempo y esfuerzo (McCarthy et al. 2004). Dado que algunas especies no asimilan de la misma manera el material ingerido de diferentes recursos (Skelly and Golon 2003), los isótopos estables constituyen un complemento a los métodos tradicionales ya que integran los recursos asimilados en el tejido del animal a lo largo del tiempo (Peterson and Fry 1987). Los cambios en la dieta conllevan un cambio gradual en las proporciones de los isótopos de C, N o S de los consumidores (McCutchan Jr et al. 2012) y por ello los isótopos estables son utilizados frecuentemente en el estudio de la ecología trófica.

CAJA 1 (continuación)

Para cada elemento, los isótopos son átomos con el mismo número de protones y electrones pero distinto número de neutrones. Los isótopos estables son aquellos que son energéticamente estables, es decir, no son radiactivos. Esto ocurre normalmente cuando el número de neutrones y de protones es similar.

El carbono existe principalmente como el isótopo de carbono-12 (98.89%) pero una pequeña fracción (1.11%) existe como carbono-13, mientras que el isótopo del nitrógeno-14 es más abundante (99.64%) respecto al nitrógeno-15 (0.36%). Los valores isotópicos muestran la relación del isótopo pesado respecto al ligero en una muestra comparada a la de un estándar (Jardine et al. 2003, Biasatti 2004) y se representan con un símbolo delta (δ) seguido del isotopo pesado (p. ej. δ^{13}C ó δ^{15}N).

El enriquecimiento en una muestra se produce por la extracción de isótopos ligeros a través del fraccionamiento y, por tanto, se incrementa la proporción de isótopos pesados (Persic et al. 2004). Los valores de δ^{13}C son más variables entre las fuentes de alimento que entre los diferentes niveles tróficos porque el enriquecimiento trófico del carbono es generalmente del 1‰, y por tanto son indicadores de la dieta del consumidor (DeNiro and Epstein 1978, 1981). En cambio, los valores de δ^{15}N indican las posiciones tróficas relativas de los organismos ya que incrementan de media en 3-5‰ desde un nivel trófico al posterior (Peterson and Fry 1987), aunque esto es variable según los ecosistemas y puede haber enriquecimientos de tan sólo 1.6‰, como ocurre normalmente en los sistemas tropicales (Kilham et al. 2009). Con estos isótopos se pueden seguir las transferencias de carbono y nitrógeno desde las fuentes (plantas o detritos, productores primarios) hacia los consumidores primarios y secundarios (Peterson and Fry 1987).

La variación isotópica entre individuos puede ser debida tanto a una variación de la dieta entre dichos individuos o a una variación en las señales isotópicas de las fuentes de alimento (Araújo et al. 2007). Los isótopos estables tienen una tasa de reemplazo relativamente lenta, variable según el tejido utilizado, en comparación con la frecuencia de alimentación; por ello, son muy útiles para inferir el aporte de carbono y nitrógeno en la dieta durante largos periodos de tiempo (Tieszen et al. 1983, Dalerum and Angerbjörn 2005). Normalmente, las proporciones de los isótopos de carbono y nitrógeno se representan en una misma gráfica para entender el flujo de energía desde los niveles tróficos más básicos (la amplitud de carbono utilizada) hasta los más altos (la altura de la cadena trófica a lo largo del eje vertical δ^{15}N). Asimismo, con los análisis isotópicos se puede identificar la importancia relativa de cada recurso en la dieta –especialmente importante para organismos omnívoros– utilizando modelos mixtos en diversos programas destinados a este fin.

Sistemas acuáticos temporales

Los ecosistemas de agua dulce constituyen un modelo de estudio muy adecuado para indagar en la ecología de las comunidades ya que suelen estar delimitados espacialmente y sus componentes pueden ser manipulados con relativa facilidad (p. ej. Wilbur 1997, Blaustein and Schwartz 2001). Estos hábitats son abundantes a lo largo de todo el planeta, son complejos, dinámicos y heterogéneos en cuanto a sus características físicas y biológicas (Blaustein and Schwartz 2001, Gómez-Rodríguez et al. 2010a). Entre los medios acuáticos, se encuentran los que **alternan fases acuáticas con periodos de desecación**, habitados por organismos acuáticos capaces de resistir los periodos secos. Los medios acuáticos temporales **comparten flujos de energía con otros sistemas** (Regester et al. 2006) y entre ellos se producen **múltiples interacciones ecológicas** (Blaustein and Schwartz 2001). A pesar de ser imprescindibles para muchos organismos, especialmente los de ciclo de vida bifásicos como los insectos y los anfibios, son medios que han sido históricamente menospreciados y poco estudiados, de los que todavía no se tiene demasiado conocimiento respecto a la estructura y dinámica de las redes tróficas que se producen en ellos (Sabo et al. 2009, Opsahl et al. 2010).

La temporalidad de un cuerpo de agua está determinada por la longitud del periodo de inundación, es decir, el hidroperiodo. Las lagunas temporales son cuerpos acuáticos, normalmente de tamaño pequeño o mediano, caracterizados por tener una fase de desecación recurrente con longitud variable y por su imprevisibilidad en cuanto a forma y duración (Williams 1997). Los organismos que habitan en los medios acuáticos temporales presentan una variedad de estrategias de supervivencia para adaptarse a esta imprevisibilidad y a la desecación. Algunas de estas estrategias son la sincronización de sus ciclos de vida con la duración del periodo de inundación, la alternancia de una fase de vida larvaria acuática -donde adquieren los recursos necesarios para crecer- con una fase adulta terrestre, la formación de estructuras de resistencia o la dispersión hacia medios más permanentes (Wiggins et al. 1980, Williams 2006).

Las lagunas temporales se caracterizan por una gran variabilidad a nivel estacional y a nivel interanual tanto en sus características ambientales (p. ej., desecación y cambios asociados en la química del agua) **como biológicas** (p. ej., aumento de la depredación o la competencia) (Williams 2006). Por tanto, la estructura de las comunidades presentes en estos medios estará condicionada por la interacción de estos factores, siendo en consecuencia esperable la variación en la riqueza y la diversidad de especies de las comunidades acuáticas de invertebrados (Florencio et al. 2013) o anfibios (Werner et al. 2007, Gómez-Rodríguez et al. 2010b).

En general, los medios acuáticos permanentes contienen un mayor número de especies que los medios acuáticos temporales (Brooks 2000), pero los temporales presentan muchas especies de invertebrados, anfibios y plantas que son raras en los medios permanentes y muchas de ellas son especies amenazadas especializadas en el uso de estos peculiares hábitats (Williams 1997, Céréghino et al. 2008). Estas especies a veces se presentan en estos medios en densidades muy altas, incluso con mayor diversidad de especies, como es el caso de las larvas de anfibios (Starrett 1973, Halliday 2005). Desafortunadamente, las lagunas temporales están entre los ecosistemas más amenazados por destrucción de hábitat y cambio climático (Williams et al. 2009) a pesar de que tienen un papel muy importante en la conservación de la biodiversidad. Aunque actualmente están catalogados como hábitats prioritarios de la Unión Europea, su interés y necesidades de protección aun no están suficientemente evidenciadas. De hecho, los medios acuáticos temporales ***ni siquiera aparecen*** como tales en la directiva marco del agua europea como hábitats singulares que merecen una especial atención (Céréghino et al. 2008). La conservación de los medios acuáticos temporales no sólo requiere preservar la integridad de cuerpos de agua individuales, sino que se debe también maximizar su conectividad para favorecer la dispersión de los organismos, y mantener la diversidad de especies (Oertli et al. 2008).

En los ecosistemas de agua dulce la estructura de la comunidad puede estar en gran parte determinada por una combinación de factores abióticos, como la temperatura, la turbulencia, el pH, la permanencia del hábitat, o la disponibilidad de luz, carbono, nutrientes y oxígeno, así como por factores bióticos dependientes de interacciones ecológicas (Brönmark and Hansson 2005). Los consumidores y los factores tanto bióticos como abióticos interaccionan de diferentes maneras influyendo en el crecimiento, supervivencia, comportamiento o ecología de los organismos, afectando finalmente a la estructura y dinámica de las comunidades (Power 1992). Las comunidades pueden estar condicionadas por factores “bottom-up” (i.e. ascendentes, p. ej. recursos abióticos), procesos “top-down” (i.e. descendentes, p. ej. herbivoría o efectos indirectos en cascadas tróficas) o incluso influenciados por ambos fenómenos (McQueen et al. 1986, Shurin et al. 2006, Stoler and Relyea 2013). Los consumidores alteran de forma directa o indirecta la composición del grupo de productores primarios, y este efecto puede ser desproporcionadamente grande si los consumidores son especies clave (Paine 1966, Woodward et al 2008) y el sistema está dominado por un control “top-down”. Por lo tanto, comprender correctamente la dinámica trófica de los organismos es fundamental para **comprender la estructura y funcionamiento de la comunidad y la ecología de los ecosistemas** (Paine 1980, Woodward 2009).

Los **macrófitos acuáticos son importantes productores primarios de los sistemas acuáticos temporales**, pero además pueden alterar los parámetros físico-químicos del medio, producir gran cantidad de materia orgánica (Wetzel 2001) y proporcionar una gran complejidad física al hábitat (Scheffer et al. 1993, 2001). Hay una enorme variedad de macrófitos de diferentes tipos y tamaños (Deil 2005), lo que favorece la creación de diferentes microhábitats en los medios acuáticos aptos para las diferentes especies de organismos que habitan en ellos. Las importantes fluctuaciones en el nivel de agua de las lagunas temporales pueden dar lugar a variaciones en la composición de macrófitos. Debido a los grandes cambios y la imprevisibilidad de este tipo de medios, es característico de la vegetación acuática la producción de semillas resistentes que forman un **banco de semillas** que se acumula entre ciclos hidrológicos y del cual germinan cuando las condiciones son beneficiosas para su establecimiento (Gauthier et al. 2004). Estas semillas se encuentran mayoritariamente en la superficie del sedimento, pero pueden acumularse durante años y quedar enterradas a profundidades considerables.

El fitoplancton y el zooplancton conforman a su vez elementos clave de la producción primaria y de los consumidores primarios, respectivamente, en las lagunas temporales. Tanto el uno como el otro proporcionan alimento a los macroinvertebrados y vertebrados que se encuentran en los medios acuáticos temporales. Dentro de los vertebrados, y dependiendo de la temporalidad y la situación geográfica, se pueden encontrar en las lagunas anfibios, reptiles, aves –tanto migratorias como residentes-, así como peces, cuando el medio pueda llegar a conectarse ocasionalmente a otros medios permanentes.

Los anfibios en los sistemas acuáticos temporales

Los anfibios son un grupo único de organismos presentes en prácticamente todos los biomas de la Tierra que constituyen un **modelo idóneo para el estudio de la ecología de comunidades** (Morin 1986, 1987, Babbitt et al. 2003). La mayoría de los anfibios se caracterizan por su ciclo de vida bifásico, con una etapa de desarrollo larvario en el medio acuático y una fase adulta terrestre, aunque existen diversos modos reproductivos en este grupo, incluyendo desarrollo directo o cuidados parentales (Wells 2007). Un aspecto a destacar en la ecología de los anfibios con larvas acuáticas es la **transferencia de nutrientes** entre niveles tróficos y entre barreras ecosistémicas (hábitat acuático y terrestre) (Waringer-Löschenkohl and Schagerl 2001). Los sistemas acuáticos temporales constituyen por tanto hábitats especialmente aptos para los anfibios, donde comúnmente se desarrollan desde la fase de huevo o larva hasta la metamorfosis (Semlitsch 2003, Wells 2007, Gomez-Mestre et al. 2012). Además, en muchos sistemas acuáticos temporales mediterráneos las larvas de los anfibios suponen una **gran parte de la biomasa total**, ya que son normalmente los únicos vertebrados presentes (p. ej. Gibbons et al. 2006).

Las larvas de anfibios constituyen un **importante recurso como presas** en las comunidades acuáticas, pero además pueden tener un efecto importante y duradero al afectar a la producción primaria, el ciclo de nutrientes, la descomposición de la materia orgánica, la dinámica de las poblaciones de invertebrados y el flujo de energía entre el ambiente acuático y el terrestre (Whiles et al. 2006, Regester et al. 2008). Asimismo, las larvas de anfibios están muy afectadas por interacciones competitivas y con depredadores, así como por el medio físico, como por ejemplo por la disponibilidad de agua (Morin 1983, Wilbur 1987, Wellborn et al. 1996, Halverson et al. 2003, Egan and Paton 2004).

Aunque las **larvas de anfibios** pueden alimentarse de una gran cantidad de recursos y muchas son omnívoras o incluso carroñeras (McDiarmid and Altig 1999), las de **anuros** son normalmente **importantes consumidores primarios**, no sólo de fitoplancton o perifiton, sino también de plantas vasculares acuáticas (Díaz-Paniagua 1985). Los papeles funcionales de los herbívoros son a menudo difíciles de definir ya que tienen **efectos directos** con los productores primarios a raíz de su consumo (top-down), pero además pueden tener **efectos indirectos** con otros factores que regulan la productividad primaria como el ciclo de nutrientes (bottom-up), afectando considerablemente la estructura y la dinámica de la estructura de la red trófica y de la comunidad en general (Power 1990, Kupferberg 1997, Flecker et al. 2002, Vanni 2002, Knoll et al. 2009). La herbivoría por parte de larvas de anfibios puede tener un importante papel en los ecosistemas acuáticos, afectando potencialmente a la composición de las comunidades de plantas, a su fenología reproductora o incluso podrían contribuir a la dispersión de sus semillas. Sin embargo, hasta la fecha no existen datos respecto a este papel potencial que pueden tener las larvas de anuros sobre los macrófitos acuáticos, por lo que es una prioridad profundizar en las investigaciones acerca de este aspecto. Por otra parte, **las larvas de anfibios urodelos** (tritones y salamandras) son eficientes **carnívoros** (Díaz-Paniagua et al. 2005, Wells 2007) y pueden alterar considerablemente la **composición de las comunidades de zooplancton**, y consecuentemente, a través de efectos cascada, afectar a la composición y abundancia del fitoplancton y a la producción primaria general del ecosistema (Costa and Vonesh 2013a) (**Imagen 1**).

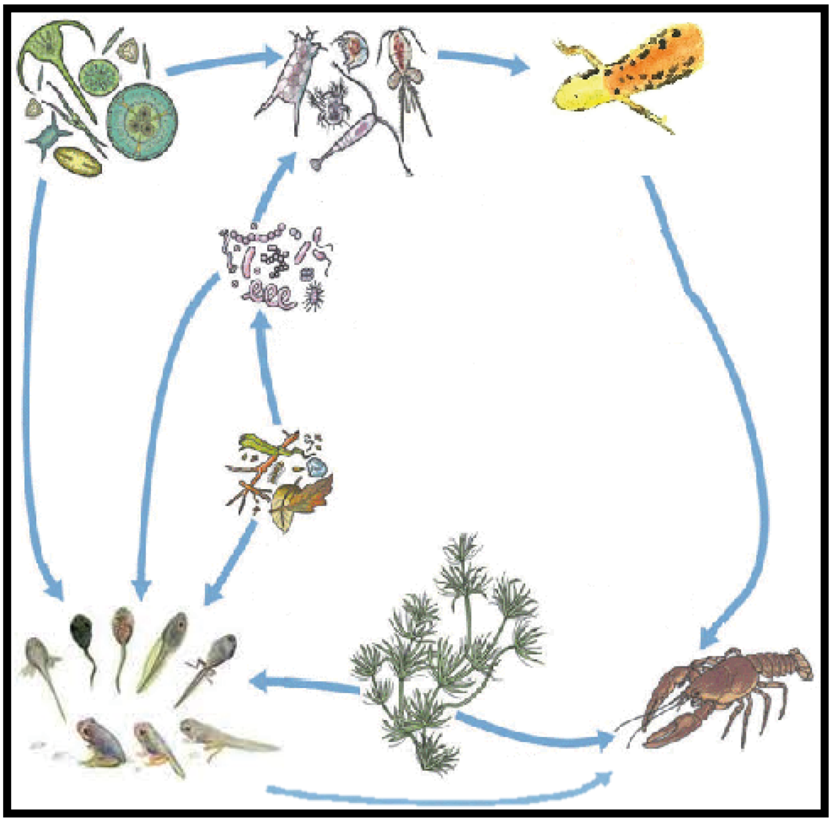


Imagen 1. Cadena trófica simplificada de una laguna temporal donde se observa que las larvas de anfibios anuros se alimentan de diversas fuentes de alimento (abajo izquierda) mientras que la fuente principal de las larvas de urodelos es el zooplancton. Los depredadores de anfibios pueden a su vez ser competidores con los anfibios por los mismos recursos y la compleja red de interacciones puede verse modificada.

El estudio de la ecología trófica de larvas de anfibios ha suscitado un interés renovado asociado a la aplicación de nuevas técnicas basadas en isótopos estables (ver **Caja 1**). Así, los isótopos estables están siendo utilizados cada vez con mayor frecuencia para determinar su estado trófico, las variaciones ontogénicas en la dieta, o las variaciones producidas en el estado trófico por diferentes factores ambientales (Trakimas et al. 2011, Schriever and Williams 2013, Arribas et al. 2015, San Sebastián et al. 2015).

Competencia y depredación en larvas de anfibios

Como se ha comentado anteriormente, la depredación ha sido un factor muy importante en la evolución de muchas características de los anfibios, incluyendo los **comportamientos y fenotipos de defensa**. Las larvas de anfibios son presas de un gran número de depredadores de todo tipo: peces, tortugas, aves, pequeños mamíferos, otros anfibios, y sobre todo, invertebrados acuáticos en las lagunas temporales donde normalmente los peces están ausentes.

Los depredadores invertebrados suelen ser generalistas que de forma oportunista se alimentan de anfibios cuando los encuentran disponibles. Entre los más importantes se pueden mencionar a las sanguijuelas, los cangrejos de agua dulce, las chinches acuáticas y las larvas de insectos como escarabajos acuáticos u odonatos (**Imagen 2**) (Wells 2007). Las ***larvas de ditíscidos*** son depredadores muy voraces que son capaces de devorar presas mayores a su propio tamaño (Lundkvist et al. 2003) y en el caso de larvas de anfibios, hasta 20 renacuajos por día (Warfe and Barmuta 2004). El consumo directo por parte de los depredadores **reduce la densidad de las larvas** lo cual, a su vez, **puede reducir la competencia** entre los individuos de una misma especie o entre especies cuando las larvas se encuentran en alta densidad (Wilbur 1997), que favorece finalmente **el incremento de tamaño de los individuos supervivientes** (p. ej. Morin 1986).

Imagen 2. Depredadores comunes de anfibios presentes en las lagunas temporales de Doñana. De izquierda a derecha: larva de escarabajo acuático (*Dityscus circumflexus.)*, larva de libélula (*Anax sp.*) y el invasor cangrejo rojo americano (*Procambarus clarkii*). Fotos: A. Portheault y H. Garrido.

Los renacuajos pueden además responder de manera indirecta mediante **cambios fenotípicos** ante la presencia de depredadores, con los que han compartido una historia evolutiva conjunta, y por las señales de alarma de otros renacuajos (Anholt and Werner 1995, Relyea 2001, Van Buskirk 2001). Estos cambios plásticos en morfología, comportamiento, o la combinación de ambos, son específicos dependiendo del tipo de depredador (Van Buskirk 2001). Por ejemplo, algunos cambios morfológicos incluyen el aumento de la pigmentación en la cola o cambios en la coloración para ser crípticos o tener un fenotipo de defensa (Van Buskirk and Schmidt 2000), la disminución del tamaño del cuerpo respecto al tamaño total, o el incremento de la altura de la cola con respecto al cuerpo (Van Buskirk 2001). Los cambios comportamentales incluyen el aumento del uso de refugios o la disminución de los niveles de actividad y pueden afectar últimamente a rasgos de las historias de vida de los individuos (Werner and Anholt 1993, Van Buskirk 2001).

Al igual que responden ante los depredadores, los renacuajos también son capaces de responder a sus competidores alterando su morfología o incrementando su actividad alimenticia (Relyea 2002b). **La combinación de competencia y depredación puede por tanto producir un cambio de dieta en las larvas de anfibios** al modificar sus microhábitats o su anatomía (longitud intestinal, número de filas de dientes, tamaño del disco oral, etc.) (Relyea and Auld 2004, 2005).

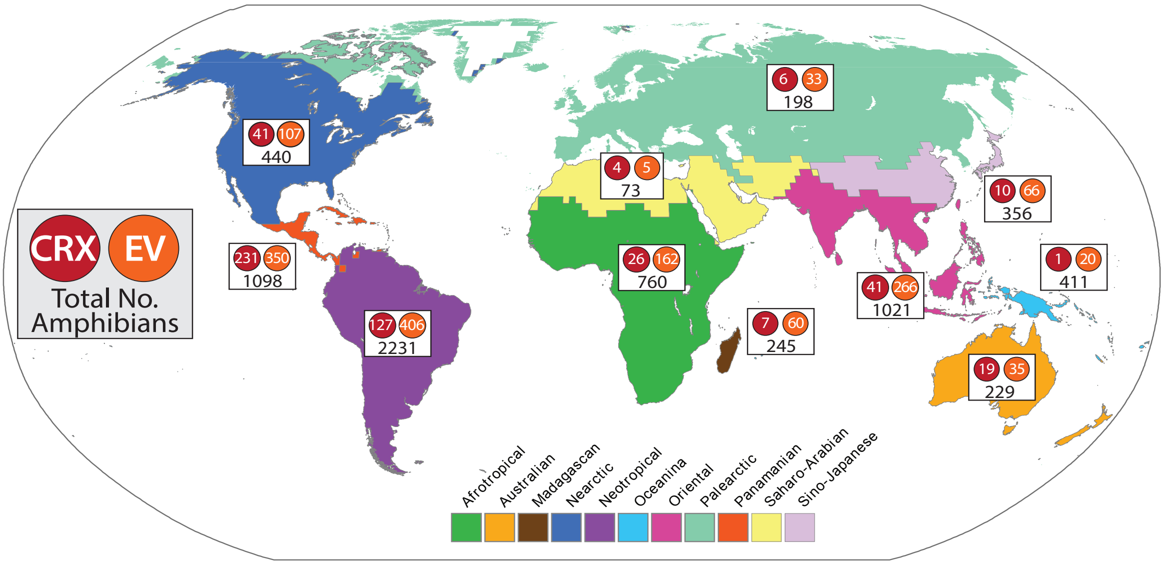
Sin embargo, la introducción y establecimiento de especies exóticas en un área supone una gran amenaza para la fauna nativa (Reynolds 2009, Kovacs et al. 2012) que puede llegar a desequilibrar el ecosistema (Maezono and Miyashita 2003). En el área de Doñana, una de nuestras zonas de estudio, hay varias **especies acuáticas exóticas que pueden producir graves consecuencias en las comunidades**, como por ejemplo el cangrejo rojo americano, ***Procambarus clarkii*** (**Imagen 2**), cuyos principales efectos han sido resumidos por Geiger et al (2005).

Fue introducido en las inmediaciones de Doñana en 1974 (Habsburgo-Lorena 1978), extendiéndose posteriormente por las marismas y en menor medida por las zonas arenosas del Parque (Díaz-Paniagua et al. 2014). Produce grandes cambios físicos en el ambiente y altera los recursos disponibles para otras especies en el medio acuático. Su efecto es especialmente perjudicial para las comunidades de macrófitos ya que son una de sus principales fuentes de alimento, en especial cuando no tienen disponibilidad de presas animales (Duarte et al. 1990, Momot 1995).

Además, se ha observado que **las larvas de anfibios no pueden reconocer las señales de depredadores invasores como el cangrejo americano** y por lo tanto no pueden producir defensas antidepredatorias ante ellos (Gomez-Mestre and Díaz-Paniagua 2011). Esto implica que su efecto sobre las larvas de anfibios y, por efectos cascada en otros niveles tróficos, puede llegar a ser mucho más acusado que el de los depredadores nativos.

Amenazas actuales de los anfibios y su situación como organismos clave en los ecosistemas acuáticos

En general, las características de las historias de vida de los anfibios y reptiles los hacen vulnerables a las perturbaciones ambientales, mayoritariamente como consecuencia de las actividades humanas, que están provocando **acusados declives globales** en sus poblaciones (Araújo et al. 2006). En particular, los anfibios son un grupo único de vertebrados que ha existido en la Tierra durante más de 300 millones de años y que incluyen más de 7000 especies conocidas. En las dos últimas décadas, se han extinguido unas 168 especies y alrededor de 2500 tienen poblaciones en declive (**Figura 1**) siendo actualmente **los vertebrados más amenazados del planeta** (Wake and Vredenburg 2008). Las causas de este declive generalizado incluyen el aumento de la radiación ultravioleta, especies introducidas que son potenciales depredadores de anfibios, pérdida de hábitat, enfermedades y cambio climático, por lo que estos números posiblemente continúen aumentando (Alford and Richards 1999, Stuart et al. 2004).



AmphibiaWeb: Wallacean Biome Map of Amphibian Species in Decline. [web application]. 2014. UC Regents, Berkeley, CA.
Available: http://amphibiaweb.org/declines/. (12 May 2014).

Figura 1. Número de especies de anfibios extintas, extintas en libertad o amenazadas de forma crítica (en rojo) y amenazadas o vulnerables (en naranja) a lo largo del planeta, sobre el total de especies (en caja). Fuente: AmphibiaWeb.org

Como se ha descrito en esta introducción, los anfibios son especies clave en la cadena trófica de los sistemas acuáticos, pero se conoce muy poco sobre su papel funcional como consumidores y también hay relativamente pocos estudios sobre las interacciones entre las larvas de anfibios y otros niveles tróficos (Wilbur 1997, Whiles et al. 2006, Costa and Vonesh 2013a) que potencialmente pueden cambiar la estructura y dinámica de las comunidades acuáticas. Los cambios en niveles tróficos relativamente inferiores debidos a perturbaciones puntuales o continuas, pueden tener importantes consecuencias que se transmiten a través de la cadena trófica y por consiguiente afectan a toda la estructura de la comunidad (Eby et al. 2006, Jonsson et al. 2006). Por lo tanto, y teniendo en cuenta su situación de declive generalizado, es **esencial caracterizar el papel funcional de los anfibios en los sistemas naturales con el objetivo de anticipar y mitigar los efectos de sus acusados y previsibles declives poblacionales**, que podría finalmente ocasionar importantes consecuencias ecológicas, económicas y de salud mundial que aún no llegamos a comprender (Mohneke and Rodel 2009).

OBJETIVOS DE LA TESIS DOCTORAL

El objetivo general de esta tesis es profundizar en el **estudio del papel funcional de las comunidades de larvas de anfibios en los ecosistemas acuáticos temporales, teniendo en cuenta sus interacciones ecológicas con competidores y depredadores**. Los vertebrados que normalmente se utilizan para explicar la estructura de las comunidades acuáticas son los peces y sin embargo los anfibios son los principales, y frecuentemente los únicos, vertebrados en medios temporales, donde alcanzan una biomasa importante y donde además ocupan distintos niveles tróficos. A pesar de ser especies clave en muchos ecosistemas, su papel en la estructura de las comunidades acuáticas ha sido escasamente estudiado.

Por tanto, este objetivo general se divide en varios objetivos específicos y son los siguientes:

1. Determinar el efecto de las larvas de anfibios en la estructura y funcionamiento de las comunidades de medios acuáticos temporales mediterráneos, centrándose principalmente en las comunidades de zooplancton y de macrófitos.

2. Evaluar las variaciones en este efecto a través de las interacciones ecológicas con competidores y depredadores, tanto por consecuencias denso-dependientes como por denso-independientes.

3. Investigar la interacción y la importancia relativa de competencia y depredación en la estructura de comunidades de anfibios neotropicales.

4. Conocer la plasticidad en el uso de los recursos tróficos y la partición de nicho trófico de las larvas de anfibios mediterráneos y neotropicales dependiendo de las condiciones ambientales y de la presencia de competidores y depredadores.

5. Determinar el efecto de las larvas de anuros sobre la estructura de la comunidad de plantas, analizando las consecuencias de la herbivoría y de la ingestión de semillas y esporas de la vegetación acuática.

Los sistemas de estudio escogidos para desarrollar los objetivos de esta tesis doctoral han sido las comunidades de las lagunas temporales del Parque Nacional de Doñana (Huelva, España) como sistema mediterráneo con influencia Atlántica; y las comunidades de las lagunas del Parque Nacional Soberanía (Gamboa, Panamá) como sistema neotropical.

ÁREAS Y ESPECIES DE ESTUDIO

Lagunas temporales de Doñana

El Parque Nacional de Doñana se localiza en el suroeste de España en el margen derecho de la desembocadura del río Guadalquivir, entre las provincias de Huelva, Cádiz y Sevilla. Dentro del Parque se pueden diferenciar tres unidades geomorfológicas a grandes rasgos: un área de marisma, un sistema de dunas móviles paralelo a la costa y un sistema de dunas estabilizadas que comprende casi un tercio del área total del Parque (Siljestrom and Clemente 1990). El clima del área es Mediterráneo con influencia Atlántica, con veranos secos y calurosos e inviernos suaves. La precipitación media anual es de 544.6 (±211.3) mm y ocurre principalmente en otoño e invierno, que contribuye a la formación de más de 3000 lagunas temporales (Díaz-Paniagua et al. 2010). Doñana se considera un área de interés especial para la conservación de los anfibios (Santos et al. 1996) debido principalmente a la gran disponibilidad y variabilidad de lugares óptimos para su reproducción (Díaz-Paniagua et al. 2006). Las lagunas temporales mediterráneas presentan un periodo de desecación recurrente en el tiempo, que suele coincidir con el verano. En Doñana, el hidroperiodo es muy variable entre lagunas dependiendo de la profundidad del nivel freático y entre años según la meteorología de cada año, pues depende del patrón de las lluvias y de la cantidad de precipitaciones que se producen (Gómez-Rodríguez et al. 2010a). El momento de inundación de las lagunas en Doñana es bastante impredecible, pero suele ser aproximadamente sincrónico para la mayoría de las lagunas excepto para las más efímeras que sólo se inundan en años de intensas precipitaciones (Díaz-Paniagua et al. 2010) (**Imagen 3**). Las lagunas se sitúan sobre suelos arenosos muy permeables, por lo que su ciclo de inundación depende sobre todo de las oscilaciones del nivel de las aguas subterráneas, que al elevarse con las lluvias, interceptan la superficie del terreno formando las lagunas (Serrano et al. 2006, Gómez-Rodríguez et al. 2009). Solamente hay dos lagunas que se puedan considerar permanentes en el área, Santa Olalla y La Dulce, aunque Santa Olalla llegó a desecarse tras un periodo de sequía prolongada, y La Dulce se viene secando en años aislados secos (Díaz-Paniagua and Aragonés en prensa, Serrano and Zunzunegui 2008).

Imagen 3. Laguna temporal de corto hidroperiodo y Laguna del Zahillo, una laguna peridunar de largo hidroperiodo del Parque Nacional de Doñana. Fotos: CDP y RA.

Es necesario señalar que los macrófitos acuáticos son elementos clave en esta área e incluyen más de 70 taxones de macrófitos sumergidos (Murillo et al. 2006) y hasta 200 especies diferentes incluyendo la vegetación de las lagunas que no es estrictamente acuática. Entre los macrófitos sumergidos encontramos diferentes grupos, siendo los más comunes las plantas de la división Spermatophyta (62%) como *Callitriche* sp., *Ranunculus* sp., *Myriophyllum* sp., *Potamogeton* sp., o *Lemna* sp (**Imagen 4**). También son abundantes las algas Chlorophyta (26%) como *Chara* sp. (**Imagen 4**) o *Nitella* sp. Menos abundantes, pero también presentes, son los musgos (Bryophyta, 8%) como *Riella* sp., y los helechos (Pteridophyta, 4%) como *Isoetes vellatum* o la invasora *Azolla filiculoides* (Murillo et al. 2006). Esta invasora forma espesas alfombras en la superficie del agua que impiden el paso de la luz hacia las zonas inferiores y por tanto el correcto desarrollo de los macrófitos, además de incrementar la eutrofización al ser capaces de fijar nitrógeno y de consumir el oxígeno del agua mediante su actividad respiratoria (Garcia-Murillo et al. 2007).

Imagen 4. Macrófitos abundantes en las lagunas temporales de Doñana. De izquierda a derecha: *Callitriche obtusangula, Ranunculus peltatus, Myriophyllum alterniflorum* y *Chara connivens*. Fotos: CDP

En cuanto a la diversidad de la fauna en las lagunas temporales de Doñana (**Imagen 5**) se puede destacar la gran riqueza de zooplancton, con más de 100 especies entre microcrustáceos y rotíferos (Fahd et al. 2009). También encontramos en el área una gran riqueza de macroinvertebrados, conformando 124 taxones (Florencio et al. 2009). Destacan por número de especies los coleópteros acuáticos, en especial los de la familia Dytiscidae con 21 especies, pero también algunas especies de Odonatos catalogadas como vulnerables según las categorías de la IUCN (Verdú et al. 2011). Las lagunas de menor hidroperiodo presentan una menor riqueza pero una mayor singularidad de especies (Díaz-Paniagua et al. 2010). En las lagunas de Doñana también se encuentran algunos reptiles como la culebra de agua (*Natrix maura*) o los galápagos autóctonos (*Mauremys leprosa* y *Emys orbicularis*). Respecto a los anfibios, se pueden encontrar en el área 11 de las 13 especies presentes en el suroeste de España (Diaz-Paniagua et al 2006). De éstas, ocho son las más abundantes (*Hyla meridionalis, Pelobates cultripes, Bufo calamita, Discoglossus galganoi, Pelodytes ibericus, Triturus pygmaeus, Lissotriton boscai* y *Pleurodeles waltl*) y se reproducen en las lagunas temporales; la rana común, *Pelophylax perezi*, se reproduce en lagunas más permanentes o en aquellas excavadas artificialmente que persisten durante el verano con agua; y dos especies (*Bufo spinosus* y *Alytes cisternasii*) son más escasas y están restringidas a determinadas zonas del Parque (Díaz-Paniagua et al. 2006). Además, numerosas aves pasan una gran parte de su tiempo alimentándose en estas lagunas y también son zonas de abastecimiento para muchos mamíferos como los jabalíes, la nutria o la rata de agua.

Imagen 5. Algunos individuos de la fauna que se encuentra en las lagunas temporales de Doñana. De izquierda a derecha: un copépodo que forma parte del zooplancton (*Hemidiaptomus roubaui*), un macroinvertebrado (*Notonecta viridis*), el galápago leproso (*Mauremys leprosa*) y la ranita meridional (*Hyla meridionalis*). Fotos: A. Portheault, CDP, RA.

Sistemas acuáticos neotropicales

La variedad y la riqueza de especies en sistemas neotropicales (región tropical del continente americano, desde el norte de México al norte de Argentina y Chile) es mucho mayor que en las zonas templadas (Gaston 2000), y esto también es cierto para los sistemas acuáticos (p. ej. Margalef 1983). La estabilidad del clima en los trópicos permite un mayor rango de adaptaciones morfológicas y fisiológicas y por tanto conlleva a una mayor especialización de las especies y una mayor diversidad de las comunidades (Fischer 1960, Schemske et al. 2009). Las precipitaciones anuales son muy superiores y la estación lluviosa es menos impredecible que en sistemas mediterráneos, por lo que el solapamiento de especies en el medio acuático, como es el caso de los anfibios, es bastante frecuente durante este período.

Por el contrario, los mecanismos que gobiernan la dinámica de las redes tróficas en los trópicos no están tan claros como en sistemas templados (Lazzaro et al. 2003, Menezes et al. 2010), aunque se sugiere que las interacciones bióticas son más importantes que en las regiones templadas (Schemske et al. 2009). Además, muchos cuerpos acuáticos en las regiones neotropicales están realmente amenazados (Rolon and Maltchik 2006).

El área de estudio en sistemas neotropicales para esta tesis es Gamboa, en el centro de Panamá, que se encuentra inmerso en el Parque Nacional de Soberanía. En esta área se conocen 62 especies de anfibios (1 cecilia, 2 salamandras y 59 anuros). El clima de la zona es el típico de las tierras bajas en el trópico, con una temperatura anual casi uniforme que varía más diariamente (con una media de 22 y 30 grados por el día y por la noche respectivamente) que estacionalmente (entre 23 y 26 grados del mes más frío al más caluroso). El periodo de lluvias se produce entre mayo y diciembre, pero la época seca presenta normalmente un periodo de lluvias y la época de lluvias presenta un periodo seco (Ibañez et al. 1999), sumando alrededor de 2150 mm de precipitaciones anuales. Las lluvias comienzan normalmente de forma gradual y terminan de forma abrupta. Por lo tanto, con las lluvias se forman pequeñas charcas de escasos días de duración, lagunas temporales que permanecen inundadas durante la época de lluvias o cuerpos de agua más permanentes que se mantienen inundados prácticamente todo el año (**Imagen 6**). En todos ellos se desarrollan multitud de formas de vida. Las plantas acuáticas, tanto flotantes, como sumergidas o emergentes, son abundantes en muchos cuerpos de agua. Además, hay grandes grupos de macroinvertebrados (larvas de insectos, arañas, etc), peces, numerosos anfibios, y reptiles como caimanes o cocodrilos. También en algunos cuerpos de agua más grande se puede encontrar un grupo de mamíferos, los manatíes. Los cuerpos de agua suelen estar rodeados de abundante vegetación pero también hay zonas más abiertas en donde llega abundante radiación solar.

Imagen 6. Sistemas acuáticos temporales de zonas neotropicales en Gamboa, Panamá. Fotos: RA.

Especies del estudio: representación de la comunidad de anfibios en ambientes mediterráneos y neotropicales

En Doñana se pueden encontrar 11 especies de anfibios: 8 de ellas corresponden a anuros y 3 a urodelos. De estas, se escogieron 6 especies representativas -5 anuros y 1 urodelo- para los experimentos realizados en mesocosmos (ver **Caja 2**), todas ellas abundantes en el área. Estas especies son el sapo de espuelas (***Pelobates cultripes***), la rana común (***Pelophylax perezi***), la ranita meridional (***Hyla meridionalis***), el sapillo pintojo ibérico (***Discoglossus galganoi***), el sapo corredor (***Bufo calamita***) y el tritón pigmeo (***Triturus pygmaeus***) (**Imagen 7**). Todas estas especies han sido utilizadas para el desarrollo de los estudios de los dos primeros capítulos y, además, las 4 primeras fueron también utilizadas para responder al último de los objetivos que conforman el cuarto capítulo de esta Tesis.

El sapo de espuelas, *Pelobates cultripes*, se reproduce principalmente en lagunas temporales de gran dimensión, aunque también se puede encontrar en lagunas más pequeñas (Diaz-Paniagua 1990, Gómez-Rodríguez et al. 2012). Las larvas de esta especie alcanzan un tamaño corporal mucho más grande que las de las otras especies encontradas en Doñana (Díaz-Paniagua et al. 2005). El periodo de reproducción comienza después de las primeras lluvias del otoño o invierno y el desarrollo larvario puede extenderse más 6 meses, alcanzando las larvas un tamaño corporal máximo (sin incluir la cola) alrededor de 40mm (Díaz-Paniagua et al. 2005). *Discoglossus galganoi* puede reproducirse varias veces en un año en charcas poco profundas, normalmente formadas tras intensas lluvias o en las formadas en las proximidades de lagunas más

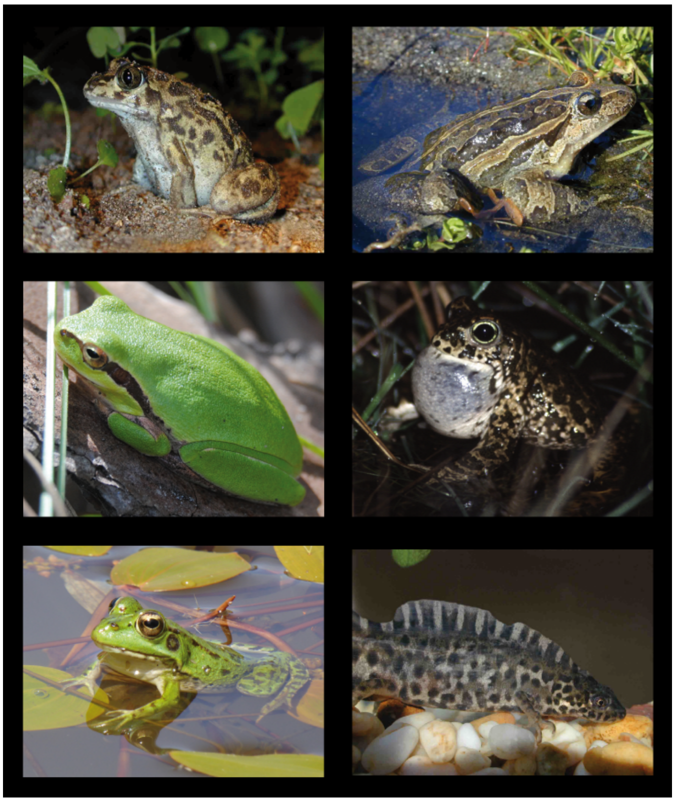


Imagen 7. Adultos de las 6 especies de anfibios estudiados en esta tesis doctoral. De izquierda a derecha y de arriba a abajo: *P. cultripes, D. galganoi, H. meridionalis, B. calamita, P. perezi* y *T. pygmaeus*. Fotos: AP, CDP y RA.

grandes. Las larvas se pueden encontrar desde diciembre hasta mayo y alcanza como máximo un tamaño corporal aproximado de 12mm (Díaz-Paniagua et al. 2005). *Hyla meridionalis* pone los huevos en pequeños paquetes o individualmente y el periodo larvario dura aproximadamente entre 2 meses y medio y 3 meses. La reproducción comienza generalmente en enero y se extiende por varios meses por lo que se pueden encontrar larvas en las lagunas temporales en estados de desarrollo muy diferentes. El tamaño máximo de las larvas es aproximadamente 18mm (Díaz-Paniagua et al. 2005). *Bufo calamita* realiza sus puestas en largas cadenas de huevos, que deposita durante un periodo muy corto tras lluvias intensas en invierno o primavera. Los sapos corredores prefieren charcas poco profundas con escasa vegetación y zonas soleadas. Las larvas son pequeñas (longitud máxima sin cola: 12,5 mm) y pueden ser observadas en Doñana desde febrero. *Pelophylax perezi* se diferencia de los demás anuros porque empieza a reproducirse más tarde que las otras especies, generalmente a partir de Abril. Sus larvas pueden encontrarse mayoritariamente en el fondo de las lagunas, alimentándose de detritos y también en la columna de agua. Completan su desarrollo en las lagunas durante el verano o a veces, incluso en el año siguiente, alcanzando un tamaño máximo (sin cola) de unos 27 mm (Díaz-Paniagua et al. 2005). Las larvas de todas estas especies de anuros se alimentan principalmente de algas, fanerógamas y detritos (Díaz-Paniagua 1985). *Triturus pygmaeus* es una de las especies más abundantes de Doñana. La dieta de sus larvas está constituida principalmente por crustáceos planctónicos (93%) y el periodo en el que se encuentran larvas en las lagunas puede durar desde Febrero hasta el final de Julio (Díaz-Paniagua et al. 2005).

CAJA 2. Mesocosmos: experimentación en condiciones seminaturales

Las investigaciones de esta Tesis se desarrollan en su gran mayoría en mesocosmos, ampliamente utilizados en el campo de la ecología experimental desde hace varias décadas, en particular en el medio acuático (p. ej. Peacor and Werner 1997, Wilbur 1997, Arribas et al. 2014). Los mesocosmos suelen ser tanques o cubetas de gran capacidad, en los que se simulan las condiciones ambientales en condiciones controladas con la máxima fiabilidad. La finalidad para este estudio es simular las condiciones, la estructura y los niveles tróficos de una laguna. Para ello, en los mesocosmos utilizados para responder a los objetivos de la presente tesis, se introduce en primer lugar el sustrato característico del hábitat de las especies que serán estudiadas, como arena y sedimento del fondo de una laguna temporal en el área de Doñana u hojarasca en el caso de charcas en los trópicos. El sustrato normalmente contiene formas de resistencia de vegetación, plancton e invertebrados que, tras la inundación con agua de lluvia, permitirá el desarrollo de gran parte de los organismos característicos de un cuerpo de agua temporal (plancton, vegetación, con excepción de los invertebrados voladores. Asimismo, en muchas ocasiones se inocula el agua con microorganismos de otros cuerpos de agua, con el fin de incrementar los recursos disponibles en los mesocosmos y la diversidad de fitoplancton y zooplancton, como es en el caso de los mesocosmos del área de Doñana.

Tras un período adecuado de desarrollo de estas formas de vida, se introduce en cada tanque experimental o mesocosmo las especies objeto de estudio y en las abundancias adecuadas para responder a los objetivos de la investigación. Además, estos experimentos permiten añadir cualquier factor modificado que permita cumplir los objetivos así como la eliminación de organismos clave y sus depredadores, tal y como se explicará en los capítulos de la Tesis Doctoral. Cada tratamiento (situación experimental) se replica aleatoriamente en una serie de mesocosmos para asegurarse la repetibilidad y evitar cualquier situación estocástica no deseada que pudiera darse en uno de los mesocosmos.

Sin embargo, la experimentación en mesocosmos ha sido también criticada por algunos autores, debido a su potencial para sobreestimar efectos ecológicos y a la posibilidad de que no reflejen ciertos procesos naturales de forma correcta (Skelly 2002, Rubbo et al. 2006). Esta observación debe tenerse en cuenta, aunque en muchas ocasiones los experimentos ecológicos permiten comprobar una gran variedad de hipótesis concretas y con un grado de repetibilidad que sería muy difíciles de alcanzar en condiciones totalmente naturales. Por lo tanto, los experimentos en mesocosmos constituyen una opción acertada para indagar en mecanismos y consecuencias ecológicas en cuanto al papel funcional de ciertas especies o incluso interacciones ecológicas entre individuos (Werner 1998, Chalcraft et al. 2005).

En Panamá la diversidad de anfibios es muy superior y la etapa reproductiva puede extenderse durante toda la estación lluviosa (mayo-diciembre), por lo que el solapamiento en el tiempo y en el espacio es todavía mayor que en los sistemas templados. Para responder a los objetivos referentes a los sistemas neotropicales utilizamos, en el tercer capítulo, 5 especies de anuros muy frecuentes en el área de estudio y que normalmente coexisten en los cuerpos de agua, cuyas condiciones intentamos simular en los mesocosmos del estudio. Una de estas especies es la rana de ojos rojos (***Agalychnis callidryas***), que deposita sus puestas sobre las hojas de la vegetación que se localiza encima de los medios acuáticos (**Imagen 8**). Los huevos eclosionan sin perturbaciones en 6-7 días (Gomez-Mestre et al. 2008) y las larvas caen al medio acuático. Los renacuajos de esta especie se alimentan de fitoplancton o perifiton en la mitad de la columna de agua (Wells 2007). ***Dendropsophus ebraccatus*** tiene puestas terrestres o acuáticas dependiendo del grado de sombra de las lagunas (Touchon and Warkentin 2008, **Imagen 8**) y los huevos eclosionan aproximadamente en 3.5 días. Los renacuajos son bentónicos y se alimentan de algas filamentosas y perifiton (Wells 2007). ***Dendropsophus microcephalus*** y ***Scinax ruber*** depositan sus puestas en el agua y los huevos eclosionan en 2-3 días. La rana Túngara ***Engystomops pustulosus*** deposita sus huevos en nidos de espuma que se mantienen flotando sobre los cuerpos de agua en los que se reproduce (Ryan 1985). La espuma protege los huevos fertilizados hasta su eclosión después de 3 o 4 días. Las larvas de *A. callidryas* y de *S. ruber* son considerablemente más grandes que las del resto de las especies del e studio (obs. pers.), seguidas en tamaño de las de *D. microcephalus*.

Imagen 8. En la parte superior, amplexo de la ranita de ojos rojos (*Agalychnis callidryas*) y dos puestas de esta especie en una hoja dispuesta sobre de un cuerpo de agua y en la parte inferior amplexo de la ranita del reloj de arena (*Dendropsophus ebraccatus*) y una puesta terrestre de esta especie en la parte derecha. Fotos: RA

ESTRUCTURA DE LA TESIS DOCTORAL

La Tesis consta de 4 capítulos que responden a los objetivos planteados previamente, con los que se pretende aportar la información necesaria para entender el papel clave que representan las larvas de anfibios en los medios acuáticos temporales, donde mantienen además gran cantidad de interacciones con otros organismos produciendo efectos de gran importancia sobre el ecosistema.

En el **Capítulo 1** se estudia el efecto de una representación de la comunidad de larvas de anfibios de áreas templadas mediterráneas, las lagunas temporales de Doñana, evaluando su papel sobre la estructura y funcionamiento de las comunidades características de estos medios acuáticos. Se determina su impacto sobre la abundancia y composición de macrófitos y de zooplancton, así como sobre diversos parámetros físico-químicos del agua. Además, se evalúa dicho efecto en distintas condiciones de densidad de larvas y teniendo en cuenta la presencia o ausencia de una especie de anfibio cuya larva es desproporcionalmente más grande que las del resto de la comunidad. Por otro lado, se evalúan los efectos directos e indirectos de depredadores nativos e invasores en el medio y en la comunidad de larvas de anfibios. Se analiza también el efecto de los mismos tratamientos (alta o baja densidad, presencia de las larvas de mayor tamaño, presencia de depredadores nativos e invasores), así como los cambios que se producen en las condiciones ecológicas del medio, en la ecología trófica de las larvas de anfibios. Por ello, en el **Capítulo 2** se identifica mediante isótopos estables el potencial solapamiento y la partición de los recursos alimenticios según la variación de las condiciones en los mesocosmos que se han observado y descrito en detalle en el primer capítulo. Se utilizan análisis de los isótopos estables de carbono (C) y (N) para evaluar las posibles variaciones en el posicionamiento trófico de los anfibios y en la asimilación de los recursos en la dieta debido al aumento de la densidad y a la presencia de depredadores y competidores. Esto nos permitirá entender cómo las larvas de anfibios pueden hacer frente a los cambios producidos naturalmente o por causas humanas en los ecosistemas acuáticos gracias a la plasticidad trófica. La frecuencia de estudios de isótopos en anfibios continúa aumentando en los últimos años, pero en este capítulo se ha realizado la aproximación al estudio de una comunidad de anfibios en lugar de enfocarse en una o dos especies, cuantificando el solapamiento o partición del nicho trófico entre especies que pertenecen al mismo o a diferentes niveles tróficos.

En el **Capítulo 3** se examinan los efectos simultáneos, así como la importancia relativa, de depredación y competencia en la estructuración de una comunidad de larvas de anfibios neotropicales, utilizando un diseño experimental similar al del primer capítulo. Se evalúan los efectos de una especie de anfibio cuya larva se conoce también por su capacidad competidora en combinación con los efectos directos o indirectos de dos tipos de depredadores comunes en los cuerpos de agua del centro de Panamá. Además, se utilizan también isótopos estables para estudiar el efecto tanto de los depredadores como de la composición de la comunidad de anfibios en el nivel trófico de las larvas de anfibios. Así, se pueden estudiar con perspectiva las interacciones ecológicas de sistemas neotropicales en comparación con las de sistemas mediterráneos.

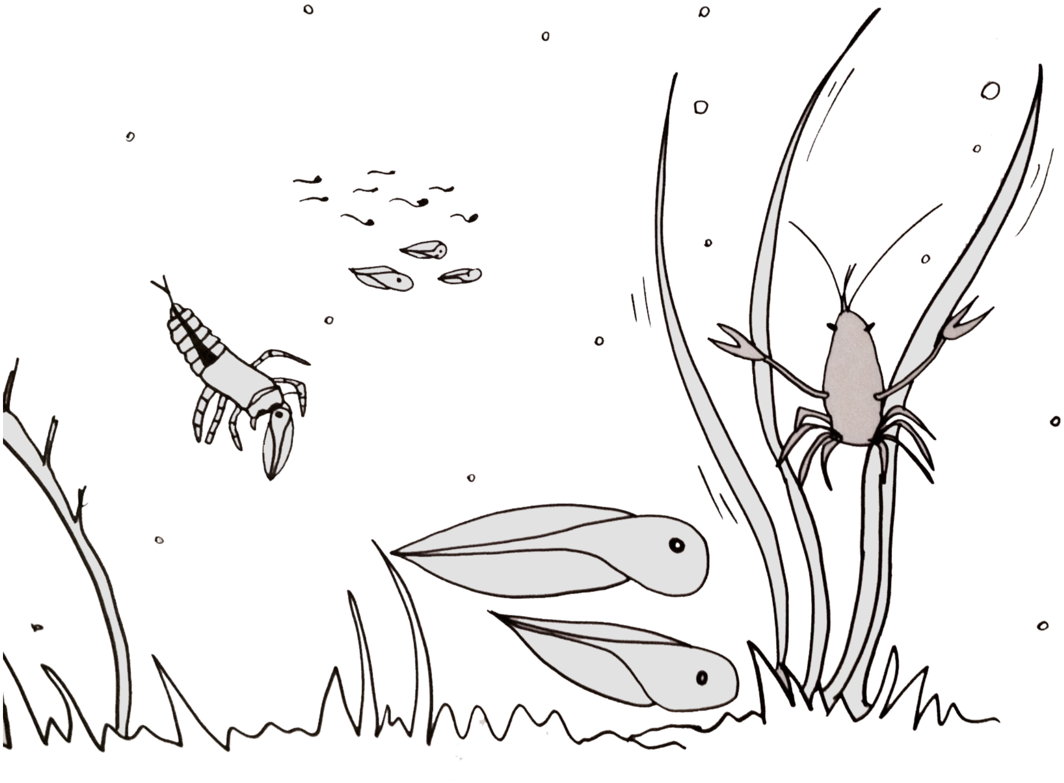
Por último, y dado que en el primer y segundo capítulo se observa un gran consumo de macrófitos acuáticos por parte de las larvas de anfibios, en el **Capítulo 4** se investiga por un lado la herbivoría de las larvas de anuros y por otro se muestra su capacidad para transportar semillas de plantas acuáticas y esporas de macroalgas. Estos son los primeros datos que demuestran el potencial de las larvas de anuros en la dispersión y germinación de semillas por lo que esta Tesis abre un interesante campo de investigación en las relaciones planta-animal. Además, se detallan las preferencias en la herbivoría de cada especie y la relación con las semillas transportadas, así como la probabilidad de germinación de éstas.

Algunos capítulos de esta Tesis se encuentran publicados en revistas científicas internacionales mientras que otros están en vías de publicación.

Se incluye finalmente una **Discusión General**, en la que a modo de síntesis se destacan los resultados más relevantes de todos los capítulos y son posteriormente discutidos de una forma global, planteándose futuras líneas de investigación enfocadas a seguir avanzando en el conocimiento sobre la ecología de los anfibios, con la finalidad de incrementar el esfuerzo que requiere la conservación de este grupo tan amenazado. Para finalizar, la Tesis se cierra enumerando las **Conclusiones Generales** más significativas que han brindado esta investigación.

Capítulo 1

Ecological consequences of amphibian larvae and their native and alien predators on the community structure of temporary ponds[1]



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[1] Arribas R, Díaz-Paniagua C, Gomez-Mestre I (2014). Ecological consequences of amphibian larvae and their native and alien predators on the community structure of temporary ponds. *Freshwater Biology 59(9): 1996-2008*

RESUMEN

Las conexiones entre los consumidores y los recursos en las redes tróficas son complejas y afectan a la estructura y el funcionamiento de los ecosistemas. En este capítulo evaluamos la influencia de los anfibios como consumidores en la estructura y funcionamiento de las lagunas temporales, determinando su impacto en la abundancia de macrófitos, en la diversidad de zooplancton y en la química del agua.

El efecto de los anfibios puede ser modulado por interacciones con depredadores o competidores que alteran la densidad o el comportamiento de los renacuajos. Por lo tanto, también investigamos los efectos en larvas de anfibios y en ecosistemas acuáticos de depredadores nativos e invasores derivados de su depredación, y por tanto de su consumo, y los que no son derivados de su consumo.

La alta densidad de anfibios disminuyó la biomasa de macrófitos y la diversidad de zooplancton y aumentó la turbidez del agua y el contenido de nutrientes. Estos efectos fueron atribuidos en su mayor parte a los renacuajos de la especie herbívora más grande, el sapo de espuelas (*Pelobates cultripes*). Sorprendentemente, en la ausencia de sapos de espuelas los anfibios tuvieron un efecto positivo en la biomasa de plantas.

El cangrejo invasivo (*Procambarus clarkii*) alteró la estructura de la comunidad de forma similar que la alta densidad de anfibios y causó una mayor mortalidad de larvas de anfibios que los depredadores nativos.

El gran impacto de herbivoría de los sapos de espuelas y del cangrejo invasivo fue patente en el siguiente ciclo hidrológico.

ABSTRACT

Connections between consumers and resources in food webs are complex and affect the structure and functioning of ecosystems. We assessed the influence of amphibians as consumers on the structure and functioning of temporary ponds, determining their impact on macrophyte abundance, zooplankton diversity and water chemistry.

The effect of amphibians may be modulated by interactions with predators or competitors that alter tadpole density or behaviour. Therefore we also investigated the consumptive and non-consumptive effects of native and invasive predators on amphibian larvae and pond ecosystems.

High amphibian density decreased macrophyte biomass and zooplankton diversity, and increased water turbidity and nutrient content. These effects were largely attributable to tadpoles of the largest herbivorous species, spadefoot toads (*Pelobates cultripes*). In the absence of spadefoot toads, amphibians unexpectedly affected plant biomass positively.

Invasive crayfish (*Procambarus clarkii*) altered community structure in similar ways to high densities of amphibians, and caused greater mortality of amphibian larvae than did native predators.

High herbivorous impact of spadefoot toads and invasive crayfish carried over to the following hydrological cycle.

INTRODUCTION

Extensive research has been conducted on ecological interactions within amphibian guilds, but few studies to date have explored the role of amphibian larvae in structuring aquatic communities. Fewer still have studied how larval density and trait-mediated indirect effects can modify the impact of amphibians on aquatic systems (but see Schiesari et al. 2009, Whiles et al. 2010, Costa and Vonesh 2013a). This is despite the fact that amphibians seem to have the potential to determine the structure and dynamics of aquatic communities (Buck et al. 2012), and to have profound and long-lasting effects on primary production, nutrient cycling, leaf litter decomposition, dynamics of invertebrate populations and energy flow between aquatic and terrestrial environments (Wilbur 1997, Whiles et al. 2006, Regester et al. 2008, Costa and Vonesh 2013a).

Amphibian larvae are common prey for a suite of vertebrate and invertebrate predators, and predation can also alter the outcome of the ecological interactions of their prey (Morin 1986). However, amphibians respond to predators by activating adaptive plastic changes in their morphology, behavior and/or timing of ontogenetic switch points (Anholt and Werner 1995, Relyea 2001, Van Buskirk 2001). Such plastic responses increase amphibian survival (Lima 1998, Van Buskirk and McCollum 2000) but often result in reduced activity and lower growth rates (Skelly 1997, Miner et al. 2005), suggesting altered consumption rates on trophic resources such as periphyton, zooplankton and macrophytes (e.g. Costa and Vonesh 2013a). Different types of predators, however, may have different consumptive and non-consumptive effects, and native predators are more likely to exert non-consumptive effects than invasive predators. Activation of appropriate plastic defenses critically depends on the accurate detection of predator cues, and local prey may fail to recognize invasive predator cues and hence fail to activate their defenses against them (Polo-Cavia et al. 2010, Gomez-Mestre and Díaz-Paniagua 2011). This lack of innate recognition of invasive predators relates to a lack of joint evolutionary history between local prey and novel predators, and it may confer a high disruptive potential on invasive predators.

We tested experimentally the effects of amphibian density and guild composition on the structure and dynamics of Mediterranean temporary ponds. Using an array of mesocosms we assessed the effect of amphibians on macrophyte diversity and biomass, phytoplankton abundance, zooplankton abundance and diversity, and water chemistry. Macrophytes constitute an essential element in aquatic food webs, because they are a source of energy for many herbivores, including tadpoles, but also they provide shelter for zooplankton, a substrate for periphyton development, and decrease water turbidity by reducing sediment suspension (van Donk and van de Bund 2002). Zooplankton abundance and composition is also a determining factor for primary production and is strongly dependent upon water turbidity and the presence of organisms at higher trophic levels (Carpenter et al. 1987, Des Roches et al. 2013). We expected amphibians to change water physicochemistry, the composition of zooplankton, and to decrease macrophyte biomass through direct herbivory. We studied the effect on community structure of the local amphibian guild as a whole, but also tested the effect of western spadefoot toad (*Pelobates cultripes*) tadpoles specifically, because these are much larger and have a longer larval period than the other amphibians we investigated (Díaz-Paniagua et al. 2005). Consequently, we expected a disproportionately greater effect on community structure of spadefoot toad tadpoles than the rest of the anuran guild. Furthermore, we studied the role of native and invasive predators in modifying the effect of the anuran guild by comparing both consumptive and non-consumptive effects of dytiscid beetle larvae (*Dytiscus circumflexus*: native predators) and red swamp crayfish (*Procambarus clarkii*: invasive predators). We experimentally distinguished non-consumptive predator effects (derived from caged predators altering tadpole behavior and/or morphology) from the combination of non-consumptive and consumptive effects (from free predators reducing tadpole density), which could alter tadpole trophic ecology (Caut et al. 2013) and hence their role in the aquatic food web. We expected uncaged invasive and native predators to reduce amphibian survival, hence allowing survivors to grow bigger. Direct tadpole consumption would reduce the net effect of amphibian larvae on community structure. We expected crayfish to have a bigger effect on community structure than dytiscid beetle larvae, because they are larger predators, local tadpoles do not seem to recognize its chemical cues, and they are omnivores with a potentially high direct impact on macrophyte biomass. We expected caged native predators fed on tadpoles to provide predator cues and tadpole alarm cues in the experimental ponds, and thus to exert non-consumptive effects on tadpoles via behavior modification. However, given the lack of innate recognition of alien crayfish cues, we did not expect such non-consumptive effects from caged red swamp crayfish.

METHODS

Study system

The study was carried out in Doñana National Park (37º00′N, 6º38′W), located in the southwest of Spain on the right bank of the Guadalquivir River mouth. This area contains more than 1100 temporary ponds within a 6794 ha area (Díaz-Paniagua et al.

2010), which are usually flooded in autumn or winter (Díaz-Paniagua et al. 2010). Most amphibian species in the area use temporary ponds as breeding habitats (Díaz-Paniagua et al. 2005). A total of 11 amphibian species are found in the park. In our study we included the six species most commonly found in temporary ponds (Diaz-Paniagua 1990) to have a representative characterization of the amphibian larval guild of the area (**Image 1**). We included five anuran species: the western spadefoot toad, the Mediterranean treefrog (*Hyla meridionalis*), the Iberian green frog (*Pelophylax perezi*), the Iberian painted frog (*Discoglossus galganoi*) and the natterjack toad (*Bufo calamita*). We also included a urodele, the pygmy marbled newt (*Triturus pygmaeus*), which is very abundant in Doñana and whose larval diet is largely comprised of planktonic crustaceans (93%; Díaz-Paniagua et al. 2005).

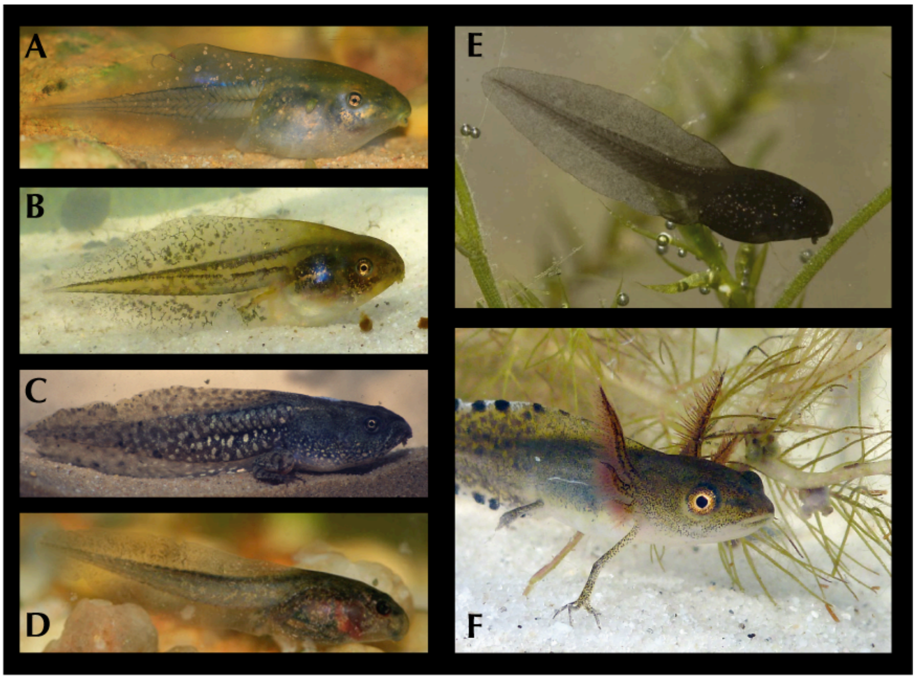

Image 1. Amphibian larvae included in the experiment.
(A) *P. cultripes*,
(B) *H. Meridionalis*,
(C) *P. perezi*,
(D) *D. galganoi*,
(E) *B. calamita* and
(F) *T. pygmaeus*.

As native predators we used dytiscid beetle larvae (*Dytiscus circumflexus*), which are found in the Park. We used red swamp crayfish as an invasive predator and a potential competitor for amphibians. Red swamp crayfish were introduced in the neighbourhood of Doñana National Park in 1974 (Habsburgo-Lorena 1978) and have since expanded into many aquatic habitats throughout the park. The crayfish are voracious predators of amphibian eggs and larvae (Cruz and Rebelo 2005, Portheault et al. 2007) and also exert strong effects as primary consumers of macrophytes and as filter-feeders (Gutiérrez-Yurrita et al. 1998, Cronin et al. 2002). Thus, red swamp crayfish are not just tadpole predators (Cruz and Rebelo 2005, Ficetola et al. 2011b), they are also competitors that use many of the same resources that tadpoles consume (mostly macrophytes and algae; Caut et al. 2013).

Experimental array

We established an experimental array of mesocosms at Doñana Biological Reserve (RBD) consisting of 8 treatments: (1) No amphibian larvae (*No Amph*): absence of amphibians or their predators; (2) Low amphibian density (*Low*): presence of low density of larvae of six amphibian species; (3) High density of amphibian larvae (*High*): a three-fold increase with respect to initial density in the Low treatment; (4) Absence of *P. cultripes* (*No Pc*): as in Low treatment but excluding *Pelobates cultripes*, the largest species with the longest developmental period; (5) Caged native predator (*NatC*): Low density of amphibian larvae exposed to one caged *Dytiscus* larva; (6) Free native predator (*NatF*): Low density of amphibian larvae exposed to a single free ranging *Dytiscus* larva; (7) Caged invasive predator (*InvC*): Low density of amphibian larvae exposed to one caged red swamp crayfish; and (8) Free invasive predator (*InvF*): Low density of amphibian larvae exposed to a single free ranging red swamp crayfish. We considered low density to be 3 individuals per tank for *P. cultripes*, *T. pygmaeus*, *D. galganoi* and *P. perezi*, and 10 individuals per tank for *H. meridionalis*. Tadpole densities fell well within the range commonly observed during field surveys in the Park (Diaz-Paniagua 1990). Each treatment was replicated 12 times for a total of 96 tanks distributed in 12 randomized complete blocks (**Image 2**).

Image 2. Mesocosm array at Doñana Biological Reserve where we performed the experiment.

The experiment lasted for 10 weeks between 24 March and 2 June 2011. Mesocosms consisted of 500 L rounded plastic tanks, 100 cm high and 120 cm in the upper diameter. To replicate the conditions of the natural ponds in Doñana, we added 105.6 ± 0.86 (mean ± SE) kg of sand to each tank to provide a low-nutrient substrate for macrophytes to root in, resulting in a 20 cm deep layer. We added an extra 13.7 ± 0.1 kg of sand to build a sandy ramp in each tank to provide tadpoles a shallower and warmer area to bask within the tank. We then added sediment (5.63 ± 0.18 kg) from the basin of a dry temporary pond where several amphibian species usually breed, and an extra 1.125 kg of an evenly combined mixture of sediment from nine other temporary ponds in the area. Mesocosms were filled with 250 L of well water and we added a 5 L inoculum of pond water collected from two temporary ponds that contained zooplankton and phytoplankton. Furthermore, we planted several species of aquatic macrophytes to provide spatial complexity and as a food base for the aquatic community. Thus, we added in each tank 25-30 stems of *Myriophyllum alterniflorum*, 8-10 stems of *Ranunculus peltatus*, 40-50 stems of *Callitriche obtusangula* and four stems of *Mentha pulegium*. These species provide a good representation of the macrophytes present in the temporary ponds of Doñana (Díaz-Paniagua et al. 2010).

The tanks were covered with fiberglass window screens that were opened for several hours a day to allow colonization by flying insects. Each tank also contained a floating, lidded 1 L plastic bucket with holes drilled in the bottom, where predators were held in tanks assigned to caged predator treatments.

Introduction and removal of amphibians and predators

We collected the six species of amphibian larvae by dip-netting in a number of different temporary ponds within the RBD. Larvae of each amphibian species were haphazardly assigned to each experimental tank from a species pool of field-collected larvae. We added larvae to the tanks following the natural breeding phenology of each species (Díaz-Paniagua 1988): On 24 March we introduced *P. cultripes* and *B. calamita*, on 29 and 30 March we introduced *H. meridionalis* and *T. pygmaeus*, on 8 and 9 April we introduced *D. galganoi* and finally on 17 May we introduced *P. perezi*. All anuran larvae were between 25 and 30 Gosner stages (Gosner 1960), and newt larvae were between 40 and 44 stages (according to the development stages of *Pleurodeles waltl* in Shi and Boucaut 1995). We also collected predators (crayfish and dytiscid larvae) from ponds within the RBD, and haphazardly allocated them to either caged or free predator treatments. Because predator and alarm cues may degrade after ~ 48h (Peacor 2006) we fed each caged predator two amphibian larvae every three days throughout the experiment, randomly drawing from one of the six amphibian species used for this study, to ensure a constant source of the appropriate cues. Tadpoles fed to caged predators were kept in additional outdoor tanks prepared in the same way as the experimental tanks.

We checked the tanks twice daily for metamorphs (i.e. Gosner stage 42) from the time we observed the first metamorph until the end of the experiment. We digitally photographed every group of amphibian larvae and every free or caged predator entering each tank at the beginning and at the end of the experiment. We also measured larval body length and total length, snout-to-vent length (SVL) of metamorphs, and predator body length with ImageJ 1.46r (NIH, USA). At the end of the experiment we also recorded body mass (BM) of all metamorphs and remaining larvae. We also recorded the date of forelimb emergence (i.e. Gosner stage 42; Gosner, 1960). Nonetheless, our design precluded comparisons of larval period across species, because different species were incorporated into the experiment at different times, according to their breeding phenology.

Physicochemical parameters

We recorded pH, dissolved oxygen and electrical conductivity with a YSI 6600 V2-4 multiprobe four times during the course of the experiment. We also recorded turbidity at the end of the experiment with a turbidimeter. We took a 330 mL water sample from every tank at the end of the experiment on 30 May to determine the level of chlorophyll-*a* (Chl-a, μg/L, tricromatic method; Holmhansen and Riemann 1978) and the concentration of four dissolved nutrients in the water (ammonium, phosphate, nitrite and nitrate). Nutrient concentrations were determined with a multi-channel Seal Analytical AutoAnalyzer, Norderstedt, Germany. Water samples were filtered through Whatman GF/F 47-mm glass microfiber filters 0.7 μm in pore size, using a low-pressure vacuum pump. Filtered water was bottled and frozen until analyzed. Filters were kept for chlorophyll-*a* analysis.

Sample collection

At the end of the experiment (30-31 May), we collected zooplankton samples by filtering five liters of water from the water column from each tank through a 100 μm plankton net. We preserved the samples in a 70% ethanol in order to quantify the zooplankton species abundance. We identified the species in our samples under an inverted microscope using published taxonomic accounts of aquatic invertebrates (Galindo et al. 1994).

At the end of the experiment we harvested all plants remaining in the tanks. We removed the excess water with a manual centrifuge and recorded the fresh weight of every plant species per tank: *Myriophyllum alterniflorum, Ranunculus peltatus, Callitriche obtusangula* and two species of charophytes. To test for carry-over effects of experimental treatments on plant biomass onto the next hydrological cycle, we let the tanks dry naturally over the summer and then refilled them in the autumn with well water to mimic pond re-flooding. Plants grew naturally from seed in each tank after reflooding and were allowed to grow for seven weeks. We then harvested all plants in each tank, separated them by species, and weighed them.

Data analysis

We excluded two tanks from all data analyses (one replicate belonging to the native caged predator treatment and one to low density treatment) due to extremely low outlier values in plant biomass as high numbers of *Triops mauritanicus* emerged from the sediment in these tanks and strongly affected water quality and depleted macrophyte biomass from the beginning of the experiment. No *B. calamita* survived in the experiment and only 9 metamorphs of *D. galganoi* were observed emerging throughout the experiment, mostly in the treatment without *P. cultripes*. Experimental tanks may not constitute ideal habitats for these two species that usually breed in shallow areas of short-lasting ponds, despite the basking ramp we had explicitly added. Thus, differences among treatments in survival and size at metamorphosis were not estimated for these species.

We used generalized linear mixed models (GLMM) to test for the effect of experimental treatments on our response variables using the GLIMMIX procedure (Schabenberger 2007) in SAS. 9.2 (SAS-Institute, Cary, NC, USA). For all dependent variables, we calculated the mean within each experimental tank and used tank means for statistical analyses. In the case of survival, we modeled the number of survivors per tank out of the initial number of individuals. We tested the effect of experimental block as a random factor, and removed it from the model when it was not significant.

Response variables were transformed to meet parametric assumptions as follows: total plant biomass and *Myriophyllum* biomass were squared-root transformed whereas phosphate and chlorophyll concentrations were log transformed; we used the inverse for all other nutrients and conductivity, and we ranked the variable turbidity. Morphological measurements were normally distributed and homoscedastic for all species (except for *P. perezi* body mass, which was then square-root transformed). We found no among-treatment differences in initial size of any of the species studied, but initial size had a significant effect on survival of *H. meridionalis* and *P. perezi*. We therefore included initial size as a covariate in analyses of survival.

Normally distributed variables were modeled with a gaussian error distribution and an identity link function. The biomass of the three other plants (*Ranunculus*, *Callitriche* and *Chara*) was analyzed using integers and fitted a model using a negative binomial distribution and a log link function. We modeled amphibian survival assuming an underlying binomial error distribution and a logit link function, including initial size as a covariate to control for any possible effect of species differences in size. Since our experimental design was not fully factorial, we specified multiple contrasts through planned comparisons to test specific hypotheses. We therefore tested for effects of presence/absence of amphibians and the effect of increasing their density with the following contrasts: *No Amph-Low, No Amph-High* and *Low-High*; we tested the effect of *P. cultripes* comparing *Low-No Pc*; and we tested the effect of predators with that of the amphibian presence at low density (*Low*-Predators) and with different combinations of native and invasive caged and free predators (Table 1). Given that several of these multiple comparisons (15 comparisons total) were not orthogonal, we corrected the observed p-values to minimize the false discovery rate (FDR; Benjamini and Hochberg 1995, Garcia 2003).

Table 1. Contrasts performed to test for effects of amphibian larvae and their predators on temporary ponds. Effects of amphibians are tested by its presence/absence -Low/No Amph-, increased density -High- or exclusion of *Pelobates cultripes* -No Pc-. We also used the contrasts shown on the right to test the effects of predators on the amphibian guild and ultimately on the trophic web (NatC=Caged Native predator; NatF= Free Native predator; InvC=Caged Invasive predator; InvF=Free Invasive predator).

CONTRASTS	
Effects of amphibians on temporary ponds	**Effects of predators on amphibians and on temporary ponds**
No Amph - Low	Low - NatC
	Low - Nat F
No Amph - High	Low - InvC
	Low - InvF
Low - High	NatC -NatF
	InvC - InvF
No Amph -No Pc	NatC -InvC
	NatC- InvF
Low - No Pc	NatF - InvC
	NatF - InvF

We calculated the abundance of each zooplankton species per liter of water and also estimated the diversity of aquatic invertebrates in each sample. We estimated the Shannon-Wiener diversity index using the package VEGAN within R (R Core Development Team, Vienna, Austria).

We also conducted a multivariate analysis with PRIMER- E (Clarke and Gorley 2006)(Clarke and Gorley 2006) including the physicochemical variables in addition to plant biomass at the end of the experiment. We failed to collect physico-chemical data from eight tanks and these had to be discarded in the multivariate analysis since the software requires full matrices. The variables were log and squared-root transformed and normalized to obtain a dissimilarity matrix using Euclidean distance for environmental variables prior to performing a non-metric multidimensional scaling (NMDS) ordination (Clarke and Gorley 2006). An NMDS ordination was also performed with the four main groups of zooplankton (cladocerans, copepods, rotifers and ostracods) after having log-transformed the data, standardized the samples dividing by total count and obtained a resemblance matrix using the Bray Curtis similarity index.

RESULTS

Effects of amphibians in temporary ponds

Amphibian larvae had a considerable effect on water physicochemistry (Fig. 1; see Appendix A and B). Turbidity increased 3.66 fold in the presence of amphibians at low density (*No Amph-Low*; $F_{1,86}$ = 10.85; P = 0.0024) and 6.41 fold at high amphibian density (*No Amph-High*; $F_{1,86}$ = 20.58; $P < 0.001$). When *P. cultripes* was absent, however, turbidity was not different from tanks without amphibians (Fig. 1A). Amphibian larvae decreased oxygen concentration by 15.9 % at low density (*No Amph-Low*; $F_{1,75}$ = 10.91; P = 0.001) and 26.2 % at high density (*No Amph-High*; $F_{1,75}$ = 27.38; $P < 0.001$; Fig. 1C). Amphibians increased ammonium concentration by over a two-fold difference at both densities, although it was not significant (*No Amph-Low*; $F_{1,78}$= 2.89; P = 0.09; Fig. 1E). In presence of amphibians, we also observed increased water conductivity in low ($F_{1,86}$ = 5.95; P =0.031) and high larval density ($F_{1,86}$ = 19.42; $P <$ 0.001; Fig. 1B). We observed a marked effect of *P. cultripes* on water chemistry (*Low-No Pc*; Fig. 1): tanks with amphibian larvae but lacking *P. cultripes* had on average 18% more dissolved oxygen ($F_{1,75}$ = 13.07; $P < 0.001$), 62% less ammonium ($F_{1,78}$ = 6.24; P = 0.0029), and 45% less phosphate ($F_{1,78}$ = 8.07; P = 0.017) than those with *P. cultripes*.

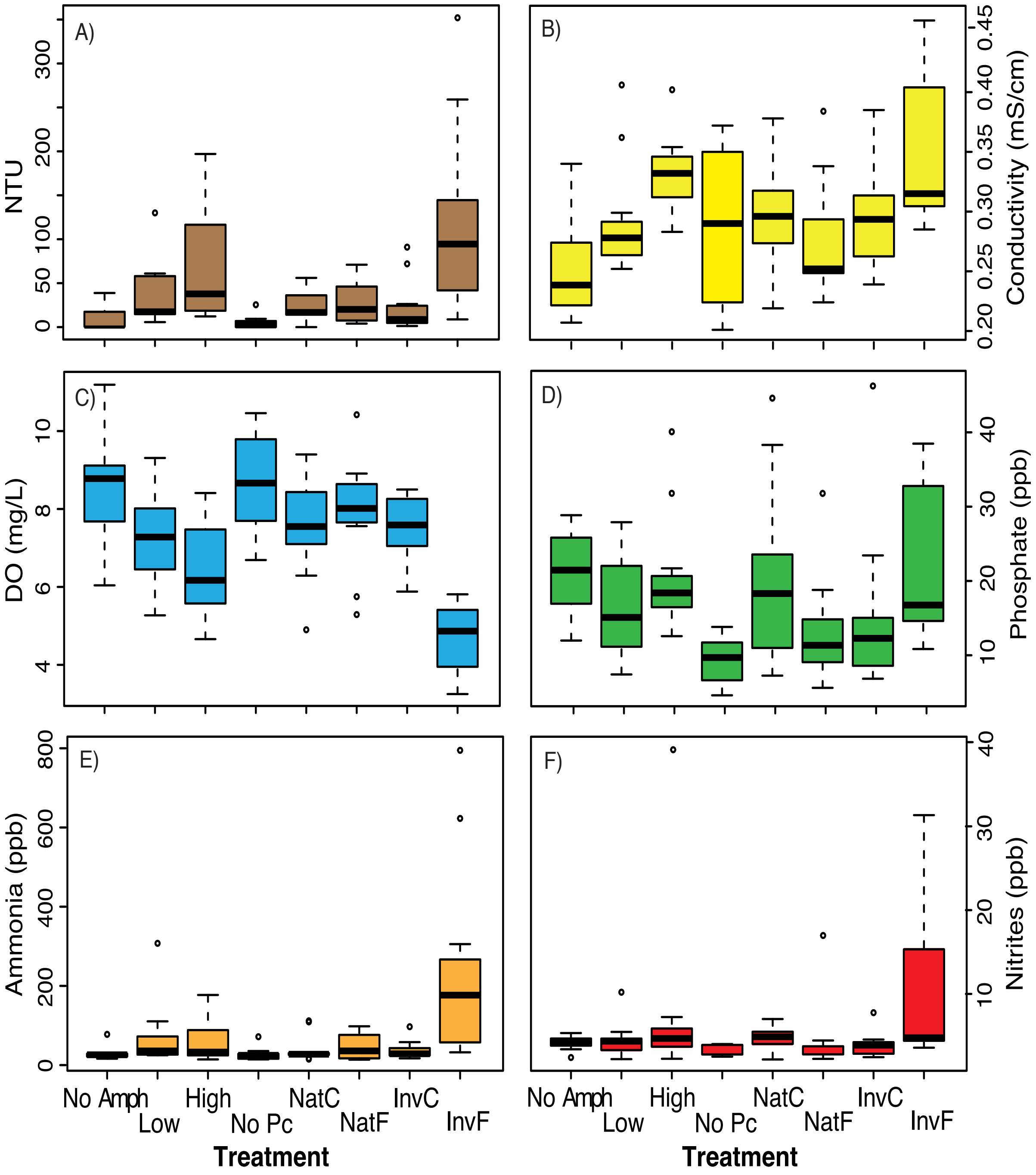

Fig. 1. Physicochemical parameters in the experimental tanks at the end of the experiment: (A) turbidity, (B) conductivity, (C) dissolved oxygen, and three dissolved nutrients (D-F) in the water. The boxes represent the first and third quartile and the band near the middle of the box represents the median. Possible outliers are represented with open circles.

We observed a significant increase in chlorophyll-*a* concentration, an indirect measure of phytoplankton biomass, when the amphibian guild was at high density compared to tanks lacking amphibian larvae ($F_{1,78}$ = 4.84; P = 0.0308), whereas no significant increase was detected at low amphibian density or when *P. cultripes* was absent (Fig. 2A; Appendix A).

Regarding the aquatic vegetation, by the end of the experiment there was about 50% less plant biomass in tanks with a low density of amphibian larvae than in tanks without amphibians ($F_{1,86}$ = 14.95; P < 0.001) and about 85% less plant biomass at high amphibian density ($F_{1,86}$ = 64.84; P < 0.001; Fig. 2B). Two of the macrophyte species, *C. obtusangula* and *R. peltatus*, were completely consumed in treatments containing amphibian larvae, whereas *M. alterniflorum* was never depleted. Charophyte biomass did not vary across treatments. When *P. cultripes* was excluded but all other amphibians were present, macrophyte biomass actually increased by an average of 45% compared to tanks without amphibians ($F_{1,86}$ = 4.42; P = 0.038; Fig. 2B).

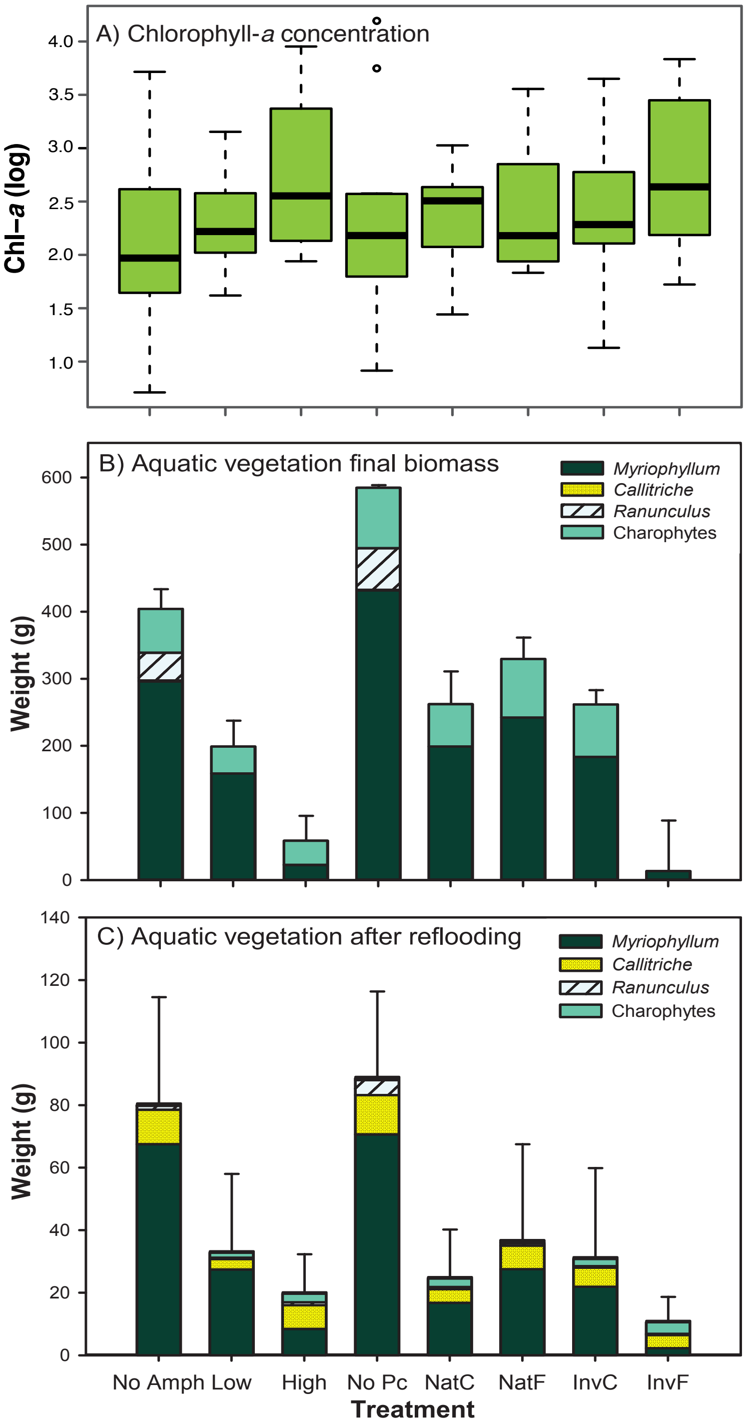

Fig. 2. (A) Variation in chlorophyll-*a*, an indirect measure of phytoplankton. (B) Biomass of the three most abundant macrophytes plus charophytes remaining in each treatment at the end of the experiment and (C) after re-flooding the tanks. Bars represent the contribution of each species to the final biomass and error bars represent the standard error of the total plant biomass.

The presence of amphibian larvae reduced zooplankton diversity by an average of 12% when at low density, although the effect was only significant when amphibian density was high, reducing zooplankton diversity by 25% on average ($F_{1,85}$ = 9.63; P = 0.003; Appendix B and C). Thus, the zooplankton changed in composition at high amphibian density, increasing its copepod abundance (mainly due to a greater number of nauplii and copepod larvae; *No Amph-High*; $F_{1,85}$= 19.98; $P < 0.001$), whereas cladoceran abundance was reduced (*No Amph-High*; $F_{1,85}$ = 15.03; $P < 0.001$) and ostracods were almost absent (*No Amph-High*; $F_{1,85}$ = 11.44; P = 0.004; Fig. 3A). The presence of the amphibian guild also increased the abundance of copepods (*No Amph-Low*; $F_{1,85}$ = 4.71; P = 0.033). At low amphibian density we also observed a trend towards decreased number of cladocerans, although the effect was non-significant (*No Amph-Low*; $F_{1,85}$ = 2.58; P = 0.112). In the absence of *P. cultripes*, however, zooplankton composition was similar between treatments with and without amphibians. Rotifers were not significantly affected by any of the treatments, although there was a trend of increased density in the absence of *P. cultripes* (*No Amph-No Pc*; $F_{1,85}$ = 2.94; p = 0.09; Fig. 3A).

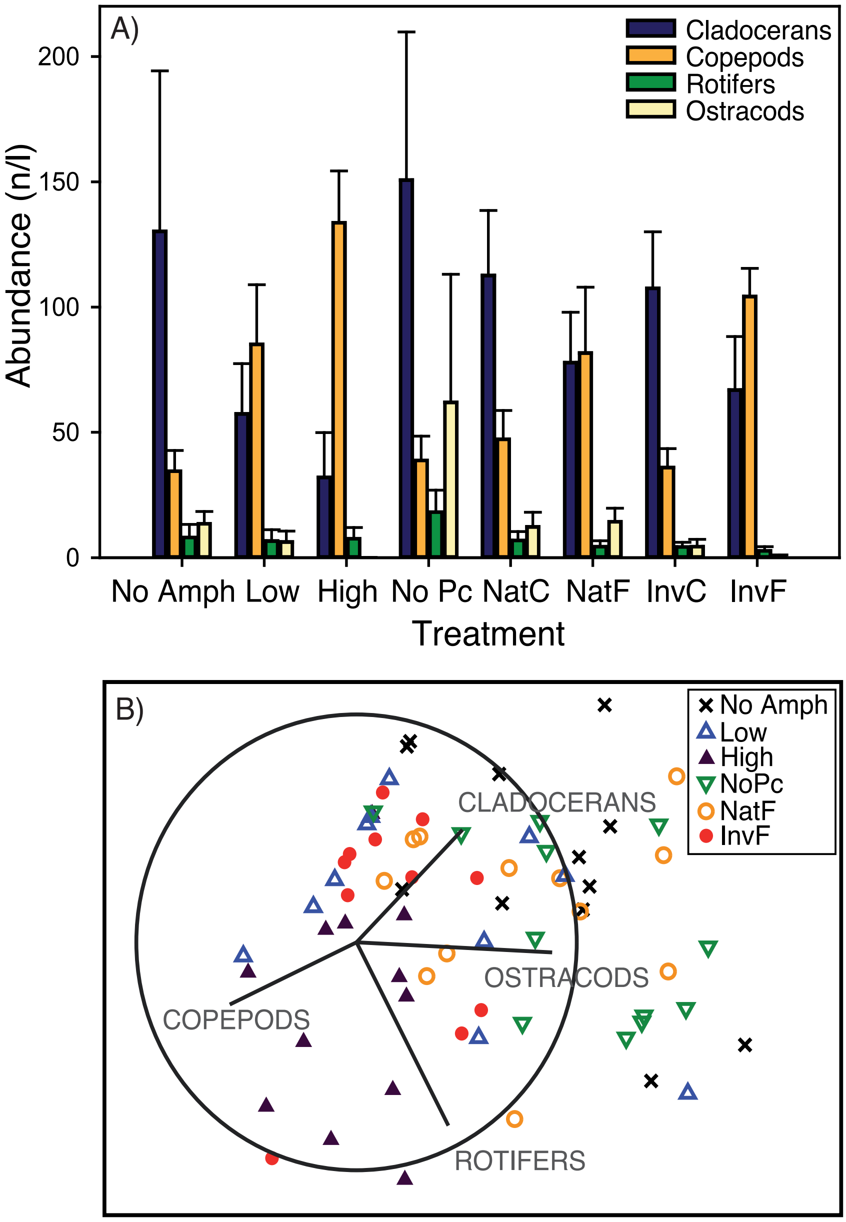

Fig. 3. (A) Abundance of cladocerans, copepods, rotifers and ostracods in each treatment. (B) Results from non-metric multidimensional scaling (NMDS) ordination including the 4 major groups of zooplankton after standardizing the data.

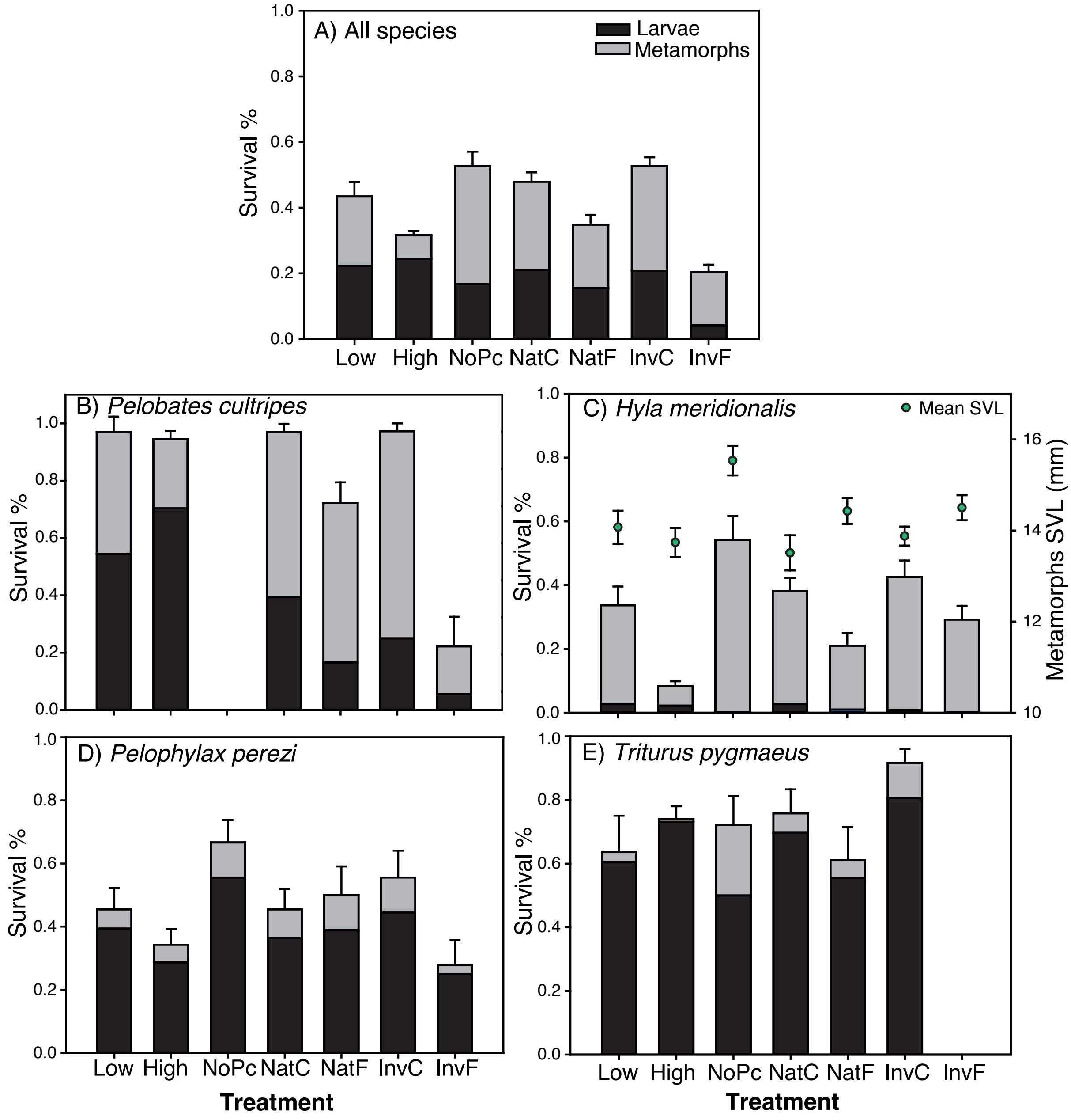

Fig. 4. (A) Overall survival for all 5 species included in this study. (B-E) Metamorphic and larval survival across treatments for the various amphibian species. Bars indicate overall survival across treatments (+ SE) considering both life stages, each column partitioned to indicate the proportion of individuals remaining as larvae (in grey) or metamorphs (in black). Metamorphic snout-vent length is only provided for *H. meridionalis*, as it was the only species for which most individuals had metamorphosed by the end of the experiment (mean ± SE).

Because the different amphibian species were added to the tanks sequentially, to mimic reproductive phenology, among species comparisons of survival, weight at, or time to metamorphosis could not be made. Overall, amphibian survival at low density was 43.6% and 31.6% at high larval density (*Low-High*; $F_{1,75}$ = 10.87; *P*=0.002), whereas it increased to 53% when *P. cultripes* was excluded (*Low-No Pc*; $F_{1,75}$ = 4.01; *P* = 0.049; Fig. 4A). At low density, survival of *H. meridionalis* increased when *P.cultripes* was excluded (*Low-No Pc*; $F_{1,74} = 8.17$; $P = 0.009$; Fig. 4C), but high density reduced its survival (*Low-High*; $F_{1,74} = 41.51$; $P < 0.001$). Although not statistically significant, the same trend was observed for *P. perezi* (Fig. 4D). *Pelobates cultripes* and *T. pygmaeus* had high survival in all treatments without predators (Figs. 4B & 4E).

The species varied in the extent to which they had completed larval development by the end of the experiment. Thus, most *H. meridionalis* individuals had completed metamorphosis (90% of survivors), whereas a high proportion of *P. cultripes* remained in the larval phase (55.6% of survivors). For *T. pygmaeus* and *P. perezi*, the proportion of metamorphs was still very low (3% and 5%, respectively; Fig. 4). *Pelobates cultripes* had the lowest proportion of metamorphs at high density, and those that metamorphosed emerged at a smaller body size (18.3% reduction in SVL; $F_{1,48} = 27.6$; $P < 0.001$; Fig. 4B) and lower body mass (52% reduction; $F_{1,49} = 43.86$; $P < 0.001$) than at low density.

In addition to the negative effects of *P. cultripes* on survival of other amphibian species, we also found that 44% of *D. galganoi* larvae that reached metamorphosis came from tanks where *P. cultripes* was absent. Coexistence with *P. cultripes* reduced SVL of the emerging *H. meridionalis* metamorphs by 8% (SVL in *Low-No Pc*; $F_{1,68} = 10.83$; $P = 0.005$; Fig. 4C), but size at metamorphosis was unaffected in all other species.

Effects of native predators on amphibian larvae and pond communities

The presence of native predators (dytiscid larvae) had no effects on water chemistry, phytoplankton, aquatic vegetation or zooplankton diversity and composition (Figs. 1, 2 & 3; Appendix A and C). Presence of free dytiscid larvae reduced overall amphibian survival from 43.6% to 34.85% (*NatC-NatF*; $F_{1,75} = 8.87$; $P = 0.005$; Fig. 4A). However, *P. cultripes* metamorphs were 6.5% longer ($F_{1,48} = 4.27$; $P = 0.018$) and 18.5% heavier ($F_{1,49} = 7.89$, $P = 0.018$) in the presence of free native dytiscid larvae than when dytiscid larvae were caged. There was also a marginally significant increase in *H. meridionalis* size at metamorphosis ($F_{1,68} = 3.92$; $P = 0.052$; Fig. 4C).

Effects of the red swamp crayfish on amphibian larvae and on pond communities

The red swamp crayfish affected most parameters (see *Low- InvF* comparisons in Figs. 1, 2 & 3; Appendix A and C). When crayfish roamed free in the tanks, turbidity increased 67% on average ($F_{1,86} = 6.86$; $P = 0.01$) while oxygen concentration was reduced by 35.5% ($F_{1,86} = 35.89$; $P < 0.001$). Phosphate ($F_{1,78} = 5.24$; $P = 0.0297$) , ammonium ($F_{1,78}$

= 8.8; P =0.004) and nitrite ($F_{1,78}$ = 4.52; P = 0.036) concentrations were higher in tanks with free crayfish compared to tanks with caged crayfish (Fig. 1; Appendix A).

We found a non-significant trend towards increased chlorophyll-*a* in the presence of free crayfish (Fig. 2A). Tanks containing free crayfish had practically no plants at the end of the experiment, as plant biomass was reduced by 95% compared to the absence of predator treatment (*Low-InvF*; $F_{1,86}$ = 54.94; P < 0.001; Fig. 2B).

Zooplankton diversity did not vary among treatments with and without predators (see data in Appendix A). However, copepods were more abundant when crayfish were free than when they were caged ($F_{1,85}$ = 11.08; P = 0.033). Ostracods were not abundant in either treatment with crayfish, but their abundance was lower in tanks with freely roaming crayfish (*InvC-InvF*; $F_{1,85}$ = 3.96; P = 0.049; Fig. 3A).

Free crayfish reduced amphibian survival from 43.6% to 20.4% (*Low-InvF*; $F_{1,75}$ = 29.74; P < 0.001; Fig. 4A). *Pelobates cultripes* experienced the highest overall survival rate and only suffered high mortality when predators were free, especially crayfish (*Low-InvF*; $F_{1,63}$ = 23.66; P < 0.001; Fig. 4B). Crayfish reduced survival in *H. meridionalis* by 32% (*InvC-InvF*; $F_{1,74}$ = 3.89; P = 0.052; Fig. 4C) and by 50% in *P. perezi* (*InvC-InvF*; $F_{1,74}$ = 5.8; P = 0.018; Fig. 4D), and caused complete mortality of *T. pygmaeus* (Fig. 4E).

Pelobates cultripes tadpoles remaining at the end of the experiment in tanks with free roaming crayfish had smaller SVL (16% reduction; *Low-InvF*; $F_{1,38}$ = 8.52; P = 0.015) and mass (34% reduction; *Low-InvF*; $F_{1,38}$ = 5.89; P = 0.025).

Persisting effects over the next hydrological cycle

The effect of amphibian larvae on plant biomass persisted in the next hydrological cycle, after tanks dried up and were re-flooded in the autumn, without adding any more amphibians. Tanks that had contained amphibians at low density during the experiment, had about 60% less plant biomass seven weeks after re-flooding than tanks that had not contained amphibians (*No Amph-Low*; $F_{1,75}$ = 25.34; P < 0.001). Amphibian density also had a carry-over effect, as tanks that had contained high density of amphibians had about 75% less plant biomass after re-flooding than tanks that had contained no amphibians (*No Amph –High*; $F_{1,75}$ = 47.27; P < 0.001; Fig. 2C and Appendix B). The impact of free roaming crayfish on plant biomass also persisted after re-flooding, as plant biomass was 67.7 % lower in tanks that had contained free crayfish than tanks that had not (*Low-InvFree*; $F_{1,75}$ = 11.5; P = 0.001; Appendix A and C). The carry-over effect of amphibians and crayfish was evident for *M. alterniflorum* (Fig. 2C), whereas biomasses of *R. peltatus* and *C. obtusangula* only varied between low amphibian density and absence of *P. cultripes*, increasing in the absence of *P. cultripes*.

Combined effects of amphibians and predators on community structure

NMDS on turbidity, nutrients, oxygen, chlorophyll-*a*, and plant biomass at the end of the experiment provided an overall view of the effects of the experimental treatments (Fig. 5). NMDS grouped tanks that did not contain amphibians with tanks that contained amphibians but not *P. cultripes* (Fig. 5). Tanks in these two treatments (No Amphibians and No *P. cultripes*) had a higher oxygen concentration, lower turbidity and chlorophyll-*a*, and greater plant biomass. At the other end of the gradient, tanks containing a high density of amphibians and tanks containing free roaming crayfish were also grouped, determined by their higher nutrient content (except for nitrates) and higher conductivity and turbidity. Tanks with caged predators or only amphibians in low density were located between these two extremes. Likewise, NMDS grouped tanks according to zooplankton composition so that tanks with high amphibian density showed a higher abundance of copepods and rotifers whereas tanks with no amphibians or with amphibians but excluding *P. cultripes* had a predominance of cladocerans and abundant ostracods, similar to tanks with free native predators.

Tanks with free invasive crayfish, however, were in an intermediate position with similar abundances of copepods and cladocerans, as in the case of tanks with low amphibian density or with caged predators (Fig. 3B).

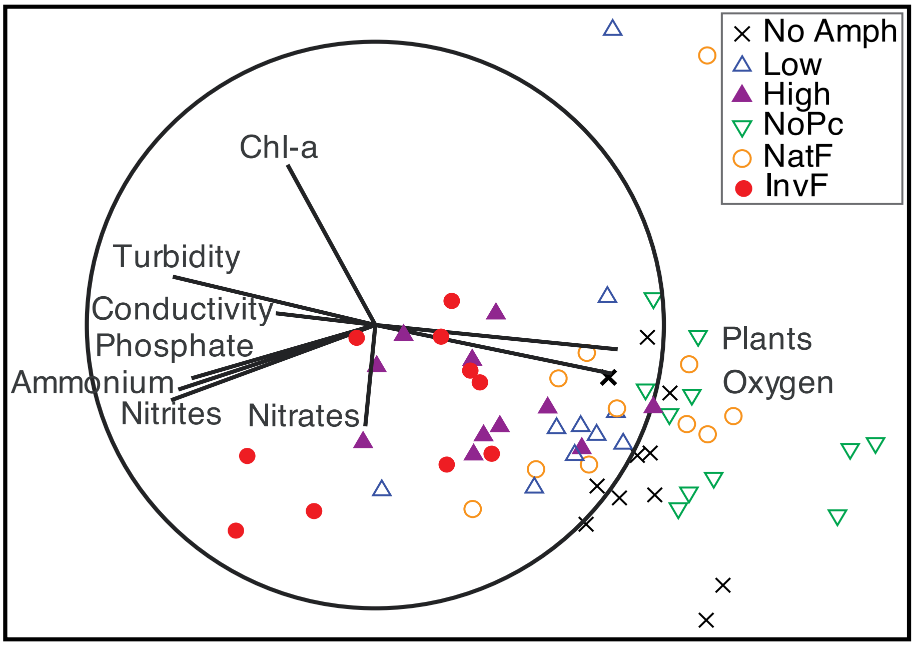

Fig. 5. Non-metric multidimensional scaling (NMDS) ordination involving key parameters of community structure.

DISCUSSION

Effects of amphibians on temporary ponds

Our results show that amphibian larvae can have an important impact as key primary consumers on the structure of freshwater communities, and that individual amphibian species may differ in the magnitude of their effects, and even exert opposite effects. In this study, amphibians affected pond water quality, algal abundance, plant biomass and zooplankton diversity. These effects were magnified at high amphibian density, which also caused almost complete depletion of aquatic vegetation, directly or indirectly induced algal proliferation (as indicated by increased chlorophyll-*a* content) and increased water turbidity and nutrient content, and decreased oxygen.

Nutrients and grazing interact to control periphyton productivity and growth of submersed macrophytes (Marks and Lowe 1989). Water turbidity affects nutrient dynamics, in particular of nitrate and ammonium, which can in turn produce algal blooms that shade aquatic macrophytes and reduce carbon exchange at leaf surfaces, constraining their growth or even causing their death (Sand-Jensen and Borum 1991). Amphibians also markedly reduce macrophyte biomass. Macrophytes constitute food resources for tadpoles and have essential roles in freshwater ecosystems because they increase water clarity and the complexity of the physical habitat (Scheffer et al. 1993, 2001), critical for amphibian survival. The sharp decrease in plant biomass, however, could not be attributable to all tadpole species, but was largely caused by those of spadefoot toads. The reduction in macrophyte biomass carried over to the next hydrological cycle, as tanks that had contained high amphibian density during the experiment had substantially fewer plants growing in them upon refilling in the autumn. Hence, macrophytes may have been consumed prior to flowering, fruits may have been damaged, seed production may have been reduced by plants under tadpole herbivory, or several of these factors may have combined to result in reduced plant biomass after re-flooding.

In the absence of *P. cultripes*, however, the rest of the amphibians had a positive effect on macrophyte abundance, directly or indirectly. To the best of our knowledge, this is a novel and unexpected effect of amphibians on community structure. It is known that grazers such as isopods, amphipods and gastropods, together with nutrient enrichment, have a strong positive effect on macrophyte production (Neckles et al. 1993). Kupferberg (1997) found that tadpole grazing removed epiphytes that shaded filamentous green algae and transformed them into excreted nutrients, hence facilitating microalgae growth and productivity, although this effect differed according to tadpole species. The reduced turbidity, increased oxygen concentration, and decreased ammonium and phosphate in the water in tanks without *P. cultripes* can also be indirect factors benefitting aquatic macrophytes, or conversely these factors could result from abundant healthy macrophytes.

Of all the species comprising the amphibian guild in Donana, *P. cultripes* clearly stands out in terms of its disproportionate contribution to the observed amphibian-driven alterations of the aquatic environment. Tadpoles of *P. cultripes* are much bigger than the other species in our system, breed early in the season, and have the longest larval period (Díaz-Paniagua et al. 2005), which also makes them unrivalled competitors in Mediterranean guilds (Richter-Boix et al. 2007). Large herbivorous tadpoles thus have the potential to alter the life history and possibly the population dynamics of aquatic plants. Large tadpoles can forage disproportionally more not only due to allometric differences in size-dependent filtering ability and consumption rates (Wassersug 1975), but also because the smaller tadpoles are displaced and have reduced access to valuable resources (Richter-Boix et al. 2004).

The presence of amphibian larvae also affected zooplankton composition. Urodele larvae can decrease zooplankton biomass and affect its density and composition (Holomuzki et al. 1994). In our study, the reduced zooplanktonic diversity observed at high amphibian density could have direct and indirect causes. Direct predation by newt larvae is likely to have been the primary factor in reducing zooplankton diversity. Additionally, anuran larvae, as primary consumers, could also have exerted resource competition over zooplanktonic species for algae (Mokany 2007), contributing to reduce its diversity, especially at high density. Moreover, the decrease in macrophyte biomass and consumption of preferred phytoplankton may also have favored the proliferation of other algae (Kupferberg 1997). In line with this idea, we found increased chlorophyll-*a* at high amphibian density, which, together with the increase in turbidity and detritus, may have influenced the shift in zooplankton composition from cladoceran to copepod dominance.

Effects of predators on amphibian larvae and on community structure

Free dytiscid larvae greatly reduced amphibian survival, mostly affecting *P. cultripes* and *H. meridionalis*. By reducing the number of individuals, predators release surviving larvae from competition resulting in their increased size (Morin 1983, 1986), as we detected in surviving *P. cultripes* and *H. meridionalis* metamorphic individuals. However, predator consumption did not reduce the impact of amphibian larvae on community structure, and non-consumptive effects were almost negligible in this study.

Invasive red swamp crayfish had a large impact on the environment, and directly caused changes in water quality, nutrient content and macrophyte biomass. Red swamp crayfish strongly affect various trophic levels in freshwater systems, via both consumptive and non-consumptive processes (e.g. macrophyte cutting or uprooting; Anastácio et al. 2005, Gherardi and Acquistapace 2007). In our study the presence of freely roaming crayfish in the tanks increased ammonium, phosphate and nitrite in accordance with previous studies (Angeler et al. 2001). This release of nutrients into the water may be produced through bioturbation (Angeler et al. 2001) or excretion (Evans-White and Lamberti 2005). Increased nutrient levels could be caused by feeding of caged predators (Costa and Vonesh 2013a) but that effect was not found in our study. On the other hand, Matsuzaki et al. (2008) found that crayfish reduced the concentration of dissolved ammonium probably due to its rapid uptake by phytoplankton, showing the complexity of possible trophic connections within freshwater systems. We also observed increased copepod abundance in the presence of freely roaming crayfish. Another study suggested that crayfish may have positive effects on zooplankton biomass via changes in phytoplankton abundance (Dorn and Wojdak 2004), but we found no evidence for crayfish effects on algal abundance.

The negative impact of invasive crayfish on amphibian survival was much stronger than the overall impact of native predators. Red swamp crayfish are much bigger than any other invertebrate in the area and native amphibians lack a joint evolutionary history with it. Hence, amphibian larvae from Doñana cannot recognize the cues from this invasive species and consequently fail to trigger inducible anti-predator defenses against them (Gomez-Mestre and Díaz-Paniagua 2011). This double role of invasive crayfish as competitors and generalist predators renders them very harmful in aquatic communities (Ficetola et al. 2012), with the potential to markedly reduce local amphibian densities (Cruz and Rebelo 2005, Cruz et al. 2006).

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Appendix A. Chemical parameters measured in tanks at the end of the experiment: Turbidity (NTU), Chlorophyll-*a* (µg L^{-1}), Shannon Diversity Index for the zooplankton (cladocerans, copepods, rotifers, and ostracods), total final plant biomass remaining in the tanks (g), total plant biomass after re-flooding (g), dissolved oxygen in the water (mg L^{-1}), conductivity (mS cm^{-1}) and four dissolved nutrients (ammonium, phosphate, nitrate and nitrite, ppb). Data represent mean ± se for every treatment (number of replicates per treatment, N=12, except for Low and NatC, N=11).

Treatment	No Amph	Low	High	No Pc	NatC	NatF	InvC	InvF
Turbidity (NTU)	10.49 ± 4.16	38.43 ± 11.1	67.21 ± 18.87	4.85 ± 2.09	24.79 ± 5.32	27.79 ± 6.97	21.84 ± 8.42	116.17 ± 29.23
Chlorophyll-*a* (µg L^{-1})	11.57 ± 3.23	11.30 ± 1.82	20.57 ± 4.33	16.55± 5.71	11.75 ± 1.40	13.64 ± 2.66	13.26 ± 2.79	20.22 ± 4.07
Zoo Shannon Div.	1.70 ± 0.1	1.51 ± 0.08	1.28 ± 0.06	1.56 ± 0.08	1.50 ± 0.15	1.47 ± 0.09	1.46 ± 0.12	1.57 ± 0.06
Total Plants (g)	404.34 ± 29.23	198.88 ± 37.06	58.69 ± 21.44	584.78 ± 75.51	262.03 ± 38.65	329.59 ± 48.95	261.78 ± 31.98	13.27 ± 3.91
Total Plants re-flooding (g)	80.10 ± 9.81	32.81 ± 7.46	19.71 ± 3.51	88.62 ± 7.87	24.56 ± 4.58	35.33 ± 9.16	30.93 ± 8.22	10.58 ± 2.21
Diss. Oxygen (mg L^{-1})	8.57 ± 0.37	7.28 ± 0.37	6.47 ± 0.35	8.69 ± 0.35	7.67 ± 0.4	7.92 ± 0.39	7.53 ± 0.23	4.75 ± 0.24
Conductivity (mS cm^{-1})	0.25 ± 0.01	0.29 ± 0.01	0.33 ± 0.01	0.29 ± 0.02	0.30 ± 0.01	0.27 ± 0.01	0.29 ± 0.01	0.35 ± 0.02
Ammonium (ppb)	29.52 ± 4.76	71.32 ± 26.35	60.86 ± 14.41	26.98 ± 4.7	95.92 ± 51.08	47.86 ± 9.27	37.38 ± 6.86	240.4 ± 72.4
Phosphate (ppb)	20.95 ± 1.71	16.72 ± 2.1	20.78 ± 2.33	9.26 ± 0.94	22.68 ± 3.96	13.18 ± 2.09	15.14 ± 3.28	22.19 ± 3.08
Nitrate (ppb)	9.3 ± 2.81	10.96 ± 3.53	8.21 ± 1.53	11.1 ± 1.64	8.97 ± 1.26	6.87 ± 1.14	11.31 ± 2.45	9.61 ± 1.21
Nitrite (ppb)	4.13 ± 0.23	4.52 ± 0.66	7.64 ± 3.04	3.32 ± 0.17	10.9 ± 6.07	4.46 ± 1.21	3.83 ± 0.43	10.74 ± 3.08

Appendix B. Statistical results of the GLMM to test the effect of amphibians on different ecological variables of temporary ponds. We used five different contrasts to test for such effects. Degrees of freedom (df), F value (F) and p value (*p*) are shown for every contrast. Numerator df was equal to 1 in all statistical contrasts and therefore not shown in the table.

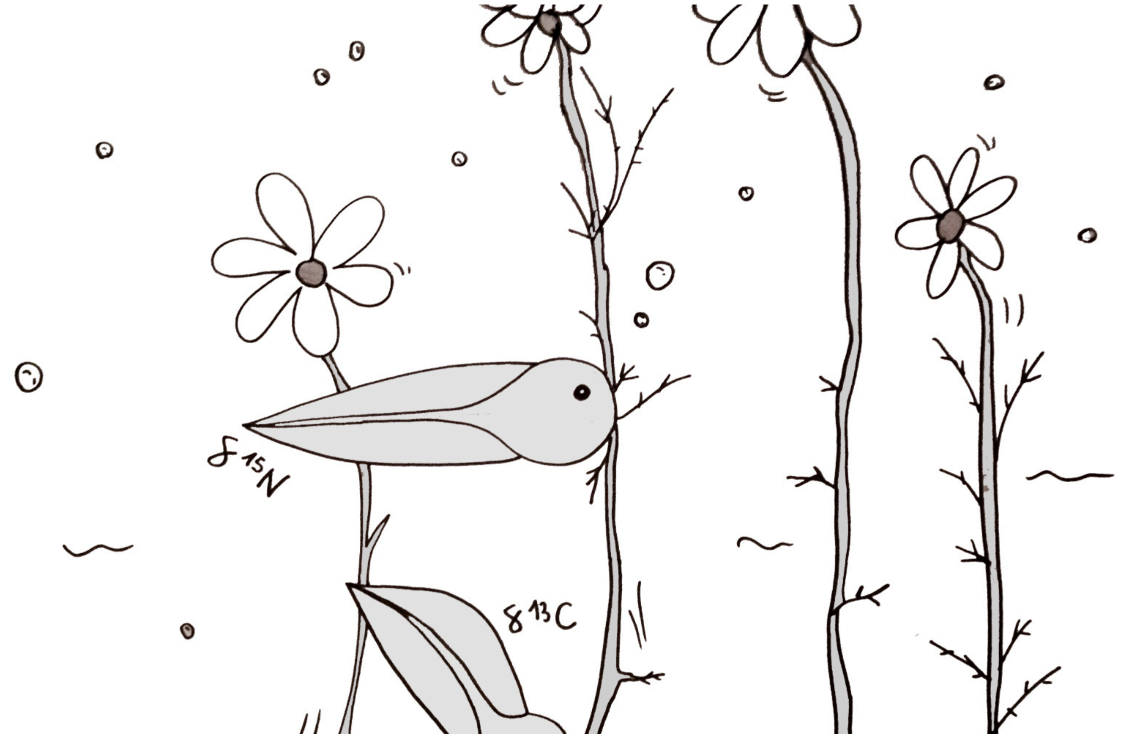
Effects of amphibians on temporary ponds		**No Amph - Low**	**No Amph - High**	**Low - High**	**No Amph -No Pc**	**Low – No Pc**
Turbidity	df	86	86	86	86	86
	F	10.85	20.58	1.31	1.02	18.33
	p	**0.0024**	**0.0002**	0.2564	0.3157	**0.0002**
Oxygen	df	75	75	75	75	75
	F	10.91	27.38	3.25	0.1	13.07
	p	**0.0015**	**0.0001**	0.0755	0.7496	**0.0006**
Conductivity	df	86	86	86	86	86
	F	5.95	19.42	3.5	3.06	0.53
	p	**0.031**	**0.0005**	0.0649	0.0838	0.4679
Ammonium	df	78	78	78	78	78
	F	2.89	1.59	0.22	0.67	6.24
	p	0.0932	0.2105	0.6415	0.416	**0.0029**
Phosphate	df	78	78	78	78	78
	F	1.77	0.01	1.48	8.07	8.07
	p	0.1871	0.9075	0.2272	**0.017**	**0.017**
Nitrite	df	78	78	78	78	78
	F	0.02	0.39	0.56	2.22	1.73
	p	0.889	0.5361	0.4576	0.1402	0.1926
Nitrate	df	78	78	78	78	78
	F	2.26	0.00	2.26	0.00	2.26
	p	0.137	0.9997	0.1369	0.9997	0.1369
Macrophytes	df	86	86	86	86	86
	F	14.95	64.84	16.07	4.42	35.08
	p	**0.00025**	**0.00014**	**0.00014**	**0.0385**	**0.00014**
Macrophytes after reflooding	df	75	75	75	75	75
	F	25.34	47.27	2.81	0.83	35.1
	p	**0.00021**	**0.00021**	0.0981	0.3644	**0.00021**
Chlorophyll-*a*	df	78	78	78	78	78
	F	0.39	4.84	2.31	0.39	0
	p	0.5319	**0.0308**	0.1329	0.5341	0.9853
Zooplankton Shannon Diversity	df	85	85	85	85	85
	F	1.95	9.61	2.67	0.99	0.18
	p	0.166	**0.0026**	0.1057	0.3223	0.6731

Appendix C. Statistical results of the GLMM to test the effect of native and invasive predators on different ecological variables of temporary ponds. We used seven different contrasts to test for such effects. Degrees of freedom (df), F value (F) and p value (*p*) are shown for every contrast. Numerator df was equal to 1 in all statistical contrasts and therefore not shown in the table.

Effects of predators		**Low - NatC**	**Low - Nat F**	**Low - InvC**	**Low - InvF**	**NatC - NatF**	**InvC - InvF**
Turbidity	df	86	86	86	86	86	86
	F	0.57	0.38	3.24	5.46	0.02	17.89
	p	0.4535	0.54	0.0754	**0.0218**	0.8781	**0.0002**
Oxygen	df	75	75	75	75	75	75
	F	1	3.02	0.62	35.89	0.51	48.25
	p	0.3198	0.0866	0.4338	**0.0001**	0.4794	**0.0001**
Conductivity	df	86	86	86	86	86	86
	F	0.1	1.07	0.03	5.55	1.83	5.04
	p	0.7548	0.3041	0.8721	**0.031**	0.1793	**0.0328**
Ammonium	df	78	78	78	78	78	78
	F	0.7	1.36	1.19	3.25	0.1	8.8
	p	0.4047	0.2464	0.2781	0.0752	0.7571	**0.004**
Phosphate	df	78	78	78	78	78	78
	F	1.19	1.8	0.88	1.68	6.02	5.24
	p	0.2797	0.1842	0.3513	0.1986	**0.0246**	**0.0297**
Nitrite	df	78	78	78	78	78	78
	F	0.76	1.08	0.61	4.52	3.74	8.89
	p	0.3852	0.3012	0.436	**0.0366**	0.0567	**0.0057**
Nitrate	df	78	78	78	78	78	78
	F	2.16	0.02	2.26	2.26	1.87	0.00
	p	0.1461	0.8917	0.1369	0.1369	0.1758	1.00
Macrophytes	df	86	86	86	86	86	86
	F	1.86	6.66	2.36	32.65	1.41	54.94
	p	0.1763	**0.0128**	0.1284	**0.00014**	0.2384	**0.00014**
Macrophytes after reflooding	df	75	75	75	75	75	75
	F	1.12	0.01	0.05	11.5	1.38	10.61
	p	0.2939	0.928	0.8325	**0.00154**	0.2445	**0.002**
Chlorophyll-*a*	df	78	78	78	78	78	78
	F	0.05	0.17	0.05	2.16	0.04	1.6
	p	0.8307	0.6847	0.8159	0.1461	0.8513	0.2096
Zooplankton Shannon Diversity	df	85	85	85	85	85	85
	F	0	0.05	0.12	0.25	0.04	0.73
	p	0.9843	0.8287	0.729	0.6216	0.8444	0.3957

Capítulo 2

Stable isotopes reveal trophic partitioning and trophic plasticity of a larval amphibian guild[2]



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[2] Arribas R, Díaz-Paniagua C, Caut S, Gomez-Mestre I (2015). Stable isotopes reveal trophic partitioning and trophic plasticity of a larval amphibian guild. *PLoS ONE* 10(6): e0130897.

RESUMEN

Las lagunas temporales son sistemas altamente variables donde la disponibilidad de los recursos y la estructura de la comunidad cambia ampliamente a lo largo del tiempo, y, consecuentemente, la red trófica es altamente dinámica. Los anfibios juegan un papel crítico tanto como consumidores como presas en las comunidades acuáticas y aún así, todavía hay poca información sobre el estado trófico de la mayoría de los anfibios. Incluso más importante, se sabe muy poco de hasta qué punto pueden alterar su ecología trófica en respuesta a las condiciones cambiantes del medio.

Investigamos experimentalmente los efectos del aumento de la densidad de anfibios, la presencia de competidores dentro de la comunidad y la presencia de depredadores nativos e invasores (tanto libres como enjaulados) en el estado trófico de una comunidad de anfibios Mediterráneos, todo ello mediante el uso de isótopos estables. Observamos variaciones en los valores isotópicos de carbono y nitrógeno (δ^{13}C y δ^{15}N) entre las especies de anfibios y entre los tratamientos, así como diferencias en sus fuentes de alimento. Los macrófitos fueron la fuente de alimento más importante para los renacuajos del sapo de espuelas (*Pelobates cultripes*) y también fueron relativamente importante para todos los anuros de la comunidad. La alta densidad de larvas y la presencia de los renacuajos de *P. cultripes* redujo la biomasa de macrófitos considerablemente, forzando a los renacuajos a incrementar su ingesta de detritos, algas y zooplancton, resultando en valores menores de δ^{13}C. Los depredadores nativos (ditíscidos) solo cambiaron la señal isotópica de los tritones mientras que los invasores, el cangrejo rojo americano, tuvo un enorme impacto en las condiciones ambientales y afectaron enormemente a los valores isotópicos de los anfibios. Los cangrejos forzaron a los renacuajos a incrementar su ingesta de detritos y otros recursos con bajo valor de δ^{13}C. Encontramos que la alimentación oportunista de los anfibios estaba ampliamente condicionada por competencia intra- e interespecífica mientras que los efectos del depredador no derivados de su consumo no fueron muy notables. La determinación de la plasticidad trófica de los anfibios nos puede ayudar a comprender los cambios naturales y antropogénicos de los ecosistemas acuáticos y a evaluar la habilidad de los anfibios a adaptarse a las diferentes condiciones ambientales.

ABSTRACT

Temporary ponds are highly variable systems where resource availability and community structure change extensively over time, and consequently the food web is highly dynamic. Amphibians play a critical role both as consumers and prey in aquatic communities and yet there is still little information on the trophic status of most amphibians. More importantly, little is known about the extent to which they can alter their trophic ecology in response to changing conditions.

We experimentally investigated the effects of increased amphibian density, presence of intraguild competitors, and presence of native and invasive predators (either free or caged) on the trophic status of a Mediterranean amphibian guild, using stable isotopes. We observed variations in δ^{13}C and δ^{15}N isotopic values among amphibian species and treatments and differences in their food sources. Macrophytes were the most important food resource for spadefoot toad tadpoles (*Pelobates cultripes*) and relatively important for all anurans within the guild. High density and presence of *P. cultripes* tadpoles markedly reduced macrophyte biomass, forcing tadpoles to increase their feeding on detritus, algae and zooplankton, resulting in lower δ^{13}C values. Native dytiscid predators only changed the isotopic signature of newts whereas invasive red swamp crayfish had an enormous impact on environmental conditions and greatly affected the isotopic values of amphibians. Crayfish forced tadpoles to increase detritus ingestion or other resources depleted in δ^{13}C. We found that the opportunistic amphibian feeding was greatly conditioned by intra- and interspecific competition whereas non-consumptive predator effects were negligible. Determining the trophic plasticity of amphibians can help us understand natural and anthropogenic changes in aquatic ecosystems and assess amphibians' ability to adjust to different environmental conditions.

INTRODUCTION

Studying the diet of individuals within a community provides key ecological insight about food web structure essential to understand trophic relationships, ecological roles, and niche partitioning (Paine 1980, Boon and Bunn 1994, Schindler et al. 1997). This information is of utmost importance to understand the structure of natural communities and to predict the consequences of ecological disturbances such as habitat modification, global climate change, or introduction of invasive species (Gillespie 2013, Richter-Boix et al. 2013). Interactions among individuals and with the environment modulate variations in community diversity, structure, and dynamics (Polis 1994), and also influence niche width of the interacting species at an ecological and evolutionary scale (e.g. Ackermann and Doebeli 2004, Bolnick et al. 2010). Trophic niche partitioning within aquatic communities has been studied through the analysis of functional groups in macroinvertebrates (e.g. Wallace and Webster 1996, Tomanova et al. 2006) and fish (e.g. Chouinard 1998), and more recently via determination of isotopic signatures (Vander Zanden and Rasmussen 1999, Vander Zanden et al. 1999, Beaudoin et al. 2001, Newsome et al. 2007, Turner and Edwards 2012).

Amphibian larvae are pivotal agents in structuring aquatic communities, acting as primary consumers, as prey for both vertebrate and invertebrate predators, but also as predators themselves (e.g. Wells 2007). Thus, amphibian larvae can have profound effects on nutrient availability, zooplankton composition, and macrophyte biomass (Arribas et al. 2014). Surprisingly, despite their potential cascading consequences for the rest of the community, relatively little work on their trophic ecology has been conducted (but see Whiles et al. 2006, Altig et al. 2007, Schiesari et al. 2009, Costa and Vonesh 2013b). Tadpoles of many species are considered herbivores that also feed on detritus, bacteria, plankton or fungi (Hoff et al. 1999), although our knowledge about what tadpoles really eat has been put into question (Altig et al. 2007). The diet of larval urodeles has been studied for particular species, all of which are carnivorous and can have a great impact on aquatic communities (see review in Wells 2007). In contrast, the diet of anuran larvae has seldom been described and actual direct information about diet composition has only been described for a limited number of species (Altig et al. 2007). Instead, their trophic niche has rather been inferred from their mouth morphology or their most frequent position in their aquatic habitats (Wassersug 1980, Wells 2007, Altig et al. 2007). Tadpole morphology shows a wide spectrum of feeding specializations, which allows classifying them into functional groups, ranging from typical herbivorous feeding on phytoplankton to cannibalistic species, including species with endotrophic larvae that entirely rely on yolk (Wells 2007, Altig et al. 2007).

Despite general predictions about the trophic status or ecological specialization at the species level according to behavior, preferred habitats, and structure of the mouthparts, trophic ecology of amphibian larvae is likely to be complex and contingent upon the environmental conditions experienced. Most amphibians breed in highly dynamic aquatic systems, like temporary ponds or flooded river banks which show extensive variation in space and time regarding availability of potential resources for amphibian larvae (Polis et al. 1995). To complicate things further, trophic ecology of tadpoles may not only vary due to high environmental dynamism, but also because tadpoles respond plastically to such environmental fluctuations by altering their own behavior and morphology (Anholt and Werner 1995, Relyea 2000). Their trophic status may consequently be rather plastic and change in response to interactions with competitors and predators. In amphibian larvae, competitors and predators induce changes in morphological traits that could have direct implications in their diet, such as gut length, oral disc size, beak width, and tooth rows (Relyea and Auld 2005). Tadpoles also reduce their activity rate or increase the use of refugia in the presence of predators (Anholt and Werner 1995, Richardson 2001), which will cause changes in their diets if they start feeding in different parts of the water column, shifting from a macrophyte-based diet to a periphyton- or a phytoplankton-based one. However, recently introduced predators that did not share a joint evolutionary history with tadpoles do not trigger such behavioral responses (Polo-Cavia et al. 2010, Gomez-Mestre and Díaz-Paniagua 2011). Despite the great potential importance for aquatic systems of plastic changes in the trophic ecology of amphibian larvae (Peacor and Werner 1997, 2000, Pfennig et al. 2006), there is still a lack of information regarding how ecological factors may alter diets within local amphibian guilds (Wells 2007). Traditional methods to determine diet composition such as gut contents, fecal analysis, or foraging observations only give information about the most recent ingestion, although they can be very informative if sampling is extensive over time and across age and size classes. However, stable isotope analysis can further provide an account of the diet that the organisms have had over a longer period of time and may help revealing the specific resources used by consumers (Layman et al. 2007b). Stable isotopes have been recently used in amphibian larvae, as for example in assessing ontogenetic diet variations or their implications in food webs (e.g. Trakimas et al. 2011, Schriever and Williams 2013). Carbon isotopes (δ^{13}C) identify major energy sources, and nitrogen isotopes (δ^{15}N) indicate trophic position within a food web (Vander Zanden and Rasmussen 1999).

The present study constitutes a follow up to a comprehensive mesocosm experiment designed to assess the effect of larval amphibians on physico-chemical and biotic characteristics of ponds under different ecological conditions [18]. We experimentally manipulated amphibian larval density and the presence or absence of a large tadpole species that acts as a strong competitor. Moreover, we also manipulated the presence/absence of native and invasive predators, kept either caged or freely roaming to distinguish their non-consumptive effects on the trophic status of amphibians. Free predators would exert both direct density-dependent effects and non-consumptive effects whereas caged predators would only have the potential to have non-consumptive effects, mostly via induction of altered behavior in the tadpoles. We reported that amphibian larvae at high density, as well as invasive crayfish had marked effects on the aquatic environment, altering water physico-chemistry, plant biomass, and zooplankton abundance [18]. At high larval density, amphibians decreased macrophyte biomass, reduced zooplankton diversity, caused increased water turbidity, and increased the presence of nutrients in the water (Arribas et al. 2014). The large spadefoot toad tadpoles (*Pelobates cultripes*) were the main contributors to these changes. Free-roaming invasive crayfish (*Procambarus clarkii*) caused analogous effects to that of high amphibian density, and they also caused greater amphibian mortality (79.6%) than native dragonflies did (65%) (Arribas et al. 2014). These changes in pond characteristics would be expected to imply substantial changes in the abundance of trophic resources available to the amphibian larvae, consequently causing shifts in their trophic status.

Our aim in this study was to assess the trophic plasticity of a guild of amphibian larvae under different ecological conditions. For this purpose, we used stable isotopes to analyze how the changes in pond characteristics due to amphibian density and predator presence would be reflected in diet shifts of the amphibian species studied. We hypothesized that amphibian larvae would alter their carbon source and present a lower trophic level (lower δ^{15}N values) with increased larval density and when faced with strong competitors like *P. cultripes* tadpoles. We expected lower δ^{15}N values due to reduced availability of resources and an increase in the relative consumption of detritus. We also expected shifts in the carbon source of amphibian larvae and higher δ^{15}N values in the presence of native predators, because they reduced amphibian density thus relaxing inter- and intraspecific competition. We expected this to result in the maintenance of a greater variety of resources available for amphibians. On the other hand, we expected lower δ^{15}N values in amphibian larvae exposed to caged native predators due to induced lower foraging activity. However, we expected no such changes in trophic status in the presence of caged invasive predators due to lack of innate recognition. Nonetheless, the dramatic impoverishment of the environmental conditions produced by free-roaming crayfish (Arribas et al. 2014) would force amphibians to seek alternative carbon sources.

METHODS

Study system

This study was carried out in Doñana National Park, located in the southwest of Spain on the right bank of the Guadalquivir River mouth (37º00′N, 6º38′W). The climate of this area is Mediterranean with Atlantic influence, with hot and dry summers and mild winters. One half of the park is a sandy area with an important temporary pond network and the other half is an extensive seasonal marsh (more details in Arribas et al. 2014). The amphibian community is composed of eleven species (eight anurans and three urodeles), of which the most abundant are those that use temporary ponds as breeding habitats. We included in our study the larvae of six of the most abundant species (five anurans and one urodele) to have a good representation of the community of the area. The anurans include in their diet a variable proportion of detritus, algae and phanerogams. However, these anurans range from mainly bottom-dwelling tadpoles feeding on detritus and periphyton (the natterjack toad, *Bufo calamita;* the Iberian painted frog*, Discoglossus galganoi;* and the Iberian green frog, *Pelophylax perezi*) to water column feeders, mainly on macrophytes (the western spadefoot toad, *Pelobates cultripes*) or filtering algae (the Mediterranean treefrog, *Hyla meridionalis*) (Díaz-Paniagua 1985). The larvae of the urodele, the pigmy marbled newt (Triturus pygmaeus), mainly feed on planktonic crustaceans (Díaz-Paniagua et al. 2005).

Experimental array

We established an experimental mesocosms array at the central area of the park consisting of 7 treatments (see Table 1 for an overview): 1) Low amphibian density (*Low*): presence of low density of larvae of six amphibian species; 2) High density of amphibian larvae (*High*): three-fold low density number; 3) Absence of *P. cultripes* (*No Pc*): same as in the Low treatment but excluding *Pelobates cultripes*, the largest species with the longest developmental period in the area; 4) Caged native predator (*NatC*): Low density of amphibian larvae exposed to a single caged *Dytiscus* larva. Caged predators provide chemical cues but do not reduce amphibian survival by predation; 5) Free native predator (*NatF*): Low density of amphibian larvae exposed to a single free ranging *Dytiscus* larva, which can provide chemical cues and reduce amphibian survival; 6) Caged invasive predator (*InvC*): Low density of amphibian larvae exposed to a caged red swamp crayfish; 7) Free invasive predator (*InvF*): Low density of amphibian larvae exposed to a single free ranging red swamp crayfish; and 8) Control (*No Amph*): absence of amphibians and predators.

Table 1. Overview of the experimental treatments specifying name, acronym, number of replicates and details of each treatment.

Treatment name	**Acronym**	**Replicates**	**Details**
Low amphibian density	*Low*	12	Presence of 6 species of amphibian larvae at low density
High amphibian density	*High*	12	3-times the density of amphibian larvae in the Low treatment
Absence of *P. cultripes*	*No Pc*	12	Same as in low density but without the presence of one species (*Pelobates cultripes*)
Caged native predator	*NatC*	12	Low density of amphibian larvae together with a caged *Dytiscus* larva
Free native predator	*NatF*	12	Low density of amphibian larvae together with a free *Dytiscus* larva
Caged invasive predator	*InvC*	12	Low density of amphibian larvae together with a caged red swamp crayfish
Free invasive predator	*InvF*	12	Low density of amphibian larvae together with a free-roaming red swamp crayfish
Control	*No Amph*	12	No amphibians and no predators

We considered low density to be 3 individuals per tank for *P. cultripes*, *P. perezi*, *D. galganoi,* and *T. pygmaeus*, 10 individuals per tank for *H. meridionalis*, and 15 individuals per tank for *B. calamita*. The high-density treatment increased three-fold each species' density. Tadpole densities were chosen according to the natural range of abundances in the natural ponds in the area (Diaz-Paniagua 1990).

Each treatment was replicated 12 times for a total of 96 tanks distributed in 12 randomized blocks.

The experiment lasted for 10 weeks between March 24th and June 2nd 2011. Mesocosms were 500-L plastic tanks, with an upper diameter of 120 cm, and covered with tightly fitting lids of fiberglass window screening. Tanks were filled three weeks prior to the onset of the experiment as described in Arribas *et al.* (2014), with a combination of sand and pond sediment from nine different temporary ponds prior to fill them with well water, replicating the natural conditions of ponds in Doñana. In addition, we added pond water as inoculum for zoo- and phytoplankton, and planted in each tank the same amount of three species of abundant aquatic macrophytes of the area (*Myriophyllum alterniflorum, Ranunculus peltatus*, and *Callitriche obtusangula)* to provide greater spatial complexity and nutrient availability for the aquatic community.

The six species of amphibian larvae, crayfish, and dytiscid beetle larvae were collected by dip-netting in different temporary ponds within the park. We introduced them in the experimental tanks following the natural breeding phenology of the species (Diaz-Paniagua 1988) and according to the rainfall patterns of the season. Thus, we first introduced *P. cultripes* and *B. calamita*, then *H. meridionalis* and *T. pygmaeus*, then *D. galganoi*, and lastly *P. perezi* (see Arribas et al. 2014). Amphibian larvae were similar in initial size and were randomly distributed across tanks (see A1-A4 Tables). Predator cages consisted of lidded 1L plastic buckets with small holes drilled at the bottom to allow predator cues to diffuse throughout the tank. Depending on the treatment, we kept these cages empty or included either a dytiscid larvae in the native predator treatment or a red swamp crayfish in the invasive predator treatment. Two amphibian larvae randomly taken from the six species of the study were introduced in the cages with predators to feed them during the experiment. We checked the tanks every day to record metamorphosis date of individuals of each species (i.e. Gosner stage 42, (Gosner 1960)) until the end of the experiment, when tanks in some treatments had been depleted of macrophytes and when natural ponds had begun to dry up. At that point, we removed every larvae or metamorph remaining in the tanks. At the end of the experiment, we observed steep reductions in amphibian survival in treatments where invasive and native predators were free, as well as under high amphibian density.

Isotopic analysis

We could only take samples for isotopic analyses from four out of the six species of amphibians, because there were no survivors of *B. calamita* and very few survivors of *D. galganoi* at the end of the experiment. We collected one or two individuals from each species and tank, when available, and euthanized them by immersion in MS-222. The anesthetic MS-222 is commonly used for euthanizing aquatic organisms and no effects have been reported on stable isotope measurements (Frédérich et al. 2010, e.g. Caut et al. 2013). We collected individuals as they metamorphosed and larvae from tanks from which we had not yet recovered metamorphic individuals. Metamorphs and larvae of each species collected were kept at -20 ºC until processed for isotopic analysis. Most individuals processed of *H. meridionalis* had already metamorphosed by the end of the experiment, whereas most individuals of *P. perezi* were still in the larval stage (see Table 2), as is usually the case in natural ponds.

Table 2. Number of individuals (metamorphs and larvae) used for isotopic analyses in each species and treatment.

Species	***Hyla meridionalis***	***Pelobates cultripes***		***Pelophylax perezi***		***Triturus pygmaeus***	
Treatment	Metamorphs	Larvae	Metamorphs	Larvae	Metamorphs	Larvae	Metamorphs
Low	10	7	9	7	1	7	2
High	1 (+ 5 larvae)	10	8	9	2	9	1
No Pc	11	-	-	9	2	12	2
NatCaged	12	4	10	8	3	10	1
NatFree	9	4	12	7	3	7	2
InvCaged	11	5	11	8	3	10	3
InvFree	7	2	5	7	0	0	0
TOTAL	66	87		69		66	

We determined carbon and nitrogen isotopic values ($\delta^{13}C$ and $\delta^{15}N$) from muscle samples of amphibians, dissecting tail muscle from tadpoles and newts, and leg muscle from anuran metamorphs (n = 288, but see Table 2 for specific number of individuals processed per species and development stage). Each sample was kept in a separate labeled eppendorf tube, oven-dried at 50 ºC to constant mass and ground to a fine and homogeneous powder with mortar and pestle.

All possible resources for amphibians in the mesocosms were collected one month after the experiment started from six additional tanks randomly chosen out of the twelve tanks with the same conditions but containing neither amphibians nor crayfish or dytiscids (*No amph*) [18]. In this study, we considered detritus, algae, zooplankton, three species of macrophytes, and one species of charophytes that emerged from the sediment, as possible carbon sources for consumers. We considered these food sources appropriate to perform the isotopic analysis as we have previous and detailed information on the diet of the species studied. The anuran species studied are mainly herbivorous, feeding on algae, macrophytes, fungi and detritus with a small fraction of the diet contributed by animal matter and bacteria (Díaz-Paniagua 1985). In contrast, larval newts are carnivorous, feeding mainly on zooplankton (Díaz-Paniagua et al. 2005), although other particles may accidentally be ingested. For this reason, we included all seven food items to calculate the relative contribution of each source to the diet of tadpoles, but only included zooplankton and detritus as possible food sources for newts.

For detritus samples, we carefully collected 4 cm^2 of the upper 1 cm sediment layer in each additional tank. In order to collect the finer top particles, we centrifuged the sample for 5 minutes at 1100 G in 15 mL conic Falcon tubes and preserved the first 3 mm of the pellet for analyses. We then divided the sample in two, and treated one of them sequentially with HCl 0.1M and 1M to remove any carbonates (Harris et al. 2001). We cleaned this sample with several centrifugations with distilled water until the supernatant was clear. The isotopic values for $\delta^{13}C$ were obtained after treating them with HCl, and for $\delta^{15}N$ were obtained from the other untreated part of the sample. To collect zooplankton, we filtered 5 L of water from each of the additional tanks through a 100 μm net and kept the individuals in the filter with dechlorinated tap water. We then took each water sample, centrifuged them, and kept only the portion of the water column containing swimming zooplankton. For algae, we filtered two extra liters of water from these tanks through a smaller mesh size (60 μm), placed the water in a glass container and allowed it to evaporate in an oven at 50 ºC, and then scraped the remains from the walls and the bottom for isotopic analysis of all the microalgae found there. All samples of potential resources were dried up in the oven at 50 ºC to constant mass prior to isotopic analysis and then ground to a fine and homogeneous powder.

We weighed 0.3 ± 0.01 (mean ± SD) mg of amphibian muscle, 2.16 ± 0.05 mg of algae, 1.02 ± 0.14 mg of zooplankton, 10.6 ± 0.73 mg of detritus, and 1.66 ± 0.04 mg of macrophytes and placed them into tin capsules for $\delta^{13}C$ and $\delta^{15}N$ determinations. These weights depend on nitrogen concentration of the samples as well as on the resolution of the spectrometer. Isotopic analyses were carried out at the Laboratory of Stable Isotopes at Doñana Biological Station. All samples were combusted at 1,020 ºC using a continuous flow isotope-ratio mass spectrometry system by means of 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 ($\delta^{13}C$) and atmospheric N2 ($\delta^{15}N$), using the equation $\delta^{13}C$ or $\delta^{15}N = ((R_{sample} / R_{standard}) - 1) \times 1000$, where R is $^{13}C/^{12}C$ or $^{15}N/^{14}N$. Replicate assays of laboratory standards routinely inserted within the sampling sequence, and previously calibrated with international standards, indicated analytical measurement errors of ± 0.1‰ and ± 0.2‰ for $\delta^{13}C$ and $\delta^{15}N$, respectively. We calculated the mean and standard deviation for the seven sources included in this study, for $\delta^{13}C$ and $\delta^{15}N$ as well as for %C and %N (elemental composition). Post et al. (Post 2002) recommended correcting the $\delta^{13}C$ values for lipid content when the C/N ratio of the tissue is > 3.5 in aquatic animals. Following this approach, we normalized $\delta^{13}C$ values to account for lipid variation in $\delta^{13}C$ (mean C/N = 3.51) following the equation in Caut et al. (2013), derived for amphibian tadpoles: $\delta^{13}C_{normalized} = \delta^{13}C_{untreated} - 1.11 + 0.37\ (C/N)$.

Statistical Analyses

We performed statistical analysis with SAS 9.2 (SAS-Institute, Cary, NC, USA) and R 3.0.2 software (R Core Team 2013). We evaluated the significance of diet differences between individuals at the tadpole stage and individuals at the juvenile stage by means of two-sample t-tests. We used generalized linear mixed models (GLMM) to test for the effect of experimental treatments, species, or 'treatment x species' interactions on the mean values of $\delta^{13}C$ or $\delta^{15}N$ for each amphibian species and tank. Values of $\delta^{13}C$ were log-transformed to meet parametric assumptions whereas $\delta^{15}N$ values met the assumptions and no transformation was necessary except for the species *P. cultripes*, where we ranked the variable (ties method = minimum) prior to analysis with general linear models. We tested for differences in isotopic values among treatments using the GLIMMIX procedure from SAS statistical package (Schabenberger 2007). In all models fitted, we first tested the effect of experimental block as a random factor, but finally removed it from the model since it was never significant. Normally distributed variables were modeled with a Gaussian error distribution and an identity link function. Since our experimental design was not fully factorial, we specified multiple contrasts through planned comparisons to test specific hypotheses: we tested for effects of increasing amphibian density with the contrast *Low-High*; we tested the effect of the big sized tadpoles *P. cultripes* comparing *Low-No Pc*; and we tested the effect of predators with that of the amphibian presence at low density (*Low*) and comparing caged and free predators. Given that several of these multiple comparisons (8 comparisons total) were not orthogonal, we corrected the resulting *p*-values to minimize the false discovery rate (FDR, (Benjamini and Hochberg 1995, Garcia 2003)). The FDR is a simple and powerful method for controlling type I error when multiple comparisons are carried out, keeping the proportion of those errors low (Verhoeven et al. 2005).

We checked for differences on the isotopic values and on C and N content of the potential food sources by means of a non-parametrical Kruskal-Wallis test (K-W). The relative isotopic contribution of each food item to a consumer's diet was calculated using the Bayesian stable isotope mixing model Stable Isotope Analysis in R (SIAR package, Parnell et al. 2010). The model calculates the range of all possible sources' contributions for systems where the number of potential sources can be greater than n + 1, n being the number of isotopes analyzed. Isotopic models typically use the mean δ^{13}C and δ^{15}N values and standard deviations for each type of diet, corrected for the discrimination factor of the consumer (the increase in consumer isotopic ratio compared to its diet, noted Δ^{13}C and Δ^{15}N). Concentration dependence or groups were not included in the models. Discrimination factors depend on several sources of variation (e.g. taxa, site, tissue; see review in Caut et al. 2009). Previous laboratory work showed significant relationships between δ^{13}C and δ^{15}N of diets and the corresponding Δ^{13}C and Δ^{15}N of the tissues of amphibian tadpole fed on these diets (Caut et al. 2013). For both tadpoles and metamorphs, we calculated the diet-dependent discrimination factors corresponding to each potential diet item with the equations $\Delta^{13}C = -0.35\ \delta^{13}C - 7.70$ and $\Delta^{15}N = 0.53\ \delta^{15}N - 0.37$ from Caut et al. (2013).

RESULTS

General isotopic differences among species and treatments

We collected between 7 and 18 individuals per species per treatment (nested within tank), summing up a total of 288 individuals for determination of isotopic values. The number of individuals analyzed for each species is shown in Table 2 (larval and metamorph sizes are described in A1-A4 Tables for each species). Values of δ^{13}C and δ^{15}N did not differ between tadpole and metamorph stages within species (all t-tests $P>0.09$) and we therefore pooled all samples within species regardless of stage for further analyses.

We found significant species-by-treatment interactions for both isotopes (δ^{13}C: $F_{16,262} = 3.54$; $P<0.0001$; δ^{15}N: $F_{16,262} = 4.66$; $P<0.0001$) indicating that the effect of experimental treatments on the diet varied among species. Muscle δ^{13}C and δ^{15}N values differed among species (δ^{13}C: $F_{3,262} = 13.33$; $P<0.0001$; δ^{15}N: $F_{3,262} = 81.1$; $P<0.0001$) and among treatments (δ^{13}C: $F_{6,262} = 23.83$; $P<0.0001$; δ^{15}N: $F_{6,262} = 8.07$; $P<0.0001$). Pooling across treatments, we found higher δ^{15}N values in *P. cultripes* and *T. pygmaeus*, and the lowest values in *P. perezi*. For δ^{13}C, we also found the lowest values in *P. perezi* (Fig. 1).

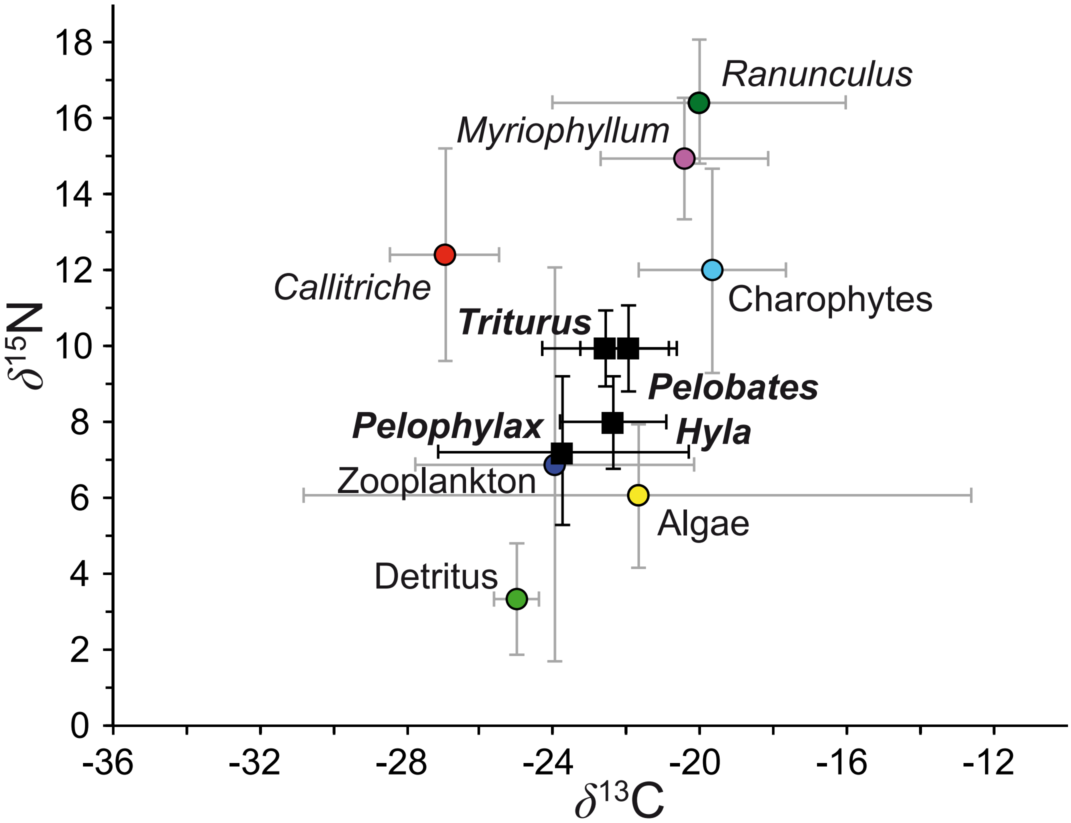

Fig. 1. Stable isotope biplot of each amphibian species and sources of the experiment. δ^{15}N and δ^{13}C values (mean ‰ ± SD) of the amphibians included in this study (*Hyla meridionalis, Pelobates cultripes, Pelophylax perezi,* and *Triturus pygmaeus*) pooled across all experimental treatments for a general view of differences in trophic status among species. Resources are also shown as means ± SD. The discrimination factors were added to the sources and not subtracted to the consumers to allow different discrimination factors to be assigned to different sources.

The effect of larval density and species interactions on trophic niche

The isotopic values of each of the four amphibian species changed between low and high density treatments, especially for δ^{13}C. All species showed a decrease in 8-16 % of δ^{13}C values [(final δ^{13}C value-initial δ^{13}C)/initial δ^{13}C] at high density than at low density (*Low-High*, all $P<0.05$, Fig. 2 and S5 Table). However, δ^{15}N values were not significantly affected by high density (all $P>0.05$). Only *P. cultripes* showed a trend towards a decrease in δ^{15}N when at high density, although it was marginally non-significant (*Low-High*: $F_{1,81} = 2.98$; $P = 0.088$; Fig. 2).

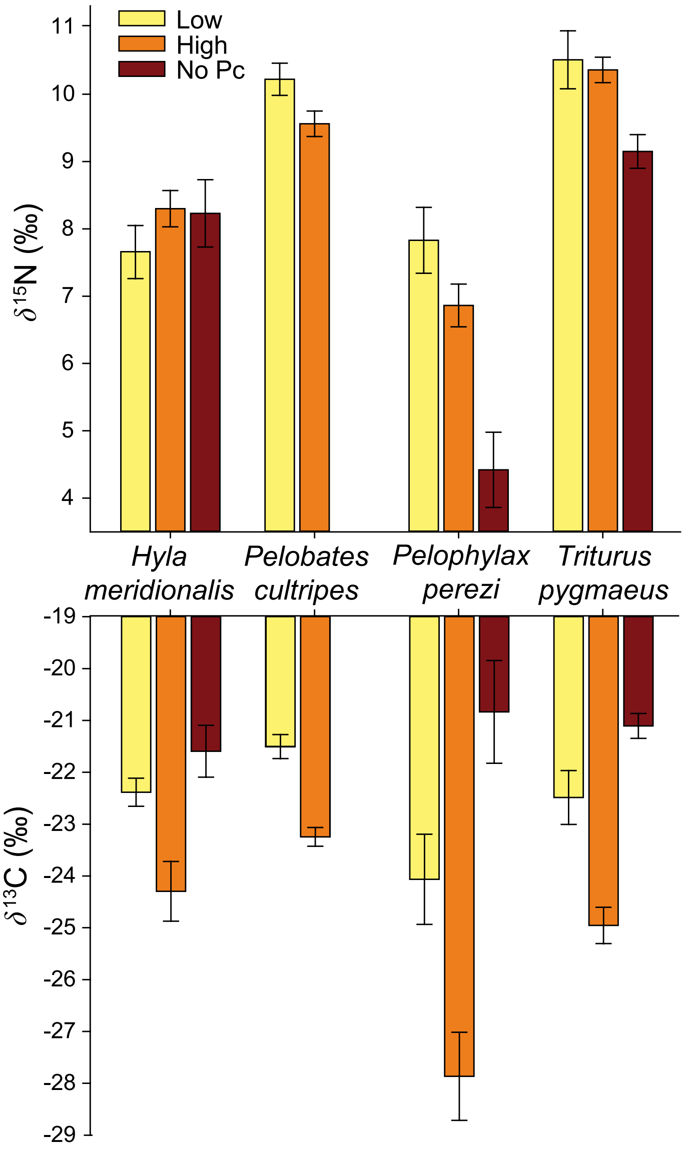

Fig. 2. Stable isotopic values for each amphibian species in the density treatments of the experiment. δ^{15}N and δ^{13}C values (mean ‰ ± SE) of *H. meridionalis, P. cultripes, P. perezi,* and *T. pygmaeus* muscle in three different density treatments: low density of amphibian larvae (*Low*), a three-fold increase of the density from the low treatment (*High*), and exclusion of the competitive species *P. cultripes* (*No Pc*).

The exclusion of *P. cultripes* caused differences in δ^{13}C values in all other species, with higher δ^{13}C values for *P. perezi* (*Low-No Pc*: $F_{1,62} = 28.7$; $P<0.0001$), *T. pygmaeus* (Low-No Pc: $F_{1,60} = 6.97$; $P = 0.01$) and *H. meridionalis* (*Low-No Pc*: $F_{1,60} = 7.57$; $P = 0.0078$), indicating that their carbon source was different in the absence of spadefoot toads. We found lower δ^{15}N values in individuals of *P. perezi* and *T. pygmaeus* when *P. cultripes* larvae were absent (*Low-No Pc* contrast; *P. perezi:* $F_{1,62} = 10.7$, $P = 0.0034$;

and *T. pygmaeus:* $F_{1,60} = 11.68$; *P* = 0.0011; Fig. 2). Instead, we observed no changes in nitrogen values among treatments for *H. meridionalis* (*Low-NoPc*: $F_{1,60} = 0.25$; *P* = 0.62).

Predator effects: type of predator and lack of non-consumptive effects

Predators influenced the isotopic signatures of carbon and nitrogen (Fig. 3 and S5 Table). Native predators only exerted significant changes in isotopic values in the newts. We found higher δ^{13}C value in *T. pygmaeus* when native dytiscid were free in the tanks compared to when they were caged (*NatC-NatF*: $F_{1,60} = 8.95$; *P* = 0.006) although these changes were not significant in the absence of predators (*Low-NatF*: $F_{1,60} = 2.5$; *P* = 0.12). Free crayfish, however, caused the biggest differences in isotopic values. We found significantly lower δ^{13}C in the presence of free crayfish compared to the absence of predators for *H. meridionalis* (*Low-InvF*: $F_{1,59} = 4.21$; *P* = 0.045) and for *P. cultripes* (*Low-InvF*: $F_{1,81} = 5.24$; *P* = 0.025), or when crayfish were caged (*InvC*-InvF: $F_{1,59} = 4.97$; *P* = 0.044 and $F_{1,81} = 6.77$; *P* = 0.015 for *Hyla* and *Pelobates* respectively). On the contrary, *P. perezi* showed higher δ^{13}C in the presence of free crayfish, both compared to the absence of predators (*Low-InvF*: $F_{1,62} = 4.86$; *P* = 0.031) and when the crayfish was caged (*InvC-InvF*: $F_{1,62} = 6.25$; *P* = 0.02).

We detected a non-significant trend towards lower δ^{15}N values in tanks with free invasive crayfish for *P. perezi* (*Low-InvF*; $F_{1,62} = 3.09$; *P* = 0.08), but crayfish did not affect *H. meridionalis* or *P. cultripes*. There were no survivors of *T. pygmaeus* when free crayfish were free and thus no isotopic analyses could be conducted. However, δ^{15}N values for newts were lower when crayfish were caged compared to the absence of predators (*Low-InvC*; $F_{1,60} = 3.94$; *P* = 0.0518, Fig. 3).

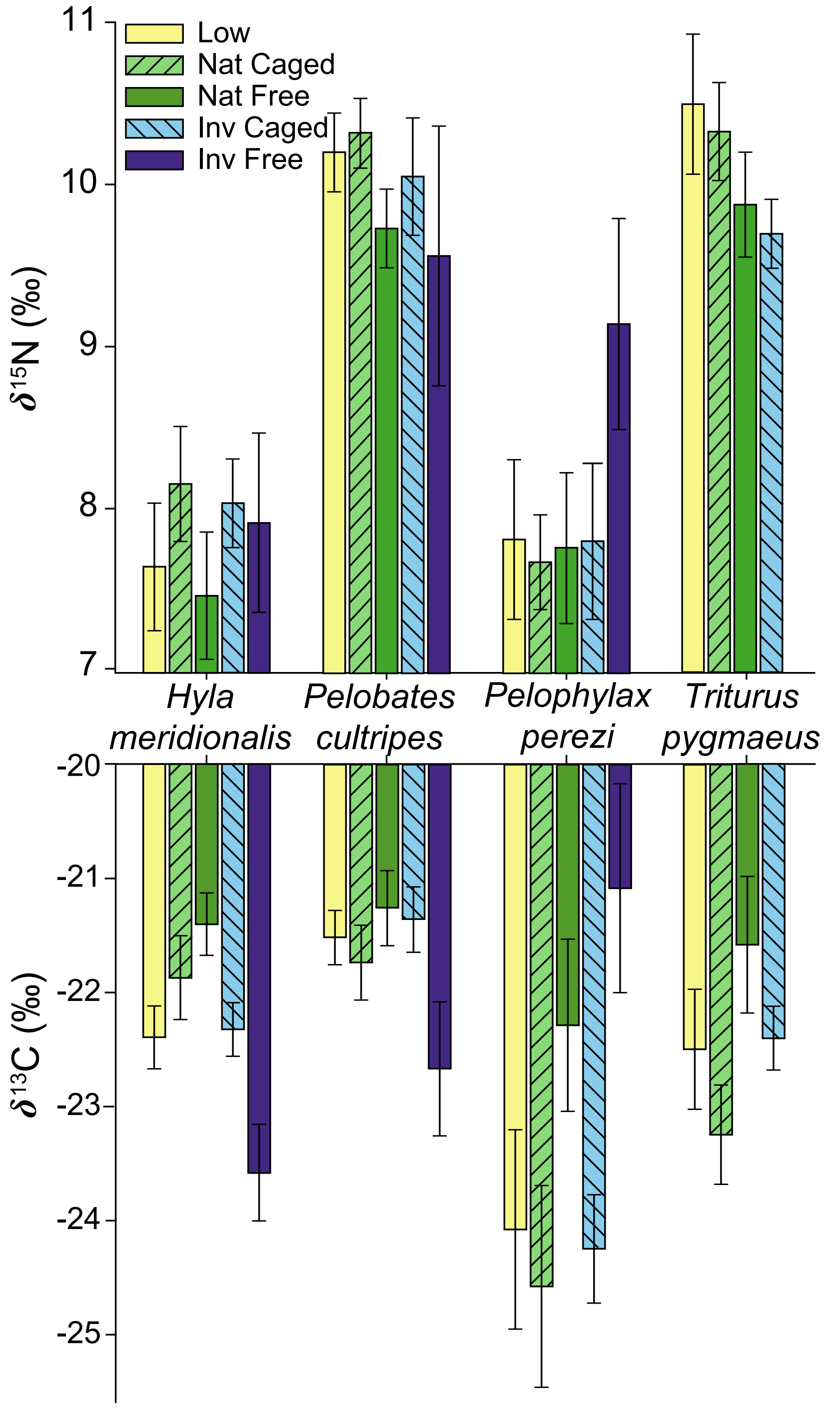

Fig. 3. Stable isotopic values for each amphibian species in the presence / absence of predators. δ^{15}N and δ^{13}C values (mean ‰ ± SE) of *H. meridionalis, P. cultripes, P. perezi,* and *T. pygmaeus* muscle in the presence of native dytiscid larvae (free or caged, *Nat Free* or *Nat Caged*), or in the presence of invasive red swamp crayfish (free or caged, *Inv Free*,or Inv Caged), compared to absence of predators (*Low*).

Dietary contributions of the different sources to different amphibian larvae

The food sources available for tadpoles in our tanks differed in their δ^{13}C (K-W χ^2_6 = 28.62, $P<0.001$) and δ^{15}N (K-W χ^2_6 = 32.45, $P<0.001$) as well as in the percentage of C (K-W χ^2_6 = 36.68, $P<0.001$) and N (K-W χ^2_6 = 33.54, $P<0.001$) (Table 3). We used a different discrimination factor for each food source in the model input, as Δ^{13}C ranged from -0.85 to 2.67 and Δ^{15}N from 0.91 to 4.93 (Table 2).

Table 3. Stable isotope composition (δ^{13}C, δ^{15}N), elemental composition (%C, %N) and discrimination factor (Δ^{13}C, Δ^{15}N) of the potential food sources for amphibian larvae in the experimental arrays of the study.

	Source	δ^{13}C ‰	δ^{15}N ‰	% C	% N	$\boldsymbol{\Delta}^{13}$C	$\boldsymbol{\Delta}^{15}$N
	Detritus	-26.6±0.32	2.42±0.68	5.66±0.36	0.55±0.04	1.61±0.11	0.91±0.36
	Algae	-	4.20±0.75	7.89±1.79	0.44±0.13	-0.17±1.63	1.86±0.4
	Zooplankton	-25.02±1.5	4.68±2.05	23.26±11.5	2.07±1.43	1.06±0.52	2.21±1.09
Macrophytes	***Myriophyllum***	-	9.95±0.72	44.5±0.74	3.36±0.34	-0.85±0.36	4.93±0.38
	Callitriche	-	8.35±1.19	45.74±1.98	2.43±0.19	2.67±0.22	4.06±0.63
	Ranunculus	-	10.98±0.84	41.33±0.74	2.26±0.4	-1.06±0.68	5.45±0.45
	Charophytes	-18.38	8.08±1.15	20.6±1.42	0.91±0.09	-1.26±0.3	3.91±0.61

The mean ‰ ± SD with lipid correction and δ^{15}N is specified for each source, as well as the mass fraction (%) and the discrimination factor (Δ) for C and N. Food sources were taken from control tanks containing no amphibians or predators. Sample size equaled 6, except for algae δ^{15}N (n = 3) and zooplankton δ^{15}N (n = 5).

The contribution of the different sources to the diet of amphibians revealed differences among species and among experimental treatments (see Fig. 4 and S6-S9 Tables).

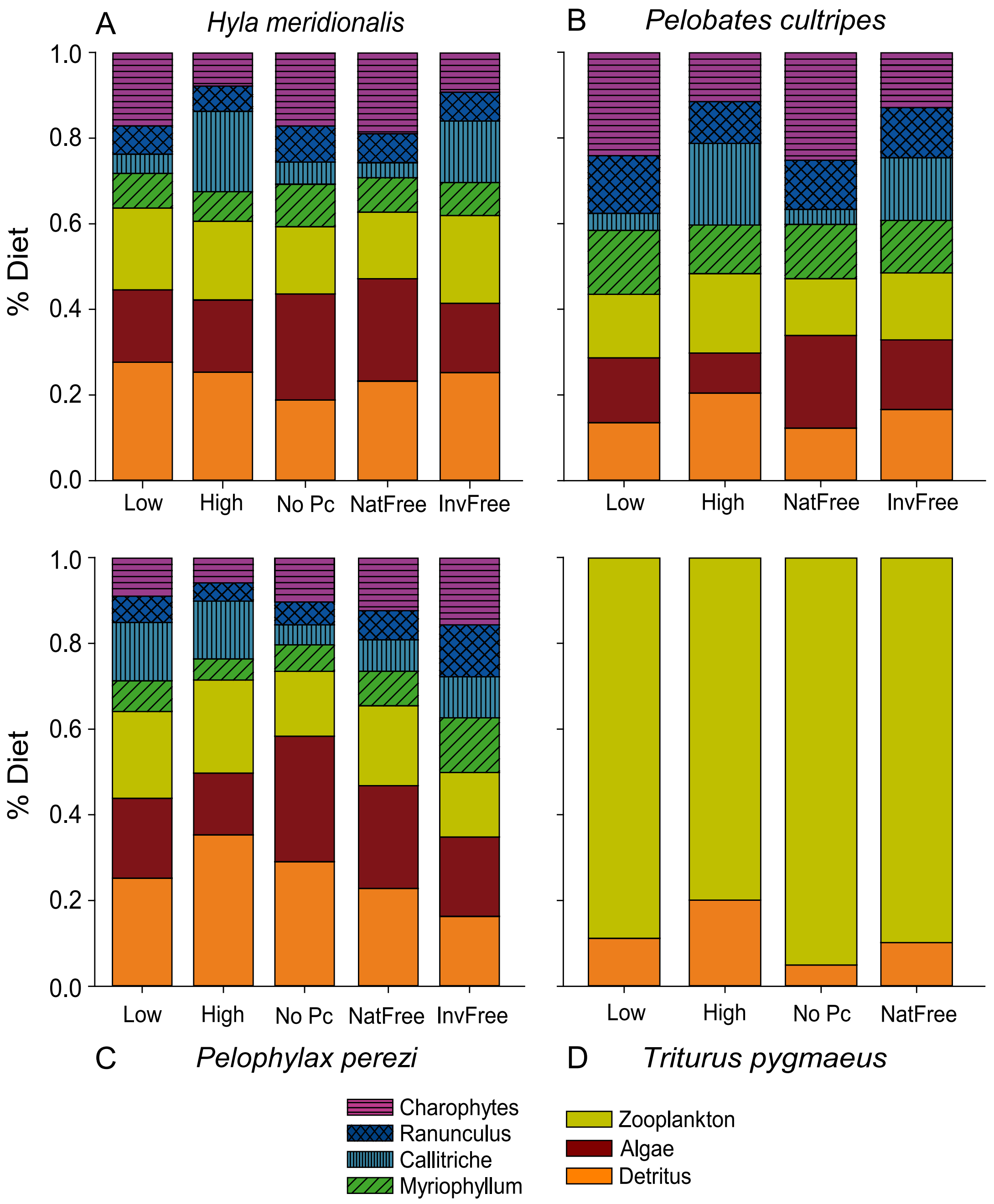

Fig. 4. Potential contribution of different food sources to the diet of each amphibian species. Mean estimated proportion of each of the seven potential sources (detritus, algae, zooplankton, and four types of macrophytes) in the diet of three anuran species under five different ecological scenarios: low and high larval density, absence of spadefoot toads (*P. cultripes*), presence of free native dytiscid beetles, or presence of invasive red swamp crayfish. In the case of newts (*T. pygmaeus*), only two sources were considered since they do not consume macrophytes or algae. Also, no newts survived the presence of free crayfish.

Detritus, algae and zooplankton appeared in the diet of all anurans, but their proportions varied among different treatments (Fig 4). Macrophytes (*Ranunculus*, *Callitriche* and *Myriophyllum*) and Charophytes were the main component of the diet in *P. cultripes*, with more than 50% of the diet in all treatments (Fig 4B). In the other anuran species, macrophytes were also important food sources, but they were not predominant as in spadefoot toad tadpoles. Only in the case of *P. perezi* exposed to free invasive crayfish did macrophytes reach similar importance in the diet (ca. 50%; *InvFree*, Fig 4C). High larval density caused all species (except *H. meridionalis*) to increase the proportion of detritus ingested. It was in detriment of the proportion of algae ingested for the species *P. cultripes* (Fig. 4B) and *P. perezi* (Fig. 4C). In contrast, at high larval density, the proportion of detritus did not vary for *H. meridionalis* (Fig. 4A) but it increased the ingestion of one of the macrophyte species (*Callitriche*), which was also the most consumed macrophyte for other anuran species in this treatment (*High*). *Hyla meridionalis* had similar diets when they were at high density as when invasive crayfish were present. However, the diet of *P. perezi* varied between these two treatments (*High* and *InvFree*), with a remarkable increase in macrophyte consumption and lower proportion of detritus when they coexisted with invasive free predators (Fig. 4C). The presence of crayfish, however, did not seem to affect the general composition of the diet of *P. cultripes*, where only the proportion of macrophytes assimilated changed in respect to the absence of predators. In the absence of *P. cultripes*, tadpoles of *H. meridionalis* slightly increased the proportion of algae in a similar way as occurred when native predators were present (Fig. 4A). Although almost negligible, the proportion of macrophytes also increased in comparison to when the entire community was present (Fig. 4A). The diet of *Pelophylax perezi*, however, shifted to a lower consumption of macrophytes and a remarkable increase of algae, which in this treatment contributed to diet in a similar proportion as detritus. The presence of native predators increased the proportions of algae in the diets of the three anuran species, which was more evident in the case of *H. meridionalis* and *P. cultripes*.

Since *T. pygmaeus* is a carnivorous species, we only analyzed the variation of zooplankton in relation to detritus as potential food sources. Detritus had a minor contribution to the diet in the absence of *P. cultripes* (No Pc), but considerably increased in the high-density treatment (Fig. 4D).

DISCUSSION

Analyzing the trophic position of amphibian larvae

Differences in δ^{15}N and δ^{13}C among species and treatments revealed differences among species in their trophic niche, as well as showed substantial flexibility in the use of alternative food sources within species depending on the environmental conditions experienced.

Newts presented high values of δ^{15}N, indicating that they fed mostly on animal matter. This is congruent with previous gut content analyses, which showed their carnivorous habits, with a strong preference for cladocerans (Díaz-Paniagua et al. 2005). Tadpoles are mainly herbivorous in which macrophytes and algae are important food sources, complemented with detritus and zooplankton. This composition coincides with the description of a previous study based on the description of gut contents of the same species (Díaz-Paniagua 1985). However, in relation to gut analyses, our isotopic studies reveal a higher importance of macrophytes and zooplankton than expected, since they appeared at lower frequencies in gut analyses. Nevertheless, macrophytes and zooplankton are the food sources with higher carbon and nitrogen concentrations (Table 3) and consequently have higher incidence in the assimilation onto tadpole tissues. In fact, these differences illustrate the main advantage of isotopic analyses over gut content analysis for diet determination (Layman et al. 2007b), as it shows the importance of particular resources with high assimilation.

Among tadpoles, spadefoot toads (*P. cultripes*) had the most enriched δ^{15}N and δ^{13}C values, close to the high values of larval newts. Tadpoles of this species caused a great impact on the pond structure, mainly due to their high plant biomass consumption (Arribas et al. 2014). The high prevalence of macrophytes in their diet thus explains the elevated isotopic signature of this species.

In general, the trophic structure of tadpoles in our study shows a large tadpole species that stands out as the prime macrophyte consumer (*P. cultripes*), and two species (*H. meridionalis* and *P. perezi*) in which other resources have higher importance than macrophytes, but show a wide variation in their proportions related to changes in their availability among treatments. All tadpoles included an additional amount of zooplankton as have been previously reported in their diet (Díaz-Paniagua 1985) and could be the result of scavenging, as suggested by the fact that they often co-vary with detritus consumption.

Diet shifts under varying conditions

The amphibian species studied did not equally respond to variations in the environmental factors manipulated, like larval density or predator presence. Changes in tadpole feeding may be related to changes in relative abundance of appropriate food items or to the quality of food sources (Altig et al. 2007). The inferred changes in diet through stable isotope analysis are consistent with observed changes in the environmental conditions across the experimental treatments, such as altered macrophyte biomass, changing zooplankton composition, and algal abundance.

High larval amphibian density, presence of *P. cultripes* tadpoles, and free-roaming invasive crayfish had caused increased nutrient availability in the water column, increased algal abundance, and caused macrophyte depletion (Arribas et al. 2014). High larval density also increased the proportion of copepods in relation to cladocerans (Arribas et al. 2014), which may be one of the explanations for the slightly different diet composition of larval newts at high density (Fig. 4D). The considerable reduction of plant biomass forced herbivorous tadpoles to increase their feeding on detritus, and probably this lack of macrophytes also enhanced the prey capture of larval newts from the detritus. Impoverishment of environmental conditions in the presence of *P. cultripes* included plant depletion and shifts in zooplankton composition (Arribas et al. 2014) and these changes were reflected in altered diets of other tadpoles, as we had hypothesized. In general, when *P. cultripes* was absent, macrophytes were more abundant and tadpoles showed enriched δ^{13}C. However, we observed that the two other tadpole species increased the proportion of algae, but only *H. meridionalis* slightly increased the proportion of macrophytes in the diet whereas *P. perezi* reduced it in the absence of *P. cultripes*. These differences are related to different availability of resources as well as to intrinsic differences in the general food habits between these species. The absence of the strong competitor, *P. cultripes,* may result in higher availability of algae and also in lower availability of detritus, as the presence of *P. cultripes* increases the proportion of detritus in the bottom of the tanks due to their high consumption of macrophytes. However, under the same availability of macrophytes, one species increased while the other reduced macrophyte consumption. These differences are in agreement with different feeding behavior described for these two species (Díaz-Paniagua 1987) as well as with their diet description based on gut contents, where detritus was one of the most consumed food resources for *P. perezi* (Díaz-Paniagua 1985). In addition, the morphology of the species also indicates that *P. perezi* is better adapted to feed at the bottom of the ponds, while *H. meridionalis* mainly feeds on the water column (Díaz-Paniagua 1985), where they probably also consume macrophytes as a complementary alternative resource. Newts also increased the proportion of zooplankton in their diet when *P. cultripes* was absent, showing higher δ^{13}C but substantially lower δ^{15}N values. This may be attributed to the observed differences in species composition of zooplankton (Arribas et al. 2014), since cladocerans increased in relative abundance with respect to copepods, and cladocerans typically show lower δ^{15}N values than copepods (Karlsson et al. 2004). Spadefoot toad larvae can thus be considered a strong competitor that markedly reduces the availability of resources that would otherwise be readily consumed by the rest of the amphibian guild. We expected native predators to cause an increase in the trophic level of surviving amphibians, since they would have access to more abundant and diverse food sources (including those with higher δ^{15}N values). Our results do not support our prediction since released competition via predation did not significantly alter the diet of amphibian larvae.

In accordance with our expectations, freely roaming crayfish produced a considerable change in the trophic status of tadpoles, with *H. meridionalis* and *P. cultripes* showing a slight decrease in δ^{13}C values whereas *P. perezi* experienced a similar increase as when *P. cultripes* was absent from the tanks. Invasive crayfish have a high disruptive potential of the trophic webs of aquatic systems (Anastácio et al. 2005, Gherardi and Acquistapace 2007). In our mesocosms, crayfish had a similar effect to that of high density of tadpoles, but had different consequences in different species. Only the diet composition of *H. meridionalis* was similar in both treatments. Also, the presence of crayfish did not affect the diet of *P. cultripes,* which was in general similar to that in the low-density treatment. The composition of the macrophytes, however, was more similar to the high-density treatment. Crayfish largely contributed to deplete plant biomass and impoverished water quality (Arribas et al. 2014), presumably forcing tadpoles to increase their ingestion of sediments or to feed on C-depleted resources. This also happened in the presence of *P. cultripes*, mainly at high densities. However, tadpoles of *P. perezi* did not modify their diet in the same way as in high density; instead they increased the proportion of plants in their diet in the presence of crayfish, considerably increasing their δ^{15}N. Crayfish commonly uproot or cut plants to favor the growth of bacteria and fungi that complement their diet (Geiger et al. 2005). This feeding behavior can increase the plant material included in sediments, favoring their consumption by detritivorous bottom dwellers such as *P. perezi*. Therefore, crayfish act not only as predators of amphibian larvae (Cruz and Rebelo 2005, Ficetola et al. 2011, Nunes et al. 2013) but also compete with some of them (Caut et al. 2013) for resources like algae and macrophytes. They may in turn favor more detritivorous species. These effects were not found when crayfish were caged, and the isotopic values of tadpoles were similar to those in the absence of predators. Nevertheless, larval newts showed a decrease in $\delta^{15}N$ with caged crayfish, which was likely due to the reduction in copepod abundance in favor of cladocerans caused by caged crayfish (Arribas et al. 2014).

Our results show that the anuran species studied are not specialists of specific types of food but rather opportunistic herbivores and/or detritivores. However, despite some degree of niche partitioning, the species studied were at the same time capable of exploiting a variable proportion of different food resources (algae, zooplankton, macrophytes, detritus), depending on the ecological scenarios. Thus, which food source predominates in each species' diet is largely conditioned by both intraspecific and interspecific competition, whereas we found little evidence for non-consumptive, trait-mediated predator effects. This ability of amphibian larvae to alter their trophic niche depending on the ecological scenario may be determinant in their capacity for exploiting widely divergent or fluctuating conditions in the large array of aquatic systems they inhabit.

CONCLUSION

The combination of ecological experiments and stable isotope analyses offers a powerful approach to test the effect of environmental changes on the trophic adjustment of interacting species and its consequences for food web structure. Our results show that the anuran species studied are not specialists of specific types of food but rather opportunistic herbivores and/or detritivores. However, despite some degree of niche partitioning, the species studied were at the same time capable of exploiting a variable proportion of different food resources (algae, zooplankton, macrophytes, detritus), depending on the ecological scenarios. Thus, which food source predominates in each species' diet is largely conditioned by both intraspecific and interspecific competition, whereas we found little evidence for non-consumptive, trait-mediated predator effects. Changes in diet due to different ecological interactions have direct or indirect consequences on the local structure of an ecosystem, as the food web interactions are also likely to be altered. The observed shifts in the amphibians' diet in response to environmental changes demonstrate that amphibians are greatly affected by intraspecific density, interspecific competition, and invasive species. This ability of amphibian larvae to alter their trophic niche depending on the ecological scenario may be determinant in their capacity for exploiting widely divergent or fluctuating conditions in the large array of aquatic systems they inhabit. Such shifts in the trophic niche of larval amphibians may strongly affect the structure and dynamics of the food web of temporary ponds, especially if such changes occur at a fast rate due to the introduction of invasive species or other factors causing rapid declines of amphibian populations.

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Appendix A. Initial total body length of the amphibian larvae and final total body length of the amphibian larvae or metamorphs included in each of the experimental treatments of the experiment. Total length is expressed in mm (TL, mean ± SE). We specify the final TL and number (n) of all individuals in the experiment, and also for those individuals only used for the isotopic analysis for: *Hyla meridionalis* (**Table A1**), *Pelobates cultripes* (**Table A2**), *Pelophylax perezi* (**Table A3**), and *Triturus pygmaeus* (**Table A4**).

Table A1	***Hyla meridionalis***				
		Experiment		Isotopic analysis	
Treatment	**Initial TL**	**Final TL (tadpoles)**	**Final TL (metamorphs)**	**Final TL (tadpoles)**	**Final TL (metamorphs)**
Low	23.06 ± 0.61 (n=120)	34.17 ± 1.74 (n=3)	14.11 ± 0.31 (n=34)	(n=0)	12.96 ± 0.18 (n=10)
High	24.21 ± 0.36 (n=360)	25.13 ± 0.98 (n=8)	14.03 ± 0.29 (n=22)	24.07±1.37 (n=5)	12.3 (n=1)
No Pc	23.96 ± 0.6 (n=120)	(n=0)	15.22 ± 0.16 (n=65)	(n=0)	15.33 ± 0.44 (n=11)
Nat Caged	24.75 ± 0.66 (n=120)	30.66 ± 2.7 (n=3)	13.96 ± 0.26 (n=39)	(n=0)	12.96 ± 0.46 (n=12)
Nat Free	23.27 ± 0.54 (n=120)	(n=1)	14.27 ± 0.28 (n=24)	(n=0)	14.04 ± 0.38 (n=9)
Inv Caged	23.78 ± 0.64 (n=120)	32.3 (n=1)	14 ± 0.19 (n=50)	(n=0)	13.05 ± 0.4 (n=11)
Inv Free	23.26 ± 0.6 (n=120)	(n=0)	14.51 ± 0.23 (n=35)	(n=0)	14.03 ± 0.72 (n=7)

Table A2	***Pelobates cultripes***				
		Experiment		Isotopic analysis	
Treatment	**Initial TL**	**Final TL (tadpoles)**	**Final TL (metamorphs)**	**Final TL (tadpoles)**	**Final TL (metamorphs)**
Low	31.05 ± 1.32 (n=36)	98.08 ± 1.96 (n=18)	30.22 ± 0.8 (n=14)	92.82 ± 3.02 (n=7)	30.29 ± 0.83 (n=9)
High	28.77 ± 0.85 (n=108)	68.92 ± 1.13 (n=76)	24.59 ± 0.29 (n=26)	70.48 ± 3.8 (n=9)	24.74 ± 0.85 (n=9)
No Pc					
Nat Caged	32.07 ± 1.35 (n=36)	85.79 ± 3.84 (n=13)	30.21 ± 0.8 (n=19)	92.6 ± 1.28 (n=4)	30.53 ± 1.25 (n=10)
Nat Free	31.91 ± 1.65 (n=36)	96.57 ± 4.46 (n=6)	32 ± 0.94 (n=20)	93.8 ± 7.66 (n=4)	32.22 ± 1.16 (n=12)
Inv Caged	31.84 ± 1.58 (n=36)	92.5 ± 2.08 (n=9)	30.54 ± 0.57 (n=26)	90.62 ± 5.29 (n=5)	30.43 ± 0.83 (n=11)
Inv Free	31.88 ± 1.52 (n=36)	82.13 ± 7.25 (n=2)	30.62 ± 0.55 (n=6)	89.88 ± 0.5 (n=2)	30.27 ± 0.83 (n=5)

Table A3	***Pelophylax perezi***				
		Experiment		Isotopic analysis	
Treatment	**Initial TL**	**Final TL (tadpoles)**	**Final TL (metamorphs)**	**Final TL (tadpoles)**	**Final TL (metamorphs)**
High	22.02 ± 1.26 (n=108)	30.67 ± 1.26 (n=31)	16.2 ± 0.65 (n=6)	28.82 ± 2.43 (n=9)	16.15 ± 1.45 (n=2)
No Pc	22.31 ± 2.17 (n=36)	45.65 ± 1.93 (n=20)	18.9 ± 0.45 (n=4)	47.12 ± 2.73 (n=9)	18.4 ± 1.2 (n=2)
Nat Caged	21.77 ± 2 (n=36)	36.13 ± 2.73 (n=12)	17.63 ± 1.39 (n=3)	34.05 ± 3.15 (n=8)	17.63 ± 1.39 (n=3)
Inv Caged	22.65 ± 2.25 (n=36)	40.62 ± 2.54 (n=16)	18.1 ± 0.62 (n=4)	41.27 ± 2.93 (n=8)	18.1 ± 0.62 (n=3)

Table A4	***Triturus pygmaeus***				
Species		Experiment		Isotopic analysis	
Treatment	**Initial TL**	**Final TL (tadpoles)**	**Final TL (metamorphs)**	**Final TL (larvae)**	**Final TL (metamorphs)**
Low	30.6 ± 0.6 (n=36)	52.5 ± 0.93 (n=19)	53.45 ± 0.95 (n=2)	52.54 ± 1.86 (n=7)	53.45 ± 0.95 (n=2)
High	31.67 ± 0.42 (n=108)	44.11 ± 0.54 (n=79)	52.4 (n=1)	49.14 ± 1.46 (n=9)	52.4 (n=1)
No Pc	31.66 ± 0.71 (n=36)	50.95 ± 0.92 (n=18)	54.11 ± 0.88 (n=8)	53.45 ± 1.42 (n=12)	55.1 ± 0.5 (n=2)
Nat Caged	32.81 ± 0.58 (n=36)	53.05 ± 0.84 (n=23)	55.5 (n=1)	53.67 ± 1.64 (n=10)	55.5 (n=1)
Nat Free	32.02 ± 0.89 (n=36)	54 ± 1.24 (n=20)	49.15 ± 2.05 (n=2)	56.38 ± 2.55 (n=7)	49.15 ± 2.05 (n=2)
Inv Caged	31.8 ± 0.88 (n=36)	52.97 ± 0.67 (n=29)	51.42 ± 0.41 (n=4)	54.81 ± 1.28 (n=10)	51.3 ± 0.56 (n=3)
Inv Free	32.56 ± 0.8 (n=36)	(n=0)	(n=0)	(n=0)	(n=0)

Appendix B. Stable isotopic values of carbon and nitrogen (mean ± 1 SE) of the different amphibian species included in the experiment, shown by treatments. The range of isotopic values (min – max, in parentheses) and the number of individuals per treatment (n) is also shown. Carbon values are corrected for lipids to account for variation (mean C:N = 3.51), following the equation for amphibian tadpoles in Caut et al (2013).

Treatment	δ^{13}C ‰				δ^{15}N ‰			
	H. meridionalis	*P. cultripes*	*P. perezi*	*T. pygmaeus*	*H. meridionalis*	*P. cultripes*	*P. perezi*	*T. pygmaeus*
Low	-22.4 ± 0.27 (-24.14, -20.93) n=10	-21.52 ± 0.23 (-22.88, -19.6) n=16	-24.08 ± 0.87 (-29.19, -21.66) n=8	-22.5 ± 0.52 (-24.85, -19.93) n=9	7.66 ± 0.39 (5.69, 9.12) n=10	10.22 ± 0.24 (8.37, 11.58) n=16	7.83 ± 0.49 (6.15, 10.04) n=8	10.51 ± 0.43 (9.18, 13.4) n=9
High	-24.45 ± 0.67 (-26.67, -22.09) n=6	-23.26 ± 0.18 (-25.21, -22.02) n=18	-27.88 ± 0.85 (-31.19, -24.74) n=11	-24.97 ± 0.35 (-26.92, -22.8) n=10	8.41 ± 0.29 (7.83, 9.49) n=7	9.56 ± 0.19 (7.4, 10.78) n=18	6.86 ± 0.32 (5.32, 8.55) n=11	10.36 ± 0.19 (9.37, 11.33) n=10
No Pc	-21.61 ± 0.50 (-24.44, -19.39) n=11		-20.85 ± 0.99 (-26.74, -17.26) n=11	-21.12 ± 0.24 (-22.42, -19.35) n=14	8.23 ± 0.50 (4.23, 10.35) n=11		4.42 ± 0.56 (2.08, 7.47) n=11	9.15 ± 0.25 (7.79, 10.7) n=14
Nat Caged	-22.02 ± 0.35 (-24, -20.11) n=12	-21.74 ± 0.32 (-24.26, -19.12) n=14	-24.58 ± 0.88 (-28.68, -18.38) n=11	-23.25 ± 0.43 (-25.79, -21.23) n=11	8.13 ± 0.33 (6.27, 10.18) n=11	10.34 ± 0.21 (9.21, 12.01) n=14	7.69 ± 0.29 (6.34, 9.62) n=11	10.34 ± 0.3 (8.48, 12.01) n=11
Nat Free	-21.41 ± 0.27 (-22.5, -20.27) n=9	-21.26 ± 0.32 (-24.78, -19.24) n=16	-22.29 ± 0.75 (-27.86, -20.03) n=10	-21.59 ± 0.59 (-24.49, -19.79) n=9	7.48 ± 0.39 (4.81, 8.72) n=9	9.75 ± 0.24 (8.49, 11.62) n=16	7.78 ± 0.46 (5.89, 10.83) n=10	9.89 ± 0.32 (8.75, 11.38) n=9
Inv Caged	-22.33 ± 0.23 (-23.45, -20.98) n=11	-21.36 ± 0.28 (-23.97, -19.74) n=16	-24.25 ± 0.47 (-26.69, -21.99) n=11	-22.41 ± 0.27 (-23.7, -20.25) n=13	8.05 ± 0.27 (6.75, 9.29) n=11	10.07 ± 0.36 (7.65, 12.38) n=16	7.82 ± 0.48 (6.18, 12.07) n=11	9.71 ± 0.21 (8.34, 11.09) n=13
Inv Free	-23.59 ± 0.42 (-24.95, -22.18) n=7	-22.67 ± 0.58 (-25.53, -21.06) n=7	-21.09 ± 0.91 (-25.72, -19.06) n=7	- - n=0	7.93± 0.55 (6.22, 9.69) n=7	9.58 ± 0.8 (6.5, 12.56) n=7	9.16 ± 0.65 (5.39, 10.63) n=7	- - n=0

Appendix C. **Potential food sources of the amphibian larvae included in the experiment.** Percentage (mean ± SD) and 95% confidence interval of each potential food source contributing to the diet of the different amphibian larvae included in the experiment in the density treatments and in the predator-free treatments for *H. meridionalis* (**Table C1**), *P. cultripes* (**Table C2**), *P. perezi* (**Table C3**), and for the newt *T. pygmaeus* (**Table C4**). Values reported resulted as output from SIAR models

Table C1	***Hyla meridionalis***									
Treatment	**Low**		**High**		**No Pc**		**NatFree**		**InvFree**	
Source	%	95%	%	95%	%	95%	%	95%	%	95%
Detritus	0.28 ± 0.09	0.11-0.44	0.25 ± 0.09	0.09-0.43	0.19 ± 0.08	0.015-0.34	0.23 ± 0.09	0.05-0.4	0.25 ± 0.09	0.08-0.43
Algae	0.17 ± 0.07	0.018-0.3	0.17 ± 0.09	0-0.33	0.25 ± 0.09	0.06-0.43	0.24 ± 0.09	0.06-0.41	0.16 ± 0.08	0.003-0.3
Zooplankton	0.19 ± 0.11	0-0.37	0.18± 0.09	0.002-0.34	0.16 ± 0.09	0-0.32	0.16 ± 0.1	0-0.33	0.21 ± 0.11	0.001-0.4
Myriophyllum	0.08 ± 0.06	0-0.2	0.07 ± 0.05	0-0.18	0.1 ± 0.07	0-0.23	0.08 ± 0.06	0-0.2	0.08 ± 0.06	0-0.19
Callitriche	0.05 ± 0.04	0-0.12	0.19 ± 0.08	0.018-0.33	0.05 ± 0.04	0-0.14	0.04 ± 0.03	0-0.1	0.14 ± 0.07	0.006-0.27
Ranunculus	0.07 ± 0.05	0-0.17	0.06 ± 0.05	0-0.15	0.08 ± 0.06	0-0.21	0.07 ± 0.06	0-0.18	0.07 ± 0.05	0-0.17
Charophytes	0.17 ± 0.09	0.005-0.33	0.08 ± 0.06	0-0.2	0.17 ± 0.09	0.001-0.33	0.19 ± 0.1	0.0022-0.36	0.09 ± 0.07	0-0.22

Table C2	***Pelobates cultripes***							
Treatment	**Low**		**High**		**NatFree**		**InvFree**	
Source	%	95%	%	95%	%	95%	%	95%
Detritus	0.14 ± 0.07	0.005-0.26	0.2 ± 0.07	0.08-0.33	0.12 ± 0.07	0.0007-0.24	0.17 ± 0.07	0.01-0.29
Algae	0.15 ± 0.06	0.02-0.26	0.09 ± 0.05	0.0006-0.18	0.22 ± 0.07	0.06-0.35	0.16 ± 0.08	0.005-0.29
Zooplankton	0.15 ± 0.09	0-0.3	0.19 ± 0.09	0.008-0.33	0.13 ± 0.08	0-0.28	0.16 ± 0.08	0.0007-0.3
Myriophyllum	0.15 ± 0.08	0-0.29	0.11 ± 0.07	0-0.24	0.13 ± 0.08	0-0.27	0.12 ± 0.08	0-0.25
Callitriche	0.04 ± 0.03	0-0.1	0.19 ± 0.05	0.1-0.28	0.04 ± 0.03	0-0.09	0.15 ± 0.07	0.006-0.27
Ranunculus	0.13 ± 0.07	0.0007-0.26	0.1 ± 0.07	0-0.21	0.11 ± 0.07	0-0.25	0.12 ± 0.07	0-0.24
Charophytes	0.24 ± 0.1	0.05-0.44	0.12 ± 0.07	0-0.25	0.25 ± 0.11	0.04-0.47	0.13 ± 0.08	0-0.26

Table C3	***Pelophylax perezi***									
Treatment	**Low**		**High**		**No Pc**		**NatFree**		**InvFree**	
Source	%	95%	%	95%	%	95%	%	95%	%	95%
Detritus	0.25 ± 0.09	0.066-0.44	0.35 ± 0.11	0.14-0.57	0.29 ± 0.13	0.025-0.55	0.23 ± 0.09	0.034-0.4	0.16 ± 0.08	0.005-0.3
Algae	0.19 ± 0.1	0-0.36	0.14 ± 0.11	0-0.34	0.29 ± 0.14	0.022-0.57	0.24 ± 0.11	0.017-0.44	0.18 ± 0.09	0.004-0.34
Zooplankton	0.2 ± 0.11	0-0.4	0.22 ± 0.13	0-0.45	0.15 ± 0.1	0-0.34	0.19 ± 0.11	0-0.37	0.15 ± 0.09	0-0.29
Myriophyllum	0.07 ± 0.06	0-0.18	0.05 ± 0.04	0-0.13	0.06 ± 0.06	0-0.18	0.08 ± 0.06	0-0.2	0.13 ± 0.08	0-0.26
Callitriche	0.14 ± 0.08	0-0.28	0.14 ± 0.08	0-0.28	0.05 ± 0.04	0-0.14	0.07 ± 0.06	0-0.19	0.1 ± 0.07	0-0.23
Ranunculus	0.06 ± 0.05	0-0.16	0.04 ± 0.04	0-0.11	0.05 ± 0.05	0-0.16	0.07 ± 0.05	0-0.17	0.12 ± 0.08	0-0.25
Charophytes	0.09 ± 0.07	0-0.22	0.06 ± 0.05	0-0.16	0.1 ± 0.08	0-0.26	0.12 ± 0.08	0-0.27	0.16 ± 0.09	0-0.3

Table C4	***Triturus pygmaeus***							
Treatment	**Low**		**High**		**NoPc**		**NatFree**	
Source	%	95%	%	95%	%	95%	%	95%
Detritus	0.11 ± 0.1	0-0.36	0.2 ± 0.16	0-0.52	0.05 ± 0.05	0-0.15	0.1 ± 0.1	0-0.33
Zooplankton	0.89 ± 0.1	0.64-1	0.8 ± 0.16	0.48-1	0.95 ± 0.05	0.85-1	0.9 ± 0.1	0.67-1

Capítulo 3

Predation and competition differentially affect amphibian guild structure and trophic niche of a Neotropical tadpole community[3]

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[3] Arribas R, Touchon J, Gomez-Mestre I. Predation and competition differentially affect the structure and trophic niche of a Neotropical tadpole community. *Under Review in Ecology.*

RESUMEN

La competencia y la depredación alteran la supervivencia, la tasa de crecimiento, el comportamiento y los rasgos de historia vitales de los individuos, y estos efectos pueden agrandarse y tener consecuencias en la estructura y la dinámica de la comunidad. En sistemas naturales, competencia y depredación ocurren frecuentemente a la misma vez y pueden modularse entre ellas de una forma altamente dinámica. Sin embargo, la importancia relativa de competencia y depredación dependerá en gran medida del grupo local de especies, los tipos de depredadores y el grado de segregación del nicho. Investigamos experimentalmente la interacción de competencia y depredación en la estructura y el estado trófico de una comunidad de larvas de anfibios Neotropicales utilizando una aproximación con mesocosmos en el centro de Panamá. Testamos el efecto de dos tipos de depredadores (larvas de libélulas o chinches acuáticas adultas) en combinación con la presencia/ausencia de un competidor común, la rana de ojos rojos (*Agalychnis callidryas*). También distinguimos entre efectos derivados del consumo y los no derivados del consumo de cada tipo de depredador, presentándolos libres o enjaulados a los renacuajos. Las larvas de libélulas fueron depredadores mas eficientes que las chinches acuáticas, pero estos efectos no fueron uniformes para las diferentes especies de renacuajos. Todas las especies de anfibios crecieron más cuando convivieron con la presencia de una larva de libélula libre, probablemente debido a la reducción de la competencia, pero los renacuajos fueron más pequeños cuando estaban expuestos a libélulas enjauladas, indicando la existencia de efectos no derivados del consumo por parte del depredador. La presencia de depredador también alteró la posición trófica relativa de las diferentes especies de anfibios, causando aumentos o disminuciones en la toma de nitrógeno dependiendo de la especie. La competencia interespecífica de *A. callidryas* redujo la supervivencia y altero la toma de carbono de algunas especies. Nuestros resultados indican que la composición de la comunidad puede afectar enormemente al estado trófico de las larvas de anfibios y que la depredación puede tener un mayor papel estructurador en las comunidades de larvas de anfibios Neotropicales que la competencia.

ABSTRACT

Competition and predation alter survival, growth rate, behavior, and life history traits of individuals, and these effects can scale-up to have consequences on community structure and dynamics. In natural systems competition and predation often occur at the same time and can modulate each other in a highly dynamic fashion. Nevertheless, the relative importance of competition and predation will depend largely on the local assemblage of species, the type of predators, or the degree of niche segregation. We experimentally investigated the interplay of competition and predation on the structure and trophic status of a Neotropical larval amphibian guild using a mesocosm approach in central Panama. We tested the effect of two types of predators (dragonfly nymphs or adult water bugs) in combination with the presence/absence of a common competitor, the red-eyed treefrog (*Agalychnis callidryas*). We also distinguished between consumptive and non-consumptive effects of each type of predator by presenting them to tadpoles caged or freely roaming. Dragonfly larvae were more efficient predators than water bugs, but these effects were not uniform for all tadpole species. All amphibian species grew bigger when raised in the presence of a free dragonfly nymph, presumably due to reduced competition, but tadpoles were smaller when exposed to caged dragonfly nymphs indicating the existence of non-consumptive predator effects. Predator presence also altered the relative trophic position of the different amphibian species, causing species-specific increases or decreases in nitrogen intake. Interspecific competition from *A. callidryas* reduced survival and altered carbon intake for some species. Our results indicate that community composition can greatly affect the trophic status of larval amphibians, and that predation may have a greater structuring role than competition within Neotropical larval amphibian guilds.

INTRODUCTION

The structure and dynamics of ecological communities is ultimately determined by just four basic processes: selection, drift, speciation, and dispersal (Vellend 2010, Kozak and Wiens 2012). Species can become incorporated into communities by speciation or dispersal, whereas drift and selection are responsible for fluctuations in the relative abundance of species in a community. Stochastic and deterministic factors influence temporal and spatial species turnovers that in turn determine which species interact at any given time and their relative abundances (Legendre et al. 2005), introducing broad-scale variation in community composition (Berg and Bengtsson 2007, Soininen 2010). Bioclimatic and local abiotic variables act as deterministic factors of community composition, depending on the tolerance limits of each species. On top of these factors, ecological interactions such as predation and competition are decisive in structuring community composition. Predation and competition directly affect trophic relationships among species, which are key in dictating species coexistence (Pimm 1982, Morlon et al. 2014). Therefore, more direct consideration of trophic interactions may be needed to accurately explain community composition.

Determining trophic relationships among interacting species is far from trivial. Although community ecology has often viewed species as fixed entities that interact in easily predictable ways (Ackerly 2003), the outcome of trophic interactions is highly context dependent because a set of interacting species may produce different community structures and dynamics under slightly different scenarios. For instance, hierarchical relationships among competing species within a guild can be intensified due to restricted food availability causing trophic overlap (Gómez-Campos et al. 2011). However, competition can also have the opposite effect, increasing niche partitioning through ecological diversification (Svanbäck and Bolnick 2007). Predators further complicate understanding trophic relationships, since they can relax or even reverse effects of competition (Relyea 2000, Gurevitch et al. 2000). Lastly, alterations in ecological interactions are not exclusively mediated by density-dependent effects but also by non-consumptive effects (Werner and Peacor 2003, Schmitz et al. 2004). This is because organisms often show a high degree of plasticity in their behavior, morphology, and life-history traits in response to competitors and predators (Werner and Peacor 2003, Prasad and Snyder 2006, Zanette et al. 2011). Plastic changes in the phenotype of interacting species may alter ecological interactions (Schmitz et al. 2004), for instance reducing the strength of competition (Relyea 2000, Mowles et al. 2011). The reverse can also occur and competition can change the strength of trait-mediated indirect effects (Bolnick and Preisser 2005).

Aquatic communities are amenable to experimental approaches because they can be reasonably well replicated in outdoor mesocosms to achieve a good combination of realism and experimental control (Wilbur 1997, Nystrom et al. 2001). Larval amphibians in particular make good model organisms for studying ecological interactions. Competition and predation have been extensively studied in temperate amphibian guilds (Morin 1983, Wilbur 1997), whereas tropical amphibian guilds have been far less studied, despite their greater diversity and hence increased potential for complex ecological interactions (Azevedo-Ramos et al. 1999, Hero et al. 2001). Little research has examined the combined effects of predation and competition on tropical anuran guilds (but see Gonzalez et al. 2011), and studies of the trophic ecology of tropical amphibians is similarly scarce (but see Whiles et al. 2006, Costa and Vonesh 2013b). Most of our perception about niche partitioning in tropical amphibians has been inferred from mouthpart morphology or behavior of a limited number of species (reviewed in Wells 2007, Altig et al. 2007).

We studied the consequences of consumptive and non-consumptive predator effects and competition on the trophic status, growth and survival of a tropical amphibian guild. Utilizing an amphibian guild of four core species, we conducted a mesocosm experiment manipulating the presence of a large tadpole with a high potential for competition (red eyed treefrog, *Agalychnis callidryas*; Gonzalez et al. 2011), and the presence of either caged or free-roaming predators (either water bugs or dragonfly nymphs). We assessed the trophic status of each amphibian species by means of stable isotopes to determine the degree of trophic overlap or trophic partitioning within the guild. Moreover, we also examined trophic plasticity of each species in response to different conditions experienced using stable isotopes (Post 2002, Caut et al. 2013). Stable isotopes reveal long-term assimilation of resources instead of a snapshot of the resources ingested, such as gut content analyses or behavioral tests might provide. Stable carbon isotopes ratios (δ^{13}C) identify major sources of energy, and stable nitrogen isotopes ratios (δ^{15}N) can be a powerful tool to estimate trophic position within a food web, as consumers are usually enriched by 3–4 ‰ in δ^{15}N relative to their food sources (DeNiro and Epstein 1981, Layman et al. 2012), and about 2 ‰ in tropical aquatic systems (Whiles et al. 2006). Changes in isotope ratios reflect variation in the resources an organism consumes (nutrient flow, Layman et al. 2012).

We hypothesized that all tadpole species within the guild would experience strong competition from *A. callidryas*, whose tadpole grows bigger than the other species and feeds mostly by filtering algae from the water column (Costa and Vonesh 2013a). We expected that other tadpoles co-habiting with *A. callidryas* would feed on periphyton or decomposing leaf litter at the bottom rather than from algae in the water column. This would result in altered carbon sources and in lower δ^{15}N values, as periphyton and leaf litter are considered low quality food and tend to show lower δ^{15}N than algae (see Schriever and Williams 2013). Alternatively, strong competition causing low food availability could increase consumer's δ^{15}N due to an increase of catabolism of their tissues (Bowes et al. 2014). Values of δ^{13}C would also be affected as algae and detritus have different isotopic signals and are variable depending on the environment.

We predicted that predators would reduce competition from *A. callidryas*, benefiting smaller tadpole species and shifting the carbon source of survivors. Free roaming predators were expected to exert direct consumptive effects, reducing competition, increasing body size of survivors and increasing the availability of higher trophic resources (i.e., increasing δ^{15}N). The two predators were chosen for their different niches and thus potential for different effects on tadpole trophic ecology. Water bugs generally remain near the top of the water column; we therefore expected them to cause tadpoles to feed mostly on leaf litter or periphyton in the bottom of the mesocosms, thereby showing a decrease in δ^{15}N. Dragonfly nymphs are more likely to remain in the leaf litter or on the walls of the mesocosms, potentially causing tadpoles to filter more algae from the water column, thereby increasing the trophic status of survivors (higher δ^{15}N). Lastly, we also hypothesized that indirect trait-mediated effects of predators would decrease tadpole growth through reduction of foraging activity, and constrain them to a lower trophic status (lower δ^{15}N) as tadpoles would tend to spend longer times in the bottom of the tank seeking refuge.

METHODS

Study system

The study was conducted at the Smithsonian Tropical Research Institute in Gamboa, Panama (9º7′17′′N, 79º42′11′′W) between 13 June 2012 and 13 July 2012. We included five species in this experiment that are locally abundant at the study site and that co-occur and breed throughout the rainy season (May-December at our study site). The red-eyed treefrog *Agalychnis callidryas* lays arboreal clutches that hatch in 6–7 days if left undisturbed (Gomez-Mestre et al. 2008). Tadpoles of this species feed primarily on phytoplankton or periphyton while suspended in the mid-water column (Warkentin 1999, Wells 2007). The pantless treefrog *Dendropsophus ebraccatus* lays eggs terrestrially and in water depending on the shade above the pond (Touchon and Warkentin 2008) and they hatch in ca. 3.5 days. Tadpoles are benthic grazers that feed on filamentous algae and periphyton along the water column (Costa and Vonesh 2013a). The small-headed treefrog *Dendropsophus microcephalus* and the red-snouted treefrog *Scinax ruber* lay clutches in the water and the eggs hatch in 2–3 days. The Tungara frog *Engystomops pustulosus* generates a floating foam nest (Ryan 1985) that protects the fertilized eggs until hatching after 3–4 days. The predators used for this study were aeshnid dragonfly larvae (Family Aeshnidae) and giant waterbugs *Belostoma sp*. (Family Belostomatidae), both of which are commonly found in the ponds where frog usually breed in Gamboa (Touchon and Vonesh, *in press*) (**Image 1**).

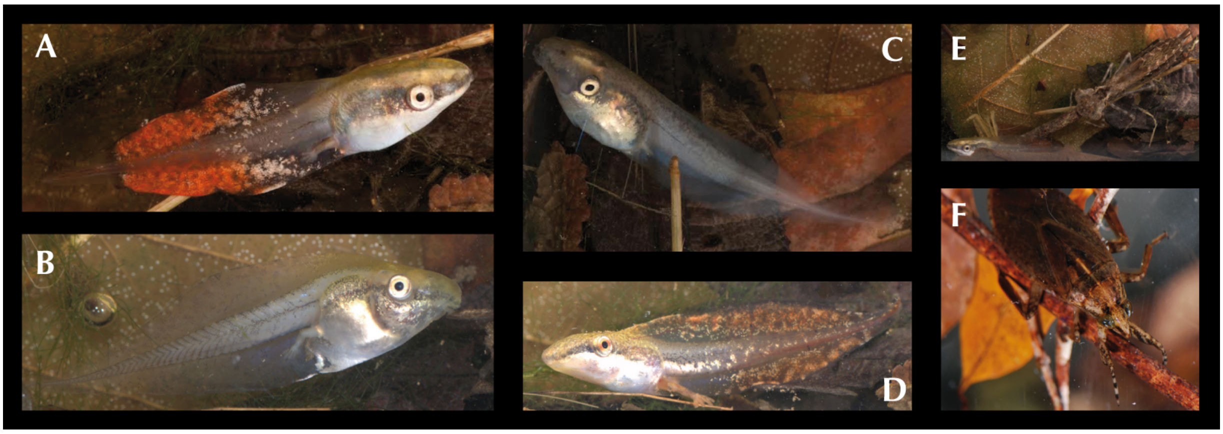

Image 1. Tadpoles (A-D) and predators (E-F) of the species included in this experiment (except for Tungara frog): A) *D. microcephalus*, B) *S. ruber*, C) *A. callidryas*, D) *D. ebraccatus*, E) *Anax* sp., and F) *Belostoma* sp. Photos: J. Touchon.

Experimental Design

To examine the interactions between competition with *A. callidryas* and predation on growth and survival of the larval amphibian community, we conducted a 2x5 factorial mesocosm experiment where we crossed presence of *A. callidryas* with one of five predator treatments: a no predator control (NP hereafter), either free roaming dragonfly larvae or adult water bugs (D-free and WB-free, respectively) or caged dragonfly larvae or adult water bugs (D-cage and WB-cage, respectively). Each treatment combination was replicated eight times for a total of 80 experimental tanks distributed in eight fully randomized spatial blocks (**Image 2**). This design allowed us to distinguish the competitive effect of *A. callidryas* and the consumptive and non-consumptive effects of predators (freely roaming or caged). Each mesocosm contained 10 tadpoles per species and therefore mesocosms with *A. callidryas* contained 50 tadpoles in total, whereas mesocosms lacking *A. callidryas* had 40 tadpoles.

Image 2. Mesocosm array at Gamboa, Panama, where the experiment was performed.

Our experimental mesocosms were arranged in an open field next to secondary forest and consisted of 400L round plastic tanks (0.75m diameter and 0.8m high) with screened drain holes at a height of 0.75m. Approximately one month before the experiment we added ca. 7000 cm^3 of loosely packed dry soil and leaf litter to each tank and filled them approximately one third full with collected rain water and aged tap water. The soil used was collected during the dry season from a dry pond that fills only during the wet season ("Bridge Pond" from Touchon and Vonesh, *in press*). All amphibian and predator species are known to occur in this pond. We covered the tanks with window screen to prevent colonization by unwanted organisms and allowed rainwater to fill the tanks by the beginning of the experiment. This process allowed a natural food base for the aquatic community to develop, thereby mimicking the conditions of many ponds in the area. To provide spatial complexity, we added three plastic pots containing plants transplanted from Bridge Pond. Each pot contained four individual plants and did not supply food in tanks. The plants were completely submerged beneath the surface of the water but grew and were alive throughout the entire experiment. Since our tanks contained a mixture of vegetation and soil and had been allowed to fill naturally, they contained a substantial amount of visible periphyton at the start of the experiment. No supplementary food was added to mesocosms during the experiment and tadpoles consumed only what had developed naturally.

Each tank contained two predator cages made of a fine mesh that were suspended at the top of the water column on the same side of each tank. All cages contained a stick for predators to perch on. Identical empty cages were included in tanks lacking a caged predator treatment. Dragonfly larvae and adult water bugs were collected from ponds in the area and haphazardly assigned to either free or caged predator treatment. The average body length of dragonflies was 18.2 ± 0.27 mm (mean ± SE here and throughout, n = 32) and that of water bugs was 40.38 ± 0.37 mm (n = 32). Caged predators were fed every other day with two tadpoles of any one of the five amphibian species included in the experiment, selected on a rotating basis, to ensure consistent alarm cues (Peacor 2006). We replaced predators that died as necessary.

To ensure that all eggs would hatch at approximately the same time, we collected clutches of *A. callidyras* first, then collected *D. ebraccatus* one day later, and clutches of *D. microcephalus*, *S. ruber* and *E. pustulosus* one day later (i.e., two days after collecting *A. callidyras*). This allowed all tadpoles to be essentially the same age post-hatching (two days) at the start of the experiment. All tadpoles and predators were dorsally photographed just before introducing them into the mesocosms. The experiment lasted for 4 weeks and we removed the remaining tadpoles and predators with dip nets between 10 and 13 July. Immediately upon removal, we individually photographed tadpoles and recorded body mass. All photographs included a ruler for scaling the image and to obtain measurements of predators' body length and tadpoles' body length and total length (body plus tail). For individuals that had already metamorphosed, we recorded snout-vent length (SVL). Photographs were measured using ImageJ 1.46r (NIH, USA). Initial total length was 11.8 ± 0.01 mm for *A. callidryas*, 6.5 ± 0.004 mm for *D. ebraccatus*, 5.4 ± 0.003 mm for *D. microcephalus*, 6.1 ± 0.004 mm for *S. ruber* and 8.3 ± 0.009 for *E. pustulosus*. There were no differences in initial size within species across treatments (all $p > 0.12$). At the end of the experiment, all individuals not used for stable isotope analyses were released at the site of collection.

Stable Isotopes Analysis

We performed analyses of stable isotopes on one individual per species per tank when available, from 6 of the experimental blocks. Isotopic data came from tadpoles (n = 178) for all species except *E. pustulosus* (n = 47), where most individuals had metamorphosed by the end of the experiment. The isotopic signature is not likely to differ between tadpoles and newly metamorphosed juveniles, in part because amphibians do not feed during metamorphosis (Arribas et al., *in press*). We euthanized individuals by immersion in MS-222, dissected tail muscle or leg muscle as appropriate, and then froze samples at -20 ºC until processing. Samples were oven-dried at 50 ºC to constant dry weight and ground to a fine and homogeneous powder with mortar and pestle. Weighed samples (0.3 ± 0.003 mg per sample) were processed at the Stable Isotopes Laboratory at Doñana Biological Station. Samples were combusted at 1,020 ºC using a continuous flow isotope-ratio mass spectrometry system by means of 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 ($\delta^{13}C$) and atmospheric N2 ($\delta^{15}N$), using the equation $\delta^{13}C$ or $\delta^{15}N = ((R_{sample}/R_{standard}) - 1) \times 1000$, where R is ^{13}C:^{12}C or ^{15}N:^{14}N. Laboratory standards run every nine samples, and calibrated with international standards, indicated measurement errors of ± 0.1 ‰ and ± 0.2 ‰ for $\delta^{13}C$ and $\delta^{15}N$, respectively. Amphibian $\delta^{13}C$ values were normalized to account for lipid variation in $\delta^{13}C$ (mean C:N = 3.41) following the recommended equations in Caut et al. (2013): $\delta^{13}C$ normalized = $\delta^{13}C$ untreated - 1.11 + 0.37 C:N.

Statistical analysis

We conducted all statistical analysis using R 2.15.3 (R Core Team 2013). We used generalized linear mixed models (glmer function in package lme4) to test for significant effects of the different treatments on survival, growth, and diet. We first performed an overall model including the three-way interaction among competition with *A. callidryas* (two levels, presence/absence), risk of predation (all 5 predator treatments), and amphibian species (four levels), with block as a random factor. In order to further understand the nature of our treatments (and because we had significant two- and three-way interactions in the overall model [see results]), we conducted several follow-up analyses. First, we evaluated the competitive effect of *A. callidryas* in the non-predatory environment. Second, in tanks containing *A. callidryas* (i.e., the complete amphibian guild) we tested separately the effects of caged predators (considering only control, WB-cage and D-cage treatments) and free predators (considering only control, WB-free and D-free treatments). Third, in the event of a significant interaction between predator treatment and amphibian species, we performed contrast analyses to examine differences between types of predators (dragonfly vs. water bugs). In all models, we used likelihood ratio tests to evaluate the significance of each predictor. Given that these multiple comparisons were not orthogonal, we corrected the *P*-values to minimize the false discovery rate (FDR; Benjamini and Hochberg 1995). Moreover, we used specific contrasts to check for effects of competition in the different amphibian species with respect to predation when the three-way interaction was significant.

We analyzed tadpole survival with generalized linear mixed models fitting a binomial error distribution using a logit link function, and analyzed tadpole size fitting a Gaussian error distribution. When appropriate, assumptions of normality and homoscedascity of errors were assessed by means of Shapiro-Wilks and Bartlett tests respectively in the simplified model, and we examined qq-plots and checked for overdispersion in the mixed effects models.

We occasionally found undesired dragonflies of various species in our mesocosms, accidentally added with the sediment or leaf litter. We included the number of unwanted dragonflies as a covariate in the analyses of tadpole survival, which was not significant ($\chi^2 = 2.257$; $P = 0.133$) and we thus removed it from the model. We also tested if the initial length of tadpoles explained differential survival among species, using tank means of initial total length for each species. We found no effect of initial body length on survival and the covariate was removed from the final model ($\chi^2 = 1.47$; $P = 0.22$). We analyzed growth using measurements of tadpoles' total length (final TL - initial TL), using tank means to compare growth among treatments and species. We log-transformed growth to meet parametric assumptions. In testing for differences in isotopic signatures, we first tested for an association between tadpole size (TL) and isotopic values for each species using linear regressions. We then used linear mixed models to test for the effects of predator presence, competition, and species on isotopic value ($\delta^{13}C$ or $\delta^{15}N$). Lastly, we performed the above analyses on each species separately to test the effect of the competitor *A. callidryas* and the effect of free and caged predators (Appendix A).

RESULTS

In the overall model, survival was not affected by the three-way interaction between competition, predation and tadpole species ($\chi^2 = 5.32$; $P = 0.95$) but was strongly affected by predator treatment ($\chi^2 = 262.93$; $P < 0.001$), tadpole species ($\chi^2 = 198.24$; $P < 0.001$), and the interaction between them ($\chi^2 = 189.65$; $P < 0.001$). Neither the 'competition x species' interaction nor the 'competition x predation' interaction were significant (both $P > 0.1$).

The three-way interaction between competition, predation and tadpole species was not significant for mean tadpole growth (final TL - initial TL) (χ^2 = 7.37; P = 0.5). Growth was affected by predator treatment (χ^2 = 17; P = 0.002), competition (χ^2 = 5.23; P = 0.02) and tadpole species (χ^2 = 286.27; $P < 0.001$), and no interactions were significant (all $P > 0.3$). We excluded *E. pustulosus* because most individuals had already metamorphosed and their size was not comparable to the other species, which were tadpoles.

The only significant predictor for $\delta^{13}C$ isotopic values in the overall model was tadpole species (χ^2 = 101.04; $P < 0.001$). For $\delta^{15}N$ values, however, the three-way interaction between competition, predation and tadpole species was significant (χ^2 = 22.65; P = 0.031) indicating that the combined effects of predation and competition varied for different tadpole species (see *Among species variation* below). Statistical results for the effects of competition and either free or caged predators on amphibian survival, growth and stable isotopes are shown in Table 1.

Total body length (TL) of all species (except *E. pustulosus*) was positively related to $\delta^{13}C$ (Pearson's r = 0.31, $P < 0.001$) but no correlation was observed between size and $\delta^{15}N$. Within species, TL was marginally positively related to $\delta^{13}C$ in *S. ruber* (Pearson's r = 0.29, P = 0.06), but no such correlation was found in other species. $\delta^{15}N$ decreased with size in *A. callidryas* (Pearson's r = -0.53, P = 0.009) but increased with size in *S. ruber* (Pearson's r = 0.34, P = 0.03) and *E. pustulosus* (Pearson's r = 0.36, P = 0.02). In particular, *D. ebraccatus* fed at the highest trophic level (highest $\delta^{15}N$ values) but had relatively small tadpoles.

Effects of competition

In the absence of predators, competition from *A. callidryas* did not significantly affect survival of the other amphibian species (see Table 1 for statistical results). Although not significant, *A. callidryas* tended to improve survival of all species except *D. ebraccatus* (Table 2). Similarly, the presence of *A. callidryas* did not significantly affect growth or stable isotopes of the other species (Table 1, all $P > 0.12$).

Table 1. Community responses (for survival, growth and stable isotopes) to competition and predation. Results for the general statistical models are expressed using likelihood ratio tests. Analysis were performed in all amphibian species for the scenery of predation (n = 5) except for growth (n = 4) where we excluded *E. pustulosus* as the majority of individuals were metamorphs and not comparable in length with tadpoles. Analysis for competition compared a non-predatory environment with or without the species *A. callidryas* so that n = 4 for all variables except growth (n = 3). Values in bold indicate $P < 0.05$.

	Survival		**Growth**		**δ^{13}C**		**δ^{15}N**	
Statistical Models	χ2	*p*	χ2	*p*	χ2	*p*	χ2	*p*
Competition								
Competition*Species	0.5814	0.9	7.371	0.13	2.669	0.445	2.35	0.5
Competition	0.7074	0.4	3.475	0.66	2.303	0.129	1.4726	0.225
Species	29.024	**<0.0001**	1.7846	**<0.0001**	14.666	**<0.0001**	34.535	**<0.0001**
Predation: Free Predators								
Predation*Species	72.867	**<0.0001**	6.355	0.385	4.803	0.778	19.525	**0.012**
Predation	204.09	**<0.0001**	11.297	**0.0035**	5.083	0.078 *	9.976	**0.007**
Species	84.468	**<0.0001**	107.79	**<0.0001**	34.283	**<0.0001**	80.386	**<0.0001**
Predation: Caged Predators								
Predation*Species	8.994	0.3428	1.703	0.945	14.734	0.064 *	14.9	0.06 *
Predation	5.074	0.079 *	18.91	**<0.0001**	1.45	0.484	8.248	**0.016**
Species	83.495	**<0.0001**	82.094	**<0.0001**	35.515	**<0.0001**	98.83	**<0.0001**

Effects of free predators

Both free water bugs and free dragonfly larvae greatly reduced tadpole survival. However, not all tadpole species were equally prone to predation, as noted by the significant "predator x species" interaction (Fig. 1, Table 1). Dragonfly predation varied greatly; *E. pustulosus* was the most consumed (94%), followed by *A. callidryas* (91%), and *S. ruber* (86%), whereas *D. ebraccatus* and *D. microcephalus* were less impacted (60% and 49% mortality, respectively) (see Appendix A for mean ± SE % survival in the different treatments). Water bugs also negatively impacted tadpole survival; the most affected species was *E. pustulosus* (80%), followed by *S. ruber* (51%), *D. ebraccatus* (50%), and *D. microcephalus* (36%) whereas the least affected species was *A. callidryas* (24%).

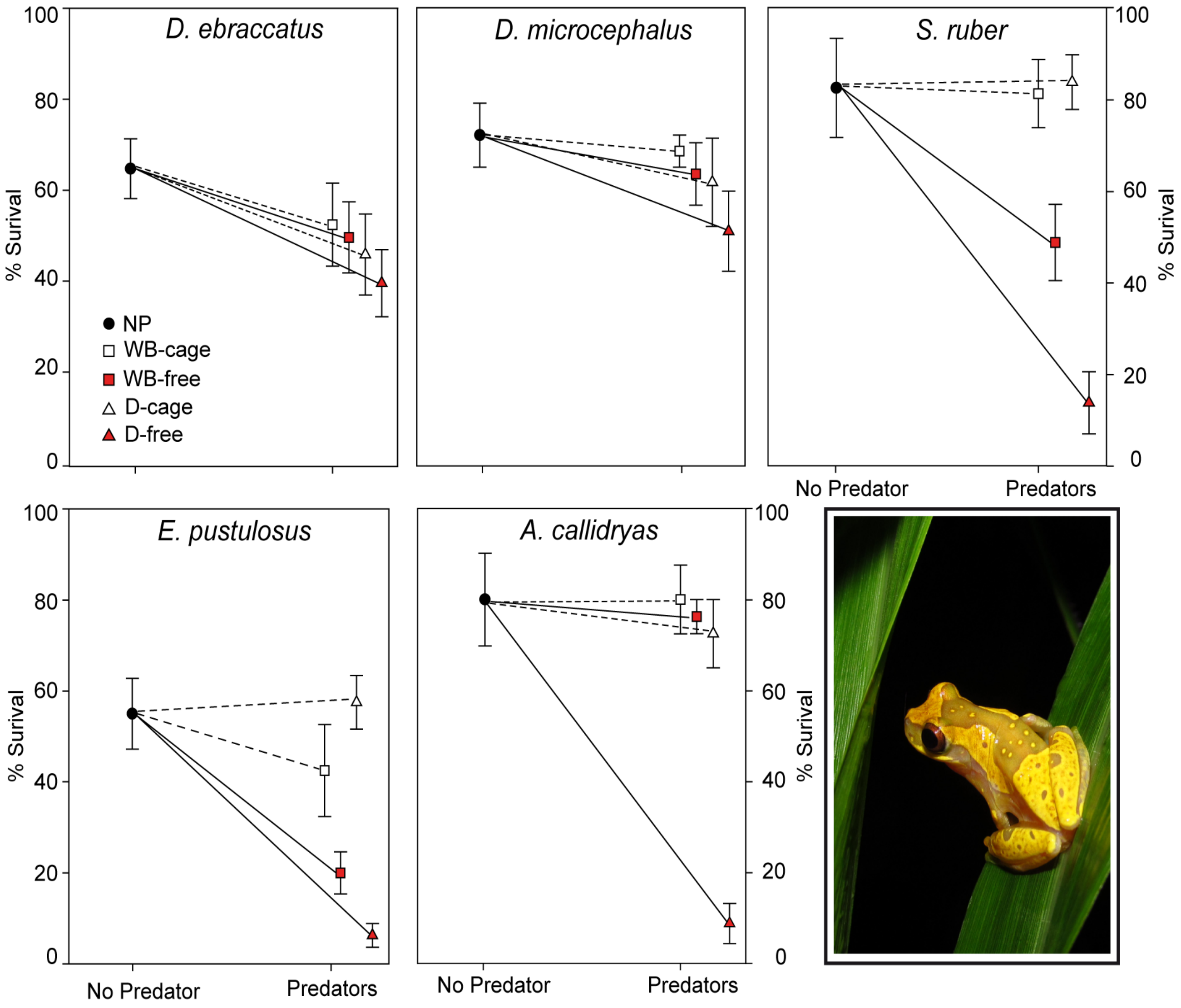

Fig. 1. Survival (mean ± SE, %) of: A) *D. ebraccatus*; B) *D. microcephalus*; C) *S. ruber;* D) *E. pustulosus;* and E) *A. callidryas* in a non-predatory environment (left, No Predator) and in each of the 4 predatory treatments (right) of this experiment. Treatments with water bugs (WB) are represented with squares and dragonflies (D) are represented with triangles. Open symbols and dashed lines represent caged predators and filled symbols represent free predators. On the bottom right, adult of the hourglass treefrog, *D. ebraccatus*.

Free predators had a significant effect on tadpole growth (Table 1, Fig. 2). However, in general, the two predators exerted opposing effects. For example, *D. ebraccatus* growth increased 20.5% with free dragonflies whereas free water bugs decreased growth by 6.8% (compared to predator-free controls). Similar patterns were seen for *A. callidryas* and *S. ruber* (Table 2) although only the effect of dragonflies on *S. ruber* was statistically significant (z = 2.22, *P* = 0.04, Table B4). Lastly, predator effects were not uniform across amphibian species, as free dragonflies reduced total length, weight, and leg length in *E. pustulosus* (all $P < 0.003$).

Free predators did not significantly affect the values of δ^{13}C, but had varying effects on δ^{15}N values (see Table 1). Free dragonflies decreased δ^{15}N of *D. microcephalus*, *S. ruber* and *A. callidryas* but slightly increased δ^{15}N of *D. ebraccatus* (Fig. 3 and Appendix B). Free water bugs greatly increased δ^{15}N in *D. ebraccatus*, but decreased δ^{15}N for *A. callidryas* and *S. ruber* (Fig. 3). Neither δ^{13}C nor δ^{15}N values varied with predation in *E. pustulosus*.

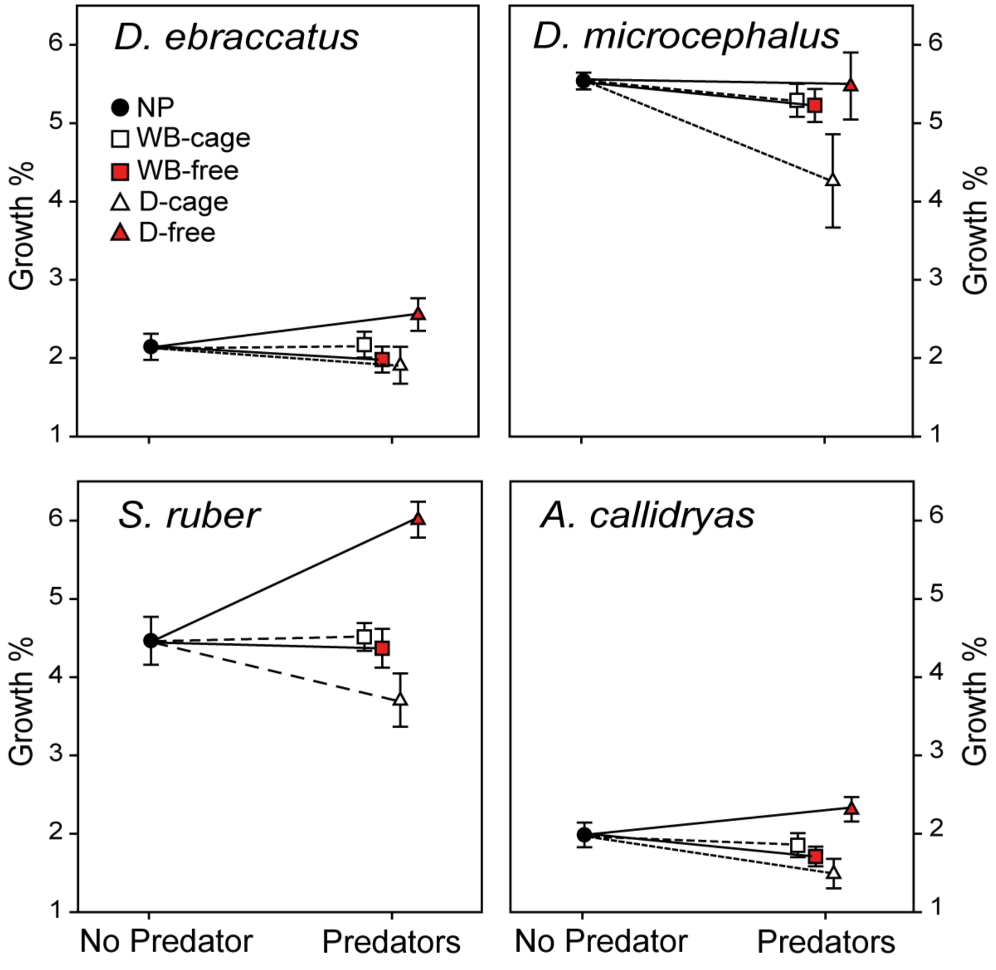

Fig. 2. Percentage of growth during the experiment in respect to the initial size (mean ± SE) of: A) *D. ebraccatus*; B) *D. microcephalus*; C) *S. ruber;* and D) *A. callidryas* in a non-predatory environment (left, No Predator) and in each of the 4 predatory treatments (right) of this experiment. Treatments with water bugs (WB) are represented with squares and dragonflies (D) are represented with triangles. Open symbols and dashed lines represent caged predators and filled symbols represent free predators.

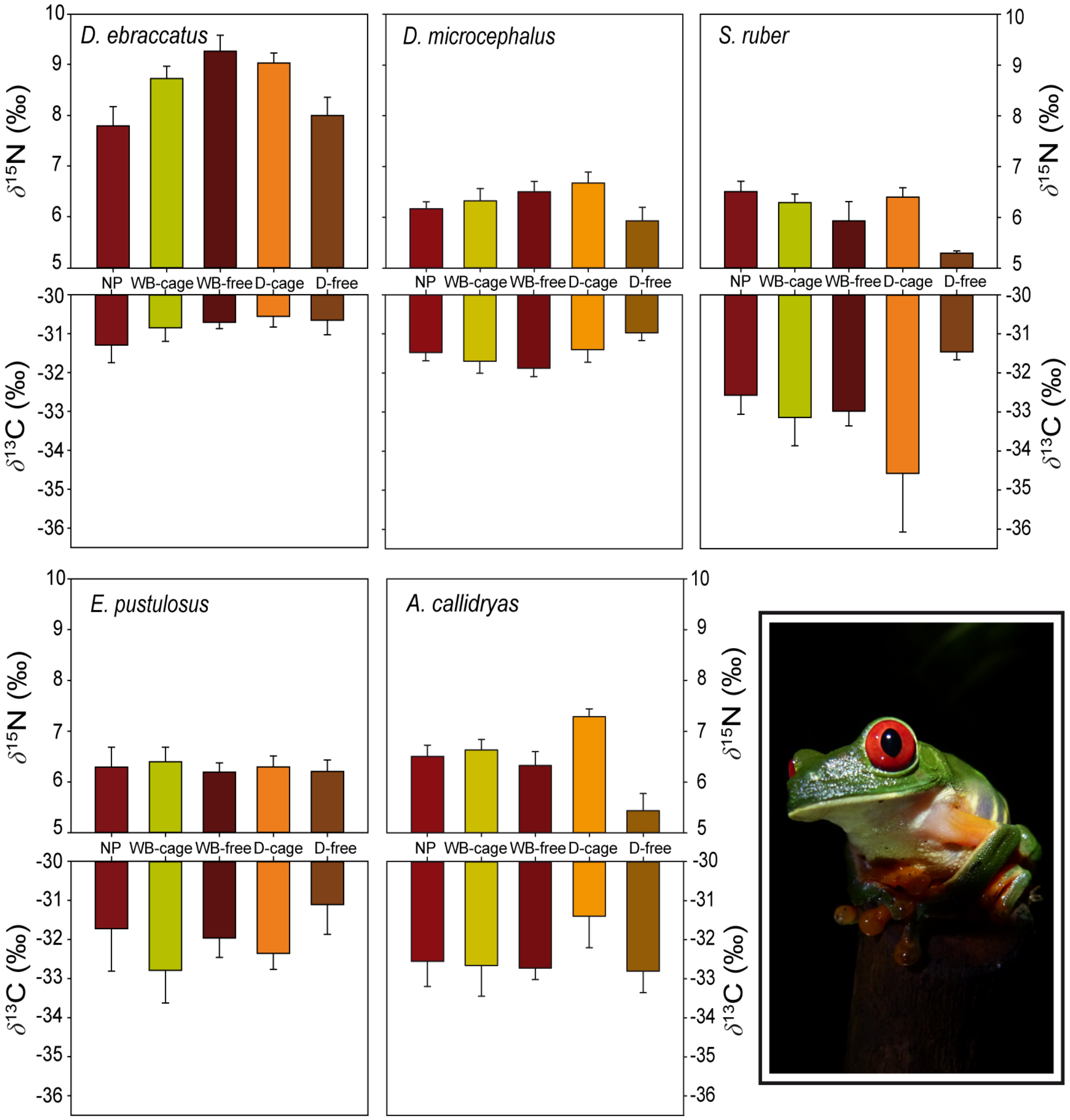

Fig. 3. Stable isotopic values (mean ± SE) for each tadpole species included in the mesocosms: A) *D. ebraccatus*; B) *D. microcephalus*; C) *S. ruber;* D) *E. pustulosus;* and E) *A. callidryas*. $\delta^{13}C$ and $\delta^{15}N$ values are represented with bars in different colors for each predatory treatment. On the bottom right, adult of the red eyed tree frog, *A. callidryas*

Effects of caged predators

Caged predators did not significantly affect amphibian survival. Although species survival varied overall (Fig. 1), survival was unaffected by the presence of caged dragonflies or water bugs (Table 1, and Appendices A and B). Caged predators did, however, have a significant effect on tadpole growth (Fig. 2 and Table 1). Caged dragonflies had the largest effect, considerably decreasing growth of all tadpoles (t = -2.68, $P = 0.02$) and the SVL of *E. pustulosus* metamorphs (t = -2.68, $P = 0.048$). Growth in the presence of caged water bugs did not differ from the control (t = -0.32, $P = 0.94$) (see Appendices A and B).

Furthermore, caged predators differently affected the stable isotope values of different tadpole species (Fig. 3 and Appendix B), as indicated by the marginally significant 'caged predator x species' interaction for both δ^{13}C and δ^{15}N ($P = 0.06$, Table 1). Looking at tadpole species individually, caged dragonflies slightly reduced δ^{13}C of *S. ruber* by 6.14% (n.s.) but increased the value of δ^{13}C of *A. callidryas* by 3.56% ($P = 0.04$). Similarly, caged dragonflies increased the value of δ^{15}N of *A. callidryas* (by 12%, $P = 0.03$), *D. ebraccatus* (by 15.9%, $P = 0.004$) and *D. microcephalus* (by 8.3%, $P = 0.005$). Caged water bugs only had a significant effect of increasing δ^{15}N in *D. ebraccatus* (by 11.9%, $P = 0.017$).

Relative importance of predation and competition

The largest tadpoles at the end of the experiment were *A. callidryas*, followed by *D. microcephalus* and *S. ruber*. Mean growth of each tadpole species after four weeks is given in Appendix A. The effect of predation on survival and growth was stronger than the effect of competition, as indicated by their effect sizes (Table 2). However, not all amphibians responded in the same way. Only *D. ebraccatus* suffered from competition from *A. callidryas*, exhibiting reduced survival and decreased growth rate (Table 2). The presence of *A. callidryas* instead increased survival and growth of the other amphibian species (Table 2). Effects of competition on stable isotope signatures were more equivocal, again with the exception of *D. ebraccatus*, where the presence of *A. callidryas* greatly reduced δ^{15}N values (Table 2). Having *A. callidryas* in the mesocosms also greatly increased the survival of *E. pustulosus*.

Table 2. Magnitude and effect size of competition and predation on survival, growth, and stable isotopic values of the amphibian community. For *A. callidryas* the effect of predation is shown. For the effect of competition, we calculated the percentage of increase or decrease between the treatments with and without *A. callidryas* in a non-predatory environment. For the effect of predation, we calculated the percentage of increase or decrease between the treatments with and without free dragonflies or free water bugs where all 5 species were present (including the competitor *A. callidryas*). Effect size is expressed with Cohen's *d*.

	Survival			**Growth**		
Species	**Competition**	**Free dragonflies**	**Free water bugs**	**Competition**	**Free dragonflies**	**Free water bugs**
D. ebraccatus	- 1.88 %, 0.065	- 38.46 %, 1.273	- 23.07 %, 0.737	- 15.08 %, 0.808	+ 20.51 %, 0.798	- 6.82 %, 0.316
D. microcephalus	+ 5.05 %, 0.165	- 29.04 %, 0.972	- 11.73 %, 0.435	+ 4.96 %, 0.437	- 0.38%, 0.024	- 5.21 %, 0.727
S. ruber	+ 6.45 %, 0.186	- 83.33 %, 2.691	- 40.9 %. 0.687	+ 2.75 %, 0.116	+ 25.55 %, 1.91	- 2.6 %, 0.16
E. pustulosus	+ 10 %, 0.776	- 88.63 %, 2.964	- 63.63 %, 1.93	-	-	-
A. callidryas	-	- 89.06 %, 3.21	- 4.69 %, 0.173	-	+ 8.98 %, 0.505	- 13.99 %, 0.884
All species	+ 8.1 %, 0.777	- 66.17 %, 1.864	- 27.05 %, 0.751	- 0.31 %, 0.01	+ 6.17 %, 0.193	- 0.075 %, 0.257
	$\delta^{13}C$			**$\delta^{15}N$**		
D. ebraccatus	+ 1.27 %, 0.332	- 2.04 %, 0.702	- 1.87 %, 0.837	- 9.6 %, 1.25	+ 2.58 %, 0.926	+ 18.77 %, 1.93
D. microcephalus	- 0.75 %, 0.192	- 1.6 %, 1.042	+ 1.28 %, 0.776	- 0.51 %, 0.081	- 3.88 %, 0.495	+ 5.37 %, 0.786
S. ruber	- 3.63 %, 0.539	- 3.4 %, 1.273	+ 1.26 %, 0.371	+ 0.51 %, 0.035	- 18.62 %, 3.4	- 8.82 %, 0.726
E. pustulosus	- 2 %, 0.314	- 1.94 %, 0.315	+ 0.75 %, 0.126	- 4.82 %, 0.355	- 1.39 %, 0.129	- 1.59 %, 0.147
A. callidryas	-	+ 0.76 %, 0.189	+ 0.52 %, 0.139	-	- 16.37 %, 1.9	- 2.72 %, 0.308
All species	- 0.81 %, 0.14	- 2.33 %, 0.585	+ 0.26 %, 0.065	- 6.6 %, 0.438	- 1.54 %, 0.098	+ 4.08 %, 0.234

Free predators exerted the greatest impact on amphibian survival, primarily on *E. pustulosus* and *S. ruber* (Table 2). Dragonflies had a greater effect on survival than water bugs. The two predators had opposite effects on amphibian growth, with free dragonflies increasing growth of all species except *D. microcephalus*, whereas free water bugs reduced growth of all species. The greatest effect sizes were produced by dragonflies increasing growth of *S. ruber* (Cohen's *d* = 1.91) and by water bugs decreasing growth of *A. callidryas* (Cohen's *d* = 0.88).

The two predators tended to have opposite effects on carbon, with free dragonflies decreasing and free water bugs increasing δ^{13}C. The exceptions were *D. ebraccatus* and *A. callidryas*, where both predators caused decreases in *D. ebraccatus* δ^{13}C and increases in *A. callidryas* δ^{13}C (Table 2). Predators clearly impacted the trophic level tadpoles were feeding as indicated by the decrease in δ^{15}N values with both types of predators. In particular, dragonflies decreased δ^{15}N of *S. ruber* and *A. callidryas* by 18.6 and 16.4 %, respectively, whereas water bugs increased δ^{15}N of *D. ebraccatus* by 18.8 % (Table 2). Although not statistically significant, competition and predation shifted the isotopic values of δ^{13}C and δ^{15}N in opposite ways in *D. ebraccatus* (Table 2). Moreover, the significant 'competition x predator x species' interaction on δ^{15}N indicates the effect of competition on trophic ecology is dependent upon the tadpole species and the predatory environment. For example, *A. callidryas* in the absence of predators did not have an effect on δ^{15}N of *D. microcephalus* whereas it produced a significant decrease of δ^{15}N in the presence of a free dragonfly (Table 3).

Table 3. Effects of competition on δ^{15}N, with respect to predation. We specify when the diet of a species is not affected by *A. callidryas* (No effect) and when the presence of either free or caged predators affects the isotopic values of δ^{15}N. Significance levels were set at $p<0.05$***, $0.075<p>0.05$** and $0.1<p>0.075$*.

Treatment	***D. ebraccatus***	***D. microcephalus***	***S. ruber***	***E. pustulosus***
No predator	Decrease**	No effect	No effect	No effect
Caged Belostoma	No effect	No effect	No effect	No effect
Free Belostoma	No effect	No effect	Decrease*	No effect
Caged Dragonfly	Increase*	No effect	No effect	No effect
Free Dragonfly	Decrease**	Decrease***	Decrease**	No effect

DISCUSSION

Competition and predation are two key deterministic factors for the structure and dynamics of ecological communities (Paine 1966, Vellend 2010, Arribas et al. 2014). These two factors often occur at the same time and the mere presence of predators or competitors, in addition to their density-dependent effects on prey, may force organisms to plastically shift their phenotype (e.g., behavior, feeding structures, metabolism) thereby altering the realized trophic niche of the species (Levin 2009, Caut et al. 2013). The relative importance of competition and predation is largely dependent upon factors such as the biogeography, the type of predators and competitors, whether the habitat is permanent or ephemeral, or the degree of niche segregation (Azevedo-Ramos et al. 1999, Gurevitch et al. 2000, Chase et al. 2002). In our experiment, we quantified the effects of predation and competition on the trophic ecology of a Neotropical larval amphibian guild.

Predators can greatly affect differential prey survival and alter their growth trajectories by non-randomly consuming individuals of different size classes (Claessen et al. 2002), inducing novel behavioral or morphological phenotypes (Lima 1998), or through thinning and ensuing release of resources for the remaining individuals (Van Buskirk and Yurewicz 1998, Brodin and Johansson 2002). These effects of predators may have cascading consequences for community structure (Post et al. 2008; Arribas et al 2014).

Trophic ecology of tropical amphibians is dependent upon environmental conditions and the nature of ecological interactions. We found that predation played a more important role than competition in shaping the structure of our tropical tadpole guild. Freely roaming predators greatly reduced survival of all amphibian species. Dragonfly nymphs had a very strong impact on amphibian survival, reducing survival of three species by more than 80% and subsequently increasing growth of the surviving larvae of all species 9–26% compared to predator free controls (Table 2). Belostomatid water bugs were less effective predators than dragonflies, preferentially consuming *E. pustulosus* and *S. ruber*, and generally reducing growth in all species. Most individuals of *E. pustulosus* had already metamorphosed by the end of the experiment, and predation by water bugs may have occurred during metamorphosis, when amphibians become highly vulnerable (Vonesh and Warkentin 2006). Water bugs and dragonflies are common predators in the study area and are known to impact *A. callidryas* and *D. ebraccatus*, although previous studies have always been conducted in less diverse communities and less complex physical environments (McCoy et al. 2012, Touchon and Wojdak 2014). Given the high predation effects previously documented on these two tadpole species, it appears the presence of multiple tadpole prey species in the environment reduces the predation risk to others. For example, dragonflies rapidly decimated *D. ebraccatus* in one study (Gonzalez et al. 2011), consuming more than 90% of tadpoles in only eight days, whereas during our four-week experiment only 38% of *D. ebraccatus* tadpoles were consumed. In view of these results, it is clear that realistic estimates of predation rates need to take into account other biotic elements of the natural community.

Direct consumption is not the only way that predators can affect community structure. Predators can have many non-lethal, indirect effects on morphology, behavior, or life history which also have important consequences for community dynamics (Lima 1998, Agrawal 2001). Contrary to free dragonflies, the non-lethal presence of caged dragonflies reduced tadpole growth, as the perceived risk of predation through chemical cues typically causes reduced activity and lower metabolism (Van Buskirk and Yurewicz 1998, Werner and Peacor 2003). We did not detect indirect effects of water bugs, although phenotypic responses have been seen in other studies (McIntyre et al. 2004, Vonesh and Warkentin 2006).

Like predators, competitors can negatively affect individuals, and competition itself can be negatively affected by predators (Gurevitch et al. 2000). Contrary to our expectations, all species except *D. ebraccatus* survived somewhat better in the presence of *A. callidryas* although this was often not statistically significant (Table 2). However, this was only in the absence of predators; dragonflies or water bugs generally reversed the effect of competition on survival (Appendix A). *Dendropsophus ebraccatus* was also the only species whose growth was negatively impacted by competition from *A. callidryas*, similar to previous studies (Gonzalez et al. 2011). This may indicate that *A. callidryas* competes differentially with certain species in the guild and others may even benefit from its presence, perhaps by reducing effects of *D. ebraccatus* on other species.

Shifts in trophic status

Stable isotopes constitute a valuable tool to evaluate predator or competitor impacts on the trophic status of individuals, and represent the combination of food sources an organism has accumulated over time (Kilham et al. 2009). Values of δ^{15}N are usually enriched between 2 and 5‰ relatively to their prey (Post 2002) so that greater values are indicative of a higher trophic position (i.e., consuming organisms higher in the food web; (Fry 2006, Newsome et al. 2007). However, the trophic discrimination of nitrogen and carbon stable isotopes (change in isotopic ratios between consumer and resource, Δ) in tropical aquatic systems differ from their temperate counterparts, with lower Δ^{15}N (1-2%) and higher Δ^{13}C (1-1.6%) values (Kilham et al. 2009). Stable isotopes helped us identify that tadpoles altered their feeding sources in response to the presence of predators and competitors, although different species responded in different ways. For example, *D. ebraccatus* fed at a higher trophic level with free predators, whereas *A. callidryas* and *S. ruber* fed at a lower trophic level under the same conditions. Similarly, competition with *A. callidryas* caused *D. ebraccatus* and *E. pustulosus* to feed at a lower trophic level, whereas competition had essentially no effect on *D. microcephalus* and *S. ruber*. Thus, the same predator or competitor can have very different, even opposing, effects on different tadpole species.

The interacting effects of predation and competition on tadpoles' trophic status varied across species. These effects were primarily seen for nitrogen, but one species (*S. ruber*) also showed significant changes in carbon. An example of the interplay between predation and competition can be seen when looking at the effects of *A. callidryas* on the smaller *D. ebraccatus* (Table A1). In the absence of predators, *A. callidryas* drove down the trophic status of *D. ebraccatus*. However, free water bugs removed the effect of competition and caged dragonfly nymphs reversed it. Similarly, *A. callidryas* had no effect on *S. ruber* feeding in the absence of predators, but when coupled with either free water bugs or dragonflies, competition caused a large decrease in trophic status. Our results demonstrate that competition and predation interact to affect feeding ecology in complex ways. Certainly thinning of dominant species drives some of these patterns, but thinning alone does not appear to fully explain our results. Clearly there are inherent interspecific differences in trophic status and feeding. For example, the two most closely related species (the two *Dendropsophus* species) fed at very different trophic levels, with *D. ebraccatus* generally feeding at the highest carbon and nitrogen levels of all five species (Appendix A).

We observed a stronger change in δ^{15}N than in δ^{13}C values. Changes in isotopic values of consumers are often representative of the variety of food sources in the habitat, and the food sources available in our mesocosms may not have been diverse enough to allow a measurable carbon isotopic change (Post 2002, Layman et al. 2007a). Furthermore, interpretation of isotopic values should be done with caution, as precise trophic position requires knowledge of the baseline sources of the system (Post 2002), which we could not obtain (Layman et al. 2007a).

In summary, we demonstrate that predation and, to a lesser extent, competition, have strong top-down effects structuring a guild of Neotropical amphibian larvae. Predation and competition differentially alter growth, survival and the trophic niche of multiple amphibian species, with potential downstream ramifications for resource assimilation and energy flow between aquatic and terrestrial habitats. Longer-term studies of trophic alterations and structure in aquatic communities are needed to better clarify the potential ecological consequences and cascading effects of amphibian losses in food webs across the world, given that they are the most endangered group of vertebrates (Stuart et al. 2004, Wake and Vredenburg 2008).

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APPENDIX A. Survival, growth (final TL-initial TL, except for *E. pustulosus* where we show final TL of metamorphs), and stable isotopic values of the amphibian species included in each experimental treatment: with or without *A. callidryas* and within different predator scenarios. Survival is expressed in percentage (mean ± SE) and growth and final TL in milimeters (mean ± SE) calculated from all survivors from each tank. Initial number of individuals is 10 for each species (in 8 replicates=80 individuals) and we show the final sum of individuals (n) per species and treatment (only shown in the survival, but it is the same for all parameters measured).

	D. ebraccatus		***D. microcephalus***		***S. ruber***		***E. pustulosus***		***A. callidryas***
	Survival								
Treatment	*Agalychnis*	No *Agalychnis*	*Agalychnis*	No *Agalychnis*	*Agalychnis*	No *Agalychnis*	*Agalychnis*	No *Agalychnis*	
Control	65 ± 6.55 (n = 52)	66.25 ± 7.05 (n = 53)	72.22 ± 6.97 (n = 57)	68.75 ± 7.89 (n = 55)	82.5 ± 10.81 (n = 66)	77.5 ± 7.96 (n = 62)	55 ± 7.79 (n = 44)	50 ± 9.64 (n = 40)	80 ± 10.18 (n = 65)
Caged Belostoma	52.78 ± 9.07 (n = 42)	61.25 ± 6.93 (n = 49)	68.75 ± 3.5 (n = 55)	69.09 ± 6.41 (n = 56)	81.25 ± 7.42 (n = 65)	76.25 ± 9.99 (n = 61)	42.5 ± 10.13 (n = 34)	42.5 ± 9.01 (n = 34)	80 ± 7.56 (n = 64)
Free Belostoma	50 ± 7.79 (n = 40)	56.25 ± 5.65 (n = 45)	63.75 ± 6.8 (n = 51)	77.5 ± 6.48 (n = 62)	48.75 ± 8.33 (n = 39)	55 ± 5.67 (n = 44)	20 ± 4.63 (n = 16)	20 ± 5 (n = 16)	76.25 ± 3.75 (n = 61)
Caged Dragonfly	46.25 ± 8.85 (n = 37)	52.5 ± 7.26 (n = 42)	61.94 ± 9.63 (n = 49)	70 ± 8.45 (n = 56)	83.75 ± 5.96 (n = 68)	81.25 ± 10.43 (n = 65)	57.5 ± 5.9 (n = 46)	57.5 ± 12.06 (n = 46)	72.5 ± 7.5 (n = 58)
Free Dragonfly	40 ± 7.32 (n = 32)	48.75 ± 6.39 (n = 39)	51.25 ± 8.75 (n = 41)	67.5 ± 8.81 (n = 54)	13.75 ± 6.8 (n = 11)	7.5 ± 2.5 (n = 6)	6.25 ± 2.63 (n = 5)	3.75 ± 1.83 (n = 3)	8.75 ± 4.41 (n = 7)
	Growth (except in *E. pustulosus*, SVL)								
Control	14.04 ± 1.09	16.53 ± 1.09	29.73 ± 0.58	28.32 ± 1.5	27.29 ± 1.66	26.56 ± 2.69	10.31 ± 0.1	9.65 ± 0.17	23.39 ± 1.2
Caged Belostoma	14.17 ± 1.07	15.18 ± 1.08	28.3 ± 1.02	29.72 ± 0.8	27.49 ± 1	28.89 ± 1.21	9.75 ± 0.23	10.3 ± 0.16	21.87 ± 1.85
Free Belostoma	13.08 ± 1.06	13.73 ± 1.1	28.18 ± 0.9	29.8 ± 1.13	26.58 ± 1.45	28.28 ± 1.15	10 ± 0.16	9.97 ± 0.17	20.12 ± 1.41
Caged Dragonfly	12.35 ± 1.47	14.61 ± 1.35	22.92 ± 3.02	27.77 ± 1.21	22.88 ± 1.93	26.96 ± 1.93	9.1 ± 0.13	9.55 ± 0.15	17.66 ± 2.02
Free Dragonfly	16.92 ± 1.44	17.92 ± 0.85	29.61 ± 2.28	33.14 ± 1.12	34.26 ± 0.75	27.9 ± 2.5	8.83 ± 0.2	8.49 ± 0.56	25.49 ± 1.7

	D. ebraccatus		***D. microcephalus***		***S. ruber***		***E. pustulosus***		***A. callidryas***
	Stable Isotopes: δ^{13}C								
Treatment	*Agalychnis*	No *Agalychnis*	*Agalychnis*	No *Agalychnis*	*Agalychnis*	No *Agalychnis*	*Agalychnis*	No *Agalychnis*	
Control	-31.29 ± 0.45	-30.9 ± 0.58	-31.48 ± 0.21	-31.72 ± 0.84	-32.57 ± 0.49	-33.8 ± 1.22	-31.72 ± 1.09	-32.37 ± 0.66	-32.56 ± 0.64
Caged Belostoma	-30.85 ± 0.35	-30.72 ± 0.33	-31.7 ± 0.31	-31.7 ± 0.53	-33.14 ± 0.72	-32.63 ± 0.74	-32.79 ± 0.84	-31.76 ± 0.66	-32.66 ± 0.79
Free Belostoma	-30.71 ± 0.16	-31.28 ± 0.43	-31.88 ± 0.21	-31.92 ± 0.3	-32.98 ± 0.5	-34.97 ± 0.92	-31.96 ± 0.5	-32.4 ± 0.64	-32.72 ± 0.29
Caged Dragonfly	-30-55 ± 0.27	-30.67 ± 0.38	-31.4 ± 0.32	-31.46 ± 0.21	-34.57 ± 1.5	-33.25 ± 0.81	-32.36 ± 0.41	-32.16 ± 0.44	-31.4 ± 0.81
Free Dragonfly	-30.66 ± 0.37	-30.4 ± 0.36	-30.97 ± 0.2	-30.98 ± 0.31	-31.46 ± 0.2	-34.2 ± 1.75	-31.11 ± 0.76	-32.09 ± 1.49	-32.8 ± 0.55
	Stable Isotopes: δ^{15}N								
Control	7.79 ± 0.37	8.62 ± 0.23	6.17 ± 0.14	6.2 ± 0.22	6.5 ± 0.2	6.47 ± 0.51	6.29 ± 0.39	6.61 ± 0.38	6.5 ± 0.22
Caged Belostoma	8.72 ± 0.24	8.25 ± 0.49	6.33 ± 0.24	6.19 ± 0.23	6.29 ± 0.17	5.7 ± 0.39	6.4 ± 0.29	6.56 ± 0.2	6.63 ± 0.21
Free Belostoma	9.25 ± 0.31	9.16 ± 0.16	6.5 ± 0.2	6.38 ± 0.18	5.93 ± 0.38	6.88 ± 0.26	6.19 ± 0.18	6.46 ± 0.39	6.33 ± 0.27
Caged Dragonfly	9.03 ± 0.19	8.2 ± 0.36	6.68 ± 0.21	6.51 ± 0.29	6.4 ± 0.18	6.82 ± 0.44	6.3 ± 0.22	6.29 ± 0.19	7.28 ± 0.15
Free Dragonfly	7.99 ± 0.36	8.8 ± 0.17	5.93 ± 0.27	6.39 ± 0.16	5.29 ± 0.04	6.5 ± 0.61	6.21 ± 0.22	5.8 ± 0.23	5.44 ± 0.34

Appendix B (Tables B1-B5). Responses (in survival, growth and stable isotopes) to competition and predation of each species of the amphibian community. Results for the general statistical models are expressed using likelihood ratio tests. Analysis were performed in all amphibian species (n = 4) except for growth and total length (TL) (n = 3) where we excluded *E. pustulosus* as the majority of individuals were metamorphs and not comparable in size with tadpoles. Values in bold indicate *P* <0.05.

Table B1	***A. callidryas***							
Variable	**Survival**		**Growth**		**δ^{13}C**		**δ^{15}N**	
Statistical Models	*z*	*p*	*z*	*p*	*z*	*p*	*z*	*p*
Predation: Free Predators								
NP vs. WB-Free	-0.611	0.541	-1.71	0.13	-0.12	0.967	-0.51	0.611
NP vs. D-Free	-7.63	**<0.0001**	0.66	0.51	-0.06	0.967	-2.65	**0.024**
WB-Free vs. D-Free	-7.3	**<0.0001**	1.925	0.13	0.04	0.967	-2.15	**0.0476**
Predation: Caged Predators								
NP vs. WB-Cage	-0.023	0.344	-0.71	0.48	-0.24	0.813	0.48	0.63
NP vs. D-Cage	-1.23	0.344	-2.43	**0.04**	2.18	**0.043**	2.52	**0.035**
WB-Cage vs. D-Cage	-1.2	0.344	-1.72	0.13	2.39	**0.043**	2.09	0.055 *

Table B2	***D. ebraccatus***							
	Survival		**Growth**		**δ^{13}C**		**δ^{15}N**	
Statistical Models	*z*	*p*	*z*	*p*	*z*	*p*	*z*	*p*
Predation: Free Predators								
NP vs. WB-Free	-1.94	0.078 *	-0.6	0.55	1.88	0.09 *	3.27	**0.0016**
NP vs. D-Free	-3.17	**0.0045**	1.47	0.21	2.03	0.09 *	0.41	0.682
WB-Free vs. D-Free	-1.29	0.197	2.07	0.11	0.17	0.86	-3.27	**0.0016**
Predation: Caged Predators								
NP vs. WB-Cage	-1.51	0.195	0.06	0.95	0.799	0.425	2.52	**0.017**
NP vs. D-Cage	-2.37	0.053 *	-1.16	0.37	1.542	0.369	3.22	**0.0038**
WB-Cage vs. D-Cage	-0.87	0.384	-1.22	0.37	0.9	0.425	0.9	0.368

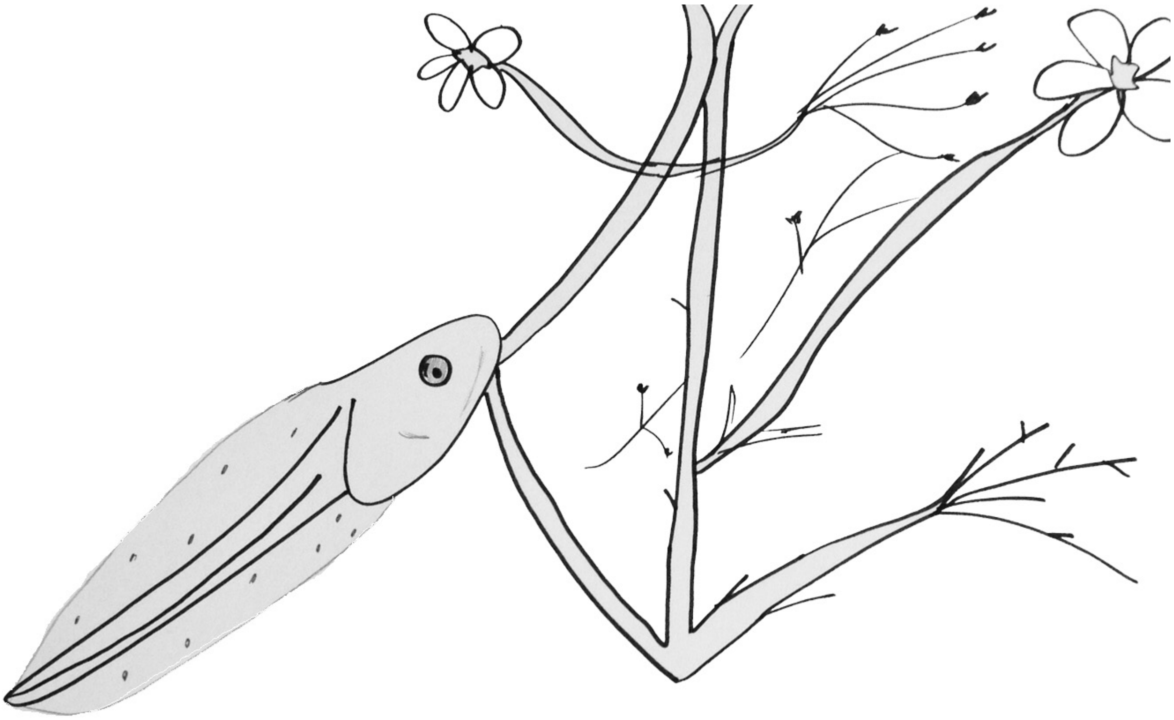
Table B3	***D. microcephalus***							
	Survival		**Growth**		**$\delta^{13}C$**		**$\delta^{15}N$**	
Statistical Models	*z*	*p*	*z*	*p*	*z*	*p*	*z*	*p*
Predation: Free Predators								
NP vs. WB-Free	-1.17	0.24	-0.76	0.735	-1.49	0.135	1.3	0.289
NP vs. D-Free	-2.76	**0.017**	-0.34	0.735	1.814	0.1	-0.9	0.366
WB-Free vs. D-Free	-1.64	0.15	0.42	0.735	3.23	**0.0037**	-2.14	0.097*
Predation: Caged Predators								
NP vs. WB-Cage	-0.48	0.63	-0.4	0.693	-0.83	0.66	0.99	0.32
NP vs. D-Cage	-1.37	0.51	-2.525	**0.035**	-0.007	0.994	3.12	**0.005**
WB-Cage vs. D-Cage	-0.9	0.55	-2.13	**0.0497**	0.77	0.66	2.2	**0.042**

Table B4	***S. ruber***							
	Survival		**Growth**		**$\delta^{13}C$**		**$\delta^{15}N$**	
Statistical Models	*z*	*p*	*z*	*p*	*z*	*p*	*z*	*p*
Predation: Free Predators								
NP vs. WB-Free	-4.54	**<0.0001**	-0.22	0.82	-1	0.314	-1.33	0.27
NP vs. D-Free	-7.66	**<0.0001**	2.22	**0.04**	1.18	0.314	-1.97	0.147
WB-Free vs. D-Free	-4.74	**<0.0001**	2.26	**0.04**	1.83	0.2	-1.08	0.28
Predation: Caged Predators								
NP vs. WB-Cage	-0.235	0.814	0.18	0.86	-0.83	0.408	-0.85	0.71
NP vs. D-Cage	0.25	0.814	-1.99	0.069*	-2.38	0.052*	-0.38	0.71
WB-Cage vs. D-Cage	0.48	0.814	-2.17	0.069*	-1.66	0.145	0.38	0.71

Table B5	***E. pustulosus***							
	Survival		**SVL**		**δ^{13}C**		**δ^{15}N**	
Statistical Models	*z*	*p*	*z*	*p*	*z*	*p*	*z*	*p*
Predation: Free Predators								
NP vs. WB-Free	-4.42	**<0.0001**	-0.75	0.45	-0.21	0.834	-0.256	0.99
NP vs. D-Free	-5.66	**<0.0001**	-3.42	**0.002**	0.47	0.834	-0.23	0.99
WB-Free vs. D-Free	-2.45	**0.014**	2.79	**0.008**	0.65	0.834	-0.01	0.99
Predation: Caged Predators								
NP vs. WB-Cage	-1.615	0.159	-0.87	0.38	-1.35	0.53	0.27	0.87
NP vs. D-Cage	0.327	0.744	-2.68	**0.02**	-0.93	0.53	-0.16	0.87
WB-Cage vs. D-Cage	1.935	0.159	-1.61	0.16	0.5	0.614	-0.45	0.87

Capítulo 4

Tadpole-plant interactions: differential herbivory across species and potential role of tadpoles as seed dispersers[4]



[4] Arribas R, Díaz-Paniagua C, Gomez-Mestre I. Tadpole-plant interactions: differential herbivory across species and potential role of tadpoles as seed dispersers. *In preparation*

RESUMEN

Las larvas de anfibios constituyen una parte importante de la biomasa total de los sistemas acuáticos y se sabe que muchas larvas de anuros son consumidoras primarias, alimentándose especialmente de fitoplancton y perifiton. Sin embargo, también se sabe que los renacuajos consumen plantas acuáticas, aunque se sabe muy poco acerca de las consecuencias ecológicas de las interacciones planta-renacuajo. Aquí examinamos las preferencias alimenticias de cuatro especies de renacuajos que divergen ampliamente en su ecología trófica y en su uso de microhábitats dentro de las lagunas. Demostramos que los renacuajos de diversas especies consumen cantidades considerables de biomasa de plantas y muestran fuertes preferencias hacia especies de plantas específicas. Las especies de anfibios difirieron en gran medida en la cantidad de plantas consumida, ya que las especies con el mayor tamaño de nuestro grupo de estudio, el sapo de espuelas (*Pelobates cultripes*), consumió casi el doble de biomasa de plantas que los renacuajos de cualquier otra especie. También presentamos la primera evidencia de transporte de semillas o esporas de macrófitos acuáticos viables en los intestinos de los renacuajos. Determinamos la frecuencia con que los renacuajos de cuatro especies de anfibios transportan semillas en sus intestinos en condiciones naturales en las lagunas. Esta frecuencia varió entre especies desde el 9.4 % al 32.8%, con la mayor especie de renacuajo transportando la mayoría de semillas (*P. cultripes*, con el 84.2% de semillas transportadas del total de semillas). Las semillas obtenidas de las heces de los renacuajos fueron secadas para simular un nuevo ciclo hidrológico de las lagunas temporales, y tras la reinundación observamos un amplio rango de éxito de germinación. En la mayoría de los casos, la probabilidad de germinación se mantuvo similar o aumentó con respecto a la germinación de las semillas no ingeridas. Las larvas de anuros parecen tener por tanto no sólo un importante papel como herbívoros, sino también un papel desconocido previamente como dispersores de semillas, revelando por tanto un alto potencial para modular la dinámica de poblaciones de las plantas acuáticas.

ABSTRACT

Amphibian larvae constitute an important part of the total biomass of aquatic systems and many anuran larvae are known to be primary consumers, especially feeding on phytoplankton and periphyton. However, tadpoles are also known to consume aquatic plants (macrophytes), although little is known about the ecological consequences of tadpole-plant interactions. Here we examined feeding preferences of four species of tadpoles widely divergent in their trophic ecology and their use of microhabitats within ponds. We showed that tadpoles from several species consume substantial amounts of plant biomass and show strong and shared preferences towards specific plant species. Amphibian species differed greatly in the amount of plants consumed, as species with the largest body size within the studied guild, spadefoot toads (*Pelobates cultripes*), consumed almost twice as much plant biomass as tadpoles of any other species. We also present the first evidence of tadpole transportation of viable seeds or spores of aquatic macrophytes in their guts. We determined the frequency of tadpoles carrying seeds in their guts in natural ponds for all four amphibian species. This frequency ranged across species from 9.4% to 32.8%, with the largest herbivorous species accounting for the majority of seeds transported (*P. cultripes*, accounted for 84.2% of seed transport). Seeds retrieved from tadpole feces were dried up to simulate a new hydrological cycle of temporary ponds, and upon re-flooding we observed a broad range of germination success. Anuran larvae thus seem to have not only an important role as herbivores but also a previously unknown role as seed dispersers, therefore revealing a high potential for modulation of population dynamics of aquatic plants.

INTRODUCTION

The interactions between plants and animals comprise a great variety of complex relationships that have a key role in ecosystem dynamics. Some of these interactions may be only beneficial for one of the interacting species, as in the case of herbivory, or may be mutually beneficial, such as in pollination, frugivory, or nutrient cycling by soil invertebrates (Herrera and Pellmyr 2002).

Main herbivores are typically large ungulates and the most common pollinators are insects that commonly feed on pollen. Herbivory can determine the structure, diversity and dynamics of plant communities (Huntly 1991), affecting other plant-animal interactions such as pollination (Herrera 2000) or changing the normal reproductive phenology of the plant (Pilson 2000).

Plants often depend on wind, water, or animals to transport their seeds or spores and ensure dispersal and successful establishment of seedlings (Ridley 1930, Traveset et al. 2014). Thus, dispersal is a fundamental feature of plant life cycles that has deep repercussions for the distribution of single species and for community structure (Herrera and Pellmyr 2002).

Seeds falling next to the parent plant are bound to experience higher resource competition, suffer higher seed predation, or suffer diseases related to high densities (Fenner 2000). In most cases, seeds are dispersed only over relatively short distances from the parent plant (Willson 1993). However, many plants widely depend on animals to disperse their seeds (zoochory) and seeds can be transported long distances. Seeds can be externally transported while passively attached to hair or feathers of animals (Sorensen 1986) or when animals actively pick seeds and store them as future resources (Vander Wall 1990). More commonly, herbivorous animals can ingest fruits or seeds. The germination probability (% of seed germination) and germination rate (time required to germinate) of such seeds increases in many cases as the chemical processes during digestion help in dormancy breakage, which benefits plant germination (Traveset and Verdú 2002). A large number of vertebrates are known to disperse seeds through endozoochory, such as birds and mammals (Howe 1986), and, less frequently but still very important locally, reptiles and fish (Olesen and Valido 2003, Calviño-Cancela et al. 2007, Horn et al. 2011). In the case of amphibians, to the best of our knowledge there is only one record of seed dispersal, and that is by adults of the neotropical treefrog *Xenohyla truncata*. Despite feeding mostly on various kinds of arthropods, frogs from this species have been observed consuming fruits of *Araceae* or *Erythroxylaceae* plants (da Silva et al. 1989) and their seeds were found in their feces.

There is, however, a considerable lack of data regarding interactions between plants and amphibians. Although adult amphibians are thought to be largely carnivorous, aquatic anuran larvae are considered primary consumers, mostly feeding on detritus, bacteria, plankton or fungi (Hoff et al. 1999), but their diets have seldom been described in detail (Altig et al. 2007). However, it has also long been known that anuran larvae include macrophytes (aquatic plants and macroalgae) in their diet (Díaz-Paniagua 1985). Amphibian larvae often account for a very large fraction of the total animal biomass in wetlands (Gibbons et al. 2006, Werner et al. 2007, Peterman et al. 2008), and they can have a strong impact on nutrient cycling, leaf litter decomposition and patterns of primary production (Whiles et al. 2006). Thus, due to their abundance and ecological importance, amphibians have a strong potential for affecting the structure and dynamics of aquatic communities (Costa and Vonesh 2013, Arribas et al. 2014). However, the functional role of tadpoles as herbivores and seed dispersers has been overlooked, but tadpole-plant interactions could have a high ecological relevance in aquatic systems worldwide.

Tadpole-plant interactions can be positive or negative for either one of the interacting species. For example, it has been shown that litter from invasive plants negatively affect tadpole performance (Maerz et al. 2005, 2009). This negative impact was largely due to increased soluble phenolics and changes in the C:N and N:P ratio rather than on plant origin *per se* (Cohen et al. 2012). Recently, we have shown that macrophytes are an important food source for several amphibian species in Mediterranean temporary ponds (Arribas et al. 2014, 2015), and that particular species like spadefoot toads (*Pelobates cultripes*) may cause considerable herbivorous impacts that may even carry over to the next hydrological cycle (Arribas et al. 2014). However, major questions regarding tadpole-plant interactions remain unanswered. Here we address some of them: Do tadpoles show preferences among plant species? How does the impact of herbivory vary across local anuran species? Do tadpoles feed solely on vegetative parts of the plant or do they also consume fruits? If so, do they transport seeds in their guts and are any of those seeds viable?

Aquatic macrophytes play essential roles in freshwater communities because they provide food for herbivorous animals, increase water clarity, or provide shelter and spatial complexity (Scheffer et al. 1993, Engelhardt and Ritchie 2002). These aquatic plants often have different reproductive strategies than terrestrial plants. In particular, the community of plants living in temporary ponds is adapted to the alternation of flooded and dried seasonal periods by means of an abundant and heterogeneous seed bank that ensures plant diversity and influences community dynamics (Bonis et al. 1995, Brock and Rogers 1998, Aponte et al. 2009). The seeds buried in the sediment of aquatic systems constitute a rich and perennial seed bank (Bonis et al. 1995) that acts as a reservoir to ensure the maintenance of the submerged macrophyte community during the flooded period of the hydrological year (Grillas et al. 1993).

Dispersion in aquatic systems is thought to occur mainly through wind, water currents, and zoochory (Ridley 1930, Smits et al. 1989). Among animals, the main dispersers are waterfowl, fish, and macroinvertebrates (Cook 1988, Figuerola and Green 2002), although there are some other cases where reptiles are involved, such as aquatic turtles carrying seeds of water lilies (Calviño-Cancela et al. 2007). Some ingested seeds may not be viable after passing through the digestive system of animals as a result of breakage. Nevertheless, other studies have shown that a great number of seeds continue being viable, or even enhance germination capability, after several hours in the digestive tract of fish (Pollux et al. 2006) or birds, which additionally allows longer-distance dispersal of those seeds during migratory flights (Figuerola and Green 2002, Charalambidou et al. 2003).

Here, we studied the feeding behavior of four common herbivorous tadpoles in a Mediterranean area in southwestern Spain, and assessed their potential for seed dispersal. The species studied are mainly herbivores in which macrophytes and algae are important food sources, complemented with detritus, zooplankton and some portion of fungi and bacteria (Díaz-Paniagua 1985, Arribas et al. 2015). We conducted a microcosm experiment offering a selection of four common aquatic macrophytes to the different tadpole species and assessed their consumption and feeding preferences. As species differed considerably in average tadpole size and behavior, we expected feeding preferences to vary among amphibians, and also predicted that herbivorous impact would increase with tadpole size. We assessed whether macrophyte consumption was related to seed transport by tadpoles and the probability of germination of those seeds. We also hypothesized that larger tadpoles would carry a greater number of seeds in their guts and of greater size than smaller species. Finally, we determined for the first time the frequency of tadpoles transporting seeds under natural circumstances in temporary ponds, and estimated the germination success ratio of the transported seeds.

METHODS

Study site and organisms

This study was carried out in Doñana National Park (37º00’N, 6º38’W), located on the right bank of the Guadalquivir River mouth, in southwesters Spain. More than 3000 temporary ponds may be found in the Park, which are usually flooded in autumn or winter and dry out in summer (Gómez-Rodriguez et al. 2011). A total of 11 amphibian species (8 anurans, 3 urodeles) inhabit the Park and most of them use temporary ponds as breeding habitats (Díaz-Paniagua et al. 2005). We included in this study 4 out of the 8 herbivorous tadpoles found in Doñana: the western spadefood toad (*Pelobates cultripes*), the Mediterranean treefrog (*Hyla meridionalis*), the Iberian painted frog (*Discoglossus galganoi*) and the Iberian green frog (*Pelophylax perezi*). The tadpoles of *P. cultripes* have the longest larval period, reaching the biggest size in relation to other anuran species in this area (Díaz-Paniagua et al. 2005).

As there is large interannual variation in timing and duration of flooding, the submerged macrophyte communities of Doñana are dependent on the seedling emergence from the seed bank (Grillas et al. 1993), which permits the survival of aquatic communities after drought (Brock et al. 2003). Common macrophytes in aquatic habitats of the study area include *Myriophyllum alterniflorum* DC, *Ranunculus peltatus* Schrank, *Juncus heterophyllus* Dufour, and several species of the genus *Callitriche*. Also, several species of charophytes algae are also commonly found in temporary ponds (Díaz-Paniagua et al. 2010).

Macrophyte consumption by tadpoles

To investigate the macrophyte consumption and preferences of the four different species of tadpoles selected for this study, we set up 11 aquaria (80x30x45 cm) of 80-L capacity in a field laboratory at Doñana Biological Reserve. We carried on the experiment in the spring of 2013 and ran it at room temperature during one week. Trials were run separately for each tadpole species. We provided the aquarium with sand, previously washed and rinsed to avoid turbidity, as a low-nutrient substrate for macrophytes. We collected from the field four of the most abundant macrophytes commonly found in temporary ponds (*Myriophyllum alterniflorum, Ranunculus peltatus, Callitriche* sp., and *Chara connivens*). We rinsed the plants, removed the excess water with a manual centrifuge and recorded the fresh weight of each species per aquarium prior introducing them into the aquaria and at the end of the experiment as a proxy of consumed plant biomass. We planted 15.45±0.02 mg (mean ±SE, here and throughout) in small pots filled with sediment from the basin of a temporary pond. After filling the aquaria with 70 L of well water, we introduced two independent pots of each plant species and randomly distributed them on each aquarium making up a total of 8 pots.

The number of tadpoles of each species added to the aquaria during the experiments was adjusted so that each tank had a similar tadpole biomass, regardless of the species. Therefore, each tank held one *P. cultripes* tadpole (mean±SD: 2.23±0.07 g), or five *H. meridionalis* tadpoles (sum of five tadpoles: 2.69±0.08 g), or 3-5 *P. perezi* (sum: 2.63±0.04 g). We could not collect enough *D. galganoi* tadpoles to match the average biomass of *P. cultripes*, so tanks with *D. galganoi* only held 12-13 tadpoles (sum: 1.54±0.02 g). We recorded tadpole body mass, as well as body length and total length at the beginning and at the end of the experiment in order to estimate the increase in size and mass during the experiment. Body and total length were measured using the program ImageJ 1.46r (NIH, U.S.A) on scaled photographs.

Furthermore, we observed the tadpoles' behavior during four out of the seven days that tadpoles of each species were in the aquaria. We recorded the time spent feeding in each plant species during a period of 20 minutes for one individual tadpole in each aquarium twice a day (morning and evening). We considered feeding when the tadpole actively grazed on a plant leaf, stem or fruit. We used the program JWatcher v. 1.0 (Blumstein et al. 2000) to quantify time invested feeding on each plant. After one week all tadpoles were returned to their original pond.

Seed transport

After recording behavioral data in the aquaria, tadpoles were placed individually in 250 mL labeled plastic containers filled with well water and maintained unfed for 48 h in order to quantify the number of seeds ingested and transported at any given time. Each plastic container was built with a false bottom using a mesh with grid of 1 cm to prevent coprophagy while allowing feces to sink to the bottom. After the 48 h period all tadpoles were weighed and digitally photographed to measure their body length. Feces recovered from each container were individually passed through a labeled filter paper and allowed them to dry up in the sun to prevent molding.

We investigated the role of amphibian larvae as seed transporters in natural conditions using a similar procedure. For this purpose, we collected a total of 1,042 tadpoles of the same four species by dipnetting in seven temporary ponds of the area between March and June 2013. Tadpoles were collected at different developmental stages, ranging from 26 to 40 Gosner (Gosner 1960), and their total length was recorded in order to test for a relationship between tadpole size and the amount of seeds transported. All tadpoles were then released in their pond of origin and filtered samples were checked for seeds, determining the number of seeds per plant species present in each sample. Samples were kept in dark and dry conditions until next spring, simulating the dry phase of the hydrological cycle of temporary ponds.

Seed germination

We also determined how many of the seeds present in tadpole feces germinated over the first two weeks after re-flooding (germination probability). We placed each sample (i.e., all feces from one tadpole) in a Petri dish and filled it with carbon-filtered dechlorinated water, simulating pond re-flooding after rainfall. We kept the Petri dishes in incubators at 20 ºC with a photoperiod of 12L:12D for 2 weeks and checked them every 24h to monitor germination and prevent desiccation.

We recorded the total number of seeds that germinated in every Petri dish after the 2 weeks period. We determined the genus and species of each plant species *de visu* when possible. However, in order to ensure the species of the seedlings, as *Callitriche* spp. seedlings are not easy to distinguish, we first collected common plants from different ponds (including the seven ponds where the tadpoles were originally from) for DNA extraction and sequencing in order to have an available library of consensus sequences for the most common macrophytes of the study area (Table 1).

Table 1. Adult plant species that were used to provide a library of consensus sequences for this study.

ID	Plant species
M1	*Ranunculus peltatus*
M2	*Ranunculus tripartitus*
M3	*Myriophyllum alterniflorum*
M4	*Callitriche brutia*
M5	*Callitriche obtusangula*
M6	*Callitriche truncata*
M7	*Callitriche stagnalis*
M8	*Potamogeton* sp.
M9	*Illecebrum* sp.

We also preserved 100 seedlings germinated in the laboratory in dry conditions for DNA extraction, to double check the accuracy of our *de visu* species identification, and to be able to distinguish among morphologically similar seedlings, such as those from the different *Callitriche* species.

Genetic identification of seedlings

Dry seedlings and tissue sample from known adult plants were placed in individual tubes with beads to break the cell wall and we extracted DNA using the QIAamp DNA Micro Kit (Qiagen) for small volumes of tissue in accordance with the manufacturer's instructions. We stored the DNA at -20 ºC until amplification. Species identification was based on fragments of the chloroplast gene rbcL, encoding the large subunit of RUBISCO (ribulose-1,5-bisphosphate carboxylase/oxygenase) and the non-coding region trnH-psbA using primers available in the literature (Table 2).

Table 2. Primers used for the chloroplast gene rbcL and the non-coding region trnH-psbA for the genetic identification of seedlings.

rbcLa-F	ATGTCACCACAAACAGAGACTAAAGC	Levin et al. 2003
rbcLa-R	GTAAAATCAAGTCCACCRCG	Kress et al. 2009
psba-F	GTTATGCATGAACGTAATGCTC	Sang et al. 1997
trnH-R	CGCGCATGGTGGATTCACAATCC	Tate and Simpson 2003

For rbcL amplification we used 2 µl of plant DNA as a template (4-6 ng/µl) in a 25-µl reaction mixture (17.7 µl dH20, 2.5 µl buffer, 1.25 µl 50 mM MgCl2, 0.125 µl 10 mM dNTPs, 0.25 µl of each primer, 0.8 µl BSA, and 0.125 of TaqTM DNA polymerase). PCR conditions for rbcL amplification were a first cycle at 94°C for 4 min, 35 cycles of 94°C for 30 sec, 53°C for 30 sec, 72°C for 60 sec followed by a final extension at 72°C for 10 min. For trnH-psbA amplification we used 1 µl of plant DNA (4-6 ng/µl) in a 25-µl reaction mixture (16.225 µl dH20, 2.5 µl 10X buffer, 1.25 µl 50mM MgCl2, 2 µl 2.5 mM dNTPs, 0.75 µl of each primer, 0.4 µl BSA and 0.125 µl TaqTM DNA polymerase). PCR conditions for trnH-psbA amplification were a first cycle at 94°C for 3 min, 33 cycles of 94°C for 45 sec, 53°C for 45 sec, 72°C for 60 sec followed by a final extension at 72°C for 7 min.

PCR products were then cleaned using an enzymatic mixture containing exonuclease I (3 units) and Antarctic phosphatase (1 unit). The products were sequenced on an Applied Biosystems 3130XL genetic analyzer using the Big Dye terminator v1.1 Cycle Sequencing kit following the manufacturer's instructions. Sequences were edited, assembled and aligned using the software Sequencher 4.5 (Gene Code Corporation, Ann Arbor, MI) for phylogenetic analysis and correct assessment of the seedlings to the known adult plants.

Statistical analysis

We conducted all statistical analyses using R 2.15.3 (R Core Team 2013). For the feeding preferences experiment, we first checked the macrophyte consumption across all tadpole species with a Wilcoxon test of paired samples. We then used linear mixed-effects models (lmer function in package lme4; Bates et al. 2014) with aquaria as a random factor to check for differences in plant consumption (difference between initial and final biomass) among different tadpoles species and also within species. Response variables that did not meet parametric assumptions were either log-transformed or squared-root transformed, whereas we ranked the response variable in the within-species analyses. We also checked for differences in size and mass of tadpoles at the beginning and at the end of the experiment with paired t-tests. We conducted linear regressions between initial tadpole biomass and consumed plant biomass and also between tadpole biomass and number of seeds transported [ln(number of seeds+1)]. We used likelihood ratio tests for all models to evaluate the significance of each predictor.

Furthermore, we performed a compositional analysis (package "compositions" in R) to assess the proportion of time spent by tadpoles feeding/grazing in each of the four plants included in the aquaria. For this purpose we removed the behaviors that did not include grazing on a plant and set to 1 the proportions of time grazing during four consecutive days with the function "acomp". We also used a compositional analysis test to assess differences in dietary proportions among species of amphibians, using in this case the ingested plant biomass of each plant species (plant consumption) as the response variable.

In order to assess if the number of seeds and seedlings carried by tadpoles was different among species, and if a particular plant species was predominant among seedlings, we performed generalized linear models with Poisson distribution and log link. To assess the seed transport and dispersal in the field, we used generalized linear mixed-effects models fitting a Poisson distribution and a log link to test which plant species was more successfully transported within and among amphibian species, including pond as a random factor. We also assessed if the number of seeds was associated with the size (length and body mass) of tadpoles by means of Pearson correlations. For this purpose, the number of seeds was log transformed as ln(number of seeds+1).

RESULTS

Macrophyte consumption by tadpoles

Total plant biomass was significantly reduced at the end of the feeding trial experiment by an average of 30% across all species (*W*=820, *P*<0.001). The amount of plant biomass consumed depended on the plant species (χ^2=140.6; *P*<0.001) and the amphibian species (χ^2=31.59; *P*<0.001). Not all tadpole species showed identical preferences for the different plant species, as indicated by significant 'plant x amphibian' interaction (χ^2 =85.65; *P*<0.001). With a similar initial tadpole mass, *P. cultripes* consumed more plant biomass than the other amphibian species (Fig. 1). Overall, the most consumed plant was *Callitriche brutia* except for *D. galganoi* (Fig. 1). The following most consumed plant was *Ranunculus* peltatus whereas the least consumed one was *Myriophyllum alterniflorum*, which was never consumed by *D. galganoi* or *H. meridionalis*. However, *D. galganoi,* the smallest species of the four tadpoles, mainly fed on charophytes instead of phanerogams (χ^2 =44.2; *P*<0.001), consuming on average 5.36±0.18 g of *Chara connivens*.

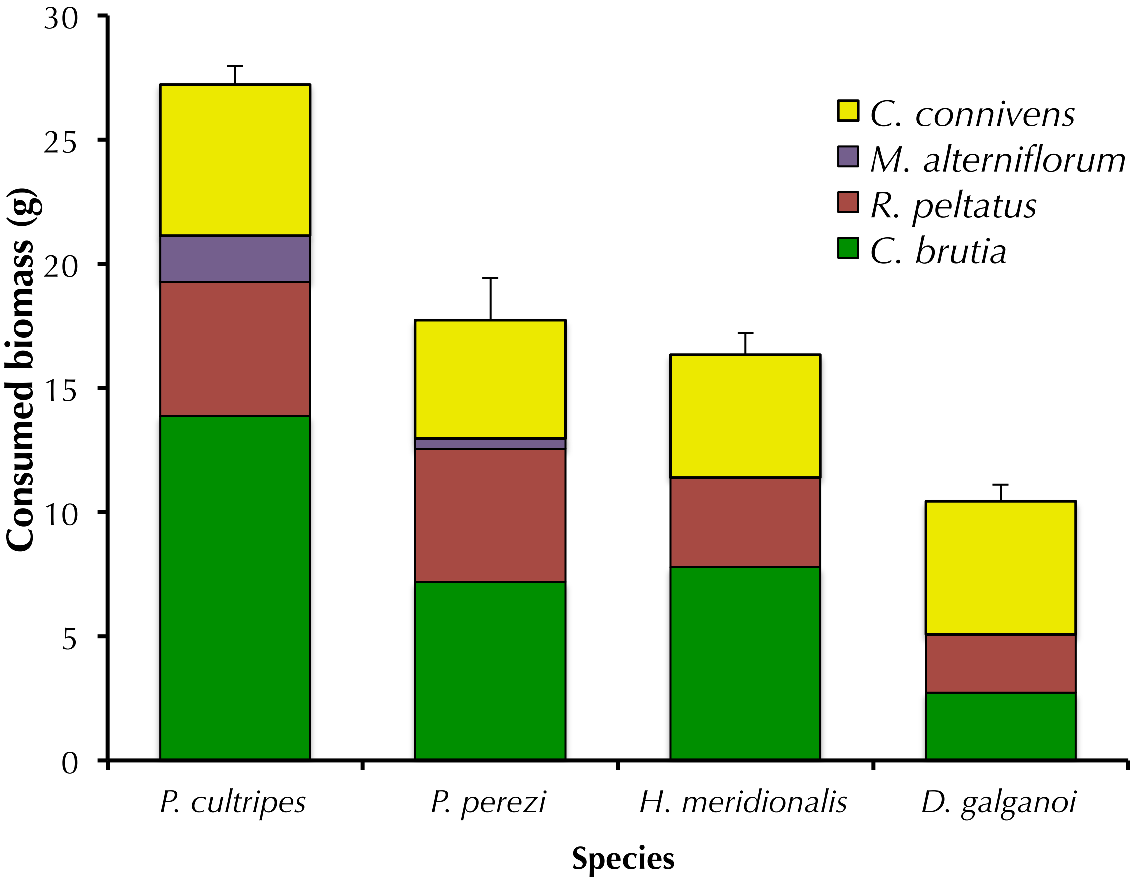


Fig. 1. Plant biomass (g) consumed by tadpoles of each amphibian species in the aquaria. Every stacked bar indicates the biomass consumed of each plant species and the standard error of the total biomass.

The compositional analysis of the diet regarding the plant biomass consumed showed differences among tadpole species ($F_{9,108} = 5.24$; $P < 0.001$).

However, the compositional analysis considering the proportion of time spent for each amphibian on the different plants did not show significant differences among tadpole species ($F_{9,102} = 1.3$; $P = 0.24$). We did not observe a clear relation between the total time spent in the macrophytes and the macrophyte consumption. In this sense, *D. galganoi* was the species that consumed the least macrophyte biomass despite spending the most time on the macrophytes whereas *P. perezi* spent less time in the macrophytes than the other tadpoles (Fig. 2) but consumed virtually the same amount of macrophytes as *H. meridionalis* (Fig. 1). However, in 3 out of the 4 species (all except *D. galganoi*) the time spent in the plant *C. brutia* was higher than in the others and this plant was also proportionally more consumed than the other macrophytes.

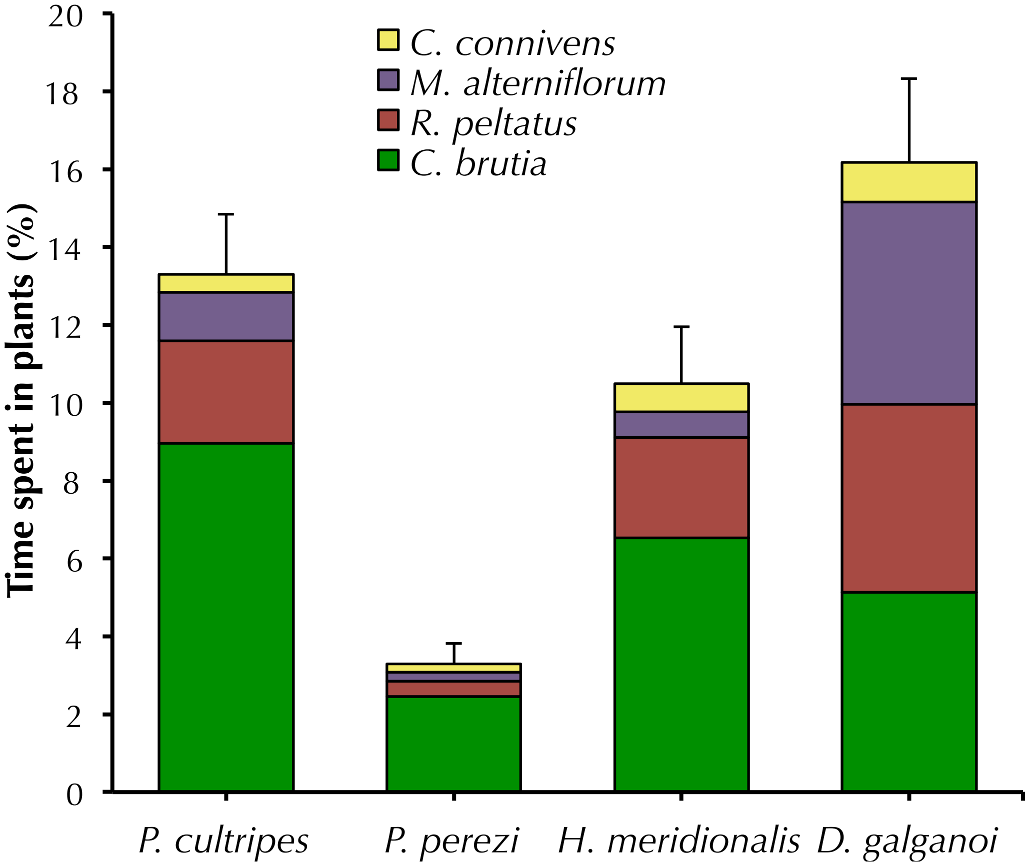


Fig. 2. Average time spent in each species of plant per species of amphibian tadpole. The time is expressed as a proportion of the total observational time across all the experiment in the aquaria.

Moreover, tadpoles considerably increased in body mass and body size after one week in the aquaria. The most notable increase was perceived in *P. cultripes*, which gained in average across all tadpoles 2.75±0.19 g, i.e., more than 2-fold increase in mass ($F_{1,20}$ = 119.39; $P < 0.001$), and increased 9.97±0.36 mm in length, i.e., 44.5% bigger ($F_{1,20}$ = 119.39; $P < 0.001$). The other species gained in average between 2.6 and 34.2% of weight and increased between 0.4 and 8.7% in length (Table 2).

Table 2. Initial tadpole mass and body size of the tadpoles included in the aquaria to determine macrophyte consumption. We show the mean ± SE for each species. The number of tadpoles per species is indicated between brackets.

Species	**Initial mass**	**Final mass**	**% mass increase**	**Initial length**	**Final length**	**% length increase**
Pelobates cultripes	2.23 ± 0.07 (N=11)	4.98 ± 0.36 (N=11)	123 %	22.66 ± 0.37	32.75 ± 0.41	44.5 %
Pelophylax perezi	2.63 ± 0.04 (N=60)	3.53 ± 0.11 (N=53)	34.2 %	15.07 ± 0.26	16.16 ± 0.33	7.2 %
Hyla meridionalis	2.69 ± 0.08 (N=55)	2.76 ± 0.14 (N=53)	2.6 %	13.09 ± 0.19	13.14 ± 0.31	0.4 %
Discoglossus galganoi	1.54 ± 0.02 (N=65)	1.76 ± 0.07 (N=46)	14.3 %	8.37 ± 0.09	9.1 ± 0.09	8.7 %

Seed transport by tadpoles under controlled conditions

In general, 29.29% of tadpoles from the aquaria transported seeds in their guts, adding up to a total of 892 seeds transported across all tadpole species (see Table 3 for details of the number of seeds of each macrophyte transported by tadpoles). The proportion of individuals transporting seeds varied among species (χ^2 = -53.46; $P < 0.001$). All *P. cultripes* tadpoles carried seeds (100%), whereas the proportion was reduced for the other species: 10 out of 17 *P. perezi* (58.8%), 12 out of 28 *H. meridionalis* (42.86%), and only 2 out of 46 *D. galganoi* tadpoles (4.35%) transported seeds (Fig. 3). As only 2 individuals of *D. galganoi* carried very little number of seeds, we removed this species for subsequent analyses. Number of seeds transported per tadpole depended both on the amphibian species (χ^2 = 18.68; $P < 0.001$) and on the plant species (χ^2 = 767.5; $P <$ 0.001). The interaction "plant x amphibian" was also highly significant (χ^2 = 235.8; $P <$ 0.001*)*.

Although all species were offered identical arrays of plants with the same plant availability, the proportion of seeds transported differed among plant species. However, we did not observe an association between the macrophyte consumption and the amount of seeds of different macrophytes transported by each tadpole species, except in the case of *H. meridionalis*, where the number of *R. peltatus* seeds and biomass consumed was highly correlated (Pearson r = 0.84; $P = 0.03$) (see Figs. 1 and 3).

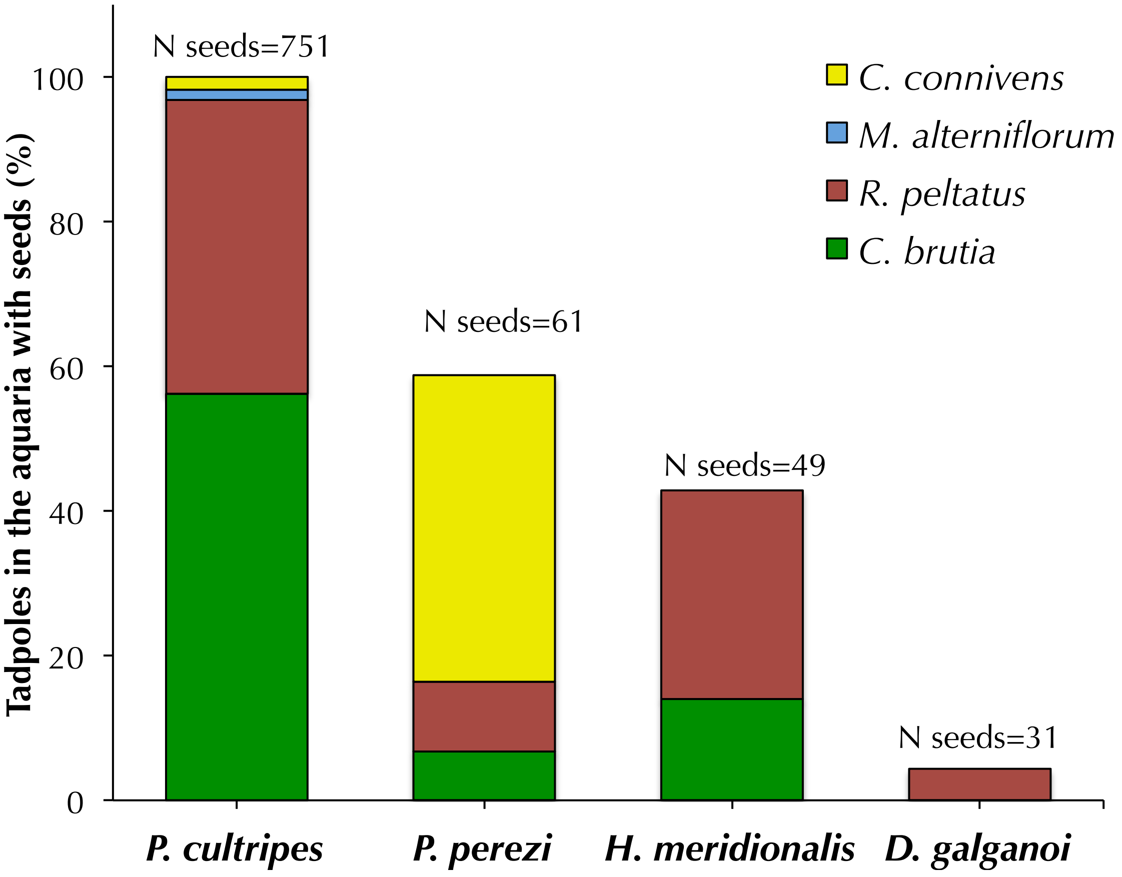


Fig. 3. Proportion of tadpoles of each species carrying seeds and percentage of each macrophyte species transported, specified with different colors. The number of seeds transported (n) by each tadpole species is presented over the bars. The total number of individuals per tadpole species is n=11 (*P. cultripes*), n=17 (*P. perezi*), n=28 (*H. meridionalis*) and n=46 (*D. galganoi*).

Tadpoles of *P. cultripes* carried many more seeds than any other tadpole species (Table 3-Aquaria and Fig. 3; χ^2 = 27.6; $P < 0.0001$). Mean seed number (among individuals that transported seeds) for *P. cultripes* was 68 per individual, composed of 56% of *C. brutia* seeds, 41% *R. peltatus*, 13% *M. alterniflorum* and 1.7% *C. connivens* (charophytes) (Fig. 3). On the other hand, the amount of seeds transported by the other tadpole species did not vary (all $P > 0.25$).

Compared to *P. cultripes*, we observed that the other species transported fewer species of macrophytes and the genus of the seeds transported differed among them. Both *C. brutia* and *R. peltatus* appeared in *P. perezi* and *H. meridionalis* whereas the 2 individuals of *D. galganoi* only transported seeds of *R. peltatus*. Mean seed number for *P. perezi* was 6 per individual, composed of 11.5% *C. brutia* seeds, 16.4% *R. peltatus,* and 72.13% spores of *C. connivens*. Regarding *H. meridionalis*, mean seed number was 4.1

per individual that belonged in 32.65% to *C. brutia* and 67.35% to *R. peltatus* seeds (Fig. 3).

The largest species of the anuran guild, *P. cultripes*, carried more seeds than the other tadpole species (**Image 1** for pictures of fruit and leaf consumption). However, we did not find any significant correlation between size and number of seeds within any of the amphibian species (all correlation tests with $P > 0.1$).

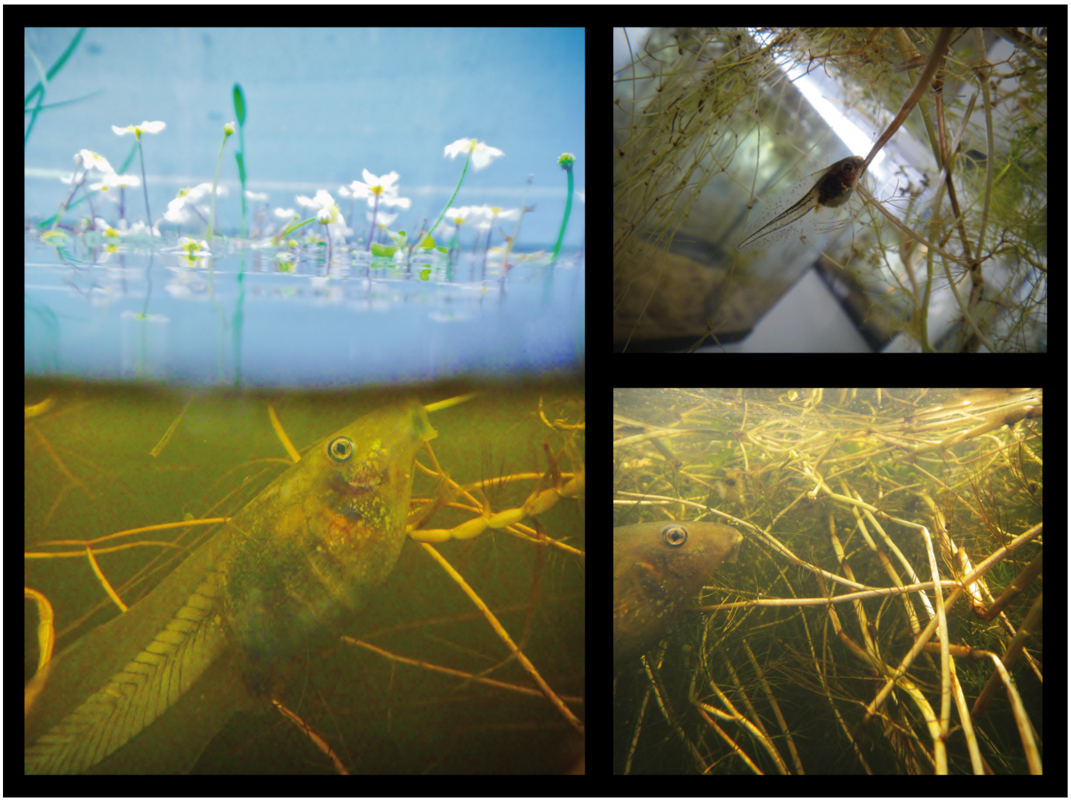


Image 1. A tadpole of the species *P. cultripes* (left) and of the species *H. meridionalis* (top right) consuming fruits of *R. peltatus*.

Seed transport by tadpoles in natural ponds

We found 832 seeds across the 1042 feces of the wild caught tadpoles of the four species (Fig. 4). The tadpole species was a significant predictor of the number of seeds transported (χ^2 = 39.6 $P < 0.001$), which also depended on the genus of the plant transported (χ^2 = 392.3; $P < 0.001$). The interaction tadpole species*seed species was also a significant predictor of the number of transported seeds (χ^2 = 39.9; $P < 0.001$).

The great majority of seeds (660, 79% of the total number of seeds) retrieved from feces collected in natural ponds were transported by *P. cultripes*, who showed the highest incidence of individuals carrying seeds (32.8%). 16.6% of the individuals of *H.*

meridionalis transported 154 seeds and 9.4% of the individuals of *P. perezi* carried 16 seeds, whereas only 2 tadpoles of the species *D. galganoi* (18.18% of the individuals) transported two seeds (Fig. 4). Thus, the sample size for *D. galganoi* is too low for discussing the results any further.

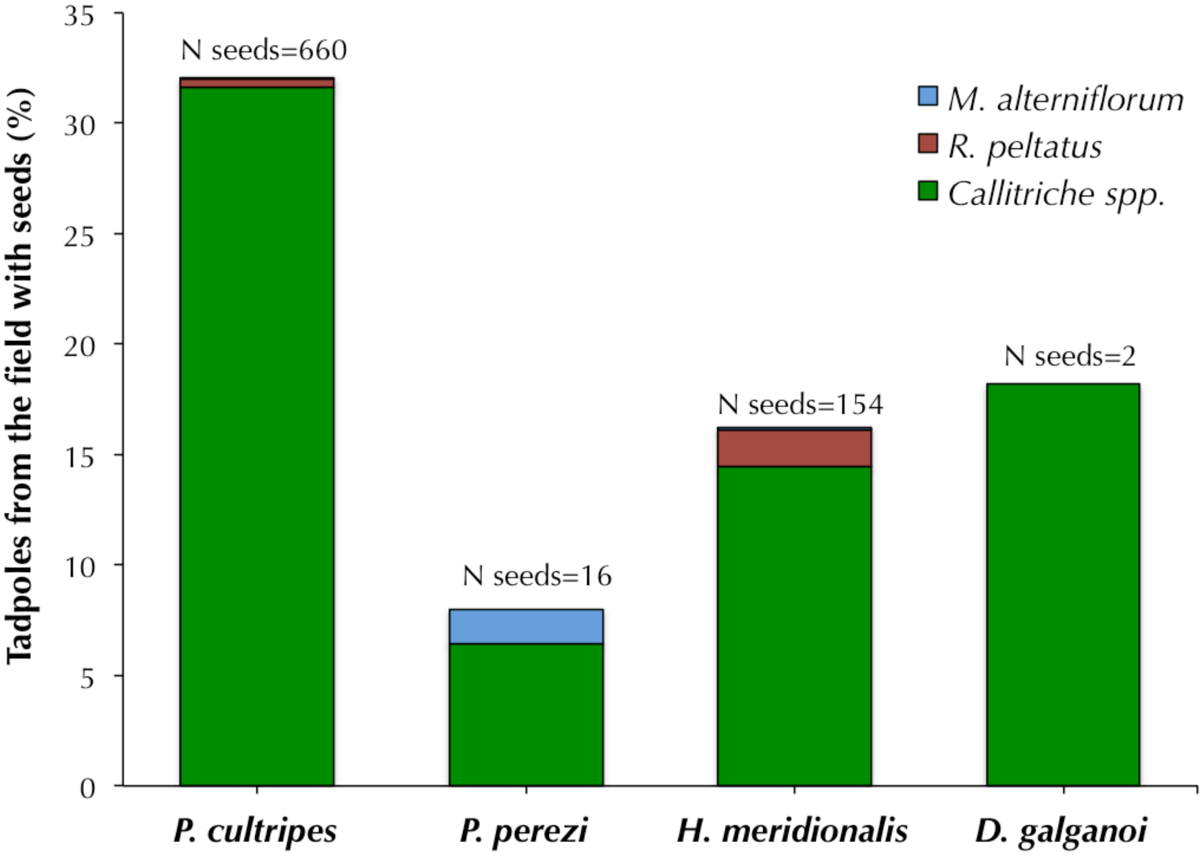


Fig. 4. Percentage of individuals of every species of amphibian tadpoles from the field that carried seeds. The percentage of each macrophyte species is specified with different colors. The number of seeds transported (n) by each tadpole species is presented over the bars. The total number of individuals per tadpole species is n=351 (*P. cultripes*), n=128 (*P. perezi*), n=542 (*H. meridionalis*) and n=11 (*D. galganoi*).

Callitriche seeds of different species, not identifiable *de visu*, appeared in the feces of all tadpole species but smaller amounts of seeds from other plants (*M. alterniflorum* and *R. peltatus*) were also present (Fig. 4). We did not find spores of charophytes in tadpoles collected from the field. Within the individuals transporting seeds, the average number of seeds per individual in *P. cultripes* was 5.7±0.7, mostly belonging to *Callitriche* species (96.5% of the seeds), 2.4% to *R. peltatus* and 1% to *M. alterniflorum*. In the case of *P. perezi*, the mean number of seeds per individual was 1.3 ± 0.14. In

68.75% of the cases seeds belonged to *Callitriche* spp., but we also found a relatively high proportion of seeds of *M. alterniflorum* (31.25%). Regarding *H. meridionalis*, the average number of seeds per individual was 1.7 ± 0.09 and we found a higher proportion of seeds from *Callitriche* spp. (87%), followed by seeds of *R. peltatus* (10.4%) and a very low proportion of seeds of *M. alterniflorum* (2.6%). However, the only two seeds found in the low number of feces we could obtain from *D. galganoi* corresponded to the genus *Callitriche* (Fig. 4).

There was not a significant correlated trend between tadpole body mass of all species and number of seeds transported (correlation test with $P > 0.2$) although tadpole body length was slightly correlated with the number of seeds (Pearson $r = 0.18$; $P = 0.009$). However, within species, tadpoles of *P. cultripes* and tadpoles of *H. meridionalis* that carried greater number of seeds belonged to an intermediate size and weight within the individuals captured (*P. cultripes*: 22-23 mm and 1.5 g; *H. meridionalis*: 13-15 mm and 0.4-0.6 g).

Seed germination

The number of seedlings (germinated seeds) was dependent upon tadpole species ($\chi^2 = 10.5$; $P = 0.005$) and upon type of plant ($\chi^2 = 14.15$; $P = 0.003$).

Genotyping of a subsample of all seedlings emerged confirmed our *de visu* identification of *Ranunculus peltatus*, *Myriophyllum alterniflorum*, and *Callitriche* spp (see **Image 2**). Moreover, sequence homology allowed us to distinguish among various species of *Callitriche* present in the study area, whose seeds or seedlings are not distinguishable *de visu*. Within the seedlings identified as *Callitriche*, 53.76% corresponded to *Callitriche brutia*, 46.23% to *Callitriche stagnalis*, and 1% to *Callitriche truncata*. Furthermore, two seedlings (out of 100) remained unidentified as their blast against species in our species library or other plant species reported in GenBank indicated less than 70% similarity.

Apart from seeds, we often found in the feces small pieces of leaf material, zooplankton eggs, and ostracods, some of which hatched from diapause during the 2-week period when the feces were flooded to account for seed transport.

Germination probability of *Callitriche* spp. was high for all tadpole species (more than 85% in *P. cultripes* and 100% in *P. perezi* and in the 2 only seeds transported by *D. galganoi*) except for *H. meridionalis* that was 41% (Table 3). In contrast, the other macrophyte species had lower germination probabilities, except for *M. alterniflorum* seeds consumed by *P. perezi* (60% germination). The germination probability of the seeds of this plant consumed by *P. cultripes* was lower (14.3%). For *R. peltatus*, the probability of germination ranged between 31.25% when ingested by *P. cultripes* to 6.25% in seeds ingested by *H. meridionalis* (Table 3).

Table 3. Number of total seeds of each macrophyte species ("Seeds") and germination ("Germ %") of the seeds transported by the different tadpoles from the field and from the individuals in controlled conditions in the aquaria.

Field	*Callitriche* spp.		*R. peltatus*		*M. alterniflorum*	
	Seeds	Germ %	Seeds	Germ %	Seeds	Germ %
P. cultripes	637	88.4	16	31.25	7	14.3
P. perezi	11	100	0	-	5	60
H. meridionalis	134	41	16	6.25	4	0
D. galganoi	2	100	0	-	0	-

Aquaria	*Callitriche* spp.		*R. peltatus*		*M. alterniflorum*		*C. connivens*	
	Seeds	Germ %	Seeds	Germ %	Seeds	Germ %	Seeds	Germ %
P. cultripes	422	38.4	305	7.21	11	9.1	13	46.2
P. perezi	7	71.43	10	40	0	-	44	0
H. meridionalis	16	37.5	33	0	0	-	0	-
D. galganoi	0	-	31	0	0	-	0	-

On the other hand, the germination of the seeds transported by tadpoles under controlled conditions in the aquaria differed from the germination found in field-collected tadpoles (Table 3). The number of seedlings was also dependent upon tadpole species ($\chi^2 = 47$; $P < 0.001$) and plant species ($\chi^2 = 25.35$; $P < 0.001$). Seeds of *Callitriche* and *Ranunculus* consumed by *P. perezi* had higher germination than seeds consumed by other tadpoles (Table 3). They also ingested *Chara* spores in the aquaria, but they did not germinate. Germination of the seeds of three plant species ingested by *P. cultripes* were lower than those found in wild tadpoles, while the germination of the consumed charophytes spores was 46%. Germination of seeds of *Callitriche* ingested by *H. meridionalis* in the aquaria were in average lower than in field tadpoles, although it was not statistically significant ($z = 0.41$, $P = 0.68$), while no *Ranunculus* seeds germinated.

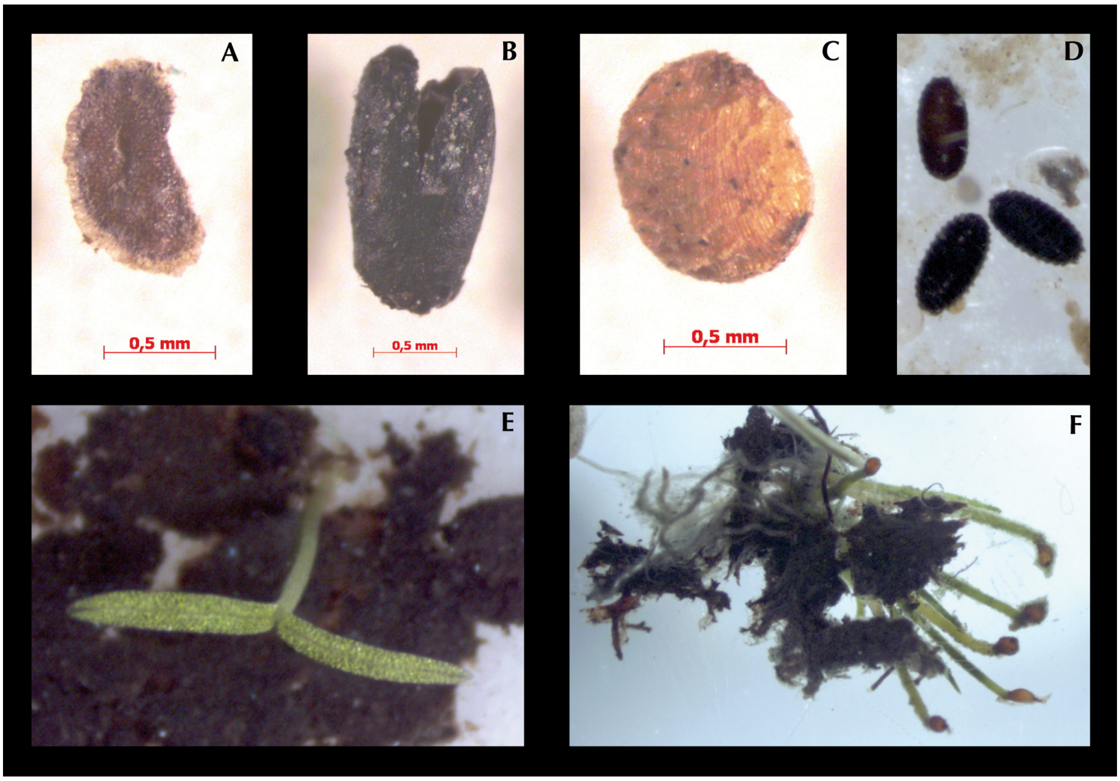


Image 2. On the top, seeds of the different species found in the tadpole feces. A) *Callitriche* sp, B) *M. alterniflorum*, C) *R. peltatus*, D) Chara connivens. On the bottom, feces of tadpoles with one seedling of *Callitriche* sp. (E) and seedlings of *R. peltatus* (F).

DISCUSSION

Tadpoles are important consumers of macrophytes, and within our amphibian guild spadefoot toad tadpoles (*Pelobates cultripes*) stand out in terms of their capacity to consume aquatic plants and transport seeds in comparison to other tadpole species. This is, to the best of our knowledge, the first documentation that tadpoles of different species transport viable seeds of aquatic plants.

Our experimental conditions offered similar proportions of common pond macrophytes, of which *Callitriche* was the preferred plant of three out of the four tadpole species. Because tadpoles may consume different parts of the plants, they can also ingest their seeds, which were commonly found in their feces. However, the proportion of *Callitriche* seeds transported by tadpoles was only very high for *P. cultripes* whereas *P. perezi* had higher proportions of *Chara* spores and *H. meridionalis* had higher incidence of *Ranunculus*. These results suggest that, despite similar availability, tadpoles of different species prefer feeding on seeds of particular species or, alternatively, avoid seeds of particular species. Moreover, our observations of plants consumed in the aquaria revealed that the proportions of plants consumed by each tadpole species did not necessarily coincide with the proportion of seeds transported and either with the proportion of time spent in a plant. Tadpoles may spend more time in certain plants because they may be feeding just on the periphyton attached to the leaves and stems. Also, seed characteristics (e.g., size, hardness of seed coat) and characteristics of the tadpoles (e.g., mouth size, feeding preferences, feeding behavior, microhabitat preferences) can greatly affect the probability of seed ingestion and their dispersal (Traveset 1998). Large tadpoles (such as *P. cultripes*) may ingest all parts of the plants, including seeds or fruits, whether ripe or still immature, or may search for particularly nutritive or easily consumed parts, as soft leaves or relatively fleshy fruits. This could explain the high number of *Ranunculus* seeds transported, which have a thicker pericarp than the seeds of the other aquatic plants of our study. On the other hand, tadpoles commonly complement their diet by feeding on sediment from the bottom of the pond (Díaz-Paniagua 1985, Altig et al. 2007, Arribas et al. 2015), which usually includes both plant matter and a high number of seeds that form the seed bank of aquatic plants (Grillas et al. 1993). This is a common adaptation of macrophytes to survive the annual dry period in temporary systems (Brock et al. 2003). and may contribute to seed consumption by tadpoles in natural conditions, such as the tadpoles we collected from the field. A high proportion of tadpoles in natural ponds transported seeds, and tadpoles of all species transported primarily *Callitriche* seeds, perhaps reflecting a higher presence in the seed bank. It seems unlikely, however, that feces collected during our aquarium-based study would have included seeds from sediments since there was only a small portion of sediment added in the plant pots and the main substrate was sand. Instead, seeds must have been consumed directly from the plants themselves. For example, the small but considerable proportion of *Ranunculus* in *H. meridionalis* tadpoles suggests the preference of this tadpole to feed on these ripe seeds, in accordance with our observations in aquaria.

Our results show that feeding and gut passage does not necessarily destroy the seeds ingested by tadpoles, as many of them germinated upon re-flooding in the laboratory. Tadpoles therefore may constitute an important vector of local seed dispersal, even if constrained by the extent of the pond basin. Seeds are frequently transported only short distances (Wilson et al. 1993). For example, ants constitute the main group among invertebrates that disperse seeds and fruits in terrestrial habitats and their mean global distance of dispersal is just 0.96 m (Gómez et al. 2005). This short dispersal within populations affects the spatial distribution of individuals and thus the environmental conditions experienced (Bolker and Pacala 1999).

Tadpoles are often known to congregate along the shore of a pond, which coincides with the area of establishment of macrophytes and where high seed concentrations have been reported (Wilson et al. 1993). Nevertheless, temporary ponds are extremely variable water bodies, exhibiting a wide inter-annual variation in their flooded area and depth, in relation to variations in annual rainfall (Díaz-Paniagua et al. 2010). Thus, a particular aquatic macrophyte may be within the flooded area one year but may be stranded beyond the flooded area in a different year of low rainfall, or very deep in the water during a particularly rainy year. In these cases, seed dispersal by tadpoles within the pond basin may contribute to increase the successful plant establishment through different areas of the ponds across different annual cycles. Moreover, tadpoles have relatively long guts (McDiarmid and Altig 1999) so the retention time may be long enough to allow for relatively distant movements within the pond area.

Aquatic plants can present all reproductive modes known in plants (Barrett et al. 1993). Although clonal propagation is an important way of dispersal for aquatic plants (mostly perennial), seeds are essential for annual species to survive the harsh drought conditions (Barrett et al. 1993). There are few studies about germination probability of non-ingested seeds of aquatic plants, and results are variable (from 0% to more than 70%) depending on the species (Pollux 2011). Our preliminary data on germination probabilities of some of the aquatic plants included in this study reveals that natural germination is not high. *Ranunculus peltatus* seeds incubated at a constant temperatura of 20ºC reached a germination of just 2.98% and the rate of *Callitriche brutia* was 46.9% (unpublished data).

The germination probability notably increased after gut passage in the case of *Ranunculus* seeds, which reached 31% germination after being consumed by *P. cultripes* tadpoles. Also, a high increase was observed for *Callitriche* seeds ingested by the same species and by *P. perezi*, revealing that tadpoles may improve germination in these aquatic plants. There are no other cases reported of tadpoles improving seed germination.

Nevertheless, the increase in the probability of germination cannot be similarly extrapolated to all our studied species. At least, not all tadpole species had a similar effect on seed germination. Seeds can be affected either positively or negatively by gut passage (Traveset 1998). In aquatic habitats, seed coat of certain plants passing through the gut of fish may induce breakage of dormancy and increase germination and germination rate (Pollux et al. 2006). The highest germination probabilities in our study corresponded to seeds of all macrophytes ingested by *P. perezi*, either in the field or in aquaria. Also, *P. cultripes* notably increased the germination of all seeds ingested in the field. However, seeds ingested by *H. meridionalis* did not seem to notably increase the natural germination probability. These important differences among tadpole species may be related to the chemical processes affecting seeds in the guts, which may be favorable for the seeds after being ingested by *P. perezi*. In contrast, another type of digestion not affecting seed germination seems to characterize *H. meridionalis* tadpoles. A contradictory result was observed for *Chara* spores ingested by *P. perezi* and *P. cultripes* in the aquaria. While there was a germination of 46% of spores ingested by *P. cultripes,* no germination resulted from spores ingested by *P. perezi*. The transit of the spores through the guts of *P. cultripes* may be less aggressive than in *P. perezi* and therefore increase the probability of germination of the spores which have less protection than the seed's pericarp.

The increase in seed germination favored by tadpole ingestion may result in early plant emergence, which could occur soon after filling in temporary ponds. Early emergent plants may have a competitive advantage (faster seedling growth and thus higher fecundity) over other aquatic plants whose seeds have not passed through animals' guts, affecting the composition of the vegetation in a similar fashion as terrestrial herbivores do (Pacala and Crawley 1992). Amphibians in our study area are characterized by a temporal segregation of species, among which *P. cultripes* tadpoles are early breeders, with eggs and tadpoles exploiting pond resources since their initial phase (Díaz-Paniagua 1988), when there is still a low productivity. The growth period of early breeding tadpoles and plants co-occur when the first rains of the autumn results in the flooding of the temporary ponds. The increase in seed germination favored by tadpole ingestion (in the previous pond cycle) could contribute to an early emergence of seedlings, which may have a particular value as food availability for early breeding species like *P. cultripes*. In contrast, this is not so evident for late breeding species as *P. perezi*, with tadpoles typically developing in ponds in late spring and during summer. At this time of the season, many plants are not still alive, and these tadpoles mainly feed on the thick layers of sediments from the bottom of the ponds (Díaz-Paniagua 1985, Arribas et al. 2015). The increase in seed germination caused by these tadpoles would not directly benefit both plants and tadpoles, but it can increase the annual aquatic plant production that finally would contribute to higher plant detritus that constitute the main food resource of *P. perezi* tadpoles.

Lastly, we cannot discard the possibility that tadpoles indirectly contributing to a wider dispersal of aquatic plants. With respect to long distance dispersal, although often rare (Nathan 2006), it is considered to play a determinant role in large-scale processes such as colonization of new unoccupied habitats or gene flow among populations (Levin et al. 2003b, Nathan et al. 2008). Long distance dispersal also typically contributes to population fitness, as a seedling far away from a parent contributes more to population growth (Howe 2000) or to avoid sibling competition (Rousset and Gandon 2002). Dispersal through the water is only possible between water bodies that have a physical connection (Barrat-Segretain 1996) and longer seed dispersal among water bodies is only possible by wind or by animals like birds (Figuerola and Green 2002, Mueller and Van der Valk 2002). Tadpoles are commonly preyed upon by mammals or shorebirds, including herons or ducks (Wells 2007), which frequently move across widely distant wetlands. Therefore, tadpoles may also be part of a multi-step dispersal of seeds when such birds feed on seed-eating animals and then disperse such seeds to other locations. This secondary dispersal would provide another mean of long-distance dispersal for aquatic seeds. For example, many aquatic invertebrates such as zooplankton are dispersed far away by birds, which may be even having a role in the introduction of non-native species to areas out of their range (Green and Figuerola 2005). Also, piscivorous birds contribute to dispersal of the seeds carried by fish and it has been a recent focus of dispersal ecology (e.g., Green et al. 2008). Aquatic plants, in particular, have a very wide geographical distribution which is likely to be explained by long distance dispersal via migratory water birds that move continuously among water bodies (Green et al. 2002). However, safe sites for seeds may not be suitable habitats for seedlings (Schupp 1995). Thus, short-distance dispersal by tadpoles may provide seeds a more appropriate location for seeds to be settled with detritus, and the feces might provide a proper nutrients bed for seeds germination and survival, as it occurs in seed dispersed by fish (Anderson et al. 2009, Pollux 2011). The benefits of long distance dispersal may thus be outbalanced by the costs of not reaching suitable establishment sites, as the dissimilarities with the native place increase with dispersal distance (Nathan 2006).

In summary, tadpoles are important consumers of both the vegetative and reproductive parts of macrophytes, and this plant consumption and seed dispersal have important consequences in the ecology, distribution and persistence of the vegetation of aquatic habitats (e.g., De Souza-Stevaux et al. 1994). Moreover, amphibians are the most threatened vertebrates in the world, with around 43% of them have declining populations (Stuart et al. 2004). In just the Mediterranean area around 25% of amphibian species are threatened, mainly as a cause of habitat loss, pollution and invasive species (Cox et al. 2006). Therefore, it is essential to explore and conserve what may be an important, and previously ignored, plant-animal interaction, as ongoing amphibian declines are likely to have important but still unpredictable changes in the structure and functioning of aquatic systems. More research is necessary to assess the dispersal ability of tadpoles from other geographical areas, including other factors like dispersal distances and germination under different environmental conditions or seasonal dynamics.

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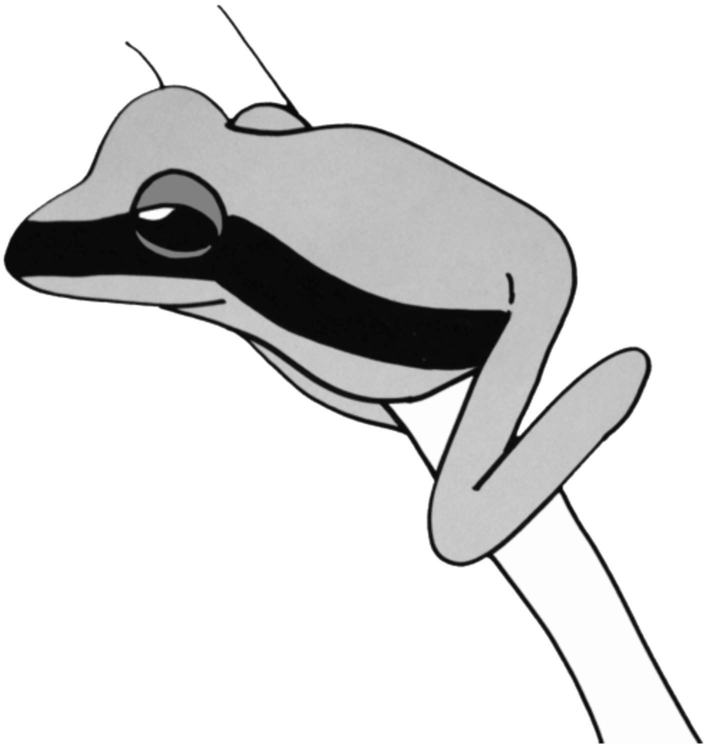
Appendix

Table A1. Consumed biomass (mean±SE) of every plant species by each tadpole during one week of experiment in the aquaria. The number of replicates of the aquaria is also indicated(n).

Species	n	*Callitriche*	*Ranunculus*	*Chara*	*Myriophyllum*
Pelobates	11	13.87±0.22	5.41±1.04	6.08±0.18	1.86±0.32
Hyla	12	7.78±0.36	3.62±1.63	4.93±0.45	0
Discoglossus	5	2.74±0.43	2.33±1.55	5.36±1.28	0
Pelophylax	12	7.2±1.02	5.36±1.04	4.76±0.35	0.4±0.25

DISCUSIÓN GENERAL Y SÍNTESIS



DISCUSIÓN GENERAL Y SÍNTESIS

La presente Tesis ha contribuido al avance del conocimiento de los múltiples papeles que juegan las larvas de anfibios en los ecosistemas acuáticos temporales. Las larvas de anuros son importantes consumidores primarios, que no sólo se alimentan de algas y perifiton, sino que además llegan a consumir grandes cantidades de macrófitos. Por otro lado, las larvas de urodelos son importantes consumidores secundarios. En su conjunto, tienen un gran potencial para alterar la composición físico-química del agua, la biomasa de las plantas, la densidad de algas y la composición de zooplancton. Algunas especies de larvas de anuros son detritívoras, mientras que otras pueden alimentarse mayoritariamente en la columna de agua, pero su estado trófico puede verse afectado por la competencia y la depredación. Dado que las larvas de anfibios, además de consumidores, son presa frecuente de depredadores abundantes en sistemas acuáticos temporales, sus efectos pueden variar conforme al tipo e impacto de dicho depredador. Las larvas de anfibios son organismos clave en los ecosistemas acuáticos y esta Tesis manifiesta los numerosos papeles funcionales que presentan, matizando y cuantificando las complejas interacciones entre larvas de anfibios y otros niveles tróficos o especies de la comunidad acuática.

Influencia de las larvas de anfibios en lagunas temporales

En una comunidad de anfibios del área Mediterránea, las larvas de anfibios ejercen un papel importante en los ambientes acuáticos y sobre las comunidades que los habitan (Capítulo 1). Además de alterar parámetros de la físico-química del agua como la turbidez, el oxígeno o la conductividad, la presencia de larvas de anfibios puede producir un aumento de las algas en el medio acuático. Muchos de estos efectos son denso dependientes, siendo más notables cuando las larvas se encontraban en altas densidades. Los anfibios también tienen un fuerte impacto sobre la biomasa de las plantas, que son un recurso alimenticio más importante para los anfibios anuros de lo que hasta ahora suponía la comunidad científica (Wells 2007). En definitiva, las larvas de anfibios pueden alterar considerablemente la composición vegetal de las lagunas, y consiguientemente la estructura de los microhábitats, lo que llega a afectar al funcionamiento de toda la comunidad, tanto a nivel de disponibilidad espacial como de alimento.

Asimismo las larvas de anfibios tienen un efecto notable en el zooplancton, ya que contribuyeron a la reducción de su diversidad así como al cambio de la composición de las comunidades de zooplancton, pasando de una predominancia de cladóceros en ausencia de larvas de anfibios a una predominancia de copépodos cuando determinadas especies de anfibios estaban presentes. Por una parte, los urodelos afectan a directamente a la composición específica de la comunidad zooplanctónica mediante la depredación. Las larvas de tritones, cuando se encuentran en densidades altas, reducen la diversidad de zooplancton, cambiando su composición, ya que los tritones presentan preferencia por los cladóceros y los consumen en grandes cantidades (Díaz-Paniagua et al. 2005). Sin embargo, las relaciones ecológicas son complejas y sin duda caben otras explicaciones posibles. Por un lado, los cambios en la físico-química del agua provocados directa o indirectamente por los anfibios, pueden haber influido en el cambio de la composición y en la menor diversidad del zooplancton. Así, también las especies eminentemente herbívoras, como las larvas de *P. cultripes*, pueden producir cambios en la composición del zooplancton, ya que alteran la disponibilidad de su alimento, la estructura espacial y las características físico-químicas del ambiente. Además, las larvas de anuros al igual que el zooplancton son consumidores primarios y por tanto pudieron competir por las algas (Mokany 2007), contribuyendo así a la reducción de la diversidad del zooplancton. Esta cuestión podrá ser objeto de estudio en próximos trabajos de investigación.

Sin embargo, los papeles ecológicos de las especies no son intercambiables, ya que algunas especies pueden ser únicas respecto a su función (Flecker 1996, Vanni et al. 2002, Taylor et al. 2006). Una de las conclusiones de este trabajo es que todas las especies de anfibios no ejercen el mismo papel ni el mismo impacto, sino que dentro de un grupo que podría considerar relativamente homogéneo, como las larvas de anuros, en el que todos son eminentemente consumidores primarios, se aprecian importantes diferencias. Por lo tanto, no se puede generalizar sobre los papeles funcionales de las diferentes especies ni concluir que son funcionalmente redundantes en un ecosistema. Algunas especies pueden tener por tanto efectos desproporcionadamente grandes en los productores primarios y en otros consumidores, lo que les confiere una posición única en la cadena trófica de una laguna. Esto se evidencia con el gran impacto que producen las larvas de *Pelobates cultripes* sobre diferentes parámetros del ecosistema acuático (Capítulo 1) así como en el mayor consumo de macrófitos y mayor capacidad para consumir y transportar sus semillas (Capítulo 4) y su mayor capacidad para modificar la ecología trófica de otras especies de anfibios con las que coexiste (Capítulo 2). De hecho, en ausencia de esta especie, el resultado de la interacción renacuajo-planta para el resto de la comunidad de anfibios cambia de signo, pasando a tener un efecto neto positivo sobre la biomasa de macrófitos. La magnitud de los efectos puede por tanto diferir entre las distintas especies de la misma comunidad o incluso ser opuestos. Sin embargo, no se puede generalizar entre sistemas ya que en los trópicos no se observó un efecto tan marcado de las larvas de la rana de ojos rojos, *Agalychnis callidryas* (Capítulo 3), una especie que inicialmente era esperable que ejerciera un gran efecto de competencia sobre otras dados los resultados de estudios previos.

A modo de esquema, en la Ilustración 1 se integran la magnitud y dirección de los efectos principales observados en las especies mediterráneas estudiadas.

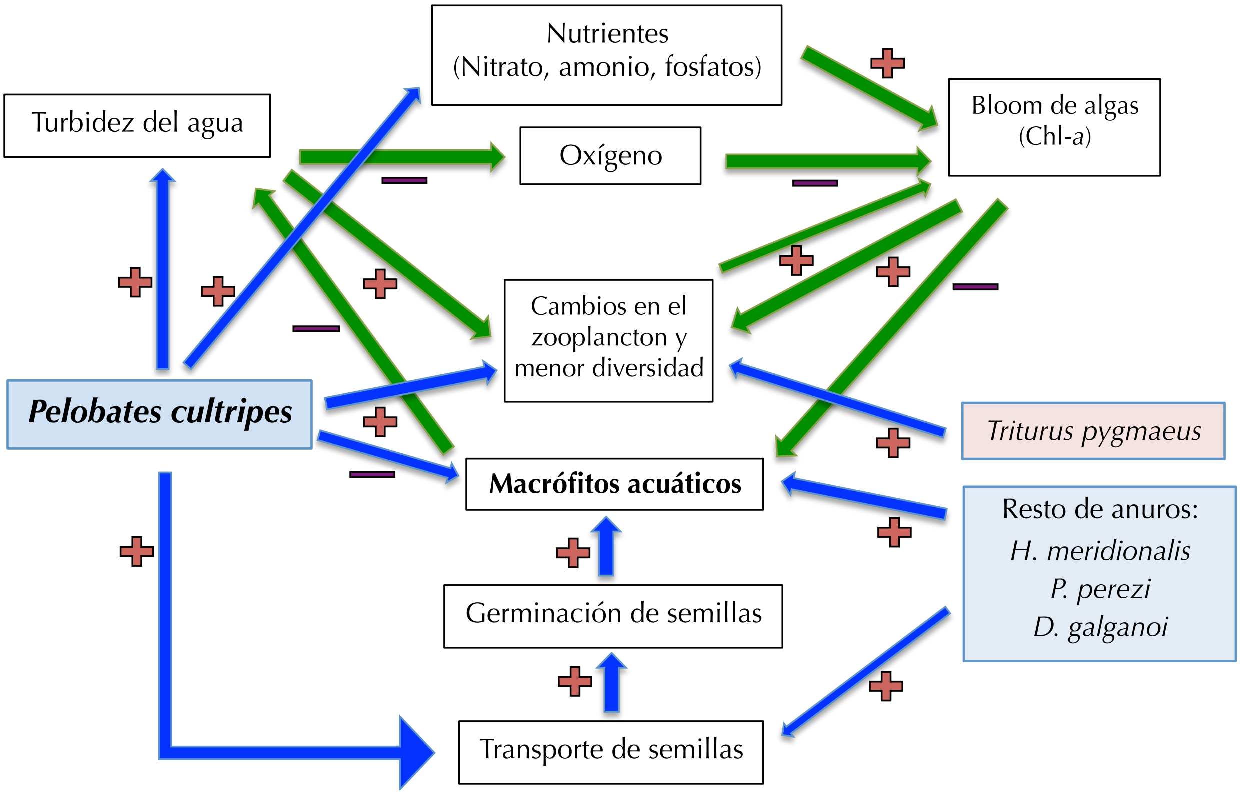


Ilustración 1. Esquema simplificado de las complejas relaciones ecológicas que se dan en el estudio del primer y cuarto capítulo de esta Tesis. Las flechas indican la dirección del efecto y el símbolo positivo o negativo indica como afecta al siguiente factor según aumenta el factor que le precede. Las cajas con fondos de color representan a las larvas de diferentes especies de anfibios.

La importancia de la competencia y la depredación en la estructura y en la plasticidad trófica de las comunidades

La competencia y la depredación están entre los factores bióticos más importantes que regulan y determinan la estructura y el funcionamiento de las comunidades ecológicas (Holt and Polis 1997, Sih et al. 1998), interactuando el uno con el otro de múltiples formas (Gurevitch et al. 2000).

Además de producir cambios en la supervivencia y en el tamaño de los individuos de una comunidad, la competencia intra- o interespecífica y la depredación también alteran las interacciones tróficas, como hemos observado en las larvas de anfibios de ambientes mediterráneos y de ambientes tropicales (Capítulos 2 y 3). La combinación de experimentos ecológicos y análisis de isótopos estables nos ha permitido abordar de una forma muy completa tanto el estudio de los efectos de los cambios ambientales en el ajuste trófico de las especies que interaccionan, como sus consecuencias para la estructura de la cadena trófica. Los isótopos estables son una técnica normalizada en los estudios de dieta de los organismos o en movimientos migratorios que ha sido de gran ayuda en los avances sobre la ecología trófica (Jackson et al. 2012, Layman et al. 2012, Perkins et al. 2014). Los valores isotópicos reflejan los recursos asimilados durante el periodo en el que el tejido se forma, lo que en larvas de anfibios muchas veces incluye el periodo larvario al completo, excepto en larvas con una etapa de desarrollo excepcionalmente largas como *P. cultripes*. Los cambios en la dieta debidos a diferentes interacciones ecológicas tienen consecuencias directas o indirectas en la estructura local de un ecosistema, dado que lo más probable es que las interacciones tróficas resulten también alteradas. En el caso concreto del sistema Mediterráneo estudiado, se observa que existe una gran variabilidad en la adquisición de recursos por parte de las larvas de anfibios, produciéndose en muchos casos un solapamiento entre fuentes de alimento, lo que normalmente se resuelve por diferencias temporales o espaciales de nicho permitiendo la coexistencia de las especies (Case and Gilpin 1974, Harrington et al. 2009). Los cambios en la dieta de los anfibios en respuesta a las condiciones ambientales y a la competencia y la depredación (Capítulo 2) demuestran que los anfibios están fuertemente afectados por la densidad, la competencia interespecífica y las especies invasoras. La competencia por parte de *P. cultripes* llevaba a los otros renacuajos a cambiar su fuente de carbono hacia recursos con una firma isotópica de carbono menor. El cangrejo rojo americano produjo diferentes cambios en los recursos que utilizaban los anfibios según la especie.

Los anfibios presentan una gran plasticidad y capacidad adaptativa a cambios ambientales, por lo que normalmente las comunidades pueden incorporar los cambios producidos por una especia invasora para minimizar sus efectos (San Sebastián et al. 2015). Por esta razón, seguramente cada especie responde de la manera más idónea para ella. Sin embargo, si dichos cambios en el nicho trófico de las larvas de anfibios ocurren a una tasa muy rápida debido a la introducción de especies invasoras u otros factores que producen un rápido declive de las poblaciones de anfibios, entonces toda la estructura y dinámica de la cadena trófica de las lagunas temporales podrían verse irreversiblemente afectadas.

En sistemas Neotropicales, la interacción ecológica con mayor influencia en la variación del estado trófico no es tanto la competencia sino la depredación (Capítulo 3). Al contrario de lo que ocurre en sistemas templados, aquí observamos que el esperado efecto competidor de la rana de ojos rojos (*A. callidryas*) sobre los otros anuros de la comunidad no es tan obvio. Tan sólo podemos determinar que esta especie compite de manera diferente con ciertas especies de anuros de la comunidad utilizada en el experimento y que, en muchas ocasiones, los depredadores revirtieron el efecto de la competencia en la supervivencia o tamaño del resto de los anfibios. Al introducir dos tipos de depredadores con diferentes estrategias y diferente localización en la columna de agua esperábamos que sus efectos sobre los renacuajos difirieran y en algunas ocasiones ocurrió así. Por ejemplo, en los tratamientos en los que se usaron libélulas como depredadores, la mayoría de las especies de anfibios eran más grandes mientras que en los que contenían hemípteros ocurría al contrario. La ecología trófica de las diferentes especies también se vio afectado por la interacción de competencia y depredación, seguramente por el aumento de tamaño derivado de la depredación selectiva (hacia individuos de menor tamaño) o por la disminución de la densidad y por tanto el aumento de los recursos disponibles para los supervivientes (*thinning*). Todos estos factores pueden tener efectos cascada en los ecosistemas, y por tanto es esencial tener en cuenta la mayor cantidad de elementos bióticos posibles para conseguir una visión más realista de lo que está sucediendo en una comunidad.

Durante el transcurso de esta Tesis se observó una serie de diferencias sustanciales entre los dos sistemas biogeográficos estudiados. Sin embargo, es necesario destacar que los resultados obtenidos son específicos del sistema estudiado y que por tanto las diferencias no son necesariamente generalizables a diferencias entre sistemas mediterráneos *vs*. tropicales. La mayoría de los estudios de anfibios se centran en Europa y Norteamérica, y para ampliar nuestro conocimiento de la estructura trófica a escalas globales es necesario expandir la cobertura geográfica de los trabajos. Algunos estudios anteriores sugieren que hay una mayor tasa de omnivoría en sistemas cálidos que en los templados (Lazzaro 1997), pero todavía hay pocos trabajos sobre ecología trófica en los sistemas acuáticos tropicales y por tanto es interesante continuar con las investigaciones en este campo. Además se pueden también integrar en el futuro aspectos de la biogeografía, estudios climáticos o la historia filogenética de las especies para poder obtener una información más detallada sobre las relaciones tróficas.

La herbivoría en renacuajos y su ignorado papel en el transporte de semillas

Las larvas de anfibios anuros son herbívoros clave en muchos ecosistemas acuáticos como lagunas y arroyos (Dickman 1968), donde la vegetación acuática puede llegar a ser muy abundante. Sin embargo, las **interacciones planta-renacuajo** todavía permanecen poco estudiadas, a pesar de que en esta Tesis se muestra que pueden tener una gran influencia en la estructura y dinámica de la vegetación acuática, dada la gran biomasa que representan los renacuajos en muchas ocasiones en un sistema acuático. A pesar de que las larvas de anfibios anuros se consideran generalistas, así como redundantes en su funcionalidad en muchos casos, hemos observado que los macrófitos acuáticos constituyen una parte importante de su dieta (Capítulo 2). Sin embargo, hasta el momento se desconocía cuál es la preferencia de cada especie de anfibio por estos productores primarios y qué consecuencias ecológicas pueden tener en estos macrófitos, como por ejemplo en su germinación y dispersión de sus semillas, o en su abundancia relativa en el siguiente ciclo hidrológico. Uno de los resultados más interesantes y novedosos de esta Tesis es conocer la viabilidad de las semillas digeridas por renacuajos, y la gran proporción de semillas que los individuos capturados en la naturaleza son capaces de transportar (Capítulo 4). Esto tiene consecuencias sobre la estructura de la comunidad de macrófitos acuáticos, pues al alterar las tasas de germinación se altera también la composición específica en la comunidad. El consumo de semillas contribuye también a la dispersión de los macrófitos, aunque los renacuajos no pueden ejecutar grandes desplazamientos, excepto cuando son transportados por depredadores. La importancia y dimensión de la dispersión que pueden hacer los renacuajos ofrece una cuestión abierta a futuros trabajos, puesto que los estudios de dispersión de semillas por vertebrados en sistemas acuáticos están enfocados en su gran mayoría a aves acuáticas, reptiles y peces (Figuerola and Green 2002, Calviño-Cancela et al. 2007). La dispersión de semillas por parte de renacuajos ha sido ignorado hasta ahora en las relaciones planta-animal, pero la dispersión por peces también fue desconocida hasta hace relativamente poco tiempo (primeras observaciones a finales de la década de los 70). Sin embargo, probablemente comenzó con los peces en la era Paleozoica hace 300 millones de años (Tiffney 2004), aunque sólo ha empezado a considerarse importante en las últimas décadas.

La probabilidad de germinación de las semillas transportadas por los renacuajos capturados directamente del campo fue mayor que en los renacuajos mantenidos en los acuarios durante una semana, lo que puede explicarse por el hecho de que los renacuajos podrían haber consumido semillas mientras estaban inmaduras en las plantas, ya que el sustrato en el fondo de los acuarios estaba constituido por arena y apenas había sedimento de lagunas. Por el contrario, los renacuajos pueden consumir con el detrito muchas semillas maduras que ya han caído al sedimento de las lagunas. Los renacuajos además incrementaron la probabilidad de germinación de muchos de los macrófitos acuáticos respecto a la tasa de germinación que presentan estos macrófitos sin haber sido consumidas (Capítulo 4).

Así, los efectos de los anuros encontrados en sistemas mediterráneos podrían estar ocurriendo en otros sistemas a lo largo del planeta, aunque probablemente con variaciones debido a su diversidad geográfica y por tanto las funciones únicas de los anfibios de un área determinada. Muchos macrófitos acuáticos en particular tienen una distribución muy amplia que en parte puede ser explicada por la dispersión a largas distancias a través de las aves acuáticas migratorias que continuamente se mueven entre cuerpos de agua (Green et al. 2002, Santamaría 2002). Las aves consumen directamente frutos de la vegetación como alimento (Vander Wall 1990) y éstos pueden engancharse a sus patas o plumas de forma pasiva (Sorensen 1986); pero además, aves piscívoras y aquellas que incluyen en su dieta otros organismos como larvas de anfibios, pueden dispersar las semillas transportadas por sus presas. Este transporte directo o indirecto de semillas atraviesa barreras de dispersión, lo cual es especialmente importante si los períodos de mayor migración coinciden con la fructificación de las plantas (Hanya 2005).

En cuanto a la herbivoría, observamos en primer lugar en el Capítulo 1 el importante papel que tienen algunas especies como *P. cultripes*, que se refleja también en los subsiguientes ciclos hidrológicos. Al contrario que la presencia de la larva de esta especie, con un tamaño desproporcionadamente grande, la comunidad de pequeños anuros puede favorecer el crecimiento de las plantas acuáticas, lo que también se refleja en el siguiente ciclo tras la inundación. Los macrófitos juegan un doble papel ya que son recursos alimenticios para muchos herbívoros pero además influyen en la supervivencia, no solo de los anfibios, sino de todos los organismos que habitan en un sistema acuático puesto que reducen la turbidez del agua y por tanto controlan los *blooms* de algas, además de incrementar la complejidad del hábitat aportando refugios y soportes vitales con su compleja estructura física (Scheffer et al. 1993, 2001).

Como hemos resaltado anteriormente, en ausencia de *Pelobates cultripes* las características físico-químicas del medio no eran diferentes de las del control sin anfibios, lo que puede deberse a la gran abundancia de macrófitos. Por el contrario, dichas condiciones ambientales pueden ser factores beneficiosos para el saludable desarrollo de los macrófitos (ver Ilustración 1). Sin duda, el ramoneo no abusivo de los renacuajos en los epífitos de los macrófitos y los aportes de nutrientes por las excreciones favorecen indirectamente al mayor desarrollo de los macrófitos, tal y como documenta Kupferberg (1997).

En el Capítulo 4 de esta Tesis se investigaron las preferencias de las larvas de anfibios anuros por los macrófitos acuáticos y se observó que unos son consumidos mayoritariamente respecto a otros, principalmente los del género *Callitriche*. Las plantas de este género pueden ser más sabrosas para los anfibios por su composición química o puede que la encuentren más blanda y más fácil de digerir, dado que en el experimento del primer capítulo también encontramos un mayor consumo de esta planta y aparecía con una alta frecuencia en el análisis isotópico de la dieta en el segundo capítulo. De nuevo observamos que la especie de anuro con la larva más grande, *P. cultripes*, y por tanto con mayor capacidad competitiva, es la que consume mayor biomasa de esta planta. Probablemente su alto consumo altera considerablemente la historia de vida y dinámica de las plantas acuáticas.

Para finalizar, es importante enfatizar que esta tesis supone un gran avance en el conocimiento de los papeles funcionales de las larvas de anfibios, algunos de ellos totalmente ignorados hasta ahora pero que tienen un gran potencial en la estructuración de las comunidades acuáticas. Además, esta Tesis abre prometedoras líneas de investigación en el campo de la ecología de comunidades y dispersión de semillas por larvas de anfibios, no sólo en el ámbito nacional sino que también es extrapolable al internacional. Así, se pueden abordar estas cuestiones utilizando diversas especies de anfibios representativas de diferentes áreas biogeográficas y observar la capacidad intraespecífica para transportar semillas y su probabilidad de germinación. Estos futuros estudios deberían además incluir información más detallada sobre la probabilidad de ingesta de las semillas, la tasa de germinación o tiempo de retención intestinal, para así poder calcular las posibles distancias de dispersión dentro de un cuerpo de agua, de forma similar al detallado estudio de la dispersión en peces llevado a cabo por Pollux (2011).

El conocimiento de los detalles que aportan los capítulos de la presente Tesis puede ser de gran ayuda para la toma de futuras decisiones en la gestión de la conservación de la biodiversidad y las relaciones ecológicas, dado que los anfibios son el grupo de organismos más amenazados del planeta y por tanto es de gran importancia evidenciar el papel que ocupan en las comunidades acuáticas para plantear las consecuencias ecológicas derivadas de la disminución o extinción local de sus poblaciones.

CONCLUSIONES

1.- Las larvas de anfibios ejercen importantes funciones en la estructura de las comunidades de los sistemas acuáticos. Las larvas de anuros son importantes consumidores primarios mientras que las de urodelos son eficientes consumidores secundarios. Los anfibios con frecuencia representan una gran parte de la biomasa total de un sistema acuático temporal, y por tanto es probable que sus interacciones con otros niveles tróficos conlleven considerables consecuencias ecológicas. También son presa de una variedad de depredadores que pueden modular las interacciones competitivas dentro de la comunidad de anfibios. Sin embargo, existen diferencias importantes que caracterizan la función de cada especie en el ecosistema. Ante el declive generalizado de sus poblaciones, es fundamental comprender el papel que desarrollan las larvas de anfibios en los ecosistemas acuáticos, donde pueden tener repercusiones ecológicas derivadas de su extinción local.

2.- Las larvas de anfibios de ecosistemas mediterráneos, estudiadas en Doñana, pueden producir un marcado efecto en las características físico-químicas del agua, aumentando la turbidez y la concentración de nutrientes como el amonio, principalmente cuando se encuentran en altas densidades. Las larvas de anfibios también pueden contribuir en el aumento de la biomasa de algas y alterar la composición de la comunidad de zooplancton, favoreciendo a los copépodos en detrimento de los cladóceros.

3.- Las larvas de anuros presentan frecuentemente una alimentación generalista, consumiendo detritos, algas, materia animal y una parte importante de macrófitos. La proporción de cada una de estas categorías alimenticias en la dieta es variable en función de la especie de anfibio y las condiciones ambientales. En concreto, la presencia de depredadores o la intensidad de la competencia durante su fase larvaria pueden alterar la ecología trófica de las larvas de anuros. Además, la mayoría de especies presentan una preferencia por determinadas especies de macrófitos como los del género *Callitriche*. El efecto que ejercen los anfibios sobre las plantas acuáticas se extiende de un ciclo hidrológico al siguiente, donde la biomasa total de macrófitos es menor en caso de haber sido expuesta a la presencia de anfibios en alta densidad. Esto se debe particularmente a la intensa herbivoría que realizan las larvas del sapo de espuelas, *Pelobates cultripes*, que probablemente se produce antes de que los frutos de las plantas estén maduros, imposibilitando su germinación en el siguiente ciclo.

4.- Estos efectos son característicos de cada especie, siendo de mayor importancia los debidos a *P. cultripes*. Las larvas de esta especie alcanzan un tamaño mucho más grande que las de otras especies con las que coexiste, y por tanto consume una mayor cantidad de macrófitos. En ausencia de esta especie, el bajo impacto de herbivoría realizado por el resto de la comunidad de anfibios tuvo un efecto positivo sobre la biomasa de la comunidad de plantas. El consumo del perifiton adherido a los tallos de las plantas, unido a las mejores condiciones ambientales respecto a turbidez y oxígeno en ausencia de *P. cultripes*, son probablemente los factores que, ejercidos por el resto de la comunidad de larvas, favorecen el crecimiento de los macrófitos.

5.- La interacción de la competencia y la depredación puede dar lugar a complejas interacciones ecológicas que alteran la historia de vida de las larvas de anfibios y su estado trófico. En nuestros sistemas de estudio, la competencia resultó más importante en la alimentación oportunista de los anfibios mediterráneos, mientras que la depredación tuvo un efecto preponderante en los anfibios del área Neotropical. En el ecosistema mediterráneo observamos un aumento del uso de recursos con menor valor isotópico de carbono (δ^{13}C) en alta densidad, en presencia de *P. cultripes* y de cangrejo rojo americano. En Panamá, los depredadores alteran el crecimiento y la supervivencia de las larvas, así como su estado trófico. Dependiendo de los depredadores, la composición de la comunidad de anfibios puede también afectar a su estado trófico.

6.- Los efectos de los depredadores sobre las larvas de anfibios pueden ser directos o indirectos. El cangrejo rojo americano, un depredador invasor en sistemas acuáticos mediterráneos al cual las larvas de anfibios no reconocen de manera innata, reduce considerablemente la supervivencia de los anfibios. También produce severas alteraciones sobre la físico-química del medio acuático, aumentando la turbidez y los nutrientes y reduciendo las plantas prácticamente en su totalidad. Su impacto también es notable en el siguiente ciclo hidrológico. Sin embargo, los efectos indirectos de este depredador invasor y de un depredador nativo (larva de escarabajo ditíscido) apenas son notables en la estructura de la comunidad o en el estado trófico de los anfibios.

7.- En este estudio se incluyen las primeras observaciones con respecto al transporte de semillas y esporas de macrófitos acuáticos por parte de larvas de anfibios anuros. Entre el 9.4% y el 32.8% de las distintas especies de larvas de Doñana transportaban semillas ingeridas directamente de la planta o con los detritos del sedimento. El principal consumidor de macrófitos, *P. cultripes*, portaba el 84.2% del total de semillas transportadas. Muchas de estas semillas germinaron en un plazo de dos semanas, aumentando en algunos casos la probabilidad de germinación con respecto a la de las semillas sin ingerir y permaneciendo similar en otros. Las larvas de anuros pueden por tanto contribuir a la dispersión directa de semillas dentro de un cuerpo de agua.

CONCLUSIONS

1.- Amphibian larvae exert important functional roles on the community structure of aquatic systems. Anuran larvae are important primary consumers whereas urodele larvae are efficient secondary consumers. Amphibians often represent a great amount of the total biomass in temporary aquatic systems, and therefore their trophic interactions with other trophic levels are likely to bear important ecological consequences. Amphibians are also prey to a variety of predators, both vertebrates and invertebrates, which can modulate the competitive interactions within the amphibian guild. However, there are remarkable differences in the ecological impact of different species of amphibian larvae. Given the generalized decline of their populations, it is essential to fully understand the functional role of amphibian larvae in aquatic ecosystems, as their local extinction can produce severe ecological impacts.

2.- Amphibian larvae from Mediterranean ecosystems, studied at Doñana, can produce a marked effect in the water physicochemistry, increasing turbidity and nutrient concentrations as ammonium, mainly at high larval densities. Amphibian larvae can also contribute to the increase of algal biomass and alter the zooplanktonic community composition, favoring copepods to the detriment of cladocerans.

3.- Anuran larvae often present generalist feeding habits, consuming detritus, algae, organic matter, and a considerable portion of macrophytes when available. The proportion of each of these categories in the diet is variable depending on the amphibian species and the environmental conditions experienced. In particular, the presence of predators or the intensity of competition during the larval phase can alter the trophic ecology of anuran larvae. Furthermore, most of the species studied show shared preference for certain macrophyte species, particularly *Callitriche spp*. The effect of amphibian larvae on the aquatic plants carries-over to the next hydrological cycle, where the total macrophyte biomass is lower after having been exposed to high larval density. This is due in particular to the intense herbivorous impact of spadefoot toad larvae, *Pelobates cultripes*, which may be present and actively consuming macrophytes before fruits have a chance of ripening, and therefore precluding seed recruitment and germination in the following cycle.

4.- These effects are species-specific and the most important are those produced by *P. cultripes*. The larvae of this species reach a disproportionally bigger size compared to the other species they cohabit with, and therefore they are capable of consuming a greater amount of macrophytes. In absence of this species, the low herbivorous impact made by the rest of the amphibian community had in fact a positive effect on the plant biomass. Such a tadpole-driven positive effect on macrophyte growth is likely to be determined by the consumption of periphyton growing over the plants stems and the better environmental conditions in the absence of *P. cultripes* regarding turbidity and oxygen.

5.- The interplay between competition and predation can lead to complex ecological interactions that alter the life history of amphibian larvae and their trophic status. In our study systems, competition was more important in the opportunistic feeding of Mediterranean amphibians, whereas predation had a prevailing effect on the amphibian guild of the Neotropical area. In the Mediterranean system we observe an increase in the use of resources with lower carbon isotopic value ($\delta^{13}C$) at high larval densities, and in the presence of *P. cultripes* and the red swamp crayfish. In Panama, predators alter growth and survival of amphibian larvae, as well as their trophic status. Depending on the predatory environment, species composition within the amphibian guild also affects their trophic status.

6.- Predators can produce direct or indirect (non-consumptive) effects on amphibian larvae. Red swamp crayfish, an invasive predator in Mediterranean aquatic systems whose chemical cues local amphibian larvae cannot innately recognize, considerably reduces amphibian survival. Invasive crayfish also cause profound alterations on the water physicochemistry, increasing turbidity and nutrients but reducing plants biomass almost completely. Its impact is also considerable in the following hydrological cycle. However, the indirect effects of this invasive predator and also a native predator (dytiscid beetle larvae) are almost negligible on the community structure or on the trophic status of amphibian larvae.

7.- This study includes the first observations regarding the transport of seeds and spores of aquatic macrophytes by anuran larvae. Between 9.4% and 32.8% of the different anuran larvae from Doñana transported seeds, directly ingested from the plant or consumed with the detritus from the sediment. The main consumer of aquatic macrophytes, *P. cultripes*, accounted for 84.2% of the seeds transported. A great amount of these seeds germinated within a two-week period, increasing in many cases the germination probability or remaining similar to the germination success of the non-ingested seeds. Anuran amphibian larvae can thus contribute to the direct seed dispersal within a water body.

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No faltaron tampoco amigos de Madrid, incluso de mi Erasmus, que se acercaron a verme a Sevilla. Bea ha venido cada año, ha conocido cada piso al que me he mudado, desde los Bermejales, donde Javi me acogió sin dudar, hasta la Alameda. Cuando estaba conmigo, me hacía olvidar los agobios que me caracterizaban cuando veía como se iba de rápido el tiempo. Nunca han faltado unas palabras de ánimo de mi hermana adoptiva, que en cualquier momento y lugar conseguían arrancarme mil y una sonrisas. Y también muchas risas cuando nos fuimos con Manu y Grego a la playa a compartir tardes de mus como en los viejos tiempos. Sabes que estoy infinitamente agradecida por sentirte siempre cerca. Y gracias por no dudar a ponerte a dibujar renacuajos (primera y única vez en tu vida!) para introducir cada capítulo. Denise no dudó en acercarse a Sevilla cuando estaba por España y así aprovechar la oportunidad de ver el mar, nuestro aliado en la relajación. A pesar de la distancia, tantos años de mejor amiga (con 50 mejores más!) se reflejan en cada encuentro. Ana compartió conmigo la experiencia del mítico Territorios y su alegría se me contagiaba para ver siempre el lado positivo de las cosas y arreglar el mundo en un segundo. Tú sabes que yo me voy contigo a cualquier lado y con muy poco a ser feliz. Marta e Irene, mis gemelas aventureras, me acompañaron de expedición a Doñana cuando apenas empezaba mis andanzas, y fueron ellas de las que más apoyo me brindaron. Natalia vino en el mejor momento y disfrutamos de la vida de esta ciudad como nunca en los corralones, aunque era difícil dar con tapas veganas a pesar de los infinitos sitios por los que pasábamos. Alda me acompañó en uno de los cumpleaños que he pasado aquí y siempre recupero con ella la parte más artística que parece que a veces se olvida. La reunión del grupo del 1st floor, Maria, Romain, Fred, Sam, Camille, Trini y Carlos coincidió con la visita de los Erasmus de mis compis de piso, y la casa de Quevedo se llenó de vida con nada menos que con 15 personas. Y esto también lo encontramos divertido, como tantas cosas durante aquella etapa en Pensión Quevedo los 5. Con María, Pablo, Néstor y Grego salíamos a tomar tapas o cervezas por la Alameda y por ellos conocí a Nolo y a Alegría. Slackline en cualquier parque, circo, música, Cokuiris, y sobre todo… alegría en esencia. Mis amigas de la Autónoma, Silvia, Payus, Clara, Irene y Eva, también pasaron por aquí y pasamos momentos inolvidables por las calles de Sevilla, incluso algunas vinieron a conocer el Pichilín. Sé que muchos de vosotros no pudisteis venir de visita, pero las navidades o un fin de semana dan para mucho y hacíais un hueco para encontrarnos un ratito por Madrid. Sois muchos (Julio, Alba, Yago, Tere, Paula, Javi, Titu, Alfredo, Nerea, Isa, Miguel, Sandra… y un largo etcetera) y de verdad que cada momentito que me dedicabais era un empujón más para avanzar.

Entre la Reserva y la EBD he pasado más tiempo que en mi propia casa y además del trabajo me ha brindado la suerte de haber conocido a personas realmente estupendas. En el Palacio no puedo olvidar la alegría de las cocineras, y Paqui se convirtió en mi segunda madre allí. En el campo y en las noches del Palacio coincidí con Gemma, Mamen, María León, Basti, Francesca, Paloma, Miguel o Marcello, sin olvidar toda la ayuda de David, Fabián, Ricardo, Irene o Inga, y la de mis voluntarias y a la vez amigas Lola y María Cuenca. Sin duda, toda la ayuda de los voluntarios que han puesto su granito de arena en este proyecto ha sido indispensable para terminar así de satisfecha. Además, sin los dibujos de la portada de Lola esta tesis no sería igual de especial. Tenían que ser de ella, que conoce lo que es hacer una tesis tanto como yo pero sin hacerla y que me ha escuchado y comprendido durante prácticamente todo el camino. Mil gracias de nuevo.

De vuelta en la EBD tengo que referirme especialmente a todos mis compañeros del grupo de las 3, en el que me conocen por los "contraplanes", por hacer "un rosas" en un viaje a Cazorla, por llegar siempre algo tarde o por proponer cervezas o barbacoas que a veces caen en el olvido. Tanto los nuevos como los más veteranos habéis sido como una gran familia. Puedo mencionar el slackline y las cartas con Edu, Sol, Nati y Ale (además Ale también mi compañero de café en la EBD y de los que son capaces de seguirme con los multiplanes), los juegos de mesa con J, los talleres de reparación de pinchazos de bicis con Rafa y Fran, aprender lo que es un huerto de verdad con Antonio y Alazne, las quedadas en el Piola propuestas por Mar y Mari, los compañeros en común con David, los zumos de frutas, la piscina y los primeros festivales con Irene, o las charlas filosóficas o de risas en el despacho con Pablo y Jesús. Pero Pablo también es mi hermano de tesis, mi compañero de muchas aventuras por el campo y por las cámaras, gracias por absolutamente todo, desde escuchar una queja hasta aconsejarme que tamaño de letra es mejor. Además de los cafés al sol con tostada entera de jamón y zumo de naranja con Lucas, las conversaciones contigo y todo el apoyo al compartir el recorrido por este lugar han sido muy importantes para mí. Las manualidades y las cenitas con Noa y Martina me ayudaron a mantener mi mente inquieta y atenta a otras cosas con las que sólo se permite disfrutar. Gracias por todo. Sois muchos más, cachorros y no tan cachorros, y la gente seguirá yendo y viniendo, pero vosotros sabéis lo importantes que sois en mi vida y todo el ánimo que me habéis transmitido.

Fue casualidad que me encontrara a mi compañera más fiel, Yla, durante un fin de semana en el que Wouter me invitó a participar como monitora de un seguimiento de anfibios. Mi último año de tesis no hubiera sido lo mismo sin el cariño incondicional que puede llegar a ofrecer un peludo tan pequeño, y que lo saben todos los que alguna vez han compartido su vida con uno, como Manuela y Marta de la perripandi. Los paseos con Yla y Maus por el parque han sido esenciales para desconectar, descubrir mil y una cosas, y simplemente disfrutar del momento, lo que tantas veces se nos pasa por ir demasiado rápido.

Además, durante estos años decidí probar la danza y jamás imaginé que allí encontraría a una de las personas más especiales de mi vida. María, las conversaciones infinitas contigo están abrazadas por una magia especial. Aún sin tener ninguna conexión con mi trabajo siempre has sabido entenderme y ofrecerme el abrazo o las palabras adecuadas en cada momento. Gracias por todos y cada uno de los momentos, en Sevilla y en cada viaje que seguiremos haciendo para descubrir el inmenso mundo. Y ya sabes, lo más lejos a tu lado.

Y porque se acostumbra a dejar para lo último lo más importante, vuelvo atrás ya que al poco de empezar la tesis, apareció Grego, que al poco tiempo leía la suya. Me enseñó un video de un charrancito ofreciéndole a la hembra el pez que recién pescó lanzándose en picado al mar. Aunque sobran las palabras, gracias infinitas por haber recorrido todo este camino a mi lado, aguantando tanto los buenos como los malos momentos. Y esto era sólo el comienzo del viaje, aún nos quedan muchos sitios donde volar.

Todos y cada uno de vosotros sois un pedacito de esta tesis, y perdóname si no he mencionado tu nombre, porque sabes que has estado en algún momento presente aquí y sin duda te lo agradezco de corazón.

Gracias de nuevo a todos los que siempre estuvisteis dispuestos a brindarme una ayuda, en cualquier forma, porque sabéis que esta tesis tiene mucho de vosotros.

Podrán cortar todas las flores,

pero no podrán detener la primavera.

Pablo Neruda