

Breeding activity of the agile frog *Rana dalmatina* in a rural area

M. Biaggini, I. Campetti, C. Corti

Biaggini, M., Campetti, I., Corti, C., 2018. Breeding activity of the agile frog *Rana dalmatina* in a rural area. *Animal Biodiversity and Conservation*, 41.2: 405–413, Doi: <https://doi.org/10.32800/abc.2018.41.0405>

Abstract

Breeding activity of the agile frog Rana dalmatina in a rural area. Rural landscapes can host many protected species that are constantly threatened by agriculture intensification and abandonment of traditional managements. Amphibians are severely affected by both processes due to loss and alteration of aquatic and terrestrial habitats. We monitored the breeding activity of *Rana dalmatina* in a lowland rural area focusing on spawning sites in open habitats, namely ditches amid traditional arable lands and pastures with varying vegetation features, size, and distances from woodlots. Egg clump density and clump size differed between sites, probably depending on environmental and ecological factors (i.e., larval competition, food availability, and predation). The sites next to woodlots showed the highest clump density (up to 0.718 n/m²). Our observations indicate that the maintenance and correct management of water bodies connected to traditional rural activities can be key to amphibian conservation in agricultural areas.

Key words: Agriculture, Amphibians, Breeding sites, Conservation, *Rana dalmatina*

Resumen

Actividad reproductiva de la rana ágil Rana dalmatina en una zona rural. Los territorios agrícolas pueden ser refugio de varias especies protegidas que, sin embargo, están constantemente amenazadas por la intensificación de la agricultura y el abandono de las prácticas tradicionales. Los anfibios están gravemente amenazados por ambos procesos, a causa de la pérdida y la alteración de los hábitats acuáticos y terrestres. Estudiamos la actividad reproductiva de *Rana dalmatina* en una zona rural de tierras bajas, centrándonos en los sitios de desove en hábitats abiertos, es decir, zanjas entre tierras arables y pastos con diferente vegetación, tamaño y distancia a las parcelas forestales. La densidad y el tamaño de las masas de huevos difirieron según el sitio, probablemente como resultado de factores ambientales y ecológicos (p. ej., la competición entre las larvas, la disponibilidad de alimentos y la depredación). Los sitios cercanos a las parcelas forestales mostraron la densidad más alta (hasta 0,718 n/m²). Nuestras observaciones indican que la conservación y el mantenimiento adecuado de las masas de agua destinadas a las actividades rurales tradicionales pueden ser esenciales para la conservación de los anfibios en zonas agrícolas.

Palabras clave: Agricultura, Anfibios, Sitios reproductivos, Conservación, *Rana dalmatina*

Received: 30 XI 17; Conditional acceptance: 23 I 18; Final acceptance: 26 II 18

Marta Biaggini, Claudia Corti, Museo di Storia Naturale dell'Università degli Studi di Firenze, Sezioni di Zoologia "La Specola", Via Romana, 17, 50125 Firenze, Italy.– Irene Campetti, Dipartimento di Biologia dell'Università di Pisa, Via Luca Ghini 13, Pisa, Italy.

Corresponding author: M. Biaggini. E-mail: marta.biaggini@virgilio.it

Introduction

Most European countries are widely characterized by cultural landscapes that have been deeply modified by human activities over the centuries. Agriculture, in particular, has played a major role in shaping large extensions of territories (Emanuelsson, 2009). Agricultural lands in Italy occupy over 40% of the total surface area and about 30% of the Natura 2000 network, and they include many endangered habitats and species (<http://www.crea.gov.it/lagricoltura-italiana-conta-2016>; <http://www.minambiente.it/pagina/rete-natura-2000>). However, due to the intensification of cultivation practices, agriculture is now a major threat for biodiversity worldwide (Wake, 1991; Foley et al., 2005). Therefore, monitoring biodiversity and planning conservation actions in rural landscapes is essential to develop effective environmental protection policies.

Amphibian populations are declining worldwide (Stuart et al., 2004; Gallant et al., 2007) due to several threats such as habitat loss and fragmentation (Blaustein and Wake, 1990; Cushman, 2006), emerging infectious diseases (Daszak et al., 2003), and climate change (Corn, 2005; Pounds et al., 2006). Moreover, amphibians are particularly vulnerable to land use changes and habitat alterations due to their complex lifecycle that requires both aquatic and terrestrial habitats at different life stages and, after metamorphosis, throughout the seasons (i.e., for breeding, hibernating) (Beebee, 1996; Alford and Richards, 1999). For these reasons, they can be considered good indicators in human-altered environments.

The agile frog, *Rana dalmatina* Fitzinger, in Bonaparte, 1838 is a brown frog, broadly distributed in central and southern Europe, and listed in Habitat Directive 92/43/EEC (Annex IV). It is an explosive breeder; in early spring, females lay a single egg clump in some type of water body or, less frequently, in slow-flowing watercourses. Such breeding features make it particularly easy to monitor the population density and the breeding trend of this species through standardized egg counts (Grossenbacher et al., 2002; Rossi et al., 2016). *R. dalmatina* has terrestrial habits and occurs in various environments, mainly deciduous forests, but also open habitats like grasslands within forested areas, from sea level up to 1,500 m a.s.l. (Speybroeck et al., 2016). In some areas, the lifecycle can involve both forested (terrestrial post-metamorphic phase) and open habitats (breeding period, larval phase). As such, *R. dalmatina* may be considered a useful target species for studies on environmental conservation in lowland landscapes where alternation of both kinds of habitats often occurs. In these areas, most females spawn in open habitats (frequently within 200 m from forests) where egg and larval development can be favored by higher temperatures (Ponsero and Joly, 1998; Wederkinch, 1988). In particular, *R. dalmatina* can also be found in agricultural areas near forests or residual woodlots (Pavignano et al., 1990; Hartel et al., 2009). Consequently, waterbodies, such as permanent or ephemeral ponds, ditches and watercourses included in the matrix of agricultural land uses can acquire key importance for the species' persistence at a local

scale. The risk of alteration and loss of both breeding sites and habitats suitable for dispersion and survival of the post-metamorphic phase is particularly high in rural areas where the intensification of agricultural practices can strongly threaten *R. dalmatina*.

In this study, we monitored *R. dalmatina* female breeding activity and tadpole development in a rural area in central Italy, partially included in a Natura 2000 site (special area of conservation, SAC IT5120101). In a previous study (Biaggini and Corti, 2011), we observed that, in the same area, the species also spawned in ditches and watercourses outside the forested patches. We focused on these breeding sites in order to: i) verify if and how rural habitats are exploited by *R. dalmatina* for the breeding activity; ii) identify the main threats affecting the species in a rural lowland area; and iii) suggest possible conservation actions. In particular, in the study area, one of the two woodlots frequented by *R. dalmatina* is fenced and included in a WWF oasis and is thus at least partially protected. Fields and grasslands, on the contrary, are involved in economic activities (sheep pasture, arable lands) and the SAC still lacks a management plan. Among amphibians, *Lissotriton vulgaris*, *Triturus carnifex*, *Bufo bufo*, *Hyla intermedia*, *Pelophylax lessonae-esculentus complex*, also occur in the same open habitats (Biaggini and Corti, 2011). Therefore, the need for conservation proposals for the rural habitats is pressing.

Material and methods

Study area and sampling

The study area (43° 46' N, 10° 37' E, 1.71 km²) is partially included in the SAC IT5120101 'Ex alveo del Lago di Bientina', a large reclaimed area dominated by agricultural lands that are periodically flooded, and smaller patches of wetlands and wet woods.

From mid-February to the end of May 2017, we visited the area once a week in search of egg clumps in all potential breeding sites, focusing on open habitats. We detected five breeding sites (table 1) where we counted egg clumps during each visit, by walking along the ditch banks and back again. For each egg clump found, we recorded the following variables: position (using a GPS device), distance of the clump from the water surface (spawning depth), water depth, the three main diameters of clumps (two horizontal, one vertical) and egg diameter (with and without envelope, using a caliper. For each clump, we considered the mean diameter of three eggs). We estimated the number of eggs per clump from the ratio between the volume of the clump (assimilated to an ellipsoid) and the volume of the egg (assimilated to a sphere). Considering that eggs and egg clumps tend to expand with maturation, we took measures on newly laid clumps (six days at most). We assessed the distance of each clump from the nearest woodlot (distance from the wood) using Google Earth aerial images and tools (www.google.com/intl/it/earth/).

Statistical analyses

We used the whole dataset for descriptive statistic (egg and clump size) and to compare egg clump density between the five breeding sites found in the study area (table 1). For the other analyses, we considered only sites 1, 2, 3, for which larger samples were available (table 1).

We performed a Friedman test to compare clump density between the five breeding sites, using the ratio between the number of clumps recorded during each sampling, and the studied surface for each watercourse. For the following pairwise comparisons, we used the Wilcoxon matched pairs test.

Focusing on sites 1, 2 and 3 to explore the relationships between the main reproductive variables, water depth, and the distance from the nearest woodlot, we performed a Spearman rank correlation between egg size (considered with envelope hereafter), number of eggs per clump, spawning depth, water depth, and distance from the wood.

To verify whether spawning activity varied between ditches, we compared the egg size, the number of eggs per clump, and the spawning depth in the three breeding sites, using log-linear models. We adjusted egg size for the number of eggs per clump and vice versa, based on Spearman rank correlation results. When analyzing the spawning depth we assumed a Poisson distribution of the data due to the high number of egg clumps recorded on the water surface (spawning depth equal to zero); spawning depth was adjusted for water depth and distance from the nearest woodlot (covariates), based on Spearman correlation results.

Results

The spawning activity occurred from mid-February to mid-March, peaking during the first two weeks (fig. 1). We measured 141 clumps. Eggs had a mean diameter (\pm SD) of 2.04 ± 0.18 mm, and 10.14 ± 1.99 mm considering the envelope (table 1). We estimated that clumps contained from about 90 to 1,480 eggs, with a mean value of 578.19 ± 295.55 (table 1).

The density of egg clumps (n/m^2) varied significantly between the five breeding sites (Friedman test: $n = 8$, $\chi^2 = 23.771$, $p < 0.001$). Site 2 had the highest density, differing significantly from all sites but 1 (Wilcoxon tests: 2 vs 1, $Z = 1.400$, $p = 0.161$; 2 > 3, $Z = 2.028$, $p = 0.042$; 2 > 4, 5, $Z = 2.201$, $p = 0.028$), followed by site 1 (1 > 3, 4, 5, $Z = 2.521$, $p = 0.012$), and 3 (3 > 4, 5, $Z = 2.201$, $p = 0.028$); site 4 and 5 had comparable low densities (4 vs 5, $Z = 1.604$, $p = 0.109$) (fig. 1, table 1).

Spearman correlation revealed that in the biggest clumps eggs tended to be smaller (correlation between egg size and number of eggs per clump: $n = 137$, $r = -0.642$, $p < 0.001$, fig. 2); both egg and clump sizes were not correlated with any other variable ($p > 0.05$ in all cases). The spawning depth was positively correlated with water depth ($n = 137$, $r = 3.149$, $p = 0.002$) and with the distance from the woodlots ($n = 137$, $r = 2.660$, $p = 0.009$). These two last variables

were highly correlated ($n = 137$, $r = 0.448$, $p < 0.001$) because the deepest ditch was also the furthest from woodlots (table 1). No other correlations were significant ($p > 0.05$).

Log-linear models revealed that egg size (adjusted for the number of eggs per clump) did not vary between breeding sites, while clumps contained significantly fewer eggs at site 2 than at sites 1 and 3, when controlling for egg size (even if significance was low, table 2, fig. 3). The spawning depth was greater at site 3, with respect to sites 1 and 2, and the distance from the wood had no significant influence on the model (table 2).

Discussion

In the present study, we further verified that *Rana dalmatina* exploits rural habitats for spawning in lowland environments (Pavignano et al., 1990; Hartel et al., 2009). Eggs were laid in slow running waters, both in drainage ditches with no aquatic or semi-aquatic vegetation and completely in the open, and in ditches rich in semi-aquatic plants and with trees and bushes on their banks (table 1). The two breeding sites with the highest clump density were next to a woodlot: in site 2, in particular, we recorded a very high clump density, $0.718 n/m^2$ (table 1) compared with values available in literature. Ponsero and Joly (1998) recorded a maximum clump density of $0.30 n/m^2$ in a marsh, Hartel (2005) a maximum of $0.026 n/m^2$ in a rural area, and Romano et al. (2016), a maximum of $0.47 n/m^2$ in a woodland. In the study area, the breeding sites exploited by *R. dalmatina* in open habitats were the same as in 2010 (Biaggini and Corti, 2011), with the exception of site 2, where no egg clumps were found during the first observations.

Clumps varied in size, from few eggs to almost 3,000, with the mean number of eggs per clump being nearly 600. In previous studies, Ponsero and Joly (1998) found a mean of 316 to 406, Weddelling et al. (2005) 950, Bernini et al. (2007) 800, and Solský et al. (2014) 1,300. In the largest clumps, eggs tended to be smaller and vice versa, suggesting a possible trade-off between egg number and size, as found in other *R. dalmatina* (Weddelling et al., 2005) and *Rana* spp. populations (e.g., Cummins, 1986). When comparing clump and egg size between the three main breeding sites, we found that clumps in site 2 contained, on average, fewer eggs (when correcting for egg size), while we found no differences in egg size. According to Weddelling et al. (2005), clump size does not depend on age, or size, or somatic condition of females. Alternatively, the lower number of eggs per clump in site 2 could be a response to some environmental constraints. Site 2 was the ditch with the highest clump density and the only one lacking aquatic vegetation, contradicting the more commonly observed association between *R. dalmatina* breeding sites and the presence of abundant aquatic vegetation (Pavignano et al., 1990; Kecskés and Puky, 1992; Hartel, 2008). Moreover, no bushes or trees occurred on the banks of the ditch, just grasses. Consequently, site 2 was also the only site lacking

Table 1. Main features of *Rana dalmatina* spawning sites and breeding characteristics in the study area: BS, breeding site; Land uses, nearby land uses; Vegetation, semi-aquatic vegetation and vegetation along the ditch banks; Width, mean width (\pm SD) (m); Depth, mean water depth (\pm SD) (cm); NClump, maximum number of clumps (number of measured clumps); DClump, maximum egg clump density (N/m²); NEggs, Estimated number of eggs per clump (mean \pm SD, range); EggSz, mean egg size (\pm SD) (cm, with envelope); SpDepth, mean spawning depth (\pm SD, and range) (cm); Dist, mean distance of clumps from wood edge (\pm SD) (m).

BS	Land uses	Vegetation	Width	Depth
1	Deciduous woodlot, arable lands. On the SCI boundaries	Dominated by <i>Iris pseudacorus</i> and <i>Arundo donax</i> from the end of March	1.482 \pm 0.205	15.800 \pm 4.165
2	Deciduous woodlot, arable lands. Outside the SCI	Absent	0.533 \pm 0.052	19.230 \pm 4.938
3	Sheep pasture and abandoned pastures managed with periodic cutting. On the SCI boundaries	Dominated by <i>Iris pseudacorus</i> , <i>Arundo donax</i> , <i>Phragmites australis</i>	1.552 \pm 0.383	31.727 \pm 4.968
4	Abandoned pastures managed with periodic cutting. Outside the SCI	Mainly <i>Carex elata</i>	2.267 \pm 0.441	19.333 \pm 8.042
5	Sheep pasture. Inside the SCI	Shrubs, mainly <i>Rubus</i> spp., <i>Crataegus monogyna</i>	1.228 \pm 0.225	18 \pm 3.606

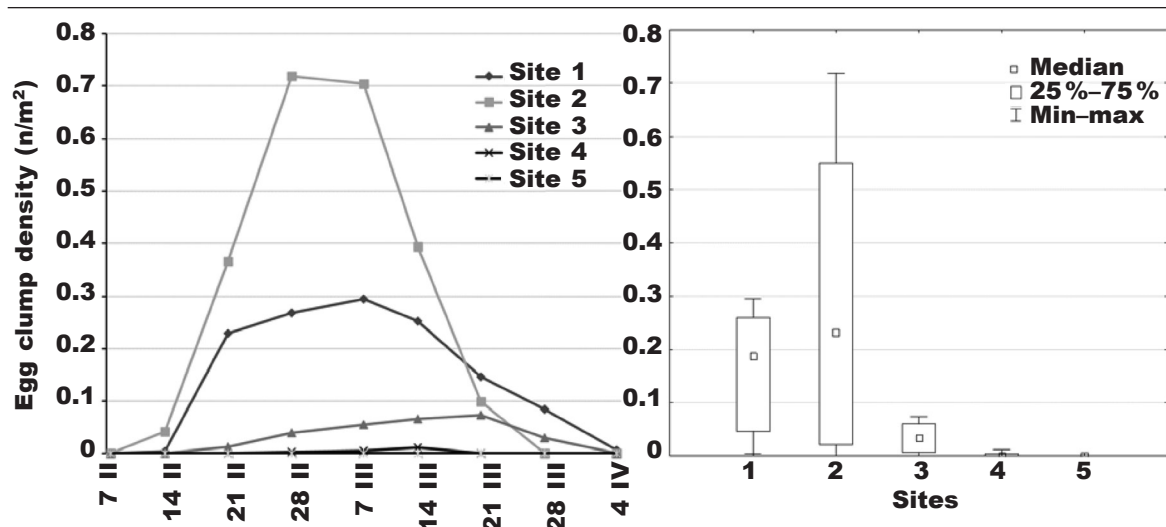


Fig. 1. Egg clump density at the various breeding sites: trend throughout the spawning period (left); comparison between sites (right).

Fig. 1. Densidad de las masas de huevos en los diferentes sitios reproductivos: tendencia durante el período de puesta (izquierda); comparación entre sitios (derecha).

Tabla 1. Principales características reproductivas de Rana dalmatina y principales características de sus sitios de desove en la zona del estudio: BS, sitio de desove; Land uses, usos de las tierras cercanas; Vegetation, vegetación semiacuática y vegetación en el lecho de la zanja; Width, anchura media (\pm DE) (m); Depth, profundidad media del agua (\pm DE) (cm); NClump, número máximo de masas de huevos (número de masas medidas); DClump, densidad máxima de las masas de huevos (N/m^2); NEggs, número estimado de huevos por masa (media \pm DE, rango); EggSz, tamaño medio de los huevos (\pm DE) (cm, con envoltura); SpDepth, profundidad media de la puesta (\pm DE, rango) (cm); Dist, distancia media de las masas de huevo a las zonas forestales (\pm DE) (m).

NClump	DClump	NEggs	EggSz	SpDepth	Dist
129 (84)	0.295	614.767 \pm 276.366 (130–1391.602)	0.976 \pm 0.158	0.875 \pm 1.893 (0–8)	10
51 (41)	0.718	471.811 \pm 299.564 (91.594–1367.187)	1.085 \pm 0.271	1.158 \pm 2.428 (0–9)	40.634 \pm 12.369
89 (12)	0.073	685.634 \pm 335.169 (330.625–1481.481)	0.954 \pm 0.094	6.417 \pm 5.760 (0–16)	224.750 \pm 18.733
4 (4)	0.011	249.278 \pm 78.342	1.075 \pm 0.050	0	325.232 \pm 13.722
11	< 0.001	–	–	0	149.957 \pm 16.106

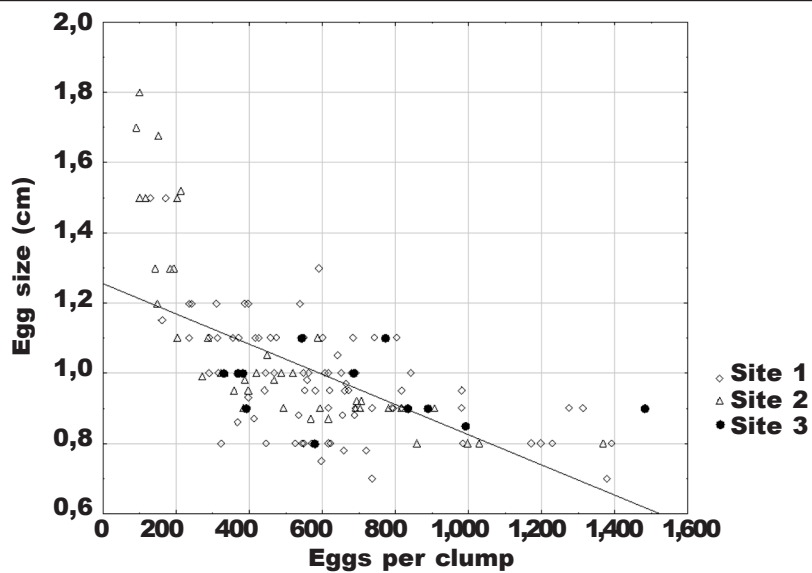


Fig. 2. Correlation between egg size and number of eggs per clump.

Fig. 2. Correlación entre el tamaño de los huevos y el número de huevos por masa.

Table 2. Comparison of egg size, clump size and spawning depth between the three main breeding sites: * comparisons significant at $p < 0.05$.

Tabla 2. Comparación del tamaño de los huevos, el tamaño de las masas y la profundidad de puesta entre los tres sitios reproductivos principales: * comparaciones significativas con $p < 0,05$.

	Wald stat.	p
Dependent variable: egg size		
Intercept	92.888	< 0.001
Number of eggs per clump	208.915	< 0.001
Water course	0.017	0.991
Goodness of fit. Deviance: stat/DF = 0.003; Pearson χ^2 : stat/DF = 0.003; AIC = -381.169		
Dependent variable: number of eggs per clump		
Intercept	3218.385	< 0.001
Egg size	160.633	< 0.001
Water course	5.909	0.048
Comparisons between water courses: 2 > 1, 3*		
Goodness of fit. Deviance: stat/DF = 0.138; Pearson χ^2 : stat/DF = 0.138; AIC = 123.863		
Dependent variable: spawning depth		
Intercept	0.028	0.868
Distance from wood	3.641	0.056
Water depth	25.886	0.000
Water course	6.231	0.044
Comparisons between water courses: 3 > 1, 2*		
Goodness of fit. Deviance: stat/DF = 3.157; Pearson χ^2 : stat/DF = 4.243; AIC = 547.843		

evident debris –the tadpoles' food source– on the bottom. These observations suggest intraspecific larval competition was probably high at this site (in addition, at the end of March, *Hyla intermedia* also spawned at this site). Larval density can have detrimental effects on metamorphosis, influencing the development rate, size and survivorship of larvae (e.g., Wilbur, 1976; Semlitsch and Caldwell, 1982; Petranka and Sih, 1986; Murray, 1990). At high densities and/or low food levels, larvae generally grow more slowly and are smaller at metamorphosis, both because of reduced resources, and because of the production of growth inhibitors (Licht, 1967; Wilbur, 1977; Steinwascher, 1978). As such, high larval competition can induce females to choose alternative breeding sites (Hartel, 2008) or, to lay fewer eggs (Resetarits and Wilbur, 1989). A similar mechanism could be hypothesized at site 2 but an extended sampling is clearly necessary to verify and understand the observed pattern.

Nonetheless, some other features at site 2, such as the presence of fewer predators and favorable thermal conditions (e.g., Resetarits and Wilbur, 1989; Petranka et al., 1994; Laurila and Aho, 1997; Binckley and Resetarits, 2003; Ficetola and De Bernardi, 2004) may have made it an attractive breeding site. If, on

one hand, the absence of vegetation surely reduced the availability of food and shelter for tadpoles, it could also have reduced the presence of predators, which, among invertebrates, are usually associated with more abundant vegetation (Gunzburger and Travis, 2004). Indeed, during sampling, we did not observe potential predators (such as, Odonata or Dytiscidae larvae), at site 2 on any occasion, with the exception of the allochthonous species *Procambarus clarkii* (adult individuals), which was present in the whole area but was not observed spawning in this ditch. In the other *R. dalmatina* breeding sites, on the contrary, we recorded *P. clarkii* larvae (at all sites), *Gambusia affinis* and, sporadically, Dytiscidae. Previous studies have shown that the choice of the breeding site of anuran females can be driven by the attempt to avoiding larvae predators (Resetarits and Wilbur, 1989; Petranka et al., 1994; Laurila and Aho, 1997; Binkley and Resetarits, 2003).

Moreover, site 2, like site 1, was characterized by shallow water, which is usually an advantageous feature for the development of both embryos and larvae, because temperature can quickly rise, hastening the growth processes and easing survival rates (Semlitsch, 2002).

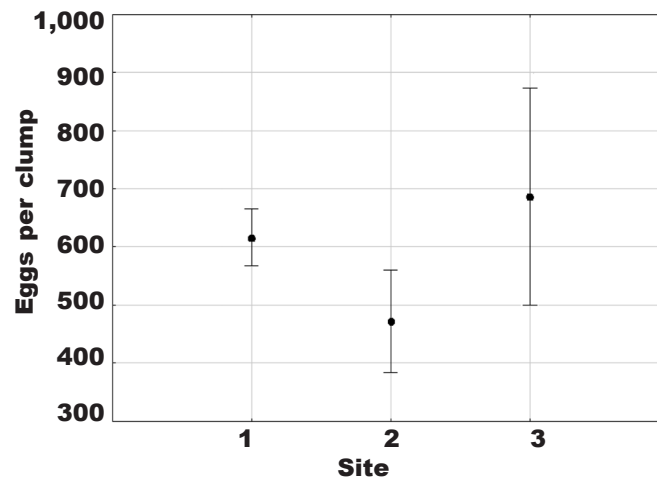


Fig. 3. Comparison of the estimated egg number per clump between sites 1–3 (mean values \pm SD are shown).

Fig. 3. Comparación del número estimado de huevos por masa entre los sitios 1–3 (se muestran las medias \pm DE).

Most importantly, sites 1 and 2 were located next to wet woodland (dominated by *Quercus rubur*, *Alnus glutinosa*, *Robinia pseudacacia*), the presence of which guarantees an optimal habitat for metamorphosed juveniles and adults (Bernini et al., 2007). The positive relationship between the occurrence and density of *R. dalmatina* and the presence of deciduous woodlands has been widely observed (e.g., Pavignano et al., 1990; Hartel et al., 2009), and it is particularly strong at a small spatial scale (Ficetola et al., 2009). In a study in a floodplain, Ponsero and Joly (1998), found that most egg clumps were laid within 50–100 m from the wood.

Most breeding sites in the study area dried up before the end of April, and the only site that guaranteed tadpoles' complete metamorphosis was site 1 (where we observed the first metamorphosed juvenile in less than three months, at the end of May). This caused the loss of 54% of the breeding effort (in terms of egg clumps) in the surveyed area. An analogous situation was observed in 2010 (Biaggini and Corti, 2011), when most of the drainage ditches and the flooded portions of fields dried out before larval metamorphosis (within April). On that occasion, a very large amount of *Procambarus clarkii* (in early life stages) was also recorded, especially at site 3, where although an egg clump density of 0.067/m² was recorded at the beginning of March, we did not observe *R. dalmatina* larvae at the end of April (Biaggini and Corti, 2011). In 2010, we hypothesized a possible influence of *P. clarkii* in *R. dalmatina* loss of fitness, as observed in other areas (Ficetola et al., 2011). In spring 2017, all egg clumps developed without evidence of predation and the main cause of mortality was the early drying up of the breeding sites. It is possible that the red swamp crayfish may have

different impacts over time, due to natural population fluctuations (Martelloni et al., 2012).

The variability in the breeding activity of the agile frog over the years (Cummins, 1986) and the species relatively high longevity (Sarasola–Puentes et al., 2011) underscore the need for long-term monitoring in order to understand the population trends of the species, and to identify the involved biotic and abiotic factors in a certain area. Our observations, however, can provide some prompts for the conservation of the species in rural environments. The high clump densities recorded and the persistent spawning activity in artificial ditches over the years, suggest that the minor water bodies strictly connected to agricultural activities can be attractive breeding sites for *R. dalmatina*. In view of this, their maintenance and correct management may be of key importance for the persistence of amphibian populations, at least in traditional agricultural areas. The management of such water bodies must take several requirements into account. In the study area, the hydroperiod of *R. dalmatina* breeding sites should be extended to guarantee the metamorphosis of larvae. However, maintaining the ephemeral nature of ditches is important to lower the presence of potential predators and invasive species (e.g., Ficetola et al., 2012). The observed preference of the agile frog for a site without aquatic vegetation could indicate an opportunistic behavior of this species, probably shifting its more common habits to avoid predation. Should this be the case, the increase of sites similar to site 2, by simply managing already existing drainage ditches in the fields next to woodlots, would enhance the availability of breeding sites suitable for *R. dalmatina* and less fit for *P. clarkii*. Finally, promoting the control of red swamp crayfish abundance and dispersion could have positive effects for all the amphibian species in the area.

Acknowledgements

Animal manipulation was approved by *Istituto Superiore per la Protezione e la Ricerca Ambientale* (Prot. 68754/T-A31 del 28/11/2016). We thank M. Ferrer and A. Gosá for their useful comments.

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