

## Comparative reproductive biology of European cave salamanders (genus *Hydromantes*): nesting selection and multiple annual breeding

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**Abstract.** Information on the life history of European cave salamanders remains limited. Despite a handful of studies carried out both under natural and controlled conditions, one of the least known aspects concerns the reproductive and breeding behaviour. Here we present information on the breeding behaviour of all the eight European *Hydromantes* species collected during four years of intensive monitoring (2014–2017). We provide information on the breeding phenology, suggesting that the breeding seasons are likely linked to environmental variables. Nesting sites were used repeatedly in different years by different females. Our data indicated a seasonality of mating activity and the possibility of sperm storage. Furthermore, we report on the presence of a single large-sized oviductal egg in *H. imperialis*. Finally, we documented oviparity in *Hydromantes sarrabusensis*, the only species that was suggested to be viviparous.

**Key words.** *Speleomantes*, Plethodontidae, cave, Amphibia, Urodela, mating behaviour, Mediterranean, biospeleology, seasonality.

### Introduction

European cave salamanders (genus *Hydromantes*; see WAKE 2013 for discussion on nomenclature) include eight species distributed in Italy and in a small part of South-Eastern France (LANZA et al. 2006a, SILLERO et al. 2014). The two main characteristics of these amphibians are the lack of lungs and direct development (LANZA et al. 2006b). Lack of lungs forces these animals to rely on cutaneous and bucco-pharyngeal respiration only (LANZA et al. 2006b), which occurs efficiently only in specific environmen-

tal conditions, i.e., high moisture and cool temperature (SPOTILA 1972). Underground habitats (e.g., caves, mines, cracks and other epikarst-environments) are therefore optimal refuges from adverse outside environmental conditions (FICETOLA et al. 2012, CULVER & PIPAN 2014, LUNGH<sup>1</sup> et al. 2014a). Even though underground detection of salamanders is higher in summer, these habitats are exploited throughout the year, suggesting that they are extremely suitable for these species (LUNGH<sup>1</sup> et al. 2015a); moreover, besides the suitable microclimate, they mostly lack predators (PASTORELLI & LAGHI 2006, MANENTI et al. 2016, SAL-

VIDIO et al. 2017a), and, therefore, represent an optimal choice to carry out a delicate life-phase such as reproduction.

Giving the elusive habits of *Hydromantes*, many of their life-history traits are still poorly known, and reproduction is among the less known traits. During the last years, several studies on the biology and physiology of these salamanders have been performed to better understand their reproduction (all related papers are gathered in the latest review written by LANZA et al. 2006b). Sexual maturity in *Hydromantes* is generally reached at the third or fourth year, while in the big-sized Sardinian species is likely reached a bit later (SALVIDIO 1993, SALVIDIO et al. 2017b). Courtship has been observed throughout the year, while the deposition of spermatophores was observed only in autumn (for further details on courtship see also BRUCE et al. 2000, LANZA et al. 2006b). However, given that in these species the pachytene spermatocytes do not degenerate during the cold season, it is likely that European cave salamanders can mate throughout the year (MERTENS 1923, LANZA 1959).

Besides some physiological analyses, only a few studies performed under controlled conditions were focused on *Hydromantes*' breeding behaviour (DURAND 1967, LANZA et al. 2006b, ONETO et al. 2010, ONETO et al. 2014). Recently some authors monitored clutches in nature, improving the knowledge on this topic and allowing comparison with findings obtained in controlled conditions (PAPINUTO 2005, LUNGI et al. 2014b, LUNGI et al. 2015b, MURGIA et al. 2016). The first field report on the breeding behaviour was provided by PAPINUTO (2005), who monitored one clutch of *H. genei* in an abandoned mine. The female was observed curled up on her eggs for nearly five months until hatching occurred. This behaviour not only allows the transfer of her skin secretion to the eggs providing protection against bacteria and fungi, but also protects the nest from intruders (LANZA et al. 2006b, ONETO et al. 2014). Monitoring activities on clutches was also carried out on *H. italicus*, *H. flavus* and *H. imperialis* (LUNGI et al. 2014b, LUNGI et al. 2015b, MURGIA et al. 2016). Findings of these studies were generally consistent with those observed for *H. strinatii* in controlled conditions (ONETO et al. 2010, ONETO et al. 2014). Embryos require a long time to develop before being ready to hatch, and during this span of time the mother rarely leaves the clutch unattended (LANZA et al. 2006b, LUNGI et al. 2014b, ONETO et al. 2014). After hatching, the mother provides parental care to the hatchlings for some weeks before they leave the nest (ONETO et al. 2010, LUNGI et al. 2015b). MURGIA et al. (2016) also observed that the highest density of nest sites was found in a section of a cave showing the most stable microclimate, characterized by high humidity levels (close to saturation) and a yearly fluctuation of temperature of only 2°C.

Nevertheless, many aspects of the breeding biology of cave salamanders remain poorly known. a) Does *Hydromantes* breed more than once a year? All studies on European cave salamanders carried out in nature report that

only one breeding season occurs during the warmer periods of the year (PAPINUTO 2005, LUNGI et al. 2014b, LUNGI et al. 2015b, MURGIA et al. 2016). b) Do cave salamanders show nest site selection? Observations performed in nature described the location chosen by females to nest, but it is not known whether and how females select the breeding site, nor if nest-site fidelity occurs. The present long-term study attempts to answer the above mentioned questions, adding unpublished insights to the behaviour of these salamanders. Furthermore, for the first time oviparity in *Hydromantes sarrabusensis* is reported, a species that was thought to be viviparous (LANZA et al. 2006b).

## Materials and methods

### Data collection

During a period of four years (2014–2017) we performed extensive field activities with the aim to cover as much as possible the distribution range of the eight European *Hydromantes* species (LANZA et al. 2006a). Overall, we surveyed > 150 underground sites (caves and mines) where the salamanders were present. Twenty-two of them (seven for *H. flavus*, three for *H. supramontis*, three for *H. imperialis*, three for *H. genei*, three for *H. sarrabusensis* and three for *H. ambrosii*) were repeatedly surveyed during the entire study period. Surveys on *H. flavus* were performed during all seasons, while for the other species they were mainly concentrated in spring/early summer and autumn. During each survey, we first checked if salamanders were performing courtship behaviour. These salamanders adopt the so called “vaccination”: after mounting on the female, males use their pre-maxillary teeth to produce scratches on the females skin in order to transfer the hedonic secretions produced by their mental gland directly in the female circulatory system; all these actions are performed with the purpose to increase the female interest and make her willing to collect the spermatophora (LANZA et al. 2006b). We then measured snout–vent length (SVL, using a plastic transparent rule) to the nearest mm in females salamanders, considering gravid those showing fully developed eggs (~3–5 mm in diameter) visible in the abdominal cavity; when possible, we also recorded salamanders' distance from the cave entrance. Using Linear Mixed Models, we tested whether gravid females occupy different cave areas when compared to the non-gravid ones. For this analysis we used the square-root transformed distance from the cave entrance as dependent variable, female condition (gravid/non-gravid) and species identity as dependent variables, and cave identity as random factor. We then used Linear Models to test whether gravid females showed larger size. To avoid pseudo-replication, for this analysis we only used the females collected during the survey where the highest number of individuals was measured, plus individually marked females (LUNGI & VEITH 2017). In this analysis, SVL was used as dependent variable, while condition (gravid/non-gravid), species identity and the interaction between these two, as independent variables.

Table 1. Number of females with visible abdominal eggs of eight species of *Hydromantes* observed from different caves per month. Last columns show the number of surveyed caves, the total number of surveys performed and the total number of females observed per each species. Each cave was surveyed max once per month; x = no surveys performed.

Species	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	N caves	N surveys	N females
<i>H. strinatii</i>	x	x	x	x	x	7	x	x	x	x	x	x	1	1	40
<i>H. ambrosii</i>	x	x	x	x	x	10	x	x	x	x	x	x	4	10	113
<i>H. italicus</i>	x	x	x	x	x	3	x	x	1	x	x	x	5	5	27
<i>H. flavus</i>	1	0	0	12	2	1	0	0	4	3	0	0	10	47	282
<i>H. supramontis</i>	x	x	x	0	1	x	x	x	2	x	x	x	3	10	82
<i>H. imperialis</i>	x	x	9	1	7	1	6	x	25	x	x	x	7	14	157
<i>H. sarrabusensis</i>	x	x	x	x	7	x	x	x	1	x	x	x	2	8	46
<i>H. genei</i>	x	x	x	x	3	x	x	x	3	x	x	x	3	10	40

### Extended monitoring

*Hydromantes imperialis*. One cave located in the Oristano Province was monitored for 3 years (2014 to 2016). During 2014, the monitoring of clutches started in early summer and lasted until late summer, when nests became empty (LUNGI et al. 2015b). In 2015 and 2016, surveys always started in January and ended when nests were empty: we performed 19 surveys in 2015 and 9 in 2016. We used an endoscope to check for mothers and eggs inside rock cracks, when inspection by bare eyes was not possible. On September 14<sup>th</sup>, 2016, four gravid females were marked using Visual Implant Elastomers (SALVIDIO 2013).

*Hydromantes sarrabusensis*. On May 17<sup>th</sup>, 2016, we found in two sites some females with externally visible developed eggs. One female per site, apparently in advanced pregnancy, was placed in a terrarium on site. Soil and stones were placed in the terrarium to imitate the natural substratum and holes were drilled to allow air circulation. Females were regularly fed with invertebrates captured on site (Diptera, Coleoptera and Orthoptera).

## Results

### Gravid females

Out of 787 observed females, 112 from 29 different underground environments were observed carrying eggs in their abdomen (Table 1). The number of externally visible eggs ranged from six to ten (Fig. 1A); in two cases (in *Hydromantes imperialis*) females showed just one single large egg (~10 mm, Fig. 1B). Gravid females were found at an average depth ( $\pm$  SD) of 19.99 m  $\pm$  2.90 (N = 57, min = 3 m, max = 102 m); no significant differences between the position of gravid and non-gravid females were found (condition-gravid,  $F_{1,523} = 0.592$  and  $P = 0.441$ ; no significant differences between species:  $F_{6,26} = 1.30$ ,  $P = 0.29$ ). The smallest gravid females observed for the mainland species *Hydromantes strinatii* and *H. ambrosii* was 52 mm (SVL), while for the Sardinian species was 56 mm long (*H. flavus* and *H. imperialis*). The average SVL of gravid females was significantly larger than for those in which we did not detect

eggs (N of females analysed = 454; ANOVA:  $F_{1,438} = 59.16$ ,  $P < 0.001$ ) (Fig. 2). SVL was significantly different among species ( $F_{7,438} = 8.7$ ,  $P < 0.001$ ), but the interaction between species and egg presence was not significant ( $F_{7,438} = 0.71$ ,  $P = 0.655$ ), indicating that the pattern is the same across all the species.

### Courtship, breeding behaviour and clutches

From May to September, couples of different species were observed performing the so called “vaccination” (Fig. 3A–B). In two occasions, we observed the exchange of a spermatophore. On September 17<sup>th</sup>, 2014, a female and a male of *H. italicus* were found sharing the same crack in a cave located in the Prato Province. The female showed visible developed eggs in her abdomen and a spermatophora attached to the cloaca. On May 28<sup>th</sup>, 2017, in a cave located in the Oliena area, a female without any visible egg was observed with a spermatophora attached to the cloaca.

One female of *H. genei*, one of *H. supramontis* and two of *H. ambrosii* were observed close to oviposition on September 11<sup>th</sup> 2016, in a cave located in the Carbonia-Iglesias Province, on May 18<sup>th</sup>, 2017, in a cave located in the Oliena area, and on June 22<sup>th</sup>, 2017, in a cave located in La Spezia Province, respectively. During manipulation an egg was almost extruded from the cloaca (Fig. 1C–D). On September 7<sup>th</sup>, 2016, a large number of *H. supramontis* hatchlings were found in a cave located in the Dorgali area; more than 70 small salamanders (total length 25–30 mm) were found on the walls and on the cave floor. Finally, on April 24<sup>th</sup>, 2017, a female *H. supramontis* was found guarding a litter of young in a cave in the Baunei area (Fig. 4A).

### Prolonged monitoring

*Hydromantes imperialis*. Seven clutches were found in 2015 (Table 2; the first one on March 22<sup>nd</sup>). The first hatchlings were observed on August 21<sup>st</sup> and all nests were empty on September 30<sup>th</sup>. In 2016, nine clutches were found (Table 2): the first on February 21<sup>st</sup>, the first hatchlings ap-



Figure 1. Gravid females of various cave salamanders species. (A) *Hydromantes flavus* with eight visible developed eggs; (B) *H. imperialis* showing a single large-sized egg. Females of (C) *H. genei* and (D) *H. supramontis* almost dropping an egg from their cloaca.

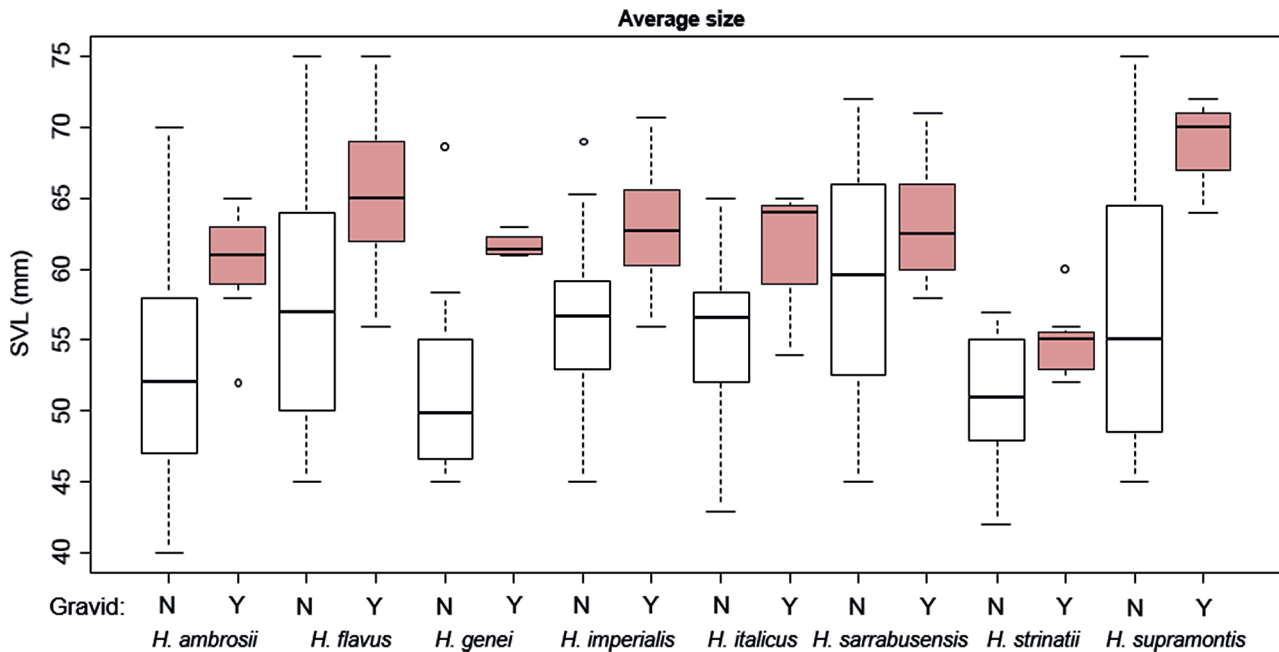


Figure 2. Boxplots showing average SVL (mm) of non-gravid (N, white) and gravid (Y, pink) females for each *Hydromantes* species (*H. strinatii*, N = 33 (N) and N = 7 (Y); *H. ambrosii*, N = 103 (N) and N = 9 (Y); *H. italicus*, N = 21 (N) and N = 4 (Y); *H. flavus*, N = 97 (N) and N = 11 (Y); *H. genei*, N = 17 (N) and N = 4 (Y); *H. imperialis*, N = 48 (N) and N = 24 (Y); *H. sarrabusensis*, N = 24 (N) and N = 6 (Y); *H. supramontis*, N = 43 (N) and N = 3 (Y)).



peared on July 19<sup>th</sup>, all nests were empty on August 14<sup>th</sup>. In 2017, the first oviposition was recorded on March 8<sup>th</sup>. The cave was occupied by a few males (N = 10), by gravid females (carrying externally visible eggs, N = 17) and by females who already oviposited (N = 3) (Table 2) on this date. Ten nests were counted on April 29<sup>th</sup> (Table 2). Overall, 13 different nesting sites were detected (Table 2) and the majority were used for more than one season: ten were used at least twice, while three of them were used consecutively for three or even four times (Table 2). All the nesting sites were located between 15 m and 30 m from the cave entrance and showed variable shapes (holes, cracks, cervices) and positions (height from the ground).

*Hydromantes sarrabusensis*. On April 22<sup>nd</sup>, 2017, one female produced a clutch of 14 eggs in the terrarium (Fig. 4B), while the second female was still retaining the eggs in her abdomen.

### Discussion

In *Hydromantes*, body size of gravid females is highly variable; the body size of the smallest gravid females recorded

in this study for both mainland and Sardinian species, was smaller than observed by SALVIDIO (1993) during individual dissections performed on one of the smallest *Hydromantes* species, *H. strinatii*. The case of *H. imperialis* females carrying a single large egg is noteworthy (Fig. 1B) since the clutch size for *Hydromantes* reported by LANZA et al. (2006b) varies from 6 to 14 eggs; however, there are no reports on the correlation between clutch and egg size, nor for unusual large eggs. A dissimilarity in the number of fertilised and unfertilised eggs has been never reported; therefore, additional studies are urgently required to understand the significance of single large eggs.

Our observations on the mating behaviour mostly correspond to the literature, describing aseasonal courtship (LANZA et al. 2006b). We observed mating throughout the year, and both gravid and non-gravid females collecting spermatophores. If mating occurs throughout the year, but egg laying (at least for Sardinian species) is mostly restricted to spring (Table 2), it likely indicates that *Hydromantes* females may be able to store sperm, as known for other plethodontids (ADAMS et al. 2005). ONETO et al. (2010) rearing wild salamanders for breeding experiments report-

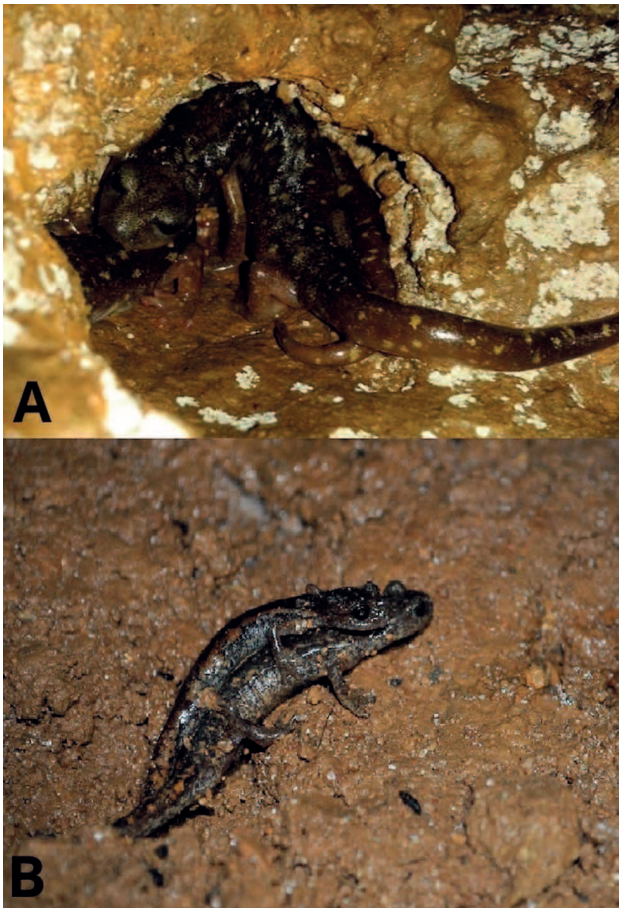


Figure 3. *Hydromantes imperialis* (A) and *H. ambrosii* (B) during courtship.



Figure 4. (A) Female of *H. supramontis* attending the nest with her brood and (B) Female of *H. sarrabusensis* with her clutch.

Table 2. Data on monitored clutches of *Hydromantes imperialis*. First column = numbered nests; per each year columns = date at which eggs were observed for the first time, date at which hatchlings were observed for the first time, date at which the nest was empty; wide dash = nest unused during the monitoring year; “n.o.” = hatchlings not observed; “Failed” = breeding was interrupted. Data for 2014 were taken from LUNGHİ et al. (2015).

Nest site	2014			2015			2016			2017
	First eggs observation	Hatchlings	Empty	First eggs observation	Hatchlings	Empty	First eggs observation	Hatchlings	Empty	First eggs observation
1	15.VI	28.VIII	25.X	18.IV	n.o.	27.VII	21.II	n.o.	11.V	12.III
2	15.VI	25.VIII	20.IX	4.IV	21.VIII	30.IX	5.III	n.o.	19.VII	8.III
3	15.VI	28.VIII	13.IX	–	–	–	–	–	–	–
4	15.VI	16.VIII	27.IX	–	–	–	–	–	–	29.IV
5				22.III	Failed		1.VI	n.o.	24.VI	
6				28.III	Failed		6.IV	n.o.	24.VI	
7				18.IV	26.VIII	30.IX	5.III	19/07	14.VIII	8.III
8				18.IV	21.VIII	26.VIII	1.VI	n.o.	19.VII	
9				26.IV	n.o.	27.VII	–	–	–	
10				–	–	–	5.III	n.o.	11.V	2.IV
11	–	–	–	–	–	–	6.IV	n.o.	11.V	25.IV
12	–	–	–	–	–	–	11.V	n.o.	24.VI	8.III
13										29.IV
14										1.V
15										25.V

ed that they captured “apparently gravid females” in June 2007 with visible fully developed eggs. The females were placed in a terrarium without any contact with males. Egg laying occurred after more than four months, and 45 weeks later authors observed the first hatchlings. Two females of the present study were observed picking up a spermatophora each: one had visible fully developed eggs, while the other did not show any egg presence in her abdomen. In plethodontids male competition for reproduction is well documented (HOUCK et al. 1985, VERRELL 1991, HOUCK & VERRELL 1993), and females have the opportunity to mate with several males by storing their sperm (HOUCK & ARNOLD 2003). Therefore, according to our observations, sperm storage may also occur in European *Hydromantes*.

Another interesting information concerns the time of egg retention. The recapture of the marked gravid female (*H. imperialis*) and the observation of the two *H. sarra-busensis* females kept in the terrarium show that fully developed eggs are carried over a very long period (up to 12 months), confirming the observations reported for other *Hydromantes* species (ONETO et al. 2010). Females need to allocate a large amount of resources for reproduction, and the long period lasting from fecundation to egg deposition is followed by more than six months of clutch attendance and parental care (LUNGHİ et al. 2014b, LUNGHİ et al. 2015b; Table 2). Therefore, the reproductive cycle is extremely long and likely lasts at least two years. Considering the limited lifespan of these salamanders (max. 11 years in captivity; SNIDER & BOWLER 1992) and that sexual maturity is reached at around the third-fourth year (SALVIDIO 1993), it implies that females can only complete a limited number of breeding cycles during lifetime. This limits the potential

growth rate of populations and is an additional concern for the conservation of these endemic species.

*Hydromantes* salamanders are usually reported to lay eggs in springs while hatching mostly occurs in late summer – early autumn (LANZA et al. 2006b, LUNGHİ et al. 2015b, MULARGIA et al. 2016, SERRA & ARGIOLOS 2016). Most of our observations match the above schedule (see Table 2), although in some cases a different seasonal pattern occurred. A female of *H. genei* was observed in September close to lay eggs (Fig. 1C), while previous studies on this species reported clutches in spring only (STEFANI & SERRA 1966, PAPINUTO 2005). For *H. supramontis* we observed hatchlings in a nest on late April (Fig. 4A) and a female close to lay eggs in late May (Fig. 1D); the only available information on this species reports an egg clutch observed in February (MULARGIA et al. 2016). These data suggest that breeding activity may be not constant, and thus might occur in different periods. Given that these observations of non-standard breeding activity were performed in different caves, such differences might be related to local environmental condition (e.g., temperature, humidity). However, additional data is needed to confirm this hypothesis.

Given the length of the *Hydromantes* breeding cycle (LANZA et al. 2006b, ONETO et al. 2010, LUNGHİ et al. 2015b, MURGIA et al. 2016), the choice of the breeding period has a strong impact on the breeding success. It is generally assumed that caves are characterised by a relatively stable microclimate with limited seasonal variation. However, recent analyses suggest that the impact of climate seasonality on underground communities may be strong (LUNGHİ et al. 2017). The earliest observations of *H. imperialis* clutches ranged between late February (in 2016) to



late March (in 2015; Table 2), suggesting some variation in the phenology of *Hydromantes* among years. Meteorological data showed that February 2015 was much colder than February 2016 [average air temperature: 2015:  $9.4 \pm 0.1^\circ\text{C}$ ; 2016:  $12.8 \pm 0.1^\circ\text{C}$ ; data from the nearest weather station (Cagliari; www.eurometeo.com)], suggesting that meteorological conditions could affect breeding phenology. Underground microclimate is partially affected by the variation of outdoor conditions (LUNGHİ et al. 2015a), and this might influence underground activity because of the peculiar physiology of *Hydromantes* (SPOTILA 1972, LANZA et al. 2006b, LUNGHİ et al. 2016). However, in the above mentioned cave the nesting area has a rather stable microclimate (LUNGHİ et al. 2015b, MURGIA et al. 2016).

In the visited cave, several nesting sites were used in different years, suggesting nesting site selection (Table 2). This is the first time that such behaviour was observed in *Hydromantes*. The repeated use of nesting sites cannot represent site fidelity, because it is unlikely that a female breeds for two consecutive years. Our data suggest that different females use the same nesting site, as the majority of nests were used multiple times (Table 2). The reason of such behaviour is still unknown and might be related to favourable environmental conditions that drive all females to breed together in such a reduced space (LUNGHİ et al. 2015). Breeding site defence might be an alternative hypothesis. Females actively defend their offspring against intruders (ONETO et al. 2014), and laying eggs in deep crevices might improve egg protection. In the study cave, the number of such sites may be somehow limited, thus increasing the probability that the most suitable ones are repeatedly used.

Until the present study, the breeding biology of *H. sarrabusensis* was uncertain. Only a single report on a captive female suggested ovoviviparity, mostly based on the observation of a newborn salamander (LANZA & LEO 2001). Such information is strongly in contrast with the available knowledge related to the other *Hydromantes* species, which are known to be oviparous. Conversely, our data indicate that *H. sarrabusensis* is oviparous and thus shares the reproductive mode of its congeners (Fig. 4B). The case of a record of “viviparity” might have been the result of stress caused by the prolonged cooled condition at which females were kept.

The study of underground habitats is extremely challenging, especially because animals exploit small environments that humans cannot access and where observations are extremely difficult to obtain. Therefore, only the future collection of a large amount of data will allow a comprehensive understanding of the breeding biology of subterranean cave salamanders. As we have presented novel interesting information on the breeding behaviour of European cave salamanders, we hope to stimulate future investigations.

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