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Original Citation:

In vivo sexual discrimination in Salamandrina perspicillata: a cross-check analysis of annual changes in external cloacal morphology and spermic urine release / VIGNOLI L.; SILICI R.; BRIZZI R.; BOLOGNA M.. - In: HERPETOLOGICAL JOURNAL. - ISSN 0268-0130. - ELETTRONICO. - 20:(2010), pp. 17-24.

Availability:

This version is available at: 2158/609090 since:

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12 October 2024

In vivo sexual discrimination in *Salamandrina perspicillata*: a cross-check analysis of annual changes in external cloacal morphology and spermic urine release

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In *Salamandrina*, the lack of visible external sexual dimorphism makes the sexing of individuals difficult without sacrifice. The cloaca of *Salamandrina* in both males and females appears externally as a slit on an unswollen surface, a trait which is consistent throughout the year. Nonetheless, a slight divarication of its borders allows the recognition of three morphs (A, B and C), respectively characterizing male cloaca (all phases), female cloaca without protruding oviductal papillae (courtship phase) and female cloaca with prolapsed oviductal papillae (oviposition phase). Figures and schematic diagrams are provided to illustrate the differences in detail, which are all recognizable to the naked eye or by means of a hand magnifier. In addition to morphology, another reliable method of sexing salamanders is urine examination, albeit only during the courtship and post-courtship phases. Applying these methods for sex determination, we found a male-biased operational sex ratio in two populations, ranging from 6.6:1 (autumn–winter) to 14:1 (May). Males were confined to terrestrial environments, whereas females were also found in water during oviposition. *Salamandrina perspicillata* was active throughout the year, except during the hottest months (July–August).

Key words: cloaca, salamander, sex determination, sex ratio, sexual dimorphism

INTRODUCTION

Sexual dimorphism, defined as the morphological differentiation between males and females of the same species, is a conspicuous feature of gonochoric animals (Andersson, 1994; Fairbairn, 2007). Depending on the species, sexual dimorphism may include differences in body size, shape and other traits (secondary sex characteristics – Arnold & Duvall, 1994; Fairbairn, 2007). Sexual dimorphism may be attributable to sexual selection (Darwin, 1871; Vitt & Cooper, 1985; Shine, 1988), differences in ecology between the sexes (Shine, 1989; Anderson & Vitt, 1990), or both (Hedrick & Temeles, 1989; Sandercock, 2001; Pearson et al., 2002; Fontenot & Seigel, 2008). Among amphibians, sexual dimorphism in body size and morphology has been widely explored with regards to male combat and sexual selection. Females are often larger than males, presumably due to fecundity selection, whereas males are larger than females in cases when they engage in intrasexual combat (Shine, 1979; Woolbright, 1983; Halliday & Verrell, 1986; Fontenot & Seigel, 2008). During courtship and mating, urodeles show a swelling of male cloacal glands controlled by testicular hormones (Duellman & Trueb, 1984; Sever, 1991a, b; Kupfer, 2007). However, some caecilians and salamanders lack any distinct external sex dimorphism (Silverstone, 1975; Hayek & Heyer, 2005; Kupfer, 2009) and the identification of gender is unclear. Sexing via cytogenetic analysis is often hampered by sex chromosomes which, if present, are generally weakly differentiated (Duellman & Trueb, 1984; Hayes, 1998). Sexing based on behavioural differences requires direct observations, which often prove difficult.

The Italian endemic genus *Salamandrina* Fitzinger, 1826, which includes only two vicariant species (see Angelini et al., 2007, for a review), lacks evident secondary sexual characters. Probably due to fecundity selection, males are on average smaller and more slender than females (Vanni, 1981; Romano et al., 2009), but the large overlap between males and females in these traits precludes their use for sex determination. Previously documented morphological features are not suitable for *in vivo* discrimination of sex (e.g. cytogenetic: Mancino & Barsacchi, 1966; lateral line organs: Delfino et al., 1984). Lanza (1983) stated that an examination of urine (spermic vs non-spermic) could be helpful in discriminating between males and females, although this approach needs experimental confirmation (Angelini et al., 2007). Hitherto the absence of external characters discriminating between sexes in the genus *Salamandrina* has limited the investigation of basic population demographic parameters (but see Romano et al., 2009 for preliminary data) and of the species' reproductive and courtship behaviour. Behavioural traits in *Salamandrina* species were previously examined only in females (recognizable during the oviposition period, Romano et al., 2008 on *S. perspicillata*), whereas anecdotal field observations were made on individuals of uncertain sex (Utzeri et al., 2005). Behavioural traits involving intersexual interaction (courtship, mating, male-female attraction, etc.) have been neglected up to now (Silici et al., unpublished).

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Recently, Romano et al. (2009) observed distinctive cloacal features (visible to the naked eye) among sexes in autumnal individuals and proposed a method of *in vivo* discrimination that has yet to be tested for individuals from other periods of the year. Here we provide additional information on *S. perspicillata* cloacal morphology supplied by optic and electronic scanning microscope explanatory photos as well as by spermic urine examination to distinguish sexes *in vivo* throughout the entire year. The method is based on the recognition of the typical features characterizing male and female cloacal chambers according to the reproductive phase, visible *in vivo* after simple cloacal slit divarication. Moreover, we report the application of the method to two salamander populations, while providing the operational sex ratio throughout the year. As a case study, we used *S. perspicillata* Savi, 1821, a threatened species belonging to a genus strictly endemic to the Apennine peninsula (Italy), which has been studied by some of us from an ecological point of view (e.g. Vignoli & Bologna, 2001; Vignoli et al., 2001; Della Rocca et al., 2005).

MATERIALS AND METHODS

We collected individuals of *S. perspicillata* from two populations in October 2008–June 2009 (Table 1). Population A inhabited an oak (*Quercus cerris*) wood surrounding a small tributary of the Crémera river within Vejo Regional Park (latitude 42.105455, longitude 12.405161; Latium region, central Italy). Population B inhabited a beech (*Fagus sylvatica*) forest surrounding a small brook within the WWF reserve of Guardiaregia (latitude 41.398789, longitude 14.549980; Molise region, central Italy). We sampled salamanders during the daytime under a clear sky. Samplings in the selected areas were performed during both morning (0800–1200) and afternoon (1400–1800) to evaluate possible differences in diel activity and between the sexes. The sampling effort was standardized in terms of the number of man-hours. All the individuals were released into the collection site two months after capture, having previously been used in other experiments on behaviour (Silici et al., unpublished). We defined four behavioural and ecological phases according to prevalent activity: 1) courtship (October– November) when animals are in terrestrial activity (feeding and mating); 2) post courtship (December–January) when salamanders have probably ended mating activity but can be still found continuing feeding activity on land; 3) laying phase (February–early May) when ovipositing females are found in water and males on land; 4) pre-aestivation (late May–June) when a few salamanders are found on land, but showing reduced activity. Generally, during the summer individuals remain under a variety of substrates, e.g. stones, fallen branches, litter, etc.

Before the morphological analysis of the cloacal region, all the individuals were kept isolated in small boxes in an air-conditioned room at 18 °C in the *stabularium* of Roma Tre University, Dipartimento di Biologia Ambientale. Together with the cloaca inspection, we performed an examination of urine. A microscope slide was prepared for each individual. Fresh urine samples (at least three drops) from each salamander, obtained by means of slight pressure on the cloaca sides, were smeared on to clean slides. The slides were air-dried for 1–2 h and then the presence of free sperm or spermatophore structures were detected under a binocular microscope (Leitz, model Laborolux) using 20–100× lenses. For 16 individuals collected in October 2008 from population A we performed three replicates of urine inspection at three-day intervals. The observation of spermic urine necessarily tends to be ambiguous in discriminating between sexes, since sperm may be from either the Wolff ducts in males or sperm storage in the spermatheca of females. Besides the presence/absence information, we also used a rough quantitative estimate of free sperm in spermic urine. It is known that males stored sperm in Wolff ducts from autumn to spring, whereas females can store sperm in spermatheca for months after mating (Brizzi et al., 1985). It is highly unlikely that mated females released a massive number of sperm in the urine because the sperm remain agglutinated in the spermatheca (Brizzi et al., 1988; Sever, 2002).

Table 1. Synopsis of the individuals studied with indications of population, sample date, behavioural phase, type of analysis (cloacal and urine inspections), and number of individuals analysed (*n*). *For 16 individuals we performed three replicates of urine inspection.

Population	Date	Phase	Cloacal morph (*n*)	Urine (*n*)
A1	Oct 2008	Courtship	40	40*
A2	Nov 2008	Courtship	22	22
A3	Jan 2009	Post-courtship	30	30
B1	May 2009	Laying	25	9
B2	May 2009	Pre-aestivation	15	7

For examining external cloacal morphology, we took photographs of the cloacal region under an optical stereomicroscope (Olympus, model SZ11) using 4–20× lenses. Cloacal *rima* were observed both in natural conformation and with gland outlets and other internal structures exposed by divaricating the cloacal lips using forceps. Data on internal cloacal morphology of males and females refer to previous studies by Brizzi et al. (1988, 1989, 1990, 1995), here with the addition of appropriate drawings and unpublished images performed by scanning electron microscope (model Philips 515).

We used chi-square tests to analyse the diel activity pattern between sexes. Statistical analyses were performed by Statistica (Statsoft, version 7.0).

RESULTS

We collected a total of 157 salamanders, 102 individuals from Vejo (autumn and winter) and 55 individuals from Guardiaregia (late spring). We used 132 individuals for cloacal morphological inspection and 108 individuals for urine inspection.

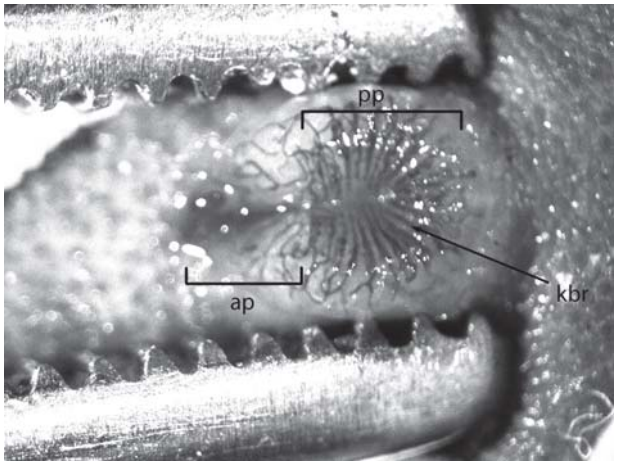

Fig. 1. Cloacal morph A. Details in the text. Abbreviations: ap = anterior region; kbr = knife blade ridges; pp = posterior region.

Cloacal morphology on external inspection

The cloacae of 132 salamanders were observed both in natural conformation and with divaricated lips. The external appearance of the cloacal region was relatively uniform among individuals from all the study periods, with some salamanders showing a slight swelling and others without any protuberance around the vent. The inspection of the internal features performed by divaricating cloacal *rima* revealed three distinct morphs.

Morph A (Fig. 1): a cloacal cavity consisting of two regions according to the cephalo-caudal axis; the anterior characterized by several ridges, richly irrorated, and a rough surface probably due to the presence of glandular outlets, and the posterior with a lower number or no presence of the above structures.

Morph B (Fig. 2): a cloacal cavity lightly rugose and without any visible glandular structures.

Morph C (Fig. 3): a cloacal cavity with a richly irrorated surface, well evident prolapsed oviductal papillae and no visible glandular structures.

Morph A occurred in 87 of the 107 individuals collected from October to May, when only in terrestrial environments. Morph B characterized 14 of the 92 individuals collected from October to January in terrestrial environments. Morph C was observed in 31 of the 70 individuals collected from February to May in both aquatic and terrestrial environments.

Internal cloacal morphology

Male cloaca. According to the cephalo-caudal body axis, the male cloaca of *S. perspicillata* consists of two consecutive regions: a cloacal tube and a cloacal chamber, as schematized in Figure 4. The first is tubular in shape, follows the posterior end of the gut and receives the outlet of the urinary bladder and urogenital papillae. The cloacal chamber is a larger cavity opening to the exterior by means of the vent. In both regions peculiar structures are found on the walls, consisting of characteristic outgrowths (folds, reliefs, ridges), in which the various cloacal glands involved in spermatophore assemblage open. By external examination of the cloaca, particularly after vent divarication, only some characters of the cloacal chamber become evident, the cloacal tube being very internal. As shown in Figure 5, performed under SEM, the anterior ventral portion of the cloacal chamber contains "knife-blade" ridges, arranged in groups of 9–10 on both lateral walls and covered by ciliated epithelium (the latter not visible to the naked eye). Minute papillae, corresponding to the outlets of the ventral glands (typical of the male cloaca of most salamandrids), are aligned along the medial margin of each ridge. The ridges gradually flatten as they converge towards the vent and disappear in the posterior third of the cloacal chamber where the wall surface appear slightly rugose (Fig. 5). In strong contrast with the highly cornified epidermis surrounding the cloacal orifice, the epithelium of the internal cloacal cavity appears vascularized, particularly in correspondence with the knife-blade ridges.

This cloacal chamber arrangement consists of two regions: 1) the anterior one showing knife-blade ridges, and 2) the almost flat posterior one, corresponding to cloacal Morph A described above (compare Fig. 1 with Figs 4–5).

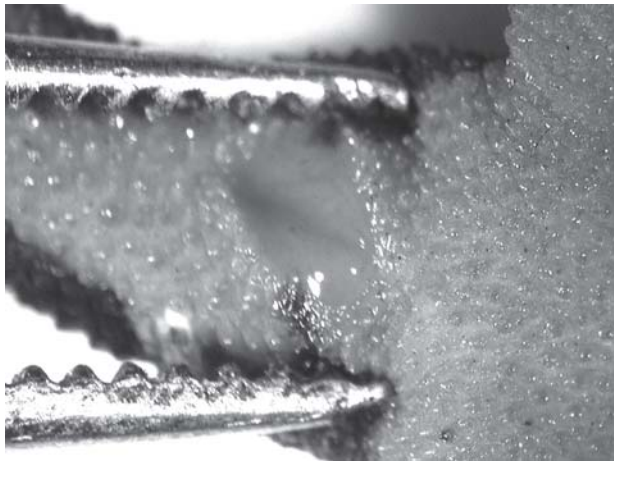

Fig. 2. Cloacal morph B. Details in the text.

Fig. 3. Cloacal morph C. Details in the text. Abbreviations: ov = oviduct end tracts; is = irrorated surface.

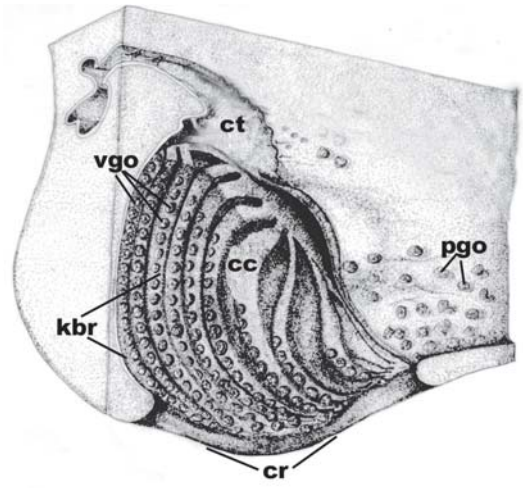

Fig. 4. Three-dimensional reconstruction of the cloacal chambers in male *S. perspicillata*, which appears cut on the sagittal plane. Details in the text. Abbreviations: cc = cloacal chamber; cr = cloacal rima; ct = cloacal tube; kbr = knife blade ridges; pgo = pelvic gland outlets; vgo = ventral gland outlets.

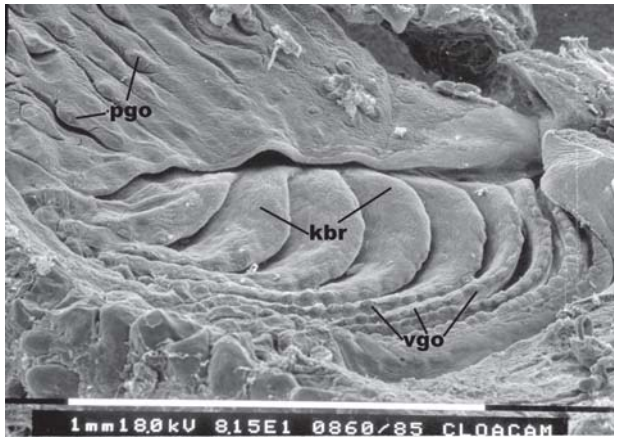

Fig. 5. A detail of the cloacal chamber with rough walls in male *S. perspicillata*. S.E.M. 100x. Details in the text. Abbreviations: kbr = knife blade ridges; pgo = pelvic gland outlets; vgo = ventral gland outlets.

Female cloaca. This is also made up of both a tubular region, receiving the openings of oviducts and ureters, and a chamber surrounding the vent. The cloacal tube contains two conspicuous structures: the oviductal papillae (Fig. 6). During the laying season, these papillae project ventrally and caudally up to the anterior half of the cloacal chamber and appear collapsed and very wide in diameter, related to oviposition. Their distal outlets are also obvious on external inspection (Morph C, Fig. 3). In contrast to the clear presence of the oviductal papillae, the female cloacal chamber is also provided with shallow folds or ridges (Fig. 7), only scarcely evident to the naked eye. In addition, the outlets of both spermatheca and ventral glands, occurring in the anterior half of the cloacal chamber, are very small pores, without any particular relief or papilla. Despite this, the female cloacal epithelium also appears richly vascularized. Morph B (lack of any glandular structures and regular folds with no prolapsed oviductal papillae; Fig. 2) observed in 14 of the 92 individuals collected from October to January in terrestrial environments, corresponds to females outside the laying season.

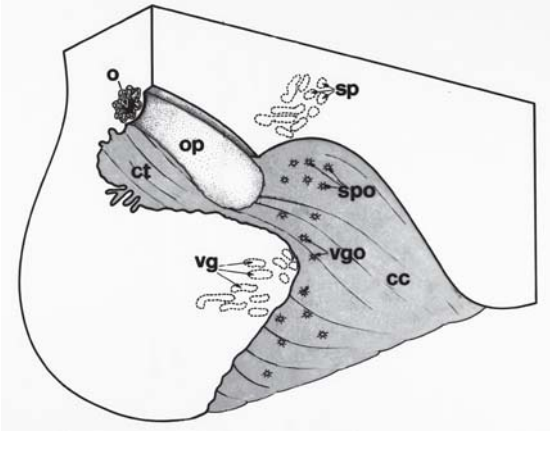

Fig. 6. Diagrammatic representation of mid-sagittal section through the cloacal region of female *S. perspicillata*. The dotted profiles represent spermathecal and ventral gland tubules localized in the wall thickness. Details in the text. Abbreviations: cc = cloacal chamber; ct = cloacal tube; o = oviduct; op = oviductal papilla; sp = spermathecae; spo = spermathecal outlets; vg = ventral glands; vgo = ventral gland outlets.

Fig. 7. The cloacal region on a para-sagittal plane of female *S. perspicillata*, showing its main internal configuration with the two typical regions: the cloacal tube (only its caudal portion is visible) and the wide cloacal chamber represented by various structural complications of the walls. S.E.M. 100x. Details in the text.

Table 2. Inspection responses from morphological and urine analyses in individuals of *Salamandrina perspicillata* from the study populations. Abbreviations: A=morph A; B=morph B; C=morph C; S=spermatic urine; NS: non spermatic urine. * = number of individuals used for cloacal and urine inspection. Numbers in parentheses: individuals on which both cloacal and urine inspections were performed.

		Inspection response	
Population	Phase	Cloaca*	Urine*
A1	Courtship	28A/12B	29S/11NS
A2	Courtship	20A/2B	19S/3NS
A3	Post-courtship	25A/5C	26S/4NS
B1	Laying (individuals in water)	0A/25C (6C)*	0S/6NS
B2	Pre-aestivation (individuals on the ground)	14A/1C (9A)*	2S/7NS

Urine observation

We inspected urine samples belonging to 108 salamanders. Spermic urine was predominant in individuals in courtship and post-courtship phases, whereas almost all (87%) of the individuals collected in May produced non-spermic urine. For individuals in the courtship phase (October–November) spermic urine was found in 48 samples and non-spermic urine in 14 samples. For the 16 individuals (33%) examined in this phase, only one provided inconsistent results for three repeated urine inspections (a sample with spermic [a single sperm] and the remainder with non-spermic urine). In individuals in the post-courtship phase, 26 out of 30 presented spermic urine. No individuals collected in water during the laying phase (n=9) had sperm in the urine, and only two collected on land presented spermic urine (very few sperm were visible).

Cross observation

A synopsis of the cloacal–urine cross observations is reported in Table 2. The urine samples analysed from autumnal (courtship) individuals showed an almost complete (97.9%) correspondence between the presence of sperm (spermic urine) and the characterization of cloacal morphology (morph A). Moreover, 21.4% of autumnal morph B individuals showed few sperm in the urine. Individuals from the post-courtship phase showed a total correspondence between urine and morphological inspections (spermic urine and morph A) except for one (morph C) with spermic urine. During oviposition activity we found only cloacal morph C individuals with no sperm in the urine, whereas on land two out of nine individuals (all morph A) presented spermic urine.

Sex ratio and ecological inferences

The operational sex ratio was male-biased in both the populations analysed and during all activity phases. The Vejo population showed a sex ratio of 6.6:1 during autumn and early winter. The Guardiaregia population in the late laying and early pre-aestivation phases showed an overall sex ratio of approximately 2:1. Moreover, if we computed the sex ratio considering terrestrial and aquatic habitats separately, we found respectively a sex ratio of 14:1 and no males in water. There was no difference in the number of individuals (males and females) observed between morning and afternoon (for all tests, $\chi^2 \leq 0.157$; df=1; $P \geq 0.692$).

DISCUSSION

This is the first attempt to describe in detail the external morphology of the cloaca in *S. perspicillata* during the whole adult life cycle, and it is one of the first studies aiming to discriminate between the sexes *in vivo* (see Romano et al., 2009 for the autumnal season). On the basis of our observations (confirming previous literature: Brizzi et al., 1989), the cloaca of *S. perspicillata* in both males and females appears externally as a simple slit located on an unswollen surface, a trait which was consistent throughout the year. In most European urodeles, cloacal lips and swellings are easily identifiable as secondary sexual characters allowing easy discrimination between the sexes (Duellman & Trueb, 1994). In *S. perspicillata*, the lack of any visible external sexual dimorphism hindered any attempt to sex individuals without sacrificing them (Vanni, 1981; Brizzi et al., 1989). All the morphological features described in this paper were easily recognizable by means of a stereomicroscope or a hand magnifier by divaricating cloacal margins; such features can therefore be useful in distinguishing males and females in the field and in every season, without any sacrifice or damage to the individuals.

Cloacal examination and urine analysis

External observation of cloacal morphology allowed the recognition of three clearly differentiated morphs (A, B and C). Compared with evidence under SEM, morphs A, B and C could be easily assigned respectively as follows: A) male cloaca (consistent feature through all phases); B) female cloaca, lightly rugose, without protruding oviductal papillae (namely during autumn/winter courtship phase); and C) female cloaca with prolapsed oviductal papillae, evident during the spring, pre- or post-oviposition phase. This latter feature is noticeable since it may be observed not only in aquatic samples (definitely female), but also in terrestrial individuals (males or females). Thus, only the actual recognition of swollen oviductal papillae (shown in this paper) can prevent these female structures being mistaken for male cloacal ridges, particularly when individuals are collected and examined in the field. To confirm our findings, the external and internal cloacal features described here match those reported in previous papers (Brizzi et al., 1988, 1989, 1990; Romano et al., 2009 for individuals from autumn). In particular, the glandular outlets aligned on the richly irrorated ridges observed in the cloacal anterior region of Morph A correspond to the male "knife-blade" ridges described by Brizzi et al. (1988) (see also the same structures in Figs 4–5; this study). Concerning the external cloacal morphology of individuals assigned to morph B, the lack of any prominent structure is consistent with our observations on the fe-

male cloaca outside the laying season when the oviductal papillae do not project as far as the cloacal chamber. Nonetheless, the lack of "knife-blade" ridges rules out this morph's corresponding to the male cloaca. On external observation, the cloaca of individuals belonging to Morph C consisted of a cavity with an epithelium richly vascularized and not organized into any well defined structures (as "knife-blade" ridges) apart from rather shallow folds or ridges. Moreover, none of the latter was particularly evident to the naked eye. In this same morph, however, clearly visible oviductal papillae projected from the cloacal tube, their distal outlets reaching the cloacal chamber.

Urine examination is a reliable method of sexing salamanders only during the courtship and post-courtship phases (from October to January). In these phases, males usually release into the urine a large number of sperm that they accumulate in the Wolff ducts from early autumn to April (Brizzi et al., 1989). Qualitative (spermic versus nonspermic urine) and quantitative aspects (number of sperm observed) need to be taken into account and replicates for single individuals are required for urine examination to be used as a method to discriminate between sexes with confidence. The failed correspondences between morphological analysis and urine examination in the individuals examined from the courtship and post-courtship phases can easily be avoided if all individuals presenting spermic urine with very few sperm are eliminated from the analysis. These individuals are probably already mated females, which release remaining sperm that are not agglutinated in the spermatheca. During the pre-aestivation phase, the correspondence between morphological and urine analyses was very scant. This could be due to the discontinued presence of sperms in Wolff ducts from May to September (Brizzi et al., 1989).

Sex ratio and ecological inferences

The operational sex ratio (i.e. the ratio of sexually active males to fertilizable females ready to mate; Emlen & Oring, 1977) was male-biased in both populations. For the Vejo population, the sex ratio was 6.6:1 during autumn and early winter (courtship and post-courtship phases). In late laying and early pre-aestivation phases (May), the Guardiaregia population presented a roughly 2:1 sex ratio. If we excluded the individuals found in water (all females), the sex ratio was 14:1. Although Ramorino (1863) found aquatic prey in male stomachs, the presence of males in water was never confirmed (e.g. Vanni, 1981; Della Rocca et al., 2005). At Guardiaregia Reserve, we surveyed both terrestrial and aquatic environments and found that males were confined to the terrestrial environment, generally being observed in the litter and under stones or branches. The male-biased operational sex ratio observed is in agreement with Romano et al. (2009; calculated from three surveys) and could be explained by 1) a proportion of females skipping annual breeding opportunities (Vignoli et al. unpublished); 2) age at maturation (there are no data on *S. perspicillata*, but see Bjorklund, 1991); 3) mortality (Hairston et al., 1983); 4) asynchronous arrival (Arntzen, 2002; Salvidio, 2008); 5) spatial variation when searching for mates (Quinn et al., 1996); or 6) a combination of several factors. Although the male-biased sex ratio was consistent throughout the year, more data are needed to formulate sound conclusions on this aspect.

Our observations revealed no difference in day-time activity between morning and afternoon and between the sexes. However, this is the first study in which the activity of both sexes of *S. perspicillata* has been monitored throughout the whole year in terrestrial and aquatic environments. We found females and males (the latter exclusively in terrestrial environments) active from October to early June. The method for discriminating sexes in the field that we report in this paper can help clarify further ecological characteristics of this species and contribute to further field and captive research.

ACKNOWLEDGEMENTS

The Italian Ministry of the Environment gave us the authorization for the temporary capture of amphibians (authorization n. DPN-2009-0005106). No animals were damaged or killed during this project and all were handled according to the standards of the Italian Ministry for Scientific Research and Technology. We would like to thank the administrations of Vejo Regional Park and WWF Italia which permitted research in Vejo Park and in the Guardiaregia Reserve respectively. Particular thanks should go to Luca Luiselli who recommended the Vejo area for this research, and to Marco Zuffi and an anonymous referee for useful suggestions on the submitted draft.

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Accepted: 22 November 2009