

## Sex-biased dorsal spotted patterns in the fire salamander (*Salamandra salamandra*)

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**Abstract.** Sexual dichromatism has been reported for several amphibian species. Generally, in the animal kingdom, males are more colourful than females. This more pronounced colouration in either quantitative (area of pigmentation) or qualitative (hue, saturation, brightness) form probably plays a role in courtship or mate attraction, but one common polytypic species *Salamandra salamandra* is still unexplored in this conjunction. We investigated the dorsal patterns of wintering salamanders captured from December 2012 to April 2013 in six underground shelters in Slovakia. We recorded significant size differences between the sexes in all of the morphometric characteristics evaluated, and using free image analysis software, we provide first evidence of the existence of sexual dichromatism in this species, as males exhibit significantly larger yellow spots on the dorsum and tail than females. No significant sex-related differences in the number of spots and their qualitative colour parameters (hue, saturation, brightness) were detected, though. In both sexes, spots along the vertebral line predominate. The dominance in colouration in females on the left-hand side and the weak correlations between snout–vent lengths and spot characteristics were also surveyed, as was the total area covered with spots.

Key words. Caudata, colour patterns, image analysis, pigmentation, sexual dimorphism.

### Introduction

Sexual dimorphism is widespread in the animal kingdom and describes phenotypic differences between males and females of the same species (FRAYER & WOLPOFF 1985, SHORT & BALABAN 1994, FAIRBAIRN et al. 2007). Identifying sexually dichromatic traits helps to understand behavioural patterns associated with sexual selection or reproductive success as well as the evolution of life history traits and mating systems (KUPFER 2007, POKHREL et al. 2013). Sexual dichromatism has been reported for several amphibian taxa, mostly anurans (e.g., TODD & DAVIS 2007, DAVIS & GRAYSON 2008, BELL & ZAMUDIO 2012).

The development of graphic software has facilitated measuring and recording differences not only in skin colour over the entire surface of an individual (e.g., DAVIS & GRAYSON 2007), but also colour quality patterns (hue, saturation, brightness) or quantity (spotted area) of each spot on the dorsal and ventral sides. These techniques have been applied to amphibians; an example relevant to the Caudata is a study of adult red-spotted newts, *Notophthalmus viridescens*. Here, sexual dichromatism was observed using an image analysis software approach: the significantly more intensely red dorsal spots in males than in females

were otherwise invisible to the naked eye. This was the first evidence of a sex-related difference of pigmentation in this species (DAVIS & GRAYSON 2008). Generally, the dorsal spots of some amphibian species have been evaluated not only in terms of sexual dimorphism, but also in further perspectives, e.g., subspecies delimitation, sexual dichromatism, or fluctuating asymmetry (RIVERA & SIMÓN 1999, WRIGHT & ZAMUDIO 2002, TODD & DAVIS 2007, DAVIS & GRAYSON 2007, DAVIS & MAERZ 2007, POKHREL et al. 2013).

The fire salamander, *Salamandra salamandra* LINNAEUS, 1758 (Salamandridae) is one of the most common caudate species in Europe and occurs in various habitats (KLEWEN 1988, EGEE-SERRANO et al. 2006, MANENTI et al. 2009, JABLONSKI et al. 2013, KUZMIN et al. 2013). Sexual dimorphism in body size and shape is relatively well known in this species, with females being altogether larger and having larger interlimb distances (LABUS et al. 2013). In contrast, males have a slender body with longer limbs and a markedly swollen cloaca during the breeding season (KALEZIĆ et al. 2000). These external features as well as the presence of a receptaculum seminis in the female's cloaca are commonly used for identifying sexes in this species (FRANCIS 1934, OPATRŇY 1983). However, the very similar appearance of the sexes and absence of specific secondary

Table 1. List of investigated wintering sites of *Salamandra salamandra*.

Wintering roost	Type	Altitude [m]	Orographic unit	Coordinates		No. of individuals (females/males)
Bobačka cave	cave	811	Spišsko-gemerský kras Mts.	48°46.9'N	20°06.3'E	10 (6/4)
Veterná diera	cave	441	Košická kotlina Basin	48°53.8'N	21°20.7'E	8 (5/3)
Ticha Voda	gallery	855	Volovské vrchy Mts.	48°46.2'N	20°36.3'E	20 (6/14)
Teplá	gallery	370	Revúcka vrchovina Mts.	48°36.7'N	20°10.1'E	16 (8/8)
Ružín water reservoir	gallery	587	Čierna hora Mts.	48°50.0'N	21°02.8'E	13 (4/9)
Čremošna	gallery	588	Vihorlatské vrchy Mts.	48°53.9'N	22°11.1'E	1 (0/1)

sexual characteristics in males during the breeding season can cause considerable difficulties in identifying sexes.

Dorsal patterns in salamandrids have been studied in terms of their supposedly aposematic function (WELLS 2007), but sexual dimorphism in this characteristic is only little known. In one endemic species, the Corsican fire salamander, *Salamandra corsica*, a significant increase in spots, which changed into irregular shapes with increasing snout-vent length (SVL), was discovered using image analysis. This brought one of the first pieces of concrete evidence of a post-metamorphic pattern change in amphibians (BEUKEMA 2011). Furthermore, GIESENBERG (1991) recorded a post-metamorphic development of the dorsal pattern in *S. salamandra terrestris*. Nevertheless, this polytypic species is still poorly explored in terms of dorsal pigmentation and its sexual dimorphism.

We used an image analysis approach to study sexual dimorphism in the dorsal pattern of the fire salamander. Further, we examined differences in the position and number of spots and whether a dominant side with a greater surface area covered with spots existed between sexes. Finally, we investigated the relationship between the length of an individual and the number, area covered, circularity and perimeter of the spots.

### Material and methods

Salamanders were captured from December 2012 through April 2013 during wintering in six underground shelters in Slovakia (Tab. 1), where the nominate subspecies, *Salamandra s. salamandra* occurs. We found a total of 68 individuals (29 females, 39 males). Salamanders were individualised using a marking method based on unique dorsal spotted patterns (OPATRŇÝ 1983). To avoid pseudo-replication, each individual was subjected to colour analysis only once. Sexes were identified by examining the external morphology of the cloaca and the presence of a receptaculum seminis in females (FRANCIS 1934, OPATRŇÝ 1983). Pictures of the salamanders were obtained using a fully opaque wooden 'black box' (40 × 30 × 30 cm) with precise cutouts for the camera (Canon EOS 1100D with 18–55 IS II lens and Zeikos CPL5 filter) and flashlight in the lid of the box. Each individual was placed on the bottom of the box vertically un-

der the camera. This photographic method ensured maintaining standardised lighting conditions. After having been photographed, the individuals were measured with callipers and released. We recorded their TL (total length), SVL (the distance from the tip of the snout to the posterior margin of the cloaca), RPL (right parotoid length), and HW (the widest part of the head). Photographs were analysed with Image J software (ABRAMOFF et al. 2004, SCHNEIDER et al. 2012). Using the program's polygon selection tool, we manually selected all spots on the dorsum and tail of each individual to minimise distortion effects. Subsequently, we added pictures into the ROI (region of interest) manager list and calibrated pictures converted to an HSB stack – a three-slice (hue, saturation and brightness) stack – from which we could obtain these colour characteristics for each selected spot (Fig. 1). Hue describes the attribute of pure colour and therefore distinguishes between colours; saturation characterizes the shade of the colour, i.e., how much white is added to the pure colour; and brightness describes the overall brightness of the colour. Furthermore, we obtained the parameters of each spot's shape (circularity and perimeter). Patch circularity is defined as  $4\pi$  (area/perimeter<sup>2</sup>) and ranges from zero (infinitely elongated polygon) to one (perfect circle), and perimeter as the length of the outside boundary of the selection. Total body area of an individual (TBA = dorsum + tail without head and limbs) and the area of yellow spots were measured in mm<sup>2</sup>, from which we calculated the percentage of the total area covered with spots (total spots area, TSA) per individual. We also counted the number of spots and their positions using predefined categories: (1) spots on the right-hand side, (2) spots on the left-hand side, and (3) spots in the vertebral part of the body (passing through the vertebral line). In addition, we checked whether the amount of spot-covered area was different on the left- vis-à-vis the right-hand side of each individual.

Sex-related differences in physical parameters, spot characteristics and their colour were examined with t-tests. Analysis of spot positions in salamanders was conducted using the Kruskal-Wallis test. The relationships between SVL and the previous parameters were analysed using Pearson ( $r_p$ ) and Spearman ( $r_s$ ) correlations. All tests were conducted with GraphPad Prism 5 software (GraphPad Software, Inc.).

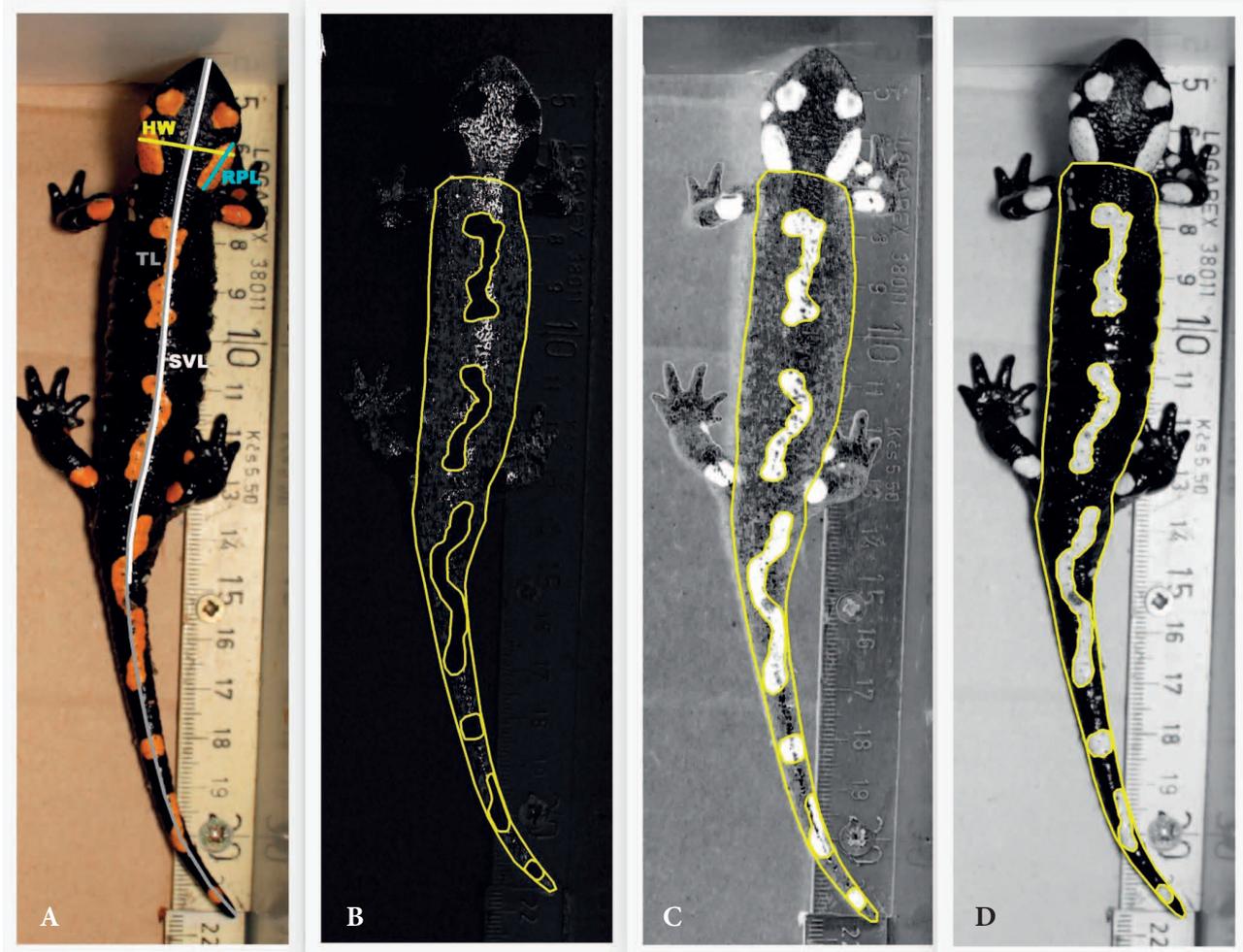


Figure 1. Picture in RGB colour with the basic biometric characteristics (TL – total length, SVL – snout-vent length, HW – head width, RPL – right parotoid length) marked and a HSB stack of the photographs of fire salamanders converted to three slices (hue, saturation, brightness) with outlined spots and the total body area of the individual during measuring. A) RGB stack; B) hue; C) saturation; D) brightness stack.

### Results

Females generally had significantly larger TLs (mean females 17.4 cm, males 16.9 cm;  $t = 1.997$ ,  $p < 0.05$ ) and SVLs (mean females 10.7 cm, males 10.2 cm;  $t = 2.255$ ,  $p < 0.05$ ) than males. We furthermore recorded significant differences in other biometrical parameters, namely RPL (mean females 1.4 cm, males 1.3 cm;  $t = 2.739$ ,  $p < 0.01$ ) and HW (mean females 2.2 cm, males – 2.1 cm;  $t = 3.824$ ,  $p < 0.01$ ). There was a strong positive relationship between SVL and each HW ( $r_s = 0.5639$ ,  $p < 0.01$ ) and RPL ( $r_s = 0.5113$ ,  $p < 0.01$ ) as well as between TL and TBA ( $r_s = 0.7672$ ,  $p < 0.01$ ) (Fig. 2), suggesting that individuals with a larger TL had a larger body surface area. No significant correlation was recorded between TL and the number of spots ( $r_p = 0.08321$ ,  $p = 0.4999$ ).

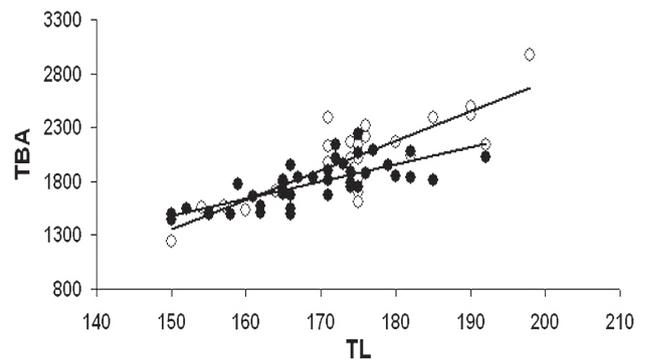


Figure 2. Relationship between total length and total body area covered with spots in the captured individuals. TL – total length [mm]; TBA – total body area covered with spots [mm<sup>2</sup>]; white circles – females; black circles – males.

A strongly significant sex-related difference in TSA was found ( $t = 4.304$ ,  $p < 0.01$ ) with a larger proportion of yellow colour in males than in females (Fig. 3). We did not record a significantly different result in the number of spots between the sexes ( $t = 0.9227$ ,  $p = 0.3595$ ); however, their most common positioning was in the vertebral part of the body, with a higher significance in males (females: Kruskal-Wallis test  $H = 7.016$ ,  $p < 0.05$ ; males:  $H = 31.24$ ,  $p < 0.01$ ) (Fig. 4). The area covered with spots was biased to the left-hand side in females ( $t = 2.169$ ,  $p < 0.05$ ), while no such difference was noted amongst males ( $t = 0.8123$ ,  $p = 0.4192$ ).

Correlation tests between SVL and spot characteristics (circularity and perimeter) produced variable results. In males, we did not record significant relationships (circularity:  $r_s = -0.009029$ ,  $p = 0.9565$ ; perimeter:  $r_s = 0.02792$ ,  $p = 0.8697$ ). The same was recorded in males with regard to the relationship of the total area bearing spots ( $r_p =$

$0.08324$ ,  $p = 0.6144$ ) and the number of spots ( $r_p = 0.2676$ ,  $p = 0.0996$ ). In females, a weak correlation was found in the circularity of the spots ( $r_p = -0.4302$ ,  $p < 0.05$ ) and the perimeter ( $r_s = 0.4616$ ,  $p < 0.05$ ). There was also a strong positive relationship between TSA and SVL ( $r_p = 0.6096$ ,  $p < 0.01$ ), suggesting that larger females had a larger spot coverage. No significant correlation was recorded with the number of spots in females ( $r_p = -0.09231$ ,  $p = 0.6339$ ).

Using image analyses, we found and quantified a wide range of skin colour values with regard to saturation, hue and brightness for male and female salamanders, but we found no sex-dependency in the observed colour parameters (saturation:  $t = 0.6864$ ,  $p = 0.4949$ ; hue:  $t = 1.170$ ,  $p = 0.2464$ ; brightness:  $t = 1.043$ ,  $p = 0.3006$ ).

## Discussion

We confirm the presence of significant size differences in all morphometric characteristics of fire salamanders, as has been recorded by previous authors from different portions of the species' geographic range (SHINE 1989, ROMANO et al. 2009, KALEZIĆ et al. 2000, LABUS et al. 2013).

Although we did not record any sex-related differences in the number of spots and their qualitative colour parameters (saturation, hue, brightness), significant differences were recorded in the total area covered with spots on the dorsum and tail of individuals. Males were more yellow in appearance, because of the markedly larger area of the dorsal surface that was covered with yellow spots. Inversely, a lower quantity of yellow pigmentation was found in females. This result corresponds, for example, with patterns recorded for the North American marbled salamander, *Ambystoma opacum* (TODD & DAVIS 2007, POKHREL et al. 2013). Similarly, more colourful males do exist, for instance, in numerous other vertebrates including amphibians such as newts and frogs, but likewise in fish, lizards and birds (ZAHAVI 1975, LOCATELLO et al. 2006, TODD & DAVIS 2007, DAVIS & GRAYSON 2007, 2008, BOURCE et al. 2011, DUBEY et al. 2011, BELL & ZAMUDIO 2012, POKHREL 2013). This phenomenon is commonly interpreted in various vertebrates (e.g., fishes, birds) as an intraspecific signal serving mate attraction (DUGATKIN & GODIN 1998, DILL et al. 1999, PRYKE et al. 2001, SAKS et al. 2003, DAVIS & GRAYSON 2008, SIMONS et al. 2012, STAFSTROM & HEBETS 2013) in that the amount and brightness of carotenoid-based colours are indicators of an individual's quality (HILL 1990, GODIN & DUGATKIN 1996, NICOLETTO & KODRIC-BROWN 1999, ALONSO-ALVAREZ et al. 2012). Maintaining this trait may be costly and thus indicates social dominance, good condition, good nutritional status, low parasite load, high behavioural vigour, high viability etc. (GODIN & DUGATKIN 1996). For example, HERMANSKI et al. (2013) found a strong relationship between the amount of yellow pigmentation in post-metamorphic fire salamanders and nutritional conditions during their larval phase. Analysing the predation behaviour, PRZYREMBEL et al. (1995) recorded that salamanders have a trichromatic colour vision based

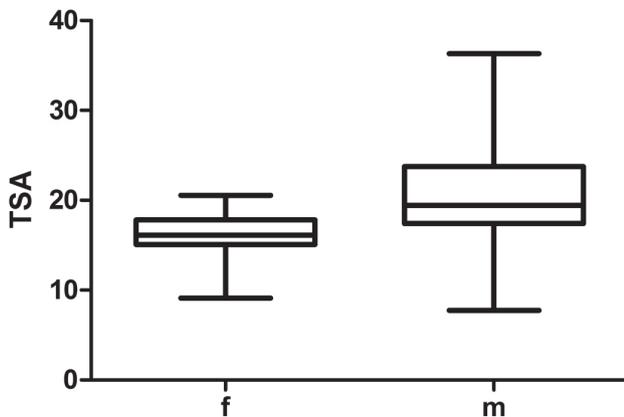


Figure 3. Differences in the total area covered with spots between sexes. f – females (min.–max. = 9.1–20.6%, mean = 15.8%); m – males (min.–max. = 7.8–36.3%, mean = 20.5%); TSA – total area covered with spots [%].

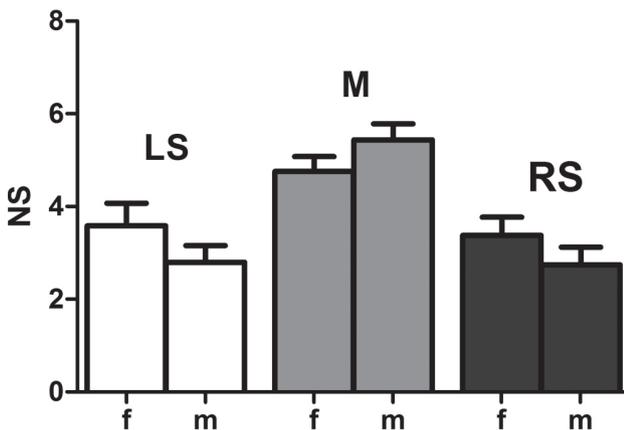


Figure 4. Observed spot positions in salamanders. f – females; m – males; NS – number of spots; LS – left-hand side; RS – right-hand side; M – vertebral.

on three types of photoreceptors that are maximally sensitive around 450, 500 and 570 nm. It is therefore possible that females have an ability to distinguish between shades of yellow colour and thus recognize and assess the yellow pattern of a male individual.

In some amphibians, sexual dimorphism in the proportion of coloured dorsal surface area or colour parameters (hue, saturation, brightness) of dorsal spots is well documented (e.g., HOFFMAN & BLOUIN 2000, TODD & DAVIS 2007, DAVIS & GRAYSON 2008, DOUCET & MENNILL 2010). We could therefore assume that the quantity of yellow pigmentation rather than its quality (hue, saturation, and brightness) may be a sexually dimorphic feature in this species, as the latter is not necessarily obvious to the naked eye. The question arises whether there is a relationship between this pattern and successful sexually motivated behaviour, such as the rival-combats of males in *S. salamandra* (KÄSTLE 1986), or whether more yellow males represent higher-quality individuals in terms of reproductive fitness.

In many species, dorsal patterns serving as sexual signals generally increase not only the visibility of the males to females, but equally their visibility to predators (GODIN & McDONOUGH 2013). In the fire salamander, however, the black and yellow dorsal pattern is also associated with skin glands whose excretions may cause serious toxic effects in occasional predators (CARRETERO & ROSELL 1999); therefore, we assume that in this species (more precisely, in the population studied by us), a markedly more intense pigmentation is not a handicap for males, but conversely a benefit in this regard. An improved protection from predation might make a more intensely coloured male specimen more attractive to a female, since her offspring stand a chance of 'inheriting' this improved protection. Furthermore, with regard to the relation between predation and various colour types, DAVIS & MILANOVICH (2010) observed different levels of stress hormones in two morphs of the red-backed salamander, *Plethodon cinereus*, with higher levels being found in lead-phase forms than in red-stripe forms as a possible consequence of the former being more exposed to attacks from predators.

Furthermore, less intense yellow pigmentation, i.e., a greater proportion of black colour in females, should be linked to an increased heating rate, because melanistic individuals are thought to absorb more solar radiation and thus reach optimal body temperature faster than lighter individuals (CLUSELLA-TRULLAS et al. 2008). A trend towards more melanistic females was also observed by POKHREL et al. (2013). Body temperature is essential for the physiological and behavioural performance of ectothermic animals (HOCHACHKA & SOMERO 2002, VENCES et al. 2002). Therefore, increasing the heating rate could play a vital role in the development of offspring inside the females and their birth, during which the female expends a lot of energy. Melanistic animals are mostly associated with higher altitude as a form of adaptation to cooler climate conditions, e.g., mountain species such as the Alpine salamander, *Salamandra atra*, which is completely black and

gives birth to only two, but fully metamorphosed young in what are practically inhospitable conditions (WAKE 1993). Thermoregulation provides ectothermic animals with the possibility of using diverse habitats in terms of different temperature conditions and actively control temperature-sensitive physiological processes (GÜIZADO-RODRÍGUEZ et al. 2010). A larger proportion of black colour for better absorption of solar radiation in females could also benefit a larger body size, because larger individuals heat up more slowly than smaller ones. POKHREL et al. (2013) assumed the same situation, i.e., more black pigmentation on the dorsum to enhanced heat conservation in females, in the marbled salamander.

In terms of the position of spots, the vertebral part of the body was the most commonly covered place in both sexes. We assume that this part of the body may be more conspicuous than the sides and have greater benefits for individuals in certain situations, such as mate attraction or deterring predators. It would be interesting to monitor the rate of fluctuating asymmetry in dorsal patterns as a proxy for differences in the average state of adaptation and coadaptation between populations (GRAHAM et al. 2010).

Left-hand side dominance in the yellow colouration of females could be caused by their preferring to expose one side for greater aposematic effect for deterring predators, but more data and appropriate behavioural tests are required for investigating this issue.

Additionally, weak correlations between the SVL and spot characteristics on the one, and between snout-vent length and total area covered with spots on the other hand may suggest that the dorsal pattern might change with age, as has already been observed by BEUKEMA (2011) in *Salamandra corsica*. Further research is needed to confirm such claims, however.

Our study represents the first quantitative and qualitative analysis of dorsal patterns relative to sex and produces evidence that a sexual dimorphism exists in dorsal spot characteristics in *Salamandra salamandra*.

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