ORIGINAL RESEARCH

A methodological workflow for quantitative colouration and colour pattern comparison reveals taxonomic and habitat-level differences in the polymorphic fire salamander (*Salamandra salamandra*)

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Abstract

The study of colour, including physical properties and patterns, is an essential step in comprehensively understanding the role, evolution and diversification of this trait involved in functions like survival, performance, reproductive success and communication. While quantitative studies on colour have primarily focused on colour attributes, such as hue or brightness, the quantitative assessment of how colours are distributed across an organism's body (i.e. pattern) has received less attention despite its high functionality irrespective and together with colour. This gap is particularly noticeable in amphibians, especially in highly polymorphic, continuously variable species. In this study, we use the urodele *Salamandra salamandra* as a study model to apply an analytical approach for extracting and quantifying colour proportions and patterns variation using the recently developed R packages *patternize* and *recolorize* to precisely describe and compare colour distribution at both taxonomic and habitat levels. We first assessed the potential application of this workflow in taxonomic studies by examining its accuracy and sensitivity in differentiating morphs, using *S. s. bernardezi* and *S. s. gallaica* subspecies. Subsequently, we applied the same analytical approach to compare colour and colour pattern shifts among population of *S. s. bernardezi* within an urban–forest system. Our results demonstrated that this methodological workflow effectively detects differences in colour patterns at the taxonomic and habitat levels, confirming its utility in different fields, from systematics to eco-evolutionary studies. Furthermore, our results revealed an increased prevalence of melanistic colouration and a less homogeneous colour pattern within urban settings, contributing to the limited number of studies about amphibian colouration in anthropic habitats. We outline and discuss alternative hypotheses to explain these patterns, although further investigation is necessary to address several new questions that arise from our results.

Introduction

Colour is a highly functional trait that mediates organisms' relationship with their environment, playing a crucial role in their biology, survival, performance, reproductive success and communication (Cuthill et al., 2017), but also frequently associated with other morphological, behavioural, physiological or life-history traits (McKinnon & Pierotti, 2010). Consequently, the study of colour variation allows the exploration of the underlying mechanisms driving the origin and evolution of phenotypic diversity (Maan & Sefc, 2013; Martin & Orgogozo, 2013; Orteu & Jiggins, 2020; Protas & Patel, 2008), for

which the development of appropriate tools capable of addressing the complexity of this trait has been fundamental (Endler, 1990; Endler & Mappes, 2017; Kemp et al., 2023; Pérez-Rodríguez et al., 2017).

Two distinct components can be extracted from an organism's colour: colour itself, which is determined by the visual properties (i.e. hue, brightness and saturation) of the light transmitted from the pigments or nanostructures in a specific body area; and pattern or the spatial arrangement of patches (i.e. spots, stripes or any marking) with varying colours, sizes, orientation, number and shapes (Endler, 1990; Shawkey & D'Alba, 2017). Although the combined effect of both colour

components encodes important visual information (Qvarnström et al., 2014; Spottiswoode & Stevens, 2010), it has also been demonstrated that each of them may play a significant biological role on its own. For instance, colour brightness and hue influence both predator learning and attack rates irrespectively of colour patterning in aposematic species (Hegna et al., 2011, 2013), but also affects sexual selection, denoting individual quality or status (Kenyon & Martin, 2023; Maan & Cummings, 2008), while darker colouration has been related to an improved thermoregulation performance (Clusella Trullas et al., 2007) and UV radiation protection (Brenner & Hearing, 2008). Colour pattern has also been demonstrated to be a highly functional trait nondependent upon the displayed colours. A specific pattern can act as a warning signal to deter predators (Preißler & Pröhl, 2017; Valkonen et al., 2011; Wüster et al., 2004), increases individual saliency at close distances in aposematic preys (Barnett et al., 2018), be associated to different escape behaviours (Rojas et al., 2014), influences sexual selection (Acord et al., 2013), serves as a badge of quality and status, or allows interspecific recognition (Mason & Bowie, 2020; Pérez-Rodríguez et al., 2017). Moreover, while the visual information provided by the colours of an individual would depend on receivers' ability to discriminate them, pattern features are expected to be accessible in more diverse visual environments (Robertson et al., 2022) and for a wider variety of taxa, as for instance highly-contrasted aposematic patterns for colour-blind predators (Prudic et al., 2007). Thus, although the overall colour perception would ultimately depend on receivers' visual system and environmental conditions, such as lighting conditions, visual angle and background, and transmission medium (Endler, 1990), colour components might differ in the scope of information transmitted, highlighting the importance of addressing both of them when studying colour variation and their underling mechanisms.

Quantitative studies on colour have mostly addressed physical colouration properties (i.e. hue, brightness and saturation), while far fewer studies have quantitatively addressed colour pattern variation, despite the current availability of a variety of analytical tools to quantify and analyse different patterns properties (e.g. Chan et al., 2019; Endler, 2012; Endler et al., 2018; Todd et al., 2005; Valvo et al., 2021; Van Belleghem et al., 2018; van den Berg et al., 2020). Indeed, such frameworks have allowed addressing diverse questions about specific processes underlying colour pattern variation between and within species (e.g. Hemingson et al., 2019; Lorioux-Chevalier et al., 2023; Yuan et al., 2022; Zhou et al., 2021). This gap in the quantitative study of both colour components is taxonomically widespread, but particularly striking in amphibians, a renowned group for its remarkable diversity in coloration and patterns. Colour in amphibians mediates in a variety of functions such as antipredator responses, conspecific communication, physiological regulation and UV protection (Rojas et al., 2023; Rudh & Qvarnström, 2013). Quantitative studies investigating colour pattern variation in amphibians have demonstrated the importance of considering the precise distribution of colours across an animal's body in: (i) species discrimination (Angelini et al., 2010; Vörös et al., 2007); (ii) intraspecific diversification (Costa et al., 2009); (iii) individual identification (Renet et al., 2019;

Schulte et al., 2024); (iv) the description of sexually dimorphic patterns (Ancillotto et al., 2022) and (v) the evolutionary processes underlying colour variation (Wollenberg et al., 2008). Furthermore, quantifying colour patterns would enable the expansion of studies to systems exhibiting continuous variation in colour patterning, where discrete categories cannot be defined, or their delimitation rely on subjective, non-biologically based criteria.

That is the case of the urodele fire salamander (*Salamandra salamandra*, Linnaeus, 1758), in which its exceptional colour polymorphism and polytypism have attracted taxonomical (Beukema et al., 2016), ecological (Aguilar et al., 2023; Preißler et al., 2019; Sanchez et al., 2019; Velo-Antón & Cordero-Rivera, 2017) and evolutionary (Burgon et al., 2020; García-París et al., 2003) interest. It consists of a striped or blotched patterning of yellow colour on a black background commonly used as a diagnostic character to differentiate individuals of *S. s. bernardezi/fastuosa/terrestris* (striped pattern) from the remaining 11–12 subspecies (see Burgon et al., 2021; Gippner et al., 2024; Mulder et al., 2022). However, those patterns are intermixed across contact and introgression zones (Alcobendas et al., 1996; Bas & Gasser, 1994; Brejcha et al., 2021; Donaire-Barroso & Rivera, 2015, 2018) and variation in colour pattern has also been reported within subspecific level (Beukema et al., 2016; Brejcha et al., 2021; Donaire-Barroso et al., 2014, 2016; Najbar et al., 2018). Some subspecies (e.g. *S. s. gallaica*) also show grey, orange, or red areas (Velo-Antón & Buckley, 2015), or even brownish and yellowish morphs within *S. s. bernardezi* in a narrow geographical area in northern Spain (Beukema et al., 2016). The resulting conspicuousness of the combined yellow and black colours, together with the presence of toxins (Lüddecke et al., 2018), has traditionally been associated to an aposematic antipredator strategy in *S. salamandra* (but see Preißler et al., 2019), while dark colouration has also been associated to thermoregulation benefits in a species of the same genus, the alpine salamander, *S. atra* (Bonato & Steinfartz, 2005; Žagar et al., 2018).

Despite the high colour variation observed within *S. salamandra*, which is highly rare among salamanders, studies addressing it on adults were mostly descriptive and defined discrete categories (e.g. Aguilar et al., 2023; Bas & Gasser, 1994; Beukema et al., 2016; Brejcha et al., 2021; Najbar et al., 2018; Pasmans & Keller, 2000). On the other hand, quantitative studies on colouration have related colour physical properties with inter and intrasubspecific communication (Aguilar et al., 2023) and environmental conditions such as larval environment and food availability (Barzaghi et al., 2022; Sanchez et al., 2019). Among the studies that quantitatively examined colour pattern variation within *Salamandra* spp., some employed manual procedures to collect data on the number, size, shape, and location of yellow spots, aiming to assess sexual dimorphism (Balogová & Uhrin, 2015), ontogenetic change (Beukema, 2011), or subspecies comparison (Bosch & López-Bueis, 1994). Others employed an automated measurement method to quantify yellow-to-black proportions in salamanders' body (Caspers et al., 2020; Preißler et al., 2019; Sanchez et al., 2019), following the approach described by Sanchez et al. (2018). However, those methodologies prevent obtaining

a general perspective on how colours distribute across the body, nor identifying in which regions each colour is consistently expressed or not (i.e. colour pattern) (Van Belleghem et al., 2018). Indeed, to the best of our knowledge, only one study quantitively addressed colour patterning among a few populations in *S. s. bernardezi* (Burgon et al., 2020), but it lacks a standardized procedure that test its applicability to study body colour variability across populations and species.

In the present study we apply an analytical workflow to not only extract and quantify colour proportions but also colour pattern variation, with which to precisely describe and compare colour distribution at both taxonomic and habitat levels. Our objective is two-fold: (1) to propose a methodological workflow to quantitatively study colour and colour pattern variation in urodeles using *S. salamandra* as study model and (2) to investigate, through a quantitative analysis, shifts in colour and colour pattern variation in urban salamander populations. To evaluate the actual applicability at the taxonomic level, we first tested the methodological sensitivity to segregate colour morphs at the intraspecific level in our system, comparing two phenotypically differentiated subspecies, *S. s. bernardezi* and *S. s. gallaica* (Fig. 1b,c). By quantitatively assessing the differences between these phenotypically contrasting subspecies, we contribute to validating colour as a trait for describing intraspecific variability in this group. Then, we applied the same analytical approach to compare *S. s. bernardezi* populations in an urban–forest system, the city of Oviedo and surrounding areas (north of Spain; Lourenço et al., 2017). Intraspecific comparative studies between urban and non-urban (e.g. rural or forest areas) populations have allowed testing hypothesis about the eco-evolutionary processes affecting colour diversity in animals (see Kreling, 2023 for a review) and plants (Walker, 2023). Nevertheless, there is a notable scarcity of studies investigating the impact of urbanization on colour evolution in amphibians, especially when compared to taxa such as birds (Leveau, 2021). Thus, this study not only aims to assess the applicability of the described methodological framework in eco-evolutionary studies, but also contributes to the limited number of studies addressing the relationship between colour and urban environments in amphibians.

Materials and methods

Study system and sampling

To test the applicability of this methodological approach at the taxonomic level we sampled 59 male individuals of the subspecies *S. s. gallaica* from two forest populations in the north-west of the Iberian Peninsula (Portugal) in autumn 2021 (Fig. 1a), which were compared with 55 *S. s. bernardezi* males from two forest populations sampled around Oviedo city between October and November in 2020 (subspecies dataset: $N_{total} = 114$) (Table 1).

To test the potential effect of urban environments on salamanders' colour, 10 urban and nine neighbouring forest populations of *S. s. bernardezi* were sampled in the Iberian city of Oviedo (Asturias, Spain) (Fig. 1a–d). A total of 363 adult males were captured in Oviedo (habitats dataset: $N_{urban} = 178$; $N_{forest} = 185$) during rainy nights between October and November in 2020 (Table 1). To control for sexual dimorphism (Balogová & Uhrin, 2015) and because they are more abundant and active during the sampling period, only adult males were sampled. Populations within the city of Oviedo inhabit a wide variety of isolated and diverse habitat patches, such as city parks, backyards and stony courtyards (Lourenço et al., 2017). Conversely, the habitats immediately outside the city boundaries mainly consist in rather continuous forest areas dominated by deciduous forests and scrublands, and some rural areas. All forest populations were within a radius of nine kilometres from the city centre to keep geographic environmental variation between habitat groups to a minimum (Fig. 1d).

After collection, animals were numbed using anaesthesia (benzocaine; Ethyl 4-aminobenzoate; Sigma-Aldrich, Darmstadt, Germany. Product number: E1501. Ref.: 12909) by skin absorption following the protocol described in Alarcón-Ríos et al. (2017) to facilitate handling and standardized data collection. We obtained pictures of the dorsal view of the body using an OLYMPUS TOUGH TG1 camera. The objective was placed parallel to the body surface of standardized positioned animals (Fig. 2), which were gently dried out with soft paper to reduce reflections to the minimum. All pictures included a scale, white background, and a colour standard (ColorChecker, X-Rite®). Sharp shadows and light gradients were minimized using a diffuse lighting setup and taking pictures within a white box and adjusting exposure time.

After recovery from anaesthesia, all animals were released at the place of capture within the following 24 h. Salamanders were captured and processed under the collection permits provided by the regional government of Asturias, Spain (N° Expte: AUTO/2020/671) and the Instituto da Conservação da Natureza e das Florestas, Portugal (LICENÇA N° 543/2021/ CAPT). All applicable national and institutional guidelines for the care and use of animals were followed.

Colour and pattern data acquisition

To extract and quantify colour and colour pattern from adult individuals we applied a workflow that uses *patternize v. 0.0.3* (Van Belleghem, 2022; Van Belleghem et al., 2018) and *recolorize v. 0.1.0.* (Weller, 2023; Weller et al., 2024a) R packages in the statistical R environment (R Development Core Team, 2022), which is adapted from the examples and code in (Weller et al., 2024a, 2024b) and in https://hiweller.rbind.io/post/recolorize-patternize-workflow/ for batch-image processing. Applied workflow consists of three main steps: (1) prior image adjustment, (2) alignment and masking and (3) colour segmentation (Fig. 2). The same workflow was applied to the comparison between subspecies (hereafter 'subspecies') and between habitats (hereafter 'habitats') datasets, adjusting the parameters for each dataset accordingly.

Images pre-treatment: resolution and colour calibration

As applied quantitative colour analyses are based on the assignment of a colour value to each pixel conforming an image, all images were standardized to the same resolution
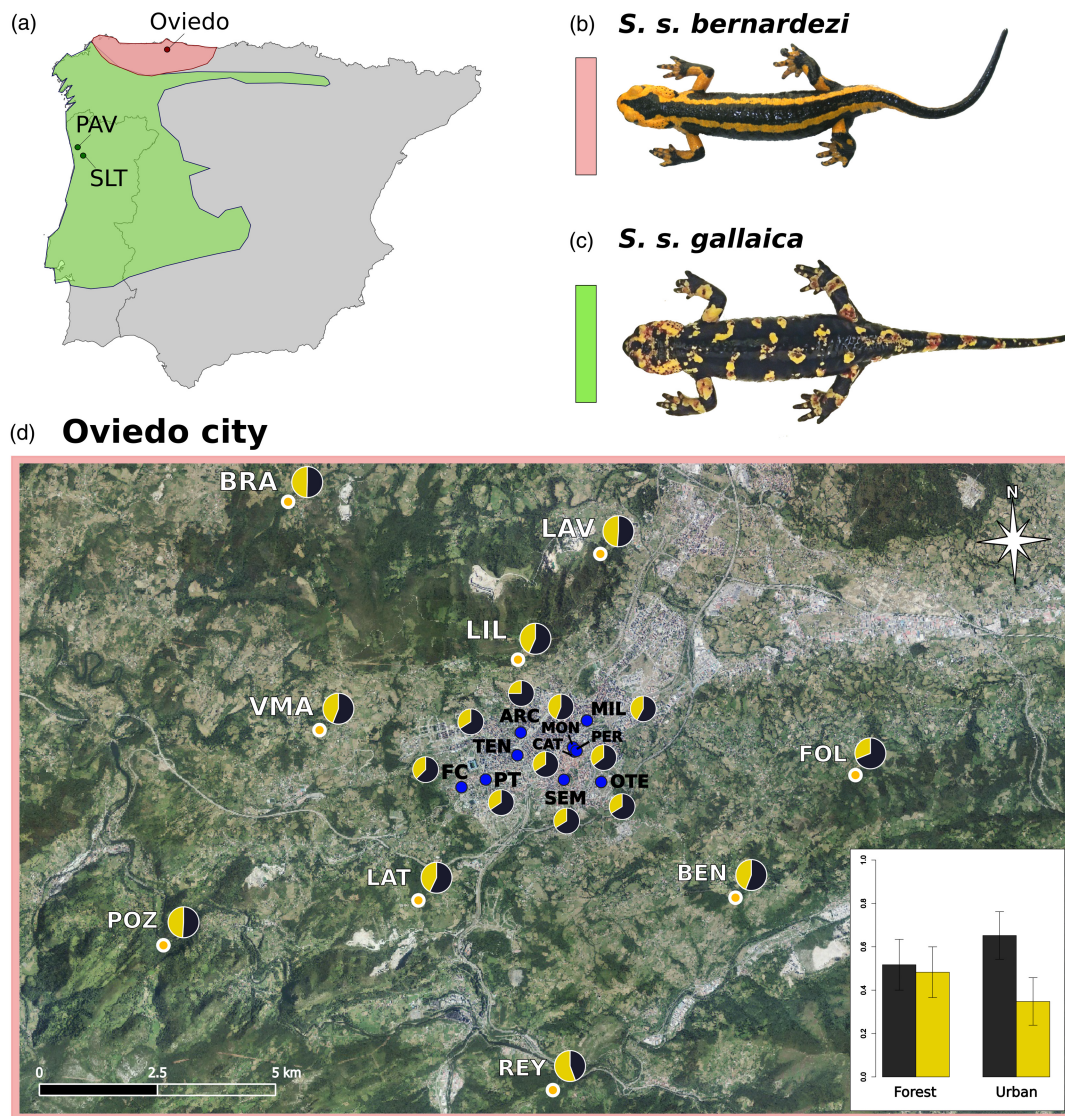
Figure 1 (a) Map of the Iberian Peninsula displaying the distribution areas of subspecies *S. s. bernardezi* (red) and *S. s. gallaica* (green). Points indicate the locations of studied populations: Oviedo city, Avioso Park (PAV), and Senhora do Salto (SLT). (b, c) Example of the characteristic phenotype of *S. s. bernardezi* (b) and *S. s. gallaica* (c). Pictures are not scaled. (d) Map of Oviedo city and its surrounding area, including the locations of sampling populations within (blue points) and outside (orange points) the urban area. Pie charts denote the mean proportion of yellow and black colour in the dorsal body for each population. The inset includes the barplot of the mean colour proportions by habitat, with standard deviation (SD) bars.

(i.e. n° pixels/mm) to ensure comparability across individuals. Image resolution was measured in ImageJ (Schindelin et al., 2012; Schneider et al., 2012) and the smallest resolution was used to rescale each image to the same pixel by millimetre ratio (12 px/mm) using the function *recolorize::readImage*. Although it was not strictly necessary (Weller et al., 2024a), we calibrated colour to facilitate posterior batch segmentation using the function *patternize::colorChecker*. Colour standard corners were landmarked using the software tpsDig2 (Rohlf, 2015).

Alignment and masking

In this second step, two important stages were done simultaneously. One involves the alignment of homologous anatomical structures across different individuals applying a landmark-based transformation alignment. We defined 16 homologous discrete points in the dorsal body of salamanders (Fig. 2) using tpdDig2 (Rohlf, 2015). Then we used those points and a preselected reference individual (POZ32_POZ) to superimpose all images through the translation, rotation and rescaling of images (see

Table 1 Summary table detailing the studied populations, their codes, subspecies, habitat types, as well as sample sizes

Subspecies	Population	Code	Habitat	N
S. s. bernardezi	Facultad Biología	FB	Urban	22
S. s. bernardezi	Plaza de Toros	PT	Urban	15
S. s. bernardezi	Club de Tenis	TEN	Urban	18
S. s. bernardezi	San Pedro de los Arcos	ARC	Urban	18
S. s. bernardezi	Jardines Seminario	SEM	Urban	18
S. s. bernardezi	Calle Muérdago-Otero	OTE	Urban	21
S. s. bernardezi	Campus del Milán	MIL	Urban	17
S. s. bernardezi	Patio Catedral	CAT	Urban	33
S. s. bernardezi	Patio Peregrinos	PER	Urban	8
S. s. bernardezi	Monasterio Las Pelayas	MON	Urban	8
			Total urban	178
S. s. bernardezi	San Miguel de Lillo	LIL	Forest	17
S. s. bernardezi	Villamar	VMA	Forest	14
S. s. bernardezi	Bendones	BEN	Forest	19
S. s. bernardezi	Pozoval	POZ[a]	Forest	32
S. s. bernardezi	Latores	LAT	Forest	17
S. s. bernardezi	Brañes	BRA[a]	Forest	23
S. s. bernardezi	Soto del Rey	REY	Forest	22
S. s. bernardezi	Laviada	LAV	Forest	15
S. s. bernardezi	La Folguera	FOL	Forest	26
			Total forest	185
			Total *S. s. bernardezi*	363
S. s. gallaica	Avioso Park	PAV[a]	Forest	35
S. s. gallaica	Senhora do Salto	SLT[a]	Forest	24
			Total *S. s. gallaica*	59

[a]Populations used in taxonomic comparisons test.

Van Belleghem et al., 2018 for details) using he function *patternize::alignLan*.

The second stage involves the removal of background and structures of no interest using a predefined mask using this same function. The presence of certain structures like the tail or the limbs may be problematic, both to accurately locate homologous landmarks and to standardize their position regarding the rest of anatomical structures during sample alignment. As most part of an animal's surface is concentrated in the salamanders' trunk, we defined an elliptical mask between hind limbs posterior limit and the fore limbs anterior limit on the reference individual and used it to crop all images (Fig. 2). This procedure standardizes masking across samples by consistently positioning the mask with respect to the landmarks within each individual, allowing for batch-image processing and avoiding the bias that could be introduced by defining the mask manually for each image (Weller et al., 2024a).

Colour segmentation

Image colour-based segmentation consists in a categorization of pixels from an image by colour classes creating a numeric matrix, or zone map, that represents the actual pattern and that can be later used to measure variation in different aspects (Weller et al., 2024a). Importantly, producing scientifically meaningful zone maps requires that colour elements classification, based on human perception, relies on a biological basis.

In *S. salamandra* black and yellow colours differ in the type of chromatophore cells, with yellow skin attributed to the presence of xantophores and iridiophores, while black colour is exclusively the result of a high density of melanophores (Burgon et al., 2020). Intermediate colours (i.e. brown, grey or red) arise from the combination at different densities of those three chromatophores (Burgon et al., 2020; unpublished data).

We first extracted and clustered colours from each image using a histogram method, with red channel divided into four bins and red and blue channels into two bins each, using the function *recolorize::recolorize2*. Then, we grouped discretised colours from all images by similarity calculating the Euclidean distance matrix between colours centres in CIE Lab colour space using the function *recolorize::hclust_color* and generated a universal palette of four colour centres (i.e. clusters) for 'habitats' and 10 colour centres for 'subspecies' (e.g. Fig. 2). Differences between 'subspecies' and 'habitats' were due to variations in colour between subspecies (i.e. the existence of red and greyish areas). Finally, we mapped each original image to the same set of colours using the function *recolorize::imposeColors*.

As a final step, we adjusted the segmentation output to remove redundancies in colour clusters (e.g. different shades of yellow and black) or errors (i.e. light reflection). To do so, we combined colours using the function *recolorize::mergeLayer*. In the case of the 'subspecies' dataset, colour layers were merged differently in each subspecies and, when
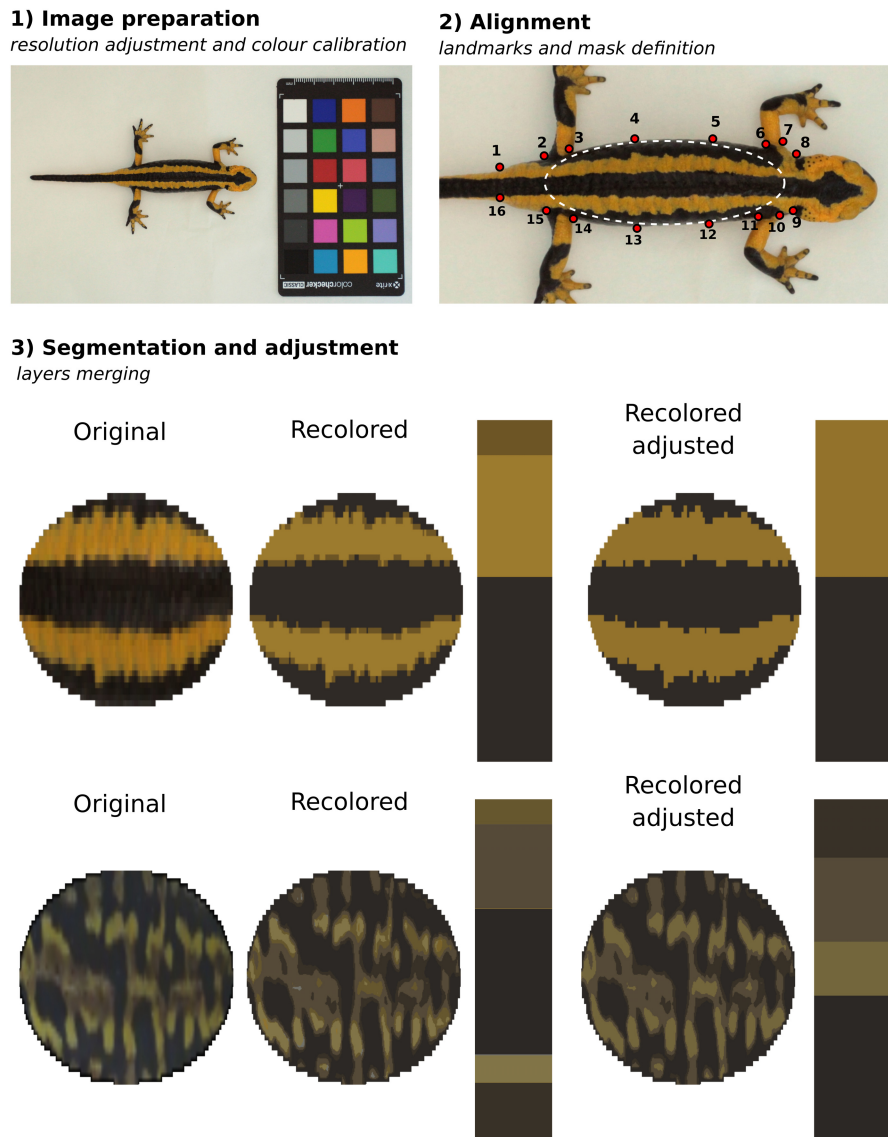
Figure 2 Example of the general workflow for the extraction of colour proportions and colour patterns from the dorsal body views in *S. salamandra*. Image preparation and alignment steps, including landmarks (1, 16: cloaca; 8, 9: head–trunk intersection; 2–3, 6–7, 10–11, 14–15: fore and hindlimbs–trunk intersection; 4–5, 12–13: fourth and eighth left and right lateral grooves) and mask positioning in *S. s. bernardezi*. Segmentation is shown in both studied subspecies, *S. s. bernardezi* in the upper row and *S. s. gallaica* in the lower row. First segmentation resulted in four and 10 colour clusters for each subspecies respectively although displayed individuals do not show all of them. After adjustment through layer merging, the zone maps consisted of two colour clusters for *S. s. bernardezi* and four for *S. s. gallaica*. In the latter, no-black colours were clustered together for further analyses (see the text).

necessary, individually corrected using the toolbox in *recolorize*. In *S. s. bernardezi*, we grouped all layers corresponding to black or yellow colours. In *S. s. gallaica* we first grouped layers into four clusters: black, yellow, grey, and red (Fig. 2). Then, to allow comparison with *S. s. bernardezi* and posterior pattern analysis, we merged all layers of 'no black' colours (i.e. yellow, grey and red), resulting in two final clusters (i.e. 'black' and 'no-black'). As a result, both 'subspecies' and 'habitat' datasets, consisted of zone maps of two clusters each (Fig. 2).

Methodological accuracy

The ellipse used to crop images is assumed to be representative of the whole dorsal body colour and pattern. To confirm it, we tested its representativeness by using the complete body, including limbs, tail and head. We removed the background by hand from 100 random images of the urban *S. s. bernardezi* dataset after resolution and colour calibration adjustment using GIMP 2.10.34. (The GIMP Development Team, 2023). Then, we

colour-segmented them using the same parameters as with the ellipsis (i.e. red-green-blue channels divided in four, two and two bins respectively). We grouped segmented colours into seven clusters, and refined segmentation by posterior colour cluster merging. Once we had all images segmented into two colour clusters, corresponding to yellow and black colour, we extracted the number of pixels belonging to each colour from the zone maps and calculated their proportion. Finally, we tested the correlation between the proportion of black extracted when using ellipse-masked images and whole-body images using permutation tests with 1000 resampling cycles without replacement.

Taxonomical applicability

To assess taxonomic differences in colour traits we first evaluated the sensitivity of applied methodology to segregate phenotypes in *S. salamandra*. We performed a Principal Component Analysis (PCA) to visualize differences between subspecies in black colour pattern using the function *patternize::patPCA*. Then, we extracted the resulting principal components (PC) scores of the main axis of variation and compared them between subspecies, and populations within each of them, using a permutational ANOVA with subspecies and population nested within subspecies as factors.

All permutational ANOVA, including subsequent ones, were conducted using the package *RRPP* v. 1.4 (Collyer & Adams, 2018, 2023) and statistical significance was assessed based on 10 000 random permutations, using Residual Randomization (Collyer et al., 2015).

Colour and pattern variation between habitats

Colour variation

Within 'habitats', we calculated colour proportion in segmented images by counting pixels for each colour relative to the total number of pixels within each ellipse. As our sample only presents two colours that are complementary, hereafter we only present methods applied to black colour. Variability among populations within urban habitats has been seen to be an important component of phenotypic variation (Alarcón-Ríos et al., 2024; Thompson et al., 2022), and thus considering inter-population disparity when comparing habitats is important. Thus, we tested whether the proportion of black colour differed between habitats and across populations within each habitat running a permutational ANOVA with habitat and population nested within habitat as factors.

Colour pattern analysis

To visualize and characterize the main variations in black colour pattern between urban and forest populations we performed a PCA and generate predicted colour pattern changes along principal axes of variation (PC1 and PC2) in the function *patternize::patPCA*. To consider colour pattern variation within each habitat, the PC scores of the main axis of variation were compared between habitats, and populations within each of them, using a permutational ANOVA.

Finally, to improve the visualization of colour distribution in the individuals from each habitat we plotted the heatmaps for each colour layer (i.e. black and yellow colour) using the function *patternize::plotHeat*.

Results

Methodological accuracy and taxonomical applicability

The proportion of black colour extracted when using an elliptical mask and when using the whole body are highly correlated (cor: 0.94, $P = 0.001$) in the subsample analysed. We confirmed that the described method is appropriate to analyse phenotypic variation at the taxonomic level. The two phenotypically distinct subspecies segregate along the PC1 axis (Fig. 3), which is related with the extent and the spotted and/or striped arrangement of yellow areas (Fig. 3). This grouping is statistically supported by the significant differences in PC1 scores between subspecies (Table 2a).

Colour pattern variation between habitats

The PCA showed that the two principal components cumulatively explained 30.5% of variance (Fig. 4a). Individuals from different habitats segregate mostly along the second axis (PC2), which is related with a higher predicted black colour all over the dorsal area in urban populations (minPC2) against a low predicted expression of black in such area in individuals from forest areas (maxPC2) (Fig. 4a). Differentiation in dorsal pattern between habitats are also supported by the significant differences in PC2 scores, which remain significant despite the high variability in dorsal pattern across populations within habitats (Table 2b). The tendency towards narrower yellow bands, and a higher presence of black colour in the inter black stripe areas in urban salamanders are also supported by the heatmaps of individuals from different habitats (Fig. 4b).

It is worth mentioning that the variation in PC1 in the habitats dataset is probably the result of asymmetries derived from image capture. Although efforts were made to keep the camera lens as parallel to the animal's body surface as possible, sometimes the position of the individuals was slightly tilted to one side, as animals were not completely asleep but numbed (see Materials and Methods section).

Colour variation between habitats

Despite the existence of variability in black colour proportion across populations within each habitat, salamanders from urban areas present significantly higher proportions of black colour (65.21% ± 10.95) in the dorsal part of their body than those individuals from nearby forests out of the city (51.72% ± 11.69) (Table 2c; Fig. 1d).
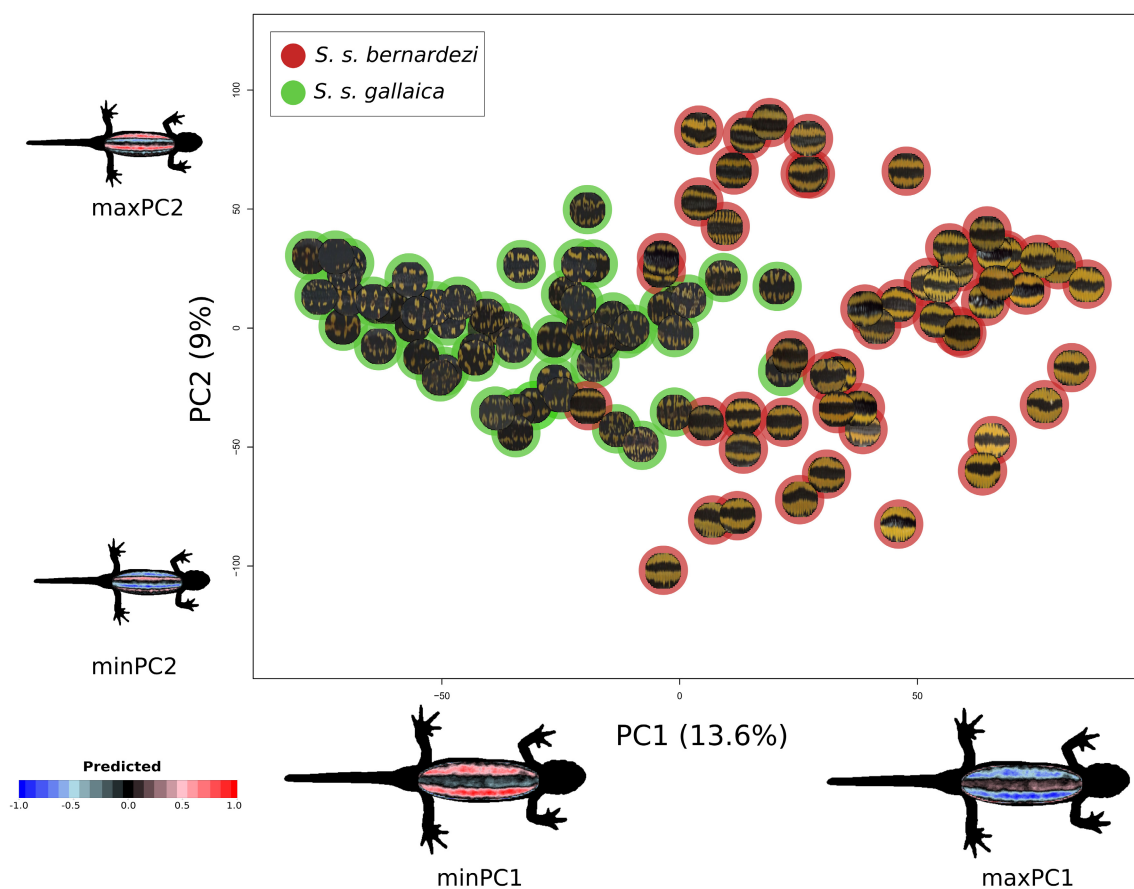
Figure 3 Principal Components Analysis of black colour pattern variation between *S. s. bernardezi* (red) and *S. s. gallaica* (green). The PCA confirms that both subspecies differ in colour pattern along the main axis of variation (PC1), which relates to the extension of black colour all over the dorsal surface. Almost all individuals present an underlying striped pattern, with a predicted value close to zero that does not contribute to variance for the PCA analysis (shades of black within the ellipse). In the predicted colour pattern changes, negative values (shades of blue) represent the absence of the pattern, predicting mostly striped individuals (maxPC1, *S. s. bernardezi*). Positive values in the predicted colour pattern plot (shades of red) represent a higher predicted expression of the pattern, resulting in individuals with black colour more widespread across the dorsal body (minPC1, *S. s. gallaica*).

Discussion

Overall, our results show this methodological workflow as a useful tool to quantitatively assess colour proportions and colour pattern variation, as well as to describe the magnitude and direction of colour phenotypic variation in natural populations of a polymorphic taxa, the urodele *S. salamandra*. We demonstrated that it is appropriate to detect differences in colour pattern at both taxonomic and habitat levels, further reinforcing its applicability in taxonomic and eco-evolutionary studies.

Applicability of the workflow for quantitative studies of colour pattern

The use of digital photography allows gathering information about colour variation in an inexpensive, objective, accessible and repeatable way (McKay, 2013; Stevens et al., 2007). In

the context of our polymorphic study system, having tools that enable the quantitative description of colour traits variability would allow for an integrative revision – combining molecular, ecological and phenotypic data (e.g. Beukema et al., 2016; Burgon et al., 2020) – of the taxonomic status of currently described subspecies, some of which show subtle genetic and phenotypic differences (Burgon et al., 2021; Gippner et al., 2024; Velo-Antón & Buckley, 2015). This study only included individuals from two of the most phenotypically differentiated subspecies (i.e. strictly striped vs. highly spotted) to test the sensitivity of the methodological workflow to segregate morphs, but differences between subspecies are not always so evident and colour morphs usually overlap across subspecies and vary gradually in contact zones (Bas & Gasser, 1994). For instance, the subspecies *S. s. bernardezi*, *S. s. fastuosa*, and *S. s. terrestris* generally present a striped patterning, while remaining subspecies present a spotted pattern, that vary in the number, size, shape, and

Table 2 Results of permutational ANOVAs used to test for differences in *S. salamandra* dorsal body: (a) PC1 scores of black colour pattern between *S. s. bernardezi* and *S. s. gallaica* and sampling populations nested within them; (b) PC2 scores of black colour pattern of *S. s. bernardezi* between urban and forest habitats and population of origin nested within them; (c) black colour proportion of *S. s. bernardezi* between habitats and population of origin nested within habitat

	d.f.	SS	F	Z	P
(a) PC1 scores of patterns between subspecies					
Subspecie	1	148 673	148.884	2.143	**0.018**
Population	2	1997	1.534	0.776	0.225
Residuals	110	71 623			
(b) PC2 scores of patterns between habitats					
Habitat	1	105 779	28.728	3.280	**0.001**
Population	17	62 596	3.616	4.411	**<0.001**
Residuals	344	350 305			
(c) Dorsal black proportion between habitats					
Habitat	1	1.650	42.532	3.628	**<0.001**
Population	17	0.659	3.350	4.262	**<0.001**
Residuals	344	3.984			

Significant terms ($p < 0.05$) are highlighted in bold.

disposition of yellow colour patches (see Velo-Antón & Buckley, 2015 for a review on Iberian subspecies). Although the high polymorphism and polytypism within this species make it challenging to use colour as a diagnostic trait (Beukema et al., 2016; Brejcha et al., 2021; Donaire-Barroso et al., 2014, 2016; Najbar et al., 2018), the variety of colours and their spatial arrangement have been used as a trait for species description and delimitation within the *Salamandra* genus (Degani, 1986; Trevisan, 1982), although there is a general lack of thorough studies characterizing colour patterns in the studied species. This emphasizes the need of tools that allow an objective and quantitative description of colour distribution and arrangement for subspecies description, as have been done with other phenotypic traits such as snout shape (Alarcón-Ríos et al., 2020). Indeed, one of the main strengths of the proposed workflow is its potential to uncover patterns within continuously variable groups, which may otherwise be overlooked by observer-based classifications, and testing differences (if any) among them.

This study also confirms the applicability of the proposed methodological workflow in eco-evolutionary studies as it successfully detects differences in colour traits, both colour proportions and patterning, between populations from highly contrasted habitats. First, it is sensitive enough to detect differences between populations that are geographically close (the maximum distance between populations is 15 km) but ecological and evolutionarily differentiated (Lourenço et al., 2017). And secondly, it enables handling several individuals in a more time efficient way than other manual image processing procedures, ensuring large enough sample sizes to detect significant differences when present. Importantly, our results underscore the need for systematic and standardized image capture for data analysis, with particular attention to the position of the

study subject, which could be especially relevant when sample size is small. Nevertheless, the clear pattern of differentiation between the studied groups confirms the validity of this workflow to study colour traits in our system. Altogether, a more comprehensive description of colour traits would allow us to test more specific hypothesis exploring the role of colour in this species and to uncover the evolutionary mechanisms underlying different phenotypic trajectories in combination with environmental and genomic data (e.g. Burgon et al., 2020; Winchell et al., 2023). For instance, different ecological, such as larval developmental environment or trophic resources availability (Barzaghi et al., 2022; Caspers et al., 2020; Sanchez et al., 2019), and evolutionary drivers, such as predation or sexual selection (Caspers et al., 2020; Preißler et al., 2019) have been related to the amount of yellow colouration in *S. salamandra*, although not explored in relationship to colour pattern. Additionally, some ecotypes have been proposed in relationship to specific environments (e.g. insular melanic forms, Velo-Antón & Cordero-Rivera, 2017), but not quantitatively addressed yet. This methodology may also be useful at the individual level, as colour patterning in *Salamandra* changes ontogenetically (Beukema, 2011; personal observation). Its implementation in ontogenetic studies would enable the quantification of how colour pattern changes across an animal's lifetime and better explore the underpinning determinants, including the functional role of colour and the molecular basis of ontogenetic change and phenotypic evolution in complex life-cycle organisms (Rojas et al., 2023).

Differentiation in colour proportions and colour pattern of urban individuals

In the example of the urban–forest system examined here, we found that urban salamanders are darker than those from forests. This pattern primarily arises from the wider black stripes and the more frequent occurrence of black colour within areas that are supposed to be yellow in the standard striped morph, resulting in discontinuous – and sometimes almost absent – yellow bands (Fig. 4). These findings align with the observed trend within urban and anthropically altered areas towards increased melanism in birds, reptiles, insects (reviewed in Leveau, 2021), and other amphibians (Anderson et al., 2019; Burraco & Orizaola, 2022). The higher occurrence of alternative phenotypes in cities, including increased melanism, has been linked to different evolutionary processes, both adaptive and non-adaptive that stem from the ecological conditions that characterize these urban habitats (Kreling, 2023). With the data at hand, however, it is not possible to determine the exact mechanisms underlying observed trends in the amphibian urodele *S. salamandra*. Consequently, we will outline various hypotheses proposed to explain the increased prevalence of melanistic forms within urban settings and analyse their potential application to our species considering the accumulated knowledge regarding colour evolution and function in Fire salamanders and the peculiarities of urban populations.

The biological and ecological significance of colour traits makes it plausible that detected differences between habitats
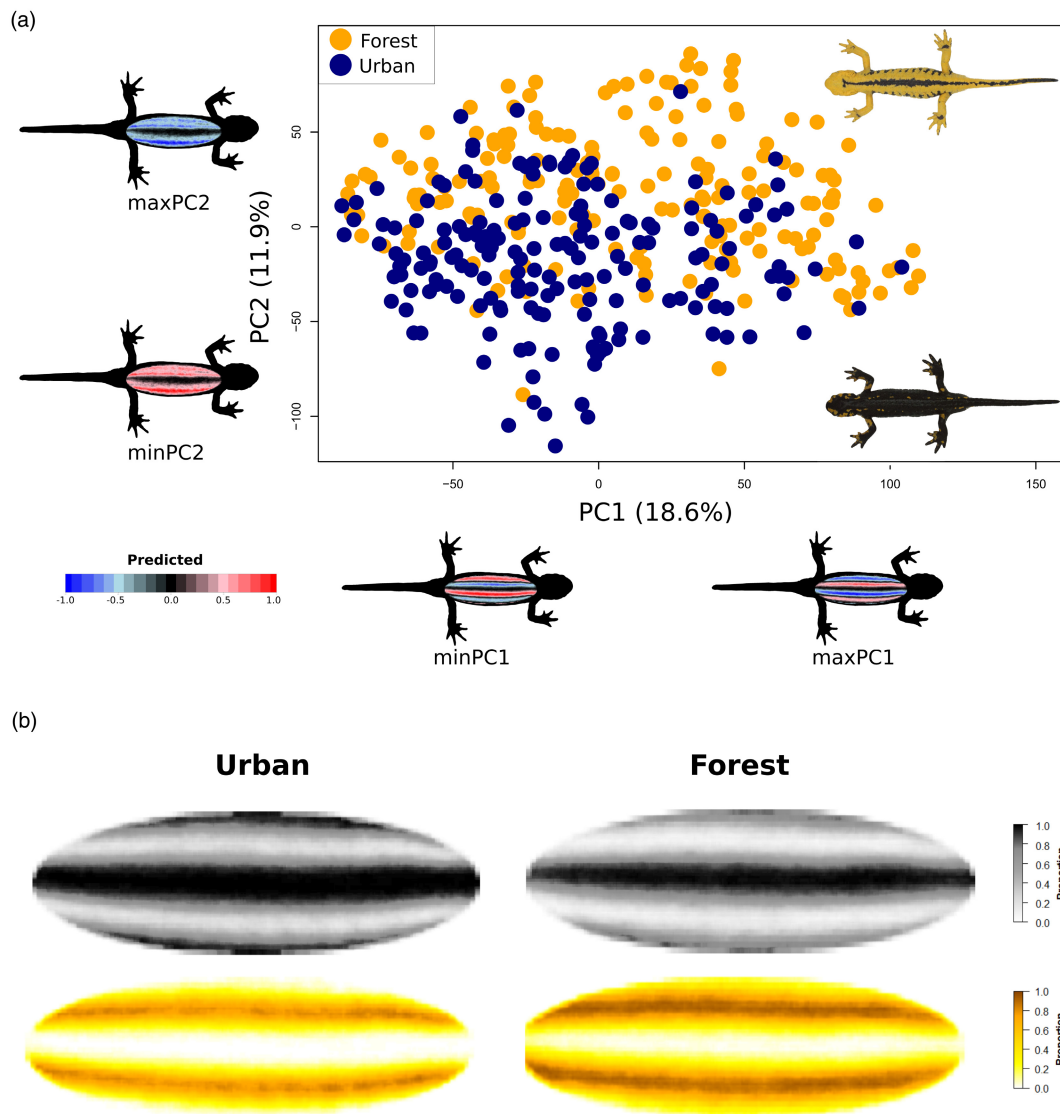
Figure 4 (a) Principal Components Analysis of black colour pattern variation in dorsal colouration between urban (blue) and forest (orange) individuals of *S. s. bernardezi*. The PCA confirms that, despite some overlap, individuals from each habitat type differ in colour pattern along the second axis of variation (PC2), which relates with the presence of black colour in the interstrip areas. All individuals present a clear dorsal black strip, with a predicted value close to zero (shades of black within the ellipse) that does not contribute to variance for the PCA analysis. In the predicted colour pattern change, negative values (shades of blue) represent the absence of the pattern, predicting a clearly defined striped pattern in forest individuals (maxPC2). Positive values (shades of red) represent a higher predicted expression of the pattern, implying a higher presence of black colour in the interstrip areas across urban individuals (minPC2). Real images of the individuals with extreme values along PC2 are displayed within the PCA plot. (b) Heatmaps for the visualization of variation in each colour pattern expression between urban and forest individuals. The darker the colour, the more frequent the pattern is in the sample. Urban individuals present a consistently wider black dorsal stripe than forest individuals (above). Conversely, wider yellow bands in forest individuals are more consistently expressed than in urban ones (below).

have emerged as adaptative responses to environmental differences associated with urbanization (Alberti, 2015; Grimm et al., 2008; Szulkin et al., 2020). Indeed, despite the high variation within each habitat, overall significant differences between them still arise, which points to the existence of adaptive processes driving colour traits differentiation within the city. In this sense, factors usually altered in urban areas that have been suggested as selective forces of melanin-based colorations include pollution (Goiran et al., 2017), stress (Almasi et al., 2010), temperature (Clusella Trullas et al., 2007), diet (Britton & Davidowitz, 2023), intra and interspecific interactions (Kenyon & Martin, 2023; Vroonen et al., 2013), as well

as perception environments, such as background traits (Yamamoto & Sota, 2020).

Some of these selective forces can be hypothesized to be operating in urban populations of *S. salamandra*. For instance, differences in trophic resources availability between habitats emerges as one of the candidate drivers to explain the observed patterns. First, differences in the trophic niche of salamanders between urban and forested areas has been described, with a lower prey richness within the city and a prevalence of preys typically associated to anthropogenic areas (e.g. less annelids, but more arthropods) (Marques et al., 2022). Secondly, food availability has been pointed out as a factor with a significant positive effect on the proportion of yellow and, consequently, negative effect on black colouration in this species (Barzaghi et al., 2022; Caspers et al., 2020; Preißler et al., 2019). Therefore, the higher proportion of black observed in urban salamanders could be reflecting a reduction in the resources they have access to in this habitat or other energetic constraints (see below). However, further studies on trophic resources availability and exploitation, as well as energetic trade-offs with other traits are needed to determine the role that diet may have on increased melanism within the city.

Two other related selective pressures that may favour the adaptive evolution of darker and less clearly striped morphs among urban salamanders includes changes in the biotic community, mostly predators, and in the perception environment. On the one hand, urbanization filters and homogenizes urban species assemblages (Aronson et al., 2016), potentially leading to the modification or even exclusion of *S. salamandra* predators. A tendency to melanisation was observed in insular environments where the predator community is reduced (Velo-Antón & Cordero-Rivera, 2017), although the high abundance of a single predator, *Rattus rattus*, in these islands (Velo-Antón, 2024) may compensate the reduction in predators diversity. On the other hand, the effectiveness of a colour-based signal would depend on the conditions in which it is perceived, including background, transmission medium and lighting conditions (Driessens et al., 2017; Endler, 1990, 1993; Orton et al., 2018). Altogether, urban changes in receivers' (i.e. predators) presence, density and identity, heterogeneous and novel backgrounds, as well as the alteration of environmental parameters that affects colour perception or individuals' behaviour (Delhey & Peters, 2017; Perry et al., 2008) arise as potential factors influencing colour differentiation within these anthropized areas, although further studies specifically testing such hypothesis are still needed.

Alternative adaptive hypotheses to explain the increase of melanism in urban areas lack clear evidence in our system. For example, if the increase in melanin content is related to a detoxification function and immune or stress response as occur under industrial pollution in the seasnake *Emydocephalus annulatus* (Goiran et al., 2017), it remains to be studied in *S. salamandra*. Additionally, the urban heat island hypothesis (Oke, 1973), predicts an elevated local environmental temperature, especially at night. Considering that darker morphs are better at heating and absorbing solar radiation and thus advantageous in cold environments (Clusella Trullas et al., 2007), a trend towards lighter morphs would be expected within the

city. Indeed, the nocturnal habits of the fire salamander reduces the likelihood of solar radiation playing a significant role in thermoregulation in this species (e.g. Hastings et al., 2023). Therefore, the observed increased proportion of black in the studied urban populations can hardly be attributed to an adaptation to a differentiated thermal environment. Nonetheless, other consequences on colour variation of warmer temperatures within cities can be hypothesized. For instance, in the polymorphic urodele *Plethodon cinereus*, higher developmental temperatures favour the occurrence of darker morphs (i.e. unstriped) (Evans et al., 2020). Also, the higher temperatures within the urban environment may facilitate a broader window of activity, thereby increasing energy expenditure with potential repercussions on other traits, such as coloration (see above).

Finally, even though adaptive processes may explain observed differentiation alone (Johnson et al., 2018), non-adaptive evolutionary processes such as genetic drift and genetic ancestry should also be considered (Kolbe et al., 2012; Pita-Aquino et al., 2023). Indeed, drift and demographic processes have been described as the driving mechanisms shaping contemporary genetic patterns across salamander populations in Oviedo (Lourenço et al., 2017), and cannot be completely ruled out as in the differentiation patterns of other phenotypic traits (Alarcón-Ríos et al., 2024).

Conclusions

Describing and quantifying colour variation in species showing continuous phenotypic variation can be impractical or highly subjective without standardized and validated methods, especially for certain colour traits such as patterns. Thus, the methodological workflow described in this article for the study of dorsal coloration in urodeles, based on the implementation of *patternize* (Van Belleghem, 2022; Van Belleghem et al., 2018) and *recolorize* (Weller, 2023; Weller et al., 2024a) R packages, emerges as a highly applicable tool across fields ranging from systematics to the evolution of coloration in this group.

The method has proven useful for the comparative analysis between subspecies with contrasting colour phenotypes, but also between urban and forest populations of the urodele *S. salamandra*, revealing the potential applicability of this workflow in taxonomic and eco-evolutionary studies in this and other polymorphic groups. Finally, this study contributes to the limited body of knowledge about amphibian colouration in anthropic habitats and open several new questions to be addressed in the future.

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Author contributions

L. Alarcón-Ríos and G. Velo-Antón conceived the study; L. Alarcón-Ríos, D. Álvarez and G. Velo-Antón carried out sampling and data collection; L. Alarcón-Ríos designed methodology, analysed the data, and led the writing of the manuscript to which all authors contributed critically and gave final approval for publication.

Data availability statement

Data used in the present study can be found in Figshare https://doi.org/10.6084/m9.figshare.25205717.v1.

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