Automatic quantification of colour proportions in dorsal black-and-yellow coloured amphibians, tested on the fire salamander (*Salamandra salamandra*)

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Animal colouration has important biological functions, such as providing key signals for intra- and interspecific communication. Colour patterns are subject to natural and, more specifically, sexual selection and they are, therefore, often included in studies of evolutionary and behavioural biology (e.g., Kodric-Brown, 1985; Engen et al., 1986; Storfer et al., 1999; Caro, 2005; Siefferman and Hill, 2005; Cuthill et al., 2017). Several species of amphibians present conspicuous dorsal and ventral colour patterns made up by two highly contrasting and sharply delimited colours. A prominent example for this is the group of European, Near Eastern, and North-African fire salamander species (*Salamandra salamandra, S. algira, S. corsica,* and *S. infraimmaculata*) that are typically patterned black and yellow (Thiesmeier and Grossenbacher, 2004; Seidel and Gerhard, 2016). Although this pattern is known to have an at least partially heritable basis, the amount of black and yellow varies greatly between life stages of an individual, within and between populations of the same species, and also between species (Thiesmeier and Grossenbacher, 2004; Balogová and Uhrin, 2015; Seidel et al., 2016).

Analysing the black-yellow pattern can be useful for several purposes; on the one hand, the unique pattern of spots allows software-based identification of individuals in mark-recapture studies (e.g., using AmphIdent, www.amphident.com; Wild-ID, dartmouth.edu/faculty-directory/douglas-thomas-bolger; or APHIS, imedea. uib-csic.es/bc/ecopob; see Matthé et al., 2017 and Goedbloed et al., 2017 for reviews). On the other hand, quantifying the relative proportion of each colour can be useful to study the processes that govern the evolution and ontogeny of colouration (e.g., Boulenger, 1921; Summers and Clough, 2001; Wright and Zamudio, 2001; Balogová and Uhrin, 2015). For this second type of studies, digital photographs of the animals are typically taken for later analysis. However, processing of digital pictures to determine the proportion of certain colours can be time-consuming when using traditional programs like ImageJ (Schneider et al., 2012), GIMP (www.gimp.org), or Adobe® Photoshop®.

We here report on our development of a method for the automated measurement of fire salamanders' black and yellow colour areas from digital images. We propose two alternative procedures: (1) using the commercial software NeuroCheck 6.0, which allows visualisation of all image processing steps and therefore can be supervised by the researcher, and (2) using a newly-developed, free Python script, which allows batch processing of images.

Materials and Methods

Using NeuroCheck 6.1, as a first step, all pixels are normalized (using a function incorporated into the software) based on their brightness. Next, the image is segmented according to references of yellow variations uploaded by the user, and transformed into a binary black-and-white image, with white areas corresponding

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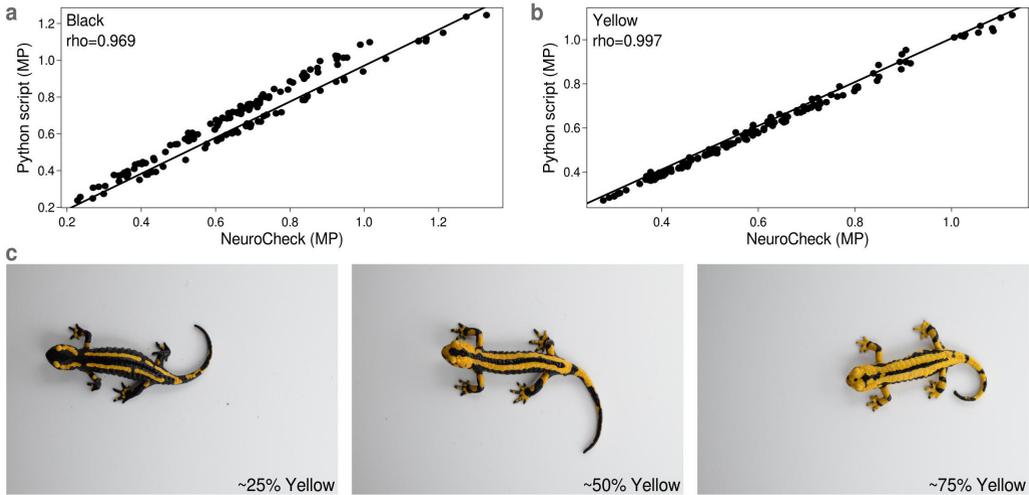
Figure 1. Correlation of the results obtained using NeuroCheck and Python script measuring black and yellow areas in 145 digital fire salamander images. (a) Correlation of black pixel quantification. (b) Correlation of yellow pixel quantification. Spearman's Rank Correlation Tests produced a *P*-value of < 0.0001. MP = megapixels. Slight differences in the measurements done with NeuroCheck and the Python script arose as pixels belonging to black or yellow might be counted as background in the Python script because light is reflected on the skin as white to gray, and the Python script does not take into account the position of ana individual pixel, such as if the pixel is in the main shape of the salamander or not. The two clouds of dots in graph (a) represent two sets of photos with different lighting in which larger or smaller areas of glare were counted as background. (c) Examples of the fire salamander images used showing different proportions of black and yellow.

to areas previously displayed as yellow. As a result of this transformation, the total area of the now white surfaces is recorded by the software and saved into an Excel spreadsheet. This area is then excluded from the original image, and the contrast of the remaining portions of the image is repeatedly optimized (using the "Look-up" function of NeuroCheck) based on the RGB colour model, which allows the black surface of the salamander to be contrasted against any background or shadows. Subsequently the image is binary-transformed, leaving now only the black surfaces of the salamander to be measured and exported into the spreadsheet.

Because NeuroCheck is a commercially sold software, we also developed a free Python script. This script is simpler, in the sense that pixels are recognised as belonging to one of four categories, background (white to dark gray), yellow, black, or other (not assigned) based on their values for the RGB colour model, without any prior optimization of the image. Instructions for the use of this script are shown in Appendix 1, and the script can be downloaded from https://github.com/eu-sanisa/Measure_salamander_colors.py.

Because NeuroCheck is a non-automated method,

results obtained using this software can be visually evaluated by the researcher, who can manually handle uncertainties if necessary. In contrast, the Python script is automated and therefore its effectiveness requires *a priori* evaluation. To this end, we took .jpg formatted images of 145 fire salamanders (*Salamandra salamandra*) on a white background, using a Nikon D3300 camera (24.2 megapixel resolution), and compared the results obtained using both types of software.

Results

The results using NeuroCheck and the Python script correlated strongly (Spearman's Rank Correlation Coefficient for black pixels, $n = 145$, $\rho = 0.969$, $P < 0.0001$; yellow pixels $n = 145$, $\rho = 0.997$, $P < 0.0001$; Fig. 1). Difficulties arose when processing images that contained shadows that were difficult to recognize as part of the background, or light reflection from the moist skin of the salamanders, which led to areas where distinction between black and yellow became difficult. This particularly affected the measurement of black areas by the Python script, because the glare is counted as grey-white background (Figure 1a). In an ideal

situation all pictures should be taken under standardised conditions of light and equipment, including the use of a highly contrasting background (i.e., white) and the same camera and camera settings (e.g., light sensitivity and exposure time); salamanders should be in the same position (see Stevens et al., 2007 for more details). From our experience in analysing our images with the two methods presented here, illumination is a key factor to consider when taking scientific images of fire salamanders for analysis.

Discussion

The procedures presented here were developed using images without any kind of pre-processing (examples in Fig. 1). However, because the salamanders tend to pose their limbs in different positions, even if the body is as straight as possible, we strongly recommend pre-processing the pictures by manually cropping out the limbs and the background for studies in which the proportions of black and yellow are to be compared among groups, such as populations, or treatments. This can be achieved using any traditional image-processing program.

The measurement of the black and yellow proportions can also be done using such programs but the assessment of one image would take several minutes (i.e., in our experience 10–15 minutes per image, hence 2–3 days for 100 images). In comparison, using the Python script presented here the measurement of one image (approx. 8 MB in size) takes only about 40 s, and the times are even shorter – about 30 s – for images in which the limbs and background were cropped out. This means 100 images in high resolution can be automatically measured in approximately 1 h of computing time on a stand-alone desktop computer, and the process does not require supervision or manual work by the researcher. Furthermore, the output of the Python script is a tab-delimited table summarizing the results for all images, ready for statistical analysis.

Of the two alternatives we applied to fire salamander images (NeuroCheck and the Python script), both have different advantages: (1) the Python script is fully automated but in its present version restricted to images on a white background of animals with black and yellow pattern, such as fire salamanders, *Dendrobates leucomelas*, *Ambystoma maculatum*, or *Atelopus zeteki*; (2) NeuroCheck has a more sophisticated procedure to handle background noise, such as shadows, and since all steps can be visually supervised, uncertainties such as light reflections or dirt on the background can be

manually handled, and this software can be used for animals with any two contrasting colours.

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Appendix 1

The following text presents the usage of a Python script for the assessment of the black-and-yellow dorsal colouration in fire salamanders (*Salamandra salamandra*). The Python script can be downloaded from https://github.com/eu-sanisa/Measure_salamander_colors.py

System requirements.—Python, and the Python modules argparse, os, and Image (from PIL).

This program was developed in a Linux environment, however, Python is a universal programming language and therefore can be used in Microsoft Windows and Macintosh OS.

Usage.—By default, the user can just place the program file 'Measure_salamander_colors.py' in the same folder that contain the pictures, and run it from the command line:

python Measure_salamander_colors.py

This will generate a table with results in the same folder.

Optional arguments are listed in Table 1, and can also be accessed by running:

python Measure_salamander_colors.py -h

Table 1. Optional arguments of the script 'Measure_salamander_colors.py'.

Argument	Description	Defaults
-directory	Path to folder containing the photos	Actual folder
-extension	Extension of photos files	.JPG
-background	Minimum R, G, or B value for Background	120
-black	Maximum R, G, or B value for Black	120
-output	Name of results table	Salamander_color_results.csv

The following line is an example how to use the optional arguments.

python Measure_salamander_colors.py -extension .jpg -output My_results.txt

Results.—Tab-delimited table containing the following information:

Sample (i.e., image name), number of background pixels, number of total pixels of the salamander's dorsum, number of black pixels, percentage of black in the salamander, number of yellow pixels, percentage of yellow in the salamander, ratio of black to yellow, number of other pixels (i.e., pixels that were not identified as background, black, or yellow).