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Can ventral pattern be used for individual recognition of the vulnerable Pyrenean brook newt (*Calotriton asper*)?

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Individual recognition of animal species is a prerequisite for capture-mark-recapture (CMR) studies. For amphibians, photo-identification of body pattern is a non-invasive and less expensive alternative than classical marking methods (e.g. passive integrated transponder). However, photo-identification is effective only if the patterns are (i) sufficiently variable between individuals, and (ii) stable over time. This method also depends on the observer's judgment. In the present study, we assessed the effectiveness of an automatic algorithm (AmphIdent) to recognise ventral colour patterns of the Pyrenean brook newt (*Calotrion asper*), endemic to the Pyrenees Mountains of France. To assess the performance of the tested method, 113 individuals from two different streams were marked with passive integrated transponders (PIT-tags). We used false rejection rate (FRR), false acceptance rate (FAR) and true acceptance rate (TAR) as metrics to evaluate performances of photo-identification. Mean FRR was 7.3 %, FAR was 5.2 %, and TAR was 92 % across both streams, both sexes and all the observers. FAR was significantly different between sexes, while FRR and TAR were significantly influenced by the interaction between the sex and the stream. Despite these differences, our error rates are among the lowest values found in the literature for both amphibian and non-amphibian computer-assisted photo-identification. We found that poor-quality reference pictures could lead to an increasing difficulty to achieve a correct match when time since first capture rose. Consequently, individual photo-identification using AmphIdent software is a reliable tool to aid in the monitoring the Pyrenean brook newts, provided that pictures are taken with care, reference images are regularly updated and observers are properly trained to use the software and interpret images.

Keywords: AmphIdent, natural marking, computer recognition, amphibian monitoring, pattern changes

INTRODUCTION

Capture-mark-recapture (CMR) studies provide important knowledge about demography, life cycles, movements and ecological characteristics of species (Nichols, 1992; Wilson et al., 1999; Honeycutt et al., 2019). Such information is crucial to implement the most appropriate and effective conservation strategies for species or populations (Govindarajulu et al., 2005; Lyet et al., 2008). Capture-mark-recapture studies with amphibians often use invasive techniques to individually mark animals, such as passive integrated transponder (PIT) tagging (Zydlewski et al., 2006; Cucherousset et al., 2008), coloured elastomer subcutaneous marking (Simon, 2007; Josephson et al., 2008) or the archaic method of toe-clipping (Phillott et al., 2007). However, these methods may be of concern due to potential welfare and ethical issues (Narayan et al., 2011). Furthermore, some tags may be lost from animals if the operational mode is not optimal (e.g. anaesthesia and post-operating surveillance) and can affect survival (Reeves & Buckmeier, 2009), growth (Davis & Ovsaka, 2001; Mazel et al., 2013), and movements (Schmidt & Schwarzkopf, 2010).

A non-invasive alternative to traditional marking techniques is photo-identification. This method relies on natural marking (e.g. spots, stripes, scales or scars) present on animal's body which are compared to an image databank of known individuals. This method is increasingly used in CMR studies to provide reliable demographic data on wildlife populations (Mizroch et al., 2004; Cheney et al., 2014). Identification "by eye" is feasible with a small set of pictures (Silver et al., 2004; Langtimm, 2004). For larger datasets, recent technical advances have enabled the development of photo-matching algorithms of two types: (i) feature-based (detection of distinctive features within the pattern), and (ii) pixel-based (comparison of pixel values between two images) photo-matching algorithms. Photo-matching identification has been used for several taxonomic groups such as mammals (Bolger et al., 2012), reptiles (Sacchi et al., 2010;), chondrichthyans (Dureuil et al., 2015), osteichthyans (Chaves et al., 2016), insects (Caci et al., 2013; Romiti et al., 2017; Díaz-Calafat et al., 2018) and amphibians (Šukalo et al., 2013; Drechsler et al.,

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2015; Morrison et al., 2016). In addition to being less invasive for animals, photo-identification method has the advantage of being cheaper and less demanding in materials than traditional marking methods. Its main drawback is the time required to handle animals and to analyse pictures.

Photo-matching identification requires that body patterns are sufficiently variable between individuals and stable enough over time, at least over the study period (Dodd, 2010). These two assumptions are crucial to avoid misidentifications and consequently incorrect estimates of the population parameters (Renet et al., 2019). Moreover, identifying animals through their natural body markings involves a higher risk of subjective assignment than more invasive methods (e.g. reading a PIT tag code). Although the photo-matching software compares a picture with all patterns present in a database to sort them by similarity order, the final diagnostic decision about whether this is a new capture or a recapture indeed comes down to the observer's judgement. Marshall & Pierce (2012) suggested that observer subjectivity is a substantial source of errors in photo-matching studies, while Cruickshank & Schmidt (2017) emphasised a learning effect of the observers in matching identification. However, deviations induced by an observer are seldom considered in studies using computer-aided matching software (Bolger et al., 2012; Cruickshank & Schmidt, 2017).

Most amphibians exhibit natural body marks (e.g. coloured and contrasted patterns, spots) and photo-identification has been successfully applied to several species (e.g. the Jollyville Plateau Salamander *Eurycea tonkawae*, the Iberian midwife toad *Alytes cisternasi*, the marbled salamander *Ambystoma opacum*) (Gamble et al., 2008; Ribeiro & Rebelo, 2011; Bendik et al., 2013). Drechsler et al. (2015) proposed two new promising amphibian candidates to test the effectiveness of photo-matching identification including the Pyrenean brook newt (*Calotriton asper*). This amphibian is endemic to the Pyrenean mountain range (France, Spain and Andorra) and lives in cold and well oxygenated freshwaters (Martínez-Rica & Clergue-Gazeau, 1977; Serra-Cobo, 1989; Arrayago et al., 2005; Montori, et al., 2008; Amat et al., 2011). It is listed in the Appendix IV of the European Council Directive on the Conservation of natural habitats and of wild fauna and flora (Habitats Directive 92/43/CEE, May 21st 1992), in the appendix II of the Berne Convention (JORF of August 28th 1990 and August 20th 1996), and in the national Red List of amphibians of metropolitan France as vulnerable species (IUCN France, 2015). Its conservation suffers from a marked lack of knowledge about its biology and ecology, as well as the factors that influence it directly or indirectly (Dalibard et al., 2020). Population status and trends across its distributional range in the Pyrenees are also poorly known as CMR studies are difficult to implement due to the absence of non-invasive tools to identify individuals. Yet, adults display contrasted black and yellow-orange ventral patterns that could potentially make them good candidates for individual photo-identification (Fig. 1).

In this study, we tested and measured the accuracy of computer-aided photo-identification for two populations of the Pyrenean brook newt. Specifically, our objectives were: (i) to implement the AmphIdent software for the individual identification of adults of the Pyrenean brook newt, a software specifically targeted to amphibians, (ii) to assess the performance of this software for the Pyrenean brook newt by comparing the results obtained with this method to the monitoring of PIT-tagged individuals and (iii) to measure the ability of AmphIdent to recognise individuals over time.

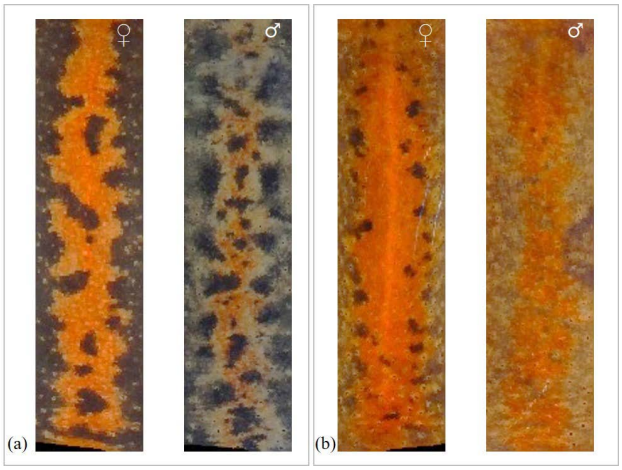

Figure 1. Examples of the ventral patterns of adult female (♀) and male (♂) Pyrenean brook newts, sampled in two streams monitored for the present study: (a) Salau and (b) Fougax (France). The part of the ventral pattern analysed by AmphIdent starts from above the anterior legs and ends at the cloaca.

MATERIAL AND METHODS

Field data collection and individual marking

Two populations of Pyrenean brook newt (*Calotriton asper*) were sampled during Summer 2018 in two streams located in Ariège county in France (thereafter named Fougax and Salau). Summer is the period when the species is the most active, which maximises the chance of capturing many individuals (Nicol, 1990). The first sampling was carried out on 4 June 2018 for Fougax (elevation: 700 m) and on 26 June 2018 for Salau (elevation: 1,250 m), in order to mark individuals with electronic PIT-tags. This invasive marking was necessary to validate the pattern recognition by computer-assisted photo-identification. Search for Pyrenean brook newt was conducted by experienced observers from downstream to upstream by looking under rocks and shelters within river-bed, especially riffles and pools, along a 200 m transect within each stream. A total of 59 and 54 adult Pyrenean brook newts were caught in Fougax and Salau, respectively. They were then transported and housed to the Station d'Ecologie Théorique et Expérimentale (SETE, Moulis, France; coordinates: 42°57'29.82"N, 1°05'11.27"E) located about 1 hour drive from both streams. Individuals were kept in captivity for a 48 hour period in 80 x 40 x 35 cm aquariums (maximum 20 animals per aquarium, males and females

Figure 2. Photography set up for ventral pattern recognition of the Pyrenean brook newt, located close to the river bank (a and b). Individuals are placed in the glass box placed on a drilled support, between the barrier and the side of the glass box, and covered by a white background (c and d). They are photographed from below with a tripod-mounted camera placed at a standardize distance of 30 cm below the glass box.

were separated). To reflect wild conditions and minimise stress, we kept aquariums at 15 °C and added artificial shelters. After this acclimation period, each animal was placed ventral surface down in a 20 x 10 x 5 cm glass box (with a thin layer of water) to be photographed (Fig. 2). In addition to reduce animal stress, the layer of water minimises water droplets under the animal, which could distort the pattern via a magnifying glass effect. A polystyrene barrier was placed inside the glass box to keep the animal straight. Once the animal was still and straight (from a few seconds to 1-2 min depending on the animal), four pictures of the ventral pattern were taken from below at a standardised distance of 30 cm. Pictures were taken with a camera Nikon Coolpix AW110 © stabilised on a tripod. The camera's flash was always used. Each animal was then anesthetised by placing a spot of EMLA ointment (5 % Lidocaine 2.5 % and Prilocaine 2.5 %; Astra-Zeneca GmbH Laboratories, Germany, EMLA) in a cutaneous squared surface of 1 x 1 cm, on the left side. Once the animal was considered surgically anesthetised (i.e. loss of 'withdrawal reflex' and 'righting reflex', Mitchell, 2009), an electronic PIT-tag (Biolog Tiny 10268 – R02-0717 –, tag size: 1.4 x 8 mm, needle size: 1.75 mm; from BIOLOG-ID, FR) was inserted subcutaneously on its left side, between the front and hind legs. Anesthesia and recovery duration were noted for each animal. Permission for animal marking was issued by DREAL Occitanie (Prefectoral decree n°2017-s-02 from 30 March 2017 to 30 October 2020). Both anaesthesia and PIT-tag marking were conducted during 2 days by a qualified person (user establishment agreement n° B09583; nominative authorisation n° A09-1) in compliance with ethical standards. Once the animal was awake, it was kept in captivity for 3 or 4 days, depending on the day of marking, to ensure the PIT-tag was not lost and that no post-operatory complications occurred. Newts were fed with Tubifex worms ad libitum before and after the anaesthesia and marking. Finally, all captured and marked Pyrenean brook newts were released 7 days after being captured, on 11 June 2018 for Fougax and on 2 July 2018 for Salau, in the 200 m transect where they were captured.

Recapture of PIT-tagged Pyrenean brook newts

The next step was to sample the same two 200 meter-long transects throughout the summers of 2018 and 2019 in order to recapture PIT-tag marked individuals. Between June and September 2018, we searched for individuals on seven occasions in Fougax (on 21 June, 6 July, 19 July, 2 August, 22 August, 4 September and 28 September) and five occasions in Salau (on 11 July, 26 July, 8 August, 28 August and 11 September), between 0930 and 1400 with a consistent sampling effort (i.e. two samplers for two hours surveying the 200 meter-long transect). Between June and September 2019, we searched for individuals on four occasions in Fougax (on 4 June, 4 July, 2 August and 13 September) and three occasions in Salau (22 July, 8 August and 12 September). Fewer sampling occasions were carried out in Salau than in Fougax, as Salau is inaccessible before July due to a risk of late snowfall episodes. Two samplings of two hours were conducted per occasion, distant in time of at

least one-hour, without animal release between them. Individuals captured were placed in plastic freezer bags. Each animal was photographed four times in the field, using the same material and procedure as in laboratory. To control for varying amount of sunlight when taking pictures, we placed a white background on the top of the glass box (Fig. 2) and we used a sunshade to cover the entire photographing set up in open area (i.e. in Salau). We then scanned each individual for detecting the presence of a PIT-tag and we determined its sex. With two experienced observers to perform these tasks, less than two minutes per individual were required. After manipulation, each individual was replaced in its plastic freezer bag, and stocked in a cool box containing water. Pictures and measures were performed outside the stream, at a standing place on the riverbank quickly accessible from everywhere along the 200 meter-long stream transect (Fig. 2). At the end of the two sampling occasions, all the individuals were released back at the exact location where they were caught. For each sampling occasion, including the initial laboratory marking, a single picture of each individual (the straightest and with the least camera glare) was selected to represent the animal in the database.

Although the two streams studied seem to be uninfected by emerging diseases such as the chytridiomycosis caused by *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorens* or the ranaviruses (Miaud, 2013; Martinez Silvestre et al., 2018; pers. comm.), we minimised as much as possible the risk of disease transmission. All the material used for sampling were disinfected between the sampling occasions by spraying a solution of VIRKON disinfectant (Virkon S powder, concentration: 1 %, time of action: 30 minutes). New freezer plastic bags where captured individuals were stocked during sampling were also used.

Photo-identification with AmphIdent

AmphIdent is an automatic photo-matching software using cross-correlation comparisons and straightening transformation of pictures (Matthé et al., 2008). The first step is to define and extract the pattern zone from the original picture. As the resolution across pictures was the same due to the standardised distance between the glass box containing the animal and the camera, pictures did not need to be resized before the extraction. Thus, less than one minute per picture was required to perform the extraction step. This step consists in adjusting the automatically generated body contour points (i.e. from the location of the anterior legs to the cloaca) into a common rectangular reference space. Second, the algorithm compares the full extracted pattern with all existing images in the reference database. As no spot pattern is exactly the same, even between pictures of the same individual, the algorithm uses transformation on each pixel's position to transform one pattern into the other. Pairwise comparisons between the extracted pattern and all the patterns in the reference database provide similarity scores, which correspond to the number of matching pixels between the two images. Finally, the observer compares "by eye" the 20 best images proposed by AmphIdent, which are sorted according to their similarity score, to either (i) choose the matching image (i.e. recapture), or (ii) decide that there is no match in the reference database (i.e. new individual).

Assessment of AmphIdent performances

Reference image databases and test datasets

Given the dispersal ability of Pyrenean brook newt (in the range of several hundred meters; Montori et al., 2008) and the straight-line distance between the two streams (60 km), we assumed that recaptures were impossible between streams, and thus created one separate reference image database for each stream. Reference image databases (and then test datasets) were separated for males and females given that sex identification is easy in situ and reliable in the Pyrenean brook newt. This categorisation enabled us to limit the number of images in the reference database and thus the computing time. The Fougax reference database includes 59 pictures (22 females, 37 males), and the Salau reference database 54 pictures (22 females, 32 males), which correspond to the pictures taken before PIT-tagging (i.e. one for each marked animal). These four databases are the reference for the photo-matching performance analysis (see Figure 1 for examples of reference pictures).

To assess performances of photo-identification software, rates of false rejection (FRR), false acceptation (FAR) and true acceptation (TAR) are traditionally calculated. False rejection rate is the failure to identify the same individual between two captures whereas false acceptance rate is the incorrect matching between two captures of two different individuals. True acceptance rate is the success of matching the same individual between two captures. To compute these rates, a test dataset was built for each stream and each sex including all the pictures of the PIT-tagged recaptured newts taken during the sampling occasions of summer 2018 (Table 1). The pictures of PIT-tagged recaptured newts give the opportunity to evaluate false rejection errors (i.e. FRR), and the proportion of PIT-tagged recaptured newts which have been correctly matched with AmphIdent (i.e. TAR). A random selection of pictures of Pyrenean brook newts captured during sampling occasions, but not marked with PIT-tags in laboratory, named "unknown", was added to the test dataset (Table 1). The "unknown" animals are

Table1. Number of pictures selected for the four test datasets used for the assessment of AmphIdent performances, two for each sex (males and females) into each stream (Salau and Fougax). For each sex into each stream, PIT-Tagged corresponds to the number of pictures of recaptured newts (i.e. individuals with PIT-tag) and'Unknown' is the number of pictures of individuals without PIT-Tag.

Stream	Salau		Fougax	
Sex	Males	Females	Males	Females
PIT-Tagged	19	11	46	23
'Unknown'	6	3	13	9
Total	25	14	59	32

not present in the reference images database and give the opportunity to assess false acceptation errors (i.e. FAR). The number of pictures of "unknown" individuals depended on the number of pictures of PIT-tagged recaptures in the dataset (about 25 % according to Drechsler et al., 2015). The Fougax test dataset included 91 pictures, while the Salau test dataset contained 39 pictures (Table 1).

We differentiated FAR into FAR1 and FAR2, which correspond respectively to (i) the rate of false matching between an animal tested and an "unknown" animal (i.e. there is no match in the reference images database but the observer has assigned one among the "unknown" individuals), and (ii) the rate of false matching between an animal tested and a PIT-tagged animal (i.e. a match exists in the reference images database but the observer has not assigned the good one).

During summer 2018, a total of 41 adult Pyrenean brook newts marked with PIT-tags were recaptured at least once in Fougax and 21 in Salau, representing about 70 and 39 % of marked individuals in each stream, respectively. Among the PIT-tagged recaptured animals, 54 and 67 % were recaptured only once in Fougax and Salau, respectively.

Photo-identification exercise by multiple observers

We asked 10 volunteer observers to implement the photo-identification process using AmphIdent. Observers were scientists and students in zoology, but all inexperienced with photo-identification software. Nine observers were trained in AmphIdent during a course of two hours, where they could perform tests with the software. As the tenth volunteer could not attend the course, he was given an accelerated course before implementing the photo-identification test.

Each observer was asked to compare all the pictures of the four test datasets one-by-one to the corresponding reference images database. To improve matching-recognition, they had to look for a correct match within the top 20 highest-ranking candidate matches. For each picture tested, the observer had to record the unique code and rank (from one to 20) of the image from the reference database matching the best according to them. If the observer found no image from the reference database matching the tested picture, he/she had to record it as well. Time was not limited but the observers were recommended not to spend more than 5 minutes per picture tested. Five minutes is the maximum time we estimated to compare the 20 candidates to the tested picture, even for complex patterns. Thus, recognition effort was standardised, providing against potential bias between observers. The observers were not informed about the "tag" and "unknown" pictures in order to enable identification errors (i.e. FAR and FRR).

Computation of performances metrics

Once all the observers had performed the photo-identification exercise, FRR, FAR (i.e. sum of FAR1 + FAR2) and TAR were computed to assess the performances of AmphIdent. FRR was the number of false rejections (i.e. not recognising a PIT-Tagged individual while it is present in the test dataset) divided by the number of "PIT-Tagged" pictures in the test dataset. FAR1 was the number of type 1 false acceptances (i.e. assigning a wrong but not PIT-Tagged individual from the test dataset) divided by the number of "unknown" pictures in the test dataset. FAR2 was the number of type 2 false acceptances (i.e. assigning a wrong but PIT-Tagged individual from the test dataset) divided by the number of "PIT-Tagged" pictures in the test dataset. TAR was calculated as the number of true matches (i.e. assigning the correct PIT-Tagged individual from the test dataset) divided by the number of "PIT-Tagged" pictures in the test dataset. These rates (%) were calculated separately for each sex within each stream and for each observer.

The computation of performance metrics was repeated 20 times, each time with a different top k highest-ranking, with k ranging from 1 to 20. For k ranging from 1 to 20, the rank of the best matching picture identified by the observer when examining the top 20 highest-ranking was compared to k. If the rank was greater than k, the criteria "no match" (i.e. false rejection) was assigned to the picture tested. Else, the rank was recorded. For instance, if the observer found a correct match ranked at the 18th position within the top 20 highest-ranking candidates for one picture tested, "no match" was recorded for k ranging from one to 17.

Statistical analyses

To assess the potential effect of the sex and the stream on performance metric values while controlling for the observer effect, we used a linear mixed-effect model (LMM) with the lmer function of the lme4 package of the R software (R Core Team 2018) (Bates et al., 2015). In the model fitted, the response variable was one of the three performance metrics (i.e. FRR, FAR, TAR) while the explanatory variables were the stream and the sex included as fixed effects. The observer was included as a random effect. We tested for the effect of the two explanatory variables and their interaction, using restricted maximum likelihood (REML) estimation method (Bolker et al., 2009). A significance threshold of 0.05 was chosen for all conclusions derived from statistical tests.

Assessment of AmphIdent performance over time

Study design

We tested the ability of AmphIdent to recognise potential changes in ventral patterns over time in the same two streams as above during a two-year period (from June to September in 2018 and 2019). The pictures of the individual marked with PIT-tag in June 2018 recaptured at least once in summer 2018 and/or in summer 2019 (156 pictures: 113 pictures for Fougax and 43 pictures for Salau), were gathered in a new dataset (hereafter named "time dataset"). A total of 71 unique PIT-Tagged individuals (45 from Fougax and 26 from Salau) was recaptured at least once over the two-year period: 39 % were recaptured once, 28 % were recaptured twice, 17 % were recaptured three times and 15 % were recaptured between four and seven times. We tested the ability of AmphIdent at recognising the PIT-Tagged recaptures of

the time dataset (i.e. recaptures recent or distant from the date of the artificial marking) given that all these PIT-Tagged recaptures correspond to individuals present in the reference image database. If (i) the ventral pattern is sufficiently stable over time, and (ii) AmphIdent and the observer performs well, all the 156 pictures should have a match in the reference database.

The comparison between the pictures of the time dataset and the respective reference image database was done by a single observer, who was the one who had the lowest error rates during the previous described photo-identification exercise. To avoid a potential observer bias, the observer was informed that all pictures of the time dataset had a match in the reference databases.

For each of the 156 pictures of the time dataset, the observer assigned the "1" value if the picture of the animal matched with one of the top 20 ranked images in the reference database (i.e. true acceptation). They also recorded the rank of the matching image. Else the "0" value was assigned (i.e. false rejection).

Statistical analyses

We used a generalised linear mixed model (GLMM) to relate the binary variable (1) true acceptation, (0) false rejection, to the interaction between the time since artificial marking (i.e. with PIT-tags) and the stream, and the interaction between time since artificial marking and the sex of the individual. We controlled for non-independence between several PIT-tagged recaptures of a single individual by including the individual as a random effect in the model. The glmer function of the lme4 library of R was used.

We also tested whether the rank at which PIT-Tagged recaptured individuals were photo-identified (among the top 20 images) increased over time. For this analysis, we kept only the PIT-Tagged recaptured animals for which the image matching was found within the top 20 of the reference database (i.e., "1" value in the previous analysis, 144 pictures). We used a LMM to test the effect of the interaction between the time since artificial marking and the stream, and the interaction between the time since artificial marking and the sex of the individual, on the rank assigned to each picture. As previously, individual was included as random effect to account for several recaptures of a single individual.

RESULTS

Assessment of AmphIdent performances

The proportion of PIT-tagged recaptured newts which have been correctly identified (TAR) with AmphIdent, both sexes combined, was always better when analysing the first 20 images proposed by the software (top 20) than when analysing a lower number of images, with no plateau reached, for both Fougax (nindividuals = 69; nobservers = 10; mean ± SD TAR = 0.89 ± 0.11) and Salau (nindividuals = 30; nobservers = 10, mean (±SD) TAR = 0.95 ± 0.05) (Fig. 3). Consequently, only the metrics results obtained using the top 20 will be shown hereafter. On average among all the observers, streams and sexes (n = 40), false rejection rate (FRR) was 7.3 ± 8.4 % (ranging from 0 to 30 %), false acceptance rate (FAR) was 5.2 ± 7.1 % (ranging from 0 to 22 %; mean FAR1 = 4 %; mean FAR2 = 0.8 %), and true acceptance rate (TAR) was 92 ± 9.2 % (ranging from 65 to 100 %).

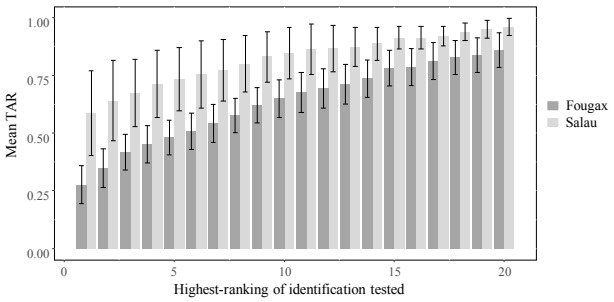

Figure 3. Relationship between the highest-ranking of identification tested and the mean proportion of correct identification (TAR) among all the observers (n=10) for Fougax (dark grey; 69 pictures) and Salau (light grey; 30 pictures). Black bars show the standard deviation of TAR across pictures and observers.

The interaction between the sex and the stream (Table 2) significantly influenced FRR and TAR. False rejection rate was significantly smaller for females than males in Fougax but there was no difference between sexes in Salau (Table 2). True acceptance rate was significantly higher for females than males in Fougax but there was no difference between sexes in Salau again (Table 2). False rejection rates and true acceptance rates were also significantly different between streams but for males only, with higher FRR and lower TAR values in Fougax than in Salau (Table 2). False acceptance rate varied significantly between sexes, being smaller for females than males when both streams were combined but was similar for the two streams (Table 2).

Table 2. Results of the linear mixed-effect model relating performance metrics to the sex (F: females, M: males) and stream (FOU: Fougax, SAL: Salau): differences of least squares means (means of terms, estimate) with p-values (* p < 0.10, ** p < 0.05, *** p < 0.01) and standard error (SE), for the FRR, the TAR and the FAR. For each difference term, the estimate is computed from the value of the first part of the difference term. For example, an estimate of 0.031 in the difference term "Sex(F):Stream(SAL) - Sex(M):Stream(SAL)" for the FRR, means that females from Salau has a FRR higher of 0.031 than males from Salau.

Difference Term	FRR	TAR	FAR
	Estimate ± SE	Estimate ± SE	Estimate ± SE
Sex(F) - Sex(M)	-	-	-0.047 ± 0.02*
Stream(FOU) - Stream(SAL)	-	-	0.036 ± 0.02
Sex(F):Stream(FOU) - Sex(M):Stream(FOU)	-0.148 ± 0.02***	0.154 ± 0.02***	-
Sex(F):Stream(SAL) - Sex(M):Stream(SAL)	0.031 ± 0.02	-0.031 ± 0.02	-
Sex(F):Stream(FOU) - Sex(F):Stream(SAL)	-0.038 ± 0.02	0.025 ± 0.02	-
Sex(M):Stream(FOU) - Sex(M):Stream(SAL)	0.141 ± 0.02***	-0.16 ± 0.02***	-

Table 3. Contingency table showing the number of pictures of PIT-tagged recapture from the time dataset for which the match was found in the reference images database (1) or not (0) for both streams and sexes (F : female; M : male).

Stream	Match	Fougax		Salau	
Sex		F	M	F	M
	0	0	7	2	4
	1	38	68	17	20
	Total	38	75	19	24

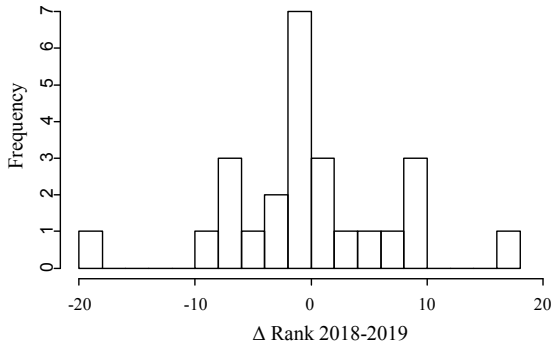

Figure 4. Histogram showing the distribution of the differences between the rank (i.e. position of the identified individual in the top 20 highest-ranking candidate matches) obtained in 2018 and 2019, for individuals recaptured both in 2018 and 2019 (most distant recaptures), all streams and sexes combined. A negative difference in rank indicates that the rank of matching in 2018 was lower than the rank of matching in 2019 for a single animal.

AmphIdent performance over time

For 12 of the 71 Pyrenean brook newts marked with PIT-tags that were recaptured at least once during summer 2018 or summer 2019, one (n = 11) or two (n = 1) pictures of PIT-Tagged recapture were not matched with the images of the reference databases, corresponding to only 13 pictures of the entire time dataset (n = 156; Table 3). However, the GLMM indicated that the time since artificial marking (p = 0.013) and the stream (p = 0.041), significantly influenced the probability of matching with the reference images databases. A negative relationship was found between the time since artificial marking and the probability of matching (slope estimate = -0.004, SE = 0.002, z value = -2.133). The probability of matching was found to be smaller for Pyrenean brook newts recaptured in Salau (estimate = 3.060, SE = 1.225, z value = 2.496) than in Fougax (estimate = 4.180, SE = 1.297, z value = 3.222).

We did not find any evidence that the time since artificial marking has significantly affected the rank at which individuals were photo-identified (p = 0.309). However, the mean rank obtained for Salau (estimate = 3.606, SE = 1.088, t value = 3.313) was significantly lower (p = 0.036) than for Fougax (estimate = 6.328, SE = 0.705, t value = 8.974)

When focusing only on PIT-tagged individuals recaptured both in 2018 and 2019, and comparing the position (i.e. rank) of a re-identified individual in the top 20 highest-ranking candidate matches between the earliest recapture of 2018 and the latest recapture in 2019, the difference between the positions obtained was not significant (Wilcoxon signed-rank test: p = 0.876; Fig. 4).

DISCUSSION

Photo-identification is an efficient tool in capture-mark-recapture studies and is relevant for many species (Drechsler et al., 2015; Chaves et al., 2016) including protected species with conservation issues (Rowat et al., 2009; Bendik et al., 2013). At present, many automatic software programs are available to assist in wildlife pattern recognition (e.g. APHIS, AmphIdent, I3S, Wild-ID; Moya et al., 2015; Matthé et al., 2008; Bay et al., 2008; Bolger et al., 2012). However, as the platforms use different algorithms, their accuracy can be unequal for a given species (Morrison et al., 2016; Matthé et al., 2017; Renet et al., 2019). Assessing the performance of a photo-identification software for a particular species is thus essential to ensure the quality of a CMR dataset. This study evaluated the performance of AmphIdent software for the individual recognition of adult Pyrenean brook newts. Our results show that AmphIdent provides on average low error rates (false rejection rate = 7.3 %, false acceptance rate = 5.2 %), and a relatively high recognition rate (true acceptance rate = 92 %). These rates are consistent with other studies applying AmphIdent to photo-identify amphibians. For example, Drechsler et al. (2015) reported an FRR of 2 % for the great crested newt *Triturus cristatus*, and Matthé et al. (2017) found that FRR ranged from 0 % to 10.4 % for four species of amphibian. Finally, Goedbloed et al. (2017) found that FRR for the near Eastern fire salamander (*Salamandra infraimmaculata*) ranged from 0 % with a standardised pictures dataset (i.e. using a standardised photographic procedure, with a consistent distance between the camera and the animal) to 35 % with a non-standardised dataset.

Many studies stress that rising the quality of the pictures can significantly reduce the FRR. For example, Bendik et al. (2013), using another photo-identification software (i.e. Wild-ID), reported a FRR of 15 % for poor-quality pictures and thus concluded that automated photo-identification was not efficient for the Jollyville Plateau salamanders. Using another camera type, they improved the quality of the pictures and decreased greatly the FRR (0.7 %). In the same vein, Morrison et al. (2016) found differences in FRR between two photo-identification software programs (i.e. Wild-ID and Hotspotter) for the Wyoming toad (*Anaxyrus baxteri*), with an FRR of 47 % and 64 %, respectively. They also highlighted that the FRR was improved with higher quality cameras with faster automatic focusing speed. For the photo-identification of the Pyrenean brook newt, we acknowledge that improving the quality of the pictures is an objective readily reachable that would likely reduce error rates (e.g. using portable photography studio to better reduce the variations of ambient

light). In addition, for FRR equalling about 10 %, it is recommended to compare the focal picture to a larger number of pictures (e.g. increased number of pictures to compare to 20, as we did), in order to make sure that the majority of the recaptures can be identified (Chaves et al., 2016; Cruickshank & Schmidt, 2017). Except that comparing more pictures would increase the time needed to analyse the pictures, this recommendation seems also relevant and easy to follow.

Unlike FRR, very few studies using AmphIdent for amphibians have considered FAR and TAR. As far as we know, Drechsler et al. (2015) are the only ones to have computed the FAR for the great crested newt photo-identification, but they found a FAR of 0 % indicating no false acceptation errors. Using other software, FAR is often reported very low, with a maximum value of 1.8 % reported by Bendik et al. (2013) for the Jollyville Plateau salamanders, using Wild-ID. Lastly, TAR ranged between 89.6 % and 100 % in the multi-species study of Matthé et al. (2017) using AmphIdent. This value is also consistent with our results.

In this study, we show that FRR varies significantly between two Pyrenean brook newt populations. Individuals from Salau were indeed easier to recognise than those from Fougax, for both sexes. Our experience with the Pyrenean brook newt suggests that this difference between streams is due to different contrasts in patterns, colour and size of spots (Fig. 1). Pyrenean brook newts from Salau have darker skin than those from Fougax (personal observation). Consequently, contrast with the yellow-orange pattern is more pronounced and could explain why individuals are more easily recognised in Salau (i.e. lower FRR) than in Fougax.

Our results indicate that within a population, the FRR could also be different between sexes. In Fougax, males were significantly harder to recognise than females, which was not the case in Salau (Fig. 1). However, this difference between sexes could mask a difference in the age of the individuals, which was not estimated with accuracy in this study. The Pyrenean brook newt is a long-lived species (>20 years; Clergue-Gazeau, 1971; Montori, 1988) and colour pattern could change over life cycle. Coloration strategies for sexual selection, predation avoidance or thermoregulation are known to change across life stages, in response to changes in competition relationships or environmental conditions (Landová et al., 2013). Thus, if the oldest individuals tended to be either only males or only females in the reference images databases, the difference found between sexes must be interpreted with caution. Finally, as the conspicuousness and contrast of a pattern is dependent on multiple factors, it is difficult to predict the effect of the location, the sex or the age on the performance of individual pattern recognition and the consequences on the estimation of population size, for long-term monitoring programs.

When studying wildlife population dynamics, false negative errors (e.g. FRR) and false positive errors (e.g. FAR) can differently affect inferences about demographic parameters (Royle & Link, 2006; Miller et al., 2011). Using natural markings, Stevick et al. (2001) showed that false negative errors positively bias abundance estimates, as one unrecognised recaptured individual leads to the creation of a new individual in the reference database. Renet et al. (2019) reported a 3 % over-estimation of population size of the cryptic salamander *Hydromantes strinatii*, with a FRR of 4.3 % (using the top 10 matching pictures with Wild-ID software). For the Pyrenean brook newt, we assume that over-estimation of population size would also likely occur when FRR is high. The larger FRR found for Fougax population could lead to a larger over-estimation of population size or other demographic parameters (e.g. survival rates; Morrison et al., 2011) than in Salau population. Consequently, we recommend estimating the error rates on a larger number of Pyrenean brook newt populations, with a large diversity of ventral patterns, before estimating demographic parameters for this species. We also found a FAR higher (5.2 %) than those found in the literature about amphibians. False positive errors result in large biases when estimating demographic parameters, as they lead to over-estimation of capture probability (Schwartz & Stobo, 1999), due to falsely assigning recaptures to known individuals. False positive error rates are mainly influenced by the observer experience and training (Carlson et al., 1990; Agler, 1992), or the quality of the pictures (Bendik et al., 2013). The relatively high FAR we found suggests that observers must therefore have a good training before being able to use the methodology proposed here.

In this study, we controlled for the observer effect when comparing the FRR, the FAR and the TAR values obtained for each sex and stream. Few studies have explored the possible deviation in error rates caused by the observers, while subjectivity is one of the main acknowledged drawbacks of these methods (Marshall & Pierce, 2012). Cruickshank & Schmidt (2017) compared the performance of photo-identification “by eye” and using a photo-matching software. They emphasised that computer-aided photo-identification reduced the variability in error rates between observers. Cruickshank & Schmidt (2017) highlighted a learning effect in the photo-matching identification, that is, an observer can remember a pattern already encountered, and thus spends less time in the identification process and performs better. This last statement is in accordance with the need to be sufficiently trained using the AmphIdent software and analysing pictures before performing a full CMR study based on natural marking.

One of the most important prerequisites in CMR studies is to have an equal chance to re-identify an individual at all sampling occasions. Using photo-identification methods, this assumption first implies that the body pattern must not change over time. Our results about the performance of AmphIdent to identify Pyrenean brook newts recaptured several months after their first capture revealed that the body patterns were increasingly difficult to match when time elapsed between the first capture and the recapture rose. Even if one individual has already been recaptured and identified once, we cannot thus assume that this individual will be recognised later. Surprisingly, the probability to find a match is lower for Salau than for Fougax, whereas Pyrenean brook newts from Salau are the easiest to identify through their

ventral patterns according to the FRR, FAR and TAR metrics. Pictures that failed to match with the reference images database and particularly those from Salau were of poor quality because of an improper camera's flash or an inaccurate camera setting, resulting very likely in these identification errors. Consequently, we would recommend that standardised and high-quality pictures are crucial to conduct CMR studies based on individual photo-identification. In the same vein, Mettouris et al. (2016) reported that difference in body conditions over time, such as the weight and gravidity of the individuals, or reproductive status of females, could lead to changes in ranking position during the identification process, without patterns changing. We found one picture from Salau failing to have a match with the reference images database, which was one gravid female at the time of recapture (i.e. with a higher body mass, and subsequently, a distorted ventral pattern). To limit the effect of potentially different body conditions of individuals and the effect of the time elapsed between several recaptures, a solution could be (i) to provide more than one reference image for each individual (but only ventral images as the Pyrenean brook newt has no pattern on the dorsal and lateral sides), and (ii) to regularly update the reference images of the individuals (i.e. each time it is recaptured). Most photo-identification software provide this option, including AmphIdent. Chaves et al. (2016) highlighted that when two reference images of the lionfish *Pterois volitans* were provided, matching probability could reach 100 %. Thus, this solution could substantially improve the performances of photo-identification software such as AmphIdent and reduce error rates.

The robustness of the individual re-identification also depends on the size of the reference database (Matthé et al., 2017). In the present study, we used reference databases of 59 and 54 pictures for Fougax and Salau, respectively, to assess the performances of AmphIdent, that is quite small compared to other studies assessing photo-identification performances in amphibians recognition. Šukalo et al. (2013) used the lowest sample size reported in the literature on amphibians, with 159 individuals of fire salamanders from two populations. Matthé et al. (2017) used much larger databases, with for example 4 063 images of the yellow-bellied toad or 12 488 images of the marbled salamander. However, these databases gathered pictures from many surveys and studies, which does not necessarily reflect the real population size. Matthé et al. (2017) also found that AmphIdent could perform accurately even when increasing the size of the database (i.e. from 500 to 12 488 individuals). This finding was true for the four amphibian species studied. This suggests that AmphIdent could keep good performances in identifying individuals of the Pyrenean brook newt, even with larger databases.

Like many amphibian species, the Pyrenean brook newt requires urgent consideration in conservation strategies, but knowledge about its biology and ecology is lacking (Dalibard et al., 2020). To date, the population dynamics of this species have been very little studied, partly due to the lack of non-invasive methods to identify individuals which is the level required to study population dynamics. This study emphasises that photo-identification assisted by the AmphIdent software performs well for the Pyrenean brook newt, provided that pictures are taken with care, reference images are regularly updated and observers are trained to use the software and to interpret images of ventral patterns. This method has many advantages compared to more traditional marking methods. First, it makes possible to sample all the Pyrenean brook newts found in the stream, and thus to study population of potentially large size, compared to toe clipping or PIT-tagging which can only be applied to a limited number of individuals due to money or time constraints. Second, the sampling is made to limit stress of newts and handling is limited as much as possible. Third, the material needed to take pictures is very simple, hand-made, light to carry, re-usable and easy to use. Lastly, the total cost is limited to a digital camera and a license for AmphIdent software. Other photo-identification software like Wild-ID or I3S pattern are free to download and use but their performance for the studied species should be assessed before conducting a full CMR study based on photo-identification. The major drawback of the method proposed here is the time required to analyse all the pictures taken in the field (estimated not to exceed 5 to 7 minutes in total for each captured animal, including both the time needed to take pictures in the field and to analyse them with AmphIdent). But this time becomes shorter with experienced observers. Furthermore, as emerging pathogens (e.g. *Batrachochytrium salamandrivorans*) have particularly impacted European newt populations in recent years and pose an important conservation challenge in the Pyrenees (Martinez Silvestre et al., 2018; Dalibard et al., 2020), there is a need to improve the decontamination protocol during sampling occasions (e.g. disinfection of the glass box between each individual, individuals placed in separate plastic bags). Thus, we propose that environmental managers and professionals who manage the territories where the Pyrenean brook newt is present, implement photo-identification method using AmphIdent after a sufficient training, but also within the respect of biosecurity measures to limit pathogens transmission. As long as proper disease-prevention protocols are followed, this would enable them to account for this threatened species in their practices, without deploying oversized and expensive means and within the respect of animal welfare.

Conflicts of interest

The authors declare no conflicts of interest

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