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*Front cover: Aspicer viper (*Vipera aspis*) from Oriolo Romano near Rome, Italy where a population of this species has been monitored since 1987. See article on page 137*

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Declining occupancy rates in the hibernacula of aspic vipers (*Vipera aspis*) in Italy and France; evidence for climatic effects?

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Hibernation is a key aspect of the physiological ecology of temperate zone reptiles and where suitable dens are present, communal hibernation and long-term den fidelity may be expected. We studied long-term communal occupancy of hibernation dens in Italy and France by aspic vipers, *Vipera aspis*. Long-term trends were evaluated using regression analysis of the annual numbers of *V. aspis* at dens as dependent variables against year as the independent variable. The regression coefficients were tested against a 0 hypothetical coefficient, indicative of population stability. The results indicated that in Italy den numbers were stable from 1987 - 2000. However, after this period to 2017 there was a steep decline in den occupancy as indicated by a negative regression coefficient that differed significantly from 0. These declines correlated with increasing temperatures and shortened hibernation periods from 2000 and agreed with the general decline in viper numbers at the study area. At the smaller den in France, *V. aspis* numbers declined significantly during the period of observation and the den was abandoned by the 5th year. This was attributed to absence of females due to mortality of one of the two females and parturition in the second female. However, in contrast to the situation in Italy the general population in the locality was apparently stable over the period of observation.

Key words: Aspic viper, *Vipera aspis*, long-term hibernation den occupancy, climate change.

INTRODUCTION

In a seminal study, Reading et al. (2010) presented evidence of a worldwide decline in snake numbers. Included in this study were the vipers, a clade of snakes that are under greater-than-expected threat in comparison to other reptiles (Uetz & Hošek, 2015; Maritz et al., 2016). Vipers typically have “slow” life-histories, including infrequent and sophisticated breeding and thermoregulatory strategies (e.g. Bonnet et al., 1999; Lourdais et al., 2004; Lorigou et al., 2013), which may render them particularly sensitive to environmental changes. For example, long-term studies on aspic vipers in Italy (*Vipera aspis*) revealed considerable phenological changes in response to climate warming (Rugiero et al., 2013). Climate effects also concern the winter period because the population ecology of reptiles in temperate regions is intrinsically linked to hibernation, with some species spending almost half the year in winter dormancy (Fitch, 1960; Brown, 1992; Shine & Mason, 2004; McCartney et al., 1989) and remain close to dens after emergence (Duguay, 1958). Selection of winter dens in reptiles also involves locating suitable thermal and hydric

environments (Gregory, 1982) but in certain landscapes, appropriate dens may be limited and shared behaviour in site selection may result in communal hibernation (Viitanen, 1967; Reed et al., 2012).

Knowledge of long term occupancy of snakes at hibernation dens is therefore a key aspect of their ecology in temperate regions (e.g. Viitanen, 1967, Presst, 1971; Zuffi et al., 1999; Altweg et al., 2005; Meek, 2014). The aspic viper is commonly found over a large area of southern and central Europe (Arnold, 2002; Speybroeck et al., 2017) and in most of its range, undergoes winter hibernation and frequently occupies the same winter dens long term. With a maximum total length at around 85cm (Corti et al., 2010), it is a capital breeder that reaches maturity after around 3 to 4 years (Bonnet et al., 1999). In this paper, we report on long term winter den occupancy of *V. aspis* based on data from hibernation dens in two areas of Europe: one set (thirty years of records) consisting of four hibernacula in an area in the Tolfa Mountains north of Rome, Italy and a second (five years of records) situated in a fragmented landscape in Vendée in western France.

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MATERIALS AND METHODS

The study locality in Italy was at Oriolo Romano, a hilly locality about 450 m above sea level situated 50 km north of Rome (central Italy; coordinates: 42.194587, 12.122658), where a free-ranging aspic viper population has been monitored consistently and in detail between 1987 and 2017 (Luiselli & Agrimi, 1991; Reading et al., 2010; Rugiero et al., 2012, 2013). The area is characterised by a grassy pasture bordering an oak (*Quercus cerris*) forest patch, with the hibernacula of vipers being situated at the ecotones between forest and pasture and in hedgerows mostly of *Rubus* sp. bushes. Four different hibernacula were repeatedly used by vipers and were monitored across the years. These hibernacula were located close to each other (less than 300 m distance) and hence are treated as one hibernation complex and hereafter defined as A, B, C and D.

In Italy, field observations started in February 1987 and continued throughout each active season. Surveying was between 08.30 and 12.00h in the morning and between 15.00 and 18.00h (CET) in the afternoons and usually completed within 90 minutes. Detection was made by visual encounter by walking alongside both sides of the hedgerow surrounding the dens at a distance of 4–6m. Grassy and wooded areas surrounding the various hibernacula were also searched within a radius of 5–30m. Snakes were individually marked by ventral scale-clipping and temporarily dorsally painted with a white number allowing the surveyors to identify individual snakes that were already captured, thus avoiding further recaptures.

In France, observations were made at a hibernaculum in Vendée, in western France (46°27'N;1°53'W) (Meek, 2014). The area surrounding the hibernaculum was mainly agricultural land, small urban areas and patches of woodland, usually connected by hedgerows. The den area was situated at the northern end of a hedgerow system and formed by a series of discontinuous drainage pipe remnants of approximately 1 m diameter. A canopy of European ash (*Fraxinus excelsior*) with a dense understory of bramble (*Rubus fruticosus*) covered the den area. The terrain surrounding the hibernaculum was mostly cultivated land including a nearby farmstead and woodland. A combination of autumn leaf fall, drifting soil from agricultural land resulted in debris entering the drainage pipes, leaving only limited openings less than 15 cm at the top of the pipes for snake entry. The full extent of the hibernaculum chamber was unknown. Most visits to the den area were carried out twice daily but were dependent on weather. Surveying commenced in March 2013 after discovery of the den and continued throughout each active season until November 2017. Surveying was conducted between 09.20 and 10.50h in the morning and between 15.30 and 18.40h (CET) in the afternoons and usually completed within one hour. Detection was by visual encounter by walking alongside both sides of the hedgerow surrounding the den at a distance of 4–6 m. This included hedgerows to the north/north-east, west and south. Areas of approximately 5–10 m of farmland to the east and west of the hedgerows and the grassy areas next to the road were also searched for

snakes. These areas had little vegetation except during the summer months of 2015. Each hedgerow was surveyed once during a visit. Snake identification was by photographs and their locations recorded and plotted on an aerial satellite map (Google Earth) to determine their approximate distances from the den entrance along with dates of observation.

Statistical analysis

Statistical analyses were performed by Minitab software, with alpha set at 5%. To determine temporal trends in den occupancy, regression analysis was applied to the data sets with year treated as the independent variable and number of snakes at the den as the dependent variable. This produced equations of the form:

$$\text{number at the den} = b + m\text{year},$$

where m is the regression coefficient and b the y-intercept. Population stability would be indicated when $m = 0$ with tests for departures from this theoretical value made by comparison of the true values of m with $m = 0$ using a t -test at $n-2$ degrees of freedom (Bailey, 2008). A positive regression coefficient would indicate population increase, a negative coefficient population decline. The \pm values for m are the standard errors. Other statistical tests, for example emergence and entrance from hibernation, was through the use of non-parametric Mann-Whitney U -tests.

RESULTS

Italy

Surveying started February 1987 and continued throughout the active year until November 2017. Sampling intensity varied substantially across years and seasons: it was 2–3 times a week during the pre- and post-hibernation phases in some years, but once a week during the same phase of annual activity in other years. Overall, the number of yearly field days was similar across years (Table 1, and see Rugiero et al., 2013 for more details). There was no correlation between annual numbers of individual snakes observed and annual field effort ($r = 0.301$, $n = 30$, $p > 0.05$). Overall, the number of vipers varied year-by-year, ranging from 3 to 14 (Table 1, Fig. 1). These numbers corresponded to the total number of individuals that were observed around each den during late autumn to the beginning of the spring period. During the sunniest and warmest days, the various individuals were often encountered in the open. These numbers also correspond well to the maximum number of individuals observed on any day for each spring.

Annual changes of snake numbers at each den throughout the period 1987–2017 using regression analysis indicated significant declines with negative values of m for Hibernation Den A; -0.101 , $t = 3.42$, $p = 0.0002$, B; -0.165 , $t = 10.6$, $p < 0.0001$ and D; -0.137 , $t = 6.4$, $p < 0.0001$ but although m was negative in C (-0.122), it did not differ significantly from 0 ($t = 1.5$, $p = 0.14$). Combining all den data against year gave, $m = -0.43 \pm 0.044$. Removal of the non-significant Den C data

Table 1. Annual numbers of individual aspic vipers observed in each of the four hibernacula (A, B, C, D) during the period 1987–2017 at Oriolo Romano, central Italy. Data are shown as the means of annual means of individual snakes along with means of their standard deviations. Annual numbers of snakes observed during the period 1987–2000 was significantly higher than 2001–2017, Mann-Whitney *U*-test, $W = 343$, $p < 0.00001$, despite a marginally significantly greater number of days searching during the latter period $W = 170$, $p = 0.03$.

Year	Den A mean± st. dev	Den B mean± st. dev	Den C mean± st. dev	Den D mean± st. dev	Total mean± st. dev	Days in field mean± st. dev
1987-2000	3.8±1.7 range 1 - 6	4.1±0.73 range 3 - 5	1.4±0.63 range 0 - 2	4.1±1.2 range 2 - 6	13.4±1.7 range 11 - 17	11.6±1.2 range 10 - 14
2001-2017	1.6±0.9 range 0 - 3	1.0±0.7 range 0 - 2	1.0±0.8 range 0 - 3	1.6±0.8 range 1 - 3	5.4±1.8 range 3 - 9	12.6±1.4 range 9 - 15

Table 2. Number of days delay in the emergence of *V. aspis* from hibernation and delay of entry into hibernation at Oriolo Romano, central Italy. The results are shown as the mean of annual means of individual snakes along with means of their annual standard deviations across the years.

Time period	Mean delay in emergence post 20 February (days)	Mean of annual standard deviations	Mean delay in entering hibernation post 10 October (days)	Mean of annual standard deviation
1987-2000	28.3 range 19.6-36.2	1.3 range 0.5-2.6	24.2 range 0.5-2.6	1.8 range 0.5-3.8
2001-2017	19.3 range 16.8-24.8	1.3 range 0.5-2.1	42.8 range 33.0-47.4	2.4 range 0.4-3.9

made little difference to the general trend and gave for Dens A, B and D together;

$$\text{number at the den} = 860 - 0.40 \pm 0.044 \text{year}, (r^2 = 0.73, t = 9.12, p < 0.0001),$$

with r^2 the adjusted value. Closer inspection indicated there were two distinct trends; 1) from 1987–2000 the population was stable with the value of $m = 0.31 \pm 0.11$, which was not significantly different from 0 ($t = 0.26$, $p = 0.80$) and 2) from 2001 to 2017 snake numbers declined as indicated by a value of $m = -0.245 \pm 0.06$, which was significantly different from 0 ($t = 3.81$, $p = 0.002$). There was no significant difference in the variance during the periods of both stability and decline (Levens test; $W = 0.096$, $p = 0.76$). The trends, along with the lines predicted by the regressions are shown in Fig. 1.

Temporal sightings

The yearly patterns of the length of hibernation and the time of beginning of spring activity of vipers at the studied hibernacula are already presented in Rugiero et al. (2013). In summary, there was a progressive delay of snakes entering hibernation and earlier dispersing from hibernacula in spring, this pattern being consistent with an increase in temperature recorded at the study area (Rugiero et al., 2013). The number of days of delay (after 10th October) in entering hibernation increased after 1998 (over 40 days compared to less than 30 days, and often even less than 20 days in the early years of study, with a significantly positive trend throughout ($r^2 = 0.815$, $p < 0.00001$). There was also a significant decrease of the onset of annual activity throughout the years ($r^2 = 0.744$, $p < 0.00001$), from >28 days (including >30 days in five out of eleven years between 1987 and 1997) to 17.4–24.8 days from 1998 to 2011 (Rugiero et al., 2013; Table 2). The earlier emergence and later entrance into hibernation during the period 1987–2000 were significant; emergence, Mann-Whitney *U*-test, W

$= 309.5$, $p = 0.0001$; entrance, ($W = 136.5$, $p = 0.0009$; Table 2).

France

A total of six individuals were identified from photographic records, two females and four males. In total, 93 snake location observations were made during morning ($n = 58$) and afternoon ($n = 35$). One of the females, observed regularly during the spring of 2013, was killed on 28 May (likely by a raptor, based on body remains) and a second gave birth in the autumn 2015 after mating in April 2015. Snake numbers at the den declined between 2013 and 2016 with the last viper seen April 2016 (Meek, 2013; 2016) and hence viper presence was only observed for a period of four years becoming absent by spring 2017 (and also in spring 2018). The regression for numbers at the den versus year was negative ($m = -1.5 \pm 0.1$, with the test against 0, $t = 15.0$, $p = 0.001$). However, in contrast to the Italian study area, regression analysis of long-term trends of road mortalities against year in the surrounding area from 2005 – 2017 gave $m = 0.01 \pm 0.09$, was not significantly different from the 0 regression coefficient required for population stability ($t = 0.11$, $P = 0.91$; Fig. 2).

Temporal sightings

First sightings in spring around the den were: 28th March (2013), 12th March (2014), 4th April (2015) and 21st April (2016). Final spring sightings were 28th May (2013), 28th April (2014) 22nd April (2015) and 27th April (2016), after which it is presumed the snakes moved to summer home ranges. Only females were sighted at the den area in autumn between first sightings 5th September (2015) and 30th September (2014).

DISCUSSION

The trends for *V. aspis* in Italy showing significant decline in hibernaculum occupancy, were in good agreement with the general decline in population numbers of vipers

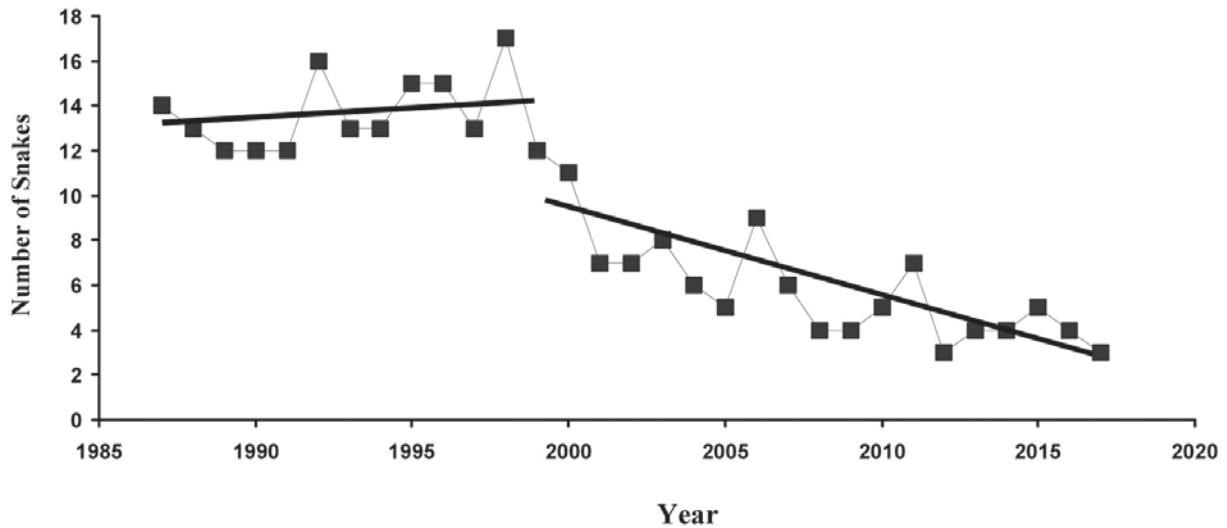


Figure 1. Relationships between year and number of vipers at the hibernaculum at Oriolo Romano, central Italy. The lines running through the data are derived from regression analysis of long term trends of numbers of *V. aspis*. See text for statistical details.

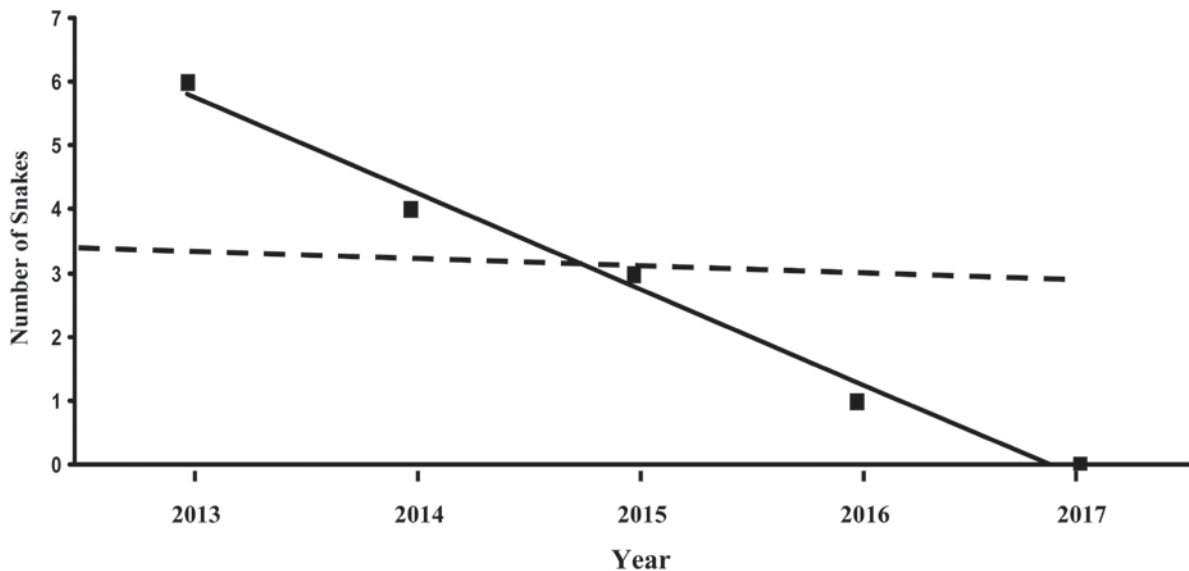


Figure 2. Relationships between year and number of vipers at the hibernaculum in Vendée, western France. The continuous line running through the data is based on regression analysis of *V. aspis* den occupancy from 2013–2017. The broken line represents long term trends of road mortalities of *V. aspis* in the surrounding area, which have been employed as a proxy to identify population trends. See text for further details.

at the study area (see Reading et al., 2010). These have been possibly linked to global warming by impacting on the long-term winter phenology of vipers in the area (Rugiero et al., 2013). For example, data from www.meteo.it indicate average temperatures previous to 2000, when numbers of vipers at the dens were stable, were significantly lower (median = 13.9°C), than throughout the period of viper decline from 2001 to 2017 (median 16.1°C; Mann Whitney *U*-test, $W = 126$, $p = 0.0002$). Why would increasing temperatures impact negatively on population numbers? The reproductive strategy and behaviour associated with reproduction is complex in *V. aspis*. Females are capital breeders delaying

reproduction until they have amassed large energy reserves that frequently result in only a single litter during their lifetime (e.g. Bonnet et al., 1999; Bonnet et al., 2002). The frequency of reproduction in *V. aspis* is temperature dependent, especially including the length of the active season and also food supply (e.g. St Giron, 1996; Bonnet et al., 2002). Gravid females also increase basking time, operate at higher body temperatures and thermoregulate very precisely around thermal set points (Lourdais et al., 2004; Lorioux et al., 2013). Disruptions to this sophisticated thermoregulatory strategy from shortened hibernation period impacts on female reproductive output (Bonnet et al., 2002) and potentially

the population as a whole. However, whilst we recognise that links between viper declines and climate change are at present tentative it should be noted that a climatic link between a decline in body condition with increasing temperatures, especially milder winters, has been found in common toads, *Bufo bufo* in England. The effects were a reduction in female body size, with fewer eggs being laid annually (Reading, 2007).

Increased natural mortality both in hibernation and during the active year may account for some of the observed changes in snake numbers. For example, high winter mortality is known from other snakes including *V. berus* (18–47%; Viitanen, 1967) and *Sistrurus catenatus* (11–43%; Harvey & Weatherhead, 2006). Movement away from the dens in Italy is possible but this is unlikely because the area was rigorously explored and marked individuals from each of the four dens were found in the other hibernacula sites. Indeed, when snakes do not show temporal consistency in den fidelity, the alternate den is usually in the same vicinity of that used the year before (e.g. Harvey & Weatherhead, 2006; Burger & Zappalorti, 2015). Fidelity to hibernation dens varies; for example, some pine snakes (*Pituophis melanoleucus*) regularly changed hibernacula whilst others used the same den year after year (Burger et al., 2012). In the Italian study area, den fidelity was not observed in sympatric rat snakes *Zamenis longissimus* and *Elaphe quatuorlineata*, whereas it was observed more regularly in whip snakes *Hierophis viridiflavus* (Luiselli et al., unpublished observations).

The use of road mortality data as a proxy to estimate numbers of the general population in France is nothing new and has given reliable population estimates in several species, including, mammals (e.g. Mallick et al., 1998; Widenmaier & Fahrig, 2006), amphibians (Meyer et al., 1998; Hartel, 2008) and snakes (Capula et al., 2014; Rugiero et al., 2018). Although such data give only relative estimates, they have value in that they represent independent samples facilitating avoidance of double counts and autocorrelation. At the study locality in France, long-term data (2005 – 2017) has indicated general population stability in *V. aspis*, which contrasted with the decline in occupancy of the hibernation den. This was possibly a result of eventual absence of females at the den due to predation on one female during the first spring of observations (Meek, 2013) and parturition in the remaining female in the autumn of 2015, not seen after the spring of 2016 (Meek, 2016). The latter may be explained by high post parturition female mortality in *V. aspis* due to starvation (Bonnet et al., 2002; Lorioux et al., 2013). A possibility is that males may optimally select hibernacula where females are present to facilitate mate acquisition in spring.

Climate change is emerging as a potential threat for many species of amphibians and reptiles (e.g. Araujo et al., 2006), but disentangling the key drivers in population dynamics is difficult due to natural population change. In this respect one of the most valuable requirements are long-term populations monitoring programs to accumulate reliable data-bases. Long-term monitoring of *V. aspis* winter den occupancy in Italy has enabled

the detection of a decline in *V. aspis* numbers, which track similar declines of the general population and has been associated with a general rise in temperatures. However, limited funding and the time factors involved in long term monitoring are a constant challenge, but are fundamental for credibly identifying population trends.

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Telescoping turtles: a comparison of smartphone telephoto magnifiers to non-invasively observe and identify freshwater turtles

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Sampling freshwater turtles using traditional trapping methods can present significant economic investment to researchers. However, collecting baseline data on turtle relative abundance and species presence requires limited investment and can be non-invasive. Recent advances in performance of readily available smartphone cameras enable collection of high quality digital photos of wildlife, accessible to both researchers and citizen scientists. We report on the feasibility of using several low cost and lightweight telephoto lens attachments for smartphones to identify turtles from various observational distances. All three magnifiers provided a reliable, effective method for counting turtles with increased standard image resolution, with the number of basking turtles correctly enumerated and identified increasing with decreasing distance to observers (Spearman rank correlation = -0.719). The most consistently usable images for species identification were taken with 10X at distances under ~15 m and in urban pond settings where individuals are potentially less easily startled or where ambient noise is common. Ultimately, these magnifiers can be successfully incorporated into university outdoor biological laboratories, undergraduate research and community citizen science programs.

Key words: conservation, *Trachemys*, emydidae, freshwater ecology, survey techniques

INTRODUCTION

Amphibians and reptiles are facing declines across many of their natural habitats (Stuart et al., 2004; Bohm et al., 2013). Monitoring wildlife using emerging digital technologies has provided affordable methods for researchers to assess populations of a variety of flora and fauna using non-invasive methods (Schofield et al., 2008; Davis et al., 2017). Citizen science programmes using volunteers in research have recently been recognised as important in providing valuable data on occurrence and reporting of species (Burr et al., 2014; Pescott et al., 2015). Moreover, as the widespread use of smartphones with high quality digital cameras develops, incorporation of these technologies into research projects may allow researchers with new and valuable tools to monitor presence, abundance and behaviour of common herpetological species (Dickinson et al., 2012; Scott, 2016; Todd et al., 2016).

Observation and identification of pond turtles presents challenges, as many basking or surface swimming turtles will readily retreat or flee once observers are detected (Moore & Seigel, 2006). Current techniques for non-invasive surveying of freshwater turtles includes the use of binoculars (Lambert et al., 2013), spotting scopes (Lindeman, 1999) and, more recently, trail camera traps (Bluett & Schaubert, 2014)

that allow observation from far distances and timely collection of turtle basking behaviour at multiple sites (Vogt, 2012). However, few if any studies have assessed the use of recently available telephoto smartphone lens for turtle identification and enumeration. Moreover, the use of telephoto lens attachments for smartphones could provide an assortment of important biological data in studies of freshwater turtles including evidence of competition for basking sites, identifying signs of disease and collecting data on co-occurrence of species and possibly even behaviour interactions among species present.

Here, we provide a comparison of clarity and resolution of digital images used for identification for common south-eastern U.S.A. basking pond turtles using multiple lightweight telephoto smartphone attachments (8X, 10X, and 12X). We also report on ideal observational distances for documenting basking behaviour using smartphones in urban environments.

MATERIALS AND METHODS

Study site

We enumerated and identified turtles to species level with smartphone telephoto attachments at two ponds (Fig. 1). Study site 1 was located on Wingate University campus, a small private university in Union County, North

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Carolina. Study site 2 was located at the Arboretum Pond in a more urban site (directly behind a large shopping complex) in Charlotte, North Carolina. Site 1 is a partially forested ~55,200 m² pond, while the arboretum pond is ~22,000 m² urban pond. Both sites have a limited amount of downed woody debris/rocks for basking habitat based on a combination of visual surveys of basking sites, which makes observation of common basking turtles ideal given the limited basking sites available. Common basking pond turtles of the south-eastern United States and readily observed in our study sites include the yellow-bellied slider (*Trachemys scripta*), the eastern river cooter (*Pseudemys concinna*) and the painted turtle (*Chrysemys picta*; Palmer & Brasswell, 2013).

Sampling techniques

We conducted visual surveys of turtles basking daily during peak basking times (~1200-1400) on the 17th [Site 1] and 26th of May 2017 [Site 2] during early summer. Surveys were made by three observers (all authors working as a group) carefully walking the periphery of the lake and visually scanning from the bank for the presence of basking turtles, or turtles swimming near the surface with at least half of their heads protruding above the surface. We defined basking as any individual turtle lying quiescent on an object above the water surface or lying in shallow water with any part of the carapace above the water surface according to Obbard & Brooks (1978). For each individual observation of basking, we attempted to obtain at least one to three usable digital photos with a Samsung Galaxy S7 phone equipped with either an 8X, 10X, or 12X clip on telephoto attachment for smartphones (each ~75 g and under £15 GBP) at a resolution of 72 dpi (4031 pixels width X 3024 pixels height) and saved as JPEG files. In addition, for each individual observation of basking, two photos were taken without any telephoto attachment for comparison (smartphone camera only). Smartphones were secured to a 127 cm tripod with a cellphone mount adapter. All photos were taken by the first author with the same phone for all telephoto magnifications with all authors present. Positive species identification was confirmed by comparing smartphone photos with reference photos taken by one observer with a Nikon Coolpix 21X zoom digital camera. We used a Nikon 8397 laser rangefinder to measure the distance of basking turtles from our point of observation and digital capture to the nearest meter. Temperature of basking habitat surface was recorded using an Etekcity digital infrared (IR) thermometer to the nearest 0.1° C.

Data Analysis

We qualitatively ranked photos based on; 1) ability to count total number of turtles basking (identify how many turtles were present in an image) and 2) positive identification of turtles down to probable species based on size and morphological traits. All photos were manually examined on a laptop using digital zoom and enlarging for inspection of morphological traits useful for identification. To identify turtle species, we used an assortment of physical characteristics including shape,

scute arrangement, size and contour of carapace, head colour and markings on head; and overall body size (Conant & Collins, 1998; Buhlman et al., 2008). We excluded field photos in which any turtles moved during photo capture or if images were of poor resolution. Observations and image review were conducted by all authors. Images were examined by each author initially separately, then the number of turtles per image and probable species were compared as a group with all authors present. To validate the number of correct turtles enumerated and correct species identification, we compared images captures with no telephoto smartphone attachment, or photos taken with only a smartphone (0X), and those with 8X, 10X, and 12X with those taken at the same time with a Nikon Coolpix 21X zoom digital camera. The 21X images allowed for direct comparison of images taken with various telephoto attachments with a known, correct reference species identification and number of turtles. We used spearman rank correlation in R to determine the relationship between the number of turtles correctly identified across all 4 types of smartphone magnification photos (0X, 8X, 10X, and 12 X) and the distance of basking turtles to observers.

RESULTS

On the 17th and 26th of May 2017, we observed a total of 12 instances of basking turtles (singular and group) totaling 28 individuals across both sites. Out of these, we selected nine basking observations across both sites for image analysis (total of 121 images) in which turtles were present for all series of telephoto lens. In only one instance (basking identification number four or B4), was the species of reference turtle unknown using the Nikon Coolpix 21X zoom digital camera (Table 1). During three occasions, turtles moved off basking structures during photo capture. Therefore, we excluded these three instances of basking from analyses. Most turtles were basking on partially submerged logs, one artificial basking structure, or exposed rock. We successfully identified three species of turtles basking at our study sites using smartphone telephoto lens and reference photos taken with 21X zoom handheld camera, including painted turtles (*C. picta*), yellow bellied sliders (*T. scripta*;) and river cooter (*P. concinna*; Table 1). The closer an observer was to a turtle resulted in increased positive identification and in all instances, telephoto smartphone attachments provided greater image resolution than photos taken with only the smartphone (no lens attachment) (Fig. 2). At short to mid distances, the quality of the image enabled identification, whereas turtles farther away from observer couldn't be accurately identified due to low image detail (low image quality per pixel). The majority of pictures taken with the 10X consistently provided ideal images, allowing for positive identification (ideal spatial image resolution or picture clarity) of basking turtle number and in some cases down to species if observer was within ~ 15 m of basking turtle. The number of turtles correctly identified to species using 8X, 10X, and 12X telephoto lens was 44.4%, 77.8% and 33.3%, respectively. The number of basking turtles correctly enumerated varied across



Figure 1. Study site 1 located on Wingate University campus in Union County, North Carolina ($34^{\circ}59'13.71''\text{N}$, $-80^{\circ}25'49.98''\text{W}$; bottom) and study site 2 located at Charlotte Arboretum Shopping Center in County, North Carolina ($35^{\circ}5'42.18''\text{N}$, $-80^{\circ}46'49.68''\text{W}$; top). Study site 1 is a small rural $\sim 55,200\text{ m}^2$ pond with surrounding land partially forested, while study site 2 is a smaller sized $\sim 22,000\text{ m}^2$ urban pond (compliments of Google Earth). Basking locations denoted by sun icon.

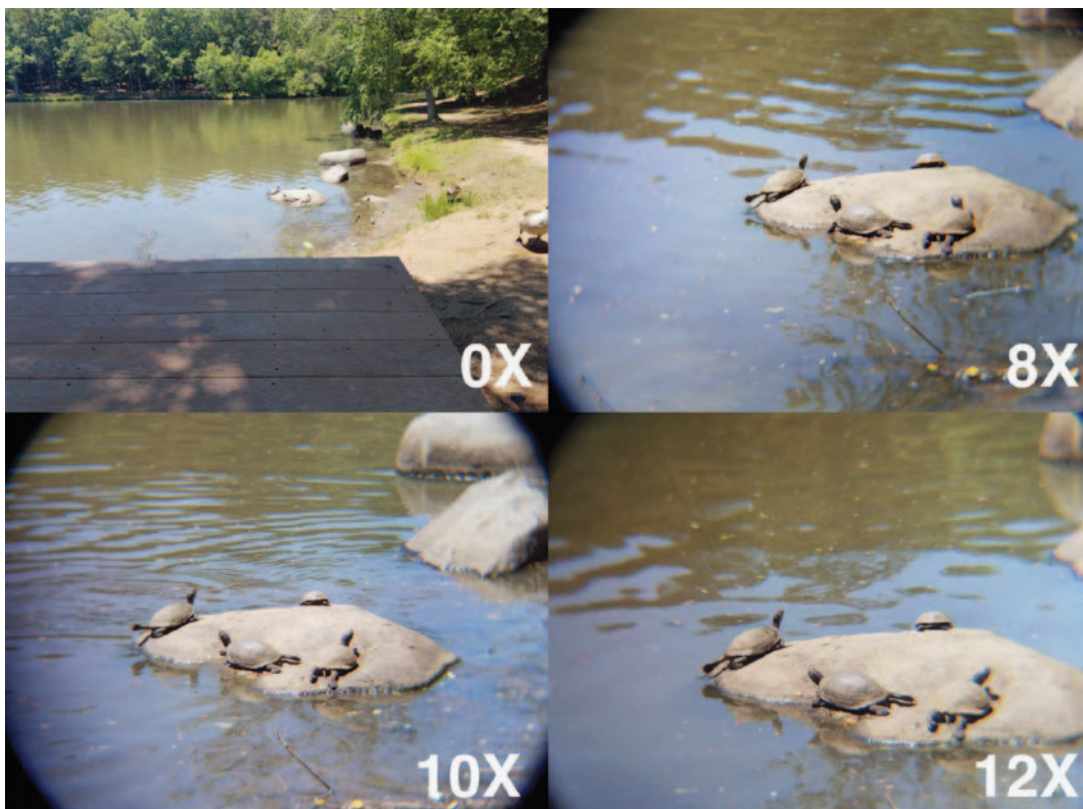


Figure 2. Comparison of image quality of the same four *Trachemys scripta* individuals (B8, or Basking identification number eight) observed from 8 m at site 2 (Top left: smartphone no attachment or 0X, top right: 8X telephoto, bottom left: 10X telephoto, bottom right: 12X telephoto). Note slight variation in resolution of telephoto attachments 8X, 10X, and 12X, with 10X providing most clear spatial resolution for all four turtles with the least amount of blurring. Photo: Escobar, J.

Table 1. Basking identification number, number of turtles identified by traits using telephoto attachments (0X or smartphone with no attachment, 8, 10, & 12 X), total number and species validation (using 21X zoom camera), and distance (basking turtles to observer) during study. Basking identification number corresponds to nine observations of turtles basking in which photos were captured for all telephoto attachments. Species identification (ID) positive when noted.

Basking identification number	Number of turtles identified by traits using telephoto attachments	Total number and species validation	Distance (m)
B1	0X: 1 turtle 8X: 2 turtles 10X: 2 turtles, carapace shape & yellow stripe on head visible, ID positive 12X: 2 turtles, reduced clarity	2 (<i>Trachemys scripta</i>)	17
B2	0X: 0 turtles 8X: 3 turtles 10X: 3 turtles; distinct carapace & neck extension visible, ID positive 12X: 2 turtles	3 (<i>T. scripta</i>)	47
B3	0X: 0 turtles 8X: 0 turtles 10X: 1 turtle, head stripe visible 12X: 0 turtles	1 (<i>T. scripta</i>)	58
B4	0X: 0 turtles 8X: 2 turtles 10X: 4 turtles 12X: 3 turtles	4 turtles, species unknown	50
B5	0X: 1 turtle 8X: 1 turtle, marginal scutes & head coloration visible, ID positive 10X: same as above 12X: same as above	1 (<i>T. scripta</i>)	37
B6	0X: 0 turtles 8X: 1 turtle, ID positive 10X: same as above 12X: same as above	1 (<i>T. scripta</i>)	59
B7	0X: 5 turtles (depending on observer) 8X: 6 turtles, 10X: 6 turtles, carapace patterns & shape, colors well defined, ID positive 12X: 6 turtles, central neck pattern visible	6: 2 (<i>Pseudemys concinna</i>) & 4 (<i>T. scripta</i>)	31
B8	0X: 4 turtles, neck patterns & shell blurry 8X: 4 turtles, similar to above 10X: 4 turtles, well defined color & neck patterns, ID positive 12X: 4 turtles, similar to smartphone & 8X	4 (<i>T. scripta</i>)	8
B9	0X: 1 turtle, ID not possible 8X: 1 turtle, ID positive 10X: 1 turtle, ID positive, Scute pattern on carapace clear 12X: 1 turtle, ID not possible, carapace blurry	1 (<i>T. scripta</i>)	5

telephoto type (10X = 100%, 8X = 77.8%, 12X = 66.7% & 0X = 33.3%). We did observe greater vignetting (shading around edges) in all telephoto attachments. Turtles at site 2 (urban pond with a fountain present) were more likely to remain basking compared to site 1, based on our limited observations of turtles abandoning basking sites and fleeing once authors on bank approached turtles at site 1. Basking temperature averaged 28.0° C for the nine observations of basking and ranged from 25.5° C to 29.3° C, while average observation distance of photo capture to basking turtles was 34.7 m. Spearman rank correlation for the number of turtles correctly identified in telephoto analysis and distance of basking turtles to observer was -0.719 ($p = 0.035$, degrees of freedom = 7), indicating a strong correlation or that the number of turtles correctly identified increases as observation distance decreases.

DISCUSSION

The prevalence of “digiscoping” (use of a digital camera held up to spotting scopes or binoculars) for observation of birds (Larson & Craig, 2006) is less frequently applied for herpetological surveys. However, traditional spotting scopes used for observation of turtles may be large, expensive and difficult to carry in the field. We recommend incorporation of affordable lightweight telephoto attachments for conservation monitoring projects to increase turtle species identification in sites where turtle

species are typically found within ~15 m or less observed from the bank, or at urban sites where turtles do not readily flee their basking sites or are seen swimming near the water’s surface. In these scenarios, we anticipate telephoto attachments may be helpful validating total numbers of turtles, but potentially aiding in both species identification and depending on proximity, sexing turtles if male’s anterior claws are viewable, as we observed at site 2. Moreover, we unexpectedly observed 10X telephoto attachment for smartphones to provide a more accurate species identification than a 12X, possibly due to either the specific manufacturer resolution, quality of lens, and focal length (relationship between total area captured in image and magnification). Alternatively, 10X may provide the ideal telephoto smartphone attachment for the optimal observer viewing distance near the water to most of the basking turtle sites in our study.

Previous studies in birds have found improved readability of long term tags with digital cameras over traditional telescope surveys (Saunders et al., 2011). However, care must be taken when relying on photographic identification of species. This includes taking multiple photos to increase the likelihood of obtaining high quality usable photos. Studies in small cetaceans recommend a minimum of five photos per observation for increase probability of identification even with a traditionally large (~300 mm) telephoto lens (Wursig & Jefferson, 1990). In addition, it is important to use photos in which

individual animal markings are more clearly visible or in which individuals are correctly oriented as has been noted in studies relying on photographic identification in fish (Marshall & Pierce, 2012).

While the use of digital cameras, spotting scopes and binoculars clearly has advantages for viewing turtles from distances greater than 15 m, telephoto attachments need further testing as digital smartphone camera technologies improve. Citizen scientists have helped to track monthly changes in bird distribution using smartphones (Pimm et al., 2015) and it is likely the use of smartphone technology will increasingly be applied to herpetological surveys. The increased use of smartphone photo identification by researchers, citizen scientists and volunteers if concomitantly incorporated into reporting smartphone applications to wildlife agencies can go a long way to address gaps in knowledge in monitoring common and rare herpetological species (Reisser et al., 2008; Tingley et al., 2016).

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Rediscovery of the golden-striped salamander *Chioglossa lusitanica* of Sintra, Portugal

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The southern distribution limit of the Iberian endemic and threatened golden-striped salamander (*Chioglossa lusitanica*) is located about 170 km NE of Lisbon, Portugal. In 1943 Anthero Seabra reportedly introduced a few specimens in the Sintra mountains, about 20 km NW of Lisbon, but the exact introduction site is not known. The existence of a reproducing population in Sintra became a recurrent topic among herpetologists and, despite the efforts of several individuals and teams, was not confirmed until now. After a fortuitous finding of one individual, we report here the results of a monitoring program involving photoidentification of adults and juveniles conducted during the autumn and winter of 2015/16 and 2016/17. We found a reproducing population living along a 107 m stretch of a single stream. Phenology and larval sizes were similar to those of other populations. Notable aspects of this population are its small size (estimated at 339 ± 35 individuals) and confinement to a very small area, the low proportion of individuals that were recorded moving along the stream and the very short distances travelled by those individuals, and the large size of several adults, including the longest individual recorded so far.

Key words: amphibia, assisted migration, caudata, historical introduction, isolate

INTRODUCTION

The golden-striped salamander, *Chioglossa lusitanica* Barbosa du Bocage, 1864, is endemic to North-western Iberian Peninsula, typically living near small brooks with fast-flowing, well-oxygenated water and dense surrounding vegetation, in mountains where annual precipitation exceeds 1000 mm (Arntzen, 1981). Adults are mainly nocturnal, sheltering in caves, or under rocks or leaf litter, during the day. In Portugal, reproduction may occur between September and May depending on climatic factors, mainly precipitation (Arntzen, 1981; Sequeira, Ferrand and Crespo, 2003).

Golden-striped salamanders have a slender body and an exceedingly long tail that corresponds up to two thirds of its total length (Arntzen, 1994). Other unusual traits that distinguish it from other European caudates are a protractile tongue, extreme lung reduction and tail autotomy (Bocage, 1864; Arntzen, 1981). Due to their unique morphology, ecology and endemism, *C. lusitanica* is a species of high conservation interest, yet threatened by habitat destruction and agrochemical pollution of streams. It is listed as vulnerable in both the IUCN Red List of Threatened Animals (Arntzen et al., 2009) and the Red Data Book of Vertebrates of Portugal (Cabral et al., 2005).

The geographical distribution of *C. lusitanica* has been studied by several researchers (Arntzen & Teixeira, 2006) and is currently well documented, at least in Portugal

(Loureiro et al., 2008). The species occurs from Asturias and Galiza in Spain to north-western and central Portugal (Fig. 1.2) with the Alvéolos Mountains, just north of the Tejo river, as the southern-most location (Loureiro et al., 2008). However, there are three historical indications of population isolates in the south (Fig. 1.2): i) the single record south of the river Tejo, in Elvas, for which it is now clear that arose from misreading a faded label (Crespo, 2008); ii) "La Serrota" in Ávila, Central Spain (Pérez-Arcas, 1874); iii) the Sintra mountains (Seabra, 1943). In 1943, the zoologist Anthero Seabra captured "a few specimens" (the exact number is unknown) of golden-striped salamanders in the Buçaco mountains, central Portugal and introduced them in the Sintra mountains (Seabra, 1943). These mountains are about 170 km SW of the recorded species distribution limit and 20 km NW of Lisbon (Fig. 1.1). Seabra (1943) mentions that environmental conditions at Sintra were similar to those of the northern mountains where the species was common, and would therefore probably be adequate for the establishment of a reproducing population.

Even though many a herpetologist has tried to find *C. lusitanica* in Sintra mountains since then, it has remained elusive (Almaça, 1959; Malkmus, 1979; Teixeira et al., 1998; Loureiro et al., 2008). Around 1990, Gaston-Denis Guex found one single individual in Sintra while looking for *Salamandra salamandra* (Arntzen, 1999). Unfortunately, the exact location of that individual was not recorded. In 2015, Nuno Reis and João Martins

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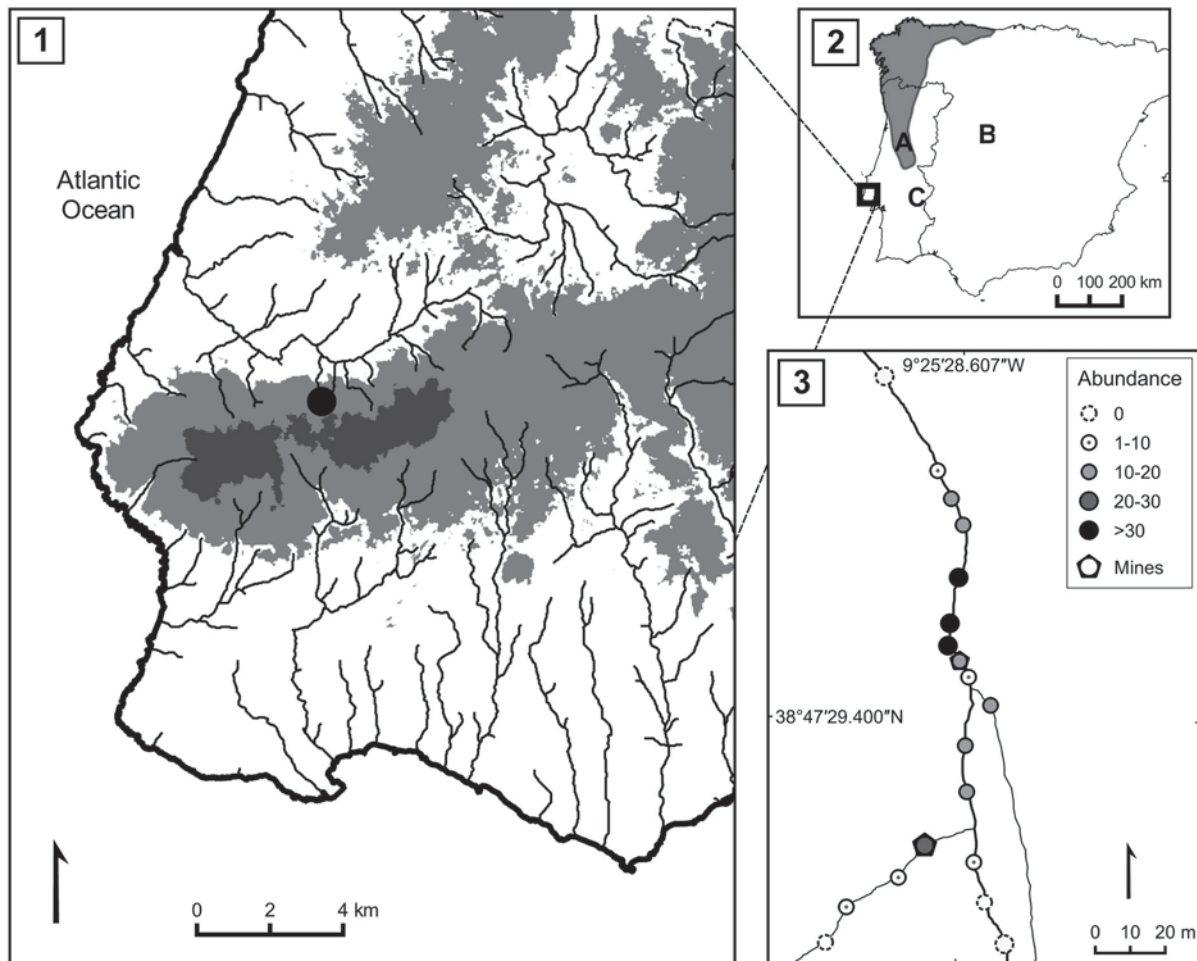


Figure 1. (1) Sintra mountains hydrographic network and topography (light grey – above 150 m; dark grey – above 350 m a.s.l.); (2) Current distribution of *C. lusitánica* on the Iberian Peninsula (grey); A – Buçaco; B – La Serrota; C – Elvas; (3) Local distribution and abundance (number of individuals first found in each stream section) of *C. lusitánica* along the streams.

photographed one adult golden-striped salamander that they found in a stream near Sintra. We report here on the distribution, population size, phenology, movements and body size distribution of this *C. lusitánica* population.

METHODS

Study area

The field survey was conducted in Sintra mountains from November 2015 to April 2016 (Year 1) and from October 2016 to April 2017 (Year 2) in the two streams that flow along the valley where the first individual was spotted (valley coordinates: 38°47'44 N, 9°25'25 W to 38°47'19 N, 9°25'30 W - Fig. 1.2). Altitude of the surveyed area varies between 82 m and 281 m a.s.l. The climate is Mediterranean with Atlantic influences, with a hot dry summer and a mild rainy winter. The average annual precipitation is 727 mm and average annual temperature is 15.3° C (data from Colares meteorological station, 2.3 km NW of the surveyed area; SNIRH, 2018). Both streams flow along large granite boulders, forming small caves and inner pools; one of the streams is fed by waters flowing out of two abandoned mine galleries. Non-native invasive trees, such as *Pittosporum undulatum* and *Acacia* sp., are the dominant vegetation along the banks. In addition, *Eucalyptus globulus* and the native *Quercus*

suber, *Castanea sativa* and *Pinus pinea* are present in low density. The sub-arboreal stratum is composed by mosses, vines, shrubs, ferns and leaf litter.

A total of 37 visits (23 on the first year and 14 on the second) were conducted at dusk and during the night. The time between visits was 6.32 ± 5.36 days in the first year and 11.57 ± 7.96 days in the second. About 1 km of stream plus a 3-meter band bordering each margin were prospected; additional prospecting for eggs or developing embryos was conducted on sheltered locations, such as under large boulders and crevices, or in the mines. Water temperature and pH were measured in all visits and ranged from 11° C to 15° C and from 6.2 to 6.8 respectively.

Distribution and population structure

Sixty survey points were defined along the two streams according to landscape features, and each captured individual was assigned to the nearest point. If no individuals were found after searching for 30 minutes and 3 sampling days, it was assumed that none was present in that stream section. This procedure was repeated on the beginning of the second year at all survey points, regardless of its status on the first year. The distribution map depicted in Figure 1.3 was produced using QGIS software, version 2.14 Essen.

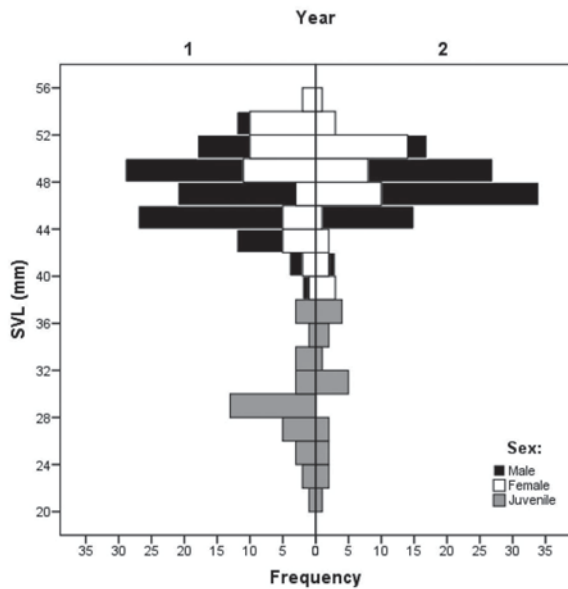


Figure 2. Histogram of body size classes (SVL, mm) on Years 1 and 2

Snout-vent length (SVL) of all captured individuals was measured from snout to the insertion of the hind limbs, using Fiji (Schindelin et al., 2012) software tools over dorsal photographs, with animals photographed in a natural, curved position. Several pictures of each individual were measured; the measurement error was 0.47 ± 0.37 mm for adults (1% of their average length). Individuals with $SVL \geq 38.0$ mm were considered adults (Arntzen, 1981). Adults and juveniles were weighed with a digital scale to the nearest 0.01 g. Sex identification was based on the presence (males) or absence (females) of cloacal swelling and any unusual observations (e.g. body injuries) were registered.

The sex-ratio was calculated by dividing the number of males by the number of females. Body condition was estimated using the scaled mass index proposed by Peig & Green (2009). This index standardises the mass of the salamanders to an average SVL (46.9 mm for males and 48.4 mm for females, in our sample) using the scaling relation between log mass and log SVL.

Photo-identification was based on the unique dorsal pattern. Growth rate was calculated from the difference between final SVL and initial SVL of a recaptured individual divided by the number of days between captures. We grouped the data as: "autumn" - October and November; "winter" - December and January; "spring" - February to April (this final interval included three months as there were few captures in April). We then tested for differences in physical condition among seasons in each sex with ANOVA, after checking for homocedasticity. Net displacement distance was assumed as the distance between the first and final points of capture for each individual, and were calculated within each year and between years. Results are expressed as mean \pm SD; α was set to 5%. Statistical analyses were conducted with the software SPSS (version 24).

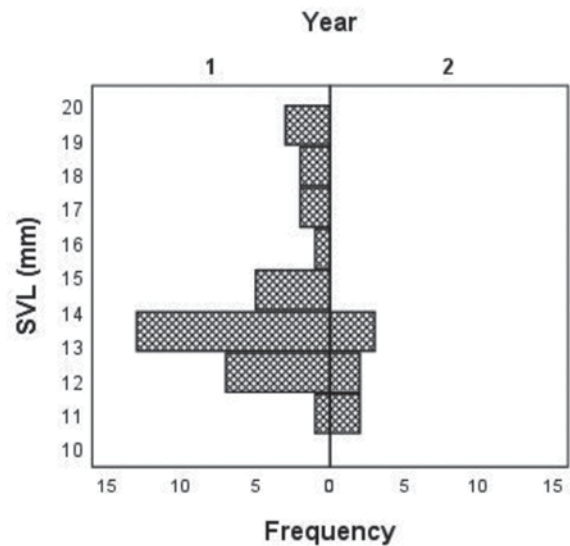


Figure 3. Histogram of body size classes (SVL, mm) for larvae on Years 1 and 2

Population size estimate

To estimate population size, we used the open-population POPAN model incorporated into the software programme MARK (White & Burnham, 1999). As sampling was not constant thought time, capture histories were built by aggregating 30 successively captured adults within each year, resulting in 10 groups (six in the first year and four in the second). A goodness-of-fit (GOF) test was run to test the capture-mark-recapture assumptions of the model using the program RELEASE GOF (Burnham et al., 1987), also included in MARK. Based on the result of TEST 2 + TEST 3 of RELEASE, a *post hoc* variance inflation factor (\hat{c}) was estimated by $[(\text{TEST 2} + \text{TEST 3}) / \text{df} = \chi^2 / \text{df}]$. Since $\hat{c} = 2.88$, the most parsimonious model was identified using the Quasi-Akaike Information Criterion (QAIC) (Burnham & Anderson, 2002).

A POPAN model can estimate four parameters: the survival rate (φ), the probability of capture (p), the probability of an individual to enter the population studied ($pent$) and the size of the population (N). Given the short migration distances and the limited scope for emigration or immigration (see Results), we considered $pent = 0$ and modeled φ and p as constant (.) or time varying (t). The following functions were used: sinus or logit link function for φ and p and log-link and identity link function for N .

RESULTS

Distribution and population structure

A total of 308 different golden-striped salamanders (225 adults, 40 juveniles and 43 larvae) were found along a continuous section of 107 m in one of the streams and its tributaries, which represents 11% of the total length of the prospected streams. Within this section there are two main nuclei separated by 50 m (Fig. 1.3), both near the mines. No other individuals were found in any of the adjacent streams.

Table 1. POPAN models for the *C. lusitanica* population in the Sintra Mountains. Model-choice criteria: corrected Quasi-Akaike Information Criterion (QAICc), and difference of QAICc values from the best fitting model (Δ QAICc). w_i - model weight. K - number of estimated parameters. For model descriptions, see Methods.

Model	QAICc	Δ QAICc	w_i	K
$\varphi(t) p(\cdot)$ (logit link for φ , sinus link for p , identity link for N)	216.516	0.000	0.870	3
$\varphi(t) p(t)$ (logit link for φ and p , log-link for N)	221.654	5.138	0.067	13
$\varphi(\cdot) p(t)$ (logit link for φ and p , identity link for N)	221.920	5.404	0.058	12
$\varphi(\cdot) p(\cdot)$ (logit link for φ and p , identity link for N)	226.916	10.400	0.005	3

Males were smaller than females ($t_{228} = 3.55$, $P < 0.001$; Fig. 2). This was also seen in their median SVL (47.0 mm for males and 49.6 mm for females). With an SVL of 55.8 mm, the largest female captured was the largest *C. lusitanica* individual ever recorded, 1.8 mm longer than the previous maximum, found at Galicia, about 350 km N of Sintra (Brizzi et al., 1999). Despite its large size, this individual grew 3.53% of its initial SVL in 33 days. The smallest juvenile was recorded in year 1 with SVL of 20.2 mm (Fig. 2). Larval SVL varied from newly hatched (9.9 mm) to pre-metamorphic (19.2 mm), with a bimodal distribution in year 1 (Fig. 3). There were low frequency injuries such as tail autotomy (24 individuals). Other anomalies were also rare: unusual short toes (16 individuals), skin deformities (3 individuals) and bloat-like disease (1 individual).

Matings were only seen in November (both years) and gravid females were seen from late October to early January. In the first year, most of the adults were found in March (22.98%), while in the second year most adults were found in October (38.23%) and March (14.13%). The sex-ratio in the first year was 1.60 and in the second year was 1.39. Males were more frequent during autumn and winter - sex ratios of 2.00 in November and of 3.09 in December of the first year; of 2.29 in October of the second year. Females were more frequent only in late spring, with sex ratios of 0.50 and 0.67 in April of the first and second years, respectively. The body condition of males was significantly higher in spring than in the other seasons (ANOVA, $F_{(2, 131)} = 8.71$, $P < 0.001$; Tukey HSD test, $P < 0.001$ for Autumn vs Spring; $P = 0.02$ for Winter vs Spring). This pattern was not found in females (ANOVA, $F_{(2, 83)} = 1.02$, $P = 0.36$).

Newly hatched larvae (near their egg capsules) were seen in January (year 1) and in November (year 2). Egg capsules and developing embryos ($N=41$) were found underneath rock crevices in one of the mines and in the stream adjacent to it. At the stream, larvae were more frequent in February of year 1 (48.6% of the larvae of that year) and in November of year 2 (50%).

Movements and population size estimate

Considering the adults, recaptures represented 36.8% of captures by the end of the first sampling year and 46.2% by the end of the second; only 31.1% of the adults captured in the second year had been captured in the first. At the final visit, 10 out of 17 adults were new individuals. As

for juveniles, recaptures constituted 14.71% the captures at the end of the first year and 11.11% at the end of the second. No juveniles were recaptured between years.

Most recaptures occurred on the same stream section - only 8% of the salamanders moved between stream sections in the first year (three juveniles, six males and one female), and 4.7% (five males) in the second. Considering the animals captured in both years, displacements were more frequent - 36.36% of the recaptures (seven males and five females) were found in different stream sections. Most movements were upstream (eight out of ten in the first year, four out of five in the second year and seven out of 12 between years). The maximum net distance moved was 48.2 meters downstream in the first year, by a male (average 15.38 ± 12.99 m), 26.3 meters upstream in the second, also by a male (average 23.73 ± 20.16 m) and 69 meters downstream between years, again by a male (average 16.86 ± 7.27 m).

The GOF test results showed that the data did not follow all assumptions (TEST 2: $\chi_{11}^2 = 13.28$, $P = 0.27$; TEST 3: $\chi_{11}^2 = 50.07$, $P < 0.001$; TEST 2 + TEST 3: $\chi_{22}^2 = 63.37$, $P < 0.001$). However, if \hat{c} values are ≤ 3 , the lack of fit is acceptable (Lebreton et al., 1992). The most parsimonious model considered a constant capture probability (of 0.1) and a survival probability dependent of time (Table 1). In this model, survival between visits was estimated to vary from 0.82 to 1. This population was followed for only two years and therefore it is not possible to estimate annual survival. Population size was estimated to consist of 339 ± 35 individuals, which corresponds to 3.2 salamanders per linear meter of the occupied stream section.

DISCUSSION

Finding a reproducing population of an iconic West-Iberian endemic salamander so close to Lisbon and in an area regularly surveyed by several herpetologists (including *R. Rebelo* and *E. Crespo*, both senior authors of this paper) over several decades is remarkable. The simplest explanation for its presence is the introduction reported by A. Seabra in 1943, although Seabra does not refer to the exact location of the introduction. However, the fact that the Sintra Mountains are an interglacial refuge for other Iberian north-western species, such as *Lacerta schreiberi* Bedriaga, 1878, a lacertid that maintains an isolated population in Sintra, in habitats similar to those of *C. lusitanica* (Brito et al., 1996), points at the possible presence of a relic population of this

salamander (Alexandrino et al., 2007). In fact, climate and orography-based distribution models predicted the suitability of the north-facing slopes of Sintra mountain for *C. lusitanica* (Arntzen & Teixeira, 2006), corroborating the opinion of Seabra (1943). Genetic analyses will most likely contribute to identifying the origin of this isolated population.

The Sintra salamanders live near abandoned mines and subterranean streams, microhabitats that maintain high ambient humidity and constitute optimal shelter and reproduction sites for the species (Sequeira, Ferrand & Crespo, 2003) and that have been regularly identified as summer refugia for local populations (Arntzen, 1981; Arntzen, 1994; Sequeira et al., 2001; Arntzen, 2015). The population size structure shows a continuous distribution across juveniles and adults, indicating successful reproduction over the years. The bimodal larval size distribution of the first year's sampling is consistent with the sizes of the first and second-year larval cohorts of the northern populations (Arntzen, 1981; Lima, Arntzen & Ferrand, 2000). Our very small sample of metamorphs and small juveniles does not allow for statistical comparisons with other populations; nevertheless, the sizes of the pre-metamorph and of the smallest juveniles at Sintra were similar to that reported by Arntzen (1981) for two populations near Porto. The very large body sizes of some adults may result from long lifespans, high growth rates (possibly influenced by the relatively higher autumn and winter temperatures at Sintra, when compared to the condition in the NW of the Iberian Peninsula), or both.

Chioglossa lusitanica can be either remarkably faithful to a favourable site (e.g., mines) or to migrate rapidly over long distances (Arntzen, 1994). Migrants can constitute a relatively high proportion of a population (up to 49%; Arntzen, 1994), but this was not found at Sintra, where less than 10% of the individuals were found to move between stream sections within the same year. Both the average and maximum distances moved at Sintra are much shorter than at the two northern populations studied by Arntzen (1994), where displacements over 350 metres were registered in just one night; they are however, similar to the values recorded by Sequeira et al. (2001), also near Porto, but in a more intensively human-modified habitat (abandoned farm with dry-stone walls). The higher sedentarism of the Sintra salamanders may be due to the inhospitable habitat surrounding the stream, considering both microclimate and vegetation cover (Vences, 1993).

Reproduction at Sintra occurred from November through January, coinciding with the known reproductive season for this species in its main distribution range (Vences, 1990; Sequeira, Ferrand & Crespo, 2003). The physical condition of males was significantly higher at the end of the activity season, suggesting that they were accumulating reserves for aestivation (Arntzen, 1981). For females this trend was not noticed, as the variability in their condition was much higher, most likely due to the egg development and laying processes. The first observation of eggs and larvae occurred much later in the first than in the second year, which may have been

due to the very strong, torrential rains of October 2015 (150 mm, twice the normal precipitation for that month; SNIRH, 2018). The torrential rains may have inhibited egg laying and/or driven the larvae to sheltered, inaccessible pools. However, the strong stream flow did not displace all the larvae downstream, as a fair number of larvae from the second year cohort was seen later at the stream.

Low human disturbance and absence of water or soil contamination have probably contributed for the continued existence of the Sintra population. Arntzen (2015), showed that larval survival is a crucial factor for the maintenance of *C. lusitanica* populations. We could not estimate larval survival up to metamorphosis, but our data on larval size distribution and metamorph sizes, although very limited, do not support the hypothesis of a poor habitat for larval development. On the other hand, the terrestrial habitat at the occupied site is not favourable for the salamanders and may hamper population increase and expansion, as a eucalyptus cover was already shown to be associated with less food resources for *C. lusitanica* (Vences, 1993), and eucalyptus plantations were related to a severe local decline (Arntzen, 2015). Our estimate of population size revealed a small effective, especially when compared with populations from the main distribution range that reach abundances of 11-12 salamanders per meter of stream (in some locations up to 16-17 salamanders per meter of stream) (Arntzen, 1981; Teixeira et al., 1998). In other locations, the size of the breeding population was found to consist of 1/5 to 1/6 of the total population (Arntzen, 2015) and a similar pattern may explain the low probability of capture, the relatively high number of new individuals captured up to the final visit and the violation of the TEST 3 of RELEASE, which is affected by heterogeneity in capture rates (White & Cooch, 2012). Continued sampling may ultimately reveal a larger population.

CONCLUSION

It is startling how amphibian populations continue to be rediscovered, even near dense populated areas like Lisbon. This population may have remained undetected for more than 70 years (and much more, if it is a remnant from glacial eras). The rediscovery of species and populations of amphibians previously thought to be extinct or nonexistent is not uncommon, as the case of the iconic Mallorcan midwife toad, *Alytes muletensis*, which was considered extinct until its discovery in 1980 (Mayol & Alcover, 1981), or the more extreme rediscovery of *Latonia nigriventer* Mendelssohn & Steinitz, 1943 in Israel (Biton et al., 2013). Although the origin of this population remains uncertain, *C. lusitanica* is a threatened and protected species. If future studies reveal that the Sintra population results from a 70-year old introduction, an interesting conservation dilemma will result, adding to the current discussion on whether the conservation of introduced species in their non-native range is justifiable (e.g., Gibson & Young, 2017), even in the cases where they reveal an invasive character (Marchetti & Engstrom, 2015). Furthermore, the long-

term survival of this single population of a species restricted to favourable microhabitats and with so many exquisite ecological requirements bring some hope for future reintroductions, or even assisted migration to sites previously unoccupied, but deemed climatically favourable for other amphibian species.

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Ranging behaviour of adders (*Vipera berus*) translocated from a development site

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Translocation of animals from sites scheduled for development is a widespread but controversial intervention to resolve human-wildlife conflicts. Indeed, reptiles are very frequently the subject of such translocations, but there is a paucity of information on the fate of such animals or how their behaviour compares to residents. In 2014, a population of adders (*Vipera berus*) was translocated from a development site in Essex, UK. A sample of snakes was fitted with external radio tags and tracked for a period of 10 days during the spring. This exercise was repeated during the summer using a combination of translocated and resident individuals. Translocated males exhibited significantly greater average daily movements than resident conspecifics. Furthermore, all translocated males undertook long-distance, unidirectional movements away from the release site. In contrast, all translocated females remained within 50 m of the point of release. One of the males returned to the donor site, crossing large areas of unsuitable habitat in doing so. Translocated males also maintained significantly larger total ranges than resident conspecifics. No differences in range sizes were observed between translocated and resident females. The dispersal of male snakes from the release site may increase the risk of mortality of translocated snakes and reduces the likelihood of establishing a new population. Interventions to encourage the establishment of new home ranges within the boundaries of release sites may include mechanisms to prevent dispersal immediately following release.

Key words: relocation, viper, radio-telemetry, reptile, human-wildlife conflict

INTRODUCTION

The translocation of animals from sites scheduled for development is a widespread but controversial intervention to resolve human-wildlife conflicts. The purpose of such 'mitigation translocations' is: (1) to prevent threatened wildlife being harmed through development activities; and (2) to encourage the establishment of a self-sustaining and viable population at sites protected from future development (Griffith et al., 1989). Over recent decades, the number of 'mitigation translocations' appears to be increasing exponentially and outnumbers translocations specifically carried out for conservation purposes (Germano et al., 2015).

Reptiles are frequently the subject of mitigation translocations despite questions being raised about their suitability for the practice (Dodd & Seigel, 1991; Germano & Bishop, 2008). European reptile populations have declined markedly over the past two decades (Cox & Temple, 2009; Reading et al., 2010). In the UK, all three native species of snake are legally protected from harm under the Wildlife and Countryside Act 1981. However, with the exception of the smooth snake (*Coronella austriaca*), this protection does not extend to their respective habitats. So if adders (*Vipera berus*) or grass snakes (*Natrix helvetica*) are found to be present on a site

scheduled for development, developers simply need to relocate individuals to an alternative site. Unfortunately, legislation does not require that translocations are reported to national recording schemes nor does it include any provisions for post-translocation monitoring. As such, it is not known how many translocations are undertaken annually nor whether translocation is effective in conserving snake populations.

The ecology and behaviour of the adder and grass snake have been relatively well-documented. Both species exhibit clear and predictable annual movement patterns, implying that they are aware of both the spatial and temporal availability of local resources (Phelps, 2004). It follows that translocation - and thus the introduction of novel stimuli - is likely to disrupt these patterns. Currently, there are no rigorous studies of how adders or grass snakes respond to translocation. However, there are comparable studies from North America, Asia and Australia that allow some broad inferences to be drawn (Plummer & Mills, 2000; Nowak et al., 2002; Butler et al., 2005; Lee & Park, 2011; Barve et al., 2013). The removal of visual and chemical cues has been shown to have a disorientating effect on snakes (Hare & McNally, 1997; Plummer & Mills, 2000; Nowak et al., 2002). Individuals are forced to spend a greater proportion of their time exploring their environment, often at the expense of

foraging and breeding behaviours (Wolf et al., 1996). Furthermore, studies of translocated snakes showed that individuals typically travelled further and occupied larger home ranges than their resident conspecifics (Reinert & Rupert, 1999; Butler et al., 2005; Lee & Park, 2011; Barve et al., 2013). Such long distance movements are often ascribed to the influence of philopatry; indeed, long-distance movements are likely to be more prevalent in species that exhibit strong homing behaviours (Sullivan et al., 2015).

As the human population in the UK continues to grow, more land will be required to satisfy the demand for housing. This in turn will exacerbate the need for and frequency of translocations. It is therefore important to understand how snakes respond spatially to novel environments. As the adder appears to be undergoing a decline in the UK (Baker et al., 2004), it is particularly important to ensure that translocation is effectively reducing impacts and ultimately conserving the species in the short-term.

The aim of this study was to ascertain the effects of translocation on the spatial ecology of adders. Specifically, for the first time we sought to determine whether adders translocated from a development site moved further and occupied larger home ranges than resident conspecifics.

MATERIALS AND METHODS

Study Site

The study site was a disused golf course located in the county of Essex, south-east UK. Planning permission was granted for the redevelopment of a 15 ha portion of the 75 ha site (Fig. 1). The remainder of the site was either to be retained as a golf course (14 ha) or re-graded and landscaped to become a country park (46 ha). Although the principal function of the country park was to provide residents with accessible 'open space', it was also designated as a receptor site to accommodate displaced wildlife. Both the donor and receptor sites were situated on the disused golf course albeit at opposite ends and separated by a minimum distance of 500 m.

Experimental Design

A total of 45 adders were translocated from the footprint of the development. Of these, eight snakes (six males and two females) were fitted with an external radio transmitter (1.1 g PicoPip tags) in April 2014. The tag attachment method followed the protocol described by Gent and Spellerberg (1993). This approach has been successfully applied to the tracking of adders in Britain and is well-suited to short-duration studies (Ujvari & Koros, 2000). The processing and study of adders was undertaken in accordance with the University of Kent's Ethics Policy, itself compliant with guidance from the Association for the Study of Animal Behaviour (2006).

The eight snakes were tracked for a period of 10 days following release. The location of each snake was recorded on three occasions each day: morning (08:00 – 10:00 hrs), afternoon (13:00 – 15:00 hrs) and evening (18:00 – 19:00 hrs). The frequency of sampling was balanced against the risk of causing disturbance;

Ujvari and Koros (2000) recommend at least two hours between sampling viper species to allow the resumption of original behaviours. The location of the adders was recorded to 4 m using a handheld GPS device (Garmin™). A further 10-day tracking period was undertaken in late August 2014. This block of telemetry involved two translocated adders and four non-translocated adders that were residents at the receptor site.

Data Analysis

A maximum of 30 sampling occasions for each animal were recorded. However, several tags detached or malfunctioned resulting in fewer fixes for those individuals. Those adders with fewer than 15 sampling occasions were excluded from range analysis.

Movements were measured as a straight line between successive locations; in reality, these measurements would be underestimates as snakes rarely travel in a straight line (Whitaker & Shine, 2003). Snakes were scored as 'active' when the distance moved exceeded 4 m, the minimum resolution of the GPS device. Upon completion of each 10-day study, the average distance moved per day was calculated. A two-way ANOVA was used to test for differences in average movement by sex and status (translocated vs. resident).

With the exception of one male for which too few fixes were obtained for range analysis, the 100% Minimum Convex Polygon (MCP) and 95% Harmonic Mean were calculated for each snake using BIOTAS® v. 2.0 (Ecological Software Solutions, 2005). Following Butler et al. (2005), the MCP and 95% harmonic mean were calculated as a proxy for 'total' and 'home' ranges respectively. Individual ranges were compared for differences between sex and translocation status using a two-way ANOVA, and ranges were plotted using ArcGIS (v.10.3) (ESRI, 2014).

RESULTS

Fourteen adders, comprising ten translocated and four resident individuals, were tracked. Following a series of tag malfunctions, data were collected from 10 individuals including six and four translocated and resident adders respectively. Of these, adder AD4 was excluded from the range analyses due to too few data points.

Although the sample size was small, two-way ANOVA showed that males had higher mean daily movements than females ($F_{1,6}=53.58$; $P<0.001$) and that translocated adders on average moved further than residents ($F_{1,6}=79.92$; $P=0.006$). A significant interaction was also detected ($F_{1,6}=55.3$; $P=0.012$) indicating that the male and females responded differently to translocation. Indeed, translocated males moved between 1.1 and 2.6 times further than resident conspecifics (Table 1).

Estimates of total (MCP) and home ranges were calculated for the translocated adders and their resident conspecifics (Table 2). A two-way ANOVA revealed highly significant differences in MCP size by sex ($F_{1,5} = 59.97$; $P < 0.001$) and translocation status ($F_{1,5} = 32.41$; $P < 0.001$). Males held total ranges that were significantly larger than females and translocated adders exhibited total ranges that were larger than their resident conspecifics (Figures

Table 1. Mean daily movements of translocated and resident adders

Adder ID	Survey period	N	Sex	Movement (m)*
Translocated snakes				
AD1	April 2014	30	M	21.07 (SD 11.6)
AD2	April 2014	18	M	17.04 (SD 10.2)
AD4	April 2014	6	M	23.36 (SD 13.6)
AD5	April 2014	27	F	4.41 (SD 1.4)
AD8	April 2014	27	F	3.71 (SD 0.6)
AD11	August 2014	12	F	5.35 (SD 0.9)
Resident snakes				
AD9	August 2014	30	F	3.83 (SD 0.5)
AD10	August 2014	30	F	2.01 (SD 0.5)
AD13	August 2014	27	M	9.03 (SD 1.9)
AD14	August 2014	27	F	4.8 (SD 1.0)

Table 2. Range sizes in translocated and resident adders. Total Range = MCP, Minimum Convex Polygon; Home Range = 95% harmonic mean.

Adder ID	Survey period	Sex	Range size (ha)	
			Total range	Home range
Translocated snakes				
AD1	April 2014	M	2.38	3.02
AD2	April 2014	M	2.57	6.38
AD5	April 2014	F	0.04	0.23
AD8	April 2014	F	0.02	0.06
AD11	August 2014	F	0.01	0.03
Resident snakes				
AD9	August 2014	F	0.03	0.09
AD10	August 2014	F	0.006	0.05
AD13	August 2014	M	0.19	0.52
AD14	August 2014	F	0.05	0.55

provided as supplementary material). Furthermore, the interaction between translocation status and sex was highly significant ($F_{1,5} = 41.38$; $P = 0.001$) indicating that males and females respond differently to translocation, with translocated male adders increasing their total range more than translocated females.

The analyses also identified significant differences in home range sizes by sex ($F_{1,5} = 9.85$; $P = 0.012$) but not by translocation status ($F_{1,5} = 1.25$; $P = 0.29$). However, significant differences between males and females was detected ($F_{1,6} = 5.87$; $P = 0.038$). As previously, translocated males increased their home range sizes whilst translocated females did not.

DISCUSSION

Adders increased their movements post-translocation, but unlike other studies this response was exhibited solely by males. Although the sample sizes were small the movement patterns were compelling, and to our knowledge this is the first study to report that behavioural

responses of snakes to translocation differ between the sexes. Translocated males undertook daily movements almost double those of the resident males and over three times those of the females (both translocated and resident). Indeed, all three telemetered males migrated away from the receptor site, crossing areas of unsuitable habitat in the process. As would be expected, the increased movements also resulted in larger range estimates.

There is a growing body of literature that criticises the use of mitigation-translocations (Dodd & Seigel, 1991; Reinhert & Rupert, 1999; Fischer & Lindenmayer, 2000; Butler et al., 2005; Germano & Bishop, 2008). The increased movements associated with translocated snakes can incur significant costs to the individual. Locomotion is energetically expensive, particularly so when it results in a behavioural shift from foraging to exploration. This combination of increased movements and reduced foraging is likely to have a deleterious effect on body condition (Reinert & Rupert, 1999) including diminutions in fecundity (Luiselli, 1992). Increased movements have also been associated with elevated risks of mortality (Andren, 1985; Madsen & Shine, 1993; Plummer & Mills, 2000; Butler et al., 2005). Highly mobile individuals are more likely to encounter predators or enter high-risk areas (Madsen & Shine, 1993; Shine & Fitzgerald, 1996). In a meta-review of published snake studies (which included the adder), Bonnet et al. (1999) reported that the highest levels of mortality coincided with dispersing neonates, males undertaking mate searching and females migrating to egg-deposition sites. Similarly, young adders experienced mortality rates of between 88% and 92% whilst undertaking dispersal (Prestt, 1971; Phelps, 2004). The results of this study provide further, albeit qualified, support to this criticism. The act of translocation appeared to disrupt typical courtship activities during April, a key period for breeding. Adders AD1 and AD2 undertook large, unidirectional movements back towards to the donor site where the former was observed courting a large female. It is not understood why the adders left the receptor site, which contained a high density of females (both translocated and resident), to return to the donor site. Male adders are able to detect females over considerable distances and would have been aware of the presence of females. Phelps (2004) described the formation of sub-groups within two adder populations across which there was no genetic exchange. If such a subdivision was present in the Essex adder population, it could explain why the males left the receptor site and perhaps resumed breeding activities with females in the donor site.

When developing mitigation strategies for adders, and in particular when designing receptor sites, it is important to account for this additional area requirement. However, given the absence of any published values for home ranges of adders in the UK, it is inconceivable that developers or their consultants are incorporating this important metric into mitigation strategies at present. Consideration should be given to whether short- or long-distance translocation would be the more appropriate technique. To answer this, further studies

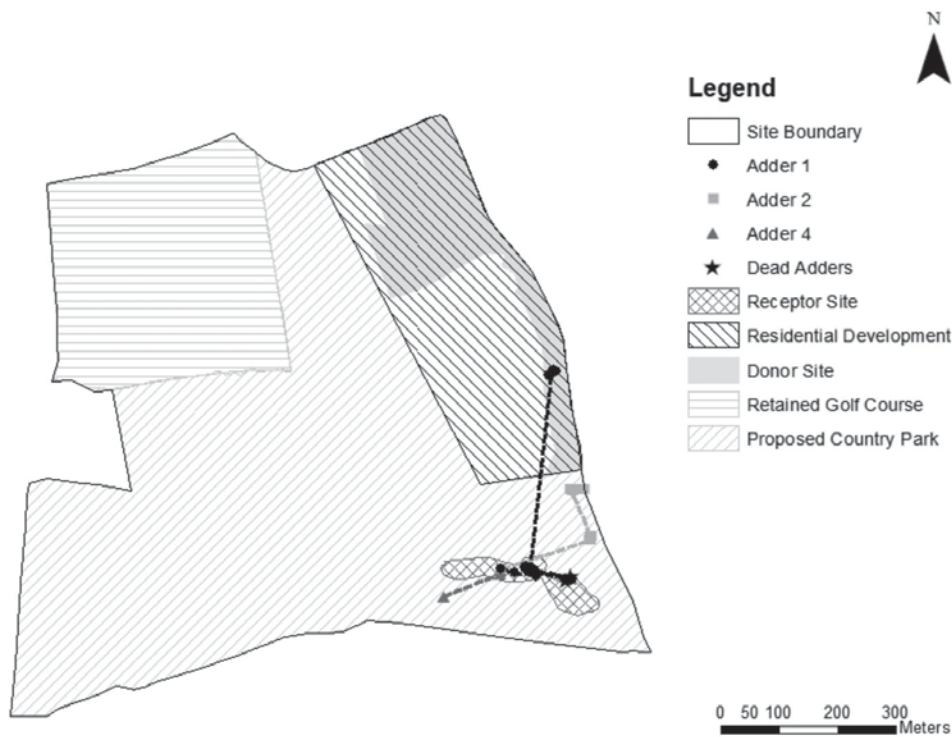


Figure 1. A map depicting the disused golf course and the proposed development plans. Following relocation from the donor, all male adders appeared to have left the receptor site. In contrast, female adders remained within 50 m of the point of release.

would be required to ascertain what constitutes ‘typical’ movements and home ranges for adders in the UK. Maintaining adders within their current range has clear benefits, such as the negation of extended movements and associated risks of mortality. Other factors, such as the transmission of disease and the mixing of locally adapted alleles, would also be mitigated. Conversely, the loss of part or all available habitat (including key features such as hibernacula and corridors) or the introduction of anthropogenic pressures (including domestic pet predation) could result in localised extirpation of retained populations. Any reductions in suitable habitat would correspondingly reduce the carrying capacity and thereby increase competition. Moreover, the introduction of high risks factors, such as a busy road, close to known adder population foci could adversely affect the population.

Homing behaviours could be managed through the construction of physical barriers such as strategically placed fences or, for more permanent developments, concrete walls (Kyeck et al., 2007). Similar barriers could also be used to mitigate the propensity of translocated male adders to cross unsuitable and potentially unsafe habitat. However, the purchase and installation of fencing can be costly, extending to tens of thousands of pounds (Lewis, 2012). As a cheaper alternative to fencing, developers often intentionally degrade habitat making it unsuitable for reptiles. This ‘buffer’ is often considered to be sufficient for excluding reptiles from otherwise unfenced development sites. Whereas this approach may be effective for more sedentary species, such as viviparous lizards, the current study has demonstrated that its effectiveness cannot be guaranteed for adders.

Whether this translocation was a success or not depends on how success is defined. Forty-five adders were removed from the footprint of the development where the risk of harm was considerable. However, previous studies have indicated that the proportion of animals in a population that is detected and moved in a translocation is typically low (Platenberg & Griffiths, 1999; Germano et al., 2015). Consequently, any adders that remained undetected within the donor site along with those that returned post-translocation are likely to have been harmed through construction-related activities. In this context, for those individuals that were moved and did not return to the donor site, the translocation should be considered a success in the short-term at least. Although the current study did not include survival analyses, it is possible to infer increased risks of mortality post-translocation. A clear association exists between increased movements and mortality (Andren, 1985; Madsen & Shine, 1993; Plummer & Mills, 2000; Butler et al., 2005). Male adders, which exhibited both increased mean movements and ranges, would have experienced an increased risk of encountering predators or inhospitable habitat features. Indeed, two dead adders were recovered from adjacent to the release site (Fig. 1); both exhibited extensive musculoskeletal damage indicative of large mammals i.e. domestic cats, badger (*Meles meles*) or red fox (*Vulpes vulpes*). As both adders were decapitated, it was impossible to ascertain whether they were part of the translocated population. Longer-term comparisons of survival between natural and translocated populations obtained using capture-mark-recapture analysis would provide further information on the effects of translocation on snakes.

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A ring-species or a ring of species? Phylogenetic relationship between two treefrog species around the Yellow Sea: *Dryophytes suweonensis* and *D. immaculatus*

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Phylogenetic patterns due to glaciation are still understudied in North East Asia (NEA). Furthermore, the effects of the Last Glacial Maximum on phylogenetic patterns are less explicit in NEA than for other regions of the northern hemisphere due to topographically homogenous landscapes in general. Here, we aim to assess the phylogenetic status of the *Dryophytes suweonensis* and *D. immaculatus* treefrog clades. We used concatenated partial mitochondrial 12S and 16S gene fragments, with a combined length of 678 bp for *D. suweonensis* (n = 32) and *D. immaculatus* (n = 5), collected from the Republic of Korea and downloaded from GenBank (originating from the People's Republic of China). *Dryophytes suweonensis* formed an apparently monophyletic clade whereas *D. immaculatus* was divided in two clades. Our results also demonstrated the continuous genetic variation through haplotypes forming a ring around the Yellow Sea. It is therefore difficult to conclude on either a ring-species or a ring of species around the shallow Yellow Sea, which acted as a land-bridge several times during recent geological times. We recommend the use of other data such as call characteristics and morphology to determine the species or sub-species status of these two clades.

Key words: *Dryophytes suweonensis*, *Dryophytes immaculatus*, species divergence, *Hylidae*, Yellow Sea

INTRODUCTION

The impact of glacial cycles on speciation events in Europe and North America has been clearly demonstrated for a number of species (Hewitt, 2000; Knowles, 2001; Veith et al., 2003; Avise, 2007). For instance, the *Hyla arborea* complex, like many other species (Hewitt, 2000), survived glaciation cycles through the use of peninsular refugia in southern Europe (Stöck et al., 2008; Stöck et al., 2012). However, this kind of study is generally missing in North East Asia, here limited to the south by the Yangtze River and around 100° west by the monsoon weather (Wang & Lin, 2002; Lee et al., 2005), where the continuous mainland landscape was not as conducive to speciation events as those in other regions of the northern hemisphere. The major impermeable geographical barrier to gene flow on the East Asian mainland is the Himalayan range to the south and the adjacent southern edge of the Tibetan Plateau. These topographical patterns delimit a southern edge of species distribution, as visible for instance in *Bufo gargarizans* (Yan et al., 2013; Borzée et al., 2017c), *Rana chensinensis* (Kuzmin et al., 2004b) and *Pelophylax nigromaculatus* (Kuzmin et al., 2004a). However, glaciation cycles did influence speciation events for peninsular populations, as demonstrated in the split between *B. gargarizans* and *B. bankorensis* (Chen et al., 2013; Yu et al., 2014), and

Hyla chinensis and *H. simplex* (Hua et al., 2009).

Due to the sea level rise and recession, the Korean peninsula was alternatively isolated and linked to the Japanese Archipelago and the Chinese mainland. During glacial oscillations, the peninsula became totally isolated and acted as an isolated refugium during the last glacial maximum (LGM), even for good dispersers such as racoon dogs (*Nyctereutes procyonoides*; Kim et al., 2013). The Korean Peninsula was not covered by glaciers but was colder and drier during glacial periods (Kong, 2000; Yi & Kim, 2010), and acted as one of the two refugia during the late Pleistocene, together with current central China. For instance, the water frog *Pelophylax nigromaculatus* (Zhang et al., 2008) and the clawed salamander *Onychodactylus koreanus* (previously assigned to *O. fischeri*; Poyarkov et al., 2012) benefitted from these refugia (Yoshikawa et al., 2008). The Korean peninsula itself is divided by non-crossable landscape elements, leading to genetic diversification within *P. chosonicus* (Min et al., 2008), *Hynobius* spp. (Baek et al., 2011; Min et al., 2016), and *Dryophytes japonicus* (Dufresnes et al., 2016).

Glaciation cycles lead to species specific speciation scenarios, with some clear examples of segregated species separated by the Yellow Sea, such as *P. chosonicus* in Korea and *P. plancyi* in China (Liu et al., 2010). However, the same climatic variations also resulted in repeated

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Table 1. Specimen information used in this study. For each specimen, species identity, GenBank accession numbers for each partition, specimens with voucher code (if available), and locality are included. *indicates an update from *Hyla* to *Dryophytes* (Duellman et al., 2016), independently of the name present on the GenBank upload. RoK stands for Republic of Korea.

Taxon	GenBank No.		Specimen voucher no./isolate no.	Locality
	12S	16S		
<i>Hyla arborea</i>	DQ055835	DQ055814	-	Croatia, Kamesnica, Donja Korita
<i>H. chinensis</i>	KP742571	KP742700	IOZCAS4796	China: Zhaowu, Fujian
<i>Dryophytes femoralis</i> *	DQ055838	DQ055819	MVZ137344	USA: Emanuel, Georgia
<i>D. immaculatus</i> *	KP742584	KP742712	CIBLJT060811	China: Conghua, Guangdong
	KP742585	KP742713	CIBLJT060812	China: Conghua, Guangdong
	KP742586	KP742714	SCUM0606003	China: Jingzhou, Hubei
	KP742587	KP742715	SCUM0606004	China: Jingzhou, Hubei
	KP742588	KP742716	SZ100604	China: Sangzhi, Hunan
	KP742589	KP742717	CIBLJT070515	China: Conghua, Guangdong
<i>D. japonicus</i> *	KP742597	KP742724	CIBLJT070602	China: Hailin, Heilongjiang
	KP742598	KP742725	CIBLJT070607	China: Shenyang, Liaoning
	KP742599	KP742726	ZISPRussia101001	Russia: Ussuriysk Distr.
	KP742600	KP742727	ZISPRussia101002	Russia: Ussuriysk Distr.
<i>D. suweonensis</i> *	MG282190	MG282222	BHV383	Geumchon, RoK
	MG282191	MG282223	BHV387	Geumchon, RoK
	MG282192	MG282224	BHV390	Geumchon, RoK
	MG282193	MG282225	BHV391	Geumchon, RoK
	MG282194	MG282226	BHV393	Geumchon, RoK
	MG282195	MG282227	BHV394	Geumchon, RoK
	MG282196	MG282228	BHV396	Geumchon, RoK
	MG282197	MG282229	BHV397	Geumchon, RoK
	MG282198	MG282230	BHV398	Geumchon, RoK
	MG282199	MG282231	BHV399	Geumchon, RoK
	MG282200	MG282232	BHV400	Geumchon, RoK
	MG282201	MG282233	BHV403	Geumchon, RoK
	MG282255	MG282234	SUR12	Cheonan, RoK
	MG282212	MG282245	SUR32	Cheonan, RoK
	MG282213	MG282246	SUR33	Cheonan, RoK
	MG282214	MG282247	SUR34	Cheonan, RoK
	MG282215	MG282248	SUR35	Cheonan, RoK
	MG282216	MG282249	SUR36	Cheonan, RoK
	MG282217	MG282250	SUR38	Cheonan, RoK
	MG282218	MG282251	SUR39	Cheonan, RoK
	MG282219	MG282252	SUR40	Cheonan, RoK
	MG282220	MG282253	SUR43	Cheonan, RoK
	MG282221	MG282254	LTR1	Iksan, RoK
	MG282202	MG282235	LTR2	Iksan, RoK
	MG282203	MG282236	LTR3	Iksan, RoK
	MG282204	MG282237	LTR4	Iksan, RoK
	MG282205	MG282238	LTR5	Iksan, RoK
	MG282206	MG282239	LTR6	Iksan, RoK
	MG282207	MG282240	LTR8	Iksan, RoK
	MG282208	MG282241	LTR9	Iksan, RoK
	MG282209	MG282242	LTR10	Iksan, RoK
	MG282210	MG282243	LTR11	Iksan, RoK
<i>Smilisca fodiens</i>	AY819387	AY819519	MVZ132994	Mexico: Sonora
<i>Pseudacris nigrita</i>	AY819386	AY819518	MVZ145454	USA: North Carolina

gene exchanges over large geographic areas, such as for *B. gargarizans* in North East Asia (Borzée et al., 2017c). Intermediate and unresolved cases also exist, such as for the *D. suweonensis/immaculatus* complex (Li et al., 2015; Dufresnes et al., 2016; Borzée et al., 2017a), for which studies on clade divergence and relationship with the sister clade *D. japonicus* are not consistent (Riehl et al., 1995; Hill, 2009; Li et al., 2015; Dufresnes et al., 2016).

The origin of the Eurasian Hylinae (Rafinesque, 1815;

Smith et al., 2005; Faivovich et al., 2005; Hua et al., 2009; Wiens et al., 2005; Wiens et al., 2010) is consistent with the Savage (1973) hypothesis of a late Cenozoic invasion. The northern oriental Hylid populations belong to two main groups, namely “*H. arborea* group” (*sensu stricto* Anderson & Green, 1991) and “*D. japonicus* group” (*sensu stricto* Hua et al., 2009). In North East Asia, *D. japonicus*, *D. suweonensis* and *D. immaculatus* were recently moved from the genus *Hyla* to *Dryophytes* (Duellman et al.,



Figure 1. Ranges and sampling sites for *Dryophytes suweonensis* and *D. immaculatus*. The range based on data from AmphibiaChina (<http://www.amphibiachina.org/>) is drawn from the most external localities and in a way that avoids sharp angles in the polygon created. Map generated through ArcMap 10.5 (Environmental Systems Resource Institute, Redlands, California, USA), with Service Layer Credits & Sources to Esri, USGS, NOAA, DeLorme, USAS and NFS.

2016). The *Hyla* clade expanded from Northern America through the Bering pass (Anderson & Green, 1991; Borkin, 1999; Duellman, 2001) 28 to 23 mya (Smith et al., 2005) and diverged between 22 to 18 mya into the Asian and European clades (Riehl et al., 1995; Smith et al., 2005). The *Dryophytes* clade, comprising *D. japonicus*, *D. stephensi* (Dufresnes et al., 2016), *D. suweonensis* (Kuramoto, 1984; Riehl et al., 1995) and *D. immaculatus* (Hua et al., 2009), reached Asia during a second expansion by the “*D. eximia* taxa” (Anderson & Green, 1991; Borkin, 1999; Faivovich et al., 2005) from the American continent (Hua et al., 2009) between 18.9 and 18.1 mya (Smith et al., 2005). Divergence between species of the ‘*D. japonicus* group’ started 24 mya (Riehl et al., 1995), resulting in the split between *D. japonicus* and *D. immaculatus* 14 mya (Hill,

2009), and the divergence between *D. japonicus* and *D. suweonensis* between 6.4 and 5.1 mya (Li et al., 2015; Dufresnes et al., 2016).

The *Dryophytes* and *Hyla* Asian genera are largely parapatric (Hua et al., 2009), with an area of overlap from 28 to 33° N latitude (Hoffmann, 2001; Hua et al., 2009). This contact zone may have led to potential competition, such as between *H. chinensis*, occurring together with *D. immaculatus* (Anderson & Green, 1991; Hua et al., 2009). Other ambiguities have been noted, such as the homoplasy of calls properties between *D. japonicus* and *H. hallowelli* (Kuramoto, 1980), while *D. japonicus* and *D. suweonensis* calls are distinct despite their phylogenetic promiscuity (Riehl et al., 1995; Faivovich et al., 2005; Hua et al., 2009).

Since Gunther (1958), and Kuramoto (1980), two

clades of treefrogs have been identified on the Korean peninsula: *D. japonicus* and *D. suweonensis* (Yang & Park, 1988; Yang et al., 1997; Lee et al., 1999; Duellman et al., 2016; Dufresnes et al., 2016). On the opposite side of the Yellow Sea, the Chinese mainland is populated by *D. japonicus* and *D. immaculatus*. Here, we hypothesize that the *D. suweonensis/immaculatus* complex is composed of two divergent species at the extremities of their ranges, but less clearly differentiated when in proximity.

MATERIAL AND METHODS

Species description

Dryophytes suweonensis populations have been recorded as declining throughout the last decade (IUCN, 2017) and are today restricted to a narrow land strip between the Yellow Sea and the western edge of the Baekdudaes Range (Roh et al., 2014; Borzée et al., 2016b; Borzée et al., 2017b). This spatial distribution in relation with urban development lead to the isolation of populations and poses a threat to the survival of the species in the long term. *Dryophytes immaculatus* is described as a lowland species from the Chinese mainland (Xie, 2017; AmphibiaChina, 2018), with a much broader range of habitats than *D. suweonensis*, and expected to be more tolerant to habitat modification (Xie, 2017).

Field sampling

Field sampling was conducted over three localities, encompassing the totality of the known range of *D. suweonensis* (Borzée & Jang, 2015; Borzée et al., 2016b; Fig. 1). This was necessary as *D. suweonensis* populations are fragmented due to landscape barriers such as Metropolitan Seoul and the Geum River (Borzée et al., 2015a; Borzée et al., 2017b). The three localities were Geumchon (North of Seoul, $n = 12$, annotated as BHV on subsequent tables and figures), Cheonan (South of Seoul, $n = 10$, annotated SUR), and Iksan, a non-connected population on the southern edge of the species' range ($n = 10$, annotated LTR). A minimum distance of at least 90 km separates the adjacent localities. The samples at the two northern localities, Geumchon and Cheonan, were collected in 2013 (Table 1) under the Ministerial authorisation number 2013-16, while the Iksan locality was sampled in 2014 under the permits 2014-04, 2014-08 and 2014-20. Due to the endangered status of the species, we followed the strict ethical regulation dictated by the permits.

Each locality was sampled once only to prevent pseudo-replication (Table 1). Localities were presumed to be independent of each other, as the dispersion range of most amphibian species is below 10 km per year (Smith & Green, 2005), and that of hylids not over more than a couple kilometres per season (Vos et al., 2000; Smith & Green, 2005; Arens et al., 2006). Genetic material was acquired through oral (= buccal) swabs (Broquet et al., 2007), frozen within 24 h at -20°C .

Molecular work

Mitochondrial DNA was extracted with the Enzyomic® Genomic DNA Extraction Kit (Tissue; Genomics Extraction

Kit protocol V2013-1; Seoul, Korea) for Geumchon and Cheonan samples and the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) for Iksan samples, following the instructions of the manufacturer.

Because *D. suweonensis* and *D. japonicus* are difficult to identify based on morphology only (Borzée et al., 2013), each individual was identified as belonging to *D. suweonensis* based on mtDNA Cytochrome c oxidase subunit I (COI) sequencing, following the protocol developed by Jang et al. (2011). These sequences were not used for further analysis as not sequenced for *D. immaculatus*. Then, as sequences of *D. immaculatus* were available from GenBank for partial mitochondrial ribosomal 12S and 16S genes (accession numbers KP742584 through KP742589 for 12S, and KP742712 through KP742716 for 16S, Table 1), we sequenced all individuals for part of these genes. For 12S, new primers were designed with Geneious v.9.1.6 (<http://www.geneious.com>, Kearse et al., 2012; plug-in Primer3 v.2.3.4) based on *D. immaculatus* sequences from GenBank: DIS-12S-F: CCC AAG ACA CCT AGC TAC GC; and reverse DIS-12S-R: TGG CTT TGA AGA GGG TGA CG. For 16S, the primers used were: 16SA-L CGC CTG TTT ATC AAA AAC AT and 16SB-H CCG GTC TGA ACT CAG ATC ACG T (Vences et al., 2005; Jeong et al., 2013), completed by specifically designed primers overlapping with the 16SA and 16SB primer set, also designed with the Geneious plug-in Primer3 (Koressaar & Remm, 2007; Untergasser et al., 2012): DIS-16S-F: GTA AGG GCC CCA ACG TAG TC and DIS-16S-R: AGG GAT GCT GTA GTT AGG GGT.

PCR reactions using published primers 16SA-L and 16SB-H were run following the protocols by Jeong et al. (2013). All newly designed primers were used in PCRs with each reaction containing 14.5 μL of distilled water, 2.0 μL of (10x) buffer, 1.6 μL of dNTPs (final concentration of 0.07 mM), 0.4 μL of each primer, 0.1 μL of Takara Taq polymerase and 1 μL of DNA. The thermocycler (SimpliAmp Thermal Cycler; Applied Biosystems by Life Technologies; Carlsbad, California, United States) was programmed to 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 58°C for 30 s and 72°C for 1 min, with a terminal elongation at 72°C for 5 min (12S) and 10 min (16S). PCR products were then run on a 1.5 % agarose gel during a 12 min electrophoresis, and pre-stained with MaestroSafe dye (Maestrogen; Las Vegas, Nevada, USA). PCR products were subsequently cleaned-up with the PCR Clean-Up Kit (LaboPass PCR, PCR Purification Kit; Cat. No. CMR0112; Cosmogenetech; Seoul, Republic of Korea) and sent to Macrogen Inc. (Seoul, Republic of Korea) for direct sequencing with both forward and reverse primers on an ABI PRISM 3100 automatic sequencer (Applied Biosystem Inc., USA).

Molecular analysis

The partially sequenced mitochondrial 12S and 16S ribosomal RNA genes, 628 bp and 485 bp, respectively, were proofread, edited, assembled, and concatenated with Geneious v9.1.6 (Biomatters Limited, Auckland, New Zealand). Despite the 16S sequences being originally 485 bp long, only 50 bp were used in the analysis, due to short overlap with the selected reference sequences

for *D. immaculatus*. Details on specimen vouchers, GenBank accession codes, and sampling sites of the all sequences used for this study are listed in Table 1. The alignment was performed using MUSCLE (Edgar, 2004), implemented through the Geneious plug-in, with a maximum of 10 iterations following default parameters, and further revised manually when needed. The final alignment had a sequence length of 677 bp, and the new sequences were deposited in GenBank (accession number for 12S: MG282222 to MG282255, and 16S: MG282190 to MG282212).

Estimation of genetic variables

We computed the haplotype diversity, Fu's *F* (Fu, 1997) and Tajima's *D* test for neutrality (Tajima, 1989) to infer whether the genes chosen, although slow evolving, were under selection or at mutation-drift equilibrium. The software DnaSP was used to estimate these three indices (Librado & Rozas, 2009).

In order to refine the relationships between and within the two clades, we created a haplotype network in TCS (Clement et al., 2000) with a fix connection limit at 500 steps (estimated) for the concatenated partial 12S and 16S, with all other parameters set as defaults (Clement et al., 2000). PopART (Population Analysis with Reticulate Trees; <http://popart.otago.ac.nz>) was used to visualise the haplotype network.

Construction of phylogenetic tree

Three types of phylogenetic trees were constructed to resolve the phylogenetic relationship between the two clades. We first employed jModelTest 2.1.10 (Darriba et al., 2012; Guindon & Gascuel, 2003) to estimate the best-fitting substitution model of the concatenated 12S and 16S rRNA genes (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). We chose the General Time Reversible model with the addition of invariant sites and gamma distribution of rates across sites (GTR+I+G) for the reconstruction of the phylogeny (AIC data not shown).

We then created a PhyML tree with the PHYML plugin in Geneious (Guindon & Gascuel, 2003), as it implemented a fast and accurate heuristic for estimating maximum likelihood phylogenies. We applied the GTR substitution model with 50,000 bootstraps and all other variables as default. We also added *Pseudacris nigrita* and *Smilica fodiens* as outgroups to the analysis (GenBank accession numbers in Table 1), such as suggested by Li et al. (2015).

We then constructed two phylogenetic trees to clarify the relationship between the two species. We first ran a RAxML tree (Geneious plugin; RAxML 7.2.8) with the model selected with 50 000 bootstrap replicates and all other variables set as default. *Pseudacris nigrita* and *S. fodiens* were also integrated as outgroups to the analysis. We finally ran a Bayesian Inference phylogenetic analysis with a single Markov Chain Monte Carlo (MCMC) analysis of 1.1 million iterations, each with 4 heated chains on MrBayes 3.2.6 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). One tree was saved every 200 generations, and prior distribution default parameters were used. The final 50% majority rule consensus tree

was saved. For this model, 18 species extracted from Li et al. (2015) were used as outgroups, and we added four *Dryophytes japonicus* individuals to the analysis (all GenBank accession numbers in Table 1).

Osteological comparison

In order to further assess variations between the two clades, we conducted a meta-analysis on osteological information extracted from the literature. Fei et al. (2012) and AmphibiaChina (2018) provided information on the snout-vent-length (SVL) and head morphology, that was compared with the data extracted from Borzée et al. (2013). Besides, Dang et al. (2017) described the bone structure of hands for several anurans in China, while Kim et al. (2017) described the bone structure of *D. suweonensis* in the South Korea. We extracted the corresponding pictures (Fig. 1 and Fig. 2 respectively), enlarged them to 300 % and measured the length of metacarpals and phalanges for fingers 2, 3 and 4. Because scale bars were not provided for one of the figures, and because metacarpals are known to be a phylogenetically informative feature (Dang et al., 2017), we created a ratio by dividing the length of phalanges by that of the metacarpal of the same finger. We then compared the ratios obtained for the two species.

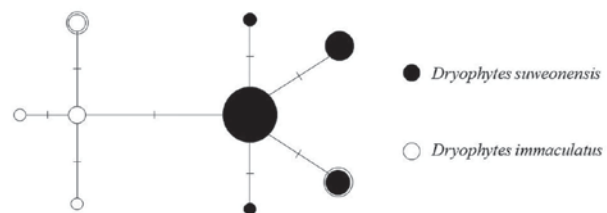


Figure 2. Haplotype network constructed with PopART overlapped on sampling localities for *D. immaculatus* (open circles) and *D. suweonensis* (solid circles). The population-specific haplotype found in Geumchon, north of Seoul for *D. suweonensis*, and Conghua, southernmost locality in China for *D. immaculatus*, are circled. The size of markers is representative of sample size ($1 \leq n \leq 22$).

RESULTS

Our results highlight the differences, and divergence albeit potentially recent, between the *D. suweonensis* and *D. immaculatus* clades. *Dryophytes suweonensis* formed a monophyletic clade in both Maximum Likelihood and Bayesian Inference phylogenies. The absence of shared haplotypes at the extremes of the ranges for both species implies the two species are distinct in terms of sequence structure for the concatenated 12S and 16S rRNA. Furthermore, the range of the two species, *D. suweonensis* extracted from Borzée et al., 2017b and *D. immaculatus* extracted both from the IUCN (Xie, 2017) and AmphibiaChina (2018), show the absence of overlap in distribution. Additionally, the published ranges for *D. immaculatus* are not properly overlapping, and sequences for one of the individuals harvested from

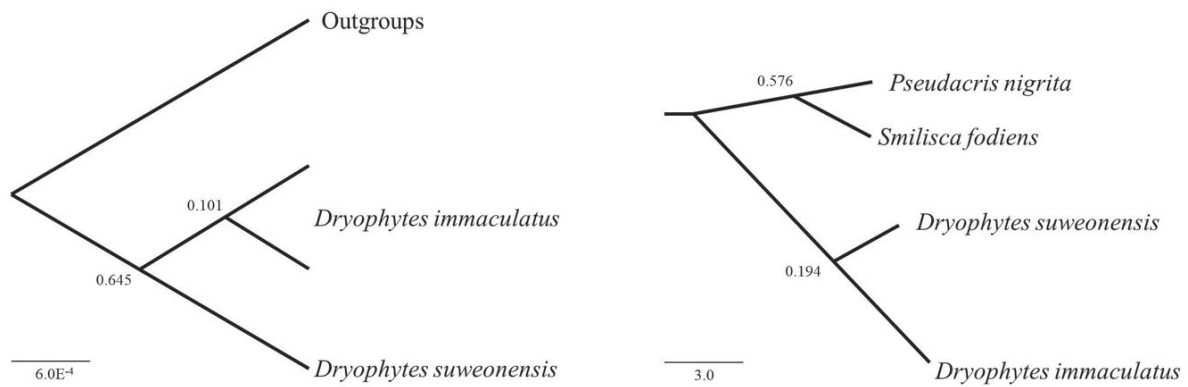


Figure 3. Simplified Maximum Likelihood Phylogenetic tree built with the PHYML plugin in Geneious (A) highlighting the segregation between the two *Dryophytes* clades and supporting the monophyly of *D. suweonensis*. Simplified Maximum Likelihood Phylogenetic tree built with the RaxML plugin in Geneious (B), with both species presented as monophyletic. Branch distances represent nucleotide substitution rate and scale bar represents the number of changes per nucleotide position.

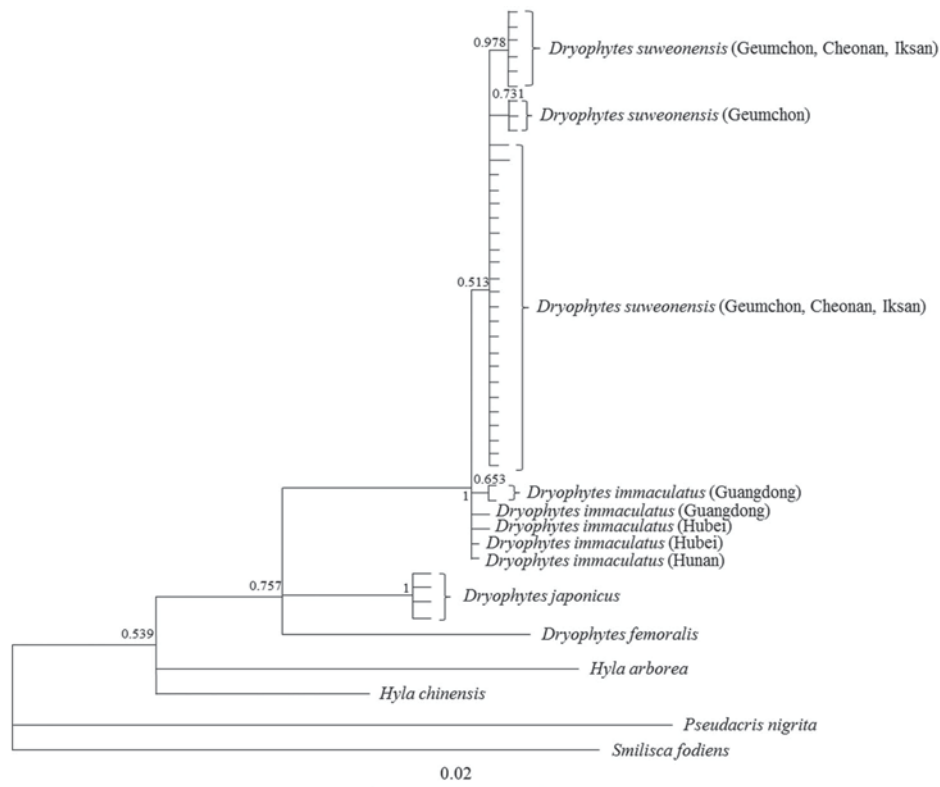


Figure 4. Bayesian Inference phylogenetic tree with a single Markov Chain Monte Carlo. This analysis also highlights the monophyly of *D. suweonensis*, despite some isolated branches. Branch distances represent nucleotide substitution rate and scale bar represents the number of changes per nucleotide position.

GenBank (isolate CIBLJT060812; Table 1) is about 800 km south of the southernmost point of the known species' range.

Estimation of genetic variables

The haplotype diversity calculated through DnaSP for *D. suweonensis* showed low genetic variability with only five haplotypes ($Hd = 0.54$, variance = 0.00812). Fu's F ($F = -1.15$, $p > 0.10$) and Tajima's D test for neutrality ($D = -0.99$, $p > 0.10$) were not significant, thus not supporting any clear pattern of demographic dynamics.

The conflicting values of Fu's F and Tajima's D may indicate a low sample size or recent population reduction or bottleneck.

The haplotype network created (Fig. 2) highlights the non-overlap of common haplotypes between the two species, but also the same degree of variation (one nucleotide difference) between and among species. In addition, one of the *D. suweonensis* haplotypes is only found at the locality north of Seoul, while the isolated locality in Iksan does not display unique haplotypes. We found the same pattern for the southernmost locality for

D. immaculatus, with all individuals from Conghua, the southernmost site, displaying the same haplotype (Fig. 2).

Phylogenetic analysis

The three models used with the phylogenetic analysis of the two concatenated mitochondrial genes were congruent for the monophyly of *D. suweonensis*, although variations were visible within the *D. immaculatus* clade, and the branching of the outgroups. The Maximum Likelihood tree constructed through PhyML highlighted the segregation between the two clades (Fig. 2A), and at the same time supported the non-monophyly of *D. immaculatus*. In contrast, the Maximum Likelihood tree constructed through RAxML suggested the early divergence between the two species, while both species were presented as monophyletic (Fig. 2B). The final Bayesian Inference analysis is congruent with both analyses on the monophyly of *D. suweonensis*, despite a posterior support of 0.512, and the phylogenetic relationships reflected by the RAxML analysis (Fig. 3).

Osteological comparison

The data provided by AmphibiaChina (2018) states that for *D. immaculatus*, male SVL is 31 mm, which is larger than *D. suweonensis* where male SVL is 293 ± 013 mm (Borzée et al., 2013). Oppositely, the cranial features extracted follow the same ratios, with the inter-nostril distance (IND) being lower than the distance between the anterior corner of the eyes (EAD), while being comparatively equal to the eye length (EL). No values are given for *D. immaculatus*, but the values for *D. suweonensis* are $IND = 0.25 \pm 0.02$; $EAD = 0.59 \pm 0.03$ and $EL = 0.24 \pm 0.02$. The ratios representative of the finger bone lengths are consistent between the two species. However, the ratios were higher for all phalanges for all fingers in *D. suweonensis*, indicating longer phalanges compared to metacarpals in *D. suweonensis* than in *D. immaculatus* (Table 2).

Table 2. Osteological comparison for the metacarpal and phalange bones of *Dryophytes suweonensis* and *D. immaculatus*, based on data extracted from Dang et al. (2017) and Kim et al. (2017). The values in the table are the ratios calculated from the length of the phalanges divided by the length of the metacarpal of the same finger.

Finger	<i>D. suweonensis</i>			<i>D. immaculatus</i>		
	2	3	4	2	3	4
Phalange 1	0.67	0.73	0.62	0.62	0.71	0.54
Phalange 2	0.35	0.61	0.60	0.11	0.58	0.59
Phalange 3		0.32	0.37		0.28	0.31

DISCUSSION

The results of our phylogenetic analyses are congruent in the monophyly of the *Dryophytes suweonensis* clade. However, and despite highlighting the absence of haplotype overlap between *D. suweonensis* and *D. immaculatus*, the haplotype network clearly shows the

relatedness of the two clades, and the same genetic variation between and within clades. The phylogenetic analyses also highlight the low variation between the two clades, however, a generally similar level of variation is found between the closely related *Hyla molleri* & *H. orientalis* (Stöck et al., 2012) and there is an even lower variation between *H. intermedia* and *H. perrini*, a new cryptic species (Dufresnes et al., in review), previously called “clade N” by Canestrelli et al. (2007a, 2007b) and “new taxon 2” by Stöck et al. (2008, 2012). The pattern described here could very well be the one of a ring species, although more variable markers such as microsatellites, and additional sampling between the sites currently available, would be required to confirm such a claim. This pattern is consistent with the fact that landscape features usually are adequate predictors of genetic variations (reviewed by Storfer et al., 2007), such as seen here with the Yellow Sea creating a barrier between the two clades.

Despite being based on a single samples and averages for the two species, the osteological comparisons highlight differences between the two clades. While *D. suweonensis* is smaller than *D. immaculatus*, the bones of its hands are comparatively longer. This is likely related to variations in their breeding ecology: *D. suweonensis* calls while holding on leaf and vegetation, with the metacarpals and phalanges wrapped around the blade of the leaf (Borzée et al., 2016a). The breeding ecology of *D. immaculatus* has not been described, but based on these results, we expect differences between the two species. Li et al. (2015) and Dufresnes et al. (2016) recommended the synonymy of *D. suweonensis* (Kuramoto, 1980) and *D. immaculatus* (Boettger, 1888), under the name *D. immaculatus* as junior synonym. We, however, call for further analysis before this conclusion. Clearly, additional basic ecological and behavioural research on *D. immaculatus* is required before being able to answer the question, as even the range of the species is largely undescribed.

To better determine the status of species or subspecies of the two populations, we recommend a Total Evidence Analysis that would include call properties, morphometrics and genetic information, such as conducted for the phylogeny of *Dendropsophus elegans* (Forti et al., 2017). It has been suggested that intraspecific variation in call properties are not adequate to recover phylogenetic history due to selective factors related to sexual selection. However, inter-species phylogeny is accurately described by acoustic properties due to genetic isolation processes, in relation to species recognition and ecological pressure (Fonseca et al., 2008; Forti et al., 2017). Call variables are expected to be significantly different for species diverged for at least 6.5 million years (Forti et al., 2017). The alternation between periods of divergence and contact between *D. suweonensis* and *D. immaculatus* may have occurred repeatedly since the Triassic (Haq et al., 1987), with the repeated resurgence and decline of the Yellow Sea (Jingtai & Pinxian, 1980; Oba et al., 1991; Liu et al., 2009). Accordingly, the two clades may have been partially isolated, starting the process of speciation through isolation. Then, they were

brought back in contact during glacial maxima and the creation of the land-bridge over the Yellow Sea, before being isolated again. This is supported by the fact that the Korean Peninsula is known to have acted as a refugium for the ancestor of *Pelophylax* and *Onychodactylus* species (Zhang et al., 2008; Yoshikawa et al., 2008), and thus enabled the survival of clades that had previously colonised the area.

Alternatively, the two clades may be part of a ring-species, as individuals were not isolated in different refugia during LGM. This proposition is supported by the absence of ice sheets on the Korean peninsula circa 126,000 years ago (Kong, 2000; Walker et al., 2009; Yi & Kim, 2010), partially due to the presence of a shared monsoon regime because of the uplifting of the Himalayan range (An et al., 2001; Harris, 2006). This is also supported by the fact that the Korean peninsula was last connected to the Chinese mainland during the late Pleistocene (Millien-Parra & Jaeger, 1999). In this case, the clustering of individuals in a single clade implies the repeated gene flow between the Korean Peninsula and the Asian mainland. Besides, such clustering is supported by the shared paleo-river basin due to the southward flow of the Amur River, west of the Sakhalin Islands, due to the ice sheet blocking its current bed (Grosswald & Hughes, 2005). Furthermore, the paleo-Yangtze and paleo-Yellow rivers were closer than they are today and merged with the paleo-Han river (Ryu et al., 2008).

Within each of the Korean Peninsula and China, the north/south genetic structure was unexpected because of the continuous low plains allowing for easy dispersion of individuals, such as exemplified by *D. suweonensis* (Borzée et al., 2015b). This may indicate strong philopatry of *D. suweonensis*, leading to a larger genetic divergence than expected and population management for *Dryophytes* spp. needs to take into account the origin of individuals for introduction and translocations plans (Gascon, 2007). This is especially true if the individual *D. suweonensis* isolated on the phylogenetic tree (Fig. 3) are not monophyletic with the main *D. suweonensis* clade, due to hybridisation with *D. japonicus* (Borzée et al., 2015b).

We cannot confirm here the clade status of the two species, but we clearly highlight variations between the two clades. Thus, based on the genetic and osteological data available, and as the ranges of two species do not overlap and are consequently reproductively isolated, we recommend the preservation of the two clades at the species level until clear data becomes available. Considering the two species as the same entity would be a hastily made decision that would not be based on rigorous scientific knowledge. It would also decrease the amount of research conducted on these organisms as it would extend the range of the species, leading the Korean populations to a non-threatened level, and resulting in the loss of interest, and research fund, generally attributed to endangered species.

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Interviews on the status of West African forest tortoises (genus *Kinixys*), including preliminary data on the effect of snail gatherers on their trade

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The forest hingeback tortoises *Kinixys homeana* and *Kinixys erosa* are two of the most declining African chelonians. Although the population size trends of these species have received attention in some specific areas of West Africa, an overall perception of their declining trajectories are still largely unexplored. We used interviews with rural people (hunters, farmers and snail gatherers) in order to explore the general perception that these experienced people have on the population trends of these threatened tortoises. Overall, we interviewed over 2000 people in three West African countries (Côte d'Ivoire, Togo and Nigeria), which mostly supported the notion that these tortoises are heavily declining in Togo and Nigeria, but less so in Côte d'Ivoire. In addition, many respondents suggested that snail gatherers are the main providers of tortoises to the bushmeat trade. Indeed, our market surveys revealed that, in Nigeria, there was a significantly positive correlation between number of wild snails traded by individual sellers and numbers of sold tortoises in their 'shops'.

Key words: Chelonians, *Kinixys erosa*, *Kinixys homeana*, interviews, snail trade, Côte d'Ivoire, Togo, Nigeria

INTRODUCTION

Standardised or informal interviews with indigenous communities or with selected people (e.g., hunters, farmers, fishermen, etc.) from local villages have been widely employed by conservation biologists (Gadgil et al., 1993; Gros et al., 1996; Gros, 1998; Gilchrist et al., 2005; Pan et al., 2015) in order to obtain information on threatened species at the local scale. In this regard, these studies valued local ecological knowledge for scientific reasons (e.g., Begazo & Bodmer, 1998; Aiyadurai et al., 2010; Rist et al., 2010). Local ecological knowledge (LEK) can also be informative for detecting population trends of target species (e.g., Meijaard et al., 2011; Akani et al., 2013). Therefore, it is generally assumed that LEK can be usefully integrated with complementary scientific knowledge to improve species management.

In this paper, we used interviews with a large number of rural people for uncovering the population trends of threatened species from a logistically difficult region of the world. Target species of our study are two forest tortoises (genus *Kinixys*: Testudinidae), *Kinixys homeana* and *Kinixys erosa*. Hinged tortoises (genus *Kinixys*) are endemic to tropical Africa, and are characterised by

having hinged carapace (Branch, 2007). *Kinixys homeana* and *K. erosa* habitat is typically tropical forests (Branch, 2007; Luiselli & Diagne, 2013, 2014). Indeed, both *K. homeana* and *K. erosa* are known to inhabit exclusively remnant mature moist forests in the region (Luiselli & Diagne, 2013, 2014). Heavy over-exploitation of the forest, habitat loss and massive harvesting for domestic consumption in West Africa have caused a considerable decrease in populations of the two species in the last two decades (Luiselli, 2003a, 2003b; Luiselli et al., 2006). Another major problem that impacts on these species is that they are also heavily exploited through opportunistic capture by snail-gatherers (Akani et al., 2015b), and also a target of the international pet trade (Auliya et al., 2016). Due to all these factors, in the IUCN/SSC TFTSG Red List Workshop for African chelonians (held in Lomé, August 2013), *K. homeana* was assessed as Critically Endangered (CR) and *K. erosa* as Endangered (EN), and there was a wide agreement among experts that both species are seriously threatened (for example, Luiselli and Diagne, 2013, 2014). However, these red list status updates have not yet been published in the IUCN (2018) Red List web site.

Whereas studies on the occurrence of *Kinixys* tortoises

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in bushmeat markets have already been carried out (e.g., Luiselli, 2003a; Luiselli & Diagne, 2013, 2014), there are no studies exploring the effects of snail gathering on the tortoise trade in the West African regions. Snail gathering is a substantial economic activity in many areas of West Africa (e.g., see Adaigho & Nwadiolu, 2016; Amani et al., 2016), and thus its effects on tortoise trade may be considerable despite being neglected so far.

One of the prerequisites for LEK surveys is that the species is distinct from any other species in the same landscape and can be distinguished easily by the interviewees from whom LEK is being gathered (e.g., McKelvey et al., 2008; Newing, 2010; Turvey et al., 2015). In our case, the interviewees have distinct vernacular names for the two species in comparison with other chelonian species, but some people consider the two taxa (that are indeed similar from the morphological point of view and, anyway, similarly threatened) as a single species. Since the two species show a nearly identical distribution in West Africa (Luiselli & Diagne, 2013, 2014), are almost invariably sympatric and syntopic (Luiselli et al., 2008), and both are declining and of high conservation concern (Luiselli & Diagne, 2013, 2014; Turtle Taxonomy Working Group, 2017), any eventual decline/increase of population sizes detected by local people would indicate a shared pattern between the two species.

Our aims in this paper are twofold:

(1) Understanding whether people from forest areas of three West African countries (Côte d'Ivoire, Togo, and Nigeria) consider that *Kinixys* populations are stable, increasing or declining in the surroundings of their settlements;

(2) providing preliminary data on a possible novel threat for forest tortoises, i.e. the opportunistic hunting made by snail gatherers for the bushmeat markets of southern Nigeria.

MATERIALS AND METHODS

Study areas

The field study was conducted in south-western Côte d'Ivoire (Bas-Sassandra region), not far from the political border with Liberia, south-western Togo (Missahohe forest area, surroundings of Kpalimé and Badou) and southern Nigeria (Niger Delta region, Bayelsa and Rivers State) (Fig. 1). The general habitat of these regions is similar, being characterised by forest-plantation mosaics, with remnant forest patches that become more isolated year-by-year (e.g., Akani et al., 2014; Luiselli et al., 2015), thus severely fragmenting the connectivity of the tortoise populations in this part of Africa (e.g., Segniagbeto et al., 2015).

Protocol

Local markets and small human settlements sparsely spread in the forest zones of Côte d'Ivoire, Togo, and Nigeria were regularly visited in order to gather data on the bushmeat trade and its correlates with human societies between February 2013 and December 2017 (e.g., see Akani et al., 2015a; Luiselli et al., 2017a, 2017b). Overall, 51 settlements were visited during this study.

All these settlements were located inside the known distribution range of the two *Kinixys* species (Luiselli & Diagne, 2013, 2014), and were widespread across most of the distribution range of the two target species in Nigeria and Togo (whereas for Côte d'Ivoire we surveyed a much smaller portion of the tortoises' ranges). We conducted face-to-face semi-formal semi-structured interviews (Newing, 2010) with only hunters, farmers, snail gatherers, and bushmeat market sellers that may have had direct experience with tortoises and are therefore considered as 'experts' (Davis & Wagner, 2003) (Table 1). Thus, people not directly involved in activities within forest patches were not considered in our analysed data. Hunters and farmers were pooled in our statistics because the same people often conducted both the activities. During interviews, we also obtained profiles of the respondents' age, profession, education, and gender. Our sampled interviewees were dominated by men aged 21-35 years because this was the category mostly conducting activities in bush/forest and thus the most experienced with tortoises. In particular, we focused our surveys and our interviews in sites of small human settlements situated inside, or in the immediate surroundings of, forest patches (Fig. 1), in order to maximise the chances to meet with people already experienced with these chelonians. Coauthors and assistants fluent in the local language undertook the surveys. No minors were interviewed (youngest was 21 years of age), and all interviewees were informed of the aims of the project and their consent was obtained before proceeding. Their identity was kept anonymous in order to assure full privacy to the interviewees and to minimise the risk of obtaining false answers. All interviews were conducted in the local language. Overall, our interviews followed the ethical guidelines developed by the British Sociological Association. In all cases, the discussion with the interviewees was not pre-arranged, but was performed opportunistically when appropriate people were met during our surveys. In all cases, we noted the response after we conducted the interview with the respondent. We conducted single interviews for the various tortoise species.

In the interviews, we asked each interviewee, although not using a formal questionnaire, the following questions:

- 1) Are terrestrial tortoises present near your village?
- 2) If present, do you consider the tortoises to be plentiful or rare?
- 3) Has the population increased, decreased, or stayed the same over the last ten years?

Together, the three answers would indicate stable, increasing or declining populations according to the perception of the respondent.

In question (1), the possible answers were 'yes' or 'no'. Concerning question (2), the possible answers were 'plentiful' or 'rare'. The scope of this question was not to understand the interviewees' perception on whether the tortoise populations are declining (that is covered by question (3)) but to get a non-quantified indication on whether the apparent population status is still good at the local level (answer: plentiful) or whether these reptiles are apparently uncommon (answer: rare). Obviously, 'plentiful' or 'rare' is subjective. So, we asked

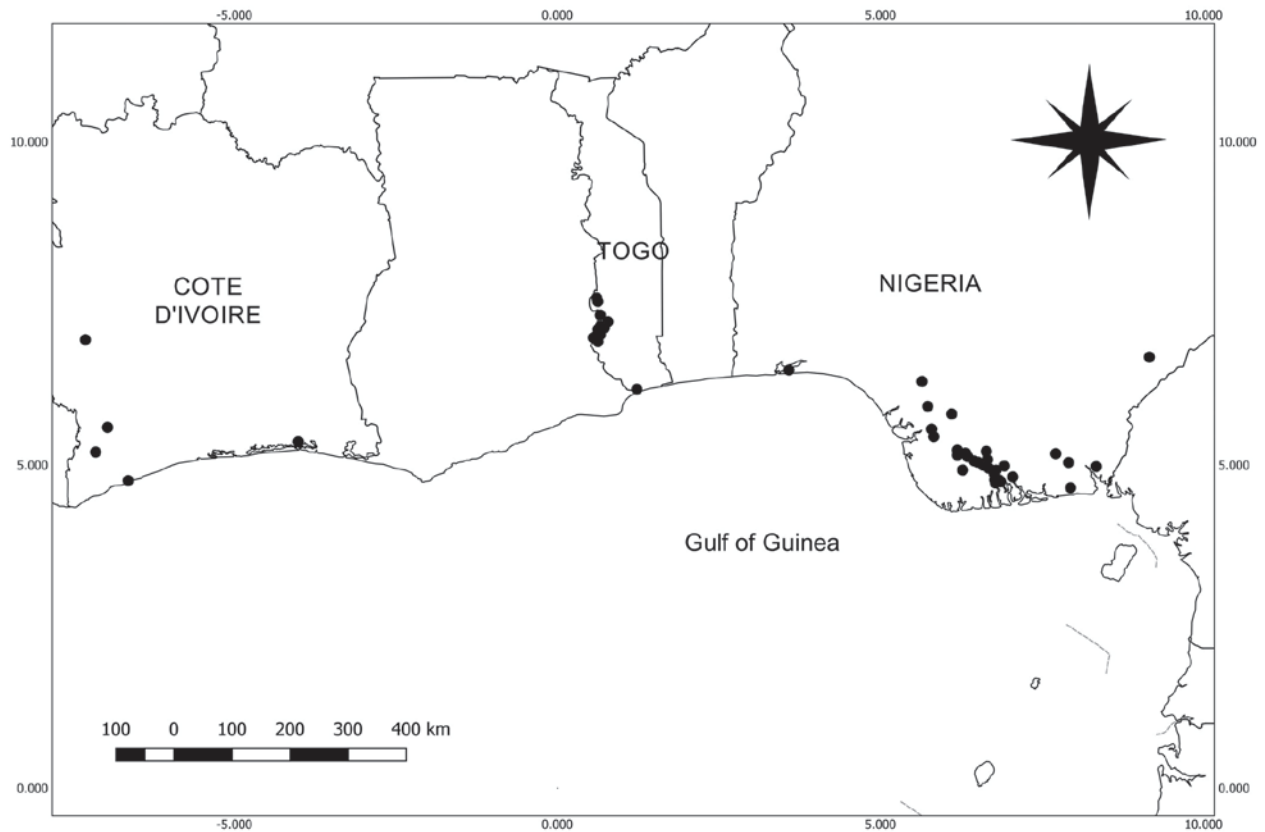


Figure 1. Map of the study area showing the sites where interviews were performed

Table 1. Distribution of the various categories of interviewees by country, by sex and by age class. Numbers would indicate the numbers of interviewed people.

	Nigeria	Togo	Côte d'Ivoire
Number of settlements	31	13	7
Number of people	1033	703	341
Men	774	562	240
Women	259	141	101
21-35 ys old	781	511	233
36-50 ys old	153	107	66
>50 ys old	99	85	42
Hunters/farmers	911	543	256
snail gatherers	35	131	65
Bushmeat market sellers	87	29	20

the respondents to grossly quantify their 'estimates': we defined 'plentiful' when the respondent said that he/she encountered tortoises at least once every month, and 'rare' when the encounter rate was less than that. In almost all cases, the interviewees did provide answers that were easy to classify within one of the two categories, as those claiming that tortoises are 'plentiful' stated that tortoises can be seen on almost a daily basis, whereas those claiming that they are 'rare' said that tortoises can be observed much less than once every month (often, 3-4 individuals per year). When respondents were uncertain between 'plentiful' and 'rare' ($n = 41$ cases),

we deleted their answers from our analyses. Note that in many cases, the respondents stated that freshwater species (*Pelomedusa subrufa*, *Pelusios* spp.) were much more common than forest tortoises in the forest patches surrounding their villages.

During the early years of our field research in West Africa (years 1996-2003), we noticed in > 30 instances that several tortoises for sale in local markets were carried there by snail gatherers (*Achatina* and *Arcachatina* sp.) (Luiselli & Diagne, 2013, 2014; Akani et al., 2015b). In the years 2016-2018, we then decided to explore quantitatively, albeit in a preliminary way, this potential threat for tortoise survival in West Africa. In order to explore this issue, we acknowledge that the best protocol should have been to select a few specific markets in the country and select shops at random to be systematically surveyed and identify whether they sold tortoises. However, this approach was not possible due to logistic constraints, as the snail sellers are unhappy to be visited and disturbed on a very regular basis for a time-consuming operation such as counting their snails. In this regard, snail sellers are usually more disturbed by scientists than how bushmeat sellers are because counting the traded snails requires more time and is more invasive towards the routine selling activities than just examining the fewer bushmeat carcasses that are sold.

Therefore, we decided to focus only on the 'shops' selling tortoises in the explored markets. So, when in a given survey day and in a given shop, we found *Kinixys* specimens for sale, we contextually recorded whether

also snails were traded and, if so, we counted the number of snails that were there for sale. Thus, in each shop, we counted in the same day both the number of tortoises available for sale and the number of traded snails. Our assumption was that, if tortoises are collected especially by snail gatherers, it would have been uncovered a positive relationship between number of traded tortoises and number of traded snails per shop. Indeed, following this rationale, the number of traded snails per shop would be a proxy of the search intensity made by the seller/provider of the seller for gathering snails in the field. These data were collected very early in the morning (0700 – 0800 hr) in order to avoid that our counts were affected by snails and tortoises sold out. Unfortunately, the sample size of cases was small and referred only to the Niger Delta (Nigeria). In many cases, in fact, snails are provided by snail farms (that represent a lucrative novel business in the region; e.g. see <https://www.vconnect.com/nigeria/list-of-snail-farming_c96>, last accessed on 08 January 2018), and so it was not possible to indiscriminately count all the snails exposed for sale in the markets. Thus, we firstly identified 'shops' with tortoises for sale, then we asked whether there were also snails and, if so, whether they were farmed or collected from the wild, and if the latter, we counted also the snails for contrasting their number with that of the traded tortoises.

Statistical analyses

Frequencies of the different types of answer were compared by observed-versus-expected χ^2 test. Variables were tested for normality by Shapiro-Wilk W test, and when non-normal ($P < 0.05$), they were log-transformed to achieve normality. After this, parametric tests were used. The relationship between number of traded snails and number of traded tortoises in each 'shop' was analyzed by Pearson's correlation coefficient. All tests were performed by the software PAST version 3.0, with alpha set at 5%.

RESULTS

Population trends as perceived by local people

Question 1: are terrestrial tortoises present or not in the surroundings of your village?

Overall, 2,077 independent interviews were performed, including of 341 in Côte d'Ivoire, 703 in Togo and 1033 in Nigeria. Despite 37.2% of Ivorian, 16.7% of Togolese and 20.4% of Nigerian respondents not knowing whether tortoises are found around their settlements, the majority of respondents considered hingeback tortoises to be present (62.8% in Côte d'Ivoire, 83.9% in Togo, and 79.6% in Nigeria). These inter-country differences were statistically significant ($\chi^2 = 16.36$, $df = 2$, $P < 0.001$), with the percentage of 'yes' respondents in Côte d'Ivoire being lower than in the two other countries.

Question 2: do you consider the tortoises to be plentiful or rare in the surroundings of your village?

After considering only the respondents that confirmed the presence of tortoises in the surroundings of their settlements ($n = 214$ in Côte d'Ivoire, 590 in Togo, and 822 in Nigeria), most of them considered the tortoises

to be rare: 66.8% in Côte d'Ivoire, 81.0% in Togo, and 86.0% in Nigeria. The differences among countries were statistically significant ($\chi^2 = 27.64$, $df = 2$, $P < 0.0001$), with responses 'rare' being more dominant in Togo and Nigeria than in Côte d'Ivoire.

Question 3: Do you think that tortoises are as plentiful as before, more plentiful than before or less plentiful than before?

Most of the interviewees considered the sighting frequency of these tortoises to be decreasing, with fewer people considering them to be stable and almost nobody thinking that these tortoises are nowadays more easily encountered than before ($\chi^2 = 105.39$, $df = 3$, $P < 0.0001$) (Fig. 2). In this regard, however, it should be pointed out that many interviewees had no precise opinion (Fig. 2).

The differences in the frequency of answers by country were statistically significant ($\chi^2 = 199.68$, $df = 6$, $P < 0.0001$), with a comparatively lower frequency of respondents suggesting a declining trend in the Côte d'Ivoire than in the other two countries.

Effects of snail gatherers on tortoise trends

Interestingly, in the market 'shops' of Nigeria in which we counted snails for sale, we found a significant positive correlation with the number of traded tortoises (both *Kinixys* species pooled; $r^2 = 0.746$; $P < 0.000$; Fig. 3).

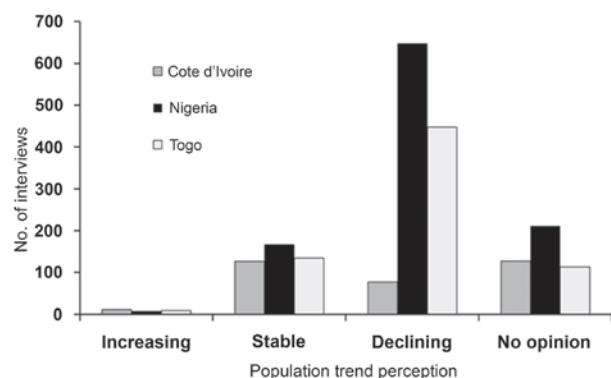


Figure 2. Distribution of the answers by interviewees concerning the temporal trend in the frequency of sightings of the hingeback tortoises in the surroundings of their village, during the last 10 years, in three West African countries.

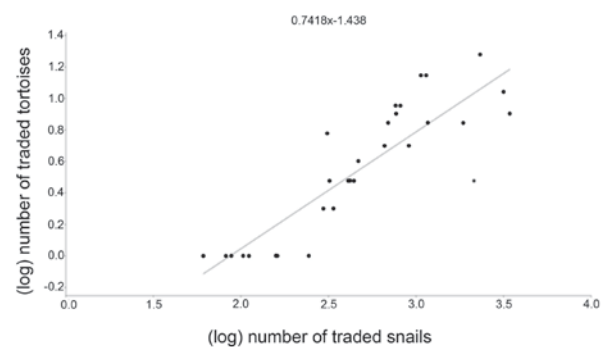


Figure 3. Relationship between the number of traded snails and that of traded tortoises in the surveyed bushmeat markets, years 2016-2018. Variables were log-transformed to achieve normality. For the statistical details, see the text.

DISCUSSION

Methodological shortcoming

Another threatened tortoise species (the Vulnerable *Kinixys nogueyi*) may occasionally occur sympatric or contiguously parapatric with the two target species (Ségniagbeto et al., 2015), and can possibly be confused with the two targets from some interviewees. This would introduce some potential biases to our respondents' answers, and the resulting precision may be lowered (O'Donnell et al., 2010). However, this latter species has different morphology and habitat usage (being a Guinea savannah species; Segniagbeto et al., 2015). This latter species is more habitat generalist than the two target species, and may be also sympatric or contiguously parapatric with them (Ségniagbeto et al., 2015). Therefore, it cannot be ruled out the possibility that the true abundance of the two target species is even less than what appeared from the performed interviews.

Population trends as perceived by local people

Although the answers made by interviewees cannot be considered as a straightforward evidence of any population size trend (Anadón et al., 2009), it is noteworthy that the perception of the rural people was that: (i) *Kinixys* tortoises still inhabit the forested surroundings of several villages in all the three countries; (ii) there was a general agreement that the tortoises are uncommon in most areas, and (iii) the tortoises were declining, with a comparatively lower number of respondents considering these chelonians to be declining in Côte d'Ivoire than in the other two countries.

Consistently among countries, very few interviewees stated that *Kinixys* tortoises are more easily found nowadays than in the past (3.2% in Côte d'Ivoire, 0.7% in Nigeria, and 1.3% in Togo). This fact is unexpected given that several hunters and collectors usually tend to minimise declining patterns of their target animals and human perception of rarity is skewed and scale-dependent (Hall et al., 2008). Thus, this fact would really indicate an overall declining pattern for these species. Obviously, since our interviews could not discriminate between *K. homeana* and *K. erosa*, the declining trend should be attributed to both species, as also indicated by field ecological studies in some sectors of their range (Luiselli et al., 2008; Luiselli & Diagne, 2013, 2014).

The comparison with Togo and Nigeria is very interesting: in these two latter countries (where indeed a heavy decline of the population sizes of the two target species has been observed by detailed field studies; e.g. see Luiselli et al., 2016), the great majority of the respondents considered the species in decline, whereas the same was not true in Côte d'Ivoire. Considering that (i) some field studies showed a heavy decline of *K. erosa* and *K. homeana* in Nigerian sites where these species are hunted for subsistence (Luiselli, 2003b), and that (ii) there is a consensus among reptile traders operating in Togo, Ghana, and Benin that the abundance of *K. erosa* and *K. homeana* has declined over the years (Auliya et al., 2016), the hypothesis that they are less threatened in Côte d'Ivoire than elsewhere should be considered for

further studies, in order also to enhance the conservation perspectives of these species in West Africa. Up to now, there are still few geo-referenced field records for the two target species in the Côte d'Ivoire (Iverson, 1992; Maran, 2004), but it is certain that several additional sites can be easily discovered by intensifying field efforts. Indeed, four new presence sites for *K. homeana* and five for *K. erosa* have been discovered during recent field surveys in the southern regions of the country (Luiselli et al., 2018, unpublished report to Turtle Conservation Fund). We do not know why Ivorian populations of these tortoises should be less threatened than Togolese and Nigerian populations. It seems unlikely that deforestation rates/habitat alteration at a large scale may have any effect in explaining this pattern, as deforestation rate during the period 2001-2015 has been much greater in Côte d'Ivoire (5.81%) than in Nigeria (0.50%) and Togo (0.58%) (data source: www.globalforestwatch.org; last accessed on 12 March 2018). Also, snail gatherers are common all throughout the West African countries (e.g. Sodjinou et al., 2002; Adaigho & Nwadiolu, 2016; Amani et al., 2016), and thus it is likely that small-scale factors (i.e. overhunting and excessive snail gathering at the village scales) may be responsible for the observed patterns.

Effects of snail gatherers on tortoise trends

Despite its logistical constraints (see methodology), our study demonstrated a clear positive relationship between number of traded snails (when these came from the wild and not from farms) and number of traded tortoises. In our opinion, this is the first clear demonstration that *Kinixys* tortoises are often located while people search for snails (as also suggested by Luiselli & Diagne, 2013, 2014; Akani et al., 2015), and that, therefore, snail searchers (generally men and farmers; Amani et al., 2016) may be at least as dangerous, if not even more dangerous, than normal hunters for the continued survival of these chelonians. Indeed, (i) people hunting for larger animals may give low priority in collecting small animals like tortoises when they are occupied with time-consuming searches of much larger and economically more attractive wildlife; and (ii) snail gatherers may collect high numbers of tortoises, especially by wet season and including also the hidden and inactive specimens, because they scout exactly the same microhabitats that are preferred by tortoises (snails and tortoises share the same microhabitat preferences; Akani, Luiselli & Eniang, unpublished observations). Small scale opportunistic catches of threatened species due to similar habitat shared by a common species with commercial interest were already observed in aquatic ecosystems, for instance for fish (e.g., Palmeira et al., 2013), sea turtles and cetaceans (e.g., Bard et al., 2002; Panagopoulou et al., 2017), penguins and seabirds (Anderson et al., 2011), but not in terrestrial tropical ecosystems.

While the hypothesis that snail collectors are driving tortoise harvesting is, in our opinion, reasonable, we should highlight that we just presented here a correlation between the number of snails and tortoises, which does not constitute proof without a more detailed examination of the strategy and economics behind snail and tortoise

collecting. Unfortunately, the data available on the economics behind snail and tortoise collecting are too few to stress any final considerations in this issue. Based on the evidence presented in this paper, we plan to investigate more in detail the relationships between snail searching and tortoise catches not only in Nigeria, but also in other West African countries, in the years to come.

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