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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; Lorioux 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 (Duguy, 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 ± 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±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;

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

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±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

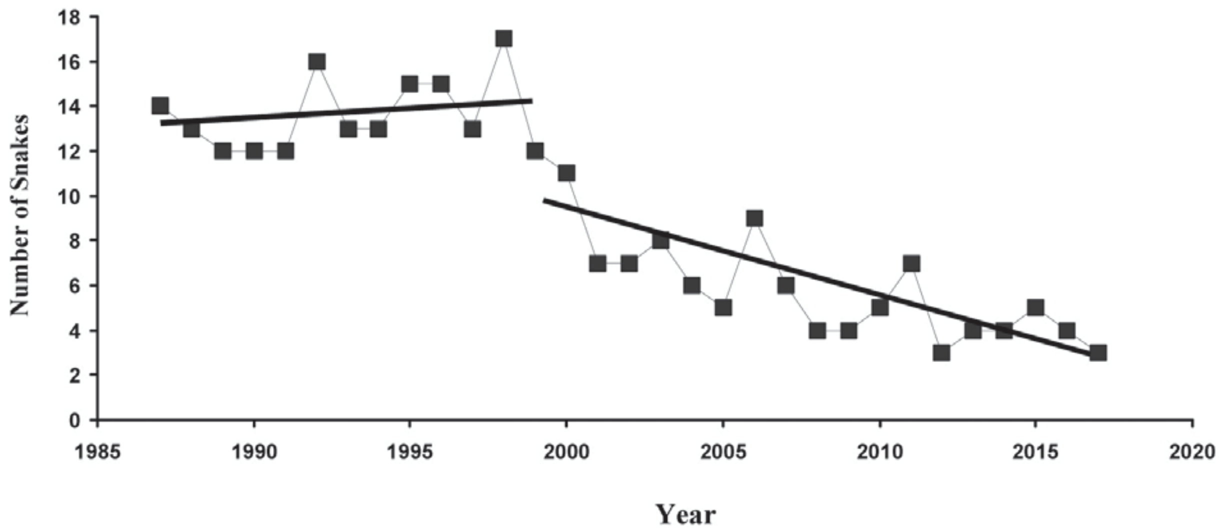

Figure 1. Relationships between year and number of vipers at the hibernaculum at Oriolo Romano, central Italy. The lines running through the data are derived from regression analysis of long term tends of numbers of *V. aspis*. See text for statistical details.

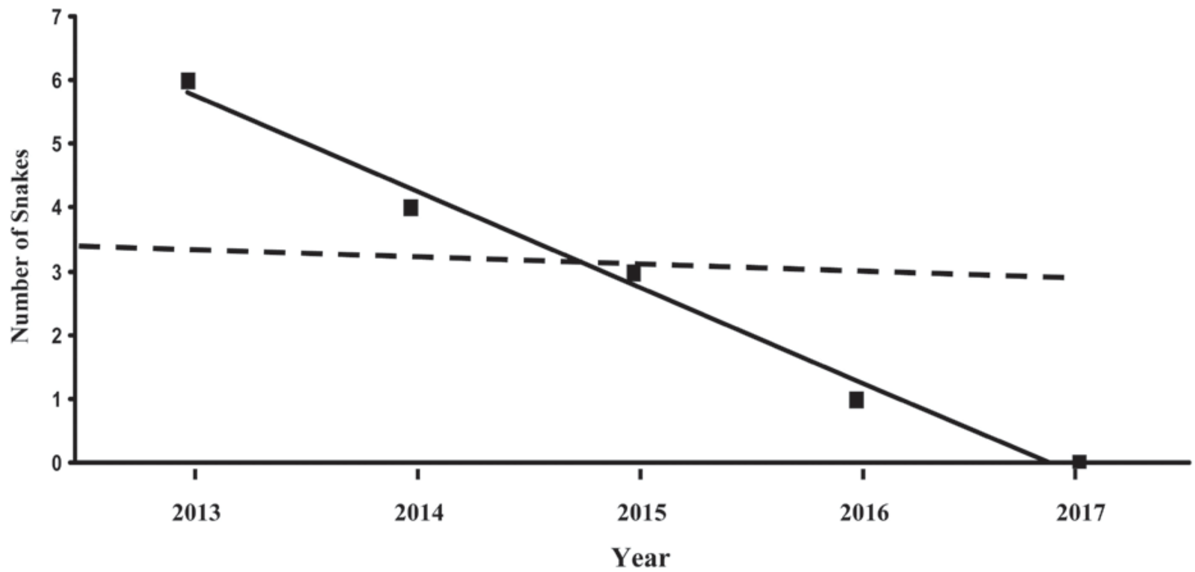

Figure 2. Relationships between year and number of vipers at the hibernaculum in Vendée, western France. The continuous line running through the data is based on regression analysis of *V. aspis* den occupancy from 2013–2017. The broken line represents long term trends of road mortalities of *V. aspis*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