

# Growth, longevity and age at maturity in the European whip snakes, *Hierophis viridiflavus* and *H. carbonarius*

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**Abstract.** Age and size at maturity are major life history traits, because they influence lifetime fecundity. They represent the outcome from complex interactions among environmental pressures (abiotic and biotic) and individual characteristics. They are also difficult to measure in natural populations and thus they are rarely appraised, especially in reptiles due to the elusive nature of juveniles. Using skeletochronology to circumvent these difficulties, this study aims to compare age structures, longevity, age-size relationships, growth curves, age and size at maturation and potential reproductive lifespan in three populations of the European whip snake (two *Hierophis viridiflavus*, one *H. carbonarius*). We measured the body size and counted the skeletal growth marks on 132 specimens, accidentally killed or from museum collections (72 from NW France [Chizé]; 28 from Tuscan Archipelago [Montecristo], Italy; 32 from S Italy [Calimera]). General patterns of age at maturity and longevity were consistent with previous studies based on recapture investigations. Strong differences among populations suggest local adaptation to contrasted environmental conditions. These results suggest that skeletochronology is a useful technique that can be applied opportunistically in snakes (e.g., using road-kills) in order to collect otherwise unavailable data that are essential to address fundamental questions regarding longevity, life-history traits and to perform population viability analyses.

**Keywords.** Insularity, snakes, *Hierophis*, sexual maturity, skeletochronology.

## INTRODUCTION

Iteroparous species with indeterminate growth (e.g., many fish, squamate reptiles) exhibit strong variations in most life history traits in response to environmental factors; body size is highly variable among and within populations for example (Wimberger, 1992; Madsen and Shine, 1993; Ryser, 1996; Rohr, 1997; Zuffi et al., 2007; Warner, 2014). Considering versatile life history traits, age at maturity is pivotal because fitness is particularly sensitive to changes in this trait (Stearns, 1992). Similarly, body size exerts strong effects on fecundity, survival, and thus on fitness (Shine, 1988, 1990). Age and body size at maturity are inextricably linked because

they both depend on juvenile growth rate which in turns depends on the trophic and climatic conditions experienced by individuals (Sinervo and Adolph, 1994; Webb et al., 2003). Fast juvenile growth promotes early maturation, large body size, and thus likely increases individual potential reproductive lifespan (Ryser, 1996; Day and Taylor, 1997; Bronikowski and Arnold, 1999). However, many examples suggest that there may be costs associated with fast growth; strong metabolic increase or reduced longevity in early-breeders for instance (Beaupre and Zaidan III, 2001; Blouin-Demers et al., 2002).

Sexual maturity entails a shift in the allocation of resources used to sustain growth during juvenile phase to reproduction (and secondarily to growth in many

reptiles) during adulthood (Shine and Charnov, 1992; Day and Taylor, 1997; Rohr, 1997; Wapstra et al., 2001; Stanford and King, 2004). There is a marked decrease of growth at maturity, although reduced growth may persist through life in many species with marked differences among individuals (Congdon et al., 2003). As a result, marked intra-population differences of body size set at maturity are usually maintained in individual later age-classes (Madsen and Shine, 1993; Shine, 1994; Zuffi et al., 2011). Individuals that mature at a small size remain small for the rest of their life; thereby counter balancing the fecundity advantages of early maturation (Halliday and Verrell, 1988). Overall, it is expected that the physiological processes that determine maturity, and thus the average age and body size of adults, should maximize lifetime reproductive success through differential adjustments in response to environmental conditions (Stearns and Crandall, 1981; Stearns and Koella, 1986; Bernardo, 1993; Ford and Seigel, 1994; Wapstra et al., 2001).

Studies that compare populations of the same species throughout different parts of its distribution range are valuable to understand how environmental factors shape age and size at maturity along with related life-history attributes (e.g., age-size relationship, longevity, population age structure; Mateo and Castanet, 1994; Nobili and Accordi, 1997; Lima et al., 2000; Miaud and Guillaume, 2005). Organisms that display important intra- and inter-population variations in body size are of particular interest because these variations may correlate with growth rate and age at maturity (Alcobendas and Castanet, 2000; Miaud et al., 2001; Kutrup et al., 2005). Unfortunately the relationships between growth rate, body size and age are not easily assessed in the field. Indeed, long-term mark-recapture surveys must be set up to collect the raw data necessary to calculate growth rate and to examine key life history traits. But in most reptile species juveniles escape observations (Pike et al., 2008; Bjørndal et al., 2013). Growth rate has been rarely measured accurately before sexual maturity (Bonnet et al., 2011) and the exact age of monitored individuals is seldom known in the field (Lagarde et al., 2001). This lack of information poses major difficulties to perform analyses. For example, for a given species a single body size for maturity is usually extracted from the literature, used to assign individuals into age categories, and then used to perform various analyses (e.g., regarding population viability, demography). Possible variations caused by inter-individual, geographic, and time heterogeneity are systematically neglected. These limitations apply with force in snakes due both to the extremely elusive nature of immature individuals (that precludes estimating accurately the age of individuals) and to the marked pheno-

typic plasticity of these organisms (Madsen and Shine, 1993; Bronikowski, 2000).

Skeletochronology offers a reliable alternative (Halliday and Verrell, 1988). This approach is based on counting the skeletal growth marks (SGM) that are successively deposited on the growing bone (Castanet et al., 1992). Although this method does not require long term population monitoring, major prerequisites are nonetheless important. Continuous growth and a lack of bone remodelling are essential characteristics. The relationships between age and SGM count is accurate in species where an active season precisely alternates with a period of inactivity (e.g., hibernation); it has been validated through mark-recapture in several reptiles (Lagarde et al., 2001). Many snakes from temperate climates fulfil the above conditions, are spread across a wide range of habitats, and thus they represent suitable candidates to examine the influence of environmental factors on the relationships between age and body size.

The aim of this study was to compare the mean age, mean longevity, the age-size relationship, growth curves, age and size at maturation of individuals sampled in three populations of two closely related species of whip snakes (two populations *Hierophis viridiflavus* and in one population *H. carbonarius*; Mezzasalma et al., 2015). The three populations are situated in distant parts of the distribution range of the species characterised by contrasted environmental conditions (one forested site in temperate climate zone and two sites in the Mediterranean climate zone; one continental and one insular). We thus expected marked differences among populations in the traits observed.

## MATERIALS AND METHODS

### *Study species and study sites*

The taxonomy of the European whip snake (*Hierophis* [*Coluber*] *viridiflavus*) has been recently revised (Rato et al. 2009; Mezzasalma et al., 2015). Depending upon the study and criteria, it has been suggested to differentiate several subspecies or species. The debate is not closed because the taxonomic boundaries between species and subspecies are often tenuous. We considered that we sampled three populations and two species, *H. viridiflavus* and *H. carbonarius*, of the European whip snake; but we emphasize that we could not rule out the possibility that one species and two subspecies were sampled. Thus, for conciseness (and cautiousness) we refer to three populations of the 'European whip snake' hereafter.

The three populations studied are widely spread across the distribution range of the species: (1) Forest of Chizé, France (46°07'N, 00°25'W). The site is close to the northern limit of the distribution range of *H. viridiflavus*; (2) Montecristo Island, Tuscan Archipelago, Central Italy (42°19'54"N, 10°18'38"E) is

situated more than 900 km south-easterly. An isolated population of *H. viridiflavus* inhabits this rocky island; and (3) surroundings of Calimera, Lecce (Apulia, Southern Italy; 40°15'N, 18°16'E). This third site located more than 700km further south-easterly is in the southern distribution range of *H. carbonarius*. More than 1600 km separate the first and third populations whereas climatic and general environmental conditions strongly contrast among all populations. Climatological data were obtained from the three meteorological stations closer to the respective study sites: Niort (30 Km from the Forest of Chizé), Lecce (15 km from Calimera) and Gorgona island (65 km from Montecristo island; Elba island is closer to Montecristo, but Gorgona resembles much more Montecristo island in overall surface, vegetation and climatic conditions). While it was possible to obtain data for Lecce only for the period between 1961 and 1990, data for France and Montecristo are referred to the same time span, between 1998 and 2006. Average monthly temperatures (°C) and average monthly rainfalls (mm) for the over cited time intervals are reported in Fig. 1.

#### Sampling snakes

Our samples were based on individuals accidentally killed (e.g., road-kills) and museum specimens and no snake was intentionally killed for this study. Used animals derived from a quite short time range (less than 10 years), in order to avoid any bias and/or constraint due to a too large time gap that could prevent a proper analysis of age estimation. A total of 132 specimens were analysed during four years: 72 whip snakes from the Forest of Chizé (mainly road kills; Bonnet et al. 1999a), 28 from Montecristo Island (museum specimens) and 32 from Calimera (museum specimens). The specimens were all alcohol preserved and came from the herpetological collections of Florence (Museo Zoologico "La Specola", Università di Firenze, Italy), Pisa (Museo di Storia Naturale, Università di Pisa, Italy), Frankfurt (Senckenberg Museum Frankfurt a.M., Germany), and Chizé lab. Preliminary analyses showed no effect of sampling date or duration of stay in alcohol before examination (i.e. alcohol did not destroy SGM, at least over several years).

Each snake was sexed (except newborns and juveniles), and measured (SVL, Snout-to-Vent Length) to the nearest mm by stretching it along a measuring tape. Snakes were considered adult on the basis of external body coloration and using minimal SVL for maturity in each population (Fornasiero et al., 2007). Very small snakes with typical neonate colouration were considered as newborns; larger immature snakes were considered as juveniles.

#### Skeletochronological analysis

SGM (skeletal growth marks) reveal temporary variations in osteogenesis rate (Castanet et al., 1992). Three categories of SGM can be recognised: opaque layers (zones), translucent layers (*annuli*) and lines of arrested growth (LAG) (Castanet et al., 1993). Zones correspond to fast osteogenesis phases made of badly spatially structured bone matrix, rich in randomly

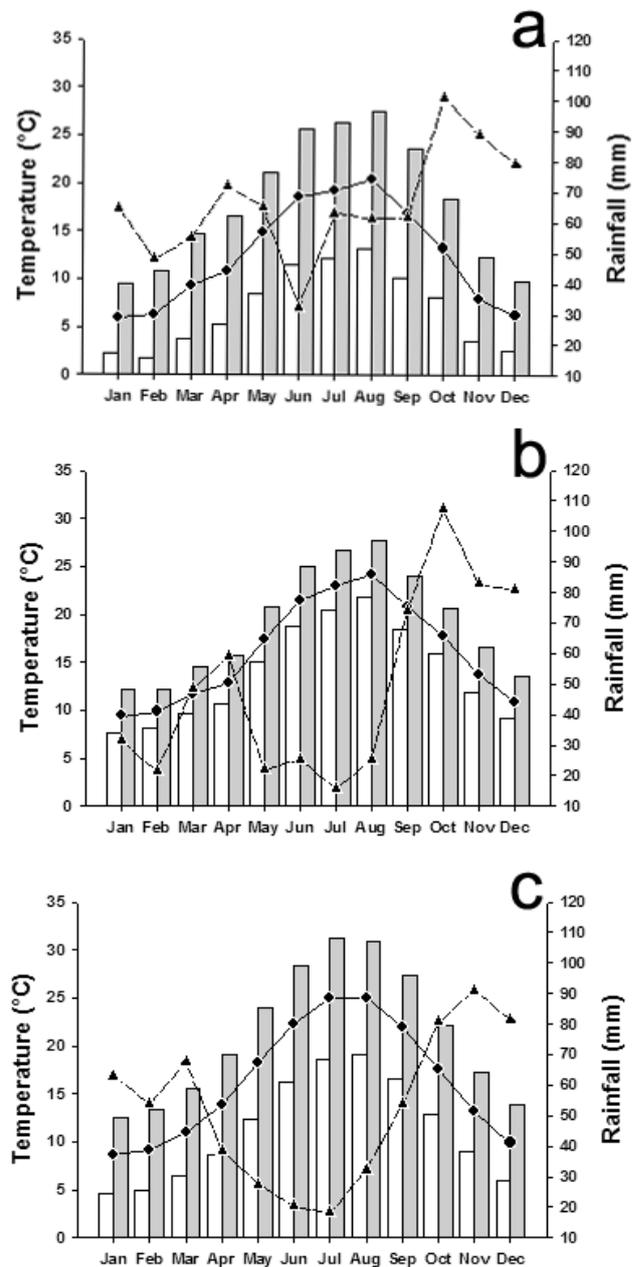


Fig. 1. Climatic features of the study sites. a) Chizé b) Montecristo c) Calimera. (□ Average min. Temperature); (■ Average Max. Temperature); (● Average monthly Temperature); (▶ Average monthly Rainfall).

distributed osteocytic lacunae. Due to their 3D structure, zones are more opaque than other marks and appear therefore dark when observed under transmitted light (Castanet et al., 1993). *Annuli* alternate with zones and correspond to periods of slow osteogenesis. Bone matrix is well structured (often being made of lamellar bone) and usually poor in osteocytes. When observed under transmitted light, *annuli* appear narrower and more translucent than adjacent zones (Castanet

et al., 1993). LAGs correspond to a temporary arrest of local osteogenesis. They are narrow, not always visible, typically bordering *annuli* or, sometimes, appearing inside them (Castanet et al., 1993). Skeletochronology is based on the assumption that growth marks are the histological expression of temporary and periodical variation in bone growth rate. SGMs may originate from endogenous rhythms, but they are influenced and synchronised by external seasonality, like the alternation of hibernation and active season in snakes from temperate climates (Castanet and Naulleau, 1974; Castanet et al., 1993). Until sexual maturity, when growth rate is high, *annuli* or LAGs are well separated by wide zones of fast-growing tissues, while the subsequent SGM appear narrower and more irregular. This pattern has been named “*rapprochement*” by Francillon-Vieillot et al. (1990).

Although caudal vertebrae can be used for age estimation of living specimens (Waye and Gregory, 1998), two flat skull bones, the supra-angular and the ectopterygoid especially, are preferred in dead individuals (Hailey and Davies, 1987). In road kills, pairs of ectopterygoids and supra-angulans (four bones) were removed. In museum specimens, the bones were removed from one side of the head only in order to preserve the external morphology and scalation of the head. The bones were stored in fresh water until organic tissues (e.g., ligaments) became soft and carefully cleaned.

#### Counting SGM

SGM counting followed the procedure reported by Castanet et al. (1993): bones were observed in toto with a binocular microscope, under transmitted light. During the reading of SGM, the bone was kept under water, in order to enhance the contrast between different growth marks. Counting was performed by two different people (or by the same observer on separate occasions), always blind to sample identity. Parallel readings on the same sample were compared, SGM were counted again in case of discrepancy. If the problem persisted, the sample was discarded. In case of divergence between ectopterygoid and supra-angular counting, ectopterygoid counting was retained because it provides more reliable results (Hailey and Davies, 1987). Sample where strong bone remodelling occurred, obviating age estimation, were discarded. Several specious countings were not retained in the analyses.

#### Measure of “*rapprochement*”

Ectopterygoids of adults presenting a clear sequence of SGM were photographed using a LEICA DC300F camera assembled with a WILD HEERBRUGG MAKROSKOP M 420 1.25x. Using an image-editing computer software, the distance from the basis of the first visible zone (corresponding to the first active season bone growth) and the basis of next zone was measured along a straight line broadly perpendicular to SGM and transecting LAGs (Fig. 2).

The thickness of each zone + *annulus* complex represents one year of bone growth (G) expressed as follow:

$$G_{n+1} = \frac{R_{n+1} - R_n}{R_1}$$

where  $G_{n+1}$  represents the increment of bone per year in relation to the increment of the first year, expressed in arbitrary units, and R (rings). The analysis was restricted to pictures where the basis of subsequent zones was clearly visible. Successive G were examined for the first 12 years in order to detect the expected drop associated with maturity (Francillon-Vieillot et al., 1990; Castanet et al., 1993). The last year before a sharp decrease in mean bone growth was considered to be the onset of sexual maturity.

#### Analyses

For most comparisons we used analysis of variance (ANOVA) when the distribution of the data did not deviate from normality (Kolmogorov-Smirnov tests) and U-Mann-Whitney tests otherwise. Mean age and longevity (maximum age recorded) were calculated for each population and each sex. The relationship between age and body size for each sex and each population was examined using Pearson's correlations, possible sex effect was assessed using analysis of co-variance (ANCOVA).

Growth patterns were estimated using Von Bertalanffy's equation,

$$SVL_t = SVL_{asympt} (1 - e^{-k(t-t_0)})$$

where  $t$  represents number of growing seasons (i.e., years),  $SVL_t$  stands for body length at age  $t$ ,  $SVL_{asympt}$  represents the estimated asymptotic body size that can theoretically be reached,  $k$  is the growth coefficient, and  $t_0$  represents the intercept at the tempo-

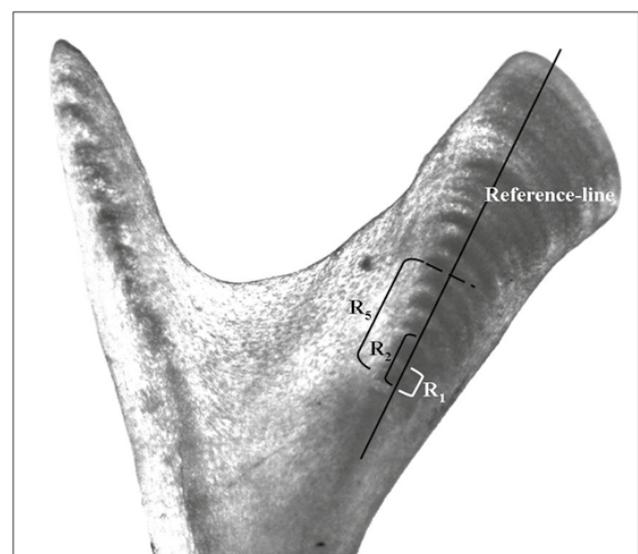


Fig. 2. Parameters for the measure of “*rapprochement*” (see text for definition).

ral axis, thus hypothetical age at size 0. The parameters  $SVL_{asymp}$ ,  $k$  and  $t_0$  and their asymptotic confidence intervals were estimated using nonlinear least-squares regressions. The Von Bertalanffy equation was fitted to age and size data for sexes within a population and for each population. Data of newborns of undetermined gender were used to build both male and female curves in all the populations. Two estimated  $SVL_{asymp}$ ,  $k$  and  $t_0$  values were considered to be significantly different (at the 0.95 level) when their confidence intervals did not overlap.

The potential reproductive lifespan was calculated for each sex and each population by subtracting the estimated age at maturity from the respective age of the oldest individual found (longevity). The estimated ages at maturity for each sex, within each population, were then substituted in the respective derived Bertalanffy's equation to obtain size at maturity. Small sample sizes precluded performing several analyses (e.g. analyses of bone growth patterns were reliable results for Chizé and Montecristo populations only).

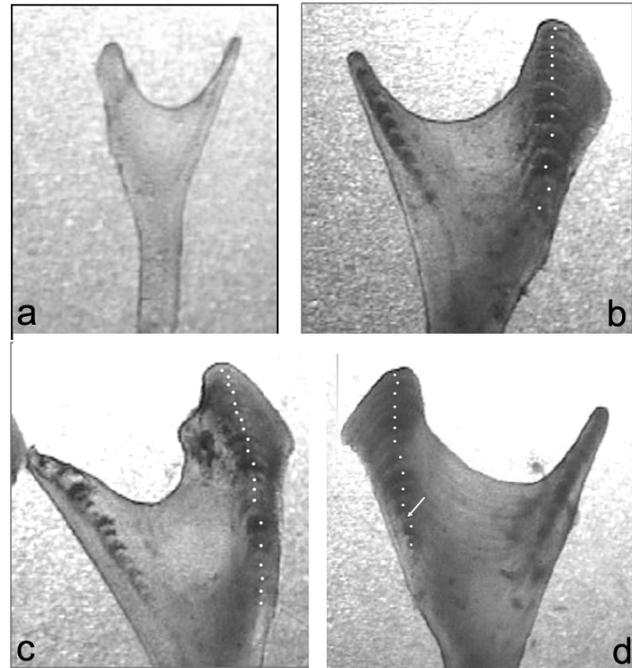
## RESULTS

### Body size, age, longevity and population age structures

**Chizé.** The whole sample ( $n = 72$ ) included 46 males, 24 females and 2 newborn specimens of undetermined sex. Sixty-five specimens out of 72 were adults, and, among these, 43 were males and 22 females. Adult body size was normally distributed (Kolmogorov-Smirnov test,  $Z = 0.632$ ,  $P = 0.820$ ) and adult males were significantly larger than females (t-test,  $t = 1.171$ ,  $df = 58$ ,  $P = 0.001$ ). It was possible to reliably estimate the age of 90.3% (65 of 72; Fig. 3) of the whole sample. Mean estimated population age was 12.1, ranging from 0 to 24 years; additional details are provided in Table 1A.

**Montecristo.** The overall Montecristo sample ( $n = 28$ ) included 14 males, 13 females and 1 specimen of undetermined sex. All determined males and females in the population were adults, the only juvenile being of undetermined gender. Adult body size was normally distributed (Kolmogorov-Smirnov test,  $Z = 0.392$ ,  $P = 0.998$ ) and there was no significant difference in mean adult body length between the sexes (t-test,  $t = 0.995$ ,  $df = 23$ ,  $P = 0.330$ ). It was possible to reliably estimate the age of 92.9% (26 of 28) of the whole sample. Estimated mean population age was 19.4, ranging from 0 to 29 years (details in Table 1B).

**Calimera.** The Apulian sample ( $n = 32$ ) included 21 males, 6 females and 5 newborns or juveniles of undetermined gender. All males were adults, while two out of the six females were subadults; on the whole the sample included therefore 25 adult specimens and seven subadults. Adult body size was normally distributed (Kolmogorov-Smirnov test,  $Z = 0.817$ ,  $P = 0.517$ ), however,



**Fig. 3.** Examples of SGM reading on ectopterygoids, Chizé sample. a) 0 yr; b) 12 yr; c) 20 yr; d) 13 yr; the arrow indicates a double zone, and white dots indicate the age in years.

**Table 1.** Sample sizes, Mean, minimum and maximum age values estimated for *Hierophis* sample. Values are reported in years.

A) Chizé	n	Mean age ( $\pm$ 1SD)	Min.	Max. (Longevity)
Whole population	65	12.06 $\pm$ 5.06	0	24
Adult males	39	14.31 $\pm$ 3.11	10	24
Adult females	19	11.42 $\pm$ 3.58	6	20
Subadults	7	1.29 $\pm$ 1.98	0	5
B) Montecristo				
Whole population	26	19.35 $\pm$ 5.87	0	29
Adult males	13	22 $\pm$ 4.58	14	29
Adult females	12	18.08 $\pm$ 3.37	11	24
Subadults	1	/	/	/
C) Calimera				
Whole population	32	15.69 $\pm$ 9.31	0	33
Adult males	21	19.9 $\pm$ 6.33	7	33
Adult females	4	18.5 $\pm$ 3.70	14	22
Subadults	7	1.43 $\pm$ 1.62	0	4

considering the small number of adult females in the sample ( $n = 4$ ), mean SVL was compared between sexes using a Mann-Whitney U test. The analysis revealed that there was no significant difference in mean adult body

**Table 2.** Results of statistical comparisons of age distributions between the different populations.

Sex	Populations compared	n1	n2	Z	Asymp. Sig.	Exact Sig.
Males	Chizé - Montecristo	39	13	2.242	< 0.001**	< 0.001**
	Chizé - Calimera	39	21	2.260	< 0.001**	< 0.001**
	Montecristo - Calimera	13	21	0.540	0.933	0.788
Females	Chizé - Montecristo	19	12	2.117	< 0.001**	< 0.001**
	Chizé - Calimera	19	4	1.435	0.033*	0.016*
	Montecristo - Calimera	12	4	0.577	0.893	0.807

length between the sexes ( $U = 23.500$ ,  $P = 0.201$ ). This result could however be the consequence of the relatively small data-set of females. Although SGMs reading was difficult in many cases, as a consequence of bone remodelling and of “rapprochement” of outer SGMs, it was possible to estimate the age in 100% of the whole sample. Mean population age was 15.7, ranging from 0 to 33 years (details in Table 1C).

In all the three populations, age values were normally distributed (Kolmogorov-Smirnov test. Chizé:  $Z = 0.969$ ,  $P = 0.305$ ; Montecristo:  $Z = 0.554$ ,  $P = 0.919$ ; Calimera:  $Z = 0.607$ ,  $P = 0.855$ ) and adult males were on average significantly older than females in Chizé and Montecristo (t-test, t. Chizé: 3.160,  $df = 56$ ,  $P = 0.003$ ; Montecristo: 2.417,  $df = 23$ ,  $P = 0.024$ ), not in Calimera (Mann-Whitney U test = 34.5,  $P = 0.577$ ). In the three populations age distributions did not differ significantly between the sexes, when considering only adult specimens (Kolmogorov-Smirnov Z test. Chizé: 1.230,  $P = 0.097$ ; Montecristo: 1.121,  $P = 0.162$ ; Calimera: 0.611,  $P = 0.849$ ). In Chizé, Exact test showed a significant tendency towards a left-shifted age distribution in adult females with respect to adult males ( $P = 0.033$ ). However, in Montecristo and Calimera this pattern was not evident (Exact test. Montecristo:  $P = 0.093$ ; Calimera:  $P = 0.653$ ).

#### Inter-population comparisons

Mean adult male SVL differed significantly among the three populations (ANOVA, with SVL as the dependent variable and population as the factor,  $F_{2,71} = 36.106$ ,  $P < 0.001$ ). A Post-Hoc test revealed that males from Chizé were larger compared to the males from the two other populations (Bonferroni Post-Hoc test, both multiple comparison  $P < 0.001$ ), while males from Montecristo and from Apulia did not differ significantly in mean body size (Bonferroni Post-Hoc test,  $P = 0.086$ ). The same analysis was then performed on adult females (ANOVA, with SVL as the dependent variable and population as the factor,  $F_{2,32} = 6.794$ ,  $P = 0.003$ ; homogeneity of variances

assumption was not met, but the ANOVA is quite robust to violations of this assumption, Zar, 1984). The Bonferroni Post-Hoc test revealed that Chizé females were larger than Montecristo females ( $P = 0.003$ ). Mean estimated age was significantly different between adult males from the three populations ( $F_{2,70} = 19.195$ ,  $P < 0.001$ ). Bonferroni Post-Hoc multiple comparison test revealed that males from Chizé were significantly younger than males from Montecristo and from Apulia (both  $P < 0.001$ ), while no differences were found between adult males from the two Italian populations ( $P = 0.575$ ). Similarly, adult females from the three sites showed significant differences in mean estimated age (ANOVA, same design,  $F_{2,32} = 16.066$ ,  $P < 0.001$ ), females from Chizé being significantly younger than females from Montecristo and Apulia (Bonferroni Post-Hoc test,  $P < 0.001$  and  $P = 0.003$  respectively). The mean age between the two Italian female samples was not significantly different ( $P = 1$ ). In Table 2 we reported results of Kolmogorov-Smirnov tests on differences in age distributions among sexes and sites: both age distributions of males and females from France are significantly shifted to the left (thus to younger ages) with respect to age distributions of Italian whip snakes. Moreover, no significant differences in age distributions were found between the two Italian populations in both sexes.

#### Relationship between age and body size

Body size was highly correlated with age both in the whole population (Pearson correlation. Chizé:  $r = 0.904$ ,  $P < 0.001$ ; Montecristo:  $r = 0.876$ ,  $P < 0.001$ ; Calimera:  $r = 0.936$ ,  $P < 0.001$ ) and considering each sex separately (Pearson correlation. Chizé:  $r_{\text{males}} = 0.882$ ,  $P_{\text{males}} < 0.001$ ,  $r_{\text{females}} = 0.838$ ,  $P_{\text{females}} < 0.001$ ; Montecristo  $r_{\text{males}} = 0.792$ ,  $P_{\text{males}} = 0.001$ ,  $r_{\text{females}} = 0.818$ ,  $P_{\text{females}} = 0.001$ ; Calimera:  $r_{\text{males}} = 0.820$ ,  $P_{\text{males}} < 0.001$ ;  $r_{\text{females}} = 0.921$ ,  $P_{\text{females}} = 0.009$ ).

Differences in mean age between the sexes were then re-analyzed taking into account this relationship. An ANCOVA performed with age as the dependent variable, SVL as the covariate and sex as the factor revealed that

there was no difference between the sexes in age in Chizé and Calimera snakes, when body size was taken into account (Chizé:  $F_{1,55} = 0.216$ ,  $P = 0.644$ ; Calimera:  $F_{1,23} = 0.007$ ,  $P = 0.935$ ). ANCOVA on Montecristo snakes, on the contrary, revealed a significant effect of gender on age ( $F_{1,22} = 6.603$ ,  $P = 0.017$ ). It has to be noted, however, that in all the three populations, the assumption of homogeneity of slopes was not met ( $P < 0.001$ ). A t-test was then performed comparing the unstandardized residuals obtained from the linear regression of the ln-transformed estimated age on the ln-transformed SVL. The result again highlighted the absence of any significant sexual difference in size-corrected age in Chizé and Calimera snakes (t-test. Chizé:  $t = 0.177$ ,  $df = 22.486$ ,  $P = 0.861$ ; Calimera:  $t = -0.331$ ,  $df = 24$ ,  $P = 0.744$ ). Males from Montecristo were on average older than females when estimated age was corrected for body size ( $t = 2.473$ ,  $df = 23$ ,  $P = 0.021$ ).

#### Inter-population comparisons

Taking into account the positive correlation between age and body size, males from the three populations exhibited significant differences in estimated age (ANCOVA, with age as the dependent variable, SVL as the covariate and population as the factor,  $F_{2,69} = 89.752$ ,  $P < 0.001$ ). Bonferroni Post-Hoc multiple comparisons were all highly significant (all  $P \leq 0.001$ ), showing that, for similar SVL values, Montecristo males were the oldest, while males from Chizé were the youngest. However, the assumption of homogeneity of slopes was not met ( $P < 0.001$ ). Yet the differences among populations were marked (Fig. 4a),

suggesting that possible effect of violation of homogeneity assumption on our main conclusions was limited. The analysis was then performed by considering the unstandardized residuals obtained from the linear regression of the ln-transformed estimated age on the ln-transformed SVL. Results confirmed that population differences in mean age, among males, were not dependent on differences in mean body sizes (ANOVA, with residuals as the dependent variable and population as the factor,  $F_{2,68} = 191.326$ ,  $P < 0.001$ ; all Post-Hoc multiple comparisons  $P < 0.001$ ; Fig. 5). Within females, age was significantly different among populations, when age-size correlation was considered (ANCOVA, with age as the dependent variable, SVL as the covariate and population as the factor,  $F_{2,32} = 41.042$ ,  $P < 0.001$ ). For similar SVL values, females from Chizé were significantly younger than females from the other two populations (Bonferroni Post-Hoc test, both  $P < 0.001$ ), while no differences were found between Italian females from Montecristo and from Apulia, even if there was a trend towards older island females and the small Apulian sample size may have influenced the result ( $P = 0.074$ ; Fig. 4b). Unstandardized residuals of the linear regression of the ln-transformed estimated age on the ln-transformed SVL were furthermore analysed, and the over mentioned result was confirmed (ANOVA, with residuals as the dependent variable and population as the factor,  $F_{2,33} = 34.870$ ,  $P < 0.001$ ). Females from Chizé were significantly younger than females from both Montecristo (Post-Hoc Dunnett test,  $P < 0.001$ ) and Calimera (Post-Hoc Dunnett test,  $P = 0.05$ ), while the difference was not significant between females from Italian populations (Post-Hoc Dunnett test,  $P = 0.149$ ; Fig. 5).

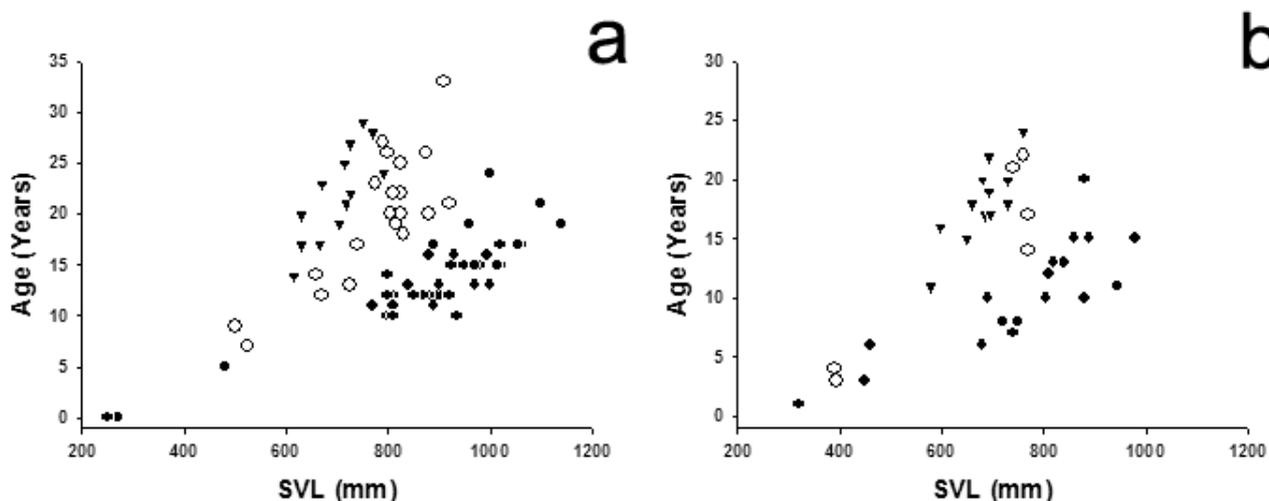


Fig. 4. Relationship between age and body size in males (a) and females (b) of the three populations. (● Chizé); (▲ Montecristo); (○ Calimera).

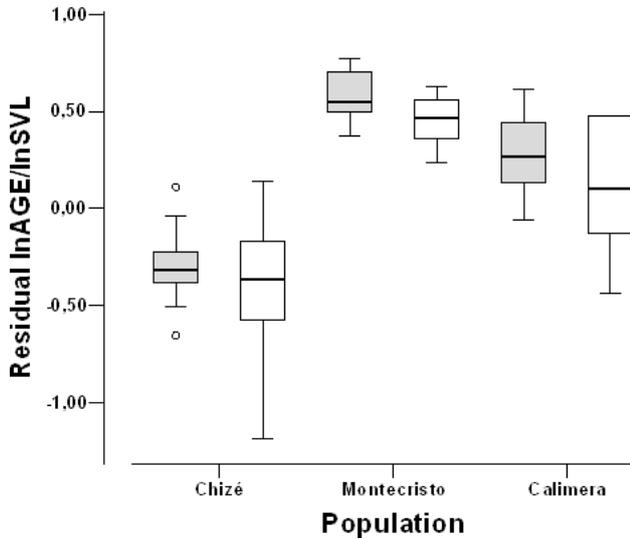


Fig. 5. Intra population and intraspecific differences in size-corrected age. Grey=males; white=females.

#### Population growth curves

In the three studied populations, derived values for  $SVL_{asymp}$ ,  $k$  and  $t_0$ , their asymptotic confidence intervals and the coefficients of correlation of the Von Bertalanffy equation fitted separately on male and female data (Table 3). In the three populations, modelled curves fit-

ted well the relation between age and body size in both sexes (correlation coefficients in Table 4). Growth curves had similar shapes for males and females (Chize: Fig. 6a; Montecristo: Fig. 6b; Calimera: Fig. 6c): even if  $SVL_{asymp}$  was higher in males and  $k$  was higher in females, these differences were not significant (confidence intervals widely overlap in all considered populations). In the snakes from Chizé, the estimated male  $SVL_{asymp}$  was very close to actual maximum SVL observed in the field (1200 mm,  $n > 1,600$  records; X. Bonnet, personal unpubl. data), while the Von Bertalanffy model slightly underestimated the asymptotic body length for females (maximum SVL observed in the field 1080 mm,  $n > 1,100$  records; X. Bonnet, personal unpubl. data). In the snakes from Montecristo the model provided a satisfactory male asymptotic size (maximum SVL observed in the field 873 mm,  $n = 53$ ) but slightly overestimated it in females (maximum SVL observed in the field 790 mm,  $n = 30$ ). In the snakes from Calimera the model slightly overestimated asymptotic sizes, both in males (maximum SVL observed in the field 920 mm,  $n = 16$ ) and in females (maximum SVL observed in the field 825 mm,  $n = 4$ ).

#### Inter-population comparisons of growth

Comparisons of growth parameters are reported in Table 3. While the estimated  $k$  and  $t_0$  for males were

Table 3. Coefficients of correlation and parameters of the Von Bertalanffy's estimated model for male and female *H. viridiflavus* from the three populations considered. 95% confidence intervals are in parentheses.

Sex	$SVL_{asymp}$	$k$	$t_0$	$R^2$
<i>Chizé</i>				
Males	1216.97 (1032.86-1401.10)	0.086 (0.052-0.119)	-2.69 (-3.85--1.52)	0.925
Females	1012.38 (811.47-1213.28)	0.123 (0.049-0.197)	-2.19 (-3.83--0.55)	0.907
<i>Montecristo</i>				
Males	843.05 (686.61-999.50)	0.068 (0.025-0.110)	-5.01 (-8.29--1.75)	0.943
Females	854.20 (637.19-1071.22)	0.071 (0.018-0.124)	-4.72 (-7.84--1.60)	0.953
<i>Calimera</i>				
Males	965.60 (843.36-1087.82)	0.073 (0.043-0.103)	-4.10 (-5.85--2.34)	0.958
Females	861.67 (690.74-1032.59)	0.095 (0.028-0.162)	-3.55 (-5.84--1.27)	0.977

Table 4. Coefficients of correlation and parameters of the Von Bertalanffy's estimated models for the three populations considered. 95% confidence intervals are in parentheses.

Population	$SVL_{asymp}$	$k$	$t_0$	$R^2$
Chizé	1178.22 (1028.90-1327.54)	0.091 (0.060-0.122)	-2.55 (-3.66--1.44)	0.901
Montecristo	820.81 (731.52-910.11)	0.077 (0.047-0.107)	-4.55 (-6.92--2.17)	0.907
Calimera	948.26 (842.03-1054.50)	0.076 (0.048-0.105)	-3.94 (-5.56--2.31)	0.954

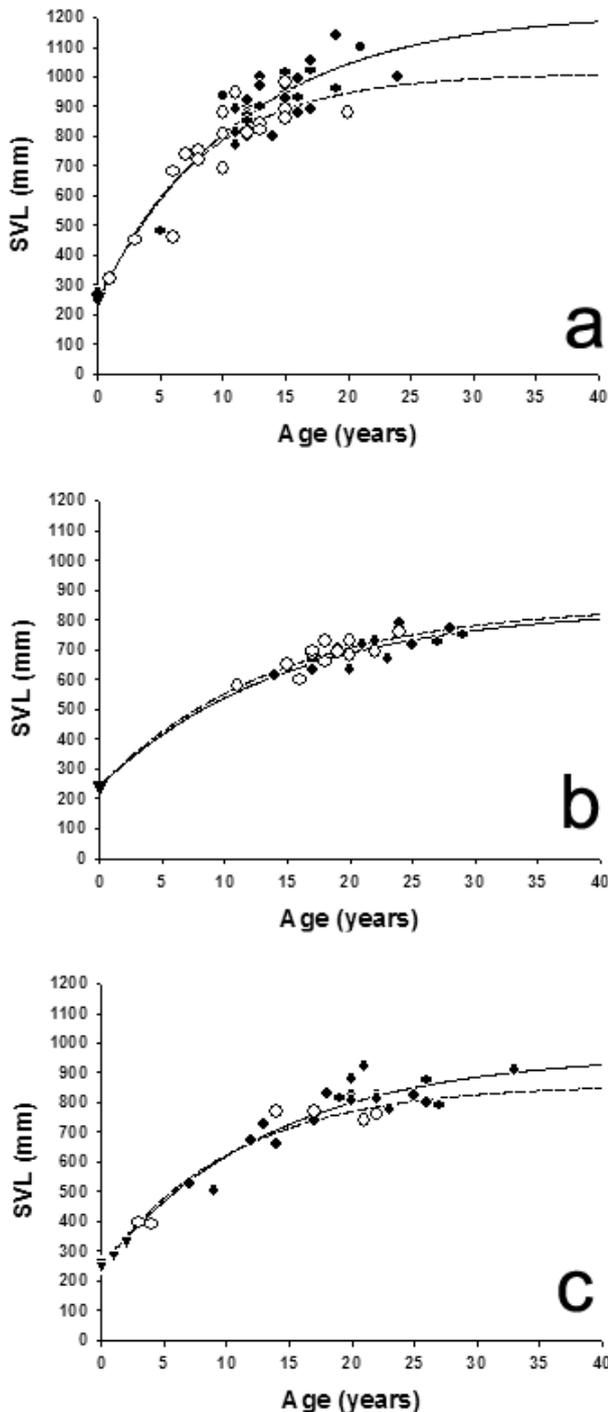


Fig. 6. Population growth curves for *H. viridiflavus* at a) Chizé; b) Montecristo; *H. carbonarius* at c) Calimera. (● males); (○ females); (▶ juveniles).

comparable across populations with a strong overlap of the 95% confidence intervals, there was a limited overlap between confidence intervals for asymptotic SVL between Chizé and Calimera (5.05%) and no overlap with Monte-

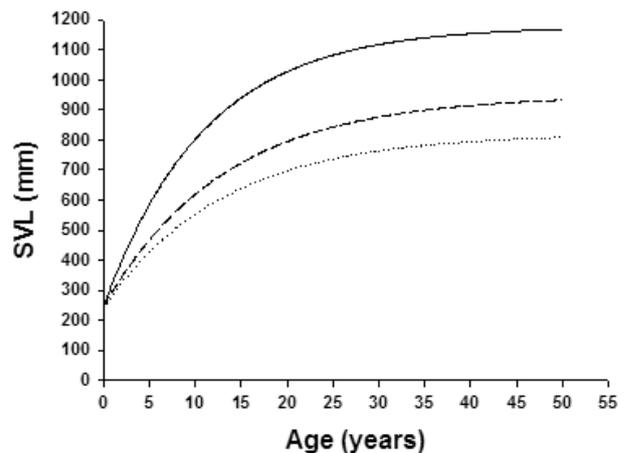
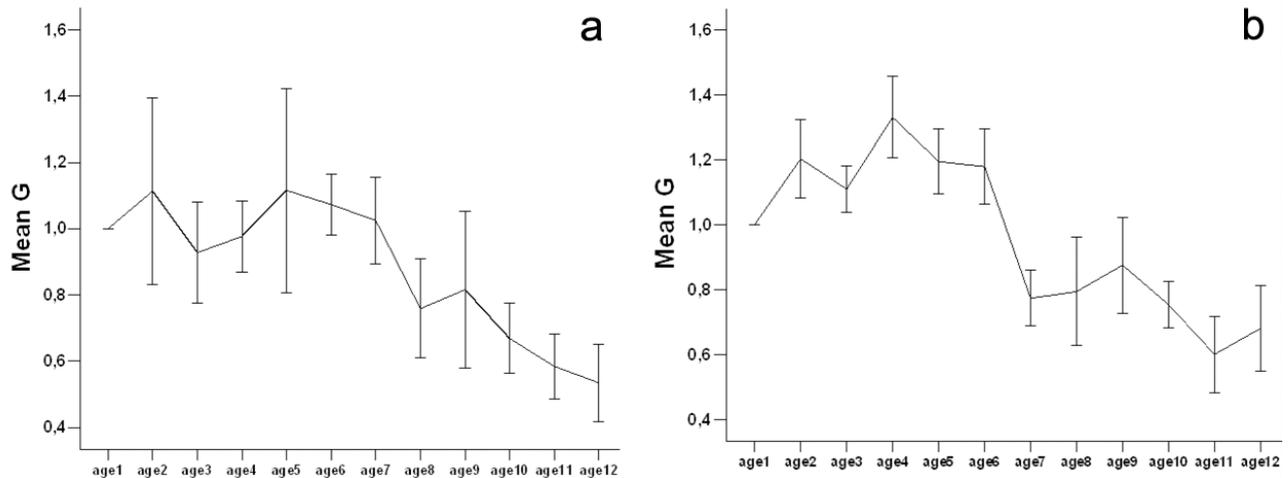


Fig. 7. Estimated Von Bertalanffy's growth curves for Chizé (—), Montecristo (....) and Calimera (- - -) populations.

cristo (see Table 3). This suggests that males from different populations attained different asymptotic body sizes following comparable growth rates. A different pattern was observed in females:  $k$  and  $t_0$  were relatively similar across populations with strongly overlapping of 95% confidence intervals, and  $SVL_{asympt}$  confidence intervals were overlapping (even if estimated values were dissimilar between populations). We note however, that the small Calimera sample limited the power of this analysis.

The absence of significant male-female differences for growth parameters within each sample allowed to estimate cumulative population growth curves by fitting the Von Bertalanffy equation to each population entire data set. Derived values for  $SVL_{asympt}$ ,  $k$  and  $t_0$ , their asymptotic confidence intervals and the coefficients of correlation of the Von Bertalanffy equation fitted for each population are reported in Table 4. Growth curves for the three sites are plotted in Fig. 6. While the growth coefficient  $k$  and the estimated  $t_0$  had similar values throughout populations, and their 95% confidence intervals strongly overlapped, there was only a negligible overlap (e.g., 2.43%) between confidence intervals of asymptotic SVL of Chizé population and of Calimera population. There was no overlap between the French versus insular populations. Moreover, the asymptotic SVL estimated for the two Italian populations overlapped only marginally, suggesting that, even if less pronounced, differences in growth pattern also exist between these two populations (Fig. 7). These results suggested that whip snakes from the Forest of Chizé (*H. viridiflavus*) follow different growth trajectories compared to the two Italian populations (*H. viridiflavus*, *H. carbonarius*) and attain larger maximal size.

Derived values of the two main growth parameters,  $SVL_{asympt}$  and  $k$ , for males, females and the whole sample



**Fig. 8.** Annual bone growth (Mean G) in males (a) and females (b) *Hierophis viridiflavus* from Chizé. Lines connect mean values, bars represent  $\pm 1SD$ . Age in years in the abscissa.

were inversely correlated within each population; this relationship was significant for the populations of Chizé and Calimera, while it was not for the population of Montecristo island (Pearson correlation,  $r = -0.999$ ,  $P = 0.035$ ,  $r = -1$ ,  $P = 0.019$  and  $r = -0.785$ ,  $P = 0.425$  respectively).

*“Rapprochement”*: age and size at maturity and potential reproductive lifespan

It was possible to clearly measure successive annual bone growth marks for 4 males and 6 females in Chizé, for 9 males and 7 females in Montecristo and for 10 males and only 2 females in Calimera.

In Chizé, the annual bone growth showed a sudden decrease between the seventh and the eighth year of age for males (see also Fig. 8a, an example for all the populations) and between the sixth and the seventh for females (Fig. 8b). It is therefore likely that males *Hierophis viridiflavus* of this population attain sexual maturity at 7 years of age, while females become reproductive when they are one year younger, at 6 years of age. In both sexes bone growth was particularly rapid during the second active season, and then it showed a decrease, followed by a subsequent increase and a slowing down until the sharp decrease in connection with sexual maturation. From these estimated values it emerged a potential reproductive lifespan of 17 years for males and 14 for females. Using the derived Bertalanffy's equation for each sex, it emerged that male size at maturity is 687.9 mm, while females reach sexual maturity at a mean size of 642.6 mm. In Montecristo, annual bone growth in males showed the highest mean value during the second

year of life, then it decreased but remained at more or less constant values until the eighth year, when it showed a strong decrease. Therefore males *Hierophis viridiflavus* of this population likely reach sexual maturity when they are 8 years old. Females showed a bone growth pattern characterised by a decrease of growth after the second active season, followed by a subsequent increase and a slowing down until sharp decrease between the sixth and the seventh years. However, mean annual bone growth slowed down under initial values only after the eighth year. It is therefore likely that island females, as island males, attain sexual maturity at 8 years of age. Substituting these values in the derived Bertalanffy's equation for each sex it emerged that male size at maturity is 495.1 mm, while females reach sexual maturity at a mean size of 507.9 mm. Calculated potential reproductive lifespan for males and females were, respectively, 21 and 16 years. In Calimera, unfortunately, as a consequence of bone remodelling, LAGs on ectopterygoids of this population were not so sharply differentiated on the bone surface and measurements of annual radius (e.g.,  $R_n$ ) were sometimes performed quite subjectively. The following results are therefore only indicative. Male annual bone growth showed the highest mean value during the third year of life, and then it suddenly decreased. The last drop off was in correspondence of the sixth active season (when mean annual bone growth slowed down under initial values). Male *Hierophis viridiflavus* of Calimera may therefore attain sexual maturity during their sixth year of life, with an estimated potential reproductive lifespan of 27 years. Bone growth pattern was obtained only for two females; hence it was not possible to estimate age at maturity for females of this population. Sub-

stituting male estimated age at maturity the respective derived Bertalanffy's equation it emerged that male size at maturity is 503.7 mm.

#### *Inter-population comparisons of age at maturity.*

Only Chizé and Montecristo populations with reliable estimated age at maturity were considered. Plotting estimated values for each sex we observed a positive trend (not significant, Pearson correlation,  $r = 0.827$ ,  $P = 0.173$ ), between the estimated age at maturity and longevity. Moreover, there was a clear positive relationship between the estimated SVL at maturity and asymptotic body length ( $SVL_{\text{asympt}}$ ). This correlation was statistically significant (Pearson correlation,  $r = 0.954$ ,  $P = 0.046$ ).

## DISCUSSION

Skeletochronology enabled us to estimate the age of most of the sampled individuals, providing novel information (otherwise unavailable) in both sexes and for wide spectrum of body sizes. Below we first examine broad patterns and then population divergences.

#### *Broad patterns*

In the European whip snake, estimated size at maturity ranged from 57% to 64% of the maximal body size (in Chizé males and Montecristo females respectively), in accordance with previous studies in snakes (Shine and Charnov, 1992). The positive correlations between estimated age at maturity and longevity, or between size at maturity and Bertalanffy's asymptotic length, also conform to the general pattern described in ectotherms in general (Shine and Charnov, 1992) and in snakes more specifically (Parker and Plummer, 1987). These relationships represent trade-offs between maturation programmes, growth patterns, and costs versus benefits of large body size (Shine and Charnov, 1992). The congruence of our results with previous studies suggests that skeletochronology provided useful information.

In the studied populations, we found a strong relationship between estimated age and body size (all  $R^2 > 0.91$ ). Following a fast growing juvenile phase, growth rate decreased with body size. Similar results have been documented in other reptile species monitored through capture-mark-recapture surveys (CMR); growth following asymptotic patterns derived from the von Bertalanffy curve (Dunham, 1978; James, 1991; El Mouden et al., 1999; Bonnet et al., 2011). However, despite a strong rela-

tionship between age and size, there was a considerable variance in age-related body size, especially among older individuals. Our study confirms that SVL is not a reliable estimator of age in snakes, especially after maturity and in large specimens. Many idiosyncratic and environmental factors influence growth in snakes, notably after maturity, and can explain the strong inter-individual divergences of trajectories (Bronikowski, 2000; Madsen and Shine, 2000; Bonnet et al., 2002; Lelièvre et al., 2013). Sex is one of those important factors (Koos Slob and van der Werff Ten Bosch, 1975; Kuwamura et al., 1994).

On average males were older and attained greater maximal longevity compared to females. In the three populations studied, male and female growth curves were not significantly different. Male *Hierophis* showed the highest values of  $SVL_{\text{asympt}}$  and the smallest values of  $k$  within each population: this could mean that i) the tendency for males to reach a larger maximum size approaching this asymptote at slower rate than females is overall present in the populations considered or that ii) males grow more rapidly than females after maturity. Yet, because they continue to grow they need longer time to reach their maximal size. In other words, the trend for a cessation of growth in females means that they reach earlier maximal SVL, but not at a faster rate. Consequently, the observed male-biased sexual size dimorphism (SSD; Fornasiero et al., 2007; Zuffi, 2007) was essentially attributable to a longer period of growth after maturity in males (King, 1989). Sexual bimaturation (sexes maturing at different ages) can also influence SSD (Shine, 1990, 1994): because growth decreases after maturity, earlier maturing sex tend to exhibit smaller mean adult sizes (e.g., Lagarde et al., 2001). Our results provide a partial support to this scenario. In the northern population (Chizé), males reached maturity one year after females, no difference was observed in the Montecristo population, and a small sample size precluded performing robust analyses in the Apulian population. Overall, a longer growth period after maturity in male European whip snakes may explain the larger mean body size in this sex. However, the respective contribution of possible underlying mechanisms remains unknown. Many males are killed during mate searching and suffer from a high-risk mortality compared to females (Bonnet et al., 1999a; Meek, 2009). This sex difference in mortality should induce a female biased SSD and thus does not fit well with the above results. However, survival in emaciated females after reproduction can be very low in snakes (Bonnet et al., 1999b). Examining the effect of sex and age on survival is necessary to clarify these issues (Bonnet et al., 2011).

Beside fundamental morphological differences (Bonnet et al., 1998) various ecological and physiological factors may generate sexual divergences in growth rate.

During the mating season male European whip snakes engage into vigorous ritual combats and display very high testosterone levels (Bonnet and Naulleau, 1996). In reptiles, this androgenic steroid stimulates sexual behaviours and growth, resulting into male biased SSD (Cox et al., 2009). Moreover, high reproductive investment controlled by high estradiol levels during vitellogenesis exhausts body reserves and can markedly hamper growth in females (Bonnet et al., 1994, 2011; Van Dyke and Beaupre, 2011). In species exhibiting male-to-male combats, selection for large body size of males tends to overrule selection for large body size to accommodate larger clutches in females (Shine, 1993, 1994). European whip snakes display all these traits; lower growth rate in females than in males is thus expected in this species but our results show no sex effect. Yet, other key factors may balance growth rates between sexes. For example, males tend to be anorexic during the mating season while females forage during the whole active period (Bonnet and Naulleau, 1996), females possess more developed attributes to process food (Bonnet et al., 1998), and thus females may assimilate greater amounts of food available for growth compared to males. Sex difference in prey selection can also influence body size (Zuffi et al., 2010). Overall, our results regarding age and size pose more questions than offering responses; CMR studies combined with eco-physiological investigations are necessary to obtain a general understanding of the determinants of SSD in free ranging snakes.

#### *Inter-population variations*

We found strong differences in mean adult body size, mean adult age, age distribution, longevity, growth rate, age and size at maturity among the three populations studied. These results are important to better interpret already documented inter population variations in body size and reproductive traits (Fornasiero et al., 2007; Zuffi et al., 2007). The most salient differences were observed between genetically close populations (*H. viridiflavus* [sub]species) respectively sampled in the northern and southern parts of the distribution range (Chizé *versus* Montecristo). The genetically distinct third population (*H. carbonarius* from Apulia) was relatively more similar to the island population (Montecristo). Thus genetic proximity did not translate into similarities in age/size life history traits.

On average, the snakes from Chizé were markedly larger and younger compared to the two other populations. Less pronounced differences in body size were found between specimens from Montecristo and Apulia. Snakes from Chizé reached larger asymptotic size without

difference in the Bertalanffy's growth coefficients ( $k$ ), and thus exhibited higher absolute growth rate than snakes from Montecristo and Apulia (Dunham, 1978; Wapstra et al., 2001; Stanford and King, 2004). Additionally, French specimens exhibited lower longevity compared to Italian snakes. Growth rate and mean body size should be higher at lower latitudes in ectotherms due to more favourable temperatures and longer activity period. On the contrary, we found a reverse trend, likely because Mediterranean, dry and arid habitats of the Italian populations offer less favourable trophic (Zuffi, 2007) and hydric conditions compared to more productive areas typical of mild oceanic climate. In fact, during drought periods snakes remain sheltered to maintain their hydro-mineral balance (Bonnet and Brischox, 2008), even entering into a prolonged estivation period (*Vipera aspis*, M.A.L. Zuffi, unpublished data). *Hierophis carbonarius* from Calimera follows an intermediate growth trajectory between the opposite extremes represented by the insular and the northern population, being however closer in this pattern to the former one.

#### *Limits of the study*

Estimating age with skeletal marks does not pose major problems to assess broad patterns for comparisons among sexes and populations because possible methodological biases will apply equally in the different groups examined. However, absolute values should be considered with caution. Inferring the exact age at maturity and exact growth rates rely on a set of assumptions.

Our results suggest that whip snakes from Montecristo Island mature at an estimated age of 8 yr, and at a minimum derived body size (SVL) of 495 and 507 mm in males and females respectively. But the smallest female with developed follicles from Montecristo measured 598 mm in SVL, leading to an estimated age of 12 yr according to our growth curves. This discrepancy might be due to insufficient sampling of reproductive females in the field ( $n = 18$ ), to imprecision in the estimates (e.g., small sample size in juveniles hampered comparing linear *versus* nonlinear functions to select the best fitting equations between age and body size) or due to other factors (e.g., a lack of perfect correspondence between reduced bone growth and maturity). Indeed, 8yr is already an elevated age for maturity for snakes, 12yr would be a remarkable value. Our results in the Chizé population suggest an estimated age at maturity of 7 yr for males and 6 yr for females, with a derived minimum size at maturity of 687 and 642 mm in SVL respectively. The smallest reproductive female found in the Forest of Chizé measured 680 mm in SVL (X. Bonnet, unpubl. data), a value

in accord with skeletochronology. However, using our results, it also suggests an estimated age of 7 yr for maturity, a value that does not fit well with CMR data. Lelièvre et al. (2013) showed that juvenile growth rate averages 0.04 cm/day in Chizé, leading to an age for maturity of 3-4 years. As above, this discrepancy might be caused by inappropriate sampling (e.g., few road-kill juveniles were found intact) or to insufficient fitting of the growth models used. Further analyses based on larger number of juveniles are required to better calibrate skeletochronology to CMR data and to take into account marked inter-individual differences in growth trajectories.

Whatever the case, the strong differences of age at maturity observed between populations likely reflect adaptation to local conditions. The markedly slow growth, delayed maturity, smaller body size and higher longevity of Montecristo snakes are expected in a dry environment where the acquisition of trophic resources (see also Zuffi, 2001) is more challenging than under mild oceanic climate. Relative clutch size, an index of energetic investment per reproductive bout is lower in Montecristo snakes, in accordance with the notion that resources availability is limited in this island (Zuffi et al. 2007). Populations respond by shifting a set of traits along a slow-fast gradient (Stearns and Koella, 1986; Wapstra et al., 2001). Using dead snakes, our results suggest a strong plasticity of major traits driven by trophic and climatic conditions. Collecting opportunistically and examining dead snakes might be useful to address key questions. Major parameters, especially before maturity, can be inferred and implemented to calibrate models that aim to examine the impact of climatic changes. Indeed currently implemented mean values do not permit to encompass the wide range of variations observed among individuals and across populations.

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