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49 Abstract

Phalangeal reduction is a common and widespread phenomenon among tortoises that has been associated with the adaptation to terrestrial life. While reduced manual digit 1 appears characteristic in almost all *Testudo* species, it is uncertain why the metacarpal I and distal carpal of the same digit are completely missing in some individuals of Hermann's tortoise (*Testudo hermanni hermanni*). To clarify this issue, we investigated the number of manual claws in six populations of Hermann's tortoise (one from the Ebro Delta in the Iberian Peninsula and five from Minorca Island), their age, sex, genetic lineage, and the substrate type that they inhabit. The number of claws was ascertained based on direct counts ($n > 1500$ individuals) and by X-rays ($n = 32$ individuals), obtaining three different phalangeal formulae: (1-2-2-2-1, D-2-2-2-1, 0-2-2-2-1). Thus, claw counts through both methodologies (direct count and X-ray) further confirm that the observed claws serve as a good proxy to assess the actual number of digits. Our results show no loss of phalanges, metacarpal and carpal bones in digit 1 associated with age, sex, or substrate, contrary to some previous authors who hypothesized a relationship between this loss and sexual dimorphism. Therefore, variations in the number of manual digits and the loss of metacarpal I and distal carpal in digit 1 in Hermann's tortoise are related to population and genetic lineage. More detailed comparisons with other *Testudo hermanni* populations from elsewhere in Europe would be required to understand the evolutionary significance concerning the intrapopulation variability in the number of digits remaining.

50 Keywords separated by ' - ' *Testudo hermanni* - Phalange reduction - Ebro Delta - Minorca Island - Phalangeal formula

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ORIGINAL ARTICLE

Intraspecific variation in digit reduction in *Testudo*: the case of the Hermann's tortoise

Àngel H. Luján[1,2,3] · Mariona Ferrandiz-Rovira[4,5] · C. Torres[6] · A. Bertolero[7]

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Abstract

Phalangeal reduction is a common and widespread phenomenon among tortoises that has been associated with the adaptation to terrestrial life. While reduced manual digit 1 appears characteristic in almost all *Testudo* species, it is uncertain why the metacarpal I and distal carpal of the same digit are completely missing in some individuals of Hermann's tortoise (*Testudo hermanni hermanni*). To clarify this issue, we investigated the number of manual claws in six populations of Hermann's tortoise (one from the Ebro Delta in the Iberian Peninsula and five from Minorca Island), their age, sex, genetic lineage, and the substrate type that they inhabit. The number of claws was ascertained based on direct counts ($n > 1500$ individuals) and by X-rays ($n = 32$ individuals), obtaining three different phalangeal formulae: (1-2-2-2-1, D-2-2-2-1, 0-2-2-2-1). Thus, claw counts through both methodologies (direct count and X-ray) further confirm that the observed claws serve as a good proxy to assess the actual number of digits. Our results show no loss of phalanges, metacarpal and carpal bones in digit 1 associated with age, sex, or substrate, contrary to some previous authors who hypothesized a relationship between this loss and sexual dimorphism. Therefore, variations in the number of manual digits and the loss of metacarpal I and distal carpal in digit 1 in Hermann's tortoise are related to population and genetic lineage. More detailed comparisons with other *Testudo hermanni* populations from elsewhere in Europe would be required to understand the evolutionary significance concerning the intrapopulation variability in the number of digits remaining.

Keywords *Testudo hermanni* · Phalange reduction · Ebro Delta · Minorca Island · Phalangeal formula

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Introduction

Turtles have successfully diversified into a variety of forms: adapted to aquatic, marine, and terrestrial habitats, despite the fact that they have been structurally constrained by the presence of the shell. Thus, this great ecological diversity correlates with different locomotor patterns that are reflected, among others, in different limb anatomies (Delfino et al. 2010). Testudinids (Testudinidae or tortoises) are an exclusively terrestrial group of turtles with almost worldwide distribution (except Oceania and Antarctica) that comprise 18 genera and 65 species (Marmi and Luján 2012; TTWG 2017). In particular, the high-domed shell and unique limb anatomy are what mainly characterize tortoises, which are adapted for terrestrial life (Ernst et al. 2000).

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Testudinid limbs are thick and robust, serving as forelimbs that are rather flat and well bent, whereas hindlimbs are short and cylindrical (Ernst and Barbour 1989). All digits in both manus and pes are short, and show short and rounded claws (Zug 1971).

Currently, the genus *Testudo* includes the five extant Western Palaearctic species, and according to the more recent molecular phylogenies (Fritz and Bininda-Emonds 2007; Vasilyev et al. 2014) and phylogenetic analysis of morphological traits (Luján et al. 2016), three subgenera are recognized (*Agrionemys*, *Chersine*, and *Testudo*). Specifically, *Chersine* subgenus is represented by a single extant species, the Hermann's tortoise (*Testudo hermanni*), which is a medium-sized species ranging in average carapace length between 13 and 21 cm (Cheylan 2001; Bertolero et al. 2011a). Hermann's tortoise populations are distributed throughout most of the European Mediterranean region, usually below 500 m a.s.l. Nowadays, two subspecies of Hermann's tortoise are distinguished: *T. h. hermanni* in Western Europe, which is in strong decline and has very restricted distributions, and *T. h. boettgeri* in Eastern Europe, which seems to be more stable and displays rather continuous distribution; however, a recent assessment has shown a worse conservation situation (see Fritz et al. 2006a; Bertolero et al. 2011a; Nikolić et al. 2018). Moreover, human impact has been reducing the favorable habitats for this taxon, especially agricultural practices and urbanization (Cheylan 1981, 2001; Vetter 2006), human-related forest fires and poaching (Cheylan 2004; Couturier et al. 2011; Santos and Cheylan 2013), and illegal animal trade (Bertolero et al. 2011a).

Digital reduction (i.e., when species show fewer digits than their related species) is a common characteristic that often occurred throughout the evolution of tetrapods (e.g., Shapiro et al. 2003; McHorses et al. 2017). Although in Hermann's tortoise, either five or four digits can occur in both forelimbs (Fig. 1 and Fig. 2a–d) and hindlimbs (Bertolero 2010, 2014), in the former the digit that tend to reduce is the first and in the latter is the fifth (see Crumly and Sánchez-Villagra 2004). We defined here the condition of a "full digit" when the members of the genus *Testudo* retains two phalanges in manus (being the distal phalanx the claw), which always occurs in the digits 2, 3, and 4 (Fig. 2a). Thereby, a digit 1 or 5 is defined as a "reduced digit" when this digit lacks the proximal phalanx but still retain the distal one (Fig. 2a). The latter occurs in all *Testudo* spp. with the exception of *T. marginata* that retains two phalanges in digit 5 and thus has a "full digit" (see Crumly and Sánchez-Villagra 2004; Hitschfeld et al. 2008). Similarly, digit 1 in at least three of five *Testudo* species (*T. graeca*, *T. hermanni* and *T. horsfieldii*) is reduced, but also it may be: a "lost digit," when the digit is completely missing (i.e., both phalanges are missing: Fig. 2b); a "lost digit and metacarpal I" which implies the additional loss of the metacarpal I (Fig. 2c); and a "lost digit, metacarpal I and distal carpal" when the distal carpal is also missing (Fig. 2d).

The first studies of the ontogeny of turtle limbs started in the late eighteenth century, but the examinations of the phalangeal formula in testudinids were restricted to anecdotal reports or were merely concerning a single species (e.g., Baur 1888; Boulenger 1889). Auffenberg (1966) was the first author to provide a functional interpretation of the adult carpus of several tortoise species. Then, the phalangeal formulae of the pes were analyzed in testudinids using a phylogenetic approach, although only four species of tortoises were examined by Zug (1971). He concluded that tortoises always have four clawed full digits in each manus; however, a reduced or lost digit occurs sometimes. Shortly after, detailed manus illustrations of both extant and fossil gopher tortoises (genus *Gopherus*) were provided by Bramble (1982). The first mention of the number of digits in *Testudo hermanni* is found in Highfield (1988), who points out that all individuals (both males and females) of *T. h. boettgeri* that he found in Bulgaria showed four full digits in their manus. However, preliminary results published by Eendebak (2001) claimed that the number of digits on *T. h. boettgeri* was strongly determined by

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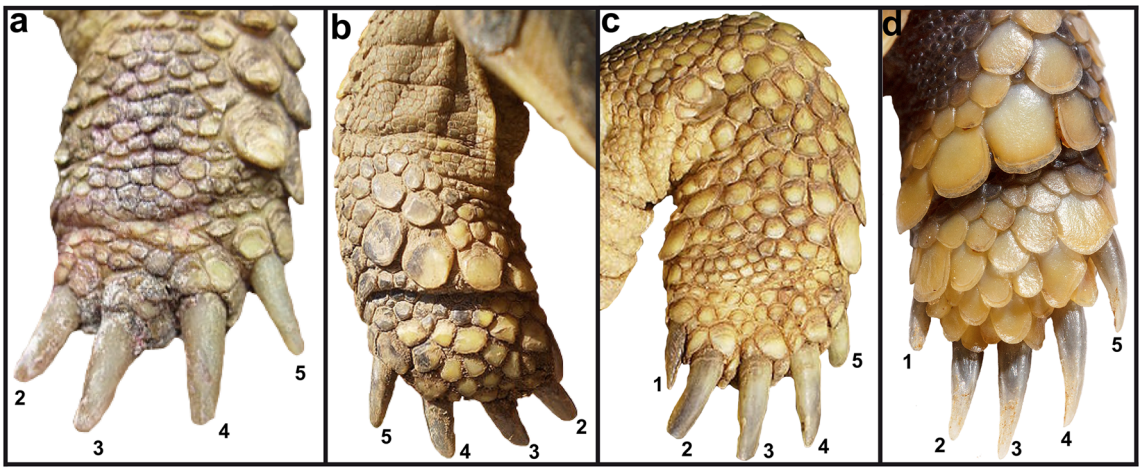


Fig. 1 External manus morphology in *Testudo hermanni hermanni* with four (**a**, **b**) or five digits (**c**, **d**): left adult manus in ventral (**a**) and dorsal (**b**) views; adult (**c**) and juvenile (**d**) left manus in ventral views. The number of the digit is denoted by numerals from one to five

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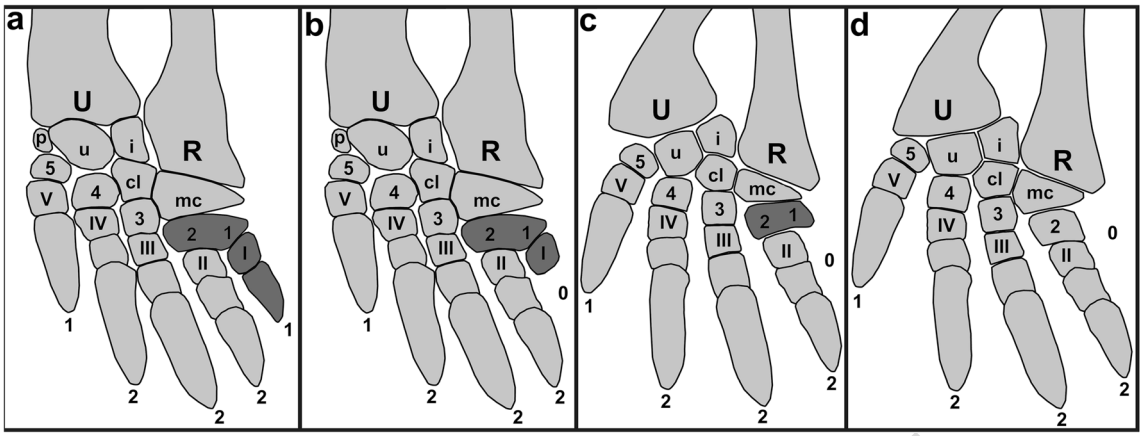


Fig. 2 Bones of the right manus of *Testudo hermanni hermanni* (**a**) including the three possible reductions of the digit 1 and the metacarpal and carpals bones (**b**–**d**). Digit 1 reduced (**a**): one phalanx is present. Digit 1 lost (**b**): the distal phalanx is missing but still retain the metacarpal I and distal carpal. Digit 1 and metacarpal I lost (**c**): only the distal carpal is present. Digit 1, metacarpal I, and distal carpal lost (**d**): no related bone of digit 1 is present (i.e., phalanges, metacarpal I, and distal carpal). *U* ulna, *R* radius, *p* pisiform, *i* intermedium, *cl* lateral centrale, *mc* medial centrale, distal carpalia (1–5), metacarpalia (I–V), and the four phalangeal formulae (**a** *1-2-2-2-1*, **b** M-2-2-2-1, **c** *D-2-2-2-1*, **d** *0-2-2-2-1*). Bones affected by the phalangeal, metacarpal, and carpal reduction are denoted in dark gray

the maternal genetic characteristics (i.e., offspring of mothers with four digits on their manus were four times more likely to have four digits themselves). Although Eendebak (2001) announced the publication of the complete study clarifying the later results on a genetic basis, this study has not been published so far; therefore, these explanations are still missing. There are remarkable results in a study focused on Hermann's tortoise from Corsica (*T. h. hermanni*) conducted by Vetter (2006), as he found sex differences related to the number of digits (44.2% of males and 59.4% of females possessed five digits), although the sample size was not provided. Of all the sampled individuals with five digits, only 4.2% of males and 12.5% of females showed a claw well developed. Moreover, adult individuals with four digits often present the fifth digit also reduced, which is clearly recognized because the latter does not possess a claw (distal phalanx). However, Vetter (2006) did not provide any hypothesis to explain these differences between sexes regarding to variations of digit 1 or 5. In a study of carpal morphology and phalangeal formulae, Crumly and Sánchez-Villagra (2004) examined in testudinids the ontogenetic stage of the individuals, as well as the phylogenetic context. Fritz et al. (2006b) proposed that the phalangeal reduction present in terrestrial and semiterrestrial geoemydids (*Cuora*) and emydids (*Terrapene*) is related with their mode of life, mainly as a consequence of adapting to walk on land (see further Minx 1992; Ludwig et al. 2007). In particular, in some highly aquatic geoemydids (e.g., *Malayemys*, *Morenia*, among other genera), there is no loss of phalanges; they even display an additional phalanx in the digit 5 of the manus and pes, which apparently seems more favorable for swimming (Ludwig et al. 2007). Finally, a unique study that was focused on carpal morphology and phalangeal reduction both in *Testudo hermanni* and *T. horsfieldii* (Hitschfeld et al. 2008) found more variation in phalangeal formula than Crumly and Sánchez-Villagra (2004). This is easily explained by the higher sample (29 individuals) studied by the former in comparison to just three specimens analyzed by the latter. Therefore, the loss of digit 1 (both phalanges), metacarpal I and distal carpal, together with the extensive fusion of carpal elements in *T. horsfieldii*, could be linked with its burrowing mode of life (Hitschfeld et al. 2008).

The main aim of this study is to characterize variation in both the number of digits and phalangeal formula in the manus and to test if this variation is related to other variables (age, sex, habitat among others), using a large sample of Hermann's tortoise obtained in wild populations. First, we investigated whether the number of visible digits corresponds with the phalangeal formula; X-rays were used because digit 5 is often atrophied, and/or digit 1 may be partially preserved as stated above, both inside the manus (see Vetter 2006). Second, we tested whether the number of digits in the manus differs between the sexes, as other authors have claimed (Eendebak 2001; Vetter 2006). Third, given that digit 1 may be atrophied, and it is more recognizable in individuals larger than 45 mm (approximately from 1 year of age), we tested whether the number of digits differs between age groups (juveniles vs. sub-adults and adults). Finally, we tested whether the number of digits in the manus differs between populations, genetic lineages, or the types of substrates that Hermann's tortoises inhabit.

Therefore, the most important contribution of this paper is that the number of individuals is significantly larger than that of previous works (here, 1669 individuals compared to three in Crumly and Sánchez-Villagra 2004; or 29 in Hitschfeld et al. 2008), allowing us to carry out reliable statistical analyzes. Secondly, our sample is very representative, including wild individuals of various ages, sexes and populations (i.e., six). This extraordinary amount of data is very important since the previous works used only museum specimens. Therefore, in most cases, it was nearly impossible to assess with confidence their subspecies, population, or sometimes even their age and sex.

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Materials and methods

Hermann's tortoise populations

This study was conducted on six wild populations of Hermann's tortoise, one in the Iberian Peninsula and five on Minorca Island (Tables 1 and 2). These populations inhabit a bioclimatic zone of evergreen oak trees, characterized by long dry summers, and wet warm winters (Cheylan 2001). The mainland Ebro Delta (DE) population is situated in the Punta de la Banya Reserve (Delta de l'Ebre Natural Park), a flat sandy salt marsh peninsula of 2514 ha, which is formed by dunes with small slopes fixed by psammophile and halophile vegetation (Bertolero 2002; Bertolero et al. 2007). On Minorca Island, Herman's tortoise occupies most of the island with a patchy distribution, mainly concentrated on the coast around the island, except in the most arid areas (Bertolero and Pretus 2012). We studied five populations near the towns of Ferreries (one population: NF), es Mercadal (three populations: RSM, DSM and DNM), and Alaior (one population: RSA). The exact locations are not specified owing to poaching concerns (Table 2). The NF population inhabits a hill formed by a consolidated sand dune with holm oak (*Quercus ilex*) and pine (*Pinus halepensis*) forest. The DNM and DSM populations inhabit dune vegetated systems. The RSM and RSA populations are located in calcareous ravines. The proportion of juveniles and sub-adults relative to adults varies between the populations and years, but all show high tortoise densities

Table 1 Comparison of number of visible digits in adult manus individuals of *Testudo hermanni hermanni* and number of digits on radiographies. Phalangeal formula is a sequence of numbers and/or letters. The formula 1-2-2-2-1 indicate that the first and fifth digit possess only a single phalanx, and the digits 2–4 possess two phalanges; in D-2-2-2-1 digit 1 only possess the distal carpal (phalanx and metacarpal have been lost), and in 0-2-2-2-1 digit 1 is entirely lost

Specimen	Number of visible digits on manus		Number of digits on radiographies		Phalangeal formula	
	Right	Left	Right	Left	Right	Left
17	5	5	5	5	1-2-2-2-1	1-2-2-2-1
25	4	4	4	4	0-2-2-2-1	0-2-2-2-1
26	4	4	4	4	0-2-2-2-1	0-2-2-2-1
34	4	4	4	4	0-2-2-2-1	0-2-2-2-1
35	5	5	5	5	1-2-2-2-1	1-2-2-2-1
36	4	4	4	4	0-2-2-2-1	0-2-2-2-1
38	4	5	4	5	0-2-2-2-1	1-2-2-2-1
39	5	5	5	5	1-2-2-2-1	1-2-2-2-1
51	5	5	5	5	1-2-2-2-1	1-2-2-2-1
66	5	5	5	5	1-2-2-2-1	1-2-2-2-1
68	5	5	5	5	1-2-2-2-1	1-2-2-2-1
91	4	5	4	5	1-2-2-2-1	1-2-2-2-1
93	5	5	5	5	1-2-2-2-1	1-2-2-2-1
101	4	5	4	5	0-2-2-2-1	1-2-2-2-1
116	5	5	5	5	1-2-2-2-1	1-2-2-2-1
127	5	5	5	5	1-2-2-2-1	1-2-2-2-1
134	5	5	5	5	1-2-2-2-1	1-2-2-2-1
135	4	4	4	4	0-2-2-2-1	0-2-2-2-1
142	4	4	4	4	0-2-2-2-1	0-2-2-2-1
146	5	5	5	5	1-2-2-2-1	1-2-2-2-1
158	5	5	5	5	1-2-2-2-1	1-2-2-2-1
166	4	4	4	4	0-2-2-2-1	0-2-2-2-1
184	5	5	5	5	1-2-2-2-1	1-2-2-2-1
187	5	5	5	5	1-2-2-2-1	1-2-2-2-1
189	5	5	5	5	1-2-2-2-1	1-2-2-2-1
1202	4	5	4	5	0-2-2-2-1	1-2-2-2-1
1235	4	5	4	5	D-2-2-2-1	1-2-2-2-1
3001	5	5	5	5	1-2-2-2-1	1-2-2-2-1
6006	5	5	5	5	1-2-2-2-1	1-2-2-2-1
6015	4	4	4	4	0-2-2-2-1	0-2-2-2-1
7000	5	5	5	5	1-2-2-2-1	1-2-2-2-1
11,071	4	5	4	5	0-2-2-2-1	1-2-2-2-1

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t2.1 **Table 2** Number of tortoises analyzed by population and ecological and genetic characteristics of each population

t2.2 Population	Habitat	Substrate	Genetic group	Males	Females	Juveniles	Total
t2.3 ED	Vegetated dunes with scrubs	Sand	Minorca 2	266	196	374	836
t2.4 RSM	Ravine	Calcareous and clay	Minorca 2	244	350	88	682
t2.5 DSM	Vegetated dunes with scrubs	Sand	Minorca 2	66	50	50	166
t2.6 NF	Forested fossil dunes	Sand	Minorca 1	214	184	34	432
t2.7 DNM	Vegetated dunes with scrubs	Sand	Minorca 1	272	192	378	842
t2.8 RSA	Ravine	Calcareous and clay	Mixed	134	96	150	380

(> 20 tortoises/ha in Minorca Island and 7 tortoises/ha at the Ebro Delta; Bertolero et al. 2018, AB unpublished data). Juveniles included in this study had a minimum shell length of 45 mm, to avoid any problems with visual digit counting due to the small size of the individual (hatchlings were not included because in some individuals, the claw of the first digit is very small, and needs careful inspection, which is difficult to see during field work).

These populations are from two distinct genetic lineages (Zenboudji et al. 2016): 1, populations NF and DNM are of continental lineage, which includes populations from Spain (Albera), France (Var), and Italy (called Minorca 1); 2, populations DE, RSM, and DSM are of insular lineage found only on Minorca and the Ebro Delta (called Minorca 2). The RSA population is an interbreed population of both lineages embedded in the area of the genetic group Minorca 2 (Massana et al., unpublished data). In the latter population, 1615 Minorcan tortoises of unknown genetic lineage were released by the NGO GOB-Menorca for conservation purposes between 1995 and 2004 (GOB-Menorca, personal communication).

Field methods and data collection

The number of claws of both forelimbs has been recorded since 2000 in the Ebro Delta population and from 2003 to 2006 in the Minorca Island populations, within the framework of a long-term research program of these populations (Bertolero 2002; Bertolero et al. 2018). Once captured, all tortoises were sexed (only sub-adults and adults), age-class classified according to the type of growth rings (juveniles of unknown sex, sub-adult and adult; Bertolero et al. 2011b) and marked individually with notches on the carapace. Additionally, from a previous study where females from the Ebro Delta were X-rayed to assess their reproductive status (Bertolero et al. 2007), radiographs of 32 individuals were carefully reviewed to assess their phalangeal formula (Table 1) to test if the number of claws could be a good proxy of the number of digits. Since the number of claws is equivalent to the number of digits (see "Results" section) we use the term number of digits throughout the text. The coding of phalangeal formulae follows Crumly and Sánchez-Villagra (2004), where, for example, 1-2-2-2-1 indicates that one phalanx is present in the digits 1 and 2, whereas two phalanges occur in digits 2, 3, and 4. The fieldwork conducted complies with Spanish laws. Handling and sampling of Herman's tortoises were authorized by the regional governments of Catalonia and the Balearic Island (Generalitat de Catalunya permit SF/058 and Govern de les Illes Balears permit CEP 08/2017).

Statistical analysis

Firstly, to test whether the number of digits that are externally displayed and the phalangeal formulae seen on the X-rays (Table 1) are equivalent, a paired sign rank test was calculated with a total of 32 females from both right and left forelimbs (i.e., $N = 64$).

Secondly, to determine whether the number of digits depended on sex, age, population, genetic lineage, or substrate type, three generalized linear mixed models (GLMM) were constructed with the number of the observed digit forelimbs (coded as 0 for four digits and coded as 1 for five digits) as the dependent variable. To investigate the effect of sex and population (model 1), this first model was constructed with sex (male vs. female) and population (DE, NF, RSM, DSM, DNM, and RSA) as fixed factors. To investigate the effect of age and population (model 2), a second model was constructed with age-class (juveniles vs. sub-adult + adult) and population as fixed factors. These two models were built separately since sex data was not available for juveniles, and thus not allowing to include the effect of sex, age, and population in a single model. Thus, the sample size of model 1 was lower (2264 forelimbs and 1132 individuals) than model 2 (3338 forelimbs and 1669 individuals). To investigate the effect of substrate types and genetic lineages, a third model (model 3) was conducted with the same dataset used in model 2 but with substrate (sand or calcareous and clay) and genetic lineage (mixed, Minorca 1 and Minorca 2) as fixed factors (3338 forelimbs and 1669 individuals) instead of the population factor. This third model was built since population (included in models 1 and 2) and genetic lineage are highly correlated and cannot be introduced in the same model. For all models,

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tortoise identity was included as a random factor (to count the right and left forelimbs). Tukey's HSD test was used for post hoc comparisons. The GLMMs were fitted with the maximum likelihood method, using the logit link function and with binomial error distribution. Analyses were carried out with the R 3.2.2 software (R Development Core Team 2015), with BSDA (Arnholt and Evans 2017), lme4 (Bates et al. 2015), and multcomp packages (Hothorn et al. 2008).

Results

We studied 3338 forelimbs belonging in 1669 individuals of six populations (Tables 1 and 2).

Does the number of digits displayed externally correspond to the phalangeal formulae?

The number of digits that are externally displayed and the phalangeal formulae seen on the X-rays did not significantly differ (S = 0; $n = 64$, $P = 1$), and thus, the number of claws could be a good proxy of the number of digits. In the 32 females studied by X-rays, we observed that all the individuals with five or four visual digits corresponded with what we observed in the radiographies (Tables 1 and 2). Indeed, individuals with five visual digits had the digits 2, 3, and 4 full (two phalanges) and digits 1 and 5 reduced (one phalanx = claw) and therefore displaying the following phalangeal formula (1-2-2-2-1: Fig. 2a). However, individuals with four visual digits had entirely lost digit 1 (both phalanges) but also the metacarpal I and distal carpal (0-2-2-2-1: Fig. 2d), with one exception only (right manus of the individual 1235: D-2-2-2-1). In this way, no individual with four digits of our sample showed a manus with the metacarpal I and distal carpal (M-2-2-2-1: Fig. 2b). We found only one specimen with four visual digits but presenting five digits in the X-rays. In this case, digit 1 was in an unnatural anatomical position (entirely blended in dorsal direction inside the manus), and therefore, it was a pathological individual.

As stated above, Hitschfeld et al. (2008) further found a fourth phalangeal formula, i.e., a "lost digit," when both phalanges are missing but the metacarpal I and distal carpal are present (M-2-2-2-1). The observed frequencies between Hitschfeld et al. (2008) and our study are significant ($Chi^2 = 12.01$, $df = 2$, $p = 0.002$; classes M-2-2-2-1 and D-2-2-2-1 pooled), with more cases of phalanges and/or metacarpal I loss in Hitschfeld's sample than in our sample from DE. However, when the three classes of phalangeal formulae recovered by Hitschfeld et al. (2008) are pooled (0-2-2-2-1, M-2-2-2-1, and D-2-2-2-1), both studies showed similar frequencies of individuals without an external digit ($Chi^2 = 0.119$, $df = 1$, $p = 0.730$).

Does the number of digits depend on sex, age, or population?

Although males tended to have a lower number of digits than females (61.5% of males and 65.6% of females had five phalanges), the gender factor was not significant (Table 3). As expected, the number of digits was not significantly different between juveniles and adults (61.3% of juvenile and 63.5% of adults had five phalanges; Table 4). Finally, the number of digits significantly differed between populations (Tukey's HSD tests, DNM vs. RSM: $p = 0.002$; DNM vs. RSA: $p = 0.002$; Fig. 3, Tables 3 and 4).

Does the number of digits depend on substrate or genetic lineage?

The number of digits was not significantly different between substrates (see Table 5). However, the number of digits significantly differed between genetic lineages (Table 5, Fig. 4). The mixed group showed the lowest proportion of individuals with five digits, Minorca 1 the lineage with a higher proportion with five digits, and Minorca 2 the one with an average proportion between Minorca 1 and the mixed group (Table 5, Fig. 4: Tukey's HSD tests, Minorca 1 vs. mixed: $p < 0.001$; Minorca 2 vs. mixed: $p = 0.06$; Minorca 1 vs. Minorca 2: $p = 0.002$).

Discussion

According to the evolutionary history of reptiles, the number of digits for a species seems to be a strongly stable feature, with the only known exceptions present in the lizard genus *Hemiergis* (Choquenot and Greer 1989) and in both tortoises of the *Testudo* and *Kinixys* genera (Crumly and Sánchez-

Table 3 Generalized linear mixed model showing the effects of sex and population on the number of digits in right and left manus (coded as 0 for four digits and as 1 for five digits) in *Testudo hermanni hermanni*. $N = 2264$ forelimbs belonging to 1132 individuals, including 1196 males and 1068 females. Abbreviations: Sex, coded as 0 for females and 1 for males; pop, population

Parameters of the selected model				Analysis of deviance		
Independent variable		Estimate ± SE	*p* value	χ^2	*df*	*p* value
	Intercept	1.34 ± 0.37	0.0002			
	Sex	− 0.62 ± 0.34	0.07	3.29	1	0.070
Pop	RSA	− 0.56 ± 0.62	0.36	19.60	5	0.001
	NF	0.80 ± 0.52	0.13			
	DE	0.80 ± 0.50	0.11			
	DSM	0.46 ± 0.80	0.57			
	DNM	1.96 ± 0.52	0.0002			

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t4.1 **Table 4** Generalized linear mixed model showing the effects of age and population on the number of digits in right and left manus (coded as 0 for four digits and as 1 for five digits) in *Testudo hermanni hermanni*. *N* = 3338 forelimbs belonging to 1669 individuals; 1074 juveniles and 2264 adults

t4.2	Parameters of the selected model				Analysis of deviance		
t4.3	Independent variable		Estimate ± SE	*p* value	χ^2	*df*	*p* value
t4.4		Intercept	1.27 ± 0.31	< 0.0001			
t4.5		Age	− 0.43 ± 0.32	0.18	1.83	1	0.18
t4.6	Pop	RSA	− 1.04 ± 0.52	0.04	32.37	5	< 0.0001
t4.7		NF	0.71 ± 0.49	0.15			
t4.8		DE	0.21 ± 0.42	0.60			
t4.9		DSM	0.09 ± 0.68	0.90			
t4.10		DNM	1.66 ± 0.44	0.0001			

Villagra 2004; Hitschfeld et al. 2008). In particular, the number of digits in the lizard *H. peronii* differs among populations of this species (Choquenot and Greer 1989). However, for tortoises, no detailed information is available in the literature regarding the population origin of the samples used in previous studies (Crumly and Sánchez-Villagra 2004; Vetter 2006; Hitschfeld et al. 2008). Thus, the present study, which focuses on *T. h. hermanni*, is the first to report intrapopulation variability in the number of visible digits in a reptilian species.

Several authors have reported that *T. hermanni* can display four or five digits in the forelimbs (Highfield 1988; Eendebak 2001; Vetter 2006; Hitschfeld et al. 2008; Bertolero 2014). Nevertheless, so far, the extent of this individual variation among and within populations has not been evaluated rigorously or in detail. Our results denote that the proportion of individuals with four visible digits varies according to populations and genetic lineage only (see below other relevant variables considered such as age, sex, or type of substrate).

Digital reduction can be achieved by two ontogenetic processes: complete loss of the digit (i.e., loss of all phalanges and related metacarpal and carpal bones) or a severe reduction (loss of all phalanges; Shapiro et al. 2007). In any case, both cases externally produce the same result in the Hermann's tortoise: showing four digits in the forelimbs. Given that the number of digits counted externally in the forelimbs of *T. h. hermanni* fits well with their real number of digits, the number of observed claws can be used as a good proxy of the real number of digits. Overall, our results confirm that 37% (*n* = 3338 examined extremities) of *T. h. hermanni* individuals have only four digits in

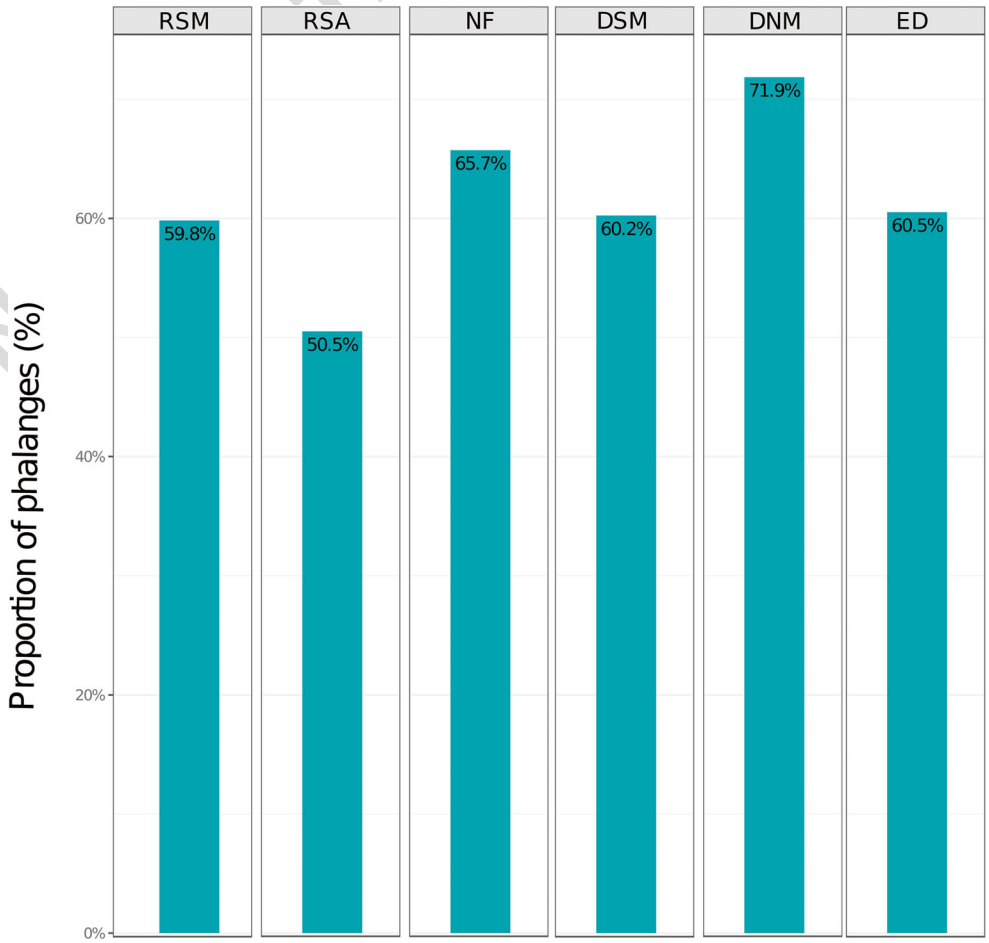


Fig. 3 Visible proportions of manus with five digits between the six studied populations (*N* = 3338 forelimbs belonging to 1669 individuals; 682 in RSM; 380 in RSA; 432 in NF; 836 in DE; 166 in DSM; and 842 in DNM)

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t5.1 **Table 5** Generalized linear mixed model showing the effects of substrate and genetic lineage on the number of digits in right and left manus (coded as 0 for four digits and as 1 for five digits) in *Testudo hermanni hermanni*. $N = 3338$ forelimbs

Parameters of the selected model				Analysis of deviance		
Independent variable		Estimate ± SE	*p* value	χ^2	*df*	*p* value
	Intercept	0.06 ± 0.40	0.87			
Substrate	Sand	0.07 ± 0.40	0.86	0.03	1	0.86
Genetic lineage	Minorca 1	2.34 ± 0.62	0.0002	16.53	2	0.0003
	Minorca 2	1.16 ± 0.51	0.02			

their forelimbs, but this percentage varies greatly among populations (range 28.1–49.5%). Even though our sample size for the phalangeal formulae is much more limited and for one population only, this reduction may merely be due to the entire loss of digit 1 (0-2-2-2-1: 31.3%, $n = 64$ extremities), or to the loss of the phalanges and/or the metacarpal I (D-2-2-2-1: 1.6%, $n = 64$ extremities). Apart from the two later variations, Hitschfeld et al. (2008) further reported a third case produced by the loss of the phalanges (M-2-2-2-1) for this subspecies but retaining the metacarpal I and distal carpal; however, it was absent in our population (DE).

Concerning digit variation, we did not find differences between ages, as expected, since digits and claws are fully developed when tortoises hatch. To date, no other works analyze age differences in the number of digits in extant and extinct Testudines. Similarly, we did not find differences between the sexes in the number of external digits, contrary to Vetter (2006) for Corsican tortoises (*T. h. h.*). Unfortunately, Vetter (2006) did not provide sample size or a statistical test; consequently, it is not possible to know if his observed frequencies are significantly different. The author also reported that only 44.2% of males showed five claws; however, our results denote that the five digits were present externally in greater proportion, although nonsignificantly different from the females, in all our studied populations (61.5% of males with five digits, all populations pooled). Furthermore, Eendebak (2001) indicated that the number of external digits has a clearly genetic component, since he observed in their enclosures that females with four digits produced 80% of hatchlings that also had four digits. Despite omitting to provide information about the sires and no further data were published to quantify his observations, our results indicate that a genetic component could be operating, since we found that lineage and population factors were the only significant ones in our analyses. However, we are not able to clearly disentangle both factors because some pairwise comparisons between the populations of the two pure lineages did not show significant differences (e.g., no significant differences between populations NF from lineage Minorca 1 and RSM from lineage Minorca 2: Table 4). Finally, the population factor does not

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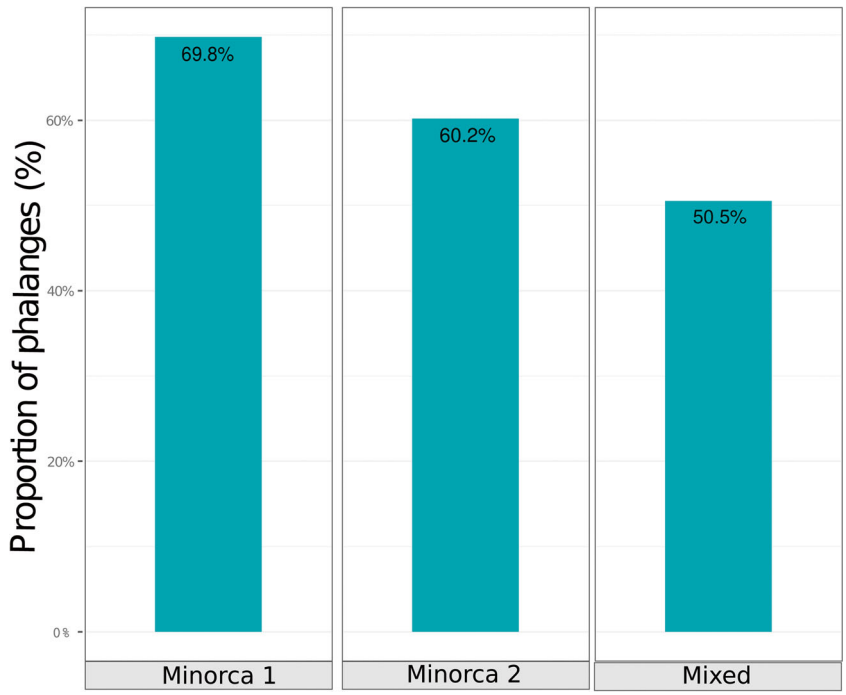


Fig. 4 Visible proportions of manus with five digits between genetic lineages ($N = 3338$ forelimbs belonging to 1669 individuals, 380 in mixed population, 1274 in Minorca 1, and 1684 in Minorca 2)

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encompass genetic characteristics only, but further ecological, environmental characteristics (e.g., substrate, but this was not relevant to explain the number of digits) and genetic drift.

Interestingly, the population with a high percentage of forelimbs with four external digits (49.5% at RSA) was also the population that showed a mixed genetic lineage (Massana et al., unpublished data). In contrast, populations of both pure lineages showed a lower frequency of four digits (30.2% for lineage Minorca 1 and 39.8% for lineage Minorca 2). Thereby, the RSA population does not on average show an intermediate percentage of forelimbs with four external digits between the two pure lineages. However, it is known that hybridization can produce phenotypic diversity (Grant and Grant 1994), and hybrids can show phenotypes that exceed those of either parental line (i.e., transgressive segregation; Rieseberg et al. 2003). Therefore, the RSA high percentage of forelimbs with phalanx reduction could be a case of phenotypic diversity resulting from the hybridization of the two well-differentiated genetic lineages (Zenboudji et al. 2016).

It has been suggested that the reduction in the number of digits in some groups of turtles (geoemydids and emydids) is related to the shift from aquatic to terrestrial locomotion (Zug 1971), since the reduction of phalanges has only been observed in terrestrial or semi-terrestrial testudinoids (see Minx 1992; Fritz et al. 2006b; Ludwig et al. 2007; Hitschfeld et al. 2008). Nevertheless, Hitschfeld et al. (2008) argued that the burrowing activity is not necessarily linked to the loss of digit 1, because the other species of *Testudo* can also lose this digit, but they do not show significant digging behavior. Despite this, it is known that the digging behavior in Hermann's tortoise can be important in dune environments, as in the Ebro Delta and Minorca populations, where tortoises dig cavities and short tunnels to rest, hibernate, or shelter from extreme weather conditions (A.B. personal observations). Our results support the Hitschfeld's hypothesis that the loss of digit 1 is not related to digging behavior since no differences between populations inhabiting in different substrates were found (sandy soil vs. calcareous and clay soils). According to Hitschfeld et al. (2008), the differences in carpal fusion patterns between *T. horsfieldii* and *T. hermanni* are actually related with their different modes of life. A more rigid hand skeleton could explain why the carpal and metacarpal bones fuse more extensively in *T. horsfieldii* (useful for excavating burrows). Conversely, fused bones in *T. hermanni* occur in the part of the hand that is exposed to the greatest mechanical strain during walking; therefore, congruent with species without extensive digging behavior.

Several studies have corroborated that many genes are involved in digit development (e.g., Hedgehog, BmP, Fgf, and Hox families: Merino et al. 1998; Litingtung et al. 2002; Sheth et al. 2012). Within these genes and regulatory factors, the *Sonic hedgehog* (SHH) gene seems to be most critical concerning the determination of the number of digits (see Litingtung et al. 2002; te Welscher et al. 2002). Even though the expression of the SHH varies between different lizard species, there is no doubt that it is more prolonged during ontogeny in lizards with more digits (Shapiro et al. 2003). The differences found in this study between both genetic lineages of tortoises and their hybrid population (RSA) in relation to individuals with only four digits may indicate gene expression differences that regulate digit formation. Only new research focused on the expression of genes involved in the development of the digits could clarify the causes of the variability observed in *T. h. hermanni*, which is beyond the scope of the present work. Moreover, the evolutionary significance of this intraspecific and intrapopulation variability in the number of digits remains an open question that merits further research.

Conclusions

Our study firstly reports on the intrapopulation variability in the number of visible digits in *Testudo hermanni hermanni*, the first tortoise species that this study has observed. Moreover, this work also shows that the number of observed claws can be used as a good proxy of the real number of digits. Contrary to previous studies, no digit variation could be attributed to sex. Neither age nor the substrate that the Hermann's tortoises inhabit could explain the digit variation. On the contrary, phalangeal reduction varied according to the studied populations and genetic lineages.

Our knowledge of *T. h. hermanni* individuals in their natural environment prove that digging behavior can be significant in the Ebro Delta and Minorca populations, and more specifically in dune environments. However, Hermann's tortoise populations in sandy substrates showed a lower percentage, although not statistically significant, of individuals with four digits than populations in calcareous or clay soils; therefore, supporting the hypothesis that the loss of digit 1 is not related to digging behavior.

In particular, the RSA population from Minorca was the only one that displayed a mixed genetic lineage and the highest percentage of forelimbs with phalanx reduction. This phenotypic diversity could be explained through a hybridization process of two well-differentiated genetic lineages. Apparently, the differences obtained between both genetic lineages of tortoises and their hybrid population (RSA) seem to indicate differences in the gene expression that regulates digit formation. However, gene expression involved in the development of the digits needs further investigation in order to better explain the intraspecific and intrapopulation variability observed in *T. h. hermanni*.

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Author contributions MFR and CT performed the statistical analyses; AB and AHL coordinated the data collection; AB, AHL, and MFR conceived and designed the experiment; AB, AHL, MFR, and CT analyzed the data; AB, AHL, and MFR wrote the paper.

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Data availability All data generated and analyzed during this study are included in this published article.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Q2. Ref. "McHorses et al. 2017" is cited in the body but its bibliographic information is missing. Kindly provide its bibliographic information in the list.

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