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RESEARCH

Captive-introduced tortoises in wild populations: can we identify them by shell morphology?

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Abstract

Direct exploitation and wildlife trade pose significant threats to global biodiversity, particularly impacting reptiles, such as tortoises, which are highly vulnerable. Moreover, the releases of captive-held animals into the wild can lead to potential negative consequences, including genetic introgression, disease transmission, and parasite spread. Therefore, diagnostic assessments are essential to evaluate the impact of release practices. In this study, we analyzed the shell morphology of *Testudo graeca* tortoises to assess the effectiveness of morphological analysis in identifying captive-introduced tortoises and quantifying tortoise releases in SE Spain. Despite being illegal, the cultural tradition of keeping and breeding tortoises at home persists in this region. Principal component analyses identified significant morphological differences between captive and wild tortoises. Captive tortoises generally exhibited larger shell sizes and greater morphological variability compared to wild tortoises. Accordingly, linear discriminant analyses accurately identified approximately 99% of wild tortoises, but the identification of captive tortoises was only 50% accurate because some captive tortoises show shell morphologies identical to wild ones. These results likely reflect the diverse origins and growth conditions of captive tortoises. Although this is likely an underestimation, our approach classified 7% of 125 wildlife tortoises across 13 natural sites such as captive-introduced tortoises. These findings highlight the potential impact of tortoise releases in wild populations and underscore the value of morphological analyses as an initial tool for diagnosing such impacts. Furthermore, integrating genetic and isotopic methods could further enhance the precision of these assessments.

Keywords Conservation management · Captive-introduced animals · Management tools · Morphological analyses · *Testudo graeca* · Wildlife trade

Introduction

Direct exploitation and wildlife trade constitute the second main driver of global biodiversity loss in recent decades (Jaureguiberry et al. 2022). The global trade industry raises significant concerns regarding unsustainable harvesting, zoonotic infections, and animal health, as well as the introduction of invasive species (Chomel, 2007; Masin, 2014). Moreover, it evokes ethical and moral considerations stemming from inequalities and even criminal actions (Warwick 2014; Brito et al. 2018; Lunstrum and Givá 2020). Among traded species, reptiles stand out due to their popularity as exotic pets, posing a significant risk to their conservation (Cox et al. 2022; Challender et al. 2023). Illustrating this, it has been estimated that online reptile trade encompasses over 35% of species (Marshall et al. 2020). Crocodiles and chelonians are particularly vulnerable, requiring urgent and

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targeted action to prevent extinctions. However, they are significantly threatened by species trade (Cox et al. 2022; Rodríguez-Caro et al. 2023).

Tortoises represent one of the most threatened groups among chelonians, with poaching and trade ranking among their primary threats (Stanford et al. 2020). According to the IUCN, over 65% of tortoise species are estimated to be threatened (Graciá et al. 2020; Turtle Taxonomy Working Group, 2021); and global data suggests an increasing number of specimens trafficked annually (Luiselli et al. 2016; Nijman and Bergin 2017). Trade and tortoise captivity also include illegal breeding, which increases the number of specimens held in captivity (e.g. Pérez et al. 2011; Edwards and Berry 2013; Graciá et al. 2020). Moreover, illegal and uncontrolled releases of captive animals into wildlife populations have been documented, with associated risks such as the spread of diseases and parasites (Feldman et al., 2006; Todd 2011; Chávarri et al. 2012; Aiello et al. 2014), as well as genetic exogamy (Zenboudji et al. 2016; Graciá et al. 2017). In recent decades, public conservation agencies have even carried out captive-tortoise releases due to the challenges of accommodating numerous animals from illegal captivity under proper welfare conditions (e.g. Pérez et al. 2012a; Edwards and Berry 2013; Graciá et al. 2020). However, reintroductions and translocations must prioritize conservation objectives, not just welfare considerations, especially when captive and wild animals come into contact (Pérez et al. 2012a; Edwards and Berry 2013; Graciá et al. 2020).

In this study, we assess the utility of shell morphology in identifying captive-introduced tortoises. Shell morphology reflects adaptation and long-term plastic growth in these long-lived animals (Dosik and Stayton 2016). Therefore, morphometric studies have been valuable for investigating sexual dimorphism in size and shape (SSD and SShD) (e.g. Djordjevíc et al. 2011; Benelkadi et al. 2022; Tiar-Saadi et al. 2022; Semaha et al. 2024) and for exploring the impact of selective pressures (e.g. Lagarde et al. 2001; Ben Kaddour et al. 2005; Rodríguez-Caro et al. 2013; Chiari et al. 2017). These analyses have also been used to understand morphological divergence among lineages (e.g. Carretero et al. 2005; Chiari et al. 2009) and testing biogeographic patterns like Bergmann's and Rensch's rules (e.g. Angielczyk et al. 2015; Werner et al. 2016; Semaha et al. 2024). However, identifying captive-introduced individuals remains challenging (e.g., Paquette and Lapointe 2007; Gerlach 2011), and there are currently few viable alternatives to morphological examination, such as genetic and stable isotope analyses. While these alternative methods are technically demanding and involve laboratory costs (e.g., Graciá et al. 2017; Hopkins et al. 2022), morphological examination may be a feasible option due to the significant impact captivity can have on shell morphology (Wiesner and Iben 2003; Gerlach 2004). Furthermore, genetic analyses may not be sufficiently sensitive if captive-introduced tortoises originate from near populations (Ogden et al. 2009) as occur with the isotopic signal on the diet, which could even be similar to wild animals if they live under semi-free conditions (Wood 2012). On the other hand, identifying captive-held tortoises by their shell morphology is challenging, but some exhibit abnormal growth patterns due to phenotypic plasticity and changes in activity periods and diet (Wiesner and Iben 2003; Gerlach 2004; Fritz et al. 2007). The most distinctive indicator of abnormal growth in captivity is pyramidal growth syndrome, where the scutes develop a pyramidal shape due to dietary imbalances and environmental conditions (Wiesner and Iben 2003; Gerlach 2004). This condition is rare or absent in wild populations (Heinrich and Heinrich 2016). However, the shell morphology of captive tortoises may resemble normal if they have been kept in captivity for brief periods or if they were captured as adults, when the tortoise growth is minimal (Rodríguez-Caro et al. 2013; Semaha et al. 2024).

Our aim is to assess the extent to which the morphology of captive and wild tortoises differs and whether it can be used as a tool for tracking the effects of introductions. We particularly focus on the spur-thighed tortoise (*Testudo graeca*), one of the most poached and trafficked animals in the Mediterranean region since at least the 1800s, with tens of thousands exported annually during the mid-20th century for the pet trade (Lambert 1969; Inskipp and Wells 1979; Highfield and Bayley 1996; Znari et al. 2005; Türkozan et al. 2008; Nijman 2010; Salinas et al. 2011; Mucci et al. 2014; Tiar et al. 2019; Segura and Acevedo 2019; Segura et al. 2020). This species is listed as Vulnerable by the IUCN (International Union for Conservation of Nature) and included in Appendix II of the CITES convention. However, in SE Spain, as in other parts of its range (e.g. Segura et al. 2020), the species is subjected to cultural practices that resulted in high levels of trade, collection, ownership, and breeding (Pérez et al. 2004, 2012b; Salinas et al. 2011; Graciá et al. 2013, 2020). After more than 20 years of research in SE Spain, some animals of this species have been observed exhibiting signs of previous captivity, both markings on the shell and abnormal size or shape. We hypothesize that studying differences in the shell morphology of wild and captive tortoises will allow to detect a significant number of introduced tortoises in certain locations in SE Spain.

Materials and methods

Study system

Testudo graeca is distributed throughout North Africa, ranging from the Atlantic coast of Morocco to Northeast Libya, with small populations found in the Doñana National Park (southwest Spain), Majorca, and parts of SE Spain (Graciá et al. 2017). The primary population of spur-thighed tortoises in Europe is located in SE Spain, where the wild population covers approximately 2600 km^2 of semi-arid coastal mountains between the provinces of Almeria and Murcia (Fig. 1). In Spain, the spur-thighed tortoise is protected at both national and regional levels (Pleguezuelos et al. 2002), and since 2015, illegal ownership has been punishable by prison sentences (Law 1/2015, of March 30). Morphologically, this population exhibits a significantly smaller adult size compared to other populations and a reversal of Bergmann's rule, with no strong effects of precipitation and temperature observed in the study area (Semaha et al. 2024).

Between 2004 and 2023, we measured the shells of a total of 318 adult tortoises, including individuals from wild populations, captive environments, and those known to have been introduced into the wild. We do not anticipate differences based on sampling year, as shell morphology reflects long-term growth. The wild dataset consisted of 137 wild tortoises of native origin (62 females and 75 males; hereafter referred to as "wild tortoises"), collected from 17 remote locations where no evidence of tortoise release was observed. These sampling locations were evenly distributed to represent the species' distribution in SE Spain and to avoid biases associated with the gradient of Bergmann's rule. The captive dataset consisted of 57 captive and known captive-introduced tortoises (30 females, 27 males; hereafter referred to as "captive tortoises"), from six sites, including zoos, wildlife recovery centers, and locations with semi-free or free conditions. These known captive-introduced

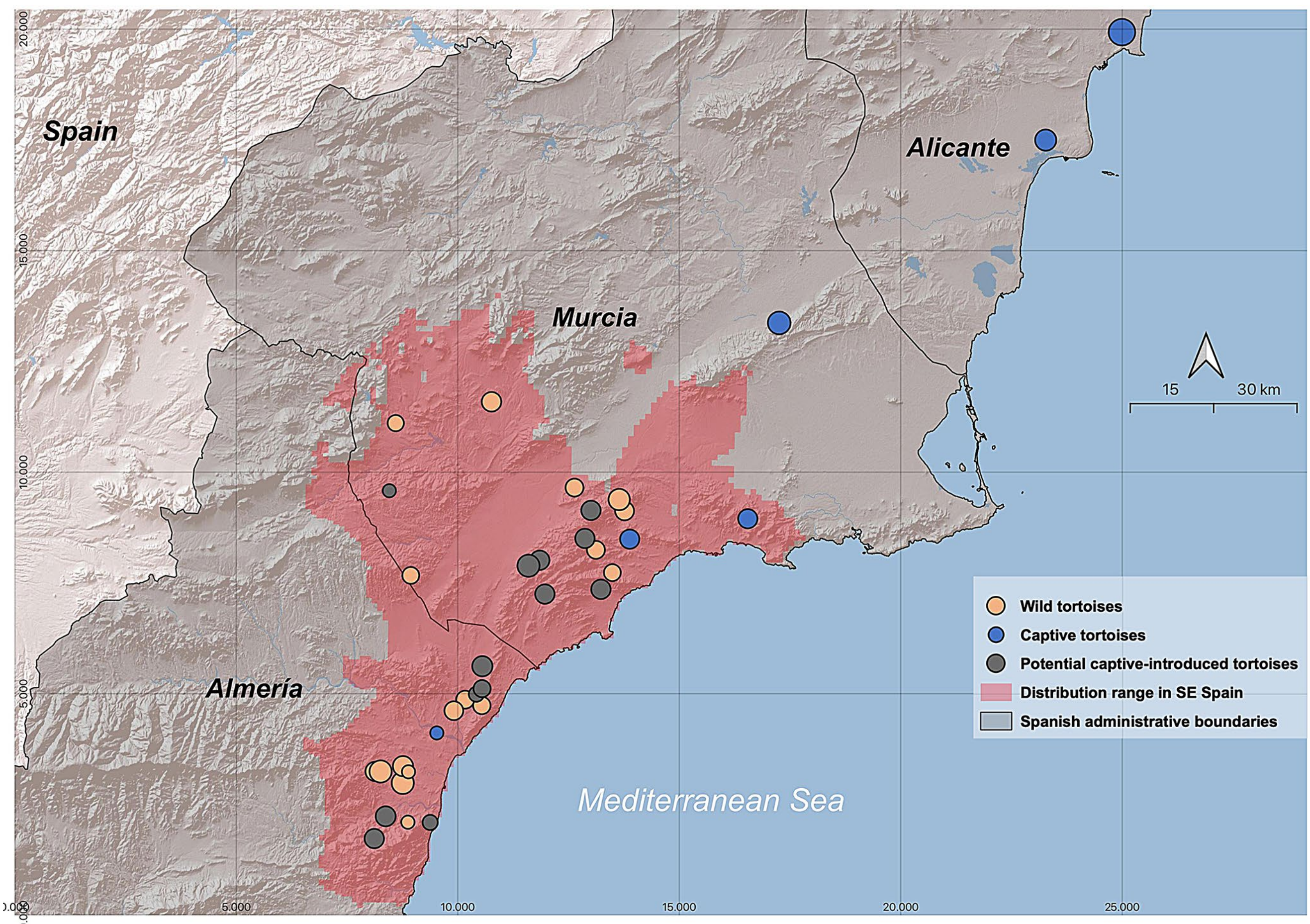

Fig. 1 Sampling distribution of *Testudo graeca* tortoises in SE Spain. The orange points represent 17 sites at the wild population, where no captive tortoises have been recorded or detected. The blue points refer to 6 sites where captive individuals were sampled (two recovery centers, a Zoo Park and 3 wild sites where captive tortoises were released). Finally, grey points refer to sites where we found some individuals with evidence of captivity, or where there are records of releases of captive tortoises. The size of the points is proportional to the number of individuals analysed at that sampling site

tortoises were sampled in areas where public conservation agencies or conservation associations had released captive tortoises, or were sampled in the wild and identified as captive-released through genetic analysis (Graciá et al. 2013). Finally, we compiled a third dataset with 125 tortoises from 13 sites suspected of containing captive-introduced individuals (hereafter referred to as “potential captive-introduced tortoises”). These sites were selected because had anecdotal evidence of tortoise releases noted by our personnel during fieldwork, including signs of abnormal growth and the presence of painted or previously marked animals. It is important to note that the wide majority of tortoises in this group are likely wild, as they were sampled within the natural distribution the species’ range.

During fieldwork, each tortoise was individually marked, weighed, its shell measured, and released at the capture site. Wild tortoises were visually located during standardized transects. Measurements were obtained using a 500 mm digital caliper. The selected biometric variables (see Fig. 2 and Table S1) included plastron length (PL), straight carapace length (SCL), carapace height (H), anal suture (AS), femoral suture (FS), anterior width (AW), posterior width (PW), femoral width (FW), and ventral suture length (VS). Sex determination was conducted based on external secondary characteristics, following the methods outlined by Willemsen and Hailey (2003).

Statistical analyses

To describe the shell morphology of adult captive and wild tortoises, we conducted two principal component analyses (PCAs), one for each sex. PCA simplifies complex multidimensional data into a set of eigenvectors or components (Klingenberg 2010; O’Higgins, 2011), facilitating the interpretation of morphological differences among groups. The PCA included all biometric measurements, and the resulting scores summarized the morphological variability of each group. We used a correlation matrix and retained only those axes that explained > 5% of deviance. Subsequently, we performed a separate multiple analysis of variance (ANOVA) for each sex, with the scores of the retained PCA axes as the dependent variable and the tortoise’s origin (captive or wild) as the independent variable.

To evaluate shell morphology as an indicator of tortoise captivity, we conducted Linear Discriminant Analysis (LDA) using all biometric measurements. LDA involves creating linear combinations of predictor variables optimized to effectively discriminate between pre-established groups. This functionality enables the assignment of new cases to these groups. Independent LDAs were performed for each sex, with captive and wild tortoises as known groups. The accuracy of LDAs was assessed by evaluating the predicted group assignments of these same tortoises. Subsequently, we conducted assignment analyses using the resulting LDA functions to classify the group of potential captive-introduced tortoises, thereby quantifying tortoise releases across the 13 sampled sites. Furthermore, we took into consideration the observations of our technical personnel when interpreting the results. All statistical analyses were performed using R project version 4.2.0.

Results

Upon analysing the 194 tortoises with known origins (137 wild and 57 captive tortoises), we confirmed the presence of morphological differences between captive and wild groups in both males and females. The PCA results effectively

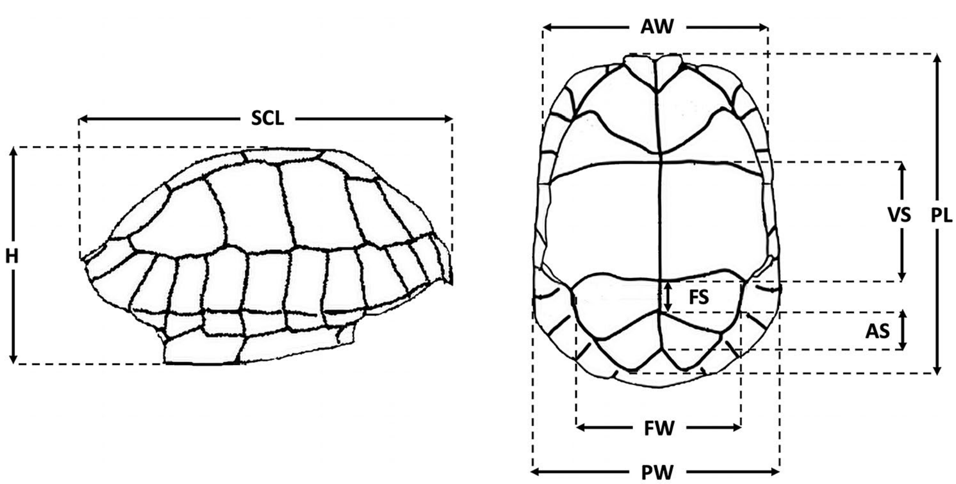

Fig. 2 Graphical representation of the nine morphometric measurements registered for *Testudo graeca*tortoises. The selected measures included plastron length (PL), straight carapace length (SCL), carapace height (H), anal suture (AS), femoral suture (FS), anterior and posterior width (AW and PW), femoral width (FW), and ventral suture length (VS). Definitions of the measures are provided in Table S1

described the species' morphology with two axes for both sexes, as illustrated in Fig. 3. In females, PC1 (83.6% deviance) primarily reflected general shell size, with larger measurements (such as SCL, PL, or H) associated with higher positive values of this axis. Meanwhile, PC2 (9.1% deviance) represented the trade-off between two suture lengths in the plastron, as depicted in Fig. 3, showing a positive relationship with FS and a negative correlation with AS. Similarly, the PC axes for males exhibited a comparable pattern, with PC1 (82.4% deviance) linked to overall size and PC2 (9.0% deviance) representing the trade-off between FS and AS (see Fig. 3). Notably, captive tortoises displayed consistently larger shell sizes in both sexes. Furthermore, significant positive correlations were observed between PC1 and the tortoise's group for both males and females ($p<0.001$ in ANOVA tests for both analyses, $F\ value=22.58$ for females and 82.91 for males). However, no significant relationship was found between PC2 and the tortoise's group for either sex. Table S2 shows the descriptive statistics of the morphological measurements used.

After describing the relationship between individuals' group and shell morphology, Linear Discriminant Analysis (LDA) was conducted to evaluate the discriminatory capacity of shell morphological measurements in distinguishing between wild and captive individuals. Separate analyses were conducted for males and females, with captive and wild tortoises serving as reference groups. The LDA exhibited a classification accuracy of 100% for wild males and approximately 67% for captive males. Similarly, for females, the LDA achieved an accuracy of approximately 98% for wild tortoises and 53% for captive tortoises. Notably, the LDA demonstrated a high level of precision in categorizing wild tortoises into their respective groups, with only one female displaying misclassification, indicated by an assignment percentage of 0.6 to the captive group.

As a result, in subsequent analyses aimed at quantifying introduced tortoises in the wild, we classified animals as captive-introduced if their assignment values to the captive group exceeded 0.6. Our findings revealed nine captive-introduced tortoises (three females and six males) out of the 125 analyzed. This represents 7% of the tortoises sampled across the 13 sites suspected of harboring introduced animals (see Fig. 4). Notably, personnel who conducted measurements documented signs indicative of captivity for eight of these nine tortoises. These signs included abnormal growth of the vertebral and costal scutes, and in two cases,

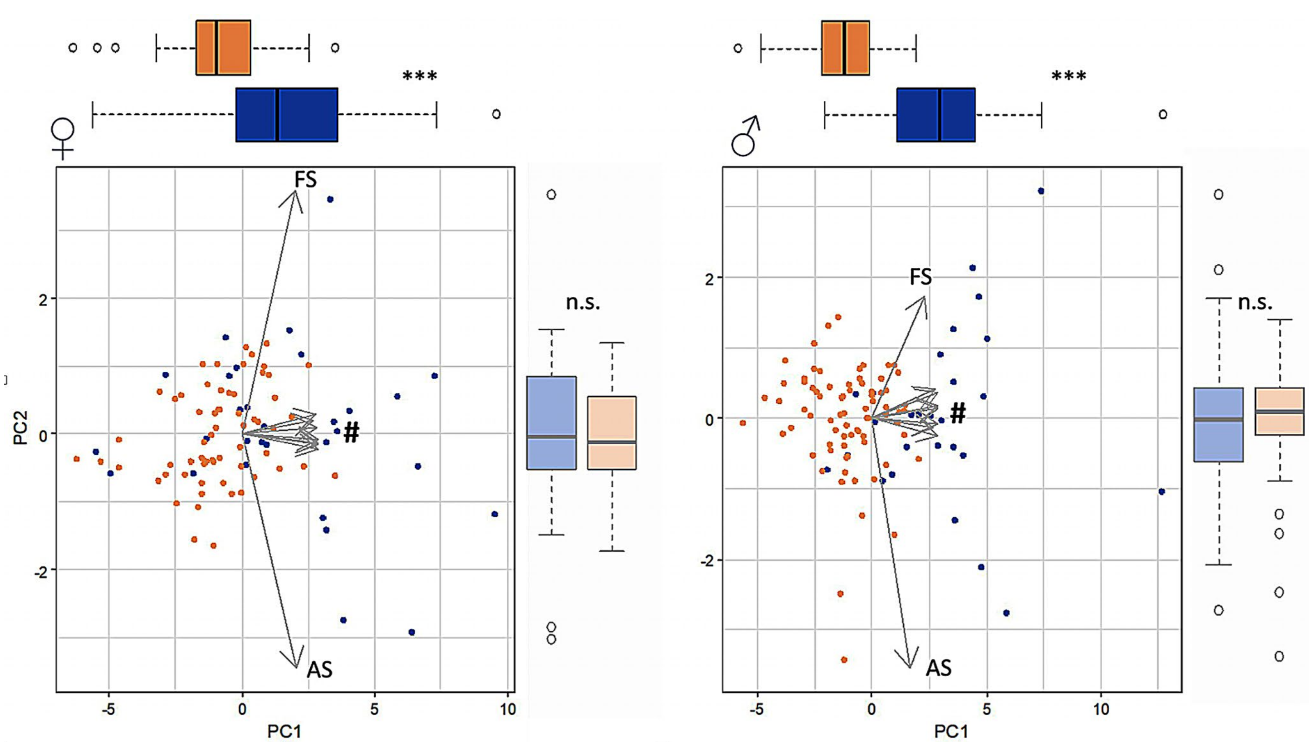

Fig. 3 Captive *Testudo graeca* tortoises are larger The PCA analyses accurately describe the morphology of females (left) and males (right) through two axes of variation. In females, PC1 accounts for 83.6% and PC2 for 9.1% of the variance; while in males, PC1 explains 82.4% and PC2 9.0% of the variance. Each dot in the 2-D space represents an individual, color-coded by origin (orange for wild tortoises and blue for captive tortoises). Arrow lengths indicate the loading of each variable on the respective principal component axis. Symbol "#" represents the following variables: PL, SCL, H, AW, PW, FW, and VS. For both males and females, PC1 exhibited a significant association with the group ($p<0.001$) in ANOVA tests, while PC2 did not show significance in either sex. The sample size for the analyses was 92 for females and 102 for males

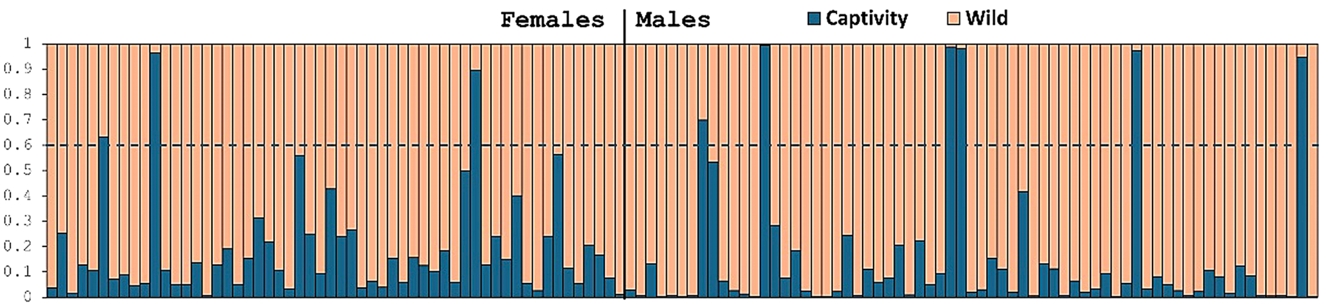

Fig. 4 The individual assignment analyses using the discriminant functions reveal 9 introduced *Testudo graeca*tortoises. The 125 tortoises sampled at sites suspected of harbouring released animals were analysed and represented by each bar, with the blue bars indicating the probability that each specimen was captive-released. Using a threshold of 0.6, we identified three females and six males as introduced from captivity

Fig. 5 Photos illustrating the diversity of phenotypes found in captive *Testudo graeca*tortoises. Left: wild tortoise sampled in SE Spain. Center: captive tortoise with abnormal morphology. Right: captive tortoise displaying a wild appearance. All individuals photographed are male

carapace marks indicating prior captivity before release. Conversely, another 20 tortoises displayed similar annotations related to abnormal growth but were not categorized as captive by the LDA.

Discussion

The identification of captive individuals within wild populations is crucial for diagnosing and, if possible, reversing the problems associated with uncontrolled and illegal trade in threatened species. This study investigated the utility of morphological shell measurements in distinguishing between captive and wild tortoises. By analyzing 194 spur-thighed tortoises (*Testudo graeca*), we revealed morphological disparities, showing that captivity primarily affects overall general size rather than specific body proportions. Captive tortoises also exhibited greater morphological variability. Despite this, our analyses identified 9 released tortoises among 125 sampled in the wild.

Size emerged as the most distinguishing trait of captivity, likely influenced by overfeeding and extended activity patterns under captive conditions, such as avoiding hibernation or estivation (Wiesner and Iben 2003; Gerlach 2004). The wild population of tortoises in SE Spain was noted as the smallest among *T. graeca* populations (Semaha et al. 2024), potentially aiding in the identification of individuals from different populations or lineages. However, it is important to note that the analyses conducted here do not account for the age of the individuals, which is desirable when comparing sizes (Semaha et al. 2024). The abnormal growth observed in captive tortoises hinders the ability to estimate their age, unlike the case with wild individuals, for which age estimation is feasible (Rodríguez-Caro et al. 2015).

While our Linear Discriminant Analyses (LDA) accurately classified 99% of wild individuals, only around 50% of captive tortoises were assigned correctly to the captive group. These results illustrate that some tortoises kept in captivity exhibit morphologies entirely similar to wild tortoises (Fig. 5). The greater variability in morphology among captive individuals underscores the intricate effects of captive conditions on tortoise morphology, highlighting the strong phenotypic plasticity exhibited by *T. graeca* tortoises (Fritz et al. 2007). But this variability also reflects the different pathways through which tortoises enter captivity in SE Spain, from those born and raised in captivity to those captured from the wild or traded (Pérez et al. 2004, 2011, 2012b, c). Tortoises captured as adults from the wild show minimal growth, so their morphology is not influenced by captivity, as they have already reached their asymptotic size before capture (Rodríguez-Caro et al. 2013; Semaha et al. 2024). In contrast, individuals born and raised in captivity experience aberrant growth during their peak growth phases (Wiesner and Iben 2003; Gerlach 2004). It has been described that the adult *T. graeca* trade accounts for approximately 20% of its total, with larger tortoises fetching even higher prices when sold (Nijman & Berjin, 2017; Tiar et al. 2019; Segura et al. 2020). Demographic effects have even been observed in wild populations due to the removal of adults, which would otherwise have very high survival rates (Znari et al. 2005; Pérez et al. 2012b, c; Tiar et al. 2019; Segura and Acevedo 2019).

The risks associated with captivity are not limited to tortoise poaching, they also involve the release of these tortoises into the natural environment due to health and genetic concerns (Chávarri et al. 2012; Graciá et al. 2013, 2017, 2020). When necessary, individuals selected for reintroductions or population reinforcements must a priori meet all the requirements of translocations aimed at conservation objectives (Pérez et al. 2012a). However, past tortoise releases often lacked clear conservation goals, focusing instead on animal welfare (Pérez et al. 2012a). The use of morphological approaches to detect introduced tortoises could be considered in the species' conservation and recovery plans, always implemented by qualified personnel to manage specific situations, such as controlling emerging disease outbreaks. In this regard, our morphological approach is safe, as it poses no risk to wild populations, but it has its limitations. The risk of misclassifying a wild tortoise as captive is approximately 1%, though it should be acknowledged that many introduced individuals will not be identifiable.

To know the current number of tortoises held in captivity, as well as the trends in poaching and releases, recent, specific studies are required. In the meantime, our findings suggest that these releases occur at a significant proportion (Pérez et al. 2012b, c), though the numbers documented should be interpreted with caution when extrapolated to the entire SE Spanish population. Our study reveals 7% of captive tortoises introduced in wild populations across 13 natural sites. But, on one hand, we have sampled sites with suspected tortoises in our analyses, thereby increasing the likelihood of finding introduced tortoises. And, on the other hand, we have found that a significant proportion of introduced tortoises may have gone unnoticed in our analyses. The frequent release of captive tortoises into the wild in SE Spain, could pose detrimental consequences for native populations, including the potential transmission of bacterial and viral pathogens (Feldman et al., 2006; Todd 2011; Aiello et al. 2014), and parasites (Chávarri et al. 2012). For example, while ticks are frequent in the African population of *T. graeca* (Gharbi et al. 2015; Tiar et al. 2016; Najjar et al. 2020; Perera et al., 2022; Laghzaoui et al. 2022; Segura et al., 2019, 2023), and we identified ticks in some recovery centers sampled for this work (pers. communication); yet no ticks were found in wild tortoises in SE Spain (Perera et al., 2022). Additionally, the release of captive tortoises may lead to the loss of natural genetic diversity through introgression across lineages (Graciá et al. 2017). Therefore, releasing domestic or captive tortoises into the wild would not only not resolve the issues caused by the mean threats of the species such as habitat loss and fragmentation, poaching, climate change or fires, but could also increase other concerns (Graciá et al. 2020). Translocations, population reinforcements, or introductions of tortoises should be regarded as management strategies incorporated into species recovery plans, meticulously designed to ensure conservation objectives, while also minimizing potential health and/or genetic risks (Pérez et al. 2012a; Graciá et al. 2020). In the development of these management programs, consideration should also be given to how manage already established that have been subjected to massive introductions of tortoises carried out by public conservation agencies (Graciá et al. 2020). Such considerations should be based on monitoring results, undoubtedly necessary to clarify potential effects on the wild population.

Our study aligns with previous research highlighting the impact of captivity on reptile morphology. Studies on crocodiles, snakes, turtles and lizards have reported similar observations, such as disparities in morphology and altered skeletal dimensions in captive individuals compared to wild ones (e.g. Schaerlaeken et al. 2012; Gerlach 2011; Drumheller et al. 2016; Ryerson 2020). For instance, wild American alligators (*Alligator mississippiensis*) display distinct morphological traits, with captive alligators characterized by broader and flatter skulls (Drumheller et al. 2016). In general, captive diets are often richer in nutrients and calories compared to wild food sources, potentially leading to faster growth and larger body sizes (Lapid et al. 2005; Furrer et al. 2004; Ritz et al. 2010). Additionally, reduced activity levels together with lower thermoregulatory costs may contribute to increased enlargement in captivity. This variability can be further influenced by factors such as immune health and parasitic infections, which can significantly alter physiological and reproductive outcomes, as have been linked to pyramidal growth in *T. graeca* tortoises (Chávarri et al. 2012). Our findings suggest that morphological analysis, which could be further enhanced through techniques like geometric morphometrics, can serve as a valuable tool for diagnosing the impacts of species trafficking. Further research is necessary to thoroughly explore also the potential of integrating morphology, genetics, and isotope analyses for such investigations (Hinsley et al. 2016; Dufour et al. 2022).

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10344-024-01893-1.

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Author contributions All authors contributed to the conception and design of the study. Fieldwork was conducted by MJS, EG, RCRC, and AG. Data analysis was performed by MJS, EG, and RCRC. MJS took the lead in writing the manuscript, with assistance from EG and RCRC. Subsequently, all authors participated in manuscript revision, contributed additional supportive information, and provided final approval for publication.

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Data availability The data supporting the findings of this study will be provided by the corresponding author upon reasonable request.

Declarations

Ethical approval The sampling protocols were approved by the Ethic Committee of the Miguel Hernandez University and Generalitat Valenciana (DBA-AGC-001-12-DBA.EGM.01.22) in accordance with approved guidelines.

Competing interests The authors declare no competing interests.

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