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A skeletochronological study of age in the Caspian pond turtle, *Mauremys caspica caspica* (Testudines: Geoemydidae) in Golestan Province, Iran

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ABSTRACT

We estimated age of the Caspian pond turtle, *Mauremys caspica caspica* in Golestan Province with the skeletochronological method, using specimens collected by nets in natural habitats such as ponds and fish farms. In adult *M. c. caspica*, lines of arrested growth (LAGs) were clearly discernable in the phalangeal and fibula bone cross-sections. The median age of *M. c. caspica* in the examined Golestan Province population (n = 22) was calculated 9.6 years (4-12) in males (n = 10), and 10.16 years (4-13) in females (n = 12) for the first time. The maximum straight carapace length (SCL_2) was in the range of 83.74–221.41 (mean ± SD = 178.39 ± 48.89) mm in males and 103.02–236.84 (193.52 ± 41.32) mm in female specimens. Significant correlation was found between body size and age ($p < 0.001$). No difference was found in age composition between the sexes. Our study showed that skeletochronological method can be successfully applied to the Caspian pond turtle based on the clear arrest of growth in the hibernation period.

Key words: Age, Caspian pond turtle, Golestan Iran, LAGs, *Mauremys caspica*, Skeletochronology.

INTRODUCTION

The Caspian pond turtle, *Mauremys caspica* (Gmelin 1774), is belonging to family Geoemydidae. This freshwater turtle is widespread throughout the Middle East (Yadollahvand & Kami 2014). Three subspecies were reported in Iran. *M. c. caspica* (Gmelin 1774) is widely distributed in Golestan, Mazandaran, Guilan, Ardabil, East and West Azarbaijan provinces; *M. c. ventrimaculata* in Fars and Esfahan provinces and *M. c. siebenrocki* in Bushehr, Kordestan, Kermanshah, Lorestan, Ilam, Khuzestan, Fars and Chahar Mahal Va Bakhtiari provinces (Fritz & Wischuf 1997; Kami *et al.* 2006; Fritz *et al.* 2007; Rastegar-Pouyani *et al.* 2008; Safaei-Mahroo *et al*. 2015).

There is limited information on age structure, age at maturity, growth, and longevity of turtle populations.

The ability to relate age to reproductive effort is integral to studies of population dynamics, which in turn can inform management strategies for those organisms considered at risk (Chaloupka & Musick 1997; Heppell *et al.* 2003a). Techniques used to estimate the age of turtles include analysis of body size (Frazer & Ehrhart 1985), weighing eye lenses, counting growth zones on plastral scutes (Gibbons 1983), and skeletochronology (Zug 1990; Klinger & Musick 1992; Thomas *et al.* 1997).

In turtles and tortoises, there are three main approaches to estimate age: skelotochronology (Zug *et al.* 1986), growth models (Carr & Goodman 1970) and growth ring counts (hereinafter GRC). Each one of the methods has its pros and cons (Curtin *et al.* 2008; Rodríguez-Caro *et al.* 2015).

Three common methods can be employed to directly validate the annual deposition of skeletal growth marks: the study of known-age animals, mark-recapture studies, and mark-recapture studies that incorporate fluorescent marking. All of these methods have been applied to turtles (Castanet & Cheylan 1979; Coles *et al.* 2001; Snover *et al.* 2007b). Although skeletochronology can provide reliable data about age, it is an invasive method which just can be used with death animals and also shows some biases (Curtin *et al.* 2008; Rodríguez-Caro *et al.* 2015). Bone

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formation and remodeling rates are hormonally controlled and synchronized to circadian patterns (Simmons 1992).

Parathyroid hormone (PTH), calcitonin, and vitamins A, C, D, and K have been found to influence rates of bone formation and remodeling (Narbaitz *et al.* 1991). In temperate climates, each year of growth during the warm season, and the subsequent slowing of growth in hibernation, commonly called Lines of Arrested Growth (LAGs), can be counted for age determination (Zivari & Kami 2017). However, there is substantial evidence that the spring surge in growth rates is also under endogenous control, such that animals maintained in captivity also demonstrate this pattern (Snover & Rhodin 2007). LAGs are generally very clear and easily studied, as suggested by Kleineberg & Smirina (1969), the first convergence in the distance between the LAGs was considered as the sign of reaching sexual maturity.

Hard bone tissues cannot grow through internal expansion, but rather they grow by appositional processes (on periosteally derived cortical bone) with the deposition of new tissue on the surface together with endosteal resorption (Enlow 1969). This process of resorption results in the loss of the innermost (earliest) growth marks and is a serious limitation in estimating age using skeletochronology. While not a serious issue for shorter-lived amphibians and reptiles, it is especially problematic in long-lived turtles, and the problem is noted to be extreme in age-estimate studies of marine turtles (Snover & Hohn 2004; Snover *et al*. 2007 b).

Freshwater turtles were the first turtles to have skeletal growth marks recognized in their long bones (Mattox 1936; Suzuki 1963; Enlow 1969). Growth parameters of *Emys orbicularis* and *Mauremys rivulata* in Mediterranean Turkey were estimated with skeletochornological method (Ciecek *et al*. 2016). There is little information on skeletochronological studies of Iranian reptiles.

This research is the first study on skeletochronology of the Caspian pond turtle, *M. c. caspica* in Iran. Our aim were to determine age of *M. c. caspica* in Golestan Province, Iran, comparing the age structure and body size, attaining the correlation between age and SCL, and understanding some life history traits of the freshwater turtle. These data are an indispensable prerequisite for designing any conservation strategies for the endangered populations in Iran.

MATERIALS AND METHODS

During morphometric study of Caspian pond turtle, 112 live specimens were collected from 14 stations in aquatic habitats including rivers, ponds, pools and fish farms in Golestan Province, Northern Iran, during 2016-2017 by hand and net. Seventeen dead specimens were also found in addition to live specimens. These dead materials and five previously-fixed specimens (10 males, 12 females) were studied to estimate the age structure of turtles. Phalangeal and fibula bones were dissected and removed from these 22 specimens.

The maximum straight carapace length (SCL) and maximum plastron length (PL) were measured using digital caliper to the nearest 0.1 mm. Sexes were determined by visual observation of morphological characters. The bones were fixed in 10% formalin and decalcified by 10% (v/v) chloric acid for 2-4 h. According to the size of bone, processed in paraffin block preparation (for 1.5 h), tissues were embedded in small paraffin block. Serial sections (7, 10, and 15 µm) were prepared using a rotary microtome, and all sections were stained with hematoxylin and eosin (H & E), then examined under a light microscope (Zivary & Kami 2017).

For each bone, we selected a minimum of three cross-sections at the mid-diaphyseal level, with the smallest marrow cavity (Cicek *et al.* 2016). The number of LAGs was counted in the periosteal bone. In addition, the skeletochronological data were compared with growth rings using the largest plastral scutes (generally pectoral or abdominal) (Germano & Bury 1998) and the central carapacial scutes, by two observers (M.Y. and H.K.), and the lower number was recorded.

Age classes and SCL were normally distributed (Shapiro-Wilk test, $P > 0.01$), thus allowing comparisons using parametric tests (t - test).

We used Spearman's rank correlation to examine the relationship between age and SCL. Data analysis was performed using SPSS 24 and Excel 2010 (Microsoft Office), and interpreted at $\alpha = 0.05$.

RESULTS

In all examined sections, lines of arrested growth (LAGs) , were clearly observed in the cross-sections (Fig. 1). In *M. c. caspica*, the mean SCL was 193.52 mm (range = 103.02–236.84 mm; N = 12; SD = 41.32) for females,

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and 178.39 mm (range = 83.74–221.41 mm; N = 10; SD = 48.89) for males. The character SCL was larger in females than in males (Table 1).

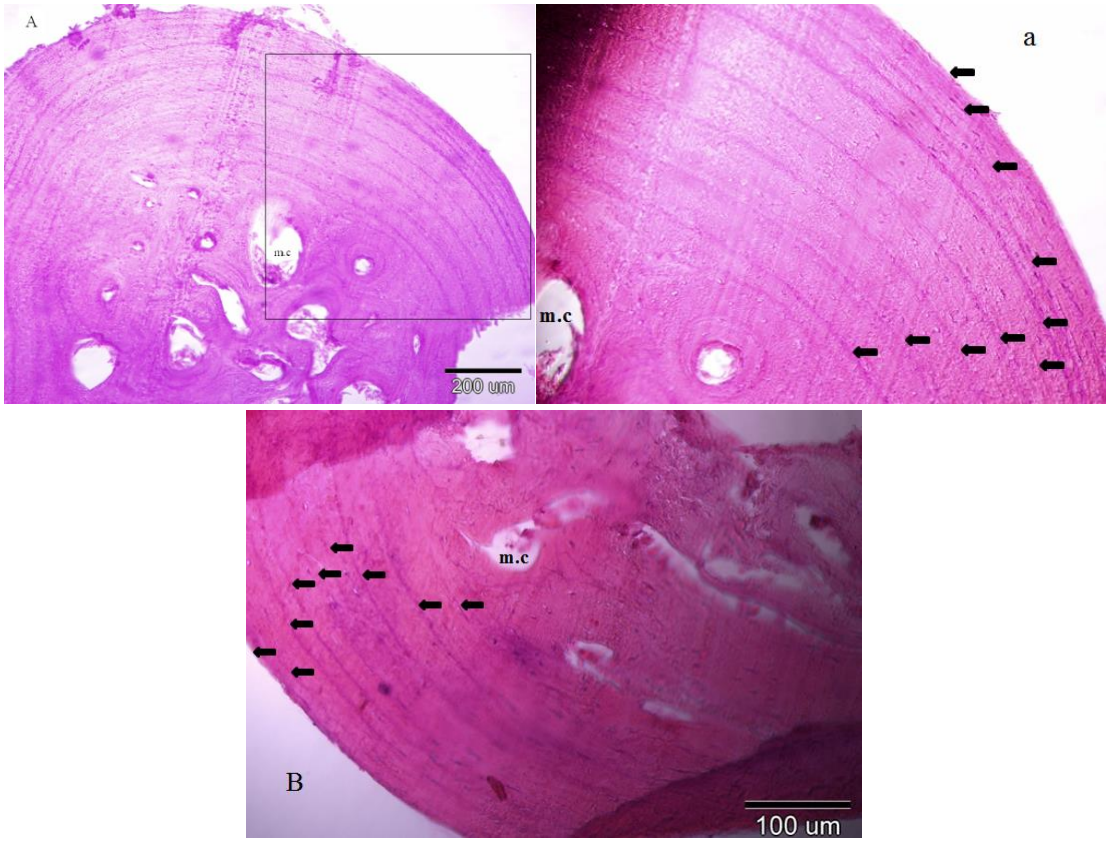

Fig. 1. Cross-section of phalange of *M. c. caspica*, Arrows: line of arrested growth (LAGs); m.c. = marrow cavity. (A) Phalangeal bone cross - section in female (SCL= 206.34 mm), 10 LAG, 10 um, 10x and (a) 20x. (B) Phalangeal bone cross section in male (SCL = 194.54 mm), 9 LAG, 7 um, H & E.

Mean plastron length (PL) was 183.25 mm (range = 99.22–219.94 mm; SD = 27.05) in females, and 155.48 mm (range = 75.6–186.34 mm; SD = 38.43) in males, hence PL is larger in females than in males. The marrow cavity was largely or entirely covered by the cancellous bone. The cancellous bone was very irregular and have small to medium trabeculae. Lines of arrested growth were clearly seen in compact bone on its outer surface. In all phalangeal and fibula cross-sections, well-defined LAGs were observed in *M. c. caspica*. The LAGs were generally in the form rings, located outside the cancellous bone. The first LAG was partially or completely resorbed by endosteal resorption in some individuals. The age range of *M. c. caspica* was between 4 and 12 years in males (mean = 9.6) and between 4 and 13 years in females (mean = 10.16) (Fig. 2). Both SCL and age showed normal distribution (Shapiro-Wilk test, $p > 0.01$).

No statistically significant difference was observed in the age structures between the sexes (Mann-Whitney U test; $p = 0.46$, $U = 49$). The Spearman's rank correlation showed significant correlation between SCL and age (Spearman's correlation coefficient, $r = 0.86$, $p < 0.001$, Fig. 3).

Skeletochronological study of age was compared with growth ring counts, revealing only a few numbers of LAGs and growth rings on carapace and plastron scutes, conformed to the age of individuals.

Our results demonstrated that growth ring counts were identical to skeletochronological study of age in 13% (2 females,1 male), while were higher than those in 9% (1 female, 1 male) specimens. In other specimens, growth ring counts were lower than skeletochronological counts.

Table 1. Age and SCL of M. *c. caspica*. Values are mean ± standard deviation and sample size (n = Number of individuals).

Age (years)	SCL (mm)	Individuals
9.6	178.39	Males (n = 10)
10.16	193.52	Females (n = 12)

SCL = Straight carapace length
mm = Millimeter
n= Number of individuals

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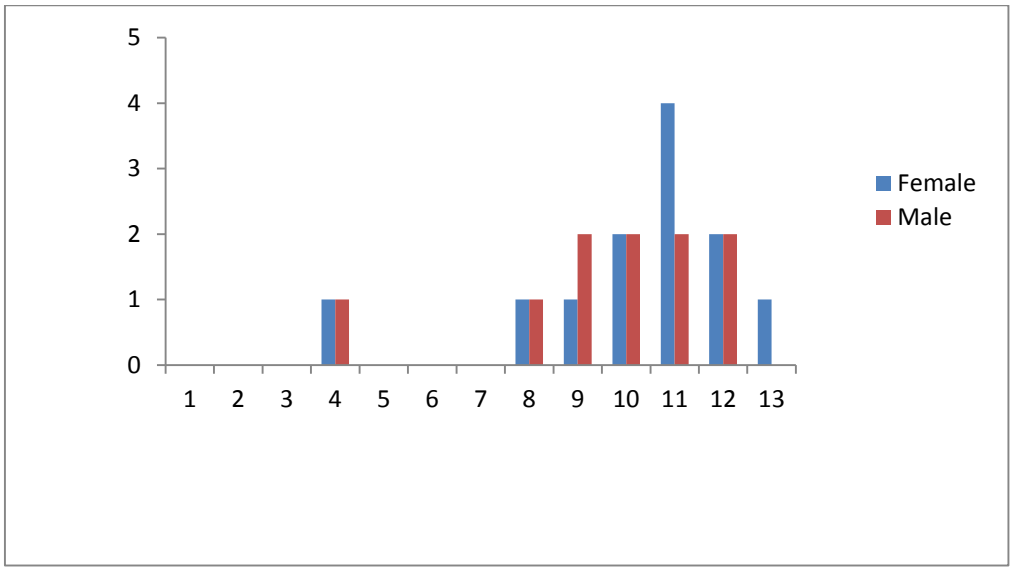

Fig. 2. Age frequency of *M. c. caspica* (adult specimens).

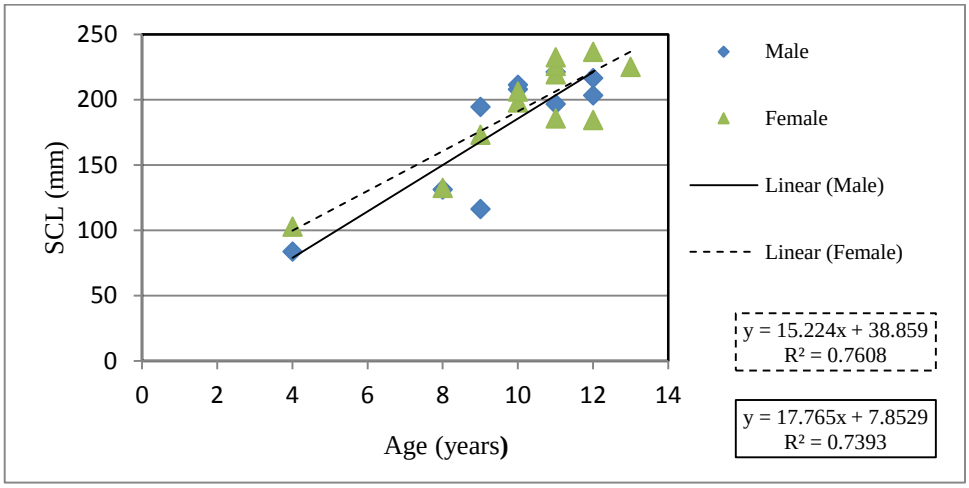

Fig. 3. Correlation between age classes and SCL in *M.c. caspica* female and male specimens from Golestan Province, Iran.

DISCUSSION

Fluctuating individual numbers and alterations in population structures often become only apparent during long-term studies. Photographic documentation of distinctive individual characters (pattern of front legs, head sides, carapace, scars, and anomalies) proved to be a powerful, non-invasive method for identifying small numbers of adult freshwater turtles (< 50) (Schneeweiss 2004). Auer & Taşkavak (2004) estimated the age of *E. orbicularis* and *M. rivulata* using plastral growth rings. This method had some disadvantages such as erosion of the growth rings. The missing rings caused false calculations in age determination studies (Germano & Bury 1998).

The body size and growth rates of freshwater turtles could be affected by several environmental factors, including thermal regimes (Germano & Bury 2009). The growth and maturation duration of freshwater turtles are influenced by ambient air and water temperatures (Brown *et al.* 1994), often vary with geographic latitude (Iverson *et al.* 1993). Thus, it is crucial to support their regeneration by conservation measures (Paul 2003).

In our results, ring production was cyclical, assuming one ring per year only in 13% of specimens. In accordance with Wilson *et al.* (2003), we were identified four critical sources of mismatches, obscuring the accuracy of GRC as an age estimator. These sources can be divided into two groups: At first, deposition of rings may not follow the usual assumed one ring per year (1:1 ratio), 1- because some individuals may deposit more than one rings in the same season during periods or in habitats with good resources availability (Aresco & Guyer 1998; Berry 2002).

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2- Some individuals may stop growing, and may not even deposit any ring for years (Turner *et al.* 1987). Secondly, even if ring deposition follows a 1:1 ratio, other factors may negatively affect the accuracy of the technique: 3- scutes worn-out from friction may make it impossible to discern individual rings, thus age may be underestimated (Litzgus & Brooks 1998; Berry 2002); and 4- identification of rings requires an observer's arbitrary decision, and repeated measures may yield different results (Germano & Bury 1998; Rodríguez-Caro *et al.* 2015).

Skeletochronology and the scute growth line counts from dead turtles can serve as supporting evidence of the annual nature of the two methods (Castanet & Cheylan 1979). Even when the scute growth line counts accurately estimate age, an advantage of skeletochronology over these counts appears with older adult animals. Growth lines of scutes could not be counted in our older specimens. As growth slows to nearly immeasurable rates in older animals, growth lines can no longer be differentiated on scutes (Snover & Rhodin 2007), hence only minimum ages can be estimated. On the other hand, a disadvantage is also found in the study of skeletochronology. One or two LAGs may not be counted due to the expansion of cancellous bone and resorption of secondary bone (Chinsamy & Valenzuela 2008).

The median age of *M. rivulata* is 11 years in Southeastern Europe and the Caspian region (Wischuf & Busack 2001). In Southern Anatolia population, recorded longevity is 12 years in two freshwater species, *M. rivulata* and *E. orbicularis* (Auer & Taşkavak 2004). In our results, the mean SCL was 193.52 mm for females and 178.39 mm for males. The median age of *M. c. caspica* was calculated 9.6 years (range = 4-12) for males, and 10.16 years (range = 4-13) for females. No statistically significant difference was observed in the age between the sexes (Mann-Whitney U test; P = 0.46, U = 49). Growth rings on carapace and plastral scutes could count up to 11 years; They were identical to skeletochronological counts in 3 specimens, were higher than those in 2 specimens, but were lower than those in 17 specimens. In Mediterranean Turkey population, the median age of *M. rivulata* was calculated 10 years (6-12) for males, and 10 years (range = 8-12) for females, while in *E. orbicularis*, 8 years (range = 5-9) for males, and 10 years (range = 5-12) for females. No statistically significant difference in age composition was observed between the sexes for *M. rivulata* (U test, $p < 0.97$) and *E. orbicularis* (U test, $p < 0.03$). Growth rings on plastral scutes could easily count up to 11 years for *E. orbicularis* and 12 years for *M. rivulata* in Southern Anatolia (Çiçek *et al.* 2016).

In our results, female body size was larger than male body size that can be the result of importance of fecundity as a factor influencing body size in female turtles (Gibbons *et al.* 1982; Brophy 2006; Yazarloo *et al.* 2017).

Given the vulnerable and endangered status of turtle populations worldwide, the need for age data to accurately characterize the status of these species is vital for conservation efforts (Heppell *et al.* 2003a, b).

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بررسی اسکلتوکرونولوژی سن لاک‌پشت خزری *Mauremys caspica caspica* (Testudines: Geoemydidae) در استان گلستان

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چکیده

با استفاده از روش اسکلتوکرونولوژی، سن جمعیتی از گونه لاک‌پشت خزری *Mauremys caspica caspica* که به وسیله تور از زیستگاه‌های آبی مختلف و استخرهای پرورش ماهی استان گلستان جمع‌آوری، بررسی کرده و در نمونه‌های بالغ *M.caspica* ، خطوط توقف رشد (LAG) به وضوح در برش‌های عرضی استخوان بند انگشت و ساق قابل مشاهده و شمارش برای برآورد سن بودند. میانگین سن در این گونه از جمعیت استان گلستان (n = ۲۲) ،۹/۶سال (۱۲-۴) در نرها (n = ۱۰) و ۱۰/۱۶ سال (۱۳-۴) در ماده‌ها (n =۱۲) محاسبه شد. بیشینه درازای مستقیم لاک‌پشتی (SCL_2) در نرها ۲۲۱/۴۱-۸۳/۷۴(۱۷۸/۳۹، ۴۸/۸۹ = SD) میلی‌متر و در نمونه‌های ماده ۲۳۶/۸۴-۱۰۳/۰۲ (۱۹۳/۵۲، ۴۱/۳۲= SD) میلی‌متر بود. در این مطالعه ارتباط معنی‌دار بین اندازه بدن و سن وجود داشت (P< ۰/۰۰۱) اما هیچ گونه تفاوتی در ترکیب سنی بین جنس‌ها یافت نشد. مطالعه اسکلتوکرونولوژی سن به وسیله شمارش خطوط توقف رشد(LAGs)، قابل اعتمادترین روش تخمین سن محسوب می‌شود و LAGs در بافت استخوانی ثبت می‌شوند که به وسیله آن سن فرد را می‌توان ارزیابی کرد.

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