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Into a box interiors: clutch size variation and resource allocation in the European pond turtle

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Abstract. In order to highlight the temporal dynamics of different stages of follicula distribution on reproductive output, we analysed the female size and the reproductive frequency of the European pond turtle, *Emys orbicularis*, using 191 ultrasonographic and 67 x-ray images (collected from 2000 to 2011 in two different localities of a coastal plain area of NorthWestern Italy). To compare digital image results we also used anatomical topography of autopsied females. Although reproductive females were significantly longer in one locality and larger in the second one, clutch size did not differ between localities. Two main clutches were produced during a year, with occasionally a third one. Shelled eggs were frequent in May, June and July, while *follicula* were present till August, with a decrease in follicular size especially in July and August. Despite the presence of a number of follicles in late summer and in autumn, a third expected clutch was only an exceptional event, differently from what happens in other sites of the species' distribution range. The permanence of follicles after the last deposition is interpreted as a possible extra energy source during the very hot and dry summers of coastal central Italy and for the hibernating phase.

Key words. European pond turtle, ultrasonography, x-rays; reproductive investment.

INTRODUCTION

Life history strategies represent the ways in which animals acquire and expend resources. The amount of energy gained with food and stored in fat bodies, liver and muscles indicates the potentiality of maximum reproductive capacity of each individual. The ecological consequences of energy storage have attracted much attention quite recently (Hom, 1988; Bonnet et al., 1998; Santos and Llorente, 2004). The amount of stored energy and its seasonal duration may play an important role in the reproduction of every organism. Furthermore, the patterns of the subsequent use of the amount of energy may represent important aspects of interspecific and intra-specific variation in life histories. In fact, differences between the endothermic and the ectothermic model of energy storage and maintenance are marked, such as in mammals versus reptiles (Else and Hulbert, 1981). Specifically, oviparous reptiles capitalize on their food income in the same season of reproduction and utilize only endogenous energy stores (Congdon and Tinkle, 1982; Bonnet et al., 1998; Santos and Llorente, 2004).

Pathways from energy income to fat storage may be resumed as food, digestion and assimilation in body reserves. Income breeders do not store lipids to any great extent. The process of the production of youngsters, involves a complex series of biochemical steps that cannot be avoided. The synthesis of vitellogenin macro-molecules involves a large amount of energy as well as their incorporation and deposition in the oocytes. Yolk deposition is a

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continuous process which requires stored substances, even from short-term storage, in order to moderate the discontinuous effects of the activities of feeding and digesting. Thus, the physiological pathways leading from resource acquisition to yolk deposition are similar among taxa, the differences depending on the magnitude and schedule of body reserve storage before reproduction (Allen, 1976; Congdon and Tinkle, 1982; Bonnet et al., 1998).

The energy budget is primarily stored in the *corpora lutea*, subsequently used for follicular formation, ovulation process and egg formation. From a series of multiple follicles, females ovulate to produce shelled eggs. In chelonians multiple clutches represent the rule: it is the case in all marine turtles, in most terrestrial tortoises and freshwater turtles. Radiography to assess reproductive status, clutch size and clutch frequency has been used for many years in Chelonians (Gibbons and Greene, 1979; Andreu and Villamor, 1986; Zuffi et al., 1999; Servan and Roy, 2004; Bertolero and Marín, 2005). The ultrasonographic technique instead has been applied only recently (Table 1). On the contrary, the post-reproductive period of Chelonians has received virtually no attention. This period could offer further information on the evolution of remnant follicular eggs of adult turtle and tortoise species, from immediately after the last egg laying to the end of the active season, prior to wintering. This contribution is aimed at describing the occurrence and the seasonal variability of developed and undeveloped *follicula*, their size and their possible function, during and especially after the reproductive period, considering also the influence of body structure (e.g. shell size and shape).

As model species we selected the European pond turtle (*Emys orbicularis*) as it is abundant in Europe (Podloucky, 1997; Sillero et al., 2014), and in many Italian areas (Zuffi, 2000; Zuffi et al., 2011), it lays multiple clutches per year (e.g. Zuffi and Odetti, 1998; Fritz, 2003, and data revision herein), and its reproductive variation strongly correlates to localities, habitat features and female size (Mitrus

Table 1. Comparative source data (in chronological order) of ultrasonographic and radiographic techniques to assess reproductive status in Chelonians

Species	Ultrasound	n	X-Ray	n	
Kinosternon subrubrum			X	3	Gibbons and Greene, 1979
Deirochelys reticularia			X	2	Gibbons and Greene, 1979
Sternotherus odoratus			X	2	Gibbons and Greene, 1979
Chrysemys picta			X	129	Congdon and Tinkle, 1982
Pseudemys scripta			X	65	Congdon and Gibbons, 1983
Emydoidea blandingi			X	90	Congdon et al., 1983
Chelydra serpentina,			X	4	Congdon and Gibbons, 1985
Chrysemys picta dorsalis			X	1	Congdon and Gibbons, 1985
Deirochelys reticularia			X	13	Congdon and Gibbons, 1985
Pseudemys concinna			X	1	Congdon and Gibbons, 1985
Pseudemys floridana			X	11	Congdon and Gibbons, 1985
Pseudemys scripta			X	88	Congdon and Gibbons, 1985
Terrapene carolina			X	8	Congdon and Gibbons, 1985
Clemmys marmorata			X	2	Congdon and Gibbons, 1985
Gopherus polyphemus			X	1	Congdon and Gibbons, 1985
Kinosternum subrubrum			X	19	Congdon and Gibbons, 1985
Sternotherus odoratus			X	9	Congdon and Gibbons, 1985
Trionyx ferox			X	1	Congdon and Gibbons, 1985
Testudo graeca			X	38	Andreu and Villamor, 1986
Mauremys leprosa			X	27	Andreu and Villamor, 1986
Chrysemys picta			X	17	Christens and Bider, 1987
Chelydra serpentina			X	68	Congdon et al., 1987
Emydoidea blandingi			X	280	Congdon and van Loben Sels, 1991
Emys orbicularis			X	15	Zuffi et al., 1999
Emys orbicularis			X	30	Servan and Roy, 2004
Cuora amboinensis	X	13			Haies and Kalb, 2010
Cuora flavomarginata	X	24	X	24	Ting-Yu et al., 2011
Emys orbicularis	X	191	X	66	Present research

and Zemanek, 1998; Zuffi et al., 1999, 2007; Kotenko, 2000; Zinenko, 2004). Despite it being among the species listed in the EU Habitat Directive and in several European Red Lists, it is much less studied than expected and should certainly be the object of further research.

MATERIALS AND METHODS

Sampled individuals were from San Rossore (average coordinate: 9 m asl, 43°42'51"N, 10°18'30"E) and from the US Army Camp Darby areas (10 m asl, average coordinate: 43°38'03"N, 10°20'11"E), both in the Natural Regional park of "Migliarino San Rossore Massaciuccoli" (Pisa, Tuscany, central Italy) (Fig. 1). The two areas are separated by the Arno river, a natural barrier that has been intensively used by humans since the time of the Second World War, including the construction of houses, a touristic harbour and anchoring hundreds of boats along the river. We are confident that no exchange of individuals has occurred since then. The two areas are characterised by a mixed wood of pines and oaks, artificial canals, ponds and marshy areas, fully described elsewhere (Zuffi and Rovina, 2006; Zuffi et al., 2007). No data nor information is however available on animal movements in this area. Thus we considered two populations as independent. Wild individuals were captured by hand and with fishing nets, placed along borders of artificial canals and ponds, from 2000 to 2011. Adult females were marked individually with notches on the marginal scutes, as described in Stubbs et al. (1984) and Zuffi et al. (1999), in order to recognize them when recaptured and avoid any pseudoreplication in analyses. We examined 191 *E. orbicularis galloitalica* adult females (secondary sexual characters described in Zuffi et al., 1999), to assess adulthood features and body size variation. Carapace and plastron measurements, body mass recording, individual marking or recognition took on average 10 minutes. We processed each individual in the field and turtles were released immediately afterwards. During ovulation and the egg-laying period, that is from early-mid May to late July (see Zuffi et al., 2007, and references herein), we re-captured marked females and we assessed their reproductive condition using manual inguinal palpation and ultrasonography (respectively to detect follicles or eggs, and quantify oviductal and shelled eggs), and X-ray techniques to measure clutch size before laying (Gibbons and Greene, 1979; Zuffi et al., 1999, 2005). Data were also collected from several females during late July and August, a period when females were reported to reproduce rarely or not reproduce at all (e.g. Mitrus and Zemanek, 1998; Zuffi and Odetti, 1998; Fritz, 2003). Ovulating and or checked adult females were brought to the vet lab and kept 24 hours at maximum in captivity. After ultrasonographic and x-ray analysis, the individuals were released. Clutch size was determined by X-ray techniques only. As a consequence, sample size of females bearing follicular eggs differed from sample size of females bearing shelled eggs. Two autopsied preserved females (Corsica, 2 May 1998, MSNT-1127 and 2 June 1998, MSNT-1128, Natural History Museum herpetological collection), belonging to the same subspecies (Fritz, 2003), were used to take direct images of the various statuses of developing follicula and of shelled eggs (Fig. 2). Follicular and oviductal egg estimation of each female was obtained by summing (from ultrasonographic images) the visible eggs on the right side plus those visible on the left side, then dividing the results by two. Even if just one single ultrasonographic image could represent most of the internal environment, we used both side images to build the average estimation, in order to minimize the risk of underestimating the whole follicular set. The reproductive phenology was considered as a whole, merging data of the two populations.

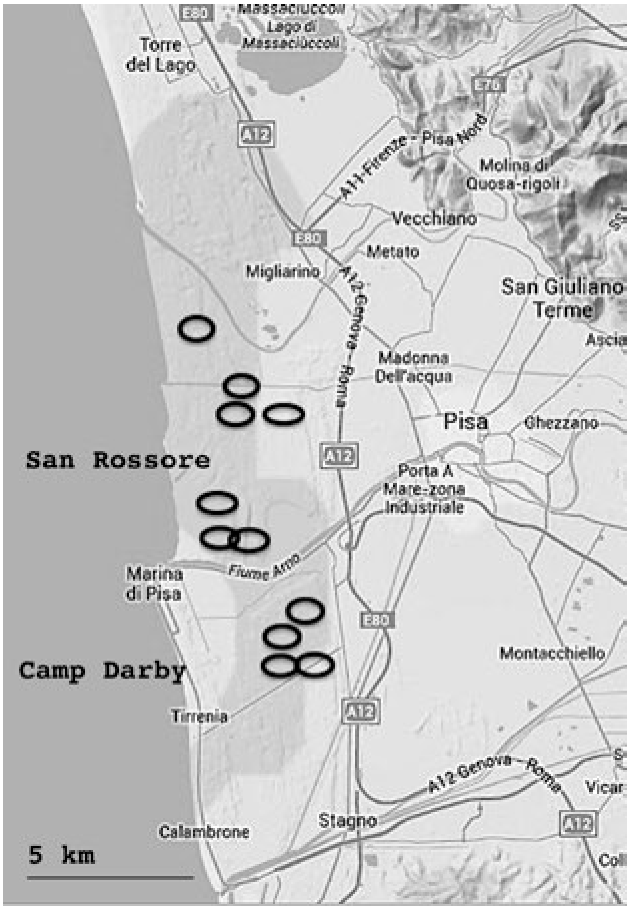

Fig. 1. Sampling areas for *Emys orbicularis* in north-western coastal Tuscany. Empty ovals indicate female sampling areas.

We measured turtles with standard methods, following Zuffi et al. (1999): variables considered were carapace length and width, plastron length and width, carapace height, tail length (from cloaca distal opening to tail tip), and body mass. Measurements were ± 1 mm and mass ± 1 g accuracy. A caliper and an electronic dynamometer were used for measurements. Biometric data of two sampling localities were analyzed for normality, then processed with the Student t-test. Ultrasound and x-ray data were analyzed between localities with the Mann-Whitney U test. Egg data were tested for normal-

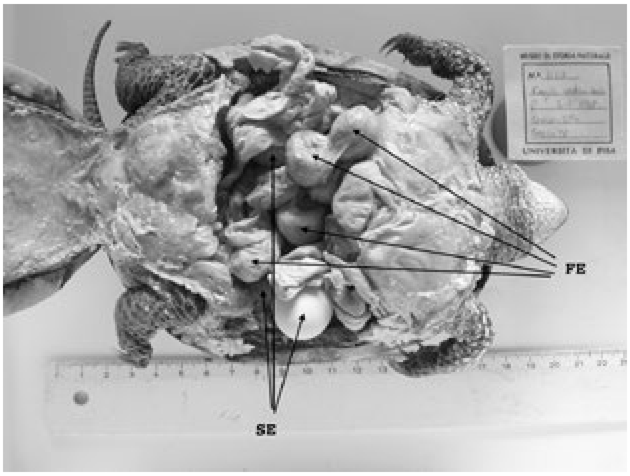

Fig. 2. Autopsied female *Emys orbicularis*, showing eggs (SE = shelled eggs) and *follicula* (FE = follicular eggs) distribution.

ity, then processed with the non-parametric Kolmogorov-Smirnov ranking test using month as factor; they were also analyzed for skewness and kurtosis (http://mvpprograms.com/help/mvpstats/distributions/SkewnessCriticalValues) to detect any asymmetry of the distribution patterns. Spearman correlation test was then applied in analyzing number of developing follicular eggs and month. Significance was selected at 0.05. Analyses were carried out with IBM SPSS 21.0.0 release.

RESULTS

Considering body size of the sampled reproductive females and sampling areas, San Rossore females were larger than Camp Darby females in terms of carapace length and smaller than Camp Darby females as regards carapace width (SR carapace length: 136.9 ± 13.7cm, CD carapace length: 132.4 ± 11.3 cm; unequal variances, Student's $t_{88,224}$, 2.174, $P = 0.032$; SR carapace width: 95.3 ± 9.4 cm, CD carapace width: 98.8 ± 7.5 cm; unequal variances, Student's $t_{86,188}$, -2.447, $P = 0.016$) with no differences in the other variables.

Number of x-ray eggs did not differ between localities (San Rossore, 6.2 ± 1.1, n = 48; Camp Darby, 5.9 ± 1.2, n = 18; Mann-Whitney U test = 1.017, $P = 0.309$). Ultrasound eggs were significantly different between localities (San Rossore, 5.3 ± 2.6, n = 152; Camp Darby, 3.3 ± 1.5, n = 39; Mann-Whitney U test = 4.017, $P < 0.0001$), while no difference was found in ultrasound egg size between localities (San Rossore, 11.7 ± 5.9, n = 151; Camp Darby, 12.7 ± 5.5, n = 39; Mann-Whitney U test = 1.096, $P = 0.273$).

Table 2. Clutch laying temporal distribution versus follicle distribution. Values are expressed in %.

Distribution	May	June	July	August
Clutch	27.0	67.7	5.4	0.0
Follicles	9.5	33.7	40.5	16.3

We found reproductive females with shelled eggs in May, June and July, with an average clutch size (x-ray) of 6.1 ± 1.1 eggs (n = 66, range 2-9). Adult females with calcified eggs were found in May (n = 9, 27%), in June (n = 25, 67.6%) and in July (n = 2, 5.4%). On the whole, developing follicular eggs were 4.9 ± 2.5 (n = 191), with an average size of 11.8 ± 5.9 mm. Developing follicular eggs were 3.2 ± 1.9 in May (range 1-8, n = 19, 9.5%), 4.2 ± 1.9 in June (range 1-9, n = 64, 33.7%), 5.4 ± 2.5 in July (range 0-16, n = 77, 40.5%) and 6.1 ± 3.2 in August (range 1-11, n = 31, 16.3%) (Table 2). The skewness was significant in May and July ($P < 0.05$ and $P < 0.01$, respectively). Difference of ultrasound eggs distribution among months was significant (rank Kolmogorov-Smirnov test = 24.708, 3 df, $P < 0.0001$; Fig. 3). Developing follicular eggs were 19.5 ± 9.1 mm in May (range 8-35.8 mm, n = 19), 14.1 ± 6.1 mm in June (range 6.2-35 mm, n = 64; Fig. 4), 9.6 ± 2.3 mm in July (range 4.5-13.8 mm, n = 76) and 7.9 ± 2.3 mm in August (range 5.3-10.9 mm, n = 31). The skewness was significant only in June ($P < 0.01$). Egg size differences among months was significant (rank Kolmogorov-Smirnov test = 63.983, 3 df, $P < 0.0001$; Fig. 5).

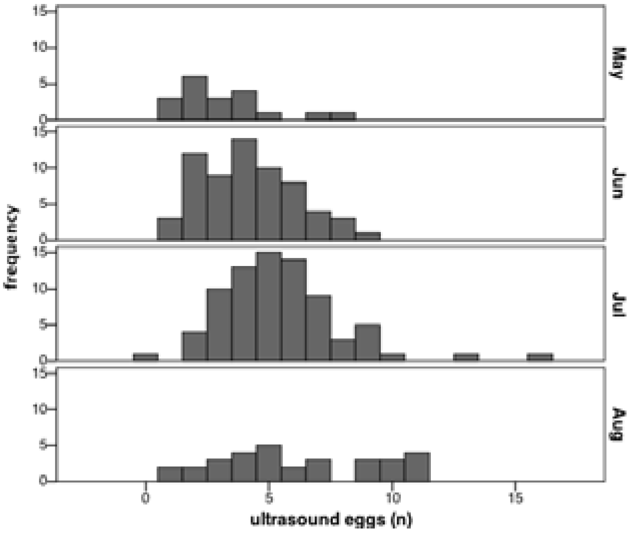

Fig. 3. Ultrasonographic distribution of developing *follicula* per month.

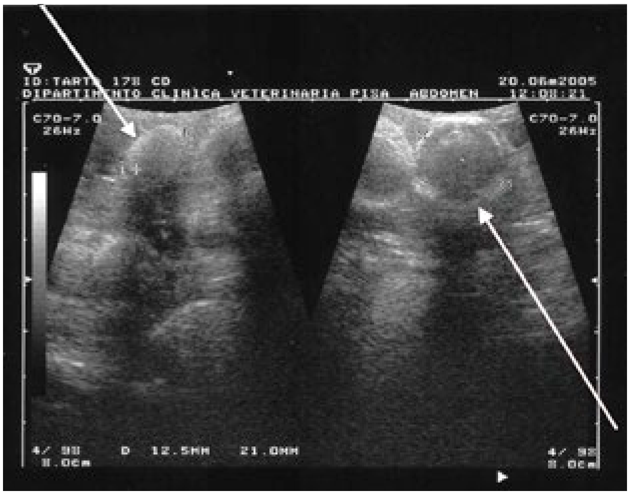

Fig. 4. Ultrasonographic image of a June female with different stages of *follicula* (on the left: developing follicles; on the right: transverse image of a shelled egg).

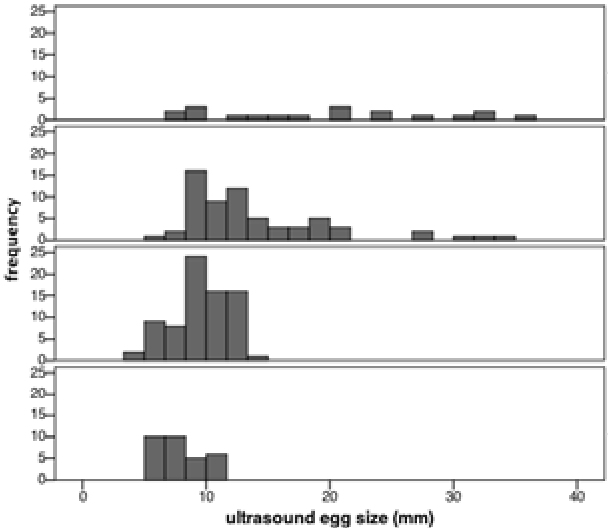

Fig. 5. Distribution of size of developing *follicula* per month determined through ultrasonography.

The number of developing follicular eggs were positively correlated with month, while size of developing follicular eggs were negatively correlated with month (Spearman ρ_{number}, 0.345, $P < 0.0001$, $n = 191$; Spearman ρ_{size}, -0.581, $P < 0.0001$, $n = 190$).

DISCUSSION

External shell morphology and size differences of reproductive females between localities are limited to carapace length and width. Our results agree only in part with previously published papers on the same populations, where differences were much more marked and significant for almost all variables (Zuffi et al., 2004, 2007). Sample size of x-rayed females is however a limited fraction of the much larger adult female sample we have been monitoring for other purposes for more than 25 years. It is evident that our sampled females are more similar in size and in reproductive output (they actually produced the same number of shelled-eggs; see results) as expected from previous results (see Zuffi et al., 2004).

In all the turtle species studied to date, the reproduction period lasts some months (Ernst et al., 1994). The constitutive elements of the clutch (e.g. follicles, oviductal eggs, *corpora lutea*) are present at the same time in the female body and characterize a reproductive feature of all Chelonians. The follicles show two distinct stages, < 10 mm diameter and > 10 mm diameter (Congdon and Gibbons, 1985). As observed (Congdon and Tinkle, 1982), the presence of discrete classes of enlarged follicles shows evidence of multiple clutches. In addition, we were also able to assess the presence of discrete classes of enlarged follicles.

In our study area adult females monitored in late summer bear a large number of follicles, especially in July. Follicle diameters range on average from 8 to 10 mm, as is the case in preovulatory females. In July we recorded only two reproducing females (with shelled eggs), while in no other cases (using manual palpation, ultrasonography, or radiography) did we have any evidence of a third clutch. A third reproduction is therefore particularly rare, if not exceptional, in this area. In fact, from previous ecological research, a maximum of two clutches was found in *Emys orbicularis* (Zuffi and Odetti, 1998; Zuffi et al., 1999), and a third deposition was only hypothesized. However, three or even more depositions have been recorded in other parts of the species' range (see revision in Fritz, 2003).

Interestingly, even if not surprisingly, the number of undeveloping follicles decreases after the last reproductive period (i.e. July, August). Reasonable questions are i) do late summer follicles have a function, and if so, which is it? and ii) what does this extra amount of energy reserve represent? Do small follicles simply represent the extra amount of undeveloped eggs?

In all probability, during the post reproductive phase, *follicula* that have not been used in reproduction may indicate the start of an absorbing process, and prior to estivation during hot summers in dry canals and ponds (Naulleau, 1991) they may represent an energy reserve to compensate for metabolic demand (Hutton et al., 1960; Huey, 1982). Alternatively, the unused follicles may remain inactive (not enlarging, not being absorbed) for the last part of the season (and of the year) and will contribute to the next year's clutch (Congdon and Tinkle, 1982).

In the painted turtle, *Chrysemys picta*, it has been found that each female shows three groups of follicles, with different developing stages, suggesting a three year cycle to complete the potential maximum reproductive output (Christiansen and Moll, 1973; Callard et al., 1978). As a member of the Emydidae family, *Emys orbicularis* could reasonably be expected to follow the same pattern recorded in North-American species. On the contrary, our ultrasonographic (and x-ray) data which are, as far as we are aware, the only available dataset for the species, seem to contradict the patterns found elsewhere, suggesting that the reproductive cycle may likely be completed within one year or at maximum in two years (many follicles of a smaller size in August, see Figs. 4 and 5). However, it remains unclear which pattern follicles do follow in autumn (September-November), prior to wintering. Keller et al. (1998) found that in south western Spain, the European pond turtle (*Emys orbicularis occidentalis*) lays multiple clutches regularly, especially during wet summers (up to five clutches in one year), suggesting how abundant rains enable turtles to reproduce frequently. However, in other parts of the species' distribution range, where multiple clutches were also recorded (Fritz, 2003), climatic data and turtle reproductive patterns were not studied or available data are still scarce or anecdotal (see Rogner, 2009). Thus, it is not simple to shed light on the biological and ecological reproductive model of the European pond turtle, *Emys orbicularis,* which appears to be highly variable, likely depending on site climatic conditions, food and water abundance and on female size.

Forthcoming research should cover analysis of any adult preserved museum specimens captured in autumn to verify the presence and the developmental stage of *follicula* and *in vivo* study of the gonadal status of adult post reproductive females.

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