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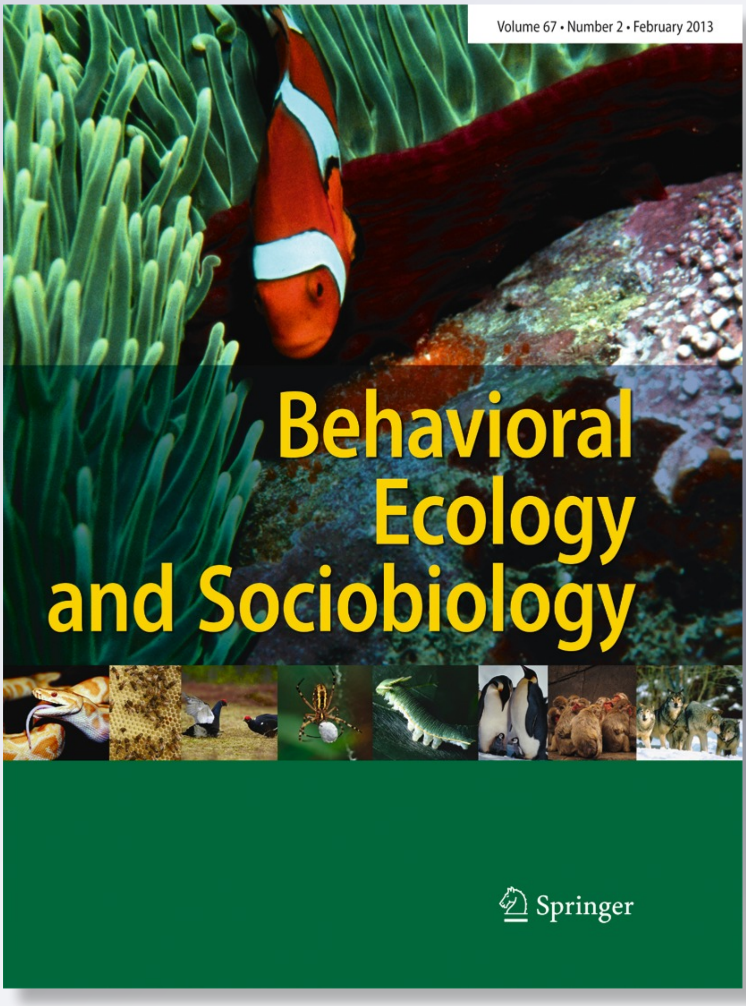

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ORIGINAL PAPER

Influence of mating order on courtship displays and stored sperm utilization in Hermann's tortoises (*Testudo hermanni hermanni*)

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Abstract Sperm storage is widespread in many vertebrate groups, and it is frequently associated with promiscuous mating systems. Chelonian species are one of the most outstanding examples of a promiscuous group capable of long-term sperm storage; specialized structures have evolved within the oviducts of these vertebrates to ensure sperm vitality across reproductive cycles. Thus far, few studies have investigated the factors regulating multiple paternity, sperm usage by females and paternity distribution in successive clutches. This study aimed to investigate the effect of mating order on male mounting and reproductive success in *Testudo hermanni hermanni*, combining behavioural and genetic data. A series of planned matings were performed, within which experimental females were mated sequentially to two different males under controlled conditions. Observations conducted during experimental matings revealed that courtship displays did not significantly differ between the first and second males to mate with a female. Interactions ending with a mount were characterized by a significantly higher intensity and occurrence of determinate courtship behaviours, for example biting and running after the female. Paternity analysis performed on hatchlings produced from experimental females revealed that 46 % of the clutches were multi-sired. A significant contribution of previous years' partners was still found, thus confirming the long-term sperm storage within the female oviduct in this species. Finally, mating order did not significantly affect the reproductive success of experimental males during the ongoing reproductive season.

Keywords *Testudo hermanni* · Mating success · Sperm storage · Female promiscuity · Paternity distribution

Introduction

The storage of spermatozoa in the female reproductive tract is widespread in numerous invertebrate (Diesel 1989; Taylor et al. 2000; Chevrier and Bressac 2002; Baer et al. 2003; Córdoba-Aguilar et al. 2003; Klowden 2003) as well as vertebrate groups. Among the latter, sperm storage has frequently been recorded in reptiles and birds (Howarth 1974; Birkhead and Møller 1993; Birkhead 1998a; Liem et al. 2001). In these taxa, natural selection seems to have operated towards female sperm storage, and in fact, females of many species of reptiles and birds have developed sperm storage tubules (SST's) to enable prolonged sperm storage in their reproductive tracts (Gist and Jones 1987; Birkhead and Møller 1992b; Birkhead 1998a). These specialized structures appear to be absent in mammals, among which long-term sperm storage is rare, with the notable exception of bats (Racey 1979).

Sperm storage has commonly been observed in species harbouring low population densities (Gist and Congdon 1998). However, due to male infertility, prolonged sperm storage has also been suggested as a means to ensure successful fertilization (Olsson and Madsen 1996; Olsson and Madsen 1998; Roig et al. 2000). Sperm storage across reproductive cycles is often associated with multiple

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matings by several males during each reproductive cycle (Birkhead and Møller 1993; Uller and Ollson 2008). Promiscuous mating systems are widespread in vertebrate groups, and extra-pair copulations are common even in species previously considered as monogamous (Birkhead and Møller 1992a; Birkhead and Biggins 1998; Griffith et al. 2002). Under a scenario of sexual conflict, sperm storage may lay the basis for sperm competition (Parker 1970), by extending the interval over which ejaculates from different males overlap within the female reproductive tract, and may also promote a post-copulatory mate choice if females are able to detect information about male quality from seminal fluids (Eberhard 1996, 1998). In many reptiles, pre-copulatory female choice is rare, and hence, females frequently mate with most courting males (Olsson and Madsen 1995).

Among reptiles, chelonian species present one of the most outstanding examples of sperm storage over long periods of time. In chelonians, SST's are located in the posterior portion of the albumin-secreting region of the oviduct (Gist and Jones 1987, 1989; Gist and Fisher 1993; Girling 2002; Xiangkun et al. 2008), and sperm remains viable in the female genital tract for as long as 3–4 years (Kuchling 1999; Pearse et al. 2001).

During a single mating season, female chelonians frequently mate with more than one male, often resulting in broods with multiple paternity (Palmer et al. 1998; Kichler et al. 1999; Pearse et al. 2002; Roques et al. 2004; Johnston et al. 2006). Turtles and tortoises, like most reptiles, do not form pair bonds or cohesive social groups; neither sex provides parental care beyond nesting, and thus, females do not receive direct benefits (e.g. parental care, nuptial gifts) from multiple matings. Polyandry may, therefore, have evolved as a mechanism for females to ensure fertilization of eggs in cases of low mate encounter probability (Olsson and Madsen 1998), to increase offspring genotypic diversity (Loman et al. 1988), to avoid genetic incompatibility (Zeh and Zeh 1996; Olsson et al. 1996) and/or to receive "good genes" (Birkhead 1998b).

Literature focusing on stored sperm utilization patterns in Chelonia and paternity distribution in their successive clutches is sparse, and studies have tended to centre on turtles (i.e. aquatic chelonians; for a review, see Pearse and Avise 2001; Harry and Briscoe 1988; Fitzsimmons 1998; Pearse et al. 2002; Theissinger et al. 2009; Sakaoka et al. 2011). Sperm storage studies in tortoises (i.e. terrestrial chelonians) have revealed the presence of multi-sired clutches (Roques et al. 2004; Johnston et al. 2006; Loy and Cianfrani 2010; Davy et al. 2011), but data concerning the paternity distribution in successive clutches are lacking.

Using a combined approach of behavioural observations and genetic parental assignment analyses, the aim of the present study was to elucidate if the mating order in *Testudo hermanni hermanni* influences male courtship displays and whether it can explain the observed paternity distribution patterns. In particular, we predicted that if a last-male sperm precedence model (Parker and Simmons 1991) exists in *T. hermanni hermanni*, the male ability to recognise whether a female has been already inseminated should be strongly selected, in order to permit males to assess and adapt the costs and intensity of their courtship. Accordingly, we predicted that the paternity should be significantly turned in favour of the last partner. Last-male sperm precedence has been frequently observed in both invertebrates (see for example Walker 1980; Harshman and Prout 1994; Franck et al. 2002) and vertebrates (Briskie 1996; Kraaijeveld-Smit et al. 2002; Raveh et al. 2010) including several chelonian species (Pearse et al. 2002; Theissinger et al. 2009). If, however, the sperm of different males is randomly mixed in the female reproductive tract (Parker 1990), we predict that fertilization should be proportional to sperm number.

Material and methods

Study species

Hermann's tortoise (*T. hermanni hermanni*; Gmelin, 1789) is one of three species of Testudinidae endemic to Europe. Living at low population densities, they maintain non-exclusive home ranges. Females are polyandrous, frequently copulating with several males during the same breeding season (Ernst and Barbour 1989; Swingland and Stubbs 1985). Matings can occur during the whole activity period (from early spring to late summer), with two peaks of male courtship activity in April–May (after hibernation) and September (before hibernation). Females usually lay two to three clutches per breeding season at intervals of 15–20 days, and eggs hatch approximately 60 days after oviposition. As in all chelonians, long-term sperm storage occurs in the female genital tract, and multi-sired clutches are frequent (Kuchling 1999; Loy and Cianfrani 2010).

The courtship and mounting behaviour of *T. hermanni hermanni*, as in most tortoise species, is elaborate and based on a multiple signalling system involving visual, olfactory and acoustic signals. The male runs after the female, biting and ramming her, trying to immobilize and force the female to copulate. The female appears to avoid face-to-face confrontation with the male by retreating on a circular or semi-circular path. Since the male walks around her, the courting pair moves circularly, the male in an outer, larger circle, and the female in an inner, smaller one. The female tends to escape during courtship; consequently, the male tries to mount her from any place on the carapace, with his front feet on the dorsal surface of the female's carapace and his rear feet on the ground. Many attempts are usually needed before a successful mount may take place. During the mount, the male fully extends his neck and emits a long sequence of simple calls (Sacchi et al. 2003). The number of vocalizations is correlated to the time spent in copulation and, possibly, to the amount of sperm released into the female genital tract. Moreover, these vocalizations display a harmonic structure with frequency and amplitude modulation typical for each species and negatively related to body size (Galeotti et al. 2005b). As male courtship is time-consuming and energetically demanding, only healthy males are able to perform successful copulations as indeed has been observed in *T. hermanni* (Galeotti et al. 2004, 2005a), *Testudo graeca* (Pellitteri-Rosa et al. 2011), and *Testudo marginata* (Sacchi et al. 2003). These studies have recorded that courtship displays and mounting calls are condition-dependent signals that reliably reflect a male's quality and strongly influence mounting success.

Planned matings and observations of mating behaviour

The study was performed on captive individuals during spring and summer 2010 at the TartaEtruria Association located in Florence (Italy). All tortoises used in the present study, 5 males and 15 females, of which 4 had not mated for 3 years, were hatched in captivity; females and males were raised in separate enclosures.

Prior to the experimental matings, all females were allowed to oviposit (henceforth oviposition occasion 1, which occurred between the 15th of May and the 20th of June 2010). Two or 3 days after oviposition occasion 1, all 15 females were sequentially mated with two different males in a prefixed order (first male = M1, second male = M2). The identity of the males that mated with 11 females in September 2009 was known, but we do have any information on the identity of males mating with the females prior to September 2009. The males that mated with the 11 females in September 2009 were not used in the experimental matings conducted in 2010. The five males used in the experimental matings were of similar age, size and physical condition (i.e. absence of diseases and parasites). Apart from the males that mated with the 11 females in 2009, the experimental M1/M2 pairs were randomly assigned to the females. Since only five males were used in the experiment, we applied a classical reciprocal design (see Boorman and Parker 1976; Birkhead and Biggins 1998), i.e. each male was used as both M1 and M2 (Table 1). Moreover, the reciprocal male mating design was combined with a resting period of at least 3 days after each mating session in order to ensure that each male (e.g. M1 and M2) was equally fertile and experienced. The experimental matings resulted in the females' production of one or two consecutive clutches, henceforth referred to as oviposition occasions 2 and 3.

Table 1 Experimental design of matings: each female was sequentially mated to two different males (M1 and M2) in a prefixed order

Female	M1	M2
1	Male 3	Male 7
2	Male 3	Male 7
3	Male 3	Male 7
4	Male 7	Male 3
5	Male 7	Male 3
6	Male 7	Male 3
7	Male 6	Male 5
8	Male 6	Male 5
9	Male 5	Male 6
10	Male 5	Male 6
11	Male 5	Male 6
12	Male 1	Male 5
13	Male 1	Male 5
14	Male 5	Male 1
15	Male 5	Male 1

Each experimental female was kept with the first partner for 2 days and with the second partner during the subsequent 2 days. Couples were placed in 2×3 m enclosures, isolated from other individuals, and observed for 6–8 h a day during the periods of maximum activity (early morning and late afternoon). During the experimental matings, for each male/female interaction (i.e. contact/encounter between the two individuals), we recorded the occurrence and duration of six behaviours performed by males which are typical of tortoise courtship (Weaver 1970; Sacchi et al. 2003), that is the number of times that the male (1) bit, (2) ran after, (3) observed, (4) smelled, (5) moved circularly around and (6) attempted to mount the female. We also recorded the number and duration of successful mounts (those associated with penile insertion) and the number of vocalizations emitted by males during each mount.

After the mating experiments, females were separated from the males and kept in an oviposition enclosure. Fourteen of the 15 females produced clutches at oviposition occasion 2, but only 4 of the 15 females produced a second clutch (i.e. at oviposition occasion 3; Table 2). All eggs, including those of the first clutches preceding the experimental matings, were collected upon laying, individually marked and transferred into an artificial incubator, and each emergent hatchling was individually marked in order to assign it to a specific female and clutch.

Multivariate and univariate analyses on behavioural data were performed using PRIMER v.6.1 (Clarke and Gorley 2006) and the PERMANOVA + for PRIMER routines (Anderson et al. 2008). Principal component analysis (PCA) on normalised data was performed to visualise

Table 2 Female reproductive output, mean clutch size and hatching success in oviposition occasions 1, 2 and 3

Female	Oviposition 1		Oviposition 2		Oviposition 3	
	No. of fertile eggs	No. of infertile eggs	No. of fertile eggs	No. of infertile eggs	No. of fertile eggs	No. of infertile eggs
1[a]	1	2	5	0	5	0
2[a]	3	0	4	0		
3[a]	0	3	0	2		
4[a]	0	3	0	3		
5	0	2	0	2		
6	4	1	5	0	3	0
7	3	1	5	0		
8	0	3	0	2		
9	0	3	0	2		
10	0	3	1	2		
11	4	0	2	2		
12	0	2	1	2	1	3
13	0	3				
14	0	1	0	2		
15	3	2	4	0		
Total	18	29	27	19	9	3
% hatched eggs	38.3		58.7		75	
Mean clutch size	3.13±0.27		3.28±0.32		4±0.57	

[a] Females that had not mated for 3 years

patterns in male–female interactions based on the six male mating behaviours. A three-way permutational multivariate analysis of variance (PERMANOVA), based on similarity matrixes computed using Euclidean distances, was applied to determine differences in male courtship displays (six behavioural variables) across groups of interactions ending or not with a successful mount ("mount", fixed and orthogonal) between the first and second male to mate ("mating order", fixed and orthogonal) and among specific males ("male", fixed and orthogonal). A two-tailed *t* test was performed to investigate any significant difference in mounting success (expressed as numbers of vocalizations) between the first and second male to mate. Further analyses were performed on the successful interactions only. Firstly, distance-based linear models (Dist-LM) and distance-based redundancy analysis (db-RDA) were performed to evaluate which of the six behavioural variables were significantly related to mounting success, calculated as "number of male vocalizations" since this parameter is strictly correlated to the time spent in copulation and possibly to the amount of sperm released into the female genital tract (Sacchi et al. 2003; Galeotti et al. 2004).

A PCA and one-way PERMANOVA were performed to test whether interactions with the 4 females that had not mated during the previous 3 years were characterized by a higher mounting frequency and courtship intensity compared to the 11 females mated in September 2009 ("time elapsed from female's last mating" as fixed factor).

Genetic analyses and paternity assignment

Samples for genetic analysis were collected using buccal swabs, following a non-invasive procedure, suitable for small-sized individuals (such as hatchlings), and avoiding the dangers associated with blood sampling (Wingfield 1999; Poschadel and Møller 2004; Broquet et al. 2007). Total genomic DNA from adults and hatchlings was extracted from epithelial cells by combining alkaline and temperature lyses. The swab was soaked in 500 μl of 50 mM NaOH at 97 °C for 10 min. In order to maximise the DNA concentration, the swab was put in a second vial and centrifuged for 5 min. This extra solution was then added to the first before adding 75 μl of 1 M Tris (pH=8.0). The average DNA concentration in 100-μl volumes was about 100–150 μg/ml. The extracted DNA was stored at 4 °C and at −20 °C for long-term storage.

All adult individuals (n=20) were screened at 11 microsatellite loci previously tested on *T. hermanni hermanni* by Cutuli et al. (2012). Since six of these loci showed less than three alleles in our experimental group, paternity assignments were performed using the remaining five polymorphic loci (Leo10, Leo56, Leo71, GmuB08 and GmuD51; Table 3).

Table 3 Level of polymorphism at each of the five loci used in the paternity analyses

	Leo10	Leo71	Leo56	GmuB08	GmuD51
Size range	178–232	121–129	197–215	209–221	129–165
N	20	20	20	20	20
Na	8	4	6	5	5
He	0.805	0.612	0.734	0.569	0.685
Ho	0.650	0.809	0.809	0.684	0.714
P	0.076	0.43	0.0001*	0.88	0.53
Male 1	178/194	125/125	203/203	209/209	137/145
Male 3	194/228	125/129	199/205	209/221	129/165
Male 5	178/228	125/129	199/205	209/209	129/165
Male 6	194/228	125/129	199/205	209/221	129/137
Male 7	194/222	125/129	199/205	209/221	137/145

The genotype of each experimental male is also presented

N number of individuals tested, *Na* number of alleles, *He* expected heterozygosity, *Ho* observed heterozygosity, *P* Hardy–Weinberg probability test

*$P<0.05$

The forward primer for each locus was 5′-labelled, and then, each locus was amplified using the PCR conditions reported in Cutuli et al. (2012). For detection of polymorphisms, labelled amplicons from the five loci were divided into two sets (Leo10-NED + GmuBo8-HEX + Leo56-FAM and Leo71-FAM + GmuD51-HEX). For each set, 1–5 µl of each PCR product was combined with water in a final volume of 10 µl for successive dimensional analysis. Sizing was performed using an ABI Prism 310 Genetic Analyzer (Applied Biosystems) with reference to the internal size standard ROX400, using GENOTYPER ver. 3.7 and GENESCAN ver. 3.7 (Applied Biosystems).

Paternity assignment was carried out using the likelihood-based paternity inference implemented in the software programme CERVUS 3.0 (Marshall et al. 1998; Kalinowski et al. 2007). As the mating history of the females prior to 2010 was not certain, all adult males were considered as candidate fathers in the paternity analyses. For each putative father–offspring pair identified by genotype comparisons, CERVUS calculated a log-likelihood (LOD) score as the natural logarithm of the ratio between the likelihood that the candidate male being the true father and the likelihood the male not being the true father. A delta score is subsequently defined as the difference in LOD scores of the two most likely candidate fathers, which was subsequently used in the paternity estimations. Simulations were then employed to determine a threshold delta score, above which identified father–offspring pairs can be considered as true relatives at a given confidence level. The threshold delta score was determined from replicate simulations as the delta score above which a given proportion of father–offspring pairs are correctly identified. This proportion represents the confidence level of the analysis, which was set to 80 and 95 % in our analysis. The simulations also include parameters such as the number of candidate fathers, the proportion of candidate fathers sampled and the estimated frequency of typing error when generating genotypes.

Results

Behavioural observations

During a total of 540 h of observation, we recorded 120 interactions between male–female pairs, 71 of which concluded with a successful mount. The entire data set was first analysed to test for differences between the overall set of male courtship displays performed in tests ending with or without a successful mount. The PCA, performed on six behavioural variables (biting, running after, observing, smelling, moving around, attempting to mount the female), revealed that successful interactions (i.e. ending with a successful mount) were markedly different from unsuccessful ones (Fig. 1). Successful interactions were mostly distributed along the negative quadrant of the II principal component (cumulated I PC and II PC explain 70.9 % of

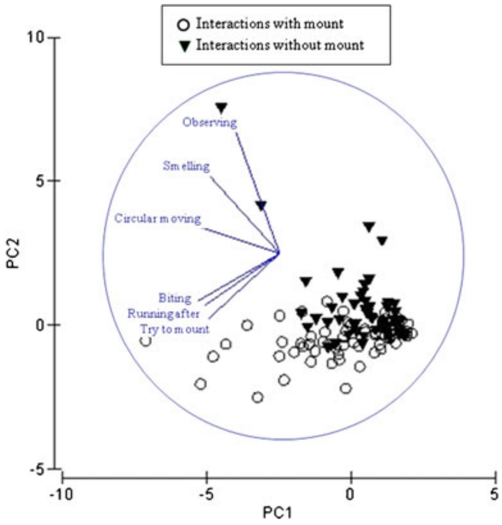

Fig. 1 Two-dimensional scatter plot of the first and second principal components of six behavioural variables in interactions ending with or without a mount. *Vectors* of the linear correlations between individual variables are superimposed on the graph

the total variance). The linear coefficients linking the original variables to the principal components revealed that successful interactions were strictly associated with three behaviours (biting, running after and attempting to mount the female). A three-way PERMANOVA performed on these 120 interactions confirmed the results of PCAs: "mating order" and "male" factors did not affect courtship displays; instead, the factor "mount" significantly influenced courtship behaviour of experimental males (Table 4).

No significant difference in mounting success (expressed as numbers of vocalizations) between the first and second male to mount was observed ($t=-0.72$; df=118; two-tailed $P=0.46$). Dist-LM and db-RDA, performed on the 71 successful mounts, underlined that the three variables "biting" ($F=11.53$; $P<0.001$), "running after" ($F=11.70$; $P<0.001$) and "attempting to mount" the female ($F=20.09$; $P<0.001$) were positively and significantly correlated with mounting success, and the variable "observing" was significantly negatively correlated with mounting success ($F=8.37$; $P<0.005$). One-way PERMANOVA showed that the time elapsed from a female's last mating significantly influenced the male's courtship intensity ($F=4.55$; df=1; $P<0.005$). In particular, PCA (Fig. 2) suggested that males spent more time courting and mounting the four females which had not mated during the previous 3 years.

Paternity analysis

Reproductive output, mean clutch size and percentages of hatching success for each experimental female, at the three ovipositions, are reported in Table 2. A total of 105 eggs were collected, of which 54 (51.4 %) successfully hatched. Two of the four females which had not mated during the three previous years were able to produce fertile eggs at the first oviposition occasion (e.g. prior to the experimental matings; Table 2), demonstrating that sperm stored in the oviduct remains viable for at least this time span. Moreover, we observed a significant variation in the proportion of fertile eggs produced by the females at the three oviposition occasions (18 out of 47 (38.3 %), 27 out of 46 (58.7 %) and 9 out 12 eggs (75 %), respectively (chi-square, 6.89; df=2; $P=0.032$)).

Table 4 Results of the three-way PERMANOVA test conducted on six behavioural variables

Source	DF	MS	*F*	*P*
Mount	1	26.37	5.195	0.0069*
Mating order	1	3.35	0.661	0.5642
Male	4	6.95	1.370	0.1688
Mount×mating order	1	9.07	1.788	0.1331
Mount×male	4	4.19	0.826	0.5696
Mating order×male	4	7.33	1.444	0.1375
Mount×mating order×male	4	8.1	1.597	0.1046
Residuals	99	5.07		
Total	118			

The value of the *F* statistic and its probability values *P* are shown

DF degrees of freedom, *MS* mean squares

* $P<0.05$

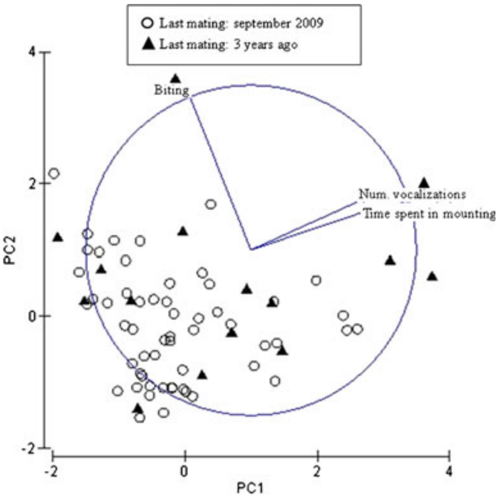

Fig. 2 Two-dimensional scatter plot of the first and second principal components of three behavioural variables in interactions involving females that mated last season and females which had not mated for 3 years. *Vectors* of the linear correlations between individual variables are superimposed on the graph

CERVUS was able to assign paternity to 38 of the 54 hatchlings (71 %) with a strict (95 %) or relaxed (80 %) confidence. However, the software indicated a "most likely candidate" father for the remaining 29 % of cases albeit with a lower confidence. The comparatively low successful paternity assignment was most likely due that the fact that the genotypes of all males partially overlapped for at least one of the five loci used in the analyses (Table 4).

In 13 of the 16 clutches, the female produced more than one fertile egg (Table 2). The paternity analyses revealed multiple paternity in 6 clutches of the 13 clutches (46 %). Four of the six clutches were sired by two males and two by three males.

The clutches from the first oviposition were entirely sired by males from the previous seasons, M00 siring 6 of 18 hatchlings (33 %) and M0 siring 12 of the 18 hatchlings (67 %). However, M00 did not sire any offspring at the two subsequent ovipositions (i.e. after the experimental matings). Remarkably, M0 sired 12 of the 27 (44.5 %) hatchlings at oviposition occasion 2 and 2 of the 9 hatchlings (22.2 %) emerging at the third oviposition occasion.

In the experimental matings (e.g. eggs produced at oviposition occasions 2 and 3), a total of 17 out of 22 eggs (77 %) were fertilized by M1 and 5 out of 22 eggs (23 %) by male M2; Fisher's exact test did not indicate any significant difference in siring success between the two experimental males (P=0.12).

Discussion

During the last decades, several theories have been proposed to explain ways in which females, including chelonian species, might use stored sperm. For example, the passive sperm-loss (Lessells and Birkhead 1990) and the sperm-displacement theories (Parker and Simmons 1991) predict that with sequential copulations, sperm from the last series of inseminations predominates within the female SSTs. In support of these models, last-male precedence has indeed been observed in painted (Pearse et al. 2002) and flatback turtles (Theissinger et al. 2009). Other hypotheses such as the fair raffle theory propose that the sperm of different males is randomly mixed in the female reproductive tract and that successful fertilization is proportional to sperm number (Parker 1990). In support of this hypothesis, paternity distribution in the loggerhead turtle (*Caretta caretta*) appears to fit such a raffle process (Sakaoka et al. 2011). Finally, first-male precedence as suggested by Birkhead (1998a) has, to our knowledge, not been recorded in any chelonians.

Our study aimed to clarify which of these sperm usage mechanisms occur in female Hermann's tortoises, particularly investigating the effect of mating order on courtship display, mounting and reproductive success of males. We predicted that, under a sperm competition scenario, if a last-male sperm precedence model was able to explain sperm utilization in this species, a male's ability to recognise whether a female has already been fertilized may be considered highly adaptive, allowing males to balance the costs and intensity of their courtship. These adaptations would result in different courtship displays and mounting success between the first and second male to mate with a determinate female. Accordingly, male paternity should be significantly skewed in favour to the last male.

Our behavioural results clearly demonstrate that different courtship displays are indeed associated with a successful mount. In particular, three behaviours (biting, running after and attempting to mount the female) were more frequent in successful interactions. Their occurrence was also significantly and positively correlated with mounting success, in accordance with observations by Galeotti et al. (2005a). Conversely, other male behaviours, such as observing, smelling and moving circularly around the female, which are generally considered to be part of the courtship display (Sacchi et al. 2003), did not differ in frequency between successful and unsuccessful interactions, and neither significantly correlate to mounting success. Thus, we suggest that these behaviours may be ascribed to as general social interactions which are not directly associated with mating. Biting, running after and attempts to mount a female are energy-demanding behaviours. The results from the present study and others (see Sacchi et al. 2003) show a positive association between the male energy devoted to courtship and the successful mounting of the female. Thus, in order to achieve successful mountings, male Herman's tortoises have to be in a good physical condition. Indeed, the observation of males involved in frequent sexual interactions shows higher hematocrit values and lower leukocyte concentrations, both indicators of health (Galeotti et al. 2005a).

Genetic analyses performed on experimental hatchlings clearly revealed the presence of multi-sired clutches, confirming that multiple paternity within the same clutch is common (see also Loy and Cianfrani 2010). Thus, as expected, polyandry in Hermann's tortoise results in sperm storage from different males within the female's reproductive tract and creates the basis for sexual conflict and sperm competition. Our results also support the findings of Kuchling (1999) and Pearse et al. (2001) that stored sperm remains viable in the female genital tract for at least 3 years, thus ensuring fertilization of eggs even in the absence of a partner.

Our analyses of behavioural data showed that males, within the same mating occasion, invest equally in courtship activity regardless of whether they are the first or the last to mount the female. Thus, males do not seem to evaluate whether females have recently mated with other partners during an on-going reproductive season, and males allocate their resources independent of the presence of rival sperm in the female reproductive tract. It has previously been shown that male Hermann's tortoises are able to detect sex and sexual maturity of potential mates by olfactory cues (Galeotti et al. 2007), but our results seem to exclude their ability of evaluating different levels of female receptivity. However, our analyses demonstrated increased courtship intensity and mounting success in interactions involving females which have not mated for several years, independent of the males' mating order. This result was most likely due to a greater co-operation by the female during mating attempts, i.e. not running away from the male, strongly suggesting her interest in receiving new and viable sperm.

As mentioned above, last-male precedence has been observed in some chelonians (Pearse et al. 2002; Theissinger et al. 2009). The results from the present study, however, did not reveal any significant difference in male paternity between the two males used in the experimental matings. Thus, mating order does not seem to affect male reproductive success, at least during a single reproductive season, and does not lend support to the last-sperm precedence hypothesis. In addition, in oviposition occasions 2 and 3, we observed considerable contribution of previous years' partners (i.e. male M0). Given that M1 and M2 achieved a similar mounting success (i.e. they spent the same time in copulation), presumably releasing a similar amount of sperm, with no significant difference in male paternity lends support to the hypothesis that male paternity in *T. hermanni hermanni* may be caused by a fair raffle (sensu Parker 1990). Moreover, our results suggest that females may actively optimize the utilization of stored sperm following a "sperm-vitality" criterion, as the relative paternity contributions of previous years' partners tended to diminish by time while that of experimental males tended to increase, suggesting that females first use the oldest, and presumably less viable, sperm present in SSTs until depletion and subsequently use newly acquired sperm. However, this pattern of sperm use could simply be a passive consequence of the long-term higher mortality in the female's reproductive tract of the sperm from the earlier inseminations than the sperm from the last inseminations, as suggested by Lessells and Birkhead (1990) and supported by our observation that hatching success was significantly smaller at oviposition 1 than at ovipositions 2 and 3. Thus, further studies elucidating variation in male fertilization success among successive reproductive seasons are needed to clarify sperm utilization in *T. hermanni hermanni*.

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Ethical standards All the experiments performed comply with the Italian and the European current laws on *Testudo* species.

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