



# Influence of male courtship intensity and male–male competition on paternity distribution in Hermann’s tortoise, *Testudo hermanni hermanni* (Chelonia: Testudinidae)

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Honest-advertisement models of sexual selection suggest that condition-dependent male secondary sexual characters could function as reliable signals of male quality, enabling females to discriminate among potential partners, both in the pre- and post-copulatory phases. In this context, many studies have revealed the importance of promiscuous mating systems and female sperm storage in determining the occurrence of such a model of sexual selection. By contrast, few studies have investigated the presence and extent of post-copulatory female choice in chelonian species. The present study aimed to investigate the effect of male size, male–male competition, and courtship intensity on paternity distribution in *Testudo hermanni hermanni*, combining behavioural and genetic data. We created experimental groups composed of two males of different sizes and three or four randomly selected females. Observations conducted during social interactions between males revealed that a hierarchy, unrelated to male size, was soon established: Alpha males were more aggressive towards competitors and courted females more intensively. Alpha males also achieved a higher mounting success than Beta males. Paternity analysis performed on hatchlings produced from experimental females revealed that male reproductive success was not correlated with male–female size ratio. Finally, despite the higher mounting success of Alpha males, paternity analysis revealed that male reproductive success did not differ between Alpha and Beta males. © 2014 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2014, 111, 656–667.

**ADDITIONAL KEYWORDS:** assignment – female choice – intrasexual competition – reproductive success – sperm storage.

## INTRODUCTION

Promiscuous mating systems and female sperm storage have important biological implications for sexual selection processes related to sperm competition and cryptic female choice (Parker, 1970; Eberhard, 1996; Birkhead & Pizzari, 2002). In recent years, theoretical and empirical studies have clarified many components of pre- and post-copulatory selection, such as competition, mate choice, sperm utilization, and sexual conflict (Birkhead & Pizzari, 2002 and Andersson & Simmons, 2006).

Honest-advertisement models of sexual selection suggest that condition-dependent male secondary sexual characters (i.e. phenotypic traits, courtship displays) could function as reliable signals of male quality, enabling females to discriminate among potential partners, both in the pre- and post-copulatory phases (Zahavi, 1975; Halliday, 1978; Brown & Brown, 1984; Simmons, 1988). Body size is the main phenotypic characteristic that, through various processes, may directly influence male mating success, such as enhancing physical prowess in sexual battles (Clutton-Brock, 1991; Andersson, 1994; Clutton-Brock & Parker, 1995). Another factor that can affect male siring success is courtship intensity; the energy allocated during the mating phase is typically

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condition-dependent because only healthy males should be able to perform elaborate and time-consuming displays and achieve successful copulations (Zahavi, 1977; Andersson, 1986).

Another mating system leading to nonrandom mating patterns and mate preferences is size assortative mating (i.e. large males typically pairing with large, often more fecund, females: Crespi, 1989; Olsson, 1993; Cooper & Vitt, 1997; Shine *et al.*, 2001). This mechanism has been demonstrated in a number of vertebrates and invertebrates in which size varies greatly among adults and sexual-size dimorphism exists (Mathis, 1991; Shine *et al.*, 2001, 2003; Sutherland, Hogg & Waas, 2007; Han *et al.*, 2010).

The topic of sperm competition and mate preferences has been extensively researched, with studies focusing in particular on model taxa such as insects and birds (Birkhead & Parker, 1997). However, although reptiles offer potentially excellent systems in which to investigate sexual selection and mate choice, little is known about the mechanisms at the basis of sperm utilization and paternity distribution. Most reptile species have a promiscuous mating system (Olsson & Madsen, 1998; Uller & Olsson, 2008), do not form pair bonds or cohesive social groups, and female sperm storage is widespread (Gist & Jones, 1987; Birkhead & Møller, 1993; Pearse, Janzen & Avise, 2001, 2002). Generally, neither sex provides parental care beyond nesting; thus, females do not receive clear direct benefits (e.g. parental care, nuptial gifts) from multiple matings. Moreover, pre-copulatory mate choice is rare and females accept copulation attempts from most males (Olsson & Madsen, 1995; Lebas & Marshall, 2001; Stuart-Smith, Swain & Wapstra, 2007); therefore, a male's contribution to female fitness is restricted to its genes. Thus, polyandry may have evolved not only as a mechanism for females to ensure fertilization of eggs in cases of low mate encounter probability (Olsson & Madsen, 1998), to increase offspring genotypic diversity (Loman, Madsen & Hakansson, 1988; Yasui, 1998) or to avoid genetic incompatibility (Olsson *et al.*, 1996; Zeh & Zeh, 1996), but also could be a solution to obtain good genes from high-quality males (Magnhagen, 1991; Madsen *et al.*, 1992; Olsson & Madsen, 1995; Houle & Kondrashov, 2002; Martín & López, 2006).

Among reptiles, chelonian species represent one of the most outstanding examples of promiscuity and female sperm storage. These features, combined with the sexual size dimorphism and elaborate male courtship display typical of most species, represent an ideal foundation for investigating the factors at the basis of male reproductive success and have important implications for the existence of a female mate choice. Previously, many studies have emphasized the

importance of male–male competition in determining hierarchies (Weaver, 1970; Berry & Shine, 1980; Kaufmann, 1992; Niblick, Rostal & Classen, 1994), and recent studies of *Testudo* species have revealed the importance of courtship intensity and male health in mounting success (Sacchi *et al.*, 2003; Galeotti *et al.*, 2004, 2005b; Pellitteri-Rosa *et al.*, 2011). However, no studies have linked these findings to male reproductive success. Biases in paternity distribution could simply be related to differences in the amount of sperm released within the female's oviduct (the loaded raffle hypothesis: Parker, 1990), or could indicate the higher quality of a male's sperm (Parker, 1970) or pre- and post-copulatory sexual selection by females in favour of specific males (Eberhard, 1996, 1998; Andersson & Simmons, 2006). Indeed, a recent study performed on *Testudo hermanni hermanni* (Cutuli *et al.*, 2013) excluded the influence of mechanical factors, such as mating order, on male reproductive success. Thus, factors such as the time spent in copulation with a female, male size and quality, and/or female cryptic choice must be taken into consideration to explain the observed patterns of sperm use by females in this species.

Using a combined approach of behavioural observation and genetic parental assignment analyses, the present study aimed to determine whether certain phenotypic traits (i.e. body size) and signals exhibited by males of *T. h. hermanni* during courtship could influence their mounting and reproductive success. In particular, we predicted that, during social interactions, larger and/or more vigorous and active males should be favoured in male–male competition and obtain more matings with females, therefore achieving a higher reproductive output. Alternatively, if size-assortative mating occurs in this species, as observed in *T. marginata* (Sacchi *et al.*, 2003), we predicted that males should achieve a higher mounting and reproductive success with females of similar dimensions. The presence of such a mating criterion could be a consequence of the sexual dimorphism (i.e. males are smaller than females) typical of many Testudinidae species (Lagarde *et al.*, 2001; Willemsen & Hailey, 2003). Because all males tend to prefer large and more fecund females (Fitch, 1970; Seigel & Ford, 1987), we hypothesize that larger males can exclude the smaller males from mating, or smaller males could simply be unable to court and mount larger females, as suggested by Olsson (1993).

## MATERIAL AND METHODS

### STUDY SPECIES

Hermann's tortoise (*T. h. hermanni*; Gmelin, 1789) is one of the three species of Testudinidae endemic

to Europe and occupies a relatively wide range of habitats, specifically thermophilous forests along coastlines, scrubs, meadows, and pastures (Ernst & Barbour, 1989). Living at low population densities, they have non-exclusive home ranges. Females are polyandrous and copulate with several males during the same breeding season (Swingland & Stubbs, 1985; Ernst & Barbour, 1989). Mating can occur throughout the entire activity period (from early spring to late summer; Mazzotti, 2006), with two peaks of male courtship activity in April/May (after hibernation) and September (before hibernation). During courtship, the male runs after the female, biting, ramming, and attempting to immobilize and force the female to copulate. The female actively attempts to avoid sexual intercourse with the male by running away, although a greater acceptance rate has been observed in females that have not mated for 3 years (Cutuli *et al.*, 2013). Consequently, many attempts are usually required 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 with the time spent in copulation and, possibly, the amount of sperm released into the female genital tract. Moreover, these vocalizations display a harmonic structure with a frequency and amplitude modulation negatively related to body size (Galeotti *et al.*, 2005a).

Females usually lay two or 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. Sperm storage tubules are located in the posterior portion of the albumin-secreting region of the oviduct (Gist & Jones, 1987, 1989; Gist & 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 & Avise, 2001; Cutuli *et al.*, 2013). Multi-sired clutches are frequent (Kuchling, 1999; Loy & Cianfrani, 2010; Cutuli *et al.*, 2013), and females are able to fertilize their eggs even when there are no available mates. Furthermore, these characteristics may lay the basis for sperm competition and/or cryptic female choice processes (Parker, 1970; Eberhard, 1996).

#### STUDY AREA, TORTOISE CAPTURE AND BREEDING

Tortoises were collected within the National Park of the Colline Metallifere Grossetane, near Massa Marittima (Grosseto, Tuscany). The climate is Mediterranean-temperate, with cold winters, rainy springs and autumns, and dry and hot summers. The vegetation is characterized by a mosaic of habitat

types, primarily mixed forest dominated by *Quercus ilex* and *Quercus cerris*, Mediterranean maquis and plantations including olive (*Olea europea*) and fruit trees.

During March/April 2011, a total of 62 wild individuals of *T. h. hermanni* were sampled in an area of approximately 12 ha (altitude ranged from 174 to 214 m a.s.l.) immediately after hibernation, to prevent individuals mating before the experiments (Cutuli, Vannini & Fratini, 2013). For each animal, the collected data were: sex, straight-line carapace length (SCL; Stubbs & Swingland, 1985), carapace width and weight, and photographs of both the carapace and plastron were also taken. For our experiments, we selected 39 adult individuals (25 females and 14 males), excluding sexually immature or diseased individuals (i.e. with parasites, mycosis, carapace fractures). Selected individuals were transferred into enclosures built on-site to allow behavioural observations and collection of eggs laid by females. Enclosures were completely enclosed by nets to avoid predation on adults and eggs. Groups of five or six individuals of the same sex were put into enclosures (3 × 3 m), in which wild bushes and artificial refuges guaranteed adequate shelter from weather for all individuals. Food (i.e. wild plants collected on-site and vegetables) and water were provided *ad libitum* once per day. At the end of the reproductive season, all of the 39 experimental individuals and hatchlings born after experimental matings were released in the area from which they were collected.

#### EXPERIMENTAL MATINGS AND BEHAVIOURAL OBSERVATIONS

Experimental matings took place in April and May 2011. We created eight experimental groups, each comprising three or four randomly selected females and two males of different dimensions; thus, each group included a larger and a smaller male (Table 1). Two experimental males were used twice in two different groups. The number of females within relative small breeding groups does not appear to affect male reproductive success, whereas the number of males available is important because of the establishment of hierarchies that may influence access to females. Because we divided the individuals into experimental groups prior to the start of reproductive activities, we can exclude the possibility that recent matings occurred after hibernation. However, because females of this species can store sperm for up to 3–4 years (Cutuli *et al.*, 2013), we could not exclude the possibility of the presence of old sperm in their oviducts.

Each group was sequentially held in a mating arena (2 × 3 m) for 3 days and carefully observed by

**Table 1.** Experimental plan of matings

Group	Females' code	Larger male	Smaller male	V $\alpha$
1	7, 3, 2	Alpha	Beta	5/7
2	18, 20, 6, 23	Alpha	Beta	11/13
3	12, 10, 14	Alpha	Beta	5/5
4	9, 1, 8	Alpha	Beta	8/9
5	17, 15, 4	Beta	Alpha	3/5
6	19, 5, 11	Beta	Alpha	6/6
7	13, 22, 24	Beta	Alpha	2/3
8	26, 27, 29	Beta	Alpha	2/2

Respective dimension and hierarchy (defined on the basis of inter-male combats) is reported for each male pair, together with the number of interactions won by the Alpha male. V $\alpha$ , Alpha's victories/male–male interactions.

two experimenters for 6 h day<sup>-1</sup> during the periods of maximum activity (early morning and late afternoon). Animals were separated after each observation session to avoid any additional intra- and intersexual interactions when observers were not present. Food, water, and shelter, as in the breeding enclosures, were provided.

For each male–male interaction, we recorded the occurrence of aggressive displays exhibited by the two competitors: the number of times a male (1) bit, (2) ran after, (3) rammed, and (4) mounted the rival. Thus, for each inter-male combat, it was possible to determine a 'dominant' (i.e. the male that exhibited aggressive behaviour) and a 'subordinate' (i.e. the male that was subject to aggressive behaviour). Subsequently, for each experimental group, we defined a hierarchy between the two males based on the outcome of all male–male interactions observed during the 3 days. Thus, we considered the male that won the majority of combats to be the Alpha male and the male that lost most combats to be the Beta male (Table 1). Aggressive interactions between males occurred rather rarely in a day because males spent most of their time smelling and observing each other or mating with females; for this reason, at least 3 days were required to establish a hierarchy within a group.

During the experimental matings, for each male–female interaction (i.e. encounter between the two individuals), we recorded the occurrence and duration of four behaviours performed by males, and typical of tortoise courtship (Weaver, 1970; Sacchi *et al.*, 2003), that were well correlated with mounting success in a previous study (Cutuli *et al.*, 2013): the number of times the male (1) bit, (2) ran after, (3) and attempted to mount the female, and (4) the number of vocalizations emitted by males during each mount.

We also recorded (5) the number and duration of successful mounts (i.e. those associated with penile insertion).

Biting is the most frequent behaviour both in male–male and courtship interactions, although it rarely causes injuries. In our experimental approach, we planned to separate animals in cases where a bite would cause a bleeding wound and to carefully check animals for injuries. However, during experimental observations, it was never necessary to intervene to protect animals from an aggressor because the mating arena had shelters and was sufficiently large to permit individuals to escape. After the mating experiments, females were transferred into an oviposition enclosure and all except five individuals produced one or two consecutive clutches, henceforth referred to as oviposition occasion 1 and 2. Twenty of the 25 females produced clutches at oviposition occasion 1, and 13 of these 20 females produced a second clutch (i.e. at oviposition occasion 2) (Table 2). All eggs were collected upon laying, individually marked on the shell using a nontoxic pen, and transferred into an artificial incubator (at 31 ± 0.5 °C and 70–80% humidity for approximately 2 months). Each emergent hatchling was individually marked to assign it to a specific female and clutch.

All of the experiments performed complied with the current Italian and European laws on *Testudo* species. Permits and ethical approvals were released by Comunità Montana Colline Metallifere (Protocol Number 7263, 26 October 2010; Protocol Number 7618, 12 October 2011) and Corpo Forestale dello Stato, Servizio CITES Territoriale, Firenze (Protocol Number 2535, 5 April 2011).

#### GENETIC ANALYSIS AND PATERNITY ASSIGNMENT

Samples for genetic analysis were collected using buccal swabs, in accordance with a non-invasive procedure suitable for small-sized individuals (such as hatchlings), and avoiding the dangers associated with blood sampling (Wingfield, 1999; Poschadel & Møller, 2004; Broquet *et al.*, 2007). Total genomic DNA from all experimental adults and hatchlings was extracted from epithelial cells by combining alkaline and temperature lysis. The swab was soaked in 500 µL of 50 mM NaOH at 97 °C for 10 min. To maximize DNA concentration, the swab was placed into a second vial and centrifuged for 5 min. This extra solution was then added to the first solution before adding 75 µL of 1 M Tris (pH 8.0). The mean DNA concentration in a 100-µL volume was 100–150 µg mL<sup>-1</sup>. Extracted DNA was stored at 4 °C and at –20 °C for long-term storage.

All of the individuals sampled in the study area were screened at the six most polymorphic



**Table 2.** Female reproductive output and hatching success at oviposition occasions 1 and 2, as well as mean clutch size for both oviposition occasions

Female	Oviposition 1			Oviposition 2		
	Fertile eggs	Infertile eggs	% Hatching success	Fertile eggs	Infertile eggs	% Hatching success
1	–	–	–	–	–	–
2	5	0	100	–	–	–
3	0	2	0	2	0	100
4	0	2	0	1	0	100
5	–	–	–	–	–	–
6	–	–	–	–	–	–
7	2	2	50	3	0	100
8	3	0	100	–	–	–
9	2	1	66.67	2	1	66.67
10	–	–	–	–	–	–
11	0	2	0	3	0	100
12	0	2	0	1	2	33.33
13	0	3	0	3	1	75
14	1	1	50	–	–	–
15	0	2	0	1	2	33.33
17	4	0	100	3	0	100
18	3	0	100	–	–	–
19	–	–	–	–	–	–
20	0	2	0	2	0	100
22	0	2	0	2	0	100
23	0	1	0	0	2	0
24	0	3	0	–	–	–
26	4	0	100	–	–	–
27	0	2	0	–	–	–
29	2	0	100	1	1	50
Total	26	27	38.3	24	9	73.8
Mean clutch size		2.65 ± 0.22			2.53 ± 0.21	

microsatellite loci (Leo10, Leo56, Leo71, Leo 76, GmuB08, and GmuD51) among the 11 previously tested in *T. h. hermanni* by Cutuli *et al.* (2012).

The forward primer for each locus was 5'-labelled and each locus was amplified using the polymerase chain reaction (PCR) conditions reported by Cutuli *et al.* (2012). For detection of polymorphisms, labelled amplicons from the six loci were divided into two sets (Leo10-NED + GmuBo8-HEX + Leo56-FAM and Leo76-NED + 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, version 3.7 and GENESCAN, version 3.7 (Applied Biosystems).

Paternity assignment was carried out using likelihood-based paternity inference implemented in CERVUS, version 3.0 (Marshall *et al.*, 1998;

Kalinowski, Taper & Marshall, 2007). 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 of the candidate male being the true father and the likelihood of the male not being the true father. A delta score was then 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 is correctly identified. This proportion represents the confidence level of the analysis, which was set to 95% (strict) and 80% (relaxed) in our analysis. The simulations also included parameters such as the number of candidate

fathers, proportion of candidate fathers sampled, and estimated frequency of typing error when generating genotypes. Because the mating history of the females prior to 2011 was unknown, we considered 'unknown males' (UM) as the most likely sires when CERVUS was not able to assign any of the experimental males of each group as the father (i.e. when the confidence level was  $< 80\%$ ).

#### STATISTICAL ANALYSIS

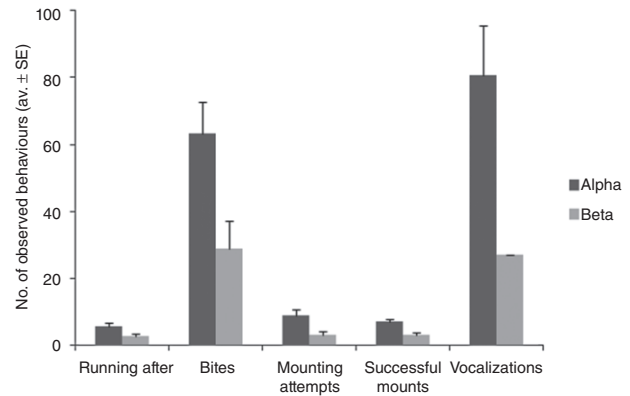
Two-tailed  $t$ -tests were performed to investigate potential significant differences in SCL and weight between the two male types (Alpha or Beta) and to determine whether there was a difference in their mounting success (number of mounting attempts, successful mounts, and vocalizations).

The homogeneity of variances among groups of behavioural variables was assessed by Levene's test. A two-way permutational multivariate analysis of variance (PERMANOVA) was performed, based on similarity matrixes computed using Euclidean distances, to determine differences in male courtship intensity (five behavioural variables) across groups of interactions involving Alpha or Beta males ('hierarchy', random, and orthogonal) and considering male/female size ratio (M/F size ratio, fixed, and orthogonal). 'Male size' was also added to the design as a covariate.

canonical analysis of principal coordinates (CAP; Anderson, Gorley & Clarke, 2008) was performed to determine which behaviours were different between Alpha and Beta males, as well as to what extent they differed.

One-way analysis of variance was applied to find differences among experimental groups in total female hatching success and hatching success of eggs sired by the experimental males only (factor 'group' fixed and orthogonal).

Further analyses were performed taking into account the reproductive success of each male type, expressed as a percentage of sired offspring in each clutch (data were arcsine transformed). First, a distance-based linear model (Dist-LM) was performed to determine whether any of the five behavioural variables were significantly related to the reproductive success that each male achieved with each fertilized females. Second, linear regressions were performed to test whether M/F size ratio, male dimension, and number of vocalizations emitted during the mounts were correlated with male mounting and reproductive success. In particular, reproductive success may be strictly correlated with the time spent in copulation and possibly to the amount of sperm released into the female genital tract, as suggested by previous studies (Sacchi *et al.*, 2003; Galeotti *et al.*,



**Figure 1.** Mean  $\pm$  SE of the five behavioural variables in interactions involving Alpha or Beta males.

2004). Finally, a two-tailed one sample  $t$ -test against the theoretical mean of 0.5 was applied to Alpha male's paternity values to determine whether these males had significantly higher reproductive success than Beta males.

Univariate analyses were performed on behavioural data using EXCEL 2010 (Microsoft) and PAST, version 2.04 (Hammer, Harper & Ryan, 2001). Multivariate analyses were performed using PRIMER, version 6.1 (Clarke & Gorley, 2006) and PERMANOVA+ for PRIMER routines (Anderson *et al.*, 2008).

## RESULTS

### MALE-MALE INTERACTIONS AND HIERARCHY

We observed a total of 47 male-male interactions, on the basis of which we established a hierarchy between the two males in each group (Table 1). There was no significant influence of SCL ( $t = 0$ , d.f. = 14,  $P = 1$ :  $t$ -test) or weight ( $t = -0.03$ , d.f. = 14,  $P = 0.9$ :  $t$ -test) on male hierarchy (i.e. the outcome of male-male competition).

### MALE-FEMALE INTERACTIONS AND MOUNTING SUCCESS

We recorded a total of 180 male-female interactions during 70 h of observations. Alpha males made more mounting attempts ( $t = 2.23$ , d.f. = 14,  $P = 0.04$ :  $t$ -test) and successful mounts ( $t = 2.25$ , d.f. = 14,  $P = 0.04$ :  $t$ -test) than Beta males. Moreover, they emitted more vocalizations during these mounts ( $t = 2.1$ , d.f. = 14,  $P = 0.05$ :  $t$ -test).

A two-way PERMANOVA performed on all of the recorded interactions indicated that the factor 'hierarchy' significantly affected courtship displays as well as the covariate 'male size', whereas the factor M/F size ratio did not influence courtship behaviour of experimental males (Fig. 1, Table 3). The results of

**Table 3.** Results of two-way permutational multivariate analysis of variance test conducted on the five behavioural variables

Source	d.f.	MS	<i>F</i>	<i>P</i>
Male size (covariate)	1	199 950	4.13	<b>0.022</b>
Hierarchy	1	52 157	10.92	<b>0.0001</b>
Male/Female size ratio	1	9007.7	3.56	0.177
Hierarchy × Male/Female size ratio	1	2298.8	0.4	0.595
Residuals	45	4776.1		
Total	49			

Significant values are shown in bold.

CAP showed that the courtship display of Alpha males was characterized by a greater intensity in all of the observed behaviours with respect to Beta males (total cross-validation results: 76% of correct assignments).

Finally, neither the time spent in sexual interactions nor mounting success of males were correlated with M/F size ratio ( $R^2 = 0.033$ , d.f. = 48,  $F = 1.65$ ,  $P = 0.20$  and  $R^2 = 0.00045$ , d.f. = 48,  $F = 0.021$ ,  $P = 0.88$ , respectively).

#### PATERNITY ANALYSIS AND REPRODUCTIVE SUCCESS

A total of 86 eggs were collected, of which 50 (58.2%) successfully hatched. Reproductive output, mean clutch size, and percentages of hatching success for each experimental female at the two ovipositions are reported in Table 2. Of the 25 experimental females, five did not lay an egg and three laid only infertile eggs. Considering the 13 females that laid two successive clutches, we observed great variation in the proportion of fertile eggs produced at the two oviposition occasions (10 out of 31, corresponding to 32.25%, at oviposition 1, and 24 out of 32, corresponding to 75%, at oviposition 2;  $\chi^2 = 11.58$ , d.f. = 1,  $P < 0.001$ ).

Only total hatching success and hatching success of eggs sired by the experimental males did not differ among experimental groups (cumulated data for both oviposition occasions,  $F = 0.516$ , d.f. = 7,  $P = 0.81$  and  $F = 0.874$ , d.f. = 7,  $P = 0.55$ , respectively; one-way ANOVA).

CERVUS was able to assign paternity to 84% of the hatchlings with a strict (95%) or relaxed (80%) confidence; in the remaining cases (16%), CERVUS did not assign any of the known experimental males in each group; thus, we considered these hatchlings as offspring of 'unknown males' from previous years.

In 16 of the 21 successfully hatched clutches, the female produced more than one fertile egg (Table 2). Fertile eggs were collected from each experimental group, and all males except two (the beta males of

groups 2 and 6) sired at least one egg (Table 4). The paternity analyses revealed multiple paternity in 12 of the 16 clutches (75%) (Table 4). Of these 12 clutches, 10 were sired by two males and two by three males (Table 4).

The contribution of males of previous years (UM) was found both at the first (11.6%) and second (20.8%) ovipositions (Table 4). Considering only the contribution of experimental males, at oviposition 1, Alpha males sired 13 (56.5%) and Beta males sired 10 (43.5%) eggs, whereas, at oviposition 2, the siring success of Alpha males decreased slightly to 10 out of 19 eggs (52.6%). Reproductive success of Alpha males was not significantly different from the mean of 0.5 (cumulated data for all females and both ovipositions:  $t = 0.087$ , d.f. = 16,  $P = 0.93$ : one sample *t*-test).

Siring success was not correlated with the number of vocalizations emitted during mounts ( $R^2 = 0.001$ , d.f. = 32,  $F = 0.031$ ,  $P = 0.85$ ). A Dist-LM performed considering both courtship display exhibited by males and the proportion of sired offspring revealed that none of the five behavioural variables recorded significantly affected male reproductive success ( $P > 0.05$  in all cases; data are not shown).

Finally, neither male dimension ( $R^2 = 0.003$ , d.f. = 32,  $F = 0.093$ ,  $P = 0.76$ ), nor M/F size ratio ( $R^2 = 0.002$ , d.f. = 32,  $F = 0.005$ ,  $P = 0.94$ ) were correlated with male reproductive success.

#### DISCUSSION

Reproductive biology of tortoises represents an ideal system for testing sexual selection models of honest-advertisement (Zahavi, 1975; Halliday, 1978). Sexual size dimorphism, promiscuous mating systems, long-term sperm storage, and elaborate courtship behaviour are widespread in tortoises, as in most chelonian species, and these characteristics have important implications for mate preferences, sperm competition, and cryptic female choice (Parker, 1970; Eberhard, 1996). Moreover, because males must usually convince females to accept mounting, it appears likely

**Table 4.** Siring contribution of experimental males and unknown males from previous years (UM) for each clutch laid by the experimental females

Group	Female	Oviposition 1			Oviposition 2		
		Alpha	Beta	UM	Alpha	Beta	UM
1	7	1	0	1	0	1	2
	3	–	–	–	1	1	0
	2	0	4	1	–	–	–
2	18	2	1	0	–	–	–
	20	–	–	–	1	1	0
	6	–	–	–	–	–	–
3	23	–	–	–	–	–	–
	12	–	–	–	1	0	0
	10	–	–	–	–	–	–
4	14	1	0	0	–	–	–
	9	0	2	0	1	1	0
	1	–	–	–	–	–	–
5	8	1	2	0	–	–	–
	17	4	0	0	2	1	0
	15	–	–	–	0	1	0
6	4	–	–	–	0	1	0
	19	–	–	–	–	–	–
	5	–	–	–	–	–	–
7	11	–	–	–	1	0	2
	13	–	–	–	1	1	1
	22	–	–	–	1	1	0
8	24	–	–	–	–	–	–
	26	2	1	1	–	–	–
	27	–	–	–	–	–	–
Total	29	2	0	0	1	0	0
		13	10	3	10	9	5

that females are the choosy sex in these species because they ultimately determine whether copulation occurs (Kuchling, 1999; Galeotti *et al.*, 2005b). For these reasons, male phenotypic traits, such as size, and signals exhibited by males during courtship may represent a cue that females can use to assess their partner's qualities.

Our study of wild individuals of *T. h. hermanni* aimed to determine whether male–male competition, male size, and courtship intensity could influence male reproductive success. In particular, we predicted, first, that larger males should be favoured in intrasexual competition, and second, that the winning males in male–male competitions should achieve a higher mounting and reproductive success over smaller and less aggressive males.

Analysis of behavioural data revealed that, under male–male competition conditions, a hierarchy between males is clearly established, influencing male access to females; Alpha males (those more aggressive with competitors) courted females more intensively and achieved higher mounting success than Beta

males. We also found that the hierarchy between males is not related to male size, and, consequently, to age, because size and age are strongly related (Loy *et al.*, 2007). Because male size was significantly correlated with courtship intensity but not with hierarchy, large males were shown to be more determined in courting females, although their hierarchy and not their size determined their mounting success.

Although the role of intrasexual competition in chelonian species is still controversial (Swingland & Stubbs, 1985; Sacchi *et al.*, 2003; Willemsen & Hailey, 2003), many studies have emphasized the importance of inter-male combats in determining hierarchies, with high-ranking individuals achieving a higher copulation success (Weaver, 1970; Berry & Shine, 1980; Kaufmann, 1992; Niblick *et al.*, 1994); the results of the present study appear to support these findings.

Because Alpha males invested more energy both during male–male combats and the courtship phase, we may hypothesize that they were in better health



condition than their competitors. This hypothesis is supported by the findings of Galeotti *et al.* (2005b), who demonstrated that *T. hermanni* males involved in frequent sexual interactions display higher haematocrit values and lower leukocyte concentrations, both of which are indicators of good health.

Considering the absence of a significant correlation between male–female size ratio and mounting and reproductive success, we can reasonably exclude the presence of size-assortative mating in *T. h. hermanni* (i.e. larger males do not mate more often with larger females). This does not accord with observations in other reptile species (Olsson, 1993; Cooper & Vitt, 1997; Shine *et al.*, 2001), including the congeneric *T. marginata* (Sacchi *et al.*, 2003). The absence of a significant correlation between male mounting success and male/female size-ratio may indicate that, in *T. h. hermanni*, all males may have access to larger and more fecund females, independent of their body size, and that larger females accept mounting by smaller males too.

Genetic analyses performed on experimental hatchlings revealed the presence of multi-sired clutches, confirming that multiple paternity within the same clutch is common in this species (Loy & Cianfrani, 2010; Cutuli *et al.*, 2013). Evidence of partners of previous years siring some hatchlings supports the findings of Kuchling (1999) and Cutuli *et al.* (2013), who found that stored sperm remains viable in the female genital tract for years, thus ensuring fertilization of eggs even in the absence of a partner. Furthermore, the percentage of hatching success significantly increased in the two successive ovipositions of the season, confirming a trend observed by Cutuli *et al.* (2013). This could be the consequence of a higher maternal investment in the last oviposition of the season with respect to the previous ovipositions. Further analyses of egg quality would allow us to confirm or refute this hypothesis.

Despite the higher mounting success achieved by Alpha males compared to Beta males, parental genetic assignment did not reveal any siring advantage for Alpha males in the near future. This may suggest that females do not operate any post-copulatory selection of sperm based on behavioural traits exhibited by males during courtship and mating; on the other hand, it may suggest that Alpha male sperm does not possess a higher competitive ability with respect to achieving egg fertilization. However, we cannot exclude the existence of a post-copulatory female choice based on other factors, such as major histocompatibility complex genotype compatibility (Olsson & Madsen, 2001), that may favour a male independently of its rank or size.

The similar reproductive success observed for Alpha and Beta males could also be a result of the adoption

of alternative reproductive strategies by Beta males, such as sneaker males and males mimicking females (Gross, 1996). However, these strategies have not been investigated or described in *Testudo* species, and were not observed during our experimental matings. Thus, it appears that the only reproductive tactic for *T. hermanni* males is to be dominant/subordinate in relation to the other males (Gross, 1996): this strategy guarantees that all males will mate with females and achieve a certain reproductive success during their life, as clearly demonstrated by the behavioural and genetic data from the present study.

Moreover, Alpha males spent significantly more time in copulation with the females, and presumably, as suggested by Sacchi *et al.* (2003), they load a greater amount of sperm into the oviducts than Beta males. This observation could lead to the hypothesis that Alpha males can sire a higher number of eggs, in accordance with the fair raffle theory (Parker, 1990), which predicts the siring probability of each male to be equal to the proportion of released sperm. However, our results do not support this hypothesis; further studies to measure the actual amount of loaded sperm within the female oviduct are necessary to confirm or refute it.

Alternatively, the actual success of a male can be calculated for the entire life span of its sperm within the female's oviduct. In this case, we do not exclude that the real siring success of Alpha males over the ongoing reproductive season may exceed that of Beta males, in accordance with a loaded raffle hypothesis (Parker, 1990). If a long-term advantage for Alpha males exists, this could be the result of better health conditions (as discussed above) and/or to a higher quality of their sperm able to remain viable for a longer period in the female genital tract. Moreover, Alpha males could transmit their genetic qualities to their offspring, eventually enhancing their survival, growth and viability (Yasui, 1997; Birkhead, 1998; Olsson & Madsen, 2001).

The present study did not reveal any evidence of direct female choice in favour of larger and more vigorous males; therefore, further studies investigating variation in sperm viability and offspring survival, as well as the existence of a genetic-based mate preference, are needed to clarify the mechanisms of the sexual selection processes in *T. h. hermanni*. Additionally, because *T. hermanni* lives at low densities, further studies of natural populations may allow the investigation of whether female sexual choice is influenced by the chance to encounter a male, as suggested by Jennions & Petrie (2000) and modelled by Kokko & Mappes (2005). Indeed, these studies suggested that, in low density populations, an optimally behaving female should first mate unselectively and then improve mate quality in later matings.

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