

Focus on Determinants of Male Fertility

Sperm traits and male fertility in natural populations

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Abstract

Male fertility has seldom been studied in natural populations because it has been assumed that strong selection would result in uniformly high values among males, and therefore mating success has been equated with fertilisation success. In contrast, male fertility has received much attention in studies of domestic livestock, where economic benefits rely on improving productivity, and in human infertility studies, where the efficiency of treatments depends on understanding which ejaculate traits explain reproductive failures and predict success at assisted conception. Despite years of efforts, no conclusive results have been obtained, probably because such studies have focused on opposite extremes of the range with little variation: domestic livestock have often been subject to strong artificial selection for high fertility, and human patients requiring treatment have compromised fertility. Recent findings from natural populations of red deer have shown that males differ markedly in their fertility, and have revealed the degree of variation found in different semen traits, both between and within males. Fertility trials have shown that male fertility is determined mainly by sperm swimming speed and the proportion of normal sperm, when sperm numbers are kept constant. Sperm design exerts a strong influence on sperm swimming speed, with faster swimming sperm having elongated heads, shorter midpieces and a longer principal plus terminal pieces in relation to total flagellum length. Thus, the large inter-male variation in sperm design found among natural populations underlies differences in sperm swimming speed which, in turn, determine differences in male fertility rates. Secondary sexual characters are honest indicators of male fertility, so males with large and elaborated antlers have larger testes and faster swimming sperm. Testosterone does not seem to mediate the relationship between antler size and semen quality, since it is associated with sperm production, but not with sperm quality or antler size. Finally, more fertile males produce a greater proportion of sons, who will inherit the semen traits which will enhance their fertility.

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Male reproductive success in natural populations

Evolution is the genetic turnover of the individuals of every population from generation to generation (Mayr 2001). In natural populations, individuals vary in their ability to survive and reproduce, and thus differ in how many copies of their genes they pass on to the next generation. Selection acts on differences in lifetime reproductive success between individuals, which are the result of a complex array of phenotypic traits, which in turn result from an interaction between genetic and environmental factors. Throughout evolution, heritable traits which enhance individual reproductive success will be selected over many generations and will spread. Two main selective forces have been recognised: natural and sexual

selection. Natural selection refers to traits which increase reproductive success through improved survival, while sexual selection refers to traits which confer advantages in terms of enhanced reproduction, either because such traits make males more attractive to females or they make males more competitive in intra-sexual (male–male) contests.

The way in which males and females maximise lifetime reproductive success is very different. Females tend to show higher levels of investment in offspring, and for this reason, they have slower reproductive rates (Clutton-Brock 1988). Differences between females in reproductive success are mainly due to differences in offspring mortality. In contrast, males tend to invest less in offspring, their reproductive rates are higher, and their reproductive success is determined mainly by the number of females

they are able to fertilise. Differences between males in lifetime reproductive success are, in most species, higher than among females, so selection on males is expected to be intense. Competition between males to mate with females is therefore strong and has led to the evolution of conspicuous traits like ornaments which make them more attractive to females, or weapons which enhance their ability to win agonistic encounters. In many species, females mate with several males, and competition between males to fertilise ova continues after copulation in the form of sperm competition. This selective pressure has favoured the evolution of larger testes, enhanced sperm production (Gomendio *et al.* 1998), faster swimming sperm (Gomendio & Roldan 1991, Birkhead *et al.* 1999, Gage *et al.* 2004) and a higher proportion of sperm ready to undergo changes required for fertilisation (Gomendio *et al.* 2006a).

Sex differences in reproductive strategies become more exacerbated the greater the asymmetry between males and females in levels of investment in offspring. Most mammals represent an extreme case, since females invest heavily through gestation and lactation, while males do not invest in offspring beyond sperm production. Thus, variation between males in lifetime reproductive success is high, male–male competition is intense, and females may be choosy about mating partners. In mammals with a polygynous mating system, males defend a harem or a territory to gain sexual access to females, and as a consequence, a few males in the population father the majority of offspring in each breeding season (red deer, Clutton-Brock *et al.* 1988; fur seals, Hoffman *et al.* 2003). Competition between males to copulate with females has led to the evolution of traits that improve the chances to win agonistic encounters with other males, such as large body size or weapons (Andersson 1994). A great deal of effort has been made to understand how selective forces have shaped behavioural, morphological and physiological traits that enhance male competitiveness before and after copulation (Andersson 1994, Gomendio *et al.* 1998). However, most of the studies have assumed that every copulation leads to fertilisation and ignored the possibility that males in natural populations may also differ in their fertility. Such view does not take into account that male reproductive success relies ultimately on the ability of his spermatozoa to fertilise.

Over the last years, our research group has undertaken a comprehensive study of natural populations of Iberian red deer (*Cervus elaphus hispanicus*) to examine if males do differ in fertility rates and, if so, which are the main determinants of male fertility. This species is a good model to examine these issues because it shows strong polygyny, so male fertility may have a considerable impact on reproductive rates within populations; intense sexual selection has led to the evolution of remarkably elaborated sexual ornaments (antlers) and strong sexual dimorphism; and it is a seasonal breeder so males have

a limited period of time to fertilise females. In addition, culling of males during the breeding season makes it possible to gain access to large sample sizes, which are representative of the degree of variation found in natural populations. Finally, we have used an experimental approach which makes use of the variation in semen quality found between males in natural populations to artificially inseminate females from a captive population where other variables can effectively be controlled.

Red deer mating system

Red deer are seasonal breeders, so males are reproductively active during a few months of the year. In the northern hemisphere, antlers are cast at the end of the winter and regrown during the spring. The velvet is shed at the end of August, when sperm production begins. Mating systems have been extensively studied in populations from northern Europe, where males defend harems during the breeding season (Clutton-Brock *et al.* 1982). The rut begins in early September and males spend most time and energy fighting other males or copulating with females; as a consequence males lose physical condition during the breeding season.

In male–male encounters, males display their antlers presumably to assess their opponents' strength, and they are used as weapons when males engage in fights. It is known that fighting success influences harem size, which, in turn, is related to copulation success (Clutton-Brock *et al.* 1982). Antler size is related to the number of calves fathered by males, and it has been assumed that this is exclusively the result of males with large antlers being able to win more fights with other males (Kruuk *et al.* 2002). The prevalence of sperm competition in red deer populations is currently believed to be limited. Nevertheless, females do not stay in the same harem during the whole breeding season, so they may copulate with several males during a given sexual cycle; this possibility is supported by the fact that red deer males have large testes in relation to their body size when compared with other species from the *Cervidae* family (Clutton-Brock *et al.* 1982).

After the breeding season, males and females separate. Females form stable groups of related individuals, while males have rather weak social relationships (Clutton-Brock *et al.* 1982). In northern Europe, winter is a critical period due to harsh environmental conditions and food scarcity, and mortality is high, particularly among weak individuals or calves which have not grown large enough. Females give birth during spring, so lactation coincides with a period of food abundance. Lactation is costly energetically, and lactating females are less likely to reproduce the following year than non-lactating females. A consequence of sexual dimorphism in body size is that mothers incur higher reproductive costs when raising male calves, since they need to sustain their more rapid growth rates by investing more in lactation.

There are some suggestions in the literature supporting the existence of female choice in red deer (e.g. Lincoln & Guinness 1973) and it has been shown that female red deer choose males on the basis of their roaring performance (McComb 1991). Thus, female mate choice in red deer has been documented, although it has not received enough attention to allow an assessment of its relevance.

In contrast to populations from northern Europe, in the Mediterranean the period of greatest environmental stress and food scarcity is not winter, but the end of the summer and beginning of autumn when the breeding season takes place. It begins after the hot and dry summer and takes place before the rains start. Thus, males have to face the high costs incurred during the breeding season under severe resource limitations. In these populations, males exhibit a flexible mating system so that some males may defend harems, while other males defend territories where food resources are concentrated and which are used by females as feeding sites (Carranza *et al.* 1995, 1996). Under these conditions, females move freely in their search for food, moving between territories and between harems rather frequently whether they are in oestrus or not (Carranza *et al.* 1996).

Determinants of fertility in natural populations

Fertility under natural and artificial selection

It is generally assumed that male infertility is uncommon in natural populations, because it would be strongly selected against (reviewed in Jennions & Petrie 1997). This assumption may apply to sterile males because they would leave no descendants and would thus be at an evolutionary dead end. However, little attention has been paid to the fact that males may show varying degrees of fertility as a result of their genetic make-up (e.g. inbreeding, Wildt *et al.* 1987, Roldan *et al.* 1998, Gomendio *et al.* 2000) or may be temporarily infertile due to environmental causes such as food scarcity, stress and pathogens (reviewed in Bronson 1989, Wallen & Schneider 2000). The few field studies that have addressed this issue have shown that reduced male fertility or temporary male infertility may be more common among natural populations than previously thought (Gray 1997, Olsson & Shine 1997, Hoogland 1998, Morrow *et al.* 2002). However, the nature of the data obtained from natural populations often makes it difficult to disentangle the role played by male and female factors, or an interaction between both as in the case of genetic incompatibility (Zeh & Zeh 1997). Data from species where sperm competition is prevalent have revealed large differences between males in semen traits, which influence their paternity success when in competition with rival males (reviewed in Snook 2005, Wedell 2007); these results suggest that a large degree of inter-male variation in sperm traits should be expected in natural populations.

Because male fertility has been dismissed as a significant component of male reproductive success, few efforts have been made to understand which semen traits determine male fertility in natural populations. In contrast, this issue has received much attention in two other contexts: livestock breeding and human infertility treatment. The economic benefits derived from maximising the efficiency of livestock breeding have led to major efforts to identify which semen trait(s) determine male fertility. Most of the studies have failed to link specific sperm traits to fertility and the results are rather contradictory or inconclusive (Colenbrander *et al.* 2003, Foote 2003, Rodriguez-Martinez 2003). On the other hand, treatment of human male infertility has led to the search for ejaculate traits that can explain reproductive failures and predict success at assisted conception. These studies have shown that, in the subpopulation of infertile men which seek medical assistance, sperm concentration in the ejaculate, sperm motility, sperm morphology and acrosomal status are some of the semen traits known to influence male fertility among patients (Drobnis & Overstreet 1992, Tesarik 1994). It is unknown, however, if these same traits would account for fertility differences among males in healthy populations.

The difficulty in identifying which semen traits determine male fertility may lie in the fact that efforts have concentrated in two areas which represent opposite extremes. Livestock breeders have been artificially selecting fertile males for many generations, which may have resulted in little variability between males, particularly on those traits more closely linked to fertility. On the other hand, clinical studies have been mainly concerned with the study of a subpopulation of males whose fertility is compromised. Semen traits responsible for such infertility may not be representative of fertility determinants in less biased populations. Thus, these results cannot be readily extrapolated to natural populations of mammals, which are likely to lie somewhere in between these two extremes. In addition, selective processes acting on natural populations are disrupted in both domestic animals and most human populations, so any potential links between semen traits, male fertility and male reproductive success may be absent in these scenarios. In any case, no information is available on the degree of variability in semen traits and male fertility in natural populations.

Fertility in natural populations of red deer

Semen collected from Iberian red deer from natural populations was used to examine the degree of variation in semen traits and relationships between them (Malo *et al.* 2005a). Relative testes size and the proportion of motile spermatozoa showed a great deal of variation, with the latter showing a range between 0 and 90% (Fig. 1A and B). These traits may show more variation than others because they are more sensitive to environmental factors. The proportion of normal spermatozoa and sperm swimming

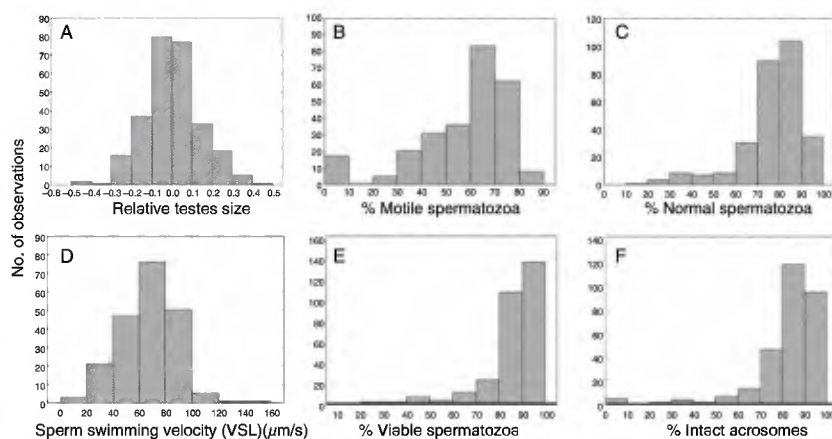


Figure 1 Frequency distribution of semen parameters in Iberian red deer from natural populations. (A) Relative testes size, (B) percentage of motile spermatozoa, (C) percentage of normal spermatozoa, (D) sperm straight-line swimming velocity, (E) percentage of viable spermatozoa and (F) percentage of spermatozoa with an intact acrosome.

velocity showed an intermediate degree of variation (Fig. 1C and D) with the proportion of normal spermatozoa showing a wide range (12–97%) and a skew towards higher values. Finally, the proportion of viable spermatozoa and the proportion of spermatozoa with an intact acrosome showed low levels of variation, with all males showing high values (Fig. 1E and F). These variables may have stabilised at high values because they are so essential for fertilisation that males with lower values have been intensively selected against in natural populations.

All semen parameters included in our study showed positive associations, meaning that males with high values in one trait also tended to show high values in other traits (Malo *et al.* 2005a). This finding contradicts the theoretical predictions that there should be trade-offs between different sperm traits, given that sperm is costly to produce (Parker 1998, Wedell *et al.* 2002). On the contrary, our results suggest that semen traits coevolve to maximise fertilising efficiency and that males in good condition invest extra resources in all semen traits. It is possible that trade-offs may only become apparent if sperm traits are experimentally manipulated, but this approach has not been used due to technical difficulties involved.

Semen traits were not associated with others to the same extent (Malo *et al.* 2005a). Sperm production was found to be closely associated with sperm swimming velocity. This finding suggests that males with high fertility in red deer populations have both high sperm numbers and sperm that swim at high speed. The proportion of motile spermatozoa and quality of motility were closely associated with the proportion of normal spermatozoa, an association which has been frequently postulated (Katz *et al.* 1982), but for which no strong data were available. Finally, sperm viability and acrosome integrity were strongly associated.

Fertility trials were carried out using spermatozoa from males from natural populations to inseminate captive females, keeping sperm numbers constant. Here, we present results with a larger data set than was originally published (Malo *et al.* 2005a) given that we have been able to add results from further inseminations. Males from

natural populations of Iberian red deer vary markedly in their fertility: some males fertilised 24% of the females while others fertilised 70%. Differences in fertility rates between males were strongly related to sperm swimming velocity as well as to the percentage of morphologically

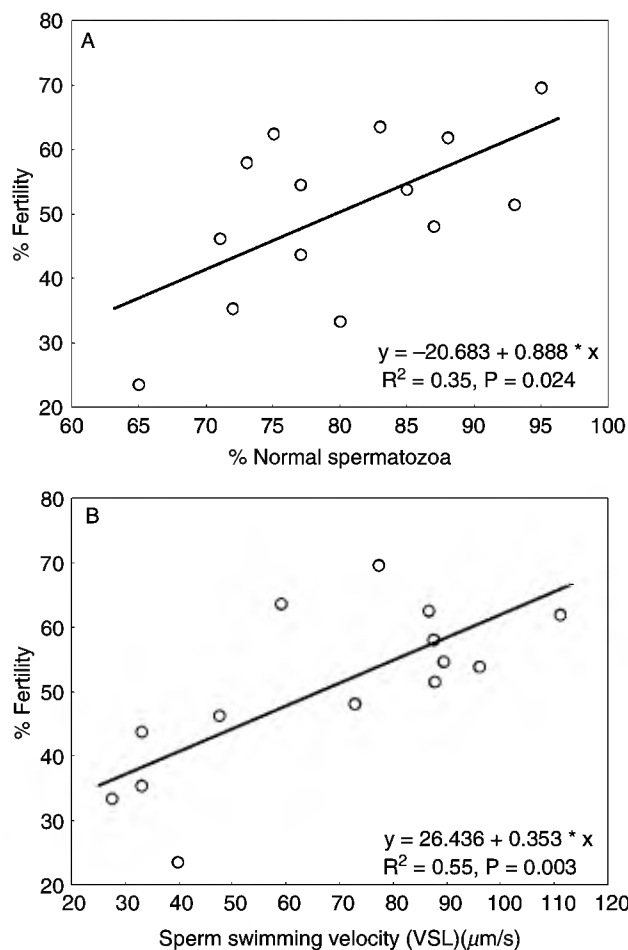


Figure 2 Relationships between *in vivo* fertility and (A) sperm morphology and (B) sperm straight-line swimming velocity, after artificial insemination of 344 red deer hinds with spermatozoa of 14 males from natural populations.

normal spermatozoa (Fig. 2A and B). However, the proportion of motile spermatozoa, the proportion of acrosome integrity and the proportion of viable sperm did not show a significant association with fertility.

From an evolutionary perspective, these findings imply that differences between males in fertility should be taken into account when considering which factors influence male reproductive success in natural populations. The variables found to determine male fertility had intermediate coefficients of variation. This raises the question as to why males differ in these crucial semen traits, namely the percentage of morphologically normal spermatozoa and sperm swimming velocity. The percentage of morphologically normal spermatozoa has been shown to decrease with inbreeding (Roldan *et al.* 1998, Gomendio *et al.* 2000), which could therefore be a primary determinant of male fertility in natural populations. On the other hand, sperm swimming velocity is related to sperm shape and size (see below).

First, these results demonstrate that it is possible to identify specific semen traits which determine fertility in natural populations, despite the lack of success when working with domestic species. Second, they show that some of the traits known to determine fertility among infertile human patients, such as acrosome integrity, are not associated with fertility in natural populations because selection has favoured uniformly high values. Third, that sperm traits believed to play an important role exclusively in the context of sperm competition, such as sperm velocity (Gage *et al.* 2004), are primary determinants of fertility in males from natural populations in the absence of sperm competition. This implies that, among mammals, there may be no specific sperm traits favoured by sperm competition, but rather that the same traits which are important for fertilisation experience an even greater selection under sperm competition.

Our findings suggest that differences in fertility between males may contribute in a significant way to generate differences in reproductive success. It is widely assumed that the large differences in male lifetime reproductive success observed among polygynous mammals, such as red deer, are mainly due to differences in their ability to win agonistic encounters with other males and defend females from other males. Our results suggest that such scenario is incomplete because once a male defends a harem or a territory its ability to fertilise plays an important role in determining his reproductive success.

Sperm design and sperm swimming velocity

In addition to the evidence gathered by us on red deer, sperm swimming velocity has been found to be a major determinant of male fertilisation success in other taxa both in non-competitive (Froman *et al.* 1999, Levitan 2000) and competitive (Birkhead *et al.* 1999, Gage *et al.* 2004) contexts. Sperm velocity may determine sperm ability to overcome physical barriers in the female tract, to

enter or leave sperm reservoirs, and to penetrate ova vestments (Froman *et al.* 1999, Suarez 2007). In addition, when spermatozoa from rival males compete, sperm velocity may determine which spermatozoa arrive first to the vicinity of the ova, and are thus more likely to fertilise.

However, recent efforts to identify which sperm phenotypic traits determine swimming velocity have been unsuccessful (Gage *et al.* 2002, Birkhead *et al.* 2005). Mammalian spermatozoa consist of the head, with a nucleus containing the highly compacted male haploid genome, and the flagellum, which is responsible for sperm motility. The flagellum is, in turn, divided into two components: a) the midpiece containing the mitochondria, which are believed to generate, by oxidative phosphorylation, the energy needed for sperm motility, and b) the principal and terminal pieces (also referred to as 'rest of the flagellum') whose beat propel the spermatozoon forward (Turner 2003).

We have examined spermatozoa of Iberian red deer from natural populations to test the hypothesis that the shape of the sperm head and the dimensions of the components of the flagellum determine sperm swimming velocity. Sperm dimensions show variation both between and within males (Fig. 3), although variation between males is greater than within males (Malo *et al.* 2006). This pattern of small variation within males and large variation between males in the size of sperm components agrees with previous studies (Gage *et al.* 2002, Birkhead *et al.* 2005).

We reasoned that the actual swimming speed achieved will be a combination of several factors: the size of the component whose movement generates the force in relation to the size of the components which have to be driven forward, and the degree of resistance offered by the head when spermatozoa swim forward. First, we predicted that sperm with elongated heads would show less resistance than sperm with rounded heads. Our results show that sperm with elongated heads do swim faster, so the effect of head shape upon sperm hydrodynamics seems to be considerable (Malo *et al.* 2006).

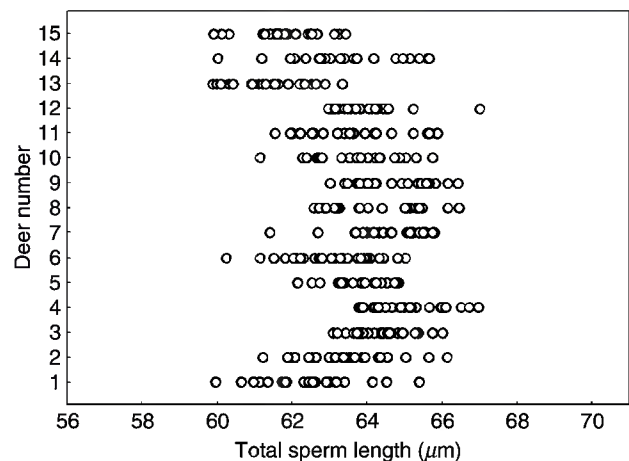


Figure 3 Total length variation in spermatozoa from 15 red deer males. A total of 25 spermatozoa were measured for each male.

Secondly, it has been suggested that the size of the midpiece is an indicator of mitochondrial loading and, therefore, of the amount of energy available to achieve higher swimming speeds (Anderson & Dixon 2002). Our results do not lend support to such hypotheses because activated spermatozoa with shorter midpieces swim faster (Malo *et al.* 2006). The role of activated motility is to propel the sperm along the female reproductive tract, through barriers such as the cervix and the uterotubal junction, until they reach the oviduct (Suarez 2007). This leaves the question open as to what the energy generated by mitochondria is needed for. One possibility that deserves further study is that oxidative phosphorylation becomes an important source of energy after sperm hyperactivation, a different type of motility which develops later and contributes to the detachment of spermatozoa from the oviductal wall, allows sperm to reach the site of fertilisation and penetrate the oocyte coats (Turner 2003).

Thirdly, we tested the hypothesis that the length of the flagellum may be a key determinant of sperm swimming velocity (Gomendio & Roldan 1991), because it is the beat of the flagellum that generates the force that drives the sperm forward and the amplitude of the waveform determines the sperm trajectory (Katz & Drobnis 1990, Turner 2003). We predicted that if the movement of the principal plus terminal piece is what propels the spermatozoon forward, the key factor should be the size of this component in relation to the length of the whole flagellum. We found support for this idea since the greater the proportion of the rest of the flagellum, when compared with total flagellum length, the faster the sperm swim (Malo *et al.* 2006). This finding suggests that the roles played by the midpiece and the rest of the flagellum should be considered jointly. In addition to the influence that size of the rest of the flagellum may have in generating the force needed for sperm movement, it may also determine the amount of energy generated. Recent studies show that most of the energy required for sperm motility is generated by glycolysis, rather than oxidative phosphorylation (Eddy 2007, Miki 2007). Glycolysis depends on a sperm-specific glycolytic enzyme which is tightly bound to the fibrous sheath (Miki *et al.* 2004), a cytoskeletal structure that extends along the principal piece of the flagellum (Eddy *et al.* 2003, Turner 2003, Eddy 2007).

In conclusion, our results show that the main determinants of sperm swimming velocity are the shape of the head and the proportions between the components of the sperm flagellum. Thus, actual swimming speed will be the result of the combined design of different sperm components. The large inter-male variation in sperm design found among natural populations underlies differences in sperm swimming speed, which, in turn, determine differences in male fertility rates. This supports the idea that sperm design is under strong selective pressures given its role in determining male fertilisation success (Roldan *et al.* 1992).

Secondary sexual characters as signals of male fertility

The concept of sexual selection was developed to explain the evolution of conspicuous traits in males, such as ornaments and weapons, which could not be explained by natural selection for increased survival (Andersson 1994). Such traits confer advantages in terms of enhanced reproduction, either because males with elaborated ornaments are more attractive to females or males with developed weapons are more competitive in male–male agonistic encounters. Thus, sexual selection can operate via two different processes: female mate choice and male–male competition. Female preferences for males with exaggerated traits has been amply demonstrated in insects, birds and fish (Andersson 1994, Jennions & Petrie 1997), while in mammals, it is assumed that male–male competition is more prevalent. According to this view, most studies on fish and birds have focused on female preferences for particular traits (referred to as ornaments), while among mammals most studies have focused on the role played by secondary sexual characters (this time named weapons) on male competitive ability. It has been suggested that differences in the intensity of female mate choice versus male–male competition between taxa are partly determined by different mechanisms of inheritance of sex chromosomes (Roldan & Gomendio 1999).

Much attention has been devoted to the benefits derived by females from choosing particular males. Females may obtain direct benefits such as access to territories of good quality, nutrients transferred by the male, and paternal care, all of which are well documented (Andersson 1994). Females could also obtain indirect benefits such as an improvement in the genetic quality of the offspring or the avoidance of genetic incompatibility. The phenotype-linked fertility hypothesis suggests that females would also benefit from choosing males with elaborated sexual ornaments if they maximise the chances of mating with fertile males (Sheldon 1994). According to this hypothesis secondary sexual ornaments would honestly advertise male fertility. In other words, more attractive males should deliver higher quality ejaculates. There have been few attempts to test this hypothesis, which have yielded contradictory results. In birds, several studies have found no relationship between male phenotype and ejaculate quality (Birkhead & Fletcher 1995, Birkhead *et al.* 1997), while correlations between plumage brightness and testis size have been reported (Merilä & Sheldon 1999). In fish, several studies found negative correlations between secondary sexual characters (or male sexual activity) and ejaculate quality (Liljedal *et al.* 1999, Pilastro & Bisazza 1999), while others found positive associations (Matthews *et al.* 1997). Recent studies on guppies have shown that, when ejaculates from rival males compete and sperm numbers are kept constant, more colourful

males have higher parentage success than their less conspicuous counterparts (Evans *et al.* 2003). The competitive advantage of more colourful males is associated with faster swimming and longer lived sperm (Locatello *et al.* 2006).

It is well established that both sperm production and sperm swimming velocity determine male fertility. We have examined, for the first time in mammals, whether secondary sexual characters signal these semen traits (Malo *et al.* 2005b). Among ungulates, male red deer possess antlers that are not only large in relation to other species, but also very complex, containing many branches and points; they are often regarded as one of the most extreme cases of exaggeration of secondary sexual characters in the animal kingdom. Antlers have been regarded exclusively as weapons and their size is believed to be important because it enhances male fighting ability. However, the possibility that antlers may signal other male attributes or that female choice may also play a role have not been considered.

To obtain a global measure that would reflect the size and complexity of red deer antlers, we used eight variables which include length of the main beam, length of the branches, three widths of the main beam at different heights and two point counts. All the measures correlate highly significantly with each other, so we obtained an overall measure of antler size and complexity by conducting a multivariate analysis. Similarly, to obtain an overall measure of sperm velocity, we performed a multivariate analysis including six objective sperm motility parameters. This analysis generated a reliable index of sperm velocity. Our findings show that the global estimate of antler size and complexity is significantly associated with both relative testes size and the overall index of sperm velocity (Malo *et al.* 2005b). Thus, antler size and complexity is an honest indicator of sperm production and of sperm velocity in male red deer, thus providing support for the phenotype-linked fertility hypothesis.

The finding that antlers are honest indicators of both male sperm production and sperm quality raises the question as to who the signal may be addressed to: other males or females? There are four possible benefits for the female that could explain the function of antlers as honest indicators of male reproductive quality. The first possible benefit is infertility avoidance. The available evidence seems to support this hypothesis, since antlers seem to be signalling reproductive traits, which have been shown to determine male fertility, i.e. sperm production and sperm swimming velocity. Secondly, antlers could signal to females a male's ability to avoid sperm depletion, since there is evidence that males can become sperm limited as a consequence of sperm production costs and sperm expenditure during reproduction (Preston *et al.* 2001, Wedell *et al.* 2002). However, sperm depletion is mainly the result of a reduction in the number of sperm available. The fact that antler size and complexity is associated with sperm

velocity suggests that signalling sperm depletion may not be the main function of antlers. Thirdly, male antlers advertise ejaculate competitiveness, which determines fertilisation success under sperm competition, although the prevalence of sperm competition in red deer populations seems to be low. Finally, elaborated sexual ornaments could advertise high quality in males, which, in turn, could be associated with better quality sperm; by mating with these males females could improve offspring quality (see for example, Evans *et al.* 2004). Both antler size (Kruuk *et al.* 2002) and semen quality are to a certain extent heritable (Humblot & Ducrocq 1996) suggesting that females may also benefit from the inheritance of both traits by their sons. These benefits would be particularly pronounced in species such as red deer in which female reproductive success is strongly influenced by their son's lifetime reproductive success (Clutton-Brock *et al.* 1982).

Another possibility which should not be ruled out is that male antlers could also be signalling to other males the ability to avoid sperm depletion and the competitiveness of the ejaculate. In this way, other males could assess not only a male's fighting ability, but also the chances that if they copulate with the same females their sperm will be defeated. Males could then use this information to decide whether competing with such a male both at the behavioural (fighting) and physiological (sperm competition) level is a good strategy.

These findings reveal a new function for male red deer antlers and suggest that, among mammals, the degree of elaboration of male secondary sexual characters may signal important aspects of male reproductive quality to females and males. Previous studies demonstrated that antler size is related to the number of calves fathered by males and it has been assumed that this is exclusively the result of males with large antlers being able to win more fights with other males (Kruuk *et al.* 2002). Our findings suggest that males with large antlers could also achieve higher reproductive success through their enhanced ability to win fertilisations both in competitive and non-competitive contexts and the possible preferences shown by females to mate with them.

Does testosterone mediate the relationship between antler size and semen traits?

It is widely assumed that testosterone influences both antler size and semen quality and, thus, that the relationship between these two traits may be an indirect consequence of the underlying influence of testosterone on both. To test this possibility, we analysed levels of testosterone during the whole year in a captive population of red deer and during the breeding season in natural populations (Gomendio *et al.* 2007).

Red deer are seasonal breeders and cast and regrow their antlers every year. In a captive red deer population,

testosterone levels remained low during antler growth, increased during antler mineralisation, reached a peak just before the breeding season started and decreased thereafter, similar to previous reports (Suttie *et al.* 1984, 1995). Thus, although testosterone may control the timing of key events in the antler cycle (such as casting and pedicle growth), the observation that testosterone levels are low during antler growth supports the current view that the presumed positive link between testosterone levels and antler size is mistaken (Price & Allen 2004). In fact, the opposite may be true, at least in red deer, since males treated with antiandrogens grow larger antlers than controls and testosterone reduces antler growth by influencing insulin-like growth factor-I binding, the latter having an important role in antler growth (Suttie *et al.* 1995). Evidence from natural populations supports this view since no relationship was found between males' testosterone levels during the breeding season and antler size (Gomendio *et al.* 2007).

The annual cycle in testosterone levels is mirrored by changes in testes size, as seen in our captive males. During the breeding season, we found that, in natural populations, differences between males in testosterone levels are closely associated with differences in testes size. These findings support the previously reported (Asher *et al.* 2000, Roelants *et al.* 2002) relationship between testosterone, testes size and sperm production. Testosterone is needed for normal spermatogenesis and seems to promote cell proliferation and prevent apoptotic cell death (Roelants *et al.* 2002).

The potential links between testosterone and semen quality are less clear and the pathways through which testosterone could influence sperm morphology, motility, acrosome integrity or swimming velocity are unknown. In our study sample, there is a significant, but weak, relationship between testosterone levels and proportion of normal spermatozoa, which is a determinant of male fertility (M Gomendio, AF Malo, J Garde, ERS Roldan, unpublished data). However, no relationship was found between testosterone and sperm swimming velocity, which has also been identified as a determinant of male fertility.

In conclusion, our results suggest that testosterone is closely associated with testes size and sperm production, but only weakly associated with other variables of semen quality. In addition, we find no relationship between testosterone and antler size. Thus, the traditional assumption that testosterone mediates the relationships between antler size, semen traits and male fertility should be examined in more detail.

Male fertility and offspring sex ratio

Sex allocation theory predicts that parents should increase the production of the sex with the higher fitness benefit (Trivers & Willard 1973). As proposed in the original hypothesis, ungulates represent good models to

test the predictions because they are sexually dimorphic in body size, male reproductive success depends on fighting ability and body size, variance in reproductive success is greater among males, and the reproductive success of sons is more strongly influenced by maternal investment during early growth which determines adult body size. Under these conditions, it is predicted that high-quality mothers will tend to produce sons, who will translate high levels of investment during lactation into high reproductive success during adulthood. Although early studies on red deer found support for this prediction (Clutton-Brock *et al.* 1984), later studies on this and other ungulate species have generated inconsistent results (Clutton-Brock & Iason 1986, Hewison & Gaillard 1999).

It has been argued that the lack of robust results among studies on vertebrate sex ratios is due to the constraints imposed by chromosomal sex determination (Maynard Smith 1978). However, the existence of extreme skews in sex ratios found in some birds and mammals shows that chromosomal sex determination is by no means an absolute constraint (e.g. Komdeur *et al.* 1997).

The Trivers and Willard hypothesis has been applied most often to mothers, who have a strong influence on offspring quality through maternal care, but it can apply to any quality that parents transmit to offspring, which has a differential effect on the reproductive success of sons and daughters. Among birds, male reproductive success is often related to secondary sexual characters, and thus sons of males with large sexual ornaments will be expected to achieve high reproductive success. There is evidence that offspring sex ratios may be adjusted in relation to the attractiveness of the father (Ellegren *et al.* 1996). Similarly, in mammals offspring sex ratio is influenced by male body mass (Røed *et al.* 2006). However, it is widely assumed that such manipulation is under female control.

The possibility that males may also facultatively adjust sex ratio has seldom been considered. In mammals, males are the heterogametic sex producing X- and Y-chromosome bearing spermatozoa, which determine the sex of the offspring. Thus, among mammals, males may have more control over mechanisms of sex determination than in other taxa. In mammals, male traits which may have a greater influence on the reproductive success of sons include male fertility.

We tested the hypothesis that more fertile red deer males produce more sons (Gomendio *et al.* 2006b). Because female effects on sex ratio exist, it was necessary to disentangle male and female effects by designing an experiment which would minimise differences between females while retaining the inter-male variation in fertility rates found in natural populations. Thus, our experimental design was aimed at eliminating several female factors known to influence sex ratios: i) we avoided the possibility that females may bias sex ratio in response to male quality by artificially inseminating females so that they had no direct experience with the males; ii) differences between females in body condition were minimised using a sample

of females which were all in good physical condition, were kept under similar environmental conditions and had access to an unlimited food supply, and iii) all females were inseminated at the same time in relation to ovulation, avoiding the confounding effects of insemination time. In contrast, using sperm collected during the rut from males living in natural populations, we ensured a representative sample of the large degree of variation in male fertility already described.

When the whole study sample is considered, a similar number of male and female offspring were produced. However, differences between males in fertility rates and in the proportion of male offspring produced were large. Male fertility rates ranged between 24 and 70% and the proportion of male offspring between 25 and 72%. More fertile males sired more sons and less fertile males sired more daughters (Gomendio *et al.* 2006b). Possible relations between sperm traits and offspring sex ratio were examined. There was a significant relationship between percentage of morphologically normal spermatozoa and proportion of male offspring sired per male, but no significant relationship was found between sperm velocity parameters and proportion of male offspring sired (Gomendio *et al.* 2006b).

Thus, of the two main determinants of male fertility, sperm swimming velocity and the proportion of normal spermatozoa, the latter was found to be associated with sex ratio. This may be because the proportion of normal spermatozoa is more likely to be inherited by sons (Smital *et al.* 2005) than sperm swimming velocity, which may be influenced to a greater extent by environmental factors (Kilgallon & Simmons 2005). Thus, males with a higher proportion of normal spermatozoa may benefit from producing sons, who will inherit the trait which will increase their fertility and will thus achieve high reproductive success. In contrast, low-fertility males will benefit from producing daughters, who will not inherit their father's poor ejaculate quality.

There are two possible mechanisms by which males may adjust sex ratio. First, high- and low-fertility males could differ in the proportion of Y-bearing spermatozoa in the ejaculate (Chandler *et al.* 2002) resulting in biases in sex ratio at birth. Second, differences between males in the competitiveness of X- and Y-bearing spermatozoa could arise through differential expression of genes carried in the sex chromosomes. Such post-meiotic expression of germ line-specific X- or Y-linked genes has been recently demonstrated (Wang *et al.* 2005). Furthermore, it has been recently shown that males with deletions in the Y chromosome produce Y-bearing spermatozoa with morphological abnormalities, which are less efficient at fertilisation resulting in sex ratio biases toward females (Ward & Burgoyne 2006). Thus, red deer males with low-fertility rates may have a lower proportion of morphologically normal spermatozoa as a consequence of genetic information on the Y chromosome, which would also impair the chances of

fertilisation of Y-bearing spermatozoa. On the contrary, males with high-fertility rates may produce more competitive Y-bearing spermatozoa.

Our experimental approach has revealed unexpectedly large differences in fertility rates between males from natural populations when females were artificially inseminated once. Are such differences in male fertility likely to occur in natural contexts? In the wild, low-fertility males have smaller testes and smaller antlers, so it is unlikely that they could compensate by transferring more sperm or by copulating more often with the same female. Thus, the differences in fertility rates found when all females are inseminated with equal sperm numbers are likely to be exacerbated when differences in sperm numbers come into play in natural contexts, and have a profound influence on male reproductive success. There is not enough information to evaluate whether male fertility rates limit female fecundity in natural populations, in other words, whether females suffer fecundity costs when they mate with low-fertility males. When females do not become pregnant in their first oestrus cycle, they come into oestrus again and have the opportunity to mate with different males. Thus, females may avoid fecundity costs by remating with other males. In spite of this, a significant proportion of females in red deer populations do not reproduce each year, and it has been assumed that this is due to the costs of the previous reproductive event or poor environmental conditions. An unexplored possibility is that low male fertility plays a role.

Our findings suggest that mammalian males can manipulate the sex ratio of their offspring, thus creating an unforeseen evolutionary scenario, which includes conflict of interests between males and females. For instance, a fertile male may benefit from producing sons, but the costs of raising a male may be high for females in poor physical condition (Gomendio *et al.* 1990). This level of conflict may improve our ability to explain biases in sex ratio at birth.

Conclusion and perspectives

More studies on natural populations of different species are needed to understand how differences between males in fertility rates translate into differences in male reproductive success, and whether male fertility may limit female reproduction. Studies on populations under different levels of genetic and environmental stress would be helpful to understand how different semen traits are influenced by these factors. Finally, comparing species with different mating systems would give us insight into how male fertility may affect reproductive rates within populations.

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