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Stallion semen quality depends on MHC matching to teaser mare

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Abstract
The major histocompatibility complex (MHC) has repeatedly been found to influence mate choice of vertebrates, with MHC-dissimilar mates typically being preferred over MHC-similar mates. We used horses (*Equus caballus*) to test whether MHC matching also affects male investment into ejaculates after short exposure to a female. Semen characteristics varied much among stallions. Controlling for this variance with a full-factorial within-subject experimental design, we found that a short exposure to an MHC-dissimilar mare enhanced male plasma testosterone and led to ejaculates with elevated sperm numbers as compared to exposure to an MHC-similar mare. Sperm velocity seemed not affected by the treatment. Overall genetic similarity between stallions and mares (determined from polymorphic microsatellites on 20 different chromosomes) played no significant role here. The MHC type of the teaser mare also affected characteristics of cold-stored sperm after 24 and 48h. As expected from ejaculate economics, sperm viability was elevated after exposure to an MHC-dissimilar mare. However, oxidative stress and the percentage of sperm with a high DNA fragmentation was mostly increased after exposure to an MHC-dissimilar mare, depending also on whether the teaser mare was in estrous or not. We conclude that males can quickly adjust ejaculate quality relative to a female's MHC, and that this male reaction to the social environment can also affect important characteristics of cold-stored semen.

Introduction

Studies of sexual selection have focused on female choice and female reproductive strategies because males usually have the higher reproductive potential and are hence expected to be less choosy than females, especially so in polygamous species with little or no paternal care (Clutton-Brock & Vincent 1991). However, the theory of ejaculate economics predicts that males invest strategically into individual ejaculates, for example, in response to the anticipated level of sperm competition or in response to the perceived quality of a female (Parker & Pizzari 2010). Empirical studies in this field have concentrated on sperm number and velocity and on how these characteristics affect fertilization success and male fitness (Olsson *et al.* 2004; Gillingham *et al.* 2009; Firman *et al.* 2013). However, non-sperm components often make up the largest part of an ejaculate. These seminal fluids typically contain proteins, hormones, antimicrobials, immunity suppressors, sugars, salts, and various cell types, including gland and immunity cells or infertile parasperm (Perry *et al.* 2013). The composition of seminal fluids is predicted to be phenotypically plastic and to evolve in response to evolutionary conflicts between and within the sexes (Dhole & Servedio 2014; Ramm & Schärer 2014; Sirot *et al.* 2015). Indeed, these fluids have been found to significantly vary between populations (Lemaitre *et al.* 2011), within populations (Mautz *et al.* 2013), and between ejaculates of individual males, for example, in response to the expected level and type of sperm competition (recent examples include Sirot *et al.* 2011; Locatello *et al.* 2013; Crean *et al.* 2014; Ramm *et al.* 2015; Yamane *et al.* 2015). Little is known about the relative costs of the different ejaculate components (Perry *et al.* 2013), but the availability of seminal fluids often limits remating before the availability of sperm does (Reinhardt *et al.* 2011). Males are therefore predicted to adaptively modify ejaculate characteristics in response to female state (Kelly & Jennions 2011) and in response to the perceived female attractiveness (Cornwallis & O'Connor 2009).

Females attractiveness can vary, for example, in egg number and egg quality in species with high female reproductive potential (including most invertebrates and lower vertebrates), with their level of promiscuity (Pizzari *et al.* 2003), with sexual ornaments (Pizzari *et al.* 2003; Cornwallis & Birkhead 2006), or with estrous stage, especially in large mammals with comparatively low female reproductive potential (Jeannerat *et al.* 2017). Apart from such generally valid quality traits, some aspects of female attractiveness are predicted to be differently perceived by different males (Wedekind 1994). MHC-linked mate preferences would be an example of the latter (Milinski 2006; Ruff *et al.* 2012; Davies 2013).

The MHC (major histocompatibility complex) is a group of polymorphic genes that play a crucial role in the adaptive immune response of vertebrates (Davies 2013) and is also "… *likely the basis for a vertebrate-wide chemosensory communication system*" (Ruff *et al.* 2012). MHC-linked signals have been found to influence individual recognition, kin recognition, mate preferences, and fertility in over 20 vertebrate species so far (Ruff *et al.* 2012), including horses (Burger *et al.* 2017a; Burger *et al.* 2017b). MHC-linked signals seem mainly based on volatile signals, at least for terrestrial vertebrates (e.g. Leclaire *et al.* 2017), and they are recognized in the main olfactory system (Spehr *et al.* 2006; Milinski *et al.* 2013) or the vomeronasal organ (Leinders-Zufall *et al.* 2004). However, neither the full pathways nor the functional significance of MHC-linked mate preferences are solved yet. Regarding the latter, MHC-linked preferences may help to reduce inbreeding and hence the fitness costs of inbreeding depression, or may directly be used to influence the MHC genotype and hence the immunocompetence of the common offspring (Milinski 2006; Ruff *et al.* 2012; Davies 2013). In both cases, MHC-dissimilar genotypes are generally expected to be preferred over MHC-similar genotypes. If used for inbreeding avoidance, similarity of the MHC may indicate relatedness, especially in small populations with low dispersal. If used in the context of host-pathogen coevolution, MHC-based mate preferences may serve to enhance the frequency of heterozygotes or of rare alleles among offspring (Penn & Potts 1999). Indeed, MHC

heterozygotes typically show superiority during coinfections (Penn *et al.* 2002; McClelland *et al.* 2003), but they may not do better against a single strain infection (Wedekind *et al.* 2005).

We use the polygamous horse (*Equus caballus*) as model to study MHC-linked male reproductive strategies, focusing here on the Franches-Montagnes breed (a Warmblood-related local breed). Under feral conditions, horses live in harems ("breeding bands") of one to very few stallions with one or several mares with or without offspring, or in "bachelor bands" formed by non-harem stallions. The dominant stallions usually sire most foals within breeding bands, but they cannot or do not always want to prevent copulations of subordinate stallions or stallions from outside the breeding band, especially if these copulations are with mares that are close kin to the dominant stallion (Kaseda *et al.* 1995; Asa 1999). Vasectomizing harem stallions, for example, leads to significantly reduced numbers of newborns, but 3 of 9 harems with vasectomized harem stallions nevertheless contained foals 3 years post treatment (Asa 1999). Sperm competition is therefore possible, and its outcome is likely to depend on ejaculate characteristics such as sperm number, their motility, or their longevity. Stallions are then expected to show plasticity in their reproductive strategies relative to their social situation (Parker & Pizzari 2010). Indeed, when simulating different types of social situations over periods of eight weeks each, total sperm number per ejaculate was affected by whether stallions were kept in proximity to other stallions (simulating a bachelor band) or to mares (simulating a breeding band) (Burger *et al.* 2015b). Moreover, when stallions were housed in proximity to one mare only and over several weeks, mean weekly blood testosterone measurements and total sperm number per ejaculate were dependent on whether the mare was MHC-similar or MHC-dissimilar (Burger *et al.* 2015a): Stallions showed higher testosterone levels and ejaculated more sperm after long-term exposure to MHC-dissimilar mares than to MHC-similar mares. Interestingly, the MHC type of a further mare that was only used as teaser during semen collection did not significantly affect ejaculate characteristics (Burger *et al.* 2015a), suggesting that the relative duration of exposure to a mare determines male reproductive strategies (e.g. that the social situation influences sperm production and hence sperm number per ejaculate). It remains to be tested whether stallions who are housed without contact to mares can still adjust sperm number and the quality of seminal fluids, for example, within moments before ejaculation, potentially linked to a "testosterone surge" (Macrides *et al.* 1975; Gleason *et al.* 2009; Petrulis 2013). Moreover, it is still unclear whether MHC matching to a mare can affect characteristics of seminal fluids and hence the long-term viability of sperm, as can be predicted from ejaculate economics.

In order to test these questions, we used short-term exposure to MHC-similar or MHC-dissimilar teaser mares of stallions that are otherwise isolated from mares to test whether MHC matching affects characteristics of fresh ejaculates, and we determined various characteristics of sperm that were cold-stored over two days following protocols that are routinely used in animal breeding. In order to control for potential between-stallion differences (and hence to significantly enhance statistical power), we used a full-factorial and within-subject study design, collecting ejaculates over several weeks and experimentally controlling for potential effects of time, order of presentation, and estrous cycles of the teaser mares.

Methods

The study took place in the Swiss National Stud, with mares and stallions separated in different stables and with a further building that was used for semen collection under experimental conditions. The animals (all Franches-Montagnes breed and unrelated to each other, age 6-18 years) were kept in always the same individual boxes bedded with straw, had daily access to a paddock, and were regularly and individually exercised (see Burger et al. (2015b) for further information about the regular housing conditions in the stud). All animals

were dewormed prior to the study. Once per week every Tuesday, stallions were individually led to the experimental set-up where a teaser mare that was either MHC-similar or MHC-dissimilar to the respective stallion was tethered close to a dummy. Because smell of feces seems to be an important source of social information (Krueger & Flauger 2011), the stallions were first exposed for 15 sec to fresh feces of the teaser mare (presented on a plate on the ground), then kept for about 30 sec in front of the respective mare's head, at a distance of about 2 m to prevent physical contact, and then led to the dummy that the stallion could mount and that allowed to collect its semen in an artificial vagina (type "Avenches"; see Jeannerat et al. (2017) for further details; extra-gonadal sperm reserves were standardized in 7 daily semen collections followed by weekly collections every Tuesday starting 4 months before the first experimental semen collection). The stallions were immediately returned to their box after semen collection. All stallions were always handled by the same person who was naïve with respect to the MHC types of the animals.

Blood samples (EDTA) were taken from each stallion about 15 min before semen collection (i.e. before exposure to the teaser mare) and immediately afterwards by jugular venipuncture. These samples were centrifuged (4000 x g for 10 min) and the plasma frozen (-80°C) for later determination of testosterone concentrations via electrochemilumiscence immunoassay (Elecsys 2010, Roche Diagnostics, Basel, Switzerland) (Janett *et al.* 2009).

The volume of each fresh ejaculate was measured after removal of the gel fraction, and total sperm number was determined in a Nucleocounter® SP-100™ (ChemoMetec, Allerød, Denmark). Semen was then diluted with INRA 96™ (IMV Technologies, L'Aîgle, France) to a density of 30 x 10^6 spermatozoa/mL. Sperm motility and velocity were determined in diluted semen with a computer-assisted sperm analyzer (CASA; HTM-IVOS, Version 12, Beverly, MA, USA) in 10 fields of a 20 µm standard count analysis chamber (Art. SC 20-01-C, Leja, Nieuw-Vennep, Netherlands). The following three velocity measures were taken: VAP (the smoothed paths the sperm heads took during an observational period), VSL (the mean distance between the sperm heads' first detected positions to their last), and VCL (the curvilinear path the sperm heads took) as in Burger et al. (2015a; 2015b).

An aliquot of semen was cooled to 4°C and analyzed for velocity, plasma membrane and acrosome integrity, membrane lipid peroxidation, and DNA integrity of sperm at 24 h and 48 h after ejaculation as described in detail in Jeannerat et al. (2017). Briefly, sperm motility was determined by CASA according to the methods used on fresh ejaculates, and all other sperm parameters were analyzed using flow cytometry (Cell Lab Quanta SC MPL, Beckman Couler Inc., Nyon, Switzerland). Plasma membrane and acrosome integrity of spermatozoa, defined as viability were measured after staining with propidium iodide (PI) and peanut agglutinin conjugated with fluorescein isothiocyanate (FITC-PNA) (Wach-Gygax *et al.* 2017), and the amount of lipid peroxidation of plasma membrane intact sperm was determined after incubation with 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (BODIPY) and PI (Gürler *et al.* 2015). The percentages of plasma membrane intact sperm with a high mitochondrial membrane potential (HMMP) or a low intracellular Ca^{2+} concentration were determined, combining PI with JC-1 and Fluo-4, respectively (Malama *et al.* 2017). Additionally, the sperm chromatin structure assay (SCSA™) performed with a Coulter EPICS XL flow cytometer (Beckman Coulter Inc., Krefeld, Germany) to analyze the percentage of sperm with a high DNA fragmentation (%DFI) (Evenson 2016; Wach-Gygax *et al.* 2017).

Two teaser mares A and B were used. For six of the stallions, mare A was MHC-similar (i.e. shared at least one ELA, see below) while mare B was MHC-dissimilar. For the remaining four stallions, mare A was MHC-dissimilar while mare B was MHC-similar. This allowed for an experimental within-subject and factorial design that was followed over a period of 7 weeks to avoid potentially confounding effects of the mares' estrous cycles. See Supplementary Fig. S1 for a schematic of the experiment. The cycles of the teaser mares were

continuously assessed by recording their behavior and in regular ultrasonography of ovaries and uterus. The mares were called in estrus when they showed typical estrous behavior, with a follicle size of >35 mm and a uterus edema at stage >2 (Ginther 1992). They were then given 1500 IU hCG intravenously (Chorulon®, MSD Animal Health GmbH, Luzern, Switzerland) on the evening before the experimental exposure to the stallion in order to induce ovulation the next day for a parallel study on potential effects of estrus on male behavior (Jeannerat *et al.* 2017) (this parallel study included six further stallions whose MHC types did not allow for the present within-subject comparison because both mares were MHC-similar to these stallions). For each combination of mare and stallion, the mare was exactly once in estrus and either 2 or 3 times in diestrus when exposed to the stallion at semen collection, i.e. the experiment was factorial with respect to estrus x MHC sharing (Fig. S1). This allowed to control for potential effects of estrus on the interpretation of possible effects of MHC matching on semen characteristics. Care was also taken to avoid potentially confounding effects of order and time by starting with five haphazardly assigned stallions each per teaser mare in the first week and strictly alternating the order of presentation during the first 6 weeks (Fig. S1).

Overall genetic similarity between stallions and mares was estimated from 20 polymorphic microsatellite loci located on 20 different chromosomes (Supplementary Table S1) (Mittmann *et al.* 2010). Genomic DNA was extracted from blood with the BioSprint robotic workstation (Qiagen) using conditions specified by the DNeasy blood and tissue kit (Qiagen). Microsatellites were amplified individually using the appropriate primers and GoTaq DNA polymerase (Promega) with the exception of AHT36, UMNe567 and UD457 which were pooled and amplified with Multiplex PCR Kit (Qiagen). PCR reactions with GoTaq DNA polymerase were performed in 10 µl using 1x GoTaq green reaction buffer, 0.5 µl DNA, 2.5 mM $MgCl_2$, a mix of 0.2 mM dNTPs, 0.5 µM of each primer and 0.25 units of GoTaq DNA polymerase. Amplification at different annealing temperatures (see Table S1 in Burger et al. (2017b)) were carried out as follows: 4 min initial denaturation at 94°C, 38 cycles with 30 sec at 94°C, 60 sec at annealing temperature and 40 sec at 72°C, final extension for 5 min at 72°C. PCR reactions with Qiagen Multiplex PCR kit were performed in 10 µl using 1x Qiagen Multiplex PCR Master Mix (3mM Mg^{++}), 1 µl DNA and 0.2 µM of each primer. Amplification was done as follows: 15 min initial denaturation at 94°C, 38 cycles with 30 sec at 94°C, 90 sec at annealing temperature of 60°C followed by 30 sec at 72°C, final extension for 10 min at 72°C. All amplicons were subsequently analyzed on an ABI-3100 sequencer. Allele sizes were determined using ROX 350 size standards and analyzed by Genemapper 4.0 (Applied Biosystems, Inc.).

Pairwise relatedness (r) after Wang (2002) were calculated as measure of genetic distance between mares and stallions, based on these 20 microsatellite loci and using the R package Demerelate (R Development Core Team 2011; Kraemer & Gerlach 2017). Table S2 gives the matrix of relatedness between mares and mares and stallions. One stallion-mare combination turned out to be extraordinarily similar on microsatellites (r = 0.298; Table S2) but shared only one MHC antigen (see below). With a pedigree analysis using the stud book (*Schweizerischer Freibergerverband*, Studbook FM, Avenches, 2017), we found only two common ancestors for this pair combination: a sire in the 4th generation of the mare and the 5th generation of the stallion, and another sire in the 7th generation of the mare and the 3rd generation of the stallion. We therefore concluded that the observed similarity on microsatellites was not due to close kinship, and we calculated mixed models with and without the outlier pair when r was included as factor. Here we present only mixed models (see below) without the outlier pair. The corresponding models including the outlier are presented in the Supplementary Material (they would not lead to qualitatively different conclusions with regard to r).

The horse's MHC includes several gene families with polymorphic loci that vary extensively in nucleotide diversity and in copy number of paralogous genes. Some of its molecular structure could recently be characterized, including several MHC class I (Tallmadge *et al.* 2010) and MHC class II genes (Miller *et al.* 2017; Viluma *et al.* 2017) of the Thoroughbred, Standardbred, and the Arabian breeds. However, many MHC genes of other breeds are not sufficiently characterized at the molecular level, including several MHC genes of the Franches-Montagnes breed we use here. MHC phenotypes were therefore determined serologically from peripheral blood lymphocytes obtained from heparinized blood samples and via Ficoll density gradient centrifugation. Equine leucocyte antigen (ELA) class I and class II were determined in microcytotoxicity tests with alloantisera detecting 18 internationally recognized (A1 - A10, W11, A14 - A20) and eight locally defined (Be22, Be25, Be27, Be28 and Be108, BeI, BeIII, and BeIV) MHC class I specificities. The ELA-C allele W21 and MHC class II alleles DW13, DW22, DW23, DBe200, and DBeVIII, as well as the locally identified, not further characterized W12, were also tested in a classical two-step microcytotoxicity test in Terasaki plates according to Lazary et al (1988). Pairs of mares and stallions were classified as MHC-similar if they shared between at least 1 ELA, and as MHC-dissimilar if they shared no ELA (Burger *et al.* 2015a; Burger *et al.* 2017a; 2017b). It turned out that six of the MHC-similar pairs shared 1 antigen, three pairs shared 2 antigens, and one 4 antigens (Table S2). When testing for possible effects of number of shared antigens, the latter two categories were summarized to one category ("≥2").

All statistical analyses were within-subjects, controlling for the strong differences between stallions that are described in Jeannerat et al. (2017). We therefore used mixed effect models and report only unbounded REML variance component estimates (Kackar-Harville corrected based on standard least squares in JMP® 11.2), with MHC similarity of the mare or relatedness r, and, in the case of cold-stored sperm, also time since ejaculation as fixed factors, and with stallion ID and all interaction that include stallion ID as random terms. Log- or logit-transformations (logit() +1) were used if graphical inspection of the data suggested significant violations of the model assumptions. We tested separately for effects of MHC similarity and for effects of relatedness r. These two fixed factors could also be combined when testing for treatment effects on blood testosterone and on characteristics of fresh sperm, but not when testing for treatment effects on cold-stored sperm (because the respective denominator degrees of freedom could not reliably be determined).

Jeannerat et al. (2017) found in the larger sample that the mares' estrous stage affected ejaculate characteristics. In the present analyses, effects of estrus are experimentally controlled for by the full-factorial and balanced study design (i.e. using means per exposures to diestrous and estrous mares, see below). The Supplementary Material shows the effects of estrus when added as a further fixed factor to the respective models.

All 70 ejaculates could be analyzed directly after collection. We could not avoid missing data for the measurements 24 h and 48 h after ejaculation (16 of 140 planned measurements), and for testosterone measurements (3 of 140 planned measurements). It turned out, however, that these missing data only concerned exposures to mares in diestrus for which we always had several replicates (Fig. S1) and there always remained at least one measurement per combination of stallion and mare, i.e. no experimental cell of the stringent factorial within-subject design was lost. The within-subject analyses were based on means per pair combination, time point, and estrous stage. Tests are directed when there was a strong *a priori* expectancy about the direction of an effect and to avoid the typical inflation of the alpha-value in one-tailed testing (Rice & Gaines 1994), otherwise two-tailed testing was used. The latter is given if directed and two-tailed testing lead to the same conclusion. Kendall's τ was used to describe correlations between measurements.

The study was conducted with stallions and mares that were used to being handled by humans, i.e. potential handling stress due to, for example, blood sampling, monitoring of

reproductive cycles, and semen collection can be considered minimal. The animals had *ad libitum* access to water and were fed three times per day. The experiments were approved by the *Etat de Vaud, Service Vétérinaire* (approval no. 2667.1).

Results

Based on the genotypes of the 20 microsatellite markers, the average pairwise relatedness r was 0.04 (95% CI = 0.04). This genetic distance was no significant predictor of MHC sharing (paired t-test on r of MHC-similar and –dissimilar pair combinations: $t_9 = 0.43$, $p = 0.68$).

Basal plasma testosterone concentrations were 3.41 ± 0.63 nmol/L and increased by 0.13 ± 0.22 nmol/L or 0.44 ± 0.20 nmol/L during semen collection when the stallions were exposed to MHC-similar or -dissimilar teaser mares, respectively (Fig. 1A; effects of MHC matching: $F = 9.4$, $p = 0.01$; see also Supplementary Table S3). The genetic distance between stallion and mare on microsatellites had no significant effect on testosterone concentration (Fig. 1c; effects of pairwise relatedness r: $F = 0.05$, $p = 0.86$; see Table S3 for further details). MHC effects on testosterone concentrations were also significant when tested as number of shared ELA (0, 1, ≥2) instead of the dichotomous sharing/no sharing ($F = 6.0$, $p = 0.02$; Table S3). An analogous mixed model including both MHC matching and r as fixed factors let to similar outcomes (effect of MHC sharing: $F = 7.0$, $p = 0.03$; effect of r: $F = 0.1$, $p = 0.72$). As observed before in a larger sample (Jeannerat *et al.* 2017), the mare's estrous stage did not significantly affect plasma testosterone concentrations (Table S3).

As expected (Jeannerat *et al.* 2017), characteristics of fresh ejaculates varied much among stallions (Table 1). Controlling for this variance in the within-subject treatment allowed testing for effects of genetic similarity between stallion and teaser mare. Fresh ejaculates contained significantly more sperm after exposure to MHC-dissimilar mares than after exposure to MHC-similar mares (Fig. 1B, Table 1a). We found no significant effects of MHC matching on ejaculate volume, total sperm motility, or sperm velocity (Table 1a), and there were no effects of relatedness r (Fig. 1d; Table 1b; Table S4) on any characteristics of fresh ejaculates. An analogous mixed model with both MHC matching and r as fixed factors led to similar outcomes (effect of MHC sharing: $F = 8.6$, $p = 0.02$; effect of r: $F = 2.8$, $p = 0.19$).

These MHC effects on total sperm numbers were confirmed in analogous models using the number of shared ELA (0, 1, ≥2) instead of the dichotomous sharing/no sharing (effect of number of shared ELA: $F = 11.8$, $p = 0.004$; Table S4). Increasing numbers of shared MHC antigens seemed to further reduce total sperm number (Table S4). An analogous mixed model with both, number of shared ELA and r, as fixed factors let to similar outcomes (effect of number of shared ELA: $F = 19.5$, $p = 0.003$; effect of r: $F = 3.7$, $p = 0.12$).

Characteristics of cold-stored sperm were again strongly dependent on stallion identity (Table 2). Overall, sperm viability and motility declined and lipid peroxidation and the velocity measure VCL increased over time (Fig. 2; Table 2). Sperm viability and lipid peroxidation were both elevated after the stallions had been exposed to an MHC-dissimilar mare at ejaculation as compared to when the stallions had been exposed to an MHC-similar mare (Fig. 2; Table 2a). Including estrous cycle into the statistical models on cold-stored sperm (24h and 48h after ejaculation) revealed also significant effects of MHC matching on the percentage of sperm with a high DNA fragmentation (%DFI), time x estrus x MHC interactions on lipid peroxidation, percentage of sperm with a high DNA fragmentation, and sperm motility (Fig. 2B, 2C; Table S5), a time x MHC effect on lipid peroxidation, and an estrus x MHC interaction on HMMP (Table S5). Most of these effects of MHC matching on lipid peroxidation and sperm motility (but not on %DFI nor sperm viability) were confirmed in analogous models using the number of shared ELA (0, 1, ≥2) instead of the dichotomous sharing/no sharing (Table S6). No significant effects of pairwise relatedness r could be found

(Table 2b). Sperm velocity seemed unaffected by the MHC type of the teaser mare (Table 2, Table S5, S6).

Sperm viability correlated negatively with %DFI both at 24h and 48h after ejaculation (Fig. S2a; Table S7). Both measures were also correlated with most other ejaculate characteristics at both time points (Table S7). In comparison, lipid peroxidation seemed to be a poorer predictor of other ejaculate characteristics: the only significant correlations we found were with sperm viability and motility at 24h, and with %DFI at 48h (Fig. S2b, c; Table S7). See Supplementary Table S7 for all other correlations between ejaculate characteristics that were determined after 24 and 48 h of cold storage.

Discussion

Although studies on sexual selection usually focus on female preferences, there are several examples of male MHC-dependent mate preferences. The link between the MHC and sexual selection was even discovered in male mice (Yamazaki *et al.* 1976) before the corresponding odor preferences could be demonstrated in male and female mice (Yamazaki *et al.* 1979), in men and women (Wedekind & Füri 1997), and in various other vertebrates (Ruff *et al.* 2012). Here we first used plasma testosterone levels as indicator of male preferences. It is not solved yet under which circumstances testosterone levels indicate mate preferences. However, male behavior towards females has often been found to be linked to this hormone (Hirschenhauser & Oliveira 2006), and Burger et al. (2015a) found that long-term exposure to MHC-dissimilar mares lead to higher testosterone levels than long-term exposure to MHC-similar mares. Here, we observed a general increase in testosterone from before to directly after semen collection. This increase was more pronounced when stallions were exposed to an MHC-dissimilar teaser mare than when they were exposed to an MHC-similar teaser mare. Interestingly, analogous effects on testosterone plasma levels could not be observed when testing the effects of estrous versus diestrous teaser mares in a larger sample that had offered more statistical power (Jeannerat *et al.* 2017). We conclude that MHC-dependent male preferences are important even in a species like the horse that has harem breeding under feral conditions.

Apart from mate choice, female reproductive strategies can include control of gamete fusion and of zygote formation (Wedekind *et al.* 1996; Rülicke *et al.* 1998; Yeates *et al.* 2009), pregnancy block (Yamazaki *et al.* 1983), and maternal support during embryogenesis (Burger *et al.* 2017b). Males seem to have less opportunity to express MHC-linked reproductive strategies, especially so in polygynous species with little or no paternal care where their choices are reduced to either avoid mating with non-preferred females or invest differentially into ejaculates. The present study focuses on the latter possibility, i.e. on ejaculate quality as indicator of male reproductive strategy. We found that stallions quickly adjust ejaculates to characteristics of a mare even if the mare was only used as teaser during semen collection. The stallions invested differentially in sperm number per ejaculate (supporting analogous findings in red junglefowl *Gallus gallus* (Gillingham *et al.* 2009) and in sand lizard *Lacerta agilis* (Olsson *et al.* 2004)). They ejaculated more sperm of higher viability when exposed to a teaser mare that was MHC dissimilar and hence predicted to be attractive than when exposed to a teaser mare that was MHC-similar and predicted to be less attractive. Using another samples of horses, Burger et al. (2015a) had found that sperm numbers per ejaculate were higher after long-term exposure to an MHC-dissimilar mare than to an MHC-similar mare, while the MHC type of the teaser mare that was present during semen collection did not seem to play a role. Our new findings suggest that teaser mares have stronger effects on stallions who are housed with no contact to mares (as in the present study) than on stallions who are housed in proximity to another mare (Burger *et al.* 2015a).

As expected from a sample of unrelated individuals, the genetic distance between stallions and teaser mares (as estimated from microsatellite markers) was not significantly linked to MHC-similarity. This genetic distance did not seem to affect stallion testosterone

levels nor any ejaculate characteristics. However, such a finding does not exclude the possibility that stallion mate preferences serve to avoid inbreeding in populations with significant kin structure. MHC-linked preferences can then be a powerful mechanism to avoid inbreeding (Ruff *et al.* 2012).

Consistent with previous analyses of MHC effects on horses (Burger *et al.* 2015a; 2017a; 2017b), we defined MHC-similar as sharing at least one ELA. As in these previous analyses, most MHC-similar pair combination shared only one ELA, but some shared two or more. When testing for possible MHC effects using the number of shared ELA instead of the dichotomous similar/dissimilar, all MHC effects on testosterone blood level and characteristics of fresh ejaculates, and many MHC effects on characteristics of cold-stored sperm, could be confirmed. The similarity of these outcomes and our limited sample size does not allow answering the question whether sharing two or more ELA induces stronger responses that sharing only one ELA. However, a recent experimental study on MHC-linked female reproductive strategies including 191 mares found no increased MHC effects with increased number of shared ELA (Burger *et al.* 2017b).

Ejaculate economics also predict that males invest differentially into seminal fluids, i.e. into the non-sperm components of ejaculates (Parker & Pizzari 2010). It remains unclear whether characteristics of cold-stored sperm can fully reveal such variation in male investment. If so, we would predict increased quality of seminal fluids and hence increased protection of aging sperm in ejaculates of males that had been exposed to an MHC-dissimilar mare. We found indeed the viability of aging sperm to be increased when males had been exposed to an MHC-dissimilar mare.

We had no clear *a priori* prediction about possible treatment effects on lipid peroxidation and the percentage of sperm with a high DNA fragmentation (%DFI), because the relationship between fertility and oxidative stress of spermatozoa is not sufficiently understood yet. On the one hand, oxidative stress is believed to be one of the major causes of damage to sperm DNA and has repeatedly been linked to male infertility and low sperm motility (Tremellen 2008; Ferramosca *et al.* 2013; Aitken *et al.* 2014). Moreover, oxidative damage to sperm DNA has been linked to miscarriage and abnormalities in developing embryos (Aitken *et al.* 2016), and sperm collected after exposure to an estrous female showed lower oxidative damage than sperm collected after exposure to a diestrous female (Jeannerat *et al.* 2017). On the other hand, recent experiments in horses revealed a positive correlation between lipid peroxidation and fertility (Gibb *et al.* 2014). Gibb *et al*. (2014) called their findings a "paradoxical relationship" and suggested that the oxidative stress they measured was simply "… a by-product of intense mitochondrial activity", because they also found positive correlations between lipid peroxidation and sperm motility and velocity. Our observations seem to partly support their hypothesis. We found significant treatment effects on lipid peroxidation, with mostly increased lipid peroxidation after exposure to an MHC-dissimilar mare. We also found positive correlations between lipid peroxidation and sperm viability and motility, and a negative correlation between lipid peroxidation and %DFI in 48h-old sperm, but there were no significant correlations between lipid peroxidation and any of the sperm velocity measures. We also found significant treatment effects on %DFI that seemed to go largely in the same direction as the effects on lipid peroxidation, i.e. we found a generally increased percentage of cold-stored sperm with a high DNA fragmentation after exposure to an MHC-dissimilar mare. The negative correlations between sperm vitality and %DFI were as expected (Samplaski *et al.* 2015).

Artificial insemination or *in vitro* fertilization programs (in humans, horses, and other animals) often depend on *in vitro* sperm storage. Our findings could therefore be of practical relevance for fertility treatment (Menezo *et al.* 2014) and for a population management that includes individual reproductive strategies (Wedekind 2002). The increased viability of sperm collected with the use of MHC-dissimilar teaser mares is likely to enhance the success rate of

artificial insemination, but the increased oxidative stress and the increased percentage of sperm with a high DNA fragmentation in such semen samples could potentially enhance the mutational load in next generations (Cotton & Wedekind 2010). It remains unclear which component explained the increased sperm viability and the mostly increased lipid peroxidation and %DFI that we observed after semen collection with MHC-dissimilar teaser mares. The possibility of artifacts induced by interactions between male differential investment into ejaculate and effects caused by the cold storage of sperm need to be further investigated.

Our findings support the prediction of ejaculate economics that males invest differentially into ejaculates relative to perceived female attractiveness. Female quality in these models is typically given by fecundity indicators like egg number, but males do not need to agree in their perception of female attractiveness, especially if MHC-linked signals are used to avoid inbreeding or to avoid MHC-homozygous offspring (Ruff *et al.* 2012). The differential investment we observed was linked to an increase in testosterone that the presence of a teaser mare induced.

Authors' contributions. D.B., H.S., and C.W. designed the study, D.B. supervised the experiments, E.J. performed the experiments, E.M. supervised the ELA typing, C.B. the microsatellite typing, and F.J. and H.B. the measurements on aging ejaculates. C.W. did the statistical analyses and wrote the manuscript that was then critically revised by all authors.
Competing interests. The authors declare that they have no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.
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Table 1: Mixed-model analyses of fresh weekly ejaculates (N = 70), with (a) MHC matching (dissimilar vs similar) between stallion and teaser mare or (b) pairwise relatedness r as fixed factors, and stallion identity (ID) and the interaction to MHC matching or to r as random factors. One pair combinations with an extreme r was excluded from the analyses on the effects of r (Table S4 provides the results on the non-reduced sample). The proportions of the total variance explained (% of total) are based on unbounded REML variance component estimates.

	Ejaculate volume		Total sperm number[1]		Sperm motility[2]		Sperm velocity[3]	
(a) Differences on MHC								
Within-subject fixed factor	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
MHC matching	0.2	0.64	9.5	**0.01**	0.2	0.70	≤ 2.8	≥ 0.13
Random factors								
Stallion ID	53.0%		81.4%		63.8%		80.1 – 89.4%	
Stallion ID x MHC	0%		0%		3.6%		0 – 0.8%	
Means ± 95% CI								
MHC dissimilar	41.5 ± 5.8 mL		10.6 ± 2.1 x 10^9		63.4 ± 8.9 %		122.3 ± 8.6 µm/s	
MHC similar	40.7 ± 6.6 mL		9.6 ± 1.9 x 10^9		65.4 ± 8.4 %		117.5 ± 9.6 µm/s	
(b) Differences on microsatellites								
Fixed factor	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
r	0.2	0.69	0.03	0.91	0.9	0.44	≤ 3.0	≥ 0.39
Random factors								
Stallion ID	57.1%		83.6%		66.8%		80.4 – 89.0%	
Stallion ID x r	0%		1.4%		0%		0%	

[1] log transformed; [2]logit transformed; [3]range over VAP, VSL, VCL, means given for VAP

Table 2: Mixed-model analyses of ejaculates after 24 and 48 hours of cold storage, with time since ejaculation and (a) MHC matching between stallion and teaser mare or (b) pairwise relatedness r as fixed factors, and stallion ID and all interactions involving stallion ID as random factors. The proportions of the total variance explained (% of total) are based on unbounded REML variance component estimates. Significant p-values are marked in bold.

	Sperm viability[1]		Lipid peroxidation		%DFI[1]		Sperm velocity[3]		Sperm motility[1]	
(a) Differences on MHC										
Fixed factor:	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
MHC matching	4.4	**0.04**[2]	4.9	**0.05**	4.8	0.06	≤ 0.3	≥ 0.57	1.5	0.25
Time since ejaculation	28.9	**<0.001**	47.3	**<0.001**	<0.1	0.87	≤ 9.1	≥ **0.01**[4]	15.3	**0.004**
MHC matching x time	0.6	0.47	9.7	**0.01**	0.4	0.55	≤ 0.5	≥ 0.51	0.1	0.71
Random factors										
Stallion ID	85.3%		10.5%		38.0%		64.8 – 69.8%		86.8%	
Stallion ID x MHC	6.7%		14.9%		7.4%		2.1 – 3.3%		7.5%	
Stallion ID x time	0%		6.3%		2.8%		0%		0.6%	
Stallion ID x MHC x time	0%		0%		0%		0%		0%	
(b) Differences on microsatellites										
Fixed factor:	*F*	*p*	*F*	*p*	*F*	*P*	*F*	*p*	*F*	*P*
r	1.1	0.34	0.1	0.78	0.6	0.48	≤ 3.6	≥ 0.06	2.9	0.13
Time since ejaculation	5.4	**0.02**	27.6	**<0.001**	<0.1	0.94	≤ 1.5	≥ 0.23	16.7	**0.003**
r x time	0.4	0.55	0.6	0.46	0.2	0.66	≤ 0.4	≥ 0.54	1.9	0.18
Random factors										
Stallion ID	7.6%		1.4%		6.4%		63.8 – 71.7%		7.5%	
Stallion ID x r	91.8%		94.5%		86.4%		0%		92.2%	
All other interactions	0%		0%		0%		0%		0%	

[1] logit transformed; [2] directed; [3] range over VAP, VSL, VCL; [4] not significant for VAP and VSL

Figure 1: Stallions' reaction to exposure to an MHC-similar or an MHC-dissimilar mare shortly before semen collection. (A) Change in mean blood plasma testosterone concentration when exposed to either an MHC-similar or to an MHC-dissimilar mare (means ± SE; change in 15 minutes before exposure to immediately after semen collection) (B) Sperm number per ejaculate (means ± SE). Panels (C) and (D) show the analogous mean changes in blood plasma testosterone concentration or mean sperm number per ejaculate, respectively, for each combination of stallion and teaser mare and relative to their pairwise relatedness r. The non-hatched lines give the regressions over all 20 means each, the hatched lines give the regressions excluding the pair with the extremely high r (filled symbol). See text and Tables 1, S3, and S4 for statistics.

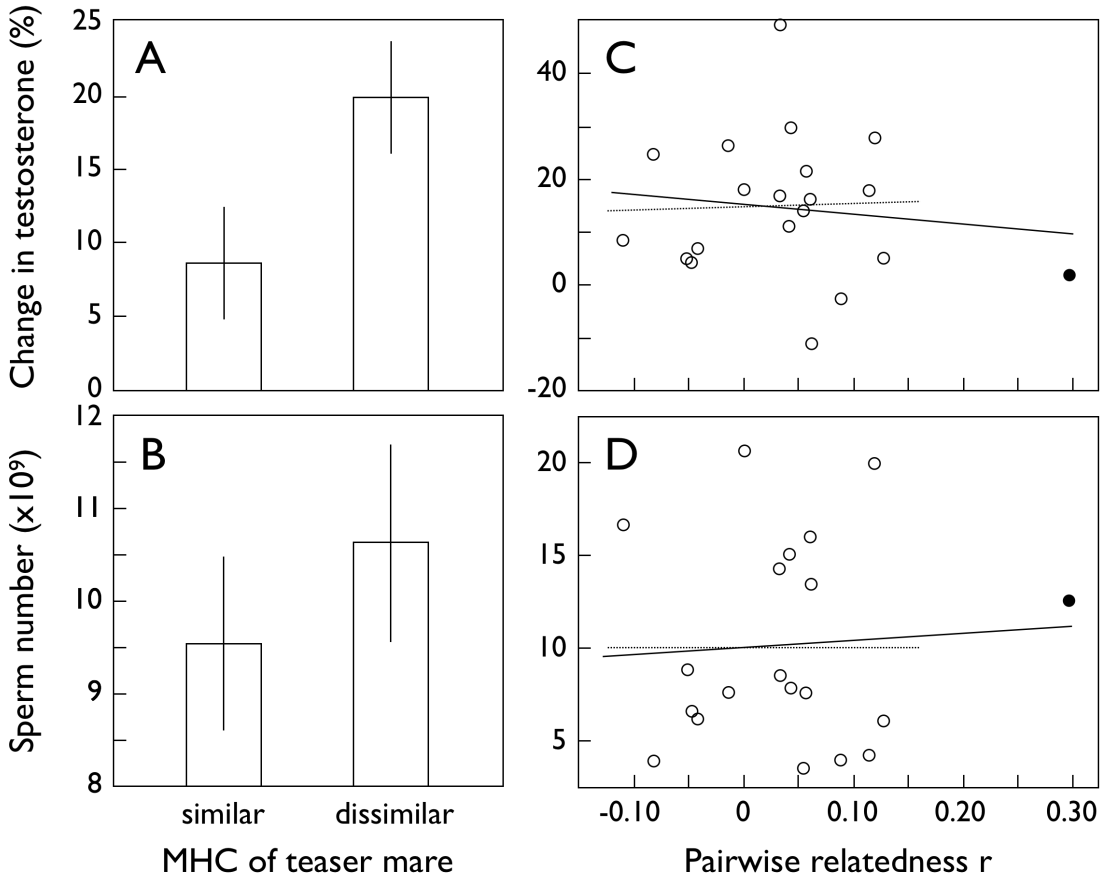
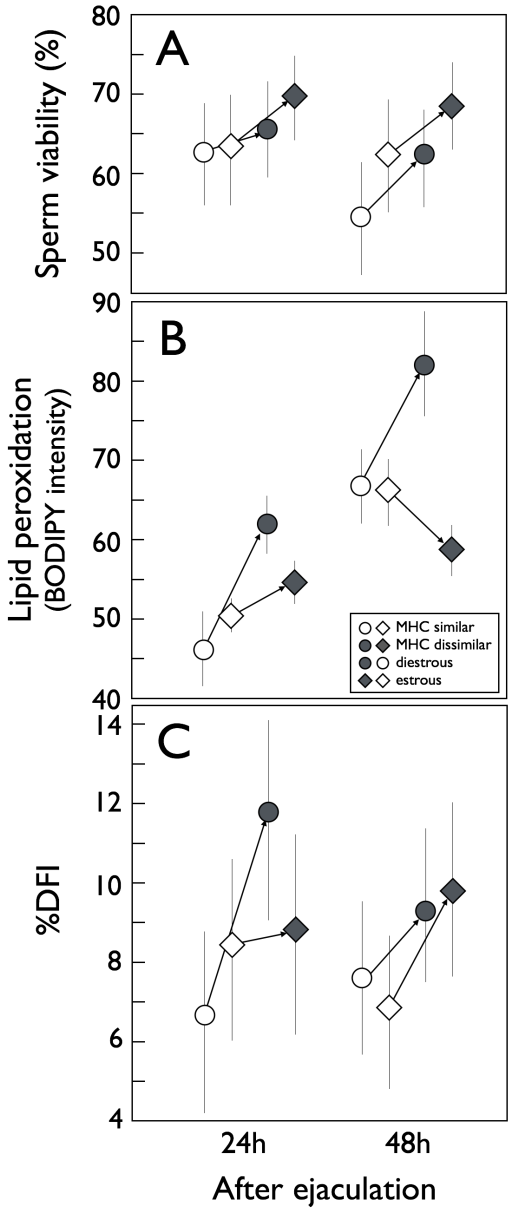


Figure 2: Performance of cold-stored sperm 24 and 48 hours after ejaculation when the stallions were exposed to MHC-similar (open symbols) or MHC-dissimilar mares (closed symbols) that were either diestrous (cycles) or in estrus (diamonds) (means ± SE). (A) Sperm viability (%; before logit transformation for statistical analyses), (B) lipid peroxidation (mean BODIPY fluorescence intensity), and (C) percentage of sperm with a high DNA fragmentation (%DFI). The arrows indicate the effects of MHC dissimilarity. See Table 2 and Supplementary Table S5 for statistics.



Supplementary Table S1. Diversity of the 20 microsatellite loci used to estimate relatedness between horses. The name of the microsatellite markers, their chromosomal location, and the number of alleles found in the present sample and in two other studies. See Burger et al. (2017) for primer sequences, annealing temperatures, fluorophores, and further information. Burger et al. (2017) tested for linkage disequilibrium and found none.

Name (chromosome)	Number of alleles		
	present study	Mittmann et al. (2010)[1]	Burger et al. (2017)[2]
VIAS-H34 (1)	6	7	11
UMNe448 (2)	4	6	6
AHT36 (3)	6	8	9
UMNe567 (5)	5	4	6
LEX065 (6)	5	5	6
HTG4 (9)	4	6	6
COR048 (10)	7	6	8
UCD457 (11)	4	10	12
COR069 (13)	5	7	8
UM010 (14)	5	7	7
HMS20 (16)	4	5	6
TKY924 (17)	4	4	5
TKY101 (18)	8	8	9
TKY582 (22)	3	7	6
UCD405 (25)	5	9	9
UM005 (26)	5	7	7
TKY315 (27)	4	8	8
TKY333 (28)	6	8	11
COR082 (29)	6	7	9
AHT34 (31)	7	7	9

[1] in 311 Warmblood horses; [2] in 148 Warmblood and Franches-Montagne horses

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Supplementary Table S2. MHC phenotypes of the mares and stallions, number of ELA shared between mares and mares and stallions, and relatedness between mares and mares and stallions.

	MHC phenotypes		ELA shared with		Relatedness[1] to	
Subject	Haplotype 1	Haplotype 2	Mare A	Mare B	Mare A	Mare B
Mare A	A2, W22	A18, BeIII	.	0	.	0.078
Mare B	A3, W12	A10, BeI, Be200, W21	0	.	0.078	.
Stallion 1	W11, BeI	Be108	0	1	-0.013	0.128
Stallion 2	Be108	BeI[2]	0	1	-0.047	-0.041
Stallion 3	A5, BeIV, W21	Be25, BeI	0	2	0.034	0.044
Stallion 4	A2, W22	Be108	2	0	-0.081	0.115
Stallion 5	A2	A20, BeIV	1	0	0.063	0.033
Stallion 6	A10, BeI, Be200, W21	Be108	0	4	-0.109	0.042
Stallion 7	A2	Be108	1	0	0.089	0.056
Stallion 8	A2	A15	1	0	0.001	0.121
Stallion 9	A2	Be108	1	0	0.298	0.062
Stallion 10	A2, W22	Be108	2	0	-0.050	0.058

[1] based on 20 microsatellite loci; [2] with an ELA that is still unknown

Supplementary Table S3: Mixed-model analysis of change in blood plasma testosterone (relative to basic levels) around ejaculation, with the mare's estrous stage and (a) MHC matching between stallion and teaser mare (dissimilar versus similar), (b) number of shared ELA between stallion and teaser mare (0, 1, ≥2), and (c) pairwise relatedness r as fixed factors, and stallion ID and the interactions as random factors. The proportions of the total variance explained (% of total) are based on unbounded REML variance components. F-values in parentheses refer to a model that include the pair combination with the extreme r = 0.298.

	Change in testosterone	
(a) Differences on MHC		
Fixed factors:	*F*	*p*
MHC (dissimilar/similar)	9.4	**0.01**
Estrous cycle	0.7	0.44
MHC x cycle	0.2	0.70
Random factors:		
Stallion ID	23.1%	
ID x MHC	0%	
ID x cycle	0%	
ID x MHC x cycle	76.9%	
(b) Differences on MHC		
Fixed factors:	*F*	*p*
Number of shared ELA	6.0	**0.02**
Estrous cycle	0.6	0.44
ELA x cycle	0.1	0.89
Random factors:		
Stallion ID	20.4%	
ID x ELA	0%	
ID x cycle	0%	
ID x ELA x cycle	79.6%	
(c) Differences on microsatellites		
Fixed factors:	*F*	*p*
r	0.05 (0.3)	0.86
Estrous cycle	0.7 (0.6)	0.42
r x cycle	0.2 (0.9)	0.67
Random factors:		
Stallion ID	13.8%	
ID x r	0%	
ID x cycle	0%	
ID x r x cycle	0%	

Supplementary Table S4: Mixed-model analyses of fresh weekly ejaculates (N = 70), with (a) number of shared ELA between stallion and teaser mare (0, 1, ≥2), and (b) pairwise relatedness r as fixed factors, and stallion identity (ID) and the interaction to number of shared ELA or relatedness as random factors. The one pair combinations with an extreme r was included here to illustrate the effect of reducing the sample in Table 1. The proportions of the total variance explained (% of total) are based on unbounded REML variance component estimates.

	Ejaculate volume		Total sperm number[1]		Sperm motility[2]		Sperm velocity[3]	
(c) Differences on MHC								
Within-subject fixed factor	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Number of shared ELA	2.1	0.19	11.8	**0.004**	0.1	0.93	≤ 1.3	≥ 0.31
Random factors								
Stallion ID	49.8%		81.4%		62.7%		79.8 – 89.3%	
Stallion ID x ELA	0%		0%		3.6%		0 – 1.4%	
Means ± 95% CI								
No shared ELA	41.5 ± 5.8 mL		10.6 ± 2.1 x 10^9		63.4 ± 8.9 %		122.3 ± 8.6 µm/s	
1 shared ELA	33.7 ± 7.1 mL		9.9 ± 2.5 x 10^9		64.2 ± 10.6 %		113.2 ± 11.1 µm/s	
≥2 shared ELA	52.3 ± 9.1 mL		9.0 ± 3.2 x 10^9		67.4 ± 13.5 %		124.7 ± 14.3 µm/s	
(d) Differences on microsatellites								
Fixed factor	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
r	0.1	0.90	2.3	0.25	1.4	0.93	≤ 1.0	≥ 0.39
Random factors								
Stallion ID	57.0%		82.2%		67.3%		56.4 – 89.4%	
Stallion ID x r	0%		0%		0%		0 – 29.5%	

[1] log transformed; [2]logit transformed; [3]range over VAP, VSL, and VCL, means given for VAP

Supplementary Table S5: Mixed-model analyses of ejaculates after 24 and 48 h of cold storage, with time since ejaculation, mare estrous stage, and MHC matching (similar versus dissimilar) between stallion and teaser mare as fixed factors, and stallion ID and the interactions as random factors. The proportions of the total variance explained (% of total) are based on unbounded REML variance component estimates. Significant p-values are marked in bold. Sperm viability = percentage of plasma membrane and acrosome intact sperm; LPO = amount of lipid peroxidation in viable sperm; %DFI = percentage of sperm with fragmented DNA; Low Ca^{2+} = percentage of sperm with low intra-cellular Ca^{2+} content; HMMP = percentage of sperm with high mitochondrial membrane potential.

		Sperm viability[1]		LPO		Sperm velocity measures[3]		%DFI[1]		Low Ca2+[1]		HMMP[1]		Sperm motility[1]	
Within-subject fixed factor:	*d.f.*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
MHC matching	1, 9	4.4	**0.04**[2]	4.9	**0.05**	≤0.3	≥0.57	4.9	**0.05**	1.2	0.30	0.1	0.72	1.5	0.25
Time since ejaculation	1, 9	28.9	**<0.001**	47.3	**<0.001**	≤9.1	**≥0.01**[4]	0.02	0.89	38.6	**<0.001**	2.4	0.16	14.9	**0.004**
Estrous stage (cycle)	1, 9	9.8	**0.01**	16.5	**0.003**	≤0.02	≥0.90	0.3	0.59	0.5	0.51	2.5	0.15	0.2	0.70
MHC x time	1, 9	0.6	0.47	9.7	**0.01**	≤0.5	≥0.51	0.3	0.57	1.6	0.23	2.3	0.16	0.2	0.64
MHC x cycle	1, 9	0.6	0.46	2.9	0.12	≤1.0	≥0.34	0.3	0.57	2.1	0.18	6.0	**0.04**	<0.1	0.97
Time x cycle	1, 9	17.6	**0.002**	19.3	**0.002**	≤3.2	≥0.11	0.6	0.44	2.2	0.17	3.0	0.12	3.1	0.12
MHC x time x cycle	1, 9	1.5	0.25	7.3	**0.02**	≤3.1	≥0.11	5.1	**0.05**	1.7	0.23	0.1	0.72	7.6	**0.02**
Random factors															
Stallion ID		87.8%		24.0%		52.8-61.6%		37.4%		86.2%		53.9%		87.0%	
ID x MHC		3.9%		0%		0%		0%		7.1%		1.9%		5.4%	
ID x time		0%		4.9%		0-0.6%		2.4%		0%		8.2%		0.6%	
ID x cycle		0%		0%		0%		0%		0.9%		9.6%		0%	
ID x MHC x time		0.4%		0%		0.4-1.8%		0%		0.4%		0%		0.9%	
ID x MHC x cycle		6.0%		64.4%		32.2-38.8%		52.2%		2.6%		0%		4.4%	
ID x time x cycle		0%		1.0%		0-0.5%		0%		0%		0%		0%	
ID x MHC x time x cycle		1.9%		5.8%		3.5-8.0%		8.1%		2.9%		26.4%		1.6%	

[1] logit transformed; [2] directed; [3] range over VAP, VSL, VCL; [4] not significant for VAP and VSL

Supplementary Table S6: Mixed-model analyses of ejaculates after 24 and 48 h of cold storage, with time since ejaculation, mare estrous stage, and number of shared ELA ("ELA") between stallion and teaser mare (0, 1, ≥2) as fixed factors, and stallion ID and the interactions with stallion ID as random factors. The proportions of the total variance explained (% of total) are based on unbounded REML variance component estimates. Sperm viability = percentage of plasma membrane and acrosome intact sperm; LPO = amount of lipid peroxidation in viable sperm; %DFI = percentage of sperm with fragmented DNA; Low Ca^{2+} = percentage of sperm with low intra-cellular Ca^{2+} content; HMMP = percentage of sperm with high mitochondrial membrane potential.

	Sperm viability[1]		LPO		Sperm velocity measures[2]		%DFI[1]		Low Ca2+[1]		HMMP[1]		Sperm motility[1]	
Within-subject fixed factor:	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*P*	*F*	*p*	*F*	*p*
Number of shared ELA	2.9	0.11	3.0	0.09	≤0.2	≥0.85	2.3	0.15	0.6	0.58	0.1	0.90	0.7	0.50
Time since ejaculation	23.0	**<0.001**	50.4	**<0.001**	≤7.6	**≥0.02**[3]	0.2	0.65	33.0	**<0.001**	3.0	0.12	14.0	**0.004**
Estrous stage (cycle)	5.1	**0.04**	2.8	0.11	≤0.2	≥0.64	<0.1	0.95	<0.1	0.94	3.8	0.08	0.03	0.87
ELA x time	0.7	0.49	4.6	**0.04**	≤3.8	≥0.06	1.0	0.41	6.9	**0.01**	1.1	0.38	0.2	0.85
ELA x cycle	0.9	0.43	1.5	0.27	≤0.5	≥0.60	0.4	0.67	3.3	0.08	3.2	0.08	0.5	0.61
Time x cycle	15.8	**0.002**	11.5	**0.007**	≤3.0	≥0.11	<0.1	0.89	0.8	0.40	2.1	0.17	5.7	**0.04**
ELA x time x cycle	1.0	0.39	4.4	**0.04**	≤2.8	≥0.10	2.5	0.12	0.9	0.44	0.7	0.50	5.6	**0.02**
Random factors														
Stallion ID	88.6%		23.4%		51.6-62.6%		37.9%		84.9%		56.2%		876.5%	
ID x ELA	3.7%		0%		0%		0%		9.0%		1.3%		6.1%	
ID x time	0%		4.5%		0-0.9%		2.6%		0.4%		8.2%		0.8%	
ID x cycle	0%		0%		0%		0%		1.5%		8.2%		0%	
ID x ELA x time	0.3%		0.2%		0.1-1.1%		0%		0%		0%		0.8%	
ID x ELA x cycle	5.4%		64.8%		32.3-40.0%		50.4%		1.4%		0%		4.4%	
ID x time x cycle	0%		1.8%		0-0.5%		0%		0%		0%		0%	
ID x ELA x time x cycle	1.9%		5.3%		3.2-8.3%		9.1%		2.8%		26.1%		1.5%	

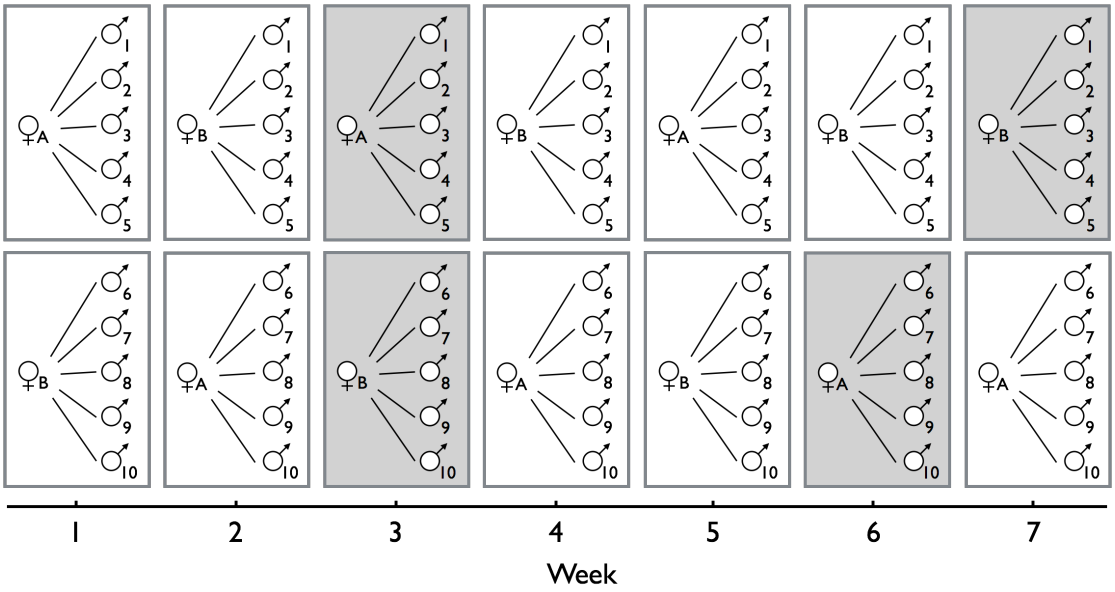
[1] logit transformed; [2] range over VAP, VSL, VCL; [3] not significant for VAP and VSL

Supplementary Table S7: Correlations (Kendall's τ) between ejaculate characteristics determined after 24 and 48 h of cold storage, Sperm viability = percentage of plasma membrane and acrosome intact sperm; LPO = amount of lipid peroxidation in viable sperm; %DFI = percentage of sperm with fragmented DNA; Low Ca^{2+} = percentage of sperm with low intra-cellular Ca^{2+} content; HMMP = percentage of sperm with high mitochondrial membrane potential. Significant correlations are marked in bold.

	Time after ejaculation	LPO	%DFI	Sperm velocity measures VAP	VSL	VCL	Motility	HMMP	Low Ca2+
Sperm viability	24h	**0.27****	**-0.42*****	**0.26****	**0.41*****	0.12 $^{n.s.}$	**0.57*****	**0.27****	**0.57*****
	48h	0.16 $^{n.s.}$	**-0.33*****	**0.32****	**0.46*****	0.14 $^{n.s.}$	**0.56*****	**0.39*****	**0.51*****
LPO	24h		-0.12 $^{n.s.}$	0.05 $^{n.s.}$	0.11 $^{n.s.}$	0.04 $^{n.s.}$	**0.18***	0.14 $^{n.s.}$	0.13$^{n.s.}$
	48h		**-0.20***	0.10 $^{n.s.}$	0.14 $^{n.s.}$	0.01 $^{n.s.}$	0.16 $^{n.s.}$	0.08$^{n.s.}$	0.13$^{n.s.}$
%DFI	24h			-0.08 $^{n.s.}$	**-0.23****	-0.02 $^{n.s.}$	**-0.38*****	**-0.26****	**-0.48*****
	48h			**-0.18***	**-0.30*****	-0.04 $^{n.s.}$	**-0.24****	**-0.25****	**-0.31*****
VAP	24h				**0.76*****	**0.73*****	0.20*	-0.03 $^{n.s.}$	0.16 $^{n.s.}$
	48h				**0.70*****	**0.66*****	0.16 $^{n.s.}$	0.25**	0.17 $^{n.s.}$
VSL	24h					**0.53*****	**0.36*****	0.09 $^{n.s.}$	**0.33*****
	48h					**0.40*****	**0.34*****	**0.35*****	**0.34*****
VCL	24h						0.07 $^{n.s.}$	-0.05 $^{n.s.}$	0.003 $^{n.s.}$
	48h						-0.02 $^{n.s.}$	0.14 $^{n.s.}$	-0.07 $^{n.s.}$
Motility	24h							**0.36*****	**0.61*****
	48h							**0.46*****	**0.46*****
HMMP	24h								**0.29****
	48h								**0.39****

* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; n.s. = not significant

Supplementary Figure S1: Schematic of the experiment. Once per week, one of the two mares was tethered close to a dummy (that was used for semen collection). Stallions were then individually exposed to this mare. After semen of the first five stallions had been collected, the mare was replaced and the experiment continued with the remaining five stallions. The order of presentation and the pair assignments were altered over the first 6 weeks, and the one of week 6 was repeated in week 7, so that by the end of the experiment, each stallion had been exposed to each mare once in oestrus (grey boxes) and two to three times in dioestrus (white boxes). The seventh experimental week was necessary because the second oestrus of mare #2 occurred one week later than expected.



Supplementary Figure S2: Correlations between sperm characteristics after cold storage for 24 hours (red dots and lines) and 48 hours (blue dots and lines). Sperm viability (logit-transformed percentage of plasma-membrane and acrosome-intact sperm (PMAI)) versus (A) logit-transformed percentage of sperm with a high DNA fragmentation (%DFI), and (B) lipid peroxidation (bodipy fluorescence intensity). (C) %DFI versus lipid peroxidation. The regression lines illustrate the directions of the significant correlations listed in Table S7.