MHC-correlated preferences in diestrous female horses (*Equus caballus*)

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ABSTRACT

Genes of the major histocompatibility complex (MHC) have been shown to influence communication in many vertebrates, possibly with context-specific MHC-correlated reactions. Here we test for MHC-linked female preferences in the polygynous horse (*Equus caballus*) by repeatedly exposing 19 mares to a group of seven sexually experienced stallions. Each mare was tested four times during two consecutive reproductive cycles, twice during estrus and twice during diestrus. Male plasma testosterone concentrations were determined from weekly blood samples, and equine leukocyte antigen (ELA) class I and II alleles were determined serologically at the end of the experiments. Perception of male attractiveness was strongly dependent on estrous cycle: mean preference scores did not correlate for mares in diestrus and estrus and varied more during estrus than during diestrus. We found elevated female interests for MHC-dissimilar stallions, but only during diestrus, not during estrus. Female preferences were not significantly predicted by mean male testosterone plasma concentrations. However, testosterone concentrations changed during the 11 weeks of the experiment. By the end of the experiment, average testosterone concentration was significantly correlated to the average number of MHC alleles the stallions shared with the mares. We conclude that the MHC affects female preferences for stallions, but non-MHC linked male characteristics can overshadow effects of the MHC during estrus.

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1. Introduction

Horse breeding under domesticated conditions differs dramatically from natural reproduction in the wild. Some of the differences may contribute to the problems commonly encountered in the equine industry [1], i.e. problems around fertility, efficiency, or safety. A better understanding of the behavioral and physiological aspects of reproduction and of intra- and inter-sexual signaling may help to improve animal well-being and the effectiveness of breeding programs [2]. Here we concentrate on possible effects of the major histocompatibility complex (MHC) on female mate preferences during estrus and diestrus.

The MHC is a group of genes that play a critical role in the immune system of vertebrates and may be the basis of a vertebrate-wide chemosensory communication system [3,4]. There is accumulating evidence from different types of studies that MHC-linked social signals facilitate cooperative behavior among kin or influence sexual selection and life history at various levels, including mate choice, gamete fusion, or even maternal decisions concerning embryo

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development and pregnancy termination [5,6]. The types of behaviors and decisions that can be MHC-mediated are very diverse, often varying from species to species or even from context to context [3].

The MHC influences sexual selection and mating preferences in amphibians, reptiles (e.g. Swedish sand lizard, *Lacerta agilis*, [7]; tuatara, *Sphenodon punctatus*, [8]), birds (e.g. ring-necked pheasant, *Phasianus colchicus*, [9]; great frigatebirds, *Fregata minor*, [10]; blue petrels, *Halobaena caerulea*, [11]) fishes (e.g. three-spined sticklebacks, *Gasterosteus aculeatus*, [12,13]; Atlantic salmon, *Salmo salar*, [14]; Chinese rose bitterling, *Rhodeus ocellatus*, [15]), and in mammals like rodents (e.g. mice, *Mus musculus domesticus*, [16,17]; tuco-tucos, *Ctenomys talarum*, [18]), monkeys (e.g. mandrill, *Mandrillus sphinx*, [19]) and humans [20,21]. In general, MHC-dissimilar males and females tend to prefer each other, i.e. disassortative mate preferences seem to be the rule.

MHC-dependent sexual selection may lead to certain kinds of MHC genotypes or simply increase heterozygosity in offspring [22–24] thereby providing an immunological advantage for the progeny [25–28] and, ultimately, fitness [14,29–32]. The MHC is of crucial importance not only for immunological resistance but especially also for kin selection and inbreeding avoidance [3,5,19,33]. MHC-based communication was recently found in the horse (*Equus caballus*): stallions reacted differently to the presence of MHC–similar or–dissimilar mares when kept pairwise over several weeks [34].

Under feral conditions, horses are polygynous and live throughout the year in fairly stable social and breeding bands so-called harems [35–37]. Harems usually consist of one and sometimes up to five stallions [38] along with between one and nine or more breeding mares and their offspring. Stallions seem to generally avoid breeding with mares born within the harem [39], but it is not clear yet whether this potential form of inbreeding avoidance is driven by male or female preferences, even if mares generally seem to play the more decisive role in mate choice [1]. Stallions who are not in a harem will often form co-called "bachelor stallion bands" that usually consist of between two and 17 stallions, typically with fluctuating group sizes [40]. Bachelors sometimes have the opportunity to mate with mares previously dispersed from a harem band. Apart from that, bachelors show no or little breeding activity. Dispersal of young mares from their natal group, a strategy hypothesized to avoid inbreeding, begins with sexual maturity and was shown to peak seasonally with sexual receptivity [41].

The objective of this study was to test for MHC-linked female behavior when exposed to several stallions during estrus and during diestrus. We also recorded male testosterone values in order to develop hypothesis about male reactions to our experimental set-up.

2. Materials and methods

2.1. Horses

19 mares without foals (mean age ± SD = 8.7 ± 3.1 years; six nulliparous with 6.3 ± 1.9, and 13 primi- or pluriparous mares with 9.8 ± 2.9 years of age) and seven sexually experienced stallions (mean age ± SD = 12.7 ± 5.8 years; all with normal fertility stud records in the years before the study) were used in this study. Before the experiments, all horses had been vaccinated and dewormed, and using the McMaster method (detection limit: 50 eggs per gram, EpG; [42]) on feces samples, no stallion with greater than 50 EpG was registered. Mares and stallions were not familiar with each other.

2.2. Study design

As experimental design we used an arena test designed for polygynous species [43–45]. The experimental stable consisted of 2×4 boxes (12 m^2) divided by a corridor (12.65 m long and 2.90 m wide). The stallions were randomly distributed to these boxes. One mare each was introduced in the corridor and her preferred whereabouts recorded. Each mare was tested in two consecutive estrous cycles both during estrus and diestrus. Estrous cycles were monitored *via* daily transrectal ultrasonographic examination during estrus until ovulation and every third day during diestrus, respectively. When at least one follicle had reached a diameter ≥35 mm, an uterus edema at stage 2 or higher was present [46], and the absence of any corpus luteum could be confirmed, 1500 IU hCG (Chorulon 1500, Intervet, Netherlands) was applied intravenously in the evening of the same day to induce ovulation, which was confirmed 48 hours later. The mare (showing behavioral estrus and receptivity when teased with an additional stallion) was then tested the day after ovulation induction ("estrus") and 5 to 12 days after ovulation, respectively ("diestrus").

For the first two test series during the mares' first estrus and diestrus, the stallions' box fronts toward the corridor were fully covered with the exception of an opening of 21.5×15.0 cm, 1.50 m above the ground. The second two tests took place without blinds (allowing full visual contact) during the mares' second estrous cycles (16 of the 19 mares were still available for the second test series). This treatment with and without blinds was used for a parallel study on visual *versus* non-visual female perception of male age and size (Burger et al., submitted manuscript).

For each test, a mare was led into the corridor and walked along the seven boxes with the stallions, halted 15 seconds in front of each stallion's box, with their head close to the small openings or to the stallion to get acquainted with each male's position, and then released in the center of the corridor. The experimenter (who was naïve with respect to the animals' MHC) left the stable and used video surveillance to record the time the mare was interacting with each stallion.

After 10 minutes, the stallion with the highest cumulative contact time was removed from the stable (after the mare had been temporarily removed to avoid direct contact). This procedure was repeated (without initial 15 seconds presentation of all remaining stallions) until only two stallions remained. The stallions were then ranked (1 for the stallion that received the highest cumulative contact time first, up to seven for the stallion that received the lowest contact time when only two stallions

Table 1
Characteristics of the stallions: mean peripheral plasma testosterone levels during the experiments (ng/mL), and mean preference ranks they received from mares during diestrus and estrus.

ID	Mean testosterone (range)	Mean preference rank (range)[a]	
		Diestrus	Estrus
A	8.2 (2.6–15.5)	3.59 (1–6.5)	3.08 (1–6)
B	6.6 (3.0–10.9)	4.59 (1–7)	3.18 (1–6.5)
C	9.0 (3.5–18.0)	3.51 (1–7)	3.66 (1–6)
D[b]	10.1 (2.6–24.0)	3.37 (1–7)	5.49 (1–7)
E	14.3 (6.0–22.5)	4.09 (1–7)	5.00 (1–7)
F	11.7 (4.2–23.8)	3.97 (1–7)	2.28 (1–5.5)
G[c]	2.6 (2.2–3.3)	3.93 (1–7)	5.21 (1–7)

[a] Low ranks indicate high attractiveness.
[b] Euthanized toward end of study because of colic (13 of 70 ranks missing).
[c] Had experienced immunological castration treatment earlier.

remained – if a mare ceased to show any preferences after the first stallions had been removed, the remaining stallions received tied ranks).

The experiments were approved by the *Etat de Vaud, Service Vétérinaire* (permit #2211). The stallions had no contact with other mares, were regularly exercised and had individual access (1 hour/day) to a separated paddock. Mares were turned out in groups in paddocks (3 hour/day). Three weeks before the end of the study, one stallion had to be euthanized due to an acute colic (Table 1). Therefore, 13 of the expected 490 preference ranks (2.7%) were missing.

2.3. MHC analysis

Equine leukocyte antigen (ELA) class I and class II were determined serologically in microcytotoxicity tests with alloantisera (i.e. sera generated in a conspecific; [47–49]) detecting 23 ELA-A (MHC class I) specificities, the ELA-C allele W21, and five MHC class II alleles. ELA of all horses

are shown in Supplementary Table S1. Stallions and mares were classified as "MHC similar" if they shared at least one ELA specificity, otherwise they were classified as "MHC dissimilar".

2.4. Testosterone analysis

Blood samples (EDTA, jugular venipuncture) were taken from stallions at the start of the experiments, and once per week from week seven on (every Tuesday, between 8:00 am and 8:30 am) to determine testosterone plasma concentrations as in Burger et al. [34,50]. The samples were immediately centrifuged (×4000*g* for 10 minutes) and the plasma frozen (−80 °C) until analysis. Testosterone was determined *via* electrochemiluminescence immunoassay with inter- and intra-assay coefficients of variation of 2.2 and 1.4%, respectively [51].

2.5. Data analysis

For statistics, we used mean preference ranks per stallion and experimental setup for mares that were MHC-similar or –dissimilar each. MHC effects were tested in within-subject analyses (MANOVA, to control for between male differences in non-MHC linked characteristics), comparing the mean preference rank a stallion received from MHC-similar mares with the mean preference rank he received from MHC-dissimilar mares. Because time spent in the experimental set up could potentially influence the animals' behaviors and hence create confounding effects, the mean time a stallion was in the experimental setup (revealed by its mean rank) and the interaction between this overall mean rank and the mean preference ranks of MHC-similar or–dissimilar mares were included in the MANOVA (and shown in Fig. 1). Pearson's correlation coefficients (r) were used if graphical inspection suggested no violation of the model assumptions, otherwise the non-parametric Kendall's τ was used.
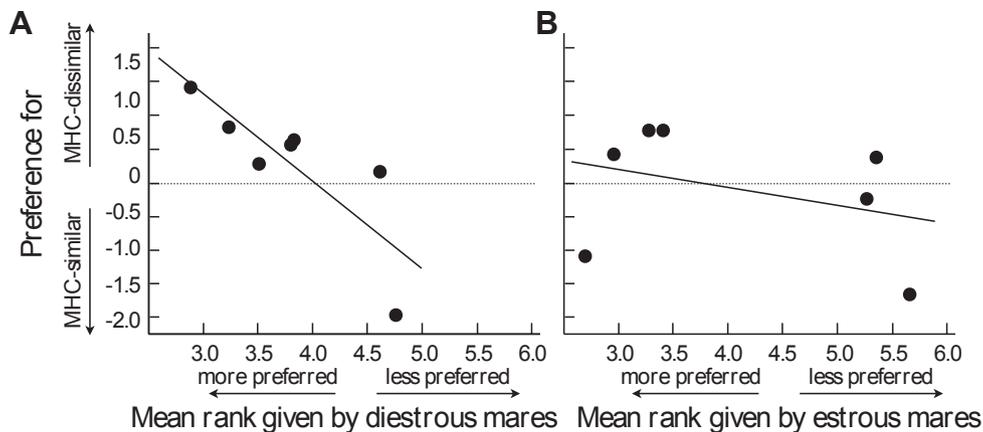
Fig. 1. Seven stallions ranked for attractiveness by 19 MHC-similar and -dissimilar mares during (A) diestrus and (B) estrus. The plot shows the difference between mean ranks each stallion received from MHC-similar and MHC-dissimilar mares (positive differences indicate preferences for MHC-dissimilarity) against the stallions' mean rank in the respective test situations to take duration of exposure into account (low ranks indicate high attractiveness and early removal from tests). The dotted line indicates the neutral expectancy that differs from 0 because of 2.7% missing data. The solid lines indicate the regressions. See text for statistics. MHC, major histocompatibility complex.
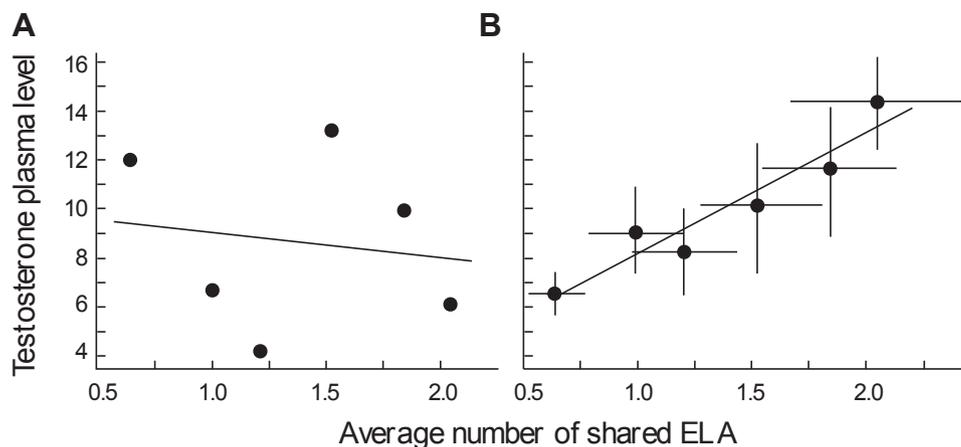
Fig. 2. Peripheral plasma testosterone levels (ng/mL) *versus* average number of MHC antigens shared with the 19 mares the seven stallions were exposed to (regression lines). (A) Testosterone before exposure to mares (r = −0.15, P = 0.78). (B) Average testosterone (95% CI) during repeated exposure to the mares *versus* their average (95% CI) number of shared MHC antigens (r = 0.95, P = 0.003). MHC, major histocompatibility complex.

3. Results

The mean ranks of attractiveness varied more during estrus than during diestrus (Brown-Forsythe test of equal variances: $F_{1,12}$ = 10.9, P = 0.006; Fig. 1A, B), and there was no positive correlation between these average ranks during diestrus and estrus (r = −0.45, P = 0.31). Rankings obtained during estrus and during diestrus were therefore analyzed separately. Because the treatment with blinds did not produce any MHC-linked effects (data not shown), we discarded the blinds as treatment factor and used mean ranks per diestrus and estrus of each mare for further analyses.

ELA sharing with stallions affected the perception of diestrous mares: stallions were on average ranked as more attractive by ELA-dissimilar mares than by ELA-similar mares, and the more dissimilar, the more attractive they were generally ranked (Fig. 1A; MANOVA: within-subject effect of ELA sharing, $F_{1,5}$ = 10.9, P = 0.022; interaction between ELA sharing and mean attractiveness during diestrus: $F_{1,5}$ = 10.0, P = 0.025). No such ELA effects could be seen during estrus (Fig. 1B; MANOVA: within-subject effect of ELA sharing, $F_{1,5}$ = 0.55, P = 0.49; interaction between ELA sharing and average rank during estrus: $F_{1,5}$ = 0.71, P = 0.44).

Table 1 lists the mean preference ranks that the stallions received from diestrous and estrous mares, and their mean testosterone blood levels. One stallion that had experienced immunological castration treatment (GnRH vaccination) 2 years before the experiments displayed extraordinary low levels of testosterone (Table 1) and was therefore excluded from testosterone-related statistical analyses. The other stallions' mean testosterone blood levels were neither significantly correlated to female preferences during diestrus (r = −0.21, P = 0.68) nor during estrus (r = −0.41, P = 0.42). Testosterone levels were never significantly correlated to stallion age (P always >0.20). As expected, there was also no correlation between testosterone and MHC sharing in the initial sampling at the beginning of the experiment (Fig. 2A). However, mean testosterone levels during the experimental exposure to mares were positively correlated to the number of MHC similar mares the stallions were exposed to (Kendall's τ = 0.93, P = 0.01) and to the average number of shared ELA (Fig. 2B; r = 0.95, P = 0.003).

4. Discussion

We found that female perception of stallions' attractiveness is dependent on the estrus cycle. Diestrous mares showed an elevated interest for MHC-dissimilar stallions. The analogous preferences could not be observed during estrus. Our observations confirm MHC-dependent social signaling in horses [34] and suggest that the importance of MHC-linked preferences depends on the estrous cycle. The relative importance of MHC-linked signals to mate choice in horses remains to be demonstrated.

During estrus, MHC preference seems to be overshadowed by non-MHC dependent male characteristics that remain to be identified. Various non-MHC-linked olfactory, behavioral, visual, or auditory characteristics can play significant roles in mate choice, as observed in other mammal species and reviewed by Clutton-Brock and McAuliffe [52]. The parallel study on visual *versus* non-visual female perception of male age and size (Burger et al., submitted manuscript) established that potential immunological effects, such as the parasitic status of our study animals, seem to play no important role in this context.

We also found that, by the end of the experiments, the average peripheral blood testosterone levels in stallions reflected their average MHC-similarity to the mares. The more MHC-similar mares the stallions were exposed to, the higher their average testosterone blood level. In another study where stallions and mares were kept in isolated pairs and over several weeks, stallions developed high testosterone levels when kept with an MHC-dissimilar mare, and significantly lower ones when kept with an MHC-similar mare [34]. Together, these findings support the conclusion

of Ruff et al. [3] that MHC-mediated behaviors are so diverse that they may well be not only species-specific but also context-dependent. In preference tests, for example, mice and humans of both sexes significantly favor odors of MHC-dissimilar individuals [16,17,20,53], while female mice when pregnant prefer the odors of MHC-similar individuals [33]. Analogous changes in preferences could be observed in women using the contraceptive pill [20,54,55]. Context-dependent interpretation of MHC-linked odors also seem to happen when a pregnancy block is induced in mice. Such a pregnancy block is more likely if a female is exposed to odors of a stimulus male that genetically differs only on the MHC to the fathering male (i.e. a male of a MHC-congenic line) than if exposed to a male from the same inbred line [56–58].

Numerous studies have found links between testosterone and the behavior males show toward other males or toward females [59]. The elevated testosterone levels we observed here were not predicted in the context of mate attraction but seemed consistent with scenarios based on perceived kin structure. A high frequency of MHC similarity within social groups could, for example, simulate a high average degree of kinship (even if >95% of all male-female pair combinations used in our experiments were unrelated regarding the last four generations). The corresponding testosterone levels may then reveal a stallion's willingness to protect and support kin. It is even possible that the perceived average level of kinship to the mares affects the socials ranks among the stallions. High testosterone levels may then reflect high social ranks within the group, as was found in bonobos (*Pan paniscus*, [60]) and bighorn rams (*Ovis canadensis*, [61]). In feral horses, harem stallions have indeed high testosterone levels if other stallions are around [62], and they are typically in constant contact to kin, because female offspring emigrate the harem definitively only at approximately 3 to 4 years of age [41]. Therefore, the typical odors that an established harem stallion perceives include those of many MHC-similar types, i.e. increasing levels of MHC sharing between mares and a given stallion may be perceived as increasing evidence of a family bond that may strengthen a stallion's rank in the group, analogous to findings in other mammals [63]. Finally, we must also consider the possibility of type I error in our findings, especially because we had no clear *a priori* expectancy about the direction of such a possible effect. Therefore, the link between testosterone and the MHC should be tested again in an independent set up.

The experimental procedure we used turned out to be useful for research on mate preferences also in horses. In contrast to other protocols that are typically based on the choice between two options [44], the protocol we used here allows for simultaneous choice among several stallions.

In conclusion, our findings confirm that (i) horses can reveal their MHC type, i.e. communication between horses can be based on MHC-linked social signals [34], and (ii) as in other species, the receiver's own MHC influences the interpretation of MHC-linked signals in horses, either because receivers have learned MHC-linked signals during ontogeny [64], or because the MHC or closely linked genes such as olfactory receptor genes [65] directly influence odor perception. Furthermore, our findings suggest that

(iii) mares' perception of male MHC-linked social signals are dependent on their estrous cycle, (iv) stallions adjust testosterone blood levels and hence their behavioral strategies to the MHC types of mares they are exposed to, and (v) stallions' reactions to female MHC-linked signals seem to be context-specific. Further studies are necessary to elucidate the most influential male parameters affecting mate choice of estrous mares. A better understanding of natural female reproductive strategies and taking them into account could help solving problems linked to current breeding methods.

Acknowledgments

Competing interests

The authors declare that they have no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.theriogenology.2016.09.015.

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Table S1
Equine leukocyte antigens (ELA) of the 19 mares and seven stallions, respectively.

Nr	ELA
Mares	
1	A2/A18 BeIII, W22
2	A3/A10 BeIII, W21, Be200, W12
3	A6
4	A5/A10, BeIII, W21, W13, Be200
5	A2/W21, BeVIII
6	A2/Be108
7	A2/A18,BeIII
8	A1/A6, BeVIII
9	A5/W11, BeIII, W21, W23
10	A3/A8
11	A15, W21, W13
12	A2/A9, W22
13	A15/A18, BeIII, W21, W23
14	A6/A10, BeIII, Be200
15	A2/A15, BeIII, W21, W22, W23
16	A2/A15, BeIII, W21, W22, W13
17	A3/A5, W21, W13, W23
18	A15/A16, BeIII, W21
19	A2/A18 BeIII, W22
Stallions	
1	A10/W11, BeIII, W21, Be200
2	A8/Be108, W21
3	Be108/W11, BeIII
4	A3/W11, BeII, BeVIII
5	A5/A18, BeIII, W21, W23
6	A10/A8, BeIII, W21, Be200
7	A2/Be108, W22