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High interindividual and intraindividual variation of oxytocin secretion in estrous mares exposed to stallions, but no significant link to mate preferences

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ABSTRACT

Oxytocin is a hormone that may not only influence reproductive mechanisms in mammals but also their social behavior, including pair bonding. We therefore tested if the concentrations of oxytocin and other hormones reveal mate preferences of 13 mares in estrus. Each mare was first exposed to two stallions (haphazardly selected out of seven) and her behavior recorded. The mare was then returned to her box (i.e., no contact to stallions during that time). Approximately 4.5 hours later, venous blood samples were collected every minute during 30 minutes preceding exposure to one of the two previously used stallions, 6 minutes during exposure, and 30 minutes after exposure back in the mare's box. The procedure was repeated in the consecutive estrus cycle, with the difference that the mare was each exposed to the other of the two stallions during oxytocin measurements. In 20 of the 26 trials, oxytocin concentrations were significantly elevated during exposure to the stallion, without significant associations to cortisol and estradiol concentrations. We found no significant association between oxytocin secretion and preferences in the previous choice situation. While estradiol concentration showed a high repeatability over the two cycles, we found considerable intraindividual differences in oxytocin and cortisol plasma concentration among the two cycles. Partially, the variation in oxytocin concentrations could be linked to the time of ovulation, with lower oxytocin plasma concentrations in mares which ovulated later than expected. In conclusion, when teasing under experimental conditions, we found high interindividual and intraindividual variation among mares in the increase of oxytocin plasma concentrations, depending on the timing of ovulation. However, oxytocin levels seemed to be no predictor of mare preference.

1. Introduction

Sexual stimulation (so-called teasing) of a mare with a stallion is a way of detecting estrus and hence represents an important tool to determine the ideal time for in hand breeding or artificial insemination in horses (*Equus caballus*). Teasing is also assumed to provoke an endogenous release of oxytocin in the estrous mare [1–3] hereby enhancing uterine contractions [1,4]. While teasing, the mare is usually exposed to a stallion that is spatially separated from the mare so that he cannot fully interact with her, for example, the two can only interact over a fence, a stall door, or a chute designed specifically for this purpose. The mare's behavioral responses to the

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stallion are then observed to assess her estrus status [5]. Estrous signs include repeatedly approaching the stallion, frequent urination, clitoral winking (rhythmic eversion of the clitoris), deviating the tail away from the perineum, and rotating the body so that the hindquarters are in front of the stallion and standing still with the hind limbs spread apart [5,6].

The nonapeptide oxytocin is a hormone that regulates several reproductive mechanisms in mammals, such as uterine contractions during parturition and milk ejection in the lactation period [7,8]. Oxytocin released within the central nervous system was also shown to play a major role in inducing maternal behavior in various species, including horses [9]. Recent studies in humans show that oxytocin also influences other behavior and behavioral strategies, for example, social recognition [10,11], trust building [12,13], reduction of stress and anxiety [14] as well as pair bonding [15]. In animal experiments, effects of oxytocin on social and affiliative behavior have been well documented (see reviews [16,17]). Oxytocin administration or restriction can result in alterations of preference formation in female prairie voles [18] and also socio-sexual behavior patterns between pair mates and the resulting mate choice between a stranger and a familiar pair mate, as found in marmosets [19].

Besides teasing, also other stimuli such as stallion vocalization, visual contact with a stallion, manual cervical dilation, artificial insemination, and mating have been shown to induce an oxytocin release in the mare [1–3,20]. On the other hand, it becomes evident, that not all mares release oxytocin in response to teasing. Measuring the concentrations of oxytocin in intracavernous sinus blood samples, Madill et al. [1] could demonstrate an oxytocin peak after teasing in only four out of five mares. In the study of Nikolakopoulos et al. [2], only six out of 10 estrous mares displayed a significant oxytocin increase in jugular venous blood samples after teasing. Reasons for this observation are not yet fully understood and have so far not been evaluated in a behavioral or evolutionary perspective. One possibility is that the variation in oxytocin levels reflects individual mate preferences. Sexual selection by female choice could be shown in a wide variety of species (reviewed in [21]). In feral harems, more than 88% of stallion-mare interactions that lead to successful copulation are initiated by the mare [22]. Studies on the interactions between mares and stallions show individual sexual preferences of estrous mares for certain stallions (D. Burger et al, unpublished work, 2016) [23].

Here, we test whether estrous mare oxytocin plasma concentrations during short-term exposure and teasing to stallions can be related to her mate preferences. In this context, the horse can be seen as a suitable experimental model, as this polygamous mammal offers by its size and manageability optimal conditions for repetitive manipulation and sample collection. This in turn allows for direct hormonal analyses, resolving difficulties in obtaining and interpreting plasma oxytocin as its release is pulsatile and therefore needing a frequent and rapid sampling to be accurately determined [8]. We therefore measured oxytocin plasma concentrations before, during, and after teasing mares with two different stallions each. The mare's reaction toward these stallions was previously recorded in a preference test. In addition, we investigated potential associations between oxytocin blood levels and stallion characteristics such as age, size and weight, or mare characteristics such as ovulation time or plasma estradiol and cortisol concentrations.

2. Material and methods

2.1. Animals and infrastructures

Twenty clinically healthy horses (13 mares and seven stallions) were used in this study. The mares were non-lactating, 8 to 20 years old (mean ± standard deviation [SD]: 12.6 ± 3.5 years), 1.52 to 1.72 m high (1.60 ± 0.06 m), weighing between 502 and 662 kg (581.2 ± 57.3 kg), and of various breeds (nine Warmblood, two Franches-Montagnes, and two Standardbred). All mares had normal perineal conformation and ovarian activity with variable reproductive histories. The stallions were 4 to 16 years old (10.6 ± 4.6 years), 1.52 to 1.61 m high (1.56 ± 0.03 m), and weighing between 477 and 610 kg (524.6 ± 41.9 kg). All of them were from the Franches-Montagnes breed and had natural service breeding experience with proven fertility.

Experimental infrastructures consisted of one stable with eight boxes each of 12 m^2, separated by 1.47-m high wooden walls and above a 2.00-m high metal grill, with a window (0.68 × 0.75 m) toward the corridor, allowing visual, olfactory, and limited physical contact with the mare. Transrectal ultrasonographic examinations were performed with a 5-MHz linear array transducer (Aquila Pro VET, Esaote, Genova, Italy).

2.2. Study design

The experiments were performed during the physiological breeding season from May to August in the Swiss National Stud in Avenches by always the same persons. In a standardized procedure (see Fig. 1), every mare was exposed to two different stallions each (out of the seven stallions). The two stallions had been haphazardly assigned to each mare and the two trials were performed in two consecutive estrus cycles.

Mares entered each trial after having been confirmed to be in estrus using behavioral observation (teasing with a stallion that was not used in later experiments) and transrectal ultrasonography (endometrial edema and preovulatory follicle ≥ 35 mm in diameter). Mares were individually stabled in clean boxes the day before the experiment. Another mare from the herd was stabled in the box beside the experimental mare to minimize eventual stress responses due to the environmental changes. At 7 PM, the experimental mare was treated with 1500 IU human chorionic gonadotropin (hCG) intravenously (Chorulon, Intervet, Boxmeer, Netherlands) to induce ovulation.

2.2.1. Preference test

At 9 AM the following day, the mares' reactions toward two stallions were studied in a preference test. For that reason, before 9 AM, the stallions were led into the experimental stalls. In the mare's first estrus cycle, the stallions'

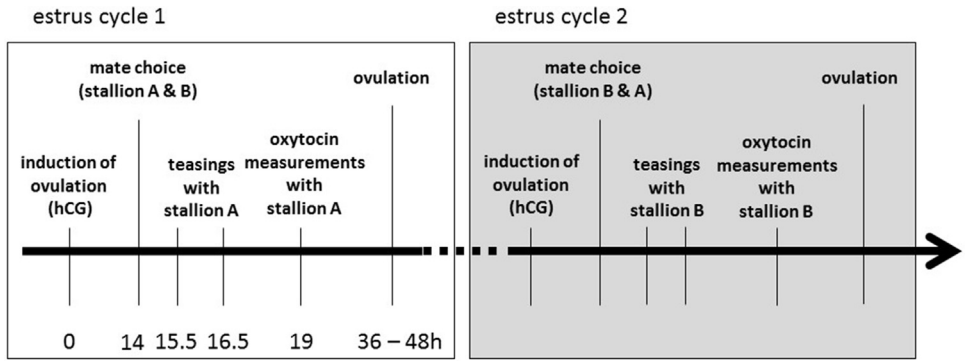

Fig. 1. Timetable of procedures.

stable boxes (left and right side at the end of the stable corridor) were assigned randomly. In the second estrus cycle, the same pair of stallions was used for the respective mare, but the stallions had switched boxes. The mare was led into the stable corridor and was presented to the stallions for 15 seconds each before the release of the mare in the middle of the corridor for totally 15 minutes. During this period, the behavior of the mares was registered by video. The interpretation of the mare's behavior was done by two independent observers who were, naïve with regard to the other measurements that were taken, including the oxytocin results. They determined the time (in seconds) the mare spent near each stallion and recorded behavioral estrus signs such as urinating, leaning toward the stallion, rotating the body so that the hindquarters were in front of the stallion and standing still with the hind limbs spread apart. After 15 minutes, the mare was led back to her stable and the stallion in always the left-sided box was removed, leaving the other stallion in the experimental stable.

2.2.2. Teasing habituation

At 10.30 AM and 11.30 AM (15.5 and 16.5 hours after ovulation induction, respectively), the mares were teased 3 minutes each with the remaining stallion to habituate them to the following procedure. During the teasing, the window of the box was left open so that the stallion could extend his head out of the stall and make physical contact with the mare. The person holding the mare was leading the mare close enough to the stallion to allow shoulder and flank contact. After the second teasing period, an indwelling catheter (13 gauge, Vygon, Ecouen, France) with extension tubing (Heidelberger extension tubing 20 cm, Cosanum, Grosswallstadt, Germany) was placed in the left jugular vein of the mare. The mare was then returned to her box.

2.2.3. Blood sampling

At 1.30 PM, the blood sampling for later hormone measurements started. Blood collection was performed every minute for a total duration of 66 minutes in the following three phases: (a) 1.30 to 2.00 PM: 30 minutes for the determination of basal oxytocin concentrations, the mare still standing in her box, (b) 2.00 to 2.06 PM (19 hours after hCG): 6 minutes for the determination of oxytocin during teasing with the experimental stallion, the mare standing in the corridor and the window of the stallion box open, allowing the stallion to have physical contact with the nose, shoulder, and flank of the mare, (c) 2.06 to 2.36 PM: 30 minutes for recording of the recovery period, the mare in her stable again. Oxytocin was assayed in all samples. Cortisol levels were determined at three time points: 1 minute before, 3 minutes after the beginning of teasing, and 1 minute after the end of teasing, respectively. Estradiol concentrations were determined at 24 minutes before the contact to the stallions during the determination of basal oxytocin concentrations.

2.2.4. Monitoring of ovarian activity

Ovarian activity was repeatedly monitored at 12-hour intervals, beginning at the time of hCG administration, and ovulation was detected ultrasonographically by the disappearance of the follicle and the presence of a corpus hemorrhagicum on the ovary.

2.3. Sample handling and analyses

Blood samples for oxytocin, cortisol, and estradiol-17β analyses were collected into tubes (Monovette 9-mL K3E, Sarstedt AG & Co., Nümbrecht, Germany) containing 1.6-mg EDTA/mL blood to prevent coagulation and were cooled immediately after sampling on ice until separation. Blood samples were centrifuged at 2000 $\times g$ for 15 minutes at 4 °C. All plasma samples were stored at −20 °C until oxytocin measurements were performed. Oxytocin plasma concentrations were determined for each time point by radioimmunoassay after extraction with SEP-PAK C18 cartridges (Waters Assoc., Inc., USA) as described by Schams [24] using antiserum raised in a rabbit. The average extraction recovery rate was 75% (n = 16 assays). The detection limit of the assay was 1.25 pg/mL. The intraassay coefficient of variation ranged from 15% to 7.5% and the interassay coefficient of variation from 22% to 9% in samples with low- (3 pg/mL) and high- (19 pg/mL) oxytocin concentration, respectively. Estradiol-17β concentrations were analyzed by means of a commercial estradiol ELISA (EIA-2693, DRG International, Inc., USA) and cortisol concentrations were determined by radioimmunoassay [25].

2.4. Statistical analyses

Mean baseline hormone concentrations were calculated from the average of the values obtained before the stallion contact (minute 1 until minute 29). Mean hormone concentrations during contact to stallion were obtained from the samples corresponding to the time of the stallion contact (minute 31 until minute 36; six blood samples). Parametric statistical models were used when graphical analyses suggested that the model assumptions were not significantly violated, otherwise nonparametrical statistical analysis was used. All analyses were performed in JMP (version 9, SAS Institute Inc., Cary, NC, USA, 1989–2007), R (version 2.14.1), and NCSS 2007 (Statistical Solutions, Saugus, MA, USA). Differences were considered significant at $P < 0.05$.

2.5. Ethical note

Animal experimentation was performed following approval from the local animal ethics committee (*Etat de Vaud, Service Vétérinaire*, approval #2538.0 and 2538.1). The animals had *ad libitum* access to water and were fed two times per day with hay or haylage, oats, barley, corn, and pellets supplemented with minerals. The stallions were regularly and individually exercised and had daily access for approximately 1 hour to a paddock without any direct contact to other stallions or mares. Mares were kept in open stable group infrastructures without any stallion contact. All horses had been dewormed before the experiments, seemed healthy, and absence of intestinal parasites could be confirmed using the McMaster method [26] with a detection limit of 50 eggs per gram on feces samples.

3. Results

3.1. Preference test

The total time spent near the stallion corresponded well with the prevalence of other behavioral parameters such as urinating, leaning toward the stallion, rotating the body so that the hindquarters were in front of the stallion, and standing still with the hind limbs spread apart (mean prevalence of these behaviors per mare vs. time spent near a stallion: $r = 0.68$, $n = 13$, $P = 0.01$), for example, the mares seemed to express on average clear preferences that seemed well quantified by the time spent near the stallion as recorded by naïve observers. Stallion age, size, or weight were no significant predictors of these preferences ($|r|$ always < 0.35, $n = 13$, P always >0.05).

3.2. Oxytocin before, during, and after teasing

During baseline recording, mean basal oxytocin plasma concentration of the mares was 3.5 ± 1.2 pg/mL (mean ± SD) and displayed a high repeatability between the two cycles (Spearman's rank order correlation coefficient on mean basal oxytocin: $r_s = 0.79$, $n = 13$, $P = 0.001$). No significant increase in plasma oxytocin concentrations was observed during baseline recordings (Fig. 2). Overall, teasing caused a significant increase in oxytocin concentrations in 20 of the 26 teasing trials (Fig. 2). Seven mares reported in both cycles a significant increase of oxytocin during teasing, whereas in the remaining six mares a significant oxytocin release was only measured in one of the two teasing trials. Maximal oxytocin plasma concentrations in the 20 trials with oxytocin release were reached 1 to 10 minutes (mean ± SD: 6.4 ± 1.9 minutes) after onset of stallion contact and were on average 16.1 ± 8.9 pg/mL (excluding mare no. 13 with extremely high maximal oxytocin concentrations; Fig. 2). Mare no. 13 had in her first estrus cycle a maximal oxytocin value of 213.2 pg/mL and in her second estrus cycle a maximal oxytocin value of 282.2 pg/mL. Her mean basal oxytocin plasma concentrations did not differ from the other mares (estrus cycle 1: 3.67 ± 0.98 pg/mL; estrus cycle 2: 4.55 ± 0.63 pg/mL). Mean oxytocin concentrations were not correlated between the two cycles ($r_s = 0.004$, $P = 0.85$). In all teasing trials, oxytocin concentrations declined close to baseline values within 30 minutes (Fig. 2).

3.3. Ovulation time

In five of the 26 teasing trials, the ovulation of the experimental mare occurred later than expected (= ovulation occurred later than 48 hours after hCG treatment): one mare ovulated 60 hours, three 84 hours, and one mare 108 hours after hCG treatment. These late ovulations were associated with significantly lower maximal ($P = 0.02$) and mean ($P = 0.03$) oxytocin concentrations during teasing (Fig. 3).

3.4. Preference test and oxytocin concentrations

Maximum oxytocin levels were no significant predictors of mate preference as determined in the preference tests, neither for the total amount of time (first cycle: $r_s = -0.24$, $P = 0.43$; second cycle: $r_s = 0.01$, $P = 0.97$; Fig. 4A) nor the relative amount of time spent close to a stallion (first cycle: $r_s = -0.10$, $P = 0.73$; second cycle: $r_s = 0.03$, $P = 0.91$; Fig. 4B). Mean oxytocin levels during exposure to the stallion (both, absolute or as difference to baseline oxytocin levels as determined before exposure) were also not correlated to the times spent near a stallion, neither in the first nor in the second estrus cycle ($|r|$ always < 0.36, $n = 13$, P always >0.05).

3.5. Teasing behavior, cortisol, and estradiol

All teasing bouts were accompanied by a display of estrous behavior such as posturing and clitoral winking. Mean (SD) plasma estradiol concentration was 22.1 ± 14.7 pg/mL. These estradiol concentrations were correlated over the two cycles ($r = 0.92$, $n = 13$, $P < 0.0001$), that is, reported a high repeatability. Mean plasma cortisol concentrations 1 minute before, 3 minutes after the beginning of teasing, and 1 minute after the end of stallion contact were 20.0 ± 5.6 ng/mL, 20.2 ± 5.4 ng/mL, and 20.0 ± 5.3 ng/mL, respectively. The respective cortisol measures were not significantly correlated over the two cycles (r always < 0.17, P always >0.05).

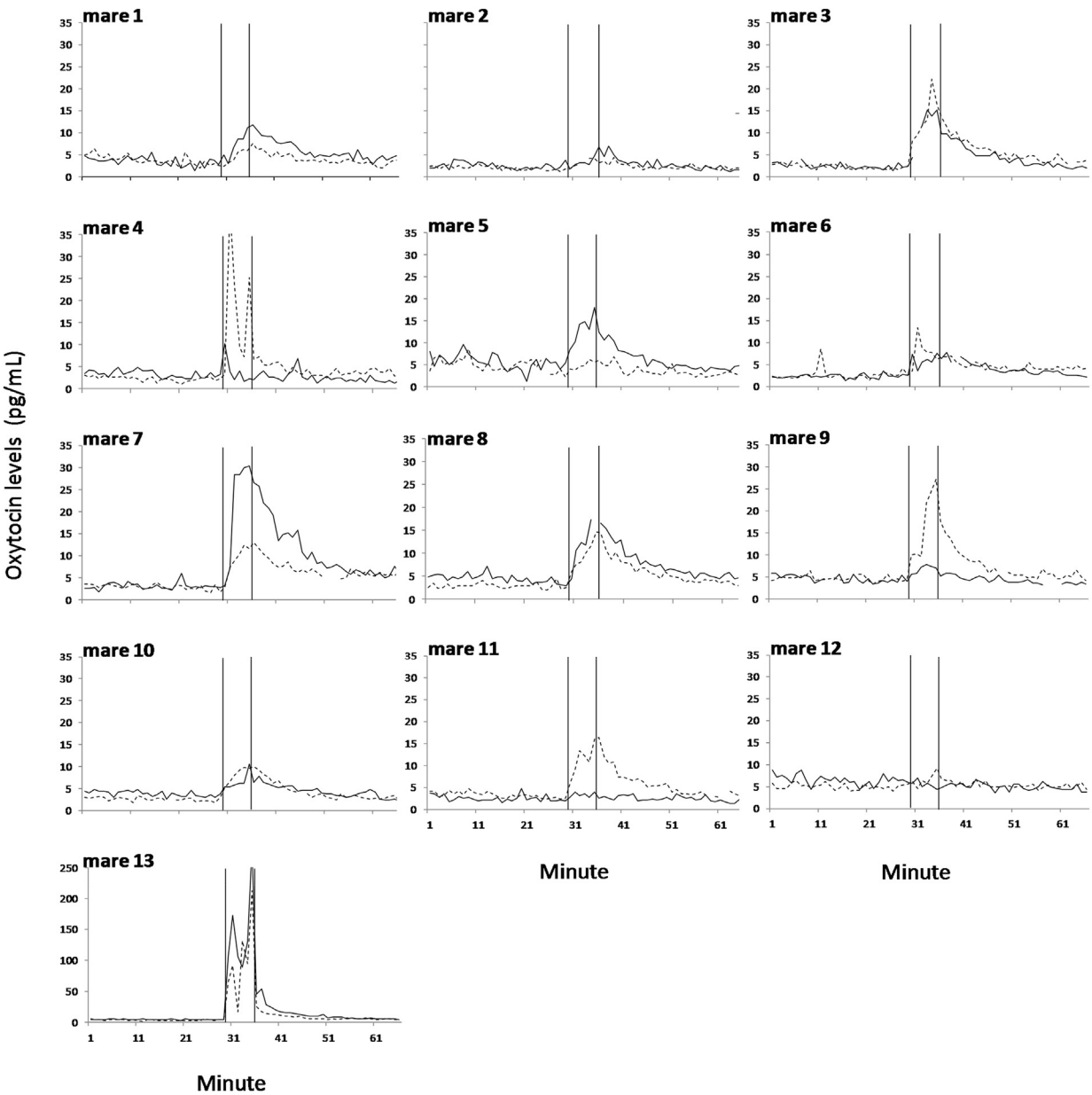

Fig. 2. Oxytocin before, during, and after teasing. Oxytocin plasma concentrations (pg/mL) of the 13 mares during 30 minutes before, 6 minutes during, and 30 minutes after exposure to a stallion, during estrus of the first (hatched line) and second cycle (nonhatched lines). Start and end of stallion contact is marked with vertical lines. Note that the scale of the Y-axes is different for mare 13.

4. Discussion

In the present study, oxytocin secretions of estrous mares reported a high interindividual and intraindividual variation in relation to stallion contact, partially linked to the time of preovulatory stage, while not being related to cortisol and estradiol concentrations nor significantly associated with short-term preferences of the mares.

Oxytocin concentrations were significantly elevated in response to teasing with stallions in 20 of 26 teasing trials, confirming earlier studies in teased horses [1–3]. Data from experiments in other species such as cattle [27], sheep [28], rabbit [29], and pigs [30] reveal a general consensus that oxytocin release is induced during the estrus period. Overall, what exactly stimulates the oxytocin release at the time of estrus is possibly a combination of physical and psychogenic stimuli with considerable species variation [31]. In humans, several studies indicate that plasma oxytocin concentrations increase in response to positive emotion and massage [32] and during sexual arousal and orgasm both in men and women [33,34]. In pigs, boar exposure at the time of artificial insemination causes an

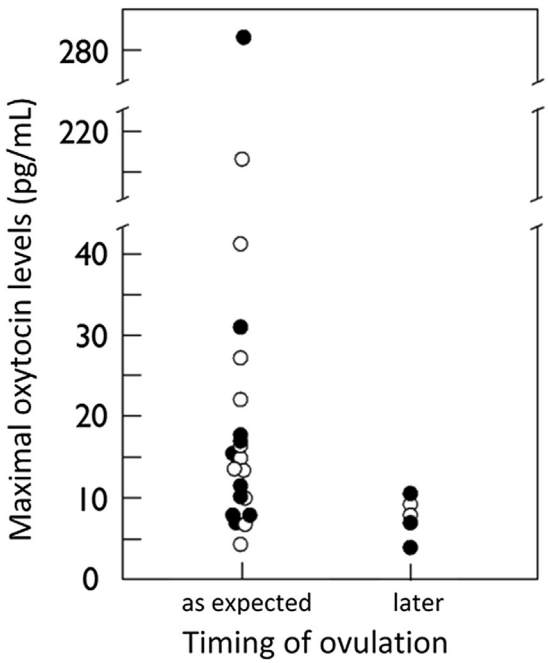

Fig. 3. Ovulation time. Maximal oxytocin concentrations (pg/mL) in mares exposed to a stallion during their first estrus cycle (open symbols) and to another one during their second estrus cycle (closed symbols) when the timing of their ovulation was as expected (36–48 hours after hCG) or later (>48 hours after hCG). hCG, human chorionic gonadotropin.

oxytocin release [35]. On the contrary, in similar studies in dairy cows no oxytocin release was detectable after exposure to a bull around the time of artificial insemination [36]. However, characteristic oxytocin peaks in this latter study may have been missed due to using a less sensitive ELISA kit for determination of oxytocin plasma concentrations and also because of blood sampling performed only at 5 minutes intervals. In our study, we chose an outstandingly sensitive RIA method and a high frequency of blood sampling to avoid missing an oxytocin peak. It must be considered that concentrations of oxytocin in the jugular vein represent only one-eighth in magnitude of the increase observed in intracavernous sinus blood samples [1,37]. However, and in agreement with our study, it was reported also in one of these studies as in another one that not all mares release oxytocin in response to teasing [1,2].

To the authors' knowledge, the high intraindividual variation of oxytocin release was not documented in horses or other mammals before. Nonetheless it was previously found that the variability of oxytocin could be explained by variations in affiliative and sexual behavior within the species of cotton-top tamarins (*Saguinus oedipus*), which varied across pair mates [38]. Unlike our study, sampling was carried out over a long period of time three times weekly for a complete ovarian cycle. However, the high intraindividual variations of mares at the time of estrus that we found in our study could not be linked to preference indicators and hence remain unexplained.

Under feral conditions, horses live throughout the year in stable social and breeding bands called harems with

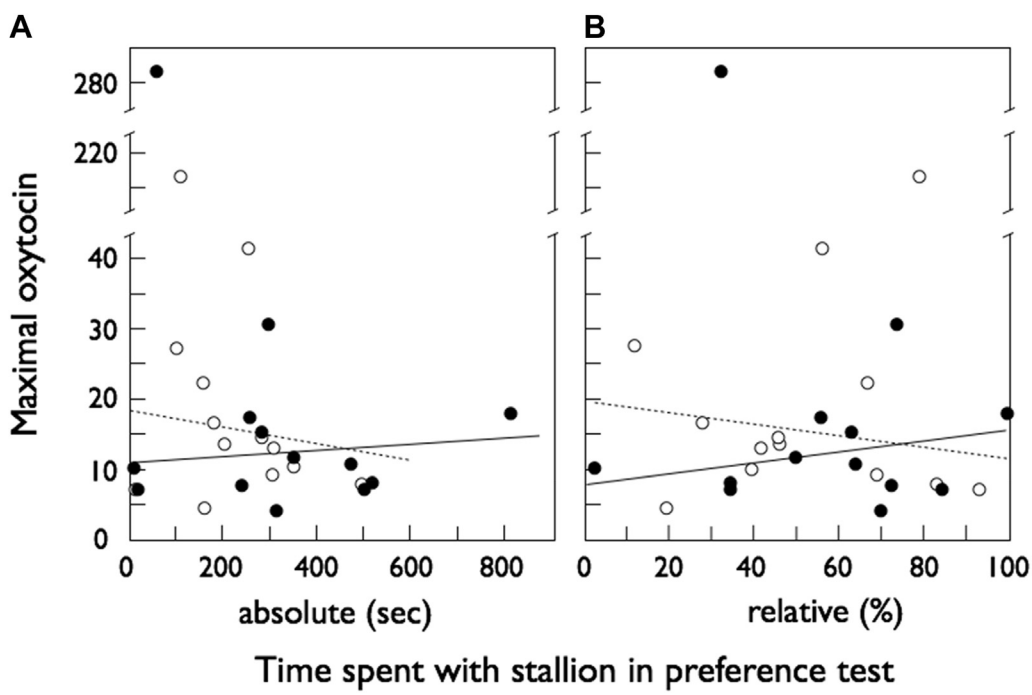

Fig. 4. Preference tests and oxytocin concentrations. Maximal oxytocin levels (pg/mL) in mares exposed to a stallion during their first estrus cycle (open symbols, hatched lines) and to another one during their second estrus cycle (closed symbols, nonhatched lines) versus (A) absolute time (in seconds) and (B) relative time (in %) spent in proximity to the respective stallion in the preference tests. Lines give the statistically nonsignificant regressions (extreme oxytocin levels > 100 pg/mL excluded each; see text for statistics).

year-round interactions between mares and stallions [39,40]. It needs to be evaluated if preference testing study designs are testing for common affiliative preference behavior in horses [41] or for sexual mate choice decisions. Our chosen analysis procedure, the mate preference ranking based on the combined assessment of time a mare spent with the "preferred" stallion (i.e., the stallion she spent more time with) relative to the total time she spent with both stallions, and the ethological evaluation of signs of behavioral estrus, should allow to focus for the latter one.

The overall percentage of mares ovulating after hCG induction within 48 hours after administration (80.7%) in this study was similar to that reported by other investigators [42,43]. Lack or delay of ovulation can be related to repeated administration of hCG and age of mares [42,43]. In five out of 26 teasing tests (= in 19.2%), ovulation occurred later than expected (>48 hours after hCG administration), with an oxytocin release during teasing being significantly lower than when ovulation occurred earlier. This leads to the presumption that the secretion of oxytocin is related to the hormonal status of the mare and is at its highest shortly before ovulation. This is in contrast to findings of Nikolakopoulos et al. [2] who found that teasing caused a significant increase in oxytocin concentrations in both estrus and diestrus, without differences either between the frequency of mares responding to teasing with increased oxytocin nor in the magnitude of response. However, in a previous study it was also shown that teasing mares during estrus led to increased uterine pressure, whereas no change in intrauterine pressure in response to teasing 2 days after ovulation was observed, for example, when serum progesterone concentrations are rising [4]. Similar results have been found for exogenous oxytocin, where the administration of oxytocin to mares before ovulation provides a greater uterine response than after ovulation [44].

Data from experiments in stallions [45–47] and species other than horses (dairy cows [48], women [49]) reveal that cortisol concentrations can increase around sexual arousal, which was not the case in the present study. It is also known that estradiol facilitates estrous behavior [50]. Despite finding a large variation of estradiol plasma concentrations in-between the mares, these values reported in the present study no intraindividual variation being highly repeatable over the two cycles.

In conclusion, we found considerable interindividual and intraindividual variations of oxytocin secretions in estrous mares during teasing without association with cortisol and estradiol concentrations. Part of the variation revealed differences in the preovulatory stage, indicating the presence of endogenous and/or environmental influences yet to be determined. Contrary to our expectations, we found no significant association between indicators of mate preferences and oxytocin secretion.

Acknowledgments

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Competing interests

None of the authors have any financial and personal relationships with other people or organizations that could inappropriately influence the study.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.theriogenology.2016.07.017.

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