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X-ray kinematics analysis of vaginal scent marking in female Syrian hamsters (*Mesocricetus auratus*)

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Abstract

Vaginal marking is a stereotyped scent marking behavior in female Syrian hamsters used to attract male hamsters for mating. Although the modulation of vaginal marking by hormones and odors is well understood, the motor control of this proceptive reproductive behavior remains unknown. Therefore, we used x-ray videography to visualize individual bone movements during vaginal marking. Kinematic analyses revealed several consistent motor patterns of vaginal marking. Despite exhibiting a diversity of trial-to-trial non-marking behaviors (e.g. locomotor stepping), we found that lowering and raising the pelvis consistently corresponded with coordinated flexion and extension cycles of the hip, knee, and tail, suggesting that these movements are fundamental to vaginal marking behavior. Surprisingly, we observed only small changes in the angles of the pelvic and sacral regions, suggesting previous reports of pelvic rotation during vaginal marking may need to be reconsidered. From these kinematic data, we inferred that vaginal marking is primarily due to the actions of hip and knee extensor muscles of the trailing leg working against gravity to support the weight of the animal as it controls the descent of the pelvis to the ground. The cutaneous trunci muscle likely mediates the characteristic flexion of the tail. Interestingly, this tail movement occurred on the same time scale as the joint kinematics suggesting possible synergistic recruitment of these muscle groups. These data therefore provide new targets for future studies examining the peripheral control of female reproductive behaviors.

Keywords

Fluoroscopy; Sex; Pheromone; Reproduction; Communication; Biomechanics

1. Introduction

Many species use scent marking as a means of conveying information between individuals, including the sex, reproductive status, individual identity, ownership, competitive ability, and health status of the marker [1]. Scent marking is particularly important for reproductive

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synchrony in solitary species such as Syrian hamsters [2]. Female hamsters scent mark by depositing vaginal secretion onto a substrate, a behavior termed vaginal marking. Converging lines of evidence suggest that vaginal marking functions as an advertisement of a female hamster's impending sexual receptivity. First, vaginal marking increases across the estrous cycle, peaking on the day prior to behavioral receptivity [3], but is not observed when females are sexually receptive [4, 5]. Second, female hamsters will preferentially direct their vaginal marks to male odors over female odors [4]. Lastly, males are highly attracted to the secretion deposited by vaginal marking [6–8].

Although studies have examined how hormones and odors stimulate vaginal marking both systemically and at the level of the forebrain [9–14], the descending motor control of vaginal marking is unknown. Unlike more complex social behaviors, vaginal marking is relatively simple and can be divided into several discrete and stereotyped behavioral events, including the lowering of the pelvis, deflection of the tail from the substrate, pelvic rotation and/or forward motion, and expulsion of vaginal secretion [4]. The stereotyped nature of these events provides tractable means for identifying the peripheral targets controlling this behavior. Indeed, previous attempts at taking a “bottom-up” approach to the study of sexually motivated behaviors, including the use of x-ray cinematography and kinematics analyses [15], have yielded substantial progress in understanding the neuromuscular control of the lordotic receptive posture in female rats [2, 16–19] and hamsters [2, 20–22].

To delineate the motor circuits involved in vaginal marking, it is first necessary to identify the relevant musculature involved in the behavior. This is difficult, however, as overlying soft tissues (fat, skin, fur) obscure skeletomuscular movements during marking. Therefore, we used digital x-ray video analysis [23] to quantify the skeletal movement patterns of the hindquarters of female Syrian hamsters during vaginal marking, focusing on the motor pattern of the marking behavior, rather than the expulsion of secretion itself. We used the resulting kinematics analysis to infer which muscle groups were active during vaginal marking behavior [15].

2. Materials and methods

2.1 Animals

Adult, female Syrian hamsters (*Mesocricetus auratus*; $n = 21$) were purchased from Charles River Laboratories (Wilmington, MA, USA) at eight weeks of age. Upon arrival at Georgia State University, subjects were singly housed in solid-bottom Plexiglas cages (43 x 22 x 20 cm) with corncob bedding (The Andersons, Maumee, OH, USA) and cotton nesting material (Ancare, Bellmore, NY, USA). To determine estrous cyclicity, the consistency of females' vaginal secretion was examined daily for eight consecutive days as described previously [13]. Only females with a consistent, four-day cycle were included in the study.

A separate group of gonadally intact, adult male hamsters (Charles River Laboratories; $n = 16$) were used to provide stimulus cages for behavioral data collection. These males were unrelated to, and had no previous contact with, female subjects. In order to provide an odor environment that would stimulate high levels of vaginal marking, stimulus cages were not changed for one week prior to pre-screening or data collection, at which time the male was removed and the soiled cage was sealed with Parafilm (SPI Supplies, West Chester, PA, USA) until use later that day.

Both subject females and stimulus males were maintained on a reversed 14-hour light/10-hour dark photoperiod (lights off at 9AM) and food and water were available *ad libitum*. All experimental procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23;

revised 1996) and approved by the Institutional Animal Care and Use Committees at both Georgia State University and the Georgia Institute of Technology.

2.2 Vaginal marking pre-screening

Prior to x-ray video data collection, female subjects were pre-screened for high levels of vaginal marking. All pre-screening tests took place during the first six hours of the dark phase on the cycle day immediately prior to sexual receptivity (proestrus). Females were placed into a soiled, vacated male stimulus cage and allowed to freely explore the cage for five minutes. The total number of vaginal marks was scored live using a hand counter. Five of the females displayed less than five vaginal marks during the pre-test and were therefore eliminated from the experiment. The remaining 16 females displayed an average of 13.44 ± 5.06 (mean $\pm$ standard deviation) vaginal marks during pre-testing and were used for further data collection.

2.3 Data collection

Data collection took place during the first six hours of the dark phase on proestrus. Female subjects and soiled stimulus cages were transported to the Georgia Institute of Technology for behavioral testing. After transport, females were left undisturbed in their cages for at least one hour prior to the beginning of data collection. During behavioral experiments, each female was placed inside of a vacated, soiled male stimulus cage and allowed to explore freely. To maximize the amount of time females spent in the line of sight of the x-ray beam during behavioral data collection, stimulus males were housed in smaller, solid-bottom Plexiglas cages (28 cm x 22 x 12 cm). The stimulus cage containing the female was positioned in line of sight of the x-ray beam and the subject was observed via a live optical video feed. To collect x-ray video data, we exposed the female to a beam of x-ray photons (80 kV, 2 mA, Monoblock 160, VJ Technologies, Bohemia, NY, USA), which passed through the cage and the female into an image intensifier (225 mm diameter, TH-9438-HX, VJ Technologies). The image intensifier converted the x-rays into visible light (548 nm wavelength) that was recorded by a digital video camera (30 Hz, A504k, Basler Vision Technologies, Exton, PA, USA) and saved to computer using commercial software (Streampix, Norpix, Inc., Montreal, Quebec, Canada). As it was not possible to anticipate when the female would vaginal mark, we activated the x-ray beam only when there was a clear sagittal view of the female and when she was not engaged in behaviors incompatible with marking (i.e. grooming or rearing), thereby maximizing optimal conditions for analysis and minimizing radiation exposure. We collected up to 15 x-ray video trials per animal lasting up to 10 seconds each. Upon completion, each animal was returned to her home cage and transported back to Georgia State University.

2.4 Data processing and analysis

After data collection, we visually assessed each video for vaginal marking behavior and cropped the videos into shorter clips that each contained a single marking event (see supplementary video online). Video clips were excluded from analysis ($n = 35$) if they did not exhibit a clear sagittal plane view of the animal during the marking behavior. In total, 16 video clips were analyzed across 8 animals (1–5 clips per animal). Given the limited amount of usable data, each vaginal mark was treated as an independent event in the analysis.

We transformed the video clips from each marking event into individual sequences of single images and then batch processed the images using commercial software (NI Vision software, National Instruments, Austin, TX, USA). Image processing consisted of two steps: distortion correction and contrast enhancement. To correct for nonlinear visual distortion, before each data collection session we captured an image of a 2-D calibration frame consisting of tantalum beads embedded in a Plexiglas sheet and ordered in a square grid

pattern (1 cm apart, accurate to within 0.00254 mm). Information to automatically correct the distortion was derived from the image of the calibration frame, and then applied to each individual video frame via batch processing [24]. We also enhanced the contrast of each image during batch processing to facilitate identification of skeletal landmarks (NI Vision software, National Instruments, Austin, TX, USA). We then reconstituted the individual images back into a video file for digitization (Adobe Premier, Adobe Systems, Inc., San Jose, CA, USA).

We manually identified and digitized bony landmarks in the sagittal plane through frame-by-frame inspection using open-source Matlab (Mathworks, Natick, MA, USA) digitizing software (DLTdataviewer) [25]. Bony landmarks included the metatarsophalangeal (MTP) joint, ankle joint, knee joint, hip joint, the base of the tail, and the tip of the tail (Figure 1A). The resulting 2-D coordinate positions of the designated bony landmarks were quantified with a custom Matlab code to characterize the limb movement patterns used during vaginal marking. We generated a linked segment geometric model of the hindlimb and pelvic anatomy from our digitized bony landmarks in the sagittal plane. 2-D sagittal plane angles were calculated for the foot segment (as a proxy for MTP), ankle joint, knee joint, hip joint, sacrum segment, and tail joint (Figure 1B). Each joint angle was centered on the landmark of the same name and bounded by neighboring linked segments formed by the two nearest neighboring digitized landmarks. Three exceptions were the hip joint, foot segment, and sacrum segment angles. The hip joint angle was defined by the knee joint, hip joint, and the base of the tail, which was visually identified by the apex of curvature of the sacral vertebrae (see Figure 1B). The foot segment angle was defined by the angle of the segment connected by the ankle joint and the MTP joint relative to the ground. The hip joint and base of the tail defined the sacrum segment angle.

As distinct trials were completed over different time intervals, joint angle trajectories were time normalized to specific stereotyped events occurring within the marking behavior so that average kinematics could be assessed. First, we identified three stereotyped events based on the height of the hip joint that could be easily quantified for every marking behavior and were correlated with the lowering of the body for scent marking: the 1st local maximum in hip height preceding vaginal marking; the minimum hip height coinciding with vaginal marking; and, the 2nd local maximum in hip height following the vaginal marking. Time normalization was then carried out over two phases: from the first stereotyped event to the second, then the second event to the third. Within each phase, trajectories were scaled in time to the mean phase duration. Finally, the two phases were concatenated together and relabeled as 0–100% of normalized time. We further calculated the magnitude and orientation of a toe-to-hip kinematic vector to provide an aggregate representation of the combined actions of all joints.

Limb kinematics, both those of leading and trailing legs, were compared across trials by identifying each limb with its posture during marking. The leading limb is that which is positioned farther ahead in the direction of travel during the entirety of marking. This generally corresponded to the late swing (limb advancement) phase and early stance phase of gait for the leading limb, and to middle and late stance phase for the trailing limb. All data were analyzed using PASW Statistics 18.0 (SPSS Inc., Chicago, IL, USA) for Windows. For each kinematic measure, paired *t*-tests (2-tailed) were used to detect significant differences between average kinematic values at two time points: 1) the first local hip height maximum vs. the local hip height minimum (first *t*-test) and 2) the local hip height minimum vs. the second local hip height maximum (second *t*-test). Multiple comparisons were corrected for using Bonferroni alpha adjustments ($\alpha_{FW} = 0.05$).

3. Results

Analyzed trials lasted 0.80 to 2.90 seconds in duration and the typical marking event within the trial lasted 0.35 ± 0.19 seconds (mean $\pm$ standard deviation). All marking events were initiated by the lowering of the pelvis from a typical standing hip height until contact was made with the floor and concluded with the pelvis rising again to standing hip height. The downward acceleration of the hip joint (0.19 ± 0.17 m/s²) was considerably slower than the acceleration expected due to gravity (9.81 m/s²), suggesting that the descent of the pelvis during vaginal marking was mediated by muscular effort, rather than free falling with gravity. A variety of ancillary behaviors, including various stages of locomotor stepping and quiet posture, were observed during marking (Figure 2). This resulted in substantial trial-to-trial variability for several of the measured kinematics trajectories. This variability was observed in the general movements of the leading and trailing limbs as well as in the same joints within each limb.

Despite this variability, characteristic trends emerged across all trials with regard to hip, knee, tail, and sacrum joint kinematics. Major events defining vaginal marking behavior corresponded with significant flexion (leading $\alpha(15) = 11.94$, trailing $\alpha(15) = -6.98$, both $P < 0.001$) followed by extension (leading $\alpha(15) = 11.14$; trailing $\alpha(15) = 6.83$, both $P < 0.001$) of both hip joints (Figure 3A). The trailing leg knee joint showed a corresponding flexion ($\alpha(15) = 6.63$, $P < 0.001$) and extension ($\alpha(15) = -5.33$, $P < 0.001$) pattern across trials, while the leading limb showed less consistency at the knee (flexion $\alpha(15) = 1.21$, $P = 0.05$; extension $\alpha(15) = -2.61$, $P = 0.02$) (Figure 3B). The flexion-extension cycle of the trailing knee corresponded with a shortening-lengthening cycle of the same leg length (Figure 4B; $\alpha(15) = 7.12$, $\alpha(15) = -6.42$, both $P < 0.001$) and a protraction-retraction cycle of the leading leg angle (Figure 4A; $\alpha(15) = 4.12$, $\alpha(15) = -3.29$, both $P < 0.001$). This indicates that in the majority of trials the trailing leg supported the body weight in stance, whereas the leading leg was swinging forward to advance the limb to its next foot placement on the ground.

Flexion of the joint at the base of the tail (Figure 5A; $\alpha(15) = 11.23$, $P < 0.001$) and small changes in sacrum segment angle (Figure 5B; $\alpha(15) = -3.91$ and 3.72 , both $P < .001$) also occurred concomitantly with flexing of the hip joint. Not surprisingly, lowering of the pelvis to the cage floor (i.e. decreased hip height, Figure 5C) corresponded with hip flexion ($\alpha(15) = -6.96$, $P < 0.001$), while ascension occurred with hip extension ($\alpha(15) = -4.12$, $P < 0.001$). In contrast, averaged data for ankle (Figure 3C) and foot (Figure 3D) joint angles showed few consistent trends with large standard deviations across trials ($P > 0.05$).

4. Discussion

4.1 Variable vs. common motor patterns of vaginal marking

These data demonstrate that vaginal marking behavior can occur during a broad spectrum of postures and ancillary behaviors. Indeed, marking behavior occurred from a resting posture and during various stages of walking (with either one or both hindlimbs on the cage floor). Although this created significant variability across trials, several consistent kinematic features emerged despite the additional behaviors. These included angles of the hip joint, knee joint, tail joint, and sacrum segment. Specifically, both legs consistently exhibited hip flexion during the descent phase, and hip extension during the ascent of the pelvis. It should be noted, however, that this similarity at the hip joint belies the fact that the two legs largely functioned in opposing roles: the leading leg acting to advance the leg forward while the trailing leg was on the ground and supporting the weight of the body. Additionally, the knee joint of the trailing leg consistently underwent flexion during the descent phase, and extension during the ascent phase. In contrast, the foot and ankle joints did not show any

consistent trends across trials, suggesting that these joints correspond to the ancillary behaviors.

4.2 Possible muscle groups involved in vaginal marking

Emergent trends in hip, knee, tail, and sacrum kinematics implicate several specific muscle groups that may be critical for vaginal marking. First, we observed a consistent pattern of flexion in the hip and knee joints during the descending phase of marking that was then followed by extension during the ascending phase. Since the body's weight must be supported at all times, generation of extensor torques at the knee and hip joints of the trailing leg are required throughout the movement. Therefore, flexion of these joints to allow lowering of the pelvis to the ground is likely controlled through active lengthening (i.e., eccentric contraction) of knee and hip extensor muscles, whereas extension of these same joints during the ascending phase is likely controlled through active shortening (concentric contraction) of the same muscles to perform work against gravity. Such movements may involve the large knee extensors that make up the quadriceps group (vastus medialis, vastus intermedius, vastus lateralis and rectus femoris) and several hip extensors (e.g., adductor brevis, biceps femoris, quadratus femoris, semimembranosus, and semitendinosus). The synergistic activation of these multiple muscles may allow for a controlled descent of the pelvis across the variety of postures and ancillary movements observed during vaginal marking.

We also observed a gradual decrease in tail joint angle (i.e. elevation of the tail) that reached maximal height during pelvic contact with the cage floor. Studies of the neural control of lordosis in female hamsters suggest that this flexion is likely mediated by the cutaneous trunci muscle, a thin broad sheet of skeletal muscle located just under the skin [26]. Indeed, stimulation of the lateral thoracic nerve, which innervates the cutaneous trunci, results in an elevation of the tail that is very similar to what we observe during vaginal marking behavior [22]. These tail movements were on the same time scale as the characteristic hip and knee joint movements, suggesting that the peripheral control of these movements is coordinated within the central nervous system. It has been suggested that the control of complex behaviors may be simplified through coordinated activation of muscle groups [27]; the verification of the presence of such a muscle synergy would, however, require further study.

We did not observe large changes in the sacrum segment angle, as would have been expected from previous descriptions of pelvic rotation during vaginal marking [4]. In fact, we observed small but significant changes (~10 degrees) in the sacrum segment angle that were greatest at the minimum hip height, but in the opposite direction as expected for pelvic rotation. This suggests that the reported pelvic rotation accompanying marking behavior may be smaller than previously reported. It is possible that these small pelvic movements instead reflect the female's attempt to optimize vaginal contact with the substrate, and therefore might be expected to be larger on less uniform substrate than a cage bottom, such as one that a female might encounter in a natural setting. Regardless of scale, these pelvic movements are likely mediated by the pelvic floor muscles (pubococcygeus and iliococcygeus), as stimulation of these muscles produces downward pelvic thrusting in female rats [28].

It is important to note that the deposition of vaginal secretion, although not detectable using the current methods, is an integral part of vaginal marking behavior. The extrusion of vaginal secretion onto a substrate is likely mediated by the smooth muscle of the vaginal wall. Indeed, stimulation of the vicerocutaneous branch of the pelvic nerve, which innervates the vaginal wall, increases vaginal wall pressure and contractions [29]. Alternatively, the extrusion of vaginal secretion may not be controlled by descending nerves, but rather by a local spinal reflex that responds to somatosensory feedback during

genital contact with the substrate. This possibility seems unlikely, however, as temporary anesthetization of the genital region, rendering it incapable of sensory transduction, does not decrease the deposition of vaginal secretion (Been and Petrulis, unpublished observations).

4.3 Conclusions

This study provides the first detailed kinematics data of a complex, multi-component scent-marking behavior. Through the use of x-ray videography, we characterized several different motor patterns of vaginal marking that would be extremely difficult, if not impossible, to accurately identify in the presence of overlying soft tissues. Further, we were able to uncover consistent changes in several joint angles that occurred regardless of the motor pattern of marking, suggesting that certain elements of vaginal marking are fundamental to the behavior, whereas other elements vary with the execution of ancillary behaviors. Although kinematics data do not provide direct information on muscle activation, we can use our knowledge of the net torques required to create movements, both with and against gravity, to predict which muscle groups are likely to mediate vaginal marking behavior, assuming minimal effort. These data therefore provide targets for electromyography, muscle stimulation, and/or neuroanatomical tract tracing studies that may further elucidate the neural control of this reproductively oriented scent marking response. As such, this study is an initial and fundamental step towards understanding the neurobiology of vaginal marking behavior and, ultimately, the insights gained from these results may be applied to the neuromuscular regulation of other stereotyped sexual behaviors in other species.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- X-ray video was used to visualize individual bone movements during vaginal marking
- Consistent and coordinated motor patterns emerged for the knee, hip and tail joints
- Vaginal marking can occur during variable postures and ancillary behaviors
- Contrary to previous reports, kinematic analyses indicated a lack of pelvic rotation
- Muscles activated during vaginal marking are possible targets for future research

Figure 1. Model of female hamster skeletal anatomy and kinematic measures

A) Six digitized bony landmarks (filled circles) of a female hamster were used to generate a linked segment geometric model of the hindlimb and pelvic anatomy. B) Foot, ankle, knee, leg, hip, sacrum, and tail joint angles, as well as leg length, leg angle, and hip height were quantified. L6, 6th lumbar vertebra; MTP, metatarsal phalangeal joint.

Figure 2. Representative vaginal marking kinematics

On all trials, vaginal marking began when the pelvis was lowered from a standing posture (hip height max 1) until contact was made with the floor (shaded area) and concluded when the pelvis returned to standing hip height (hip height max 2). Time was normalized as a function of hip height min (vertical line). A) In one representative trial, a female vaginal marks during locomotor movement, as evidenced by the opposing phases of the leading (black) and trailing (gray) limb kinematics (i.e. knee, ankle, and leg angle). B) In a different representative trial, a female vaginal marks during standing posture, as evidenced by the symmetrical phases of the leading and trailing limb.

Figure 3. Average hip, knee, and ankle, and foot joint angles

Means (solid lines) $\pm$ standard deviations (dashed lines) for leading (black) and trailing (gray) joint angles across normalized time. Significant changes between initiation of marking (time 0%) and hip height min (vertical line), and between hip height min and termination of marking (time 100%) were detected using paired t -tests. *, significant differences for leading limbs; ‡, significant differences for trailing limbs; α FW = 0.05.

Figure 4. Average leg kinematics

Means (solid lines) $\pm$ standard deviations (dashed lines) for leading (black) and trailing (gray) leg angles and leg lengths across normalized time. Significant changes between initiation of marking (time 0%) and hip height min (vertical line), and between hip height min and termination of marking (time 100%) were detected using paired t -tests. *, significant differences for leading limbs; ‡, significant differences for trailing limbs; α FW = 0.05.

Figure 5. Average sacrum, tail, and hip kinematics

Means (solid lines) $\pm$ standard deviations (dashed lines) for tail joint angles, sacrum segment angles, and hip heights across normalized time. *, significant changes between initiation of marking (time 0%) and hip height min (vertical line), and between hip height min and termination of marking (time 100%), $\alpha_{FW} = 0.05$.