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Male Mounting Alone Reduces Female Promiscuity in the Fowl

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Summary

The fertilization success of an insemination is at risk when a female has the possibility to copulate with multiple males, generating sperm competition [1] and sexual conflict over remating [2, 3]. Female propensity to remate is often reduced after copulation, and a staggering diversity of highly derived male traits that discourage female promiscuity have been investigated [4–15]. However, it is difficult to separate the effect of such specialized traits and insemination products from the more basic effect that the act of mounting per se may have on female remating. Here, we use a novel approach that separates the influence of mounting from that of insemination on female remating in the promiscuous feral fowl. Mounting alone caused a transient but drastic reduction in female propensity to remate, and—crucially—the number of sperm that a female obtained from a new male. Therefore, like other taxa, female fowl show a reduction in promiscuity after copulation, but this is entirely due to mounting alone. This effect of mounting, independent of insemination and fertilization, indicates that even copulations that deliver little or no semen, a puzzling behavior common in many species [16–24] including the fowl [25, 26], may play a crucial role in sperm competition.

Results and Discussion

The propensity of a female to remate influences the level of sperm competition faced by the ejaculate of a male [1] and is thus an important area of sexual conflict [2, 3]. Preventing females from remating with another male has led to the evolution of a diverse set of highly specialized male seminal products [4–8]. Similarly, males may discourage female promiscuity by imposing copulation costs ([9, 10] but see [27, 28]), guarding their partners [11, 12], punishing their promiscuity [13, 14], and even feeding females their own soma [15]. Recent evidence suggests that in addition to, or in combination with, these highly derived traits, the basic stimulus provided by male mounting may also influence female reproductive behavior, with important implications for sperm competition [24, 29–32]. However, despite much recent interest in promiscuity, the female response to mounting per se has not been investigated, partly as a result of the difficulty of decoupling mounting from insemination.

Here, we adopt a novel technique to separate the independent influence of mounting from that of insemination on female propensity to remate in the promiscuous feral fowl *Gallus gallus domesticus*.

We investigated the functional significance of mounting in the fowl and tested two critical predictions: that male mounting per se reduces female propensity to remate, and that it reduces the number of sperm that a female obtains from new males, thus reducing the level of sperm competition. In the fowl, like in most other bird species, males lack an intromittent organ and deposit the ejaculate on the female cloaca, without intromission and no or minimal cloacal contact [33]. Therefore, copulation typically involves mounting and—it is assumed—semen delivery. To experimentally disentangle the effect of mounting per se from the effect of insemination, we exposed female fowl to four different experimental treatments: In “inseminated,” a female was inseminated twice by each of two males; in “mounted,” a female was fitted with a harness that covered her cloaca and prevented insemination [26] and was mounted twice by each of the same two males; in “control a,” a female was exposed to the same two males but the males were prevented from mounting; and in “control b,” a female was fitted with the harness and exposed to, but not mounted by, the same two males. Therefore, the inseminated treatment detected the effect of insemination, the mounted treatment detected the effect of mounting per se, control a and control b served as controls for the effects of handling and exposure to males, and control b was a control for harness use. In each replicate trial, four females were exposed to the above treatments (one female per treatment) and released in an outdoor pen with a focal male on day 1. Female propensity to solicit and resist copulations from this male was monitored for the following 5 days.

Consistent with the idea that copulation influences female remating, females that had been inseminated on day 1 (“inseminated”) were significantly less likely to solicit a copulation from the focal male (Figure 1A) and significantly more likely to resist his copulation attempts (Figure 1B) than the females exposed to either control (which were exposed to, but not mounted by, two males in their treatment). Importantly, mounting alone was sufficient to explain the female-remating reduction associated with copulation. Mounted females, which were mounted but did not receive any semen on day 1, responded to the focal male in the same way as inseminated females: Mounted females were less likely to solicit and more likely to resist copulations than control females (Figures 1A and 1B). The effect of the experi-

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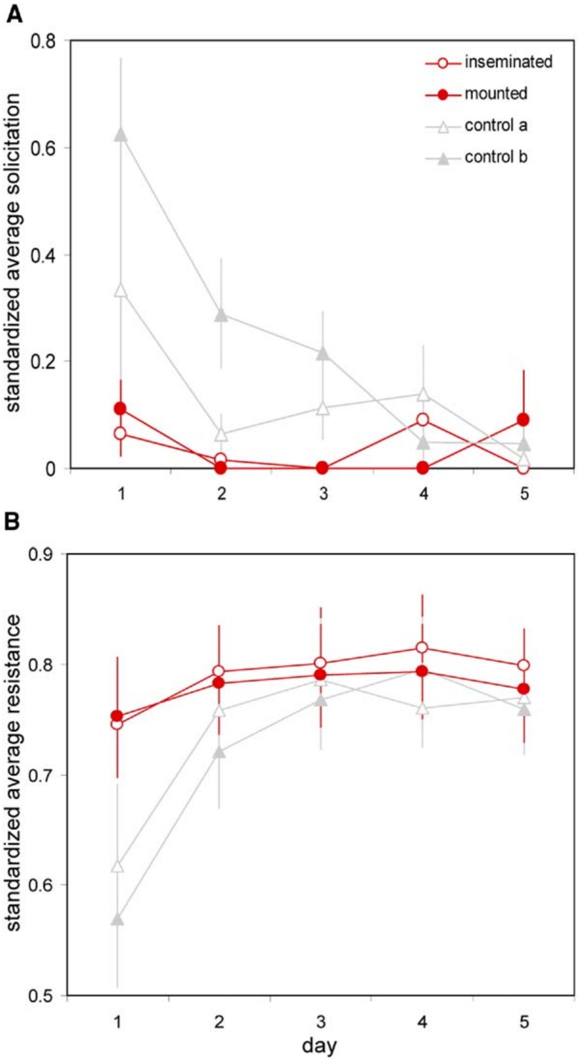

Figure 1. Mounting and Female Remating

Propensity of females exposed to four different experimental treatments (“inseminated,” “mounted,” “control a,” and “control b”) to copulate with the focal male over successive days.

(A) Mean ± standard error (SE) standardized number of female solicitations. Inseminated and mounted females were less likely to solicit copulation from the focal male than were control females. This effect diminished with exposure to the focal male (number of solicitations: treatment, $F_{3,\ 209} = 7.05$, $p < 0.0001$; day, $F_{1,\ 209} = 22.15$, $p < 0.0001$; and number of copulations, $F_{1,\ 209} = 16.86$, $p < 0.0001$ [LS means: inseminated versus mounted, $p = 0.49$; inseminated versus control a, $p = 0.006$; inseminated versus control b, $p = 0.0005$; mounted versus control a, $p = 0.0186$; mounted versus control b, $p = 0.0009$; and control a versus control b, $p = 0.172$]. Probabilities of solicitation: treatment, $F_{3,\ 209} = 7.20$, $p < 0.0001$; day, $F_{1,\ 209} = 13.43$, $p = 0.0003$; and number of copulations, $F_{1,\ 209} = 7.75$, $p = 0.0059$ [LS means: inseminated versus mounted, $p = 0.86$; inseminated versus control a, $p = 0.017$; inseminated versus control b, $p = 0.002$; mounted versus control a, $p = 0.0147$; mounted versus control b, $p = 0.002$; and control a versus control b, $p = 0.08$]).

(B) Mean ± SE standardized female resistance score. Again, there was a significant treatment effect that diminished over time of exposure to the focal male (resistance scores: treatment, $F_{3,\ 208} = 14.64$, $p < 0.0001$; number of laying females, $F_{1,\ 208} = 14.78$, $p = 0.0002$; day, $F_{1,\ 208} = 10.32$, $p = 0.0015$; number of copulations, $F_{1,\ 208} = 1076.68$, $p < 0.0001$; and day × treatment, $F_{3,\ 204} = 3.02$, $p = 0.0307$ [LS means: inseminated versus mounted, $p = 0.978$; inseminated versus control a, $p < 0.0001$; inseminated versus control b, $p = 0.0003$; mounted versus control a, $p < 0.0001$; mounted versus control b, $p = 0.0003$; and control a versus control b, $p = 0.227$]).

mental treatments on female propensity to remate with the focal male was transient. Over successive days of the trial, the probability of control females soliciting further copulations progressively declined, and the level of their resistance progressively increased until their response to the focal male approached that of inseminated and mounted females (Figures 1A and 1B). Control females obtained on average 1.74 ± 0.25 mountings a day (control a and control b combined) from the focal male, leading to the prediction that it would take more than 2 days for control females to accumulate four mountings and reach the levels of resistance and solicitation displayed by mounted and inseminated females. The behavior of control females closely fit this prediction and only became similar to that of inseminated and mounted females after days 3 (Figure 1B) to 4 (Figure 1A). This result strongly suggests that as control females accumulated copulations with the focal male over successive days, their propensity to remate declined, confirming that mounting has a strong additive inhibitory effect on future female receptivity.

We further investigated the implications of the observed effect of mounting for male reproductive success and tested whether the female-promiscuity reduction induced by mounting translated into females receiving fewer sperm from a new male. We exposed females to the same experimental protocol and removed the focal male at the end of day 2. This enabled us to estimate the number of sperm that females stored from the focal male by analyzing the number of sperm contained in the eggs [34] produced by each female for the 10 days following the removal of the focal male (day 3 to day 12). Again, there was a treatment effect on female propensity to solicit and resist copulations from the focal male during day 1 and day 2, before the focal male was removed (number of solicitations: treatment, $F_{3,\ 82} = 2.84$, $p = 0.043$. Solicitation probability: $F_{3,\ 82} = 2.62$, $p = 0.0566$. Resistance score: $F_{3,\ 82} = 3.07$, $p = 0.0326$). The behavior of mounted was again similar to that of inseminated females (least square means post hoc analysis: number of solicitations, $p = 0.478$; probability of solicitation, $p = 0.894$; and resistance score, $p = 0.993$) and differed or tended to differ from that of control females (control a: number of solicitations, $p = 0.013$; solicitation probability, $p = 0.017$; and resistance score, $p = 0.0288$. Control b: number of solicitations, $p = 0.089$; solicitation probability, $p = 0.182$; and resistance score, $p = 0.0931$), whereas the two controls were again similar to each other (number of solicitations, $p = 0.251$; solicitation probability, $p = 0.361$; and resistance score, $p = 0.600$). These behavioral differences coincided with differences in the number of sperm stored by females across treatments. Consistent with the idea that mounting per se reduces the number of sperm that a female receives from a new male, the eggs laid by mounted females contained significantly fewer sperm than those laid by females from both controls and by

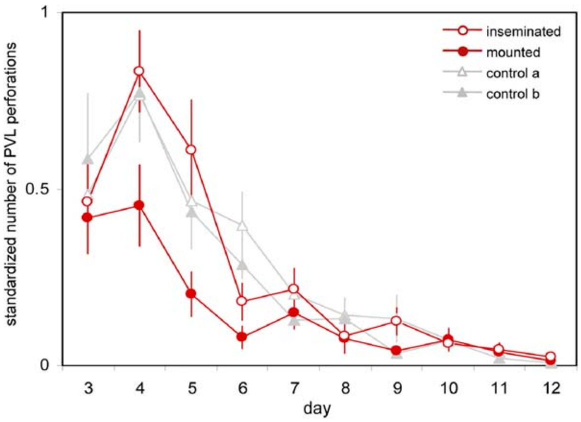

Figure 2. Mounting and Sperm Competition

Mean ± SE standardized number of sperm hydrolysis points on the perivitelline layer (PVL) of eggs laid from the day following the removal of the focal male (day 3) to day 12. Fewer sperm reached the eggs of females that were mounted but not inseminated than eggs produced by females exposed to any other experimental treatment, indicating that a female-promiscuity reduction, caused by mounting, translates into a short-term reduction in sperm-competition intensity (PVL count: treatment, $F_{3,\ 268} = 5.39$, $p = 0.0013$; day, $F_{1,\ 268} = 116.60$, $p < 0.0001$; egg number, $F_{1,\ 267} = 3.35$, $p = 0.068$ excluded through stepwise deletion of nonsignificant terms [LS means: mounted versus inseminated, $p = 0.0087$; mounted versus control a, $p < 0.0001$; mounted versus control b, $p = 0.009$; control a versus control b, $p = 0.0071$; and all other comparisons, $p > 0.10$]).

inseminated females (Figure 2). Note that although the probability to copulate with the focal male was similarly low for inseminated and mounted females, the former (but not the latter) had obtained semen during their experimental treatment. Therefore, the eggs of inseminated females contained sperm from the experimental males and the focal male, whereas the eggs of mounted females contained only sperm from the focal male. We thus expected higher numbers of sperm on the eggs produced by inseminated than those produced by mounted females. The critical comparison to test whether mounting per se is sufficient to reduce the number of sperm that a female obtains from a new male is, thus, that between mounted females and females from both controls.

Together, our behavioral and physiological results demonstrate that somatosensory stimuli generated by male mounting alone, independently from seminal products or specialized copulatory behaviors, result in a transient but marked female-promiscuity reduction, which in turn translates into females storing fewer sperm from a new male. Proximally, somatosensory stimuli associated with copulation activate female neuroendocrine (e.g., *fos*-expression) and behavioral responses in different vertebrates. In some rodents, these responses are triggered by male intromissions [35]. However, in a close relative of the fowl, the Japanese quail, *Coturnix japonica*, where intromission does not occur, similar neuroendocrine responses (activation of *fos*-like immunoreactive cells) in the female are triggered by male mounting alone [36]. It is thus possible that a neouroendocrine cascade activated by male mounting may be responsible for the marked, transient reduction in propensity to remate in female fowl. At a functional level, this response may enable female fowl to adjust mating rate by using past mountings as a cue for inseminations in order to simultaneously reduce the substantial costs often associated with copulation [37] and the risk of depleting sperm stores. Consistent with the idea that female birds use mounting as indication of insemination, female zebra finches, *Taeniopygia guttata*, paired to vasectomized males (which mounted but could not deliver semen to their partners) were not more likely to remate with a new male than females paired to intact males [38]. These results have fundamental implications for the study of sperm competition and indicate that a potential female response to mounting must be considered when studying the functional significance of sperm-competition strategies. Previous studies have focused on highly specialized male copulatory traits and seminal products. However, the present study reveals that in addition to these more derived male traits and regardless of whether a male inseminates a female, mounting alone elicits an independent and strong female response. The extent to which mounting contributes to the explanation of patterns of female remating previously attributed to more-specialized traits warrants further investigation [32]. For example, in *Drosophila melanogaster*, where copulation duration appears to be mostly under male control and sperm transfer occurs within the first 8 min of copulation, copulations often last 20 min and delay female remating more than copulations interrupted after 8 min [39]. A female mating response similar to that detected by the present study would explain why males remain in copula long after sperm transfer instead of foraging or searching for additional females. In addition, in *D. melanogaster*, an insemination typically reduces the number of sperm previously stored by a female, and this effect has been entirely attributed to male accessory-gland peptides [40–42]. However, the potential female response to mating was seldom considered. Recent evidence suggests that a female response to copulation, rather than male seminal products, may contribute to the explanation of patterns of last-male precedence in this species [30]. Consistent with this, a gene-expression study of *D. melanogaster* revealed that a set of female genes is switched on by mating alone, independently from insemination products [31].

The observed female-promiscuity reduction induced by mounting strongly suggests that even copulations delivering no (aspermic) or negligible quantities of semen may play a crucial role in sperm competition. In promiscuous species, sperm production may constrain male reproductive success, promoting the evolution of male prudence and strategic sperm allocation [26, 43]. By mounting females they have previously inseminated but not delivering additional sperm, males may, with limited sperm investment in a female, reduce the level of sperm competition faced by their ejaculates. Therefore, strategic aspermic copulation may be regarded as an extreme form of differential sperm allocation by males. Furthermore, considering that more-specialized male traits reducing female promiscuity are often costly to males [12, 44, 45], it is possible that the imposition of aspermic mounting may be an energetically cheaper and evolutionarily more parsimonious male strategy of paternity defense. Consistent with this idea, male feral fowl display highly plastic patterns of sperm allocation and invest progressively less semen and fewer sperm over successive copulations with the same female until aspermic copulations occur [26]. When males are experimentally exposed to a new female, the probability of semen delivery is rescued [26], indicating that aspermic copulations are not due to male sperm depletion but may be a male response to the sexual familiarity of a female. The same behavioral response is observed in male red junglefowl, *Gallus gallus ssp.*, the wild ancestor of the domestic fowl [26].

Nonrandom occurrence of aspermic copulations in relation to female sexual familiarity also occurs in a wild population of Adélie penguins, *Pygoscelis adeliae*, where the median proportion of aspermic copulations between social partners is 28.4%; interestingly, aspermic copulations are never observed when males copulate with extra-pair females [21]. Also, some wild species of rodents typically mount, without ejaculating additional semen, females that they previously inseminated (“post-ejaculatory copulations”) [19]. Mounting is an integral part of copulation in many species, and aspermic copulations frequently occur across a wide range of taxa [16–25], including wild species, from insects to mammals [e.g., 16, 19–21, 24]. These observations indicate three important points. First, the nonrandom occurrence of copulations delivering little or no semen suggests that this behavior may be a functional male response to sperm competition. Second, the fact that this behavior is observed in wild species and under natural conditions strongly suggests that it is functionally relevant and not a by-product of domestication. Third, the occurrence of this behavior across a wide range of taxa indicates that this trait is phylogenetically widespread. The reasons for why males mount females and yet deliver no or negligible quantities of semen have remained unresolved. The present study reveals the potential evolutionary significance of these puzzling copulations.

Male exploitation of a female response to mounting may be costly to females because it imposes mating costs and limits both the amount of sperm available for fertilization and female control of paternity. Under specific circumstances [2, 3], this sexual conflict may, in principle, set the scene for the evolution of counteracting female traits, which would enable females to control remating rates and fertilization. The antagonistic intersexual coevolution generated by sexual conflict over remating may in turn contribute to explain the evolution of puzzling male copulatory behaviors observed across different taxa where female promiscuity may be influenced by male mounting alone; these behaviors include aspermic copulations, frequent copulations among the same partners, prolonged copulations, and post-ejaculatory copulations [19, 46, 47].

In conclusion, as far as we know, these results provide the first experimental demonstration that male mounting alone reduces female propensity to remate in a bird. In the future, it will be important to elucidate the neuroendocrine mechanisms associated with the female response to mounting and establish the extent to which male mounting and female responses to mounting are influenced by social factors such as male competition, female social status, and the operational sex ratio of a population.

Experimental Procedures

Study Population

We studied a feral-fowl population (2002, 38 females and 16 males; 2003, 48 females and 36 males; and 2004, 42 females and 30 males) morphologically and behaviorally similar to its wild ancestor, the red junglefowl, *Gallus gallus ssp.*, free-ranging at the Tovetorp Zoological Research Station, Sweden [26, 48], in July–August, 2002, in April–August, 2003, and in April–May and July–August, 2004. All birds used were fully habituated to human presence, sexually mature (>8 months), nonvirgin, and sexually rested (i.e., physically, but not visually or acoustically, separated from members of the other sex: females from males for at least 14 days to ensure complete depletion of sperm in sperm storage tubules [34], and males from females for at least 2 days to enable complete replenishment of sperm supplies [25]).

Experiment 1: Female Remating

The aim of this experiment was to determine the effect of insemination and mounting per se on female propensity to remate. On day 1, during the daily copulation peak between 1600 and 1900 hr [48], each of four females was exposed to one of four treatments: One female was held by one of us (H.L.) in a soliciting position [25] and inseminated twice by each of two males in quick succession (“inseminated”); one female was handled in the same way and mounted by the same two males, but inseminations were prevented by fitting the female with a harness covering her cloaca (“mounted”) [26]; one female was held in a soliciting position and exposed to the same males, but males were prevented from mounting the female in order to control for potential effects of male exposure and handling (“control a”); and one female was exposed to the same treatment as in control a but was fitted with a harness to control for potential harness effects (“control b”). Males were kept in pairs, and social hierarchies were assessed. Two males of similar social status from two different pairs were used for each female group. One male at a time was allowed to copulate twice with the two females exposed to the inseminated and mounted treatments or to stand by the two females exposed to control a and control b for the same amount of time. After being exposed to the experimental males, one female from each treatment was released into an outdoor pen (18–50 m^2) with a focal male of the same social status as the two males to which the females were exposed in the treatment. The birds were observed until they went to roost and then between 04:00 and 10:30 hr and 15:30 and 21:00 hr, when most copulations occur [39], for the following four days (days 2–5). We recorded the number of copulations that each female solicited and ranked the level of female resistance to copulations on a scale of 1 to 6 (1 = the female crouches in front of the male and solicits a copulation, with no resistance displayed; 2 = the male approaches the female from behind with raised hackles, and the female responds by crouching; 3 = the male approaches the female, and the female walks away; 4 = the male grabs the female, the female passively accepts the copulation; 5 = the male runs after and grabs the female, and the female initially resists before accepting the copulation; and 6 = the female resists the whole copulation). Female response to a male may depend on whether a female will lay eggs in time for a current copulation to result in fertilization. We therefore monitored whether individual females laid eggs during a trial and in the 14 days following the trial. This experiment was replicated with 11 groups (n females = 42, two of which were used in two trials each; n focal males = 11).

Experiment 2: Sperm Storage

The aim of this experiment was to test whether reduced female propensity to remate translates into fewer new-male sperm reaching the eggs. We exposed groups of four females each to a protocol similar to that of the first experiment, with the difference here that females were deprived of the focal male at the end of day 2, their eggs were collected for the following 10 days (i.e., up to day 12), and maternity was assigned through yolk staining with lipid dyes fed to females [26]. The number of hydrolysis points caused by sperm on the perivitelline layer of freshly laid eggs (n = 264) was quantified [34]. In 12 out of these 16 groups, we also monitored copulation behavior with the focal male during day 1 and day 2 (i.e., until the male was removed). In these 12 groups, all females apart from one were observed copulating successfully with the focal male at least once. Only three females (mounted, control a, and control b) laid no egg containing sperm; the exclusion of these females from the analysis did not change the effects detected. This experiment was replicated with 16 groups (n females = 48, 16 of which were used in two trials each, and n focal males = 16).

Statistical Analysis

Experiment 1: Female Remating

We analyzed the effect of treatment on the following: (i) Probability of female solicitation, (ii) number of solicited copulations, and (iii) level of female resistance (cumulative daily-resistance score), in both experiment 1 (days 1–5) and experiment 2 (days 1–2). We analyzed (i) via a generalized linear mixed model (GLMM) with binomial error distribution with logit link function (probability of solicitation: 0 or 1) and (ii) and (iii) via GLMMs with Poisson error distribution and log link function because of negatively skewed error distributions. Treatment and female laying status (i.e., whether a female laid eggs during a trial or within 14 days following a trial [34]) were entered as fixed factors; number of laying females in a group, laying day, and number of copulations a female had each day were entered as covariates; and year and female identity (defined as the subject, nested within group) were entered as random factors. Defining a subject within the analysis identifies the unit upon which repeated measurements are taken (in this case, females) and thus prevents the pseudoreplication of individual females represented over successive days and in two different groups.

Experiment 2: Sperm Storage

We analyzed the effect of treatment on the number of sperm stored by a female and measured as the number of hydrolysis points generated by sperm on the perivitelline layer (PVL) of eggs laid over 10 successive days (days 3–12). We analyzed this effect through a GLMM with Poisson error distribution and log link function. Treatment was entered as a fixed factor; number of laying females in a group, laying day, number of eggs produced by a female, and number of copulations a female had were entered as covariates; and year and female identity (defined as the subject, nested within group) were entered as random factors. In all analyses, female identity is nested within group to account for intergroup variation. To represent this graphically, we presented the relative differences between treatments by standardizing the response variable (probability of solicitation in Figure 1A, level of resistance in Figure 1B, and number of PVL hydrolysis points in Figure 2) on a given day against the highest value obtained within each group across all days. All analyses were conducted in SAS version 9.1.

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