

Full title: Improved timed-mating, non-invasive method using fewer unproven female rats with pregnancy validation via early body mass increases

Short title: Non-invasive, refined timed-mating method for rats

A K Stramek¹, M L Johnson^{1,*}, V J Taylor¹

¹School of Life, Health and Chemical Sciences, The Open University, Walton Hall, Milton Keynes, MK7 6AA, UK

*Current address: Women's Health Research Group, Section of Cancer & Pathology, Leeds Institute of Cancer & Pathology, St James's University Hospital, Beckett Street, Leeds LS9 7TF, UK

Corresponding author:

Dr Vicky Taylor, School of Life, Health and Chemical Sciences, The Open University, Walton Hall, Milton Keynes, MK7 6AA, UK

Email: vicky.taylor@open.ac.uk

Tel: 01908 652274 Fax: 01908 654167

Abstract

For studies requiring accurate conception-timing, reliable, efficient methods of detecting oestrus reduce time and costs, whilst improving welfare. Standard methods use vaginal cytology to stage cycle, and breeders are paired-up using approximately five proven females with proven males to achieve at least one conception on a specific day. We describe an alternative, fast, consistent, non-invasive method of timed-mating using detection of lordosis behaviour in Wistar and Lister-Hooded rats that used unproven females with high success rates. Rats under reverse-lighting had body masses recorded pre-mating, day (d) 3-4, d8, d10 and d18 of pregnancy. Using only the presence of the oestrus dance to time-mate females for 24-hrs, 89% Wistar and 88% Lister-Hooded rats successfully conceived. We did not observe behavioural oestrus in Sprague-Dawleys without males present.

Significant body mass increases following mating distinguished pregnant from non-pregnant rats, as early as d4 of pregnancy ($10\% \pm 1.0$ increase cf $3\% \pm 1.2$). The pattern of increases throughout gestation was similar for all pregnant rats until late pregnancy, when there were smaller increases for primi- and multiparous rats ($32\% \pm 2.5$; $25\% \pm 2.4$), whereas nulliparous rats had highest gains ($38\% \pm 1.5$).

This method demonstrated a distinct refinement of the previous timed-mating common practice used, as disturbance of females was minimised. Only the number required of nulli-, primi- or multiparous rats were mated, and body mass increases validated pregnancy status. This new

breeding-management method is now established practice for two strains of rat and resulted in a reduction in animal use.

Keywords: gestation, oestrus dance, lordosis, pregnancy-prediction, reduction

Introduction

Rats are model organisms with a short oestrous cycle that are used in a multitude of research areas. For some studies, accurate timing of pregnancy or stage of offspring development is critical and it is common practise for multiple dams to be time-mated for each study dam required, which allows for the probability of most dams not conceiving during the required time window. Current practises of non-invasive time-mating involves pairing approximately five proven females with proven males to achieve at least one conception per group of five females mated. This is both time-consuming and an inefficient use of animals, either generating excess litters or requiring monitoring of the additional females for possible pregnancy. Our overall study aim was to improve on current methods and replace them with a simpler method of selecting single females in oestrus for time-mating and then accurately predicting their pregnancy status, as early as possible.

The rat oestrous cycle is 4 to 5 days long and consists of clearly identifiable stages of proestrus, oestrus, metoestrus and dioestrus^{1,2}. The cycle is divided into several phases of different length. Proestrus lasts approximately 12 hours and corresponds to the onset of mating

behaviour, known as behavioural oestrus. The female ovulates at the end of proestrus and enters the oestrus phase, which lasts approximately 26 hours. Metoestrus follows oestrus for approximately 6-8 hours, and then dioestrus ensues, a 50+ hour period that is subdivided into dioestrus I (first 24+ hours) and dioestrus II (second 24+ hours). After dioestrus, the rat returns to proestrus and starts a new cycle, if she has not conceived. Rats do not form a fully functioning corpus luteum unless they are coitally stimulated, so infertile mating can lead to a state of pseudopregnancy that can persist for approximately 10 days before the cycle resumes. The gold standard of cycle stage determination in rodents is vaginal cytology, with preference towards vaginal lavage rather than a cotton swab due to collection of an increased number of cells with undisturbed morphology³. However, it is possible to accidentally induce pseudopregnancy when conducting vaginal cytology, particularly amongst untrained users.

Female rats display behavioural signs of oestrus. When receptive to males during the peri-ovulatory phase, females have increased running activity, hopping, darting, freezing and ear quivering¹. This leads to male mounting, which in turn solicits lordosis from the female due to pelvic stimulation. Lordosis is a distinctive mating behaviour of the female rat that involves dorsiflexion of the vertebral column⁴. Our main aim was to determine if we could make use of this observed behaviour prior to oestrus to predict when females were likely to be receptive to the male to increase the individual chances of conception when pairing them for timed-mating. We also kept the animals under reverse lighting as this is more appropriate for

studying natural behaviour in nocturnal animals⁵, so observations were made during their active phase.

Pregnancy confirmation in laboratory rats in modern research facilities can involve a range of different methods, some of which may be invasive, such as taking blood samples to check for raised progesterone concentrations. Other methods involve animal separation and handling, such as ultrasound scanning for conceptus visualisation, or checking for physical changes including enlarged abdomen and eventually noticeable body mass increases, detectable by weighing. Another study aim was to confidently predict pregnancy as soon as possible following conception.

Pregnancy is a time of extensive maternal adaptation to facilitate foetal growth and development and also to build energy reserves in preparation for the demands of lactation. In rats, maternal body mass increases throughout pregnancy, starting from as early as day 3 post-conception⁶, partly resulting from increased accumulation of adipose tissue^{7, 8}, ready to support lactation. Body mass increases in pregnant dams follows increased food intake⁹. Hyperphagia during pregnancy in rodents has been reported to increase food intake by 20% during the first week of pregnancy^{10, 11}, peaking at between 50%¹⁰ and 60%⁶ by the end of pregnancy compared to nulliparous controls. Maternal leptin resistance also occurs during pregnancy; leptin increases in proportion to the adipose reserves accumulated and develops from mid-gestation to support hyperphagia and allow further adipose accumulation.

Previous work with Wistars has established body mass increases from day 14 (d14) to day 19 (d19) of pregnancy as a reliable method for recognising pregnancy and determining the exact day of gestation¹² but this is obviously a retrospective and later stage method. A further aim of this study was to see if we could track body mass increases from mating to conception and during pregnancy, to establish the earliest time point following conception, to confidently predict pregnancy status, rather than having to wait until pregnancy was physically very obvious. Additionally, we aimed to establish if these methods could be used with unproven females who had not previous conceived.

Animals, Materials and Methods

Ethical approval

This work took place in the Biomedical Research Unit and was licensed under the Home Office Animals (Scientific Procedures) Act 1986 and had approval from The Open University Ethics Committee.

Animals and housing

Female rats were housed in groups of 4 in wiretop ventilated cages (NKP, UK) within larger cabinets (Scantainer, Scanbur, Denmark) and maintained under a 12 hour reverse light cycle (lights off between 11.00 and 23.00 in Wistar room and 8.00 and 20.00 in Lister-Hooded room, for practical reasons) with free access to standard RM3 breeding diet (SDS Diets, <http://www.sdsdiets.com/pdfs/RM3-E-FG.pdf>) and water. Animals were given time to adjust to the reverse light cycle prior to the study, if needed. Intact stud male rats for breeding were

housed singly. All cages of reproductively cycling females of the same strain were kept in the same Scantainer along with one cage of male rats to keep females cycling normally.

Female nulliparous Wistars for ovarian cycle stage determination were obtained from Harlan, UK (n=53) and an in house colony was subsequently bred from more purchased animals.

Wistar rats that were also used in another pregnancy and lactation study as well as this one, were bred in-house (2 to 3 females per cage, n=54; n=3 males). To obtain initial litters from the colony, females were introduced to a male (1 to 2 females per male) in his home cage for a period of 5 days and litters (un-manipulated) were kept to maturity for future pregnancy and lactation time points. Nulli, primi and multiparous Lister-Hooded (breeding stock derived from Harlan, UK, n=91) and Sprague-Dawley rats (in-house strain, n=15) were included in the study.

Rats were regularly handled to reduce stress during regular weighing and for the subset of Wistars that underwent daily oestrous monitoring by vaginal lavage (detailed below) was conducted at 24-hourly intervals between 11.00 (lights off) and 13.00, to observe and determine cycle stages (see below and Figure 1) to confirm normal cyclicity prior to studies. All handling and data collection activities were carried out during the dark phase, so they were obtained when most physiologically relevant for natural activity and reproductive behaviour. Body masses were recorded at approximately the same time each day at the following time-points: pre-mating, day 3-4 pregnant and days 8, 10, and 18 pregnant. Rats were checked for lordosis behaviour at pre-mating and then again at each weighing time-point; checking at the later time-points was included to confirm that the behaviour seen was mating-specific, also

that the pregnancy was still viable and the dam had not lost the conceptus and returned to ovarian cyclicity and proestrus behaviour. The body mass data for one Lister-Hooded rat is missing.

Whilst handling the Wistar animals to reduce stress prior to the commencement of oestrous monitoring (details below), a distinct behaviour was observed, which was identified as an 'oestrus dance' (Figure 2). When in proestrus (confirmed by vaginal cytology and uterine masses, Figure 3, for Wistar rats subgroup/in pilot studies), gentle physical manipulation with stimulation of the back of the rat near the hind legs would result in initiation and presentation of lordosis with ear wiggling, hopping, darting and freezing, without a male rat being present or even visible, as described by Feder¹. This behavioural stimulation was repeated in Lister-Hooded rats, without vaginal cytology, the check for the 'dance' was carried out between 11.00-12.00 and took approximately 1 min per animal (see supplementary video, which is available online with this article). The oestrus dance was then used on its own, without cytology, for accurately time-mating Wistar females for the pregnancy time points of days 4 (n=7), 12 (n=7) and 18 (n=7) of pregnancy, then in a much larger group of Lister-Hooded rats for work requiring specific embryonic time points and early postnatal studies.

Oestrous cycle monitoring

Rats have an oestrous cycle of approximately 5 days. As guided by the literature^{3, 13, 14}, proestrus (Figure 1 A) was identified by the presence of a large number of spherical nucleated

cells, which resulted in a very cloudy cell suspension. Leucocytes were often present but in very small numbers, with cornified (keratinised, non-nucleated) cells being rare. A predominance of cornified cells confirmed oestrus (Figure 1 B), with a number of these cells becoming spindle-shaped in appearance. Metoestrus (Figure 1 C) was characterised by an abundance of leucocytes with a small number of cornified, particularly spindle-shaped, cells present. During dioestrus (Figure 1 D), few cells of any cell type were present, which was characteristic of the stage in itself. A dioestrus lavage would typically contain a lot of mucus, sticking leucocytes together in lines. There was no vaginal cytology undertaken in the Lister-Hooded rats.

Additionally, in 57 Wistar rats that were culled for a separate experimental purpose, the opportunity to remove their uteruses was taken and their post mortem uterine mass increases confirmed proestrus and the other ovarian stages identified by the vaginal lavage method used (Figure 3). These Wistar females were fully anaesthetised (isoflurane; IsoFlo, Abbott) and decapitated for trunk blood and tissue sample collection for other studies^{15, 16}.

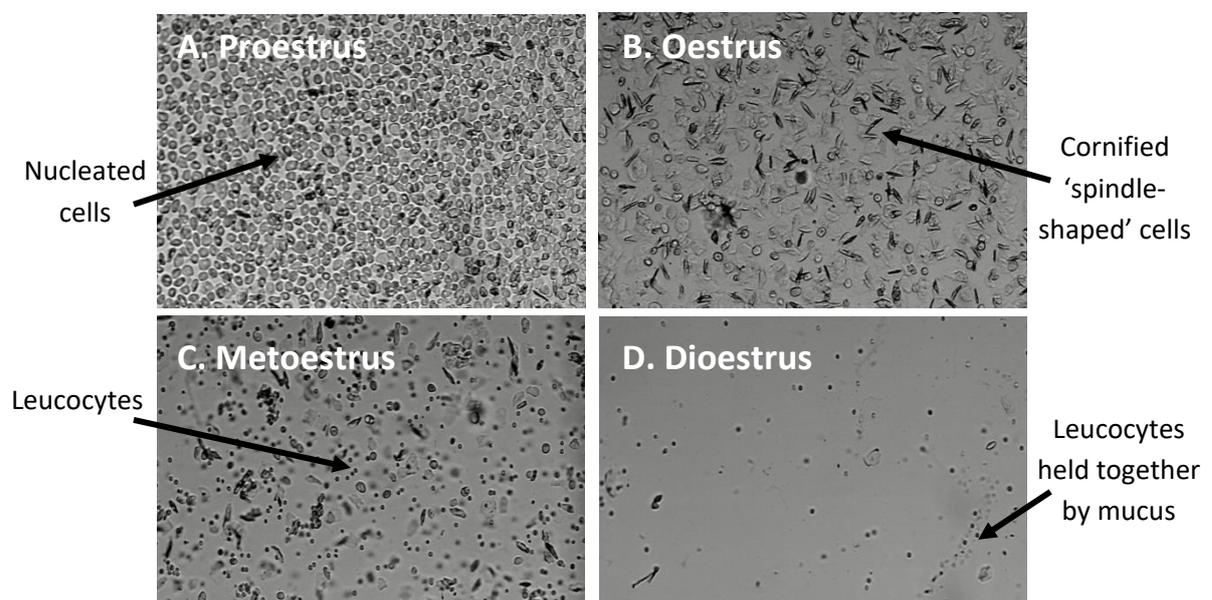


Figure 1. Representative images for each stage of the Wistar rat oestrous cycle (x100 magnification).

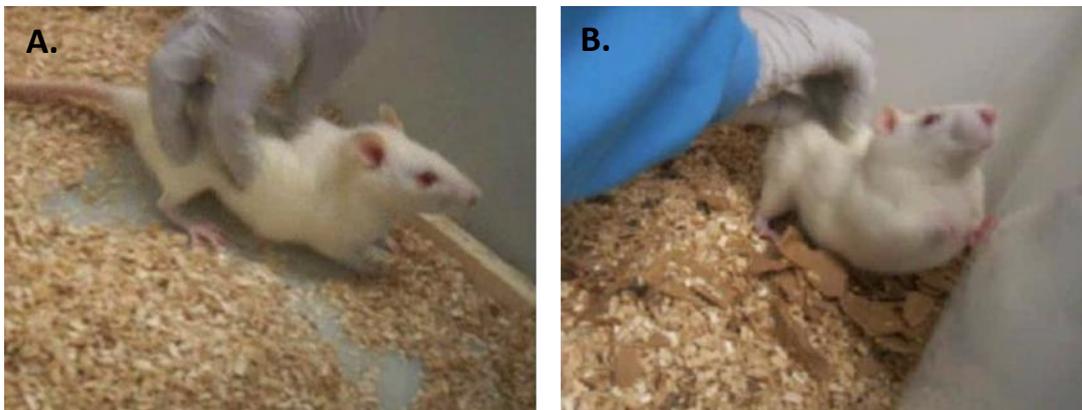


Figure 2. The oestrus dance in Wistar females

(A)-(B) the oestrus dance occurred during proestrus during the dark phase of the lighting cycle (lights turned on for photography only). See Video 1 for Lister-Hooded rat showing behavioural oestrus – note the hopping and ear twitching.

[v1]

Wistar rats have a gestation period of approximately 21 days, and it is slightly longer at 23 days for Lister-Hooded rats, so they were checked regularly close to their expected day of birth (d21

post mating) and cages were carefully checked for pups and counted, without disturbing the dams. Signs of blood were also checked for, in case of post-natal death. Pups were sexed at weaning at approximately 25 days old. Lister-Hooded rats that showed the oestrus dance and were paired with a male but failed to produce a litter (or any signs of giving birth e.g. blood) were classed as not pregnant and used as a non-pregnant body mass comparison group. Three Lister-Hooded rats were suspected of pseudopregnancy and placed into another separate group for data analysis purposes.

Statistics

Values represent mean \pm standard error of the mean (S.E.M). Statistical analysis was carried out using a one-way analysis of variance (ANOVA; uterine masses, uterine masses/body mass) and repeated measures ANOVA (body mass percentage increases following mating for nulliparous, primi/multiparous and not pregnant/pseudopregnant rat groups, with age as covariate) with Tukey post-hoc tests on normally distributed data. The ANOVA F test results are reported in the format: e.g. $F(2,27) = 18.06; P < 0.01$; where the bracketed numbers are the degrees of freedom between groups and within groups. Pearson correlations were used to determine any relationships of body mass and body mass percentage increases following mating with age at mating. All statistical tests were performed using IBM SPSS Statistics 21. $P < 0.05$ was considered statistically significant.

Results

Observations of reproductive behaviour and body mass data were obtained from three different strains of rat: Wistar, Lister-Hooded and Sprague Dawley. The initial work was carried out in Wistar rats, validated with cycle monitoring, which progressed to checking only for the oestrus dance, once the method was refined. Later, this simpler method was then used in Wistars for pregnancy and lactation time points and in two more rat strains: Lister-Hooded and Sprague Dawley. All rats were adjusted to reverse lighting conditions, then only checked to confirm the presence of an oestrus dance before being paired with a male if they did exhibit this proestrus behaviour, and taking body mass measurements at the designated time-points. Results are not presented for Sprague Dawleys as this method was not successful; females only displayed the oestrus dance once they were placed with a male in his home cage.

Behavioural observations: “oestrus dance”

The majority of females presented an obvious oestrus dance during handling in proestrus (except for Sprague Dawleys, mentioned above), every 4-5 days, so this distinctive behaviour was used as confirmation of readiness to mate/conceive. Additionally, animals were observed to have an agitated demeanour and be more physically active during proestrus. For the breeding colony of Wistars used in this study, the females demonstrated this sexual behaviour shortly after lights out and the Lister-Hoodeds, within the first six hours following lights out.

Confirmation of proestrus cycle stage in Wistars

The Wistar rats in the initial study were monitored for two (and up to four) complete ovarian cycles to ensure they were cycling normally. After they were culled at the different cycle stages, increased fluid in the uterus was used as an additional confirmation of successful proestrus identification by vaginal lavage. Intact uterus mass was found to be significantly highest at proestrus ($F(3, 39)=7.998, P<0.001$), when the uterine cavity became fluid-filled (Figure 3 A). When corrected for individual rat body mass, uteri remained significantly heavier at proestrus ($F(3, 39)=8.035, P<0.001$) than at all other cycle stages.

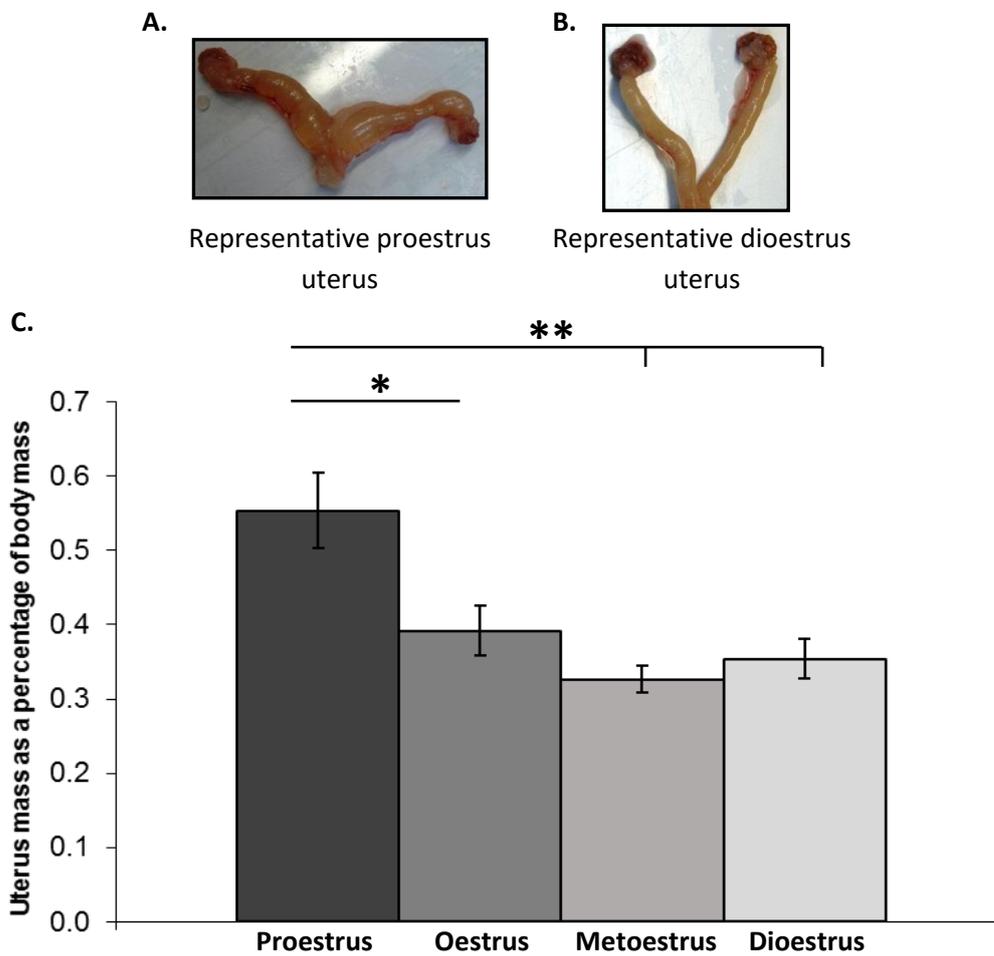


Figure 3. Uteri were significantly heavier at proestrus in Wistar rats

Uteri (not to scale) became fluid filled at proestrus (A), but not at other cycle stages (B).

(C) Intact uterus mass corrected for individual body mass (proestrus, n=12; oestrus, n=11; metroestrus, n=9; dioestrus, n=11; * $P < 0.05$; ** $P < 0.01$).

Refinement of time-mating procedure

Using oestrus dance observation alone to identify oestrus females that were then time-mated with proven males for 24-hrs, 48/54 (89%) Wistar and 80/91 (88%) Lister-Hooded successfully conceived and littered down.

Wistars

Body masses were recorded pre-mating, and during early pregnancy: d3P and d4P (but these d4 measurements were fasted overnight). For seven Wistar rats that were 212 +/- 2.63 days old (range 205-218), their pre-mating body masses were 254 +/- 6.4 g (range 223-276) and by d3P were 267 +/- 7.6 g (232-294), with no lordosis behaviour observed at this latter time-point. Their body masses had increased by 5.1% +/- 0.54 by d3P (3-7%); by d4P body masses not shown as they were fasted as a requirement for a different study.

Lister-Hooded

There were 67 nulliparous (130 +/- 6.7 days old at pre-mating weigh point, range 50-254), 10 primiparous (193 +/- 10.8 d, range 146-234) and 2 multiparous rats (236 +/- 6.0 d, range 230-242). Body masses were recorded pre-mating (d0), then throughout gestation at d4P, d8P, d10P and d18P and mean values are shown in Table 1 subdivided into groupings based on parity: nulliparous for no previous births, primiparous for one previous litter and multiparous for two previous litters. Multi- and primiparous rats were older and slightly heavier than nulliparous rats both at mating and throughout gestation, as expected. To account for variation in individual body masses, Table 2 shows the pregnancy-related body mass increases

as a percentage of initial body mass at mating/conception (d0). The 11 rats that did not conceive/with unsuccessful pregnancies had much smaller body mass changes.

Table 1. Changes in body mass after mating in Lister-Hooded rats.

Mean body masses (g) before (d0) and after mating (d1-18) in nulli/primi/multiparous Lister-Hooded rats and those that did not conceive/have a successful pregnancy.

Parity	d0	d1	d4	d8	d10	d18
Nulliparous (n=67)	205 ± 3.0	214 ± 3.0	227 ± 2.9	238 ± 3.1	242 ± 2.6	284 ± 3.3
<i>Primiparous¹</i> (n=10)	240 ± 6.4	250 ± 5.9	263 ± 6.3	278 ± 5.5	281 ± 7.3	316 ± 8.0
<i>Multiparous¹</i> (n=2)	246 ± 20.3	253 ± 23.3	267 ± 20.1	279 ± 17.4	281 ± 15.0	307 ± 19.6
Combined primi/multi (n=12)	241 ± 5.9	250 ± 5.7	263 ± 5.8	278 ± 5.1	281 ± 6.3	314 ± 7.1
Not pregnant/ unsuccessful pregnancy (n=11)	223 ± 9.9	229 ± 9.0	235 ± 7.6	234 ± 6.8	238 ± 7.0	241 ± 7.1
<i>Not pregnant without possible pseudopregnants</i> (n=8)	237 ± 6.0	241 ± 5.7	240 ± 4.3	240 ± 4.5	245 ± 3.9	246 ± 5.9
<i>Pseudopregnants²</i> (n=3)	186 ± 22.7	198 ± 22.9	214 ± 39.4	216 ± 21.1	221 ± 20.9	227 ± 21.5

¹Primi and multiparous are shown separately (in italics), then as Combined as there were only 2 multiparous and their values were similar.

²Possible pseudopregnants were separated from the not pregnant group.

The body mass of all pregnant rats increased from d1P to d4P (with no further lordosis behaviour observed) and between all later time points (Tables 1 and 2; also see Figure 4). The day following mating, all rats showed modest increases of ~4%, then by day 4 of pregnancy (when no physical signs are visible) consistent body mass percentage increases of 10% were recorded, followed by ~15-19% by days 8-10, then up to 38% by day 18 (Table 2).

Primi- and multiparous rats were heavier prior to mating than nulliparous rats (d0, Table 1; as they were slightly older). However the pattern of body mass increase (% increase of d0, Table 2) was similar for all pregnant rats until d18, when it was reduced for the rats that had already had prior litters (31% cf 38%, Table 2). Nulliparous rats had the highest body mass percentage gains, especially by d18 of pregnancy, as they were younger and still physically growing, at the same time (Table 2; re: age, see next section and Figure 5).

Table 2. Percentage changes in body mass after mating in Lister-Hooded rats.

Mean body mass differences (%) after mating in nulli/primi/multiparous Lister-Hooded rats and those that did not conceive (a>b, $P<0.001$).

Parity	d1	d4	d8	d10	d18
Nulliparous (n=67)	4% ± 0.3 ^a	10% ± 0.5 ^a	15% ± 0.7 ^a	19% ± 0.7 ^a	38% ± 1.5 ^a
<i>Primiparous</i> ¹ (n=10)	4% ± 1.2	10% ± 1.4	14% ± 1.9	17% ± 1.8	32% ± 2.5
<i>Multiparous</i> ¹ (n=2)	3% ± 1.0	9% ± 0.9	14% ± 2.4	15% ± 3.4	25% ± 2.4
Combined primi/multi (n=12)	4% ± 1.0 ^a	10% ± 1.1 ^a	14% ± 1.5 ^a	17% ± 1.6 ^a	31% ± 2.3 ^a
Not pregnant/ unsuccessful pregnancy (n=11)	3% ± 0.9	3% ± 1.2	6% ± 2.8	8% ± 3.1	9% ± 3.3 ^a
<i>Not pregnant without possible pseudopregnants</i> (n=8)	2% ± 0.7 ^b	2% ± 1.1 ^b	2% ± 1.5 ^b	3% ± 1.9 ^b	4% ± 2.7 ^b
<i>Pseudopregnants</i> ² (n=3)	7% ± 1.5	8% ± 2.2	17% ± 5.7	19% ± 4.3	23% ± 3.2

¹Primi and multiparous are shown separately (in italics), then as Combined as there were only 2 multiparous and their values were similar.

²Possible pseudopregnants were separated from the not pregnant group.

The not pregnant rat group, without 3 suspected pseudopregnant animals, showed a very slight body mass gain (up to 4%) from d0 through to d18 post mating (Figure 5) but were significantly lower than all the pregnant rat time-points ($F(2,67)=22.563$, $P<0.001$; with age as covariate). The small pseudopregnant group had similar body mass gains to pregnant rats, although the increase by d18 was reduced compared to the younger rats with successful pregnancies (23% cf 38% nulliparous cf 32% primiparous, Table 2). Although the numbers are small ($n=3$) and age range was wide (53-230 days), the pseudopregnant rats had a similar final body mass increase to multiparous rats (23% cf 25%).

Figure 4. Percentage body mass increases following mating of not pregnant and pregnant rats of different parities throughout gestation.

Percentage body mass increases throughout pregnancy from mating, for nulliparous and combined primi/multiparous pregnant and not pregnant (without three pseudopregnant) Lister-Hooded rats.

The body mass percentage increases of the pregnant rats were all significantly higher than the not pregnant rat time-points ($F(2,67)=22.563$, $P<0.001$) ($*P<0.001$, Table 2, Figure 4) from day 1 following mating. Table 3 shows the similar litter sizes per parity of the Lister-Hooded dams and sex ratio of the pups born in each litter.

Table 3. Pup totals and sex produced by nulliparous, primiparous and multiparous Lister-Hooded rats.

Data shown \pm standard error of the mean.

Parity	Total pups	Male pups	Female pups
Nulliparous (n=67)	11 \pm 0.3	6	5
<i>Primiparous (n=10)</i>	<i>11 \pm 1.1</i>	6	5
<i>Multiparous (n=2)</i>	<i>8 \pm 3.5</i>	4	4
Combined primi/multi (n=12)	10 \pm 1.1	6	5

Larger body mass increases in nulliparous rats in late gestation

Body mass increases by the end of gestation were larger in younger nulliparous rats (Table 2)

so we explored these data further. Figure 5 shows the range of ages of the nulliparous rats against their body masses at mating (d0). There was a positive correlation for nulliparous rats only ($r=0.739$, $n=67$, $P<0.001$ cf primi/multiparous $r=0.244$, $n=12$, $P=0.445$, ns), suggesting that they were still growing physically/maturing to adult body sizes despite being reproductively competent.

Figure 5. Body masses at mating (d0) of only nulliparous Lister-Hooded rats positively correlated with their age.

Body mass at mating (d0) by age (in days) in nulliparous Lister-Hooded rats. There was a positive correlation for nulliparous rats only ($r=0.739$, $n=67$, $P<0.001$).

Likewise, for nulliparous rats only, there was a negative correlation between age at mating and body mass increases throughout pregnancy: the youngest pregnant rats had the highest body mass percentage increases by d18, with the oldest having the smallest increases (d1%-d18%: $r=-0.320$ to -0.648 , $n=57-67$, $P=0.008$ to <0.001 ; Figure 6). There were no significant correlations for primi- or multiparous rats (d1%-d18%: $r=-0.274$ to -0.501 , $n=11-12$, $P=0.097-0.389$, ns).

Figure 6. Age of, nulliparous only, Lister-Hooded rats negatively correlated with body mass increases during gestation.

Relationship between age at mating (days) and percentage body mass increases from mating to day 18 of pregnancy in nulliparous Lister-Hooded rats ($r=-0.608$, $n=74$, $P<0.001$).

Discussion

We have described an alternative, non-invasive and consistent timed-mating method that uses gentle physical manipulation during handling, to instigate lordosis behaviour in female Wistar and Lister-Hooded rats. Implementing the oestrus dance to time-mate the dams was very successful and a distinct refinement of the commonly used time-mating procedure (over 5 days with male using 5 females/time point required). This new and accurate method has important welfare implications as it greatly reduced the number of animals needed and produced a pregnancy rate approaching 90% with only a single, unproven female required per time-point. This represents a further refinement as proven breeders will nearly always be used⁴ with the existing method. Additionally, the disturbance of females with either oestrous monitoring and/or to check for the appearance of a vaginal plug to confirm mating was

reduced, and then removed entirely. Females were subject to less stressful situations, as they were only paired with males when sexually receptive.

In the current study, pregnancy in Wistar and Lister-Hooded rats could be confirmed non-invasively, with confidence, as early as d4 (when visualisation of any conceptus is not yet possible) using body mass increases of 10% from prior to mating body masses, compared to the not pregnant control group. This was much earlier and an improvement on previous studies that used body mass increases from mating to d14-d19 of pregnancy¹¹. Furthermore, the unproven, nulliparous dams could be confirmed pregnant most reliably as they had the highest body mass gains towards the end of pregnancy. This could mean an improvement in study design for future studies adopting this method as they do not have to use proven breeders. They could also select study animals with less physiological variation: younger, within a reduced age and body mass range, as well as nulliparous.

This work has also revealed a further consideration for studies using unproven nulliparous rats. As they were still growing, this resulted in the youngest rats achieving the highest body mass gains from mating to d18 of pregnancy. Thus we can further predict from this work that older nulliparous rats, over approximately 20 weeks (5 months) old, are likely to have smaller body mass increases to d18P, closer to the values achieved by primi and multiparous dams. These slight body mass differences may also be a reflection of reducing litter sizes, as the multiparous dams had smaller litter sizes than the nulli and primiparous groups.

Non-pregnant animals did show small body mass increases, which could be expected to be greater in younger, growing animals but the much larger increases due to pregnancy in the other rat groups became apparent as early as the day following mating and more than doubled by day 4 of pregnancy and continued to increase further thereafter. Based on our findings for Lister-Hooded and Wistar rats that displayed the oestrus dance, we would predict that increases of greater than 3% by day 1 following mating indicate conception and over 8% by day 4 confirm a successful pregnancy diagnosis.

We do however offer one potential caveat with this body mass increase method, relevant to studies using or requiring multiparous dams. Based on our findings, albeit with very small numbers, there could be the possibility of a false positive pregnancy diagnosis in a multiparous dam when a pseudopregnancy had actually occurred (n=3) as their body mass increases did mirror the values of the multiparous dams (n=2) for the whole study period. This was surprising as it is expected that pseudopregnancy resolves by day 10 so it may have been that these pseudopregnant-grouped animals did actually conceive but their pregnancies were resorbed at a late stage, which could explain the late pregnancy body mass gains. This would need further study with larger sample sizes of pseudopregnant animals compared with multiparous dams to determine for certain.

In our study, all the rat strains were housed under reverse lighting so that they were observed and weighed during their active phase and it was simple to instigate this in separate rooms within the facility, as they each had individual light cycle monitors. After being housed in

rooms with lighting schemes that suit human workers, bought-in rats easily adapt to reverse lighting within about two weeks. Rats born under reverse lighting did not require any adjustment time. Reverse lighting rooms had red overhead lights and workers additionally used red headtorches, so ease of cleaning, feeding and other day-to-day tasks was unaffected.

We were unable to observe the behavioural signs of oestrus in Sprague Dawley rats, also studied under reverse lighting, before them being placed within the home cages of male rats, so our new methods were not replicated in all strains tested. Thus more animal and labour-intensive methods are still required to achieve the same outcomes for timed-mating in this rat strain, housed under similar conditions and it may also be the case for other different rat strains to the three studied. It remains unknown why some laboratory rat strains show overt behavioural signs of oestrus on handling whilst others do not, unless they are in close proximity to a sexually mature male rat, so this may indicate that pheromonal or other intersexual signals may have differential importance depending on genetic background. It was noted that their cytological changes were much harder to follow thus their ovarian cycles may have been irregular and less predictable. Sprague Dawleys have demonstrated more anxiety-like behaviour and reduced motor function compared with different rat strains^{17, 18}, so their natural behaviours may be more inhibited during handling. Thus testing of other rat strains to find out which ones can be gently stimulated to show lordosis behaviour will also be required before attempting to fully adopt this new method.

We have established a new robust method that combines a behavioural method to increase mating success rates and additionally validated the use of body mass increases (as % body mass since mating) across a range of ages and parities to confidently predict whether a nulli, primi or multiparous dam is, and/or remains, pregnant from day 4 of pregnancy. Based on our findings it should also be expected that the youngest nulliparous females will gain more body mass in the later stages of pregnancy, which highlights the importance of them having access to enough additional food at this stage of pregnancy, compared with primi and multiparous dams.

Setting up a study that relies on early embryonic stages requires practise of cytological methods to confirm reproductive cycle staging, but once reproductive cyclicity has been established in the breeding stock, the use of behavioural signs of oestrus, induced by gentle handling, can replace the need to undertake the previously labour-intensive and potentially disruptive procedure on the females. This refined breeding management procedure has been adopted as a new efficient practice in the BRU at The Open University for two strains of rat: Wistars and Lister-Hooded, housed under reverse lighting, which is effective for nulliparous (unproven) as well as proven females and has resulted in a reduction of rat numbers required to provide new rat litters or females at specified time points of pregnancy or lactation.

Acknowledgements

The authors thank Steve Walters, Karen Evans, Sophie Brooks and Gareth Williams for outstanding technical support and other LHCS researchers for the opportunity to monitor their

breeding stock. We are also grateful to anonymous reviewers whose comments on an earlier version have helped us improve the current manuscript. The School of Life, Health and Chemical Sciences (LHCS), The Open University, provided the funding for this work.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

References

1. Feder HH. Estrous Cyclicity in Mammals. In: Adler NT, (ed.). *Neuroendocrinology of Reproduction: Physiology and Behavior*. Boston, MA: Springer US, 1981, p. 279-348.
2. Westwood FR. The female rat reproductive cycle: a practical histological guide to staging. *Toxicol Pathol*. 2008; 36: 375-84.
3. Becker JB, Arnold AP, Berkley KJ, et al. Strategies and methods for research on sex differences in brain and behavior. *Endocrinology*. 2005; 146: 1650-73.
4. Barnett SW. *Manual of animal technology*. Oxford, UK: Blackwell Publishing, 2007, 2007.
5. Prager EM, Bergstrom HC, Grunberg NE and Johnson LR. The importance of reporting housing and husbandry in rat research. *Front Behav Neurosci*. 2011; 5: 38.
6. Cripps AW and Williams VJ. The effect of pregnancy and lactation on food intake, gastrointestinal anatomy and the absorptive capacity of the small intestine in the albino rat. *Br J Nutr*. 1975; 33: 17-32.
7. Lopez-Luna P, Maier I and Herrera E. Carcass and tissue fat content in the pregnant rat. *Biol Neonate*. 1991; 60: 29-38.
8. Pujol E, Proenza AM, Roca P and Llado I. Changes in mammary fat pad composition and lipolytic capacity throughout pregnancy. *Cell Tissue Res*. 2006; 323: 505-11.
9. Gualillo O, Caminos JE, Nogueiras R, et al. Effect of food restriction on ghrelin in normal-cycling female rats and in pregnancy. *Obes Res*. 2002; 10: 682-7.
10. Crean GP and Rumsey RD. Hyperplasia of the gastric mucosa during pregnancy and lactation in the rat. *J Physiol*. 1971; 215: 181-97.
11. Trujillo ML, Spuch C, Carro E and Senaris R. Hyperphagia and central mechanisms for leptin resistance during pregnancy. *Endocrinology*. 2011; 152: 1355-65.
12. Paronis E, Samara A, Polyzos A, Spyropoulos C and Kostomitsopoulos NG. Maternal weight as an alternative determinant of the gestational day of Wistar rats housed in individually-ventilated cages. *Laboratory animals*. 2015; 49: 188-95.

13. Goldman JM, Murr AS and Cooper RL. The rodent estrous cycle: characterization of vaginal cytology and its utility in toxicological studies. *Birth Defects Res B Dev Reprod Toxicol.* 2007; 80: 84-97.
14. Marcondes FK, Bianchi FJ and Tanno AP. Determination of the estrous cycle phases of rats: some helpful considerations. *Braz J Biol.* 2002; 62: 609-14.
15. Johnson ML, Saffrey MJ and Taylor VJ. Plasma Ghrelin Concentrations Were Altered with Oestrous Cycle Stage and Increasing Age in Reproductively Competent Wistar Females. *PLoS One.* 2016; 11: e0166229.
16. Johnson ML, Saffrey MJ and Taylor VJ. Glucagon-like peptide-1 (GLP-1) increases in plasma and colon tissue prior to estrus and circulating levels change with increasing age in reproductively competent Wistar rats. *Peptides.* 2017; 90: 55-62.
17. Ferguson SA and Cada AM. Spatial learning/memory and social and nonsocial behaviors in the spontaneously hypertensive, Wistar-Kyoto and Sprague-Dawley rat strains. *Pharmacol Biochem Behav.* 2004; 77: 583-94.
18. Turner KM and Burne TH. Comprehensive behavioural analysis of Long Evans and Sprague-Dawley rats reveals differential effects of housing conditions on tests relevant to neuropsychiatric disorders. *PLoS One.* 2014; 9: e93411.

Résumé

Pour les études nécessitant un moment de conception précis, des méthodes de détection de l'ovulation fiables et efficaces permettent de réduire les délais et les coûts, tout en améliorant le bien-être animal. Les méthodes standard reposent sur la cytologie vaginale pour déterminer l'étape du cycle, et les animaux sont accouplés en utilisant environ cinq femelles dont l'ovulation est confirmée à des mâles reproducteurs afin de parvenir à au moins une conception à un jour donné. Nous décrivons une méthode non invasive alternative, rapide et uniforme d'accouplement temporisé reposant sur la détection du comportement de lordose chez les rats Lister-Hooded et Wistar, utilisée avec succès pour se reproduire avec des femelles dont l'ovulation n'est pas confirmée. La masse corporelle de rats sous lumière inversée a été enregistrée avant l'accouplement, et aux jours (d) 3-4, d8, d10 et d18 de la gestation. En utilisant uniquement la présence de la danse de l'ovulation pour accoupler de manière temporisée les rats sur 24 h, 89 % et 88 % des rates Wistar et Lister-Hooded avaient conçu avec succès. Nous n'avons pas observé de comportement lié à l'ovulation chez les rates Sprague-Dawleys sans mâles présents.

D'importantes augmentations de masse corporelle après l'accouplement permettaient de distinguer les rates gestantes des rates non gestantes, dès le 4^e jour de gestation ($10\% \pm 1,0\%$ cf d'augmentation $3\% \pm 1,2$). Le modèle d'augmentations tout au long de la gestation était similaire pour toutes les rates gestantes jusqu'à la fin de la gestation, moment où de plus petites augmentations pouvaient être observées chez les rates primi- et multipares ($32\% \pm 2,5$; $25\% \pm 2,4$), tandis que les gains les plus élevés concernaient les rates nullipares ($38\% \pm 1,5$).

Cette méthode a démontré une nette amélioration par rapport à la méthode d'accouplement précédente couramment utilisée, la perturbation des femelles étant réduite au minimum. Seul le nombre requis de rates nulli-, primi- ou multipares a été accouplé, et l'augmentation de la masse corporelle permettait de confirmer la gestation. Cette nouvelle méthode de gestion de l'élevage est maintenant la pratique établie pour deux souches de rat et a permis de réduire le nombre d'animaux utilisés.

Abstract

Für Studien, die eine genaue Empfängnisplanung erfordern, reduzieren zuverlässige und effiziente Methoden zur Östruserkennung Zeit und Kosten bei gleichzeitiger Verbesserung des Tierschutzes. Standardmethoden stützen sich auf die Vaginalzytologie für den Zyklus, wobei Züchter ca. fünf getestete Weibchen mit getesteten Männchen paaren, um mindestens eine Empfängnis an einem bestimmten Tag zu erreichen. Wir beschreiben eine alternative, schnelle, konsistente, nicht-invasive Methode der zeitlichen Paarung durch die Erkennung von Lordoseverhalten bei Wistar- und Lister-Hooded-Ratten, bei der ungetestete Weibchen mit hohen Erfolgsraten eingesetzt wurden. Bei Ratten unter Gegenlicht wurden die Körpermassen vor der Paarung und an Trächtigkeitstagen (d) 3-4, d8, d10 und d18 aufgezeichnet. Allein unter Ausnutzung des Brunsttanzen zur zeitlich gesteuerten Paarung von Weibchen für 24 Stunden wurden 89 % der Wistar- und 88% Lister-Hooded-Ratten erfolgreich gedeckt. Wir haben bei Sprague-Dawleys keinen Verhaltens-Östrus beobachtet, wenn keine Männchen präsent waren.

Signifikante Zunahme der Körpermasse nach der Paarung unterschied trächtige von nicht trächtigen Ratten bereits ab d4 der Trächtigkeit ($10\% \pm 1,0\%$ Zunahme cf $3\% \pm 1,2$). Das Muster

der Zunahme während der Trächtigkeit war für alle trächtigen Ratten bis zur späten Trächtigkeit ähnlich, ab dann sahen wir geringere Zunahmen für primi- und multipare Ratten ($32 \% \pm 2,5$; $25 \% \pm 2,4$), während nullipare Ratten die höchsten Zunahmen verzeichneten ($38 \% \pm 1,5$).

Diese Methode zeigte eine deutliche Verbesserung der bisher üblichen terminlichen Verpaarung, da Störungen der Weibchen minimiert wurden. Es wurde nur die erforderliche Anzahl der nulli-, primi- oder multiparen Ratten verpaart, und die Körpermassenzunahme validierte den Trächtigkeitsstatus. Diese neue Zuchtmanagementmethode hat sich inzwischen für zwei Rattenstämme etabliert und hat zu einer Reduzierung der Anzahl benötigter Versuchstiere geführt.

Resumen

Para estudios que requieren una concepción programada precisa, los métodos eficientes y fiables de detección del celo reducen el tiempo y los costes además de mejorar el bienestar. Los métodos estándar utilizan citología vaginal para organizar los ciclos, y los reproductores se emparejan utilizando aproximadamente cinco hembras probadas con cinco machos probados a fin de conseguir una concepción en un día en concreto. Describimos un método alternativo, rápido, consistente y no invasivo de cría programada utilizando la detección de conductas de lordosis en ratas Wistar y Lister-Hooded donde se había utilizado hembras sin probar con un gran porcentaje de éxito. Las ratas bajo iluminación revertida se sometieron a un registro de masa corporal anterior a la cría en los días 3-4, 8, 10 y 18 del embarazo. Utilizando sólo la danza del celo para programar la cría de las hembras durante 24 horas, el 89 % de las ratas Wistar y el 88 % de las ratas Lister-Hooded concibieron fructíferamente. No observamos ningún comportamiento de celo en las ratas Sprague-Dawleys sin la presencia de machos.

Una masa corporal significativamente superior tras la cría distinguió las ratas embarazadas de las no embarazadas desde el día 4 del embarazo ($10 \% \pm 1,0$ aumento del $3 \% \pm 1,2$). El patrón de aumento durante la gestación fue similar en todas las ratas embarazadas hasta la etapa final del embarazo, cuando se registraron unos aumentos inferiores para las ratas primíparas y múltiparas ($32 \% \pm 2,5$; $25 \% \pm 2,4$), mientras que las ratas nulíparas registraron los mayores aumentos ($38 \% \pm 1,5$).

Este método demostró un refinamiento distintivo de la práctica de cría programada utilizada anteriormente, ya que la perturbación de las ratas hembras se redujo. Únicamente el número requerido de ratas nulíparas, primíparas y múltiparas se sometieron a cría, y los aumentos de la masa corporal validaron su embarazo. Este nuevo método de gestión de la cría ahora es una práctica común para dos cepas de ratas y permitió una reducción del número de animales utilizado.