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Elevated prolactin secretion during proestrus in mice: Absence of a defined surge

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Abstract

Throughout the reproductive cycle in rodents, prolactin levels are generally low. In some species, including rats, a prolactin surge occurs on proestrus with peak concentrations coinciding with the preovulatory luteinizing hormone (LH) surge. In mice, however, there are conflicting reports relating to the occurrence and timing of a proestrous prolactin surge. To gain further insight into the incidence and characteristics of this surge in mice, we have used serial tail tip blood sampling and trunk blood collection from both C57BL/6J (inbred) and Swiss Webster (outbred) mouse strains to build a profile of prolactin secretion during proestrus in individual mice. A clearly defined LH surge was detected in most animals, suggesting the blood sampling approach was suitable for detecting patterns of hormone secretion on proestrus. Despite this, levels of prolactin were quite variable between individuals. Overall both mouse strains showed a generalized rise in prolactin levels on the day of proestrus compared with levels seen in diestrus. This pattern is quite distinct from the discreet, circadian-entrained surge observed in rats.

KEYWORDS
luteinizing hormone, proestrus, prolactin

1 | INTRODUCTION

In male and nonpregnant female rodents, circulating levels of the anterior pituitary gland hormone, prolactin, are generally low. This is achieved by a tonic inhibitory input from the hypothalamus sustained by a "short-loop" negative feedback mechanism. Prolactin regulates its own secretion through action on neuroendocrine dopamine (NEDA) neurons located in the periventricular and arcuate nuclei of the hypothalamus. Prolactin action increases firing rates of these neurons, causing dopamine release. Dopamine then travels via the pituitary portal blood vessels to act on dopamine D2 receptors on lactotrophs leading to tonic inhibition of prolactin release from these cells.[1,2] In the face of this inhibitory hypothalamic tone, enhanced prolactin secretion may be achieved either through mechanisms that reduce dopamine output from the NEDA neurons, or factors that directly stimulate prolactin release (prolactin-releasing factors, PRFs).

In rats, one of the most robust episodes of prolactin release occurs during the afternoon of proestrus, with high circulating levels of estradiol inducing a surge in prolactin levels during the preovulatory stage of the reproductive cycle (proestrus in rodents).[3–7] Circulating prolactin levels are low in the morning of proestrus ~30 ng/ml and begin rising around 1200 h reaching peak levels ~250 ng/ml from 1400–1500 h. Levels then drop slightly, before being maintained in a plateau phase through the first few hours of the dark period (1900–2300 h) and reach baseline levels by 0600 h on estrus.[5,7] The mechanisms driving this estradiol-induced prolactin surge remain to be fully

characterized. Estradiol directly stimulates expression of the prolactin gene in pituitary lactotrophs,[8] but the rise in prolactin is tightly linked to the light–dark cycle, suggesting an important involvement of hypothalamic regulation. Estradiol also acts via its receptors on NEDA neurons in the arcuate nucleus of the hypothalamus to reduce dopamine output.[9,10] In addition, there is evidence for a range of other factors contributing to the proestrous prolactin surge, either by causing suppression of dopamine secretion[11,12] or acting directly in the pituitary as PRFs. These putative PRFs include, oxytocin[13] and an unidentified factor from the posterior pituitary gland.[14] It seems likely that reduced dopamine is required for the surge, with PRFs potentially involved in the circadian-timed peak secretion of prolactin. Continued low dopamine levels after ovulation are maintained by high progesterone, contributing to the plateau phase.[7,15] Interestingly, this proestrous prolactin surge coincides with the preovulatory LH surge in rats.[5,6,16] One possible common mediator is kisspeptin, which, in addition to having an essential role in induction of the LH surge, can increase prolactin secretion in the presence of high estradiol.[17] As a consequence, it has been shown to play a role in the peak phase of the proestrous prolactin surge.[17]

Because of the complexity of signals involved, the proestrous prolactin surge potentially represents a good model to investigate factors that stimulate prolactin secretion from the pituitary gland. In species other than rats, however, this surge is much less characterized, and variability in the pattern of prolactin secretion has been observed across different species[1]; For example, sheep show two prolactin surges, peak concentrations for the first surge are evident coinciding with the LH surge then again 3–5 h later after onset of estrus[18,19] and humans do not appear to have a mid-cycle rise in prolactin associated with ovulation.[20,21] Because of the prevalent use of mice as a biomedical research model, and the availability of various genetic tools, mice represent an important model for investigating neuroendocrine function. Several studies have previously investigated the pattern of prolactin secretion during proestrus in mice, but there are marked inconsistences relating to the occurrence and/or timing of a rise in prolactin levels.[22–25] Yanai and Nagasawa did not observe a proestrous rise in prolactin levels in C3H/He mice, instead they recorded the highest prolactin levels during the late afternoon of diestrus.[26] A study by Michael using mice of a random breed stock found prolactin levels begin to increase from baseline from 1100 h on proestrus, reaching peak concentration at 1900 h and returning to baseline by 0900 h on estrus.[23] DeLeon et al. recorded low prolactin levels throughout the cycle with the possible exception of late proestrus or early estrus where a small increase in the concentration of plasma prolactin was reported.[27] Similarly, Sinha et al. observed generally higher levels of prolactin during proestrus in the reproductive cycle and levels were higher at the single time point (1400 h) taken in the afternoon as compared to the single morning time point (1000 h).[28] It is possible that the variability in these studies reflects strain-specific differences in prolactin secretion. Another possibility is that patterns of secretion were missed because earlier investigations were unable to use longitudinal sampling methods to monitor prolactin secretion across time in the same animals. With the development of tail tip sampling methods[29–31] and a highly-sensitive ELISA capable of detecting prolactin in small samples (<5 μl whole blood),[32] serial blood sampling in mice is now quite possible.[30,33] Thus, here we re-investigated the pattern of prolactin secretion during proestrus using serial blood sample collection in individual mice to provide clarification of the secretion profile during the reproductive cycle. To determine whether strain differences might be present, we have compared two different common laboratory strains, one inbred (C57BL/6J) and one outbred (Swiss Webster).

2 | MATERIALS AND METHODS

2.1 | Animals

Adult female virgin C57BL/6J ($N = 34$) and Swiss Webster ($n = 10$) mice, aged 12–14 weeks were sourced from the University of Otago's colony housed at the Taieri Resource unit, Dunedin, New Zealand. All mice were group housed under controlled environmental conditions (temperature 22°C ± 1°C, lighting (12 h light, 12 h dark cycle, lights on at 0700 h and off at 1900 h) and had free access to food and water at all times. The University of Otago Animal Ethics Committee approved all experimental procedures (D36/17).

2.1.1 | Experiment 1

C57BL/6J mice ($N = 34$) were maintained under conditions described above. Mice were habituated to sample collection procedures in accordance with previously described methods.[29,30] This involved 5–10 min of training for tail tip bleeding and general handling daily for at least 3 weeks. Daily vaginal smears were collected throughout the habituation period and duration of experiment. Cytological examination of smears from individual mice was used to stage the estrous cycle and only mice showing regular 4–5 day estrous cycles were included in this study. Based on the pattern of proestrous prolactin secretion in rats, we concentrated our sampling around the known timing of the LH surge and defined an expected "surge" as a rapid (within 1–2 samples) elevation in plasma prolactin levels exceeding 2 standard deviations above the presurge baseline (surge onset), with an expected amplitude of >2.5 fold from baseline. We anticipate elevated levels will be maintained for at least 12 h and a gradual decline to baseline levels will occur over 5–6 h. Presurge baseline was calculated from the mean of the previous six measurements of plasma prolactin levels taken during the light phase on diestrus. Starting at 0900 h, whole blood samples (4 μl) were taken from the tail tip of C57BL/6J ($n = 14$) mice at specific time points (0900, 1200, 1500, 1700, 1800, 1900, 2200, and 0900 h) during diestrus and proestrus of the reproductive cycle. The tail tip was cut off with a scalpel blade <0.5 mm from the end of the tail. The tail was then gently squeezed to release a drop of blood from the tip, and a sample (4 μl) was collected with a pipette. After the sample was collected, any bleeding was stopped by gentle pressure on the tip of the tail with a gauze pad. Subsequent samples could easily be collected by wiping the tip of the tail with a damp gauze, and again gently squeezing the tail

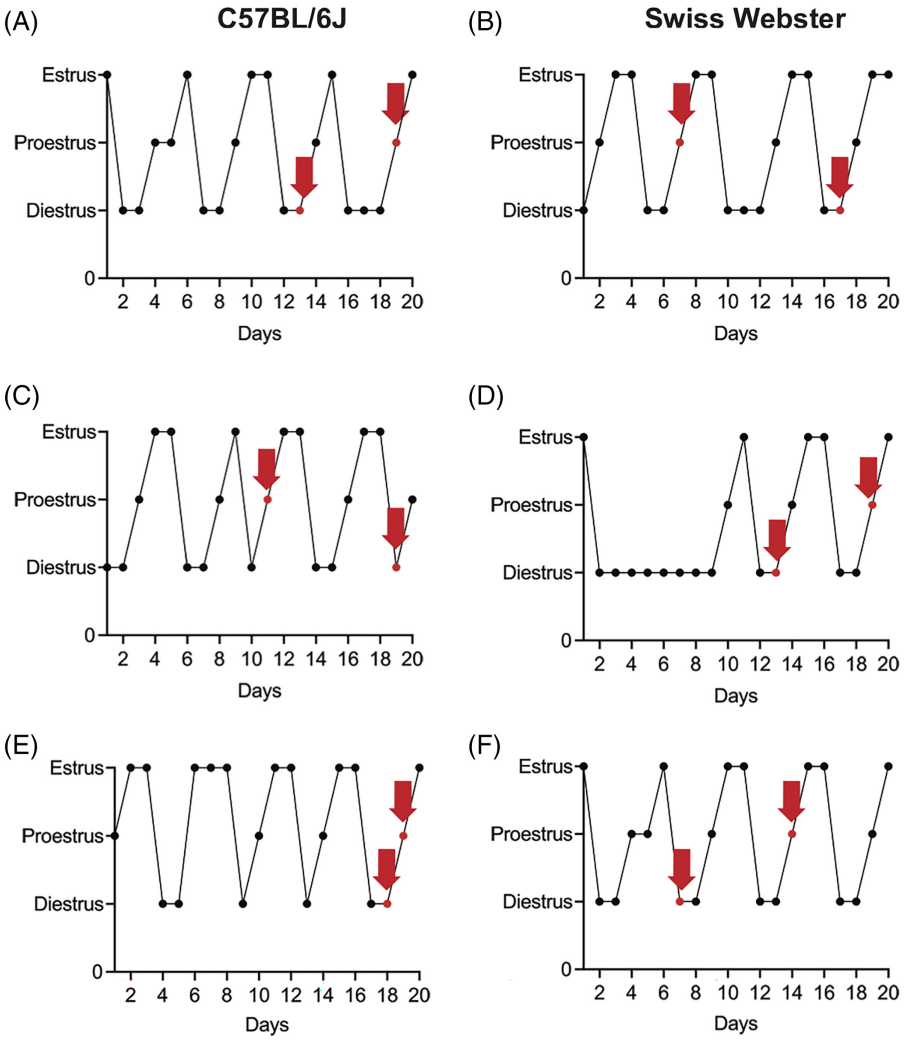

FIGURE 1 Examples of estrous cycles in C57BL/6J and Swiss Webster Mice. A, C, and E show regular 4–5 day estrous cycles in C57BL/6J mice. B and F show examples of regular cycling Swiss Webster mice. D shows the estrous cycle in a Swiss Webster mouse that has undergone a prolonged period in diestrus (8 days, pseudopregnancy) then undergone two regular estrous cycles. Red arrows and dots represent days during the cycle in which blood samples for measurements of prolactin and LH were taken. A–D, and F show tail tip blood sampling occurring on either diestrus or proestrus across subsequent cycles and E shows when tail tip blood samples were taken continuously through diestrus and proestrus

(see Steyn et al., for a full description of this sampling protocol[29]). Blood samples ($n = 14$ mice) at the specified time points described above were taken in either diestrus or proestrus (day selected at random), then in the alternate stage during the subsequent estrous cycle ($n = 8$), or continuously during diestrus and proestrus in a single cycle ($n = 6$) (Figure 1). Whole blood samples were immediately diluted 1:20 in 0.01 M phosphate buffered saline with 0.05% Tween-20 (PBS-T) and placed short-term on dry ice prior to longer term storage at −80°C. For trunk blood collection C57BL/6J mice ($n = 20$) were decapitated at either 0900 h ($n = 10$) or 1900 h ($n = 10$) on the day of proestrus. Samples were collected into heparinized 1.7 ml microfuge tubes, centrifuged and plasma collected and stored at −20°C.

2.1.2 | Experiment 2

Tail tip blood samples were also taken from Swiss Webster mice ($n = 10$) exposed to the conditions and handling regime described above. As in experiment 1, daily vaginal smears were collected during the habituation period and cytological examination performed to identify stages of the estrous cycle. Sampling was performed at specific time points during either diestrus or proestrus (day selected at random during the first sampling period) of subsequent reproductive cycles (Figure 1). Firstly, during diestrus of the first cycle and then during proestrus in the next cycle or vice versa (Figure 1). Samples were stored as previously described.

2.2 | ELISAs

Prolactin[32] and LH[30,33–35] were measured by ultra-sensitive ELISAs as reported previously. In brief, high affinity binding plates (96 well, Corning 9018) were coated with 50 µl of capture antibody diluted in 0.01 M PBS overnight at 4°C (PRL: Guinea pig anti-rat PRL (National Institute of Diabetes and Digestive and Kidney Diseases-National Hormone and Pituitary program [NIDDK-NHPP]), AFP65191, 1:2500; LH antibovine LHβ subunit 518B7 (University of California), 1:1000). The capture antibody was decanted and wells incubated with blocking buffer (5% w/v skim milk powder in PBS-T) to alleviate nonspecific binding of the capture antibody for 2 h at room temperature (RT). Mouse reference standards (PRL: 4 µg/ml, AFP6476C, NIDDK-NHPP; LH: 4 µg/ml, AFP5306A, NIDDK-NHPP) were used to generate standard curves ranging from 20 to 0.019 ng/ml (PRL) and 4 to

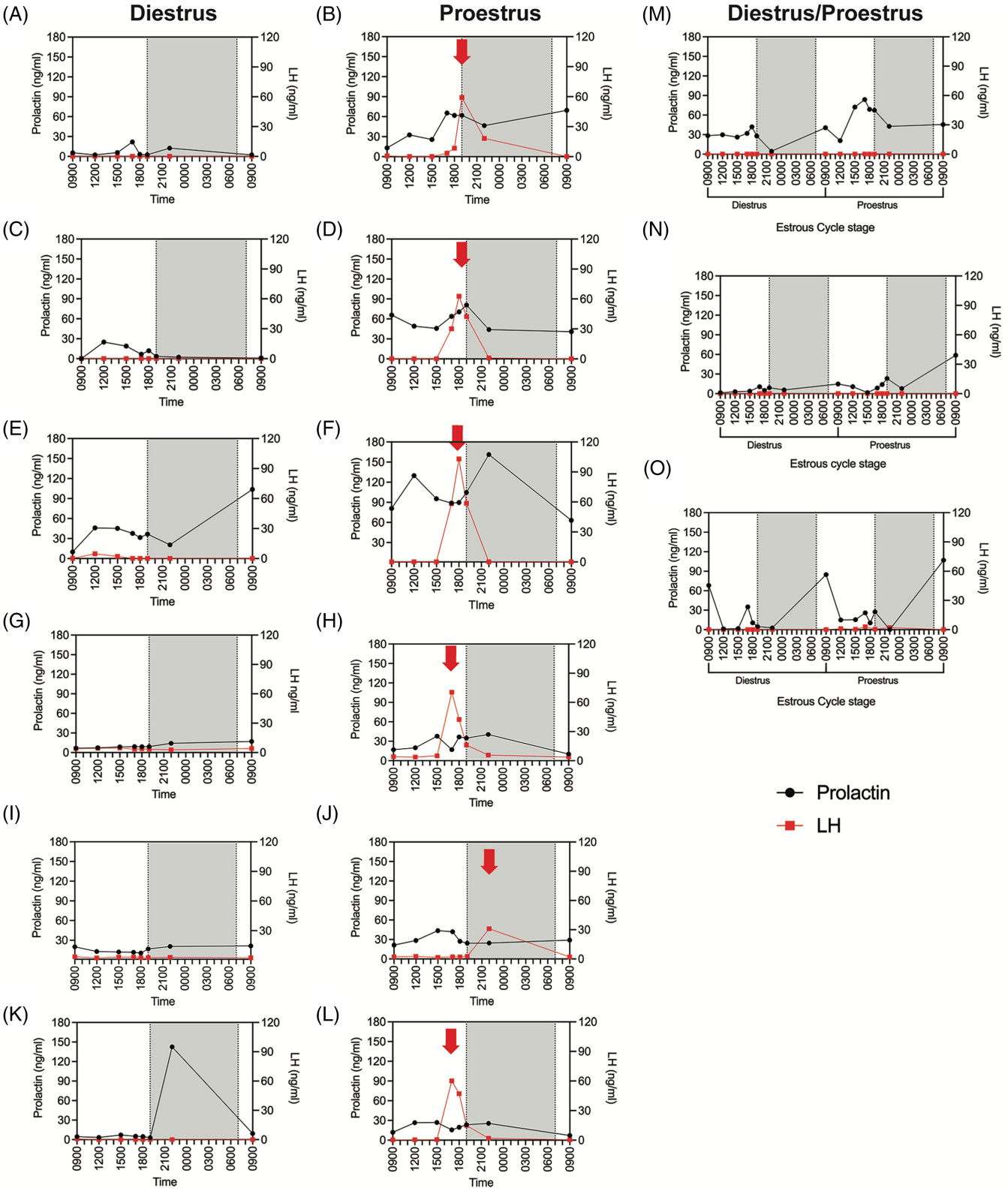

FIGURE 2 Examples of levels of circulating prolactin and LH in whole blood samples collected by the tail tip method in individual C57BL/6J and Swiss Webster mice during diestrus and proestrus of the reproductive cycle. Repeated tail blood sampling in individual C57BL/6J (A–F) and Swiss Webster (G–L) mice during either diestrus or proestrus in a single cycle. (M–O) Prolactin and LH secretion profiles from single C57BL/6J mice in which blood samples were collected over two consecutive days of a single estrous cycle. Red arrow indicates maximum LH levels recorded during proestrus

0.002 ng/ml (LH) by dilution in 0.2% (w/v) bovine serum albumin (BSA) in PBST. Standards and blood samples (1:20 (PRL); 1:40 [LH]) were loaded into appropriate wells and incubated (PRL: overnight at RT; LH for 2 h at RT). Following decanting 50 µl of detection antibody (PRL: Rabbit anti-mouse PRL, 1:50,000, AFP131078, NIDDK-NHPP; LH: Rabbit anti-mouse LH, 1:10,000, AFP240580Rb, NIDDK-NHPP) was loaded into wells and left to incubate for 1.5 h at RT. Following decanting and washing with PBS-T, wells with bound substrate were incubated with 50 µl of horseradish peroxidase (HRP)-conjugated antibody (PRL: Amersham ECL Rabbit IgG, HRP-linked Ab (from donkey), NA934, GE Healthcare Life Sciences, 1:2000; LH: Rabbit IgG, HRP-linked (from goat), P0448, DAKO Cytomation, 1:1000). After a 1.5 h incubation, 100 µl *o*-Phenylenediamine dihydrochloride (P7288, Sigma-Aldrich) diluted 1 mg/ml in citrate buffer (9.42 g of $C_6H_8O_7$ (anhydrous), 14.48 g Na_2HPO_4 (anhydrous) in 1 L ddH_2O) containing 0.05% H_2O_2 was loaded into all wells and incubated for 0.5 h at RT. The reaction was stopped with 3 M HCL and absorbance of each well read at a wavelength of 490 nm (PRL and LH) and 650 nm (LH). Intra- and interassay coefficients of variation for both PRL and LH were <10% and <15%, respectively.

2.3 | Data analysis

Prolactin and LH concentrations collected by tail tip sampling at specific time points during diestrus and proestrus were analysed by a mixed-model analysis of variance with repeated measures, using time and stage of cycle as factors, with post hoc comparisons using a Šídák's multiple comparisons test. Area under the curve (AUC) was also employed to determine the overall level of prolactin on each cycle day using the trapezium rule formula allowing for the inclusion of nonuniform points on the x-axis. Results from area under the curve were analysed with an unpaired two-tailed Student's *t* test. A Shapiro–Wilk normality test was performed prior to analysis. Prolactin and LH levels obtained from trunk blood samples were checked for normality using a D'Agostino and Pearson test and analysed by an unpaired two-tailed Student's *t* test and Mann Whitney U test, respectively. All statistical analyses were performed using GraphPad Prism 8 Software (GraphPad software, www.graphpad.com), where $p < .05$ was considered a statistically significant difference. Baseline was defined as mean prolactin levels observed during the day of diestrus in the reproductive cycle.

3 | RESULTS

Both C57BL/6J and Swiss Webster mouse strains showed 4–5 day estrous cycles (Figure 1); however, interestingly, 40% of the Swiss Webster mice used in the study also showed intermittent prolonged periods in diestrus (pseudopregnancy) lasting 8 days before returning to consecutive 4–5 day cycles (Figure 1D). Circulating prolactin levels were generally low during diestrus in both mouse strains, mean: 21 ± 3 ng/ml (C57BL/6J), 14 ±1 ng/ml (Swiss Webster) (Figures 2 and 3). Two individual Swiss Webster mice showed an acute increase in prolactin levels at 2100 h on diestrus, with levels recorded of 143 ng/ml (Figure 2K) and 173 ng/ml (not shown), respectively.

During proestrus, levels of circulating prolactin in C57BL/6J mice were higher ($n = 8$, $p = .0065$, AUC, unpaired two-tailed *t* test) and the 2 days of the cycle showed no specific time point differences, however, a significant effect of day of the cycle was apparent ($n = 8$, $p = .0122$, $F[1.000, 7.000] = 11.24$, mixed effects repeated measures analysis, Figures 2–4). There was large individual variability between animals (Figure 2A–F), and while the mean data did show an apparent peak at 1900 h (Figure 3A), this was somewhat artificial as the actual maximal value was seen at a different time in each animal (Figure 2B, D,F). The consistent observation was that levels were higher during

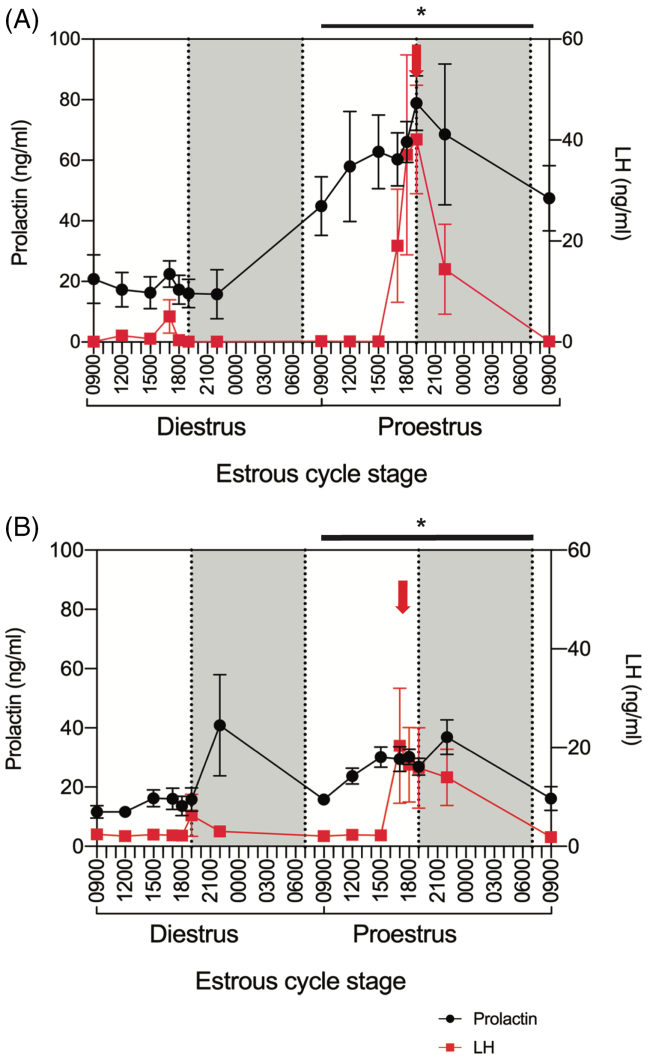

FIGURE 3 Mean prolactin and LH levels obtained via tail tip blood sampling during diestrus and proestrus. Levels of prolactin show a significantly different pattern of change across the day of proestrus as compared to diestrus in both C57BL/6J (A, $n = 8$, $p = .0122$, F(1.000 (DFn), 7.000 (DFd)) = 11.24) and Swiss Webster mice, (B, $n = 10$, $p = .0125$, F(1.000 (DFn), 105.0 (DFd)) = 6.459). A mixed effects analysis of repeated measures was used for analysis and results presented as mean ± SEM, *Significant ($p < .05$) with respect to prolactin profile during diestrus levels. Red arrow indicates mean maximum LH levels

proestrus than diestrus, and this is perhaps best reflected in the AUC analysis (Figure 4A). It should be acknowledged that our sampling strategy may have missed much shorter duration peaks, or potentially peaks occurring later during the night of proestrus (after 2200 h). By 0900 h on estrus, levels had typically returned or were projecting downward to basal levels similar to those seen on diestrus (Figure 2B, D,F). Additionally, when values in individual C57BL/6J animals were assessed there was wide variability in patterns of secretion and no clear evidence of a defined peak linked to the time of the light–dark cycle (Figure 2B,D,F).

Proestrous prolactin levels in Swiss Webster mice showed a similar secretion pattern to that observed in C57BL/6J mice, particularly in relation to variability in individual prolactin profiles (Figure 2H,J,L). Swiss Webster mice, like C57BL/6J mice showed a significant effect in the daily profile of prolactin secretion between diestrus and proestrus stages of the cycle ($n = 10$, $p = .0125$, $F[1.000, 105.0] = 6.459$, mixed effects repeated measures analysis, Figure 3B); and the 0900 h ($p = .0229$), 1700 h ($p = .0451$) and 1800 h ($p = .0210$) time points were significantly different between the two cycle stages. Overall Swiss Webster mice are exposed to higher prolactin levels during the day of proestrus as compared to diestrus ($n = 10$, $p = .0134$, AUC, unpaired two-tailed t test, Figure 4B).

Compared to the broad rise in prolactin seen during proestrus, there was a relatively well-defined LH surge observed in the evening of proestrus in both strains of mice, peaking around the time of lights off (Figure 3). Some variability was observed in individual mice in relation to timing of initial elevation in LH levels (Figure 2B,D,F,H,J,L), occurring between 1700 h and 2100 h and highest LH levels

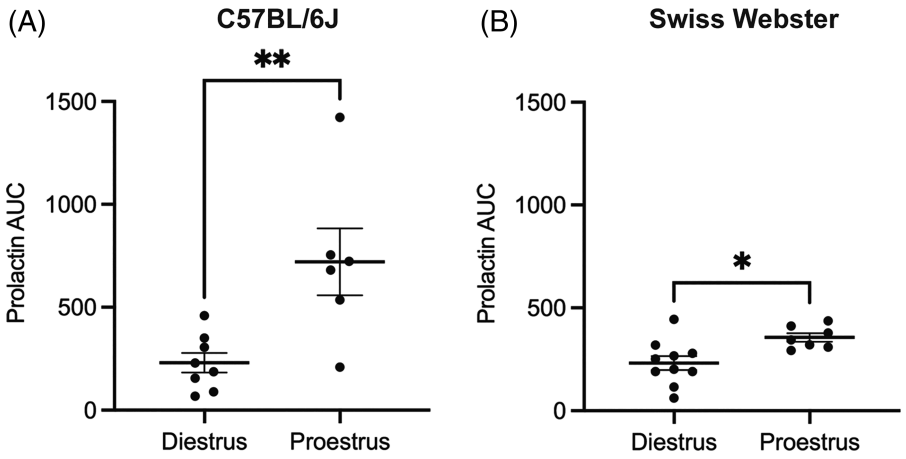

FIGURE 4 Circulating prolactin during the light phase of diestrus and proestrus stages of the mouse estrous cycle. The overall level of prolactin mice are exposed to during the light phase is higher in proestrus as compared to diestrus in both C57BL/6J (A, $n = 8$, $p = .0065$, unpaired t test) and Swiss Webster (B, $n = 10$, $p = .0134$, unpaired t test) mice. Results presented as mean ± SEM. $^{*}p < .05$, $^{**}p < .01$. Black dots represent AUC for individual mice

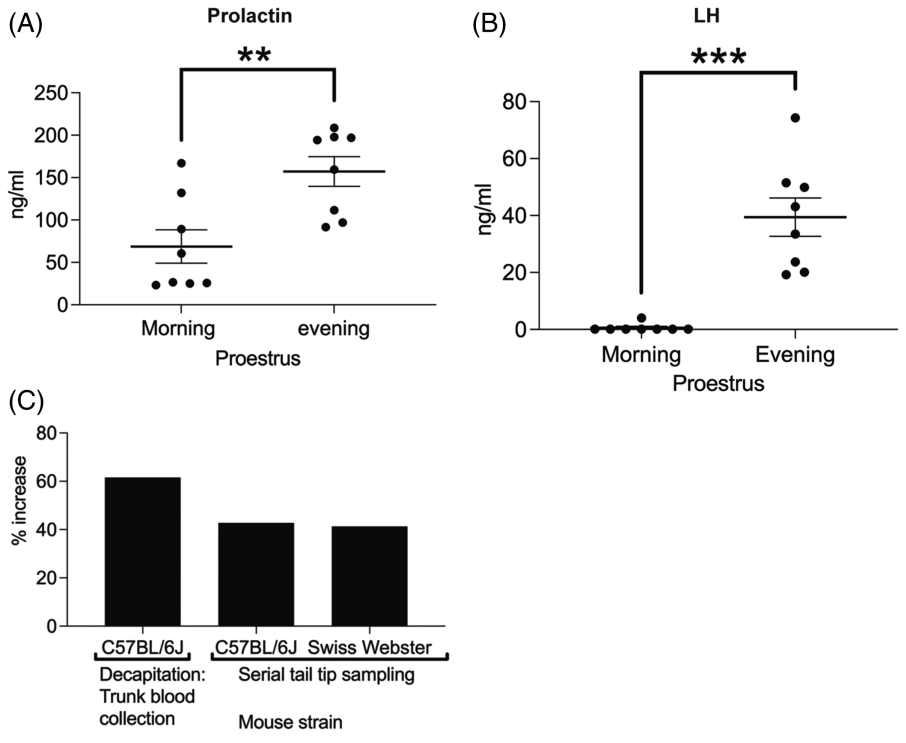

FIGURE 5 Prolactin (A) and LH (B) levels obtained via trunk blood collection following decapitation during proestrus. (A) Mice are exposed to higher prolactin levels in the evening of proestrus as compared to the morning ($n = 8$, $p = .0047$, Student's t test ($t = 3.354$, df = 14). (B) Shows a significant difference in LH levels ($n = 8$, $p = .0002$, Mann Whitney U test) on the evening of proestrus as compared to the morning. Results presented as mean ± SEM. ** $P < .01$, *** $P < .001$. Black dots indicate levels of LH and prolactin in individual mice. (C) Percentage increase in prolactin (ng/ml) levels taken from mean levels measured by the different sampling methods (tail tip sampling and trunk blood collection) at 0900 h (morning) and 1900 h (evening) on proestrus

recorded, ranged from 40 to 103 ng/ml in C57BL/6J mice and 12–70 ng/ml in Swiss Webster mice. This LH surge was observed in all mice that had tail tip blood samples taken through proestrus only (Figure 2B,D,F,H,J,L). Surprisingly, no increase in LH levels was evident in C57BL/6J mice subjected to repetitive blood sampling over 48 h throughout both diestrus and proestrus in a single cycle (Figure 2M–O), perhaps due to stress from the extended period of sampling. Consequently, these mice were excluded from the overall analysis, as were any proestrus measurements of prolactin and LH taken from mice in which an LH surge was not detected when tail tip blood sampling was performed in a single cycle stage across subsequent cycles (C57BL/6J $n = 2$, Swiss Webster $n = 3$ excluded).

To determine whether the hormone levels being measured in the serial blood samples were comparable to those seen in acutely killed animals, we also measured serum hormone levels in additional groups of animals that were euthanized by decapitation with collection of trunk blood (Figure 5). These samples showed significantly increased prolactin levels in the evening compared to the morning of proestrus ($n = 8$, $p = .0047$, unpaired Student's t test ($t = 3.354$, df $= 14$), but overall levels were somewhat elevated compared to the serial samples (Figure 5A,C). Trunk blood samples also showed clear evidence of an LH surge in the evening of proestrus, compared to the morning ($n = 8$, $p = .0002$, Mann Whitney U test, Figure 5B), and levels were identical to that seen in the serial samples (Figure 3).

4 | DISCUSSION

This study showed a gradual and prolonged elevation in prolactin levels during proestrus in mice, validated by both tail tip blood (Figure 3) and trunk blood collection (Figure 5). The pattern of secretion is distinct from the well-defined surge observed in rats that is tightly linked to the light–dark cycle, and occurs only in the afternoon of proestrus,[3–7] largely coincident with the LH surge. Interestingly, this prolonged rise in prolactin levels tracks rising estradiol levels occurring during the day of proestrus in the rodent.[5] Prolactin levels remained high throughout the day of proestrus and even into the early morning of estrus, in a strain-dependent manner (Figure 3). In rats, the proestrous prolactin surge consists of a peak, plateau and termination phase.[7] The transition through these phases is thought to be controlled by a shift from a non-dopaminergic mechanism involving one or more prolactin releasing factor(s) inducing increased prolactin levels during the peak phase; high progesterone post-ovulation maintains low dopamine levels during the plateau phase; then rising dopamine levels (dopaminergic mechanism) curtail the plateau phase and induce termination of the surge.[7] These phases observed during the proestrous prolactin surge in rats are not clearly defined in our study using mice and suggest the proestrous prolactin rise in mice may not be as tightly controlled as that observed in rats. The prolonged nature of the prolactin rise during proestrus in our study can be defined as a gradual rise and then termination. The lack of defined surge or a plateau phase in mice suggests the dynamics of the prolactin rise on proestrus are inherently different and perhaps a single-phase mechanism predominates the change in prolactin secretion. It seems likely this might be driven by a direct action of estradiol to promote prolactin synthesis in the pituitary gland,[36] facilitated by a gradual decrease in dopaminergic inhibition. Estradiol influence on dopamine levels in mice may cause the slow rise in prolactin levels during the morning/early afternoon of proestrus, then subsequent high progesterone levels after ovulation occurring in the late afternoon/early evening of proestrus,[37] may lower remaining dopamine levels further. The function of this proestrous rise remains uncertain, but recent work has identified a potential role for prolactin in olfactory function that might influence female interest in males during proestrus.[38]

Interestingly, prolactin levels in C57BL/6J mice were greater than those recorded in Swiss Webster mice (Figure 2). This observation supports a previous study in which prolactin concentrations in C57BL/6J mice were higher than C3H/St mice across all stages of the estrous cycle,[28] highlighting apparent strain differences. Higher basal prolactin concentrations have been observed in male mice as compared to male rats. Species differences in the functional connectivity of TIDA neurons is thought to be responsible for this[39] and may also contribute to the aforementioned female mouse strain differences and in fact potentially the overall species difference in proestrous prolactin secretion patterns between rats and mice.

In C57BL/6J mice, the mean peak concentrations of prolactin coincide with the timing of the LH surge (Figure 2). When evaluated in the individual animals, however, this was not evident (Figure 1), suggesting that there are largely independent mechanisms driving the two hormones during proestrus. In rats, surges of prolactin and LH occur concordantly during proestrus.[11] They are entrained to the environmental light–dark cycle via combined actions of a circadian signal and estradiol positive feedback.[11,40–44] Melatonin, a hormone produced in large quantities by the pineal gland during the dark phase may contribute to circadian regulation of the LH surge.[43–45] In rats, pinealectomy affects time of surge onset and extending photoperiod on the day of proestrus leads to a delay in termination of the surge; both of which can be corrected with melatonin treatment.[45,46] Additionally, female rats fail to show an LH surge when treated with melatonin during the late afternoon of proestrus.[45] A large number of laboratory mouse strains, however, including C57BL/6J, have low melatonin levels (potentially selected against in laboratory breeding due to its inhibitory effect on reproduction).[47] A natural point mutation in arylalkylamine*N*-acetyltransferase (AANAT), an enzyme required for acetylation of serotonin in the melatonin synthesis pathway, leads to reduced melatonin production in C57BL/6J mice.[47] This may explain why maximum LH levels were recorded over a sampling window of 5 h in individual C57BL/6J mice in the current study; as melatonin levels may not be at a threshold concentration to allow tight temporal regulation in mice. Similarly, it is possible that low melatonin production may be a factor contributing to the absence of a tightly defined surge in prolactin during proestrus in mice. Both C57BL/6J and Swiss Webster mouse strains used in the current study show comparable melatonin levels, despite their inbred and outbred strain status, however, their peak concentrations of melatonin during the dark phase vary, occurring around 0015 and 0400 h respectively.[48,49]

When we completed blood sampling from the tail tip of C57BL/6Jmice over a 48h period through both diestrus and proestrus, animals

failed to exhibit an LH surge in the expected time frame. In contrast, the LH surge could be easily detected in animals that were sampled only on the day of proestrus, or in trunk blood samples from decapitated mice. This is likely to be a stress effect. Previously, Wagenmaker and Moenter (2017) demonstrated that the preovulatory LH surge could be blocked in mice by a 5 h acute layered stress paradigm given mid-morning during proestrus.[50] This involved transferal to a novel cage, a change in environment in the novel cage, followed by restraint and exposure to predator odors.[50] Although our animals were habituated to sampling procedures, it seems that repeated exposure to short duration handling and blood sample collection over 48 h through diestrus and then proestrus caused a disruption of the preovulatory LH surge. A longer sampling window, however, would be required to clarify whether the LH surge is abolished or delayed in this study.

In summary, mice show a prolonged rise in prolactin levels on the day of proestrus, rather than a defined surge that is entrained to the light/dark cycle as seen in rats. There is considerable variation in the pattern of secretion in individual animals. Strain differences are evident in the timing of this rise, specifically when the initial increase occurs, the timing of peak concentration and the subsequent time taken to lower to baseline. The overall profile of elevated prolactin during proestrus compared with diestrus, however, was conserved between the two strains of mice. While the serial sampling methodology may not be suitable for long-term studies over multiple days, this study does suggest that this approach is suitable for defining patterns of hormone secretion within a single day.

AUTHOR CONTRIBUTIONS

Hollian R Phillipps: Conceptualization; data curation; formal analysis; investigation; methodology; project administration; resources; software; validation; visualization; writing – original draft; writing – review and editing. **Zin Khant Aung:** Investigation; writing – review and editing. **David R. Grattan:** Conceptualization; funding acquisition; supervision; writing – review and editing.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

Raw data for prolactin and LH levels during proestrus and diestrus in mice available in the Dryad Digital Repository: doi: 10.5061/dryad.rjdfn2zd4.

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