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Spontaneous voiding by mice reveals strain-specific lower urinary tract function to be a quantitative genetic trait

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Yu W, Ackert-Bicknell C, Larigakis JD, MacIver B, Steers WD, Churchill GA, Hill WG, Zeidel ML. Spontaneous voiding by mice reveals strain-specific lower urinary tract function to be a quantitative genetic trait. *Am J Physiol Renal Physiol* 306: F1296–F1307, 2014. First published April 9, 2014; doi:10.1152/ajprenal.00074.2014.—Lower urinary tract (LUT) symptoms become prevalent with aging and affect millions; however, therapy is often ineffective because the etiology is unknown. Existing assays of LUT function in animal models are often invasive; however, a noninvasive assay is required to study symptom progression and determine genetic correlates. Here, we present a spontaneous voiding assay that is simple, reproducible, quantitative, and noninvasive. Young female mice from eight inbred mouse strains (129S1/SvImJ, A/J, C57BL/6J, NOD/ShiLtJ, NZO/H1LtJ, CAST/EiJ, PWK/PhJ, and WSB/EiJ) were tested for urination patterns on filter paper. Repeat testing at different times of the day showed minimal within-individual and within-strain variations, but all parameters (spot number, total volume, percent area in primary void, corner voiding, and center voiding) exhibited significant variations between strains. Calculation of the intraclass correlation coefficient, an estimate of broad-sense heritability, for each time of day and for each voiding parameter revealed highly significant heritability [spot number: 61%, percent urine in primary void: 90%, and total volume: 94% (afternoon data)]. Cystometrograms confirmed strong strain-specific urodynamic characteristics. Behavior-voiding correlation analysis showed no correlation with anxiety phenotypes. Diagnostically, the assay revealed LUT symptoms in several systems, including a demonstration of voiding abnormalities in older C57BL/6J mice (18–24 mo), in a model of protamine sulfate-induced urothelial damage and in a model of sucrose-induced diuresis. This assay may be used to derive pathophysiological LUT readouts from mouse models. Voiding characteristics are heritable traits, opening the way for genetic studies of LUT symptoms using outbred mouse populations.

animal model; bladder; heritability; lower urinary tract symptoms; micturition

LOWER URINARY TRACT (LUT) symptoms (LUTS) are a range of problems associated with storage, voiding, and/or discharge of urine that become increasingly prevalent with aging. LUTS includes overactive bladder, stress incontinence, overflow incontinence, urinary frequency, urgency, and pelvic pain, symptoms afflicting millions of Americans with attendant human and financial costs. Because LUTS is heterogeneous and poorly understood, treatment is empiric and of limited effectiveness. To improve treatment, it is necessary that we begin to understand the causes and, in particular, the molecular, cellular, and neural mechanisms that predispose to initial symptoms and then worsen with aging. Treatment for these disorders is likely to improve dramatically once we have a better understanding of the pathophysiological mechanisms in specific patient groups.

Two of the usual approaches to understanding mechanisms of disease, human genetics and animal models, have been unavailable or relatively uninformative in LUTS. Because LUTS occurs in middle and old age, it takes the greater part of a human lifetime to develop. Add to this the uncomfortable reality that it is inherently embarrassing, and it becomes clear why LUTS is markedly underreported both to medical professionals and family members. Therefore, LUTS phenotypes are extremely difficult to track within families. Furthermore, mechanistic understanding of the causes of LUTS and its development is hampered by the lack of good animal models. While it is a simple matter to induce bladder injury or inflammation in rodents, the degree to which such models recapitulate LUTS in humans is questionable. To develop an approach to gaining insights into the genetic basis for predisposition to LUTs, we sought an assay that would allow us to observe the emergence of naturally occurring age-related LUTS in mice.

Other investigators have noted the utility of observing urine spots deposited by mice on filter paper (14, 21, 28), particularly in investigations of social behavior (4) or in genetically modified animals (11, 14). However, it is not clear how reproducible spotting patterns might be in individual mice or within inbred strains and, therefore, whether they represent a useful measure of LUT function for population studies. This question motivated us to begin to examine more closely the reliability and reproducibility of urine spot deposition. In early studies, we noticed that C57BL/6J mice tended to urinate repeatedly in one or two discrete locations over a period of several hours. It was usually at the edge or in the corner of the cage, suggesting conscious volition and urinary continence. Intriguingly, mice engineered with conditional deletion of urothelial integrins not only exhibited abnormal cystometrograms (CMGs; intravesical pressure measurements) with evidence of detrusor overactivity but also voided haphazardly in numerous locations, suggesting an inability to control voiding, i.e., a form of incontinence (17). Jointly, these observations indicated that impairment to the signaling elements that control storage and voiding reflexes led to a failure of voiding function, which could be detected and to a large degree quantitated in voiding patterns and in CMGs.

The clear advantage of assaying void patterns on filter paper compared with CMGs is that the animal is unharmed by the test, can be retested with or without experimental interventions, and followed for its entire lifetime. To investigate rigorously the reproducibility of voiding patterns and determine whether

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they represented a reliable assay of LUT function, we analyzed spontaneous voiding patterns in female mice of eight genetically distinct but inbred strains of mice for reproducibility, strain effects, and diurnal effects. The strains chosen for testing were the eight founder strains of the Collaborative Cross (CC) population, a large panel of new recombinant inbred mouse strains that was designed to address some of the perceived shortcomings of available mouse strain resources for genetic mapping (6). Since the CC founder strains have also been extensively phenotyped for behavioral and metabolic parameters (20), there exists a large body of valuable information that can be compared with the voiding data to identify potential mechanistic correlations.

MATERIALS AND METHODS

Mice. Mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The strains tested were as follows: 129S1/SvImJ, A/J, C57BL/6J, NOD/ShiLtJ, NZO/H1LtJ, CAST/EiJ, PWK/PhJ, and WSB/EiJ. These eight strains were chosen because they are the founder strains for both the CC and Diversity Outcross (DO) populations of mice developed for indepth genetic mapping (6). Phenotype information on each strain may be found at http://jaxmice.jax.org/strain/000646.html where the number (in this example 000646) is The Jackson Laboratory stock number for the strain. In previous studies of voiding patterns, behavioral issues such as dominance, aggression, and stress were found to alter voiding patterns markedly (4, 24, 32). Issues of dominance and fighting are far more prevalent in male mice than in female mice. In addition, male mice are known to mark territory with their urine (15, 21, 22). Therefore, to avoid these potential confounding variables, experiments were performed in female mice of 6–16 wk of age. All animal experiments were carried out in strict adherence with National Institutes of Health guidelines for animal care and use and with the approval of the Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center.

Spontaneous void spot assay. Individual mice were gently removed from standard polycarbonate "shoebox"-type communal cages and placed in a fresh identical plastic cage with precut Whatman grade 540 acid-hardened filter paper (catalog no. 1540-320) taped to the bottom. While Whatman no. 1 paper can be used, some mice aggressively tear/eat the paper, resulting in data loss. We found that grade 540 paper had greater resistance to tearing. The paper was cut to dimensions of 28.5 × 17.5 cm, which is slightly larger than the bottom area of the cage, allowing tape to be placed on filter paper that encroaches partially up the sides of the cage. We found it was sufficient to place tape around the corners or to tape corner to corner on two edges to secure the paper. The corners were rounded off with scissors to conform to the cage shape before taping. Enrichment in the form of a 1-in. square nestlet was also added to the cage to diminish the incidence of filter destruction. Mice were provided with standard dry mouse chow for the duration of the assay, but water was withheld during the testing period. Water dripping from bottles was found to impair the integrity of the filter paper and altered the urine spot dimensions. The micturition cages were then kept in a quiet area for 4 h (the duration of the testing period per day). The placement of mice into and removal from cages was done calmly to avoid startling the animal and causing reflex urination. At the end of the study period, animals were returned to their normal housing, the filter paper retrieved from the cage, and urine spots on the filter paper imaged using ultraviolet light on a transilluminator. Since mice were maintained in a 12:12-h light-dark cycle facility, the night testing was performed in the dark with the aid of a small red light for the placement of mice in cages and then later retrieval. During the 4-h test period, the light was extinguished. Figure 1*A* shows an example of a filter paper exposed to ultraviolet light. Filters were photographed and saved as grayscale TIFF images; images were analyzed using the Fiji version of ImageJ software (http://fiji.sc/wiki/index.php/Fiji).

Image analysis. Using ImageJ, multiple digital grayscale images were opened and converted to stacks for batch processing. Initially, the scale was set for the image based on the known length of the filter, the global option was checked, and a macro was then run that consecutively converted images to a stack, inverted, auto-thresholded using the "Max entropy" or "Yen" method, and converted to binary. Thresholded images in the stack were checked for artifacts and were individually corrected if thresholding had omitted areas of urine or highlighted tape marks. Using the "analyze particles" function, excluding all particles of <6.6 mm^2 (corresponding to 0.5 μl urine) faithfully captured urine spots while excluding small bright spots that might be due to claw or tooth marks. The results table, which contains the area of each individual spot and total number of spots, was exported as a flat file for further data processing. It should be noted that data extraction from these analyses is semiquantitative since overlapping urine spots are not counted separately and quantitation of repeat voids in the same area tend to underestimate the area and hence the volume of urine deposited. Figure 1*B* shows the ImageJ figure generated after thresholding of the image in Fig. 1*A* and particle analysis with the "show outline" option selected. From inspection, it appears there may be three separate voiding events at the *top right* and two events at the *bottom left*. However, the outline following the thresholding procedure does not distinguish these, and the data output is for two large spots. As a matter of convention, we designated the largest urine spot area as the "primary void" (Fig. 1*B*, arrow). Figure 1, *C* and *D*, show how the urine deposition pattern can be further refined to quantitate urine location, with Fig. 1*C* showing corner voiding (arbitrarily defined as 5% of the total filter area/corner) and Fig. 1*D* showing center voiding (arbitrarily defined as 40% of the total filter area in the center). Urine spot areas can be converted to volumes by construction of a standard curve that relates known volumes of mouse urine pipetted on the filter paper (Fig. 1*E*) to areas obtained from image analysis. Volume and area are linearly related up to 100 μl with $R^2 = 0.996$ (Fig. 1*F*). Figure 1 also emphasizes the sensitivity of the technique with 1 μl urine clearly visible.

CMGs. Cystometry was performed with PBS infusion (25 μl/min) as previously described (13, 17). Mice were anesthetized by a subcutaneous injection of urethane (1.4 g/kg from 250 mg/ml solution in PBS) 30–60 min before surgery. At the time of surgery, the mouse was further anesthetized with a continuous flow of isoflurane (3% induction, 1.0% maintenance). Once the pedal reflex was absent, a 1-cm midline abdominal incision was performed. Flame flanged polyethylene-50 tubing was implanted through the dome of the bladder with the use of a 25-gauge × 1.5-in needle (luer fitting removed) slid inside the tubing. Once the tubing was implanted, it was secured in place with 8-0 silk purse string surgical suture. The incision site was sutured closed around the tubing using sterile 5-0 silk, and mice were then placed into a Bollman mouse restrainer, where they were allowed to stabilize for 30–60 min. The catheter was connected to a pressure transducer (and syringe pump by side arm) coupled to data-acquisition devices (WPI Transbridge and AD Instruments Powerlab 4/35) and a computerized recording system (LabChart software). The bladder filling then commenced, after which voiding occurred naturally through the urethra. Analyses of bladder capacity, baseline and voiding pressures, and micturition efficiency were made for ~90 min per mouse.

Heritability estimates. We estimated variance components for strain and residual error by fitting a linear mixed model to each measured parameter including strain as a random effect. The intraclass correlation coefficient, an estimate of broad-sense heritability (H^2), was calculated for each time point (morning, afternoon, and night) and for each of the spontaneous void spot assay phenotypes (urine spot number, urine area in the primary void, urine area in the corners, urine area in the center, and total urine volume) using the "lme4" library in R (http://www.r-project.org, version 3.0.2). These heritability esti-

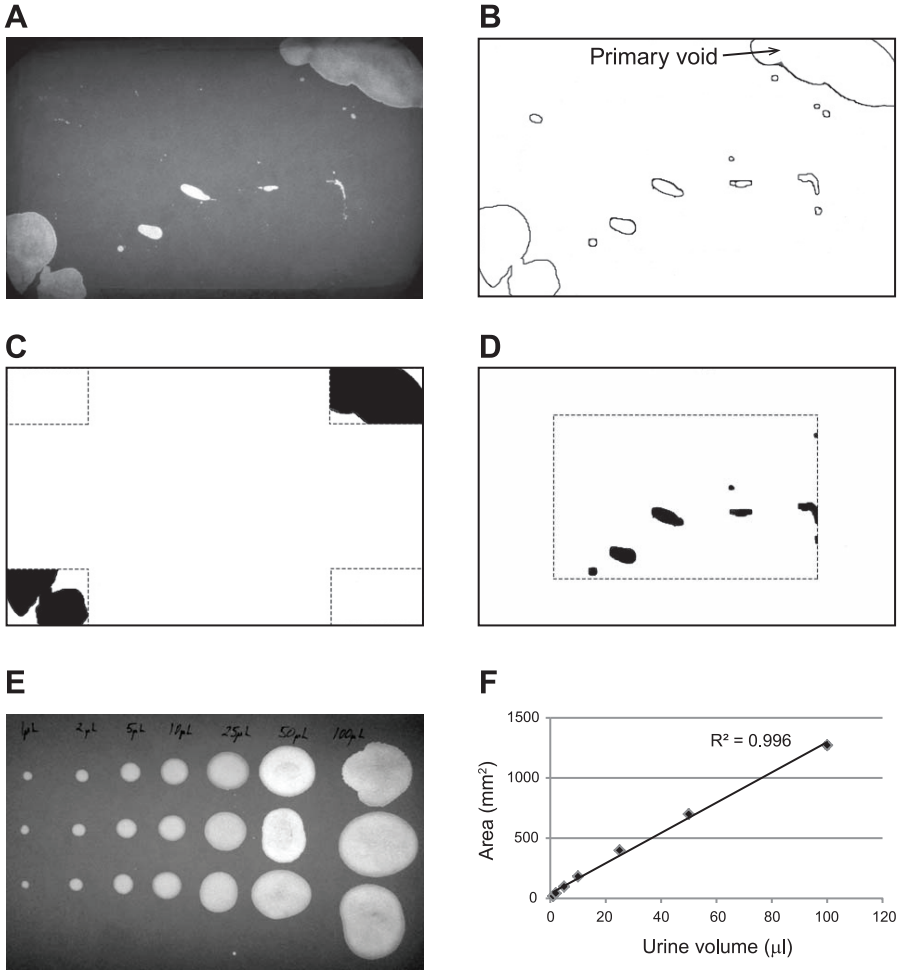

Fig. 1. Image processing of void spot filters. *A*: photographic image of a urine-stained filter paper on an ultraviolet illuminator. *B*: thresholded and outlined image. *C*: corner voids are indicated by dotted regions and represent a total of 20% of the filter paper area. *D*: center voids are indicated by the dotted region and represent 40% of the total area. *E*: controlled delivery of urine in different volumes (1, 2 5, 10, 25, 50, and 100 μl) onto filter paper repeated in triplicate. *F*: image processing and quantitation of the spot areas from *E* allowing construction of an area-to-volume standard curve.

mates were calculated using data collected on the CC founder strains, as shown in Fig. 3. In addition, the intraclass correlation coefficient was calculated for the phenotypes of "interval" and "pressure" using CMG-measured data from these same CC founder strains of mice (see Fig. 4).

Behavioral correlation analysis. Behavioral traits of CC founder mice, as quantified in Logan et al. (20), were compared with our derived voiding parameters to determine whether urination was primarily determined by behavioral responses. Pearson's correlations were run using AnalystSoft StatPlus (Vancouver, BC, Canada), which links to Microsoft Excel. Each voiding data set for each time point (morning, afternoon, and night) was run individually against the data shown in Table 2 (percent time in light of a light-dark box, percent time immobile in the open field, and visual cliff distance bottom ratio as measurements of anxiety) from Ref. 20. StatPlus produced *P* values.

RESULTS

Reproducibility of the assay. We tested four individual mice of eight strains on 5 consecutive days at each time of the day (morning: 9 AM–1 PM, afternoon: 1–5 PM, and night: 7–11 PM). Repeated testing of individual mice gave reproducible patterns. Figure 2, *top*, shows assays conducted on 4 consecutive days on two individual mice of different strains. Quantitative analysis of the voiding patterns of these two strains is shown in the graphs in Fig. 2, *bottom* (four mice of each strain, five assays on each). The data for each mouse were averaged and represents $n = 1$. The three parameters shown are the total urine spot number, percent area of urine present in the primary void, and total urine area on a filter. 129S1/SvImJ mice tended to void in a single location, which is then by convention labeled the primary void. As such, the number of spots was low (~3 spots/filter), and, correspondingly, the percent area in the primary void was close to 100%. Interestingly, the total urine deposited was significantly lower for 129S1/SvImJ mice than for NOD/ShiLtJ mice. The data shown illustrate that important quantitative comparisons can be made between genetically different strains and that voiding phenotypes are reliably consistent within individual animals and within strains.

As noted above in *Image analysis*, there is a certain loss of information due to overlapping spots and multiple micturition events in the same place that our simple algorithm could not differentiate. Two or more spots that overlap are outlined as a single "particle" (note the *bottom left* corner in Fig. 1*B*). Likewise, the area/volume of urine is likely to be underestimated when the mouse urinates several times in one place. Therefore, the quantitative differences we discern underestimate the true degree of variation between strains.

Genetic variation of voiding function. Voiding behavior did not vary with time of day for most strains (Fig. 3). However, we observed that C57BL/6J mice, the most widely used strain in biomedical research, had significantly greater urine spot deposition at night (Fig. 3*A*). The same pattern of greater

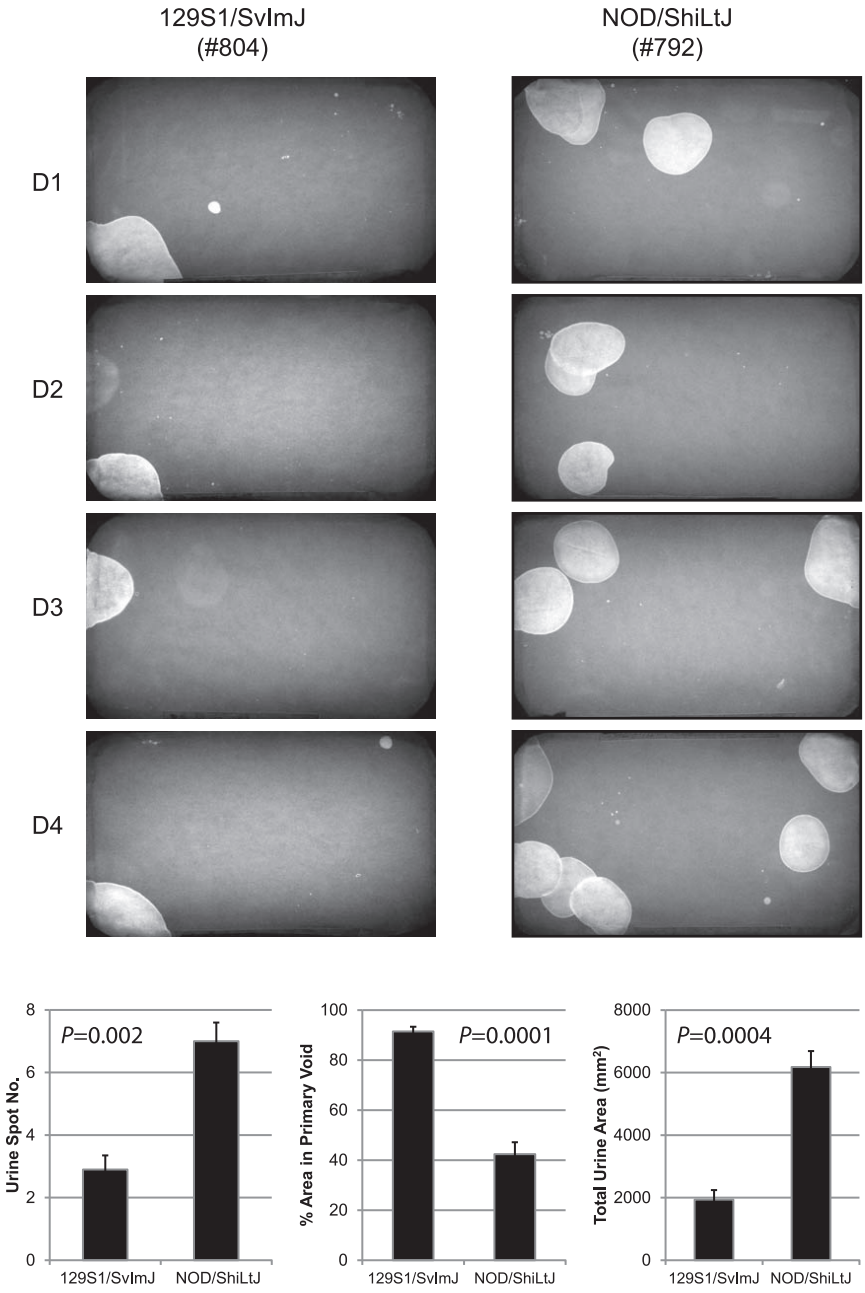

Fig. 2. Individual mice exhibit reproducible patterns of urination that are strain unique. *Top*: photographs showing filters collected over 4 days [*days 1–4* (D1–D4)] from two individual mice (*mouse 804* and *mouse 792*) of two different inbred strains. *Bottom*: quantitative data extracted from four mice of each strain assayed on 4 consecutive days in the afternoon. All three parameters (urine spot number, percent area in the primary void, and total urine spot area) were highly statistically different by Student's *t*-test (*P* values are indicated).

nocturnal micturition was also observed for PWK/PhJ mice, a distantly related wild-derived strain.

Other features of note in the data include WSB/EiJ strain afternoon percent area in the primary void (Fig. 3*B*) and NZO/H1LtJ strain nighttime urine volume (Fig. 3*C*). On the whole, measured parameters were consistent within a strain. The variation between strains was noteworthy. As examples, WSB/EiJ mice deposited only 5 spots on average, whereas PWK/PhJ mice urinated discretely over 20 times ($P < 0.002$ by ANOVA across strains for all times of the day; Fig. 3*A*). In terms of how consistently a strain chose to void in one place (i.e., the primary void), CAST/EiJ mice were promiscuous with the deposition of their urine (only ~25% in a primary location), whereas 129S1/SvImJ mice were quite fastidious, preferring to go in one spot 60–90% of the time, depending on the time of day ($P \leq 0.0005$; Fig. 3*B*). Total urine volumes also showed between-strain variations, which were greater than anticipated. WSB/EiJ mice released estimated quantities of urine of ~150 μl in 4 h, whereas NOD/ShiLtJ and NZO/H1LtJ mice urinated 500–600 μl during daylight hours ($P \leq 3 \times 10^{-8}$; Fig. 3*C*). Inspection of the corner versus center deposition patterns revealed the expected inverse correlation (Fig. 3, *D* and *E*) with mice that put more in the center having correspondingly lower distributions in the corners. A statistical summary of the interstrain variation for all parameters is shown in Table 1 and strongly suggests that the micturition phenotype is a genetically heritable trait. To determine the percentage of this intrastrain variance that could be attributable to heritable factors, we calculated the intraclass correlation coefficient, a surrogate estimate of H^2, for each phenotype and for each time

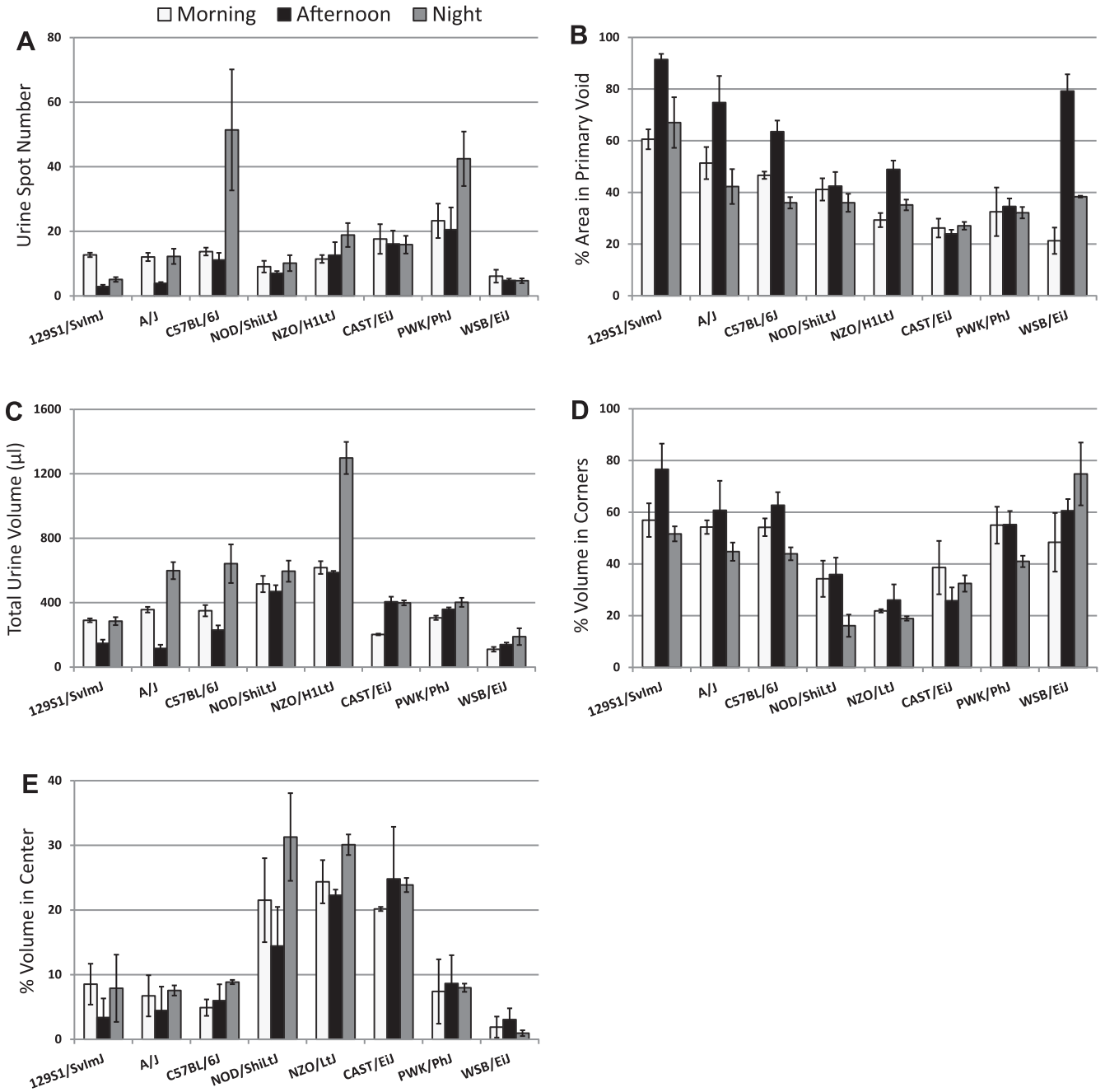

Fig. 3. Void spot data from all eight Collaborative Cross (CC) founder strains assayed in the morning, afternoon, and night. *A*: urine spot number. *B*: percent area in the primary void. *C*: total urine volume. *D*: percent volume in the corners. *E*: percent volume in the center. Each bar represents 3–5 consecutive assays/mouse averaged and 3–4 mice/strain averaged. Error bars are SEs.

point (morning, afternoon, and night). Overall, these results suggested that the traits measured by these assay are reasonably heritable, with the exception of the percent volume in the center at the afternoon time point only. These results suggested that total urine volume was the most heritable phenotype, with the intraclass correlation coefficient ranging from 88% for the night time point to 94% for the afternoon time point (Table 1).

We performed cystometry under urethane anesthesia on each of the mice to investigate bladder urodynamics and determine the extent to which CMGs correlate with urine spot patterns. Since the CMG is a terminal procedure, each mouse was assayed a single time. Figure 4*A* shows the characteristic cystometric pressure profile for each strain. From the three to four tracings obtained from each strain, quantitative data were extracted. Figure 4, *B–E*, shows the intercontractile intervals (time between voids), pressure amplitudes generated, voiding durations (defined as the width of the descending voiding peak at 50% maximal pressure), and weights of mice. The data were very reproducible within strains and showed characteristic variations between strains. The heritability of the cystometric traits, reflecting their relative within-strain to between-strain

Table 1. *Intraclass correlation coefficients and probabilities (ANOVA) for spontaneous void spot assay phenotypes*

	Morning	Afternoon	Night
Urine spot number, %			
Intraclass correlation coefficient	0.58	0.61	0.61
P value	$1.6e^{-3}$	$1.4e^{-3}$	$1.1e^{-3}$
Percent urine in the primary void			
Intraclass correlation coefficient	0.79	0.90	0.61
P value	$5.6e^{-5}$	$1.5e^{-8}$	$5.2e^{-4}$
Total urine volume			
Intraclass correlation coefficient	0.89	0.94	0.88
P value	$2.8e^{-9}$	$3.0e^{-12}$	$3.1e^{-8}$
Volume in the corners			
Intraclass correlation coefficient	0.46	0.76	0.90
P value	$1.1e^{-2}$	$3.6e^{-4}$	$1.3e^{-7}$
Percent volume in the center			
Intraclass correlation coefficient	0.51	0.11	0.75
P value	$2.0e^{-3}$	$3.2e^{-3}$	$1.2e^{-5}$

variations, was quite high (intercontractile interval $H^2 = 0.48$ and pressure amplitude $H^2 = 0.63$). A/J mice had the shortest intercontractile interval at 4 min, whereas NOD/ShiLtJ mice filled for 13 min before voiding initiated (Fig. 4*B*). Likewise, the pressure amplitude varied between strains, with 129S1/SvImJ mice generating very powerful bladder contractions reaching 50–60 cmH_2O (Fig. 4*C*). The voiding durations did not vary much for six of the strains, but, as shown in the profiles in Fig. 4*A*, 129S1/SvImJ and NOD/ShiLtJ mice exhibited unusual bladder contraction kinetics at voiding and, as a consequence, took longer to develop peak or threshold pressures, which then signal neural pathways to allow urethral sphincter opening. ANOVA demonstrated statistically significant differences between the inbred strains, further suggesting genetic regulation of these phenotypes.

Certain strains tended to be more aggressive in chewing and destroying the filter paper. As noted in the MATERIALS AND METHODS, the use of Whatman no. 540 acid-hardened filter paper reduced their physical ability to degrade the substrate. Interestingly, however, as another example of a genetically determined behavior, NZO/H1LtJ mice chewed and ate the filter paper in a highly specific manner. Figure 5 shows the filters of four individual NZO/H1LtJ mice after 4 h. Each mouse exhibited the same propensity to eat/chew only in areas where it had urinated. These mice are known to be both diabetic and hyperphagic (16). Glucose spilled in the urine may motivate the behavior.

Impact of aging, urothelial injury, and polyuria on spontaneous voiding patterns. We performed these experiments in C57BL/6J mice, because this strain is used extensively in aging research and is a background strain for many genetic manipulation studies. Figure 6 shows representative voiding patterns and CMGs of young (6 wk old) and old (18–24 mo) mice. The void spot patterns of young mice (Fig. 6, *A* and *B*) exhibited the characteristic pattern with large primary voids near one corner. Old mice exhibited a spectrum of patterns, two of which are shown in Fig 6, *C* and *D*, and these individual filters highlight in one case higher spot counts and in the other case more spots with larger than normal urine volumes. A CMG from an aged female (24 mo; Fig. 6*F*) showed abnormal urodyamics with large numbers of prevoiding contractions. Not all old mice tested showed abnormal spotting or urodyamics, consistent with a variable onset in these age-related phenotypes. There was a significant overall increase in urine spot number with aged mice (Fig. 6*E*), but, as noted, some mice had high spotting indexes, whereas others were near normal.

We investigated the effect of urothelial injury induced by intravesical instillation of protamine sulfate on C57BL/6J mice (18, 19). Two groups of five female mice were tested; one group received 100 μl PBS via intravesical instillation, whereas the other group received 100 μl of 10 mM protamine sulfate for 15 min at *time 0*. Mice were tested on filter paper 48 and 24 h before instillation and 1, 24, and 48 h postinstillation. Mice that received protamine sulfate exhibited an irritated bladder phenotype characterized by high numbers of urine spots at 1 h postprotamine ($P < 0.01$), but by 24 h urine spot deposition was back to normal (Fig. 7). The filter images shown in Fig. 7 illustrated the typical pattern from a protamine-treated animal. These data illustrate that the void spot assay can be used to assay potential models of LUTS.

When young mice were subjected to sucrose diuresis (replacement of drinking water with 5% sucrose solution for 8 wk), urine volumes increased markedly (Fig. 8, *B* compared with *A*), but the mice continued to void largely in single spots and predominantly in the corners. The CMGs for the diuretic mice (Fig. 8*D*) showed normal smooth filling and voiding curves and normal pressures; however, intercontractile intervals were much longer, indicating bladder enlargement as a result of the constant diuresis (compare with Fig. 8*C*). Similar to the findings of others who have used this model in rats and mice, the slope of the filling phase demonstrated greater compliance in diuretic mice (8, 31, 33). Despite the stress of increased urine volumes, diuretic animals, like humans with osmotic diuresis, remained continent and retained normal voiding patterns. Furthermore, the assay could detect functional volume-related changes that may occur in response to a pathological process like partial outlet obstruction or diabetes-induced diuresis, for example.

Correlation with anxiety-like behavioral traits. To minimize social behavioral input such as territorial marking or hierarchical dominance responses, we confined the study to virgin female mice housed with no exposure to male mice for the duration of these experiments. An additional aspect of urination could relate to psychological states such as anxiety. Several studies have examined the genetic basis for anxiety using open-field and a light-dark box apparatus (12, 24), where time spent in the open field or light is the quantitated parameter. The CC founder strains used in this study were examined for anxiety-related quantitative traits as previously described by Logan et al. (20). Their data indicated that A/J and 129S1/SvImJ strains were more timid or anxious than other strains. We used open-field, light-dark box, and visual cliff data from Ref. 20 and correlated them with all voiding measures using Person's *r* correlation. The open-field test consisted of a large opaque Plexiglas box with a dark gray floor. Each mouse was placed into the center of the arena and allowed to explore for 20 min. The light-dark box consisted of an insert evenly dividing the open-field apparatus into light-dark compartments. The compartments were separated with a sliding door that is closed during placement of mice into the chamber. Mice were placed into the dark compartment, and a 20-min recording began when the lid was closed. For the visual-cliff data, an avoidance test was conducted in open-field boxes with clear

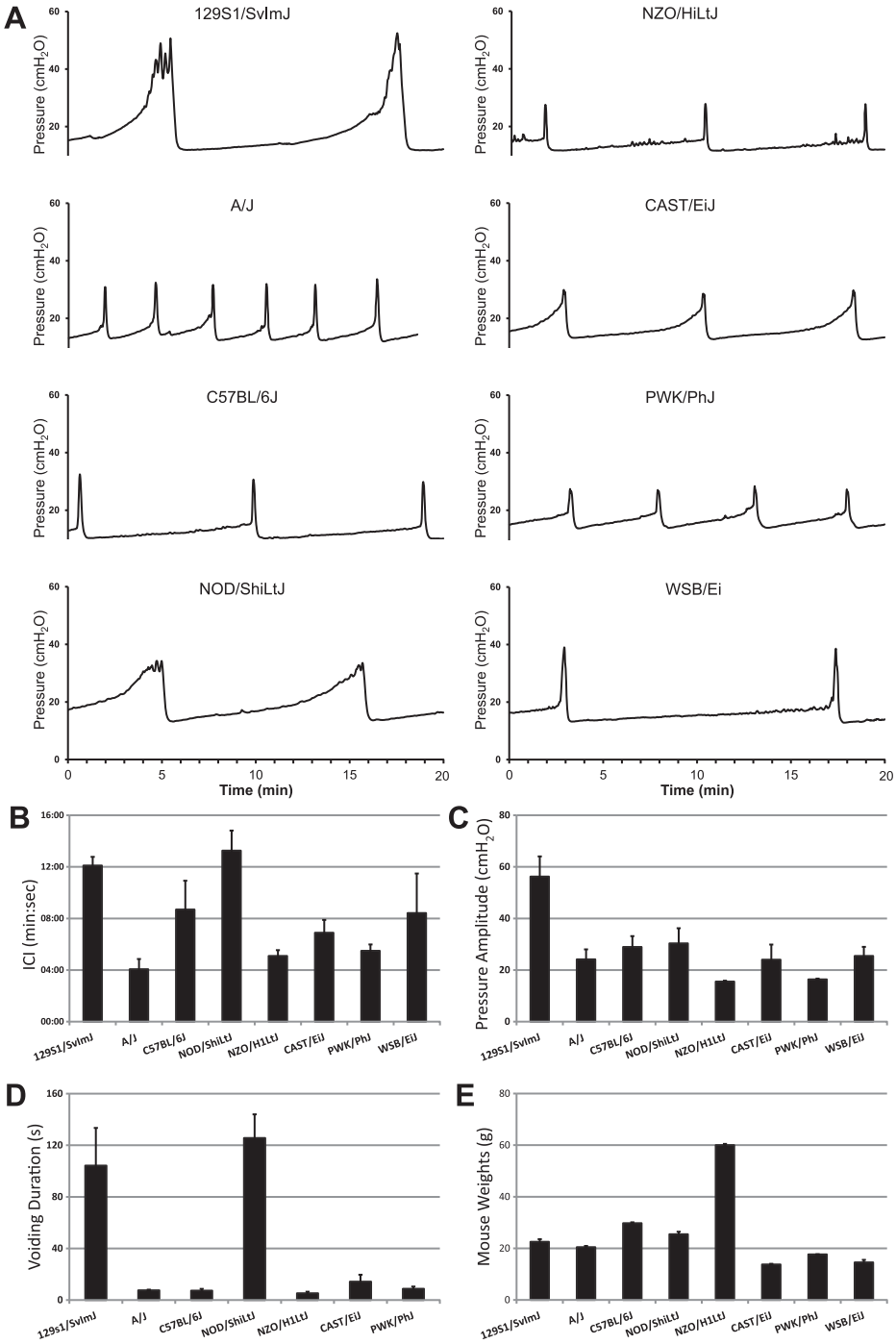

Fig. 4. Cystometry on all eight CC founder strains. *A*: representative cystometrograms (CMGs) from each strain. All tracings are displayed on the same *x*- and *y*-axes. *B*: summary data for intercontractile intervals (ICIs). *C*: summary data for pressure amplitude, defined as peak void pressure minus baseline pressure immediately after micturition. *D*: voiding duration, defined as the width of the voiding peak at 50% maximal pressure amplitude. *E*: mouse weights at 12–16 wk of age. Data were obtained from 3 or 4 mice of each strain; error bars are SEs.

Plexiglas bottoms that were secured, so half of the floor overhung the tabletop to create an appearance of a ledge dropoff. A checkerboard tablecloth draped from the tabletop to the floor served to enhance the visual appearance of the cliff. Parameters such as distance travelled, number and duration of transitions within the arenas, duration spent immobile, and defecations (anxiety behavior), among others, were recorded. We found no significant correlations between any of the voiding parameters (not shown) and therefore concluded that anxiety in these mice did not contribute significantly to their voiding patterns. While a few of the P values were <0.05, they represented no more than would be expected by chance.

DISCUSSION

The long-term goal of our experiments is to develop a robust animal model of LUTS and determine whether there are genetic factors that predispose to these symptoms. The discovery of genes or gene variants implicated in acquiring LUTS may provide clues to its causes and may, in the future, help tailor therapies to individual patients. Since it would be extremely difficult to identify genetic tendencies favoring the development of LUTS in

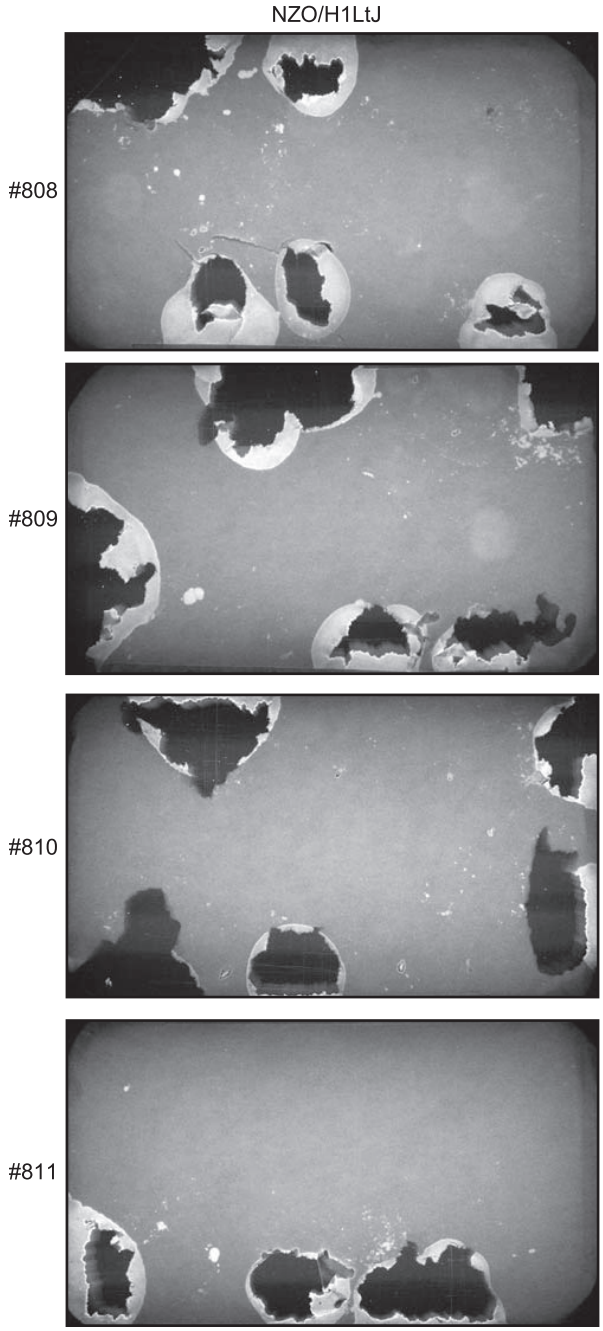

Fig. 5. Individual filters from four NZO/H1LtJ mice.

humans, mice may offer a better option. Our findings with C57BL/6J mice indicate a normal voiding pattern at a young age and a tendency to develop abnormal voiding with age. On this basis, it is possible that mice can serve as a model for the development of LUTS with aging. Mice may be ideal for this effort, because there are large-scale, longitudinal aging studies involving genetically well-characterized and heterozygous mice currently ongoing (5, 29), and these studies include extensive phenotypic analysis, which has not, to date, included studies of voiding function. In addition, studies examining interventions designed to reduce the effects of aging on numerous phenotypes are currently ongoing as part of the National Institute on Aging Interventions Testing Program (http://www.nia.nih.gov/research/dab/interventions-testing-program-itp) as well as part of numerous other research programs. To exploit these resources in the study of LUTS, we have developed an assay of spontaneous voiding in mice and a means to quantify voiding. Because the assay is noninvasive, it can be repeated over the life of the mouse, allowing us to determine when in the lifespan voiding function changes in an individual animal.

Void spot assays are easy to perform and, using optimal filters and nestlets, can be done on any strain of mouse. It is worth stressing the great advantage of an assay that can be repeated many times on the same mouse, since more precise quantitation can be achieved by averaging over repeated tests. Among the strains tested, we found no significant difference between the first void spot assay and subsequent spot assays, indicating that triplicate measurements are sufficient as a means to quantitate mean LUT function/individual animal. For most strains, the results vary little with time of day. However, since there was some variation among some strains, investigators who wish to use this method should check to see whether their strain shows such variation. This study demonstrated the high heritability of quantitative parameters obtained from the void spot assay, which reflects intrastrain consistency and large interstrain differences in voiding behavior. Spot deposition frequency and percent deposition in a primary location are quantitative traits that can be measured noninvasively and are amenable to further genetic analysis, including quantitative trait mapping.

CMGs confirmed at the urodynamic level that different strains had quantifiable and consistent differences in their micturition kinetics and pressure profiles and thus lend further weight to our conclusion that genetics underpins this physiology. One caveat to these experiments is that cystometry was performed under urethane anesthesia, which is known to alter some aspects of voiding but spares the voiding reflex (1). The literature on effects of urethane in rodents is somewhat contradictory, with some studies in rats showing reduced capacity in anesthetized compared with awake animals (3, 26), whereas in mice, mean voided volumes were larger under urethane anesthesia (27). There are a number of methodological reasons that could underlie the disparity, ranging from the depth of anesthesia to bladder damage from catheter implantation. Many investigators, for example, perform conscious cystometry in mice or rats 2–3 days after catheter implantation, whereas others wait a week. It has been shown in several studies that recovery of normal bladder cystometric function takes 6 days or more (23, 34). In general, however, peak pressures, flow rates, and micturition pressure thresholds appear untouched by urethane, and, overall, this has led to speculation that differences in voiding between urethane and awake animals may be attributable to suppressed afferent sensory function, with central/efferent traffic largely unaffected (27).

In our experiments with urethane-anesthetized mice, the pressure profiles within strains were very consistent, giving us confidence that we were measuring reproducible behaviors. This is supported by the data shown in Fig. 4, *B–D*. Linear regression analyses between void spot parameters and cystometric parameters found there was no significant correlation by Pearson's correlation analysis between peak bladder pressures and urine spot number and total urine volume, nor was there any correlation between intercontractile interval and spot num-

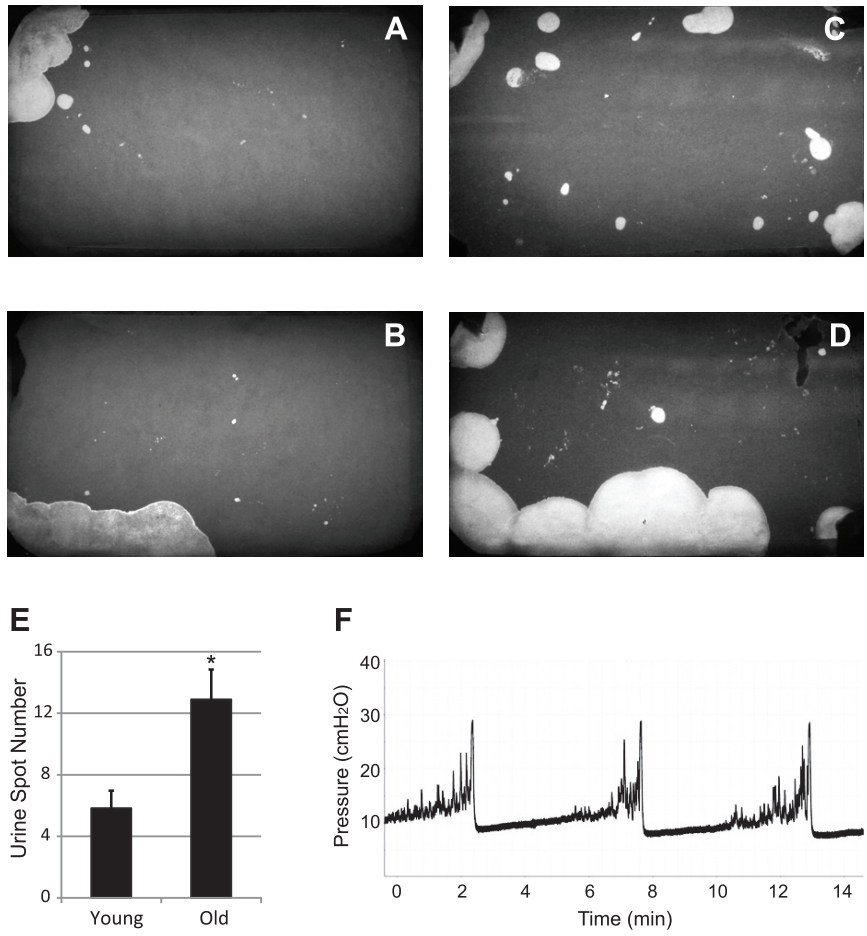

Fig. 6. Effect of age on void spot data and CMGs. *A* and *B*: void spot filters from two 6-wk-old C57BL/6J mice. *C* and *D*: void spot filters from two 24-mo-old C57BL/6J mice. *E*: urine spot number summary data from young and old mice ($n = 5$). *$P < 0.05$. *F*: CMG from an old mouse.

ber, total volume, or percent urine in the primary void (not shown). This was true for any time of the day. Thus, the assays, while complementary, tend to measure separate phenomena in young, healthy mice. Void spotting is a complex socially and genetically determined behavior, whereas cystometric pressure/time tracings evaluate neuromuscular reflexes in response to a biomechanical stimulus that mimics normal bladder filling.

While we did not test our strains for fluid consumption, we were able to perform a correlation analysis on fluid intake versus urine spot number and total urine volume based on

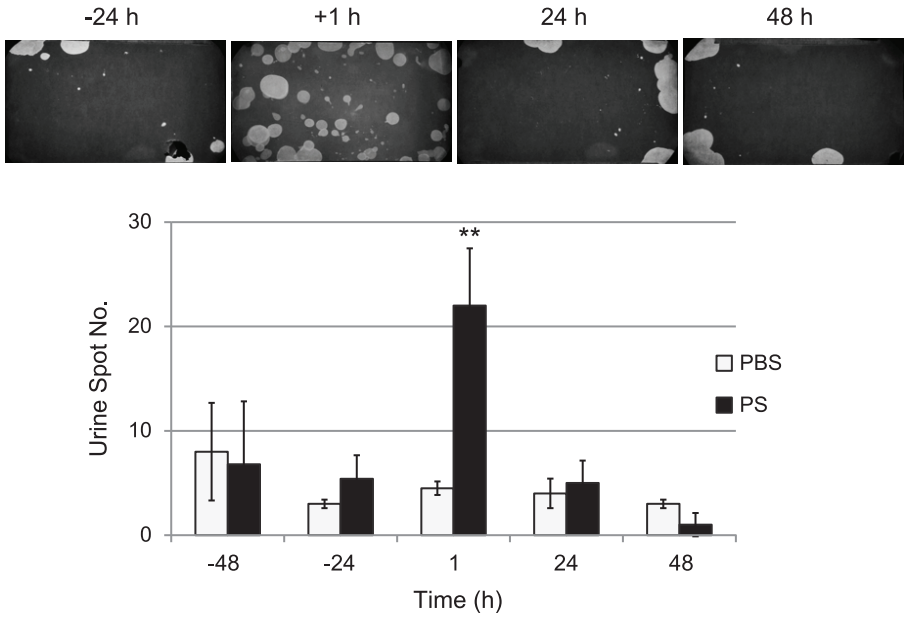

Fig. 7. Effect of urothelial injury on urine spotting. Urine spotting was assayed in 10 female C57BL/6J mice for 2 days before they received intravesical PBS ($n = 5$) or protamine sulfate (PS; 10 mM, $n = 5$). Mice were then reassayed on filter paper at 1, 24, and 48 h after instillation. *Top*: filter paper images showing the effect of PS administered at *time 0* in one mouse. *Bottom*: graph showing the data summary for urine spot number at different time points. **$P < 0.01$.

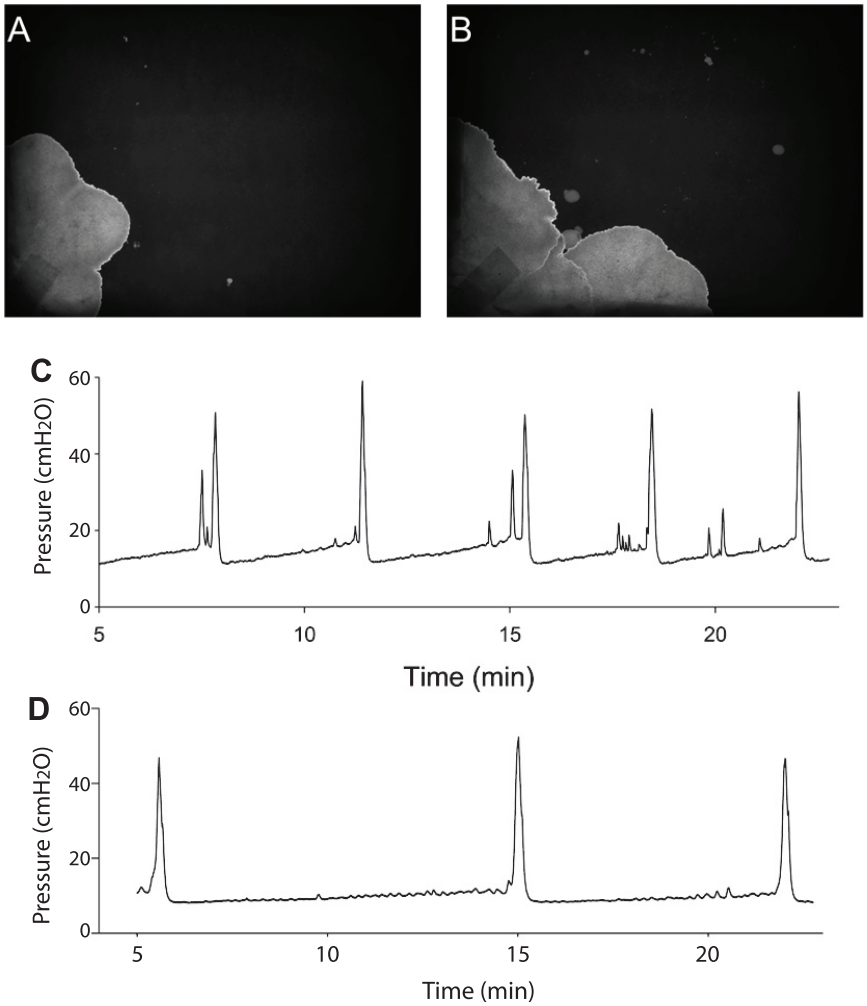

Fig. 8. Effect of enforced diuresis on void spots and CMGs. C57BL/6J mice were given water (control) or 5% sucrose to drink for 8 wk. *A*: void spots from a control mouse. *B*: void spots from a 5% sucrose-fed mouse. *C*: CMG from a control mouse. *D*: CMG from a 5% sucrose-fed mouse.

strain-specific metabolic information found in the Jackson Laboratory Mouse Phenome Database (http://phenome.jax.org/db/q?rtn=meas/catlister&req=Dmetabolismqqq204). Five of the strains we tested had fluid intake per 24 h per body weight data available. The highest regression coefficient for fluid intake and spot number was for afternoon data, and it was not significant, suggesting that mouse spotting frequency was not a function of drinking volume. This suggests volitional voiding, not merely reflex voiding at a threshold volume, and is also supported by the observation that CMGs did not correlate with voiding frequency. Conversely, total urine volume was significantly correlated with fluid intake ($P = 0.02$). This finding is consistent with our observation that sucrose diuresis, which is associated with increase fluid intake, resulted in no change in urine spot number or location but did impact urine volume.

In contrast to the results from young healthy mice, mice with naturally occurring or induced bladder perturbations could be shown to exhibit voiding dysfunctions, as shown by both voiding spots and CMGs. Our preliminary experiments on a small number of aged C57BL/6J mice revealed a mixed picture, with some mice displaying abnormal numbers of spots and altered urodynamics. This was not uniformly seen, perhaps consistent with environmental influences on the development of LUTS and/or variability in age at which LUTS appear. C57BL/6J mice are a relatively long-lived strain, with female mice having a median lifespan of >850 days (35) or 28 mo. Our study only extended out to 24 mo of age, and we may have not captured all age-associated incidences of LUTS-like symptoms in our animals. Our study does, however, provide proof of principle that the assay can be usefully used in large-scale studies of age-related LUTS as age-associated changes in LUTS-like symptoms were observed.

The assay also proved quite reliable in reporting on the effect of an acute insult to the bladder urothelium. The high numbers of urine spots 1 h after protamine sulfate treatment likely reflected pain or irritation due to loss of urothelial barrier function and stimulation of submucosal high-threshold afferents. Surprisingly, the micturition frequency returned to baseline levels within 24 h, indicating an efficient and robust urothelial differentiation program, which, while likely not complete, restored some protection to sensory afferents from urine leakage.

Mice subjected to osmotic diuresis-induced polyuria exhibited large, single voiding spots and longer intervals on CMGs, but the void spot assays showed no loss of outlet control, and the CMGs revealed smooth tracings at normal pressures. In contrast, a recent study of genetically modified mice carrying a urothelium-specific deletion of integrins exhibited dramatically abnormal voiding patterns, with symptoms resembling incontinence, while cystometry confirmed bladder overactivity (17).

Each of these uses of the void spot assay reveal that it can provide a relatively coarse grained but highly informative physiological readout of LUT function.

Since a fairly rich literature on mouse urine marking behavior is available, a consideration of other variables is warranted. The urine of mammals contains potent pheromones and other molecules capable of eliciting behavioral responses in both their own as well as other species (30). Seven species of rodents, for example, including *Mus musculus*, have been shown to have a strong preference for urinating in the same place of a cage that had been marked with their own or conspecific urine (7). Social rank in male mice correlates with urination patterns, with dominant males marking a shared cage copiously, whereas subordinate males urinate in smaller areas (10). Male mice also alter their voiding pattern, increasing their output substantially in the presence of a female mouse, whereas female mice in the presence of another female mouse show little alterations in urination behavior (25). Sexual maturity in juvenile female mice is accelerated in the presence of male mice who increase their rate of urination (2, 9). There is some question as to how important estrus cycling is in female mouse urination. One study found no differences in urine deposition between diestrus and proestrus female mice, whereas another study did show significant differences in spot number during the estrus cycle. We did not control for that variable, and it is possible that some of the variability we noted is due to changes in hormone levels or other estrus-induced parameters. In addition, abnormal urination patterns and irreversible bladder remodeling occur in response to repeated exposure to stress (4, 32), indicating some plasticity in voiding phenotypes that can be revealed by filter spotting. To avoid many of these confounders and to determine the fidelity of the phenotypic readout we used only virgin female mice, minimized stress, and did not expose female mice to male mice.

Our finding that spontaneous voiding behavior and CMGs vary by strain reveals a strong genetic influence on voiding function. It therefore appears likely that genetics play an important role in when and whether an individual mouse develops voiding dysfunction as it ages. Mouse strains are, by definition, inbred and homozygous at all genetic loci. In contrast, DO mice, a population of mice descended from the same eight founder strains as CC mice, are randomly segregating at all genetic loci, making each mouse a unique combination of alleles. Because of the breeding strategy used to make and maintain the DO population, they are ideally suited for precise genetic mapping. Serial void spot studies in such mice may help identify genetic loci that are capable of inducing a tendency to voiding dysfunction during aging. The identification of the genes underlying these loci could provide insights into the etiology of LUTS with age in humans and potentially provide novel mechanisms for treatment and or interventions.

In summary, we have developed and characterized a simple, reproducible, noninvasive, and quantitative measure of spontaneous voiding function in mice. While useful, it has limitations, and deeper analysis of voiding pathologies will likely continue to require more invasive approaches, such as cystometry. As such, it will not replace CMGs but will complement them by providing simple-to-obtain preliminary data and a means of monitoring changes with time. Our results show that this assay detects abnormalities of voiding within a strain, whether caused by environment (induced diuresis), genetic mutation, or aging. We have shown that all aspects of voiding function measured in this study are heritable traits, suggesting that the phenotypes measures by the void spot assay developed herein could be used as quantitative traits appropriate for genetic analyses. We have shown that void spot assays may correlate with CMGs within a strain but do not correlate well between strains. This study sets the stage for future efforts to identify the genetic loci driving voiding dysfunction using genetically segregating populations of mice, such as the DO population.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

DISCLAIMER

The contents of this article are solely the responsibility of the authors and do not necessarily represent the official views of the NIH.

AUTHOR CONTRIBUTIONS

Author contributions: W.Y. and J.D.L. performed experiments; W.Y., C.A.-B., B.M., W.D.S., G.A.C., and W.G.H. analyzed data; W.Y., C.A.-B., B.M., W.D.S., G.A.C., W.G.H., and M.L.Z. edited and revised manuscript; W.Y., C.A.-B., B.M., W.D.S., G.A.C., W.G.H., and M.L.Z. approved final version of manuscript; C.A.-B., B.M., W.D.S., G.A.C., W.G.H., and M.L.Z. interpreted results of experiments; J.D.L. and W.G.H. prepared figures; G.A.C., W.G.H., and M.L.Z. conception and design of research; W.G.H. and M.L.Z. drafted manuscript.

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