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Kathy Sharp

*College of William and Mary*

Donna Bucci

*College of William and Mary*

Paul K. Zelensky

*College of William and Mary*

Alanna Chesney

*College of William and Mary*

Wendy Tidhar

*College of William and Mary*

*See next page for additional authors*

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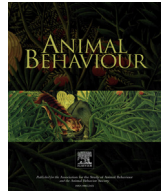
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**Authors**

Kathy Sharp, Donna Bucci, Paul K. Zelensky, Alanna Chesney, Wendy Tidhar, Paul D. Heideman, and David R. Broussard



## Genetic variation in male sexual behaviour in a population of white-footed mice in relation to photoperiod



Kathy Sharp<sup>a,1</sup>, Donna Bucci<sup>a,2</sup>, Paul K. Zelensky<sup>a,3</sup>, Alanna Chesney<sup>a,4</sup>,  
Wendy Tidhar<sup>a,5</sup>, David R. Broussard<sup>b,6</sup>, Paul D. Heideman<sup>a,\*</sup>

<sup>a</sup> Department of Biology, College of William and Mary, Williamsburg, VA, U.S.A.

<sup>b</sup> Department of Biology, Lycoming College, Williamsport, PA, U.S.A.

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In natural populations, genetic variation in seasonal male sexual behaviour could affect behavioural ecology and evolution. In a wild-source population of white-footed mice, *Peromyscus leucopus*, from Virginia, U.S.A., males experiencing short photoperiod show high levels of genetic variation in reproductive organ mass and neuroendocrine traits related to fertility. We tested whether males from two divergent selection lines, one that strongly suppresses fertility under short photoperiod (responder) and one that weakly suppresses fertility under short photoperiod (nonresponder), also differ in photoperiod-dependent sexual behaviour and responses to female olfactory cues. Under short, but not long, photoperiod, there were significant differences between responder and nonresponder males in sexual behaviour and likelihood of inseminating a female. Males that were severely oligospermic or azoospermic under short photoperiod failed to display sexual behaviour in response to an ovariectomized and hormonally primed receptive female. However, on the day following testing, females were positive for spermatozoa only when paired with a male having a sperm count in the normal range for males under long photoperiod. Males from the nonresponder line showed accelerated reproductive development under short photoperiod in response to urine-soiled bedding from females, but males from the responder line did not. The results indicate genetic variation in sexual behaviour that is expressed under short, but not long, photoperiod, and indicate a potential link between heritable neuroendocrine variation and male sexual behaviour. In winter in a natural population, this heritable behavioural variation could affect fitness, seasonal life history trade-offs and population growth.

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Natural populations contain important interindividual variation in reproductive traits (Bronson, 1989; Williams, 2008), underlying physiological traits (Bronson, 1989; Bronson & Heideman, 1994; Heideman & Pittman, 2009; Prendergast, Kriegsfeld, & Nelson, 2001) and related reproductive behavioural traits (Rhen & Crews, 2002). To understand variation in reproductive and life history traits, it is necessary to relate genetic variation among individuals to phenotypic variation in structure, physiology and behaviour

(Williams, 2008). Genetic variation may also exist in phenotypic plasticity, the ability to alter phenotype in response to the environment (Lessells, 2008). Traits that are known to have genetic variation and phenotypic plasticity in natural populations provide opportunities to test relationships among physiological traits, behaviours and phenotypes (Feder, Bennett, & Huey, 2000).

In many temperate-zone rodents, reproduction is a phenotypically plastic trait: reproduction occurs in spring, summer and/or autumn, but reproduction and sexual behaviour are suppressed in the short photoperiods of winter (Bronson, 1989; Campbell, Finkelstein, & Turek, 1978; Miernicki, Pospichal, & Powers, 1990; Morin & Zucker, 1978; Park et al., 2004; Powers et al., 1989). When descendants of wild-caught individuals are tested in the laboratory, short photoperiod is the major environmental treatment that causes reproductive suppression (Bronson & Heideman, 1994). In many populations, some individuals show complete phenotypic plasticity by fully suppressing gametogenesis and reproduction in winter, some are intermediate, and others lack

\* Correspondence: P. D. Heideman, Department of Biology, College of William and Mary, Williamsburg, VA 23187, U.S.A.

E-mail address: [pdheid@wm.edu](mailto:pdheid@wm.edu) (P. D. Heideman).

<sup>1</sup> E-mail address: [kathy\\_r\\_sharp@yahoo.com](mailto:kathy_r_sharp@yahoo.com) (K. Sharp).

<sup>2</sup> E-mail address: [dmbucci@wm.edu](mailto:dmbucci@wm.edu) (D. Bucci).

<sup>3</sup> E-mail address: [pauzele@yahoo.com](mailto:pauzele@yahoo.com) (P. K. Zelensky).

<sup>4</sup> E-mail address: [alannachesneydvm@gmail.com](mailto:alannachesneydvm@gmail.com) (A. Chesney).

<sup>5</sup> E-mail address: [wtidhar@yahoo.co.uk](mailto:wtidhar@yahoo.co.uk) (W. Tidhar).

<sup>6</sup> E-mail address: [Broussar@lycoming.edu](mailto:Broussar@lycoming.edu) (D. R. Broussard).

phenotypic plasticity, showing no response to short photoperiod (Bronson, 1989; Bronson & Heideman, 1994; Prendergast et al., 2001). The variation in response to short photoperiod has a genetic basis (Desjardins, Bronson, & Blank, 1986; Heideman & Pittman, 2009; Prendergast et al., 2001), including genetic variation in gonadal development and in physiological traits in the reproductive neurons and hormones of the hypothalamic–pituitary–gonadal (HPG) axis (Blank & Ruf, 1992; Heideman & Pittman, 2009; Mintz, Lavenburg, & Blank, 2007; Prendergast et al., 2001). An important question is whether there is genetic variation in behaviour that is related to variation in the development of reproductive organs and availability of gametes, and whether behavioural and morphological variation might cause differences in the number of offspring and size of litters.

In this study, we tested for heritable differences in sexual behaviour and phenotypic plasticity of reproduction using two selection lines derived from a wild population (Heideman, Bruno, Singley, & Smedley, 1999; Heideman & Pittman, 2009). One line has been artificially selected for small immature testes, ovaries and/or uteri under short photoperiod (8:16 h light:dark cycle), and is defined as responder to short photoperiod ('responder'). A second line has been artificially selected for large reproductive organ mass under short photoperiod, or 'nonresponder' (see Methods for selection criteria). After three generations of selection, responder mice had significantly smaller reproductive organs than nonresponder mice at age 70 days under short photoperiod (Heideman et al., 1999). With continued selection, approximately 90% of the responder individuals were prepubertal or peripubertal under short photoperiod at age 70 days, as indicated by gonadal size. At the same age, approximately 80% of nonresponder mice had large gonads under short photoperiod, and gonads were similar in size to mice raised under long photoperiod (LD 16:8 h) in a control line (Heideman & Pittman, 2009). Both selection lines undergo reproductive suppression under short relative to long photoperiod (Heideman & Pittman, 2009), but only in the responder line does reproductive suppression under short photoperiod commonly result in low levels of spermatozoa: azoospermia or oligospermia (Broussard et al., 2009). These selection lines have been shown to differ heritably in reproductive traits (reproductive organ size, number of immunoreactive GnRH neurons, levels of luteinizing hormone: Avigdor, Sullivan, & Heideman, 2005; Heideman et al., 1999, 2010) as well as some, but not all, nonreproductive traits that may affect fertility (ad libitum food intake, metabolic rate, physical activity, amount of iodomelatonin binding in the brain: Heideman & Pittman, 2009; Heideman et al., 2010; Kaseloo, Crowell, & Heideman, 2014; Kaseloo, Crowell, Jones, & Heideman, 2012; White et al., 2014).

We tested the hypothesis (H1) that there is variation in male sexual behaviour that is expressed under short but not long photoperiod. We also tested whether variation in male sexual behaviour under short photoperiod is related to genetic variation in sperm count or mass of reproductive organs. We predicted that under short photoperiod, measures of male sexual behaviour would be lower in the responder line than in the nonresponder line, but that the lines would not differ under long photoperiod. We also predicted that measures of sexual behaviour would be positively correlated with sperm count and mass of reproductive organs. This is particularly important because short photoperiod alone may not suppress male sexual behaviour. In many populations, males can be moderately to severely oligospermic in winter, but not azoospermic; if these individuals can display sexual behaviour and mate, they may be capable of insemination and fertilization. In Siberian hamsters, *Phodopus sungorus*, under short photoperiod, 50% of males with regressed testes maintained sexual behaviour to ejaculation (Park et al., 2004). In our study, therefore, an alternative

hypothesis (H2) is that reproductive behaviour under short photoperiod is independent of selection line, testis size or sperm count. Two predictions are possible under H2. First, all males may reduce sexual behaviour under short photoperiod, because sexual behaviour may be more strongly inhibited by short photoperiod than by low availability of spermatozoa. Second, azoospermic males under short photoperiod may reduce or eliminate sexual behaviour, while oligospermic males may have sufficient activation of the reproductive axis to exhibit normal sexual behaviour.

In a second experiment, we tested for heritable variation in phenotypic plasticity of reproductive maturation in the presence of olfactory cues from mature females. It is possible that exposure to olfactory cues may signal potential reproductive opportunities that accelerate the reproductive development of males under short photoperiod. Daily exposure to these cues accelerates male maturation in some mammals (Rissman, Taymans, & Wayne, 1990; Vandenberg, 1971; Whitsett & Lawton, 1982) but not all (Bediz & Whitsett, 1979). Exposure to female olfactory cues has been shown to accelerate puberty in male white-footed mice, *Peromyscus leucopus*, under long photoperiod (Terman, 1984); here we ask whether a similar acceleration would occur under short photoperiod. One hypothesis (H1), based on results from Terman (1984) and Whitsett and Lawton (1982) is that all young males from both responder and nonresponder lines will accelerate reproductive maturation under short photoperiod when presented daily with soiled bedding from the cages of mature females. An alternative hypothesis (H2) is that there is genetic variation in this response, such that only nonresponder males will accelerate reproductive development under short photoperiod.

Heritable differences in male sexual behaviour and reproductive organs could cause differences in litter size between selection lines. Therefore, in a third experiment, we tested for variation in litter size between our nonresponder and responder selection lines. Because mice in the responder line are strongly reproductively inhibited by short photoperiod, all litters were conceived under long photoperiod. We tested two conditions for each line: litters were retained under long photoperiod after birth (mimicking late spring–early summer births and inducing a phenotype typical under long photoperiod), or litters were transferred at birth to short photoperiod (mimicking late summer–early autumn births in which mice experience shortening photoperiod, and inducing a phenotype typical under short photoperiod). Under this hypothesis, enhanced male sexual behaviour or higher sperm counts in the nonresponder line might result in fertilization of a higher proportion of ova in the nonresponder line relative to the responder line. Changes in the number of pups per litter might also affect the mass of pups; therefore, we also compared the lines for total mass of litters and the average mass of pups in a litter.

Finally, these experiments did not use replicated selection lines, and so it is not possible to distinguish whether differences between selection lines might be due to genetic drift as opposed to selection. The objective here was not to test for causes of differences between selection lines, but rather to test for ecologically relevant genetic variation that might be present in a natural population.

## METHODS

### *Selection Lines of Peromyscus leucopus*

Detailed descriptions of the selection lines used in this study are available elsewhere (Broussard et al., 2009; Heideman et al., 1999; Heideman & Pittman, 2009); here we provide a brief description. Two artificial selection lines and an unselected control line were established in 1995 from 208 offspring of 48 wild-caught mice. These 208 offspring were conceived under long photoperiod

and transferred at birth to short photoperiod. At an age of 67–73 days, body mass and reproductive development was recorded. Mice were selected at random from these offspring to establish the unselected control line. From the remaining mice, a line artificially selected to be reproductively suppressed under short photoperiod (responder line) was formed using males that were azoospermic or severely oligospermic (length  $\times$  width of one testis  $<24 \text{ mm}^2$ ) and females with immature ovaries (lacking visible follicles or corpora lutea and  $\leq 2 \text{ mm}$  in greatest length) and small uteri ( $<0.5 \text{ mm}$  diameter). Similarly, males with large testes (length  $\times$  width of one testis  $>32 \text{ mm}^2$ ) and females with mature ovaries (with visible follicles or corpora lutea and  $\geq 2 \text{ mm}$  in greatest length) and large uteri ( $>1 \text{ mm}$  diameter) were selected to establish a selection line that had little or no reproductive response to short photoperiod (nonresponder line). The control line was maintained as an unselected outbred line, while artificial selection in the responder and nonresponder lines was continued for 10 subsequent generations. When raised under long photoperiod, males and females reach adult body mass at age 70 days, but become sexually mature at age 46–60 days (Broussard et al., 2009). In contrast, when transferred to short photoperiod within 3 days of birth and raised under short photoperiod, males that are affected reproductively by short photoperiod do not begin to become photorefractory until about 18–20 weeks of age (Broussard et al., 2009). As adults, breeders are commonly fertile for 2 years or longer.

Within three generations of founding the lines, most individuals in the nonresponder line matured by age 70 days when raised under short photoperiod, while those in responder line did not (Heideman et al., 1999; Heideman & Pittman, 2009). The lines differ in mass of the testes and seminal vesicles under both long and short photoperiod (nonresponder  $>$  responder under either photoperiod) (Avigdor et al., 2005; Heideman & Pittman, 2009). Photoperiod affects reproduction in both selection lines: in both the nonresponder and responder lines, the mass of testes and seminal vesicles is smaller under short photoperiod relative to long photoperiod (Avigdor et al., 2005; Heideman & Pittman, 2009). Testes of males in the nonresponder line under short photoperiod are still within the normal range for long photoperiod for the founder population or the control line (Heideman et al., 1999). Additional information on the two selection lines and control line has been published elsewhere (Broussard et al., 2009; Heideman, 2004; Smale, Heideman, & French, 2005).

#### *Ethical Note*

Surgeries and handling procedures were developed from the literature on assessing sexual behaviour in small rodents, especially in nondomesticated rodents; this included consultation with colleagues and our consulting veterinarian. We adapted procedures to minimize stress and discomfort in our colony (e.g. providing cotton nesting material, various rodent chews, tubing for hiding), particularly during pilot tests. We used detailed follow-up observations (e.g. physical examination, regular observation for huddling or lethargy, monitoring body mass for evidence of decreased food intake, monitoring pelage smoothness as an indicator of normal grooming, and observing tail vertebral prominence or pulling up a flank skin fold as an indicator of possible dehydration) to consider changes to surgeries, hormonal treatments, vaginal lavage and pairing procedures for behaviour tests. We made modifications if observations indicated potential problems, including potential for injury or preventable stress. During surgeries, depth of anaesthesia was assessed by a rapid pinch of the skin on the flank with forceps, using very lightweight forceps to prevent any risk of tissue damage. We adjusted anaesthesia by monitoring breathing rate, with a

target at or slightly below one breath per second. We altered the frequency of hormonal priming of females after our assessments detected uterine infections in three females. After modification, the problem did not recur. We monitored for stress by watching for unusual behaviours (e.g. huddling in cage corners or aggression). Prior to finalizing the procedure to record behavioural observations, we observed newly paired animals for progressively longer periods to monitor the potential for stress or injury due to aggression between paired individuals.

Approval of animal subjects for this study was provided under protocols IACUC-9812, IACUC 0219 and IACUC 0429 from the Institutional Animal Care and Use Committee of the College of William and Mary.

#### *Experiment 1: Variability and Phenotypic Plasticity in Sexual Behaviour and Reproductive Organs*

Litters from responder and nonresponder lines (generations F7 to F10) were born under long photoperiod (lights off 1700 hours) at an ambient temperature of  $22 \pm 3 \text{ }^\circ\text{C}$ . Approximately 2 days after birth, the male was removed and the female and pups were either left under long photoperiod or transferred to short photoperiod (lights off 2100 hours) with constant temperature ( $22 \pm 3 \text{ }^\circ\text{C}$ ). At weaning (23 days of age), male mice were separated and housed individually in polyethylene cages ( $27 \times 16 \times 13 \text{ cm}$ ) and provided with food (LM-485, Harlan Teklad, Madison, WI, U.S.A.) and water ad libitum. Sample sizes were 17 nonresponder and 16 responder under long photoperiod, and 13 nonresponder and 12 responder under short photoperiod. At age 8–12 weeks (after normal age of maturity under long photoperiod, but before refractoriness to photoperiod), reproductive status was assessed by lightly anaesthetizing with isoflurane (30% anaesthesia in an induction chamber and 2% maintenance with a precision vaporizer; Abbott Laboratories, Chicago, IL, U.S.A.; duration of anaesthesia was approximately 3–5 min) and using callipers to measure the length and width of the right testis through the scrotum ( $\pm 0.1 \text{ mm}$ ). Measurements were taken by one individual (P.D.H.) who was blind to both line and treatment. The length and width of the testis was used to estimate testis size (Broussard et al., 2009). The right ear of each male was tagged (Size 1 Monel, National Band and Tag Co., Newport, KY, U.S.A.) as a permanent identification marker. The presence of the tag on the right ear identified the male during behavioural observations.

Sexually mature female mice (age  $>50$  days but  $<1$  year;  $N = 32$ ) used as stimulus females in behavioural testing were housed under long photoperiod and selected at random from all three lines (responder, nonresponder, unselected control). Adult females from the three lines under long photoperiod appeared to be similarly receptive to mating during pilot testing, and the use of all three lines minimized the potential for bias due to behavioural differences among females in the three lines. Stimulus females were ovariectomized by sterile surgery under isoflurane anaesthesia (Abbott Laboratories, North Chicago, IL; isoflurane in an induction chamber at 30% followed by 2% maintenance; duration of surgery and anaesthesia was approximately 10–15 min). Mice were treated with an antibiotic (Ampicillin at  $10 \text{ mg/kg}$ ) and an analgesic/anti-inflammatory agent (Flunixinamine  $20 \text{ mg/kg}$ ) and were given supplemental heat and monitored until recovery.

Prior to each behavioural test, stimulus females were primed with exogenous hormones to induce oestrus using methods established elsewhere (Dewsbury, 1975). Briefly, an intramuscular injection (i.m.) of  $0.06 \text{ mg}$  of oestradiol benzoate in peanut oil vehicle was given 72 h before testing, followed 6 h before testing by  $0.60 \text{ mg}$  of progesterone in peanut oil vehicle ( $0.05 \text{ ml}$ , i.m.). This protocol produced reproductive behaviours identical to those seen



in naturally occurring oestrus in nonovariectomized mice (Dewsbury, 1975).

During pilot experiments, sexually naïve adult males under long photoperiod exhibited few sexual behaviours during the first night of exposure to a hormonally primed, receptive female. However, on the second exposure to a novel female on the following night almost all males displayed sexual behaviours. For this reason, testing for sexual behaviour included an initial night for experience ('experience night') when data were not collected, directly followed by a second night ('test night') in which males were paired with a novel female and behaviours were recorded for analysis. Males that failed to display sexual behaviour during the test night ( $N = 20$  of 58 males) were given a second test night ('supplemental night') 3–5 days later. In the closely related deer mouse, *Peromyscus maniculatus*, a single day was sufficient for 80% of males to recover the ability for sexual behaviour to ejaculation, but 20% of males needed more time for recovery (Dewsbury, 1983).

The range of ages for behavioural testing of males was 10–18 weeks. The average age at testing was 13 weeks (91 days), with no significant differences in age at testing among treatment groups. The lower age limit was chosen because the typical age of maturity of this species under long photoperiod is 8 weeks; the upper age limit was chosen because males require more than 18 weeks of photoperiod treatment to begin refractoriness to the effects of photoperiod (Broussard et al., 2009).

#### *Behavioural testing*

We tested males from the two selection lines (nonresponder and responder) under short photoperiod (short-day treatment) and long photoperiod (control treatment). Pairings of males with hormonally primed females were recorded for 8 h (2100–0500 hours) during the dark phase in dim red light (25 W bulb) using a 1/3-inch CCD black and white camera (Lorexpro, CVC6981P) and a four-head VCR (Panasonic PV-VS4821). The 8 h recording period included the entire dark phase for control mice and the middle 8 h portion of the dark phase for short-day mice. In pilot tests, mature males displayed the most reproductive behaviour in the middle of the dark period, which is consistent with observations by Dewsbury (1975). We recorded the number of mount attempts, number of intromissions, number of ejaculations, latency to first mount attempt, latency to first intromission and latency to first ejaculation following Dewsbury (1975). We added an additional measure, the number of pursuits, in which the male closely pursued the female for a distance of at least 25 cm.

On the test night, one male and one stimulus female were placed in separate halves of a 10-gallon glass aquarium ( $51 \times 25$  and 30 cm high) separated by a wooden divider, following a protocol adapted from Burns-Cusato et al. (2004). Each mouse had access to food, water and pine shavings with cotton bedding material. Following a period of habituation (3.5–7 h), the divider was removed and behavioural recording began immediately (2100 hours). Mice were separated on the following day. After separation, the observation chamber was washed with hot soapy water and wiped with 70% ethanol. Because wooden components (divider and water bottle stand) can retain odours, residual scent was minimized by scrubbing between trials with soap and hot water.

On the day following testing, females were checked by vaginal lavage with saline for the presence or absence of motile spermatozoa. A clean pipette tip was used for gentle lavage of the vagina with 200  $\mu$ l of saline, following which the rinse was scanned under a microscope for the presence of spermatozoa. The presence of spermatozoa provided an independent indicator of sexual behaviour to ejaculation. This independent measure was valuable because it was difficult to distinguish between behavioural ejaculation and intromission without ejaculation.

Four individual observers were given a training period that included tests for consistency of observations. Videotapes were prescanned by a single individual to identify the initiation of reproductive behaviour. Following observer training, the number and timing of sexual behaviours were recorded by one observer blind to both line and photoperiod for the 120 min following initiation of sexual behaviour. Interobserver reliability was tested during observer training until they achieved 95% consistency. In addition, 13% of the trials were scored a second time by the lead observer (K.S.) as a check on continued consistency at approximately 95%. In two of 78 tests (one nonresponder and one responder male), sexual behaviour was initiated when there were fewer than 120 min remaining in the recording; in these cases the number of behaviours was prorated according to the time remaining on the tape. Analysing the results with or without these two individual tests did not affect the results of statistical testing.

#### *Fertility measures*

After the final behavioural test, male mice were euthanized with an overdose of CO<sub>2</sub>. Body mass, wet mass of paired testes and wet mass of seminal vesicles emptied of contents were recorded. Seminal vesicles were emptied because the volume of the fluid contents can vary depending upon recent ejaculations. Sperm counts were performed on one testis and one cauda epididymis as follows. Organs were individually homogenized in 1.0 ml of sperm-grinding solution (5% Triton-X in 0.9% NaCl saline) and diluted to 2.0 ml volume with a 1.0 ml rinse of the homogenizer. The number of mature sperm heads were counted in four diagonal haemocytometer squares ( $0.25 \times 0.25$  mm; 0.1 mm depth) and presented as an estimate of the total number of sperm in one testis or one cauda epididymis. If the density of sperm heads was too dense for accurate counting, a subsample was diluted and counted. For graphical presentation, the estimates were doubled to estimate total sperm in both testes or in both cauda epididymides.

#### *Assessment of error rates*

In two of 78 tests, behavioural observations were not consistent with the results of vaginal lavage for spermatozoa, suggesting an error rate of 3% (2 of 78) for the detection of sexual behaviour followed by insemination. We repeated the statistical analyses on behaviours after removing these two cases. Those results and conclusions were qualitatively identical for analyses on the numbers of mice performing sexual behaviours, the numbers positive for spermatozoa and for the frequency of behaviours. Based on the details of these cases, one was treated in our final analysis as negative for behaviour and spermatozoa, and the other as positive for both.

#### *Experiment 2: Phenotypic Plasticity of Maturation in Response to Olfactory Cues from Mature Females*

Male mice from the F6 and F7 generations from the responder and nonresponder lines were raised under short photoperiod, weaned at age  $22 \pm 1$  days, and caged singly with ad libitum food (LM-485, Harlan Teklad) and water until measurement of body mass and testis size at age  $70 \pm 3$  days. At  $80 \pm 5$  days of age, males were assigned to receive soiled bedding from mature females (responder:  $N = 11$  males; nonresponder:  $N = 10$  males) or a control-bedding treatment (responder:  $N = 13$  males; nonresponder:  $N = 12$  males). Within each line, males from soiled-bedding and control-bedding groups were matched for testes size (length  $\times$  width of testis), age and body mass. Data were collected in four runs, each of which was balanced between the control- and soiled-bedding treatments. Twelve adult female mice housed individually under long photoperiod provided bedding soiled with

urine that served as the olfactory stimulus. For 4 weeks, equal amounts of visibly soiled bedding were collected daily from the cage of each stimulus female and replaced with the same amount of fresh bedding. Bedding from all 12 females was mixed, and 200 ml from the mixed bedding was placed in the cage of each male in the experimental groups. Control mice were taken daily to the handling area, cage tops removed, and shavings disturbed in order to match activity and disturbance of the treatment groups. Transfer of bedding and the disturbance of the control treatment occurred 0–3 h before lights out on each day. At approximately 4-day intervals, excess bedding was removed from the cages of mice in the experimental groups.

#### *Experiment 3: Heritable Variation in Litter Size and Mass*

In the F8 to F13 generations of the two selection lines of our breeding colony, we recorded the number of pups at weaning ( $N = 290$  litters) to test for differences in litter size between selection lines. For many litters we recorded the average body mass and total body mass of pups in the litter at weaning. Because mice in the responder line do not breed well under short photoperiod, all litters were conceived and born under long photoperiod, simulating summer conditions. We tested two conditions: (1) litters born and retained under long photoperiod experience summer-like photoperiods and develop a long-photoperiod-typical phenotype; (2) litters born under long photoperiod and transferred within 3 days of birth to short photoperiod experience winter-like photoperiods and develop a short-photoperiod-typical phenotype.

#### *Data Analysis*

Data were analysed using R (v 2.14.0) with RStudio (v 0.97.449) running on a Macintosh computer. Tests for effects of selection line and photoperiod on body mass, reproductive organ masses, sperm counts and specific class of behaviour were conducted using type III ANOVA or, when covariates were included, ANCOVA. Because reproductive organ mass and sperm count are often associated, we assessed the strength of these associations using the Pearson's product–moment correlation coefficient. We tested for differences in the proportion of males showing sexual behaviour and in insemination rates among treatment groups using the Cochran–Mantel–Haenszel test with continuity correction (Agresti, 2002). We used the 'fisher.exact' test in R to test for effects of photoperiod on behaviour in each selection line. To assess which specific morphological variables or sperm counts might be most useful to predict male sexual behaviour for future studies, we conducted stepwise linear models for sexual behaviour. For males that initiated sexual behaviour, we tested for differences in number of genital grooming events, latency to initiation of sexual behaviour, latency to the first mount attempt, number of pursuits, number of mount attempts, latency from first mount attempt to first intromission, number of intromissions, latency from first mount attempt to first ejaculation and number of ejaculations. In experiment 2, ANOVA was used to test for effects of odour treatment and selection line. In experiment 3, ANOVA was used to test for differences in litter size or mass between selection lines in relation to photoperiod. In experiment 1, in preliminary statistical analyses of body mass, reproductive organ mass, sperm counts and sexual behaviour, we used linear models including as factors (1) generation, (2) family, (3) age in days of males at the time of testing or (4) age in days at the time of collection of reproductive organ masses and sperm counts. None of these additional factors had significant effects ( $P > 0.25$  for all tests); therefore, they were not included in the final analyses.

The threshold for statistical significance was  $P < 0.05$ . When multiple statistical tests were made in relation to the same hypothesis, there was a possibility that false positives would occur. We tested for potential false positives in a series of related statistical tests using the false discovery rate control (Glickman, Rao, & Schultz, 2014), setting the false discovery rate at 5% (i.e. fewer than 5% of the probability values accepted as statistically significant would be accepted in error).

## RESULTS

#### *Experiment 1: Variability and Phenotypic Plasticity in Sexual Behaviour, Reproductive Organs and Body Mass*

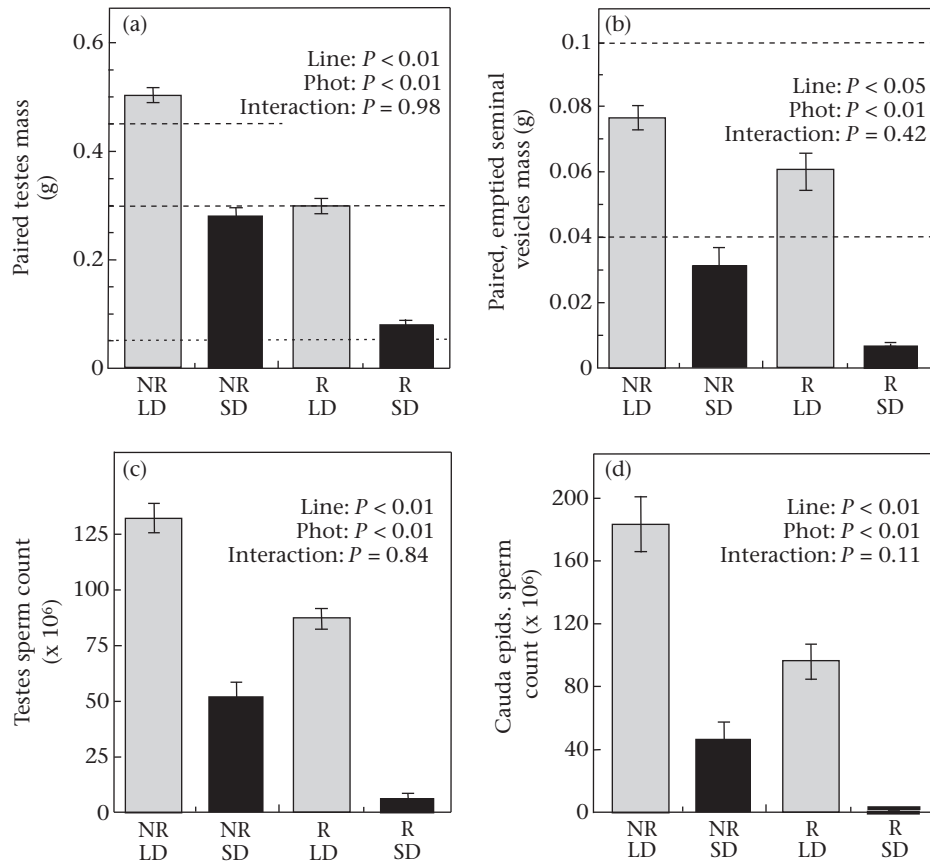
Testes and seminal vesicles were larger in nonresponder mice than in responder mice (Fig. 1a, b, Table 1). In both lines, testes and seminal vesicles were significantly larger under long photoperiod than under short photoperiod (Fig. 1a, b, Table 1). Although testes mass of nonresponder mice in the short-day group was similar to that of responder mice in the control group (Fig. 1a), nonresponder mice in the short-day group had smaller seminal vesicles (Fig. 1b). Body mass of adult males was slightly lower under short photoperiod than under long photoperiod, but did not differ significantly by line (photoperiod:  $F_{1,54} = 8.59$ ,  $P = 0.005$ ; line:  $F_{1,54} = 0.67$ ,  $P = 0.42$ ; interaction:  $F_{1,54} = 2.24$ ,  $P = 0.14$ ).

Sperm counts from the testes and cauda epididymides were higher in nonresponder mice than in responder mice and higher under long photoperiod than under short photoperiod (Fig. 1c, d, Table 1). Only a few mice lacked countable heads of developing spermatozoa in the testes and cauda epididymis and were classed as azoospermic; all of these were responder mice in the short-day group. When analysed with testes mass included as a covariate, testicular sperm counts were significantly lower under short photoperiod than under long photoperiod (Table 1). In other words, testes had fewer sperm per unit mass under short photoperiod than under long photoperiod (compare Fig. 1a and c).

Paired testes mass was correlated significantly with the mass of paired seminal vesicles ( $r = 0.88$ ,  $N = 58$ ,  $P < 0.0001$ ), testicular sperm count (Fig. 2) and cauda epididymal sperm count ( $r = 0.85$ ,  $N = 58$ ,  $P < 0.0001$ ). Nearly all (95%) mice with paired testes mass greater than 0.13 g had at least 10 million spermatozoa in the cauda epididymides (Fig. 2). Males with paired testes mass below 0.13 g had few or no spermatozoa in the cauda epididymis.

#### *Sexual behaviour*

The incidence of sexual behaviour with a receptive female during the test night was greater than 75% for males in the control groups and in the nonresponder short-day group (Fig. 3a). In contrast, only one of 12 males in the responder short-day group (<10%) showed male sexual behaviours during the test night (Cochran–Mantel–Haenszel on line, photoperiod and sexual behaviour:  $\chi^2_1 = 7.78$ ,  $P = 0.005$ ; Fig. 3a). With exact permutation  $2 \times 2$  tests ('fisher.exact' in R), responder mice differed under short and long photoperiod ( $P < 0.001$ ) and differed from nonresponder mice under short photoperiod ( $P = 0.0006$ ). When data from the test night and supplemental night were combined, over 90% of males in the nonresponder and responder control groups and in nonresponder short-day group displayed sexual behaviour (Fig. 3a). In contrast, only 42% of males in the responder short-day group displayed sexual behaviour during the test night and the supplemental night (Monte Carlo simulation with 'fisher.exact' test in R:  $P = 0.0001$ ; Fig. 3a). For both responder and nonresponder control groups, approximately 50% of stimulus females tested positive for spermatozoa at the time of vaginal lavage on the following day



**Figure 1.** Mass of paired (a) testes and (b) emptied seminal vesicles and estimated sperm count for paired (c) testes and (d) cauda epididymides of male *P. leucopus* from a photoperiodically nonresponder (NR) and responder (R) selection line reared under long photoperiod (LD) or short photoperiod (SD) and tested for sexual behaviour. For all four variables, the effects of selection line and photoperiod were statistically significant ( $P < 0.01$  for all). In (a), the two dashed lines at paired testes mass 0.45 and 0.3 g indicate the range of testes sizes in a comparable reference sample from an unselected control line in LD ( $N = 13$ ). The dotted line in (a) at paired testes mass 0.05 g indicates the approximate testis mass for azoospermia (this study; see Fig. 2). In (b), the two dashed lines at paired seminal vesicle mass 0.1 and 0.04 g indicate the range of paired seminal vesicle sizes in a comparable reference sample from an unselected control line in LD ( $N = 13$ ). For (c) and (d), sperm counts from a single testis and single cauda epididymis were multiplied by two. Values are means  $\pm$  SE.

(Cochran–Mantel–Haenszel test on line, photoperiod, and insemination:  $\chi^2_1 = 2.77$ ,  $P = 0.09$ ; Fig. 3b).

When considering only data from the test night, there were too few individuals displaying sexual behaviour in the responder short-day group for analyses of the frequency of sexual behaviours. When behavioural data were combined from the test night and the supplemental night, there were no significant differences among groups for any of these variables: latency to initiate sexual behaviour (line:  $F_{1,45} = 0.01$ ,  $P = 0.99$ ; photoperiod:  $F_{1,45} = 0.80$ ,  $P = 0.38$ ; interaction:  $F_{1,45} = 0.13$ ,  $P = 0.72$ ); pursuits (line:  $F_{1,45} = 0.16$ ,  $P = 0.69$ ; photoperiod:  $F_{1,45} = 1.09$ ,  $P = 0.30$ ; interaction:  $F_{1,45} = 0.22$ ,  $P = 0.64$ ); mount attempts (line:  $F_{1,45} = 0.02$ ,  $P = 0.90$ ;

photoperiod:  $F_{1,45} = 0.11$ ,  $P = 0.74$ ; interaction:  $F_{1,45} = 0.51$ ,  $P = 0.48$ ); intromissions (line:  $F_{1,45} = 0.08$ ,  $P = 0.78$ ; photoperiod:  $F_{1,45} = 0.44$ ,  $P = 0.51$ ; interaction:  $F_{1,45} = 0.05$ ,  $P = 0.83$ ); ejaculations (line  $F_{1,45} = 0.56$ ,  $P = 0.46$ ; photoperiod:  $F_{1,45} = 0.33$ ,  $P = 0.57$ ; interaction:  $F_{1,45} = 0.10$ ,  $P = 0.76$ ).

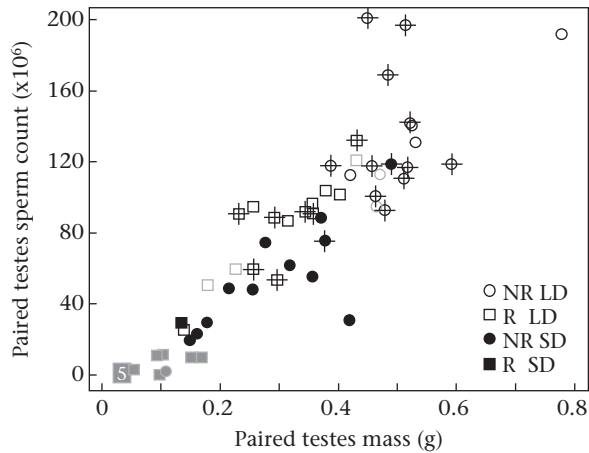
All variables for reproductive organ mass and sperm count were significantly related to insemination or sexual behaviour in males ( $P < 0.01$  for all). Using stepwise linear models, the best predictor for detectable insemination was cauda epididymal sperm count ( $F_{1,56} = 23.82$ ,  $P < 0.0001$ ), after which no added variables were significant. Significant predictors for sexual behaviour on the test night were cauda epididymal sperm count ( $F_{1,56} = 12.62$ ,

**Table 1**  
Results from ANOVA (and, for testicular sperm count with testes mass as covariate, ANCOVA) for body mass, reproductive organ mass and sperm counts of mice from nonresponder and responder selection lines housed under long and short photoperiods

	Line		Photoperiod		Interaction	
	$F_{1,54}$	$P$	$F_{1,54}$	$P$	$F_{1,54}$	$P$
Body mass	0.67	0.42	8.59	<b>0.0049</b>	2.24	0.14
Testes mass	41.56	<b>0.0001</b>	43.66	<b>0.0001</b>	0.01	0.99
Seminal vesicles mass	6.53	<b>0.013</b>	55.25	<b>0.0001</b>	0.66	0.42
Testes sperm count	24.15	<b>0.0001</b>	50.70	<b>0.0001</b>	0.04	0.84
Cauda epididymis sperm count	23.39	<b>0.0001</b>	20.88	<b>0.0001</b>	2.52	0.12
Testes sperm count (with testes mass as covariate)	0.42	0.52	7.31	<b>0.009</b>	0.09	0.98

Effects that were significant at  $P < 0.05$  are indicated in bold.





**Figure 2.** Sexual behaviour and insemination in relation to sperm count and mass of the testes ( $r = 0.90$ ,  $P < 0.0001$ ) of male *P. leucopus* from a photoperiodically non-responder (NR) and responder (R) selection line reared under long photoperiod (LD) or short photoperiod (SD). Points marked in black represent males that displayed sexual behaviours on the test night and/or inseminated the stimulus female. Symbols marked with a + indicate the subset of individuals for which the stimulus female was positive for spermatozoa on the day following testing. Points marked in grey represent males that neither displayed sexual behaviours on the test night nor inseminated the stimulus female on the test night. Sperm counts from a single testis and single cauda epididymis were multiplied by two.

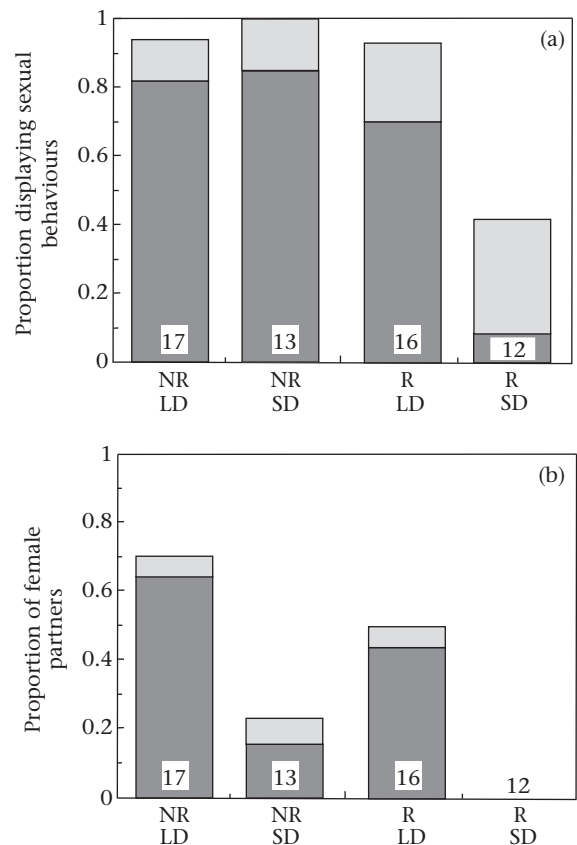
$P < 0.001$ ), seminal vesicle mass ( $F_{1,56} = 10.96$ ,  $P < 0.005$ ) and selection line ( $F_{1,56} = 6.24$ ,  $P < 0.05$ ). However, because both testes mass and seminal vesicle mass were highly correlated with cauda epididymal sperm count, the mass of either organ accounted for almost as much variation in sexual behaviour or insemination as did cauda epididymal sperm count.

The likelihood of sexual behaviour and insemination was related to testes mass and sperm counts (Fig. 2). Above a paired testes mass of 0.225 g and sperm counts from the testes and cauda epididymides above 40 million, males appeared approximately equally likely to display sexual behaviour and inseminate females (Fig. 2). Nearly all control males met these criteria. In contrast, males with a paired testes mass of 0.130–0.225 g and a testicular sperm count in the range of 20–40 million were unlikely to inseminate females detectably, but were likely to display sexual behaviour (Fig. 2). Males with paired testes mass below 0.130 g and a sperm count from the testes below 20 million and from the cauda epididymides below 10 million were unlikely to display sexual behaviour (Fig. 2). Finally, below a paired testes mass of about 0.050 g, males were azoospermic and did not display sexual behaviour (Fig. 2).

#### Experiment 2: Response of Testes and Seminal Vesicles to Soiled Female Bedding

The testes and seminal vesicles of nonresponder males under short photoperiod exposed daily for 4 weeks to soiled bedding from mature females were larger than those of nonresponder controls (Fig. 4). In contrast, the testes and seminal vesicles of responder males with soiled bedding were similar in size to the responder controls (Fig. 4).

For testes, there was a significant effect of selection line and presence or absence of soiled bedding, with a marginally nonsignificant interaction (line:  $F_{1,42} = 23.19$ ,  $P < 0.0001$ ; soiled bedding:  $F_{1,42} = 7.04$ ,  $P = 0.011$ ; interaction:  $F_{1,42} = 3.31$ ,  $P = 0.08$ ; Fig. 4a). For seminal vesicles, there was a significant effect of soiled bedding and the interaction term, but not selection line (line:  $F_{1,41} = 2.35$ ,  $P = 0.13$ ; soiled bedding:  $F_{1,41} = 24.07$ ,  $P < 0.0001$ ; interaction:  $F_{1,41} = 12.64$ ,  $P = 0.001$ ; Fig. 4b). The significant interaction was due



**Figure 3.** (a) Proportion of male *P. leucopus* from a photoperiodically nonresponder (NR) and responder (R) selection line reared under long photoperiod (LD) or short photoperiod (SD) that displayed sexual behaviour during a test night with a hormonally primed, sexually receptive female (dark grey bars). Twenty males that failed to display sexual behaviour during the test night were retested in a supplemental test 4 days later ( $N = 3$  NR LD, 2 NR SD, 4 R LD, 11 R SD); those males that displayed sexual behaviour on the second night are indicated in light grey. (b) Proportion of receptive females in which spermatozoa were detected by vaginal lavage on the day following a test night for sexual behaviour. Sample sizes of test males in each group are indicated by numbers at the base of the bars.

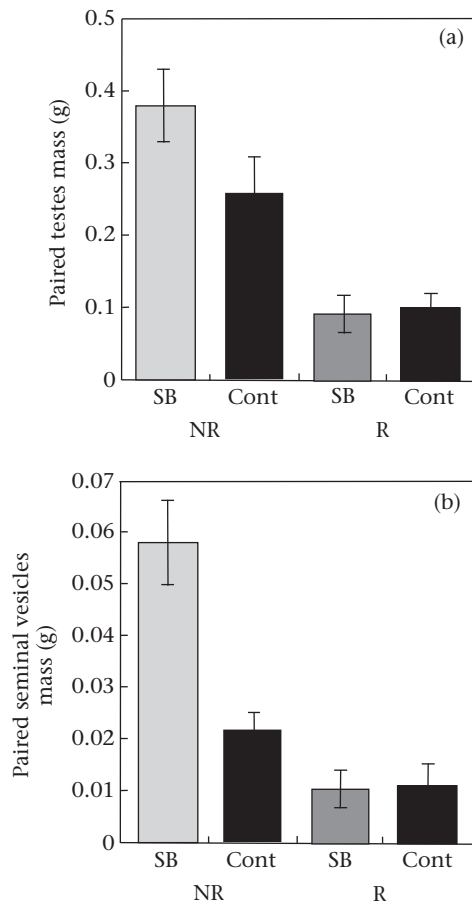
to doubled mass of seminal vesicles in the soiled-bedding treatment of nonresponder males relative to nonresponder controls, but lack of effect of soiled bedding on the responder line relative to controls (Fig. 4b).

#### Experiment 3: Litter Size and Mass under Long and Short Photoperiod

The selection lines did not differ significantly in litter size at weaning (line:  $F_{1,366} = 0.76$ ,  $P = 0.38$ ; photoperiod:  $F_{1,366} = 6.21$ ,  $P = 0.01$ ; interaction:  $F_{1,366} = 0.79$ ,  $P = 0.38$ ), total mass of litters (line:  $F_{1,201} = 0.85$ ,  $P = 0.36$ ; photoperiod:  $F_{1,201} = 0.54$ ,  $P = 0.46$ ; interaction:  $F_{1,201} = 0.83$ ,  $P = 0.36$ ) or average mass of pups in litters at weaning (line:  $F_{1,201} = 0.85$ ,  $P = 0.36$ ; photoperiod:  $F_{1,201} = 0.21$ ,  $P = 0.65$ ; interaction:  $F_{1,201} = 0.57$ ,  $P = 0.45$ ; Table 2). Even though the effect of photoperiod on litter size was  $P = 0.01$ , when probability values were evaluated as a group by the false discovery rate control (Glickman et al., 2014), this single  $P$  value less than 0.05 among nine  $P$  values did not reach statistical significance.

## DISCUSSION

Our results demonstrate heritable variation in male sexual behaviour under short photoperiod. Short photoperiod caused



**Figure 4.** Mass of paired (a) testes and (b) seminal vesicles of male *P. leucopus* from a photoperiodically nonresponder (NR) and responder (R) selection line exposed under short photoperiod either to soiled bedding (SB) from mature females, or to a control (Cont) treatment of daily disturbance to cage bedding for 4 weeks. In (a) the effect of selection line and treatment with soiled bedding were both statistically significant ( $P < 0.01$  for both), and the interaction term was marginally nonsignificant ( $P = 0.08$ ). In (b), the effect of treatment with soiled bedding and the interaction term were statistically significant ( $P < 0.01$  for both). Values are means  $\pm$  SE.

lower reproductive organ mass and sperm counts in both lines (Fig. 1), indicating that both lines detect and respond to a seasonal photoperiod signal. Male sexual behaviour was strongly reduced only in the responder line under short photoperiod (Fig. 3a), in which male sexual behaviour did not occur on the test night below defined thresholds of reproductive organ mass and sperm count (Fig. 2). For the subset of responder males under short photoperiod that had the largest reproductive organs and displayed sexual behaviour, behaviours were not different from males in the other

groups. In our second experiment, there was evidence of an effect on testes mass of olfactory cues from females (Fig. 4a). However, olfactory cues from females did not affect the two lines in the same way, as soiled bedding increased seminal vesicles significantly in nonresponder males but not in responder males (Fig. 4b), with a similar pattern of effects on testis mass (Fig. 4a). Overall, our results imply that heritable variation in male sexual behaviour may be expressed under short photoperiod, but not long photoperiod, in wild populations of *P. leucopus*. Other studies have shown variation in behaviour that is related to variation in reproductive responsiveness (Gorman, Ferkin, Nelson, & Zucker, 1993), suggesting that this type of behavioural variation might be common.

In other mammals, male sexual behaviour under short photoperiod is related to reproductive organ size and spermatogenesis (Awoniyi et al., 1993; Campbell et al., 1978; Morin & Zucker, 1978; Park & Rissman, 2007; Park et al., 2004; Powers et al., 1989), but there is variability within populations in the relationship between male sexual behaviour and testis size (Park & Rissman, 2007; Park et al., 2004). For example, under short photoperiod, 50% of male Siberian hamsters maintained sexual behaviour through ejaculation despite testicular regression (Park et al., 2004). The results of our study are consistent with the hypothesis that the likelihood of sexual behaviour and insemination is related to testis size and the availability of gametes. In our analyses, if reproductive organ mass or sperm count had been included in a linear model for sexual behaviour or insemination, the additional variables of 'photoperiod' and 'selection line' were unnecessary (and not statistically significant) for predicting sexual behaviour or insemination. We identified two thresholds of reproductive organ mass and sperm count, one for male sexual behaviour, and the other for insemination of females. Under long photoperiod, all males were above these thresholds and the selection lines did not differ in any measure of sexual behaviour. Under short photoperiod, in contrast, we observed a threshold for insemination at the lower limit of normospermy under long photoperiod (Fig. 2). Above this threshold, males were equally likely to inseminate females, while below it insemination was nearly absent. For male sexual behaviour, the threshold was lower. Males in the oligospermic range with paired testes mass above 0.13 g and sperm counts above 20 million (testes) or 10 million (cauda epididymides) were as likely to display sexual behaviour as normospermic males (Fig. 2). However, below this threshold males were azoospermic or severely oligospermic and male sexual behaviour was eliminated (Fig. 2), a result in contrast to the maintenance of sexual behaviour in hamsters that were azoospermic under short photoperiod (Park et al., 2004). Our interpretation is that males were likely to display sexual behaviour if they had any potential to produce offspring, as only males with no or almost no spermatozoa failed to display mating behaviour. Consequently, males with intermediate testes sizes may incur behavioural costs of reproduction under short photoperiod, including the opportunity costs and risk costs of mating (Lima, 1998). Only

**Table 2**

Litter size, average mass of pups at weaning and total mass of litter at weaning for litters conceived and retained under long photoperiod (long day) or transferred after birth to short photoperiod (short day) until weaning

	Nonresponder		Responder	
	Long day	Short day	Long day	Short day
Litter size at weaning	4.0 $\pm$ 0.2 (N=83)	3.4 $\pm$ 0.1 (N=85)	3.8 $\pm$ 0.2 (N=68)	3.5 $\pm$ 0.1 (N=114)
Mass of each offspring at weaning	13.9 $\pm$ 0.6 g (N=9)	13.5 $\pm$ 0.2 g (N=80)	12.8 $\pm$ 0.5 g (N=9)	13.3 $\pm$ 0.2 g (N=107)
Total mass of litter at weaning	44.1 $\pm$ 5.3 g (N=9)	45.4 $\pm$ 1.8 g (N=80)	42.7 $\pm$ 7.5 g (N=9)	45.0 $\pm$ 1.8 g (N=107)

Lines were artificially selected to be nonresponder to short photoperiod (nonresponder) or responsive to short photoperiod (responder). Values are means  $\pm$  SE. Sample size (N) is the number of litters measured (see text for statistical analysis).

azoospermic and severely oligospermic males do not attempt mating, and therefore escape these costs.

We asked whether cues from mature females would raise short-photoperiod thresholds for sexual behaviour or insemination in nonresponder or responder males. Under short photoperiod, cues from the bedding of mature females appeared to have no effect on responder males but increased the mass of testes and seminal vesicles of most nonresponder males to approach the means for nonresponder males under long photoperiod (compare Fig. 1a and b with Fig. 4). While we did not measure sperm counts in this experiment, the high correlation between testes mass and sperm count (Fig. 2) suggests that sperm count increased with testes mass. Testes mass of nearly all bedding-exposed males in the nonresponder line was above the threshold for insemination, suggesting that even under short photoperiod, males with a nonresponder phenotype and genotype might have normal fertility if exposed to mature females. The phenotypically plastic stimulation by olfactory cues in nonresponder males, but not responder males, demonstrates heritable variation in plasticity of this response. To our knowledge, heritable variation in phenotypic plasticity of reproductive responses to olfactory cues in wild-derived populations has not been reported previously, but Gorman et al. (1993) reported variation in odour preferences between responder and nonresponder phenotypes of meadow voles, *Microtus pennsylvanicus*. The photoperiod responder and nonresponder reproductive phenotypes showed variation in attraction to odours from their own versus the opposite phenotype. We did not test for preferences to odours, but it is possible that individuals might gain a fitness advantage in the response to odour cues and preferences to odour cues by maximizing interactions between reproductively active individuals. If males from our selection lines have preferences similar to those described by Gorman et al. (1993), then nonresponder males preferring the odours of mature nonresponder females would also be reproductively stimulated by those olfactory cues, while responder males would be neither attracted nor affected.

Other populations of *Peromyscus* that contain heritable variation in reproductive response to short photoperiod (Desjardins et al., 1986; Heideman & Bronson, 1991; Prendergast et al., 2001; Wichman & Lynch, 1991) also may contain heritable variation in phenotypic plasticity of reproductive response to mature females under short photoperiod. In a study on our source population, Terman (1984) reported acceleration of puberty under long photoperiod in our source population of *P. leucopus*, but did not test mice under short photoperiod. In previous studies, puberty was accelerated in young male *Peromyscus maniculatus* when housed with adult females under short photoperiod (Whitsett & Lawton, 1982), while young males from a population of *P. leucopus* were unaffected by housing with adult females under short photoperiod (Pyter, Neigh, & Nelson, 2005). Studies that show significant responses to cues from mature females under short photoperiod may have a high proportion of males that are phenotypically plastic in response to cues from mature females, and those that show no response may have a high proportion of males that lack phenotypic plasticity. Results would vary depending upon the balance of genotypes.

We hypothesized that heritable differences in male sexual behaviour, sperm count and size of reproductive organs might cause differences in litter size between the nonresponder and responder lines. For litters conceived under long photoperiod, the photoperiod in which both nonresponder and responder mice are fertile, we found no significant differences between selection lines in litter mass or number of pups per litter regardless of whether mice had been raised under long or short photoperiod (Table 2). Even though a low level of male sexual behaviour impairs fertility

in the responder line under short photoperiod, our results do not suggest that any impaired fertility occurs in the longer photoperiods of summer. In mammals, including species of *Peromyscus*, large reproductive organs, high sperm count and frequent male sexual behaviour are associated with fitness advantages when males mate with multiple females in succession or are in sperm competition (Dixon & Anderson, 2004; Fisher & Hoekstra, 2010; Ramm, Parker, & Stockley, 2005). The monogamous pairings in our laboratory colony block these potential fitness advantages, although additional effects on fitness are possible in the wild source population.

One objective of our laboratory examination of genetic variation and phenotypic plasticity was to gain insights that may be relevant in natural populations. In winter, an average of 35–55% of wild females from our source population were pregnant or lactating from 1983 to 1989 (Terman, 1993). Wild males from the source population in winter have testes sizes in the same size range (0.02–0.65 g,  $N = 33$ ; Proffitt, 2014) as those from our selection lines under short photoperiod (Broussard et al., 2009; Heideman et al., 1999; this study). We assume that sperm counts may also be similar based on the relationship found in this study (Fig. 2). In the wild, our results suggest that males at the low azoospermic or highly oligospermic end of this range would display no sexual behaviour in winter. Males with reproductive organs that are intermediate in size might display sexual behaviour, but have low rates of successful insemination. However, if intermediate males have a nonresponder genotype, then exposure to olfactory cues from mature females would increase the mass of reproductive organs to match those of males under long photoperiod (Fig. 4).

Winter reproduction is often viewed as a cost–benefit trade-off, in which the potential increase in fitness from winter reproduction is offset by a relatively high cost of reproduction in winter (Heideman, Rightler, & Sharp, 2005; Horton & Rowsemitt, 1992; Prendergast et al., 2001). Selection favouring winter sexual behaviour and reproduction would increase the frequency of nonresponders and cause population growth in winter (Nelson, 1987). Selective association of nonresponder individuals (Gorman et al., 1993) may provide odour cues that stimulate reproductive maturation (this study). Adaptation of populations to a changing climate has been hypothesized to be dependent upon the presence of heritable variation in seasonal timing of reproduction (Bradshaw & Holzapfel, 2008; Myers, Lundrigan, & Vande Kopple, 2005; Visser, 2008), including seasonality of sexual behaviour (this study). These hypotheses could be tested by investigating heritable variation in behavioural responderness in wild populations.

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