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Cross-fostering selectively bred High Runner mice affects adult body mass but not voluntary exercise

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ABSTRACT

While nursing, mammals progress through critical developmental periods for the cardiovascular, musculoskeletal, and central nervous systems. The suckling period in mammals is therefore especially vulnerable to environmental factors that may affect the "developmental programming" of many complex traits. As a result, various aspects of maternal behavior and physiology can influence offspring in ways that have lasting effects into adulthood. Several recent studies of animal models have shown that maternal effects can partially program adult activity behaviors, which has important implications for health and locomotor performance. Here, we used cross-fostering to test for possible maternal effects on adult wheel-running behavior (voluntary exercise), maximal aerobic capacity during forced exercise (VO_2max), body mass and composition, and organ masses. Subjects were from a line of mice that has been selectively bred for ~90 generations for high voluntary wheel-running behavior (High Runner; HR) and a non-selected Control (C) line. Adult HR mice run ~3-fold the daily distances of C mice and have evolved other differences associated with exercise capacity, including elevated VO_2max, reduced body mass and fat mass, and larger hearts. At birth, we fostered offspring to create 4 experimental groups: C pups to other C dams (in-foster), HR pups to other HR dams (in-foster), C pups to HR dams (cross-foster), HR pups to C dams (cross-foster). Thus, all pups were fostered to a different mother. Mice were weaned 3 weeks later, and adult testing began at ~6 weeks of age. At weaning, pups raised by HR dams were smaller than those raised by C dams for both sexes and as expected, HR pups raised by HR dams weighed less than C pups raised by C dams. As adults, mice raised by HR dams continued to have reduced body masses. As expected, adult HR mice ran approximately 3-fold more than their C counterparts and females ran more than males. However, cross-fostering did not statistically affect any aspect of wheel-running behavior (distance, duration, speed). Similarly, with body mass as a covariate, HR mice had higher VO_2max than C mice, and males had higher VO_2max than females, but cross-fostering had no effect. With body mass as a covariate, cross-fostering had variable effects on adult organ masses in a sex-specific manner. Overall, our results indicate that development of the adult High Runner phenotype does not require rearing by an HR dam, suggesting that high adult activity in humans may be independent of high maternal activity.

1. Introduction

Physical activity is essential for both the development and maintenance of physical and mental health [e.g., 1–6]. The many contributors to the levels of adult physical activity can be broadly classified as genetic versus environmental effects. Numerous studies of both humans and rodent models have demonstrated an important genetic component to physical activity, including levels of voluntary exercise [7]. Among the many environmental factors (e.g., the built environment for humans) that can influence adult activity levels, those experienced early in life, especially during critical periods, can have long-lasting effects into adulthood [8,9].

In mammals, mothers are a key component of the early-life environment, as they provide nutrition, care, and protection during critical

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periods of growth and development. Thus, variation in aspects of the mammalian maternal environment, including care *per se* (e.g., grooming), thermoregulation, and feeding via lactation (e.g., frequency of nursing bouts, milk nutritional content) has the potential to affect offspring development, with potentially persistent effects [e.g., 10–13]. For example, maternal licking/grooming behavior in rats epigenetically induces altered behavior and stress responses in offspring later in life [14].

A growing number of studies show that adult physical activity can, to some extent, be "developmentally programmed" by maternal effects [e.g., 15–20]. These studies are routinely done by using cross-fostering experiments, which are a powerful method for elucidating maternal effects in mammals, as well as possible gene × environment interactions. This approach has been applied many times using rodent models [10, 21–25] and has been used to investigate maternal differences between genetically distinct strains of rodents [e.g., 26–28], including those resulting from artificial selection. For example, Barrenha and Chester [29] cross-fostered mice selectively bred for high- and low-alcohol preference. When fostered to low-alcohol preferring dams, high-alcohol preferring mice had a significant reduction in alcohol consumption and preference across a 28-day period with 24-hour access [29].

Although cross-fostering is a common approach within rodent models, to our knowledge only two cross-fostering studies have examined possible maternal effects on adult voluntary exercise or spontaneous physical activity (SPA). For example, when mice were fostered into small-litter families (resulting in postnatal overnutrition), adults had greater adiposity and female mice, specifically, had reduced SPA [30]. In a study using an obese mouse model (viable yellow agouti A^{vy}/a), newborn mice were cross-fostered between A^{vy}/a and wildtype (a/a) dams, which resulted in reduced adult SPA in a/a females born to A^{vy}/a dams and fostered to a/a dams [31].

In the current study, we used a cross-fostering approach to test for possible maternal effects on adult physical activity in a unique animal model, which includes four replicate selectively bred High Runner (HR) lines of mice and their four non-selected Control (C) lines [32]. Mice in the HR lines have been bred for voluntary exercise on wheels on days 5 and 6 of a 6-day running period as young adults for >90 generations. Since reaching apparent selection limits between generations 17 and 27 [33], HR lines have continued running ~3-fold more revolutions per day than C mice. This wide differential in the amount of voluntary exercise should increase statistical power to detect maternal effects on activity levels, if they exist.

Given that HR and C mice differ in many additional traits, including circulating levels of multiple hormones [34–37] and reduced body fat in the HR mice [38], we reasoned that differences in maternal care might broadly contribute to the adult differences between HR and C mice (we discuss our results in the context of potential coadaptation of maternal effects below). The plausibility of maternal effects on adult HR activity levels is further supported by recent studies that have demonstrated the importance of early-life factors in the development of the HR phenotype. For instance, when given wheel access during the juvenile period (3 weeks between weaning and sexual maturity), followed by a washout period of 7.5 weeks (no wheel access), adult mice ran greater distances on wheels, demonstrating that activity levels could be modulated not only in HR lines, but in C lines as well [39]. When fed a Western diet during the same early-life period and after a similar washout period, adult wheel running was increased in HR lines, but not C, indicating that the early-life "programmability" of adult activity levels may depend on genetic background [40]. In another study, Western diet fed to HR and C dams before mating until the weaning of their pups had no overall effect on the adult wheel running of their offspring [20]. Taken together, the foregoing studies suggest that both the magnitude and direction of early-life effects on adult levels of activity in HR and C mice depend on the timing and nature of the early-life factor.

In the present study, we used one representative HR line and one C line. At birth, we cross-fostered C pups to HR dams and vice versa, as well as C pups to C dams and HR pups to HR dams (in-foster: see Table 1). We hypothesized that cross-fostering C pups to HR dams would result in higher adult wheel running and supportive traits (e.g., VO_2max, heart mass). Conversely, we expected the cross-fostering of HR pups to C dams would decrease their adult wheel running, for lack of hypothetical developmental stimuli otherwise provided by HR dams.

2. Methods

2.1. Experimental mice

Starting in 1993, four replicate lines of house mice were bred in an ongoing selection experiment for high voluntary wheel running (HR lines), based on the number of wheel revolutions on days five and six of six days of access to Wahman-type activity wheels (1.12-meter circumference) as young adults [32]. The experiment began with a population of 224 mice from the outbred Hsd:ICR strain, which was randomly mated for two generations before being randomly partitioned into eight lines. Four of these were bred randomly as Control (C) lines to the four HR lines. The current experiment used a subset of virgin male and female mice from generation 89 to produce the experimental focal mice of generation 90. For the purposes of maintaining comparability with previous generations of mice, all generation 89 males and females had previous access to a running wheel for a six-day period, as described above. All mice were fed standard mouse chow (Teklad Rodent Diet W-8604) and regular drinking water. Pregnant dams were given a breeder diet (Teklad S-2235 Mouse Breeder Sterilizable Diet 7004) through weaning. All experiments were approved by the University of California, Riverside IACUC.

To keep the number of required cross-fostering litters manageable, here we used one C line (Line 4) and one HR line (Line 7), chosen because they represented extremes in body mass among their respective linetypes. We reasoned that differences in dam body size could lead to differential cross-fostering effects, e.g., on the mass of their pups at weaning. Thus, by using lines with different body masses, they could serve as a type of positive control for offspring body masses at weaning. The wheel running behavior of these lines was representative of their respective linetypes (Table 2). The dams used in the present study were typical of other line 4 and 7 breeders of the same generation in terms of both wheel running and body mass (Table 2). Moreover, we intentionally avoided the use of HR lines 3 and 6, which express the mini-muscle phenotype, a simple Mendelian recessive allele characterized by massive alterations to skeletal muscle [41,42].

2.2. Cross-fostering

Fig. 1 presents the experimental timeline. Mice from generation 89 were sampled randomly to create a total of 60 line 4 and 60 line 7 mating pairs, with the constraint that sample size per foster group (Table 1) was equal. We created such a large number of pairings because only pups born on the same day were to be used for fostering [43,44]. Only mice that were wheel-tested (Table 2) were used for mating and fostering of pups to allow for the possibility that wheel-running itself may be important the maternal environment of HR dams. For logistical reasons, mating pairs were performed in two batches, one week apart (Fig. 1

Table 1
Four treatment groups were generated by cross-fostering between families of HR (line 7) and C (line 4) mice.

	Foster group	Birth dam	Foster dam	# of Litters
In-foster	C→C	C	C	7
Cross-foster	C→HR	C	HR	7
In-foster	HR→HR	HR	HR	7
Cross-foster	HR→C	HR	C	8

Table 2
Characteristics of generation 89 breeder females. Measurements of female breeders from generation 89 were taken as part of the on-going selection experiment. Average daily revolutions on days 5 and 6 ($n = 126$) and subsequent body mass ($n = 134$) at the time their pups were weaned are shown as least squares means and standard errors from SAS Procedure Mixed (mass with age as a covariate, wheel running with age and wheel freeness as covariates). Shown in parentheses are the corresponding values for the separate set of breeders used in the present study.

	Line	Revolutions/day	S.E.	Body mass (g)	S.E.
C	**1**	4,868	589	35.3	0.73
	2	4,248	555	35.8	0.70
	4	4,275 (3,552)	752 (855)	40.2 (40.5)	0.98 (0.44)
	5	4,901	718	32.1	0.82
HR	**3**	14,275	641	36.9	0.74
	6	16,525	585	35.7	0.68
	7	16,291 (16,378)	678 (855)	28.5 (28.0)	0.87 (0.44)
	8	16,171	606	33.2	0.70

shows one batch).

At birth, litters were standardized to eight pups from an average of ~10 [20,45] to avoid competition that might favor either HR or C pups, or other unforeseen litter effects. As sex could not be determined at birth, litter sex ratio could not be controlled (see Results 3.2.). Fostering only occurred between litters born within 24 h of one another. During the 48 h after fostering, fostered pups were checked three times daily and none were rejected by their foster mother.

As births occurred, entire litters were fostered to another dam (no pup was returned to its biological mother). Thus, we did not include a "control" group for the effects of fostering *per se*. This design was chosen to maximize the sample size in experimental groups sufficient to address our specific hypotheses (see Introduction), given logistical constraints on total sample size. We wanted to determine whether rearing by an HR dam might be necessary for some proportion of high-running phenotypic variance. We also wanted to know whether rearing by an HR dam might confer some aspect of the HR phenotype, such as higher adult voluntary wheel-running behavior.

2.3. Adult testing of fostered offspring

Physical activity is typically partitioned into voluntary exercise and spontaneous physical activity, and both components can have important effects on many aspects of health [47–54]. Accordingly, we measured both wheel-running behavior and home-cage activity as indicators of voluntary exercise and SPA, respectively [46].

At 7 weeks of age, all mice were individually housed with food and access to running wheels. For each day of wheel testing (6 days total), we recorded revolutions in each 1-minute interval over a period of 23 h. This allowed computation of the total distance run, the number of 1-minute intervals with at least one revolution (a measure of the daily running duration), and the average running speed (distance/intervals), as well as the maximum speed in any 1-minute interval [55]. We also computed average values for days 5 and 6, as these are the values used for the selection protocol [32]. Wheel freeness (an inverse measure of how difficult it is to turn the wheel) was used as a covariate in all analyses of wheel running [e.g., 55].

During wheel testing, home cages were fitted with passive infrared sensors (Talon TL-Xpress-A; Crow Electronics, Fort Lee, New Jersey, USA), protected within wire mesh, as in previous studies [39,55]. The sensors were connected to a computer with custom activity-recording software (developed by M. A. Chappell) via a digital I/O board (ICS 2313; ICS Electronics, Pleasanton, CA, USA). The sensors recorded activity three times per second and a mean value between 0 (no movement detected) and 1 (movement detected) was calculated for each minute over 23 h-periods of measurement. All analyses of SPA data used a measure of sensor sensitivity as a covariate [39,55].

2.4. Body composition

Whole-animal fat and lean masses were measured by restraining each mouse within a translucent tube before insertion into an EchoMRI-100 (Echo Medical Systems, Houston, TX, USA) for scanning. This procedure lasted approximately 1–2 min per mouse and did not require sedation or anesthesia. Body composition was measured on mice approximately one hour before the start of wheel testing and within one hour of being taken off wheels.

2.5. Maximal aerobic capacity (VO_2max)

To measure VO_2max, mice were subjected to forced exercise within an enclosed wheel metabolic chamber approximately 15 cm in diameter, with an effective volume of 900 mL [56,57]; this method yields estimates of VO_2max that are statistically indistinguishable from treadmill procedures [58]. Air was pumped into the enclosed metabolic chamber at a rate of 2000 mL per min (with instantaneous corrections applied), and the concentration of O_2 in excurrent air was passed through H_2O and CO_2 scrubbers (Drierite and indicating soda lime) and measured by an oxygen analyzer (S-3A Applied Electrochemistry, Inc., Sunnyvale, CA), the second channel of which was used to record ambient O_2 concentration.

Mice were weighed and then placed into the metabolic chamber. After an initial 1–2 min of acclimation, mice were induced to run for approximately 5 min while researchers manually accelerated the wheel until VO_2 plateaued for at least 2 min or mice stopped running. Duplicate trials were conducted, allowing a day of rest between trials, and VO_2max was taken as the highest minute of oxygen consumption during either trial, as calculated with LabHelper software (Warthog Systems, www.warthog.ucr.edu).

Similar to previous studies [57,59], we subjectively assessed trial quality, as well as tiredness at the end of each trial. Trial quality was scored between 1, least cooperative (the mouse resisted running by grasping at the pneumatic apparatus or bracing against the direction of motion) and 5, most cooperative (the mouse consistently ran with the direction of rotation). Tiredness at the end of each trial was scored on a scale of 1 (least exhausted) to 3 (most exhausted). This scale was based on how long it took for the mouse to begin moving about the chamber, with a score of 1 being 1 second or less and a score of 3 being 5 s or more). Trials where both quality and tiredness scores were less than 2 were excluded from analyses of quality, tiredness, and VO_2max (more stringent exclusion criteria, which further reduced sample size, did not alter the final statistical results). The investigator scoring each trial was recorded and used as a random effect in statistical analyses.

Trial quality and tiredness during the higher of the two VO_2max

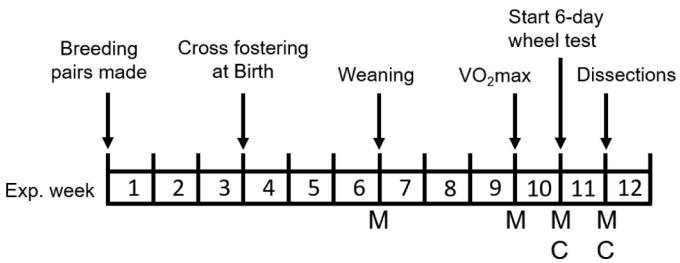

Fig. 1. Experimental timeline, starting with the pairing of generation 89 mice and ending with the dissections of generation 90 focal mice. The timeline represents experimental weeks, not age. Mice were fostered within 24 h of birth and weaned at 3 weeks of age, at the start of experimental week 7. Wheel testing (over a 6-day-period) started when mice were 7 weeks old; dissections occurred at 8 weeks of age. Note that two experimental batches (not indicated on the timeline) were offset by one week for logistical reasons, but all mice followed the outlined procedure (see Methods). M – body mass measurement; C – body composition measurement.

trials (i.e., corresponding to VO_2max) were analyzed as dependent variables [57,59], with age used as a covariate. For analyses of VO_2max, body mass and age were used as covariates, but trial quality and tiredness were not significant predictors and were not included in the final model.

2.6. Dissections

At seven weeks of age, mice were removed from wheel access for one day, then euthanized. Brains, heart ventricles, spleen, liver, left triceps surae muscle, and reproductive fat pads [60] were dissected and weighed to 0.0001 g (some organs are reported in mg for ease of viewing).

2.7. Statistical analysis

Data were analyzed as mixed models in SAS 9.1.3 (SAS Institute, Cary, NC, USA) Procedure Mixed, with REML estimation and Type III Tests of Fixed Effects. Line (selected line 7 vs. non-selected line 4) and foster line were fixed effects. To adjust for possible litter effects that would violate the statistical assumption of independence [see 61], dam ID ($n = 28$) was used as a random effect nested within line × foster line for all analyses of foster offspring, which partitioned litter variance from residual error variance [see 62]. Depending on the trait analyzed, body mass, age, wheel freeness, and/or home-cage sensor sensitivity were used as covariates. We inspected graphs of traits in relation to covariates (e.g., weaning body mass versus dam body mass) to make sure the covariate effect was not confounded with group effects, and that we did not have any obvious group × covariate interactions. (We did not include interactions between main effects and covariates in our statistical models because we had no *a priori* hypotheses concerning such interactions.) Body mass was excluded as a covariate for analyses of brain mass because we observed an anomalous negative relationship. Lean mass was excluded as a covariate for analyses of fat mass for the same reason.

We analyzed the sexes both separately and combined. We emphasize the former analyses because many sex differences have been reported previously for the traits studied, including specifically in the HR and C lines of mice [e.g., see 20,32,63,64,65], and because we had no hypotheses about sex-specific effects of cross-fostering. In all figures we show least squares means and standard errors from separate-sex analyses, along with the four p-values for differences of least squares means between the in-fostered and cross-fostered groups (i.e., the effect of cross-fostering between the HR and C lines by sex). For completeness, we present combined-sex analyses and associated least squares means in Supplemental Table 1. Supplemental material can be referenced for main effects, as well as interactions (line × foster line, line × sex, foster line × sex, line × foster line × sex). In all analyses, outliers were removed when the standardized residuals exceeded ~3.

Statistical significance was judged at $p \leq 0.05$. However, excluding the results of combined-sex analyses, nuisance variables (such as age and wheel freeness), and body mass when used as a covariate, Supplemental Table 1 includes 360 p-values for the two *a priori* contrasts of primary interest, as well as main effects of line, foster line, and their interaction. Of these 360 p-values, 88 were nominally significant at $p < 0.05$. If all null hypotheses were in fact true, then one would expect 18 p-values (0.05×360) to be < 0.05 by chance alone. In addition, these tests include a substantial amount of nonindependence because the same individuals were measured for all traits, some traits were correlated (e.g., wheel running on successive days), and many tests were interrelated (e.g., body mass and fat mass). Therefore, to compensate for nonindependence in multiple related tests, we used the positive False Discovery Rate (pFDR) procedure as implemented in PROC MULTTEST in SAS version 9.4 (SAS, Cary, NC). Based on this procedure, an adjusted critical value of 0.012 would be appropriate for controlling the false discovery rate at a 5% Type I error rate. All p-values reported in the text and tables are raw values (i.e., not adjusted for multiple comparisons), so the reader should keep this in mind.

3. Results

3.1. Characteristics of mothers

When wheel-tested at 6–8 weeks of age as part of the routine selection protocol [see Methods and 32], mice from C Line 4 ran near the bottom of the range for the C lines, whereas HR Line 7 mice ran near the top of the range for the HR lines (Table 2). As expected, dams from the C line used in the present study (Line 4) were the largest (at weaning) of any C line and those from the HR line used here (Line 7) were the smallest (Table 2).

3.2. Litter size at birth and sex ratio of fostered litters as covariates

Neither the litter size at birth nor the sex ratio of fostered litters could be controlled in the experimental design, so they were used as covariates in preliminary analyses, but subsequently removed because they were not significant predictors, with three exceptions (see Supplemental Table 1). Sex ratio (determined at weaning) had a statistically significant (1) negative effect on weaning mass in both combined- and separate-sex analyses, (2) positive effect on growth rate from weaning to sexual maturity in combined-sex analyses, and (3) negative effect on the change in body mass across six days of wheel access for male mice only (Supplemental Table 1).

3.3. Body mass, growth rate, and body composition

At weaning, pups raised by HR dams were significantly smaller than those raised by C dams for both sexes (Fig. 2A, Supplemental Table 1). In addition, HR pups raised by HR dams weighed significantly less than C pups raised by C dams (Supplemental Table 1). However, when dam mass was used as a covariate, least squares means for pups raised by HR dams were larger than those raised by C dams, and HR pups raised by HR dams no longer weighed less than C pups raised by C dams (Fig. 2B). In other words, for their body size, HR dams weaned larger pups.

As adults during experimental week 10 (measurement of VO_2max), mice raised by HR dams still weighed less than those raised by C dams, although the effect was not significant for female HR pups (Supplemental Table 1). At the beginning of experimental week 11 (just prior to adult wheel testing), mice raised by HR dams were still smaller than those raised by C dams (Fig. 3A). For lean mass, being raised by an HR dam only had a significant effect (negative) for female C mice (Fig. 3B). For fat mass the effect of cross-fostering was not significant for either sex (Fig. 3C).

Immediately following wheel testing, mice raised by HR dams continued to have less total body mass. The change in body, lean, and fat mass across wheel testing was taken as the difference between measurements (i.e., after − before). Cross-fostering did not significantly affect the change in body mass or lean mass, but HR mice did lose more total mass and lean mass across wheel testing than did C mice (Supplemental Table 1).

3.4. Maximal aerobic capacity (VO_2max)

Cross-fostering did not statistically affect VO_2max for either sex (Fig. 4), but in the combined-sex analysis mice from the HR line had higher values than C ($p < 0.0001$) and males had higher values than females ($p = 0.0496$), with no significant interactions. Body mass a significant predictor of VO_2max (Supplemental Table 1).

Trial quality was not affected by line, sex or cross-fostering. Trial tiredness (rank-transformed to achieve normality of residuals) differed by line, especially for females. C mice were tired for longer after trials than HR mice ($p = 0.0138$). Additionally, female C pups raised by HR

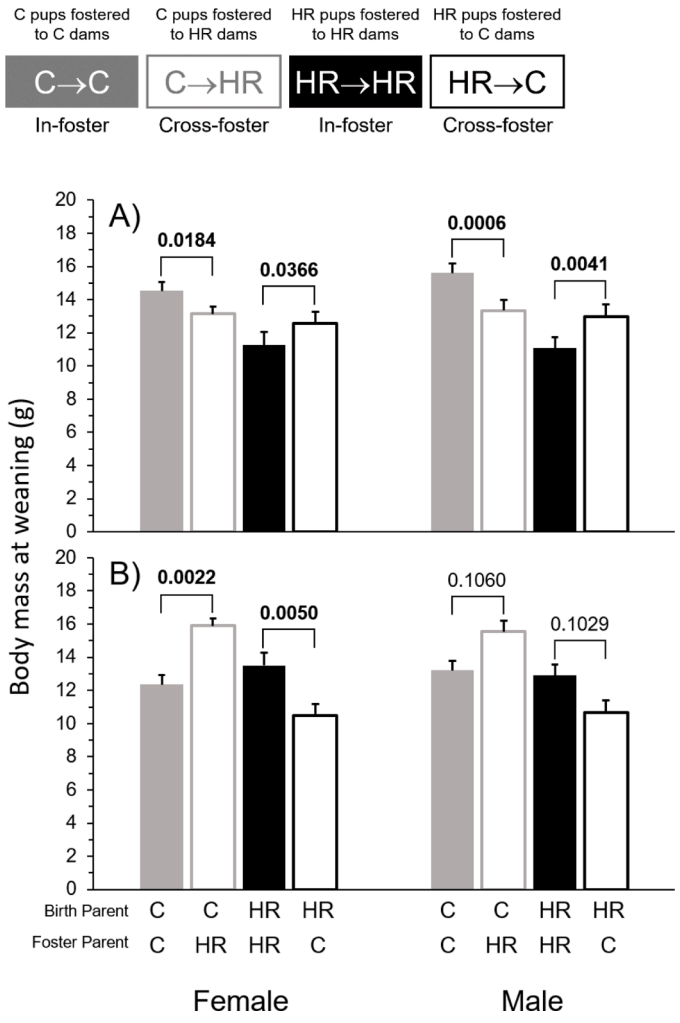

Fig. 2. Effects of cross-fostering on body mass at weaning. A) Mice reared by HR dams were smaller at weaning than those reared by C dams. This effect persisted into adulthood (see Fig. 3). B) With dam body mass as a covariate, mice reared by HR dams were larger at weaning than those reared by C dams, especially for females. Shown are least squares means and standard errors from separate-sex analyses; p-values are differences of least squares means from SAS Procedure Mixed. Statistically significant values ($p < 0.05$) are in bold. See Supplemental Table 1 for complete statistical results, including combined-sex analyses.

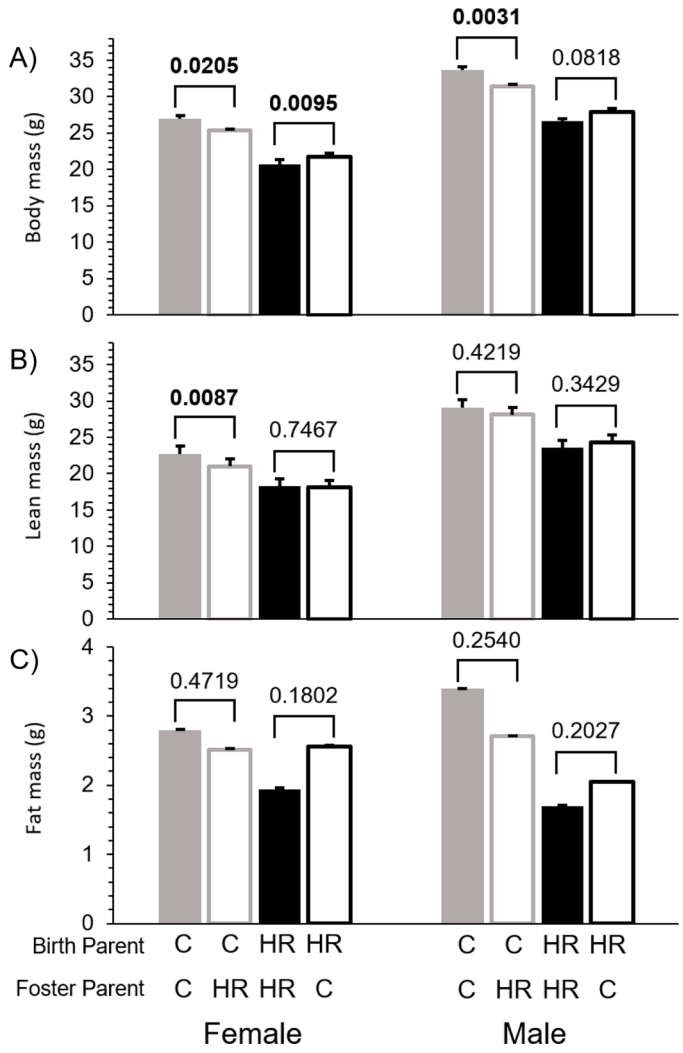

Fig. 3. Effects of cross-fostering on adult body, lean, and fat mass as measured by echo MRI, immediately prior to wheel testing. Similar to effects on body mass at weaning, adults had lower body mass (A) if they were raised by an HR dam (except male HR mice, $p = 0.0818$). For lean mass (B), the only significant effect was that female C mice had lower lean mass when raised by an HR dam. For fat mass (C), there was no significant effect of cross-fostering, though note the same pattern of effects as in adult body mass (A) and weaning (Fig. 2A). Shown are least squares means and standard errors from separate-sex analyses; p-values are differences of least squares means from SAS Procedure Mixed. P-values ($p < 0.05$) are in bold. See Supplemental Table 1 for complete statistical results, including combined-sex analyses.

dams had increased tiredness, as compared with those raised by C dams (Supplemental Table 1; $p = 0.0470$).

3.5. Wheel-running behavior

In no case did cross-fostering have a statistically significant effect on any measure of adult wheel-running behavior (Fig. 5). As expected, however, mice from the HR line ran significantly more revolutions on days 5 and 6 than those from the C line (females ran 4.5-fold and males 5.7-fold more). Mice from the HR line also ran for more minutes per day, at higher average speeds, and attained higher maximum speeds on days 5 and 6, and on all six days analyzed separately (Supplemental Table 1, Fig. 5).

3.6. Home-cage activity

Cross-fostering did not affect home-cage activity (total minutes or average intensity) on days 5 and 6 of adult wheel testing (Supplemental Table 1). For females, HR mice had greater total home-cage activity than did C mice (Supplemental Table 1; $p = 0.0416$).

3.7. Organ masses

With body mass as a covariate, cross-fostering had variable effects on adult organ masses, and all such effects were sex-specific (Fig. 6). Some differences between the HR line and C line were also observed, as well as sex differences (Supplemental Table 1).

Cross-fostering increased muscle mass among female C mice, although the effect was not statistically significant (Fig. 6B; $p = 0.0504$). Male mice had greater triceps surae muscle mass than female mice (sex $p < 0.0001$). Cross-fostering increased brain mass among female HR mice (Fig. 6C; $p = 0.0476$). Cross-fostering significantly reduced male reproductive fat mass among C mice (Fig. 6E; $p = 0.0032$). HR mice had significantly smaller male reproductive fat masses than C mice (Supplemental Table 1; $p = 0.0003$). Cross-fostering increased spleen mass in

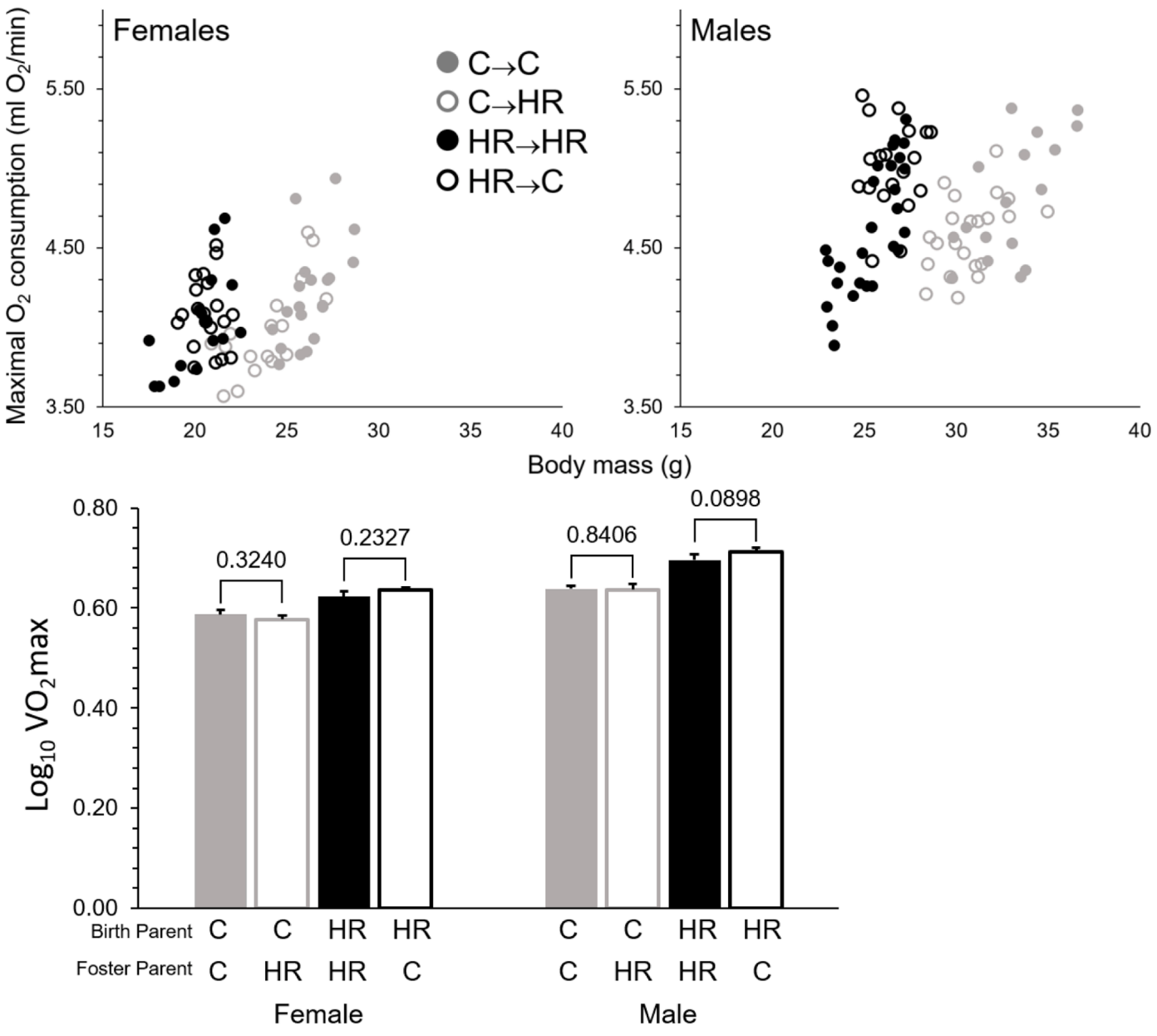

Fig. 4. Adult maximal oxygen consumption (VO_2max) during forced exercise. Body mass (g) was a significant predictor of VO_2max ($p < 0.0001$). Male mice (right plot) had greater VO_2max than female mice (left plot; $p = 0.0496$) and HR mice had greater VO_2max than C mice (female $p = 0.0005$, male $p < 0.0001$), with no statistically significant effect of cross-fostering. C mice are indicated by gray circles and HR mice are indicated by black circles. In-fostered mice are indicated by solid circles and cross-fostered mice are indicated by open circles. See Fig. 2 for more detailed explanation of legend. See Supplemental Table 1 for complete statistical results, including combined-sex analyses.

male HR mice (Fig. 6F; $p = 0.0117$). Spleen mass differed between HR and C lines among both sexes (Supplemental Table 1; female $p < 0.0001$; male $p = 0.0147$).

Cross-fostering did not affect heart ventricle mass (Fig. 6A), but HR mice had larger ventricles than C mice in both sexes (Supplemental Table 1; female $p = 0.0010$; male $p < 0.0001$). Cross-fostering did not significantly affect liver mass (Fig. 6D), but males had larger livers than females (sex $p < 0.0001$). For males, HR mice had significantly smaller livers than C mice (Supplemental Table 1; sex × line $p < 0.0001$).

4. Discussion

4.1. Coadaptation of maternal effects on adult voluntary exercise

The artificial selection protocol used to produce the HR lines has resulted in changes in allele frequencies for numerous genes that appear related to both their motivation and ability for wheel running [e.g., see 66,67,68]. In addition, selection may have caused changes in allele frequencies for genes that are expressed primarily or exclusively in mothers up to the time of weaning their pups, causing phenotypic effects in the mothers that also have effects on wheel running of those offspring when they are adults [e.g., see 69,70]. Indeed, significant divergence in wheel running between HR and C mice occurs within a few days after weaning [e.g., see Fig. 2 of 40], which is 3–5 weeks prior to normative adult wheel testing in the selection experiment. In other words, maternal traits that promote wheel running by their offspring may have evolved in response to the HR selection regime [33,44,71]. Such maternal effects are one example of early-life effects and several studies have specifically demonstrated early-life effects on adult physical activity, including in the HR mice [e.g., see 8,22,39,40,72]. In general, cross-fostering can

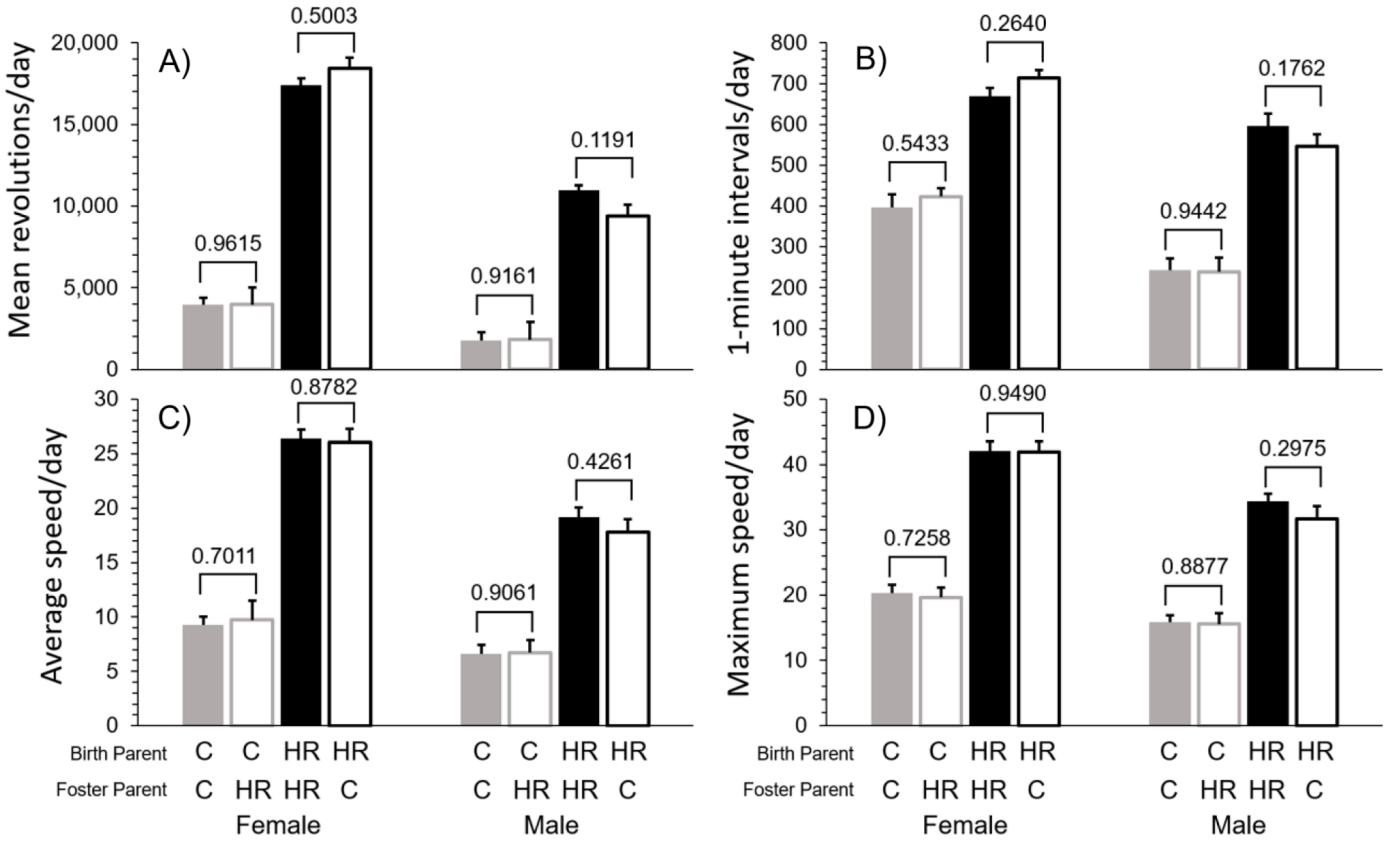

Fig. 5. Effects of cross-fostering on adult wheel-running behavior on days 5 and 6 of a 6-day test. Cross-fostering had no statistically significant effect on mean A) revolutions/day (circumference 1.12 m), B) duration of daily running (min/day), C) speed (revs/min), or D) maximum revolutions in any 1-min interval. However, for all measures, mice from the selectively bred HR line had higher values than those from the non-selected C line, and females had higher values than males. Shown are least squares means and standard errors from separate-sex analyses (see Supplemental Table 1 for combined-sex analyses); p-values are differences of least squares means from SAS Procedure Mixed. Statistically significant values ($p < 0.05$) are in bold.

affect various other behaviors, such as emotionality [73–75].

In the present study, we used cross-fostering to test whether the adult HR phenotype is influenced by the maternal environment. We recorded body mass at weaning and then, in adults, we measured maximal aerobic capacity (VO_2max: an important determinant of endurance exercise capacity, which is elevated in HR mice), body mass (reduced in HR mice), body fat (reduced in HR mice), voluntary wheel running, home-cage activity (both elevated in HR mice), and organ masses (several of which are altered in HR mice). Although we detected statistically significant effects of cross-fostering on mice at weaning and as adults (body mass, composition, and organ masses), we did not detect any effects on wheel running, home-cage activity or VO_2max. Thus, we find no evidence for coadaptation of maternal effects (via the early postnatal environment) on adult voluntary exercise during the evolution of the HR mice. However, future studies (e.g., using embryo transplants) will be required to test for possible pre-natal maternal influences.

4.2. *Effects of cross-fostering on body mass and composition*

Obesity of human [e.g., 76,77] and rodent [78,79] mothers is associated with a host of adverse health consequences in their adolescent and adult offspring, including increased risk of obesity, type 2 diabetes, non-alcoholic fatty liver disease, and altered behavior. In rodents, for example, Desai et al. [80] used a rat model involving a high-fat (HF) maternal diet during pregnancy and/or lactation, which resulted in hyperglycemia and increased systolic blood pressure in offspring. Their HF dams had elevated plasma corticosterone levels [80], which, interestingly, is a characteristic of HR mice in general [e.g., see 34,37,81]. In another rodent study, Miranda et al. [82] cross-fostered rat offspring with an obese phenotype to non-obese control dams, resulting in "rescued" body weight and food intake, among other health factors. In obese offspring raised by obese dams, males developed a wide range of metabolic disturbances, including hyperglycemia and hyperinsulinemia [82].

Although C mice are not generally viewed as obese under standard housing conditions and on standard chow, they do have more body fat than HR mice [83]. Therefore, we presumed cross-fostering effects on offspring body mass and/or composition would be apparent at weaning. As expected, pups raised by HR dams were smaller at weaning (Fig. 2A), an effect that persisted into adulthood (Fig. 3A).

The cross-fostering effects on body mass and composition (Figs. 2 and 3) might be mediated by the body mass and composition of the dams. Consistent with this idea, body mass at weaning was smaller for HR dams as compared with C dams (Table 2), but we were not able to measure body composition of either dams or their weaned offspring. However, another study in our lab did examine body composition of nursing dams from generation 84 (N. E. Schwartz et al. unpublished results). In a one-way ANCOVA with age as a covariate, nursing line 7 dams had significantly reduced body mass, lean mass, and fat mass than those from line 4 (all $p < 0.0001$). Average body masses of those dams (line 7, 29.58 g; line 4, 41.31 g) were similar to those used in the current study (Table 2).

As a statistical test of dam body mass mediation of cross-fostering effects on weaning mass, we repeated the analyses shown in Fig. 2A with dam mass as a covariate and found that the least squares (adjusted) means for pup mass were then significantly larger for those raised by HR dams (Fig. 2B). Previous studies of unmanipulated litters in earlier generations of the selection experiment (i.e., HR and C dams raising

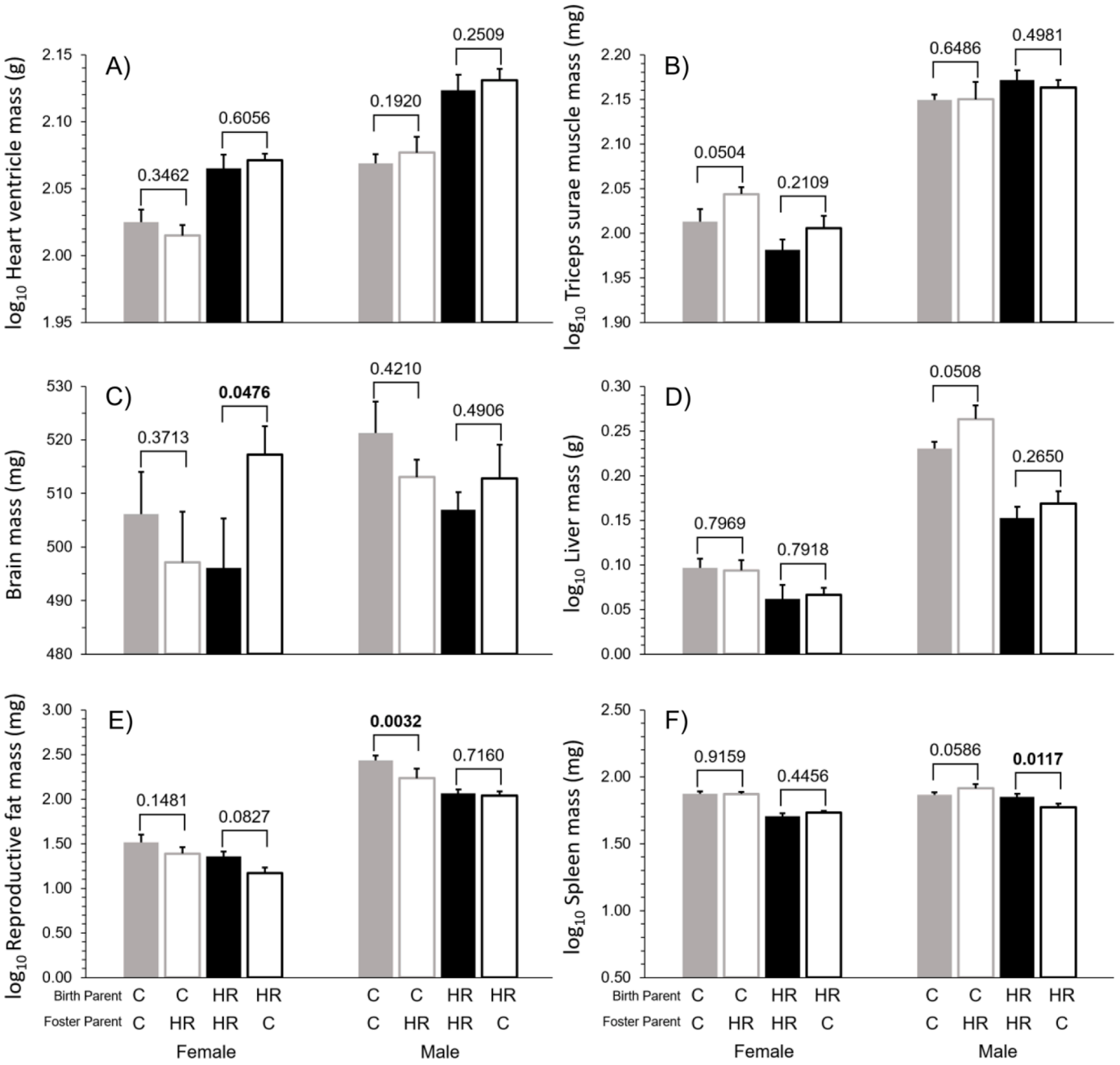

Fig. 6. Effects of cross-fostering on adult body-mass adjusted organ masses. Cross-fostering had relatively few effects on organ masses, and the effects were inconsistent between the sexes. Organ masses are log-transformed for analysis and log body mass was used as a covariate. Triceps surae (B), brain (C) reproductive fat (E), and spleen (F) masses are shown in mg rather than g for better viewing. Shown are least squares means and standard errors from separate-sex analyses (see Supplemental Table 1 for combined-sex analyses); p-values are differences of least squares means from SAS Procedure Mixed. Statistically significant values ($p < 0.05$) are in bold.

their own pups) generally did not find statistically significant differences in body mass at weaning between the complete set of four HR lines and four C lines [45,84,85], but studies of later generations have reported significantly smaller body mass at weaning for HR mice, especially for males (e.g., in geneations 73 [20] and 76 [40]), although not significant differences in lean or fat mass at weaning [20].

4.3. Sex-specific cross-fostering effects

Sex-specific cross-fostering effects have been reported for a range of traits in rodents [e.g., 19,30,31,86]. Indeed, early-life effects in general often interact with sex [e.g., see 87 and references therein]. In the present study, sex-specific cross-fostering effects were observed for weaning mass (with dam mass as a covariate), body mass and lean mass before and after adult wheel testing, brain mass, spleen mass, and reproductive fat mass (see Supplemental Table 1). Main effects of sex were nearly ubiquitous, as would be expected from numerous previous studies of, for example, adult wheel running, body mass, and composition [88]. Taken together, these results emphasize the importance of including both sexes in studies of early-life effects in general [e.g., see 20,89 for mice,90,91 for human famine studies].

4.4. Limitations of the present study

Differences among the four replicate HR lines and also among the four non-selected C lines have been documented for a variety of traits [e.g., see 20,33,34,56,92,93]. Therefore, ideally, a cross-fostering study would have included all eight lines, but such a study would require a very large number of litters to include all possible combinations. To keep the number of litters within our logistical capacities, we used only one C line and one HR line (the reasons for using these particular lines are explained in the Methods). Our results might have been different if we had used different lines, e.g., ones that did not differ so much in body size (Table 2).

The purpose of the present study was to test the specific hypotheses outlined in the Introduction concerning cross-fostering HR and C mice. We have used the term "cross-fostering" to mean the fostering of pups *between* HR and C dams. As we were not specifically interested in the effects of cross-fostering *per se* (i.e., between families within a linetype of mouse), we did not include a non-fostered control group. However, cross-fostering in and of itself is a stressful event for both mothers and their offspring, and has a variety of effects [e.g., 22,94]. For example, Eisen et al. [95] reported that fostering of mice among dams within a single inbred strain can influence lactation, with consequences for offspring. If such effects occurred in our study, and if HR and C lines responded differently to cross-fostering stress, then differential effects of maternal care by HR and C mice may have been confounded. No studies of the stress responsiveness of lactating females or of pre-weaning pups are available for these lines, but one study using 40 min of restraint stress reported a smaller increase in circulating corticosterone concentrations from baseline for HR mice [34], which could be interpreted as evidence of reduced stress responsiveness.

In the present experiment, all adult mice were tested on wheels for 6 days, followed by one day with the wheels removed, prior to dissection. Therefore, organ masses may have experienced training effects (physical conditioning), as well as acute effects that might have occurred during the intervening ~24-h period between wheel testing and dissections [e.g., see 96]. Given that HR mice run much more than C and also have greater phenotypic plasticity in response to a few days of running for some traits [e.g., see 97], our ability to detect differential cross-fostering effects on organ masses may have been affected. Nonetheless, some effects were detected (Supplemental Table 1; Fig. 6).

An interesting avenue for future research would be to test for differences in milk quantity, quality or composition between HR and C dams. Several studies have shown that inbred strains of mice differ in milk properties [98 and references therein] that may have long-lasting (even multi-generational) effects on offspring [99]. Although physical activity before and during pregnancy has effects on milk properties in mice [100], no study has demonstrated effects of altered milk content on adult physical activity levels.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.physbeh.2021.113569.

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