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Neonatal mice exposed to a high-fat diet *in utero* influence the behaviour of their nursing dam

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The behaviour of a nursing dam influences the development, physiology, and behaviour of her offspring. Maternal behaviours can be modulated both by environmental factors, including diet, and by physical or behavioural characteristics of the offspring. In most studies of the effects of the environment on maternal behaviour, F_0 dams nurse their own F_1 offspring. Because the F_1 are indirectly exposed to the environmental stressor *in utero* in these studies, it is not possible to differentiate between effects on maternal behaviour from direct exposure of the dam and those mediated by changes in the F_1 as a consequence of *in utero* exposure. In this study, we used a mouse model of high-fat (HF) diet feeding, which has been shown to influence maternal behaviours, combined with cross-fostering to discriminate between these effects. We tested whether the diet of the F_0 dam or the exposure experienced by the F_1 pups *in utero* is the most significant predictor of maternal behaviour. Neither factor significantly influenced pup retrieval behaviours. However, strikingly, F_1 *in utero* exposure was a significant predictor of maternal behaviour in the 15 min immediately following pup retrieval while F_0 diet had no discernable effect. Our findings suggest that *in utero* exposure to HF diet programmes physiological changes in the offspring which influence the maternal behaviours of their dam after birth.

1. Introduction

Maternal care plays a crucial role in shaping offspring development and physiology across taxonomic groups [1–3]. In mammals, maternal care influences pubertal onset [4], stress response [5,6], and the programming of maternal care behaviours in female offspring [7].

Maternal behaviour can be influenced by environmental and social factors. These diverse factors include exposure to endocrine disruptors [8–11], levels of anxiety in mates [12], and diet, among others. In rodents, maternal high-fat (HF) diet has been reproducibly demonstrated to cause impaired maternal behaviours. Rats fed a HF diet during pregnancy and lactation spend less time licking and grooming their pups [13], a behaviour that programmes altered hypothalamic-pituitary-adrenal responses to stress in the offspring [14]. While the mechanisms underpinning the relationship between diet and maternal behaviour have not been fully elucidated, at least some effects may be mediated through prolactin signalling. Prolactin is a key regulator of maternal behaviours, as demonstrated by the stimulatory effects of ectopic prolactin delivery in rats [15,16] and the behavioural deficiencies exhibited by prolactin receptor knockout mice [17]. Mice with HF diet-induced obesity are resistant to prolactin signalling in the hypothalamus, an important region of the brain for controlling maternal behaviour, and demonstrate an impairment in pup retrieval behaviour [18].

Maternal behaviour can also be influenced by the offspring. Maximizing the resources they can extract from their parents may be advantageous to offspring,

but for optimal reproductive success parents may benefit from a more equal distribution of resources among their offspring [19,20]. This conflict has been predicted to lead to the evolution of behaviours or other traits in offspring that influence parental care and investment. Begging behaviour in young birds, for example, is a form of communication that can modulate parental investment and the allocation of food among nestlings [21,22]. Neonatal mice stimulate maternal licking, changing of suckling position, and nest-building through the emission of low-frequency calls [23], and when separated from the nest promote maternal searching and retrieval with ultrasonic vocalizations [24,25].

Although it is clear that both the environment and the behaviour of offspring can modulate maternal behaviour, the interactions between these factors have not been extensively explored.

Previous studies of how the behaviour of a dam is influenced by her consumption of a HF diet have used an experimental design in which dams (the F_0 generation) nurse their own offspring (the F_1 generation) [13]. Thus, a dam consuming a HF diet nurses pups that were themselves exposed to the dam's HF diet during *in utero* development. Such an experimental design precludes differentiation between effects on maternal behaviour that result from direct exposure of the F_0 dam and those mediated by physiological changes in the F_1 offspring as a consequence of *in utero* exposure.

To further our understanding of the interactions between the environment, F_1 *in utero* exposure, and F_0 dam behaviour, we implemented a cross-fostering strategy in which pups born to dams on a HF diet were nursed by dams on a control (CT) diet, and the reciprocal. We also included control groups (figure 1). With this approach, we aimed to determine whether the diet of the F_0 dam or the exposure experienced by the F_1 pups *in utero* is the most significant predictor of maternal behaviour. Given that 60% of women are overweight at the time of conception in the USA, the findings of this study will build on our understanding of how nutrition affects maternal care, with potential long-term consequences for offspring physiology.

2. Methods

(a) Animals and cross-fostering

C57Bl/6 J mice were obtained from The Jackson Laboratory. This strain was chosen for its strong maternal nurturing behaviours reported by others [26] and for its responsiveness to cross-fostering [27]. All studies were approved by the North Carolina State University Institutional Animal Care and Use Committee. Animals were socially housed and maintained on a 14 h/10 h light/dark cycle at 30–70% humidity, 22°C ± 4°C. From three weeks of age, female mice (F_0 generation) were fed a 45% fat diet ('HF diet'; D12451, Research Diets Inc. (4.7 kcal g^{-1})) or a micronutrient-matched 10% fat diet ('CT diet'; D12450H, Research Diets Inc. (3.8 kcal g^{-1})). Body weight was recorded weekly. After six weeks on the diet, a glucose tolerance test was performed. Mice were fasted overnight for 16 h and basal blood glucose levels measured using an Aimstrip Plus Glucose Meter and strips through tail snips. Glucose (Sigma-Aldrich) was injected at 2 mg g^{-1} body weight and blood glucose levels were measured at 15, 30, 60, 90, and 120 min.

At nine weeks of age, F_0 females were mated to C57Bl/6 J male mice which were maintained on the 10% fat (CT) diet. To allow mating, females were placed in male cages for approximately 8 h during the light cycle for 10 consecutive days. Females were returned to their home cages for the entirety of the dark cycle, thereby ensuring continued exposure to the 45 or 10% fat diets throughout the mating period. Upon observation of pregnancy, females were individualized.

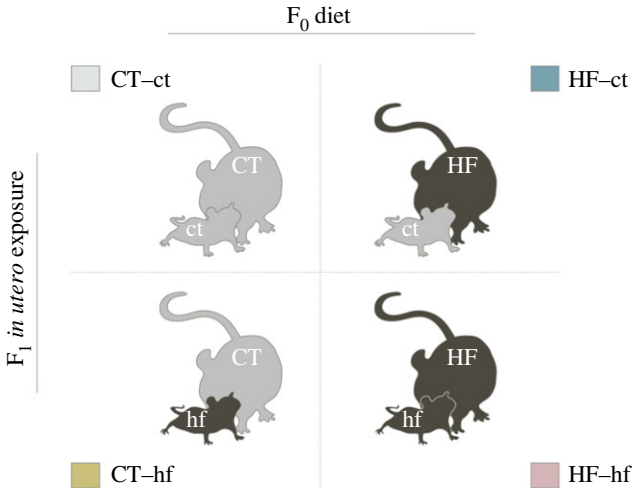

Figure 1. Experimental design. F_1 mice were cross-fostered at birth to generate four experimental groups. Group labels indicate the diet of the nursing dam (F_0) and the *in utero* exposure of the pups (F_1), in the format F_0–F_1.

On the day of birth (postnatal day 0, PND0), F_1 animals were weighed and litter sizes were normalized to five pups, maintaining an equivalent sex ratio between groups (electronic supplementary material, figure 1; $F_{3,27} = 0.683$, $p = 0.5702$). Extra pups were sacrificed and dissected. The remaining F_1 animals were cross-fostered to nurse dams who had given birth on the same day to generate four groups (figure 1). F_1 animals in all groups were cross-fostered. Groups are labelled in the format F_0–F_1, with the dam's diet in uppercase and the offspring's *in utero* exposure in lower case. CT–ct: dams consuming a CT diet nursing offspring exposed to a maternal CT diet *in utero* ($n = 9$ litters). CT–hf: dams consuming a CT diet nursing offspring exposed to a maternal HF diet *in utero* ($n = 8$ litters). HF–ct: dams consuming a HF diet nursing offspring exposed to a maternal CT diet *in utero* ($n = 10$ litters). HF–hf: dams consuming a HF diet nursing offspring exposed to a maternal HF diet *in utero* ($n = 9$ litters).

(b) Behaviour

Behavioural assays were performed during the light cycle on a subset of litters on postnatal day 3 (PND3), i.e. 3 days after cross-fostering (CT–ct, $n = 9$ litters; CT–hf, $n = 8$ litters; HF–ct, $n = 8$ litters; HF–hf, $n = 8$ litters). Analyses were performed on all animals in all groups concurrently. F_1 pups were separated from their dams for 1 h, and placed in nesting material on a heat pad. Dams remained in their home cages during this time, but were briefly removed immediately before pups were replaced. Pups were reintroduced to the home cage at the opposite end to the nest. Each dam was reintroduced to their respective cage, and a modified lid containing a GoPro camera was fitted. The time taken for each pup to be retrieved to the nest was recorded. Pups were manually replaced in the nest if they had not been retrieved within 30 min.

After all five pups had been retrieved, the behaviour of the dam was scored every minute for the subsequent 15 min. 'Interactive behaviours were classified as 'in the nest with pups, not moving', 'in the nest with pups, moving', and 'nest-building'. 'Non-interactive' behaviours were classified as 'self-grooming', 'resting', 'exploring', and 'wall-rearing'. No dam in any group was observed performing self-grooming or resting behaviours.

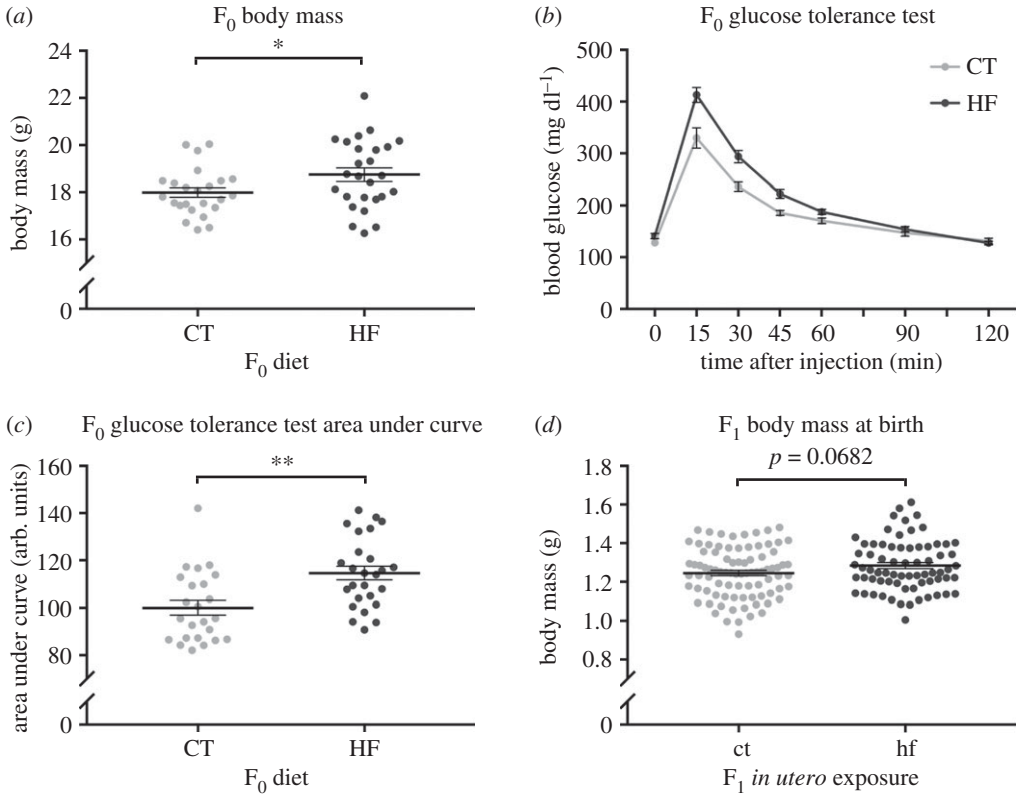

Figure 2. Effects of diet on F_0 and F_1 body mass, and F_0 glucose homeostasis. (*a*) Body mass of F_0 dams prior to mating, after consuming CT or HF diets for six weeks. (*b*) Glucose tolerance test of F_0 dams at the same time point as described for (*a*). (*c*) Quantification of the area under the curve in (*b*). (*d*) Body mass of F_1 pups on the day of birth after *in utero* exposure to a maternal control (ct) or high-fat (hf) diet. Individual data points are shown for (*a*), (*c*), and (*d*), with horizontal black lines representing means and error bars representing standard error. Data in (*b*) are presented as mean ± s.e. *$p < 0.05$, **$p < 0.005$; Student's *t*-test, two-tailed.

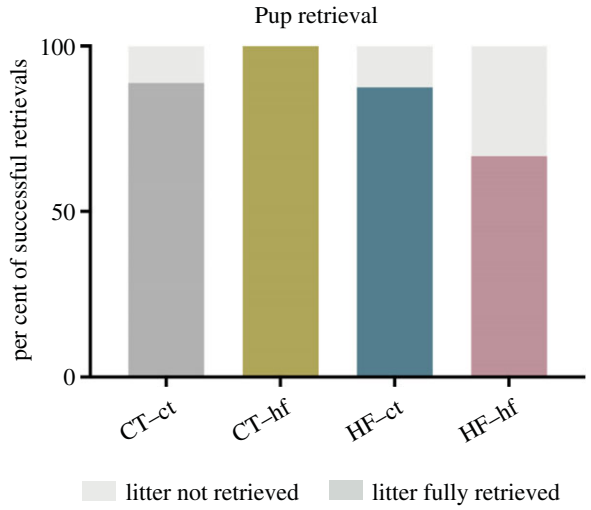

Figure 3. Pup retrieval. Per cent of dams within each experimental group that showed successful retrieval of all pups to the nest within 30 min of reunion.

(c) Statistical analyses

Means comparison by Student's *t*-test (unpaired, two-tailed) was used to compare CT diet versus HF diet groups for body mass of dams, body mass of F_1 pups at birth, litter size, and the area under the curve for the glucose tolerance test performed on F_0 dams.

For pup retrieval analysis, Fisher's Exact test was performed on all groups, testing the null hypothesis that there is no significant difference in retrieval success between groups. For maternal behaviour analyses, data were expressed as counts for each exhibited behaviour, including a count variable summing across observed interactive behaviours. For each behaviour, the mean counts of each group were compared to the CT–ct group using one-way analysis of variance (ANOVA) with Dunnett's test for multiple comparisons. The same approach was used to compare the sex ratios of groups at PND3. To determine whether F_0 diet or F_1 *in utero* exposure were significant predictors of maternal behaviour, their individual effects were analysed using a Poisson generalized linear model using CT or ct as the reference group, respectively. We also determined whether the interaction of F_0 diet and F_1 *in utero* exposure (F_0*F_1) were significant predictors. Hypothesis tests for the resulting *p*-values test that the diet coefficient does (Estimate = 0) or does not (Estimate ≠ 0) have an effect on maternal behaviour. Analyses were performed using R version 3.4.3 [28].

3. Results

(a) Mouse model

Female mice consuming the HF diet for six weeks had significantly elevated body weights ($t_{48} = 2.141$, $p = 0.04$; figure 2*a*) and impaired glucose tolerance ($t_{48} = 3.488$, $p = 0.001$ for area under curve; figure 2*b*,*c*) compared to mice consuming the CT diet. Females in both groups were mated to males consuming a CT diet. At birth, F_1 pups born to dams on the HF diet showed slightly increased body weights compared with those born to dams on the CT diet, although this was not statistically significant ($t_{153} = 1.837$, $p = 0.07$; figure 2*d*). No effects on litter size associated with diet were observed ($t_{34} = 0$, $p > 0.99$). F_1 mice were cross-fostered on the day of birth and maternal behaviours assessed on PND3, as described in the Methods (figure 1).

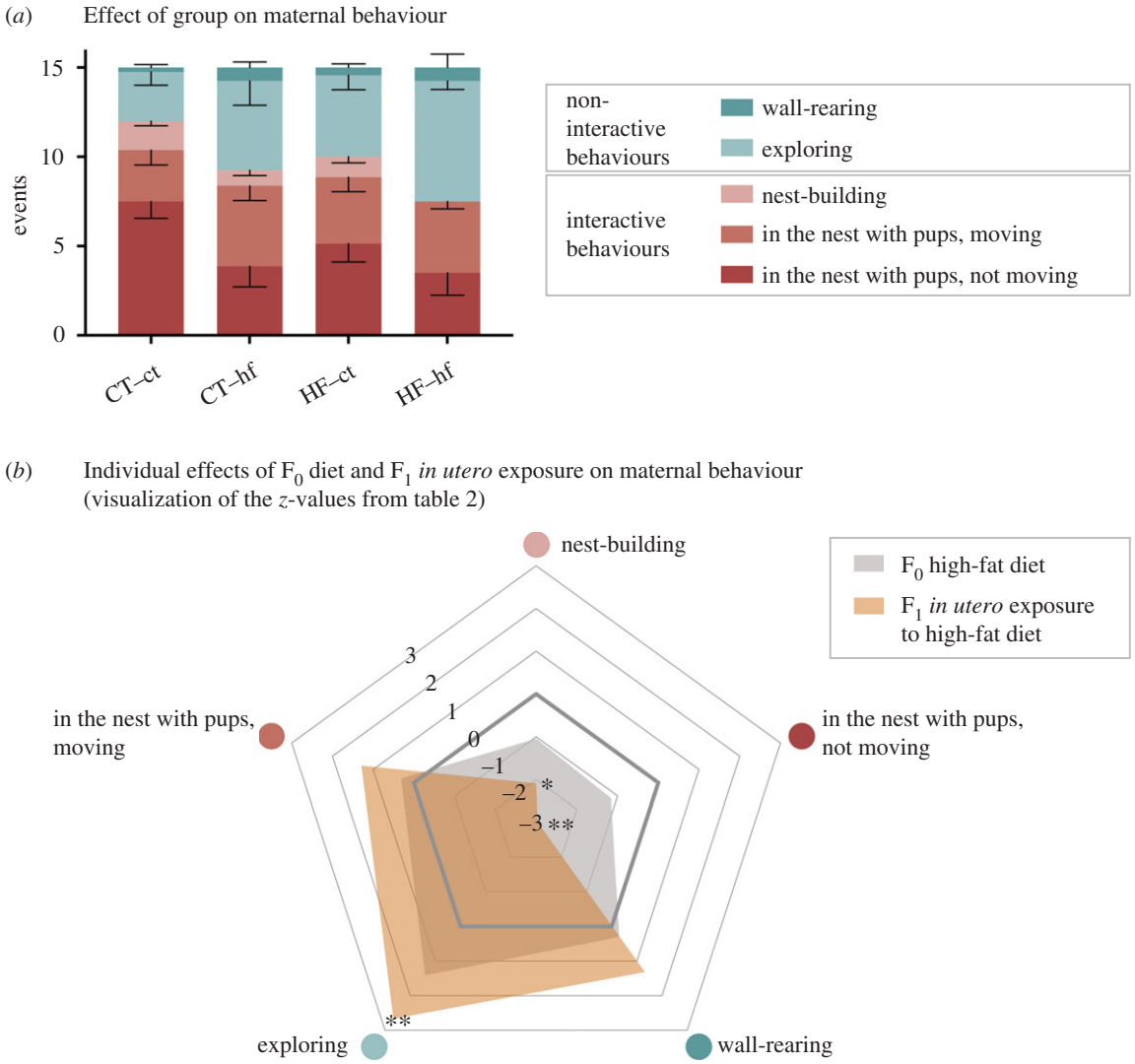

Figure 4. Post-retrieval maternal behaviour. (*a*) Effect of group on maternal behaviour. Number of time points (events) within the 15 min following reunion that dams were observed engaging in non-interactive and interactive behaviours. Error bars represent standard error. (*b*) Radar chart illustrating the predictability of F_0 HF diet and F_1 *in utero* exposure to a HF diet on specific maternal behaviours. The plot provides a graphical illustration of the *z*-scores from table 2, including the directionality of the effect. Non-interactive behaviours are grouped at the bottom of the plot (behaviours with blue shades) and interactive behaviours are grouped at the top (red shades).

(b) Pup retrieval

Following separation of the dam and pups, no significant differences were observed between the four groups in the latency to retrieve the first pup ($F_{3,25} = 0.21$, $p = 0.89$), or in the latency to retrieve all pups ($F_{3,23} = 0.17$, $p = 0.92$). Sixty-seven per cent of dams in the HF–hf group successfully retrieved all pups, compared to 88–100% of dams in other groups (figure 3), but this did not represent a statistically significant difference ($p = 0.40$).

(c) Post-retrieval maternal behaviour

Following retrieval of the last pup, dams in the CT–ct group spent an average of 12 out of 15 of the measured time points (75%) engaged in interactive behaviours (figure 4*a* and table 1), more than dams in any other group. In particular, dams in the HF–hf group spent significantly fewer time points (an average of 7.5 out of 15 time points, 50%) engaged in interactive behaviours in comparison to dams in the CT–ct group. These differences were predominantly explained by a reduction in the number of time points that dams in the HF–hf group were 'in the nest with pups, not moving' and 'nest-building' with a concomitant increase in the number of time points that these dams were observed 'exploring'.

Our data show that dams consuming a HF diet who nurse pups exposed to HF diet *in utero* (i.e. the HF–hf group) have impairments in maternal behaviour, consistent with observations by others. Interestingly, dams in the CT–hf and HF–ct groups also showed differences when compared to CT–ct dams in the number of time points spent on certain behaviours. For example, CT–hf dams were observed 'in the nest with pups, not moving' fewer times than CT–ct dams (table 1), suggesting that the *in utero* exposure experienced by the F_1 pups might contribute to influencing maternal behaviour.

To gain further insight into this possibility, we fitted the data to generalized linear models and tested the individual effects of F_0 diet and F_1 *in utero* exposure, as well as the interaction between these two terms (F_0*F_1), on maternal behaviour. F_0 diet was not a predictor of the number of time points spent on interactive or non-interactive behaviours overall ($z = -0.995$, $p = 0.32$), or indeed on any specific behaviour (table 2). Strikingly, F_1 *in utero* exposure was predictive of maternal interactive and non-interactive behaviours overall ($z = -2.071$, $p = 0.04$). F_1 exposure to a HF

Table 1. Effect of group on maternal behaviour. Values are mean ± s.e. for the number of time points dams in each group were observed engaging in each activity. *p*-values are calculated using a one-way ANOVA with Dunnett's test for multiple comparisons, comparing each group to the CT–ct group.

	CT–ct	CT–hf	HF–ct	HF–hf
interactive behaviours				
all interactive behaviours	12.0 ± 0.7	9.3 ± 1.3	10.0 ± 0.8	7.5 ± 1.2
		p = 0.120	*p* = 0.346	*p* = 0.028*
in the nest with pups, not moving	7.5 ± 0.9	3.9 ± 1.2	5.1 ± 1.0	3.5 ± 1.3
		p = 0.048*	*p* = 0.284	*p* = 0.081
in the nest with pups, moving	2.9 ± 0.8	4.5 ± 0.8	3.7 ± 0.8	4.0 ± 0.4
		p = 0.321	*p* = 0.792	*p* = 0.729
nest-building	1.6 ± 0.3	0.9 ± 0.3	1.1 ± 0.3	0 ± 0.0
		p = 0.153	*p* = 0.492	*p* = 0.006**
non-interactive behaviours				
all non-interactive behaviours	3.0 ± 0.7	5.8 ± 1.3	5.0 ± 0.8	7.5 ± 1.2
		p = 0.120	*p* = 0.346	*p* = 0.028*
exploring	2.8 ± 0.8	5.0 ± 1.4	4.6 ± 0.8	6.8 ± 0.5
		p = 0.248	*p* = 0.431	*p* = 0.059
wall-rearing	0.3 ± 0.2	0.8 ± 0.3	0.4 ± 0.2	0.8 ± 0.8
		p = 0.487	*p* = 0.952	*p* = 0.635

*$p < 0.05$, **$p < 0.01$.

Table 2. Effect of F_0 diet and F_1 *in utero* exposure on maternal behaviour. Results from the Poisson generalized linear model for predicting the given maternal behaviour using single predictors for either F_0 diet or F_1 *in utero* exposure, with CT or ct as the reference group, respectively.

	estimate	s.e.	*z*-value	Pr(>|z|)
interactive behaviours				
all interactive behaviours				
F_0 diet	−0.1207	0.1214	−0.995	0.32
F_1 exposure	−0.25531	0.12330	−2.071	0.0384*
in the nest with pups, not moving				
F_0 diet	−0.1978	0.1698	−1.1648	0.2441
F_1 exposure	−0.5306	0.179	−2.9651	0.003**
in the nest with pups, moving				
F_0 diet	0.0603	0.1955	0.3084	0.7578
F_1 exposure	0.2499	0.1943	1.2864	0.1983
nest-building				
F_0 diet	−0.4055	0.3873	−1.0469	0.2951
F_1 exposure	−0.9019	0.4317	−2.0894	0.0367*
non-interactive behaviours				
exploring				
F_0 diet	0.2549	0.1811	1.4075	0.1593
F_1 exposure	0.4850	0.1820	2.6657	0.0077**
wall-rearing				
F_0 diet	0.1542	0.5175	0.2979	0.7658
F_1 exposure	0.6931	0.5270	1.3152	0.1884

*$p < 0.05$, **$p < 0.01$.

diet during *in utero* development was associated with the nursing dam being more engaged in non-interactive behaviours and less engaged in interactive behaviours than dams nursing pups exposed to the CT diet *in utero* (table 2). This effect was predominantly explained by a reduction in the time spent 'inside the nest, not moving' ($z = -2.9651$, $p < 0.01$) and 'nest-building' ($z = -2.0894$, $p = 0.04$), with a concomitant increase in time spent 'exploring' ($z = 2.6657$, $p < 0.01$) (table 2 and figure 4*b*). F_0*F_1 interaction was not a significant predictor of any maternal behaviour (electronic supplementary material, table S1).

4. Discussion

Previous rodent studies have demonstrated that HF diet modulates maternal behaviour. In these studies, dams raised their own biological offspring which were exposed to maternal HF diet during *in utero* development, which precluded discrimination between effects of F_0 diet and F_1 *in utero* exposure on maternal behaviour. By using a cross-fostering study design, in which mice born to dams on a CT diet were fostered to dams on a HF diet, and the reciprocal, we were able to assess these effects independently.

Pup retrieval behaviour, including success at retrieving the entire litter within the experimental period and latency to retrieve pups, was not significantly affected by group, F_0 diet, or F_1 exposure, although dams in the HF–hf group showed a non-significant impairment in retrieval compared to other groups. Other studies have suggested a link between diet and pup retrieval behaviours, but this is context- and age-dependent [18,29]. For example, in one study, dams on a HF diet demonstrated impaired pup retrieval on postnatal day 7 but not on postnatal day 4 [18], with the latter time point being close to our own study.

During the 15 min immediately following pup retrieval, F_1 *in utero* exposure, but not F_0 diet, was found to be a significant predictor of maternal behaviour. Specifically, F_1 *in utero* exposure to HF diet was negatively associated with the number of time points during which the dam was observed 'in the nest with pups, not moving' and 'nest-building', and positively associated with 'exploring'. These findings suggest that alterations to maternal behaviours previously associated with the consumption of a HF diet may in part be mediated through the offspring. F_1 *in utero* exposure to polychlorinated byphenyls has been similarly reported to impact F_0 maternal behaviour [10], suggesting that our general observations may apply to a range of environmental stressors.

A parent has finite resources to invest in reproduction. Assuming identical fitness among all offspring, the best reproductive strategy would be to invest equally in each. However, offspring are likely to differ in their fitness and thus the optimal strategy is to invest more heavily in the offspring of greater quality [30]. This has been demonstrated empirically for a range of species, implying that parents can respond to cues from offspring that reflect their fitness [21,31,32].

In the context of maternal HF diet, *in utero* exposure causes reduced fitness in later life, including metabolic and behavioural dysfunction. Early physiological alterations in exposed pups may provide cues that influence the time spent on interactive behaviours by dams, representing a strategy to limit investment and enable greater resource allocation to future, potentially fitter, broods. However, the molecular nature of these cues—and how they are perceived by the dam—is unclear.

Genetic studies in mice have identified loci in offspring that can influence maternal behaviour. By fostering genetically variable mouse pups to genetically uniform dams, Ashbrook *et al*. [33] were able to map maternal behaviours as a function of genetic variation in offspring, identifying loci on chromosomes 5 and 7 that modify maternal behaviours. Together, these loci contain greater than 400 genes, and a small number of these were identified as strong candidates for modulating maternal behaviour because of their involvement in steroid hormone biosynthesis. Other loci that influence maternal behaviour have been identified through targeted approaches. Deletion of the imprinted gene *Peg3* in offspring is associated with impaired maternal behaviours, including delayed pup retrieval and increased anxiety, in wild-type nurse dams [34]. Offspring deficient for *Peg3* demonstrated a reduction in ultrasonic vocalizations upon separation from the dam, suggesting a possible mechanism through which they may influence maternal behaviour.

The findings of our study suggest that the previously described effects of diet on maternal behaviour may be partly attributed to physiological influences from offspring, motivating further study of the molecular mechanisms involved.

Ethics. All studies were approved by the North Carolina State University Institutional Animal Care and Use Committee (protocol #15-013-B).

Data accessibility. Data are provided as tables in the manuscript or in the electronic supplementary material.

Authors' contributions. M.B. and M.C. conceived the project, designed the experiments, performed the experiments, analysed data, interpreted findings, and wrote the manuscript. H.L., C.B., and J.T. performed experiments and contributed to data analysis. K.T. and D.R. analysed data and contributed to data visualization. All authors gave final approval for publication.

Competing interests. The authors declare no competing interests.

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