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Low temperatures during early development influence subsequent maternal and reproductive function in adult female mice

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Abstract

Challenging conditions early in development can have enduring effects into adulthood. The effects of low temperatures were examined on subsequent sex-specific morphology (anogenital distance [AGD]), maternal care, and reproductive performance in adult female mice. Dams (F_0) were maintained either in (1) standard laboratory room temperatures (21±2 °C) or (2) low temperatures (10±2 °C) throughout gestation. Their progeny (F_1) either remained in the temperature condition in which they were conceived or were switched to the other temperature condition at 2 days of age until weaning. Reproductive performance and maternal behaviors were assessed in adulthood. F_0 dams that were maintained in low temperatures bore larger litters as compared to F_0 animals housed in standard temperatures throughout their pregnancy. In contrast, mean litter size was reduced for all groups of F_1 females that experienced low temperatures. Infant mortality was elevated in litters of F_1 females that were exposed to low temperatures both before and after birth. Prenatal exposure to low temperatures was associated with reduced responsiveness towards the nursing young and decreased maternal aggression in F_1 animals. Prenatally treated F_1 females had longer, male-like AGDs on Day 2 following birth compared to animals not subjected to experimental manipulations. Our results indicate that exposure to low temperatures during early development impairs reproductive function and is associated with important fitness costs as evidenced by reduced offspring survival. Our findings also suggest that chronic low temperatures experienced only after birth may have less deleterious effects than exposure to a combination of pre- and postnatal or prenatal treatments alone.

Keywords: Stressors; Chronic stress; Prenatal development; Aggression; Reproduction; Maternal behavior

1. Introduction

Exposure to adverse conditions, including teratogenic substances, immobilization, or extreme temperatures during gestation has adverse consequences on physical development, physiology, and behavior of individuals. Such prenatal conditions demasculinize and feminize sexually dimorphic morphological and behavioral traits in male rodents [1–4], whereas these treatments defeminize and masculinize similar traits in females [5–10]. For example, exposure to heat and/or immobilization reduces nursing and nesting behaviors in dams [11] rendering maternal responsiveness to young more "male-like" [10]. Harsh prenatal conditions are also linked to social withdrawal in rodents [12]. Prenatally manipulated animals display more freezing and anxiety-like behaviors and engage in less exploration in novel environments than untreated animals in adulthood [12–15].

Some of these adverse consequences of prenatal treatments may be reversed or ameliorated by exposure to postnatal manipulations, such as "handling," that is, separation of pups from the mother for a limited time. Postnatal handling reduces fearfulness and freezing behaviors and also increases exploration in novel environments in adult rats and mice [16–18]. Previous studies that investigated the combined effects of pre- and postnatal treatments on offspring development and behavior typically used the "handling" procedure as a postnatal manipulation, but some other condition such as immobilization of the mother during gestation as the prenatal treatment [19]. It is not clear whether increased maternal attention alone, or increased maternal responsiveness in combination with the stress of being separated from the mother, induces the reported phenotypical changes in the

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offspring [16]. However, dams markedly increase maternal responsiveness to young, including pup retrieval and licking after "handling" [16,20,21]. Increased maternal attention in turn alters offspring responses to experimental manipulations [22]. Therefore, the contribution of prenatal and postnatal treatments individually, as well as the combined effects of these treatments on subsequent offspring development and behavior, require standardization that accounts for the postnatal mother–infant interactions. Furthermore, it seems likely that adverse environmental conditions, such as food restriction or low temperatures, would span both prenatal and postnatal developmental stages in nature.

Accordingly, the present study investigated the functional consequences of exposure to low temperatures during prenatal and neonatal development. Mother–infant interactions were also controlled in this study by employing cross-fostering manipulations, as well as maternal responsiveness tests. Reproductive performance, maternal care and aggression, and sex-specific morphology, namely, anogenital distance (AGD), were among the phenotypical changes that were examined. Exposure to low temperatures was selected in this study because low temperatures evoke stress responses such as elevated corticosterone concentrations in rodents [23]. Also, mice maintained in low temperatures differ from mice that remain in standard laboratory temperatures in terms of reproductive parameters, including litter size, lactational performance, and infant mortality [24–27]. Because many temperate zone rodents experience moderately low temperatures during the breeding season, our experimental conditions may also identify fitness costs associated with temperature fluctuations.

Based on the adverse consequences of prenatal treatments in mice, we hypothesized that prenatal exposure to low temperatures would masculinize AGDs in female mice. Furthermore, we expected that early exposure to low temperatures would impair subsequent maternal care and reproductive function in adulthood. Because prenatal and postnatal treatments may have opposite effects on the phenotype, we also predicted that the timing of the developmental condition would influence the extent and direction to which an individual would be affected.

2. Materials and methods

2.1. Animals

Twenty-four CD-1 mice from an outbred stock were procured at age 50 days from Charles River Laboratories. The mice were housed in groups of 4 for 10 days for acclimatization in our animal research facilities. Animals were maintained in standard housing at 21±2 °C temperature and 60±10% relative humidity with 16 h of light per day (LD 16:8; lights illuminated at 2400 h Eastern Standard Time [EST]), ad libitum access to food (Harlan Tekland 8640 rodent diet, Indianapolis, IN), and filtered tap water. Ten days after their arrival in our laboratory, males and females (F_0) were individually paired and housed in standard cages with conditions described above.

2.2. Experimental procedure

2.2.1. Low-temperature exposure

A week after pairing, all F_0 females were randomly assigned to either the treatment ($n=11$) or control group ($n=12$), and treatment females, along with their mates, were placed in refrigerated chambers at 10±2 °C with ad libitum access to filtered tap water and food. All animals were maintained on a 14:10 LD cycle (lights off at 1400 h EST). Control females were left undisturbed except for routine cage changes. On Day 16 post-pairing, males were removed from the females' cages. On Day 17 and onwards, females were monitored for the presence of pups. The date of birth was designated postpartum Day 0. On Day 2 following birth, litters were culled to (randomly selected) 6 pups with equal sex ratio where possible. The results of this study pertain to the female offspring only (F_1). All experimental manipulations involving the allocation of pups into their respective groups were performed on Day 2 (Table 1). The pups were allocated into groups as follows:

1. F_1 mice underwent both gestation (21 days) and lactation (21 days) in low temperatures (10±2 °C; $n=9$; group [GL]);
2. F_1 mice underwent gestation in low temperatures ($n=24$; group [G]);
3. F_1 mice gestated in standard temperatures (21±2 °C) then underwent lactation in low temperatures ($n=10$; group [L]);
4. F_1 mice maintained in standard temperatures throughout gestation and lactation ($n=24$; group [CONTROL]).

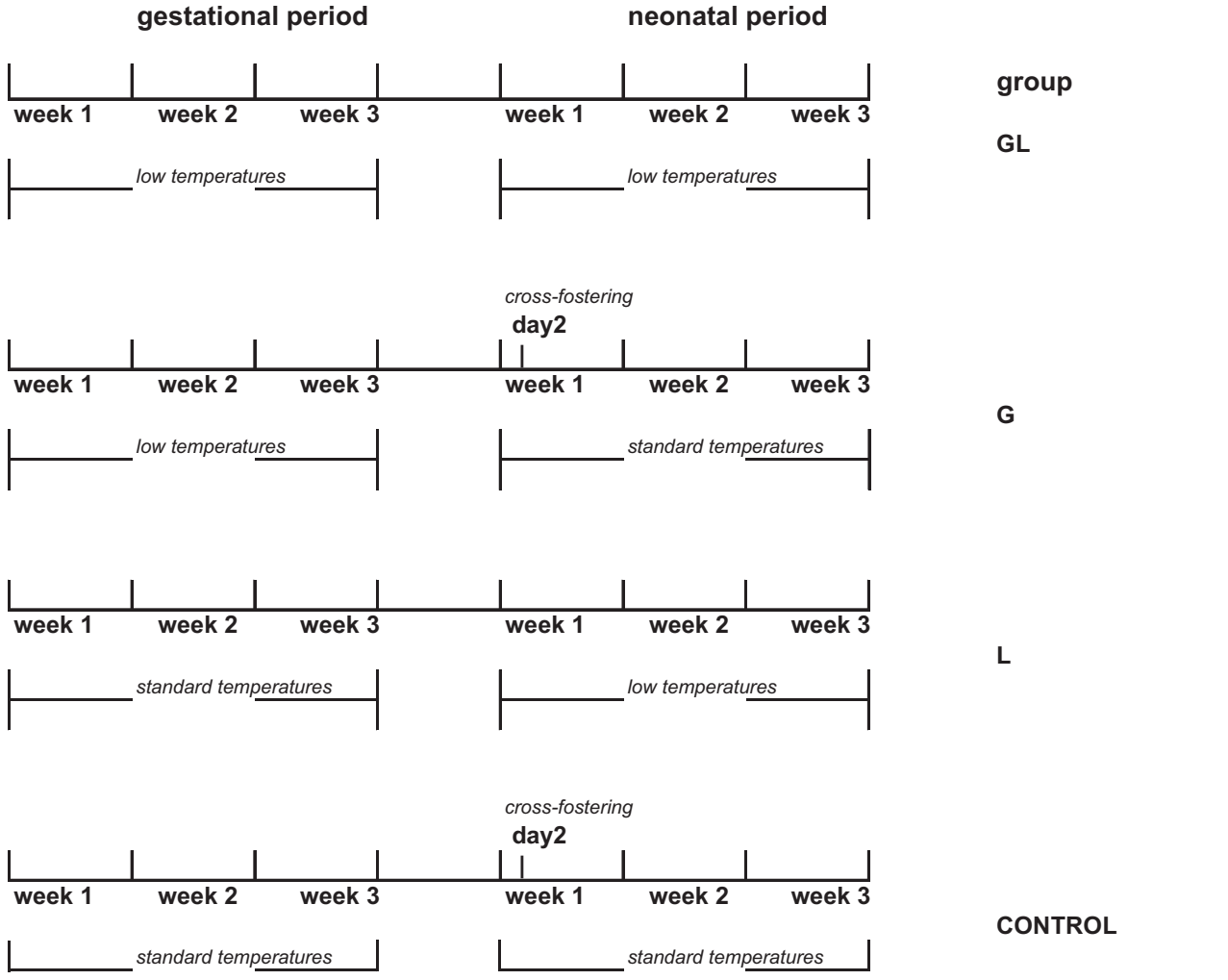
2.2.2. Cross-fostering

F_1 control pups and treatment pups that underwent gestation in low temperatures [G] were randomly allocated to cross-fostering groups on Day 2 following birth. A timetable for temperature and cross-fostering manipulations is given in Table 1. Accordingly, half of the controls were raised by F_0 dams that remained in low temperatures during gestation ($n=12$) and half of the G pups that were exposed to low temperatures during gestation were raised by F_0 control mothers ($n=12$). This procedure was aimed to partially control the effect of low temperatures on maternal care. Because GL and L mothers would be required to remain in low temperatures during experimental manipulations, mothers in all groups were additionally tested for maternal behavior, as measured by pup retrieval on Day 4 of birth. According to this testing procedure, none of the F_0 groups differed from each other in the latency and number of pups retrieved ($P>0.05$). The details of the procedure for pup retrieval are described below.

2.2.3. AGD

AGD was measured on Day 2 in all female pups before the litters were culled. For the AGD measure, the total sample size was 103 for treatment and 109 for control female pups. The measurements were made from the base of the genital papilla to the base of the anus under a dissecting microscope (×10). The same (uninformed) experimenter performed the measurements to minimize error. Litter sizes and pup body weights were also collected at this time to investigate the effect of body size on

Table 1
Timeline for temperature and cross-fostering manipulations for F_1 females



GL=animals exposed to low temperatures during both gestation and lactation; G=animals exposed to low temperatures during gestation; L=animals exposed to low temperatures during lactation; CONTROL=animals raised in standard laboratory temperatures during both gestation and lactation.

AGD. In the final analyses below, body weights alone were used to remove the effect of pup size on AGD by employing generalized linear models [28].

2.2.4. Maternal behavior: F1 females

2.2.4.1. Pup retrieval. At age 5 months, all groups of F_1 females were mated to an age-matched, non-sibling male to produce progeny. The mating procedure was the same as that of their mothers, except none of the F_1 females was exposed to additional experimenter-introduced low temperatures in adulthood. Two days after they gave birth, the cages containing all F_1 females with their pups were moved to a quiet testing room. After approximately 30 min of acclimatization, behavioral tests began. Tests were performed in the early part of the dark cycle. The dam and all of her pups were removed from the cage. Four randomly selected pups were then returned to the cage and placed randomly around the cage corners farthest from the nest. The dam was then returned to the cage immediately and several behaviors were recorded for up to 10 min [29,30]: The latency to (1) contact the first pup; (2) retrieve the first pup (the duration between the dam was put back into the cage and return of the first pup back to the nest); (3) retrieve the fourth pup (the duration between the dam was put back into the cage and return of all pups to the nest); and (4) total number of pups retrieved in a 10-min trial. If the dam did not retrieve all of the pups within 10 min, then the latency to retrieve the fourth pup was coded as 600 s.

2.2.4.2. Maternal aggression. On Day 6 after birth, all groups of F_1 females were tested on aggressive behavior towards an adult, sexually naïve, unfamiliar male intruder. All experiments were performed during the first hours of the dark portion of the day in a quiet testing room after a 30-min acclimation period. The dam was removed from her cage and the pups were taken away immediately after her removal. The dam was then returned to her cage and the intruder was introduced into the cage. The latency between her removal and the placement of the intruder was less than 3 min. Removal of pups from a dam just before an aggression test does not

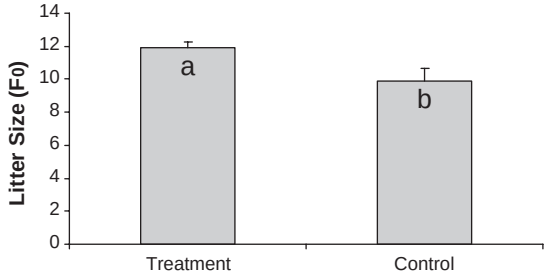


Fig. 1. Mean (±S.E.M.) litter size in treated and control dams (F_0). Group means with different letters are significantly different from each other ($P<0.05$).

decrease the attacks towards an unfamiliar intruder [31,32]. Each test session lasted for 10 min and was recorded on videotape for later analyses. Observer version 5.0 (Noldus Corp., Leesburg, VA) was used to quantify and analyze the following aggressive behaviors exhibited by the dams: (1) latency to first attack; (2) frequency of attacks; and (3) total duration of attacks towards the intruder. This set of responses was used to quantify bouts of aggression and included such behaviors as rapid thrusts towards the intruder, boxing, kicking, and biting. If the dam did not exhibit any aggression, then the latency to first attack was scored as 600 s. The rater was uninformed about the conditions of the experiment. The data were randomly checked by a second rater for consistency.

2.3. Data analysis

All statistical analyses were performed by the SAS statistical software package, version 9.1 (SAS Institute, Cary, NC). When assumptions of normality were violated in small-size samples, non-parametric tests were used in order to investigate the main effects and compare the group means. Specifically, the response variables that were not normally distributed were rank transformed and a Proc MIXED procedure was applied on these rank-transformed variables. Otherwise, group comparisons were performed with ANOVA or ANCOVA tests, where covariates (i.e., litter size or weight) were considered by using Proc GLM procedure for unbalanced data. Values of $P<0.05$ were considered statistically significant.

3. Results

Statistical analyses showed no effect of cross-fostering on maternal behavior, maternal aggression, litter size, pup survival, or pup weights ($Ps>0.05$). To reiterate, control and treatment (F_0) dams did not differ from each other in maternal behavior measured by pup retrieval tests ($Ps>0.05$). Therefore, only the GL, G, L, and CONTROL groups are presented in sections below.

3.1. Litter size, AGD, and pup weights

F_0 dams exposed to low temperatures during their pregnancy had significantly more pups than F_0 dams not exposed to experimental manipulations ($P<0.05$, Fig. 1). Mean litter size for F_0 mothers was 11.92 (S.E.M.=±0.38), whereas mean litter size for F_0 animals was 9.91 (S.E.M.=±0.72). F_1 pups of treatment mothers had significantly longer AGDs on Day 2 of birth compared to those of control dams ($P<0.0001$ with weight controlled, Fig. 2). F_1 pups born to treatment mothers had significantly lower weights on Day 2 than those born to control animals even when the litter size was controlled ($P\leq0.01$, Fig. 3(A)). As F_1 pups were allocated to their experimental groups on Day 2 of birth, mean weaning and adult weights reflect four groups, namely, GL, G, L, and CONTROL females. All F_1 treatment females had lower weights compared to controls at weaning ($P\leq0.0001$, Fig. 3(B)). In addition, L females had the lowest weaning weight among all groups (all $Ps\leq0.0001$). In adulthood, the differences among groups largely disappeared. GL and G animals had comparable weights to those of CONTROL females, whereas L females remained smaller than GL ($P\leq0.01$), G ($P\leq0.02$), and CONROL animals ($P\leq0.001$, Fig. 3(C)).

3.2. Reproductive performance and maternal behavior: F_1 females

Litter size in all experimentally manipulated F_1 females was significantly lower than that of untreated animals ($P\leq0.01$, Fig. 4). In addition, all low-temperature groups experienced infant mortality to some degree by weaning age, whereas none of the untreated females lost pups (Table 2). The effect of low temperatures on pup survival was only significant for GL females in comparison to untreated females ($P<0.05$). No effect of group on gestational length or pup weights at any age from birth to weaning was observed ($Ps>0.05$).

Maternal care was delayed among females in the GL and G groups. These mice had longer initial retrieval latencies than CONTROL animals ($Ps<0.05$, Fig. 5(A)). L and CONTROL groups did not differ from each other in this measure ($P>0.05$). The latency to retrieve the last pup in G females was also significantly lower than L females ($P<0.05$, Fig. 5(B)).

The latency to attack an intruder was significantly higher in GL females compared to G and CONTROL females ($Ps\leq0.01$). Accordingly, the frequency of attacks in GL

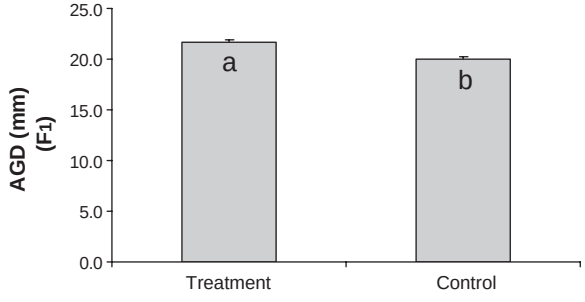


Fig. 2. Mean (±S.E.M.) anogenital distance (AGD) in 2-day-old control pups and pups exposed to low temperatures during gestation (F_1, all females). Group means with different letters are significantly different from each other ($P<0.05$).

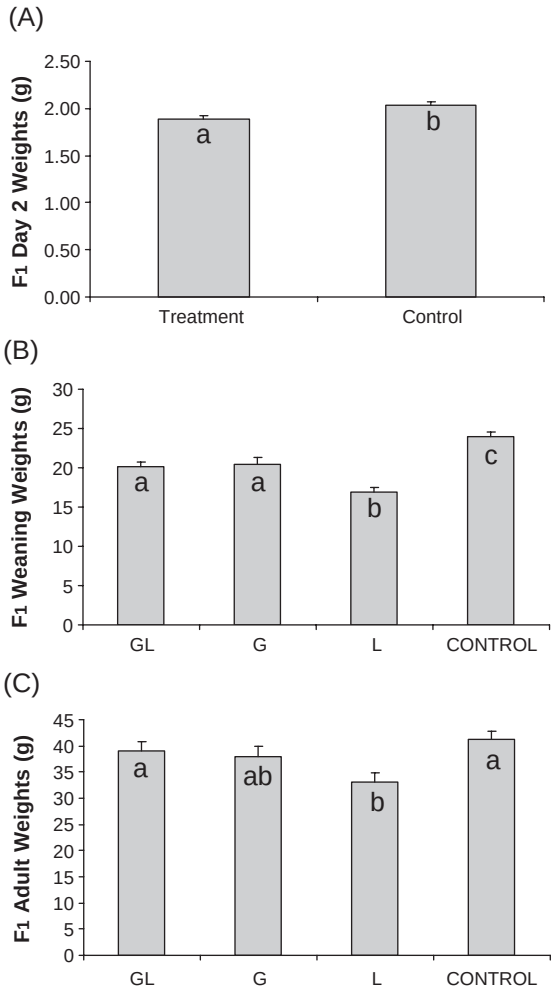


Fig. 3. (A) Mean (±S.E.M.) female F_1 pup weight on Day 2 of birth. Group means with different letters are significantly different from each other ($P<0.05$). (B) Mean (±S.E.M.) weaning weights of treated and untreated F_1 females. Group means sharing the same letter(s) are not significantly different from each other ($P>0.05$). GL=animals exposed to low temperatures during both gestation and lactation; G=animals exposed to low temperatures during gestation; L=animals exposed to low temperatures during lactation; CONTROL=animals raised in standard laboratory temperatures during both gestation and lactation. (C) Mean (±S.E.M.) adult weights of treated and untreated F_1 females.

animals was significantly reduced as compared to G and CONTROL animals ($P\leq 0.01$ and $P\leq 0.005$, respectively). In addition, GL females engaged in lower duration of attacks towards an intruder compared to G, L, and CONTROL animals

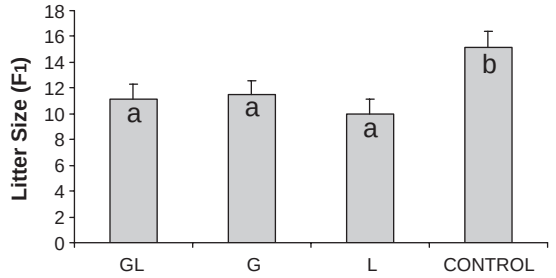


Fig. 4. Mean (±S.E.M.) litter size in treated and untreated F_1 females. Other symbols and conventions are as in Fig. 3 (B).

Table 2
F_1 Infant mortality

Group	N[a]	Percent infant mortality	S.E.M.
GL[b]	8	18	13
G	10	7	6
L	8	13	12
CONTROL[b]	9	0	0

[a] Sample size pertains to animals that produced litters.
[b] Means are significantly different from each other (two-sample median test with normal approximation $z=-1.75$, $P<0.05$). Other figures and conventions are as in Table 1.

($P\leq 0.01$; $P<0.05$; $P\leq 0.01$, respectively, Fig. 6(A)). No significant differences were observed among any other groups (Ps>0.05).

4. Discussion

The effects of low temperatures during early development were assessed on physical characteristics (i.e., AGD, body mass), as well as subsequent maternal behavior, and reproductive function in adult female mice. Overall, all dependent measures were altered to some extent by the experimental treatment conditions, suggesting exposure to low temperatures during critical periods of development may have adverse early and adult effects.

4.1. AGD

Female pups exposed to low temperatures during fetal development had longer, male-like AGDs on Day 2 of birth compared to their untreated counterparts. AGD is a sexually dimorphic trait that is correlated with several behavioral traits; males have longer AGDs than females [9,33]. Thus, the

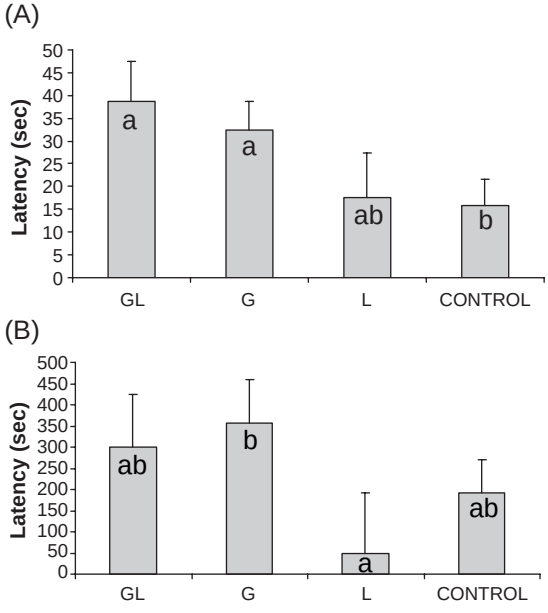


Fig. 5. (A) Mean (±S.E.M.) latency to retrieve the first pup in treated and untreated F_1 females. (B) Mean (±S.E.M.) latency to retrieve the last pup in treated and untreated F_1 females. Other symbols and conventions are as in Fig. 3 (B).

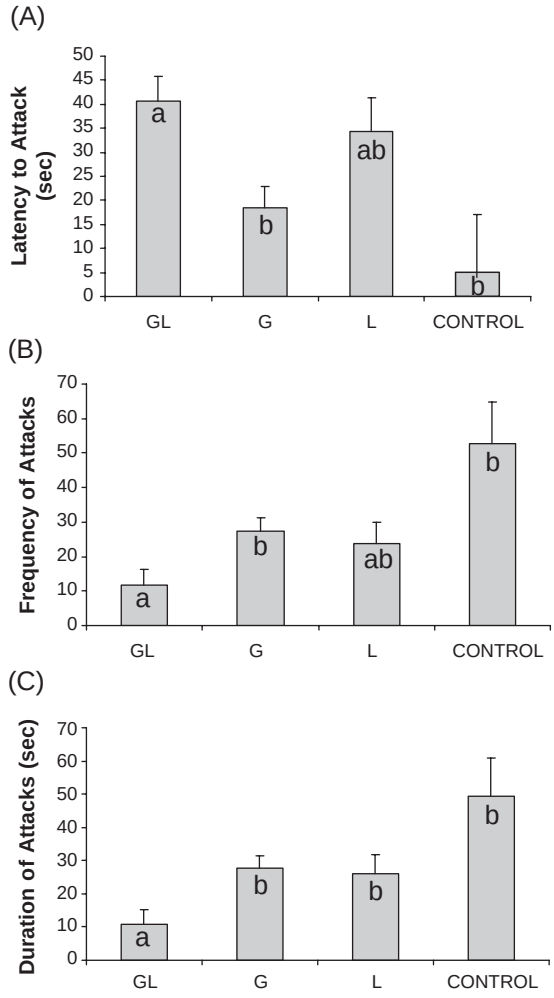


Fig. 6. (A) Mean (±S.E.M.) latency to attack a male intruder in treated and untreated F_1 females. (B) Mean (±S.E.M.) frequency of attacking a male intruder in treated and untreated F_1 females. (C) Mean (±S.E.M.) duration of attacks towards a male intruder in treated and untreated F_1 females. Other symbols and conventions are as in Fig. 3 (B).

increase in AGD is generally attributed to elevated androgen concentrations in utero [9,33,34]. Indeed, treatment with the anti-androgen flutamide reverses this effect [35,36]. In addition, prenatally stressed female mouse fetuses show increased plasma testosterone concentrations [9,33]. Prenatal stress may also affect tissue responsiveness to sex steroid hormones [37–39]. Thus, assuming that low temperatures have adverse consequences, our results confirm earlier findings on the masculinization/defeminization of sexual characters in rodents due to prenatal stress [9,33,34]. How masculinized/defeminized external genitalia and the associated changes in brain and behavior affect fitness in the wild remains unspecified. However, in the present laboratory study, there were several indications that significant fitness consequences ensued for pups exposed early to prolonged low temperatures.

4.2. Maternal care

Maternal care, as measured by the latency to retrieve the first pup, was reduced in females that experience low temperatures prenatally; i.e., GL and G females were slower to retrieve pups as compared to CONTROL animals. G females were also slower than L females in retrieving all four pups. None of the groups differed in the total number of pups retrieved. Apparently, appropriate maternal responsiveness to nursing young was delayed in both GL and G animals. In contrast, female pups experiencing low temperatures during their first weeks of life (L) displayed appropriate maternal care towards the young, similar to the levels observed in untreated females.

Because steroid hormones are implicated in sexually dimorphic behaviors and prenatal stress masculinizes/defeminizes female rodents both in terms of behavior and reproductive function [5,6], inadequate display of maternal behaviors towards the young may be attributed to an increased testosterone surge and the subsequent behavioral masculinization of the brain during prenatal development. An increase in serum testosterone concentrations in stressed female mouse fetuses is observed on Day 18 of fetal life [9]. Moreover, sex differences in pup-induced parental behavior may be eliminated by prenatal stress. Specifically, prenatal stress reduces female responsiveness to young rendering maternal behavior of the female more male-like [5]. Because females experiencing low temperatures postnatally did not differ from untreated females in any measures of the pup retrieval tests, delayed maternal responsiveness in GL and G females might be due to the low temperatures experienced during gestation only.

4.3. Maternal aggression

Maternal aggression was reduced in GL animals. Neither prenatal nor postnatal low-temperature exposure alone decreased maternal aggression in lactating females. Previous research on this topic yielded mixed results. Some studies report that prenatal stress alters and generally decreases maternal aggression in female mice [40,41], whereas other studies indicate elevated post-partum aggression in these animals [10,29].

Decreased maternal aggression in GL females may be partially attributed to increased fear and anxiety due to the adverse conditions during gestation this group experienced. Prenatal stress increases fearfulness and anxiety in rats and mice [41,42]. One possible mechanism that leads to fearfulness and anxiety after exposure to stress may be hypothalamic–pituitary–adrenal (HPA) axis dysfunction. Prenatal stress disturbs the hormonal milieu in pregnant dams and disrupts the HPA axis function and its response to stressors in both dams and their offspring [43]. On the other hand, high aggression during lactation is attributed to decreased fear and anxiety and considered adaptive to protect the offspring against infanticidal conspecifics [32,41,44]. Indeed, lactating females display less fear and anxiety than sexually naïve females in a variety of indices, such as acoustic startle and elevated plus maze [32]. In the present study, however, G females, exposed to gestational treatment alone, did not reduce maternal aggression. It may be possible that animals must be exposed to low-temperature conditions during both pre- and neonatal development for this condition to affect fear and anxiety in adulthood.

4.4. Reproductive performance

Mean litter size in all F_1 groups exposed to low temperatures was significantly reduced compared with untreated females. Also, untreated F_1 females appeared to produce larger litters than their mothers (Figs. 1 and 3(B)). The cause of this generational difference remains unspecified. Possible effects of shipping and the lab condition differences for F_0 mothers, as well as breeding in two different time periods might have contributed to this effect. Additional research is needed to explore this phenomenon.

Whereas untreated F_1 animals did not lose any pups from birth to weaning, infant mortality in GL females during this period was significantly higher than CONTROL females even when the litter size was controlled. G and L groups also experienced some infant mortality from birth to weaning.

The lower reproductive performance in GL and G females may reflect a disruption in the hypothalamic–pituitary–gonadal (HPG) axis function resulting from low-temperature exposure during their fetal development. Stress-evoked stimulation of the HPA axis adversely affects subsequent reproductive function [45–47]. Prenatally stressed females show delayed vaginal opening, longer estrous cycles and pregnancies, increased intrauterine mortality and spontaneous abortion compared to their non-stressed counterparts [6–8]. Although our study did not directly measure the mechanism involved in low-temperature exposure and stress, HPA axis, and gonadal function interactions, our results pertaining to both maternal care and reproductive performance are in accordance with the previous studies that employed such direct measures [32,48].

In sum, exposure to low temperatures during early development impaired subsequent reproductive function and was associated with other fitness costs as measured by the reduced number of surviving offspring into adulthood. Postnatal low temperatures had less deleterious effects than combined low temperatures during pre- and postnatal or prenatal development alone. Indeed, postnatally treated animals engaged in appropriate levels of maternal responsiveness towards the young. This effect was independent of the maternal care the pups themselves received during early development. Reduced number of litters in postnatally treated females is likely due to low adult body mass as low maternal weight before conception is associated with decreased number of offspring in mice [49,50]. Curiously, the mothers of GL and G females had larger litters than F_0 dams that remained in standard temperatures (L mothers were not exposed to low temperatures during their pregnancy). Because all F_0 dams were maintained in standard temperatures until the time of conception, the timing of the temperature manipulations was unlikely to cause a severe disruption in reproductive function. Our results suggest that in the absence of other stressors, such as restricted food, and of early stressors, including those experienced during early development, adult mice reproductively respond to low temperatures by adjusting the number of offspring and litter mass they produce and presumably ensure the optimum allocation of maternal resources for subsequent offspring survival. Currently, we aim to extend our findings to other species while manipulating day length and ambient temperatures in early development to investigate ensuing phenotypical variation in adulthood.

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