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# The effects of different rearing conditions on sexual maturation and maternal care in heterozygous mineralocorticoid receptor knockout mice

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A B S T R A C T

Sexual and social development is affected by a complex interplay between genetic makeup and the early-life rearing environment. While many rodent studies focused primarily on the detrimental effects of early-life stress, human literature suggests that genetic susceptibility may not be restricted to negative environments; it may also enhance the beneficial effects of positive rearing conditions. To examine this interaction in a controlled setting, heterozygous mineralocorticoid receptor knockout ($MR^{+/-}$) mice and control litter mates were exposed to a limited nesting/bedding (LN, impoverished), standard nesting (SN, control) or communal nesting (CN, enriched) paradigm from postnatal day 2–9 (P2-P9). Offspring was monitored for puberty onset between P24-P36 and, in females, maternal care-giving (i.e. as F1) during adulthood, after which basal corticosterone was measured. Different home-cage environments resulted in profound differences in received maternal care and offspring body weight. In male offspring, LN resulted in delayed puberty onset that was mediated by body weight and unpredictability of maternal care received during early development. In female offspring, rearing condition did not significantly alter sexual maturation and had little effect on their own maternal care-giving behavior. Genotype did affect maternal care: female $MR^{+/-}$ offspring exhibited a less active nursing style and upregulated fragmentation during adulthood, irrespective of early life conditions. Basal corticosterone levels were highest in $MR^{+/-}$ mice with a background of LN. Overall, we found a gene-by-environment interaction with respect to basal corticosterone levels, but not for sexual maturation or maternal behavior.

## 1. Introduction

The early-life rearing environment of mammals, including parental care, critically contributes to development and functioning later in life. For instance, aberrations in maternal care affect a wide variety of behaviors in offspring, including cognitive and social abilities (Davis et al., 2017; Gunnar et al., 2015; Levine, 2005). While early-life adversity has detrimental consequences in some individuals, others appear to be more resilient. Genetic factors have been proposed to (at least partly) underlie vulnerability to early-life stress in both humans (Hornung and Heim, 2014; Nugent et al., 2011) and rodents (Buschdorf and Meaney, 2015; Daskalakis et al., 2013). However, genetic susceptibility may not be restricted to negative environments; it may also enhance the beneficial effects of positive rearing conditions. Evidence in humans supports this for better *and* for worse concept, dubbed the 'differential susceptibility theory' (Bakermans-Kranenburg and Van IJzendoorn, 2011; Belsky and van IJzendoorn, 2017), although different patterns have been observed (Windhorst et al., 2015). To better understand differential susceptibility at a neurobiological level, we can use the unique advantages of animal models, including superior control over environment and genetic background (Knop et al., 2017; Krugers and Joëls, 2014).

### 1.1. The MR gene in differential susceptibility

A potential gene conveying differential susceptibility characteristics is the mineralocorticoid receptor (MR) gene. The MR is a high affinity receptor for cortisol (in humans) and corticosterone (in rodents) that is predominantly expressed in limbic-cortical areas such as the hippocampus, amygdala and medial prefrontal cortex; areas that additionally express the lower-affinity glucocorticoid receptor (GR) (Joëls, 2018; Reul and Kloet, 1985). MR and GR interact to mediate an adaptive response to stress; an imbalance between the two is hypothesized to increase the risk of developing psychopathology (Harris et al., 2013;

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Joëls, 2018). In humans, MR haplotypes have been shown to sex-specifically moderate the effects of childhood maltreatment on (sub-clinical signs of) depression (Vinkers et al., 2015). Moreover, the Iso/Val genotype of MR affects amygdala reactivity in individuals who experienced childhood neglect (Bogdan et al., 2012). In rodents, MR overexpression was found to mitigate the cognitive impairment seen after early-life adversity (Kanatsou et al., 2017). Gene-by-(early)-environment interactions have also been found for GR (Bet et al., 2009) and its co-regulator FKBP5 (Appel et al., 2011; Binder et al., 2008). These findings highlight the importance of hypothalamic-pituitary-adrenal (HPA) axis activity in regulating the long-term effects of early-life adversity. Since biological sensitivity to stress has been proposed as a differential susceptibility marker (Ellis et al., 2011), this network appears to be a promising target to test differential susceptibility in mice, particularly one of its key elements, the MR gene. This is further supported by the fact that in humans, an MR SNP in children has been implicated in the effect of sensitive parenting on attachment security (Luijk et al., 2011).

### 1.2. Models for impoverished or enriched environments

The effects of different rearing environments on offspring development have been studied extensively using rodent models. Exposure to an impoverished environment, in which the dam has limited access to bedding and nesting material (Rice et al., 2008), results in fragmentation and increased unpredictability of maternal care (Molet et al., 2016). This condition upregulates corticosterone levels in both the dam and offspring, affecting a wide variety of developmental outcomes during adulthood (see (Walker et al., 2017) for a review of this model). On the other side of the spectrum, early social enrichment can be modelled by utilizing the naturally occurring tendency of mice to form communal nests (Crowcroft and Rowe, 1963). Co-housing two or more lactating dams results in shared, upregulated care-giving behavior and facilitates peer interactions among pups (Branchi et al., 2006). Mice reared in this condition display enhanced sociability and exhibit markers of increased neuronal plasticity (Branchi and Cirulli, 2014). Experiments directly comparing the developmental effects of these two different rearing environments have not been conducted to date.

### 1.3. Timing of puberty onset as outcome

According to life history theory, timing of puberty onset is affected by rearing conditions as part of a reproductive strategy (Belsky et al., 1991). In females, early-life stress (ELS) has been shown to accelerate sexual maturation in both humans (Belsky et al., 2015; Mendle et al., 2011) and rodents (Cameron et al., 2008; Cowan and Richardson, 2018). Conversely, positive family relations were linked to a delay in puberty onset in humans (Graber et al., 1995). Conflicting results were reported for males, where ELS either had no effect (rats: Biagini and Pich, 2002; humans: Grassi-Oliveira et al., 2016) or delayed puberty onset in rodent models of ELS (Bodensteiner et al., 2014; Cowan and Richardson, 2018). Deviations in the timing of puberty are linked to various mental health problems including anxiety, depression and social disorders in both girls (Mendle et al., 2007) and boys (Mendle and Ferrero, 2012). Although the direction of effects is not always clear (Tremblay and Frigon, 2005), these effects highlight the importance of pubertal timing in development.

### 1.4. Intergenerational transmission

There is substantial evidence that maternal care, with its vital role in regulating social and reproductive behavior, may be transmitted across generations, altering maternal care of offspring. For instance, natural variations in received levels of licking/grooming during early postnatal rat development predict maternal behavior in adults (Champagne et al., 2003), possibly through epigenetic mechanisms (Champagne and Curley, 2009; Weaver et al., 2004). Changes in maternal care evoked by prenatal immune activation of the dam similarly transfer to the next generation (Ronovsky et al., 2017). Transgenerational effects of maternal care have also been studied using the limited bedding/nesting (Roth et al., 2009) and communal nesting (Curley et al., 2009) models, where animals reared in these conditions later in life display aberrant or improved maternal behaviors, respectively.

Overall, the aim of this study was to examine susceptibility of heterozygous MR knock-out ($MR^{+/-}$) mice to both negative and positive rearing environments, i.e. the limited bedding/nesting and a modified communal nesting model respectively. $MR^{+/-}$ mice were used to mimic the reduced functionality of MR in susceptible human haplotypes (e.g., DeRijk et al., 2008) while maintaining translationally relevant MR levels. As outcome measures we tested i) puberty onset, as a key developmental readout; and ii) maternal care (as well as basal corticosterone level) in female offspring, to monitor transgenerational effects. In line with the life history theory and previous findings in rodents, we hypothesized puberty onset to be accelerated in limited nesting/bedding reared female animals and delayed in female mice that were exposed to the communal nesting paradigm. Available literature in males is too limited to predict male puberty onset. Maternal care of adult females was hypothesized to be poor in ELS mice, while being improved in CN reared animals. Finally, in line with the differential susceptibility theory, these effects were hypothesized to be stronger in heterozygous mineralocorticoid receptor knock-out mice.

## 2. Materials & methods

### 2.1. Animals and housing

All mice were bred in our own animal facilities. Wild-type (wt) C57BL/6JOlaHsd females were originally obtained from Harlan France and bred in-house for at least two generations before experiments. Forebrain-specific MR knock-out animals were generated by using the Cre/loxP-recombination system (Berger et al., 2006). The F0 wt C57BL/6 females were bred with male $MR^{flox/flox\ -\ CaMKIIa\text{-}Cre/wt}$ mice, generating heterozygous forebrain-specific MR knock-out ($MR^{+/-}$) F1 offspring and control litter mates. Dam and litter were placed in either a limited nesting/bedding, control or communal housing condition, between postnatal day (P) 2 and 9. The F1 offspring (♂ $n = 112$, ♀ $n = 128$) of 38 breedings were tested on puberty onset. The female offspring were monitored as adults for maternal care (see Fig. 1 for a timeline of the experiment). Puberty onset and maternal care measures of F1 animals were obtained by a trained experimenter blind to rearing condition and genotype of the animals. Animals were housed under a reversed 12:12 h light/dark cycle (lights off 08:00 h, temperature 21–22 °C, humidity 40–60%) with ad libitum access to water and food. All experiments were performed in accordance with the EC council directive (86/609/EEC) and approved by the Central Authority for Scientific Procedures on Animals in the Netherlands (CCD approval AVD115002016644).

### 2.2. Breeding conditions F0

For the breeding of F0, two females were paired with a male for 4 days. Females were then co-housed until approximately one week prior to parturition. After separation, each dam was placed in a type II short Macrolon cage (21.5 × 16 cm) with a filter top and provided with a cotton Nestlet (5 × 5 cm, Technilab-BMI, Someren, The Netherlands) as nesting material. Each day at 09:00 h, animals were checked for litters, assigning the day prior to first appearance of a litter as date of birth (P0). At P2, all litters were weighed and culled or cross fostered to 6–7 pups. A maximum of 1 pup per litter was added from a different litter if a litter contained 5 pups and a minimum of 2 pups of each sex was included in each litter. Litters were randomly allocated to one of three conditions; limited nesting/bedding (LN), standard nesting (SN)

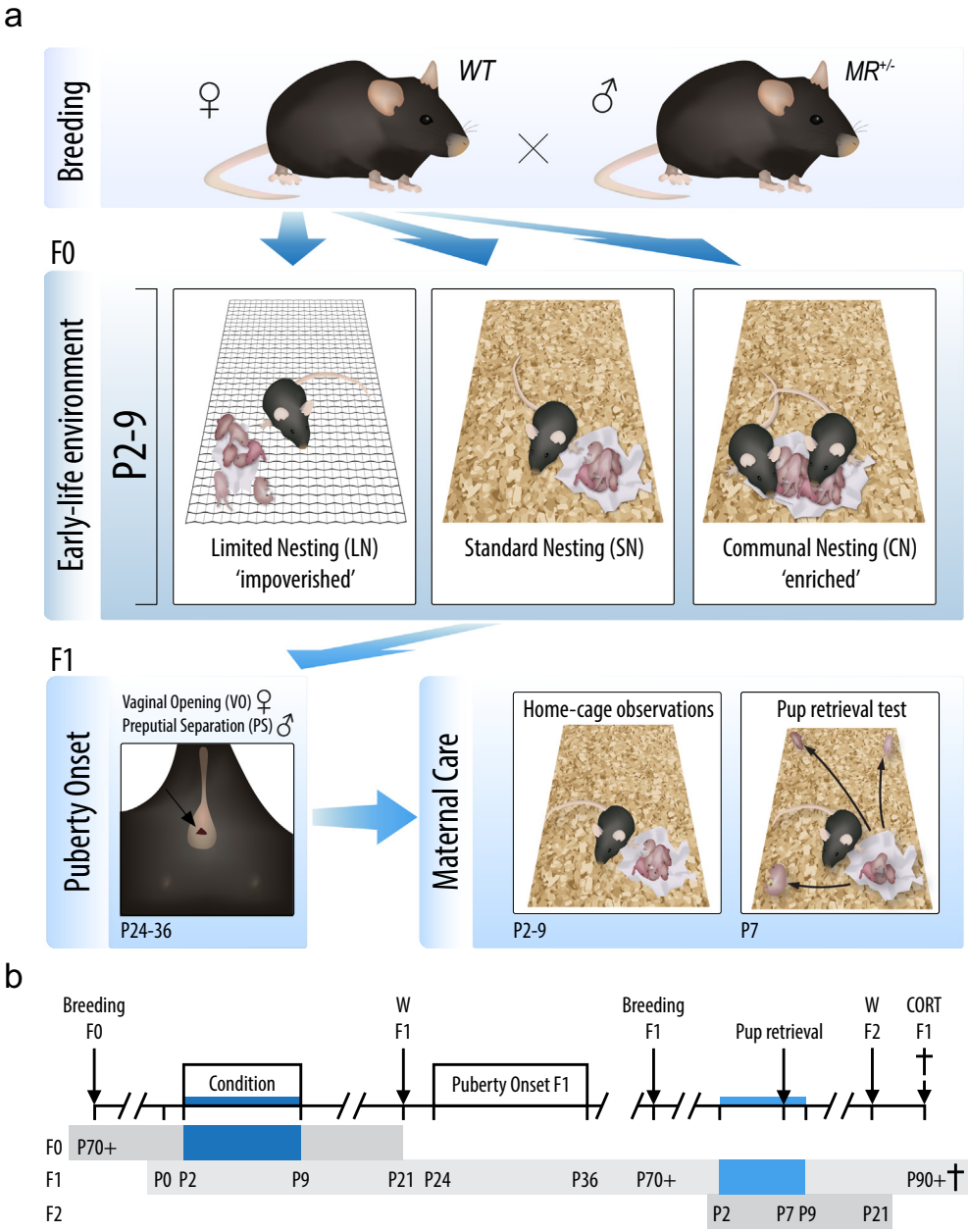


**Fig. 1.** Outline of the experiments.
(a) Study design and (b) timeline of the experiment. A wild-type female was paired with a heterozygous $MR^{+/-}$ male to obtain mixed litters. Experimental time points for each generation of mice are depicted. W = weaning. P = postnatal day. Blue lines indicate periods of home cage maternal care observations. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

or communal nesting (CN). The LN condition was performed as described earlier (Rice et al., 2008). In short, a limited amount of sawdust bedding was provided, covered by a stainless steel wired mesh. In addition, half the regular amount of nesting material was available to the dam. In the SN condition, the dam had access to a standard amount of sawdust and nesting material. The CN paradigm consisted of co-housing the experimental dam (and her genetically heterogeneous F1 litter) with another wt dam (and wt litter) in a type II regular Macrolon cage (32 × 16 cm). This other wt dam was marked using ear punches and her pups were marked with a non-toxic, non-scenting surgical marker (ArcRoyal, Ireland) on P2 and P6 to ensure correct allocation of the pups to their mother at the end of communal housing at P9. At P9, all pups were weighed and all nests returned to standard nesting conditions until weaning at P21. All cages were cleaned once between P9 and weaning. At weaning, offspring was weighed and ear punched to facilitate individual recognition and allow for genotyping.

### 2.3. Maternal care observations F0

During exposure to different rearing conditions, maternal behavior of the dams was monitored using instantaneous sampling (Liu et al., 1997). From P2-P9, maternal observations were performed three times a day, each for 75 min. The first observation took place at the end of the light phase (between 06:00 and 07:30 am), the second in the middle of the dark phase (between 12:00 and 14:00 pm) and the third at the end of the dark phase (between 16:30 and 18:30 pm). Dark phase observations were carried out in red light conditions. Within each observation period the behavior of each dam was scored every 3 min, resulting in 25 observations per period and 75 observations per day. The behaviors were identified as: arched-back nursing (ABN), passive

nursing, licking/grooming pups (LG), nest building, self-grooming on nest, feeding and self-grooming off nest. If a behavior was observed that was not covered by one of these categories, only the location of the dam (on or off nest) was scored. Observations were scored using Pocket Observer 3.3 software (Noldus, The Netherlands) on a Samsung Galaxy Note 4 smartphone and analyzed using Observer XT 10.5 (Noldus, The Netherlands).

Maternal care was evaluated using three separate approaches. First, individual maternal behaviors were analyzed using the percentage of time the specific maternal behavior was shown. For each behavior, both development over postnatal days (pooling the 3 observations per day) and circadian rhythmicity during the day (pooling the 6 days) were assessed. Second, previous studies confirmed an important role of unpredictability and fragmentation of maternal care in pup development. Unpredictability was defined as the overall entropy rate of maternal care and calculated as described earlier (Molet et al., 2016). In short, the entropy rate summarizes the probabilities that certain behaviors predict the transition to specific subsequent behaviors. The entropy rate can be regarded as a measure of unpredictability in which higher rates indicate higher unpredictability of behavior. Because transitions between off-nest behaviors might be regarded irrelevant for the pups, a separate entropy rate was calculated combining all off-nest behaviors into one category. Third, fragmentation of maternal behavior (Rice et al., 2008) was calculated by the average number of transitions from and to the nest. For the communal nesting condition in F0 dams, maternal care was calculated by averaging measures of both dams.

### 2.4. Puberty onset F1

To determine the effects of rearing conditions and heterozygous KO of MR on sexual maturation of F1 offspring, puberty onset in both males and females was determined. Female mice were examined daily from P24-P36 on vaginal opening as an external measure of puberty onset (Caligioni, 2010). In males, daily examination of preputial separation from P27-P32 was used to determine puberty onset. Mice were restrained with one hand, while gently attempting to manually retract the prepuce (Korenbrot et al., 1977). Preputial separation was defined as the potential to fully retract the prepuce and expose the glans penis.

### 2.5. Maternal care F1

After P70, breeding of F1 females with a wild-type male was performed as described for F0. F2 offspring was culled to 6 pups per litter and weighed at P2, P9, P15 and P21, in parallel with transfer to clean cages on these days. All F1 dams were placed in standard nesting conditions. Observations of maternal care-giving behavior were done as in F0 from P2-P9. To challenge maternal responsiveness, a pup retrieval test was performed at P7 between 10:00–12:00. The dam and pups were briefly removed from the home cage and sawdust bedding was leveled, leaving the nest site intact. In three corners distant from the nest a pup was placed, counterbalancing sex ratio and location of the pups across trials. The dam was replaced in the nest facing a wall and retrieval behavior was recorded for 5 min and analyzed for retrieval latencies of all three pups using Observer XT 10.5 (Noldus, The Netherlands). After testing, the three remaining pups were returned to the nest. All dams successfully retrieved all pups within 5 min.

### 2.6. Plasma corticosterone levels F1

After weaning of F2 litters, F1 dams ($n$ = 5–8/group) were decapitated between 13:00 and 17:00 and trunk blood was collected on ice in heparin containing tubes (Sarstedt, The Netherlands). To prevent effects of cage disturbance in remaining mice, a maximum of two animals per cage was used and simultaneously decapitated by two experimenters. Effort was made to distribute all experimental groups evenly across the sampling period. Blood was centrifuged for 10 min (13,000 rpm) at 4 °C and plasma was stored at −20 °C until corticosterone measurements using a radioimmunoassay kit (MP Biomedicals, The Netherlands; sensitivity 3 ng/ml).

### 2.7. Statistical analysis

All data are expressed as mean ± SEM and SPSS 23 (IBM) was used for analysis. Outlying values, defined as deviating > 3.29 SD from the mean, were winsorized (Tabachnick and Fidell, 2007). A total of 3 data points in 2 variables were winsorized. The complex samples module of SPSS was used to account for litter effects in F1 animals. Because no effect sizes are provided in this module, these are not reported. Overall ANOVA statistics and eta squared effect sizes ($\eta^2$), the explained variance as proportion of the total variance in the model, are presented in the text; post-hoc comparisons with a Tukey HSD (main effects) or Sidak (interaction) correction are depicted in the figures.

F0 maternal care was analyzed using one-way or repeated measures ANOVAs with breeding condition as the between-subject factor and postnatal day or observation (1 day and 2 night observations) as within-subject factors. A Greenhouse-Geisser correction was used for repeated measures ANOVAs. To prevent major impact of the disturbance at postnatal day 2 caused by introduction to a novel environment, this day was excluded from analysis. For F1 maternal care no interactions of genotype or condition with postnatal day or observation were found, therefore overall levels were analyzed using a two-way ANOVA. Early life experience (condition) and genotype were included as between-subject factors and all other F1 variables were similarly analyzed. As an overall index for active parenting, a principal component analysis was conducted using frequent (> 2% of time) on-nest behaviors: arched-back nursing, passive nursing, licking/grooming and self-grooming on nest. The eigenvalue was 1.68, 42% of variance was explained by the first factor and all variables loaded > 0.40, with negative load for passive nursing. The resulting PCA factor was used as an index of active parenting in F1 dams.

Pearson correlations were used for associations between puberty onset and body weight/entropy and between corticosterone and entropy in F1. Mediation analysis with rearing condition as a multicategorical independent variable, body weight and received entropy as mediators, and pubertal timing as outcome was performed using the PROCESS v3 macro for SPSS (Hayes and Preacher, 2014). To determine significance of mediation, 95% confidence intervals were calculated and deemed significant if they did not straddle zero.

## 3. Results

### 3.1. Maternal Care F0

#### 3.1.1. Individual maternal behaviors

Exposure to different early-life rearing conditions affected several aspects of maternal behavior. Maternal behavior over different postnatal days is depicted in the left panels of Figs. 2a–f and S1. Nesting condition affected arched-back nursing (ABN) ($F(2, 35) = 8.17$, $p = .001$, $\eta^2 = 0.32$, Fig. 2a). Post-hoc analysis showed that neither CN nor LN dams were different from standard housed animals, but dams in these two conditions differed from each other, with increased levels of ABN in the LN condition at the start of the week. (PND*condition interaction ($F(8.1, 141.7) = 2.95$, $p = .004$, $\eta^2 = 0.09$). A similar interaction for passive nursing (PND*condition: $F(8.8, 154.7) = 3.00$, $p = .003$, $\eta^2 = 0.14$, Fig. 2b) revealed that CN dams increased passive nursing behavior also specifically during the first part of the rearing period. Accordingly, the total time spent on nursing behavior, i.e. the sum of ABN and passive nursing, was unaffected by condition ($F(2, 35) = 0.42$, $p = .66$, $\eta^2 = 0.02$, Fig. 2c).

Condition did not alter licking/grooming behavior towards pups ($F(2, 35) = 1.32$, $p = .28$, $\eta^2 = 0.07$, Fig. 2d), but did affect the time spent on the nest ($F(2, 35) = 7.95$, $p = .001$, $\eta^2 = 0.31$, Fig. 2e). Post-

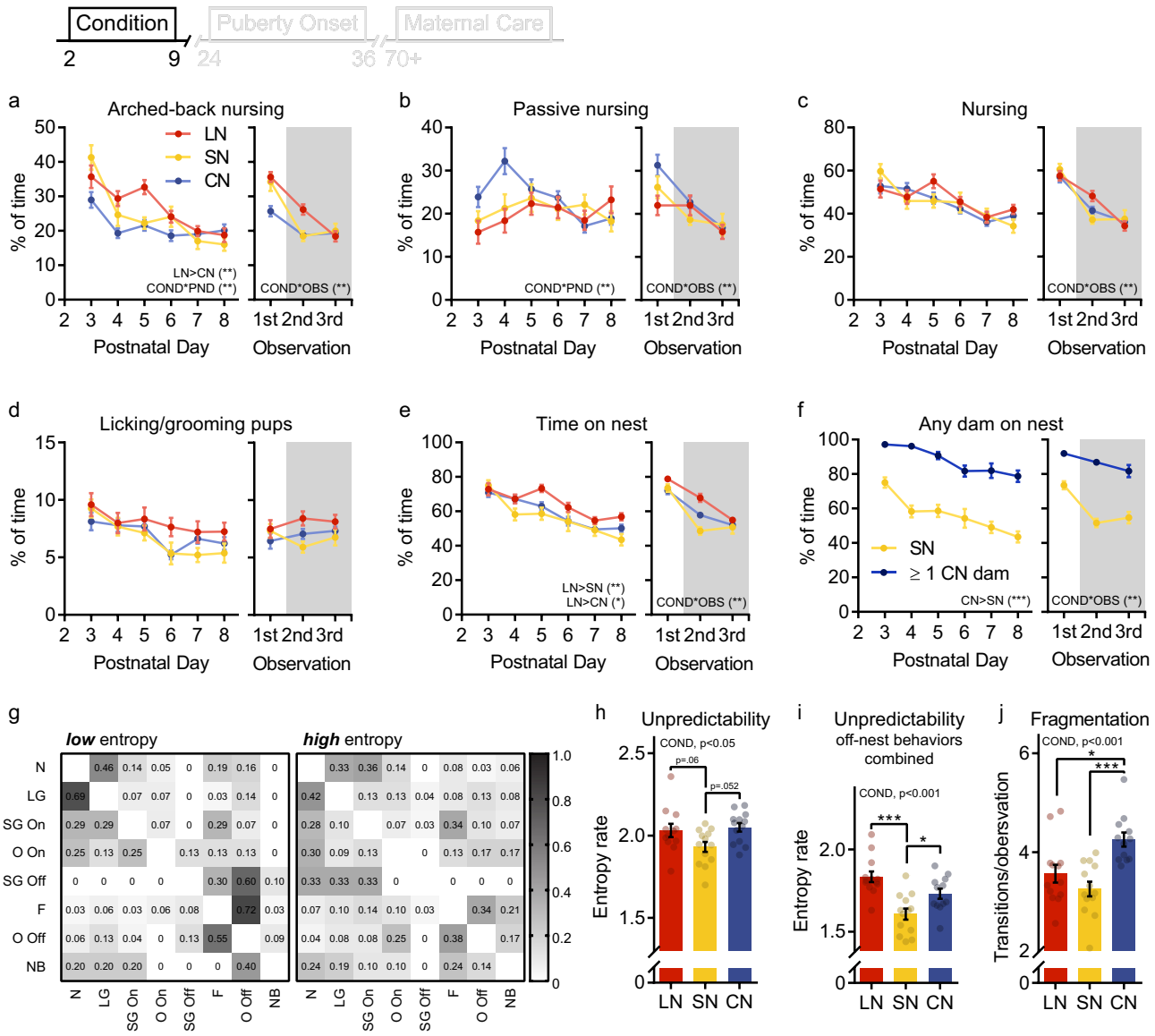


**Fig. 2.** Effect of different housing conditions on maternal care.
(a) Arched-back nursing, (b) passive nursing, (c) nursing, (d) LG and (e,f) time on nest for limited nesting (red, $n$ = 13), standard nesting (yellow, n = 13) and communal nesting (green, $n$ = 12) dams, depicted over postnatal days (left) and time of the day (right). The shaded area indicates the dark phase of the LD cycle. Data in f represents the time on nest by at least one dam from the litters perspective. (g) Example probability matrices of low (left) and high (right) entropy dams. N = nursing, LG = licking/grooming, SG On = self-grooming on nest, O On = other on nest, SG Off = self-grooming off nest, F = feeding, O Off = other off nest, NB = nest building. (h) Unpredictability of maternal care as calculated from the matrices in (g) and (i) unpredictability of maternal care when all off-nest behaviors were combined. (j) Fragmentation (on/off nest transitions) of maternal behavior. Each dot represents one dam and the average of two dams in the CN condition. Asterisks indicate interactions or post-hoc comparisons. *$p < .05$, **$p < .01$, ***$p < .001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

hoc testing revealed that LN dams spent more time on the nest, partly because they performed more self-grooming on-nest ($F(2, 35) = 27.32$, $p < .001$, $\eta^2 = 0.61$, Fig. S1b) than off-nest ($F(2, 35) = 20.18$, $p < .001$, $\eta^2 = 0.54$, Fig. S1c). However, regarding the percentage of time that the nest site had a dam present, the CN condition resulted in higher occupancy compared to the SN condition ($F(1, 23) = 135.96$, $p < .001$, $\eta^2 = 0.86$), reaching nearly permanent levels at postnatal day 3 and 4 (Fig. 2f).

### 3.1.2. Circadian rhythmicity

Differences in circadian rhythmicity of maternal care (Figs. 2a–f and S1, right panels, collapsed over days) were found for the following behaviors: ABN ($F(3.5, 61.4) = 4.65$, $p = .004$, $\eta^2 = 0.10$), passive nursing ($F(3.9, 68.5) = 3.76$, $p = .008$, $\eta^2 = 0.10$), total nursing behavior ($F(3.7, 64.0) = 3.06$, $p = .026$, $\eta^2 = 0.05$) time on nest ($F(3.6, 2.6) = 4.13$, $p = .007$, $\eta^2 = 0.06$), any dam on nest ($F(1.4, 63.5) = 22.16$, $p = .007$, $\eta^2 = 0.13$) and feeding ($F(3.3, 57.8) = 3.71$, $p = .014$, $\eta_p^2 = 0.09$). For all these behaviors, SN dams exhibited similar levels during both observations in the dark phase, whereas in particular LN mice showed a delay in displaying this dark-phase behavioral profile.

### 3.1.3. Unpredictability and fragmentation

The entropy rate, representing unpredictability of behavior, was significantly affected by condition ($F(2, 35) = 3.95$, $p = .028$, $\eta^2 = 0.18$, Fig. 2h), although the increase in both LN and CN animals compared to the SN condition did not reach significance after Tukey correction. When all off-nest behaviors were combined to better

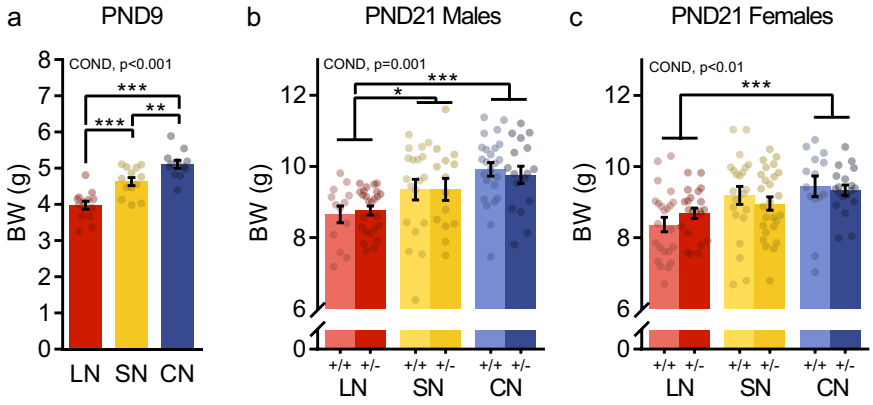


**Fig. 3.** Effects of different rearing conditions on body weight of male and female offspring.
(a) Body weight at PND 9, each dot represents the average of one litter (LN: n = 13, SN: n = 13, CN: n = 12). (b) Body weight at weaning - males. (c) Body weight at weaning - females. Each dot represents an individual. +/+: control, +/−: heterozygous MRKO. Group size: ♂: LN +/+: n = 13, LN +/−: *n* = 25, SN +/+: *n* = 19, SN +/−: *n* = 14, CN +/+: *n* = 24, CN +/−: *n* = 17; ♀: LN +/+: *n* = 23, LN +/−: *n* = 22, SN +/+: n = 22, SN +/−: *n* = 26, CN +/+: n = 14, CN +/−: n = 19). *$p < .05$, **$p < .01$, ***$p < .001$.

represent the amount of unpredictability received by the pups, the entropy rate of LN dams was higher compared to SN dams, whereas CN dams exhibited higher entropy rates compared to SN ($F(2, 35) = 12.43$, $p < .001$, $\eta^2 = 0.42$, Fig. 2i). In CN dams, an increase in transitions from and to the nest site revealed that maternal behavior of individual dams was more fragmented compared to the SN and LN condition ($F(2, 35) = 10.20$, $p < .001$, $\eta^2 = 0.37$, Fig. 2j). However, no difference between LN and SN was found.

Overall, compared to SN dams, LN resulted in more time spent on the nest not engaging in pup directed behaviors, altered circadian rhythmicity and increased unpredictability of maternal care. CN dams exhibited increased unpredictability and fragmentation of maternal care, but the presence of two dams resulted in very high nest occupancy.

### 3.2. Body weight F1

Before being exposed to different rearing conditions at P2, litter weight was comparable over experimental groups ($F(2, 35) = 1.92$, $p = .16$, $\eta^2 = 0.10$). At P9, body weight was affected by condition ($F(2, 35) = 25.76$, $p < .001$, $\eta^2 = 0.60$, Fig. 3a). LN litters weighed less than SN litters, whereas CN animals showed an increased body weight compared to both LN and SN (Fig. 3a). The overall effect of condition remained significant at weaning, both in males ($F(2, 35) = 9.25$, $p = .001$, Fig. 3b) and females ($F(2, 36) = 5.78$, $p = .007$, Fig. 3c), although post-hoc testing revealed that some differences between groups (♂: SN vs. CN, ♀: SN vs. LN and SN vs. CN) were no longer significant. The $MR^{+/-}$ genotype did not interact with condition in the prediction of body weight at weaning (♂: $F(2, 35) = 0.13$, $p = .88$; ♀: $F(2, 36) = 0.78$, $p = .47$) and no main effect of genotype was found (♂: $F(1, 36) = 0.004$, $p = .95$; ♀: $F(1, 37) = 0.010$, $p = .92$).

### 3.3. Puberty onset F1

#### 3.3.1. Males

Preputial separation was affected by condition ($F(2, 35) = 5.94$, $p = .006$, Fig. 4a). Compared to SN and CN animals, puberty onset was delayed in LN mice. There was no effect of $MR^{+/-}$ ($F(1, 36) = 0.07$, $p = .79$) and no condition*genotype interaction was found ($F(2, 35) = 0.63$, $p = .54$). Body weight at weaning was negatively correlated with puberty onset ($r = -0.56$, $p < .001$, Fig. 4b), whereas received entropy levels during the first week of life positively correlated with pubertal timing ($r = 0.29$, $p = .002$, Fig. 4c). Entropy did not predict body weight of offspring at weaning ($r = 0.11$, $p = .25$), indicating independence of these two factors. Mediation modelling with condition as the independent variable (Fig. 4d) revealed that the effects of condition on puberty onset were mediated by body weight at weaning for both the LN vs. SN contrast (95%CI = [0.09, 0.72]) and CN vs. SN contrast (95%CI = [−0.68, −0.01]). In other words, the delay in puberty onset found in LN animals was mediated through a reduction in BW gain, and the acceleration in CN mice was mediated through increased body weight at weaning. In addition, both contrasts also showed a significant mediation by entropy rates received during the early-life environment (LN vs. SN: 95%CI = [0.25, 0.82]; CN vs. SN: 95%CI = [0.29, 0.80]). Here, both LN and CN reared mice experienced elevated entropy levels compared to SN animals, contributing to a relative delay in puberty onset counteracting the effects of increased body weight in CN reared animals.

#### 3.3.2. Females

Rearing conditions had no significant effect on the timing of vaginal opening in females ($F(2, 36) = 1.10$, $p = .34$, Fig. 4e). Similar to males, no genotype effect ($F(1, 37) = 0.90$, $p = .35$) or condition*genotype interaction was observed ($F(2, 36) = 1.09$, $p = .35$). Yet, similar correlations between BW at weaning and puberty onset ($r = -0.43$, $p < .001$, Fig. 4f) and received entropy and pubertal onset ($r = 0.35$, $p < .001$, Fig. 4g) were found. As in males, entropy scores and body weight were unrelated ($r = -0.06$, $p = .52$). Although a direct effect of rearing condition on puberty onset was absent, the mediation model revealed a significant indirect effect of rearing condition via entropy rate on vaginal opening (LN vs. SN: 95%CI = [0.14, 0.72]; CN vs. SN: 95%CI = [0.18, 0.96]), whereas no indirect effect of rearing condition via body weight on puberty onset was found (LN vs. SN: 95%CI = [−0.03, 0.80]; CN vs. SN: 95%CI = [−0.81, 0.06], Fig. 4h).

### 3.4. Maternal Care F1

The principal component analysis of parenting activity in (adult female) mice that were exposed to different rearing conditions early in life revealed that $MR^{+/-}$ mice had a less active parenting style compared to $MR^{+/+}$ controls ($F(1, 70) = 17.93$, $p < .001$, Fig. 5a), irrespective of rearing condition. Considering individual maternal behaviors, $MR^{+/-}$ was related to a different nursing style compared to wild-type control litter mates (Fig. 5b–c): they spent less time on arched-back nursing ($F(1, 68) = 13.47$, $p < .001$) but showed more passive nursing ($F(1, 68) = 5.35$, $p = .02$), resulting in an equal overall amount of time spent on nursing ($F(1, 68) = 1.19$, $p = .28$, Fig. S2a). Licking/grooming the pups (Fig. 5d) and time spent on the nest (Fig. S2b) were unaffected by rearing condition, genotype or the interaction between rearing condition and genotype. In the pup retrieval test at P7, latency to retrieve all pups was similar across all experimental groups ($F(2, 70) = 1.25$, $p = .29$, Fig. S2c). The unpredictability of maternal behavior, measured as entropy rate (Fig. 5e), was not affected by rearing condition ($F(2, 68) = 0.26$, $p = .77$) nor by $MR^{+/-}$ ($F(1,68) = 1.76$, $p = .19$). A main effect of genotype on fragmentation of maternal care was found ($F(1, 68) = 4.44$, $p = .039$, Fig. 5f), with more fragmentation in $MR^{+/-}$ dams. No interaction between genotype and rearing conditions was observed for any of the maternal behaviors.

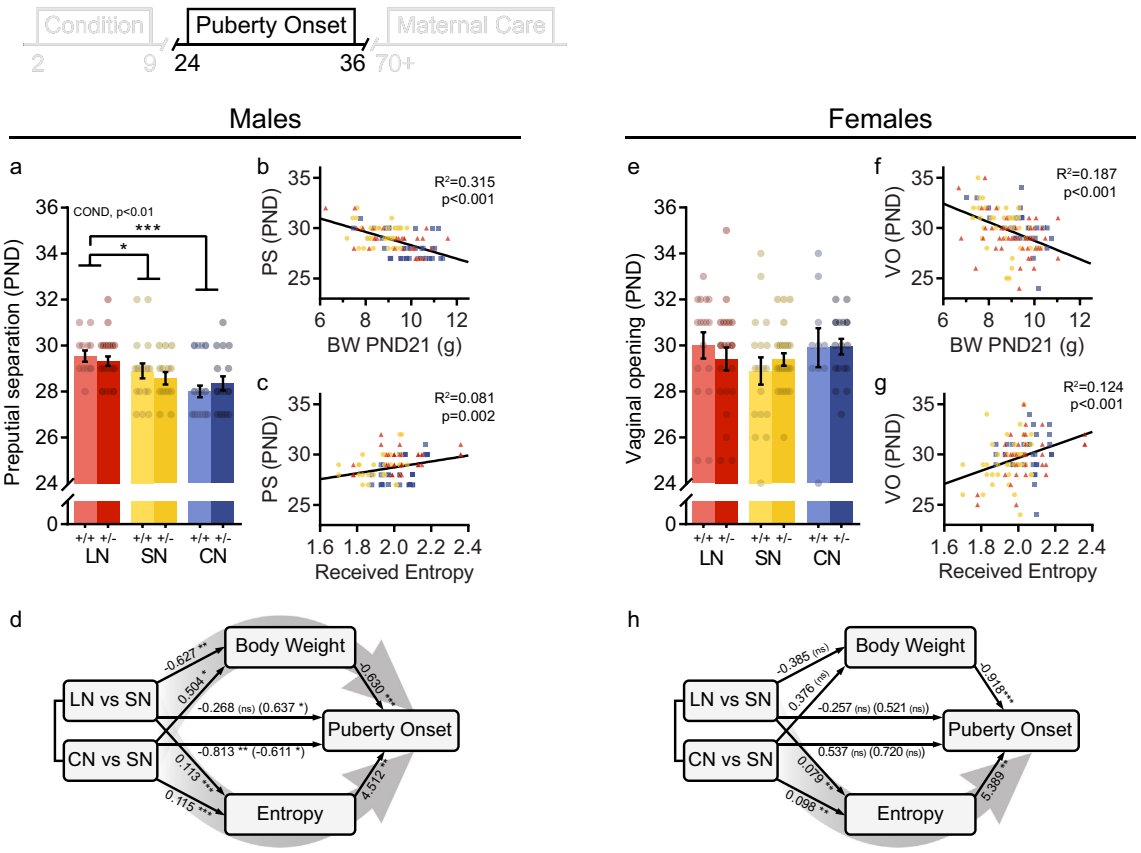


**Fig. 4.** Effects of different rearing conditions on puberty onset of male and female offspring.
(a,e) Puberty onset in male (preputial separation, left) and female (vaginal opening, right) mice. (b,f) BW at weaning and (c,g) received entropy rates during rearing correlated with puberty onset in both males and females. (d,h) Graphical representation of mediation models. +/+: control, +/−: heterozygous MRKO. Numbers represent estimated model coefficients. Grey arrows indicate a significant mediation pathway in males and a significant indirect effect in females. *$p < .05$, ***$p < .001$.

### 3.5. Corticosterone F1

We observed a significant genotype*condition interaction effect on adult basal corticosterone levels (in females) ($F(2,39) = 4.49$, $p = .018$, $\eta_p^2 = 0.18$), in the absence of a main effect of condition ($F(2,39) = 1.07$, $p = .352$, $\eta^2 = 0.04$) or genotype ($F(1,39) = 0.55$, $p = .464$, $\eta_p^2 = 0.01$). In particular $MR^{+/-}$ mice reared in a LN condition exhibited elevated plasma corticosterone levels, whereas all other groups showed similar concentrations (Fig. 6a). In addition, plasma corticosterone levels positively correlated with entropy rates of maternal care displayed by these F1 dams ($r = 0.33$, $p = .028$, Fig. 6b).

## 4. Discussion

The aim of this study was to establish an animal model that allowed detailed and controlled experiments on differential susceptibility, with emphasis on puberty onset and next generation maternal care as outcome measures. The applied paradigms, experimental manipulations of the rearing condition with limited bedding/nesting and communal nesting, both evoked alterations in several aspects of maternal care of F0 dams. Moreover, F1 mice reared in these conditions showed differences in body weight gain, where LN showed a decrease and CN an increase compared to SN animals. These effects, together with the rate of unpredictability in maternal care experienced during early development, mediated the delayed puberty onset found in LN males. However, rearing condition did not alter timing of puberty in females. In adult female offspring, heterozygous knock out of the mineralocorticoid receptor resulted in a less active parenting style and increased fragmentation of maternal behavior. Rearing conditions did not interact with genotype in the prediction of maternal behavior. However, a gene-by-environment interaction was found for basal corticosterone levels in adult females, where specifically $MR^{+/-}$ mice that had experienced early-life stress showed increased concentrations, although the typical cross-over differential susceptibility characteristics could not be observed. Finally, neither $MR^{+/+}$ nor $MR^{+/-}$ mice reared in a communal nesting environment differed significantly from SN reared animals in timing of puberty onset, maternal care or corticosterone levels.

### 4.1. Our models for impoverished or enriched environments

Similar to previous studies, the LN condition was related to decreased offspring body weight (Rice et al., 2008; Walker et al., 2017) and increased unpredictability of maternal behavior (Molet et al., 2016), although our results indicate a more pronounced effect for onnest unpredictability specifically. While the main focus of these earlier studies had been on the unpredictability and fragmentation of maternal care in the LN model, our results indicate that absolute levels of specific maternal behaviors were also affected. This discrepancy could be explained by the differences in timing of maternal observations. To our knowledge, this is the first study that observed effects of LN halfway through the dark phase, whereas other studies focused predominantly on the light phase or later part of the dark phase. LN dams deviated from SN mice in particular during the first dark phase observation, without affecting light phase and late dark phase behavior. Hence, it is possible that LN evokes alterations in maternal care specific to certain parts of the circadian rhythm. Because it has been shown that manipulation of circadian rhythmicity of dams affects pup development

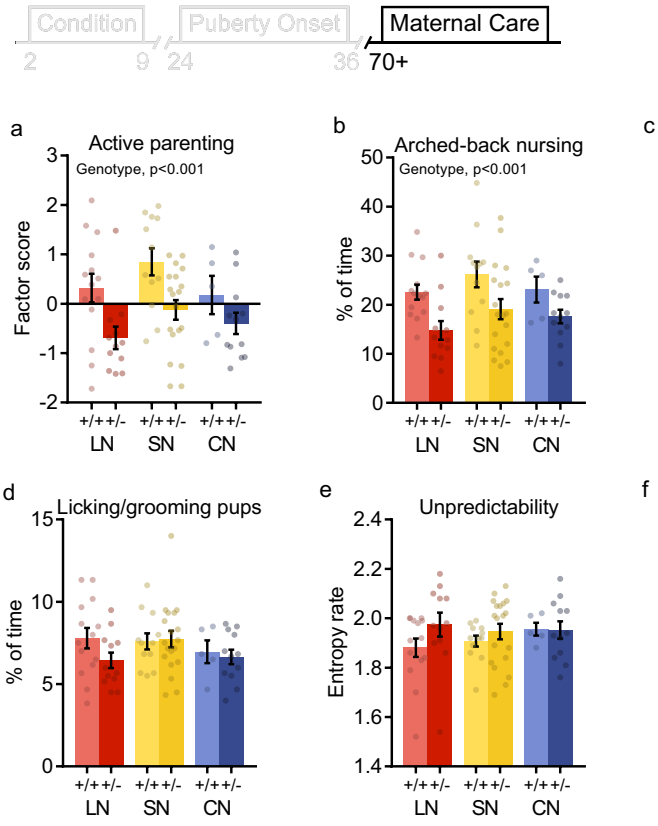


**Fig. 5.** Effects of different rearing conditions on maternal care in female offspring.
(a) Factor score for parenting quality as calculated from a principal component analysis (see Methods section for details). Overall (b) Arched-back nursing, (c) passive nursing and (d) licking grooming levels. (e) Unpredictability and (f) fragmentation (on/off nest transitions) of maternal behavior. +/+: control, +/−: heterozygous MRKO. Group size: LN +/+: n = 14, LN +/−: n = 12, SN +/+: n = 12, SN +/−: n = 19, CN +/+: $n = 5$, CN +/−: n = 12). Statistics indicate main effects.

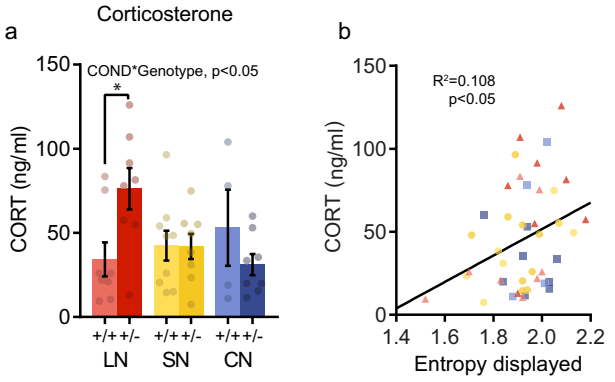


**Fig. 6.** Effects of different rearing conditions on corticosterone levels in female offspring.
(a) Basal corticosterone levels. (b) Unpredictability of maternal care positively correlated with basal corticosterone levels. +/+: control, +/−: heterozygous MRKO. Group size: LN +/+: $n = 8$, LN +/−: n = 8, SN +/+: n = 8, SN +/−: n = 8, CN +/+: $n = 4$, CN +/−: n = 8). $^{**}p < .01$.

(Hoshino et al., 2006), aberrant circadian rhythmicity in behavior of LN dams may add to the range of alterations through which the limited bedding/nesting model exerts its effects. This would require more in-depth investigation, monitoring behavior over the entire 24 h period of the day during the first postnatal week.

Interestingly, like in the LN model, we found an increase in on-nest entropy rates and fragmentation of dams in the CN condition, measures that have not been studied in communal nesting dams before. Although entropy rate could be indicative of poor maternal care in a single dam setting (Molet et al., 2016) it can be expected that in a setting with multiple nest-sharing dams the behavior of each dam is influenced by the other. This may lead to more on/off nest transitions to regulate temperature (hence, more fragmented care) and interrupted behavior (more unpredictability) on the level of the dam. However, maternal care received by the pups is not determined by unpredictability and fragmentation of the individual mothers, but rather by the overall pattern of two dams combined. Therefore, it remains to be elucidated whether the increased unpredictability of individual dams during communal nesting encodes a negative rearing environment similar to the single dam setting.

Apart from an increase in bodyweight at P9, which was normalized at weaning, we did not find differences between the communal and standard reared animals. This lack of effect of early life enrichment on the social read-outs might be partly related to the specific protocol applied. In order to synchronize the duration of communal nesting to the limited nesting/bedding model (Rice et al., 2008; Walker et al., 2017), animals were exposed to the communal nesting condition from P2-P9. Moreover, to facilitate individual characterization of maternal care in each dam, two -rather than three- dams were used. Studies with the communal nesting paradigm so far predominantly used three dams and litters in a cage, from birth till weaning (Branchi et al., 2006; Branchi and Cirulli, 2014; Curley et al., 2009). By limiting exposure to P2-P9 in our model we restricted the effects of communal nesting to maternal care alterations while peer interactions, an important component of the paradigm, may not yet have developed. In addition, neural development of specific brain regions occurs at different periods in time (Rice and Barone Jr, 2000). The development of brain networks relevant for sexual maturation and maternal care may have been unaffected by our communal nesting model. Therefore, matching the exposure time window of communal nesting to the limited nesting model may have resulted in an enrichment condition too subtle to elicit positive effects.

### 4.2. *Timing of puberty onset as outcome*

In contrast to the acceleration hypothesis of life history, but in line with previous reports in rats (Bodensteiner et al., 2014; Cowan and

Richardson, 2018), early-life stress resulted in delayed pubertal timing in males. Somewhat surprisingly, LN did not affect puberty onset in female mice, whereas others have found an acceleration in rats (Cameron et al., 2008; Cowan and Richardson, 2018). Due to the importance of body weight and leptin in mediating puberty onset in both rodents (Ahima et al., 1997) and humans (Tomova et al., 2015), it has been suggested that female resilience and male susceptibility to the effects of maternal separation on body weight may explain the sex-specific effects found in rats (Cowan and Richardson, 2018). Our mediation analysis confirms an important role of body weight on puberty onset in males, while extending the model by also taking the unpredictability of received maternal care into account. The data reported here support a similar correlation between body weight at weaning and puberty onset for both male and female mice, whereas others have found this effect in male rats only (Cowan and Richardson, 2018). This suggests a species difference in the importance of body weight in regulating puberty onset and highlights the challenges of studying puberty onset in early-life rodent models that inevitably affect body weight gain during early development. Therefore, these studies should always include body weight effects in the interpretation of results. Nevertheless, the finding that higher levels of unpredictability in maternal care experienced during early development were linked to delayed puberty onset contradicts the acceleration hypothesis of life history, at least in mice and with the presently used models.

### 4.3. The MR gene in maternal care

The less active parenting style and increased fragmentation of maternal behavior in $MR^{+/-}$ F1 dams suggests a broader role of MR in regulating complex patterns of social behavior. Although MR has been studied predominantly in relation to learning and memory (Arp et al., 2014; Berger et al., 2006; Kanatsou et al., 2017), its role in regulating emotion and social behavior is now increasingly supported (Joëls and De Kloet, 2017; Kruk et al., 2013; Ter Horst et al., 2014; Vogel et al., 2016). In humans, an MR SNP in children was found to moderate the effect of sensitive and insensitive parenting on attachment security (Luijk et al., 2011). Because parenthood elicits a wide variety of challenges (Belsky, 1986) and continuous adaptation to novel situations, the stress coping characteristics of MR may be involved in regulating parental behavior as well. Although the results presented here suggest a role of MR in regulating maternal care in mice, involvement of MR in regulating the parental aspect of human parent-offspring interactions remains elusive.

### 4.4. Gene-environment interaction

Genetic variation in the mineralocorticoid gene has been shown to interact with early environmental factors in disorders such as depression (Vogel et al., 2014; Vrijsen et al., 2015) and addiction (Rovaris et al., 2015). Although we did not find a gene-early environment interaction effect on behavioral measures such as maternal care, the increased basal corticosterone levels in $MR^{+/-}$ mice that experienced early-life adversity do support interaction effects. According to the three-hit concept of vulnerability (Daskalakis et al., 2013), a genetic predisposition interacts with the early-life environment to program an individual into an adaptive phenotype. However, a third hit later in life is needed to evoke a maladaptive response. In our study, this suggests that early-life adversity programs particularly $MR^{+/-}$ mice to develop increased basal corticosterone levels, but a strong third hit –lacking in our experiment- would be required to reveal the behavioral consequences.

### 4.5. Differential susceptibility

We aimed to study differential susceptibility in a translationally relevant way, by manipulating the living conditions of dams and pups with a background of heterozygous, rather than complete, MR knock out. Previous studies taking a similar approach have highlighted the validity of animal studies in this field (Davis et al., 2017). Moreover, recent animal work on differential susceptibility in socially monogamous prairie voles also indicates that differential susceptibility is not restricted to humans (Hartman et al., 2018; Hartman and Belsky, 2018). Although we did not find evidence to support the differential susceptibility theory with respect to the mineralocorticoid receptor and its role as susceptibility factor in the association between environmental factors and sexual maturation and maternal behavior, other candidate genes and behavioral domains remain to be investigated in controlled experimental settings. Promising candidates for future studies might be related to the dopamine-system or make use of polygenic susceptibility scores (Belsky and Van IJzendoorn, 2017; Keers et al., 2016). This would expand further on the insights presented here and enhance our understanding of the neurobiological processes related to the differential susceptibility hypothesis.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yhbeh.2019.04.001.

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