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Litter size is negatively correlated with corticosterone levels in weanling and juvenile laboratory rats

Heiko G. Rödel [a,*], Susann Meyer [a], Geraldine Prager [a], Volker Stefanski [b], Robyn Hudson [c,*]

[a] Department of Animal Physiology, University of Bayreuth, Bayreuth, Germany
[b] Animal Husbandry and Animal Breeding, University of Hohenheim, Stuttgart, Germany
[c] Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Mexico

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ABSTRACT

An animal's environment during early life can strongly affect its physiological development. For example, litter size, i.e. the number of litter siblings, has been previously shown to strongly affect early growth in many small mammal species including laboratory rats. In the present study we tested whether natural, unmanipulated litter size is also associated with differences in stress hormone levels in young Long–Evans laboratory rats. We found a negative correlation between serum corticosterone (CORT) concentrations and litter size during two different stages of juvenile life. On postnatal day 17, shortly before weaning, this relationship was apparent with respect to basal CORT values. On day 33, however, two weeks after weaning, we found this relationship only when animals were challenged by a 10-min test on an elevated plus maze, but not in control animals (basal values). Although the physiological basis of these differences is not clear, we discuss two main, not mutually exclusive possibilities: (a) delayed maturation of the HPA axis in typically lower body mass pups of large litters, and (b) that such pups, encountering greater competition for maternal resources, adjust to this presumably more stressful developmental environment by down-regulating responsiveness of the HPA axis. In conclusion, our study provides evidence that a naturally varying feature of the developmental environment of many altricial mammals — number of littermates — may contribute to individual differences in stress-related physiology. Furthermore, it suggests the need to consider litter-size effects when investigating differences in animals' stress responses.

1. Introduction

Interest has grown sharply in recent years among evolutionary biologists in individual differences in physiology and behavior. Once generally regarded as irrelevant "noise," such variation is now increasingly seen as important for life history theory and for understanding the evolution of alternative strategies and their continued maintenance within populations [37,41,55,63,70]. Of undoubted importance in accounting for such differences is the role of the early developmental environment, for example, the number of brood- or littermates with which an individual has to compete for often limited parental resources [10,33,49]. Thus, in a wide range of litter-bearing mammals, litter size is negatively correlated with milk intake and growth as measured by body mass at weaning and beyond (rodents: [8,16,20,38,47,50,56]; lagomorphs: [7,18,29,56–58]; domestic pigs *Sus scrofa*: [17] carnivores: [31,34]). Such differences can persist even into adult life and can have important consequences for an individual's reproductive success, longevity or other fitness-correlated traits [42,48,59,60,65]. Furthermore, studies in adult laboratory rats (*Rattus norvegicus*) report a relation between litter size and physiological and behavioral measures of emotionality [15,27,64].

A particularly clear demonstration of the long-term effects of early experience on individuals' physiology comes from studies of the development of the hypothalamic–pituitary–adrenal (HPA) axis and the release of the stress-associated hormone corticosterone (CORT) in rats. Animals born to mothers subjected to a stressor such as forced bouts of restraint during pregnancy, or subjected to periods of extended separation from their mother after birth, show enhanced activation of the HPA axis even as adults ([12,40,66]; but see [25]), while rats subjected to regular handling as neonates causing a mild, acute stimulation of the stress system show a long-term reduction in CORT secretion in response to stressful situations as adults [2,23,36,43,45,46,66].

Given this evidence for the effects of early experience on animals' stress physiology, we asked whether litter size, a naturally varying feature of the early developmental environment with clear effects on growth, could also affect the development of the HPA axis in young laboratory rats [43]. Young from large litters, which are usually born

* Corresponding authors. Rödel is to be contacted at the Department of Animal Physiology, University of Bayreuth, Bayreuth, Germany. Hudson, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Mexico.
E-mail addresses: heiko.roedel@uni-bayreuth.de (H.G. Rödel), rhudson@biomedicas.unam.mx (R. Hudson).

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with a lower body mass and show lower pre-weaning growth than young from small litters (reviewed above), might be subjected to more stress by having to compete for maternal resources such as milk, warmth during brooding, or anogenital licking, which in young pups is vital for voiding [9]. Accordingly, these animals might be expected to show increased responsiveness of the HPA axis.

On the other hand, young from large litters might also experience generally slower development or maturation of various physiological functions [20,42,56]. In particular, the development of the adrenocortical stress response, which is known to mature during the first postnatal weeks [28,30,61] might be a candidate for such purported litter-size effects. Consequently, one might also expect a lower functionality of the HPA axis in smaller and less developed young from larger litters.

To test these two contrasting predictions, we measured one of the most commonly used indicators of HPA-axis activity, serum CORT concentrations under three conditions: (1) in animals shortly before weaning (postnatal day 17) when they were still completely dependent on the mother's milk (rats are weaned at around postnatal day 21 [62]) and thus subjected to presumed litter-size-dependent competition, and (2) in animals which were kept after weaning in sibling groups of equal size and so with little opportunity to be differentially stressed. We took these measures about two weeks after weaning (postnatal day 33) as the increased mobility of the young allowed us (3) to introduce an additional group of animals, which were challenged with a mild and commonly used stressor, testing on an elevated plus maze [46,53,64,66]. We chose to test animals shortly before and soon after weaning, since this represents a developmental period that is likely to be particularly stressful under natural conditions as the young make the transition to independent life.

2. Methods

2.1. Animals and housing conditions

For breeding we used a total of 75 adult virgin male–female pairs of Long–Evans rats, descendants of an out-bred stock originally obtained from Harlan Winkelmann (Borchen, Germany), which started to reproduce at age 80 to 120 days. We kept the animals on a 12:12-h light/dark cycle (lights off at 01:00 h) in standard polycarbonate cages (26×42×15 cm, Macro 2808 type III, Ehret, Germany) containing wood shavings as bedding, and with free access to rat standard diet (Altromin 1324, Altromin, Germany) and water. Temperature was maintained at 21±0.5 °C, and relative humidity at approximately 50%. Male–female pairs were transferred to clean cages once a week but were otherwise not disturbed. Nevertheless, we checked the cages each day towards the end of the light phase, the period when rats most commonly give birth [26,32], for the presence of litters, and considered this postnatal day 1.

We obtained a total of 936 young from 125 unculled litters (some of the litters included in our study stemmed from the same parents, see below). Litters in which more than one pup died were excluded in order to minimize potential biases caused by postnatal litter-size reduction [18]. Of these 125 litters, 76 randomly selected litters from 49 different male–female pairs contributed to the pre-weaning CORT measures on postnatal day 17. We weighed all pups ($n=744$) on day 1 to the nearest 0.1 g, and again on day 17, with the exception of 16 pups (from 16 litters), which had died within 5 days of birth. We also determined their sex by external genital inspection at this time. Before day 17 the pups were unable to reach the overhead food trough, although they may have had access to crumbs dropped by the adults. Since we only used CORT values from maximally five randomly chosen animals per litter (see 2.3), our final sample size was 362 young from the 76 litters.

Forty-nine litters ($n=192$ young) from 42 different pairs contributed to post-weaning CORT measures on postnatal day 33 (i.e. before laboratory rats reach puberty at about 50±10 days of age [62]). We left these animals with their parents until day 20, when they were weighed and transferred to separate cages in randomly formed, mixed-sex sibling groups of four pups. We weighed pups on day 20 in order to obtain a final record of their growth during the pre-weaning, full litter phase. On day 33 they were weighed again. Procedures conformed to the ethical and animal care guidelines of Germany (where the project was carried out), and were approved by the local authority for laboratory animal care and use (government of Middle Franconia, Germany).

2.2. Behavioral testing

We removed the 33-day-old (experimental) animals individually from their home cage and, after weighing, placed them for 10 min on an elevated plus maze. Control animals were not tested. The maze was made of PVC for easy cleaning with isopropyl alcohol and water between trials. It consisted of four arms (50×10 cm, 80 cm above the floor) arranged in a cross (90° angles) with two opposite arms enclosed by 40-cm high walls, and two without walls, and all joined by a 10×10 cm central platform [46,53,64,66]. We tested in the dark phase (between 08:00 and 10:00 h) under dim red light by placing each subject on the central platform facing an open arm. We recorded the animals' behavior on video and scored the entries, defined as the animal with all four paws on one of the arms, and calculated the number of entries to open arms as absolute number and as the percentage of entries to all arms, and the time spent on open arms, closed arms or in the central zone as a percentage of the total time of the experiment.

2.3. Blood sampling

We took blood by decapitation during the dark phase between 08:00 and 10:00 h, immediately after the animals had been removed from the home cage or had been challenged with the plus-maze test. It was collected in uncoated tubes, immediately centrifuged twice and the serum frozen at −20 °C until measurement of CORT concentrations. Sampling of the four (on day 33) or five (on day 17) young per litter used for statistical analyses (see below) was always accomplished within 3 min after the home cage had been taken out of the housing room or after the animal had been taken from the maze, since the increase in glucocorticoid levels in response to a stressor usually takes place only after some minutes [67].

2.4. Serum corticosterone measurement

We measured CORT concentrations in duplicate samples by radioimmunoassay [21]. The specific antibody was kindly provided by the Institute of Pharmacology, University of Heidelberg, Germany. Inter- and intra-assay coefficients of variation were 4.0% and 5.3%, respectively, and the lower limit of detection was 5 ng/ml. Cross reactivity of the CORT antibody with other relevant steroids was 30% (desoxycorticosterone), 5.5% (testosterone), 4.4% (cortisol), and 3.2% (androstendione).

2.5. Data analysis

For our study of 17-day-old rats we obtained data from a total of 744 young from 76 unculled litters from 49 different pairs (min litter size: 2; max litter size: 18). One pup died in each of 16 litters, in all cases within 5 days of birth. Since we only used CORT values from maximally five animals per litter (see 2.3), our final sample size was 362 young from the 76 litters.

For our study of 33-day-old rats we obtained data from 192 young from 49 litters from 42 different breeding pairs (min litter size: 4, max litter size: 16; note that we did not use litters with less than 4 pups because we kept pups in groups with 4 after weaning). Of these, 113 (from 29 litters) were challenged prior to blood sampling by placing them on the elevated plus maze (experimental group), and another