Swedish University of Agricultural Sciences
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Summary

Maternal behaviour is crucial for the development of the rat pups' behaviour and stress responses later in life. There are numerous studies evaluating the effects of the dams' behaviours on the pups, but not many addressing what can be made for wellbeing of the rat dams when breeding. Few studies have addressed the question of how the commonly used rearing environment effects the dams' behaviour and in the long run how it effect the rats reared to become our research models. In this study, rats were therefore reared in two different cage systems; one cage type with elevated top and enriched by a shelf and climbable netting (RT) was compared to the type of cage most commonly used today, the makrolon type IV cage (M IV). Dams were equipped with telemetrical devices to measure activity, blood pressure and heart rate, and direct observations of their maternal behaviour were made. Urine corticosterone/creatinine quote was measured and behavioural tests (defensive withdrawal test, elevated plus maze test and open field with novel object) were performed in both dams and pups. The dams in the RT cages showed more activity and a transient increase in blood pressure, and a tendency towards less urine corticosterone levels. They also spent less time licking/grooming and arched back nursing their young. The pups showed differences in urine corticosterone levels as well as in the behavioural tests, where the RT pups had significantly less corticosterone excretion, and showed less fearful and anxiety-like behaviours in the behavioural tests. There were greater differences between the groups of female pups than between the male ones, pointing to the female pups being more sensitive to improvements of the environment. We have shown that the rat dams' activity increases and stress decreases in the RT enriched cage, and that the rat pups from the RT cages show less stress response, less fearful and less anxiety-like behaviours even though the dams spend less time licking/grooming and arched back nursing them. These findings support the argument that an enriched optimised environment and voluntary maternal separation give mentally healthier rats, possibly better as a model for mentally healthy humans.

Sammanfattning

Laboratorieråttans modersbeteende är avgörande för utvecklingen av råttungarnas beteende och deras respons för stress senare i livet. Det finns många studier som utvärderar hur moderns beteende får effekter på ungarnas beteende, men inte många riktar sig mot vad som kan göras för råttmödrarnas välbefinnande vid uppfödningen. Få studier har tagit upp frågan om hur den nu använda uppfödningsmiljön påverkar det moderliga beteendet, och i förlängningen vilken effekt det har på råttorna som föds upp för att bli forskningsmodell. I den här studien föddes därför råttor upp i två olika bursystem; en bur med förhöjd topp och berikad med hylla samt klättringsbart nät (RT) jämfördes med den mest vanligen använda buren idag, makrolon typ IV (M IV). Råtthonorna blev försedda med telemetriska sändare för att mäta aktivitet, blodtryck och hjärtfrekvens, man gjorde även direktobservationer av deras modersbeteenden. Urin-cortcosterone/creatinine-kvot mättes och man genomförde beteendetester (defensive withdrawal test, elevated plus maze test och open field with novel object) på både honor och ungar. Honorna i RT burarna uppvisade högre aktivitetsnivå och en övergående höjning i blodtryck, samt visade en tendens till lägre urincorticosterone-nivåer. De ägnade också mindre tid åt mer aktiva modersbeteenden (slicka/putsa ungarna och digivning med krökt rygg - s.k. arched back nursing). För ungarna sågs skillnader i urincorticosteronenivåer såväl som i beteendetesterna, där RT-ungarna hade signifikant lägre corticosteroneexkretion och visade färre rädslo- och ångestlika beteenden i beteendetesterna. Det var större skillnader mellan grupperna av honungar än mellan hanungar, vilket tyder på att honungarna är mer mottagliga för förbättringar av miljön. Vi har visat att honornas aktivitet ökar och deras stress minskar i RT-berikad bur, och att råttungarna från RT-burar visar mindre stressrespons, färre rädslo- och ångestliknande beteende även fast mödrarna ägnar sina ungar mindre tid åt slickande/putsande och digivning med krökt rygg. Dessa fynd stödjer argumentet att en berikad optimerad miljö och frivillig maternell separation ger mentalt friskare råttor, möjligen bättre som modell för mentalt friska människor.

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Background

When using laboratory rats as research models for humans, we must consider two important issues: Firstly, is the laboratory rat of today a reliable model, i.e. can we trust our scientific result as compatible to the human species? Secondly, are the animal needs considered when using animals at our service? Voices for animals' rights have been raised, resulting in more detailed legislation, including for the use of laboratory animals. In the European Union, the parliament and council have agreed on a new directive on the protection of animals used for research (2010/63/EU).

During decades, numerous studies have been made showing the importance of maternal behaviour for the development of the young in rats as well as in humans. The rat mother has proved to be a good model for the study of different factors that affect the maternal behaviours, such as hormonal, sensory, neural, experiential and developmental issues. (Whishaw and Kolb, 2005).

Breeding of laboratory rats commonly takes place in standard Makrolon® type IV cages (M IV). The size of this type of cage is 59 x 38 cm and 20 cm in height, which is a comparatively small cage size for grown rats, since it, for instance does not even allow the rats to fully stand on their hind legs, a very common behaviour in rats (rearing). People within the research community have been questioning the use of these rats as realistic models for healthy humans. The normal rat model in use for most studies consists of male rats from the same strain living together two or three in one M IV cage. In most countries today rats have some enrichment in their cages, normally some kind of nesting box made out of plastic and/ or wooden chewing sticks. But they are still kept in cages that are relatively small compared to their body size, and in the new EU directive mentioned above (2010/63/EU) there are no changes of the cage size minimum. It is crucial to obtain scientific data regarding animal welfare in different kinds of environments and to show how to get the most use from each animal. This would hopefully lead to scientists taking the initiative to house the animals in better conditions.

Rats kept in large cages are more active and keep themselves lean whereas rats kept in small cages are inactive and will eventually become overweight (Spangenberg 2007). When comparing overweight versus lean Sprague Dawley rats (weight controlled through energy intake and food availability), Martin *et al* (2009) found that the difference in weight also led to differences in many other measured factors, e.g. chemical, behavioural and cognitive. Also gene transcription in tissues varied significantly, as did plasma hormone levels (leptin and growth hormones) and energy-regulatory factors (for example cholesterol and 3-hydroxybutyrate). This leads to the conclusion that rats kept in small cages become overweight and sedentary and do not represent adequate models for healthy humans, but for an overweight and sedentary lifestyle. The safety and stability of the rearing environment is important to consider, since it does prepare the animal for its future challenges as an experimental animal. (Wurbel 2010). An unstable rearing environment may lead to enhanced fearfulness and increased sensitivity to stress. Since stress is an important variable in many assessments of research this can give bias-effects on the research results (Wurbel, 2001). Furthermore, Wurbel (2001) also concludes that by better understanding the environmental needs of the animals, researchers could control animal behaviour and reach refinement of their experiments. The same article raises the interesting question that cage modifications can lead to altered maternal behaviour, such as voluntary maternal separation. This is important since the behaviour of the offspring is influenced in a non-genomic way by the parental behaviour and not only by inheritance through genetic mechanisms. Variation in the rat's maternal behaviour has been shown to cause individual differences in behaviour and endocrine stress response in the pups (Francis et al 1999). Taken together, the arguments and findings above shows the importance of further studies on the effect of the rearing environment on laboratory rats as an animal model for human research.

RAT BEHAVIOUR

In The Rat by Barnett (1st ed 1963 revised 1975), the author makes an comprehensive description of the behaviour of the brown rat, *Rattus norvegicus*, the ancestor of our laboratory rat. He states that they have been bred and inbred to suit researchers' needs. This has led to more tame rats with less urge to flee or bite the human handling them. They are also less avoidant to new objects in their home environment than wild rats and they avoid fighting within the species even when clumped together in smaller areas. In the wild they live in colonies of sometimes many hundreds of individuals in the same colony.

Wild rats have a home range of about 30 metres, and they move around in the home range even when no internal or external stimulus affects them (such as hunger, threat etc). They are curious and prefer new objects compared to known features in the environment.

MATERNAL BEHAVIOURS

The brown rat has an altruistic behaviour when it comes to taking care of the pups. There is a complexity made up by hormonal, sensory, neurological factors as well as the mother rat's previous experiences. All rats build nests of some sort, but pregnant females work very intensely on their nest building and keep adjusting the nest during the whole lactation period. When pups are born the mother rat has several standard behaviours she performs: She gives milk in certain nursing postures that allow the infants to suck milk, she retrieves the pups when they fall or crawl out of the nest, she licks and grooms them, and she will also defend the nest if necessary (Barnett 1975).

By Grota and Ader (1969), the rat's maternal behaviour is defined as "specific behaviours considered by the observer to be maternal in character", and the examples of typical behaviours are nest-building, nursing and retrieving. In their study they used a special dual compartment cage, where the lactating dams could escape their young. The cage was automatically weighed and some of the dams were also studied for behaviour. The study showed that the lactating rat dam spent the most time with the rat pups (85%) the first day post partum, and then less time each day until about 30% on days 17-21. The number of visits with the pups was the same during the whole lactation period, thus the duration of each period spent with the litter was shorter. Most time with the litter was spent on mid-day- the middle of the 12 hour light period. The study also showed that nursing behaviour and "quality of the nest" decreased over time during the lactating period.

After birth of the pups, within 30 minutes the mother rat retrieves them to a nest, licks them and starts to nurse them. Retrieval of a pup is when the rat dam carries the pup to the nest or moves it to a new nest. Pup licking consists of two types of licking: pup body licking and pup anogenital licking, which ensures urination and defecation of the pup. Rat dams nurse their young in different body postures, the acknowledged postures are: hovering- the dam stands over the rat while she does something else, for example licking pups or self-grooming. Low-crouch is when her other behaviour decreases and she extends her legs and gives the pups access to the milk. High-crouch is also known as arched back nursing, where the dam will crouch her back in a high position, she extends her legs and do not engage herself in any other activity. Supine nursing is when the rat dam is lying on her side. This posture is seen when pups are older and when nest environment is undisturbed. Another frequent behaviour typical of the rat dam during lactation is nest building. When testing a rat for maternal behaviour the scientist should also record the rat's regular behaviours when she is not attending to the pups, which gives a chance to assess the dams' general activity. These behaviours are self grooming, explorative behaviours, sniffing air, digging, eating, sitting in cage corner and sleeping away from pups. (Whishaw and Kolb, 2005)

Infancy – effects of maternal behaviours and rearing environment

Maternal effects

According to Barnett (1975) the behaviour of rats is influenced by the experiences that their mother and grandmother had as young, which he says is well known and proven in several studies. This includes several factors, such as nutrition, environmental temperature and different types of stimulation. This is nowadays a new research field of its own, called epi-genetics, stating that an individual is not only a product of its genetics and its environment, but also from the environment of the previous generations, the so called grandmother effect.

Researchers are trying to find out just how the rats' maternal behaviour affects the offspring. Different types of nursing have been studied such as the so called arched back nursing (considered to be an active type of nursing) versus the supine (said to be more passive) nursing. Others have studied effects of maternal separation on rat pups. Animal caretakers' handling of the dams and pups during cage cleaning/changes must also be considered as well as the rearing environment. Very small changes in handling can have large effects on the animals and therefore also the research results. (Wurbel 2010)

Francis *et al* (1999) found that variations in the dam's licking/grooming of the pups and arched back nursing lead to variations in hypothalamic-pituitary-adrenal (HPA) stress response and in behaviour of rat pups. As adults, the pups whose mothers showed high frequencies of licking/grooming and arched back nursing, would behave less fearful and they showed modest HPA response to stress compared to pups from mothers with low frequencies of licking/grooming and less arched back nursing. The frequency of licking/grooming and arched-back nursing are highly correlated. High licking/grooming dams also have a high rate of arched back nursing. The authors further found that rat mothers who showed high licking/grooming and arched back nursing, reared female pups who when becoming mothers themselves, performed significantly higher licking/grooming compared to females whose mothers performed low levels of licking/grooming and arched back nursing. It could be argued that this was a genetic, as well as an environmental effect, but in a cross-fostering study it was proven that pups reared by high licking/grooming and arched back nursing females were less fearful and showed higher licking/grooming and arched back nursing when having their own pups than did females raised by low licking/grooming arched back nursing mothers, regardless of their born identity (Meaney 2001). Furthermore, Champagne *et al* (2003) showed that rat mothers that perform high frequency of arched back nursing also perform higher frequency of licking/grooming the first 3-8 days of the pups' lives, but not later and that there is no difference in contact time or in time spent with pups between the mothers who perform high or low frequency of arched back nursing. In another experiment, rat mothers were stressed during pregnancy and the result showed that this altered their retrieving behaviour. As adults the pups showed a low level of exploration, i.e. they needed more time to explore their environment, which may indicate that they could have a cognitive defect (Lordi *et al* 2000).

Separation effects

Natural maternal separation periods in wild brown rats are usually 15-20 minutes long according to Barnett (1975). Another field of behavioural research is standardised maternal separations (MS). These studies have been carried out in a great range of variety regarding the lengths and number of separations and the results are somewhat inconclusive, but show that there are significant effects on the rat pups later in life. Several studies have shown that when rat pups are repeatedly separated for 3 h from their dams during the early postnatal period they show increased anxiety-like behaviours and enhanced stress response when adult (see e.g. Ladd et al. 2000). On the other hand, brief, repeated maternal separation seem to give positive effects on emotionality later in life and decrease the HPA stress response (Meaney 2001). Eklund and Arborelius (2006) separated dams from the pups twice daily for the first 13 days, comparing groups with brief maternal separation (2 x 15 min = BMS), longer separation

(2 x 3 hours = LMS) and a control group not separated (AFR). The study revealed some unexpected data: In the defensive withdrawal test and spontaneous motor activity test, they showed that in male rats there were no differences, while in the females LMS decreased anxiety-like behaviour. In the motor activity test, the females who had been subjected to BMS showed less horizontal activity compared to AFR, and the LMS females had decreased peripheral activity and increased locomotion forward compared to the corresponding BMS group. Thus, significant differences between male and female rat offspring were found where female rats react by a decrease in anxiety-like behaviour and altered motor behaviour compared to males, but it is implied that the variation might lie in the expression of the emotions between the two sexes, where female rats seems to cope with their emotions in other ways than the males. The conclusion that can be drawn from maternal separation studies is that the results vary a lot and that there are sex differences.

There are a few studies made regarding the rat mothers' responses to these involuntary separations from their pups. Eklund et al (2009) showed that dams subjected to longer separation periods (4 hours on 8 random occasions during PND 1-14; MS240) showed lower peripheral activity and habituated more slowly in the spontaneous loco motor activity test, than did dams that were separated from their pups more briefly (15 minutes on 8 random occasions during PND 1-14, MS15). The MS15 dams showed more peripheral activity and less rearing compared to both MS240 and the control group (AFR), which could suggest increased anxiety-like behaviour. On the other hand, when comparing the weight of their adrenal glands six and a half weeks after weaning, the adrenal glands from the MS15 dams weighed significantly less than the adrenals from MS240 and AFR. The adrenal glands normally increase during pregnancy and alter back to normal size gradually afterwards. The MS15 dams' adrenal glands had turned back to normal size, since they were compared to the size of adrenal glands from rat dams of the same group that had failed to become pregnant, but were still in the same animal facility.

Marmendal et al (2004) have shown that dams that were separated from their pups for 4 h spent longer time within the nest after retrieval of the pups compared to dams not separated from their pups.

Handling effects

Francis *et al* (2002) found that postnatal handling, including 15 min MS damped both HPA and behavioural responses to stress and interestingly this difference could be completely abolished by environmental enrichment. According to Chapillon *et al* (2002) handled animals show lower corticosterone response in stressed situations and return more quickly to basal levels. This can possibly be linked to the fact that handling-elicited maternal behaviour (grooming, licking) may be a key determinant in the neuroendocrine effects of infantile stimulation (Durand *et al* 1998). Mothers of handled pups (postnatal handling 3-15min/d) show shorter but more frequent nest-bouts, higher overall levels of licking-grooming and arched-back nursing (Wurbel 2001).

Environmental effects

There are different aspects of the development of the pups; growth, behavioural reflexes and orientations, the amount of movement, ability to urinate/defecate etc. The pups generally increase their exploratory behaviour on their fourteenth day when their eyes open and they spend more time playing from about day eighteen (Barnett 1975).

Chapillon *et al* (2002) state that the development of the nervous system and the behaviour of an organism depend on the interactions that take place between the organism and the environment it lives in. Barnett (1975) discusses the results of some other scientists' research on environmental stimulation effects on the infant rats' development, for example Rosenzweig (1971), Daly (1973) and Levine and Mullins (1968). One of the examples is a study of rats age 25-80 days, that were reared in two different environments; enriched or impoverished (Rosenzweig 1971). The enriched environment contained other rats and objects were changed every day, while the impoverished rats lived alone in an empty cage. The weight of the cerebral cortex of the rats in the impoverished habitat was 4% lower with fewer glial cells compared to the enriched rats' brains. The impoverished rats also had lower cholinesterase activity in the cortex. Barnett states that stimulation during the neonatal period accelerates certain features of development, and stimulated animals can be said to be "less emotional" with an altered secretion of hormones. The opposite effects can be caused by prenatal stress to the mother as well as stressing the pups after birth, poor maternal care, eating disorders, physical stress (for example extreme temperature).

On the contrary, positive effects on neurodevelopment can come from optimising the environment with enrichment. Enriched environment is proven to give animals an increase of total brain weight and total cortical weight, thicker cerebral cortex, increased neuron density in the hippocampus, more synaptic contacts and more numerous dendritic branches compared to animals in standard cages. They also show a lower level of emotional reactivity than those reared in standard conditions. (Chapillon *et al* 2002).

Genetic effects (Wurbel 2001) and effects of maternal stress (Chapillon *et al* 2002) on behaviour are sometimes abolished or reversed by minor variations and enrichment of the environment. For example, rats born from dams stressed at gestational day 19 were more anxious in the elevated plus maze than offspring to unstressed control dams, but when these rats were raised in enriched environment the anxiety decreased.

Francis *et al* (2002) and Wurbel (2001) have reasoned that the prior experiences of the rats affect their ability to plasticity when it comes to positive behavioural adaptations from enriching changes in the environment. We have seen above that maternal behaviour and the rearing environment both affect the rats from pup to adult age, as well as genetics do. Champagne *et al* (2003) states that variations in maternal behaviour are associated with the quality of the maternal environment, and Wurbel (2001) also says that habitat-dependent variation in maternal care could reflect a non-genomic mode of behavioural transmission of fearfulness and stress responses. The effects on female rats appear to be different than on males, and there are inconclusive results and thoughts about what this means and how it affects the dams and their offspring (Eklund *and* Arborelius 2006, Eklund *et al* 2009).

Introduction

According to the Directive 2010/63/EU of the European parliament and of the council, laboratory animals should be kept in an environment that benefits their health and wellbeing and give them the ability to perform their natural behaviours.

Many studies have shown beneficial effects of giving rats opportunities to perform in a more natural manner. E.g. Spangenberg *et al* (2005) showed that rats housed in larger cages with a complex environmental that give more opportunity to physical activity had lower body weights and more diverse behavioural patterns. Taking this into account, together with other previous studies, a standard cage was modified so that it would give more space, possibility to perform most of the rats' general natural behaviours such as rearing, climbing, jumping, but also aiming specifically at enabling the rat dams to perform maternal behaviours in form of nesting, retrieving, nursing as well as to voluntarily get away from the pups. The altered cage made it possible to climb up on a shelf and onto the food container in the cage, which enabled the rat dams to overview the room and also to escape the pups.

The aim of this study was to evaluate the physiological and behavioural effects on rat dams and pups when reared in a new type of cage with raised top and a shelf, a more optimised environment, compared to mothers with litters reared in the standard M IV cages. The modified cage type with square netting and a shelf was designed to give the mother rats more space, opportunity to climb and possibility to use the shelf as a hide out from the pups. It would also give the pups the same opportunities to perform natural behaviours, such as climbing when they grew big enough to climb.

To evaluate the effects the different housing systems would have on stress responses and anxiety-like behaviour, physiological as well as behavioural parameters were studied in both dams and pups. The physiological parameters measured included for the rat dams: heart rate, activity, blood pressure and corticosterone in the urine. Urine corticosterone was also measured in samples from the pups. Direct observations of maternal behaviour were performed during the lactation period and by filming the dams and pups. Behavioural tests such as the defensive withdrawal test, open field, novel object and elevated plus maze of the rat dams and pups were performed after weaning to compare the rats kept in the two different housing systems.

The dams were sampled for telemetrical data at certain times during the gestation and lactation periods correlated with filming of the dams. Direct observations were also made. This study does not include any of the material from the gestation period, nor film results.

We hope that the results obtained from this study can be a step in the process towards refinement of research in combination with the interest of the Directive 2010/63/EU. If one could use rats that are mentally healthier than the ones used today, this should enable the researcher to get more reliable results as well as to use fewer animals in order to get an accurate statistical result. Improving the welfare for the laboratory animals would be a welcome bonus, as icing on the cake.

Materials and methods

Animals and housing

In the project 15 (8+7) female and six male Wistar rats (Charles River Laboratories, Germany) were used. The female rats were ex-breeders, having had two litters of pups prior to this study. After a period of acclimatisation at the new facility (> 30 days) they were equipped with telemetry transmitters (PhysioTel® PA-C40 for Small Animals, Data Sciences International, St Paul, USA). After at least 14 days of recovery they were mated to the males. After mating, the females were randomly assigned to two different housing systems. Eight dams were singly housed in standard Makrolon type IV cages (length, width, height; 59 x 38 x 20cm, floor space approximately 2240 cm^2; M IV). Seven dams were singly housed in a modified cage consisting of a bottom part of an M IV cage with an extended raised top part equipped with a shelf (length, width, height; 59 x 38 x 43cm, floor space 2240 cm^2; RT). The RT top was made of a square net and had a black plastic roof. The feeding cup was an altered rabbit cage cup in stainless steel, making it as similar as possible to the feeding system used in M IV (Figure 1). In addition, the RT cages were equipped with a net shelf alongside one of the cage walls (length x width; 44 x 9 cm) placed 25cm above the floor. The top of the feeding cup was also placed 25 cm above the floor and could also be used by the rat to sit on. The M IV and RT cages were equally enriched with a black plastic tube (Ø 6,5 x 15-18 cm) and two Kleenex tissues administered weekly. The rats were fed ad lib with standard pellets (SDS RM1, Scanbur BK AB, Sollentuna Sweden). They had ad lib access to tap water in bottles, which was changed every other day. Bedding material used was aspen shavings bedding (Beeky bedding, Scanbur BK AB, Sollentuna Sweden). Bedding was changed once a week; the nesting material was moved to the clean cage.

The males were group housed in larger net cages, but during mating, they were housed two together with one female in two M IV cages linked together, enabling the dam to choose her mate. The room temperature was kept at 22-23°C and the relative humidity varied between 23-43 %. The light in the room was on a 12 hours light (07.00-19.00h)/12 hours dark (19.00-07.00h) cycle. The lighting in the room consisted of fluorescent lamps in the ceiling, leading to some variations in light between the cages (4-48 Lux). The ventilation in the room was air changing 15 times per hour.

Figure 1. Standard Macrolon IV cage to the left, and the enriched cage with a raised top (RT) to the right.

Surgical placement of telemetrical transmitters

The surgery to equip the rat dams with the telemetry device was performed under aseptic conditions as follows: The rat was anaesthetised with isofluorane (Isoba® vet) in an anaesthetising box. The induction of anaesthesia was made with 4,8% isofluorane and the maintenance of the anaesthesia was kept individually for each rat by an assistant monitoring the animal visually, keeping the isofluorane levels between 1,2-2,5%.

After induction of anaesthesia the rat was administered buprenorphine (Temgesic®, 0,03 ml/kg s.c.) as pre-emptive analgesia. The rat was shaved at the surgical site and loose hair was removed by a vacuum cleaner, and the area was cleaned with Hibiscrub® solution.

The rat was then positioned on the operating table on a heating pad (Deltaphase®Isothermal Pad, AgnTho´s AB, Lidingö, Sweden) kept at 37˚C and the incision site was scrubbed with alcohol. An incision was made in the skin over the left loin, approximately 2.5-3cm long. The subcutaneous tissues were bluntly dissected until the femoral artery was exposed.

Using a surgery microscope the femoral artery was prepared free and the flow stopped at a distal point in the loin by ligaturing it with Prolene® suture 6-0. The flow in the artery was also temporarily tied off 1cm proximally to the first ligature using Prolene® 6-0 held by an assistant. A puncture was made in the femoral artery using a syringe tip.

The catheter of the telemetry transmitter was placed inside the femoral artery and ligatured at place by Prolene® 6-0. A small drop of tissue adhesive was put at the ligatures to make sure they stayed in place. Through blunt dissection a pocket was made subcutaneously from the loin up to the flank. The telemetry transmitter was placed in the pocket, and sutured with Prolene® suture 4-0 to the subcutaneous muscle layers.

Since the telemetry transmitter in some of the rats tended to slide ventrally from the loin towards the abdomen, we further developed the surgical technique as follows: On about half of the rats a second incision was made on the flank and the telemetry transmitter was put in a pocket in the flank before the catheter was placed in the femoral artery. Thus, the canal between the flank and the loin could be made much narrower, and the transmitter was kept better in place.

The cutis was sutured intra cutaneously using Vicryl® suture 4-0, after which the isoflourane was turned off and the rat was placed on a heating pad in its home cage to wake up. The cage was covered until the rat was fully awake, then the heat pad and the fabric were removed. The surgery lasted for about 50-90 minutes (Cvek et al, 2007)

To avoid post surgery stress and biting at the wound some distractions were used. The rat was equipped with strips of surgeon tape around its hind leg and on the fur of the back, to distract the animal from biting the wound. Furthermore, the paper surgery drape used at the surgery was placed in the cage for the rat to have as nesting material for the next 24 hours. The rat was also given its usual pelleted food soaked in water and some honey puff cereals (ICA honungspuffar, ICA Sverige AB, Solna, Sweden) to keep it busy. Using these procedures, no rats bit their wounds.

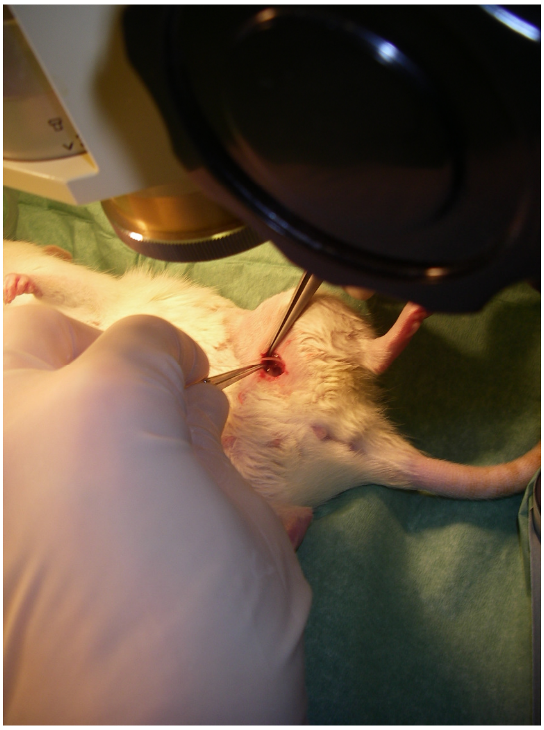

Figure 2. Surgical placement of telemetrical transmitter

Telemetry recordings

Data were recorded during lactation on five occasions for each dam, on postnatal days (PND) 1, 2 or 3, 6 or 7, 9 or 10, 12 or 13 and 19 or 20. Control data were recorded at minimum of 40 days postpartum. During recording, new values were collected every fifth minute during 24 hours. The data sampled with the telemetry device were heart rate, activity and blood pressure (mean arterial pressure, systolic pressure and diastolic pressure).

Direct observations of maternal behaviour

Direct observations of maternal nursing behaviours were made on five occasions during the lactation period. Due to practical reasons, the observation days differed a little between the rats and the days were pooled into the following groups for analysis (PND1, 3-4, 6-8, 9-10 and 11-13). When observing, the test person was sitting still in the room, and recorded the behaviour every five minutes during 90 minutes. The ethogram used for scoring the maternal behaviour contained the following behaviours; the dams’ location: With pups/without pups, Floor, shelf, climbing, on/in the plastic tube. The dams’ behaviour: Arch back nursing, supine nursing, blanket nursing, passive nursing, carrying pup, retrieval of pup, regroup pups, nest bedding, licking grooming pups, licking/grooming/scratching self, rearing, eat/drink, resting, locomotion activity, sniff, doing nothing.

Urine sampling/ corticosterone measuring

At weaning on PND 21 the dams and the pups were singly placed in an empty clean plastic container for maximum 30 minutes to get a urine sample. The urine samples were analysed for corticosterone levels using a method comparing it to the creatininee levels according to Coat-a-count (DPC, Diagnostic Products Corporation, Los Angeles, USA) and for creatininee according to Jeffe'-reaction (Photometric Colorimetric Test for Kinetic Measurements. Method without Deproteinisation, HUMAN, Gesellschaft Für Biochemica und Diagnostica mbH D-65205 Weisbaden, Germany). Unfortunately two M IV dams and two RT dams had to be excluded because they failed to urinate within the 30 minutes. Results acquired after 30 minutes could be contaminated by an increase in corticosterone due to the immediate stress of being put in the plastic container instead of the basal corticosterone level.

Behavioural studies

Behavioural tests were performed on both the dams and the pups. The tests took place after the pups had been weaned from their mothers and before they reached puberty. The tests always took place on the same day after weaning for all pups.

Defensive Withdrawal test

The defensive withdrawal test was performed on PND 25. The rat was placed in a black plastic tube (10cm × 21cm). The rat voluntarily entered the tube and the tube with the rat inside was gently placed in the corner of a plastic box (100cm × 100cm with 50 cm high walls) illuminated with dim light (approximately 100 lux). The rat was kept in the box for 10 minutes and filmed with VCR during this time. The following measures were calculated from the VCR: The time until the rat left the tube (latency), the total time the rat spent inside the tube during the session (duration), and the total number of entries into the open box. Some of the dams had to be excluded from this test since the plastic tube became aversive for them from the beginning.

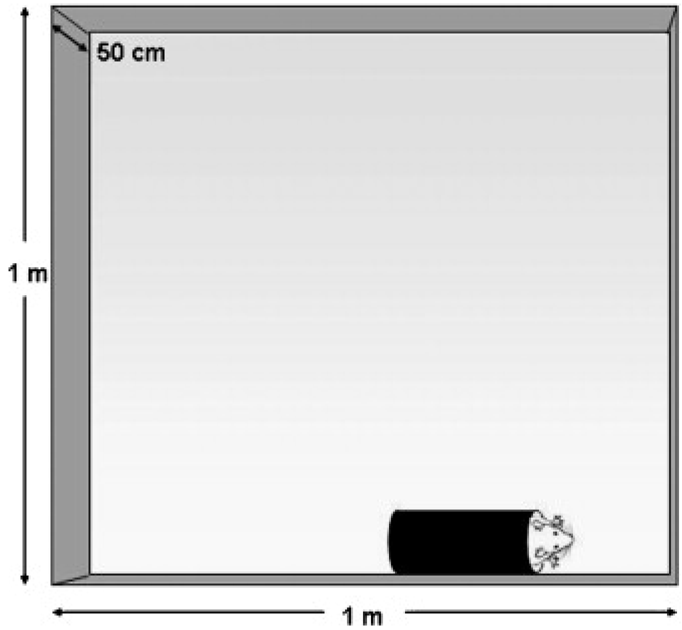

Figure 3. Schematic drawing of the defensive withdrawal test.

Elevated Plus Maze test.

The elevated Plus maze test was performed at PND 29. The rat was placed in a Plus maze, consisting of a cross-formed platform, 50 cm above the floor, with four arms (40 x 10cm long), where two of the arms had 40cm high walls. The rat was put in the centre of the Plus maze and was filmed with VCR for 5 minutes. The following behaviours were calculated from the VCR: The number of open arm entries, the number of closed arm entries, the number of total arm entries and the total time spent on the open arms.

Open Field test with Novel Object

The open field test with novel object was performed at PND 31. The rat was placed in the middle of an open arena. The open field was marked up in 15 smaller squares by black linings, and the number of crossings over the lines the rat did were measured. The crossings were further divided into peripheral and central crossings. The rats' rearing behaviours during the test were also counted. After 10 minutes a novel object (Lego® piece) was placed in the centre of the open field. The rat was filmed with VCR for an additional 5 min after the novel object was introduced. The number of total crossings as well as central and peripheral crossings was calculated during the first 10 min. The time until the rat approached the novel object for the first time and the number of visits to the novel object was calculated during the 5 min with the object. In addition, the percentage of rats in each group that did not approach the novel object at all during the 5 min was also calculated.

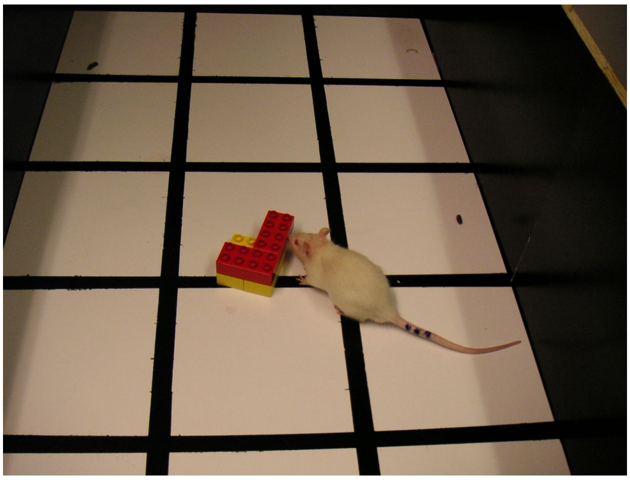

Figure 5. Open Field with Novel Object

Statistical analyses

Comparisons between rats from the different housing systems were made using t-test or a Mann-Whitney Rank Sum when the Shapiro-Wilk test for normality failed. All statistics were calculated with Sigmastat for Windows, 3.0 and GraphPad Prism 3.0. Results were considered significant at $p<0.05$, and are presented as mean ± SEM.

RESULTS

Activity, blood pressure and heart rate

On PND 2 the activity of the RT dams (7,9 ± 1,6) was significantly higher ($p=0.037$) than that of M IV dams (4,0 ± 0,8). The mean arterial pressure (MAP) was also significantly increased ($p=0.006$) in RT dams (103,3 ± 1,6 mmHg) compared to M IV dams (88,2 ± 1,4 mmHg) during PND 2, but no differences in heart rate between the differently housed dams could be detected. On the other days of measuring (PND 6, 9, 12 and 19 and 40 days after parturition) there was no difference in activity, MAP or heart rate between the two groups of rat dams.

Maternal behaviour

Data from the direct observations of the dams reveal the following: Time spent with pups and licking/grooming of pups at the different observation days showed no significant difference

(Fig. 6). But when the data from all the days were pooled together it showed that the M IV dams have a significant higher degree ($p<0.01$) of licking and grooming pups.

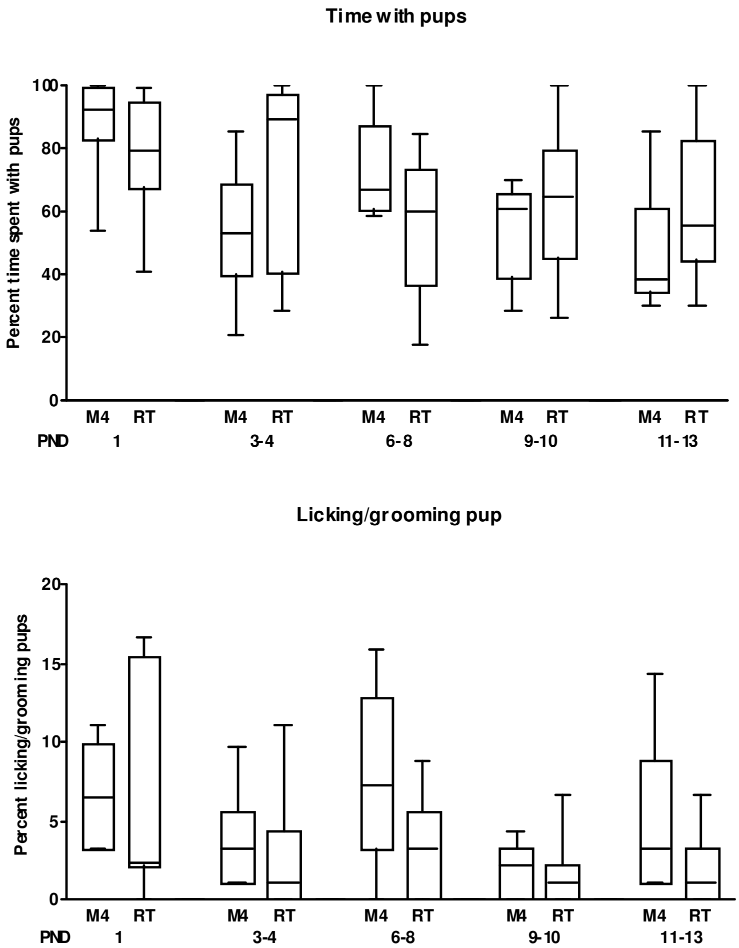

Figure 6. Maternal behaviour in dams housed in standard cages (M IV) or cages with raised tops (RT). Upper panel: Percent time that the dam spend with her pups during a 90 min time period. Lower panel: Percent time that the dams spent licking or grooming her pups. Data are shown as median ± 1st and 3rd quartiles (box). The whiskers extend to show the highest and lowest values.

Nursing behaviour: At PND 9-10 there was a tendency for a significant ($p=0.051$) difference in arched back nursing where dams in M IV cages showed a higher amount of this behaviour than dams in RT cages.: At PND 11-13 there was also a tendency for a difference in passive nursing showing that RT caged dams showed more of this nursing style than M IV dams. Again, when pooling the results from all the measuring days this revealed that there was a significant difference ($p<0.05$) for arched-back nursing with the M IV dams showing a higher degree of this behaviour (Fig. 7).

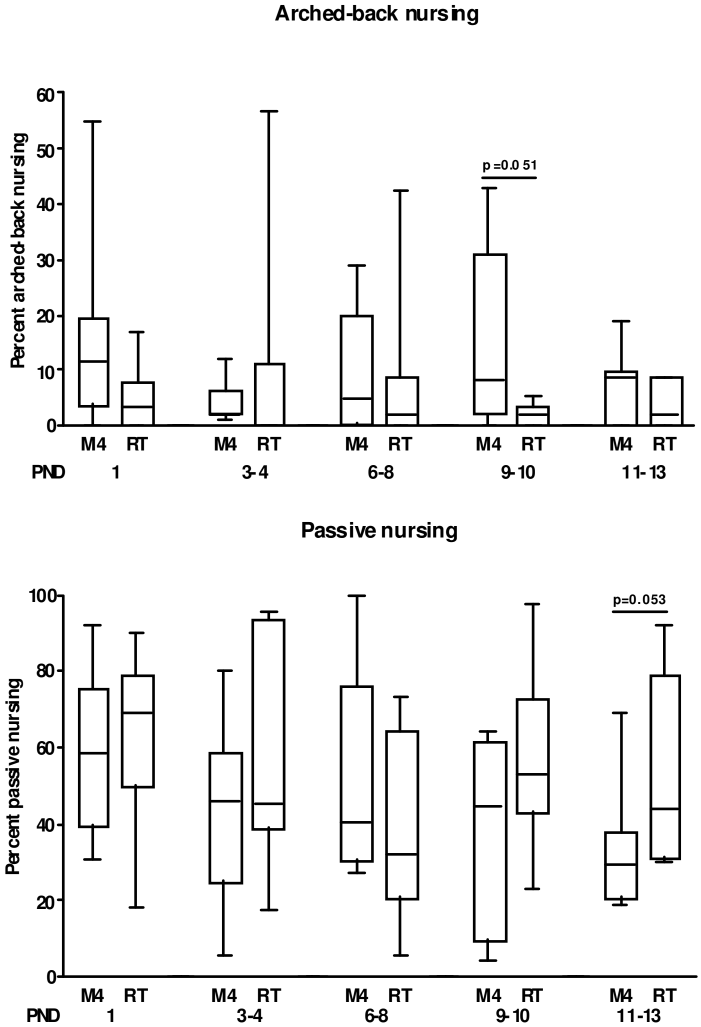

Figure 7. Upper panel: Percent arched-back nursing displayed by the dams housed either in standard cages (M IV) or cages with raised tops (RT) during a 90 min time period. Lower panel: Percent passive nursing displayed by the dams. Data are shown as median ± 1st and 3rd quartiles (box). The whiskers extend to show the highest and lowest values. Since the data were not conformed to normality Mann Whitney test was used to compare the two groups.

Urine corticosterone

The corticosterone/creatininee quote measured in urine from the dams and pups at the day of weaning (PND 21) is shown in Figure 8.

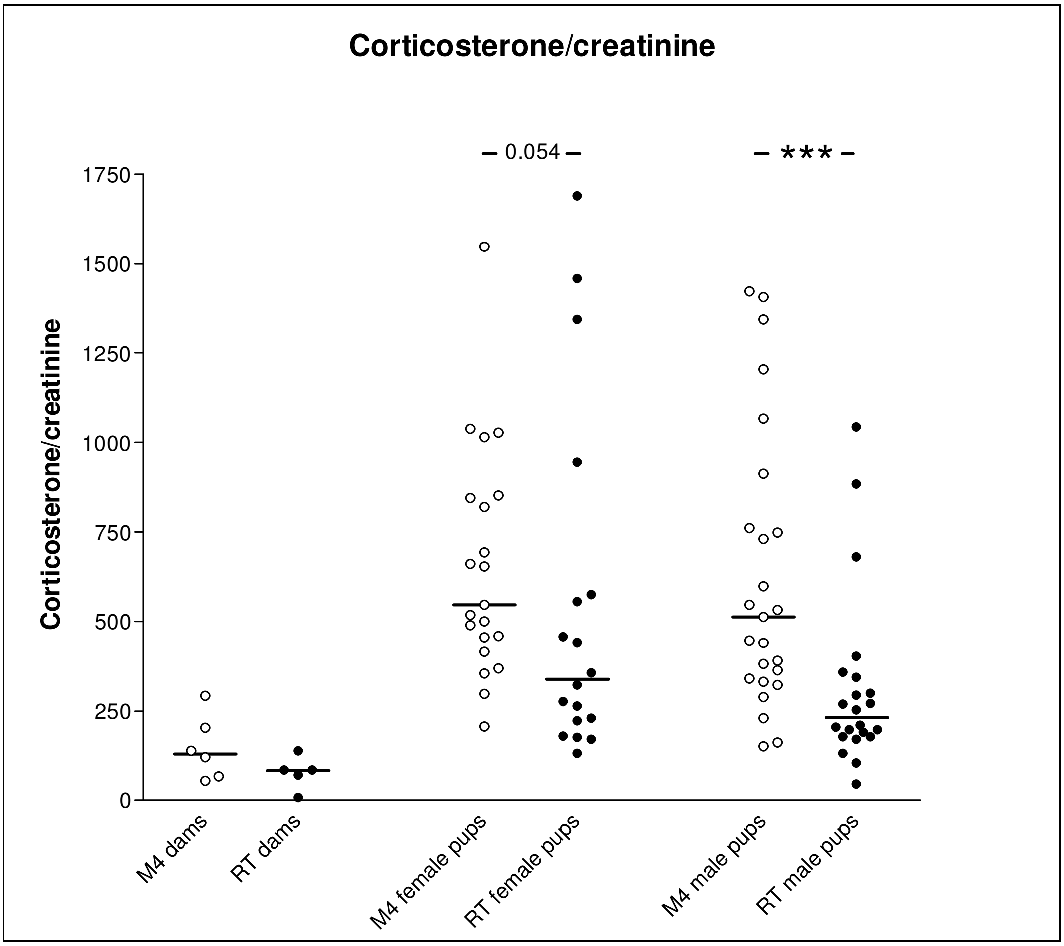

Figure 8. Scatter gram showing the corticosterone/creatininee ratio in urine from dams housed either in standard cages (M IV) or cages with raised tops (RT) and from their offspring at weaning (PND 21). The horizontal bar represents the median. Since the data were not conformed to normality Mann Whitney test was used.

We found that M IV dams had slightly higher corticosterone/creatininee ratio as compared to RT dams, although it does not reach statistical significance, probably due to too few values from dams from each group (6 in the M IV cages, 5 in the RT cages). As for the pups, RT males showed significantly lower levels of urinary corticosterone compared to M IV males. In the female pups there was a tendency (p=0.054) for lower values in the RT females as compared to M IV females (Fig. 8). When data from both female and male pups were pooled, the RT-pups had significantly lower corticosterone levels (p<0.001) compared to M IV pups.

Defensive withdrawal test

The data from the dams could not be included, because some of them showed an aversion to the plastic tube and their data had to be excluded, the data from the rest of the dams were not enough to analyse. The data from the pups show a tendency (p=0.07) for shorter time spent in the shelter (duration) in female pups raised in RT cages compared to those raised in M IV cages (Fig. 9). There was no difference between the male pups raised in the two different cage types.

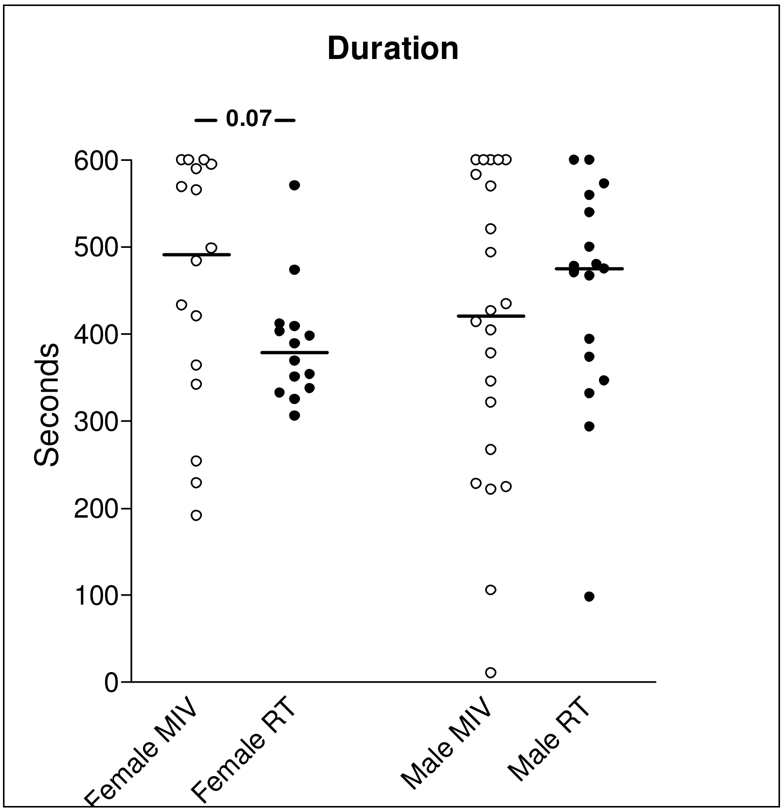

Figure 9. Scatter gram showing the time spent withdrawn in the tube (duration) in the defensive withdrawal test in 25 days old female and male pups bred either in standard cages (M IV) or in cages with raised tops (RT). The horizontal bar represents the median. Since the data were not conformed to normality Mann Whitney test was used.

Elevated Plus Maze test

No differences in the behaviour in the plus maze between the M IV and RT dams could be detected. The female pups bred in RT-cages showed a significant increase in both the number of open arm entries (Fig. 10A) and the time spent on the open arms (duration; Fig. 10B) compared to females bred in standard cages. Both these parameters measure anxiety-like behaviour suggesting that the RT female pups are less anxious compared to the M IV females. In the parameters measuring general motor activity, i.e. closed arm entries (Fig. 10C) and total number of arm entries (Fig. 10D), RT females showed significantly higher scores than M IV females.

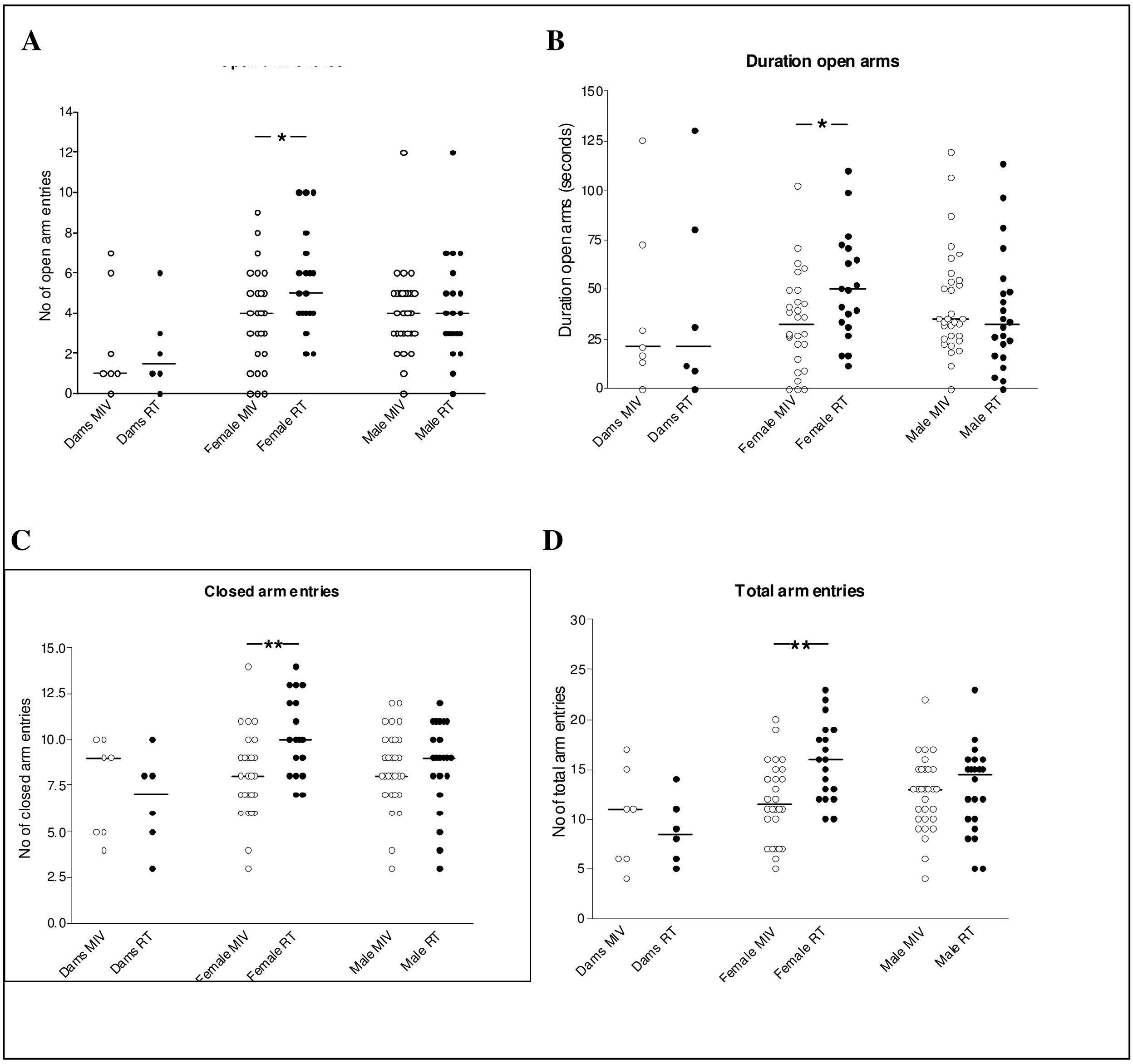

Figure 10. Scatter grams showing the number of open arm entries (**A**), the time spent on the open arms **(B),** the number of closed arm entries **(C)** and the number of total arm entries (**D**) in the elevated plus maze in dams and in 29 days old female and male pups bred either in standard cages (M IV) or in cages with raised tops (RT). The horizontal bar represents the median. Since the data were not conformed to normality Mann Whitney test was used.

Open field test

Our results reveal that male pups reared in M IV cages made significantly higher percentage central crossings than RT males, but there were no differences between female pups or dams reared in the two different cage types (Fig. 11).

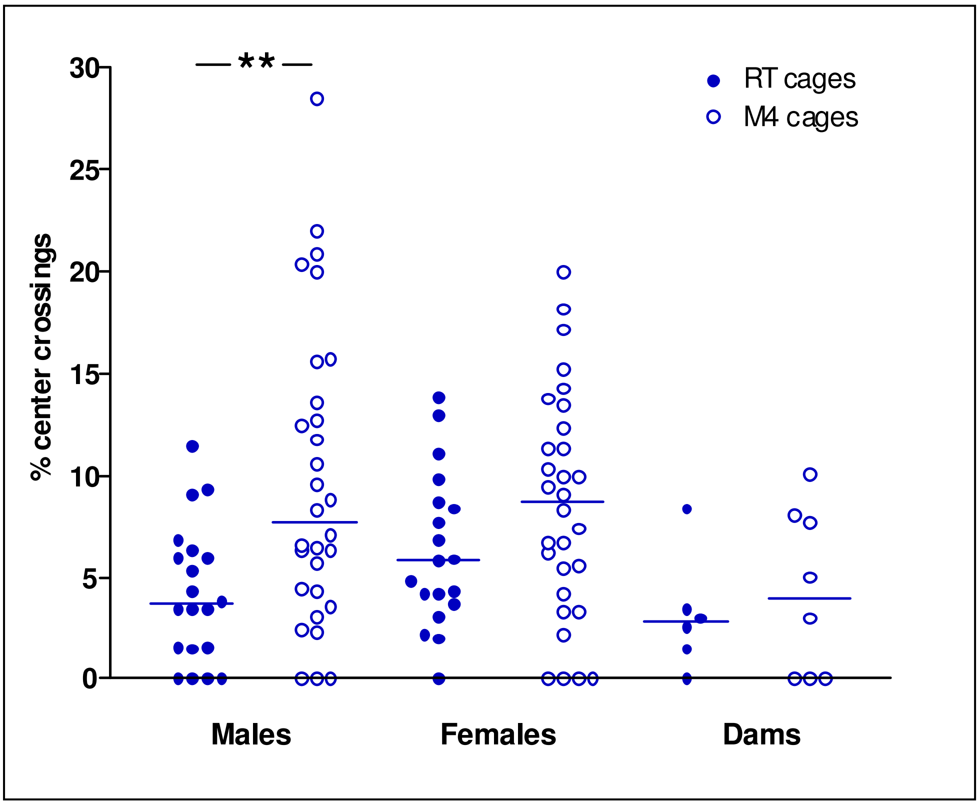

Figure 11. Scattergram showing the number of crossings of centre lines as percentage of the total number of lines crossed in the open field in dams and in 31 days old female and male pups bred either in standard cages (M IV) or in cages with raised tops (RT). The horizontal bar represents the median. Since the data were not conformed to normality Mann Whitney test was used.

Novel object test

More M IV pups, both males and females, did not approach the novel object at all compared to RT pups, whereas the opposite was observed for the dams (Fig. 12A). However, when measuring the total number of visits per pup, the females of the M IV made significantly more visits to the novel object, than did the RT females, but no differences were seen in the male pups or the dams (Fig 12B).

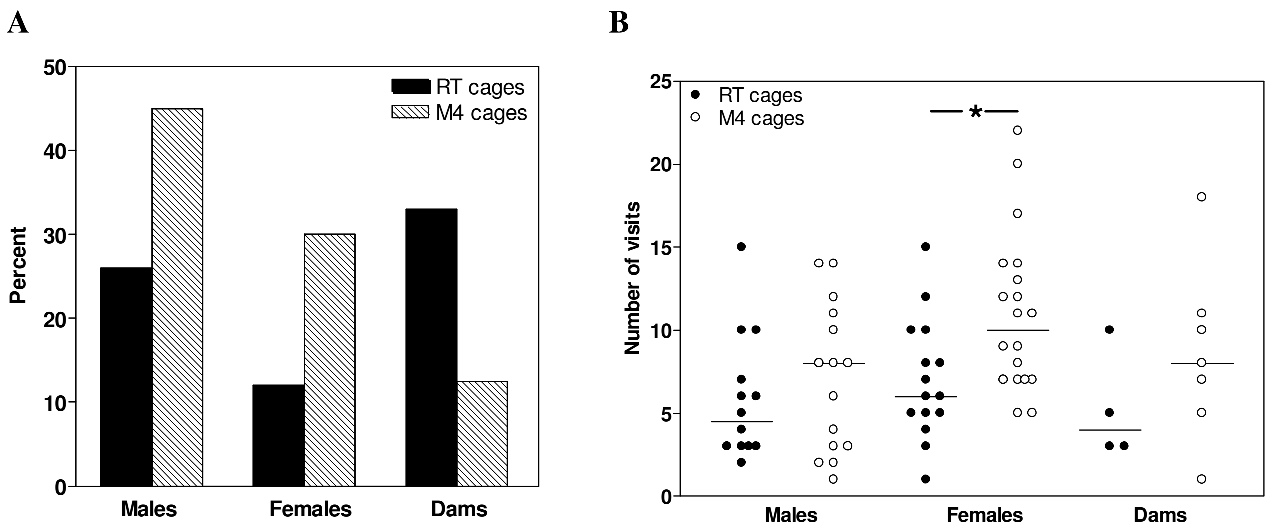

Figure 12. A. Bar graph showing the number of rats that did not approach the novel object calculated as percent of total number of rats in each group. The bars represent the median. **B**. Scattergram showing the number of visits to the novel object during five minutes in dams and in 31 days old female and male pups bred either in standard cages (M IV) or in cages with raised tops (RT).

Discussion and conclusions

The results from the present study show that laboratory rat mothers housed in enriched cages (RT cages) were significantly more active and had higher blood pressure at PND2 than mothers housed in standard cages (M IV cages). We also found that the RT dams spent less time licking/grooming their young as well as spending less time arched-back nursing them than did the M IV dams. The pups of the RT dams showed however less corticosterone excretion, and taking the results from the behavioural tests all together, they also showed less anxiety-like behaviour and less fearfulness. The differences were more pronounced in the female than in the male offspring, suggesting that female pups are more susceptible to the rearing environment.

The telemetrical data revealed that at PND 2, at the time when the dams are the most active in their nursing/caretaking of their pups, the blood pressure as well as the activity of the dams in the RT cages was significantly higher than blood pressure and activity of the dams in the M IV cages. The higher blood pressure observed in the RT dams does probably not reflect stress, but a consequence of the increased motor activity, which also activate the sympathetic nervous system. So far the blood pressure measurements show inconclusive results. We can only guess that possibly the significantly higher activity of the rat dams in the RT cages result in them having a higher MAP. More data is collected through the telemetrical system, and the plan is to further extract and analyse the data: first to evaluate if the activity is correlated to higher MAP and to compare blood pressure data when the animals are in no activity, i.e. when the rat dams are resting, and secondly to analyse the diastolic/systolic blood pressure values separately.

In this study the dams were given the opportunity to voluntary escape from the pups through expanded cage space and shelf refuges. The dams' time spent on the shelves is however not yet evaluated. When analysing the maternal behaviour we could not see any difference in the total time spent with the pups from the direct observations. The results showing that the RT dams spend less time licking/grooming and arched-back nursing their pups is in line with previous studies showing that lactating rats in a more safe and stable environment become more relaxed and show more passive maternal caretaking (Macri et al 2004). Thus, the dams in our enriched cages may be more relaxed and therefore show less active maternal care compared to the dams in standard cages. The slightly lower levels of urinary corticosterone also indicate that the RT dams may be less stressed than M IV dams, although this needs to be confirmed by analysing corticosterone from faeces.

It has been argued that pups reared with a more active nursing style (e.g. more arched back nursing) show less anxiety behaviours as adults (Meaney 2001). Our results contradict this notion at several points: in our study RT dams spend less time licking/grooming pups and arched back nursing them, and their pups excrete less corticosterone and show less fearfulness and less anxiety-like behaviours than the M IV reared pups. In our study the dams in the RT cages were given the opportunity to voluntarily separate themselves from the pups, which is not the case in the maternal separation studies, where usually the mother is left in the home cage while the pups are removed from it. This creates an artificial kind of maternal separation, whilst our procedure gave the dams opportunity to perform more natural behaviours and with possibility to control their situation by voluntarily escaping their young.

There are different ways for the rat dam to nurse her young. In many older studies, the importance of these different nursing styles has been evaluated. The different kinds of nursing have been given different importance. More recent studies show that perhaps the pattern of nursing style is not that simple, and that the consequences from the different type of nursing may not be as simple as one has believed.

Our results is in line with the study result of Macri et al (2004) on maternal separation that imply that involuntary long separation time from pups may lead to more active caretaking of the pups, but more fearful and anxiety behaviours of the pups whereas shorter separation tends to give less anxiety and less fearful behaviour in pups.

The results of our behavioural studies especially showed differences between the female pups reared in the two different cage types, where the RT reared females displayed less anxiety-like behaviours according to our results in the defensive withdrawal test and the elevated plus maze, as well as significantly higher motor activity than the M IV reared females. However in the Open field with novel object test, even if more of the RT reared females dared encounter the novel object, the M IV females made more visits. In summary, the interpretation of these results is that the female pups are considerably more sensitive to the enlargement/enrichment of the rearing environment. Thus, it would be of outmost interest to further study the difference in the maternal behaviour between the two groups of pups when grown up with focus on stress response and maternal behaviour.

Together, the urine corticosterone/creatininee levels and the data from the behavioural studies point to a distinct pattern of the pups bred in RT cages, i.e. being less stressed and showing less fearfulness than the pups bred in M IV cages.

Although we were not able to show significant results on urine corticosterone levels in the dams, the tendency was that the RT dams had lower corticosterone levels. They were also more active in the RT cages, more detailed analyses of the telemetrical data might give more clues as to whether they are actually less stressed in these cages, and more studies with larger number of dams could also be helpful to clarify this issue.

Further studies with the RT cage type would be interesting and most welcome. One direction of interest would be studies of pups bred in RT cages compared to pups bred in M IV cages. Studies of pups could be done telemetrically to obtain physiological data, direct observations could be made to evaluate the climbing/"shelfing" (comparing this to how much the M IV pups try to climb the roof of the cage), and also e.g. comparing the time pups spend fighting with each other in the different types of cages. Most important would be studies of the maternal behaviour of females who were bred in the different types of cages, and the effects on their offspring, keeping the grandmother effect in mind.

Naturally, it is difficult to evaluate exactly what it is in the new cage environment that lead to the result of the study, but the overall conclusion is that these relatively small changes in the rearing environment result in significantly less anxiety, fearful behaviour and lower stress level in the rat pups, and we have shown a tendency for less stressful behaviour in the rat dams. This gives us reason to return to the legislation: The 2010 EU directive EC Annex III Section A 3.3 b) states that "All animals shall be provided with space of sufficient complexity to allow expression of a wide range of normal behaviour. They should be given a degree of control and choice over their environment to reduce stress-induced behaviour. Establishments shall have appropriate enrichment techniques in place, to extend the range of activities available to the animals and increase their coping activities including physical exercise, foraging, manipulative and cognitive activities, as appropriate to the species. Environmental enrichment in animal enclosures shall be adapted to the species and individual needs of the animals concerned. The enrichment strategies in establishments shall be regularly reviewed and updated."

With this in mind, the findings of this study support the argument that enhancement of the animal facilities should be considered, and when it comes to the rearing facilities our study shows that the rats' mental health would benefit from larger space, climbing opportunities and opportunity of voluntary maternal separation.

Figure 13. Rat pups in RT cage

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