

# The Effects of Spaceflight on Mammary Metabolism in Pregnant Rats (44432)

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**Abstract.** The effects of spaceflight on mammary metabolism of 10 pregnant rats was measured on Day 20 of pregnancy and after parturition. Rats were flown on the space shuttle from Day 11 through Day 20 of pregnancy. After their return to earth, glucose oxidation to carbon dioxide increased 43% ( $P < 0.05$ ), and incorporation into fatty acids increased 300% ( $P < 0.005$ ) compared to controls. It is unclear whether the enhanced glucose use is due to spaceflight or a response to landing. Casein mRNA and gross histology were not altered at Day 20 of pregnancy. Six rats gave birth (on Day 22 to 23 of pregnancy) and mammary metabolic activity was measured immediately postpartum. The earlier effects of spaceflight were no longer apparent. There was also no difference in expression of  $\beta$ -casein mRNA. It is clear from these studies that spaceflight does not impair the normal development of the mammary gland, its ability to use glucose, nor the ability to express mRNA for a major milk protein.

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Mammary development during pregnancy is a complex process that involves a range of physiological and behavioral adaptations. In the rat, mammary ducts grow out from the nipple and form a ductal tree. During pregnancy the branching structure develops alveoli composed of epithelial cells surrounding a lumen into which milk is secreted during lactation (1). The mammary gland requires estrogen and progesterone during pregnancy to develop the full functional capability that is expressed during lactation. It is not until parturition, when the nursing young are born, that full expression of the milk components is achieved. Lactation begins when progesterone declines and estrogens, glucocorticoids, and prolactin increase to achieve full functional capacity, which allows the animals to secrete milk during lactation (2).

The physiological changes that occur during pregnancy

are accompanied by behavioral changes in the dam. Pregnant female rats spend a greater amount of time licking their pelvic and abdominal areas, particularly the nipples, compared to rats that are not pregnant (3). To determine whether this activity affects mammary development, Roth and Rosenblatt fitted pregnant rats with wide rubber collars that prevented self-licking and reduced the percentage of the tissue that was secretory by 30%–50% (4, 5). In a similar study, McMurtry and Anderson found that rats that wore rubber collars had significantly less wet and dried fat-free mammary tissue (6). These studies suggest that in addition to physiological changes, behavioral patterns also influence mammary development during pregnancy in the rat.

During spaceflight, both behavioral and physiological changes occur to allow an animal to adapt to their new environment. Postural changes, such as the curling required to groom the abdominal area would be difficult. There are clear shifts in fluid balance as well as marked changes in bone density and calcium metabolism and changes in muscle mass (7). It is not known how the physiological changes that occur during spaceflight will impinge on mammary development in pregnant animals. This is particularly important since pregnancy is a period when the mammary gland is undergoing marked growth and development. The objectives of this project were to determine the effect of spaceflight on functional activity of the mammary gland in pregnant rats exposed to spaceflight on Days 11–20 of gestation. Although measurements of metabolism in other tis-

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sues would have shed additional insight into the mechanisms associated with altered functional activity, the bones, muscles, blood, and other organs were shared with many other investigators and were not available for this study. Oxidation of glucose to CO<sub>2</sub>, incorporation of glucose into fatty acids, and mRNA expression of the milk protein β-casein were measured as metabolic indicators to quantify functional mammary activity. This is the first study to investigate the response of the mammary gland of pregnant animals to the microgravity environment. These types of experiments are extremely important because 1) mammary tissue is undergoing rapid developmental changes during pregnancy and 2) changes in mammary metabolism have direct effects on growth of the offspring, thus potentially altering the normal developmental pattern of offspring during spaceflight.

## Materials and Methods

**Animals.** Animal experimentation was conducted according to the guidelines of the Ames Research Center Institutional Animal Care and Use Committee (Moffet Field, CA). Timed pregnant female Sprague-Dawley rats (Taconic Farms, Germantown, NY) were shipped to Kennedy Space Center and were laparotomized on Day 9 of gestation to determine the number of implantation sites in the uterus (8). Rats having at least 10 implantation sites were selected for the experiment. The flight group (FLT;  $n = 10$ ) was housed in animal enclosure modules and placed aboard the Shuttle Discovery on Flight STS-70. They lifted off on Day 11 of gestation. A matched vivarium-housed group (VIV;  $n = 10$ ) was maintained under standard environmental conditions. A flight-delayed synchronous group (FDS;  $n = 10$ ) was time-delayed by 48 hr so that temperature and light exposure that were present on the shuttle could be mimicked on earth. The FDS animals were housed in animal enclosure modules, and conditions were mimicked based on the down-linked data received from the shuttle. A vivarium, nonlaparotomized group of animals (VNL;  $n = 10$ ) was also used. Since the VNL animals did not receive a laparotomy, the number of implantation sites could not be predetermined in these animals. Extra animals were maintained so that only animals that had 10 implantation sites at surgery or successfully delivered a litter were selected for further experimentation.

FLT rats were flown on the STS-70 from Days 11 to 20 of gestation. The first animal surgery began approximately 3 hr after landing. Animals were anesthetized with halothane, and mammary tissue was removed approximately 18 min after initiation of surgery. Four FLT and VIV animals were processed at this time. Four FDS and VNL animals were processed 48 hr later mimicking the time pattern and conditions that were established with the flight animals. The remaining animals ( $n = 6$ /trt) were housed in vivarium cages and allowed to deliver their pups. Dams were observed by using video cameras, and surgery was performed

within 3 hr after delivery of the last pup. All dams had been observed nursing the pups at least twice prior to surgery.

Inguinal mammary glands were removed from the dams and placed in Tris-sucrose buffer (250 mM sucrose, 25 mM Tris, 1 mM glutathione, 1 mM EDTA, pH 7.3). Mammary tissues were sliced into sections that were approximately 35 mm diameter and 500 μm thick using a Stadie-Riggs microtome (Thomas Scientific, Swedesboro, NJ) (9). Lymph nodes were removed from each gland prior to slicing. Mammary metabolism was measured as previously described by Bauman *et al.* (10) and Plaut *et al.* (11). Briefly, four samples per animal weighing 120 to 160 mg each were placed in Erlenmeyer flasks containing 3 ml of a medium consisting of Krebs Ringer bicarbonate buffer solution (154 mM NaCl, 154 mM KCl, 110 mM CaCl<sub>2</sub>, 154 mM KH<sub>2</sub>PO<sub>4</sub>, 154 MgSO<sub>4</sub>, 154 mM NaHCO<sub>3</sub>, pH 7.4) glucose (10 mM), insulin (1 μg/ml; Sigma Chemical Co., St. Louis, MO), and U-<sup>14</sup>C-glucose (1 μCi/flask; ICN, Irvine, CA). Blanks were prepared using the same medium but no tissue sample. The flasks were gassed with an O<sub>2</sub>:CO<sub>2</sub> (95:5) mixture for 10 sec and immediately sealed with rubber stoppers. Plastic center wells holding pieces of filter paper approximately 2 × 2 cm<sup>2</sup> were suspended from the stoppers down into each flask. The flasks were incubated in a shaking water bath at 37°C for 3 hr. Then 0.25 ml of 0.5 M H<sub>2</sub>SO<sub>4</sub> was injected through the rubber stopper into the medium to stop the metabolic activity, and 0.2 ml 1 M hyamine hydroxide was injected into the center wells to trap the CO<sub>2</sub> as NaHCO<sub>3</sub>. The flasks were returned to the water bath for 1/2 to 1 hr to allow the CO<sub>2</sub> to be released from the tissue.

**Determination of Glucose Metabolism.** Filter papers from the center wells were placed in scintillation vials. Wells were rinsed twice with 400 μl of water and the rinse water added to the vials, along with 9 ml aqueous scintillation fluid (Bio-Safe II Counting Cocktail, Research Products International Co, Mt. Prospect, IL). Tissue samples were removed from the liquid medium, put into 4 ml 5 N NaOH, and saponified by heating in a water bath at 90°C for 4 hr. This medium was acidified by the addition of concentrated HCl and then lipid extracted twice with petroleum ether. Two ml aliquots of the ether extracts were added to 8 ml organic scintillation fluid (Bio-Safe NA Counting Cocktail, Research Products International Co, Mt. Prospect, IL). All samples were counted using a scintillation counter (LS6500 Multipurpose Scintillation Counter, Beckman Instruments, Fullerton, CA). Oxidation of glucose to CO<sub>2</sub> and incorporation of glucose into lipid were corrected for the wet weight of the tissue and expressed in nmoles glucose used/100 mg wet tissue in 3 hr. The means for each treatment were subjected to analysis of variance followed by Bonferroni's method of multiple comparison (SAS).

**Northern Analysis.** Total RNA was extracted from the frozen mammary tissue ( $n = 3$ /treatment) using the single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction method described by Chomczynski and Sacchi (12). Total RNA (20 μg) was

stained with ethidium bromide on a 1% agarose gel with 0.6 M formaldehyde in 1 × 3-[N-morpholino] propanesulfonic acid (MOPS) (20 mM MOPS, 5 mM NaAcetate, 1 mM EDTA). The samples were electrophoresed in 1 × MOPS at 60 V for 5 hr and transferred overnight (13) to GeneScreen Plus (Dupont-NEN, Boston, MA). The membranes were probed with a mouse  $\beta$ -casein cDNA probe (J. M. Rosen, Baylor University, Houston, TX) labeled by random priming (Boehringer Mannheim, Indianapolis, IN) with  $^{32}\text{P}$ -dCTP (3000 Ci/mmol). The blots were hybridized overnight at 42°C in 5× sodium chloride sodium phosphate EDTA solution (SSPE) (1× SSPE = 0.15 M NaCl, 0.01 M  $\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O}$  and 1 mM EDTA), 50% formamide, 5× Denhardt's Reagent (1× Denhardt's = 0.02% ficoll, 0.02% polyvinyl pyrrolidone, and 0.02% bovine serum albumin, fraction V) and 0.5% SDS. The membranes were washed in 2× SSPE, 0.1% SDS for 15 min twice, followed by 1× SSPE, 0.1% SDS for 30 min, and 0.1X SSPE, 0.1% SDS for 15 min; all washes were done at 42°C. The membranes were exposed to autoradiographic film for approximately 24 hr at -80°C. The cDNA probe was stripped from the membranes by washing in 5 mM EDTA, 0.1× Denhardt's at 65°C for 2 hr. The membranes were then probed with 18S RNA labeled by nick translation (Boehringer Mannheim, Indianapolis, IN) with  $^{32}\text{P}$ -dCTP (3000 Ci/mmol), and the film was exposed as described above. Densitometric analyses of autoradiographs were performed on a Macintosh computer using the public domain NIH Image program (<http://rsb.info.nih.gov/nih-image/>).  $\beta$ -Casein mRNA expression was standardized with 18S expression, and the data were analyzed by one-way analysis of variance with treatment as the main effect.

**Morphology.** One 500- $\mu\text{m}$  slice from each mammary tissue sample was defatted in acetone. Tissues were stained with hematoxylin (Hematoxylin Solution, Gill, Mayer, VWR Scientific, West Chester, PA), dehydrated in alcohol, and fixed in toluene and mounted. Slides were viewed at 25× using a Zeiss Axiovert 35 inverted microscope (AZI Corp, Avon, MA), and photographs were taken with a Nikon N8008S AF camera (Melville, NY).

**Histology.** Pieces of tissue 0.5  $\text{cm}^2$  in size were placed in PBS (0.01 M phosphate buffer, 0.0027 M KCl, 0.137 M NaCl, pH 7.4) immediately after excision. The tissues were fixed in 4% paraformaldehyde for 2 hr and transferred to 30% sucrose for 2 hr. Each tissue sample was embedded in OCT (Tissue-Tek, Miles, Inc., Elkhart, IN) and stored at -80°C. Sections were cut (6  $\mu\text{m}$ ), mounted, and stained with hematoxylin and eosin. Slides were viewed at 320×, and photomicrographs were taken as described above.

## Results

A marked increase in mammary metabolic activity occurred in pregnant animals that were subjected to spaceflight compared to all other treatments (Table I). Oxidation of glucose increased 43% ( $P < 0.05$ ) compared to flight-

**Table I.** Oxidation of U- $^{14}\text{C}$ -glucose to  $\text{CO}_2$  and Incorporation into Lipid in Mammary Tissue of Rats on Day 20 of Gestation

Group*	Oxidation to $\text{CO}_2$ †		Incorporation into lipid†	
	Mean	S.E.	Mean	S.E.
FLT	925 <sup>a</sup>	36	970 <sup>a</sup>	151
FDS	645 <sup>b</sup>	91	323 <sup>b</sup>	98
VIV	698 <sup>b</sup>	72	355 <sup>b</sup>	139
VNL	694 <sup>b</sup>	21	270 <sup>b</sup>	46

<sup>a,b</sup> Means with different superscripts are different ( $P < 0.05$ ).

\* FLT-laparotomized pregnant rats exposed to spaceflight on Days 11–20 of gestation; FDS-control laparotomized pregnant rats exposed to similar conditions as spaceflight animals except time delayed by 48 hr to accommodate data retrieval from the shuttle; VIV-control laparotomized pregnant rats held in vivariums; VNL-nonlaparotomized pregnant rats held in vivariums.

† Data expressed as nmoles/100 mg wet mammary tissue in 3 hr.

delayed synchronous groups, and glucose incorporation into lipids increased 300% ( $P < 0.001$ ). By the time the animals delivered their offspring (2–3 days later), mammary metabolic activity was not significantly different between any treatment group (Table II).

The least squares means of the  $\beta$ -casein mRNA expression on Day 20 of gestation showed that there was no significant difference in  $\beta$ -casein mRNA expression between FLT and VIV of FDS animals (Table III). Similar to the Day-20 animals, there was no significant difference in  $\beta$ -casein mRNA expression in lactating animals (Table IV). When  $\beta$ -casein mRNA expression was compared to metabolic activity of the mammary gland, there was no correlation between the increase in metabolic activity on Day 20 and  $\beta$ -casein mRNA expression. The same result was found to be true in lactating animals.

There were no apparent effects on the gross morphology of the mammary glands (data not shown). Hematoxylin-stained slices from the mammary gland of all rats were densely packed with alveoli. No differences in the histology

**Table II.** Oxidation of U- $^{14}\text{C}$ -glucose to  $\text{CO}_2$  and Incorporation into Lipid in Mammary Tissue of Rats After Parturition on Day 22 to 23

Group*	Oxidation to $\text{CO}_2$ †		Incorporation into lipid†	
	Mean	S.E.	Mean	S.E.
FLT	1045 <sup>ab</sup>	184	892	253
FDS	1279 <sup>a</sup>	58	887	164
VIV	1132 <sup>ab</sup>	62	851	125
VNL	767 <sup>b</sup>	116	576	197

<sup>a,b</sup> Means with different superscripts are significantly different ( $P < 0.05$ ).

\* FLT-laparotomized pregnant rats exposed to spaceflight on Days 11–20 of gestation; FDS-control laparotomized pregnant rats exposed to similar conditions as spaceflight animals except time delayed by 48 hr to accommodate data retrieval from shuttle; VIV-control laparotomized pregnant rats held in vivariums; VNL-nonlaparotomized pregnant rats held in vivariums.

† Data expressed as nmoles/100 mg wet mammary tissue in 3 hr.

**Table III.** Expression of  $\beta$ -Casein mRNA in Rat Mammary Tissue at Day 20 of Pregnancy

Group*	$\beta$ -casein: 18S LS mean	Standard error LS mean
FLT	1.31	0.34
FDS	1.21	0.34
VIV	0.54	0.34

\* FLT-laparotomized pregnant rats exposed to spaceflight on Days 11–20 of gestation; FDS-control laparotomized pregnant rats exposed to similar conditions as spaceflight animals except time delayed by 48 hr to accommodate data retrieval from shuttle; VIV-control laparotomized pregnant rats held in vivariums.

**Table IV.** Expression of  $\beta$ -Casein mRNA in Rat Mammary Tissue After Parturition

Group*	$\beta$ -casein:18S LS mean	Standard error LS mean
FLT	1.44	0.30
FDS	0.55	0.35

\* FLT-laparotomized pregnant rats exposed to spaceflight on Days 11–20 of gestation; FDS-control laparotomized pregnant rats exposed to similar conditions as spaceflight animals except time delayed by 48 hr to accommodate data retrieval from shuttle.

of the glands were apparent. At gestation Day 20, alveolar epithelium was large and round with evidence of fat droplets and protein (acidophilic material). Histological sections from lactating animals clearly showed secretions in the lumen. However, the histological sections did not fix well. It is presumed that this is due to the large amount of fat and protein present in the mammary gland immediately after parturition.

## Discussion

We observed a marked increase in metabolic activity in the mammary glands of animals exposed to spaceflight on Day 20 of gestation. This was surprising based on our hypothesis that disruption of grooming activities coupled with endocrine and physiological changes would lead to reduced functional activity. These data lead us to believe that the metabolic response observed on Day 20 of gestation was due to an acute change in metabolic activity rather than a long physiological adaptation to spaceflight. Mammary metabolism observed in the spaceflight animals at Day 20 of gestation was similar to the metabolic rate of the animals after parturition. It is possible that signals associated with lactogenesis (the onset of milk production) were prematurely activated in those animals that were subjected to spaceflight. The endocrine signals associated with parturition include an increase in estrogen, prolactin, and glucocorticoids with a concomitant decrease in progesterone (2). Few hormonal measurements were made in these animals because whole-body perfusion of the animals was required for other investigators studying the brain. However, some blood was obtained from two of the four flight animals. Progesterone concentrations in plasma were still high in the animals exposed to spaceflight (data not shown). All animals delivered their offspring between Days 22 and 23 of

gestation. Both glucocorticoids and prolactin can rapidly change in response to stress, temperature fluctuation, and many other environmental variables (14). It is possible that re-entering the earth's atmosphere caused premature release of either the glucocorticoids, prolactin, or both hormones. These changes likely signaled the mammary gland to prepare for lactogenesis, therefore, mammary metabolic activity increased. Since mammary metabolic activity was measured approximately 3 hr after the animals had landed, this is within the time that a response to these hormones would be expected. It is likely that the signals were of sufficiently short duration not to affect parturition but they did allow the mammary gland to prepare early for expected delivery. This would explain why the effect was observed postlanding and not after parturition.

To determine whether the changes in metabolic activity were reflected in changes in lactogenesis,  $\beta$ -casein mRNA expression was measured. Since  $\beta$ -casein mRNA expression is already turned on in midpregnancy (15) and then undergoes large increases in expression at lactogenesis in response to corticosteroid and prolactin, which stabilize the mRNA transcript, it may be a good indicator of lactogenic activity (16). There were no significant differences in  $\beta$ -casein mRNA expression between FLT and FDS or VIV groups at Day 20 of pregnancy or after parturition. Although  $\beta$ -casein mRNA expression was always highest in FLT animals, large variation in  $\beta$ -casein mRNA masked any statistical difference, if one existed.  $\beta$ -casein mRNA increased significantly after parturition compared to Day 20 of gestation as expected. The original experiment was not designed to measure and compare  $\beta$ -casein mRNA expression, and it is clear that only very large differences in expression can be detected with the limited number of animals used. Another possibility is that if the metabolic activity was increased due to the release of glucocorticoids in response to the stress of reentry without the concomitant release of prolactin (Prl), then lactogenesis may not have been initiated. Casein mRNA transcription requires insulin and prolactin in the presence of hydrocortisone to maximize expression and stabilize the transcript (17).

It is also possible that, in addition to the mammary gland, overall metabolic activity increased in response to spaceflight. Since the tissues from animals exposed to spaceflight were divided among many investigators, it was not possible to measure metabolic activity in another tissue. While no other investigator performed dynamic measurements of metabolism, Johnson *et al.* (7) observed marked changes in bone density in pregnant animals subjected to spaceflight. These differences were much larger than previously observed with male rats and resembled the type of changes seen in bone during lactation (7, 18). Since the mammary gland is extremely important in calcium regulation during lactation, it is possible although very speculative at this time, that changes in mammary metabolism did occur during spaceflight and also influenced bone metabolism. Further experiments must be conducted to determine wheth-

er the observed changes in metabolic activity represent a response to microgravity or additional environmental stresses including shuttle reentry and landing.

It is clear from our study that either launch and landing or spaceflight has dramatic effects on mammary metabolic activity in pregnant rats. Future studies are necessary to delineate whether alterations in mammary metabolic activity in spaceflight would result in changes in milk composition or nutrients obtained by the pups. Observations made by others (Murakami D, personal communication) indicate that dams subjected to 2g have difficulty caring for their pups. Results from the recent shuttle missions involving lactating rats support the need for continued investigations into understanding the functional role of the mammary gland as well as the maternal behavioral responses to spaceflight.

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