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Oral Tissue Responses to Travel in Space

Maija I. Mednieks and Arthur R. Hand

Abstract

The oral cavity functions in taste, mastication, solubilization and digestion of nutrients, as well as in respiration and speech, and participates in innate and adaptive immunity. Saliva creates and regulates the environment of the oral cavity, and changes in its composition and rate of secretion have significant effects on oral tissues as well as on systemic health. The effects of microgravity on the salivary glands, mandible and teeth were studied in mice flown on US space shuttle STS-131 and STS-135 missions, and the Russian Bion-M1 biosatellite. Significant changes in morphology and secretory protein expression occurred in parotid glands; submandibular glands were affected only on the 30-day Bion-M1 mission, indicating tissue specificity of the effects due to changes in gravity which may be similar to those taking place in humans. Changes also occurred in mandibular bone and incisor teeth. Collection of saliva is a non-invasive procedure for assessing physiological status and diagnosis of several disorders and provides a simple method for monitoring astronaut health during extended spaceflight.

Keywords: salivary glands, mandible, teeth, morphology, protein expression

1. Introduction

The oral cavity is the body's portal for the intake and initial processing of food and liquids, functioning in taste, mastication, solubilization and digestion of nutrients. Additionally, the oral cavity plays a role in respiration and speech as well as in innate and adaptive immunity. The components of the oral cavity include the teeth, consisting of three types of mineralized tissue, and their supporting structures, the bones of the maxilla and mandible and alveolar processes, and the periodontal ligament. The oral mucosa, which covers the alveolar processes, tongue, palate, cheeks, inside of the lips and floor of the mouth, consists of keratinized and non-keratinized epithelium overlying the connective tissues of the lamina propria and submucosa.

Located extraorally are the major salivary glands: the parotid, the submandibular and the sublingual, which convey their product, saliva, to the oral cavity *via* long ducts. Saliva, consisting of water, electrolytes and a large number of proteins, glycoproteins and small organic substances, creates and regulates the environment of the oral cavity. Changes in composition and rate of secretion of saliva have significant effects on oral tissues as well as on general health. In addition to the major glands, numerous small minor salivary glands are present in the subepithelial connective tissue throughout much of the oral cavity.

Among the organic constituents of saliva are digestive enzymes, calcium binding proteins, a variety of growth factors and regulatory molecules, antimicrobial components and immunoglobulins, and mucins that lubricate and moisten the oral tissues [1]. Other protective components of saliva include those involved in buffering and neutralizing acids produced by oral microorganisms and ingested with food and drink. Most are produced and secreted by the cells of the salivary glands, nevertheless a number of other proteins derived from other cells, tissues and organs also find their way into saliva. The presence of these substances and the ease of, and the noninvasive means of, collecting saliva have led to a great deal of interest for its use as a diagnostic fluid. Consequently, significant progress has been made in using saliva to detect oral and other cancers, several oral and systemic diseases, and to assess physiological and environmental stressors [2].

Our early studies of rats flown on Spacelab-3 [3, 4] indicated that exposure to microgravity resulted in specific changes in salivary gland structure and biochemistry. Accordingly, the premise for our recent studies of mice flown on the US space shuttles and the Russian Bion-M1 biosatellite, was that changes in the expression of salivary gland secretory proteins appear to occur in microgravity, and that the pattern of changes detectable in saliva could be employed to assess important aspects of the physiological status of astronauts.

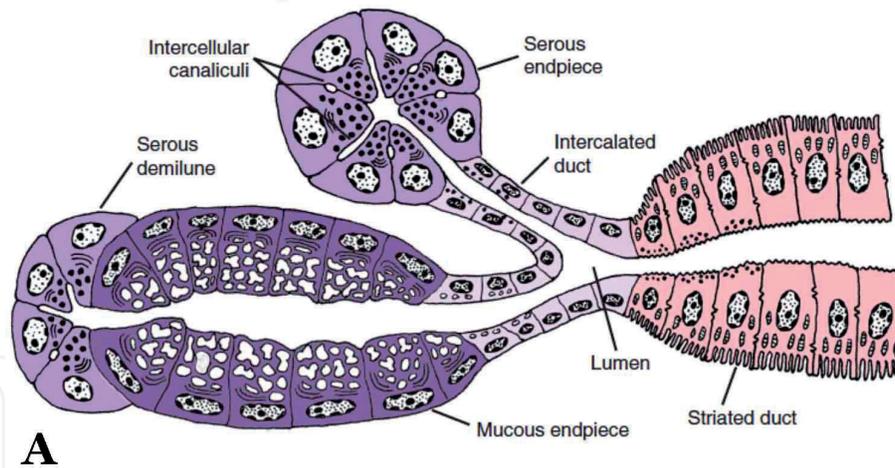
In addition to studying salivary gland tissues, we examined the effects of microgravity on the mandibles and teeth of mice flown in space. Numerous studies have been carried out documenting the effects of spaceflight on bones of the weight-bearing skeleton, especially the bones of the lower limbs and vertebrae (see reviews in [5–7]). However, only a few studies have focused on non-weight-bearing bones, such as the mandible and cranial bones, and teeth [8, 9]. Teeth, once formed, are relatively stable structures, but can be affected by changes in the oral environment. An understanding of the effects of microgravity on tooth development, which can be studied using the continuously erupting rodent incisor, is important especially in view of future possibilities of long-term space journeys and colonization of other planets.

2. Effects of spaceflight on salivary glands

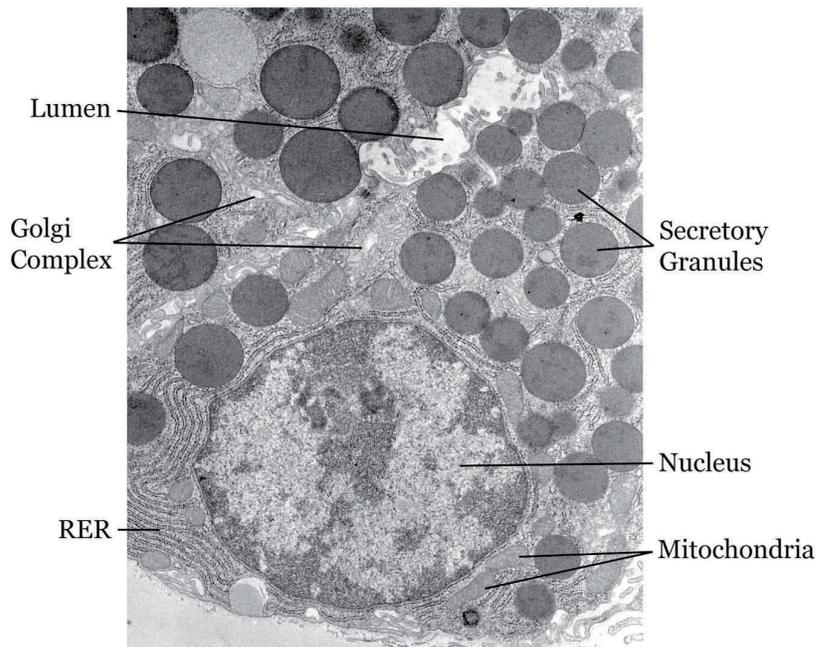
2.1 Morphology

The parenchyma of salivary glands consists of a collection of secretory end-pieces, or acini, connected to a ductal system that conveys saliva to the oral cavity [10, 11] (**Figure 1A**). The acini produce the primary saliva containing the majority of the proteins, most of the electrolytes and all of the water present in the final saliva. Serous acinar cells (**Figure 1B**) produce proteins and glycoproteins; seen in transmission electron micrographs (TEMs) the content of their secretory granules has a moderate to high density. Mucous acinar cells produce mainly mucins; their secretory granules exhibit a low density and often are fused with adjacent granules. The acinar secretory cells form a roughly spherical structure, their bases resting on the interstitial connective tissue, their apices facing a central lumen, and adjacent cells are joined by junctional complexes separating the interstitial from the luminal compartments. The cells contain abundant rough endoplasmic reticulum (RER), a prominent Golgi complex, and numerous secretory granules in the supranuclear cytoplasm. Secretory proteins synthesized by the cells are stored in the granules until their contents are released into the lumen by exocytosis. Consequently, analysis of the glandular tissue provides information about the composition of saliva.

Saliva produced in the acini flows into the initial portion of the ductal system, the intercalated ducts. These small ducts merge, forming larger ducts that



A



B

Figure 1.
 (A) Diagram showing the main parenchymal components of mammalian salivary glands. (B) Transmission electron micrograph (TEM) of a serous acinar cell of a rodent parotid gland (PG) (modified, with permission, from ([10], Chapter 11, pp. 224, 225)).

eventually empty into the main ductal component, the striated ducts. The cells of these ducts are columnar in shape, and have abundant mitochondria nestled between infoldings of the basal cell membrane. Primary saliva produced by the acinar cells is modified by the striated (and excretory) ducts principally by reabsorption of Na^+ and Cl^- and addition of a few proteins; the final saliva is hypotonic with a pH near neutrality. The striated ducts become excretory ducts as they enter the connective tissue septa that partition the gland into lobules and lobes. The excretory ducts gradually merge into larger ducts and finally form the main excretory duct that empties into the oral cavity.

In humans, the acinar cells of the parotid gland (PG) are all serous. The submandibular gland (SMG) is a mixed gland, with mostly serous acini but also some mucous acini that are capped by serous cells forming a structure called a demilune. The sublingual gland (SLG) also is a mixed gland; its acini are predominantly mucous with serous demilunes. The acini and ductal systems of the rodent PG and SLG are very similar to those of the human (**Figure 2**). The structure of the rodent SMG (**Figure 3**) differs significantly from that of the human. The acini in rodents are all seromucous and produce a number of proteins and glycoproteins as well as mucins. As the animals become sexually mature, the cells of first portion of the striated ducts

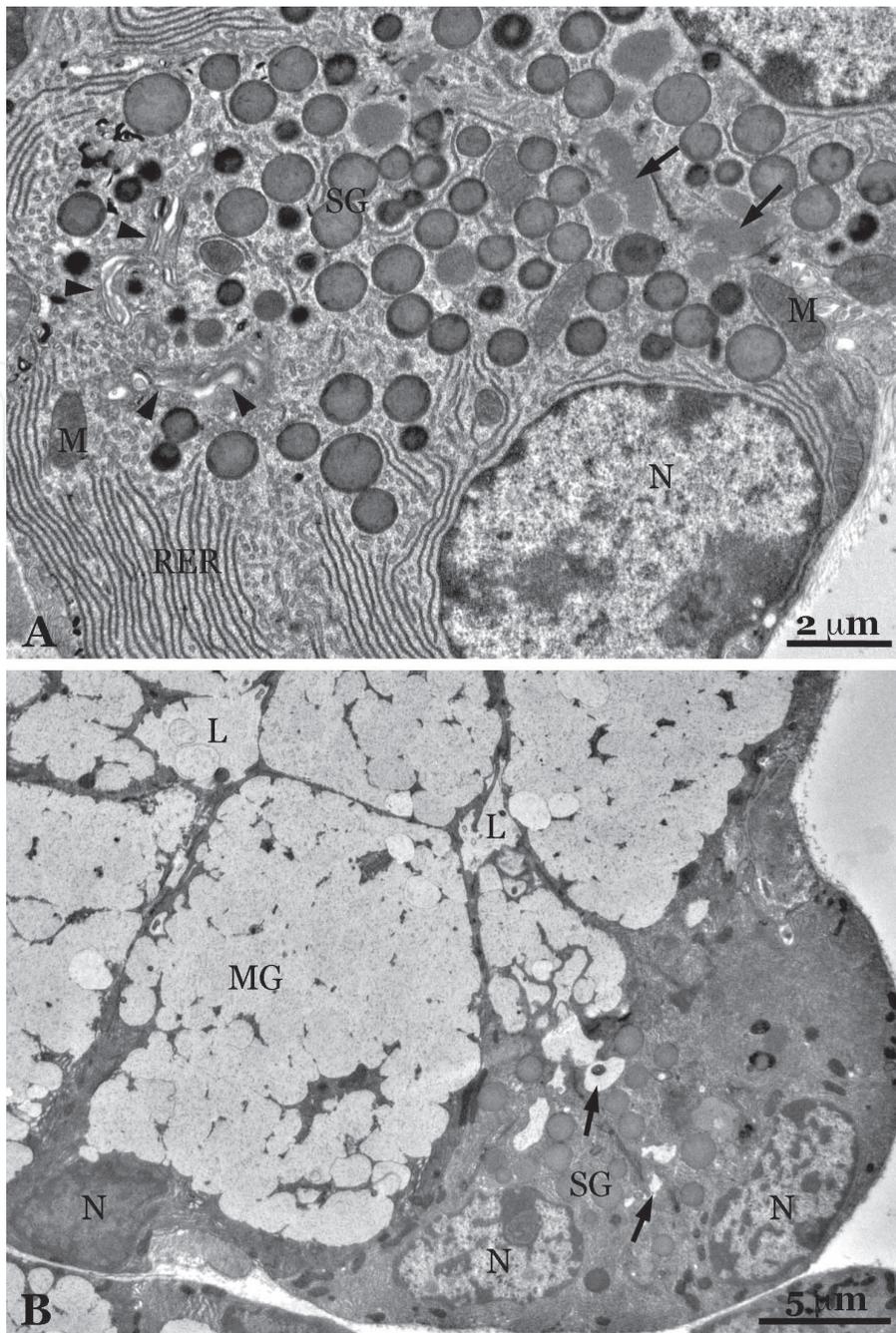


Figure 2. TEMs of (A) PG serous acinar cells from a STS-135 flight mouse, and (B) sublingual gland (SLG) mucous acinar cells and serous demilune cells from a STS-131 habitat ground control mouse. Mucous acinar cells are filled with electron lucent mucous granules (MG). Serous demilune cells (lower right) contain electron dense secretory granules (SG). Lumen (L), mitochondria (M), nucleus (N), rough endoplasmic reticulum (RER), Golgi complex (arrowheads), intercellular canaliculi (arrows).

enlarge and synthesize a number of growth factors and proteases that are stored in large secretory granules in the apical cytoplasm. The development of this portion of the duct, called the granular convoluted duct, is strongly influenced by androgens; the granular convoluted duct is, therefore, more prominent in males than in females. Another feature of the sexual dimorphism seen in the rodent SMG is the presence in females of terminal tubule cells, or granular intercalated duct cells, at the acinar-intercalated duct junction. These cells are remnants of the early development of the gland; in males they are entirely eliminated by apoptosis by about 1 month of age.

Specific morphological changes were seen in the PG after a 13- to 15-day flight on the STS-135 and STS-131 shuttle missions, and 30 days on the Bion-M1 biosatellite [12, 13]. In the acinar cells autophagic vacuoles were common, and apoptotic

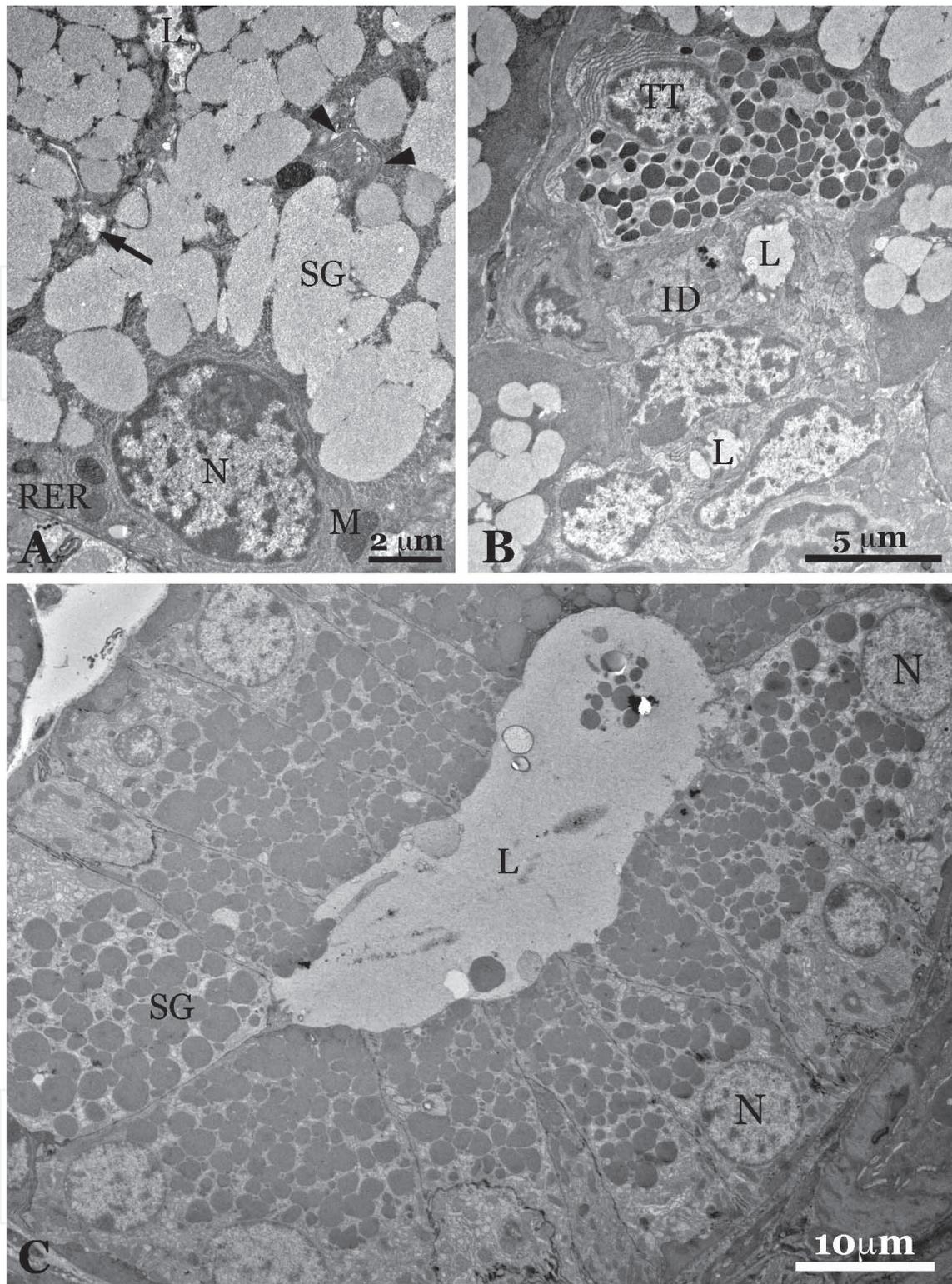


Figure 3. TEMs of submandibular gland (SMG). (A) seromucous acinar cell from a STS-131 flight mouse, (B) intercalated duct and terminal tubule cell from a STS-131 habitat control mouse, and (C) granular convoluted duct from a Bion-M1 flight mouse. Acinar cells have a basal nucleus (N) and RER, and large secretory granules (SG) with a granular content of low density. In female mice, terminal tubule cells (TT) often are present in the intercalated ducts (ID) adjacent to the acinar cells. Columnar shaped granular convoluted duct cells of the male gland have a basal nucleus and mitochondria, and numerous dense secretory granules in the supranuclear cytoplasm. Lumen (L), mitochondria (M), Golgi complex (arrowheads), intercellular canaliculus (arrow).

cells were seen more frequently (**Figure 4A**). The autophagic vacuoles often contained degenerating secretory granules as well as other organelles. Some cells in the intercalated and striated ducts had large endocytic vacuoles containing dense content in the apical cytoplasm (**Figure 4B**). Immunogold labeling showed the presence of acinar secretory proteins in these vacuoles.

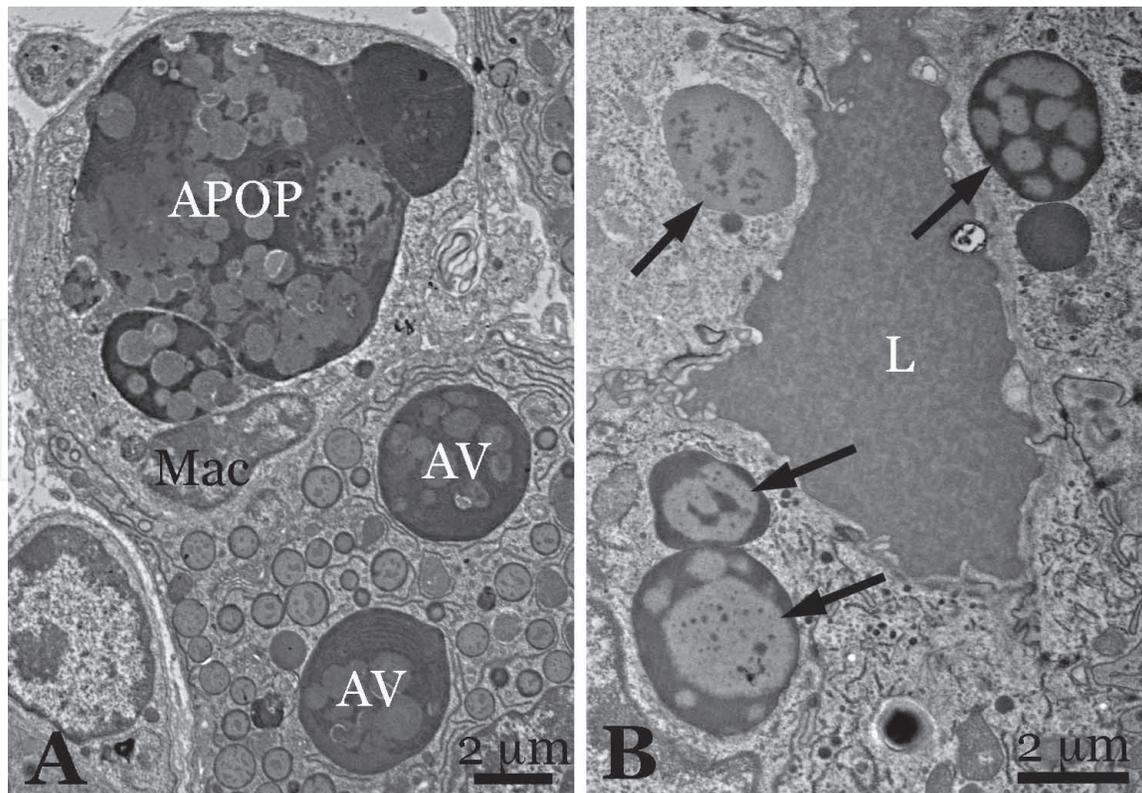


Figure 4. TEMs of PG from STS-135 flight mice. (A) An acinar cell contains 2 autophagic vacuoles (AV), and a macrophage (Mac) has engulfed an apoptotic acinar cell (APOP). (B) Intercalated duct cells contain apical vacuoles (arrows) resulting from endocytosis of secretory proteins from the lumen (L) (with permission, from [13]).

The underlying basis for these changes is believed at least in part to be the loss of neural stimulation due to reduced masticatory activity. This is indicated by experiments where withholding food for 24–48 h, or feeding rats a liquid diet, results in numerous autophagic vacuoles containing degenerating secretory granules and an increase in the number of apoptotic cells in the PG [14–17].

The mice from the Bion-M1 mission all gained weight during the flight but were fed a soft paste diet. The mice from the space shuttle missions had *ad libitum* access to NASA rodent food bars [18], but lost a small amount of weight, as did the habitat control mice (maintained on the ground for the same length of time in the same habitats as the flight mice). This suggests that the mice on the space shuttle flights may not have eaten the amount of food sufficient to maintain their body weight. Since the number of autophagic vacuoles and frequency of apoptosis was greater in flight mice than in the habitat controls, microgravity also appears to affect these processes.

Endocytosis of salivary proteins by duct cells occurs in experimental diabetes [19, 20]. This suggests that the proteins may have an altered structure that is recognized as foreign by the duct cells; these cells are capable of endocytosing foreign proteins introduced in a retrograde fashion *via* the main excretory duct [21–23]. Alternatively, duct cell function in the flight mice may have been altered.

In contrast to the PG, no morphological changes were evident in the SMG or SLG of the flight mice. Similarly, feeding a liquid diet has no apparent effect on the morphology of the rat SMG and SLG [24].

2.2 Salivary protein expression studied using two major approaches

2.2.1 Electron microscopic immunogold labeling

Relative changes in secretory protein expression can be analyzed by electron microscopic immunogold labeling. Determination of the labeling density (gold particles/ μm^2)

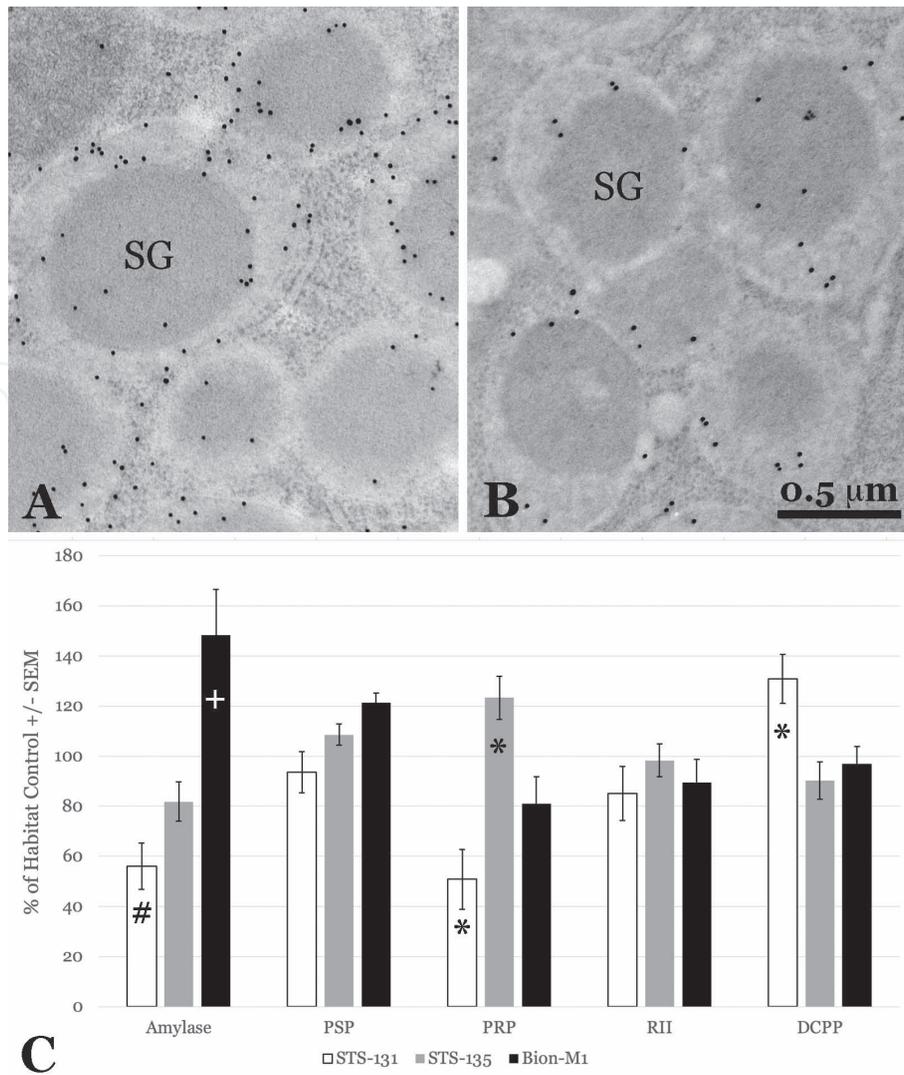


Figure 5. TEM immunogold labeling for proline-rich protein (PRP) in PG acinar cells of (A) flight and (B) habitat ground control mice from STS-135. Secretory granules (SG). (C) Quantitative TEM immunogold labeling of secretory proteins in PG of mice flown on STS-131 and STS-135, and the Bion-M1 biosatellite. α -Amylase, parotid secretory protein (PSP), PRP and the type II regulatory subunit of protein kinase A (RII) are present in acinar cell secretory granules; demilune cell and parotid protein (DCPD) is present in intercalated duct cell secretory granules. Labeling results are shown as a percentage of the corresponding habitat control mice \pm SEM. +, $p = 0.065$; *, $p < 0.05$; #, $p < 0.01$.

of secretory granules of PG acinar and duct cells showed alterations in the expression of several salivary proteins following spaceflight [12, 13] (Figure 5).

Compared to their habitat controls, expression of α -amylase, a digestive enzyme, was decreased in the parotid glands of mice flown on the space shuttles, but increased in mice flown on the Bion-M1 biosatellite. Parotid secretory protein (PSP), an antimicrobial protein [25, 26], was slightly increased in mice from the Bion-M1 flight. Proline-rich protein (PRP), a calcium and polyphenol binding protein [27, 28], was decreased in mice from STS-131, increased in mice from STS-135, and decreased, but not significantly, in mice from the Bion-M1 flight. The type II regulatory subunit of protein kinase A (PKA-RII), a stress marker secreted into saliva [29], was slightly decreased in mice from the 3 flights. Demilune cell and parotid protein (DCPD), secreted by intercalated duct cells and believed to have antimicrobial activity [30], was increased in mice from the STS-131 flight, but was unchanged in mice from STS-135 and Bion-M1 flights.

PKA-RII also is present in the nucleus, where it is involved in regulating several genes [31, 32]. No significant differences were seen in nuclear PKA-RII in parotid acinar cells of mice from the 3 flights (Figure 6).

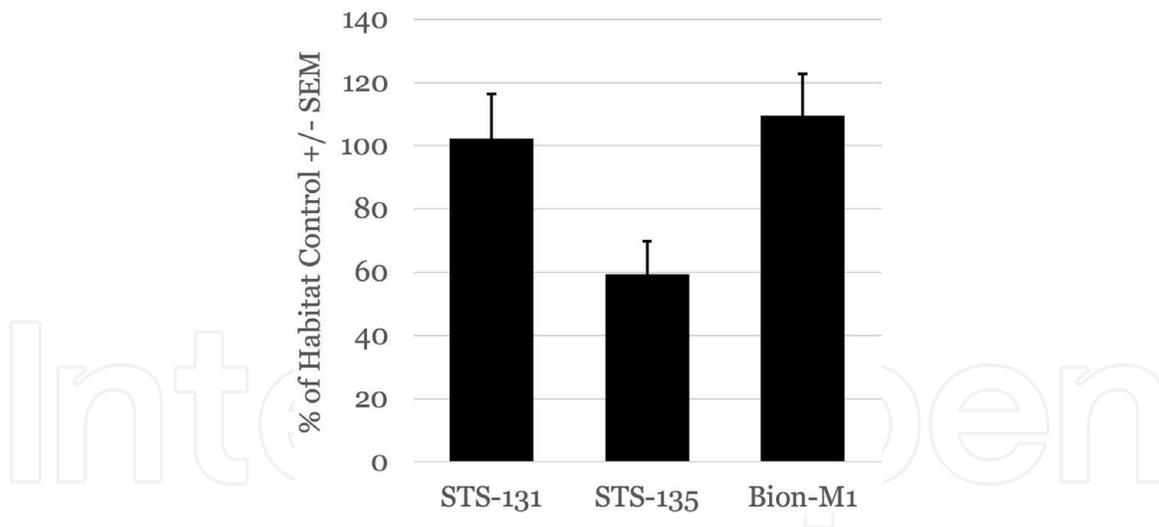


Figure 6.

Quantitative TEM immunogold labeling of nuclear PKA-RII in PG of mice flown on STS-131 and STS-135, and the Bion-M1 biosatellite. Labeling results are shown as a percentage of the corresponding habitat control mice ± SEM.

	STS-131	STS-135	Bion-M1
SABPα	114.6 ± 12.1	88.9 ± 26.3	221.9 ± 31.7*
PRP	111.1 ± 13.0	76.6 ± 9.0	134.4 ± 11.5
EGF	87.7 ± 12.4	36.3 ± 6.0	159.9 ± 4.6*
NGF	116.3 ± 23.3	89.9 ± 5.1	159.0 ± 10.6*
SMGC	83.0 ± 9.3	103.4 ± 5.3	—

Labeling results are shown as a percentage of the corresponding habitat control mice ± SEM.
 $p < 0.01$.

Table 1.

Quantitative TEM immunogold labeling of secretory proteins in SMG of female mice flown on STS-131 and STS-135, and male mice flown on the Bion-M1 biosatellite.

The expression of SMG secretory proteins was essentially unchanged in mice from the 13–15-day space shuttle flights. However, mice from the Bion-M1 flight showed significantly increased expression of an acinar cell protein, salivary androgen binding protein alpha (SABPα), a pheromone involved in mate selection [33], and the granular convoluted duct cell proteins epidermal growth factor (EGF) and nerve growth factor (NGF) (**Table 1**). PRP, present in acinar cell secretory granules of both sexes, and submandibular gland protein C (SMGC), present in terminal tubule cell secretory granules of female mice, were not significantly different from controls. In the SLG, PSP expression by demilune cells was significantly increased in mice from the space shuttle STS-131 flight, but not in mice from STS-135 [34]. The expression of both PKA-RII in demilune cells, and the acinar cell mucin Muc19, were increased, but not significantly.

2.2.2 Electrophoresis and Western blotting

Polyacrylamide gel electrophoresis (PAGE) is the separation of proteins based on mobility on a gel matrix, subjected to a charge differential between two sealed chambers, of a body fluid or tissue extract. This technique is frequently used to determine the distribution or banding pattern of protein groups in tissues under different conditions. Quantification of the intensity of the bands and their relative mobility by PAGE is carried out by densitometric analysis. Saliva is a convenient

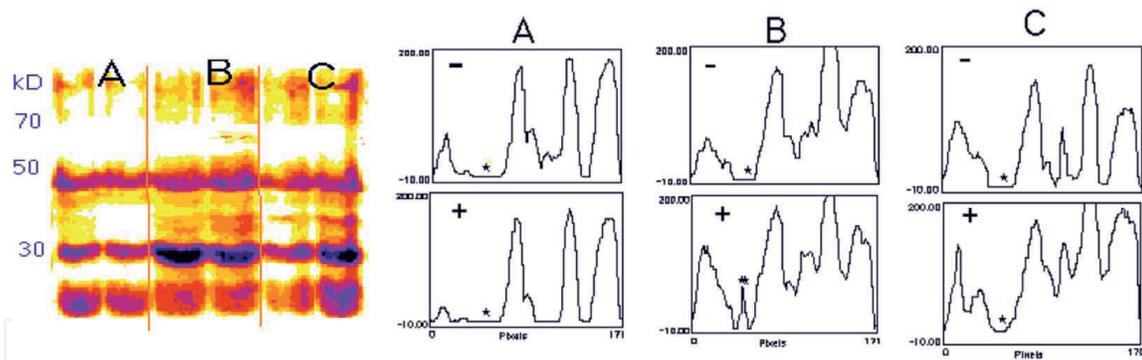


Figure 7. Electrophoresis and densitometry of saliva from three randomly selected individuals (A, B, and C) from a pool of approximately 50 test subjects. The left ordinate on the gel pattern image shows molecular based migration rates of a mixture of proteins of known size. At the right of the polyacrylamide gel electrophoresis (PAGE) banding patterns are the corresponding densitometric measurements. The top row (-) prior to, the bottom row (+) after, chewing. The asterisks show the migration distance of RII that can be identified by Western blotting.

body fluid for measuring a variety of physiological conditions or responses on the basis of variations in protein content or distribution. For example, after testing more than 50 samples, PAGE separation of salivary proteins has shown that each individual pattern is unique. Namely, no superimposable patterns were found, therefore constituting essentially a salivary protein pattern fingerprint. Shown at the left in **Figure 7**, in duplicate, are the patterns of three (A, B, C) randomly selected individuals with the corresponding densitometric profiles at the right. **Figure 7** also shows how normal chewing changes the amplitude of several protein peaks, but not the basic pattern.

The PAGE protein banding patterns of the STS mission samples showed a decrease of some of the higher molecular weight bands while the faster moving smaller bands increased when these were compared to vivarium controls. The changes were not consistent between missions and may in part be due to lack of stability of the tissue or of sample preparation.

Western blotting is a technique using antibodies generated to a specific protein for its identification among numerous others in the banding pattern. Proteins are transferred onto nitrocellulose membranes and stained with a washable dye to show the band distribution, then quantified by densitometry. The dye is removed and the membrane probed for reactivity with a specific antibody. These techniques were employed to test salivary gland extracts to measure protein patterns of flight animal samples when compared with those of ground control animals (**Figure 8**).

The results show that the RII peaks in the STS-135 flight samples (**Figure 8**) were significantly smaller than the corresponding band amplitude in either vivarium or habitat controls, middle and bottom profiles, respectively. Western blotting experiments to determine the expression of α -amylase show a significant decrease in the flight and in the habitat controls.

These results indicate that on the shorter, STS-135 flight the expression of RII was decreased, while in the longer Bion-M1 flight (**Figure 9**) an apparent stabilization or adjustment to the microgravity environment had occurred and the RII levels were not different, perhaps even increased from those of either of the controls. Both Bion-M1 and STS-135 flight and both control samples show a significant, but opposite change of a faster moving band that may represent a small protein or peptide.

The findings regarding RII, generally thought to be a stress associated protein [35, 36] responding to the conditions of spaceflight [7], varied by methods of analysis but also between flights within a particular test measure from morphometric analyses of immunogold labeling experiments. Bion-M1 Western blotting results showed no difference between RII in Bion-M1 flight and vivarium control

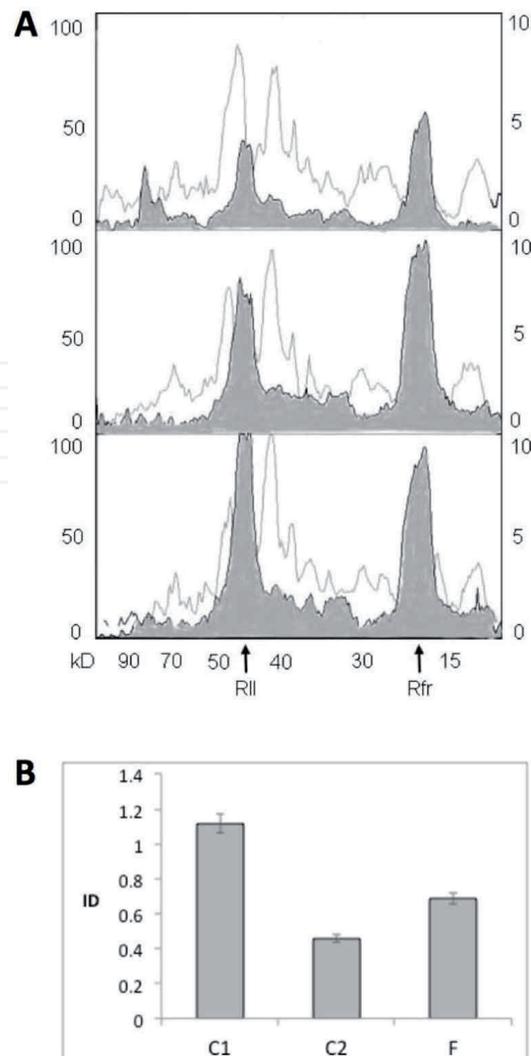


Figure 8.

Densitometric analysis of PAGE protein separation and anti-RII and anti- α -amylase reactivity in STS-135 mice. (A) PG protein samples from mice housed in, top panel, flight; middle panel, habitat; and bottom panel, vivarium type cages. The lighter curve represents the protein profile; the shaded area is the reactivity to anti-RII antibody. The ordinate axes are integrated density values of the proteins on the left-hand axis, and the integrated density values for RII and the reactivity of RII fragment (Rfr) on the right-hand axis. (B) PG α -amylase Western blotting. The ordinate shows integrated density, and the error bars show $\pm 5\%$ error. C1, vivarium control; C2, habitat control; F, flight. The values on the ordinate axis (ID) are integrated density in arbitrary units (with permission, from [13]).

mice. Measuring RII, therefore, may be a sensitive test for stress reactions during spaceflight.

The variability may be due to the sensitivity of responses affecting RII synthesis. Previous studies point to a rapid and transient increase in the cases of brief acute stress, for example lift off of a spacecraft, followed by long-term, perhaps low level, prolonged time under stress, decrease. For example, a 60-day to a year tour resulting in a decrease of RII and leveling off at concentrations below normal. Eventual physiologic adjustment to flight conditions may start to bring RII production toward normal. An apparent increase also may result from a rough landing that might have occurred in the Bion-M1 flight and the acute response obliterating any reduction due to chronic long-term flight conditions.

Tissue extract samples showed a large faster moving component, presumably an RII fragment. The fragments did resemble the densitometric changes in the flight versus the control. These differences did not completely account for the change of flight values compared to the control. There may have been smaller fragments not visible on the Western blot that could account for the decreases in flight RII.

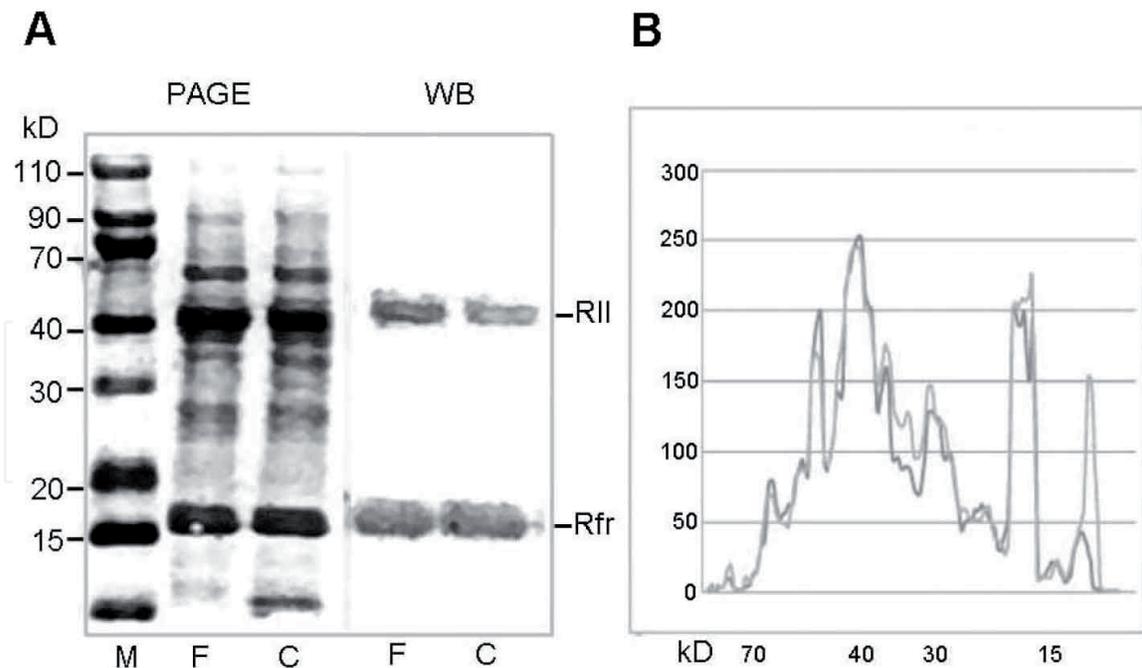


Figure 9. Electrophoresis and Western blotting of flight (F) and control (C) PG extracts from mice of the Bion-M1 mission. (A) Protein banding patterns of the flight and control animals (F and C). The left lane (M) shows the relative mobilities of marker proteins of kilodalton (kD) size from 110 to 15 marked on the ordinate. Western blotting for RII is shown in the two right most lanes with two major bands, the first at an estimated 50 kDa size (RII), the second deemed to be a fragment between 14 and 20 kDa (Rfr). (B) densitometric profile of the control banding pattern which was not distinguishable from that of the flight (with permission, from [13]).

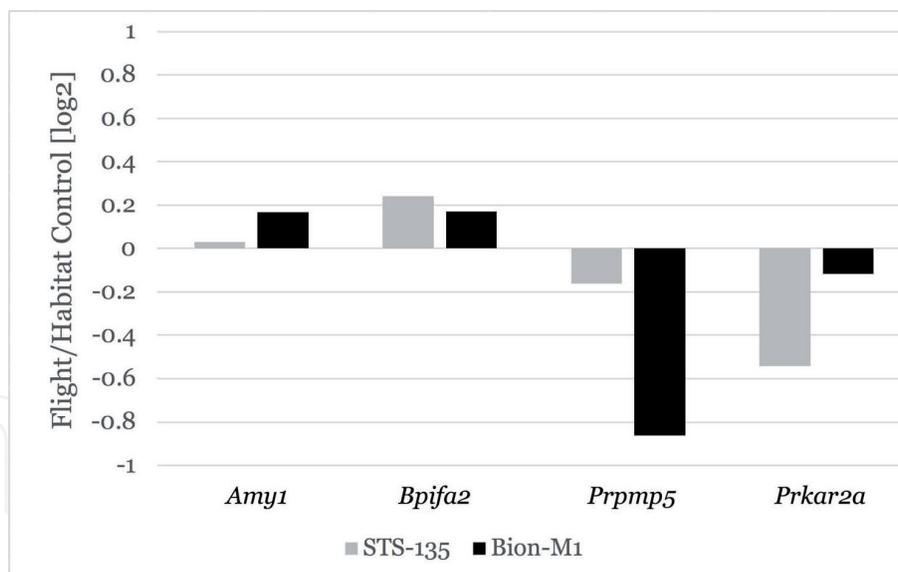


Figure 10. Microarray analysis of secretory protein gene expression in PG of mice flown on STS-135 and the Bion-M1 biosatellite. Results are expressed as log₂ of the ratio of expression in flight mice to that in habitat control mice. Amy1, α -amylase; Bpifa2, PSP, Prpmp5, PRP; Prkar2a, RII.

2.3 Gene expression

Microarray analyses of the PG showed significant changes in the expression of numerous genes of flight mice compared to habitat ground control mice. In female mice flown on STS-135, the expression of 130 genes was significantly upregulated (\log_2 ratio ≥ 1), and 75 genes were downregulated (\log_2 ratio ≤ -1), (Figure 10). In male mice flown on Bion-M1, the expression of 70 genes was upregulated, and 65 genes were downregulated.

Interestingly, significant changes in gene expression occurred in the PG of habitat ground control mice compared to vivarium housed mice. In ground control female mice housed in the NASA Animal Enclosure Modules, the expression of 50 genes was significantly upregulated, and 33 genes were downregulated. For example, Western blotting of PG of STS-135 habitat control mice showed a decrease in the expression of α -amylase (**Figure 8B**). In ground control male mice housed in the Bion-M1 habitats, the expression of 42 genes was significantly upregulated, and 169 genes were downregulated. These results indicate that the effects of the habitats must be considered when assessing the overall effects of spaceflight on animal (as well as human) physiology.

Changes in the expression of secretory protein genes (**Figure 10**), for example, PKA-RII, largely paralleled the changes seen in protein expression as determined by immunogold labeling and Western blotting (e.g., PKA-RII expression in PG from STS-135 flight mice, **Figure 8B**).

There have been a few previous studies of salivary glands of rodents following spaceflight, and saliva collected from astronauts and cosmonauts before and after spaceflight. No changes in PG morphology were seen by light microscopy in mice flown for 12.5 days on Apollo 17 [37]. The SMG of rats flown for 18.5 days on Cosmos 936 and Cosmos 1129 showed a reduced glycoprotein content as determined by light microscopic histochemical staining [38, 39]. The saliva of 18 astronauts making up the primary and backup crews for Skylab missions of 28-, 59- and 84-days was analyzed preflight and postflight for flow rate, electrolytes, protein, immunoglobulin A (IgA) and lysozyme [40]. Changes were minimal except for an increase in IgA and a decrease in lysozyme concentrations. α -Amylase in cosmonaut saliva was decreased following an 18-day flight (uncited statement in [41]). The use of saliva for monitoring drug absorption was proposed, and saliva levels of acetaminophen were determined in 1 astronaut [42]. Analyses of saliva of cosmonauts during long-term spaceflight showed levels of cortisol below baseline [43], whereas salivary cortisol biorhythms and concentrations were preserved in astronauts onboard Spacelab [44] and cosmonauts on the International Space Station (ISS) [45]. Transient increases in saliva urea and phosphate concentrations, monitored in 2 astronauts during 6 months on the ISS, were consistent with serum concentrations reported in earlier studies [46]. Although studied in astronaut plasma, not saliva, in the NASA Twin Study, EGF and NGF levels were altered during a 340-day flight and after landing [47]. Our studies and those cited above indicate the feasibility of using saliva to monitor astronaut physiology and health.

3. Effects of spaceflight on the mandible

3.1 MicroCT analyses

MicroCT was used to assess mandibular bone volume (BV) and bone mineral density (BMD) in mice from the STS-135 and Bion-M1 flights [48].

In the STS-135 mission no differences in BV or BMD were seen between the flight and habitat ground control mice. However, when compared to vivarium control mice, BV was greater in both flight mice and habitat ground control mice. Similarly, BMD was greater in both the flight and habitat control mice than the vivarium control mice, although only the habitat control values were statistically significant. The increased BV and BMD seen in the flight and habitat control mice may be due to the difference in composition and consistency of the NASA food bars [18] these mice consumed compared to the standard rodent chow eaten by the vivarium control mice. The food bars have about 20% less caloric value per gram than chow, requiring greater consumption and more masticatory effort for comparable nutrition.

In contrast, mandibular BV decreased in both flight and habitat control mice compared to vivarium control mice from the Bion-M1 mission [48]. No differences occurred in BMD among these three groups, however.

These changes in flight and habitat control mice are likely due to the difference in diet between these two groups and the vivarium mice [49, 50]. The soft paste diet consumed by the Bion-M1 flight and habitat control mice required considerably less masticatory force than chow. Previous studies have shown that reduced mechanical loading results in decreased growth of the mandible [51, 52]. The different age and sex (9 week old females vs. 15–16 week old males, respectively) of the STS-135 and Bion-M1 mice also may have contributed to the different results for BV and BMD. The STS-135 mice were skeletally immature [53, 54], and female mice are physically more active than males [55] and have a different pattern of chewing and biting activity [56].

Earlier studies examined the effects of spaceflight and simulated weightlessness on mandibular bone. Periosteal osteogenesis was reduced in areas of the mandible not covered by muscle (molar region) in rats flown for 18.5 days on Cosmos 1129 [57]. Formation of alveolar bone was reduced on the modeling (mesial or anterior) side of the first molar, indicating a slowing of the normal distal (posterior) drift of rodent molars. There also was a decrease in alveolar bone mineral and collagen in fractions representing the most mature components, with a corresponding increase in the most immature fractions, suggesting a delay in maturation. The mandibles of rats flown for 12 days on Cosmos 1887 had relatively high Ca and Mg levels but otherwise a normal composition, although their hydroxyapatite crystals were smaller in size [58].

Studies of rodents subjected to hindlimb unloading (HU) for up to 4 weeks have been used as model of exposure to microgravity [5, 59, 60]. Although the effects on the spine and hindlimbs show similarities to changes seen in animals after spaceflight, effects on the mandible do not mimic those occurring after actual spaceflight. Ten to 14 days of HU did not alter maturation of mandibular bone matrix and mineral as seen in rats flown on Cosmos 1129 [61]. Similarly, 15 days of HU did not alter the total weight, ash weight, Ca content or Ca uptake of the mandible [62]. No significant effects on the mandible were seen after 28 days of HU [63]. An earlier study, however, found an increase in the dry and ash weights of the mandible after 3 weeks of HU [64]. In HU, the mandible still closes against gravity [60], thus spaceflight induced changes in the mandible are most likely a result of hypogravity [61].

Ghosh et al. [8] studied mandibles from mice flown on STS-131 and STS-135 (from the opposite side of the same STS-135 mice we studied [48]). They also found no difference in BV between the flight and habitat ground control mice from the 13-day STS-135 mission. However, mandibular BV was decreased in flight mice from the 15-day STS-131 mission. It is unlikely that the 2-day difference in flight length would result in a significant difference in BV. As noted by Ghosh et al., it is more likely that other factors are involved, such as the age difference and resulting skeletal maturation of the mice on the two flights (23 weeks vs. 9 weeks for STS-131 and STS-135, respectively), slight differences in food consumption, and/or the relatively low statistical power due to the small number of animals. They found no difference in BMD between the flight and habitat ground control mice from the STS-131 mission, but similar to our results, BMD was increased in the flight mice from the STS-135 mission. The factors mentioned above also could contribute to the differences in BMD seen between the mice on the two shuttle flights. The difference in BV between the Ghosh et al. study and our results for the STS-135 mice may be due to the different methods used to assess BV. Ghosh et al. measured BV in a single section (6 μm) from each mandible. In our study the results from 10 serial sections (120 μm span) per mandible were averaged, providing a more representative measurement.

3.2 Bone protein expression

The expression of the bone proteins sclerostin and osteocalcin in the mandibles of mice from the STS-135 and Bion-M1 missions was studied by immunohistochemistry (IHC) [48] (**Figure 11A–C**). Sclerostin is secreted by osteocytes and other cells embedded in mineralized tissues; it inhibits osteoblast proliferation and differentiation and induces osteoblast apoptosis [65, 66]. No differences in the intensity of sclerostin staining of osteocytes were observed between the flight mice and their respective controls in either mission. The percentage of reactive osteocytes, however, varied between the 2 missions and among the 3 groups and locations within the mandible. Overall, the percentage of reactive osteocytes was greater in the STS-135 mice than in the Bion-M1 mice, and greater in flight mice than in control mice. The alveolar bone anterior to the first molar and the interradicular bone of the first molar showed the greatest percentage of reactive osteocytes, whereas the septal bone between the first and second molars had the lowest percentage.

Sclerostin synthesis is increased by mechanical unloading (i.e., rodent HU, human bed rest) [67, 68]. However, the percentage of sclerostin-positive osteocytes was unaltered in male mouse tibiae after 3 or 7 days of HU [67]. Sclerostin-positive

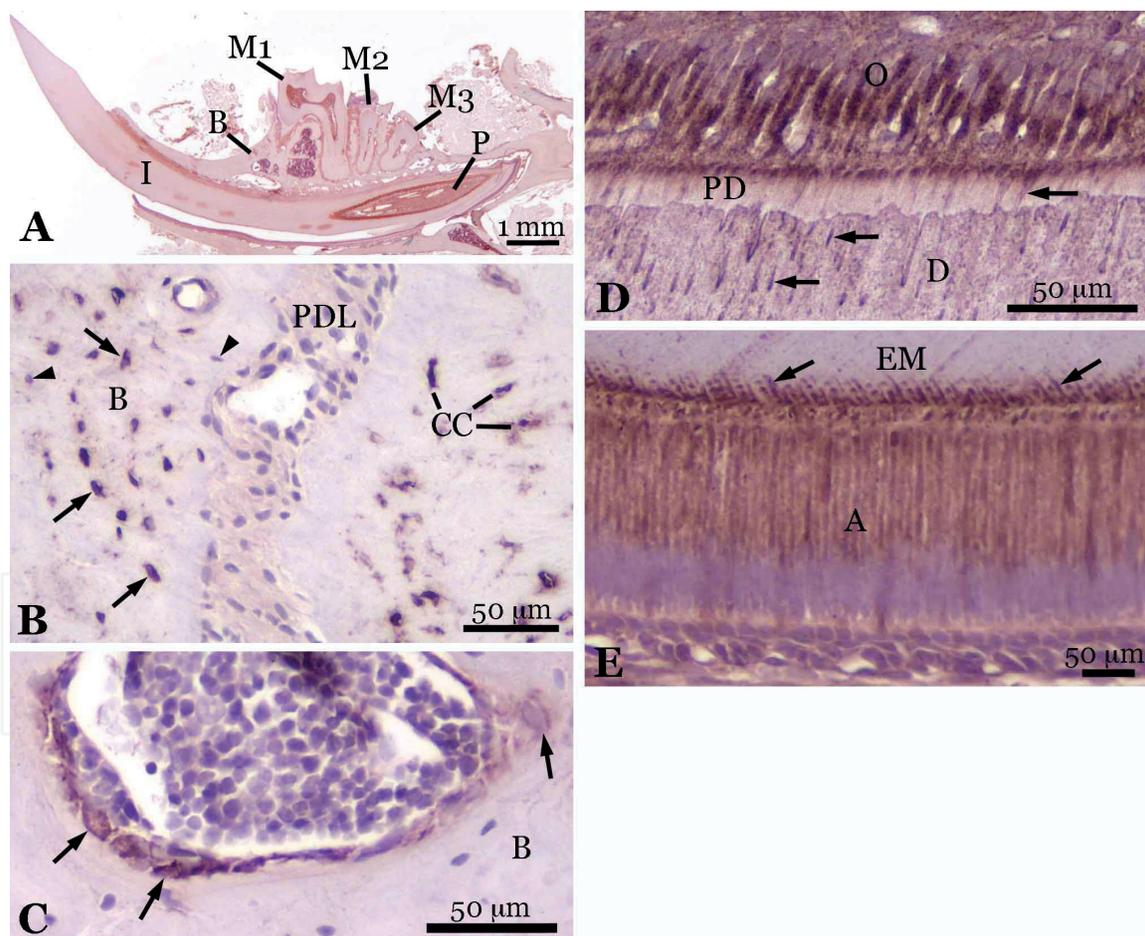


Figure 11.

Immunohistochemistry of mandibular bone and incisor teeth. (A) Sagittal section of a hemimandible from a Bion-M1 habitat control mouse, immunostained for osteocalcin. Bone (B), incisor (I), molars (M1-3), pulp (P). Note staining of odontoblasts lining the pulp chamber. (B) Sclerostin staining of osteocytes (arrows) in interseptal bone of a Bion-M1 flight mouse. A few osteocytes are unstained (arrowheads). Cementocytes (CC) in the cementum covering a molar root also stain for sclerostin. Periodontal ligament (PDL). (C) Osteocalcin staining of osteoblasts (arrows) of a Bion-M1 vivarium control mouse. (D) Immunostaining of dentin sialoprotein in odontoblasts (O) of a STS-135 flight mouse. Odontoblast processes (arrows) in predentin (PD) and dentin (D) are stained. Predentin and dentin stain less intensely. (E) Amelogenin staining of secretory ameloblasts (A) of a STS-135 vivarium control mouse. Tomes' processes of ameloblasts (arrows) are stained intensely. Enamel matrix (EM).

osteocytes increased in male rat femurs after 28 days of HU [69], and partial weight bearing (1/6G) for 21 days also increased their percentage in female mouse femurs [70]. No significant changes in serum sclerostin levels of astronauts and cosmonauts were observed after 4–6 months on the ISS [71, 72]. In the NASA Twins Study, a trend toward increased serum sclerostin levels was seen throughout the 340-day stay on the ISS [47].

Spaceflight also resulted in increases in the size of osteocyte lacunae, expression of matrix metalloproteinase (MMP) activity, and the percentage of osteocytes expressing tartrate-resistant acid phosphatase (TRAP) in pelvic bones of STS-131 flight mice, indicating osteocytic osteolysis [73]. Empty lacunae indicating osteocyte death doubled in number in the femoral cortex of Bion-M1 flight mice compared to vivarium and habitat control mice [74].

Osteocalcin is a non-collagenous bone matrix protein produced by osteoblasts (and odontoblasts). It binds calcium and has long been thought to regulate mineralization [75, 76]. More recently osteocalcin has been recognized as a major bone hormone that participates in the regulation of energy metabolism, brain development and cognition, and male fertility [77, 78]. No differences were observed in the IHC staining intensity of osteoblasts, bone matrix or osteoid between the flight mice and their respective controls from the STS-135 and Bion-M1 missions [48].

Osteocalcin synthesis in rat long bones and vertebrae decreased after short-term spaceflight (4–14 days) [79–82]. Serum and urinary osteocalcin levels increased in cosmonauts during 1 month and 6 months spaceflight [83–85]. A trend toward increased serum osteocalcin levels was seen in astronauts and cosmonauts after 4–6 months on the ISS [72], and during a 340-day flight on the ISS [47]. These findings are consistent with decreased bone formation and increased bone resorption. The lack of apparent change in osteocalcin staining in the mandible, along with the lack of or small changes in bone volume, suggests that the continued mastication of food prevents significant bone resorption.

Osteocalcin gene and protein expression also have been studied in cultured osteoblasts or osteoblast-like cells flown in space. Production of osteocalcin message after 9 days of microgravity by human osteosarcoma cells stimulated with vitamin D3 and transforming growth factor beta was only 19% of that of similarly treated cells at unit gravity [86]. The steady-state levels of osteocalcin mRNA in human fetal osteoblast cells after 17 days of microgravity did not differ from controls [87]. Osteocalcin protein expression was slightly but significantly increased in cultured human osteoblasts flown for 11 days [88]. The different results for osteocalcin expression may be due to the different cell types, different culture conditions, and different flight lengths.

The percentage of alveolar bone surface occupied by TRAP-positive osteoclasts (Oc.S./B.S. %) was increased in the mandibles of Bion-M1 flight and habitat control mice [48], indicating increased bone resorption. This correlates with the decreased BV observed in these mice compared to vivarium controls. Osteoclasts were present mainly along the mesial (anterior) surfaces of the alveolar bone, consistent with the normal physiological distal (posterior) drift of rodent molars. No differences were seen among the STS-135 mice groups, although the overall Oc.S./B.S. % was greater in these mice than in the Bion-M1 mice. This likely was due to the younger age of the STS-135 mice, as well as the increased proportion of sclerostin-positive osteocytes in these mice, as sclerostin promotes increased osteoclast formation and size [66]. In rats flown for 18.5 days on Cosmos 1129, there was a slight but non-significant decrease in alveolar bone resorption immediately after flight [57]. However, after 6 and 29 days of post-flight recovery, a significant decrease in bone formation and resorption was observed, indicating a slowing of the normal distal drift of the molars.

4. Effects of spaceflight on teeth

4.1 Incisor growth and development

The rodent mandible (and maxilla) contains, on each side, 1 incisor separated by a toothless region (diastema) from 3 molars (**Figure 11A**). The molars develop and erupt in a process similar to those of humans. The incisor, however, forms and erupts continuously, allowing the mandibular and maxillary incisors to maintain contact as the covering enamel and supporting dentin are worn away as the animal eats. The cells that form enamel, ameloblasts, originate from oral ectoderm; their stem cells are located in the cervical loop of the incisor, embedded within the bone of the mandible at the apical (posterior) end of the tooth. Ameloblasts initially deposit a partially mineralized matrix based mainly on the protein amelogenin. When the final thickness of the matrix is achieved, the ameloblasts undergo a morphologic and functional differentiation and begin to remove the matrix and add Ca and PO₄ until the enamel is fully mineralized. The cells that form dentin, odontoblasts, are derived from craniofacial ectomesenchyme. Throughout the life of the tooth, odontoblasts continually form and mineralize dentin, which has a collagen-based extracellular matrix. Odontoblasts also synthesize several non-collagenous proteins, including dentin sialoprotein (DSP), dentin phosphoprotein, osteocalcin, bone sialoprotein, osteopontin and dentin matrix protein-1 [89].

4.2 MicroCT analyses

Incisor teeth of flight and habitat ground control mice from the Bion-M1 mission had significantly greater enamel, dentin and overall volumes than the vivarium control mice (**Figure 12**). Enamel thickness in MicroCT sections at the 1st molar level was similar in all three mouse groups. In the flight and habitat control mice, however, the enamel had reached its full thickness at the 3rd molar level, and the thickness was greater in these 2 groups than in the vivarium control mice at both the 2nd and 3rd molar levels. Dentin thickness at all 3 molar levels in the flight and habitat control mice, as well as tissue density (mg hydroxyapatite/cm³), was greater than that of the vivarium control mice. Additionally, as visualized in 3D reconstruction images, incisor mineralization in the flight and habitat control mice began further posteriorly than in vivarium control mice (**Figure 12G–H**). These observations are consistent with a decreased incisor eruption rate in the flight and habitat control mice, most likely due to the soft paste diet consumed by these two groups [90–92]. In the STS-135 mice, there was a trend toward an increased incisor volume in the flight mice compared to the vivarium control mice, but no differences in enamel and dentin volumes, thicknesses or tissue densities.

Previous studies of spaceflight effects on tooth development have produced differing/conflicting results. In some investigations there were no differences between flight and control animals in Ca and P content of incisor dentin [58, 93, 94]. In other studies Ca and P concentrations were increased [57, 95, 96], or decreased [97]. These varying results are likely due to differences in age and sex of the animals, diet, length of the flight, the region of the tooth examined, as well as the methods used in these investigations.

4.3 Tooth protein expression

The expression of osteocalcin, DSP, amelogenin and PKA-RII was evaluated by IHC in the tissues and cells of the teeth [48]. Odontoblasts and some cementocytes of molar roots expressed strong reactivity for osteocalcin (**Figure 11A**); weaker

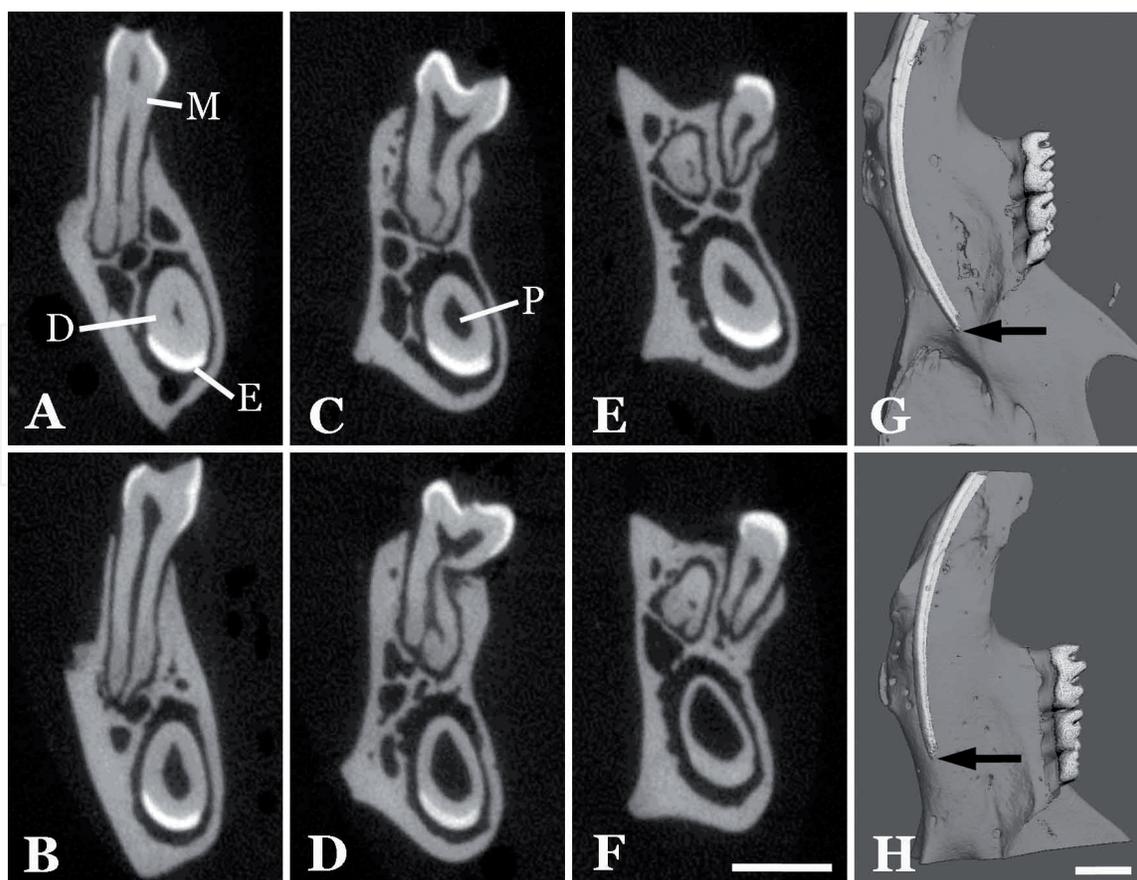


Figure 12. MicroCT images of mandibles of flight (A, C, E, and G) and vivarium control (B, D, F, and H) mice from the Bion-M1 mission. Sections at the level of the 1st (A and B), 2nd (C and D) and 3rd (E and F) molars. Incisor dentin (D) and enamel (E), molar (M), pulp chamber (P). Note the earlier appearance of mineralized enamel (images E vs. F), thicker dentin and smaller central pulp chambers in the incisors of the flight mice. Three-dimensional reconstructions of the mandibles (G and H) show that incisor mineralization begins (arrows) further posterior in flight mice than in vivarium control mice. Habitat ground control mice showed the same features as flight mice. Scale bars = 1 mm.

reactivity was seen in predentin and dentin. Semiquantitative analysis revealed no differences in staining intensities among any of the flight and control groups. Immunostaining for DSP was strong in odontoblasts (**Figure 11D**) and some cementocytes; weaker reactivity was observed in predentin, dentin, molar cementum, enamel matrix, osteoblasts and bone. Significant differences in DSP staining intensities were observed among the Bion-M1 mouse groups. Predentin and cellular cementum (and bone) stained more intensely in flight mice than in vivarium controls. Secretory ameloblasts (**Figure 11E**) and enamel matrix (especially during enamel maturation) stained positively for amelogenin. Reactivity was significantly decreased in enamel matrix of Bion-M1 habitat control mice. Both odontoblasts and ameloblasts were immunoreactive for PKA-RII. Staining intensity was decreased in odontoblasts of Bion-M1 flight and habitat control mice compared to vivarium controls, and decreased in secretory ameloblasts of STS-135 flight and habitat control mice. In addition to effects due to microgravity, the observation of decreased amelogenin and PKA-RII reactivities in the habitat control mice suggest that the habitat environment may play a role in cellular and tissue responses.

5. Effects of spaceflight on other oral tissues

There are few reported studies of the effects of spaceflight on oral tissues other than bone and teeth. Histologic examination of periodontal tissues and oral mucosa

revealed no differences between mice flown on Apollo 17 for 12.5 days and ground control mice [37]. Histologic studies of the lingual mucosa of rhesus monkeys after 30 days of simulated weightlessness showed no differences from control monkeys [98]. The masseter muscles of mice flown for 13 days on STS-135 showed no change in mass, fiber size distribution, signaling pathways, or the expression of genes associated with muscle atrophy compared to ground control mice [99]. Power output was decreased about 40% in the masseter muscles of flight mice, but maximal shortening velocity was not affected. In contrast, tibialis anterior muscles of these flight mice underwent atrophy, with loss of mass, changes in signaling pathways and gene expression, and loss of strength. In a separate experiment, mice fed a liquid diet for 2 weeks showed a decrease in masseter muscle fiber size of more than 40%. These results suggest that the load imposed by chewing hard food protected the masseter muscles from the microgravity-induced atrophy occurring in appendicular muscles, but was insufficient to preserve muscle power.

6. Conclusions

Oral tissues, particularly the salivary glands, mandible and teeth, are affected by spaceflight. The 3 major salivary glands respond differently to the effects of microgravity and the spaceflight habitats. The PG exhibits a number of morphological changes that are also seen following restriction of food, feeding of a liquid diet, and experimental diabetes. The expression of some secretory proteins is increased, whereas others are decreased; for some proteins the length of microgravity exposure may be important. A number of genes also exhibit increased or decreased expression. The response of the SMG to spaceflight appears to differ between males and females, with male glands exhibiting increases in expression of several secretory proteins and female glands remaining unaltered. The SLGs of female mice flown on the space shuttles showed a few changes in secretory protein expression. Interestingly, the expression of the stress protein PKA-RII and its gene was altered in the shorter space shuttle flights, but not the longer Bion-M1 flight. This may indicate an adaptation to the spaceflight environment, or a relatively rapid return to baseline during the longer period between landing and sample collection for the Bion-M1 mice. The ease of saliva collection makes it a potential alternative to blood for monitoring physiological responses to spaceflight and the health of astronauts and cosmonauts. Recent studies have documented substantial differences in male and female salivary gland gene expression in both mice [100] and humans [101]; therefore, additional, more comprehensive studies of the effects of spaceflight on the saliva proteome are needed.

Not considered in our studies, but important for overall oral health, are the minor salivary glands. Although they produce less than 10% of total saliva volume, these glands secrete continuously, providing mucins and antimicrobial substances that lubricate, moisten and protect the oral tissues, especially during periods when the major glands are inactive, such as during sleep. Microgravity induced alterations in their secretions could have significant effects on the teeth and oral mucosa.

The effects of spaceflight on the mandible, observed in our studies, are consistent with the observations reported by others for non-weight bearing skeletal elements. Changes in BV and bone architecture are minimal in the mandible compared to long bones of the lower extremity and vertebrae. The decrease in BV seen in the Bion-M1 flight and habitat control mice most likely was due to the soft paste diet consumed by these mice. Microgravity probably also has an effect, considering the increased proportion of sclerostin-positive osteocytes and number of osteoclasts in the mandible seen in the flight mice. Similarly, the significant changes observed

in incisor tooth volume and mineralization appear to be related to the soft diet and slowing of the eruption rate. Changes in tooth protein expression may be a combined result of microgravity, diet and the habitat environment. The change from “normal” conditions, the confined space, and different diet, must also be considered, and adequately controlled for in experimental studies.

Many of the reported animal and human studies, including ours, would be improved with a larger number of subjects, increasing statistical power and strengthening observed trends. Nevertheless, many significant changes have been documented, and should be considered in planning future animal studies and especially long-term human spaceflight. The recent NASA Twins Study [47] emphasizes the need for comprehensive studies of long-term exposure to the spaceflight environment. Advances in spacecraft and experimental design will resolve current limitations and improve outcomes of future travel in space.

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Conflict of interest

The authors have no conflicts of interest.

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