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Plasticity of the human IgM repertoire in response to long-term spaceflight

Running title: Diversity of cosmonaut IgM repertoire

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ABBREVIATIONS

ANOVA, analysis of variance; B2M, beta-2-microglobulin; BLAST, Basic Local Alignment Search Tool; CDR3, third complementarity-determining region; CST, current stress test; EVA, extravehicular activities; Geq/ml, genome equivalents par ml; GHz, gigahertz; HTS, high-throughput sequencing; HSV, herpes simplex virus; IGHD, immunoglobulin heavy chain diversity gene segment; IGHJ, immunoglobulin heavy chain joining gene segment; IGHV, immunoglobulin heavy chain variable gene segment; IGKJ, immunoglobulin kappa light chain joining gene segment; IGKV, immunoglobulin kappa light chain variable gene segment; IGLJ, immunoglobulin lambda light chain joining gene segment; IGLV, immunoglobulin lambda light chain variable gene segment; IQR, interquartile range; ISS, International Space Station; LME, linear mixed effects model; M-MLV, Moloney Murine Leukemia Virus; RAG, recombination-activating gene; RSS, recombination signal sequences; RT-qPCR, reverse transcription quantitative polymerase chain reaction ; TdT, terminal deoxynucleotidyl transferase; UBC, ubiquitin C.

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CONFLICTS OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

A. Choukér and J.-P. Frippiat designed research; S. Ghislin, J.-I. Buchheim, N. Ouzren-Zarhloul and S. Matzel performed research; J.-I. Buchheim, S. Ghislin and E. Albuisson performed statistical analyses; A. Vanet conceived the software required for this project; S. Ghislin and J.-I. Buchheim analyzed data; S. Ghislin, J.-I. Buchheim, A. Choukér and J.-P. Frippiat wrote the manuscript in conjunction with S. Ponomarev and M. Rykova.

ABSTRACT

Immune dysregulation is among the main adverse outcomes of spaceflight. Despite the crucial role of the antibody repertoire in host protection, the effects of spaceflight on the human antibody repertoire are unknown. Consequently, using high-throughput sequencing, we examined the IgM repertoire of five cosmonauts 25 days before launch, after 64 ± 11 and 129 ± 20 days spent on the International Space Station (ISS), and at 1, 7 and 30 days after landing. This is the first study of this kind in humans. Our data revealed that the IgM repertoire of the cosmonauts was different from that of control subjects ($n = 4$) prior to launch and that two out the five analyzed cosmonauts presented significant changes in their IgM repertoire during the mission. These modifications persisted up to 30 days after landing, likely affected the specificities of IgM binding sites, correlated with changes in the V(D)J recombination process responsible for creating antibody genes, and coincided with a higher stress response. These data confirm that the immune system of approximately half of the astronauts who spent six months on the ISS is sensitive to spaceflight conditions, and reveal individual responses indicating that personalized approaches should be implemented during future deep-space exploration missions that will be of unprecedented durations.

KEYWORDS: antibody, cosmonaut, spaceflight, stress, V(D)J recombination

MAIN TEXT

1. INTRODUCTION

Spaceflight-associated immune system dysregulation has long been acknowledged. Fifteen out of 29 Apollo astronauts developed bacterial or viral infections during, immediately after, or in the week after landing.¹ Recent work analyzing medical data collected from 46 astronauts who spent six months onboard the International Space Station (ISS) corroborated these initial findings.² This study revealed that 46% of them faced immunological problems, with infection being the second most frequent event.

During spaceflight, the crew experiences a multitude of unique stressors, such as microgravity, radiation, isolation, confinement, disrupted sleep and circadian rhythms, high expectations in terms of performance, and the risk of equipment failure or fatal mishaps. The combination of these stressors constitutes the space “exposome”, which exerts its altering effects on various organ systems, including the immune system.³ In addition to shifts in peripheral leukocyte distribution and function, altered cytokine levels are observed that positively correlate with latent virus reactivation in individual crewmembers.⁴ Despite the fact that immune alterations, together with limited therapeutic options, represent a serious risk to crewmembers during future long-term space missions such as the deployment of the Lunar Gateway and possible interplanetary missions,^{3,5-8} limited data are available on the antibody repertoire of astronauts. To date, most studies have quantified the total levels of circulating antibody isotypes, but these studies have produced conflicting results and have not considered the antibody binding site diversity required to ensure efficient host protection.⁹⁻¹⁴ However, some studies have suggested that humoral immunity might be affected during a long-term mission even if spaceflight does not seem to have a major impact on the numbers and proportions of circulating B-cell subsets in crewmembers staying 6 months onboard the ISS.¹³ Indeed, it was shown that a 5-month spaceflight affects amphibian antibody production in response to antigen stimulation,¹⁵⁻¹⁷ and reduces the frequency of somatic hypermutation that diversifies antibody binding sites to improve their affinity.¹⁸ These observations are supported by a study demonstrating that hindlimb unloading, a ground-based model frequently used to simulate microgravity,¹⁹ significantly reduces the number of murine pro-B cells and strongly reduces the number of pre-B cells, two maturation stages at which antibody gene assembly occurs in bone marrow.²⁰ Furthermore, two studies revealed some changes in antibody gene segment usage in mice subjected to 4 weeks of hindlimb unloading.^{21,22} Finally, it is interesting to note that reduced B lymphopoiesis was also detected in mice that spent 30 days onboard the BION-M1 biosatellite,²³ thereby suggesting that extended exposure to spaceflight conditions could modify the diversity of the antibody repertoire.

This diversity, which is essential for host protection, results from the random association of antibody gene segments: a variable (IGKV or IGLV) and a joining (IGKJ or IGLJ) gene segment to generate a kappa or a lambda light chain gene as well as a variable (IGHV), a diversity (IGHD) and a joining (IGHJ) gene segment to produce a heavy chain gene. These associations are created during B lymphopoiesis within the

bone marrow thanks to the V(D)J recombination process. V(D)J recombination requires recombination-activating gene (RAG) proteins that interact with recombination signal sequences (RSS) located at the 3' end of IGHV, on both sides of IGHD and at the 5' end of IGHJ segments, and inflict DNA breaks between coding IGHV, IGHD or IGHJ segments and noncoding RSS, leading to the formation of hairpin-sealed coding ends.²⁴ In further steps, the Artemis protein complex opens hairpin loops, eventually creating palindromic sequences corresponding to short inverted repeats of the segment terminal sequence.²⁵ This is followed by nucleotide addition through terminal deoxynucleotidyl transferase (TdT), hybridization of single stranded DNA on a few nucleotides, and ligation. The end-product is the third complementarity-determining region (CDR3) of Ig chains, a short and highly variable region responsible for much of the diversity of antigen binding sites.

Given the absence of information about the effect of extended spaceflight conditions on the human antibody repertoire, we studied the repertoire of IgM heavy chains from cosmonauts participating in long-term missions on the ISS and evaluated their stress level as well as the activities of RAG effectors required for V(D)J recombination. We focused on the IgM repertoire since IgM is one of the antigen receptors of naïve mature B-cells and is the first antibody isotype produced following an encounter with an antigen. Overall, our data show that cosmonauts respond individually to the spaceflight environment, affecting their personal IgM repertoire diversity, V(D)J recombination, and stress response.

2. MATERIALS AND METHODS

2.1 Study design

This longitudinal study was carried out in accordance with the recommendations from the ethical standards of the appropriate institutional and national committees and with the World Medical Association Helsinki Declaration of 1975 (revised in 2008). The protocol (study name: IMMUNO) for cosmonauts was evaluated by the Russian Ethical Board (Biomedicine Ethics Committee) at the Institute of Biomedical Problems (IBMP), Moscow, and was awarded the protocol number MBI-29. The protocol was also approved by the ESA Medical Board and the Russian Space Agency (Roscosmos). The local Ethical Committee of Ludwig-Maximilian University (LMU), Munich, approved the protocol for the control group (protocol numbers 025/02 and 269-15). All subjects gave written informed consent after being informed of the aim and objectives of the project. Blood and saliva were collected from 5 male cosmonauts with a median age of 48 ± 2 (IQR, interquartile range) years (ranging from 39-54 years) who were assigned to a long-term spaceflight mission (ranging from 124 to 186 days) on the ISS. Two of these cosmonauts (COS2 and COS3) took part in the same expedition. Blood and saliva collection occurred at 6 time points. Sample collection on the ground was performed at the Gagarin Cosmonaut Training Center near Moscow or at the DLR training facility in Cologne, Germany, or at the Johnson Space Center (JSC) at NASA, Houston, Texas. Baseline data collection occurred 25 days before flight (L-25, n = 5). Inflight samplings occurred after 64 ± 11 (F+64,

n = 4) and 129 ± 20 (F+129, n = 3) days spent on the ISS (indicated values are means \pm the standard deviation). Postflight sampling occurred on days 1 (R+1, n = 5), 7 (R+7, n = 5) and 30 (R+30, n = 3) after landing. A control group of 4 males with a median age of 41 ± 4 (IQR) years (ranging from 36 to 46 years) was selected according to the following criteria: (1) adult, (2) male, (3) age > 35 years, (4) no systemic pathology and (5) not receiving any daily medication. Blood and saliva were collected from these volunteers at T0 and T+8 months. On-ground-collected blood samples were either used directly for B-cell quantification or immediately stored at -80°C , as saliva samples, until further analysis. Onboard the ISS, blood and saliva samples were stored frozen inside the MELFI freezer at -100°C . Frozen samples return to Earth and to the investigators' laboratories was performed according to standard ESA protocols.

2.2 Total B-cell quantification

Relative counts (%) of B-lymphocytes (CD19^+) were determined before the flight and after landing from 1 ml of lithium heparinized fresh whole blood using the BD Multitest™ IMK kit (BD Biosciences, Heidelberg, Germany) according to the manufacturer's instructions.

2.3 RNA extraction and RT-qPCR

Total RNA was extracted from 300 μl of whole blood using the Nucleospin® RNA blood kit (Macherey Nagel, Hoerd, France) and reverse transcribed using random primers, RNaseOUT and M-MLV reverse transcriptase following the manufacturer's instructions (Invitrogen, Cergy Pontoise, France). qPCR was conducted using a Mastercycler® realplex² real-time PCR machine (Eppendorf, Hamburg, Germany). Each qPCR assay was performed in triplicate using Takyon No ROX SYBR 2X MasterMix blue dTTP (Eurogentec, Seraing, Belgium). The cycling protocol was as follows: 3 min at 95°C followed by 40 cycles of 15 s at 95°C and 30 s at the annealing temperature indicated in **Table 1**. IgM and IgD heavy chain mRNA relative expression levels were normalized to two housekeeping transcripts (ubiquitin C (UBC) and beta-2-microglobulin (B2M)) as previously described.²⁶ The stability of housekeeping transcripts was checked using BestKeeper software.²⁷ Primer pairs were designed in different exons to ensure that they could not hybridize to potential traces of genomic DNA, and their specificity was checked using a BLAST search through the U.S. National Center for Biotechnology Information database (Bethesda, MD, USA).

2.4 Stress questionnaire

The current stress test (CST) is a validated and standardized one-page paper-based self-evaluation that is completed in <1 min and is therefore easily applicable to the present study.²⁸ The CST aims to determine an individual's psychological state repeatedly under the conditions of acute and chronic stress. Because of the composition of the questionnaire, there is no carryover effect, which is a prerequisite for the application of the test in longitudinal studies. In this test, the subject must decide between six pairs of

contradictory feelings of increasing intensities. From the sum of the obtained values, the final CST score is calculated in the range from 1 (no stress) to 6 (maximal stress).

2.5 Salivary cortisol quantification

Saliva samples were collected in the morning (7:00-8:00 AM) and in the evening (7:00-8:00 PM) before food ingestion or dental hygiene measures. Saliva was collected from subjects by having them chew on a sterile cotton swab for 30-45 s (Salivette[®], Sarstedt, Germany). On ground and inflight samples were frozen and stored until analysis. Free cortisol levels were quantified with an automated immunoassay system based on the principle of electrochemiluminescence (Elecsys 2010, Roche, Mannheim, Germany) at the Institute of Clinical Chemistry, Hospital of the University of Munich, Germany.

2.6 Quantification of HSV DNA in saliva

Quantification of HSV-1/2 DNA was performed using the accredited routine diagnostics laboratory of the Max von Pettenkofer-Institute of the LMU, Munich, Germany. HSV-1/2 DNA was extracted from 200 µl of saliva using a QIA Symphony instrument and reagents (QIA Symphony DSP Virus/Pathogen Mini Kit (QIAGEN GmbH, Hilden, Germany)). Total elution volume was 90 µl. Specific HSV-1/2 sequences were amplified from 5 µl of extracted DNA using an Applied Biosystems[™] 7500 Real-Time PCR System and Applied Biosystems[™] TaqMan[™] Universal PCR Master Mix (both Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's instructions. Optimized primers and a fluorescein amidite (FAM)-labelled probe (both Ella Biotech GmbH Martinsried, Germany) amplified an 81-nucleotide sequence of the polymerase-gene in a reaction volume of 20 µl. Thermocycler settings were 50°C for 2 min and 95°C for 10 min followed by 45 cycles of 95°C for 15 s and 60 °C for 60 s. Quantification was achieved by simultaneous amplification of four HSV-plasmid-DNA standards of 5×10^2 , 5×10^3 , 5×10^4 and 5×10^5 copies/ml. The lower limit of detection (LoD95) of the PCR system was 700 Geq/ml.

2.7 Illumina sequencing

Whole blood RNA was reverse transcribed using random primers, RNaseOUT and MML-V reverse transcriptase (Invitrogen, Cergy Pontoise, France) following the instructions of the LymphoTrack[®] IGH Somatic Hypermutation MiSeq kit (InvivoScribe, San Diego, CA, USA). Then, 2.5 µl of cDNA prepared from each blood sample was mixed with 22.5 µl of IGH master mix provided in the kit (this master mix contains primers, with Illumina adapters, annealing to the leader and joining regions of IgM cDNAs thereby allowing the amplification of IgM cDNA 5'-ends) and 0.1 µl of Taq Gold DNA polymerase (Invitrogen, Cergy Pontoise, France). These mixtures were subjected to the following cycling protocol: 7 min at 95°C followed by 35 cycles of 45 s at 95°C, 45 s at 60°C, 1 min 30 s at 72°C and finally 10 min at 72°C. The quantities and sizes of the amplification products were validated using the Agilent High Sensitivity DNA

assay (Agilent, Santa Clara, USA). High-throughput sequencing of libraries was conducted using the MiSeq Illumina apparatus of UMS2008 from Lorraine University (Vandoeuvre-lès-Nancy, France) using Illumina's standard protocol. Sequences were first treated using the LymphoTrack[®] IGH SHM MiSeq software. Then, complete sequences were analyzed using IMGT/High V-quest software (<http://www.imgt.org>).²⁹ Finally, the obtained data were analyzed using a specific software (see below). Sequencing data have been deposited to the NCBI Sequence Read Archive (<https://www.ncbi.nlm.nih.gov/sra>) under accession number PRJNA627324.

2.8 Software dedicated to the analysis of sequences

A software written in Perl, an efficient editing language, was created to calculate the parameters necessary for this study. This software parsed twenty-eight different files corresponding to twenty-eight different blood samples. One to two seconds of execution was necessary to analyze a file using a 2.9 GHz Intel Core i9 processor. This execution time was proportional to the number of lines to process, with each line corresponding to one sequence. On average, there were 233 819 lines per sample. The software examined each row and defined those having a CDR3 sequence and productive functionality. A sequence was considered productive if it was in frame and did not contain a premature stop codon. The redundancy of this set of CDR3 sequences was then calculated. A new set of results consisting of one representative of each CDR3 sequence expressed one or more times was then generated. The mean number of sequences with unique CDR3 was 41 072 per sample (**Table 2**). From this set of results, the following parameters were calculated: *i*) the usage frequency of each IGHV, IGHD and IGHJ segment; *ii*) the frequency of IGHV replacement footprints as defined by Lange et al.;³⁰ *iii*) the number of nucleotides deleted on both sides of IGHD segments as well as at the 3'-IGHV and 5'-IGHJ extremities; and *iv*) associations of IGHV segments of the IGHV3 and IGHV4 subgroups with IGHJ4 to IGHJ6 segments.

2.9 Analysis of CDR3 amino acid sequences

The number of each amino acid at each position within CDR3 regions ranging from 9 to 24 AA was determined using Weblogo3 (<http://weblogo.threeplusone.com/create.cgi>).³¹ Then, dispersion indexes considering both the position and the frequency of each amino acid along CDR3 sequences were calculated as described below.

2.10 Statistics

Calculation of dispersion indexes

Dispersion indexes were used to determine whether differences in the repertoire of VDJ associations, or other criteria, exist between groups or time points.³² The following formula shows, for example, how these indexes (comprised between 0 and 1), were calculated for VDJ associations. These dispersion indexes take into account all associations whatever their frequencies of expression.

$$I(\text{treated} - \text{reference}) = 0.5 * \sum_{i=1}^n |F \text{ of VDJ}_i \text{ from treated} - F \text{ of VDJ}_i \text{ from reference}|$$

where 'F' is the frequency of a VDJ association in one group or at one time point,
'n' is the number of different VDJ associations detected in our NGS data,
'treated' corresponds to values determined for cosmonauts during or after the flight or to values determined for control subjects at T+ 8 months depending on the comparison we wish to do, and
'reference' is the mean value determined for cosmonauts before launch or for control subjects at T0.

Given the low number of subjects, the means and confidence intervals of dispersion indexes were determined for control subjects and used as references. Cosmonaut dispersion indexes were compared to these intervals, with those being outside control confidence intervals were considered different. These comparisons were used to construct heat maps in which values within confidence intervals are indicated in light gray, those above are in red and those below are in green, with color intensity reflecting the importance of the difference by comparison to the corresponding border.

Statistical analysis of data

Statistical evaluation of the data set was performed using SPSS 25 (IBM, USA). Outcome variables and their residuals were tested for normal distribution by applying the Kolmogorov-Smirnov test. Box-Cox transformation of data was performed where indicated and only when residuals of the tested variable were found not to be normally distributed. Data analysis within a single group was performed employing a linear mixed effects (LME) model, where the time points measured were regarded as fixed and subject as random effects. For cosmonaut subgroup comparisons, the LME was used where subgroup, time points and the interaction of subgroup and time point were considered fixed effects. Subjects were considered random effects. A Bonferroni correction was employed as a post hoc test. Simple comparisons between two groups were performed using an unpaired T-test for normally distributed data and a Mann-Whitney U-test for nonnormally distributed data, where indicated. A value of $p < 0.05$ was considered statistically significant.

For multiple comparisons (**Table 2**), the normality of the distribution was determined using the Shapiro-Wilk normality test, and homogeneity of variance was confirmed using the Levene test. As the normality of the distribution and homogeneity of variance were validated, one-way ANOVA was performed.

For correlations, distribution normality was assessed using the Shapiro-Wilk normality test, and then correlation was tested using the unilateral ($r > 0$) Bravais-Pearson correlation test. A unilateral test was used, as we hypothesized that dispersion indexes for the cosmonaut IgM repertoire and RAG signatures, reflecting RAG activity, would evolve in the same direction. A value of $p < 0.05$ was considered statistically significant, and a p value between 0.05 and 0.10 was considered a tendency.

3. RESULTS

3.1 B-cells and IgM/IgD mRNAs in peripheral blood

Before analyzing the cosmonauts' IgM repertoire 25 days before the flight (L-25), after 64 ± 11 (F+64) and 129 ± 20 (F+129) days spent on the ISS, and 1 (R+1), 7 (R+7) and 30 (R+30) days after landing, we determined the percentage of CD19⁺ B-cells in the lymphocyte compartment of the peripheral blood using pre- and postflight cosmonaut as well as control group blood samples (it was not possible to perform such studies with inflight blood samples) (**Figure 1**). Whereas the percentage of B-cells remained very stable over 8 months in the control group (T0: $14.48 \pm 5.96\%$ vs. T+8m: $13.23 \pm 4.92\%$, $p = 0.738$), a significant increase in B-cell percentages in cosmonauts directly after landing (R+1) compared to preflight values (L-25: $11.58 \pm 6.54\%$ vs. R+1: $16.11 \pm 6.88\%$, $p = 0.025$) was quantified. This increase was transient as it returned to the baseline level after seven days on Earth. Additionally, IgM and IgD heavy chain mRNAs were quantified in pre-, in- and postflight as well as in control frozen blood samples. **Figure 2** shows that relative expression levels of IgM and IgD heavy chain transcripts in peripheral blood were stable in control subjects, during the 6-month stay on the ISS and were not statistically increased at R+1, indicating that spaceflight would not affect our ability to assess the IgM repertoire.

3.2 IgM repertoire analysis

To determine how spaceflight conditions affect the diversity of the IgM repertoire, we constructed IgM cDNA libraries, which were subjected to high-throughput sequencing (HTS), using RNA extracted from the blood of control subjects at two time points (T0 and T+8m) and of cosmonauts at six time points (L-25, F+64, F+129, R+1, R+7 and R+30). Unique sequences were extracted from HTS data to eliminate potential PCR biases and clonal expansions. This selection was based on CDR3 sequences because it is the most diverse part of IgM transcripts. **Table 2** presents the number of obtained unique sequences in controls and cosmonauts. Eleven to fifteen percent of these sequences were nonfunctional (**Figure 3A**). This percentage of nonfunctional sequences was stable in controls and cosmonauts over time (p values always >0.05 when comparing percentages of unproductive sequences in controls and cosmonauts, regardless of the considered time point). These nonfunctional sequences were not further considered, as the purpose of this study was to determine the impact of spaceflight on IgM diversity. We also assessed the efficiency of the kit used to create our libraries by determining which functional IGHV gene segments are found in our datasets issued from the 5 cosmonauts and the 4 controls. According to the IMGT database, the genomic IGH repertoire encoded by the human IGH locus comprises 38-46 functional IGHV gene segments depending on the haplotypes. **Table 3** shows that 42-43 functional IGHV gene segments were detected in our datasets. We are therefore confident that our libraries provide an accurate picture of the IGH rearrangements. This is supported by previous studies having shown that detected IGHV gene segments assessed without amplification parallel the repertoires reported using more focused amplification methods.^{33,34}

Then, we wondered whether the usages of IGHV and IGHD subgroups and of IGHJ segments were affected. The analysis of their expression in our libraries revealed that this was not the case (**Figure 3B**). The same biases and expression frequencies were noted over time in controls and cosmonauts.

To precise this first global analysis, we studied associations between IGHV, IGHD and IGHJ gene segments in control subjects and in cosmonauts over time, and dispersion indexes were calculated to determine whether differences in the repertoire of VDJ associations exist. We first compared indexes determined for controls at T0 with those of cosmonauts before launch (L-25) and those of controls after 8 months with those of cosmonauts 7 days after landing (R+7) (**Figure 4A**). These comparisons revealed that dispersion indexes of control subjects were stable over time, thereby showing the stability of their repertoire of VDJ associations in IgM heavy chains. Consequently, means of indexes determined for controls at T0 and T+8m were used to define a reference confidence interval. However, statistically significant differences between control subjects and cosmonauts were observed at both time points, demonstrating that the cosmonaut IgM repertoire was already different from that of controls before the flight and that it was also different after landing. This difference in repertoire was corroborated by the analysis of footprints resulting from IGHV segment replacement (**Figure 4B**). IGHV replacement refers to RAG-mediated secondary recombination of previously rearranged IGHV segments. This process renews almost the entire V coding region but retains a short stretch of nucleotides as a replacement footprint at the newly generated IGHV-IGHD junction. It allows rescuing B-cells having generated, for example, nonfunctional VDJ associations following V(D)J recombination and is involved in the generation of the primary B-cell repertoire during B lymphopoiesis.³⁵

To determine the effects of spaceflight on the IgM repertoire, dispersion indexes calculated for VDJ associations in cosmonaut IgM repertoires analyzed using inflight and after-landing blood samples were compared to those determined 25 days before launch. We also compared dispersion indexes of controls at T0 and T+8m to determine normal variation in VDJ associations (used as reference). Then, cosmonaut index variations were compared to normal variation determined using controls, and differences were expressed in a heat map. **Figure 5A** shows that two cosmonauts (COS4 and COS5) were consistently above the upper limit of the confidence interval determined using controls, suggesting that their VDJ associations were more variable than those of controls. The comparison of data between COS1, 2 & 3 versus COS4 & 5, using the statistical LME model, presented a similar trend though the low number of individuals in each group must be kept in mind. The same analyses were then performed for VDJ associations involving IGHV segments of the most expressed IGHV subgroups (IGHV3 and IGHV4) and the most frequently used IGHJ segments (IGHJ4 to IGHJ6) (**Figure 5B**). These analyses confirmed that the IgM repertoire of COS4 and 5 was more modified than those of COS1, 2 and 3 during the stay in the ISS but also during the 30-day recovery period, as indicated by the abundance of orange rectangles at all considered time points indicating variations above the confidence interval determined using controls and rare green rectangles indicating less variations than in controls. Thus, the diversity of IgM heavy chains was affected in two out of the five studied cosmonauts.

Furthermore, **Figure 5B** showed that some specific associations (IGHV3-48 with IGHJ5 and IGHV3-23 with IGHJ6) are altered in all the cosmonauts.

3.3 Potential impact on immunity

To evaluate the consequences of the modifications of the IgM repertoire in COS4 and COS5, we studied CDR3 regions. Indeed, heavy chain CDR3 is a major contributor to the antibody binding site because it encodes the longest and most variable loop composing this site. It is made of the 3'-end of the IGHV, the entirety of the IGHD and the 5'-end of the IGHJ gene segments that are assembled during V(D)J recombination. We first analyzed the length of these regions and noted that this parameter is not impacted by spaceflight or during the recovery period (**Figure 6A**). The average length of IgM CDR3 was 15.7 amino acids (AA) in controls and cosmonauts, a value very close to the one (15.5 AA) determined in previous studies of IgM in healthy subjects.^{32,33} Then, we wondered whether CDR3 amino acid composition could be affected. We studied the position and the frequency of each amino acid along CDR3 sequences of 9 to 24 AA at the different time points. Dispersion indexes were calculated, and heat maps were constructed as explained above to reveal inflight and after-landing variations. **Figure 6B** revealed more changes of CDR3 AA composition in COS4 and COS5 than in COS1, 2 and 3 during the flight and recovery periods. Thus, spaceflight modified CDR3 loops composing IgM binding sites in two out of the five studied cosmonauts suggesting that the specificities of IgM binding sites are likely affected in these two subjects.

3.4 HSV reactivation

Because previous work showed that viral reactivation is frequent during short and long-term space missions,^{4,36-40} we analyzed saliva samples for the presence of herpes simplex virus (HSV) type 1 and 2 DNA but did not detect HSV-1 or -2 DNA in any of the inflight samples.

3.5 Impact on V(D)J recombination

We also wondered if these IgM repertoire changes could result from a modification of the V(D)J recombination process. To address this question, we analyzed signatures left by RAG1/2 in IgM transcripts. Indeed, when these proteins cut DNA between coding segments and RSS during V(D)J recombination, some nucleotides (nt) can be deleted at the 3'-end of IGHV, on both sides of IGHD and at the 5'-end of IGHJ segments. Consequently, we studied IGHD length as well as nucleotides trimmed off of IGHV and IGHJ gene segments. These analyses were performed at the different time points, dispersion indexes were calculated for each signature, and heat maps were established to reveal inflight and after-landing differences by comparing these data to variations over time in control subjects. The results shown in **Figure 7** indicate that COS4 and COS5 were systematically above the control confidence interval for 2 out of 3 signatures left by RAG (IGHD length and nt removed at 5'-IGHJ ends). The comparison of results between COS1, 2 & 3 versus COS4 & 5, using the statistical LME model, confirmed this difference, indicating that there was an

inflight modification of the RAG1/2 activity in COS4 and COS5. To investigate the possible link between these activity changes and inflight modifications of the IgM repertoire, Bravais-Pearson correlation coefficients were determined. Interestingly, **Table 4** shows that positive correlations between these parameters exist at the F+64 and R+1 time points and that a trend is still observed at R+7. Correlations could not be evaluated at F+129 and R+30 because the number of samples was too low.

3.6 Analysis of stress parameters

Since stress can modulate immunity,⁸ subjective and objective test methods were applied to evaluate stress in cosmonauts and controls in the morning and evening of a scheduled time point. Subjective stress levels were evaluated using the CST questionnaire. The mean CST scores were low overall, indicating a low personal stress level in both groups (**Figure 8A**). Furthermore, CST scores were stable in cosmonauts and controls over time (p values always >0.05 when comparing scores in controls and cosmonauts regardless of the considered time point). Although the morning CST score in controls at the first time point was found to be significantly higher than that in the cosmonaut group (T0: 3.20 ± 0.64 vs. L-25: 2.4 ± 0.65 , $p = 0.04$), the absolute score was not in a range suggestive of stress. All other group comparisons were not statistically significant. Furthermore, we quantified cortisol in saliva samples. Circadian differences with higher levels in the morning and lower levels in the evening were observed in controls and cosmonauts throughout the mission, indicating a preserved circadian rhythm for cortisol. No significant changes were detected over time in controls for morning cortisol. Evening values showed a statistically significant increase over time, which was not considered physiologically relevant, since the values were still at a very low level (T0: 0.03 ± 0.03 $\mu\text{g/dl}$ vs. T+8m: 0.05 ± 0.02 $\mu\text{g/dl}$, $p = 0.001$). Cosmonauts did not show any significant changes over time for morning cortisol values. However, evening cortisol values exhibited a significant increase at F+64 during the mission and shortly after return (at R+1), indicating a stress response. The significant increases observed can be attributed to COS4 and COS5, as the LME model revealed an increase in stress over time in these two subjects, which was not the case for the other three cosmonauts. Group comparisons showed no differences in morning cortisol levels, yet evening cortisol levels in cosmonauts were generally higher than control values before the mission (T0: 0.03 ± 0.03 $\mu\text{g/dl}$ vs. L-25: 0.13 ± 0.06 $\mu\text{g/dl}$, $p = 0.01$). It is worth noting that one cosmonaut (COS5) exhibited unusually high morning cortisol levels on F+64 for unknown reasons and high evening cortisol levels on R+1, which was likely due to a ballistic return to Earth; i.e., the subject was exposed to 8 g during the landing phase, resulting in an unusual acute stress load even for a trained astronaut.

4. DISCUSSION

In this study, we investigated whether a long-term space mission affects the repertoire of IgM heavy chains. To address this question, we analyzed the IgM transcriptome in the blood of cosmonauts and controls. To ensure that our transcriptomic data were not biased, we determined the percentage of blood CD19⁺ B-cells before and after the flight, studied the relative expression of IgM and IgD heavy chain transcripts in the blood before, during and after the mission, and checked the efficiency of the kit used to create libraries of IgM transcripts. These analyses revealed an increase in B-cells at R+1 which may perhaps be due to fluid redistributions that occur after landing. This increase does not correspond to an increase in transitional, mature naïve, non-switched or IgM-only memory B-cells, as no change in the relative expression of IgD and IgM was noted over time.⁴¹ In agreement with this conclusion, a previous study conducted on eight crewmembers involved in a 6-month mission to the ISS showed that spaceflight has no effect on the number and proportion of naïve, transitional and IgM⁺ B-cell subsets.¹³ Nevertheless, in that study, no increase in total B-cells was noted on the landing day (R+0). This discrepancy could result from differences in crewmembers. However, the fact that the expression of IgM transcripts was not affected by a stay on the ISS ensures that potential IgM repertoire changes are not due to a modification of this parameter. Consistent with this conclusion, no statistically significant impacts on the numbers of unique IgM heavy chain mRNA sequences were observed, indicating that the flight did not affect our ability to assess the IgM repertoire and that there was no group bias in our transcriptome analysis.

We first observed that the IGHV1, IGHV3, IGHV4, IGHD1, IGHD2, IGHD3 and IGHD6 subgroups, as well as the IGHJ4, IGHJ5 and IGHJ6 segments, were detected at high levels in IgM heavy chain transcripts of controls and cosmonauts. These results are consistent with those of previous studies of the IgM repertoire of healthy adults.^{42,43} Furthermore, the usage of these IGHV and IGHD subgroups and of individual IGHJ segments were similar between controls and cosmonauts, thereby indicating that natural biases in subgroups and segment usages were not affected by a long-term space mission. We also noted that the peripheral blood IgM repertoire of control subjects was stable over an 8-month period, as previously shown for the bone marrow plasma cell immunoglobulin heavy chain repertoire⁴⁴ and the adult peripheral blood IGHV repertoire.⁴⁵

Our data revealed that the repertoire of unique VDJ associations in IgM transcripts of cosmonauts was different from that of controls already before launch. This difference is unlikely to be due to lifetime exposure to pathogens in different countries because one cosmonaut (COS4) originated from the same country as the controls and presented the same changes in the IgM repertoire as the other cosmonauts. Vaccination history is not either a likely explanation because both cosmonauts and control subjects received immunizations recommended for adults younger than 60 without disease and against Hepatitis A and B. The only difference was that cosmonauts were also immunized against *Streptococcus pneumoniae*/Pneumococcus and *Neisseria meningitides*/Meningococcus. Since recently, ISS astronauts are

immunized against *Varicella zoster virus* (VZV). However, as an immunization against VZV was not recommended prior to December 2018, it can be expected that our subjects and all members of the control group did not receive the immunization against VZV. Our data demonstrated that the IgM repertoire of cosmonauts before flight was different from that of controls because it was much more reworked during B lymphopoiesis as more RAG-mediated secondary recombination of previously rearranged IgH gene segments occurred, as attested by the study of IGHV replacement signatures. This difference in repertoire might perhaps be due to the special regimes endured by cosmonauts to prepare them for spaceflight, as it was shown, for example, that training impacts humoral immunity.⁴⁶⁻⁴⁸

We then analyzed the effects of spaceflight on the IgM repertoire of the five cosmonauts. No obvious change was noted in COS1, 2 & 3 when analyzing their repertoire as a whole. In the same way, another study showed that the antibody repertoire of mice flown to and housed on the ISS over a short period of time (21-22 days) was not affected.⁴⁹ However, the analysis of associations involving the most expressed IGHV subgroups and the most expressed IGHJ segments revealed that some specific associations (IGHV3-48 with IGHJ5 and IGHV3-23 with IGHJ6) are altered in all the cosmonauts. Interestingly, IGHV3-48 and IGHV3-23 belong to the top 10 of the expressed IGHV segments and IGHJ6 is the second most frequently used IGHJ gene segment in the blood of healthy human adults.⁴³ Thus, changes in the expression of these associations, if confirmed by future studies, likely have a meaning that will require investigations to determine their impacts on the immunocompetence of astronauts. Indeed, IGHV3-23 is regularly over-represented in responses to many antigens.⁵⁰ It was for example shown that IGHV3-23 is utilized with either IGHJ4 or IGHJ6 in the human antibody response to the *Haemophilus influenzae* type b (Hib) polysaccharide (PS) to encode an antibody combining site that is expressed in the majority of individuals and that comprises on average more than half the serum anti-Hib PS antibody population.⁵¹

The repertoire of VDJ associations in the other two cosmonauts, COS4 and COS5, was much more affected. Changes of CDR3 AA composition and of two out of the three signatures left by RAG in IgM transcripts, indicative of a modification of the V(D)J recombination genetic machinery, were much more pronounced in COS4 and COS5 than in COS1, 2 and 3 during the flight and recovery periods. We showed that these differences are not associated to inflight HSV-1/2 reactivation, an observation in line with a previous work investigating US astronauts.³⁷ [This observation suggests a lack of immunosuppression in spite of these changes to the IgM repertoire. Differences in IgM repertoire are](#) unlikely due to immunization history as our cosmonauts were subjected to the same vaccination practice. These can neither be attributed to differences in extravehicular activities (EVAs) because COS4 did not perform an EVA, COS5 did perform three EVAs (5h 25m, 5h 38m, 7h 41m) without any reported issues, COS1 performed 1 EVA (6h 15m) without any reported abnormalities, COS2 performed 1 EVA (5h 51m) and COS3, who participated in the same mission as COS2, did not perform an EVA. Thus, EVAs were well-balanced in both groups (COS1, 2, 3 vs. COS4, 5). Furthermore, it is unlikely that these differences in repertoire resulted from mission differences, such as for example habitat microbial load or composition, because these five cosmonauts were

involved in four different missions: COS1 took part in a mission occurring six months before that involving COS2 and 3; COS4 and COS5 took part in distinct missions separated by a 10-month interval. Furthermore, the most experienced cosmonaut (COS2), who participated in 3 previous missions, was not the most affected one. Such heterogeneity of response to the space exposome has already been noted in flown mice⁴⁹ and in astronauts. Indeed, it was observed that 4/8 crewmembers involved in a 6-month mission on the ISS shed at least one latent type of herpes virus and that this was associated with elevations in salivary cortisol, indicating stress-induced activation of the hypothalamic-pituitary adrenal (HPA) axis in these four subjects.⁵² Another study showed that 61% of astronauts from ISS missions shed one or more herpes viruses in saliva or urine samples.³⁶ Thus, caveats should be strongly considered before averaging data because this process can hide individual responses. Furthermore, we observed that these modifications persisted during the whole mission and up to 30 days after landing, indicating that specificities of IgM binding sites are likely modified in these two subjects for a long period. Even if our sample size is small, it is tempting to put this observation in parallel with the study that showed that 46% of the astronauts who stayed 6 months on board the ISS encountered immunological changes.² Additionally, differences in V(D)J recombination effector signatures observed in COS4 and COS5 suggest a modification of B lymphopoiesis in these two subjects, as observed in mice that spent 30 days onboard the BION-M1 satellite.²³ Interestingly, these two subjects, as well as the BION-M1 mice,⁵³ developed a stress response, and it was shown that exposure to glucocorticoids has a rapid effect on B lymphopoiesis because the administration of corticosterone induces a loss of 30-70% in 24 h among murine early pro-B, pre-B and immature B cells.⁵⁴ Thus, an individual's stress perception and response is a likely contributor to IgM repertoire changes, as previously shown for viral reactivation.^{36,52} In line with this, it was shown that exposure to hypergravity of 2 g for 21 days induces an increase in serum corticosterone and a modification of the V(D)J recombination machinery in murine pups.⁵⁵

This study is limited by the inherently small sample size, the constraint of manned space missions, and the lack of an age- and sex-matched cosmonaut population remaining on Earth during the mission. Furthermore, we might have missed some unique rearrangements corresponding to rare B-cell clones as indicated by a study that did compare datasets generated with or without amplification⁵⁶ but also because we performed these studies using a limited volume of whole blood due to sample sharing. However, we are confident in our results, as they fit perfectly with previous independent data that have repeatedly shown that between 46 and 52% of astronauts face immune changes during a space mission.^{1,2}

In conclusion, this is the first study to show that the IgM repertoire of cosmonauts is different from that of control subjects prior to launch and that 2 out of the 5 analyzed cosmonauts present a modification of their IgM repertoire during a long-term mission on the ISS. Modifications noted in these two subjects persisted during the whole mission and up to 30 days after landing, correlated with changes of the V(D)J recombination machinery, coincided with a higher stress response, and likely affected the specificities of the IgM binding sites. These alterations of the antibody repertoire, combined with other immune alterations such as changes in cytokine production, inflammation, and immune cell population distributions and

function,^{6,8,16,57–59} could increase the clinical impact on the immune system. Challenge-type experiments will need to be done to shed light on the consequences of these modifications of the antibody repertoire on immunocompetence and to estimate the effectiveness of inflight vaccination. Finally, the observation of individual responses indicates that personalized strategies should be considered to avoid inappropriate therapeutic treatments during future deep-space expeditions. Note that the results of this study go beyond astronaut health as they are also relevant for biomedical studies that deal with stress and unloading impacts (e.g. bed ridden) on host physiology and immunity.

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CONFLICTS OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

A. Choukér and J.-P. Frippiat designed research; S. Ghislin, J.-I. Buchheim, N. Ouzren-Zarhloul and S. Matzel performed research; J.-I. Buchheim, S. Ghislin and E. Albuissou performed statistical analyses; A. Vanet conceived the software required for this project; S. Ghislin and J.-I. Buchheim analyzed data; S.

Ghislin, J.-I. Buchheim, A. Choukér and J.-P. Frippiat wrote the manuscript in conjunction with S. Ponomarev and M. Rykova.

DATA AVAILABILITY

Sequencing data from this publication have been deposited to the NCBI Sequence Read Archive (<https://www.ncbi.nlm.nih.gov/sra>) under accession number PRJNA627324.

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Table 1. Primers sequences for RT-qPCR.

Target	Sequence, 5'-3'		Annealing temperature (°C)
	Forward	Reverse	
<i>B2M</i>	ACACTGAATTCACCCCACT	CGGCATCTTCAAACCTCCAT	60
<i>UBC</i>	GGGTCGCAGTTCTTGTTTGT	TCCAGCAAAGATCAGCCTCT	61
<i>IgM</i>	CAAGGACGTCATGCAGGGC	GCAGATGAGCTTGGACTTGC	60
<i>IgD</i>	TCCGTCACCTGCACACTGAA	AGCCACATCAGGAGGATGTT	60

Abbreviations: B2M, beta-2-microglobulin; RT-qPCR, reverse transcription quantitative polymerase chain reaction; UBC, ubiquitin C.

Table 2. Numbers of unique *IgM* heavy chain sequences. Means \pm confidence intervals (CIs).

	Controls		Cosmonauts					
	T0	T+8m	L-25	F+64	F+129	R+1	R+7	R+30
Mean	46 173	53 409	36 932	54 521	36 549	34 815	35 374	35 750
(\pm 95% CI)	(\pm 21 594)	(\pm 35 002)	(\pm 16 140)	(\pm 34 342)	(\pm 62 407)	(\pm 22 736)	(\pm 17 602)	(\pm 37 632)

The normality of the distribution was determined using the Shapiro-Wilk normality test, and homogeneity of variance was confirmed using the Levene test. As the normality of the distribution and homogeneity of variance were validated, one-way ANOVA was performed. No significant differences among these numbers of unique sequences were detected; consequently, no *post hoc* test was performed.

Table 3. *Functional IGHV gene segments detected in unique sequences of cosmonauts and controls analyzed in this study. + indicates that this segment was detected.*

Subgroup	Gene segment	COS1	COS2	COS3	COS4	COS5	C1	C2	C3	C4
IGHV1	IGHV1-2	+	+	+	+	+	+	+	+	+
	IGHV1-3	+	+	+	+	+	+	+	+	+
	IGHV1-8	+	+	+	+	+	+	+	+	+
	IGHV1-18	+	+	+	+	+	+	+	+	+
	IGHV1-24	+	+	+	+	+	+	+	+	+
	IGHV1-45	+	+	+	+	+	+	+	+	+
	IGHV1-46	+	+	+	+	+	+	+	+	+
	IGHV1-58	+	+	+	+	+	+	+	+	+
	IGHV1-69	+	+	+	+	+	+	+	+	+
IGHV2	IGHV2-5	+	+	+	+	+	+	+	+	+
	IGHV2-70	+	+	+	+	+	+	+	+	+
IGHV3	IGHV3-7	+	+	+	+	+	+	+	+	+
	IGHV3-9	+	+	+	+	+	+	-	+	+
	IGHV3-11	+	+	+	+	+	+	+	+	+
	IGHV3-13	+	+	+	+	+	+	+	+	+
	IGHV3-15	+	+	+	+	+	+	+	+	+
	IGHV3-20	+	+	+	+	+	+	+	+	+
	IGHV3-21	+	+	+	+	+	+	+	+	+
	IGHV3-23	+	+	+	+	+	+	+	+	+
	IGHV3-30	+	+	+	+	+	+	+	+	+
	IGHV3-33	+	+	+	+	+	+	+	+	+
	IGHV3-43	+	+	+	+	+	+	+	+	+
	IGHV3-48	+	+	+	+	+	+	+	+	+
	IGHV3-49	+	+	+	+	+	+	+	+	+
	IGHV3-53	+	+	+	+	+	+	+	+	+
	IGHV3-64	+	+	+	+	+	+	+	+	+
	IGHV3-66	+	+	+	+	+	+	+	+	+
IGHV3-72	+	+	+	+	+	+	+	+	+	
IGHV3-73	+	+	+	+	+	+	+	+	+	
IGHV3-74	+	+	+	+	+	+	+	+	+	
IGHV4	IGHV4-4	+	+	+	+	+	+	+	+	+
	IGHV4-28	+	+	+	+	+	+	+	+	+
	IGHV4-30-1	+	+	+	+	+	+	+	+	+
	IGHV4-31	+	+	+	+	+	+	+	+	+
	IGHV4-34	+	+	+	+	+	+	+	+	+
	IGHV4-38-2	+	+	+	+	+	+	+	+	+
	IGHV4-39	+	+	+	+	+	+	+	+	+
	IGHV4-59	+	+	+	+	+	+	+	+	+
	IGHV4-61	+	+	+	+	+	+	+	+	+
IGHV5	IGHV5-10-1	+	+	+	+	+	+	+	+	+
	IGHV5-51	+	+	+	+	+	+	+	+	+
IGHV6	IGHV6-1	+	+	+	+	+	+	+	+	+
IGHV7	IGHV7-4-1	+	+	+	+	+	+	+	+	+
Total		43	43	43	43	42	43	42	43	43

Table 4 . Correlations between IgM repertoire variations observed during spaceflight and IGHD length or nucleotides trimmed off of the 5'-IGHJ ends.

	F+64	R+1	R+7
IGHD length	0.978 (p=0.011)*	0.867 (p=0.029)*	0.721 (p=0.084) [†]
Nt deleted at 5'-IGHJ ends	0.965 (p=0.017)*	0.849 (p=0.035)*	0.764 (p=0.066) [†]

Before calculating Bravais-Pearson correlation coefficient values, distribution normality was assessed using the Shapiro-Wilk normality test. * $p \leq 0.05$, [†] $p < 0.10$, for unilateral $r > 0$ statistical test.

FIGURE LEGENDS

Figure 1 *B-cell percentages before and after a long-term mission on the ISS.* Dot blots show individual B-cell percentages, as determined by flow cytometry, and mean values (red line) in cosmonauts (n = 3-5) (**A**) and control subjects (n = 4) (**B**). B-cell percentages were found to significantly increase one day after returning from space (R+1) using the LME test and Bonferroni post hoc test. * p<0.05 versus L-25. n.s. = non-significant.

Figure 2 *Relative IgM and IgD mRNA expression in peripheral blood before, during and after a long-term mission on the ISS.* Studies were performed before launch (L-25 (n=5)), inflight (F+64 (n = 4) and F+129 (n = 3)) and after landing (R+1 (n = 5), R+7 (n = 5) and R+30 (n = 3)). Dot blots show individual IgM and IgD mRNA relative expression and mean values (red line) in cosmonauts (n = 3-5) and controls (n = 4). IgM and IgD mRNAs were quantified by RT-qPCR, and their levels were normalized to two housekeeping transcripts. The relative value obtained with controls was set to 1. No statistically significant differences were found using the LME test and Bonferroni post hoc test. n.s. = non-significant.

Figure 3 *Spaceflight does not modify the percentages of functional and nonfunctional IgM mRNAs or the expression of IGHV and IGHD subgroups or IGHJ segments.* IgM repertoires were analyzed, using high-throughput sequencing, at 6 time points: L-25 (n = 5), F+64 (n = 4), F+129 (n = 3), R+1 (n = 5), R+7 (n = 5) and R+30 (n = 3). Within these repertoires, we determined the frequency of functional and nonfunctional sequences (**A**) as well as the frequency of IGHV and IGHD subgroups and of IGHJ segments in functional sequences (**B**). Data are shown as the means \pm confidence intervals. No statistically significant differences were found using the LME test and Bonferroni post hoc test. n.s. = non-significant.

Figure 4 *The cosmonaut IgM repertoire is different from that of the controls.* (**A**) Dot blots show individual dispersion indexes for unique VDJ associations in controls (n = 4) and cosmonauts (n = 5) at the beginning of the study (left panel) and 8 months later for controls or 7 days after landing for cosmonauts (right panel). (**B**) Dot blots show the individual frequency of IGHV replacement footprints at the same time points. Statistically significant differences were found using unpaired T-tests (**A**) and Mann-Whitney U tests (**B**). * p \leq 0.05; ** p \leq 0.01.

Figure 5 *Spaceflight affects the IgM repertoire of 2 out of 5 cosmonauts.* The IgM repertoire was analyzed at 6 time points: L-25 (n = 5), F+64 (n = 4), F+129 (n = 3), R+1 (n = 5), R+7 (n = 5) and R+30 (n = 3). To evaluate the impact of spaceflight on this repertoire, for each cosmonaut, dispersion indexes determined for inflight and after-landing time points were compared to those determined before the flight (L-25). These index variations were compared to the variation of indexes determined using controls at T0 and T+8m (used

as reference), and differences were expressed as heat maps in which variations within the confidence interval determined using control subjects are indicated in light gray, whereas those above or below control interval and considered to be different are in red and green, respectively. **(A)** Heat map indicating variations in dispersion indexes for all VDJ associations in the functional IgM repertoire of cosmonauts during and after the mission by comparison to before launch. P values calculated using the LME statistical model when comparing cosmonaut subgroups COS1, 2 & 3 vs. COS4 & 5 are indicated under the heat map. † indicates a tendency ($p < 0.10$). n.s. = non-significant. **(B)** Heat map indicating variations in dispersion indexes for associations involving members of the most expressed IGHV subgroups (IGHV3 and IGHV4) and the most frequently used IGHJ segments (IGHJ4 to IGHJ6) in the functional IgM repertoire of cosmonauts during and after the mission by comparison to before launch. Black rectangles correspond to unavailable samples. Values on the right indicate differences by comparison to upper or lower borders of confidence intervals determined using controls.

Figure 6 *Effect of spaceflight on CDR3*. CDR3 sequences were analyzed at different time points: L-25 (n = 5), F+64 (n = 4), F+129 (n = 3), R+1 (n = 5), R+7 (n = 5) and R+30 (n = 3). To evaluate the impact of spaceflight on cosmonaut CDR3s, we first determined the frequency of sequences according to the CDR3 length **(A)**. Each line corresponds to the mean value for each time point in control subjects and cosmonauts. We also evaluated the effects of spaceflight on CDR3 amino acid composition **(B)**. To address this question, we calculated dispersion indexes considering both the position and the frequency of each amino acid along CDR3 sequences of 9 to 24 AA. In these calculations, inflight and after-landing time points were compared to those determined before the flight (L-25). These index variations were compared to the variation of the indexes determined using controls at T0 and T+8m, and differences were expressed as heat maps in which variations within the confidence interval determined using control subjects are indicated in light gray, whereas those above or below the control interval and considered to be different are in red and green, respectively. Black rectangles correspond to unavailable samples. Values on the right indicate differences by comparison to upper or lower borders of confidence intervals determined using controls.

Figure 7 *Effect of spaceflight on signatures left in IgM mRNAs by RAG1/2*. To evaluate the impact on RAG1/2 activity, we studied the nucleotides (nt) deleted from the 3'-IGHV and 5'-IGHJ extremities as well as IGHD segment length. These signatures were analyzed at different time points: L-25 (n = 5), F+64 (n = 4), F+129 (n = 3), R+1 (n = 5), R+7 (n = 5) and R+30 (n = 3). Then, for each cosmonaut, dispersion indexes determined for each signature during and after the space mission were compared to those determined before the flight. These index variations were compared to the variation in the indexes determined using controls at T0 and T+8m, and differences were expressed as heat maps in which variations within the confidence interval determined using control subjects are indicated in light gray, whereas those above or below the control interval and considered to be different are in red and green, respectively. Black rectangles

correspond to unavailable samples. Values on the right indicate differences obtained by comparing the data to the upper or lower borders of confidence intervals determined using controls. P values calculated using the LME statistical model when comparing cosmonaut subgroups COS1, 2 & 3 versus COS4 & 5 are indicated under the heat map. † indicates a tendency ($p < 0.10$); * $p \leq 0.05$; ** $p \leq 0.01$; n.s. = non-significant.

Figure 8 *Stress markers during long-term spaceflight.* (A) Dot plots show the CST scores calculated with the paper questionnaire in cosmonauts and controls in the morning and evening. No significant differences were found over time or between groups using the LME statistical model. (B) Cortisol levels were measured in the saliva of cosmonauts and controls in the morning and evening using an automated immunoassay system based on the principle of electrochemiluminescence. Statistically significant differences were found for evening cortisol levels in cosmonauts for F+64 ($n = 4$) and R+1 ($n = 5$) versus L-25 using the LME model and Bonferroni post hoc test after Box-Cox transformation of data. Lower panels show cosmonaut subgroup analysis. COS1-3 (lower left panel) did not exhibit any significant changes in evening cortisol over time, whereas COS4 and COS5 (lower right panel) accounted for the changes observed at F+64 and R+1 versus L-25 in the whole group. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. n.s. = non-significant.

Figure 1

Figure 1

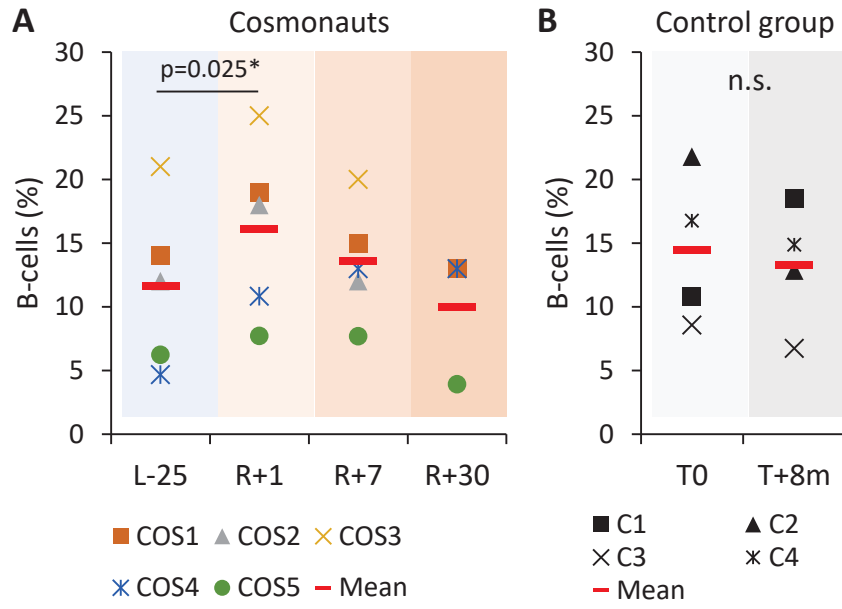


Figure 2

Figure 2

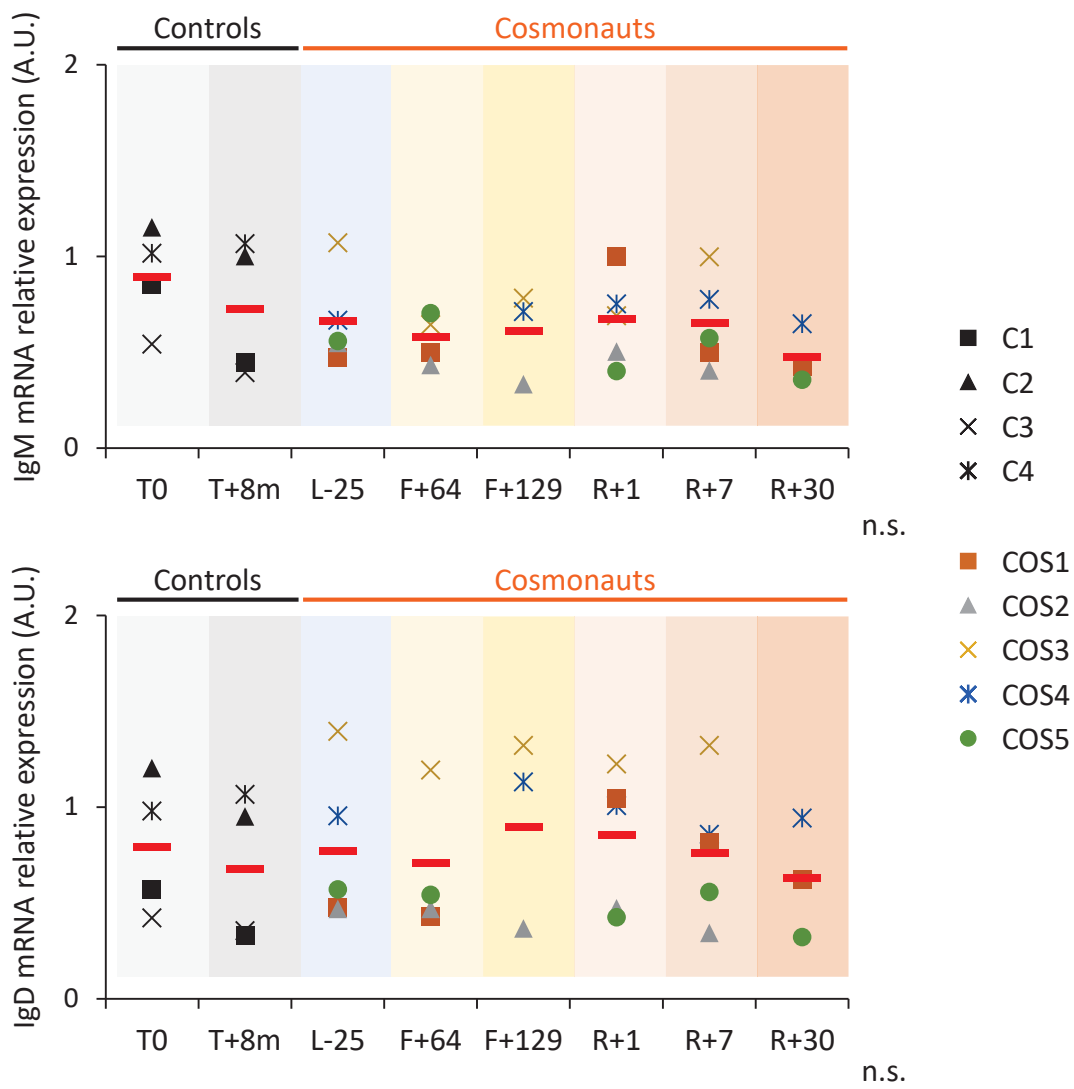
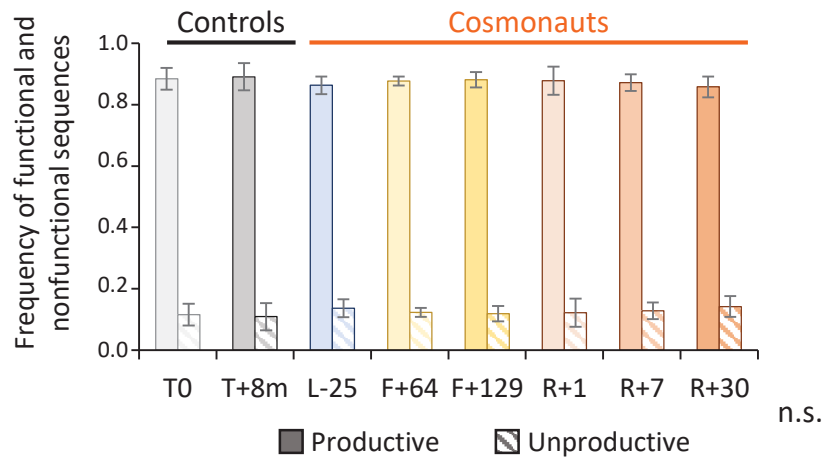


Figure 3

Figure 3

A



B

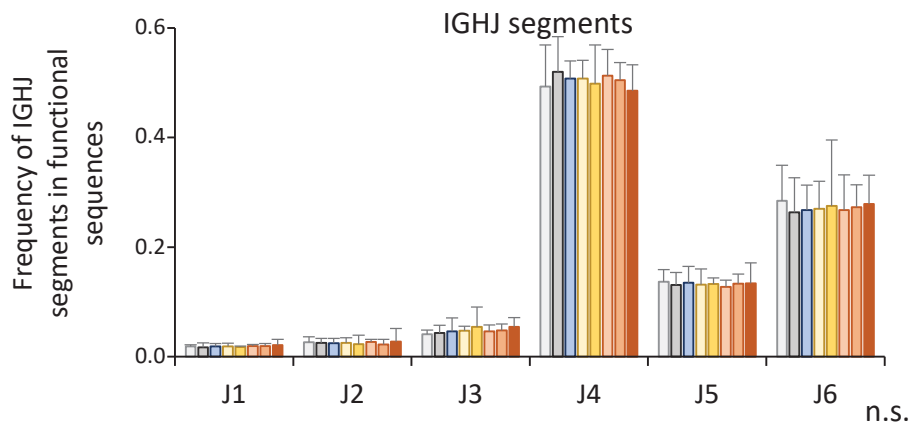
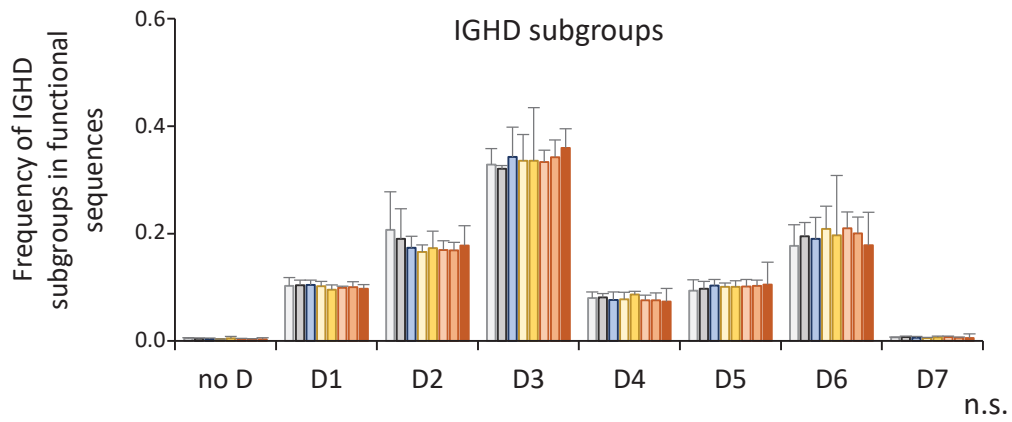
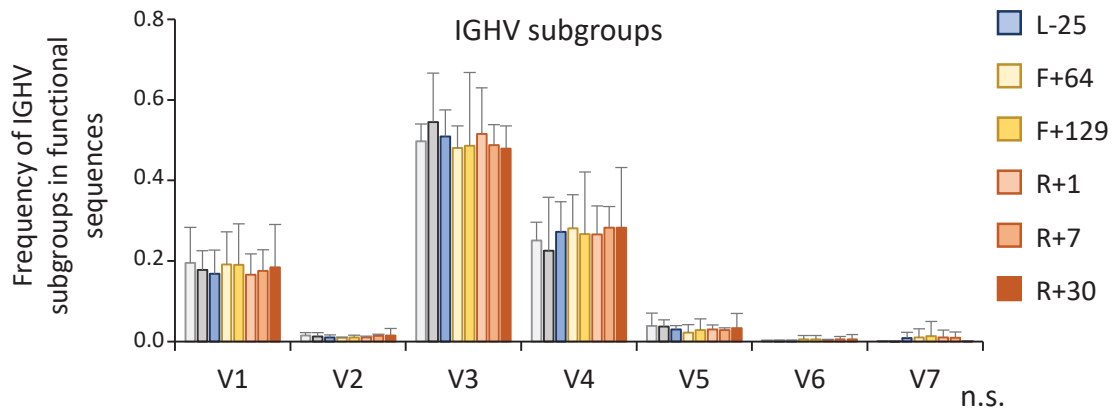
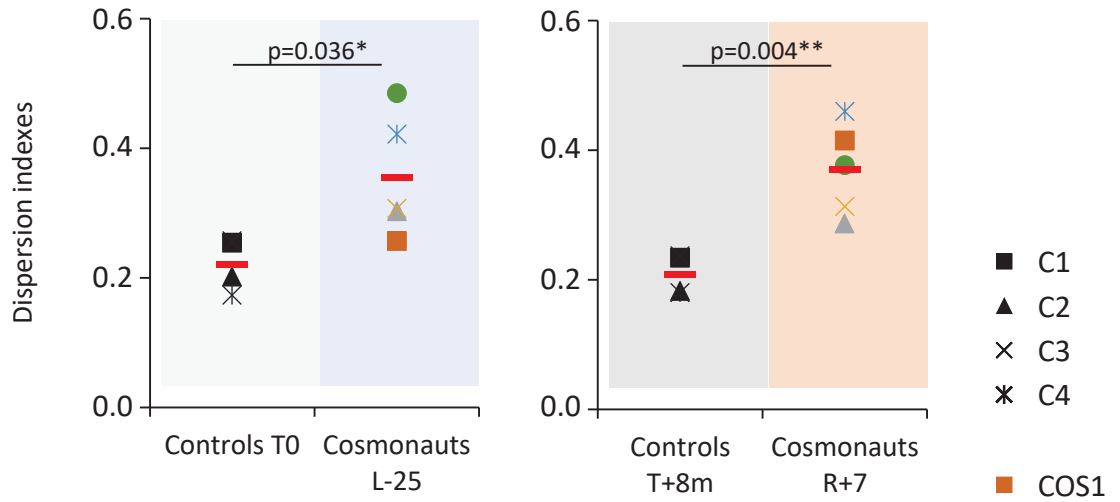


Figure 4

Figure 4

A



B

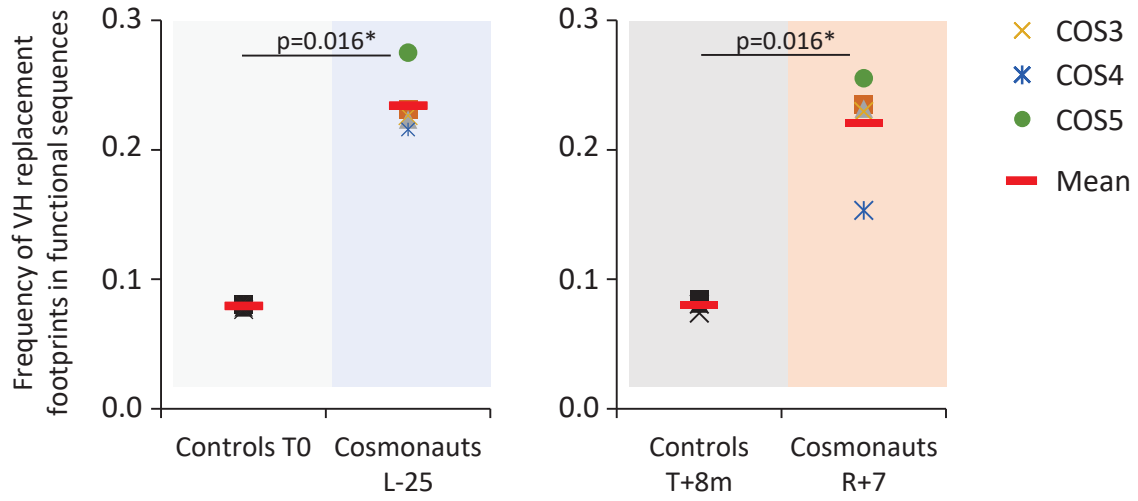


Figure 5

Figure 5A

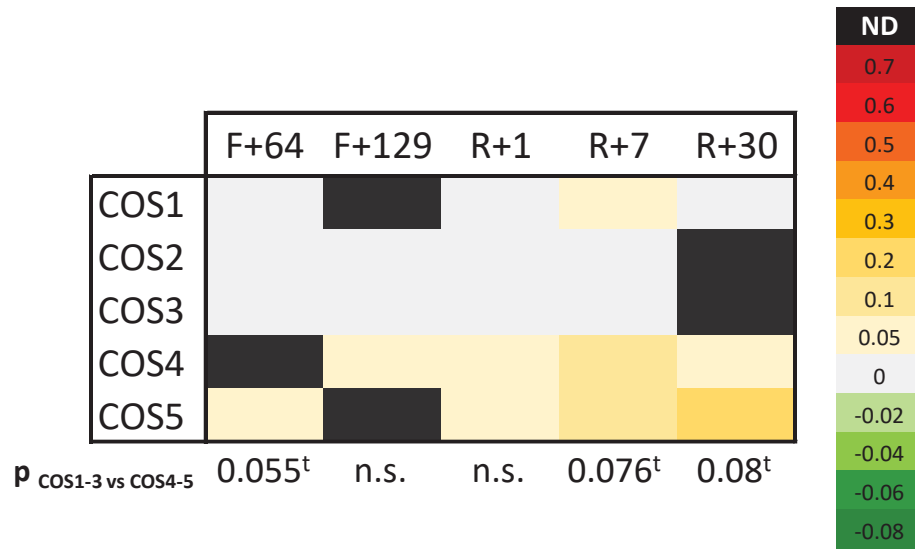


Figure 5B

Figure 5

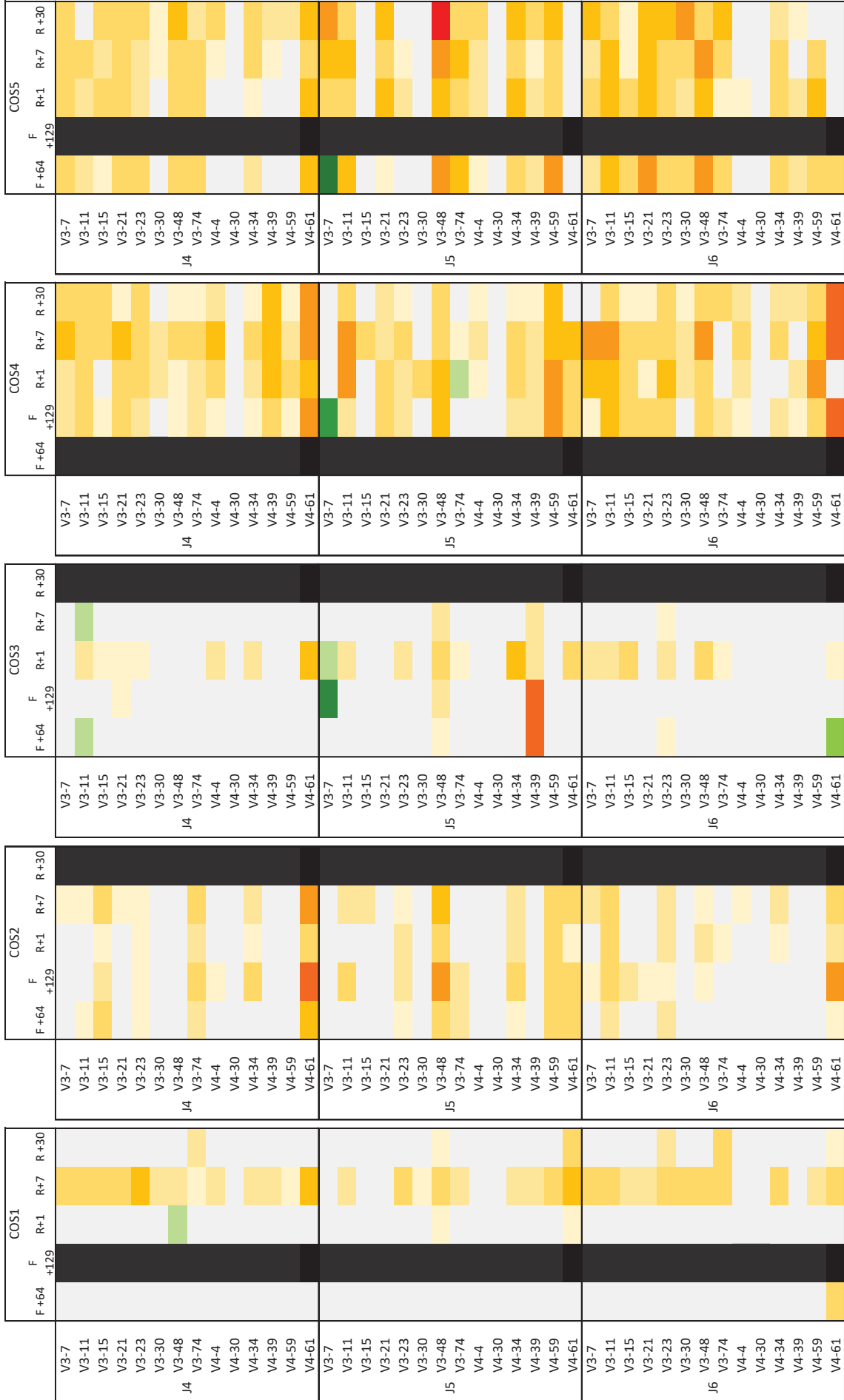


Figure 7

Figure 7

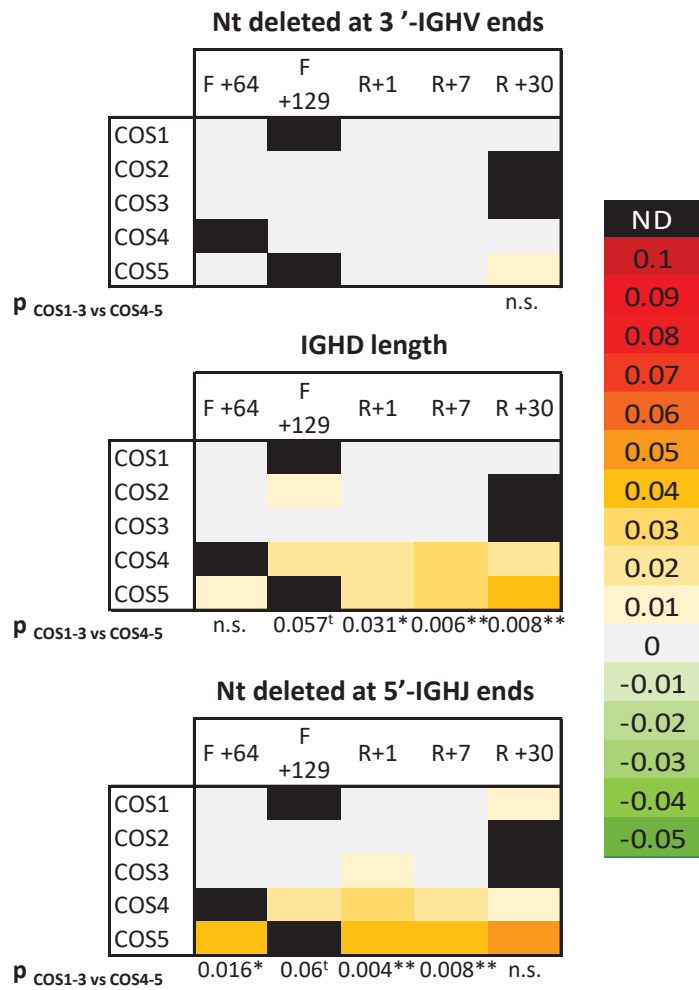


Figure 8

Figure 8

A

Stress test questionnaire

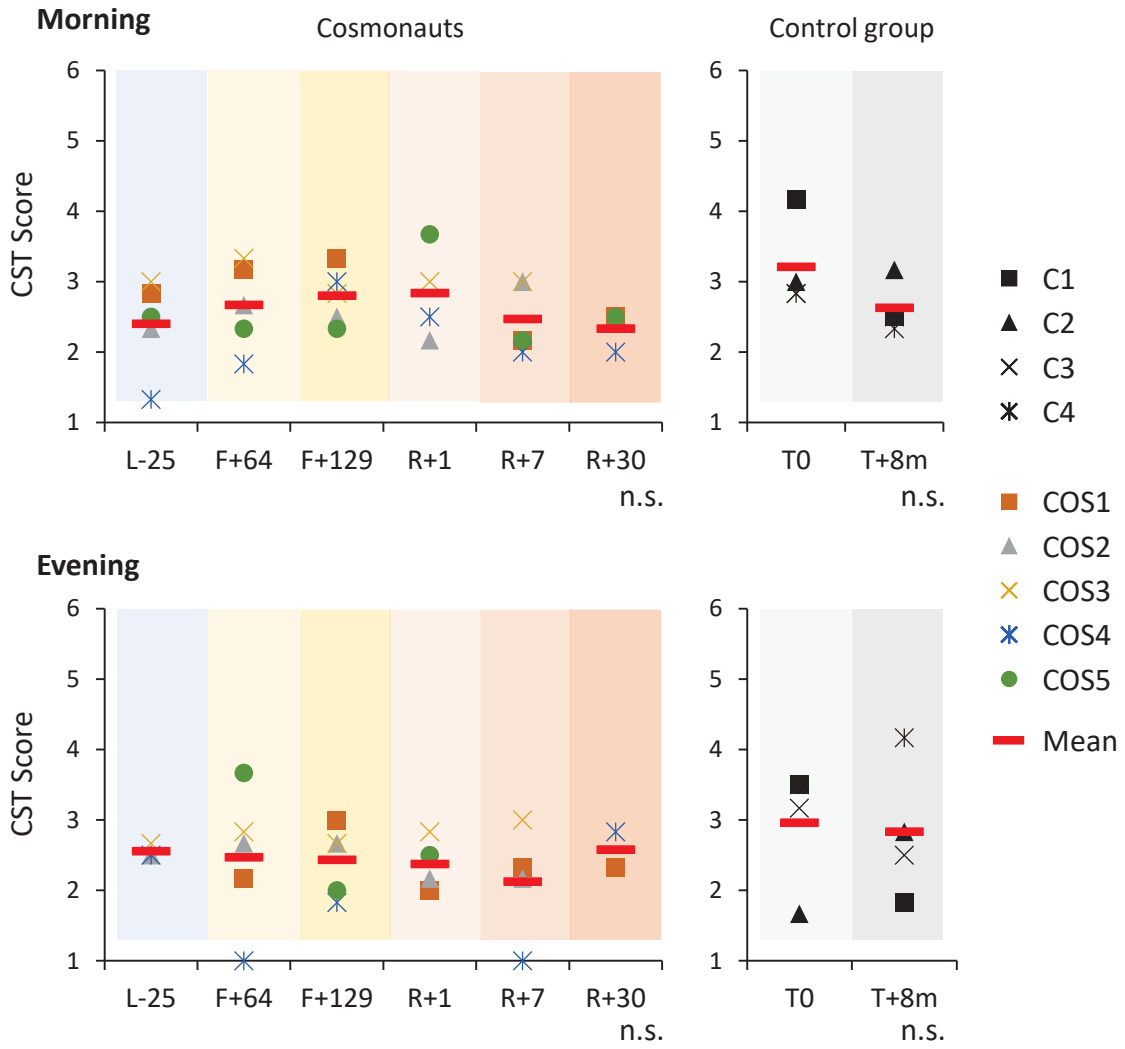


Figure 8

Figure 8

B

Cortisol in saliva

