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A neurobehavioural assessment of adaptive responses to altered gravitational environments in ground-based hypergravity and microgravity models

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Summary

Altered gravitational environment represents a unique challenge for terrestrial biological systems. The investigation of the human and animal response to unphysiological gravity, such as the permanency in space or in conditions that in some way mimic weightlessness, represents an opportunity to understand the phenomena underlying tolerance and adaptation to environmental conditions. The initial and adaptive responses to these challenges are reflected in behavioural endpoints: a careful study of these responses enables to evaluate and understand coping strategies, mechanisms underlying neuroplasticity phenomena, and the individual vulnerability to stress. Furthermore, understanding the individual responses to stress and the identification of predictive indicators of susceptibility may effectively mitigate the negative effects of altered gravity and indeed they represent a relevant emerging topic for the future of human space exploration. Reproducing environments that recreate weightlessness on the Earth is practically impossible. Among the ground-based models that are extensively employed to simulate altered gravity, hypergravity induced by rotation, the hindlimb unloading model, and the bed rest paradigm are well-established methodologies. In this conceptual and methodological framework, the aim of the present work was to investigate the neurobehavioural profile of mice exposed to hypergravity or microgravity simulated conditions in order to define possible behavioural indicators of susceptibility to un-physiological gravity. Moreover, neuroinflammatory biomarkers of resilience or vulnerability were also evaluated in human volunteers subjected to bed rest. In animal studies, behavioural adaptive responses took place during the exposure to altered gravity and the behavioural repertoire resulted compromised when returned to normogravity. Interestingly, the behavioural items manifested during the first days in the centrifuge or upon suspension could predict susceptibility of the experimental subjects to these altered environments. Moreover, evaluation of individual profile on bed rest evidenced that active coping strategy in resilient individuals is characterized by an anticipatory role of biomarkers to counteract the environmental challenge. In line with the personalized medicine approach in space, data obtained from such studies could lead to new strategies to reduce health problems associated with spaceflight.

1. GENERAL INTRODUCTION

1.1. Gravity as "Evolutionary force"

The variety and complexity of life on Earth are influenced by the changing environmental conditions (Volkmann and F. Balusˇkam 2003; Adampoulos et al., 2021). Gravity represents the only environmental parameter that remained constant throughout the history of Earth and played a pivotal role in shaping living organisms (Ross et al., 1984; Morey-Holton, 2003). Although it has been constant, it acted as a force that guided the evolution of the species, for example affecting the transition of the organisms from the sea to the land and then to the air. As life evolved into the water, the initial effect of gravity was counteracted by the buoyancy and the aquatic species developed organs, such as the swim bladder, to counterbalance the intensity of the gravitational signal. Leaving the water and exploring the land required to adapt the biological systems to the changes in gravitational loading. Land species evolved in height, changing their orientation with respect to the gravity force: they had to develop specific measures to balance fluid regulation and favour locomotion in order to counteract the gravity. Differently, in the air, birds had to develop a musculoskeletal system that could overcome the air density and gravity, and provide enough thrust before flying (Morey-Holton, 2003).

A fascinating evidence regarding the gravity as an evolutionary force comes from snakes (Morey-Holton, 2003). Existing species occupy a variety of different habitats. There are species that routinely experience gravity stress when climbing trees, others spend most of their time in a horizontal position on the land, as well as the aquatic species that might be considered to be evolutionary "deconditioned" to gravity (Lillywhite et al., 2012). It is interesting to note that, when these three species are exposed to hypergravity, the tolerance to gravity changes among them. The tree snake, continuously subjected to different orientations in relation to gravitational load, adapted better to altered gravity than the other two species, while the sea snake had the least gravity tolerance. Interestingly, the species differentiate also for the anatomical position of the heart: in the tree snakes it is located closer to the head than to either the land snake or the sea snake, suggesting a potential effect of gravity on organ position attributable to diversification of habitat demands and behaviours (Morey-Holton, 2003; Lillywhite, 2005).

Therefore, terrestrial organisms have evolved by adapting their body and biological structures to the gravitational field of Earth. Gravity has been the major force in shaping living organisms and played a pivotal role in determining the form, size and functions of physiological systems (Dubinin and Vaulina, 1976).

1.1.1. Understanding the role of gravity on physiological responses

Altered gravitational environments represent a unique challenge for terrestrial biological systems. As previously mentioned, some adaptive mechanisms to the Earth's gravitational field are obviously apparent: the vestibular system detects gravity vector and directs the musculoskeletal system to counteract the gravitational force and maintain posture or motility, or the cardiovascular system counteracts gravity when it pumps blood to the upper body while using gravity when it distributes fluid to the lower parts. These well-known adaptations become clearly evident during orbital spaceflight, where different phases of physiological changes could be investigated due to fluctuations in gravity levels faced during the mission. Living organisms experience acute hypergravity when leaving or reentering the atmosphere; they are subjected to chronic exposure to microgravity during the permanency in space, and they readapt to 1G after returning on Earth (Iwase et al., 2020). Moreover, the space environment exposes organisms to extreme physical and environmental challenges, such as acceleration forces, confinement, isolation, and radiation, and the resulted effects on physiological systems are profound and diversified (space motion sickness, bone demineralization, cardiovascular decondition, redistribution of the fluid, etc...Fig. 1)

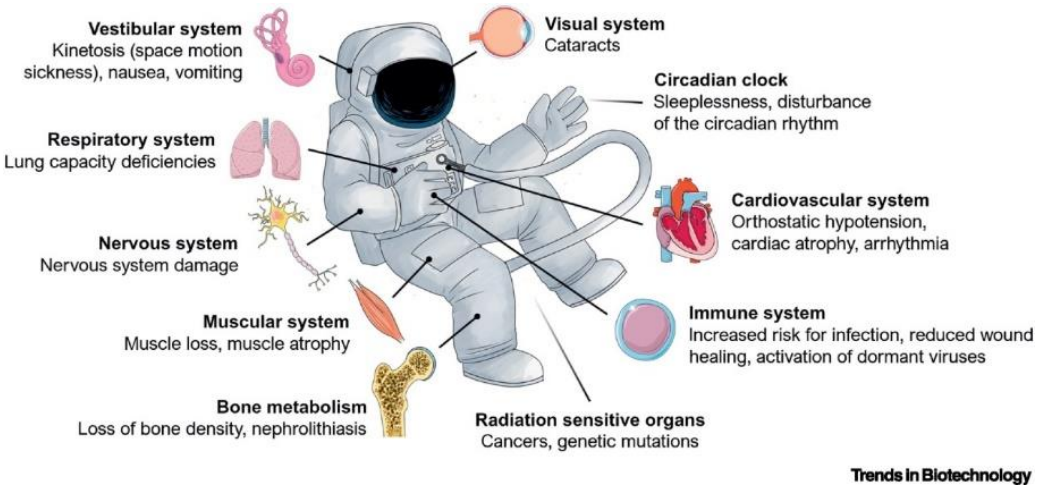

Figure 1. Physiological impairments in space environment (Moroni et al., 2022).

Only in October 1957, when the Sputnik was launched, life ventured into space, opening the frontiers towards the investigation of adaptation in microgravity (Morey-Holton, 2003). Understanding how species evolved under different gravitational forces becomes of growing interest to understand how life could emerge in other planetary systems. The issue of studying the physiological effects of altered gravity becomes of primary importance also to understand

phenomena underlying tolerance and adaptation to extreme environmental conditions. Exploiting such responses represents an opportunity to investigate coping strategies, neuroplasticity phenomena and the individual vulnerability to stress in order to study the mechanisms underlying these phenomena and to develop adequate countermeasures to preserve and reduce animal and human susceptibility in space.

1.2. Neuroscience in Space Biology

Our brain is characterized by a high degree of plasticity that promotes adaptation to different environmental conditions. It is known that central nervous system (CNS) and behavioural outcomes are affected by environmental changes, including the special conditions of space travel, such as weightlessness, radiation, acceleration, and stress induced by long confinement periods (Clement and Reschke, 2008). Among the many fields of Space Biology, Space Neuroscience seeks to investigate how the brain and mind react to the space environment or to particular space conditions simulated on the ground.

The first studies on space neuroscience date back to 1962 during the third mission on board the Russian Vostok spacecraft (Clement and Reschke, 2008). Specifically, the attention towards neuroscience studies began after the reentry of the astronauts from the second manned soviet mission, when they complained of nausea, vomiting and spatial disorientation symptoms related to weightlessness (Titov and Caidin, 1962). The "Space motion sickness" and, in general, the investigation of CNS related disorders became a significant scientific and operational concern in later missions (Lackner and DiZio, 2006). For example, it has been decided to equip the module "Columbus" of the International Space Station with an electroencephalography (EEG) system in order to evaluate CNS activity in space (De la Torre et al., 2012), and many neurocognitive tests and sensory-motor measurements became routinely usual in space to evaluate the brain activity and behavioural profile of astronauts (De la Torre, 2014).

After decades of space flight, the microgravity environment in space- and ground-based models to simulate altered gravity have become ideal laboratories to investigate the psychophysiological adjustments to gravity changes and to increase our understanding about the impact of altered gravity on CNS and behaviour (Clement and Reschke, 2008).

1.2.1. The issue of animal exposure to space environment

Animals have been invaluable in Space Neuroscience and have contributed significantly to the current knowledge of the effects of gravity on living systems (Clément and Slenzka, 2006; Ronca and Lowe, 2022). After the first living creature, the dog Laika, was launched into the space, a large variety of vertebrate and invertebrate species has been selected to carry out experiments in space. At first, very small organisms with a completely sequenced genome, such as fruit flies (*Drosophila melanogaster*), were sent into space in order to evaluate the genetic effects of cosmic radiation (Clément and Slenzka, 2006). Subsequently, mammals and non-human primates were chosen to test the physiological effects of microgravity, both before and after human missions, in order to better understand the effect of altered gravity in biological systems. Among rodents, rats and mice are

considered well-established animal models to investigate biological responses to altered gravity. Their small dimensions, known biology, completely sequenced genome, and the availability of various transgenic strains make them excellent models for space biology research. Another advantage is the short reproductive and life cycle: a permanence of rodents in space for three months means exposing them to space environment approximately for one-third of their lifespan. Such studies have the potential to investigate chronic microgravity exposure effects, evaluating the consequences on developing organisms and extrapolating information to human living in space for long periods.

For these reasons, several facilities have been designed and used to conduct experiments with rodents on the International Space Station (ISS). The payloads have been built to function autonomously and guarantee the correct food/water delivery for each animal, regulation of the light/dark cycle for the circadian rhythm, ventilation, and waste control for their survival (Ronca et al., 2019). The *Animal Enclosure Module* (AEM) was the first habitat built by NASA and designed for housing 5 rats or 10 mice (Fig. 2a). Originally, it was projected for short duration missions and subsequently was modified to support long period experiments on ISS (Globus et al., 2014). The *NASA Rodent Habitat* was an advancement of the AEM, and it was designed for long duration studies (Fig. 2a). In fact, in 2014, it was used to maintain 10 mice on ISS for 30 days (Ronca et al., 2019). One year before, 45 mice were flown for 30 days on Bion-M1 biosatellite with three mice housed in specific cylinder-shaped habitats in the Russian *Block Obespecheniya Soderzhaniya* (BOS, Fig. 2b; Andreev-Andrievskiy et al., 2014). During the same year, the Japan Aerospace Exploration Agency sent the *Habitat Cage Units* (HCUs) for 12 mice that can be installed in the Centrifuge-equipped Biological Experiment Facility in the ISS (Fig. 2c; Shiba et al., 2017). Lastly, in 2019, the Italian Space Agency in collaboration with Thales Alenia Space Italia designed and built the *Mice Drawer System* to fly 6 mice and function autonomously for more than 3 months on the ISS (Fig. 2d; Cancedda et al., 2002, 2012; Santucci et al., 2012).

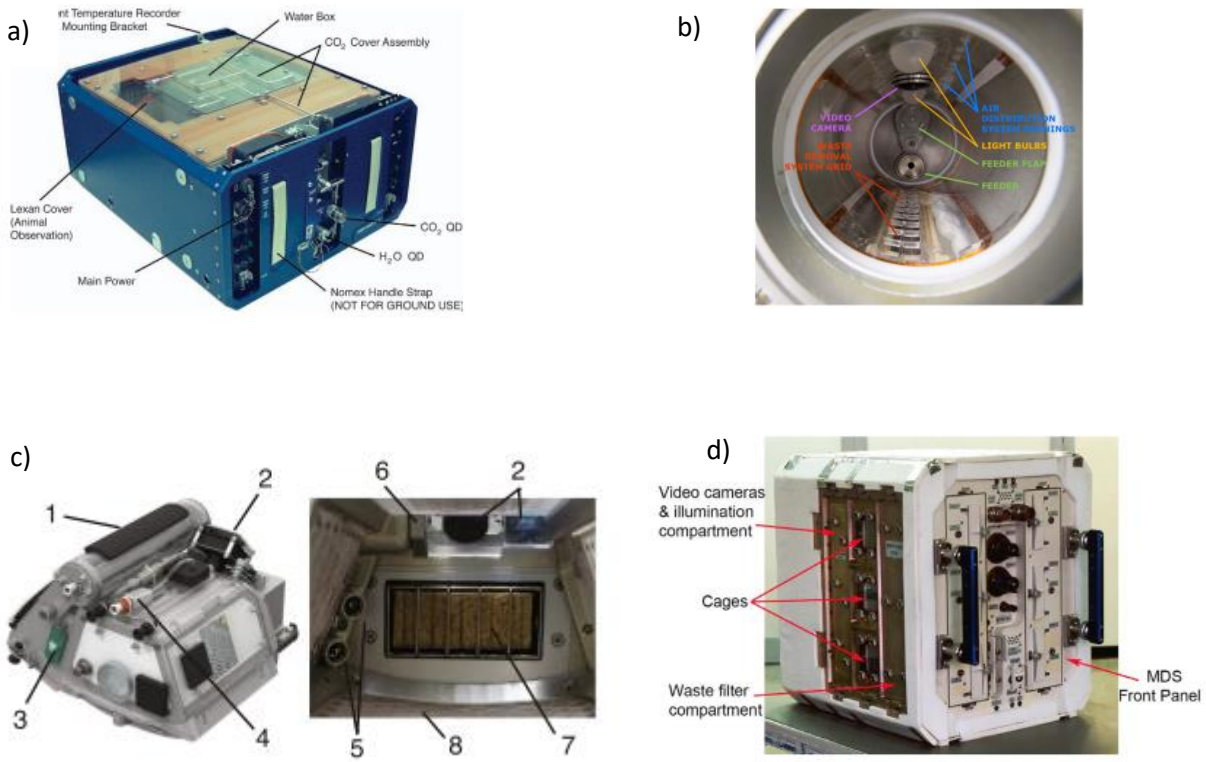

Figure 2. a) *Animal Enclosure Module* (Moyer et al., 2015*)*, subsequently modified in *Rodent Habitat* (Ronca et al., 2019); b*) Block Obespecheniya Soderzhaniya* Andreev-Andrievskiy et al., 2014; c) *Habitat Cage Units* (Shiba et al., 2017); d) *Mice Drawer System* (Cancedda et al., 2012).

1.2.2. Ground-based models for space biology studies

Reproducing environments that simulate on the Earth weightlessness condition is practically impossible. Therefore, comprehensive studies on the effects of gravity on the physiology and adaptations of organisms require necessarily the use of models that simulate conditions of altered gravity (i.e., hypergravity or hypogravity; Hemmersbach et al., 2006). The advantages of using ground-based models are related to the possibility of controlling environmental factors more effectively than can be done in space. In fact, it is possible to overcome the many challenges encountered during spaceflight experiments, confining the physiological changes observed among the various environmental conditions found aboard the spacecraft (radiation, isolation, confinement, noise, etc…; Moody and Golden, 2000; Tou et al., 2002). Moreover, due to financial and technical difficulties associated with space flight, ground-based analogues represent an excellent tool for researchers to evaluate theories without launching experiments into space (Baselet et al., 2022), and perform basic scientific studies or test hardware in preparation of real microgravity experiments (van loon J.J.W.A., 2001). Different models to simulate several effects of weightlessness on Earth are validated. In clinostats, plants or non-motile cells are rotated in three-dimension at random rates in order to inhibit the stimulation by gravity (Honson et al., 1997). In hindlimb unloading model or

hypergravity induced by rotation, it is possible to reproduce the effects of microgravity- or hypergravity-simulated conditions in animal models (Morey-Holton and Globus, 2002; van Loon et al., 2008). Similarly, head-down bed rest or water immersion paradigm in humans is usually implicated to induce cephalad fluid shift and loss of impact loading on the musculoskeletal system as found in weightlessness (Platts et al., 2009; Weber et al., 2018). Each model represents a developing system which is intended to mimic specific physiological responses to weightlessness in order to provide critical data for the design of spaceflight experiments and study mechanisms underlying adaptations to altered gravitational environments (Morey-Holton and Globus, 2002).

1.2.2.1. The paradigm of hypergravity induced by rotation

Among ground-based models to reproduce altered gravitational environments, rotation-induced hypergravity is a common paradigm usually employed to evaluate the effects of changes in the gravitational field and reproduce the effects of acceleration on living organisms (van loon. 2001). In fact, it allows to simulate both acute exposure to hypergravity, similar to hypergravity experienced by astronauts during the abandonment and re-entry of the spacecraft into the atmosphere, and biological effects after long-term altered gravity exposure.

In accordance to the "continuity principle", data emerging from space flight and hypergravity exposure demonstrated that the gravitational fields are continuous above and below the gravitational field of Earth and biological systems continue to respond similarly across the spectrum of gravity (Burton and Smith, 1999; Wade et al., 2005; van loon, 2016). Specifically, it is possible to extrapolate mathematically to 0g starting from results at various hyper-g values, providing a model that predicts the effect of weightlessness (Burton and Smith, 1999; van Loon, 2016; Fig. 3). Examples of gravity continuum can be found in different species (fruit flies, turtles, rodents, chickens and non-human primates), in reproductive performances, body mass, bone, energy stores and many physiological systems (Bergstedt, 1961; Ronca A.E., 2003; Wade et al. 2005).

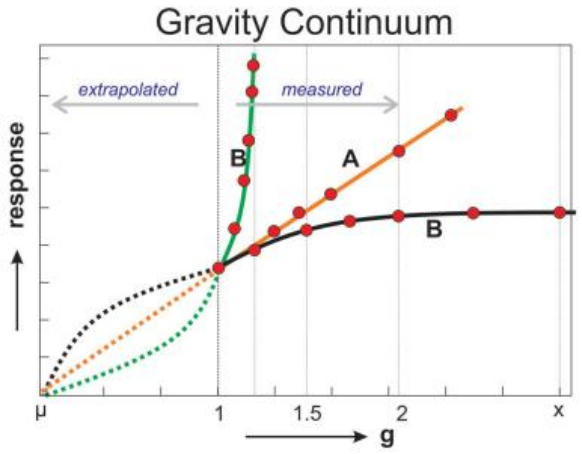

Figure 3. Representation of "gravity continuum": red dots mean various hyper-g values, while dotted lines represent extrapolated values at lower g once known higher g measurements (van Loon J.J.W.A., 2016).

According to the ''theory of equivalence'' postulated by Ernst Mach and Albert Einstein, the "*acceleration acting on a mass cannot be distinguished from the attracting force of gravity*". Therefore, if a mass is accelerated, the resultant force will be the same as an increase in the level of gravity (Wade et al., 2005).

The acceleration force to induce hypergravity is typically elicited by the use of centrifuge (Fig. 4) that, with an appropriate control for the Coriolis effect, serves to reproduce an infinite number of gradual increases in gravitational load for cellular and animal research. Mechanically, it consists of an apparatus composed of arms mounted around a central rotor that end with free swinging gondolas suitable for hosting experimental cages. In general, each gondola is equipped with a videorecording system to monitor the animal spontaneous activity during the rotation (van Loon et al., 2008).

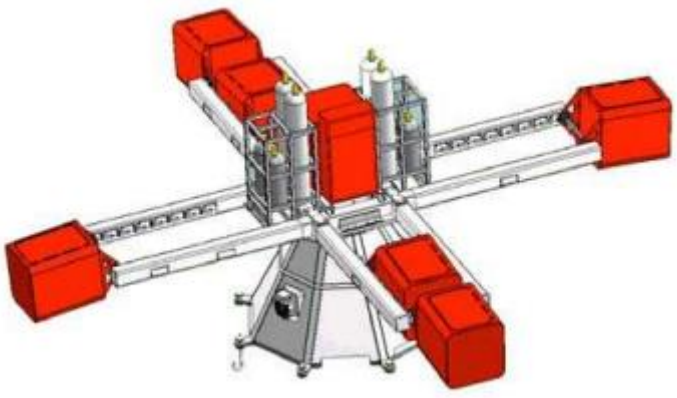

Figure 4. Large Diameter Centrifuge at ESTEC, Netherlands (van Loon et al., 2008).

1.2.2.2. The hindlimb unloading model to study microgravity simulated conditions

The hindlimb unloading model (HU) was developed to reproduce on rodents conditions analogue to those observed during spaceflight (Morey-Holton et al., 2005; Globus and Morey-Holton, 2016; Hawliczek et al., 2022), including muscle atrophy, cephalad fluid shift, eat and groom using forelimbs, and unloading of the hindlimbs (Morey-Holton and Globus, 2002). It consists of removing gravitational loading from the hindlimbs by suspending the animal by its tail (Fig. 5).

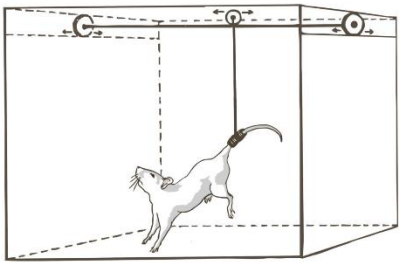

Figure 5. Representation of hindlimb unloading model.

The first paper published on this model dates back to 1979 where Emily R. Morey described the first system of hindlimb unloading on rat. The animal is attached to horizontal aluminium beam through a back harness and is free to move in a 360° using only the forelimbs (Morey et al., 1979). Subsequently, many research groups refined this model in order to ameliorate its translational validity and reduce animal suffering. For example, only one year later Musacchia and colleagues developed a different whole body suspension system where the animal was able to use its forelimbs to move only in 140° (Musacchia et al., 1980). However, in this setting, a consistent reduction in weight gain in suspended animals was observed, suggesting a systemic stress response due to the harness system. Therefore, the Musacchia technique didn't become more popular than specific orthopaedic traction tape localized on the tail (Morey-Holton and Globus, 1998, Fig. 6).

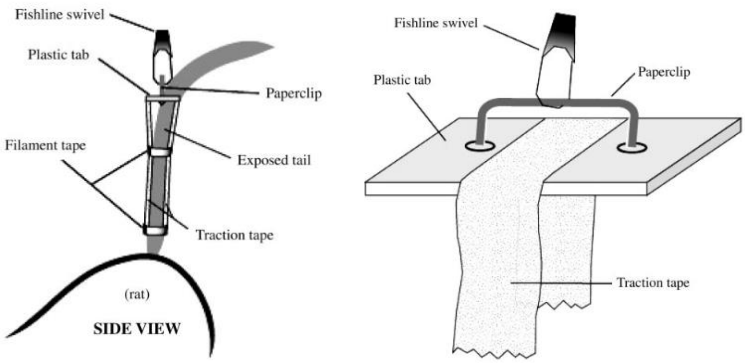

Figure 6. Traction tape to tail suspended animals.

The relevance of angle unloading was evidenced by Hargens and colleagues in 1984. They observed that when increasing the angle of unloading between the animal's body and the floor of the cage, the mechanical loading of the forelimbs was reduced, while the tail traction and the stress of the animals appeared to increase. Therefore, it has been recommended an angle of the 30° of unloading as the correct suspension procedure to provide the correct weight bearing on the forelimbs, to reproduce relevant cephalad shift and to reduce animal suffering (Hargens et al., 1984; Morey-Holton and Globus, 2002).

Finally, although comparison between hindlimb unloading and spaceflight animals revealed similar responses in many physiological systems (muscle, bone, heart, intestine, pulmonary, immune functions, Morey-Holton et al., 2005), it is worth mentioning that there are several limitations associated to this model. For example, it is possible to reproduce only a partial unloading of the animal while during spaceflight the whole body is suspended. Moreover, the position of the animal is totally unnatural, causing stress that could potentially influence the results. The HU methodology also varies greatly among research facilities, making it difficult standardization and result replication (Hawliczek et al., 2022).

1.2.2.3. The bed rest model as analog to microgravity

Since the advent of human spaceflight in 1961, the water immersion paradigm has been used to study the reduction of the force of gravity on the human body. Subsequently, due to the practical difficulties to remain in water for more than one day, the bed rest model, first introduced as a medical treatment, has been employed in space research to reproduce the effects of space flight and physical inactivity (Pavy-Le Traon et al. 2007; Parry and Puthucheary, 2015). It consists of immobilization of volunteers on the bed for a long period (typically for 10 or more days) in order to reproduce muscle atrophy, fluid redistribution, weight loss, cardiovascular and pulmonary adaptation, similar to those experienced by astronauts in space. Interestingly, in the early 1970s, a modification to this model was introduced: the head-down bed rest. Since astronauts who came back from a long mission complained that they couldn't sleep because they felt their feet falling out of bed and they tried to solve the problem by raising the foot of the bed until it felt horizontal, researchers thought that the head-down position probably best reproduced the physiological effects of the space environment (Pavy-Le Traon et al. 2007). In the head-down bed rest, subjects lay supine on a bed that has been tilted to lower the head at a determinate angle, with the international standard angle established at 6◦ (Fig. 7; Smith et al., 2011). It represents a model that reproduce an

altered gravitational environment where the subjects experienced un-physiological gravity during their longitudinal axis (Goswami et al., 2021).

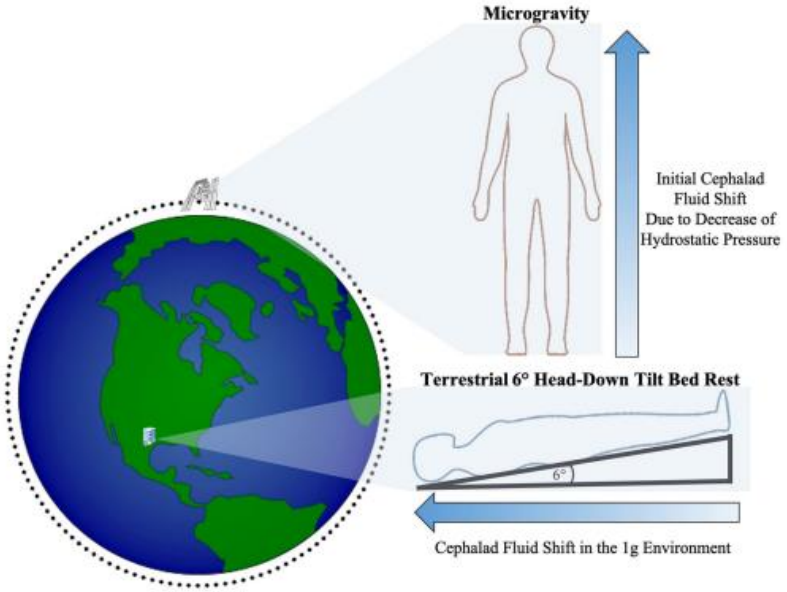

Figure 7. Illustration of the similar cephalad fluid in microgravity and in Head-Down bed rest (Ong et al., 2021).

It has been demonstrated that there are many similarities of the physiological effects between head-down bed rest and space flight. In particular, immobilization, inactivity, and confinement are reproduced in the ground-based model. The cardiac performance, the baroreflex sensitivity, and the reduced aerobic capacity are analogous to those found in space (Convertino et al., 1995). Body weight, muscle mass, and muscle strength are highly compromised in space. Interestingly, both in bed rest and spaceflight, exposed subjects also complain about neuro-ocular impairments (Ong et al., 2021), confirming the validity of this model to test potential countermeasures for space-related CNS disorders.

1.3. Neurobehavioural adaptation to altered gravitational environments

Extreme environments, such as permanence in space or conditions that in some way mimic altered gravity, impose physiological and psychological adaptations that deserve to be studied as they represent "natural" laboratories for advancing our understanding of human physiology and neuropsychology.

It is known that living organisms exposed to space flight or analogs are subjected to acute or chronic stressful conditions (Herman and Cullinan 1997). For example, the prospect of living in isolated and confined artificial environments for a prolonged period represents a quite stressful condition for humans and animals (Palinkas, 2001). Furthermore, motion sickness symptoms, spatial disorientation, alteration in sensorimotor control, or changes in the musculoskeletal system determine physical impairments that compromise the mental health of the individuals.

Adaptive responses refer to an integrated process for single or multiple stressors (e.g., gravity, photoperiod, confinement, isolation) to improve fitness to the environmental changes (Selye H., 1956; Gabriel et al., 2012; Le Roy et al., 2023). Specifically, the preservation of physical and psychological stability to promote the survival of the organism depends on maintaining of the internal stable condition (homeostasis). The response to stressful stimuli involves evolutionarily conserved mechanisms that integrate brain and body functions in order to cope with the stressors and enable adaptation (McEwan, 1998; Godoy et al., 2018). The resulting activation of neuronal and immune systems induces the release of many inflammatory or neurotrophic factors, which comprise the plasticity phenomenon and behavioural outcome at the basis of the individual coping strategies to stress (Yaribeygi and Sahraei, 2018). In this context, investigating animal behaviour in ground based models means studying the initial and adaptive response to environmental changes and synergic and intrinsic mechanisms of reaction to environmental stressors. Therefore, understanding the impact of the extreme environment on the neurobehavioural profile of the animals becomes relevant to investigate the adaptive processes in such environments as well as to develop adequate countermeasure to reduce human and animal suffering in space.

1.3.1. Behavioural responses to gravity changes

Evidence from space and ground-based research revealed that exposure to micro- and hypergravitational environment induces alteration in the vestibular stimulation compromising the postural and motor functions (Francia et al., 2004; Jamon et al., 2014). When the vestibular system is subjected to conflicting sensory information, between vestibular and other sensory systems, can lead to disorientation and to illusory spatial perception, all of which are known symptoms of motion

sickness syndrome (MS; Money, 1970; Yates et al. 1998). In a large variety of animals, MS is associated with the occurrence of emetic response, while in rodents, species unable to exhibit emesis, it is shown though different behavioural endpoints. One of them is *Pica behaviour*. It is manifested by the consumption of substances without nutritional value, such as clay or kaolin. Mitchell and colleagues (1977) pioneered the studies regarding the illness-response behaviour analogous to vomiting in non-emetic species. They reported that rats exposed to hypergravity, usually showed an increase from baseline in the post-rotational kaolin consumption (Mitchell et al., 1977; McCaffrey, 1985; Santucci et al., 2009). Since consumption of non nutritive substances in species incapable of emesis is clearly associated to several of the fundamental characteristics of MS exhibited by species capable of emesis, *Pica behaviour* is considered a reliable MS index (Francia et al., 2004). *Open eyes resting behaviour* is another index of MS in mice (Santucci et al., 2000). It is observed when mice perform no visible movements and maintain eyes open during the rotation-induced hypergravity. It is absent during the phase preceding the start of the centrifuge, while increasing rapidly after only a few minutes from the start of the rotation. This peculiar trend suggested that it could be a valid indicator of the arousal of the MS (Francia et al., 2004).

Mice exposed to hypergravity showed also reduced spontaneous activity immediately after the end of the centrifuge (Santucci et al., 2000). In agreement with previous results reported in rats (Eskin and Riccio, 1996), this reduction may suggest that the animals are experiencing MS syndrome (Santucci et al., 2000). Interestingly, it has been observed that during the hypergravity, both explorative (*digging, rearing*) and maintenance (*face washing*) behaviours were almost suppressed. In general, these behavioural changes appeared restricted in short and transient hypergravitational intervals, after which the majority of behaviours recovered after the end of the centrifugation (McCaffrey, 1985; Francia et al., 2004).

Overall, data emerging from literature showed that the duration of altered gravity represents a condition that determines differences in cognitive and emotional performances in rodents (Mitani et al., 2004). Time of exposure may represent a crucial factor that, together with the ontogenetic phase of the experimental subjects, can influence the plastic processes at the basis of adaptation phenomenon. Indeed, comparing the behavioural profile of mice exposed to different periods of hypergravity, it has been shown that mice exposed for a longer time recovered before than mice exposed for shorted periods. This could be reasonably a consequence of habituation processes, where the duration of the exposure to the altered gravity affects the recovery, probably promoting strategies to cope with the rotation-induced MS symptoms (Francia et al., 2004). Moreover, mice exposed to hypergravity at different developmental phases showed different qualitative changes in muscular force and vestibular reflexes, according to the developing period of exposure (Bojados

and Jamon, 2011). Moreover, it has been demonstrated that rats exposed to altered gravitational environments from conception until different periods after birth, reported intrauterine growth retardation, delay in postnatal motor reflexes and poor performance in tests used to asses sensorimotor coordination (Bouet et al., 2004; Nguon et al., 2006).

At adulthood, a clear effect of altered gravity has been observed in the emotional and cognitive profile of the animals (Francia et al., 2006; Guéguinou et al., 2012). Mice exposed to hypergravity showed increased anxiety-like behaviours (Wall et al., 2001; Palanza P., 2001; Kalueff and Tuohimaa, 2004: Francia et al., 2006) and impairments in the discrimination of a new spatial arrangement, as well as impaired spatial learning performance after repeated (Mandillo et al., 2003) or chronic hypergravity exposure (Bojados et al., 2014; Lee et al., 2020). These results are in line with behavioural studies in mice subjected to simulated microgravity. Rats subjected to different durations of hindlimb unloading model showed convergent impairments in spatial learning and memory (Sun et al., 2009; Wang et al., 2017; Zhang et al., 2018) and the underlying mechanism may involve specific neurotransmitters, such as cholinergic or glutamatergic domains, oxidative damage and/or neuronal apoptotic processes. Table 1 enlists an overview of some behavioural effects in rodents exposed to altered gravitational environments, with particular regard to the specifc developing phase of exposure.

Developing phase	Animal model	Behavioral observations in HG animals	Reference
Adulthood	Rat	↓spontaneous activity	Eskin and Riccio, 1996
		↓the ability to discriminate a novel object from the familiar one	Lee et al., 2020
		↓spatial memory	Horii et al., 2017
		↓spatial learning task	Mitani et al., 2004
		↓ cerebellar motor coordination	Noh et al., 2020
	Mouse	↓spontaneous activity	Santucci et al., 2000
		↑ risk assessment and defensive behaviour	Francia et al., 2006
		↓spatial learning	Bojados and Jamon, 2014
		↓ability to discriminate a new spatial arrangement	Mandillo et al., 2003
		↑ anxiety-like behaviours	Guéguinou et al., 2012
Adolescence	Mouse	↓emotional/anxiety like responses	Santucci et al., 2009
		↓spontaneous activity	Francia et al., 2004
Early postnatal development	Rat	↓development motor reflexes	Bouet et al., 2004
		↓sensorimotor coordination	Nguon et al., 2006
		↓*surfacing* behaviour	Wubbels and de Jong, 2000
		↓ exploration of pokes in a hole board, ↑fear-related responses	Thuller et al., 2002
		↓development of contact-righting, air-righting	Bouet et al., 2004
		↓motor coordination	Abe et al., 2008
		↓learning abilities	Cao et al., 2005
		↓lower forelimb force and delayed vestibular reactions	Bojados and Jamon, 2011
	Mouse	↓ the Righting Reflex, Dowel test performance	Schwartz et al., 2019
		Significant postural changes, particularly with a more extended ankle joint	Bojados *et al*. 2013

Table 1. Behavioural responses in rodents exposed to altered gravitational environments.

1.3.2. Central nervous system responses to gravity changes

The sensitivity of the mammalian CNS to altered gravitational environments involves morphological, cellular and molecular factors. A clear example comes from the neuronal substrates of MS symptoms experienced by astronauts during spaceflight or rodents during centrifuge induced hypergravity.

It has been largely demonstrated that during and/or after the spaceflight disturbances related to the vestibular system could be associated to damage in labyrinthine structure, to changes in the dimensions of the otoconial mass or to synaptic reorganization, principally in local circuits of vestibular maculae (Ross and Tomko, 1998; Kalb and Solomon, 2007). Interestingly, similar and/or opposite responses come from ground-based studies, where rodents showed a retardation in the development of neuronal circuitries in the vestibulum, a reorganization of the vestibular architecture or a reduction in otoconiae dimensions (Bouet et al., 2004). Some studies correlate the behavioural observations during and after exposure to altered gravity with structural abnormalities in the cerebellum. In particular, it has been reported that both acute and chronic exposure to hypergravity during gestation and/or early postnatal period subtly but consistently influenced cerebellar mass, alter cerebellar protein expression, and Purkinje cell proliferations (Baer et al., 2000; Sajdel-Sulkowska et al., 2001, 2005; Nguon et al., 2004). In 2020, Noh and colleagues reported that motor coordination dysfunctions after hypergravity exposure were correlated with a reduction in synaptic activity of Purkinje cells, similar to the alterations in proprioceptive and vestibular afferent inputs observed in the HU model (Krasnov et al., 2009).

As mentioned by Sadel-Sulkowska and colleagues (2009), relevant key mediators of sensorimotor impairments after altered gravity exposure, could be the cerebellar neurotrophic factors. Comparing the effects of hypergravity during different discrete developmental periods in rodents, they concluded that the alterations of cerebellar Brain derived-neurotrophic factor (BDNF) and Nerve growth factor (NGF) could contribute to the observed senosorimotor impairments. In general, the expression of NGF and BDNF has been found in the neocortex, cerebellum, hippocampus, and hypothalamus of both developing and adult CNS (Francia et al., 2004). They are key players involved in neuroplasticity phenomena in response to environmental changes, enhancing synaptic strength, altering spine density and promoting dendritic branching and axonal sprouting. Stressful events, such as altered gravity exposure, can affect the expression of these neurotrophins, which in turn act as transducers of the stressful stimuli on CNS mediating the behavioural coping response (Alleva et al., 1996). In particular, acute exposure of mice to hypergravity resulted in an increased amount of NGF in frontal cortex, hypothalamus and hippocampus (Santucci et al., 2000), while opposite results were obtained from mice exposed chronically to space environment (Santucci et al.,

2012). Indeed, it is known that following chronic stressful stimuli neurotrophic levels becomes reduced in the CNS, leading to a reduced brain plasticity and greater vulnerability for stress-related behavioural disorders (Cirulli and Alleva, 2009).

1.4. Aim of the work

The National Aeronautics and Space Administration (NASA) has announced its intention to undertake a return mission to the Moon. Furthermore, some countries are considering going to Mars, and commercial spaceflight is on the horizon (Roberts et al., 2020).
The spaceflight environment poses significant challenges to multiple organ systems, including changes in behavioural responses and cognitive performance. Therefore, understanding how the brain copes with space travel and uncovering short-, medium- and long-term health hazards has broad implications for arranging crewed space missions (Roberts et al., 2020).
In order to preserve safety in the evolving astronaut and considering the extreme variety of individual differences, the main objective of space biomedicine consists on understanding the individual risk profile in extreme environment in order to develop countermeasures that are tailored to each individual characteristic (Schmidt and Goodwin, 2013).
Although personalized medicine usually uses a person's genes, transcripts, proteins, and metabolites to optimize both safety and performance in microgravity, the investigation of neurobehavioural responses to un-physiological gravity represents a unique endpoint of complex integrated systems that could be predictive of individual susceptibility to altered gravitational environments.

Animals are sent into orbit to proactively foresee health problems in humans and, due to the limited opportunities for rodent spaceflight studies, they are extensively implicated on Earth in ground-based models of micro- or hypergravity. Indeed, the investigation of murine behavioural responses to un-physiological gravity represents an opportunity to investigate adaptation to extreme condition, deepen mechanisms underlying individual susceptibility to stress and, prospectively, develop adequate countermeasures to preserve and reduce animal suffering in space.
Given this emergent research framework, the present work of thesis was tailored to investigate neurobehavioural adaptive responses in animal and human in ground-based models of altered gravity in order to define predictive neurobehavioural indicators of vulnerability and resilience.

In Chapter n.2, we provided results collected in the international project "The MDS on LDC: Tissue Sharing Programme", aimed at integrating data from a space-related engineered payload (i.e. the Mouse Drawer System, developed by Thales Alenia Space under ASI contract) which was incorporated in the International Space Station hosting mice for 91 days with ones obtained in a 3G environment from the same payload adapted to the gondolas of the Long Diameter Centrifuge (LDC; van Loon et al., 2008) at the European Space Research and Technology Center, (ESTEC, The Netherlands). The objective of the present study was to evaluate the neurobehavioural

adaptation of mice subjected to 28 days to hypergravity induced by rotation. The behavioural profile was investigated before, during and after the end of the permanence in the centrifuge to uncover possible predictive behavioural indicators of susceptibility to un-physiological gravity. Adaptation profile upon centrifugation and spontaneous activity as well as emotional response were evaluated immediately after the end of the hypergravity exposure. Considering the relevance of microbial communities in promoting or ameliorating individual susceptibility to extreme conditions (Tesei et al., 2022), the heuristic value of preliminary investigations of fecal microbiome analysis after hypergravity exposure were also assessed.

In Chapter n.3, we investigated the behavioural adaptive responses of adult mice subjected to hindlimb unloading paradigm, which is a well-established model to explore muscle atrophy and osteoporosis caused by gravitational alterations (Milstead et al., 2004; Chowdhury et al., 2013). Mice were exposed to different durations of suspension, their behavioural repertoire was evaluated during and after the end of the suspension, and the individual ethogram has been defined, studied throughout the experimental schedule and compared with post suspension data to define predictive behavioural indicators of susceptibility to microgravity simulated condition. Moreover, by exploring the neuroplasticity phenomena underlying the adaptation to this paradigm, NGF and BDNF been also evaluated at the end of the procedure.

Lastly, in Chapter n.4, we extended the investigations on human volunteers evaluating their neuroinflammatory response during the bed rest condition, largely used in space biomedicine to reproduce adaptive physiological mechanisms similar to those experienced by astronauts in space (Pandiarajan and Hargens, 2020). In particular, the object of the study was to assess predictive neuroinflammatory biomarkers of individual vulnerability or resilience to microgravity simulated conditions correlating salivary levels of neurotrophins and inflammatory markers with psychometric responses.

1.5. References

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2. EFFECT OF 28 DAY-3G-EXPOSURE IN C57BL6J ADULT MALE MICE: BEHAVIOURAL STUDY, MICROBIOTA AND NEUROBIOLOGICAL ANALYSIS

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In preparation

2.1. Abstract

The effect of gravity levels on living systems has been recognized for many years and the issue of animal exposure to un-physiological gravity is of primary importance both to understand behavioural and physiological adaptations in such environment and to develop countermeasures attempting to minimize the suffering of animals used in space research. Several experiments with mice under microgravity conditions have already been performed and complementary on-ground reference experiments under hypergravity conditions would complement experimental results obtained under microgravity, permit the collection of experimental data under a wide range of gravity levels, and allow the extrapolation of the effects of microgravity conditions via experiments at one or higher g-levels.

In the present study, the space-related payload Mice Drawer System (MDS), which was incorporated in the International Space station hosting mice for 91 days, was accommodated inside the ESA Large Diameter Centrifuge (LDC) in order to start to utilize for a shorter period of time, i.e. 28 days, the same facility on-ground (under hypergravity conditions) and allow the comparison of scientific data collected in the same housing conditions (same food and water delivery systems, illumination, cage dimensions, ventilation etc.). Behavioural profile, neurobiological assessment of neurotrophins in the central nervous system and microbiota panels were evaluated in mice exposed to 3g hypergravity and their relative controls. Although no significative differences were observed in neurobiologial endpoints indicating a possible adaptation to the hypergravity condition, data confirm neurobehavioural changes upon centrifugation and microbiota composition. Moreover, several predictive behavioural indicators of susceptibility to un-physiological gravity were identified and correlated to neurological biomarkers.

Keywords

hypergravity, brain, behaviour, neurotrophins, microbiota

2.2. Introduction

Altered gravitational environments, such as the permanence in space or in conditions that in some way mimic wheightlessness, represent a unique challenge for terrestrial biological systems (Santucci et al., 2012). It is known that long term spaceflight determines several risks to the body, including loss of skeletal tissue (Vico and Hargens, 2018), atrophy of cardiac muscle (Shibata et al., 2023), immune systems related effects (Akiyama et al., 2020), and deficits in motor sensory system (Kourtidou-Papadeli, C., 2022; Tays et al., 2021). Animals are sent into orbit to practively foresee possible health problems in humans and research with animal models subjected to un-physiological gravity fills an important niche in understanding the phenomena underlying tolerance and adaptation to such environmental conditions. Exploiting these responses represents an opportunity to investigate mechanisms underlying neuroplasticity phenomena and the individual vulnerability to stress.

It has been largely reported that central nervous system (CNS) is deeply affected by altered gravity conditions. For example, rats exposed to microgravity showed changes in synapse morphology, with increased neurodegeneration processes in cortical regions (Clément et al., 2020), alteration in expression of immediate early genes indicative of changes in neural activity during spaceflight (Pompeiano et al., 2002; Popova et al., 2020; Holley et al., 2022; Kremsky et al., 2023), as well as impairments in synaptogenesis phenomena in response to gravitational stress (Proshchina et al., 2021; Mhatre et al., 2022; Mikheeva et al., 2023). Studies conducted on mice subjected to long-term microgravity evidenced similar alterations on neurogenesis (Naumenko et al., 2015; Popova et al., 2015; Gupta et al., 2023), apoptotic processes (Sharma et al., 2008; Pani et al.,2013) and monoaminergic systems in selected brain regions (Popova et al., 2015, 2020), with synergic effects on neurotrophin levels (Santucci et al., 2012; Naumenko et al., 2015; Ishikawa et al., 2017), inflammatory responses (Wise et al., 2005; Wang et al., 2015) or oxidative processes (Rizzo et al., 2012; Mao et al., 2020). In line with the gravity continuum paradigm, comparable results have been also observed in animals subjected to hypergravity-induced by rotation (Hariom et al., 2021). Indeed, most ground experiments employing animal models used the paradigm of hypergravity induced by rotation with the expectation that behavioural and physiological reactions to this environment might help to explain reactions to the microgravity challenges, also comparing adaptation processes at the integrated whole organism. Results collected in many years of space biology demonstarted that the identification of putative mechanisms under CNS control are critical to develop countermeasures to ensure human and animal brain during the spaceflight (Mhatre et al., 2022). Indeed, several studies investigated the impact of hypergravity on CNS, evidencing effects on brain neurotrophin levels (Bo et al., 2019; Popova et al., 2020), immune system factors (Moser

et al., 2023) or gene expression of neurotransmitter systems (Miller et al., 1989; Popova et sl., 2020; Kim and Kim, 2021). Alteration in functional, structural and morphology of brain regions have been also found after experiencing hypergravity (Noh et al., 2020; McGregor et al., 2023). Intriguingly, in a recent work published by Dubayle and colleagues (2023) it has been demonstrated that the modifications in the vestibular functions after the exposure to hypergravity induced by rotation could be also associated to a dysregulation in the endothelial cells forming the blood-barrier brain, that represents a crucial interface between brain neuronal activity and the systemic vascular system. In addition, an emerging factor in the evolution of vestibular related alterations during the exposure to un-physiological gravity is also found in the relationship between brain and intestinal functions (Matsumoto et al., 2011; Thornton et al., 2013; Wang et al., 2015; Dubayle et al., 2023). Results derived from preclinical studies strongly support the concept of brain-gut-microbiome interactions (Martin et al., 2018) in terms of bidirectional influence between brain functions and gut microbiota during the reactions to stressful stimuli (Foster et al., 2017). Sympathetic nervous system and hypothalamic-pituitary-adrenal (HPA) axis play a fundamental role in the brain-gut axis, driving communication between the CNS and the microbiota in the gastrointestinal tract (Dinanand Cryan, 2017) and supporting the concept that stress-responsiveness and susceptibility to altered environmental challenges, such as altered gravity conditions, could be influenced also by gut microbiota composition.

In a context of growing interest toward the need to define reliable predictive indicators of vulnerability to altered gravity condition in order to reduce human and animal suffering in space and continue human activity in microgravity, the investigation of the neuro-behavioural responses under altered gravity conditions play a crucial role in the analysis of the initial and adaptive responses to this environmental challenge. Therefore, the objective of the present study was to evaluate the neurobehavioural adaptation of mice subjected to 28 days of hypergravity induced by rotation, investigating their behavioural profile before, during and after the end of the permanence in the centrifuge, selecting possible predictive behavioural indicators of susceptibility to un-physiological gravity and evaluating the results in the context of the preliminary investigations of fecal microbiota. It is worth mentioning that the study was conducted accommodating the Mice Drawer System (MDS, a space-related engineered payload, which was incorporated in the International Space station hosting mice for 91 days) inside the ESA Large Diameter Centrifuge (LDC, van Loon et al., 2008) in ESTEC (European Space Research and Technology Center, Netherlands) in order to compare data collected in altered gravity on mice maintained in the same housing conditions, despite for different duration (food and water delivery systems, illumination, cage dimensions, ventilation etc.).

2.3. Materials and Methods

The experimental schedule consisted in a first pilot study concerning the collection of preliminary data in order to evaluate the responses of mice subjected to 15 days in LDC (results reported in Supplementary materials) and refine possible methods to reduce animal distress in this condition, Subsequently, on the base of the preliminary results, a second experiment was realized in order to evaluate the effects of the exposure to hypergravity for 28 days.

Animals

Upon arrival at the laboratory (University of Leiden, Netherlands), C57BL6 male mice (8-12 weeks) were housed under standard conditions in an air-conditioned room (temperature 21 ± 1 ◦C, relative humidity 60 ± 10%; 12 h–12 h light/dark cycle), in 42 cm × 27 cm × 14 cm Plexiglas cages with a metal top and sawdust as bedding. Pellet food (Mucedola Srl, Settimo Milanese, Italy (cereals 66,5%, vegetable proteins 18,2%, forage 7,5%, animal proteins 3,5%, vitamin and mineral mixture 3,2%, fats from soya oil 0,4% and amino acids 0,1% - metabolizable energy 2668 kCal/kg) and tap water were provided ad libitum.

After a period of acclimation to the housing conditions, adult C57BL6 mice with the same sex and age were weighed and randomly assigned to 3 experimental groups:

(1) Hypergravity group (MDS, n=11): mice were individually housed in two mouse drawer system (MDS) exposed to 28 days of hypergravity through the Large Diameter Centrifuge (LDC; van Loon et al., 2008) at ESTEC (Netherlands).
(2) ground control (CTR, n=12): housed individually in identical MDS cages at University of Leiden and not exposed to hypergravity.
(3) vivarium control (VIV, n=12): housed individually in normal vivarium cage at the University of Leiden as the laboratory controls.

The Large Diameter Centrifuge and MDS payloads

The LDC was an apparatus composed of 2 identical perpendicular arms mounted around a central rotor. Each arm termed in free swinging gondolas (60x60x80) where it was possible to install the two MDS payloads. The LDC had a maximum diameter of 8m for rotation and could provide centrifugal forces of maximum 20g. The mice were housed in MDS payloads and exposed to 3g for 4 weeks with a stop after 15 days for food and water supply.

Each MDS was divided into two habitats which provide all the items necessary for the life of 3 individually housed mice. In particular, each habitat was composed by three metallic cages (floor

area: 116x98 without sawdust) structured with three drinking valves for water delivery, three food envelopes, three cameras for videomonitoring the animals during the hypergravity exposure, white and infrared LED's for illumination and sensors for air composition monitoring and control (temperature, rH, CO2 and NH3). The water was delivered automatically ad libitum, while the food was delivered 2 times a day every day corresponding to 5 grams of food each day. The system permits to check anytime the water/food consumption, the habitat parameters and the animals' behaviour.

Behavioural study

The behavioural profile of each experimental subject was evaluated before, during and after the hypergravity exposure (in the results section Pre-HG and Post-HG were used to indicate the time before and after the beginning and the end of the centrifuge). Their spontaneous activity and emotive profile were assessed before and after the centrifuge in a novel object test, thus their behavioural profile across the 4 weeks of permanence in LDS was scored during the first 2 days, the intermediate 2 days and the final 2 days. After the end of the centrifuge the animals were subjected to the tail suspension test to evaluate their vestibular-related behaviour. All videos were scored using a dedicated ethological software ("The Observer 2.0"; Noldus, 1991).

Novel Object test

Mice were videorecorded in a novel plexiglass cage with sawdust (42 cm ×27cm ×14cm). Each experimental subject was placed at the centre of the cage and the spontaneous locomotor activity was videorecorded for 3 min. Frequency and duration of the following behavioural items were recorded: *crossings* (crossing the square limits with both forepaws), *wall rearing* (vertical exploration with hind-paws on the floor and forepaws on the wall of the arena), *rearing* (vertical exploration with hindpaws on the floor and forepaws in the open), *exploring* (exploration of the environment), *grooming* (self-explanatory), *inactivity* (lying flat or standing still in total absence of movement), *digging* (digging up the sawdust). Subsequently, a stimulus object (a glass marble) was placed at the centre of the half-cage, and latency, number and duration of sniffing contacts with the object were recorded for 5 min. The position of the marble was randomized for each animal and the cages used for testing the animals were changed for each experimental subject. The recording session took place between 1.00 and 5.00 pm, in an experimental room maintained at the same temperature and humidity conditions as the housing one.

Tail suspension test for vestibular-Related behaviour

After the Novel Object test at the end of the hypergravity exposure, mice were slowly suspended by their tail 30 cm above an open cage with sawdust for 30 s. The occurrence of *spinning* behaviour (mice spin in horizontal plane upon tail suspension) was detectable during the suspension. The occurrence of this behaviour was expressed as a percentage of total animals tested, independently of the length of time the phenotype was displayed (Thrushina et al., 2006).

International Tissue sharing program

According the initial conception of MDS (Cancedda et a., 2012), that was sharing tissues collected form flight mice to provide data of the effects of microgravity on multiple physiological systems, a Tissue Sharing Program was planned at the end of the hypergravity exposure. Research groups involved in the international project and with different expertise contributed to collect tissues in order to contribute with data on hypergravity effects on skeletal, cardiovascular, and immune systems, liver, kidney and brain functions. During the TSP operations every effort was made to harvest as many different samples and types of tissue as possible from the mice in order to obtain the maximum of data from this unique experiment.

Our research group was involved in the collection of brain tissues (hypotalamus, olfactory bulbs, pre-frontal cortex, cortex, hippocampus, striatum, cerebellum) in order to evaluate neurotrophin levels and inflammatory biomarkers, and feces collection for microbiota analysis.

Brain and fecal preparation before ELISA or microbiota sequencing

Brain and fecal samples were Trypsin-EDTA harvested and pellets were homogenized by ultra-sonication (Sonics, Newtown, CT, USA) in RIPA buffer (50 mM Tris-Cl, pH7.5; 150 mM NaCl; 5 mM EDTA; 1% Triton X-100; 0.1% SDS; 0.5% DOC (Sodium deoxycholate; 1 mM PMSF; 1 μg/ml leupeptin). Clear supernatants (4 °C/13,000 rpm, 20 min) were collected and used for quantification of neurotrophin, cortiosterone and microbiota levels.

Quantification of brain neurotrophin and adrenal gland corticosterone levels using ELISA

For NGF, BDNF, and corticosterone quantification, double-sandwich ELISA assays were used. In particular, 96-well Maxisorp plates (Nunc, Roskilde, Denmark) were pre-coated with the specific capture antibodies (0.4 μg/mL; R&D) overnight (4°C). Samples were diluted 1:2 in assay diluent (from the kit; R&D) supplemented with 1x protease inhibitor cocktail (Pierce - Thermo Fisher Scientific Inc. Waltham, MA USA) and loaded in parallel with the standard curve (0.32–2,000

pg/mL protein; R&D). Overnight incubation with sample was performed, and subsequently the addition of the specific detection secondary antibodies (0.15 μg/mL; R&D) and streptavidin (1:200; R&D) were carried out. Specific binding was developed by using the ready-to-use TMB substrate and Stop solution (R&D). The colorimetric signals (Optic Density, OD) were acquired at λ 450–570 nm by using the Sunrise plate reader (Tecan Group Ltd., Männedorf, Switzerland). The related target values (pg/mL) were produced using a third grade polynomial standard. The absence of cross reactivity with other neurotrophins was declared by the manufacturer for both assays.

Microbiota sequencing

DNA was extracted from the samples, and primers were used to amplify the V3-V4 region of 16S rRNA marker-gene, which was then analyzed by 2x300 paired-end sequencing. Sequencing was performed on an Illumina MiSeq platform. Bioinformatics analyses were performed in Quantitative Insights Into Microbial Ecology version 2 (QIIME2) software suite and R packages. The environmental microbiome data was analyzed by DADA2 algorithm and an in-house trained classifier for taxonomy assignment. The Silva database, release 138.1 was used to identify the bacterial flora in each sample. Assessment of alpha and beta microbiota diversity was performed using the QIIME2 platform v2023.5 with the default parameters. To test our null hypothesis statistical analysis using PERMANOVA test of alpha and beta diversity was conducted on samples grouped in the relevant variables of interest.

Statistical analysis

Continuous variables are presented as means and standard errors of means (S.E.M.). The parameters scored across the 28 days of exposure to hypergravity were analyzed by repeated measure analysis of variance (rmANOVA) with Day of exposure to hypergravity (six levels: Day1 vs Day2 vs Day 14 vs Day 15 vs Day 27 vs Day 28) as within-subjects factors. Data collected after the end of the hypergravity exposure were analyzed by two-ways ANOVA with time (two levels: before hypergravity-Pre-HG vs after hypergravity-Post-HG) as within factor and treatment (three levels: vivarium vs control vs hypergravity) as between factor.

When the interaction between treatment and another independent variable was significant, post hoc comparisons were performed using Tukey HSD test. Multiple comparisons were applied to logical sets of means according to the specific objectives of the work. Latency data, which had no normal distribution, were analyzed with the Mann-Whitney nonparametric test. To analyse the presence/absence of spinning behaviour we used the Fisher's probability test. Correlations were calculated with Pearson correlation coefficient.

Quantitative analysis of microbiota was conducted using One-way ANOVA treatment (three levels: vivarium vs control vs hypergravity) as between factor, while Ranks in one-criterion variance analysis (Kruskal Wallis) was used for Phylogenetic diversity indexes.

Comparisons were performed by the Statview software; statistical significance was set at $P < 0.05$.

2.4. Results

MDS mice recover drinking behaviour across the weeks of exposure to hypergravity

The monitoring of individual water daily consumption of mice during the exposure to hypergravity revealed that, differently form CTR mice (Fig.11 in supplementary materials), MDS mice showed a drastic drop in water consumption during the first 4 days in centrifuge compared to the first day with a subsequent gradual increase from the second week of exposure ($F_{(25,250)}$= 11,233, P<0,0001, P<0,05 after *post-hoc* comparisons between Day1 and Day3 until Day9, or Day 11 or Day 14 or Day 27; Fig.1).

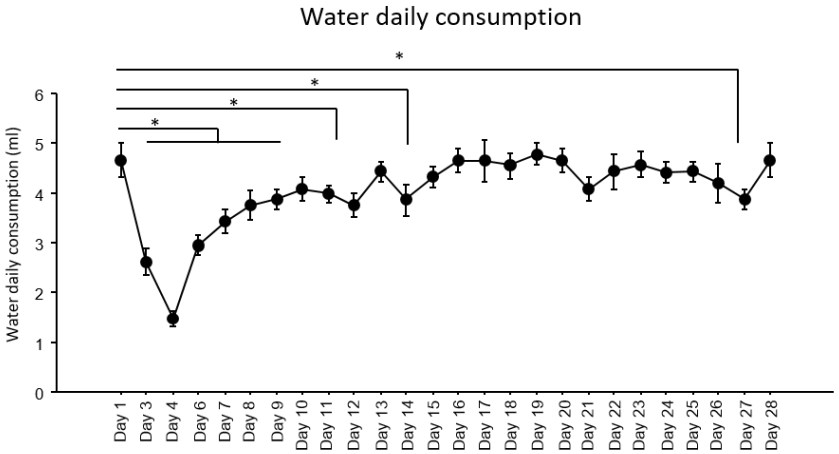

Figure 1. Water daily consumption in MDS mice. Although it has been observed a drop in water consumption during the first 4 days of hypergravity exposure, MDS recovered drinking behaviour across the weeks of exposure to hypergravity. ∗Statistically significant at p< 0.05 using repeated measure ANOVA followed by post-hoc Tukey HSD test. N=11.

Mice conserved species-specific behaviours during 28 days of hypergravity

The individual behavioural observation during the exposure to hypergravity evidenced that the species-specific behavioural repertoire was conserved with subtle changes across the days (Fig.2). In particular, the analysis of the duration of *grooming* behaviour revealed a significant increase after the second week of permanence in centrifuge ($F_{(1,10)}$= 8,483, P<0,0155) and a significant reduction in *inactivity* behaviour ($F_{(5,50)}$= 23,948, P<0,0001, P<0,0001 after *post-hoc* comparisons between Day1 and Day14 or Day15 or Day27 or Day28) compared to the Day1. Although no significant difference has been revealed, it is interesting to note a concomitantly increase in the duration of explorative behaviours (*exploring*, *head rising*, *vertical movements*) after the second week of exposure to hypergravity compared to the Day1.

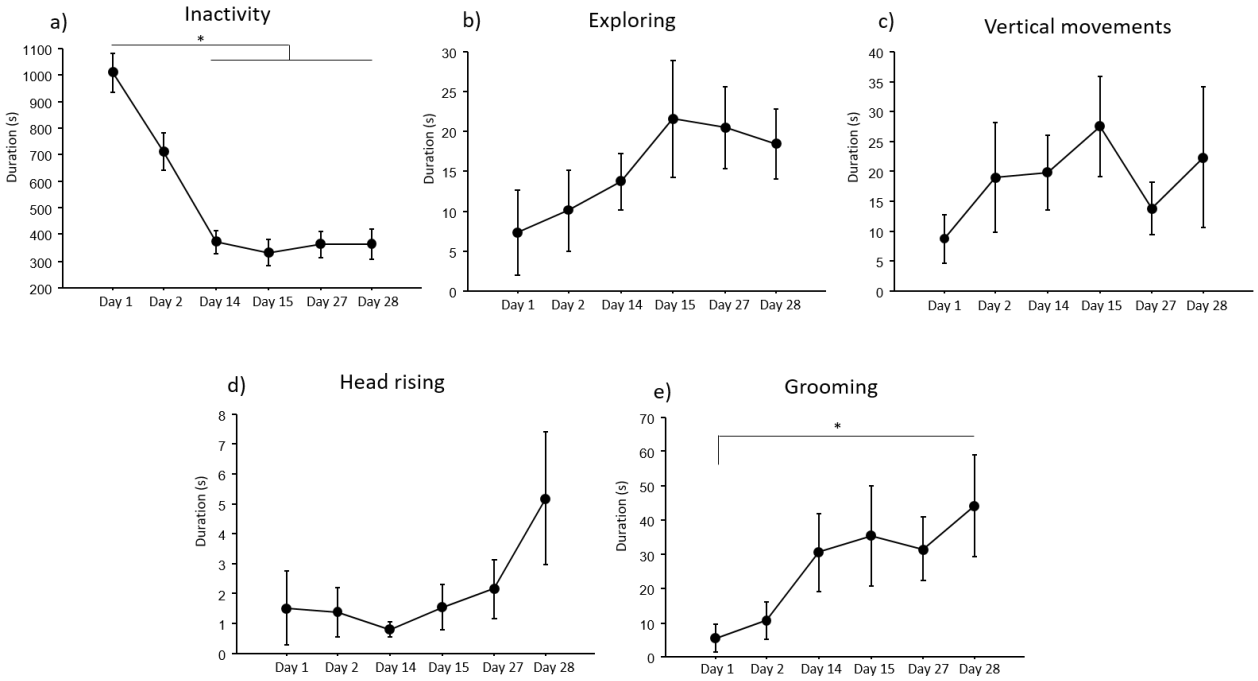

Figure 2. Behavioural analysis during the hypergravity exposure. An increase in grooming behaviour (e), a tendency to improve exploring (b), vertical movements (Wall rearing and rearing) (c) and head rising behaviours (d) and a concomitantly reduction in inactivity (a) have been observed during the permanence in centrifuge. Data are shown as means ± S.E.M. ∗Statistically significant at $p< 0.05$ using repeated measure ANOVA followed by post-hoc Tukey HSD test, N=11.

Hypergravity exposure impacts mouse body weight

The analysis of body weight evidenced that mice subjected to hypergravity lost weight after 28 days of hypergravity ($F_{(1,21)}$= 28,111, P<0,0001, P<0,05 after *post-hoc* comparisons between mice before and after exposure to hypergravity), and resulted in reduced in body weight compared to ground based controls ($F_{(1,21)}$= 28,111, P<0,0001, P<0,05 after *post-hoc* comparisons between CTR and HG mice after hypergravity exposure; Fig.3).

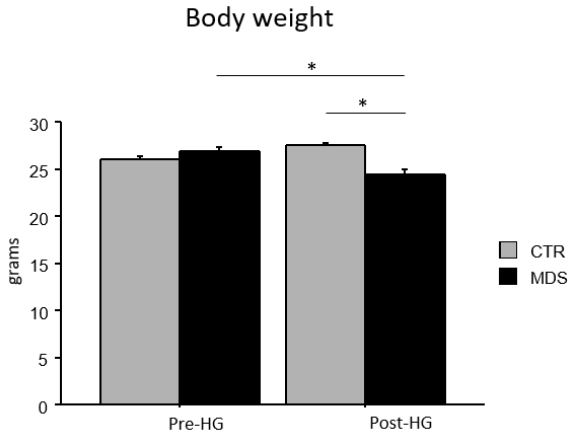

Figure 3. Body weight before and after hypergravity exposure. A reduction in body weight has been observed in mice exposed to hypergravity. Data are shown as means ± S.E.M. ∗Statistically significant at $p< 0.05$ using repeated measure ANOVA followed by post-hoc Tukey HSD test. CTR before hypergravity=10, CTR after hypergravity=10, MDS before hypergravity=11, MDS after hypergravity=11.

28 days of hypergravity affects locomotor activity and vertical movement in mice

In line with results collected during Dry Run experiment (Fig.12-13 in supplementary materials), mice subjected to hypergravity showed reduced explorative behaviours in response to hypergravity exposure (Fig. 4,5,6).

Considering the interaction between time and treatment, *post hoc* analysis revealed that HG mice exhibited reduced number of *crossings* compared to their performance before hypergravity exposure ($F_{(2,28)}$= 2,975, P=0,05; P<0,05 after *post hoc* comparison between MDS group before and after hypergravity exposure) and vertical movements (duration: $F_{(2,28)}$= 7,818 , P=0,0020; P<0,05 after *post hoc* comparison between MDS group before and after hypergravity exposure; frequency: $F_{(2,28)}$= 14,851 , P<0,0001; P<0,05 after *post hoc* comparison between MDS group before and after hypergravity exposure). Differently from CTR group (duration: $F_{(2,28)}$= 5,981, P=0,0069; P<0,05 after *post hoc* comparison between CTR group before and after 28 days; frequency: $F_{(2,28)}$= 5,774, P=0,0079; P<0,05 after *post hoc* comparison between CTR group before and after 28 days) , no gross differences were found in *digging* behaviour in mice subjected to hypergravity compared to their previous performance. Moreover, a reduction in crossings ($F_{(2,28)}$= 2,975, P=0,05; P<0,05 after *post hoc* comparison between MDS and CTR groups after hypergravity exposure or CTR and VIV groups after 28 days), duration and frequency of vertical movements (duration: $F_{(2,28)}$= 7,818 , P=0,0020; P<0,05 after *post hoc* comparison between MDS group and CTR or VIV groups after hypergravity exposure; frequency: $F_{(2,28)}$= 14,851 , P<0,0001; P<0,05 after *post hoc* comparison between MDS group and CTR or VIV groups after hypergravity exposure) and *digging* behaviour (duration: $F_{(2,28)}$= 5,981, P=0,0069; P<0,05 after *post hoc* comparison between MDS group and CTR group after hypergravity exposure; frequency: $F_{(2,28)}$= 5,774 , P=0,0079; P<0,05 after *post hoc* comparison between MDS and CTR groups after hypergravity exposure) were observed in HG mice compared to controls, selectively after the end of LDC.

Although no significant difference has been revealed, it is interesting to note that CTR and MDS mice tended to increase the duration and frequency of *Grooming* behaviour compared to their performance before the 28 days, differently from VIV mice.

During the approach to the novel object, although results collected during the Dry Run experiment revealed that mice experienced hypergravity for 15 days showed a decreased approach to the novel object (Fig.14 in supplementary materials), no significant differences were found in the duration, frequency and latency of *Object sniffing* behaviour in mice subjected to hypergravity for 28 days. Surprisingly, a significant difference in the number and duration of approach to the novel object has been observed in CTR mice before the exposure to hypergravity, selectively compared to HG or VIV mice (duration: $F_{(2,28)}$=5,601, P=0,0090, P<0,05 after *post hoc* comparison between CTR mice

and HG or VIV mice; frequency: $F_{(2,28)}$= 4,198 , P=0,0254; P<0,05 after *post hoc* comparison between CTR mice and HG or VIV mice) or between CTR and VIV group after the end of LDC (frequency: $F_{(2,28)}$= 4,271, P=0,0223; P<0,05 after *post hoc* comparison between CTR mice and VIV mice; Fig.6).

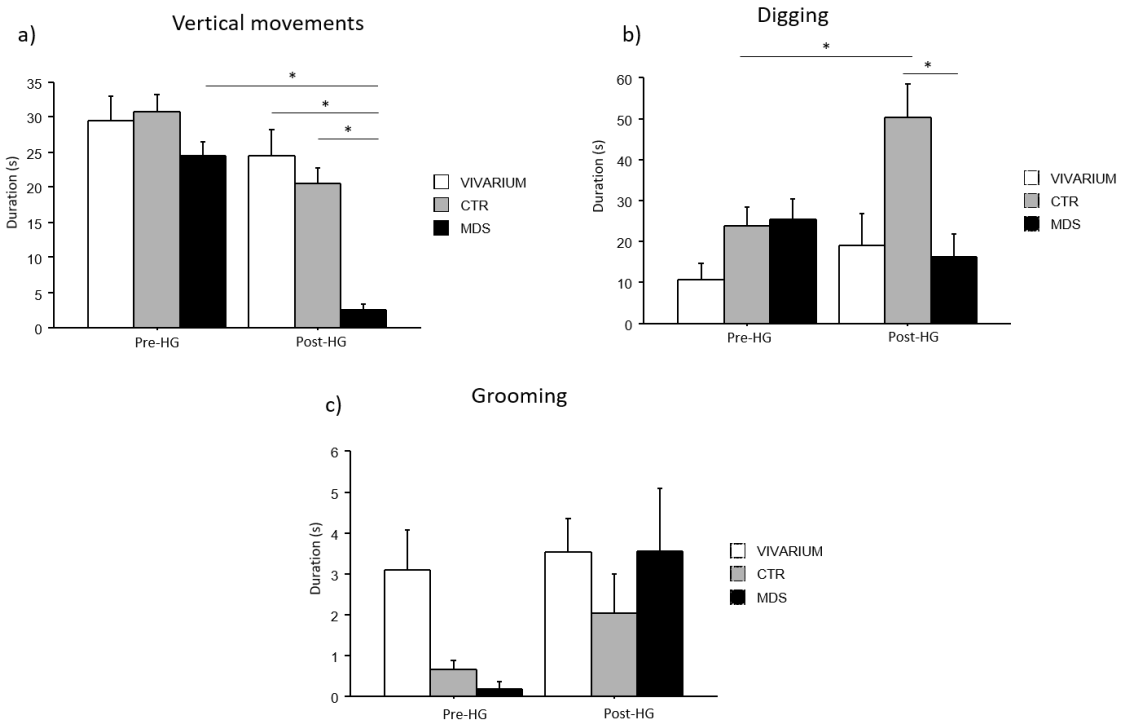

Figure 4. Behavioural analysis before and after hypergravity exposure. A reduction in the duration of vertical movements (a) and digging behaviour (b) has been observed MDS groups. Interestingly, CTR and MDS mice tent to increase the duration of grooming behaviour after LDC. Data are shown as means ± S.E.M. ∗Statistically significant at p< 0.05 using repeated measure ANOVA followed by post-hoc Tukey HSD test, MDS=11, CTR=10, VIV=10.

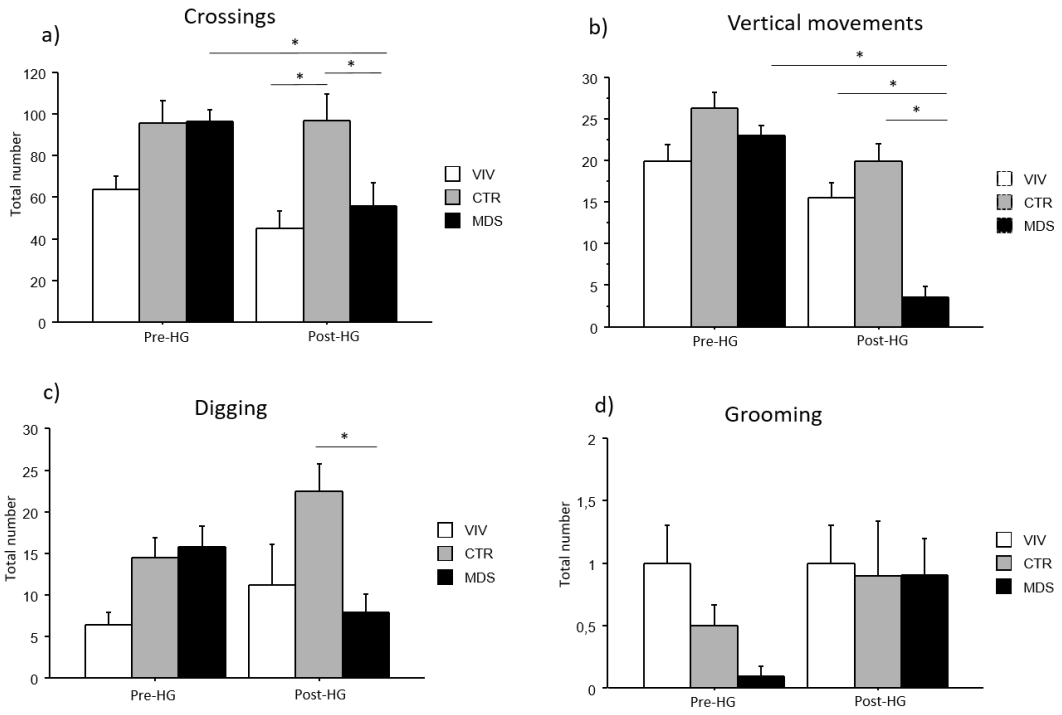

Figura 5. Behavioural analysis before and after hypergravity exposure. Decreased locomotor activity (a), frequencies of vertical movements (b) and digging behaviour (c) have been observed in MDS group. Interestingly, CTR and MDS mice tent to increase the frequency of grooming behaviour after LDC. Data are shown as means ± S.E.M. ∗Statistically significant at $p< 0.05$ using repeated measure ANOVA followed by post-hoc Tukey HSD test, MDS=11, CTR=10, VIV=10.

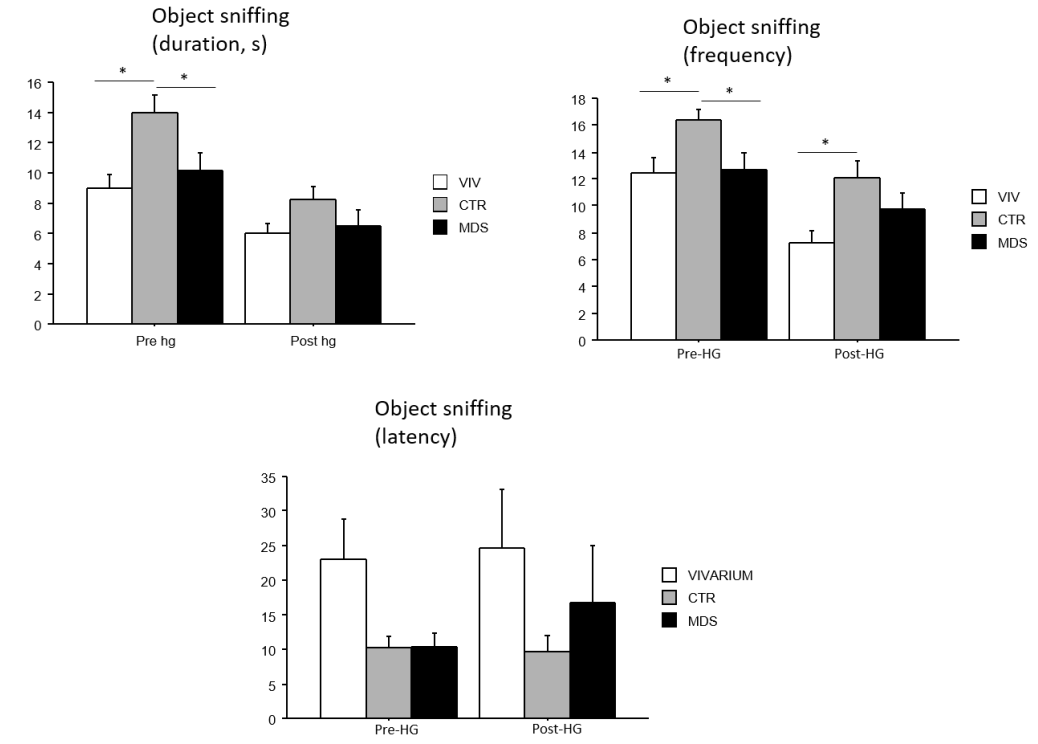

Figure 6. Effect of hypergravity on the emotional profile of mice. Although no significant differences were observed in MDS mice, increased duration (a) and frequency (b) of object sniffing behaviour has been observed in CTR mice. Data are shown as means ± S.E.M. ∗Statistically significant at $p< 0.05$ using repeated measure ANOVA followed by post-hoc Tukey HSD test, MDS=11, CTR=10, VIV=10.

28 days of hypergravity impact vestibular related behaviour in mice

Three mice subjected to hypergravity tourqued in circles immediately when tail suspended. This behaviour was very evident and in some cases very quick. Although no statistical significance was observed, it is worth mentioning that it was performed exclusively by MDS mice (Fig.7).

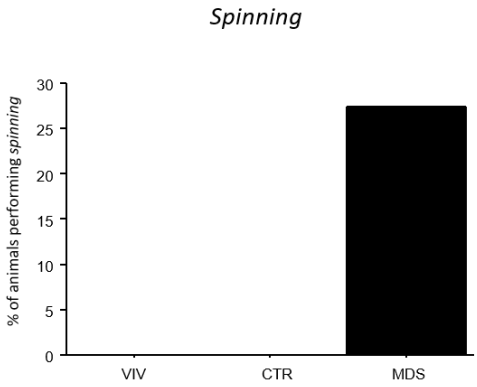

Figure 7. MDS mice displayed spinning behaviour upon tail suspension. Spinning was expressed as a percentage of total animals tested. Data are shown as means ± S.E.M. ∗Statistically significant at $p < 0.05$ using Fisher's probability test, MDS=11, CTR=10, VIV=10.

Exposure to HG did not impact brain neurotrophins levels and corticosterone in adrenal gland

The analysis of brain NGF and BDNF levels revealed that no differences were found among the three experimental groups in both neurotrophins (data not shown; it is wirth mentioning that circulating neurotrophin levels are generally in line with SNC levels, suggesting a comparable scenario between peripheral and central systems, Spillantini et al., 1989). This result differs from the prelimary evalution of NGF and BDNF levels in mouse brain after 15 days in hypergravity where a consistent increase in BDNF levels in hyppocampus has been observed (Fig.15 in supplementary materials). No differences were found also in corticosterone levels in adrenal gland (data not shown).

Some behaviours performed during the first days in hypergravity predict the individual susceptibility during post-centrifuge period

The major result of correlational analyses between behaviours performed during the first days in hypergravity and behavioural phenotype after the end of the centrifuge concerned the durations of explorative behaviours exhibited during the first two days during the exposure to hypergravity associated positively with the performance of *digging* behaviours during novel object test and negatively with neurotrophin levels from cerebellum. Significant correlations are reported in Table 1.

Table 1. Correlations of behaviours observed during the first two days in hypergravity and neurobehavioural outcome after the end of LDC.

First two days in hypergravity	Post-hypergravity exposure	r	p
exploring	*digging*	0,809	0,0015
head rising	*digging*	0,741	0,0070
Wall rearing	*digging*	0,812	0,0014
Grooming	*Head rising*	0,702	0,0138
Exploring	NGF-cerebellum	-0,881	0,05
Head rising	NGF-cerebellum	-0,881	0,05

Hypergravity affected fecal microbiota composition

The dominant phyla among all groups were *Bacteroidetes* and *Firmicutes*, followed by *Verrucomiobota*. Preliminary results evidenced that hypergravity significantly increased the proportion of *Bacteroidota* ($F_{(2,25)}$= 29,697, P<0,0001; P<0,05 after *post hoc* comparison between MDS and CTR), *Verrucomicrobiota* ($F_{(2,25)}$= 19,774, P<0,0001; P<0,05 after *post hoc* comparison between MDS and CTR), *Proteobacteria* ($F_{(2,25)}$= 4,208, P=0,0266; P<0,05 after *post hoc* comparison between MDS and CTR), *Cyanobacteria* ($F_{(2,25)}$= 41,860, P<0,0001; P<0,05 after *post hoc* comparison between MDS and CTR or VIV), and *Desulfobacteriota* ($F_{(2,25)}$= 4,831, P=0,0168; P<0,05 after *post hoc* comparison between MDS and CTR), while decreased *Actinobacteriota* ($F_{(2,25)}$= 4,753, P=0,0178; P<0,05 after *post hoc* comparison between MDS and CTR). Concerning CTR mice, a modulation has been observed in comparison to VIV mice. In particular, a significant decrease has been observed in *Bacteroidota* ($F_{(2,25)}$= 29,697, P<0,0001; P<0,05 after *post hoc* comparison) with a concomitant increase in *Verrucomicrobiota* ($F_{(2,25)}$= 19,774, P<0,0001; P<0,05 after *post hoc* comparison.

The lowest phylogenetic diversity was found in CTR mice. A significant decrease of the α-diversity was observed in all indexes used in the microbiota of CTR mice as compared to the microbiota of VIV and MDS mice (Fig.9). The observed species revealed significant differences in diversity within groups, which exhibited a decrease in species richness in CTR mice compared to VIV and MDS groups (P=0,0044). The Shannon index (P=0,0144) and Evenenes index (P=0,0269) confirmed this result with lower diversity in CTR mice compared to the other two groups.

Interestingly, Principal coordinate analysis (PCoA) based on unweighted UniFrac distances revealed demarcations between the three groups with a clear clustering between samples (Fig.10). Similarly, PERMANOVA analysis confirmed significant differences distinguishing the three ethnic groups from the whole ($p < 0.05$).

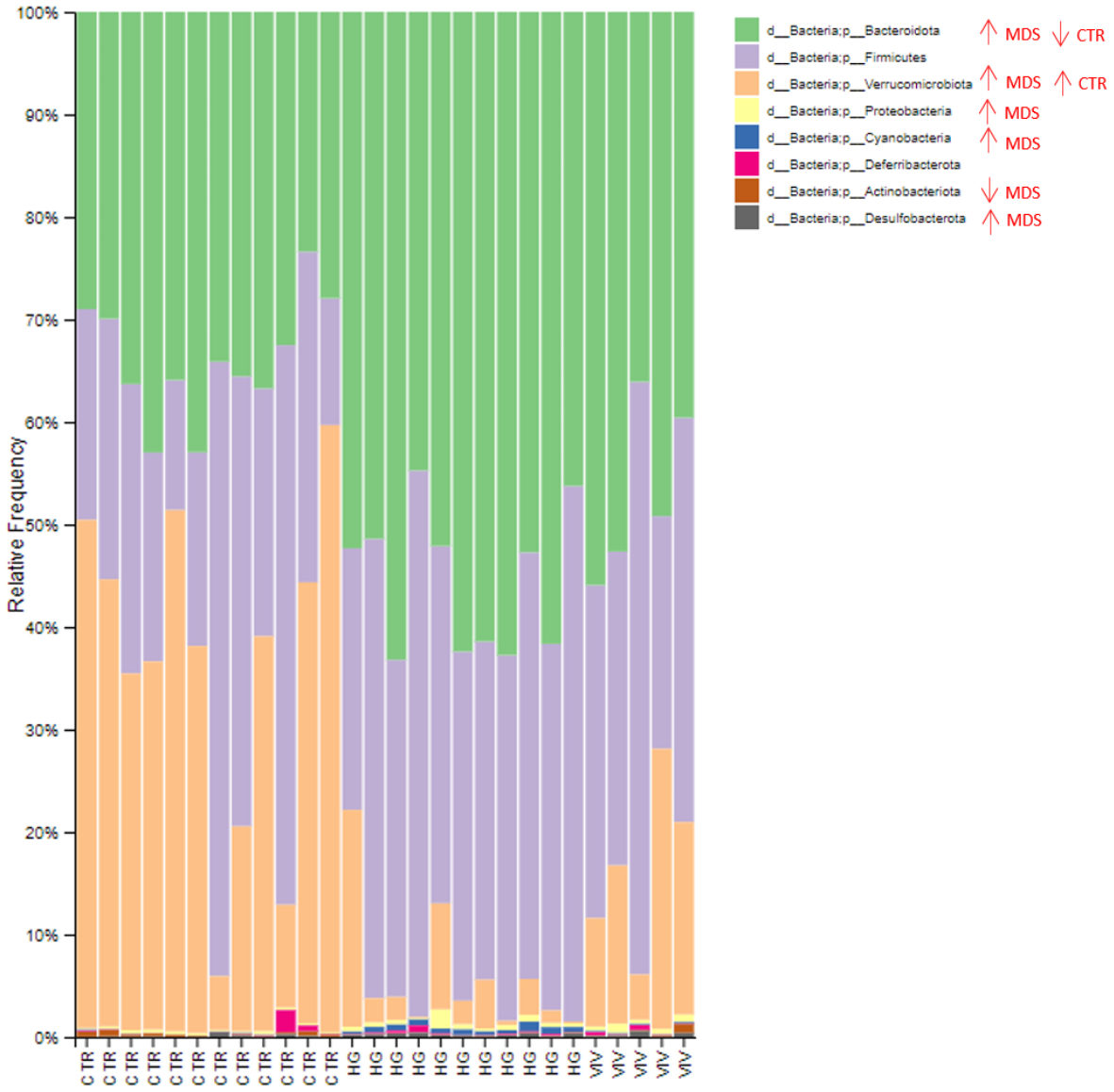

Figure 8. Fecal microbiota changes induced by hypergravity exposure. It has been reported relative frequencies of bacteria at phyla level; ↑significant increase, ↓ significant decrease. One-way ANOVA with post-hoc Tukey's test. MDS=11, CTR=12, VIV=5.

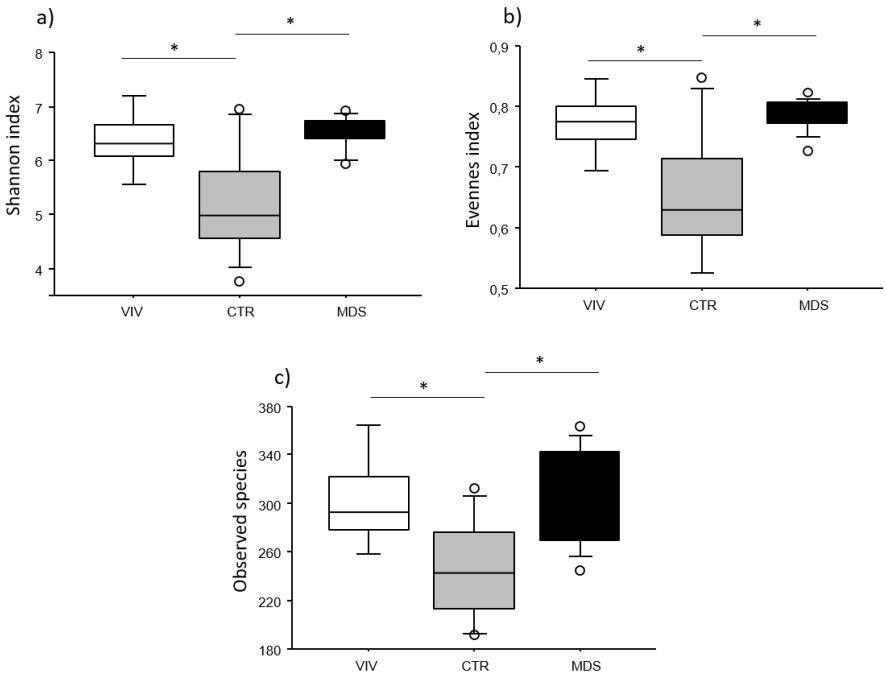

Figure 9. Phylogenetic diversity of murine fecal microbiomes. Differences were observed according to Shannon index (a), Eveness index (b) and number of observed species (c). ∗Statistically significant at $p < 0.05$ using Kruskal–Wallis test, MDS=11, CTR=12, VIV=5.

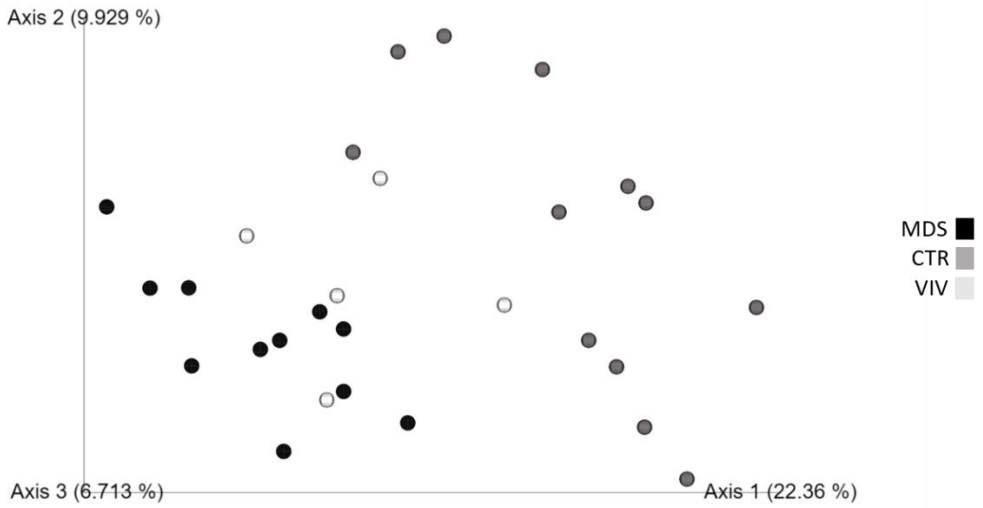

Figure 10. Principal coordinate analysis (PCoA) derived from unweighted UniFrac distances among samples from the three group. MDS=11, CTR=12, VIV=5.

2.5. Discussion

The results of the present study reported that chronic exposure to hypergravity affected the neurobehavioural repertoire of mice, both during and after rotation. Specifically, behavioural analysis across the weeks evidenced a critical time point in coping strategy during the first days in centrifuge, while a general reduction in spontaneous activity and an increase in vestibular-related phenotype were observed after the end of rotation. Interestingly, exploration of the fecal microbiota revealed that hypergravity affected bacterial composition and phylogenetic distance among micorbiota communities.

All mice subjected to hypergravity maintained good health during the permanence in centrifuge. Although, a slight reduction in body weight has been observed after the end of the hypergravity, probably due to an initial and transient hypophagia (Yuwaki and Okuno, 2004), all animals fed and drank across the weeks. The initial drop in water consumption, observed during the first days of hypergravity may be suggestive of animals experiencing motion sickness syndrome because of being subjected to rotational stimuli (Francia et al., 2006; Abe et al., 2023). Indeed, the sensory mismatch among vestibular and proprioceptive systems that characterized the progression of motion sickness symptoms, is often associated to an initial and transient absence of food or water consumption during the permanence in altered gravity (Wade et al., 2002; Abe et al., 2011; Guéguinou et al., 2012). This is in accordance with the behavioural observations during the permanence in centrifuge. Although mice conserved their species-specific behavioural repertoire, they performed reduced locomotor activity favouring resting behaviours, selectively during the first days. Subsequently, a general increase in explorative and maintenance behaviours has been evidenced, probably as index of adaptation to gravitational stress. One of the factors that could compromise the activity during the first days in altered gravity could be the plastic changes in the vestibular system or inactive behaviours induced by gravitational stress (Shimomura et al., 2021). It can be presumed that the vestibular system adapts to the new gravitational environment and the behavioural patterns through the vestibular system and the individual specific copying strategies adapt to the new environment (Bruce L.L., 2003; Jamon M., 2004; Shimomura et al., 2021). The behavioural observations immediately after the rotation and during the tail suspension test confirmed the association between behavioural modifications and vestibular system. Comparing the behavioural profile after the hypergravity to the behavioural baseline collected before the beginning of the centrifuge, it has been evidenced that mice subjected to hypergravity appeared less explorative in a novel environment, with a clear reduction in vertical movements. Since the performance of complex behaviours such as *Wall rearing* and *Rearing* require the involvement of vestibular and proprioceptive functions (Geisler, H. C., 1997), their reduction could be related to an

alteration of these systems. Interestingly, the *spinning* behaviour exhibited exclusively by hypergravitational mice supports this interpretation. As in our study, abberrant behaviours in the tail suspension test may arise as a consequence of motor deficit (Trushina et al., 2006) and may be relevant to vestibular functions (Curtin et al., 2003; Kalueff et al., 2008).

After 28 days in centrifuge mice did not show any significant increase in emotionality with reference to the control mice. In line with previous results collected by Bojador and Jamon (2011), CTR and MDS mice approached to the novel object with similar duration and frequency, although hypergravitational mice tent to show a major latency. Probably our result could be associated to the absence of differences in corticosterone levels after the end of centrifuge. In contrast to other studies where mice showed increased corticosterone levels after maximum three weeks in hypergravity (Petrak et al., 2008; Gue´guinou et al., 2012; Pulga et al., 2016; Abe et al., 2022), the absence of corticosterone modulation after almost one month could be related to a hypoactive state due to a dysregulation of neuroendocrine system (Hellhammer and Wade, 1993; Heim et al., 2000; Kudielka et al., 2006). Differently from the results collected during the Dry Run experiment where an increase in anxiety-related behaviours and hippocampus BDNF level were evidenced, the absence of alterations in emotive profile and neurotrophins levels, known to be involved in the adaptive response to stressful stimuli and acting also on the adrenal gland (Taglialatela et al., 1991), after almost one month in hypergravity, may confirm that an adrenal exhaustion took place when chronic stress overwhelms the adaptation reserve (Ahn, Y.W., 2011). Considering the behavioural observations before the beginning of the centrifuge, it is worth mentioning that CTR mice showed an increased approach to the novel object. It is important to bear in mind that the dimensions and structure of the cages varied between the animals belonging to the VIV group and those belonging to the CTR or MDS. In fact, in the last two, there was no sawdust, the dimensions were smaller, the water, and food were presented differently, all factors that can contribute to the different behavioural outcome.

In a context of growing need to individualize investigations in order to define predictive indicators of susceptibility during space missions (Pavez Lorie et al., 2021), correlations between behaviours performed during the first days in centrifuge and behavioural outcomes in normogravity revealed a positive association between explorative behaviours during 3g and *digging* behaviours during novel object test. *Digging* is a common natural rodent behaviour that comes from their ancestry in the wild, where they would forage for food (Deacon R.M.J., 2009). It represents a spontaneous species-specific behaviour provoked by a suitable substrate as sawdust, as well as could be related to an expression of anxiety (Masuda et al., 2000). In our study, the contact with the sawdust was limited to maximum five minutes of exploration, therefore it represents a too limited time to conclude that

the performance of *digging* behaviour could be related to an anxious profile of the mice, usually defined with a longer duration test (Gould T.D., 2009). On the contrary, considering the absence of sawdust during their permanence on centrifuge, the performance of this behaviour by individuals that exhibited active explorative behaviours during the first two critical days in centrifuge could underline a resilient species-specific phenotype, indicator of less susceptibility to hypergravity exposure. Interestingly, the negative correlation between explorative behaviours, which require to counteract the gravity direction, during the first days in centrifuge and the neurotrophin levels after the end of the exposure to hypergravity could suggest that in resilient mice the modulation of neurotrophin levels took place before the end of the centrifuge in order to prepare the organism to face the stressful event (Cirulli and Alleva, 2009).

In this context, considering the strict relationship between brain and intestinal functions in individual vulnerability to gravitational stress (Thornton and Bonato, 2013; Zhang et al., 2016; Turroni et al., 2020), the investigation of murine microbiota after exposure to hyprgravity could represent a valid instrument to define concrete countermeasures for spaceflight-associated illness and its consequences on health (Alauzet et al., 2019). The preliminary results of microbioma analysis revealed that mice exposed to hypergravity showed a significant community dissimilarities across the groups, alterations in fecal microbioma load and increased levels of different species within the microbial community compared to CTR mice. These observations are indicative of differential microbiota-directed effects, selectively due to hypergravitational environment in MDS group or to psychosocial stress associated probably to isolation in CTR mice. In line with results reported in human and mice exposed to chronic altered gravitational environments (Voorhies et al., 2019; Alauzet et al., 2019) and the behavioural analysis conducted during the permanence in centrifuge, it is possible that the high diversity of murine microbiota could support a more resilient profile to perturbations when exposed to altered gravity (Crucian et al., 2018). Concerning the microbiota load, an increase in *Bacteroidetes* phylum was already reported in mice exposed to hypergravity or microgravity (Ritchie et al., 2015; Alauzet et al., 2019) and their abundance could play an important role in maintaining the integrity of the interbacterial bonds in the gut (Kim et al., 2007; Gryaznova et al., 2022). Similarly, the increased phylum *Verrucomicrobiota* in centrifugated mice could potentially induce regulatory immunity to face stressful stimuli (Gryaznova et al., 2022), while the observed modulation of the phyla *Desulfobacterota* and *Cyanobacteria* deserve particular attention in the evaluation of the psycho-physiological effects to the altered gravity exposure. Although the specific mechanisms underlying the functions of microbiota in the brain through metabolites remain unclear, scientific evidence demonstrated that *Cyanobacteria* resulted responsible for the production of neurotoxic molecules (e.g., saxitoxin, microcystins or nodularin)

which, among their systemic activities, could compromise voluntary musculature, brain or cytoskeletal functions (Feurstein et al., 2011; Cestèle et al., 2000; Fischer et al., 2000; Llewellyn, L. E., 2006). Furthermore, Rao and colleagues (2021) demonstrated that the abundance of *Desulfobacterota* and brain inflammasomes significantly decreased after fecal microbiota transplantation from healthy rats, suggesting that probably there is a pathogenic impact of *Desulfobacterota*, probably harmful for CNS (Rao et al., 2021; Chen et al., 2022; Xu et al., 2023). Therefore, the demonstration that the phylum *Cyanobacteria*, known to be involved in pro-inflammatory phenomena during the pathogenesis of nurodegenerative diseases, and the phylum *Desulfobacterota*, involved ininflammatory and hypoxic processes, resulted more abundant in mice exposed to hypergravity allowed us to speculate that toxic processes took place after altered gravity exposure (Di Gioia et al., 2020) and the investigation of the other minor communities played a fundamental role to understand the subtle mechanisms that underlined the individual response to gravitational stress.

In general, data clearly indicate that exposure to unphysiological environment represents an opportunity to provide new insight in the adaptation phenomena and underlying mechanisms of the neurobehavioural basis of gravity dependent aspects. A more in-depth understanding of how mammals respond and adapt to hypergravity would also provide an opportunity to exploit the use of artificial gravity to mitigate the effects of prolonged exposure to microgravity on space vehicles, contributing to making long-term space exploration feasible.

2.6. Supplementary materials

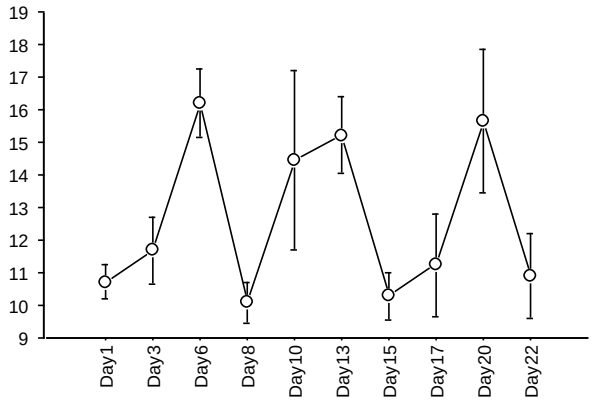

Figure 11. Water daily consumption in CTR mice. Although it has been observed oscillations in water consumption due to different time of data collection, CTR mice drank always over the baseline level at Day1. ∗Statistically significant at $p<0.05$ using repeated measure ANOVA followed by post-hoc Tukey HSD test. N=11.

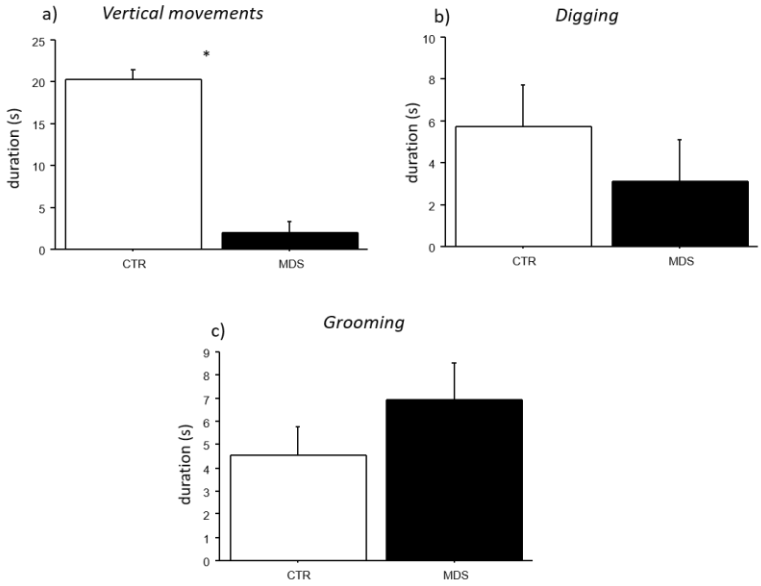

Figure 12. Behavioural analysis after exposure to hypergravity for 15 days. In line with results collected after 28 days in hypergravity, MDS mice showed a reduction in the durations of vertical movements (a) and tent to increase grooming behaviour (c) compared to CTR mice. Data are shown as means ± S.E.M. ∗Statistically significant at $p< 0.05$ using t-test, MDS=6, CTR=6.

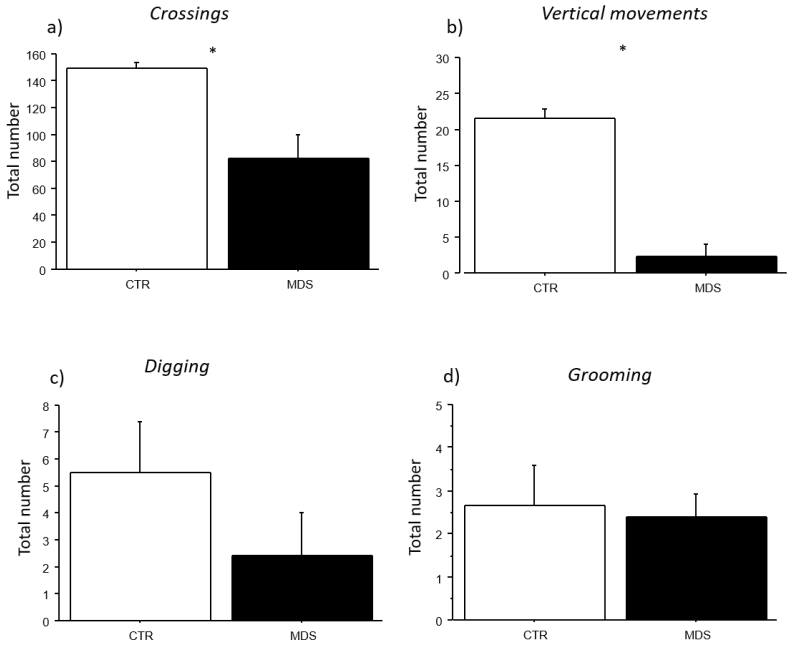

Figure 13. Behavioural analysis after exposure to hypergravity for 15 days. In line with results collected after 28 days in hypergravity, MDS mice showed a reduction in locomotor activity (a), decreased frequencies of vertical movements (b) and a similar tendendy in digging behaviour compared to CTR mice. Data are shown as means ± S.E.M. ∗Statistically significant at $p < 0.05$ using t-test, MDS=6, CTR=6.

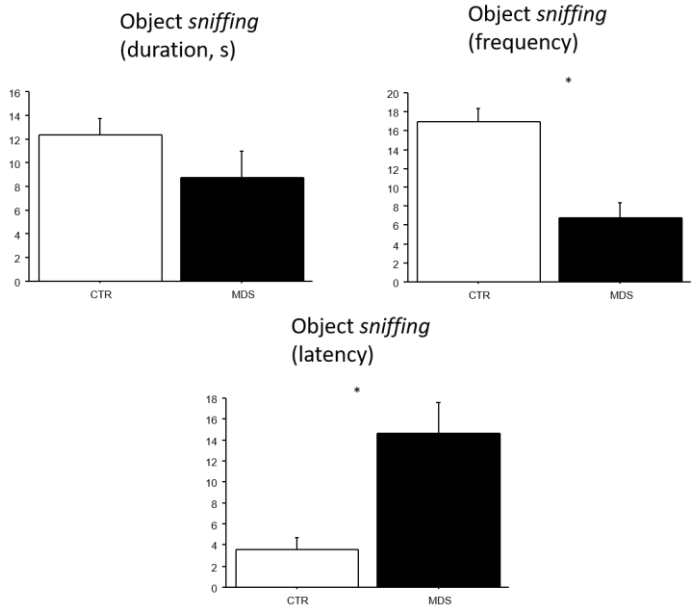

Figure 14. Effect of hypergravity on the emotional profile of mice. A reduction in the frequency of object sniffing behaviour (b) and an increase in the latency of approach to the novel object has been observed in MDS mice after 15 days of hypergravity. Data are shown as means ± S.E.M. Statistically significant at $p < 0.05$ using t-test for the duration and frequency, and Mann-Whitney test for the latency. MDS=6, CTR=6.

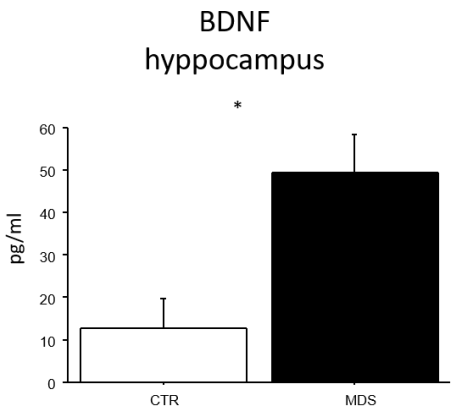

Figure 15. 15 days hypergravity decreased BDNF level in hyppocampus. Data are shown as means ± S.E.M. t-test, *P=0,05.

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3. THE HINDLIMB UNLOADING MOUSE MODEL: ETHOGRAM AND NEUROBIOLOGICAL PROFILE

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In preparation

3.1. Abstract

Understanding the phenomena underlying tolerance and adaptation to altered environmental conditions, such as un-physiological gravity, represents a valuable opportunity to study coping strategies, mechanisms underlying neuroplasticity phenomena, and the individual vulnerability to stress. In addition, animals are sent into orbit as a useful tool to preserve potential human and animal health, envisaging and developing adequate countermeasures in long-term space travel. Among ground-based experiments employing animal models to reproduce the effects of altered gravity exposure, the hindlimb unloading model is highly extensively utilized in gravitational physiology to reproduce the effects of microgravity and consists in removing gravitational loading from the hindlimbs by suspending the animal by its tail. The present study was aimed to fully characterize the behavioural and neurobiological profile of mice exposed to this paradigm, by defining a detailed HU ethogram while mice are suspended in order to identify behavioural biomarkers for individual differences in coping with the suspension paradigm and make progress in such common procedure to ensure and improve animal welfare. C57BL6 mice were subjected to tail suspension procedure and their behavioural profile studied before, during and after tail suspension exposure. Distinctive behavioural adjustments were observed during suspension. Moreover, locomotor activity, vertical movements (*rearing* and *wall rearing* behaviours) and novel object exploration, were affected after being exposed to suspension. Although no significant differences were observed in the levels of neuromodulators known to be involved in neuroplasticity phenomena in response to stress such as NGF and BDNF levels, the analysis of individual behavioural profile before and after suspension procedure revealed specific behavioural items predictive of individual resilience to suspension procedure.

Keywords

ethogram, neurotrophins, hindlimb unloading model, mouse, brain

3.2. Introduction

Animals have contributed greatly to the current database of knowledge in space biology (Clément and Slenzka, 2006). After the first living creature, the dog Laika, was launched into the space (1966), a large variety of vertebrate and invertebrate species has been selected to carry out experiments in zero-gravity (Clément and Slenzka, 2006). They are sent into orbit to proactively foresee health problems in humans and they are routinely exposed to un-physiological gravity on Earth to understand physiological adaptations in such environment and investigate possible countermeasures to reduce animal and human suffering in space.

Among groud-based models used to reproduce the effects of microgravity-simulated conditions in rodents (Song et al., 2017; Wang et al., 2021), the hindlimb unloading model (HU) is a well-established paradigm largely employed to reproduce the effects of microgravity-simulated conditions on Earth (Song et al., 2017; Wang et al., 2021). It selectively consists of unloading the rat or mouse hindquarters of rat or mouse suspending the animal by its tail, maintaining an unloading angle of the 30° in order to provide the normal weight bearing on the forelimbs and reproduce the reliable physiological responses found in microgravity. Several studies reported that many physiological changes observed in the HU model are similar to those encountered in space. HU model induces disuse osteopenia (reduced bone density; Bloomfield S.A., 2010; Sanesi et al., 2023), cephalad-fluid shift and cerebral blood flow (Hawliczek et al., 2022), as occurs in astronauts in space (Bhuyan et al., 2023). Both HU model and space flight limit the movements of the forelimbs and hindlimbs, inducing a reduction of muscle mass and reproducing the muscle atrophy only in the load-bearing muscles (Morey-Holton and Globus, 1998). Analogously, to the effects reported in space environment, the immune system is compromised, and some metabolic changes as well as cardiovascular effects confirm the translational value of the model (Globus et al., 2006; Garg et al., 2021; Siddiqui et al., 2022; Hawliczek et al., 2022). More recently, several studies attempt to demonstrate the reliability of this model as a tool to investigate also the physiology of the nervous system, with particular regard to the effects on brain neuroplasticity phenomena, gene expression of different neuronal factors, or the electrophysiological currents of the neuronal cells (Langlet et al., 2012; Salehi et al., 2016; Kulikova et al., 2017; Tahimic et al., 2019; Wang et al., 2020).

Although it is largely validated as a paradigm to mimic ground-based microgravity effects, very little is known about the neurobehavioural adaptive responses of the animals subjected to this procedure as well as about the huge variety in individual coping strategies to this unnatural posture. Indeed, results obtained from the past decades demonstrate that the complexities of the physiological adaptations to changing environmental conditions, such as un-physiological gravity,

require a shift of focus to an integrated approach in which converge a wider range of functional, structural and behavioural information (Broom D.M; 1998; Regenmortel M.H.V, 2004). In particular, the brain structure and the mind comprise complex systems characterized by mechanisms of recurrence, feedback and interconnectivity that make difficult to reduce the individual response to genetic or molecular biomarkers (Mitchell S.D., 2008; Branchi et al. 2022). Moreover, the scientific results collected in many years of space exploration have demonstrated that individuals respond differently to the condition of microgravity, and to understand the origin of this variability in such vulnerability is necessary to understand mechanisms underlying the individual differences (Schmidt et al., 2020; Pavez Lorie et al., 2021). In this context, a detailed behavioural analysis represents a useful tool to investigate the initial and adaptive response to microgravity-simulated conditions. Although neuronal circuits play a fundamental role in the investigation of mechanisms of adaptive behaviours, it has been largely demonstrated that the behavioural endpoints depend above all on interactions among the nervous system, body and environmental challenges (Chiel H.J. and Beer R.D., 1997; McEwen B.S., 2007; Branchi et al. 2022), eliciting valid indicators of tolerability to changing environmental challenges (Beery and Kaufer, 2015). In the HU model, one of the most evident weaknesses regards specifically inconsistencies in the study of the stress-related responses which require a detailed knowledge about animals' individual reactions to HU procedure. These behavioural outcomes are dependent not only on the type of stressor but also on the coping strategy that characterizes each experimental subject. Investigating such individual variation could explain why some conditions that are well tolerated by some individual may be detrimental to others, being this a key element for improving animal welfare, screening resilience to alterated gravity environment and developing countermeasures. The ethological approach requires a comprehensive evaluation of individual vulnerability and resilience to stress and also for ensuring proper care and behavioural assessment during the experimental study (Mortreux and Rosa-Caldwell, 2020). This underlines the need of defining behavioural indicators that can be used to evaluate behavioural adjustments in animals subjected to the HU procedure, as well as of enhancing the procedure to reduce psychophysical suffering.

Aim of the present study was to analyze the neurobehavioural profile of mice subjected to HU paradigm in order to identify neurobehavioural indicators of susceptibility to microgravity simulated conditions. In particular, mice were exposed to different duration of suspension procedures, their behavioural repertoire was evaluated during and after the end of the suspension, and an appropriate ethogram has been defined as a tool to both monitor the behavioural profile of the animals during the suspension and compare the adaptive outcome with post-suspension data. Moreover, since changes in neurotrophin levels in the central nervous system have been largely

reported upon exposure to gravitational stimuli and stressful experiences (Infanger et al., 2007; Berry et al., 2012; Santucci et al., 2012; Numakawa T. and Kajihara R., 2023) in association of their role as modulators of synaptic plasticity, it has been also considered of interest to investigate whether HU procedure would affect such neurobiological determinants in suspended mice.

3.3. Materials and Methods

Animals

Upon arrival at the laboratory of University of Bari, C57BL6 mice animals were housed under standard conditions in an air-conditioned room (temperature 21 ± 1 ◦C, relative humidity 60 ± 10%; 12 h–12 h light/dark cycle), in 42 cm × 27 cm × 14 cm Plexiglas cages with a metal top and sawdust as bedding. Pellet food and tap water were provided *ad libitum*.

Hindlimb unloading protocol

After a period of adaptation (1 week), adult male and female C57BL6 mice (2-months-old, n=44) were weighed and randomly assigned to six groups: three groups of normal weight-bearing control mice, each groups for each experimental group of suspended mice (CTR groups: one week (CTR1, n=6), two weeks (CTR2, n=7), and three weeks (CTR3, n=7)) and three groups of hind-limb suspended mice (HU groups: one week of suspension (WK1, n=7), one group for two weeks (WK2, n=7), one group for three weeks (WK3, n=10). The suspension procedure was realized according to recommendations by Wronski and Morey-Holton (1987). The hindlimbs of the HU group were elevated to a spinal orientation of 30° above horizontal to simulate a shift of body fluids similar to that experienced during space flight. The elevation was adjusted in order to avoid any contact of the hindlimbs with the cage floor and the forelimbs were free to ambulate in the entire range of the cage and access to food and water continuously. To maintain the animals suspended, a strip of orthopedic tape, attached to a plastic suspension bar, was applied to the sides of the tail, after spraying and letting the benzoin tincture dry on the tail skin to protect against irritation of the adhesive tape. Each mouse was then attached via the plastic suspension bar to a pulley system mounted on top of the cage.

Experimental design

In order to evaluate the neurobehavioural profile of the animals and identify possible predictive behavioural indicators of vulnerability/resilience to microgravity simulated condition, their behaviour was evaluated during and after suspension procedure. In particular, their behavioural repertoire was observed and defined across weeks of suspension for each experimental subject, and their profile was evaluated at the end of the suspension procedure assessing spontaneous behaviour in a cage and the response to a novel object. At the end of the behavioural testing, the animals were weighed and sacrified and brain samples collected in order to evaluate NGF and BDNF levels.

HU ethogram

Each experimental subject was videorecorded for 10 min across the three weeks of suspension in order to monitor the behavioural profile during the entire suspension period. According to the temporal dimension of each experimental group, mice were videorecordered and observed at the precise moment in which they were suspended ("just suspended"), and after one day (day1), one-, two-, and three weeks (week1, week2, week3) of suspension. After several times of behavioural observations, a detailed ethogram was defined and the behavioural profile of each animals scored using a dedicated ethological software ("The Observer 2.0"; Noldus, 1991). The drawings in this paper are taken directly from these videos (Fig.1).

Spontaneous behaviour and Novel Object test

To assess locomotor activity and the approach to novelty, the animals were tested in a novel object test after the end of suspension procedure. Each experiemental subject was placed at the centre of a plexiglas cage with sawdust (42 cm × 27 cm × 14 cm) and videorecorded for 7 min. In particular, after 5 min of spontaneous activity evaluation, a stimulus object (a glass marble) was placed at the centre of the half-cage, and latency, number and duration of *sniffing contacts* with the object were recorded for 5 min. The position of the marble between the two halves of the cage was randomized for each animal. Videos were scored using "The Observer 2.0" software, and frequency, duration and latency of the following behavioural items were also recorded: *exploring* (locomotor activity in the cage), *wall rearing* (vertical exploration with hindpaws on the floor and forepaws on the wall of the arena), *rearing* (vertical exploration with hindpaws on the floor and forepaws in the open), *exploring* (exploration of the environment), *grooming* (self-explanatory), *inactivity* (lying flat or standing still in total absence of movement), *digging* (digging up the sawdust). New and clean cages were used for each animal. The recording session took place between 1.00 and 5.00 pm, in an experimental room maintained at the same temperature and humidity conditions as the housing one.

Brain preparation before ELISA

Brain samples were Trypsin-EDTA harvested and pellets were homogenized by ultra-sonication (Sonics, Newtown, CT, USA) in RIPA buffer (50 mM Tris-Cl, pH7.5; 150 mM NaCl; 5 mM EDTA; 1% Triton X-100; 0.1% SDS; 0.5% DOC (Sodium deoxycholate; 1 mM PMSF; 1 μg/ml leupeptin). Clear supernatants (4 °C/13,000 rpm, 20 min) were collected and used for quantification of neurotrophin levels.

Quantification of neurotrophin levels using ELISA on brain samples

For NGF and BDNF quantifications, double-sandwich ELISA assays were used. In particular, 96-well Maxisorp plates (Nunc, Roskilde, Denmark) were pre-coated with the specific capture antibodies (0.4 μg/mL; R&D) overnight (4°C). Brain samples were diluted 1:2 in assay diluent (from the kit; R&D) supplemented with 1x protease inhibitor cocktail (Pierce - Thermo Fisher Scientific Inc. Waltham, MA USA) and loaded in parallel with the standard curve (0.32–2,000 pg/mL protein; R&D). Overnight incubation with sample was performed, and subsequently the addition of the specific detection secondary antibodies (0.15 μg/mL; R&D) and streptavidin (1:200; R&D) were carried out. Specific binding was developed by using the ready-to-use TMB substrate and Stop solution (R&D). The colorimetric signals (Optic Density, OD) were acquired at λ 450–570 nm by using the Sunrise plate reader (Tecan Group Ltd., Männedorf, Switzerland). The related target values (pg/mL) were produced using a third-grade polynomial standard. The absence of cross reactivity with other neurotrophins was declared by the manufacturer for both assays.

Statistical analysis

Continuous variables are presented as means and standard errors of means (S.E.M.). The parameters scored across the three weeks of suspension were analyzed by repeated measure analysis of variance (rmANOVA) with period of suspension (five levels: just suspended vs day1 vs week1 vs week2 vs week3) as within-subjects factors. Data collected after the end of the suspension period were analyzed by one-, two- or three- ways ANOVA with week of suspension (three levels: week1 vs week2 vs week3), treatment (two levels: control vs hindlimb unloading) or sex (two levels: male vs female) as between factors.

When the interaction between treatment and another independent variable was significant, *post hoc* comparisons were performed using Tukey HSD test. Multiple comparisons were applied to logical sets of means according to the specific objectives of the work. Latency data, which had no normal distribution, were analyzed with the Mann-Whitney nonparametric test.

Pearson correlations were applied to associate behavioural profile or neurobiological data during and after suspension.

Comparisons were performed by using the Statview software and statistical significance was set a $P< 0.05$.

3.4. Results

Suspended animals performed perculiar behavioural adjustments during suspension

Some behavioural elements and subtle changes in the typical specie-specific ethogram emerged from the observations of the mice during the suspension procedure. In particular, the postures were grouped into behavioural categories within which there was an apparent motivational similarity. The specific behavioural elements of HU ethogram are shown in Fig.1 and listed below.

Explorative behaviours*:*

- *Exploring* is performed when the animal changes the position of its body moving the forelimbs. It is usually related to a simple direction of attention towards an external stimulus, to heads towards food/water or to move around the cage. *Sniffing* is often associated with this behaviour.
- *Fully extended hindlimbs* emerges when the animal stretches its hindlimbs upward in order to bring its head towards the base of the cage. When mouse is stretching, there is usually a close contact between its head or forelimbs and some parts of the cage (walls, sawdust, food, water). For these reasons it is very often performed simultaneously to the *sniffing* behaviour.
- *Forelimbs hanging* is evidenced when the animal grabs the component of the cage with the forelimbs or the mouth. In general, it is performed rarely and could represent a behavioural strategy to reach the top of the cage and try to escape from the suspension position.
- *Vertical head rising* is observed when the animal raises its head upward. In general, the animal is in resting position and counteracts the gravity force raising its head in order to explore the environment on the top of the cage. It could be an explorative index or a self-maintaince behaviour associated to the need to change the orientation of the head and move the anterior part of the body upward. It is often performed simultaneously to *sniffing* behaviour and it could be preceded or followed by *immobility* behaviour.
- *Immobility* behaviour is defined as a posture where the mouse hangs without engaging in any actvity and keeps the forelimbs at the base of cage.

Balancing behaviours:

- *Running* is performed when the animal moves frantically its forelimbs and/or hindlimbs in order find again a balance position. It is defined “balancing behaviour” because it usually emerges when the animal has just lost balance due to the movement of the body and tries to find again a permanent resting position.

- *Alternative extended hindlimbs* is evidenced when the animal rotates its body moving unilaterally the hindlimbs in the opposite direction that guided by the head and forelimbs. This specific posture allows the animal to maintain vertical position and remains with the forelimbs on the base of the cage.

Maintenance behaviours:

- These behaviours usually occur in sequence. During *self grooming*, the animal brings the head towards the belly and begins to wash its face with the licked forelimbs (*face washing behaviour*) and drawing them over the head from just behind the ears. Very often it is accompanied by *vertical scratching* behaviour where the mouse maintains the forelimbs at the base of the cage and scratches the ear with hindlimbs.

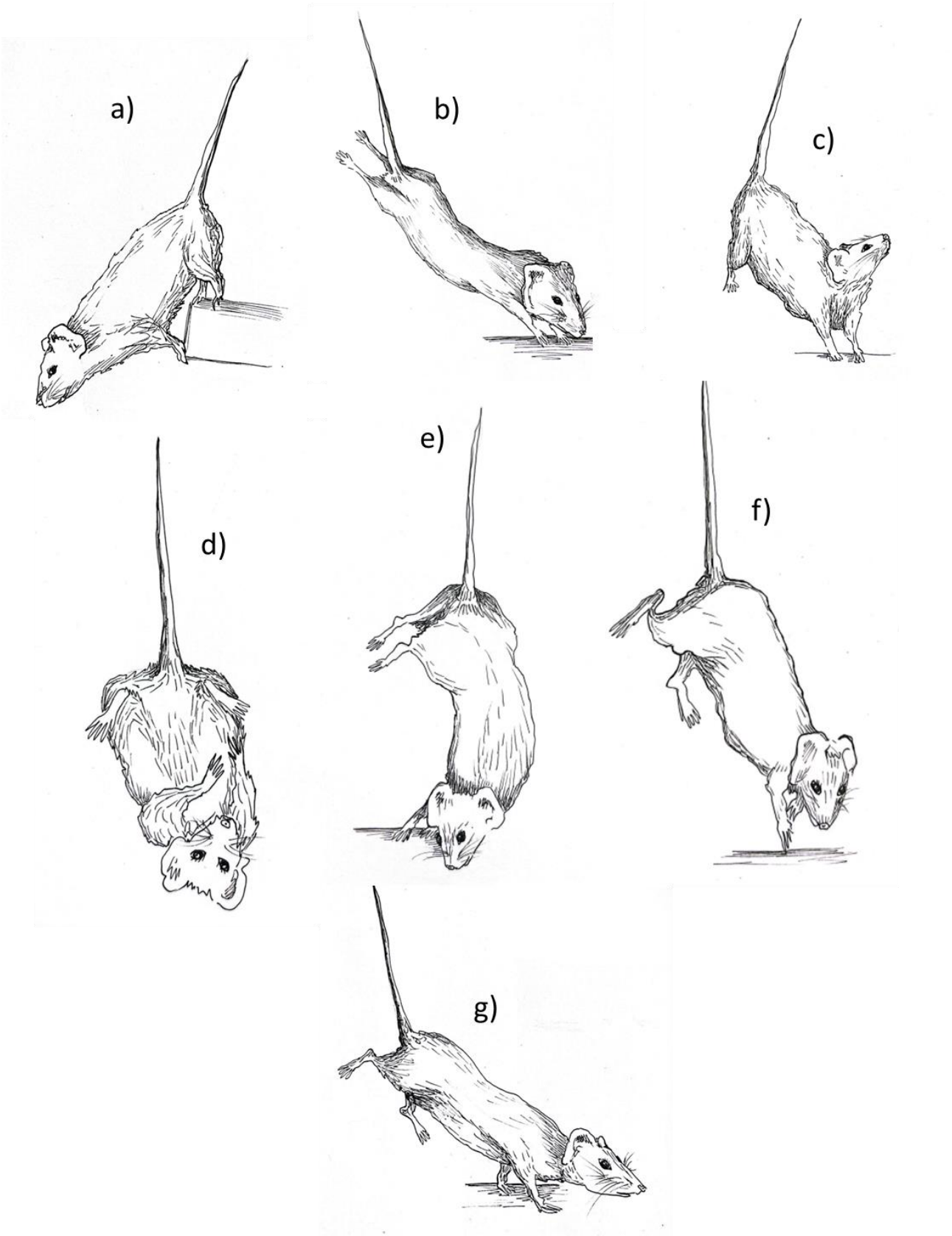

Figure 8. HU ethogram: a) *Forelimbs hanging*, b) *Fully extended hindlimbs*, c) *Vertical head rising*, d) *Self-grooming*, e) *Alternative extended hindlimbs*, f) *Running*, g) *Exploring.*

The drawings were realized by Ilaria Racca.

The behavioural analysis across the weeks evidenced a critical time point in coping strategy during suspension procedure

The analysis of the mouse behavioural repertoire across the weeks (during the day) showed a convergent critical time point of adaptive behavioural trend at day1 of suspension, during which a reduction of *explorative behaviours*, with the exception for *fully extended hindlimbs* behaviours, and a concomitantly increase in *running* behaviour and *grooming* behaviours were observed (Fig.2, Fig.3).

In particular, throughout the weeks, suspended mice showed a decrease in duration and frequency of ***explorative behaviours***, such as *Exploring* (duration: $F_{(4,28)}$=7,082, P=0,0005; frequency: $F_{(4,28)}$=13,362, P< 0,0001; P<0,05 after *post hoc* comparison between duration and frequency in just suspended mice vs day1, week1, week2, or week3), *Forelimb hanging* (duration: $F_{(4,28)}$=7,015, P=0,0005; frequency: $F_{(4,28)}$=9,877, P< 0,0001; P<0,05 after *post hoc* comparison between duration and frequency in just suspended mice vs week1, week2, or week3) and *vertical head rising* (duration: $F_{(4,28)}$=9,127, P<0,0001; frequency: $F_{(4,28)}$=7,812, P=0,0002; P<0,05 after *post hoc* comparison between duration and frequency in just suspended mice vs day1, week1, week2, or week3). Interestingly, an opposite trend has been observed in *fully extended hindlimb* (duration: $F_{(4,28)}$=4,561, P=0,0058; frequency: $F_{(4,28)}$=7,266, P=0,0004) with a significant increase in duration and frequency during week1 compared to just suspended mice or suspended for week3 (P<0,05 after *post hoc* comparison).

Concerning ***balancing behaviours***, an effect of the suspension was found in the duration and frequency of *running* (duration: $F_{(4,28)}$=7,150, P=0,0004; frequency: $F_{(4,28)}$=8,430, P=0,0001) and in frequency of *alternative extended hindlimb* ($F_{(4,28)}$=2,729, P=0,0491). Specifically, a significant increase during week1 was evidenced in duration and frequency of *running* behaviour (P<0,05 after *post hoc* comparison in duration between week1 vs just suespended mice and week3, and in frequency between week1 vas day1, week2 or week3), while a significant reduction was observed in the frequency of *alternative extended hindlimb (*P<0,05 after *post hoc* comparison between just suspended vs week1, week2, or week3).

Regarding the analysis of ***maintenance behaviours***, a progressive increase both in duration ($F_{(4,28)}$=3,596, P=0,0173) and frequency ($F_{(4,28)}$=3,945, P=0,0116) of *self-grooming* were observed in week1 (P<0,05 after *post hoc* comparison in duration between week1 vs just suspended, and in frequency between week1 vs day1 and just suspended).

Concerning sex-related differences (Fig.9 in Supplementary materials), a significant increase in ***balancing behaviours*** were observed in females at week1 in *running behaviours* (duration: $F_{(4,24)}$=

3,399, P=0,0245, P<0,05 after *post hoc* comparison in duration between females and males at week1; frequency: $F_{(4,24)}$= 3,699, P=0, 0175, P<0,05 after *post hoc* comparison in duration between females and males at week1) and at week3 in *Alternative extendend hindlimbs* (duration: $F_{(4,24)}$= 4,746, P= 0,0058, P<0,05 after *post hoc* comparison between females and males at week3).

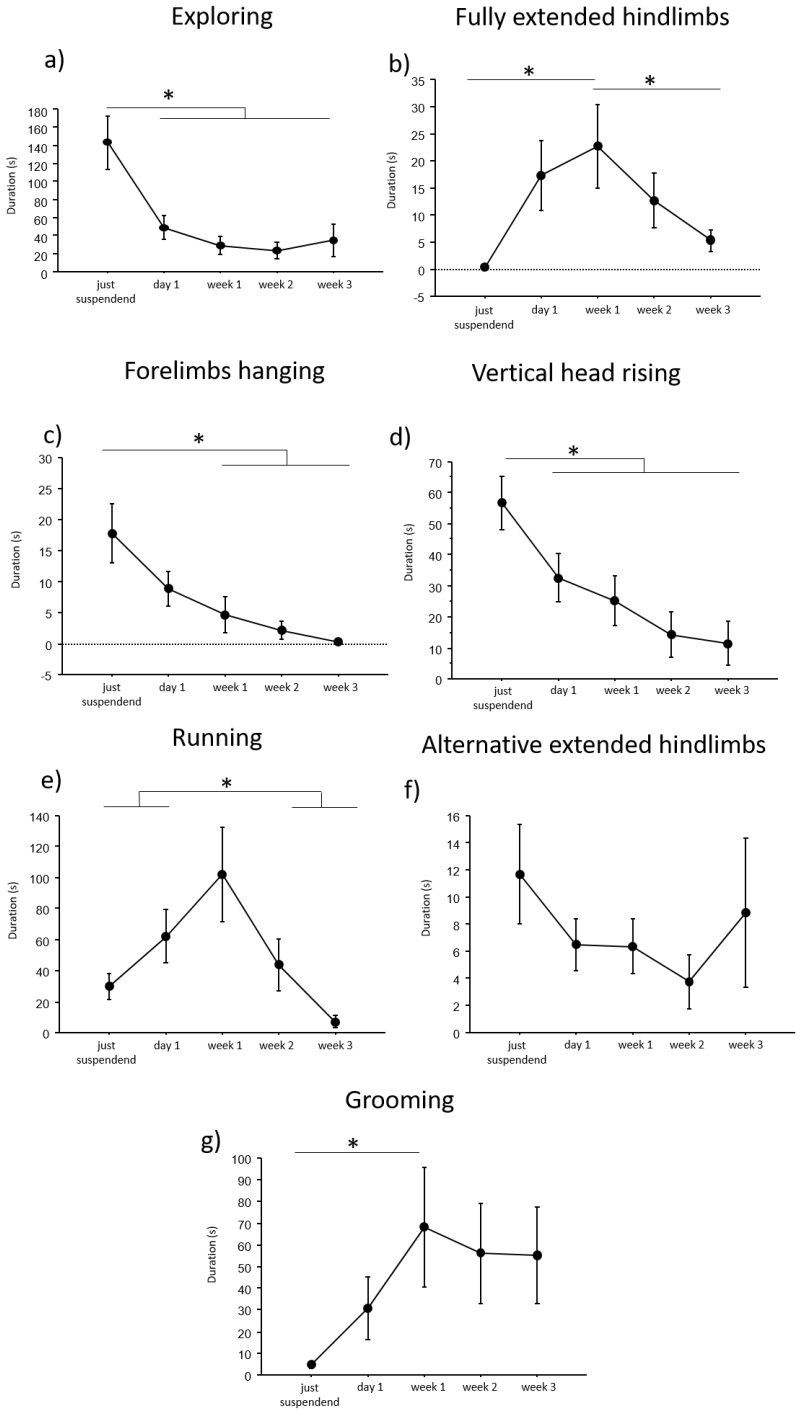

Figure 2. Behavioural analysis during suspension procedure. During the first day of suspension a reduction in duration of exploring (a), forelimbs hanging (c) and vertical head rising (d) were evidenced, while an increase was observed in the durations of fully extended hindlimbs (b), running (e) and grooming (g) behaviours. Data are shown as means ± S.E.M. ∗Statistically significant at $p < 0.05$ using repeated measure ANOVA followed by post-hoc Tukey HSD test. Just suspended=8, day1=8, week1=8, week2=8, week3=8.

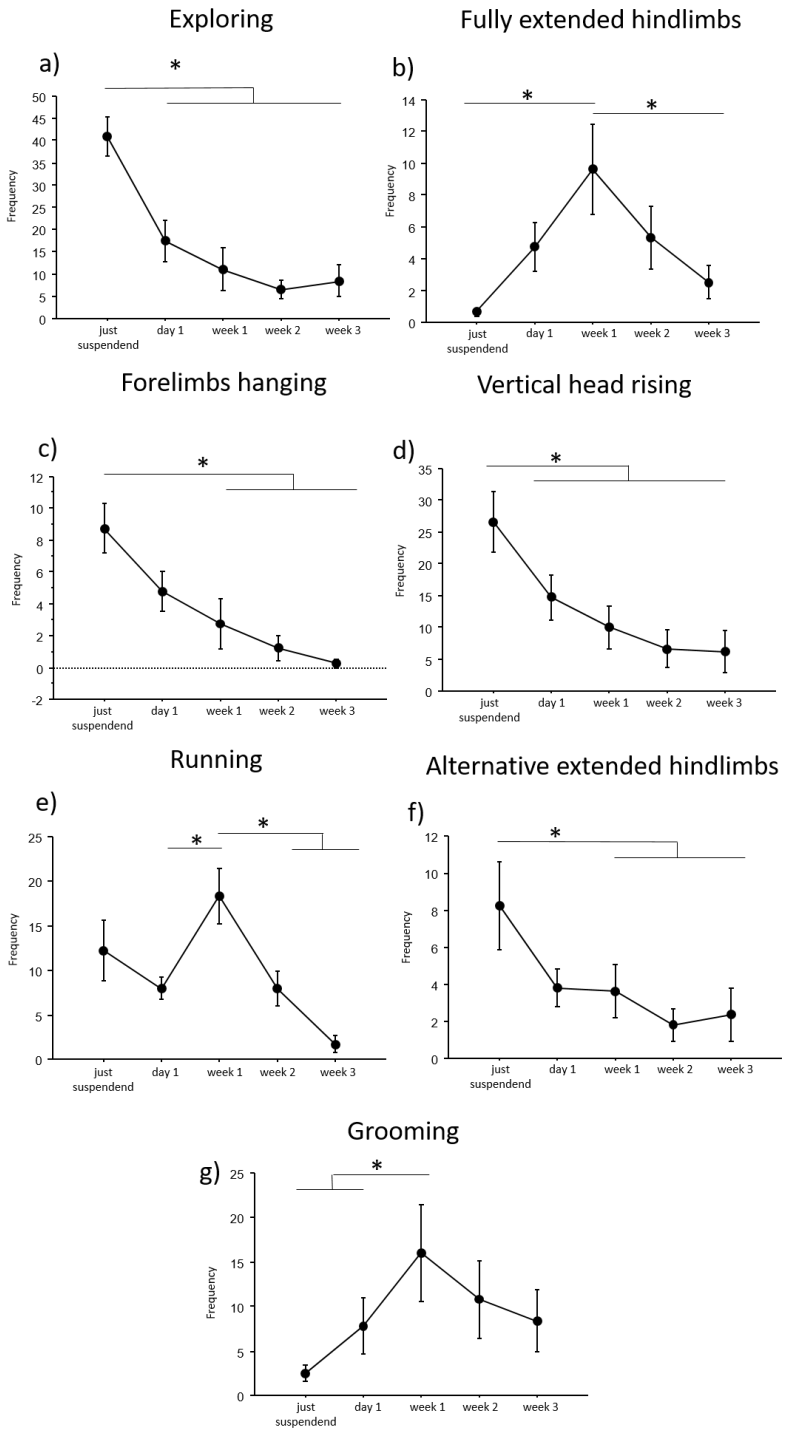

Figure 3. Behavioural analysis during suspension procedure. During the first day of suspension a reduction in frequency of exploring (a), forelimbs hanging (c), vertical head rising (d), and alternative extended hindlimbs were evidenced, while an increase was observed in the frequency of fully extended hindlimbs (b), running (e) and grooming (g) behaviours. Data are shown as means ± S.E.M. ∗Statistically significant at $p < 0.05$ using repeated measure ANOVA followed by post-hoc Tukey HSD test. Just suspended=8, day1=8, week1=8, week2=8, week3=8.

The suspension procedure affects animal body weight per se, regardless of the duration of exposure

All suspended animals showed a modest reduction in body wheight ($F_{(1,43)}$=9,892, P=0,003, P<0,05 after *post-hoc* comparisons), while no significant differences were found among the experimental groups (Fig. 4).

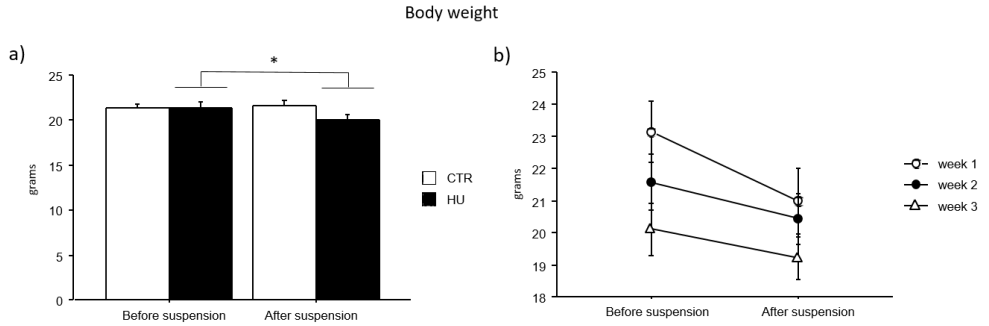

Figure 4. Body weight before and after suspension. A modest reduction in body wheight has been observed in suspended mice (a), without significant differences among the experimental groups (b). Data are shown as means ± S.E.M. ∗Statistically significant at p< 0.05 using repeated measure ANOVA followed by post-hoc Tukey HSD test. CTR before suspension=20, CTR after suspension=20, HU before suspension=24 (week1=7, week2=7, week3=10), HU after suspension=24 (week1=7, week2=7, week3=10).

The duration of suspension has a significant impact on the explorative and emotional profile of mice.

In the novel object test, the duration of some explorative behaviours was reduced in suspended mice when compared to relative controls (Fig.5, Fig.6). In particular, it has been observed a significant reduction in *Exploring* behaviour after week1 (frequency: $F_{(1,11)}$=7,484, P=0, 0194 between mice suspended for one week and relative controls), week2 (duration: $F_{(1,12)}$=27,745, P=0,0002 between mice suspended for two weeks and relative controls; frequency: $F_{(1,12)}$=4,944, P=0,0462 between mice suspended for two weeks and relative controls) and week3 (duration: $F_{(1,15)}$=39,668, P<0, 0001 between mice suspended for three weeks and relative controls; frequency: $F_{(1,15)}$=13,372, P=0, 0023 between mice suspended for three weeks and relative controls), *Rearing* after week2 (frequency: $F_{(1,12)}$=7,673, P=0,0170 between mice suspended for two weeks and relative controls) and week3 (duration: $F_{(1,15)}$= 9,261, P< 0,0082 between mice suspended for three weeks and relative controls; frequency: $F_{(1,15)}$=11,175, P=0, 0044 between mice suspended for three weeks and relative controls), *Wall rearing* after week1 (duration: $F_{(1,11)}$=5,456, P=0, 0395 between mice suspended for one week and relative controls), week2 (duration: $F_{(1,12)}$=19,277, P=0,0009 between mice suspended for two weeks and relative controls; frequency: $F_{(1,12)}$=12,291, P=0,0043 between mice suspended for two weeks and relative controls) and week3 (duration: $F_{(1,15)}$=27,517, P< 0,0001 between mice suspended for three weeks and relative controls; frequency: $F_{(1,15)}$=23,618, P=0, 0002 between mice

suspended for three weeks and relative controls), and *Head rising* after week3 (duration: $F_{(1,15)}$=5,560, P< 0,0324 between mice suspended for three weeks and relative controls; frequency: $F_{(1,15)}$=7,472, P=0, 0154 between mice suspended for three weeks and relative controls). Although no significant difference has been revealed, it is interesting to note that suspendend mice tended to increase the duration and frequency of *Grooming* behaviour compared to control mice. Concerning the interaction between week and treatment, *post hoc* analysis revealed a decrease in the duration of *Exploring* behaviour between mice suspended for three weeks when compared to mice suspended for one week (duration: $F_{(2,38)}$=5,306, P=0,0093; P<0,05 after *post hoc* comparison).

A general decrease in *object sniffing* in suspended mice with a concomitantly increase in the latency to the first approach to the *novel object* were observed (Fig.7). Specifically, an effect of treatment has been found in duration and frequency of *Object sniffing* with a significant reduction in duration in mice suspended for week3 ($F_{(1,15)}$=11,209, P=0,0044), and in frequency in mice suspended for week1 ($F_{(1,11)}$= 15,159, P=0,0025), week2 ($F_{(1,12)}$= 17,544, P=0,0013) and week3 ($F_{(1,15)}$= 25,394, P=0,0001). Moreover, mice suspended for two weeks showed an increased latency in the first approach to the novel object (U=35,000, P=0,0455). Similar trend has been observed after one and three weeks of suspension.

It is important to evidence that sex-related differences were not observed between suspended and control mice, except for *Exploring* behaviour. In particular, a significant effect of interaction between treatment and sex has been observed in the locomotor activity of mice suspended for three weeks (duration: F= 6,321, P=0,0259; frequency: F= 6,849, P=0,0213, Fig.10 in Supplementary materials).

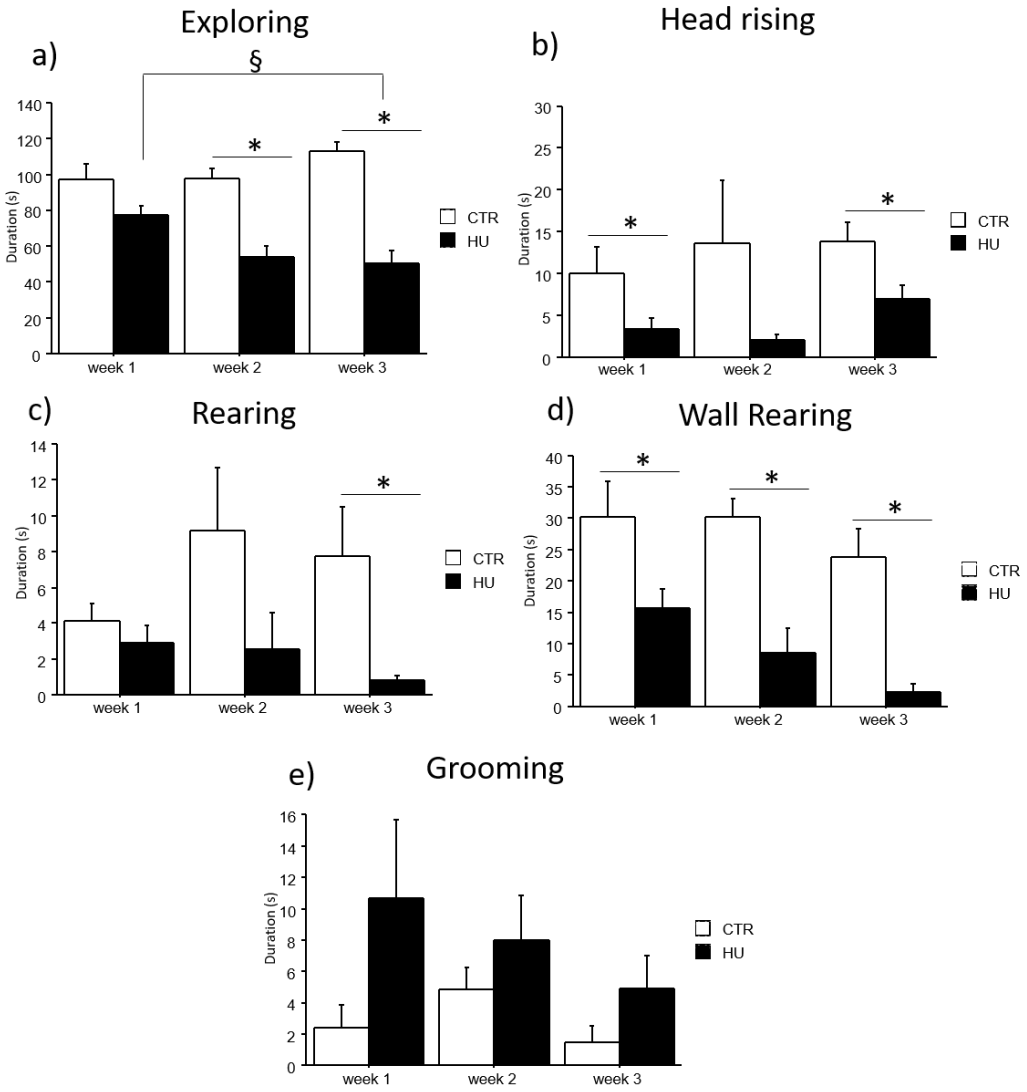

Figure 5. Behavioural effects after suspension procedure. A general reduction in the durations of explorative behaviours and vertical movements has been observed after one (b, d), two (a, d), and three weeks (a, b, c, d). Data are shown as means ± S.E.M. ∗Statistically significant at $p < 0.05$ using One-way ANOVA, § Statistically significant at $p < 0.05$ using two-way ANOVA followed by post-hoc Tukey HSD test. CTR week1=6, CTR week2=7, CTR week3=7, HU week1=7, HU week2=7, HU week3=10.

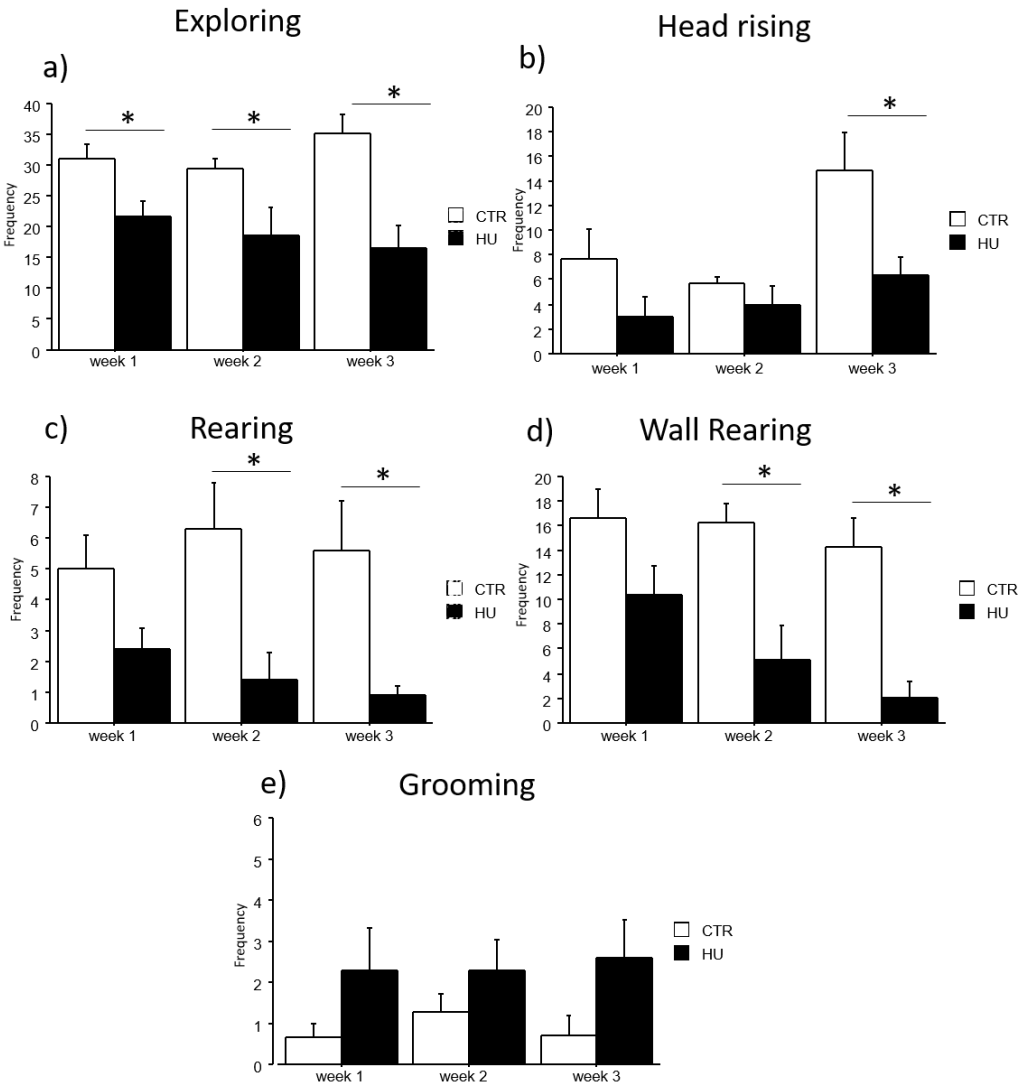

Figure 6. Behavioural effects after suspension procedure. A general reduction in the frequencies of explorative behaviours and vertical movements has been observed after one (b), two (a, c, d), and three weeks (a, b, c, d). Data are shown as means ± S.E.M. ∗Statistically significant at $p < 0.05$ using One-way ANOVA. CTR week1=6, CTR week2=7, CTR week3=7, HU week1=7, HU week2=7, HU week3=10.

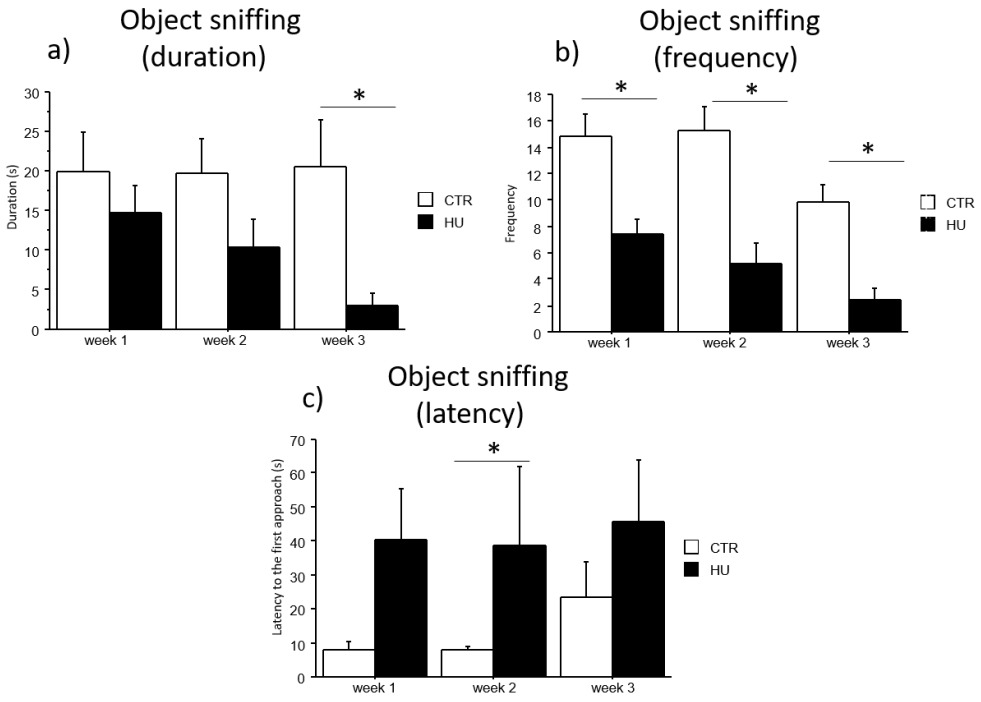

Figure 7. Effect of suspension on the emotional profile of mice. Suspended mice showed a general reduction in the object snffing behaviour (a, b) with a concomitantly increase in the latency to the first approach to the novel object (c). ∗Statistically significant at $p < 0.05$ using One-way ANOVA (a,b) and Mann-Whitney test (c). CTR week1=6, CTR week2=7, CTR week3=7, HU week1=7, HU week2=7, HU week3=10.

Brain NGF and BDNF levels were not significantly different among groups

The analysis of brain NGF and BDNF levels evidenced that the all groups (week1, week2 and week3 in control and suspended subjects) did not significantly differ. However, a trend over the weeks towards a reduction in the levels of both neurotrophins was evident, selectively in suspended mice.

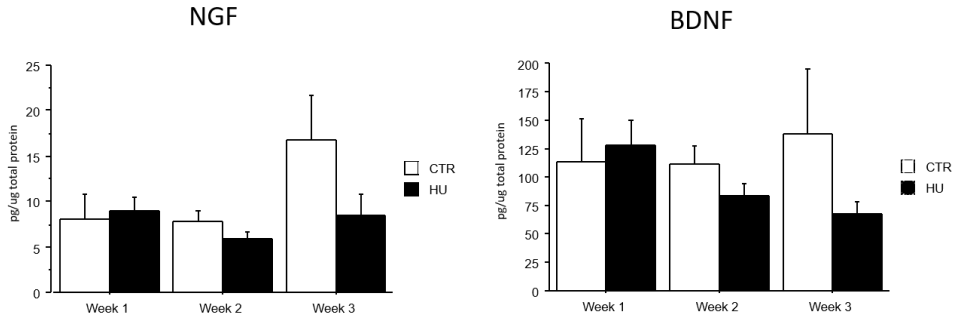

Figura 8. NGF and BDNF levels after suspension procedure. Data are shown as means ± S.E.M. CTR week1=6, CTR week2=7, CTR week3=7, HU week1=7, HU week2=7, HU week3=10.

Some behavioural items during the first day of suspension can predict individual response profile during post suspension period

Although the comparison between experimental groups revealed no differences in BDNF levels at the end of the suspension procedure, it has been shown positive correlations between that post suspension BDNF levels and behaviours performed during suspension procedure. In particular, correlational analyses between behaviours performed during suspension procedure and Novel object test revealed the following outcomes (Table 1): *Exploring* in both just suspended and Day1 mice was positively correlated with *Rearing* (r=0,729, p=0,0382) and BDNF (r=0,556, p=0,0297); *Running* during Day1 was positively correlated with *Exploring* (r=0,621, p=0,05); *Fully extended hindlimb in* just suspended mice was positively correlated with *Rearing* (r=0,647, p=0,0076); *Grooming* during Day1 was positively correlated with *Rearing* (r=0,683, p=0,0274); *Forelimb hanging* in just suspended mice was positively correlated with *wall rearing* (r=0,706, p=0,0023) and *object sniffing* (r=0,649, p=0,0074).

Table 1. Correlations between behaviours performed during the first day of suspension and the neurobehavioural outcomes collected after the end of suspension.

During early suspension	Post suspension data	r	p
exploring (day1)	*rearing*	0,729	0,03
exploring (just suspended)	BDNF	0,556	0,02
running (day1)	*exploring*	0,621	0,05
fully extended hindlimbs (just suspended)	*rearing*	0,647	0,0076
grooming (day1)	rearing	0,683	0,0274
forelimbs hanging (just suspended)	Wall rearing	0,706	0,0023
forelimbs hanging (just suspended)	object sniffing	0,649	0,0074

3.5. Discussion

The hindlimb unloading procedure strongly affects the behavioural profile of mice. In particular, a general decrease in explorative behaviours throughout the suspension weeks and a concomitant increase in *balancing* and *grooming* behaviours were clearly evident. Additionally to a slight reduction in body weight in suspended mice, index of stress response that complicates the interpretation of the behavioural results (Wronski and Morey-Holton, 1987), post-suspension data indicated a similar behavioural profile with suspended subjects showing a reduction in spontaneous activity and an increase in *grooming* behaviour.

Duration of exposure also strongly affected the behavioural profile of the mice: after the first day of suspension a slight increase in *exploring*, *balancing* and *grooming* behaviours was evident. In particular, the increase in *fully extendend hindlimbs* and the slight reduction in *forelimbs hanging* and *vertical head rising* behaviours at the beginning of suspension could be related to the attempt of escaping from the environmental novelty, including the tendency to reach the walls, and the suspension bar as well as to move around the cage. These behaviours then subsided, while *running* and *grooming* became more frequent after the first day of suspension. This change could suggest that a displacement activity or processes of dearousal due to habituation to stressful condition took place after the first day (Spruijt et al., 1992). Although it has not been observed sex related differences in the majority of the behaviours, it is worth mentioning that females performed a more marked *running* behaviour during the first week of suspension. This could suggest that the sex-related selectivity is dependent to the time of observation and the *running* behaviour emerges as behavioural indicator of sex-related susceptibility, probably due to different sex-related coping strategies.

The evaluation of behavioural profile of the mice during the novel object test revealed that suspended mice explored less the new cage and rarely approached the novel object. It is worth mentioning that the experimental subjects were isolated during the suspension period. It is known that isolation per se and the difficulty moving represent strong stressful conditions for rodents being naturally social species (Wolff J.O., 2007). Several behavioural studies reported that prolonged individual housing compromised the neurobehavioural status of the mice reducing their explorative attitude, enhancing anxiety-like behaviours and increasing behavioural responses to stress (Ahmed et al., 1995; Hall, F.S., 1998; Barrot et al., 2005; Tsvirkun et al., 2012). These effects are completely in line with results obtained in the present study: suspended mice explored the cage for less time, performed rarely vertical movements and appeared less reactive to the novelty. Moreover, we can hypotize that the subtle lack in the NGF and BDNF increase observed in suspended mice across the three weeks may confirm this interpretation. Indeed, under chronic stressful condition,

the production of adrenal-glucocorticoids is exacerbated, leading to a reduction in NGF and BDNF levels and, subsequently, to a greater vulnerability for stress-related disorders (Lakshmanan J., 1987; Schulte-Herbrüggen et al., 2006; Cirulli and Alleva, 2009). This indicates that the gravity related behavioural changes observed in animals undergone to this procedure should be investigated very carefully. Although in the scientific community it represents a recognized model to study the effects of microgravity simulated condition on musculoskeletal system, it should be taken into account that some changes in behavioural performance should be correlated also to the stressful condition experienced by the animals, due to the isolation periods and strictly connected with suspension methodology itself. In this regard, it is important to mention that this test has long been and is still currently used to evaluate manipulations that are expected to affect depression-related behaviors. The tail suspension test was, in fact, first introduced in 1985 (Steru et al., 1985) to measure the potential effectiveness of antidepressant drugs, and therefore neurobehavioral output should always be considered in the context of the adaptive response to chronic stress coupled with the effects of simulated microgravity exposure. This holds particular significance not only when assessing the impact of this experimental paradigm on learning or cognitive performance but also other physiological endpoints, since it is widely acknowledged as stress has a significant impact also on physical health, affecting the musculoskeletal, respiratory, cardiovascular, endocrine, gastrointestinal, and reproductive systems.

Tahimic and colleagues in 2019 developed a refinement of the traditional NASA single housing HU methodology (Morey-Holton and Globus, 2002; Morey-Holton; Morey-Holton et al., 2005), where suspended mice were accomodated in pairs into each cage in order to reduce at least isolation stress. Although, both single and pair HU mice showed musculoskeletal deficits compared to the normally loaded controls, surprisingly specific immune and stress-related responses were differentially affected by being in pairs. Therefore, the muscle and bone effects were basically related to gravitational changes, while neuro-immune related effects appeared differently modulated by social environments (Tahimic et al., 2019; Mhatre et al., 2022).

In this context, the possibility to define some behavioural indicators of vulnerability to suspension procedure represents an opportunity to select less susceptible subjects to the stress-related conditions to be involved in experimentations while improving animal welfare. The definition of a HU-specific ethogram clearly underlines the different trajectories in individual coping strategies during suspension procedure and highlights possible predictive behavioural indicators of resilience to stress related suspension. Indeed, present data show that active behavioural strategies during the first days of suspension are correlated with a less stressed behavioural profile after the HU suspension regardless of the duration of the exposure.

Coherently with the known muscular impairments induced by HU, all suspended mice reduced vertical movements in the novel object test (Mozdziak et al., 2001; Reidy et al., 2023). However, the analysis across the weeks evidenced that several specific behaviours such as *exploring*, *fully extended hindlimb*, *forelimb hanging* and *grooming*, are positively correlated with vertical movements after the suspension suggesting either the relevance of the individual suscetctibility in interpreting experimental data and that these behavioural items could be considered predictive behavioural indicators of resilience.

Furthermore, mice more explorative during the first two days performed more *rearing* and *wall rearing* behaviours after the end of the suspension. This could be explained by the motivational analogy underlying these behaviours: both *exploring* and vertical movements are related with the tendency to move around the cage or reach walls to increase the environmental input and be able to control and possibily escape from a pedator (Lever et al., 2006). *Rearing* behaviour in fact exploits an upright posture substantially ameliorating the capability of the subject to both sniff short or even medium-distance olfactory cues and providing the possibility to score visually the environment even in the case of minor obstacle. Such an exploratory activity is only displayed after a first phase of integration of environmental stimuli since it renders the subject more visible to potential predators, therefore it is triggered when the environmental context is considered sufficently safe, therefore signalling a lowered "anxiety" profile (Lever et al., 2006).

Interestingly, although no differences were found between CTR and HU mice at the end of the experiment, more explorative just suspended mice showed also higher levels of BDNF at the end of suspension. In general, chronic stress induce a reduction of BDNF expression in the brain due the crosstalk between glucocorticoid and neurotrophin systems (Miao et al., 2020; Murakami et al., 2005). In this case, the single correlations suggest that resilient subjects may perceive differently the stress-related challenges allowing different molecular adaptive processes (Taliaz et al., 2011; Rothman and Mattson, 2013; Leschik et al., 2022).

The predicitve value of *grooming* behaviour could be associated to the "cost" of this behaviour during the suspension. To perform this behaviour, the animal should bring the head towards the belly performing an active behaviour, showing a strong motivational boost, evidencing a possible resilient trait. This interpretation is in line also with the positive correlation found between the duration of *grooming* during the first week of suspension and the duration of post-suspension *rearing* behaviour. Interestingly, early *Running* behaviour during suspension could predict a major explorative profile after suspension procedure and could be consider another behavioural indicator of resilience. As for the explorative behaviours, the performance of *Running* during the first days of

suspension may be suggestive of active coping strategy during suspension and at the same time a decreased sensitivity to stress condition during and after the suspension.

Overall, our results evidence that suspension procedure affects the behavioural profile of mice, and the behavioural analysis during suspension represents a valid tool to assess the susceptibility of mice to this procedure. However, is worth mentioning that the interpretation of the behavioural data should take into account the different suspension methods employed. When considering animal welfare and the application of 3R principles (Russell and Burch, 1959), the possibility to standardize such methods represents a fundamental issue in refining animal suffering and reduce the number of experimental subjects. The harmonization of the procedures represents a useful tool to collect scientific data comparable among research groups and avoid unnecessary replication (Manciocco et al., 2009; Hawliczek et al., 2022).

3.6. Supplementary materials

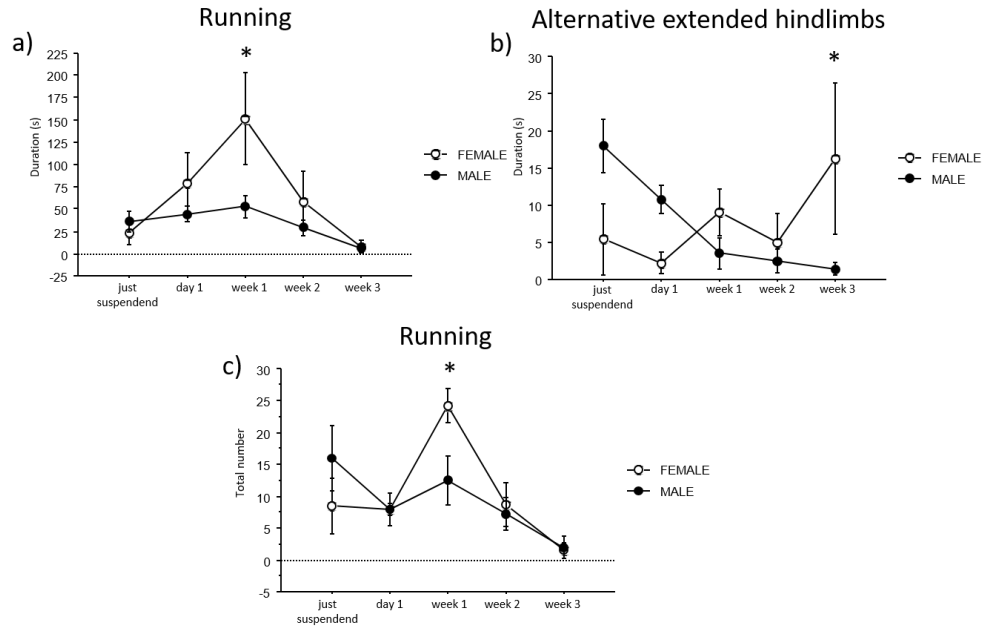

Figure 9. Sex-related differences in balancing behaviours during suspension. An increase in the duration and frequency of Running (a,c) or Alternative extended hindlimbs (b) has been observed in females. Data are shown as means ± S.E.M. ∗Statistically significant at $p < 0.05$ using repeated measure ANOVA followed by post-hoc Tukey HSD test. Females=4, Males=4.

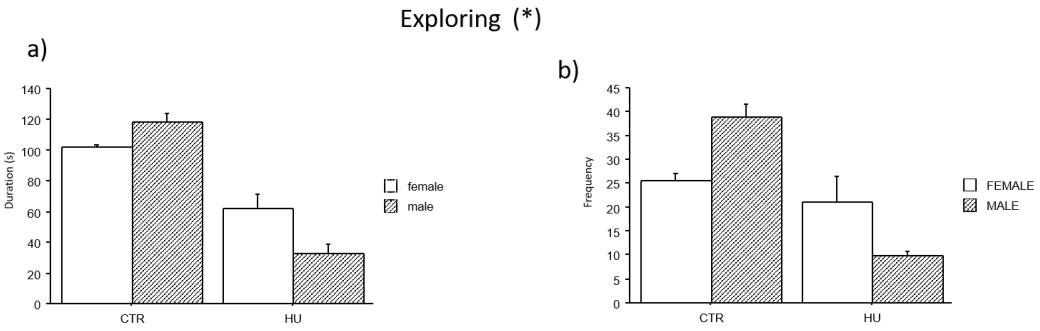

Figure 10. Sex-related differences in exploring behaviour after three week of suspension. Data are shown as means ± S.E.M. Two-way ANOVA. ∗Statistically significant interaction sex x treatment. Females=4, Males=4

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4. EFFECT OF 10-DAY BED REST ON SALIVARY LEVELS OF NEUROTROPHINS AND INFLAMMATORY MARKERS RELATED TO INDIVIDUAL RESILIENCE

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4.1. Abstract

Ground-based studies to investigate the neurobehavioural effects of gravity changes are essential for developing proper physical and cognitive countermeasures to assure safe and effective space missions and human survival in space. Bed-rest paradigm is a reliable ground-based spaceflight analogue widely employed in space biomedical research. Aim of the present study was to assess personal differences in copying with bedrest stress-related experience in order to identify behavioural aspects and potential biomarker to predict personal resilience and/or vulnerability to ground based weightlessness exposure.

Two different psychological questionnaires, the Perceived Stress Scale-10 (PSS-10) and the Resilience short scale tests, were administered at the beginning and at the end of the 10-day bed rest study. Moreover, NGF and BDNF, neurotrophic factors known to operate through multiple paths to ultimately regulate physiological homeostasis and behavioural coping, and inflammatory biomarkers were evaluated in saliva sampled in the morning.

Data do not indicate significative changes in psychological score along the 10 day bed-rest exposure, while several fluctuations were observed in the level of both neurotrophins and biomarker with specific correlation among questionnaire score and biomarker levels, confirming how identification of individual key factors is a prerequisite for pre-mission screening and for developing safe and individualized countermeasures in long-term mission.

Keywords

bed rest, salivary biomarkers, neurotrophins, resilience

4.2. Introduction

Long-term exposure to microgravity is known to functionally affect a wide range of physiological systems, including musculoskeletal system, cardiovascular response, eye-hand coordination and spatial orientation (Kourtidou-Papadeli C., 2012; Norsk P., 2021). These well-known physiological effects are often coupled with other psychological factors, difficult to dissociate from physical impairments but relevant to predict human vulnerability during spaceflight. In particular, the individual perception of environmental stressors and the individual strategies to cope with external adversities play a fundamental role in preserving the health during and after the exposure to un-physiological gravity. Well-being is a result of both state and trait differences in how individuals approach stress, and resilience is determined by a range of cognitive and personality factors (Heidemarie et al., 2014). When a stressor is perceived by an individual often results in emotional states that compromise the individual attentional capacity and mental resources (Wachtel P.L., 1968; Driskell et al., 2008), thus affecting the performance of the crew during the spaceflight. Moreover, it has been largely discussed that the individual variability in the perception of stress by the crew members as well as the great differences in the adaptive responses during and after acute or chronic stress are important indicators for limiting human suffering and preserve individual well-being under extreme conditions (Lazarus R.S., 1993; Sonnenfeld G., 1999; Gabriel et al., 2012). Indeed, systematic adjustments, which characterize vulnerable individuals, could be observed in resilient individuals with additionally active molecular mechanisms that promote positive coping strategies and protect against mental disorder. In this perspective, a personalised medicine approach, taking into account the individual reactions to altered gravitational environments, appears appropriate for selecting resilient profiles to gravitational stress and designing successful interventions during spaceflight.

Due to both financial and technical difficulties related to space missions and the few and infrequent space launches, ground-based models that simulate micro- and hypo-gravity represent a useful tool to investigate specific individual adaptive responses to altered gravity. One of these is the bed rest (BR) paradigm, largely validated in space biology and often employed in different fields of biomedical research (Pandiarajan and Hargens, 2020). It consists of immobilization of volunteers on a bed for periods of different duration in order to reproduce muscle atrophy, fluid redistribution, weight loss, cardiovascular and pulmonary adaptation similar to those experienced by astronauts in space. Even though gravitational force is not completely lost, and the skin is in contact and compressed over the bed, it is recognized as a valid model to study the varied range of effects induced by reduced physical activity (Oranger et al., 2022). Interestingly, it has been demonstrated that maintaining this position acts as a stressor on the cerebral structures (Li et al., 2015),

electophysiological activities (Marušič et al., 2014), and excecutive functions (Lipnicki et al., 2009; Liu et al., 2012; Passaro et al., 2017), and modulates endogenous neuroinflammatory mediators (Blaber et al., 2023) and plasma levels of BDNF (Soavi et al., 2015). In particular, Drummmond and colleagues (2013) reported that short-term (7 days) BR in adults increased some pro and anti-inflammatory cytokines and upregulated TLR4 proteins. Similar results were obtained by Mutin-Carnino and colleagues (2014) that found peaks of IL6 after 59 days of bed rest, while Jurdana and colleagues (2015) evidenced age-related inflammatory modifications with the IL6 more pronounced in older age group subjected to 14days of BR compared to younger adults. Moreover, accumulating evidence suggested that the immune cell mobilization under environmental challenges require also the involvement of neurotrophic mediators, such as NGF and BDNF, known to play a critical role in synaptic plasticity and differentiation and activation of immune cells (Levi-Montalcini et al., 1997; Francia et al., 2004). Crossing bi-directionally the blood-brain barrier they can act at systematic level (Passaro et al., 2017), promoting appropriate (or inappropriate) coping strategies by way of modulating attentional and/or cognitive processes while controlling the stressor itself (Francia et al., 2004).

However, very little is known about how neurotrophic responses together with inflammatory factors relate to stress adaptation during the BR condition. Analogously, it is not known if there is a relation between neuroinflammatory levels and resilience-related individual differences. Therefore, NGF, BDNF and other neuroinflammatory mediators were evaluated in salivary samples collected in volunteers subjected to acute bed rest condition and their levels were related to psychometric responses in order to select individual indicators of resilience and/or vulnerability to micorgravity-simulated condition.

4.3. Materials and Methods

Participants

The study was conducted in the General Hospital of Izola (Izola, Slovenia). Ten healthy men (20–30 years, mean 23±5 years; body mass: 77.5±10.0 kg; body height: 1.81±0.04m; body mass index (BMI): 23.5±2.5 kg·m−2) were enrolled in the study. Other two volunteers were considered as additional reserve. The sample size was determined based on statistical power analysis using the G-power3 calculator, imposing power values (1-b), a levels and effect size to reach a probability of (1-b) obtained from previous studies. The volunteers were subjected to 10 days of horizontal bed rest in standard air-conditioned hospital rooms. The evaluation of each partecipant started from two days before to the 2 days after the end of 10 days-BR. During the days preceding the bed rest period, the subjects were asked to visit the Hospital in order to become familiar to the environmental conditions and to the diet. During BR no changes from lying position, muscle stretching, or static contractions were allowed while during the two days post-BR, subjects remained in bed or wheelchair. All partecipants were constantly monitored during day and night and received a controlled diet (60% carbohydrate, 25% fat, and 15% protein) through three meals a day. Diet energy requirement was established for each individual subject taking into account their BR condition (Biolo et al., 2008).

Head down bed Rest

According to the already described methodology in Oranger and colleagues (2023), during the entire duration of the experiment, the volunteers performed all daily routine activities in bed, without being able to remove themselves from the supine position of the BR. No muscle contraction tests, or any form of physical exercise were permitted during the BR. The BR subjects slept from 10:00pm to 7:00 am. The protocol included 2 days of adaptation to the environment and diet, 2 days before the beginning of BR for collection of all control parameters, 10 days of BR and 2 days of recovery.

Psychological Questionnaire

Before the beginning and after the end of the BR period the Perceived Stress Scale (PSS) and the Brief Resilience Scale (BRS) were administered to the participants. The PSS-10 measures the degree to which a subject perceives a situation as unpredictable, uncontrollable, and overloading (Cohen S., 1983). It includes 10 items (positive and negative) concerning with feelings and thoughts related to the last month. Responses were given on a five-point Likert scale (0=never, 1=almost

never, 2=somrtimes, 3=fairly often, 4=very often). The score range included: 0–10, below the average of perceived stress; 11–14, at average of perceived stress; 15–18, above average of perceived stress; and >19, an extremely high perceived stress score (Zappella et al., 2021).
The BRS is a 6-item measure to profile the ability to bounce back or recover from stress. Responses are rated on a 5-point Likert scale (1 = strongly disagree, 2 = disagree, 3 = neutral, 4 = agree, 5 = strongly agree) and a higher BRS score outlines a more resilient participant profile.

Saliva collection

During the entire course of the experiment salivary samples were collected from each subject in order to quantify neurotrophin/ inflammatory/profibrogenic factors. Saliva samples were collected by means of specific cotton swabs (Salivette, SarstedTM, Milan, Italy). Each subject was asked to chew the Salivette cottonswab for 120 s. All samples were kept on ice and later centrifuged to collect saliva (13,000 rpm for 15 min; megafuge, Heraeus, Milan, Italy), Finally, samples were stored at −80◦C until analysis.

Biochemical analysis

A customized protein array on glass-chips was used according to the manufacturers' procedures (RayBio technology; Norcross, CA). Inter-assay normalization was conducted by including multiple positive markers and negative controls for each sub-array. The minimum sensitivity range for detection of each protein varied, ranging from 3.8 to 56 pg/mL. For hybridation, normalized protein extracts (70μg) were diluted in appropriate buffer and applied in sub-arrays. All BR salivary samples extracts were processed in parallel. After an overnight incubation at 4 °C, the array slides were washed and exposed to a biotinylated antibody mixture followed by a cy3-streptAvidin labeling solution. All steps were performed under orbital shaking (CertomaxII, Sartorius AG) following the manufacturer's recommendation and all the hybridization/washing solutions were provided by the kit. As last step, the glass-slides were washed once with MilliQ water, spin-dried and scanned in a GenePix 4100A Microarray platform (Molecular Devices LLC, Sunnyvale, Silicon Valley, CA). To obtain appropriate Cy5 (background signal) and Cy3 (specific signal) images, the slides were scanned over previously validated acquisition parameters and procedures. The images/arrays (blocks) were uniformly adjusted for size, brightness, contrast and chip-to-chip comparisons by the software and provided as 8-bit Tiff format (Axon GenePix Pro 6.0.1.25 software; Molecular Devices) that provide background-subtracted FI data (F532-B532, N factor) as of a value for spot volume representing the product of the area and the highest pixel value contained in that area. Using the SPOT tool, the specific area (corresponding to each cytokine on the array)

was manually spotted and automatically adjusted, according to prefixed acquisition parameters applied to all glass-slides of the study. All comet tails were ignored and only median signal values obtained using the same setting were used for the identification of any biomarker variation. An inter- and intra–assay coefficient of variability limit of ≤10% was set for the study, and a 1.5-fold increase or ≤0.65-fold decrease in signal intensity was considered to guarantee specific signals above background. Fluorescent signals were analyzed and fold changes were generated (pathological/control ratio). The plot of proteins included several interleukins (IL-6, IL-8, IL-18, IL-1 beta, IL-17A), Toll Like Receptor (TLR)-3, NeuroTrophin (NT)-3, NT-4, Osteopontin (OPN), Vascular Endothelial Growth Factor (VEGF)-A, VEGF-C, VEGF-D, Pigment epithelium-derived factor (PEDF), Metalloproteinases (MMP1), MMP2, MMP3, MMP7, MMP9, Erythropoietin receptor (EPO-R) and Complement component 5a (C5a). In order to minimize intra- and inter-assay variability, a single tester handled all the material and followed all the phases of the experiment.

Statistical analysis

Continuous variables are presented as means and standard errors of means (S.E.M.) and categorical variables were displayed as frequency counts. Normality of distribution was tested with Shapiro-Wilk test. The non-parametric Wilcoxon test was used to assess the effect of time on psichometric data, while neuromediators parameters were analysed by ANOVA with repeated measures with time as within factor. Correlations between continuous variables were tested by Pearson's correlation test. Comparisons were performed by using the Statview software and statistical significance was set a $P < 0.05$.

4.4. Results

BR affects salivary levels of neurotrophins and inflammatory biomarkers by highlighting a similar response across the 10 days

The analysis of BDNF levels showed a statistically significant decrease of BDNF during 10 days of BR ($F_{2,12}$= 5,544, P=0,0197; Fig. 1). *Post-hoc* analysis revealed that BDNF levels were significantly reduced in salivary samples collected during the last day (Day 12) compared to those collected during the first (Day 3) and intermediate day (Day 9). Although no significant differences were found concerning NGF level, a similar trend was observed between Day 9 and Day 12.

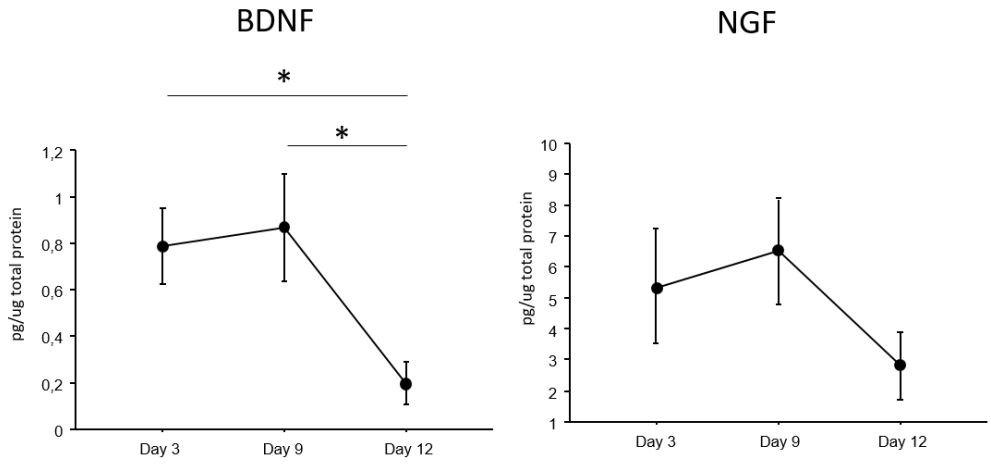

Figure 1**.** Ten days of BR decreased neurotrophins levels in salivary samples. A significant decrease in BDNF level was observed between Day 3 and Day 9 or Day 12, while a tendency in decreased NGF level was evidenced between Day 9 and Day 12. Data are shown as means ± S.E.M. One way ANOVA with *post-hoc* Tukey test, *P=0,05.

Proteins involved in immune functions and markers of neuroinflammation (vascular endothelial growth factor, VEGF; Interleukin-1, IL-1; Interleukin-8, IL-8; Interleukin-18, IL-18; Pigment epithelium-derived factor, PEDF; metalloproteinase-2, MMP-2; Toll-like receptors, TLR; Complement protein 5a, C5a) were evaluated in salivary samples. Bed rest condition leads to a significant increase in VEGF ($F_{2,16}$= 4,200, P=0,0342) and IL-18 ($F_{2,14}$=, P=0,0633) during the 10 days. Specifically, *post-hoc* analysis indicated that the amount of VEGF and IL-18 increased between the Day 3 and Day 9. Even if not statistically significant, a similar trend was observed in TLR, VEGF-C, PEDF, MMP-2 and C5a levels (Fig.2).

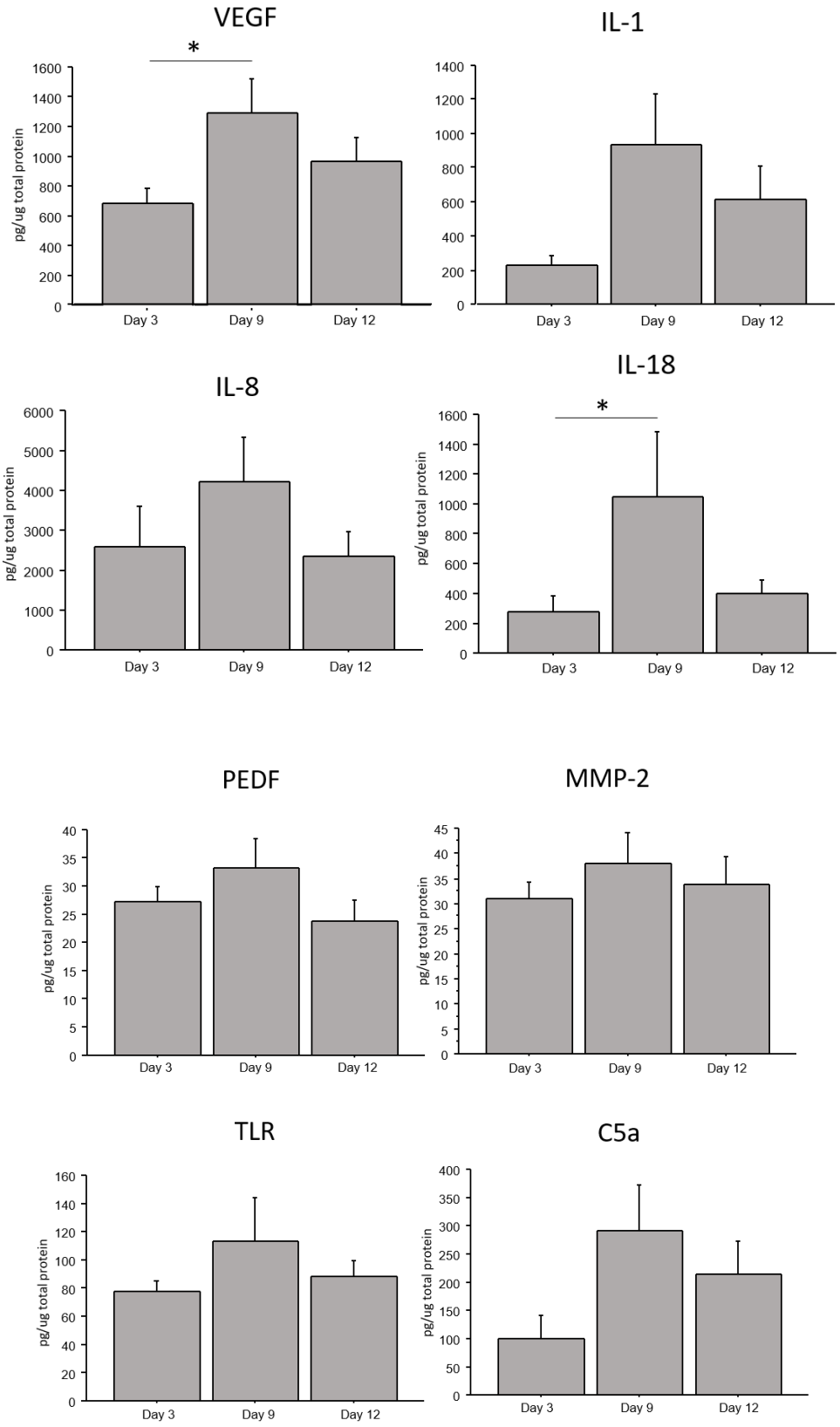

Figure 2. Ten days of BR affected inflammatory markers in salivary samples: VEGF and IL-28 significantly increased between Day 3 and Day 9 and a similar trend was observed in TLR, IL-1, IL-8, IL-18, PEDF, MMP-2, TLR, and C5a. Data are shown as means ± S.E.M. One way ANOVA with *post-hoc* Tukey test, *P=0,05.

Individual differences in resilience profile drive susceptibility to BR condition

Although the comparison of salivary biomarkers and total PSS and BRS scores collected before and after bed rest failed to reveal consistently statistical differences, it has been shown that neurotrophin and some inflammatory biomarkers are correlated with BRS and PSS scores, confirming the relevance of individual susceptibility to bed rest conditions. In particular, data evidenced how BRS scores after 10 days of BR were negatively correlated with the NGF (r=-0,7; P=0,01) levels and some inflammatory markers (IL-1: r=-0,7; P=0,04, IL-18: r=-0,7; P=0,03, IL-8: r=-0,7; P=0,03; MMP: r=-0,9; P=0,01) in samples collected during the last day of bed rest (Day 12). Interestingly, concerning PSS scores a negative correlation was found between the total score obtained at the end of BR and BDNF levels during Day 9 (r=-0,6; P=0,06).

4.5. Discussion

The results presented herein confirmed the role of neurotrophin and immune system biomarkers in the adaptive coping response to environmental challenges and, interestingly, evidenced peculiar and similar fluctuations during the 10 days of BR, with a significant reduction at the end of the BR period. In a context of growth interest toward the possibility to develop safe and individualized countermeasures in long-term missions, the specific correlation among questionnaire scores and neurobiological data underlined that neuro-immune system biomarkers could represent valid bioindicators of resilience-related individual differences.

It has been largely demonstrated that the neurotrophins represent key regulators of neuroplasticity phenomena in response to stressful stimuli (Lakshmanan J., 1997; Hadjiconstantinou et al., 2001; Baranova et al., 2015; Santucci et al., 2022). In general, the increase of NGF and BDNF during the exposure to environmental challenges could underlie some phenomena of brain plasticity as result of copying strategies to modulate attentional and cognitive processes while controlling stressor itself (Alleva and Santucci, 2001; Francia et al., 2004; McEwen B.S., 2007). Clear examples come from research carried out on animals or humans. Several studies conducted on mice demonstrated that stressful events may induce changes in brain levels of neurotrophins (Alleva and Aloe, 1989; Aloe et al., 1994; McEwen, B.S., 1999; Alleva and Santucci, 2001; Cirulli and Alleva, 2009). In response to stress, they vary across brain regions and fluctuate rapidly both immediately after a stressor and over the course of the chronic stress paradigm (Gray et al., 2013). For example, acute exposure to altered gravity has been observed to increase the amount of NGF in several brain areas of mice (Santucci et al., 2000), while exposure to hypergravity for a longer period modulated BDNF levels in the hypothalamus, hippocampus, and cerebellum (Ishikawa et al., 2017). Moreover, the evaluation of the impact of 105-day confinement in humans revealed that BDNF plays a role on the homeostatic adaptation to stressful condition (Strollo et al., 2014). Although plasma NGF levels were not significantly affected by confinement, BDNF significantly increased after 5 and 10 weeks and returned afterward to baseline values (Strollo et al., 2014). Our results evidenced a similar involvement of neurotrophins in the BR stress-related responses, with regard to the possibility that the neurotrophic action follows a typical temporal curve of homeostatic adaptation. Because the adrenal gland appears to be one of the biological targets of neurotrophins during stress-related responses, the reduction of NGF and BDNF only during the last days could suggest a sort of ‘‘adrenal fatigue’’ after long-term stimulation (Santucci et al., 2012).

Therefore, analysing the neurotrophin levels at different times during the coping response to a stressful situation may provide a more thorough understanding of the physiological consequences of the event under investigation. Indeed, although the increase in plasma BDNF levels after 14 days of

BR reported by Soavi and colleagues (2016) confirmed its involvement during BR, the evaluation of the BDNF levels in the three points observed suggests how the trend undergoes modulations concomitantly with the exposure phases. Moreover, it has been reported that different stimuli interact with the production of BDNF, including the exposure to music and watching sports activities (Brattgico et al., 2021). The 10 volunteers were constantly exposed to images through their personal computer (mainly listening to music or watching videos, including sports) indicating the importance of an evaluation, possibly non-invasive, of neurotrophins levels throughout the entire exposure period but also with a close examination of the various activities carried out by the different subjects.

Interestingly, the similar trend between neurotrophic and immune system biomarkers could elicit a convergent homeostatic mechanisms linking neuroendocrine and immune elements (Hohlfeld R., 2008; Cirulli and Alleva, 2009). Salivary levels of different immune system and inflammatory biomarkers followed a similar fluctuation across the 10-days of BR. In line with NGF and BDNF results, the increase of TLR, MMP, C5a, and IL-18 after few days of BR could underline an increased inflammatory response (Monk et al., 2007; Khokha et al., 2013; Vijay, K., 2018; Ihim et al., 2022), while the PEDF and VEGF levels could indicate a potential compensatory role in the cellular protection and survival during immune activation (Nowacka et al., 2013; Brook ET AL., 2019). An increasing body of evidence suggests that NGF, in addition to its role as a neurotrophic agent, may operate through multiple paths to ultimately regulate physiological homeostasis and behavioural coping in strictly association to immune system (Torcia et al., 1996; Kerschensteiner et al., 1999). The similar oscillation of neuro-immune system biomarkers could reflect the cross talk between the two systems where the neurotrophic expression is affected by immune cells and the immune factors they secrete, while the immunomodulatory processes also require the regulation of neurotrophin-mediated signaling pathways (Jin et al., 2019; Kim et al., 2023). Therefore, in line with different studies on animal models (Aloe and Levi-Montalcini, 1977; Ross et al., 1984; Morel et al., 2020), NGF and BDNF released during BR could be involved in potentiating of the immune responses to face this challenge. In addition, the neuro-immune fluctuation observed during BR evidenced that the adaptive response that takes place is not necessarily related to higher or lower absolute stress levels, but rather to a dynamic process of reaction to stressful stimuli (Laurent et al., 2014). In this context, attention to stress-related cognitions and personality attributes aids in understanding the short- and long-term processes giving rise to adaptive stress responding (Laurent et al., 2014). Indeed, our findings reveal no differences in the total psychometric scores before and after BR condition, while clearly indicate how the individual analysis elicited that subjects more resilient at the end of BR showed also lower levels of NGF and inflammatory biomarkers. On the

contrary, individuals who still perceived the situation as stressful at the conclusion of the BR period reported lower levels of BDNF in saliva sample collected at mid-time during the experiment, thereby confirming the modulatory role of this peptide in coping with stress

These individual profiles could suggest that neurotrophic and immune responses are related not only to the outcome of the adaptation but also with individual difference that drives adaptive responses. This supports the need to investigate individual responses to define countermeasures tailored to the individual susceptibility, confirming how identification of individual key factors is a prerequisite for pre-mission screening and for developing safe and individualized countermeasures in long-term mission. In this context, bedrest studies are widely recognized as the gold standard in space medical research when it comes to emulating the impact of weightlessness on the human body on Earth. The test subjects' bodies are affected by long-term bed rest studies in the same way that astronauts are affected by space travel. However, careful identification of individual response to this kind of stress should be evaluated and further research specifically addressed to these aspects should be performed in order to better validate experimental data.

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5. CONCLUSIONS

Extreme environments, such as space, represent crucial “natural laboratories” for the understanding of human physiology and neuropsychology (Suedfeld et al., 2009; Angeloni et al., 2020). Exploiting the adaptations in these conditions represents an opportunity to investigate mechanisms underlying neuroplasticity phenomena and the individual vulnerability to stress. Intriguingly, in a context of growing interest towards the possibility to tailor the countermeasure programmes according to the individual susceptibility to extreme environmental conditions, the definition of predictive behavioural indicators of resilience could represent reliable biomarkers of individual vulnerability (Pavez Lorie et al., 2021). Since they represent the endpoints of highly integrated systems, they emerged as sensitive indicators of impaired motor functions in mice, where subtle alterations in each of the components of such systems could be reflected in behavioural indicators. Within this context, the present work was designed to investigate possible behavioural and neurobiological biomarkers of vulnerable or resilient profiles in ground based models reproducing hypergravity and microgravity-simulated conditions.

The results collected on animals exposed to un-physiological gravity evidenced that behavioural adaptive responses took place both during the permanence in centrifuge and upon exposure to tail-suspension procedure (i.e., hindlimb unloading model). In both paradigms, animals exhibited species-specific behavioural items with selective habituation curve across the days and, selectively in the hindlimb unloading model, new peculiar behavioural outcomes were also defined. The behavioural repertoire on normogravity revealed that vertical movements and vestibular-related behaviours resulted compromised by the previous extreme experience, and, interestingly, the behavioural items performed during the first days in the centrifuge or upon suspension could predict resilience and vulnerability profile of the experimental subjects. Specifically, it emerged that behaviours falling within the explorative category and performed during the first days of altered gravity could be considered reliable behavioural indicators of reduced individual vulnerability to environmental challanges. No significant differences were observed in the brain NGF and BDNF levels, suggesting a general good level of adaptation to hypergravity conditions after one month of exposure. However, importantly, the significant correlations between neurotrophin levels and individual behavioural items during the early exposure to hypergravity or suspension underlined the functional validity of defining predictive behavioural biomarkers. In particular, suspended mice that exhibited explorative behaviours during the beginning of hindlimb unloading showed also high brain BDNF levels after the end of the suspension, while centrifugated mice that performed more explorative behaviours during the first days in hypergravity showed, instead, lower cerebellum-NGF levels after almost one month in centrifuge. The contrasting correlations could derive from

different neurobiological substrates and/or the different individual trajectories of individual coping strategies. After hypergravity, an anticipatory increase of NGF could take place as a result of appropriate coping strategies (Aloe et al., 1994; Santucci et al., 2006), while the BDNF availability at the end of suspension, known to accompanied the psycho-physiological adaptive responses after acute or chronic stress, may orchestrate lower vulnerability to suspension procedures (Yang and Zhang, 2016; Naumova et al., 2023).

In line with the results obtained in the animal models, peculiar changes in NGF, BDNF and different immune system-related biomarkers were observed across the days in humans exposed to bed rest paradigm. Interestingly, the most relevant result regarded specifically the similar peak of the biomarker levels during the intermediate phase of BR across the 10 days. In line with the findings collected from astronauts during the spaceflight (Santucci et al., 2006), NGF were higher at the beginning while BDNF increased during the intermediate phase of bed rest experience. According to their role in the neuroplasticity phenomena in response to stressful stimuli (Lindholm et al., 1994; Levi-Montalcini et al., 1997), this trend could reflect their active function during the most anxiety-related period, corresponding to anticipatory stress-related copying response to a 'unknown and potentially challenging' context and the accumulated stress from the previous days in bed rest condition. Moreover, the evaluation of individual profile evidenced that subjects more resilient at the end of the experiment showed also lower levels of neurotrophin and inflammatory biormarkes. This suggests an active coping strategy in resilient individuals with an anticipatory role of biomarkers to counteract the environmental challenge. Therefore, the current results support the pivotal role of neurotrophins during the adaptive response to stress and the dynamic neurotrophic process that accompanies the individual vulnerability to psychosocial challenges (Laurent et al., 2014).

In view of the fact that brain and peripheral metabolic functions might be entangled with individual vulnerability to environmental changes, microbial modulation after experiencing extreme conditions would be expected to have an effect on specific functions or potentially for the regulation of the system as a whole (Cowan et al., 2020). Interestingly, it has been reported that exposure to hypergravity modulated that phylogenetic diversity of fecal microbiota and, interestingly, increased the level of the phyla *Desulfobacterota* and *Cyanobacteria,* known to be involved in pro-inflammatory and hypoxic phenomena. Taken together with the behavioural results, it emerged that the investigations of bacterial communities after the exposure to alter gravity could represent a valid instrument to understand the boundary conditions for when specific neurotoxic mechanisms took

place. Moreover, in line with the personalized medicine approach in space, data obtained from such studies could lead new strategies to counteract health problems associated to spaceflight.

5.1. Future perspectives

Understanding mechanisms underlying human adaptation in space is one of the main goals of the astrobiology research. Given the remarkable similarity between the mouse and the human and due to the concrete difficulties to conducting experiments on orbit, spaceflight research is always take advantages of mice to understand individual reactions to unphysiological gravity and the underlying plasticity phenomena that occur in these conditions. Moreover, animals are sent into orbit, therefore investigating their individual susceptibility to altered gravity becomes pivotal to choose individuals more resilient and reduce animal suffering in space.

In this context, the scientific results reported in the present thesis become instrumental to investigating individual behavioural reactions in ground-based models and refining methodological approach in order to implement the translational value of the behavioural outcomes. The possibility to apply measurable indicators to follow the adaptive response of the animals and disentangle the stress related responses from adjustments associated to altered gravity conditions, become functional to deepen the phenomena underlying individual susceptibility. In particular, the behavioural repertoire observed during the experiments on animal models, especially during hindlimb unloading procedure, could help to set a methodological baseline to monitor the animals during the extreme conditions and identify species-specific behavioural elements mirroring individual differences in coping with ground-based paradigms. Future experiments could be centered on the evaluation of the effects of multiple environmental stressors typically experienced in space, such as isolation, altered gravity and radiation exposure, and it would be relevant to compare the individual susceptibility between isolated and group housed mice in order to deepen the contribution of social environment to the individual vulnerability. Moreover, it would also be important to select predictive behavioural indicators encompassing different behavioural domains related to different ages, information that was not acquired in the present work of the thesis, which deserves to be implemented. Intriguingly, the results regarding the impact of hypergravity on microbiota composition could open new perspectives to add information about the effects of altered gravity on the brain-gut axis and study the potential influence of dietary supplementation through molecules able to recover microbiota dysregulation. Lastly, it would be interesting to analyse other biomarkers from fecal samples, such as glucocorticoid levels, in order to correlate them with brain levels takeing advantage of a non invasive method to monitor stress-related responses in the experimental subjects during the exposure to altered gravity conditions.

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6. APPENDIX

Peer-reviewed publications during the PhD period

When Nerve Growth Factor Met Behavior

13

Daniela Santucci, Arianna Racca, and Enrico Alleva

Abstract

Since its first characterization in the early 1950s, the role of the polypeptidic nerve growth factor (NGF) in controlling behavior remained elusive. Since the mid-1980s, we undertook a series of experiments aimed at elucidating the biological role(s) played by neurotrophins, particularly NGF, in adult rodents. At the beginning, we concentrated on the submandibular salivary gland of the male mouse, which was known to store massive amount of NGF. We found that under specific stress conditions, the salivary NGF is released in the bloodstream: intermale fighting between isolated males was the first reported context in which salivary NGF was released, thus providing a physiological significance for its presence in the adult, territorial males. We also found that dominant males release less NGF than subordinates and provided a loop-type model which includes intermale social confrontation, adrenal gland size, and functional status, corticosterone release, a model resulting in likelihood to be stabilized in a "dominant" or a "subordinate" social status. A variety of social anxiety contexts of mammals, humans included, has been described since then, and further studies carried out on humans showed that NGF is released in the bloodstream of parachutists at their first skydiving experience and in the case of ranking high on the Passionate Love Scale (*amour fou*). Ethological data from lab rodents helped in understanding NGF function in subtly controlling social "status" of male mice: the considerations about the interplay among neurobiological, physiological, and behavioral factors in structuring the dominant vs subordinate phenotypes may well apply to other vertebrate species, specifically addressing the underlying role of neurotrophins in relating behavior and brain neuroplasticity.

13.1 Introduction

It was July 1983 when, in central Rome, in the elegant attic of Viale di Villa Massimo 3–5, following an almost 10-year-long discussion with Rita Levi-Montalcini (RLM) about the mysterious, yet likely physiological, role of NGF stored in the submaxillary "salivary" glands of adult male mice, in collaboration wither her closest fellow Luigi Aloe, we decided to attempt what is now regarded as a crucial experiment (Fig. 13.1).

The origin of NGF release into the circulation was in the early Eighty highly debated, and we

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RESEARCH PAPER

Behavioural changes in farmed sea bass (*Dicentrarchus labrax*) experimentally infected by *Anisakis* nematodes

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Abstract
Experimental studies on new host-parasite systems involving farmed fish can contribute to our understanding of the host behavioural changes associated with parasite infections, as predicted by the host manipulation hypothesis. This has applied relevance because in offshore farms, intermediate hosts of *Anisakis* nematodes may come into contact with farmed species, with an actual risk of infection. It could therefore be useful to identify behavioural indicators of infection status to monitor animal health and develop adequate countermeasures. Spontaneous activity, sociability, feeding, predatory response and boldness were evaluated in the three weeks following the infection in farmed sea bass (*Dicentrarchus labrax*) experimentally infected by *Anisakis pegreffii* larvae. Infected animals displayed a significant increase in food intake that did not affect body weight gain. They also showed increased interest towards a prey model and towards food in a risky feeding situation. While supporting the hypothesis of increased energy drain from infection that promotes increased investment in foraging rather than active host manipulation, the observed changes could constitute preliminary behavioural indicators of parasite infection in farmed fish.

Keywords *Anisakis* sp. · Aquaculture · European sea bass · Host behavioural changes · Host manipulation hypothesis · Risk-proneness

1 Introduction

Parasite infections are associated with numerous host behavioural changes (e.g. Moore 2002; Adamo 2012; McElroy and de Buron 2014; Binning et al. 2017). These changes may emerge because they confer fitness benefit on either the parasite ("adaptive host manipulation", Moore and Gotelli 1990; Moore 2002) or the host ("behavioural defence"; Hart 1990; Poulin 1995), or they may be a result of pathological side-effects of the infection, with no adaptive value to the parasite ("parasitic constraints"; Poulin 1998; Kavaliers et al. 1999; Thomas et al. 2005). Examples of host behavioural changes are the radical alteration (from aversion to attraction) in the perception of naturally occurring stimuli indicative of predator presence (e.g., cat odour, leopard urine in the case of toxoplasmosis-infected rats and chimpanzees, respectively; Berdoy et al. 2000; Vyas et al. 2007; Poirotte et al. 2016), or the alterations in the frequency at which a behaviour is performed, such as feeding rate and activity (e.g., Christe et al. 1996). In fish, parasitic infection has been reported to affect swimming speeds, escape response, predator–prey relationships as well as sexual and shoaling behaviour (Barber et al. 2000; Binning et al. 2013; Allan et al. 2020).

Trophically transmitted parasites frequently seem to adaptively manipulate their hosts' behaviour (Demandt et al. 2018). Particularly, parasites with complex life cycles, involving more than one host species, appear to have evolved strategies to increase their hosts' risk-taking behaviour in order to facilitate transmission to the next host (Lafferty

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Developmental exposure to polycyclic aromatic hydrocarbons (PAHs): Focus on benzo[*a*]pyrene neurotoxicity

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ABSTRACT

Polycyclic Aromatic Hydrocarbons (PAHs) are a class of ubiquitous organic compounds produced during the incomplete combustion or pyrolysis of organic material. Dietary source is the main route for PAH human exposure by environmental contamination, food industrial processing or domestic cooking methods. The most studied PAH is benzo[*a*]pyrene (B[*a*]P), due to its harmful and multiple effects on human health: in addition to its well-known carcinogenic effects, emerging evidence indicates that B[*a*]P also induces neurotoxicity earlier and at lower doses than B[*a*]P-induced carcinogenicity making B[*a*]P neurotoxicity relevant to human health risk assessment. Developmental neurotoxicity of B[*a*]P has indeed received increasing attention: both human and experimental studies provide evidence of detrimental effects of prenatal or early postnatal B[*a*]P exposure, even at low doses. Indeed, in some of the multi-dose animal studies, maximal adverse effects were observed at lower B[*a*]P doses, according to a non-monotonic dose-response curve typical of endocrine-disrupting compounds. In substantial agreement with epidemiological studies, both rodents and zebrafish developmentally exposed to B[*a*]P exhibit long-term changes in multiple behavioural domains, in the absence of overt toxicological effects at birth (e.g. body weight and morphologic abnormalities). Notably, most targeted behavioural responses converge on locomotor activity and emotional profile, often, but not always, leading to a disinhibitory/hyperactive profile.

1. Introduction

Polycyclic Aromatic Hydrocarbons (PAHs) are a class of ubiquitous organic compounds produced during the incomplete combustion or pyrolysis of organic material (i.e. fossil fuels, biofuels, automobile exhaust, emissions from domestic heating and cooking, and tobacco smoke) [1]. Human exposure to PAHs may occur through several routes including ingestion, inhalation, and dermal contact [2].

Among over 100 PAHs, the European Food Safety Authority [3] has defined 16 priority PAHs that are both genotoxic and carcinogenic and among these, benzo[*a*]pyrene (B[*a*]P) has been selected (with few other PAHs) as a good indicator of PAH toxicity and occurrence in food and for risk assessment.

The most studied PAH is B[*a*]P due to its harmful effects on human health: B[*a*]P exposure has been associated with mutagenesis, genotoxicity, carcinogenesis, immunotoxicity, and developmental toxicity [2]. Because of its liposolubility, B[*a*]P accumulates in several tissues, including lung, kidney, testis and brain [4], and is classified by International Agency for Research on Cancer as a carcinogenic and mutagenic agent [5]. The reproductive system is an important target of B[*a*]P [2,6].

In addition, PAHs bind covalently to DNA to form adducts, a common indicator of DNA damage associated with cancer [7]. PAH-DNA adducts can be considered markers of PAH individual exposure (up to 2–3 month period) primarily through inhalation and ingestion routes [7]. Once internalised, PAHs can be biotransformed to form monohydroxylated polycyclic aromatic hydrocarbon metabolites (OH-PAHs) which can then be excreted through urine. Urinary OH-PAH levels can thus be used as biomarkers of individual PAH exposure [8,9].

While B[*a*]P received significant attention for its carcinogenic effects, the neurotoxicity of B[*a*]P has been overlooked [10]. Emerging evidence indicates that B[*a*]P also induces neurotoxicity earlier and at lower doses than B[*a*]P-induced carcinogenicity [10], making B[*a*]P neurotoxicity relevant to human health risk assessment [11,12].

Developmental neurotoxicity of B[*a*]P has indeed received increasing attention: both epidemiological and experimental studies point to detrimental effects of early life exposure to B[*a*]P on neurobehavioural development. The current review aims to integrate findings

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7. CURRICULUM VITAE

Education and training

Ph.D. stage at the European Space Research and Technology Centre (ESTEC, NL), in the "The MDS on LDC: Tissue Sharing Programme" project. 05/02/2023-11/03/2023; 10/05/2023-20/06/2023; 17/08/2023-06/09/2023.

Ph.D. program in Neuroscience, University of Parma (PR, IT), internship at the Center for Behavioral Sciences and Mental Health, Italian National Institute of Health, Rome (IT), 01/11/2020-ongoing.

Master degree in Neurobiology (LM-06), Sapienza University of Rome (IT), 22/03/2019, 110/110 *cum laude.*

Bachelor degree in Biological Sciences (L-13), University of Tuscia, Viterbo (VT, IT), 20/10/2016, 110/110 *cum laude*.

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