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The Random Positioning Machine As Space Analog

Mechanical and Biological Characterization and Validation

Supervisors:
Prof. BOTTO Daniele, Politecnico di Torino
Prof. WOTRING E. Virginia, International Space University

Candidate:
RIO DELLA PLATA Roberta

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To my Family

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I would like to express my deepest gratitude to my dedicated and insightful tutor, Virgina Wotring, for her patience, encouragement, and belief in my abilities.

I am forever indebted to my remarkable family for their unwavering love and encouragement. Their unyielding support and belief in my potential have been the foundation of my success. Their presence in my life is a constant reminder of the strength and love that can be found within a family.

To my exceptional friends, I want to express my heartfelt appreciation. Their presence has brought joy and laughter to my life, reminding me of the importance of genuine connections. I am immensely grateful for their enduring friendship and the unwavering bond we share.

Abstract

The Random Positioning Machines (RPM) represent one of the most widely used laboratory tools to simulate microgravity, it is a device consisting of two frames, thus with two axes of rotation perpendicular to each other, which rotate at a constant speed by *randomly* varying the direction of rotation.

The following thesis discusses a possible method of studying microgravity effects using a microgravity simulator: the Random Positioning Machine. Furthermore, the study intended to verify the RPM performance on both a biological and mechanical level. The RPM was tested to ensure that it accurately simulated a microgravity environment appropriate for planarians and in order to find the characteristics of the machine that would best simulate this condition. The study and validation of the Random Positioning Machine are crucial for its continued use. It establishes the RPM as a reliable microgravity simulator, providing a scientific foundation for future research and rigorous investigation in the field of microgravity.

In order to obtain reliable results when using the RPM it is essential to use it appropriately and to understand whether and in what cases such a device really offers a reliable analog of microgravity. The object of this study is therefore to determine whether the RPM is usable as a microgravity simulator and to determine what are the correct parameters (speed and duration) that best simulate microgravity. We will attempt to validate the RPM through mathematical tools and by analyzing data collected from experiments performed on planarians.

In this study, planarians (scientific name: Dugesia spp.) were employed as the model organisms for experimental investigations, they are a wide group of flatworms, most of which live in aquatic environments and can reach the length of a few dozen milliliters. These animals are well known for their extraordinary capability to heal and regenerate themselves. We conducted the experiment by placing one animal in the lab tube and placing six tubes on the RPM, running the machine at various speeds

for varying periods of time, and then observing the results by recording animal behavior for 300 seconds.

In conducting this research, the mechanical aspects were primarily undertaken individually, with minimal external assistance. The author independently focused on the mechanical characterization and validation of the Random Positioning Machine (RPM). Conversely, for the biological component, the author sought guidance from their tutor due to their limited knowledge in the area. The tutor provided valuable support and expertise, aiding in the design and execution of the biological experiments involving planarians.

The findings of this study reveal that certain rotational speeds and exposure time did not yield favorable outcomes. These observations emphasize the importance of carefully selecting the appropriate parameters; following this, the pilot experiments were skimmed at three different speeds and over two different time (30deg/s, 45deg/s, 60deg/s and 5 hours and 20 hours). Positive results have been achieved regarding mechanical validation while from the biological point of view more studies are certainly needed and to draw more certain conclusions it is necessary to conduct experiments in a real microgravity environment, e.g. on the ISS.

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Nomenclature

Greek Symbols

μ	micro, 10E-6
π	$\simeq 3.14\ldots$

Other Symbols

μG	Microgravity
G	Average value of gravity obtained from the simulation
g	gravity acceleration $\approx 9.81\,\mathrm{m/s^2}\, Earth$

Acronyms / Abbreviations

CNS	Central Nervous System
CoR	Center of Rotation
ESA	European Space Agency
ISS	International Space Station
NASA	National Aeronautics and Space Administration
RPM	Random Positioning Machine
SMS	Space Motion Sickness

Chapter 1

Introduction

1.1 Aim of the study

The Random Positioning Machine is a widely used device in the world of microgravity condition simulations, but each device is unique and tends to be developed in-house within a research group or university [1]. What is therefore lacking is a validation of the whole system, not only related to the machine by itself but also including its application and thus use for specific applications and purposes.

The objective of this study is therefore to investigate the use of the Random Positioning Machine as a simulation tool in the context of space exploration and planarian research. This research aims to address the existing gap in knowledge and provide insights into the suitability of the RPM for planarian studies in the field of microgravity. By employing both mechanical characterization and biological approaches, this study aims to comprehensively evaluate the RPM's effectiveness in simulating microgravity conditions for planarians. The mechanical characterization aspect will involve analyzing and quantifying the mechanical properties and parameters of the RPM system, such as rotational speed, duration of the simulation, container design, and fluid motion, while the biological approach will focus on observing and analyzing planarian responses under simulated microgravity conditions. Through this **combined approach**, the study aims to contribute valuable data and understanding regarding the applicability and reliability of the RPM as a space analog for planarian research.

1.2 Space exploration

Space exploration is a challenging and perilous endeavor, necessitating meticulous preparation and caution. Due to the inherent risks and limitations associated with space missions (for further information please refer to the Appendix B), it becomes imperative to conduct thorough Earth-based experiments that simulate the conditions experienced in space. These simulations allow researchers to investigate and comprehend the physiological, biological, and physical responses of organisms in microgravity environments. By performing these experiments on Earth, we can gain invaluable insights and refine our understanding, contributing to safer and more effective space exploration endeavors. The object of this study is to determine whether the RPM is usable as a microgravity simulator and to determine what are the correct parameters to use (speed and duration) that best simulate microgravity. We want therefore to check if it possible to employ the Random Positioning Machine as an analog for conducting experiments. By utilizing the RPM, researchers aim to simulate microgravity conditions and investigate various biological or physical phenomena that occur in space, contributing to a deeper understanding of the effects of microgravity on living organisms.

1.3 Microgravity simulator and their importance

Considering the multitude of challenges associated with microgravity, simulators play a crucial role in the field of space biology and related research endeavors. These microgravity simulators are designed to replicate the microgravity conditions experienced by astronauts in space, allowing scientists to investigate the physiological and biological responses of living organisms to altered gravity environments.

The importance of microgravity simulators lies in their ability to provide controlled experimental platforms for studying the effects of microgravity on various biological processes. By creating conditions of reduced gravity, simulators enable researchers to observe and analyze changes in cellular, physiological, and behavioral aspects of organisms that would not be possible under normal gravity conditions on Earth.

These simulators offer unique opportunities to study the fundamental mechanisms underlying the adaptation and response of organisms to microgravity, thus contributing to a deeper understanding of the impacts of space travel on human health and well-being. They also serve as valuable tools for testing and validating scientific hypotheses, as well as for developing and refining space technologies and countermeasures to mitigate the adverse effects of microgravity on astronauts during prolonged space missions.

Furthermore, microgravity simulators enable scientists to conduct experiments in a controlled laboratory setting, providing cost-effective alternatives to actual space missions. They allow for repeatable experiments and provide researchers with the ability to manipulate and study specific variables related to microgravity, facilitating a better understanding of the underlying mechanisms and promoting the advancement of space biology and related disciplines.

In summary, microgravity simulators are of paramount importance in the academic realm, as they offer a means to investigate the effects of altered gravity on living organisms, advancing our knowledge of space biology, human physiology, and potential applications in space exploration and health sciences. Several microgravity simulators exist, each designed to replicate the effects of reduced gravity experienced in space.

- **Drop Towers**: Drop towers are vertical structures designed to create short periods of microgravity for scientific experiments. They consist of a tall tower with a drop zone at the top. Experiments or samples are placed in a capsule that is released from the tower's top, experiencing a brief period of weightlessness as it falls freely. This allows scientists to study the behavior of samples under microgravity conditions for a few seconds. Drop towers are used in physics, biology, fluid dynamics, and materials science research. They offer a cost-effective method to simulate microgravity, enabling researchers to conduct preliminary experiments and validate theories before more expensive platforms like parabolic flights or space missions. They provide controlled access to short-duration microgravity, helping scientists understand the effects of reduced gravity on various systems. Drop towers are crucial in advancing our knowledge of microgravity effects and aiding in the development of space technologies.
- **Parabolic Flights**: Parabolic flights involve specialized aircraft maneuvers that create short periods of microgravity for scientific research. These flights follow a trajectory called a parabola, where the aircraft ascends steeply and

then descends, resulting in a state of free-fall and weightlessness inside the cabin. Lasting around 20 to 30 seconds per parabola, researchers can conduct experiments in fields such as fluid dynamics, human physiology, material sciences, and physics. Parabolic flights offer unique opportunities to study the effects of microgravity on different systems, serving as a valuable platform for research and experimentation. While the duration of microgravity is limited compared to space missions, parabolic flights provide a cost-effective and accessible means to validate experimental setups, gather data, and investigate the behavior of various systems under reduced gravity conditions. They bridge the gap between ground-based simulations and space missions, aiding in the advancement of space-related research and technology development.

- **Neutral Buoyancy Facilities**: Neutral Buoyancy Facilities, such as large water tanks or pools, are specialized environments used to simulate the microgravity conditions experienced during spacewalks. These facilities create a neutral buoyancy environment by submerging astronauts, equipment, and mock-ups of spacecraft or structures in water. The buoyancy forces in water closely mimic the microgravity conditions of space, allowing astronauts to practice spacewalk techniques and perform experiments in a controlled setting. In these facilities, astronauts wear specialized suits and train underwater to simulate the effects of microgravity on their mobility and work tasks. They can rehearse various procedures, test equipment, and develop strategies for extravehicular activities (EVAs) without the constraints of Earth's gravity. Neutral buoyancy facilities are essential for astronaut training and mission preparation, providing a safe and realistic environment to simulate the challenges of working in space. They enable the development and refinement of spacewalk techniques, tools, and procedures, ensuring the safety and success of space missions. Additionally, these facilities serve as valuable sites for research and development in areas such as space technology, robotics, and human factors.

- **Drop Towers for Fluid Experiments**: Apart from their use in material and biological experiments, drop towers are also used to study fluid behavior under microgravity conditions. These towers are specifically designed to investigate the effects of reduced gravity on fluid dynamics and heat transfer phenomena. Drop towers are vertical structures specifically designed for conducting fluid experiments under microgravity conditions. These facilities provide short periods of reduced gravity by utilizing a free-fall mechanism. In drop tower experiments, samples or equipment are dropped from the top of the tower, allowing researchers to observe their behavior in a microgravity-like environment. These experiments are particularly valuable for studying fluid dynamics, as the absence of gravity influences the behavior of liquids and gases. Researchers can investigate phenomena such as convection, heat transfer, and fluid flow patterns without the disruptive effects of gravity. Drop towers provide a controlled and repeatable environment for studying fluid behavior under microgravity conditions, offering insights into the fundamental principles of fluid dynamics. The short duration of microgravity achieved in drop tower experiments allows for quick and cost-effective testing and validation of hypotheses before potentially advancing to longer-duration platforms or space missions. These facilities play a vital role in advancing our understanding of fluid dynamics and have practical applications in fields such as aerospace engineering, energy systems, and environmental sciences. They enable researchers to develop and refine technologies and models that account for the unique behavior of fluids in reduced gravity environments.

- **Centrifuges**: Centrifuges are devices used to simulate increased gravity levels, also known as hyper-gravity. They achieve this by rotating samples or objects at high speeds, generating centrifugal forces that mimic the effects of gravity. Centrifuges are utilized in various scientific and industrial applications to study the impact of higher gravitational forces on biological samples, materials, and physical processes. By subjecting samples to hyper-gravity conditions, researchers can investigate phenomena such as cell behavior, sedimentation, fluid dynamics, and material properties. Centrifuges play a crucial role in understanding the effects of gravity on different systems and aid in the development of technologies for space exploration, medicine, and materials science.

- **2D clinostat**: Unlike devices such as the Random Positioning Machine or parabolic flights that aim to create true microgravity conditions, the 2D clinostat provides a form of simulated microgravity called "rotational microgravity." Therefore, the 2D clinostat is not a microgravity simulator in the strict sense. The 2D clinostat operates by continuously rotating samples or organisms at a constant speed in a horizontal plane, effectively disrupting the unidirectional gravitational force experienced on Earth. This rotation causes a continuous change in the direction of gravity, resulting in a condition where gravitational forces are evenly distributed across all sides of the sample. However, there is still a small net force acting on the sample due to the rotational acceleration, which distinguishes it from true microgravity. The 2D clinostat is commonly used in biological research to investigate the effects of altered gravity conditions on plant growth, cell behavior, and other biological processes. While it does not completely replicate microgravity, it provides a useful tool for studying partial gravity conditions and understanding the responses of biological systems under altered gravity environments.

- **Random Positioning Machine**: the RPM is a widely used microgravity simulator. It rotates samples in a random or quasi-random manner, effectively canceling out the effects of gravity on the samples. The RPM is often used in biological and biomedical research to study the effects of reduced gravity on cells, tissues, and organisms.

It's important to note that each of these simulators has its own limitations and can only approximate microgravity to varying degrees. They provide valuable research opportunities, but actual space experiments remain the ultimate validation for understanding the effects of true microgravity. These are just a few examples of microgravity simulators on Earth. Depending on the specific requirements and focus of your thesis, you can explore these simulators and others in greater detail to understand their capabilities and limitations.

The selection of the Random Positioning Machine as the chosen microgravity simulator for this study was based on careful consideration of its inherent advantages and the available resources at our disposal. The RPM offers unique capabilities in reproducing reduced gravity conditions by randomizing the orientation and direction of gravitational forces acting on samples. This feature allows for the mitigation of gravity-induced biases, enabling a more accurate assessment of the effects of microgravity on mechanical and biological systems. Additionally, the RPM has been extensively used in previous research, providing a rich body of comparative data for benchmarking and validation purposes.

In this study we will use the letter g to denote the acceleration of gravity on Earth, and the letter G to indicate the average value obtained from the simulation.

Fig. 1.1 Planarian Dorata seen under the microscope

1.4 Overview of Planarians: Characteristics, Advantages, and Limitations

The animal used to conduct all the experiments is the flatworms *Planarian*, these animals usually live under rocks or water plants in freshwater ponds and rivers, the full-grown animals are on average 6-18mm long and 1-3mm wide. They can survive for several weeks without nutrition but are very sensitive to pollution. Humans are not infected with them, nor are they parasitic. Planarians can sense and respond to different forms of stimuli: light, chemicals, touch, and many studies demonstrate that they can also respond to mechanical stimulation.

Planarians are also well known for their remarkable ability to heal and regenerate themselves; transverse fission allows them to reproduce asexually. During fissioning, the worm attaches its tail to a surface and then stretches itself until it separates into two pieces, both ends regenerate the missing parts and grow into two functioning individuals. A more detailed description will be delivered in 4.1.

1.4.1 Advantages and disadvantages

Using planarians in studies related to microgravity and space biology offers several advantages and disadvantages.

Pros

- Regenerative Abilities: Planarians have exceptional regenerative capabilities, being able to regenerate entire organisms from small tissue fragments. This makes them an ideal model organism to study regenerative processes and the impact of microgravity on regeneration.

- Simplified Biology: Planarians have a relatively simple biological system compared to more complex organisms. They possess a well-characterized body plan, with easily observable tissues and organ systems. This simplicity allows for better understanding of cellular and molecular processes in response to altered gravity.
- Rapid Reproduction: Planarians have a short reproductive cycle, allowing for quick generation turnover and larger sample sizes for experiments. This facilitates statistical analysis and enhances the reliability of research findings.
- Ethical Considerations: Using planarians for experiments reduces ethical concerns, as they are invertebrates and do not possess the same level of sentience or welfare considerations as higher-order organisms.

Cons

- Limited Generalizability: Planarians are unique organisms with specific biological characteristics. Findings from studies with planarians may not directly translate to other organisms, including humans. Therefore, caution must be exercised when applying conclusions from planarian studies to broader contexts.
- Simplicity and Generalization: While the simplicity of planarian biology can be advantageous, it can also be a limitation when studying complex processes or higher-order organisms. Certain biological mechanisms may not be fully represented in planarians, leading to potential limitations in understanding the full complexity of biological responses to altered gravity.
- Environmental Differences: Space conditions differ significantly from the natural habitats of planarians on Earth. Factors such as cosmic radiation, altered atmospheric composition, and confinement in space capsules can introduce additional variables that may affect the outcomes of experiments.
- Limited Experimental Platforms: Conducting experiments with live planarians in space requires specific equipment and resources. Access to space-based

research platforms can be limited and costly, which may restrict the number of experiments that can be performed.

Despite these limitations, planarians provide valuable insights into the fundamental biology of regeneration and the effects of altered gravity. By leveraging their unique regenerative capabilities and simplified biology, scientists can contribute to our understanding of microgravity's impact on living organisms and potentially apply these findings to human health, regenerative medicine, and space exploration.

1.5 Planarians in Microgravity: Past Experiments and Insights

To the best of our knowledge, no previous studies have specifically investigated the effects of microgravity on planarians using the RPM (Random Positioning Machine). While there have been numerous investigations exploring the impact of microgravity on various organisms and biological systems, the unique characteristics and regenerative abilities of planarians make them an intriguing subject for such research. This study aims to contribute to the current body of knowledge by examining the responses of planarians to simulated microgravity using the RPM.
To date, the only experiment conducted using planarians under microgravity conditions was in the year 2016 when 15 individuals of planarians D. japonica already divided into three parts were sent to the ISS for one month. This experiment aimed to analyze the regeneration ability of planarians under microgravity conditions. Therefore, there is no experiment conducted under real microgravity conditions to refer to in order to compare the results obtained in this study, so a different strategy was pursued. Once the mechanical validity of RPM is verified, the behavior of the animals after spending a given time in simulated microgravity conditions is collected, and these results were compared with the control sample that remained under standard conditions, 1g. It should be remembered that when there will be the opportunity to compare these results with the ones obtained in space it is necessary to be very careful, indeed analyzing experimental results requires a critical examination of the difference between simulated and real microgravity in space.

Chapter 2

The Random Positioning Machine Importance, History, and Technological Overview

The present study and validation of the Random Positioning Machine hold significant implications for the ongoing utilization of this microgravity simulator in future research endeavors at our university. By systematically characterizing and validating the RPM as a reliable tool for replicating reduced gravity conditions, this study contributes to the establishment of a robust scientific foundation for its continued use. The outcomes of this research will serve as a vital reference point and guide for subsequent investigations that aim to investigate the mechanical and biological responses of various systems under microgravity-like environments.

Furthermore, the validation of the RPM within our university setting reinforces the institution's commitment to scientific rigor and ensures the reliability and accuracy of data obtained from future studies utilizing this simulator. Consequently, the findings of this study affirm the value and suitability of the RPM as a valuable research asset, enabling the pursuit of further scientific advancements and discoveries in the realm of microgravity research.

2.1 Lucerne University's RPM

The Random Positioning Machine employed in this study was generously provided by *Lucerne University of Applied Sciences and Arts - School of Engineering and Architecture*, contributing to the execution of our experimental procedures, it is called *Microgravity Incubator* and it is characterized by two rotating frames, one inside the other.

2.1.1 Design

The RPM used in this study is build in aluminum alloy, and consists of two rotating components, its main characteristic is that the inner rotating component (composed of two distinct sample holders) is attached to the outer frame and is therefore characterized by a motion composed of the rotation of itself combined with the rotation of the outer frame. The size of the RPM is small, which makes it excellent for transport and thus use from multiple research centers but at the same time neither limits its use to limited sample sizes, such as planarians or cell cultures. The outer frame has the shorter side, on which the samples holders are attached, 24 cm long, while the longer side measures 25 cm. The docking arms of the inner rotation component, on the other hand, are 10.5 cm long and have a thickness of 2 cm. These dimensions mean that hat all samples are rotated exactly around the center of rotation and are thus treated equally. In Fig.2.2 it is possible to observe in more depth at the coupling and gearing system.

As can also be seen in the figures presented earlier, there are rubber feet at the base of the RPM, which in addition to their support function also perform the task of minimizing, as much as possible, vibrations.
In Fig.2.2 it is possible to observe another key feature of the RPM utilized in this study: on the outer frame there are three cubic blocks, they have the function of counterweight, in this way, while loading the samples from only one side of the machinery it prevents the gear system from overloading going to create stresses and vibrations on the whole structure.

A final key point, as superfluous as it may seem, is related to the sheet of paper (in the figure in red color) placed at the base of the RPM, it while having no mechanical function, plays a key role from a biological point of view. Indeed,

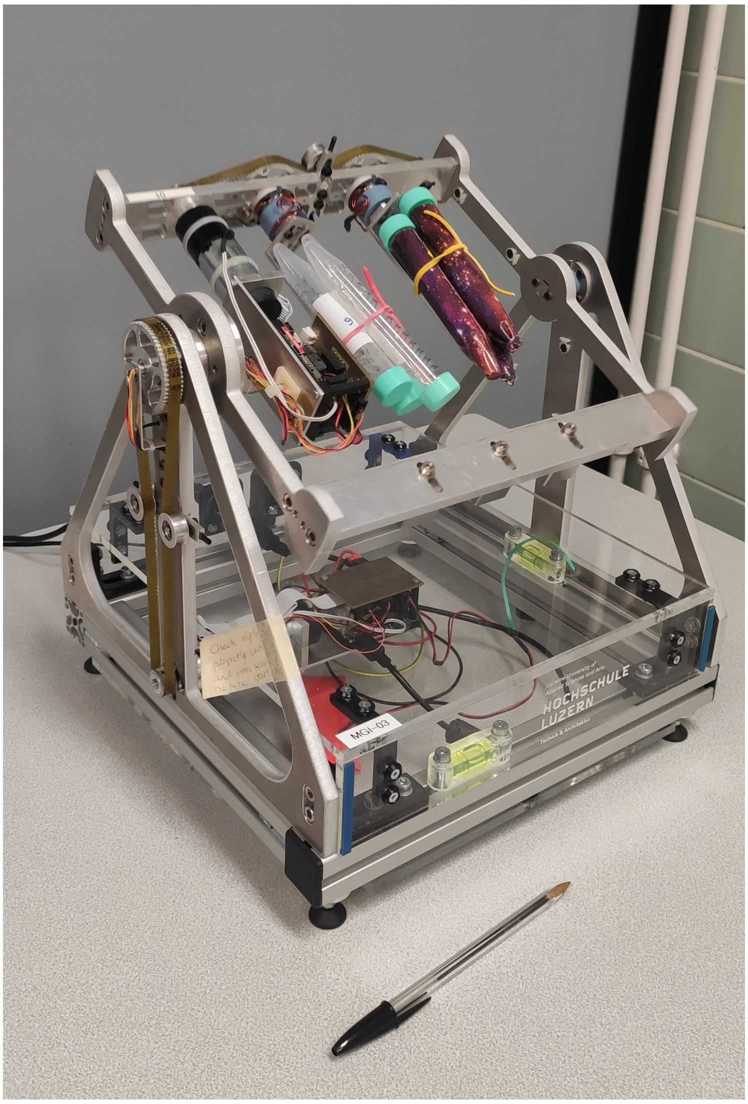

Fig. 2.1 RPM - Microgravity incubator (MGI) built by Lucerne University of applied sciences and arts

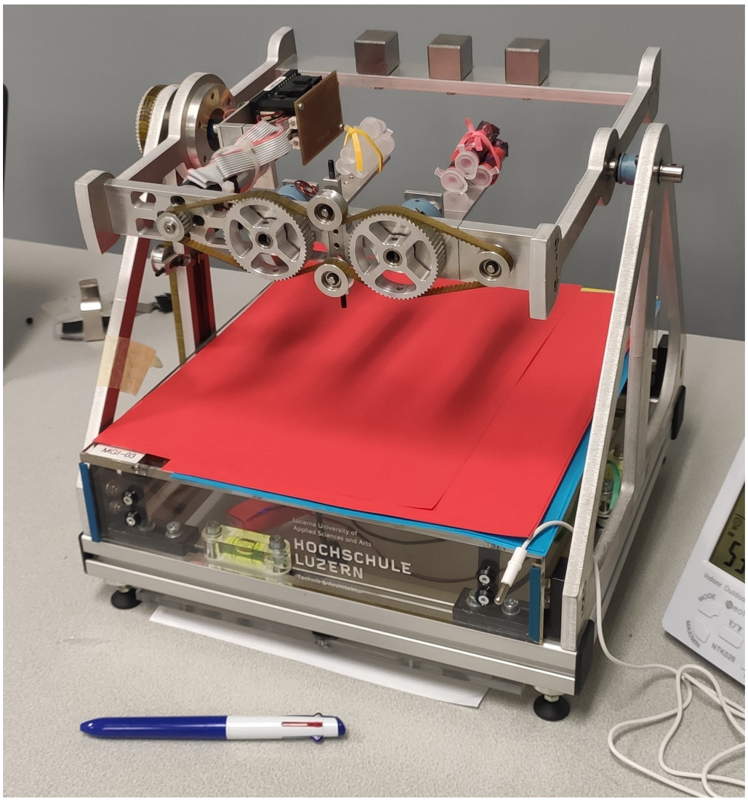

Fig. 2.2 RPM - Coupling system and gears

such a sheet has the function of shielding the RPM's LED lights since planarians are highly photosensitive and the presence of an LED could affect the outcome of the experiment.

2.1.2 Calculation of Mean Microgravity: algorithm

Simulated microgravity on an RPM is attained through the averaging of the gravity vector to zero, as previously described in section 2.1.1. By continuously reorienting the samples to random positions over time, the influence of Earth's gravity vector is distributed, resulting in a convergence of the mean gravity towards a minimal value. for the distribution of orientation we use the mean gravity over time, defined by:

$$G_{mean} = \sqrt{Gx_{mean}^{2} + Gy_{mean}^{2} + Gz_{mean}^{2}} \tag{2.1}$$

The mean gravity values in the three directions are defined as follows:

$$Gx_{mean} = \frac{\sum_{i=1}^{n} g_{x,i}}{n} \tag{2.2}$$

$$Gy_{mean} = \frac{\sum_{i=1}^{n} g_{y,i}}{n} \tag{2.3}$$

$$Gz_{mean} = \frac{\sum_{i=1}^{n} g_{z,i}}{n} \tag{2.4}$$

The letter G is used to indicate the mean value of the simulated microgravity, while g is the gravity vector on Earth.

2.1.3 Operating Parameters

The RPM offers the possibility of setting many operating parameters, those used for the purpose of this study are related to rotational speeds, the maximum accelerations allowed to reach the desired speed and on the other hand the maximum deceleration allowed to stop the system.

Table 2.1 Parameters of the Lucern Univerisity's RPM

Parameter	Range	Notes
Rotational speed	0 - 360 deg/s	-
Max acceleration/deceleration	0 - 20 deg/s^2	Allowed value at RPM during rotation changes and start/stop
Microgravity value goal	0 - 1 g	-
Duration	0 - ∞ s	Never used for a duration exceeding 20 hours

Duration of exposure is a very useful parameter for future studies, it is indeed possible to set the time for which we want to keep the RPM in motion, this parameter, however, was not set automatically rather the pauses and stops were done manually. The RPM employed in this study offers the flexibility to adjust the rotational speed according to experimental requirements. Users have the freedom to set and control the desired velocity within the operational limits of the machine. This customizable feature allows for a wide range of velocities to be explored, enabling researchers to investigate the effects of various simulated gravity conditions on biological systems. By utilizing the RPM's adjustable velocity capability, this study was able to explore different speeds and evaluate their impact on the biological responses of planarians. The maximum operational speed of the RPM used remains uncertain, as only lower speed ranges were utilized. Based on the available information and expert judgment, it is advised not to exceed a rotational speed of 360 deg/s. This conservative estimate is guided by the need to maintain the integrity of the system, ensure the safety of the experimental samples, and mitigate any potential detrimental effects of excessive centrifugal forces. Future investigations involving higher rotational speeds would require careful evaluation and validation to determine their feasibility and suitability for the desired experimental objectives.

An analogous consideration applies to the aspects of acceleration and deceleration. Similarly, due to the lack of detailed guidelines and relevant information, adjustments in acceleration and deceleration were made based on practical observations and empirical assessments. In light of this, a pre-set acceleration of 10 degrees per second square was chosen for the experimental conditions. While this decision was based on practical considerations, it is important to acknowledge the need for further investigation and calibration of acceleration parameters to ensure accurate and consistent results. The chosen acceleration value served as a starting point for this study and allowed for preliminary exploration of the effects of simulated microgravity on planarians.

The RPM utilized in this study allows for the selection of the rotating frame. The control panel enables the choice to rotate either the sample holders exclusively, the outer frame exclusively, or both frames simultaneously, providing flexibility in experimental configurations. For both frames it is possible to choose the desired speed, which means that the inner and outer frames could rotate at two separate speeds. Although the potential for differential speeds between the frames existed, it was deemed irrelevant to the objectives of the present study and therefore not explored. The RPM was indeed always used in *random walk* mode: both frames rotate at the same speed but at random time they change their rotational direction independently of each other.
As mentioned earlier there is no instruction manual nor are there any previous studies that have allowed us to know in advance the functionality and characteristics of this RPM. This lack of information is partly what prompted us to begin this study. In fact, in the following chapters we will also go on to analyze the capabilities, operating parameters and accuracy of the RPM.

2.1.4 Experimental Setup

To make the experiments possible, the samples need to be fixed on the RPM, more specifically they need to be secured on the two arms of the inner rotating component, three for each arm, figure 2.1 shows the experimental setup. It is important to note that those experiments represent the first of their kind, and as such, we approached it without prior knowledge or experience. Consequently, we refrained from making any alterations to the machine and instead focused on optimizing its performance with the available resources at our disposal. Rubber bands were employed as a means to secure the samples in place. Despite the sub-optimal aesthetics, the chosen sample holder proved to be functionally effective. In order to prioritize efficiency and avoid unnecessary time consumption, we decided not to allocate additional efforts towards improving the appearance of the holder. Instead, our focus remained on assessing the performance and outcomes of the experiment.

2.1.5 Control and Monitoring

The RPM is controlled by a `LabView` software developed by researchers from Lucerne University. As end-users of the system, we were unable to modify any settings but instead utilized the software in its existing form. As mentioned earlier what the software allows you to set is mainly related to the rotation mode, however, it is important to note that at the end of each simulation the software makes available a report file in which the operating parameters of the RPM during the entire duration of the experiment are contained. The data that are provided are the time (from zero to the end of the execution), the angular position of the two frames with respect to the neutral starting position, the instantaneous velocity, and the average gravity value reached at that instant. Those reports were used to verify the accuracy of the parameters inherent in the output and subsequently the correct functionality of the RPM itself.

2.2 History and technology of the RPM

The term "random positioning machine" typically refers to a device used in scientific research to simulate microgravity conditions. It is primarily utilized in the field of space biology and physiology to study the effects of reduced gravity on various biological systems.

The concept of using a random positioning machine to simulate microgravity conditions in biological experiments originated in the 1970s. The idea was to create an environment that closely mimicked the weightless conditions experienced by astronauts in space, allowing scientists to study the effects of altered gravity on living organisms. Early RPMs were relatively simple in design and consisted of rotating drums or platforms. The samples were attached to the platform, which rotated continuously to randomize the orientation of the samples with respect to the force of gravity. These early machines were mainly used for plant biology experiments and yielded valuable insights into plant growth and development under simulated microgravity. Over time, RPM technology advanced, leading to more sophisticated and precise devices. Modern RPMs often incorporate advanced control systems to precisely regulate the speed, direction, and duration of rotations or oscillations. This allows researchers to create specific gravity conditions, such as partial gravity or hyper-gravity, in addition to simulating microgravity.

The technology used in RPMs varies depending on the specific design and requirements of the machine. Some RPMs use electric motors and drive systems to rotate the platform, while others may employ a combination of pneumatic or hydraulic systems for controlled oscillation. The rotation or oscillation mechanisms are designed to minimize vibrations and ensure smooth movement of the samples. To monitor and control the experimental conditions, RPMs may be equipped with various sensors and feedback mechanisms. These sensors can measure parameters such as rotational speed, tilt angle, acceleration, and vibration levels. Researchers can use this data to fine-tune the RPM settings and ensure optimal experimental conditions. In addition to the mechanical aspects, RPMs often integrate with other laboratory equipment and systems. This includes environmental control systems to regulate temperature, humidity, and lighting conditions within the experimental chamber. Integrated imaging systems, such as microscopes or cameras, may also be used to observe and record the behavior of the samples during the experiments.

Overall, the history of RPMs involves the development of increasingly sophisticated devices capable of accurately simulating microgravity conditions. This technology has played a crucial role in advancing our understanding of the effects of altered gravity on living organisms and has contributed significantly to space biology research.
As we introduced above, the basic concept behind an RPM is to create a constantly changing orientation of the experimental samples with respect to the force of gravity. By doing so, it minimizes the influence of gravity on biological experiments, allowing scientists to investigate the specific effects of microgravity or altered gravity conditions. Typically, an RPM consists of a rotating platform or drum that holds the experimental samples. The platform can rotate continuously or oscillate back and forth to create a randomized orientation of the samples. The speed and direction of rotation or oscillation can be controlled to achieve the desired experimental conditions. As a result, the samples experience a near-weightless environment, similar to the conditions experienced in space. By constantly changing the orientation of the samples, the RPM ensures that gravitational forces are distributed evenly and randomly, preventing any bias in the experimental results.

Nowadays researchers use the random positioning machine to investigate various aspects of biological systems under simulated microgravity. This includes studying the effects of microgravity on cell growth, tissue development, gene expression, protein synthesis, and the behavior of organisms ranging from single-celled organisms to small animals. The insights gained from these experiments can help in understanding the impact of space travel on human health, developing countermeasures for space-related health issues, and exploring potential applications in regenerative medicine and tissue engineering.

The Random Positioning Machine is a valuable tool in space biology research for several reasons:

- Simulating Microgravity: The primary purpose of the RPM is to simulate microgravity conditions. In space, microgravity has distinct effects on living organisms, including altered physiological processes and changes in cell behavior. By using the RPM, scientists can create an environment that mimics microgravity on Earth, allowing them to investigate the specific effects of altered gravity on biological systems.
- Reduced Bias[1]: The RPM is designed to distribute gravitational forces evenly and randomly on the experimental samples. The fact that the rpm randomly chooses the time and frame at which to reverse the rotation ensures that any observed changes or effects are more likely attributable to altered gravity rather than other factors.
- Controlled Experimental Conditions: The RPM provides researchers with precise control over the rotational or oscillatory movements of the experimental samples. They can adjust the speed, direction, and duration of rotations or oscillations to create specific gravity conditions, including partial gravity or hypergravity. This control enables researchers to study the effects of different gravity levels on various biological processes.
- Accessibility and Cost-Effectiveness: Conducting experiments in space is logistically challenging and expensive. The RPM provides an accessible and cost-effective alternative for simulating microgravity on Earth. It allows scientists to conduct preliminary investigations and obtain valuable data before moving on to more resource-intensive space-based experiments.
- Incremental Research: The RPM serves as an intermediate step between ground-based studies and experiments conducted in actual space environments.

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[1] The term bias refers to systematic errors or deviations from the true value or objective of a study.In this specific case the footprint of the user who chooses the speed and direction of rotation could influence the kind of results you get.

It helps bridge the gap between laboratory research and space-based investigations, allowing scientists to gain insights into the effects of altered gravity on biological systems before conducting experiments in space.

Overall, the RPM is a vital tool in space biology research as it enables scientists to study the effects of microgravity on various biological processes and organisms. It provides controlled experimental conditions, reduces bias, and serves as an important step in understanding the impact of altered gravity on living systems.

Nowadays, there are a number of different variations of RPMs, since each research group or institution may have their own designs and modifications based on their specific research needs.

Chapter 3

Validation of the RPM

The question we attempt to answer is whether our Random Positioning Machine is a good analog for microgravity space environments. To find the answer to this problem various aspects need to be taken into account, they are all related to each other but in order to give them an answer, the problem is divided into two main aspects, the mechanical point of view and the biological one. First of all, we need to know if the RPM can properly simulate the microgravity condition. To do that it is necessary to develop a working scheme, then we try to evaluate if our machine is suitable for use in experiments with planarians.
One last question is related to the fluid-dynamic inside the tubes used, for this purpose we will follow a solely qualitative approach given the complexity of the problem and the relative difficulty in solving, even by numerical methods, the fluid motion problem in our case study. The best way to study the problem as a whole would be to have a camera that can be placed directly on the RPM in order to observe the behavior of the animals during exposure and to analyze the fluid dynamics inside the tubes but, for now, we do not have the proper equipment to do this.

3.1 Mechanical Characterization

Mechanical validation of the Random Positioning Machine means assessing its performance and functionality to ensure its proper operation and reliability. The characterization is done evaluating the performance listed below.

- **Gravity vector and rotational speed**. Determinate the appropriate speed of rotation to achieve the desired gravity vector during the experiment. This requires careful calibration and adjustment of the RPM to ensure that the centrifugal force generated accurately simulated the desired gravity levels. By meticulously controlling the speed of rotation, we aimed to replicate specific gravity conditions and investigate the corresponding effects on planarian behavior and biological responses. This represents the main point of the analysis.

- **Accuracy and precision assessment**. Evaluate the RPM's ability to achieve and maintain the desired randomization of sample orientation and direction of gravitational forces. Measure the consistency and repeatability of its movements and verify if it accurately simulates microgravity conditions.

- **Load capacity testing**. Determine the maximum load the RPM can handle without compromising its performance.

- **Vibration analysis**. Assess any vibrations or oscillations generated by the RPM during operation. Ensuring they are within acceptable limits that won't affect the integrity of the samples or the accuracy of the simulated microgravity conditions.

- **Alignment verification**. Check if the RPM is properly aligned and leveled to avoid any unintended biases or discrepancies in the simulated microgravity environment.

- **Calibration and maintenance**. Regularly calibrate the RPM to maintain its accuracy and reliability. Establish a maintenance schedule to address any mechanical issues promptly and keep the system in optimal working condition.

RPMs have been largely used to simulate microgravity, but no paper reports a better velocity than others or a deep explanation of how to reach a proper value of microgravity. What we know so far is that the most frequently used speeds can vary from 10 deg/s to 120 deg/s [2], but a method to choose the right speed has been widely neglected. Moreover, the RPM rotation is faster than the biological process under study and at the same time slow enough to avoid any unwanted effects or forces [1].

Starting from this information we decided to deepen the issue in two different directions: first of all, we attempt to understand if all used velocities allow us to reach a reasonable value of mean gravity and after which amount of time. On the other hand, we tried to find out if there is a correlation between speeds and planarians' behavior, so we could understand whether there is an optimal value of velocity that allows us to best simulate microgravity.

Gravity value

The principle of the RPM is based on gravity vector averaging to zero by constantly changing the relative direction of the sample to it, and, at the same time, avoiding any effect due to centrifugal force. The RPM rotates the samples around two perpendicular axes, the frames rotate at a constant speed and invert the rotating direction at random points in time.

This analysis is crucial because it directly relates to the effectiveness of the Random Positioning Machine in simulating microgravity conditions. In a successful simulation, the RPM should minimize or eliminate the influence of gravity, resulting in a near-zero mean value of acceleration. Verifying this trend is essential for validating the RPM's capability to create an environment that closely resembles microgravity, thus ensuring the reliability of subsequent biological and mechanical characterizations conducted using the RPM.

Therefore, the aim is to check if the mean value of gravity acceleration (G) tends to zero after a certain amount of time, to do that we used the reports generated by the RMP software, in fact with it it is possible to know the value of the G every minute on each axis and the overall value, we can then analyze data of interest using `MATLAB`. In addition, the running of the RPM was recorded and analyzed with appropriate software to verify the correctness of the reports generated by the machine itself.
To assess whether G tends towards zero over time in the context of the Random Positioning Machine, we worked on three different ways:

- Data analysis: the data acceleration we collected from the RPM during experimental runs and calculate the mean value at regular intervals or across multiple trials. Plot the mean acceleration values over time to observe the trend. If the RPM is effectively simulating microgravity, the mean acceleration values should converge towards zero as the system reaches a steady state.

- Statistical analysis: statistical methods were applied to assess whether the mean acceleration values significantly differ from zero over time. This can provide quantitative evidence of the trend towards zero acceleration and the effectiveness of the RPM in simulating microgravity.
- Sensitivity analysis: we evaluated the impact of varying RPM parameters, such as rotational speed or container design, on the mean acceleration values. Conduct sensitivity analysis to determine the parameter ranges that yield the closest approximation to zero acceleration, helping to optimize the RPM setup for improved microgravity simulation.

Study of velocity

The machine runs at a constant speed and changes direction at random times, but it is not clear which speed is best. Different velocities could have different results in the proper simulation of microgravity and therefore in planarians' behavior. Moreover, we don't know if there is a better velocity than others. Therefore, two different aspects are addressed to answer this question: the end result of G and the behavior of the animals for each different speed. To study the behavior of the animal it would be extremely useful to develop a system that allows us to study the animals while they are on the machine. In this way it would be easier to understand the fluid-dynamic and we could analyze the behavior during the exposure. That aspect, however, is not part of the scope of this study and will therefore not be pursued further.

Study methodologies and results are presented in the sections 4 and 5

3.2 Biological characterization

Our investigation focused on comparing the baseline behavior of planarians with their post-exposure behavior to the RPM, a simulated microgravity platform. By meticulously observing and analyzing the alterations in planarian behavior, we aimed to discern any discernible shifts or changes induced by RPM exposure. This analysis allows for a deeper understanding of how the RPM affects planarian behavior and serves as evidence of its potential as a space analog for planarian studies.
Due to the limited availability of real space experiments involving planarians, a direct

comparison between the results obtained from the RPM and actual space conditions was unfeasible. The scarcity of such data impeded the opportunity to establish a direct correlation between the RPM and space-based responses, highlighting the need for further space-based planarian investigations. As space-based investigations involving planarians are limited, our study lays the groundwork for potential comparisons between the outcomes of such experiments and the observed responses of planarians exposed to simulated microgravity using the RPM. This contribution is anticipated to facilitate a deeper understanding of the effects of true microgravity on planarian biology and foster advancements in the field of space biology.

To be able to understand how the behavior of planarians changes when subjected to simulated microgravity we had to take into account various aspects related to both biological and mechanical point of view. The following parameters were considered during the experimental design: rotational speed, exposure duration, fluid dynamics within the tube, flask material and shape, position of planarians within the tubes, and placement of the tubes on the RPM apparatus. These factors were meticulously accounted for to ensure controlled conditions and minimize potential confounding variables in the study of planarian responses to simulated microgravity. An essential concept is that biological systems require a minimum period of time to adapt to changes in the gravity vector, emphasizing the importance of allowing sufficient duration for planarians to acclimate to simulated microgravity conditions [3], but in the case of planarians is very difficult to define *time* because nowadays there is no a way to determining the exact age of planarians, it can indeed be challenging due to their regenerative abilities and lack of distinct aging markers.

During the study, exploratory experiments were conducted involving planarian exposure to varying durations of simulated microgravity, ranging from two hours to twenty hours. These duration choices were made without a specific underlying rationale, as the aim was to explore a range of exposure durations and assess potential effects on planarian behavior and physiology. This approach is a first attempt for a more detailed experiment plan and served as a foundation for further investigation and hypothesis formulation.

As a final point, it is also important to keep in mind that the environment has a key role when studying living creatures, we take into account and control environmental parameters such as temperature, humidity, and atmospheric composition within the RPM system. These factors indeed may influence planarian behavior and physiological responses, so maintaining consistent and controlled conditions is extremely important.

Biological validation for the use of our RPM for application with planarians was then performed following several experiments, characterized by the exact same procedure but with different exposure times, different speeds, and two different tube positions on the machine arms, as is presented in the following chapters.

3.3 Influence of fluid-dynamics

The last aspect treated concerned with fluid dynamics in the test tubes. The reason we decided, initially, to focus our attention on the fluid dynamic aspect is because we attempted to eliminate any element capable of affecting the behavior of planarians, so that we could isolate only the effects related to the simulated microgravity. During the exposure planarians are, in fact, placed in flasks filled with water, free of air bubbles, inside which the fluid is constantly moving.
As we explained before, the RPM, more specifically the "Random Walk" mode causes at random instants of time the outer frame and/or inner frame to change their direction of rotation. This means that there is no way to know exactly when the machine is going to change the direction of rotation nor in which frame.
This makes the fluid dynamic problem impossible to solve by linear methods and extremely complicated to solve by numerical methods and simulations.

For the purpose of this issue at the beginning of the study, we relied on a `MATLAB` code developed by Song et al., 2020 [4] for the study of motion inside a cylinder whose motion was simplified to an oscillatory motion on its own axis, and on papers Wuest et al., 2017 [5] at a for considerations on fluid motion in experiments on the RPM. None of those previously papers, however, examines the fluid in the same condition as we have.
Upon careful consideration, it was determined that the study of fluid dynamics within the context of this research requires specialized expertise beyond the scope of our current knowledge. Therefore, the focus of this study was directed towards the mechanical and biological aspects, where our expertise lies. In fact, our considerations took into account some aspects related to the biology of planarians. The first main observation is indeed related to the fact that planariasn have tactile receptors that would allow them to feel fluid motion mainly on the belly, which, however, is the

part of the body that is not contact with the water but rather with the surface of the vial. Second, these animals live in natural water basins, thus subject to currents and not immobile, which makes us think that the movement of the water in which they are immersed could only marginally affect the success of the experiment.

Recognizing the complexity of fluid dynamics and its potential impact on the experimental outcomes, further investigation with experts in the field is warranted for a comprehensive understanding of this aspect in future studies.

Chapter 4

Material and Method

4.1 Test animals

Planarians are flatworms belonging to the *Tricladida* group, having bilateral symmetry, dorsoventral polarity, a CNS, and a simple brain structure [6]. Two anterior cephalic ganglia and two parallel nerve cords running ventrally along the length of the animal's body form an organized nervous system [7]. Their nervous system is similar to the nervous systems of more advanced organisms, and in terms of morphology and physiology, planarian neurons are more similar to vertebrate neurons than to invertebrate neurons such as insects. Due to their variable and relatively complex behavior, as well as similarities between their nervous systems and those of vertebrates, planarians have become a popular animal model in neurobiology and pharmacology [8].

One of the most relevant characteristics is their ability to regenerate any part. Indeed, they possess a large pool of adult stem cells, called neoblasts, which are located throughout the body that provides them with the ability above [9]. Their asexual reproductive method is based on fission, as described in the opening chapter: the planarian stretches until it divides into two distinct parts, in a time of about fifteen days both and two ends reconstruct the missing part until two whole individuals are then reformed [10]. These animals are active in the dark and inactive during the light time.

Planarians move by gliding on a layer of mucus that they constantly produce using ciliated cells on their ventral side. Many studies show that when exposed to

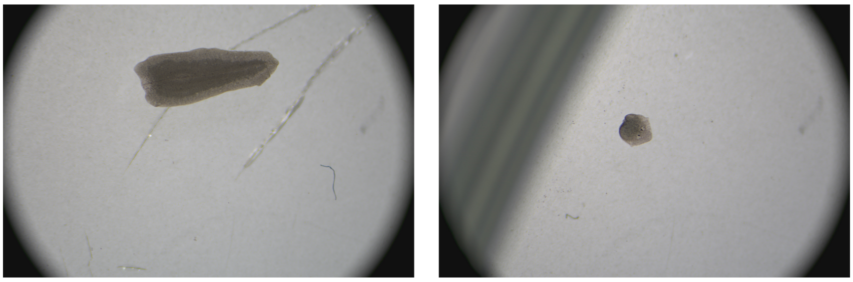


Fig. 4.1 Girardia dorotocephala: tail and head, result of natural split

a stimulus (mechanical, chemical, or electrical), planarians show clear changes in behavior. In response to stress, animals tend to produce more mucus, cease gliding normally, and assume various shapes, movements, and postures [11].

The species used to conduct the experiments is *Girardia Tigrina* (it is sometimes referred to as Dugesia). This species has a triangular-shaped head with two eyespots / two cross-eyed on the head able to see and perceive light. These creatures are about 9 up to 20 mm long and appear light brown to the eye, if examined under a microscope it can also be observed that they are filled with light-colored dots all over the body surface.

To date, the average lifetime is unknown, according to current literature, populations of Dugesia tigrina do not exhibit any signs of degenerative aging due to their ability to regenerate. Moreover, given their reproductive method that generates two individuals that are perfectly identical to each other, calculating age is not a simple process. From the information gained, individuals not used for experiments and properly fed and cleaned can be considered immortal, it has also been observed that planarians can survive several weeks without food; in fact, they become extremely thin but do not die.

4.1.1 Storage of planarians

Planarians were kept in glass tanks, normally used as food containers, filled with *Volvic*® water and stored in a dedicated room at about 21°C. To be able to perform experiments both during their day and night time, lights in the planarians' room are set to stay on from 00:00 to 12:00, in this way during the morning it is possible to perform with the animals in the light and after noon we can experiment during their active time.

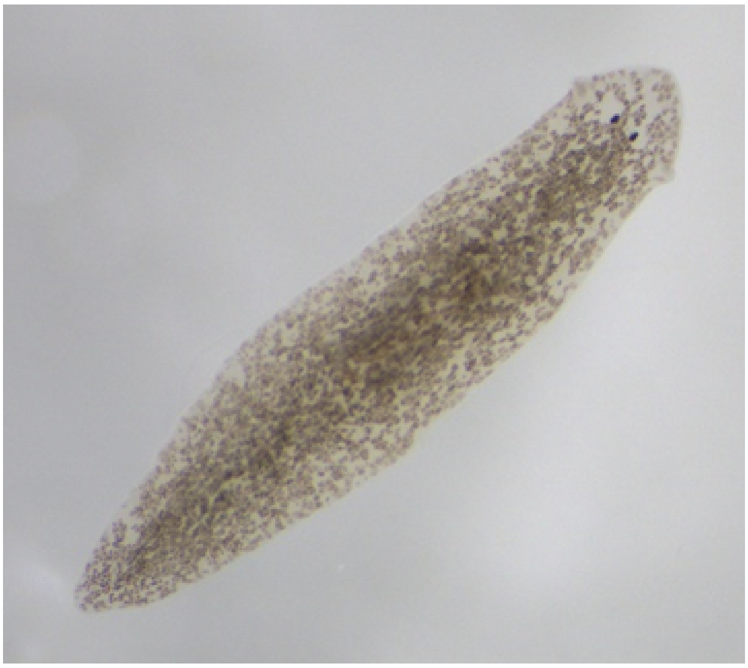


Fig. 4.2 A planarian of the Girardia tigrina species. This photo was taken using a stereo zoom microscope (Motic SMZ-171 Series Stereo Zoom Microscope). The animal is about 1.5 cm long.

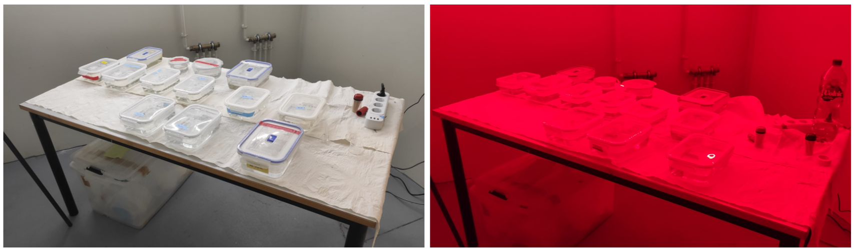


Fig. 4.3 lab during light hours (left) and dark hours (right) during which only red lights can be used for short periods

Planarians were fed by leaving in their water a piece of beef liver for two hours once per week and the tanks were cleaned the day after, the experimental sample have been starved for 1–2 weeks before utilization.

Planarians are sensitive to the electromagnetic field so we try to avoid as much as possible the use of metallic instruments that could disturb the animals and interfere with their behavior.

4.1.2 Behavior

Planarians have been observed since the early 19th century and their behavior and movement have been described and named in many different ways, for the aim of this study we rely on paper *Reho et al.,2022* [11] and consider relevant interest three categories of motions as described below:

- **Gliding**: it is the normal locomotion, utilizing ciliated cells located on their ventral side, they produce mucus and glide along a surface at a constant speed, in the past has been called swimming and normal ciliary gliding motion.
- **Scrunching**: this behavior typically indicates a state of pain or stress, in this case, the animals move by contracting and stretching their bodies, like a caterpillar, it is, therefore, possible to recognize this behavior as the body size of the planarians varies during this type of locomotion.
- **Other**: this category includes all types of behavior other than gliding and scrunching, including the movement of the head and nods, the complete contraction of the body, and twisting along the longitudinal axis.

4.1.3 Disposal of planarians

The planarians used in the RPM experiment were kept in well plates in 2mL Volvic water, once every week they were checked to see if they were alive or dead, the aim is to compare their ability to survive to observations made in other studies related to microgravity. In the case of experiments whose term does not include this type of control, planarians were placed in the laboratory's freezer.

4.2 Experimental setup

All experiments were conducted using RPM, the experiments varied in duration, time, velocity, and position of the tubes but the same procedure was followed for all of them.
Six animals were used for each experiment, which is the maximum number of tubes that could be loaded onto the RPM; however, it is important to note that the dimensions of the sample can vary from six to nine for each condition.

1. Sample preparation six tubes are opened and filled with water, the attempt is to put enough water in the tubes and in their caps to avoid any air bubbles inside when closed, in order to do this cap and tube are filled to almost overflowing, using the surface tension of water. The vessels are then left on the bench for a few minutes to allow any air bubbles to escape. After that is possible to put one animal in the tube right before closing and loading it on the machine.

2. Sample loading: a few minutes before loading, the tube is closed and checked for air inside. The sample is then ready to be loaded on the RPM, each tube is inserted in a case made of tape to shield the light, then fixed to one of the machine's arms with rubber bands. The process is repeated six times every ten minutes.

3. Sample unloading: after the end of the exposure to simulated microgravity, every 10 minutes, one sample at a time, the tubes are unloaded from the RPM, with a pipe the animal is extracted from the tube and set in the recording rig or disposed of as explained before.

The tubes are ClearLine® Centrifuge Tubes, they are made of virgin medical-grade polypropylene, with excellent transparency and a graduated scale, the tip is V-shaped, the maximum capacity is 15 mL. We also try different types of tubes, made of different materials, with a variety of tip shapes, as well as different volumes, but they were found to be of little use for our purpose; we stopped using them.
All experiments were performed in the dark, only during the preparation of the sample a red light was used, moreover, the RPM was kept protected with a cardboard box to prevent any accidental lighting and to be able to turn on the light in the lab if needed. This means that even for experiments performed during animals' light time the room was kept in the dark, this decision was made in order to always have

the exact same condition for each experiment; although aware that this may have, in part, affected the overall behavior of planarians.

4.3 Experiments

Experiments were performed with different duration, recording time, speed of rotation, and position of the tubes (distance of the tip from the Center of Rotation, CoR), as can be seen in the table 4.1.

Table 4.1 Experiments performed

Duration	2	5	18	20	[hours]
Speed	10	30	45	60	[deg/s]
Position of the tip	CoR	Ext.			

As explained earlier not all types of experiments have been successful, and the number of variables was then decreased. The final experiments were then reduced to only two durations and three different speeds as follows in Table 4.2:

Table 4.2 Experiments performed

Description	Acronym
5 hours, 30deg/s, tip external	RPM30-5h-E
5 hours, 30deg/s, tip CoR	RPM30-5h-C
5 hours, 45deg/s, tip external	RPM45-5h-E
5 hours, 45deg/s, tip CoR	RPM45-5h-C
5 hours, 60deg/s, tip external	RPM60-5h-E
5 hours, 60deg/s, tip CoR	RPM60-5h-C
20 hours, 30deg/s, tip external	RPM30-20h-E
20 hours, 30deg/s, tip CoR	RPM30-20h-C
20 hours, 45deg/s, tip external	RPM45-20h-E
20 hours, 45deg/s, tip CoR	RPM45-20h-C
20 hours, 60deg/s, tip external	RPM60-20h-E
20 hours, 60deg/s, tip CoR	RPM60-20h-C

4.4 Tracking software

In order to precisely track the motion of RPM and the movement of planarians during our experiments, we utilized a software called 'Tracker 6.0.10' This software provided us with the capability to analyze and record the kinematics of the RPM as well as the locomotion patterns of the planarians.
Tracker is developed in Java using the Open-Source Physics framework (OSP), it provides free video analysis and modeling functionality. The main functions of this software are manual and automated object tracking with position, velocity, and acceleration overlays. Builder creates kinematic and dynamic models of point mass particles and two-body systems, video filters, including brightness/contrast, strobe, ghost trails, and deinterlace filters, perspective filter corrects distortion when objects are photographed at an angle rather than straight-on, circle fitter tool fits circles to 3 or more points, steps or tracks.
In manual mode, the tracking process involves configuring the video settings appropriately and marking specific points of interest within the recorded footage. By identifying and tracking these points, the software allows the user to monitor the evolution of the object's mass or body over time. This enables precise analysis and observation of changes in position, orientation, and movement patterns. It is possible to fine more detail about the recording procedure and video setting in the following chapter (4.5).

4.5 Recording procedure and behavioral analysis

Immediately after the end of the planned time on the RPM, the planarians are unloaded from the machine, one at a time, every ten minutes to allow the second phase of the experiment: tracking and monitoring their behavior for 300 seconds. The procedure used to record the movement, track movement, and evaluate behavior is the same for all animals in the study (240).

The arena in which the animals are recorded is a circular glass dish, 145 mm diameter with a 10 mm high outer rim, the dish is filled with 50 mL of *Volvic water*, and using a pipette, the planarians are transferred from the experiment tube into the recording arena and placed as close to the center as possible. After that, the

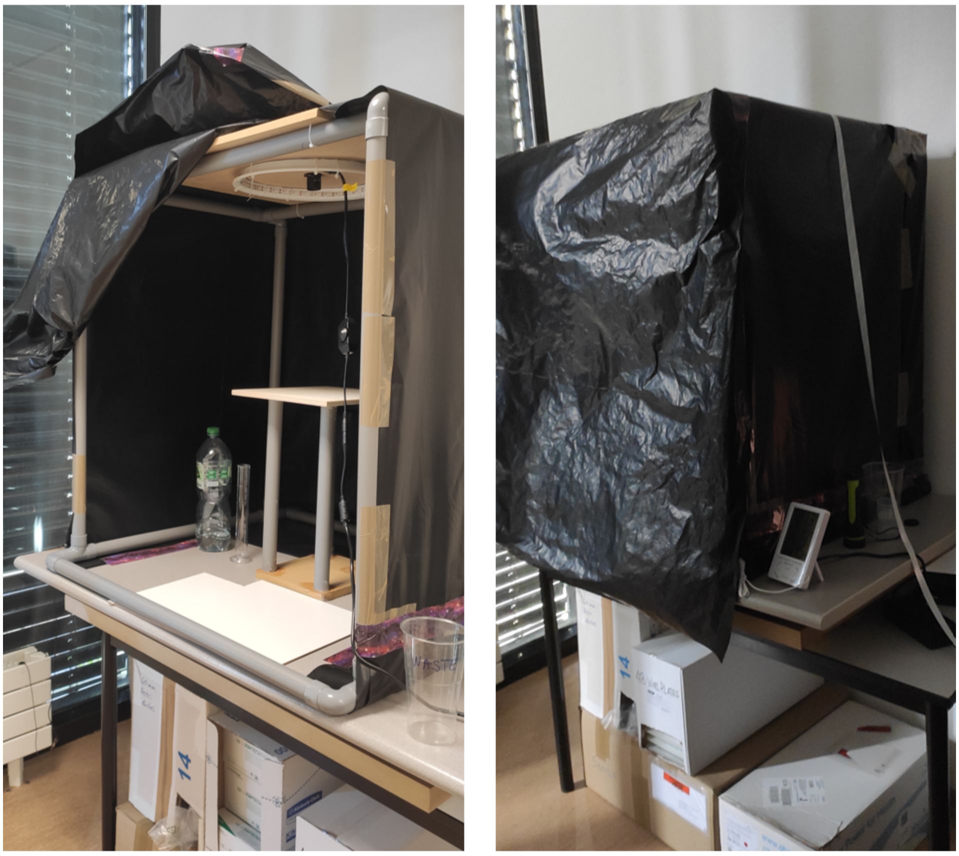
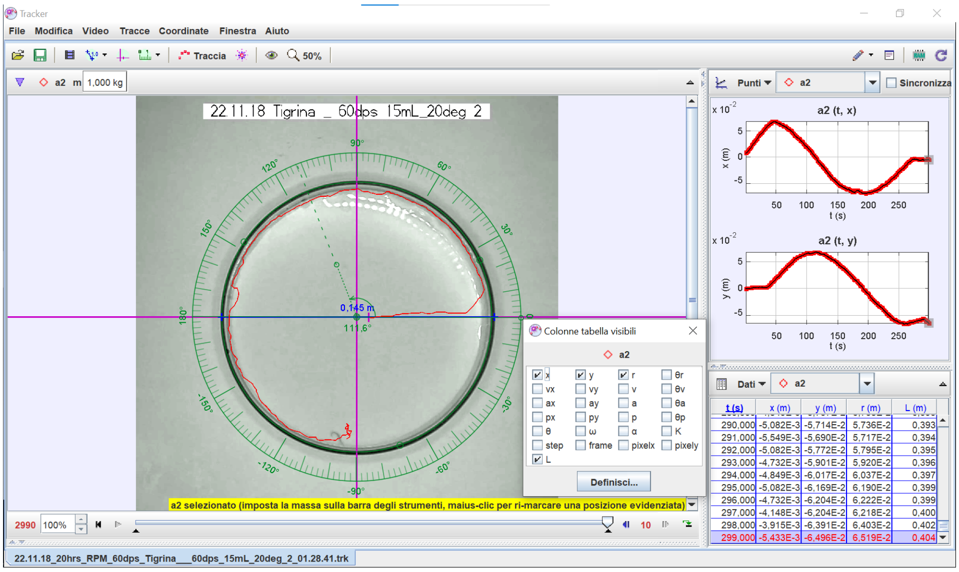


Fig. 4.4 Recording rig

recording box 4.4 is closed with the black sheet that has the function of shielding light and is capable of blocking electronic noise then the recording is started.

At this point the next step is to record the behavior for 300 seconds, to do that we use an IR camera (NB find the spec.) run by *Raspberry*, each video contains all the data necessary to uniquely identify the file: date of the recording, condition (RPM experiment or chemicals), concentration (RPM rotation speed), type of tube, temperature, and animal number. The output format obtained is .h264 but the program automatically converts that file to one with a .mp4 extension, which can then be used for analysis using the tracking software. Once we have this file format we can upload it on *Tracker* and start the analysis.

As first, the video options are changed to make the planarians more visible, this step does not interfere in any way with the results that will be obtained but makes the process easier. The analysis software does not know the actual size of the case study,



a2 selezionato (imposta la massa sulla barra degli strumenti, maius-clic per ri-marcare una posizione evidenziata)

Colonne tabella visibili

a2

☑ x	☑ y	☑ r	☐ θr
☐ vx	☐ vy	☐ v	☐ θv
☐ ax	☐ ay	☐ a	☐ θa
☐ px	☐ py	☐ p	☐ θp
☐ θ	☐ ω	☐ α	☐ K
☐ step	☐ frame	☐ pixelx	☐ pixely
☑ L			

Definisci...

t (s)	x (m)	y (m)	r (m)	L (m)
290,000	-5,082E-3	-5,714E-2	5,736E-2	0,393
291,000	-5,549E-3	-5,690E-2	5,717E-2	0,394
292,000	-5,082E-3	-5,772E-2	5,795E-2	0,395
293,000	-4,732E-3	-5,901E-2	5,920E-2	0,396
294,000	-4,849E-3	-6,017E-2	6,037E-2	0,397
295,000	-5,082E-3	-6,169E-2	6,190E-2	0,399
296,000	-4,732E-3	-6,204E-2	6,222E-2	0,399
297,000	-4,148E-3	-6,204E-2	6,218E-2	0,400
298,000	-3,915E-3	-6,391E-2	6,403E-2	0,402
299,000	-5,433E-3	-6,496E-2	6,519E-2	0,404

Fig. 4.5 Interface of the *Tracker software* used for behavior analysis

and it is, therefore, necessary to specify this information before starting tracking, to do that a simple procedure has been followed.

1. Use the "find circles" function to locate the arena
2. After locating the circle, the "axes" function is used to add a reference system to the video
3. "calibration stick" is used at this point: the ends of the rod are placed at the left or right ends of the area, identified by the intersection of the circle and the axes. Therefore, the value of the rod is changed to 0.145 m, which is the actual size of the arena

Once this procedure is finished, it is possible to focus on the actual tracking, to do this a new track of the type "mass point" is created and renamed appropriately. Videos recorded for 300 seconds contain 3000 frames, for the objective of the study it was considered sufficient to analyze the video 1 time every second, so the value of frames for each tracking step was set to 10. The "shift + click" command allows the creation of track points. It was decided to consider the head of the planarians as the mass point of interest except when an exploring behavior is observed, which is when the head is not anchored and nods around but the body is fixed, it was indeed decided not to temporarily follow the position of the head but rather click at a fixed point (center of the body) to avoid distorting the final information regarding the distance traveled. At the end of this process, the software returns all the information needed to define the motion of the analyzed object as shown in the figure 4.5 in the "visible table columns" window. The data of main interest in this study are the total distance covered **L** and the distance to the center **r** of the system.

Finished the above it is possible to save the video in .tkr format which will contain all the information obtained from the tracking, such as points, speeds, distances, and positions. In addition to tracking, a qualitative analysis of behavior is also performed. Using video playback software, such as *VLC media player* or *Windows media player*, it is possible to observe and classify the three different types of behavior described above 4.1. This procedure was performed by speeding up the video up to 4 times to be able to recognize the type of motion in general and slowing down to 0.5 when more precision is needed. For each video (300 seconds), the total duration of each behavior was calculated by timing the seconds shown on the screen by the software; any behavior with a duration of fewer than five seconds was ignored because it was too short in duration to allow classification.

4.6 Statistical analysis

As mentioned in section 4.5, outputs related to distance covered and position in the arena were evaluated, and these data were exported to external environments such as *Microsoft Excel* spreadsheets and `MATLAB` scripts to be analyzed together with the position in the tube at the end of the exposure and different type of behavior.

- **Total distance**: the distance covered by each animal during the 300 seconds of recording was measured, for each step the software increased this value by the step taken, the value of interest is solely the final total value, for each animal in each experiment, this data was saved and compared with the others. For example, animals in the "RPM30-5h-E" group (see 4.3) were compared with all other experiments with a 5-hour duration, 30deg/s speed, and positioned with the tip toward the center or outward.
- **Position**: for position analysis, it was considered relevant to distinguish the percentage of time for which the animals were on the edge or in the rest of the arena, as it is known that these worms tend to prefer places where they feel

safer (e.g., the edge of the arena) and normally tend to go there, see 4.1. Given the type of tracking that was decided to use for each animal, there are 300 frames analyzed for which 300 different positions. The maximum distance from the center is 0.145/2 m, i.e. 72.5 mm which corresponds to the outer edge of the arena, taking into account human error during tracking and uncertainty due to registration and the software utilized, it was decided to consider *side of the arena* all that area between 0.93 and 1.00 of the maximum distance from the center, thus values greater than 67.42 mm.

- **Final position in the tube**: tubes were divided into three parts, tip (T) i.e., the V-shape area, the cap (C) means the part of the tube covered by the cap, and the center (M) that is the whole remaining area, for each type of experiment, the percentage of each position was evaluated.
- **Behavior**: as explained above the behavior was analyzed by looking at the whole recording and for each action, the total time was calculated, then those data were converted into percentages.

Statistical analysis in the study of planarians' behavior relies on several commonly employed calculation tools. These include the t-test, which assesses significant differences between groups; the standard deviation, measuring the dispersion of data around the mean; the arithmetic mean, providing a measure of central tendency; and the standard error, estimating the variability of the sample mean. Complementing these tools, various graphical representations are utilized, such as scatter plots, showcasing the relationship between variables; bar graphs, facilitating comparisons between groups; and histograms, displaying the distribution of data.
The combined use of these statistical calculations and graphical tools enables us to derive meaningful insights and draw accurate conclusions from the data.

Chapter 5

Results

The investigation into the impacts of microgravity on planarians is in its nascent stage, marked by the abandonment of several initially planned experiments due to procedural errors or technical challenges encountered. These setbacks highlight the need for further refinement and optimization of experimental protocols to ensure accurate and reliable data collection. Regarding biological characterization, the data presented below are the results of the experiments in Table 4.1. The data from animals used for experiments subsequently deemed unsuitable are only considered in the section regarding survival rates. The control group in this study comprises six planarians, while the experimental group consists of nine specimens. This sample size allocation allows for a comparative analysis between the control and experimental conditions, providing a basis for assessing potential differences in planarian behavior or responses under varying experimental conditions.

5.1 Gravity vector and rotational speed and precision assesment

Figure 5.1 shows the G values achieved at different rotational speeds. Experiments during which, for the first and last hour, the RPM was paused to allow loading/unloading of the animals were used to make the graph. It is observed that the line plot of 30 deg/s and 60 deg/s are very similar and quickly reach optimal G values. The case of 10 deg/s, on the other hand, takes longer to achieve the desired G and

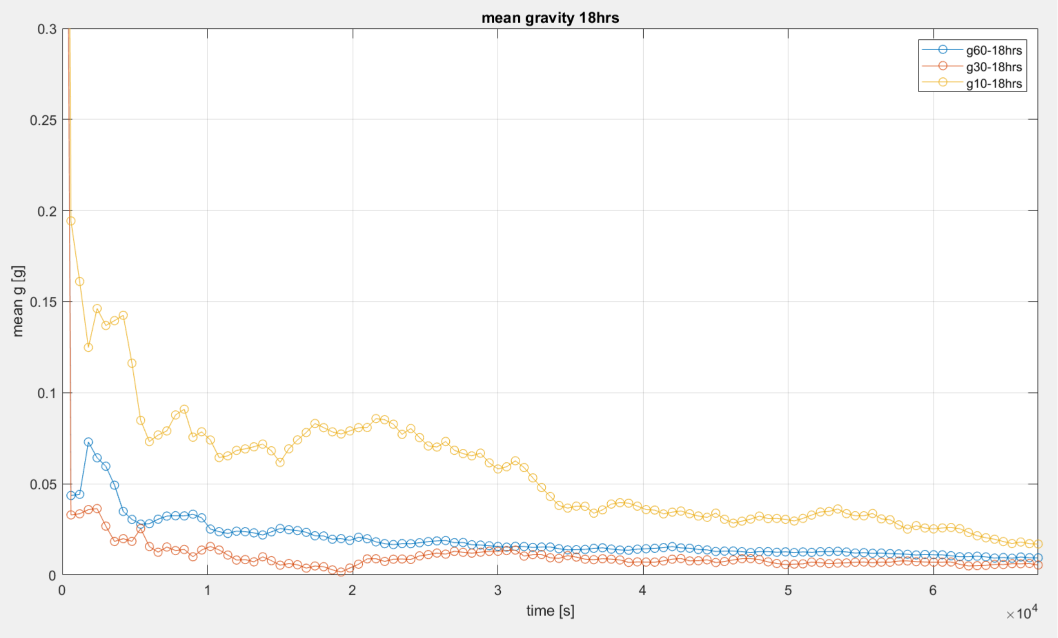


Fig. 5.1 Simulated microgravity - mean G value

throughout the simulation maintains a higher G value than the other two velocities. it is especially interesting to note that the simulated mi microgravity values obtained are not in order, in fact the top line represents the 10 deg/s curves, followed then by 60 deg/s and finally 30 deg/s. The graph 5.1 represents specific cases, but this trend is always verified.

In order to better understand the simulated microgravity values obtained, a new velocity was added: 45deg/s. It is observed in the figure that this new velocity leads to lower G values at the beginning, but thereafter the line continuously crosses with the other velocities' lines. Thus, there does not seem to be any substantial difference between the three speeds from the mechanical point of view. Figure 5.2 shows what written above, with the three colors are shown the three velocities in detail, after a transitory of about 9000 seconds all the lines drop below the desired value of 0.02g. Regarding the transition period, it was observed that all three velocities need about 10000 seconds before the mean of the G vector is sufficiently distributed among the three axis to allow the RPM to properly simulate microgravity.

Four different speeds were analyzed. It has been determined that there is no substantial difference between 30 deg/s, 45 deg/s, or 60 deg/s, however the speed of 10 deg/s does not provide the desired results, as it requires a longer period of time

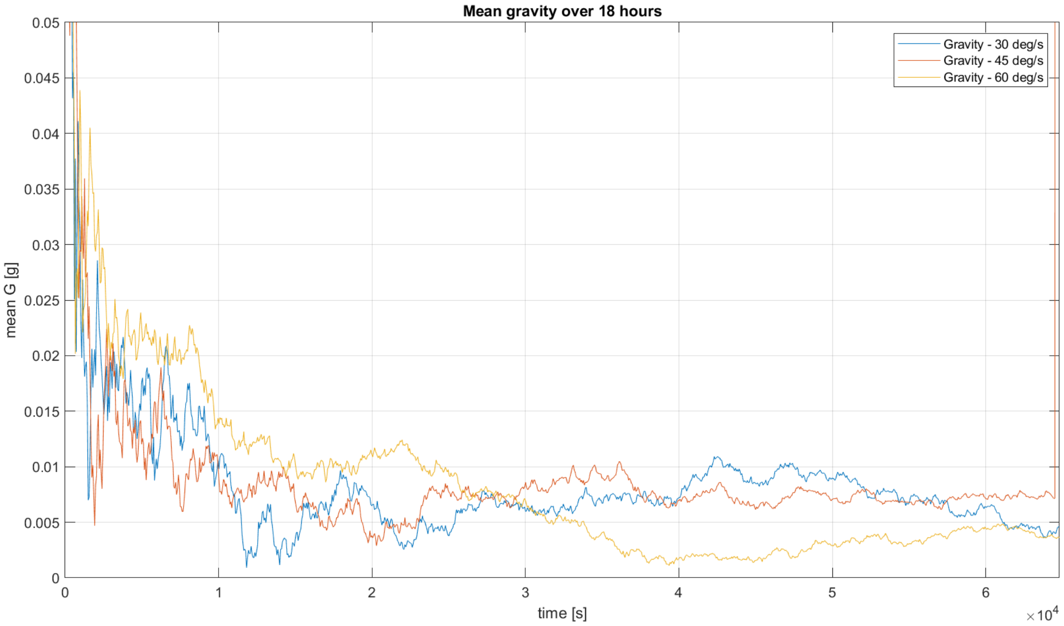


Fig. 5.2 Mean gravity over 18 hours - simulated microgravity

to reach values below 0.2g. On the other side, it was verified that the RPM works correctly, speed and decelerations in fact are consistent with those set.

As explained in Chapter 4, to load and unload animals, it is necessary to stop the RPM each time. Stopping the machine to load and unload has little effect on the result of G, so from a mechanical point of view it should not pose a problem, but as mentioned in Chapter 4.2 it is important that the change in speed be continuous and the speed of rotation itself should not allow the sample under test to be adapted. Since it is not entirely clear how such a interruption affects the results further studies are needed to develop a technique that will allow this problem to be solved. During the pause, in fact, the planarians are fixed respect to the G vector and this could affect the success of the study.

As explained in the previous chapter 4.4, the actual RPM velocities and accelerations were verified using *Tracker* software and then evaluated the position of the inner and outer frames every 0.5 seconds. The position varies continuously during the 300 seconds of machine analysis, and the angular velocities and accelerations are coherent with what is expressed by the RPM software. Figure shows, as an example, the analysis of the outer frame for the velocity of 45 deg/sec and the inner frame for the velocity of 30 deg/s.

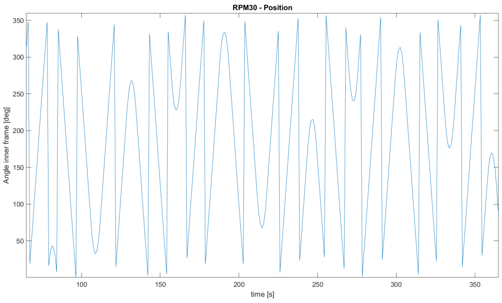


Fig. 5.3 Position of the inner frame over time, 30 deg/s

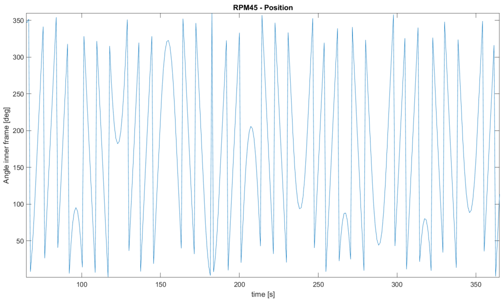


Fig. 5.4 Position of the outer frame over time, 45 deg/s

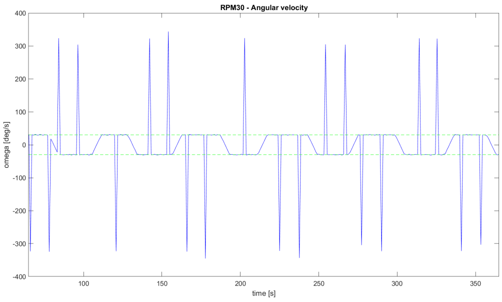


Fig. 5.5 Angular velocity of the outer frame over time, 30 deg/s

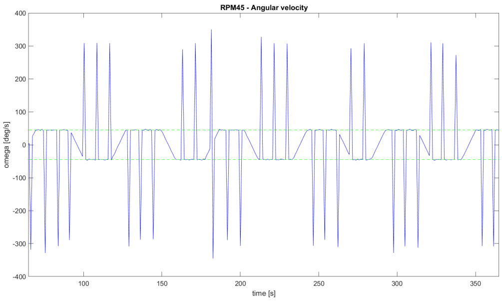


Fig. 5.6 Angular velocity of the outer frame over time, 45 deg/s

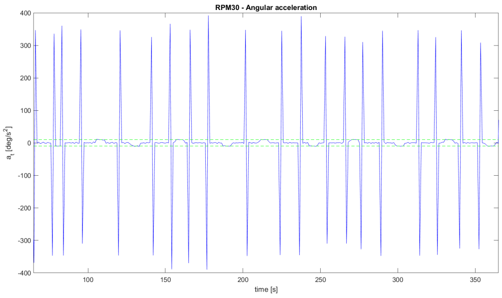


Fig. 5.7 Angular acceleration of the outer frame over time, 30 deg/s

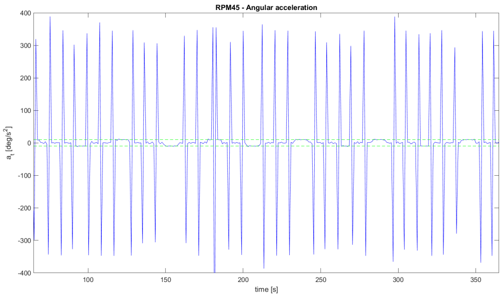


Fig. 5.8 Angular acceleration of the inner frame over time, 45 deg/s

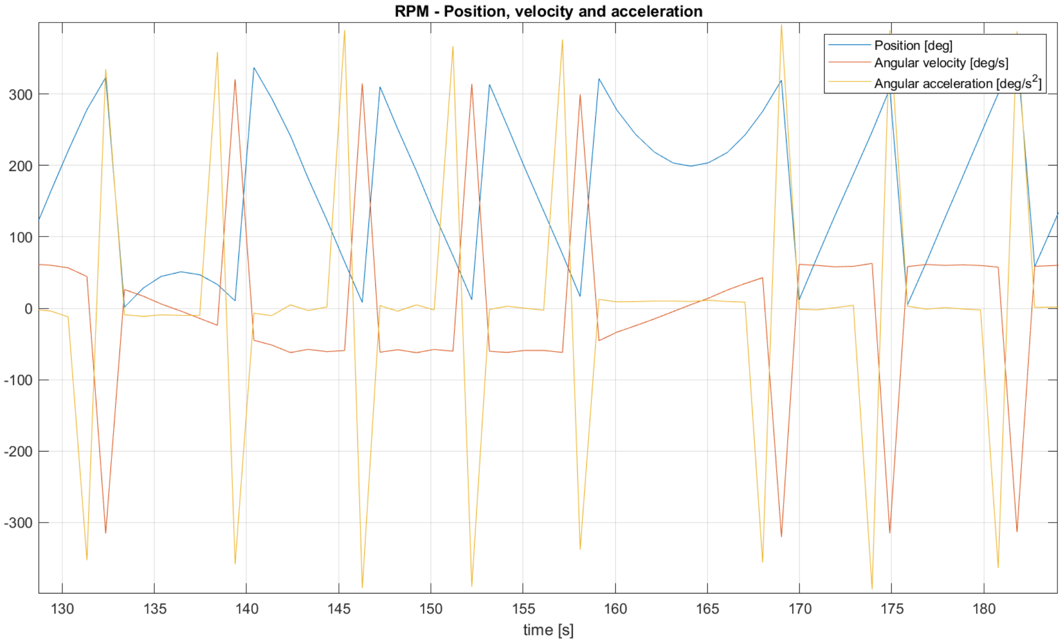


Fig. 5.9 Position, angular velocity, angular acceleration - RPM 60 deg/s - Outer frame

Figure 5.9 shows that the peak value of velocity and acceleration coincide with the point were the position reach 360° and therefore returns to 0°, thus there is a jump that is represented in the velocity and acceleration plot by the almost vertical lines of the peaks. As a final consideration, we must consider the acceleration to which they are subjected in relation to their speed and position. What we notice is that clearly acceleration increases proportionally with distance from the center of rotation and rotational speed. Considering that the maximum speed used is 60 deg/s and that our RPM is 24 cm long, so the biggest radius is 12 cm, it is possible to state that in the worst case scenario the acceleration is 0.017g, see Fig. 5.10. The centrifugal acceleration is in the order of $1e-3G$ therefore it is considered negligible.

5.2 Load capacity testing, alignment verification, and vibration analysis,

As a very first step, we made sure that the RPM was able to withstand the loads generated by the weight of the tubes themselves and by the accelerations undergone during operation. To do this we evaluated the performance in terms of velocity and gravity vector with a concentrated load on each arm of 250 grams, a mass 4.9 times

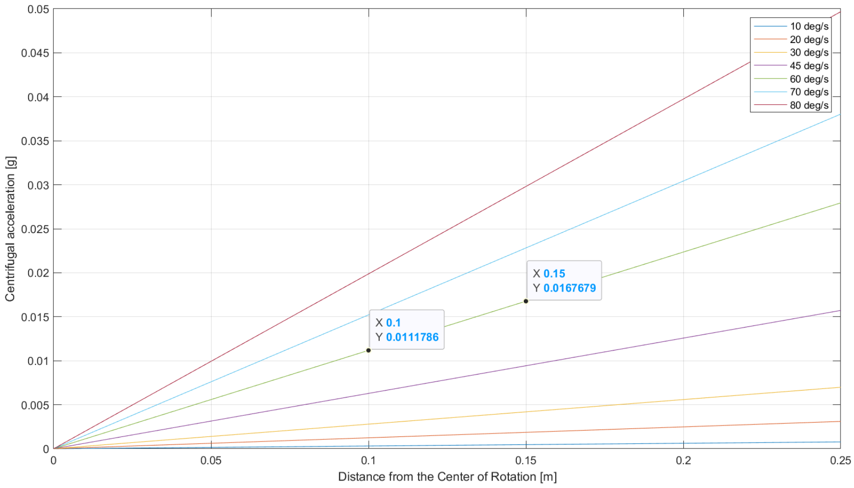


Fig. 5.10 Different acceleration for different speeds and distances from the Center of Rotation

greater than that required to perform the experiments as described in Chapter 4. The results obtained show that the performance does not differ in any way from that when unloaded and loaded with 3 tubes per arm, thus with a total weight per arm of 0.5 N, as shown in section 5.1.
Given that in order to assess the load limit of the RPM, experiments were conducted to evaluate its performance under higher-than-anticipated loads and he RPM successfully handled the increased load without experiencing any adverse effects or compromising its functionality, it is possible to establish that the intended experimental load, which is smaller in comparison, is well within the operating capacity of the RPM. These results support the decision to proceed with the determined load of 3 tubes on each arm, taking into consideration safety margins and the specific requirements of the study. for the future, it is recommended to check the performance again if the load needed for experimentation exceeds 250 grams.

In the second place it is important to verify if the Random Positioning Machine is correctly aligned and leveled to ensure that it operates without any unintended biases or discrepancies in the simulated microgravity environment. To achieve this we perform checks and adjustments to ensure that the RPM is properly aligned with the desired reference axes and leveled to prevent any tilting or misalignment. Results showed that it is necessary to perform the alignment of the two "frames" (sample holder and external frame) before starting each experiment. In fact, it was observed that without this procedure the machine does not start from the correct position and

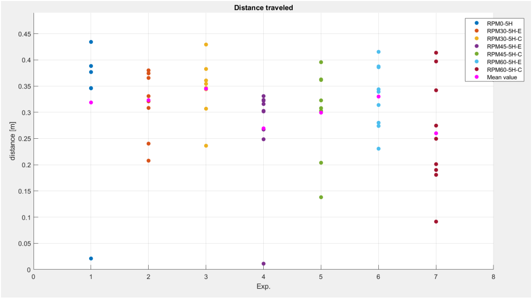


Fig. 5.11 5 hours exposure - Distance traveled by planarians. Each column represents one experiment, and each point represents one animal

during the whole use the values set as input are not respected, this clearly creates serious inaccuracies in the experiment and the results obtained.

The last aspect of mechanical validation concerns RPM vibration. As excessive vibrations could impact the accuracy and stability of the simulated microgravity environment, it is important to minimize vibrations in order to provide a more controlled and reliable experimental setup. Given the unavailability of specialized vibration measurement tools, visual observation was employed to assess vibrations in the RPM. During operation, close visual monitoring was conducted to detect any visible shaking, oscillations, or irregular movements. Furthermore, to mitigate vibrations, the implementation of dampers was considered. These measures aimed to minimize any potential vibrations and maintain a stable operating condition for the RPM, enhancing the overall reliability of the simulated microgravity environment.
Rubber feet were in fact placed under the machine to dampen any vibrations, while restrained tennis balls were used in the contact area between the table and the floor. While acknowledging that the current approach may not provide the most comprehensive assessment, it is important to recognize that further studies in the future are warranted. Despite the limitations, considering the available resources and constraints, the adopted methodology, including visual observation and the use of dampers, was deemed sufficient to address the immediate research objectives. However, it is recommended that future investigations explore more advanced techniques and equipment for a more thorough and quantitative analysis of vibrations in the RPM.

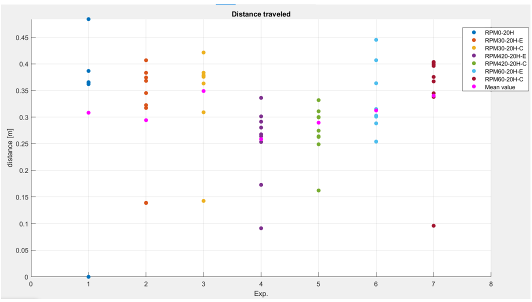


Fig. 5.12 20 hours exposure - Distance traveled by planarians. Each column represents one experiment, and each point represents one animal

5.3 Distance traveled

Below we can observe the distance traveled by each planarian during the 300 seconds of recording, in figure 5.11 it can be seen the values of each animal and in magenta the average value of each type of experiment for 5 hours duration, in figure 5.12 the distance for the 20 hours exposure. We observe that the distance displacement values are mainly distributed within the area above 0.2 m in each experiment, both in the 5-hour exposure case and in the 20-hour exposure case. We also tried to see if there was any kind of trend, so after experimenting at 30 deg/s and 60 deg/s we add 45 deg/s. In figure 5.11 and 5.12 even if there is not a clear trend but, looking at same duration experiments we can note that the mean value is always slightly bigger when the tip is pointing to the center than when it is outward.

As can also be seen in Table 5.1, the average distance traveled in each experiment is also the same for both exposures, in fact, the values are always between 0.25 and 0.35 m. The standard deviation is calculated for each experiment regarding all animals, 6 planarians were used for the control experiments, and 9 for all the other ones. Another aspect in common between the two durations is that the maximum peak value of recorded displacement corresponds to an animal belonging to the control, and the maximum average value corresponds to the rotation speed of 30 deg/s with the tip orientation pointing inward. On the other hand, the minimum average value occurs in the RPM60-C experiment in the case of 5 hours of exposure and RPM45-E in the case of 20 hours.

The overall results, however, do not show significant differences between the experiments. T-tests were conducted using the control sample for equal duration

Table 5.1 Mean distance traveled, the mean is calculated considering all animals having the same conditions

Condition	Mean Distance [m]	Standard Deviation
RPM0-5h	0.32	0.15
RPM30-5h-E	0.32	0.06
RPM30-5h-C	0.35	0.13
RPM45-5h-E	0.27	0.10
RPM45-5h-C	0.30	0.09
RPM60-5h-E	0.33	0.06
RPM60-5h-C	0.26	0.11
RPM0-20h	0.31	0.17
RPM30-20h-E	0.29	0.10
RPM30-20h-C	0.35	0.08
RPM45-20h-E	0.26	0.07
RPM45-20h-C	0.29	0.05
RPM60-20h-E	0.31	0.06
RPM60-20h-C	0.34	0.10

in each experiment, all results are greater than 0.2, thus indicating no significant difference, as can be seen in Table 5.2. This could be because they adapt very easily or because they do not spend enough time on the RPM. Ultimately, perhaps, they readjust to gravity easily, these animals in fact are often found in lakes attached below stones so it is possible that by being on their “stomachs” for a long time they have no proper sense of gravity.

5.4 Position in the recording arena

During the 300 seconds of recording, planarians can move freely in the arena, in the following plots it is possible to see their position in terms of distance from the center. Thus, animals that do not move will be represented by a horizontal line close to zero, those that stay only on the edge by a horizontal line of ordinate greater than 6.4 mm, a line with a gradient different from zero, on the other hand, indicates a move closer to or away from the arena edge.

We can observe that in the control experiments animals, starting from the center, go to the edge of the arena and keep moving along it, while in all other cases (experimental), the behavior is much more diversified. Certainly, some animals behave in the way

Condition	T-test
RPM30-5h-E	0.95
RPM30-5h-C	0.87
RPM45-5h-E	0.50
RPM45-5h-C	0.78
RPM60-5h-E	0.84
RPM60-5h-C	0.43

(a) 5-hours

Condition	T-test
RPM30-20h-E	0.83
RPM30-20h-C	0.79
RPM45-20h-E	0.33
RPM45-20h-C	0.47
RPM60-20h-E	0.96
RPM60-20h-C	0.80

(b) 20-hours

Table 5.2 T-test results for the distance covered, comparison with control samples

described above, but most of them move back to the center once they reach the edge, and so on. In figure 5.14 and 5.15 we can see the experimental samples, while in figure 5.13 we can observe the control animals. Each line corresponds to a different animal.

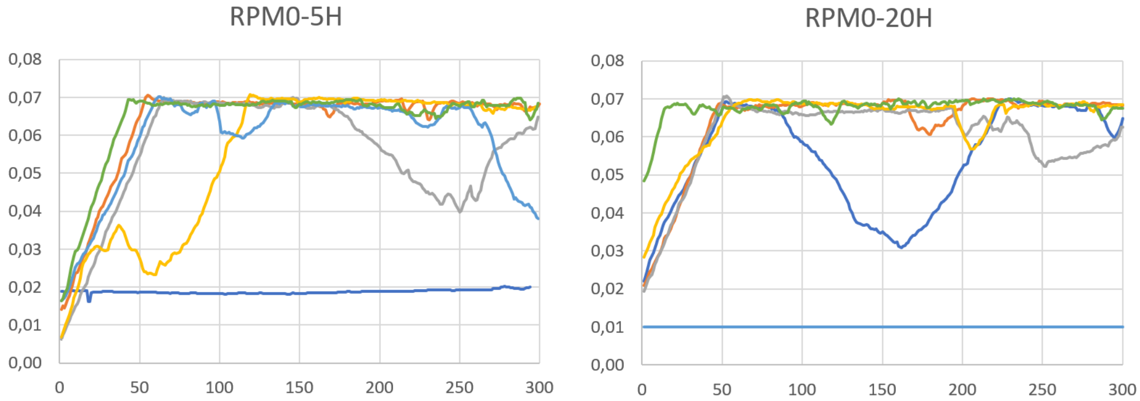


Fig. 5.13 Distance from the center of the arena over time - Control - 5 hours exposure (left), 20 hours exposure (right)

In Table 5.3 we can see the mean time spent on the side for each experiment with its standard deviation.
It is found that the standard deviation is very high, the values of individual animals are all very different from the mean value, which is therefore not representative.

With the T-test, we can compare the time spent on the edge in different conditions, as can be seen in Table 5.4.

In this case, again, all T-tests show no significant difference between the experiment and control, all values are indeed bigger than 0.2. From another point of view, it

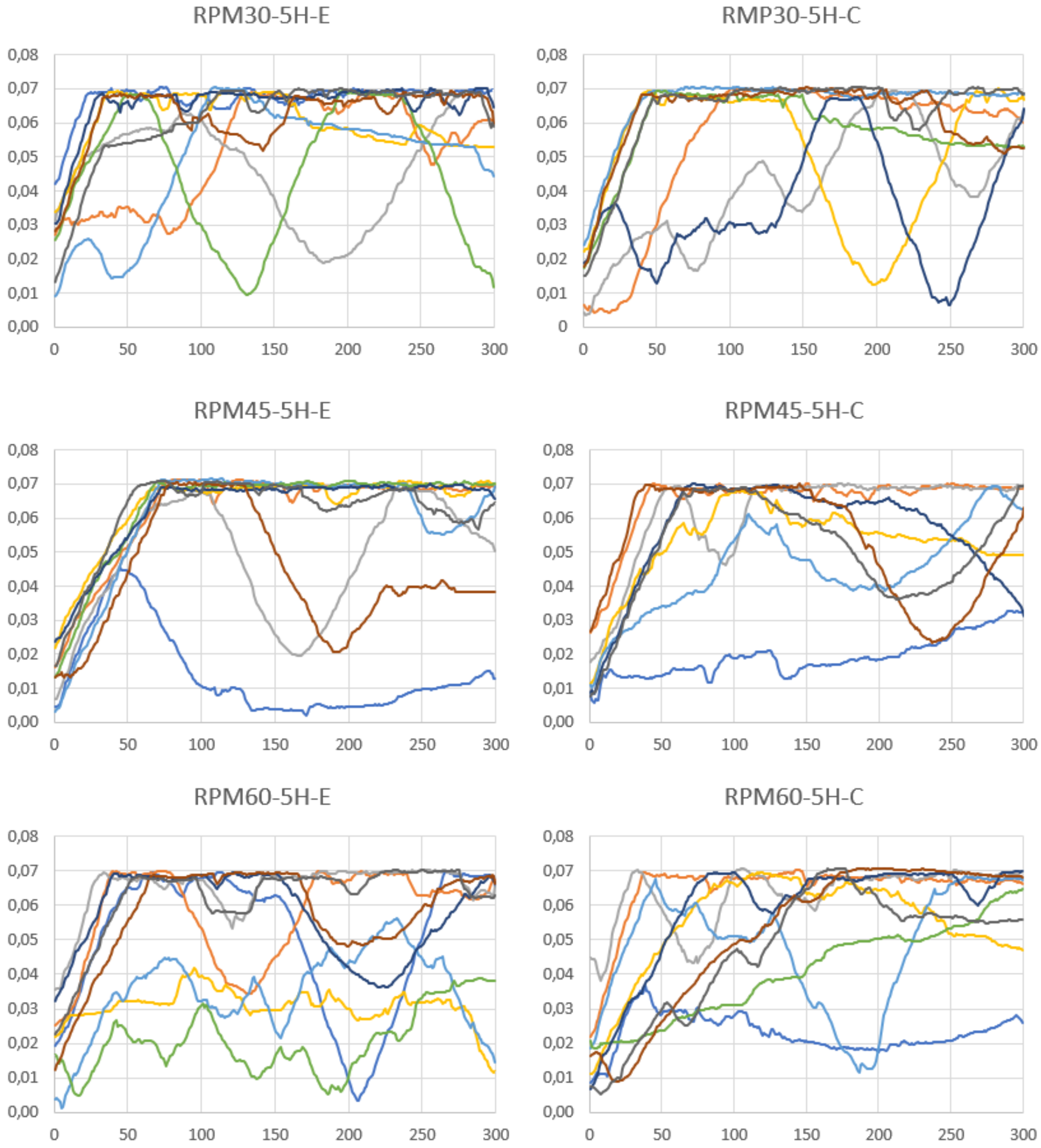


Fig. 5.14 Distance from the center of the arena over time - 5 hours exposure

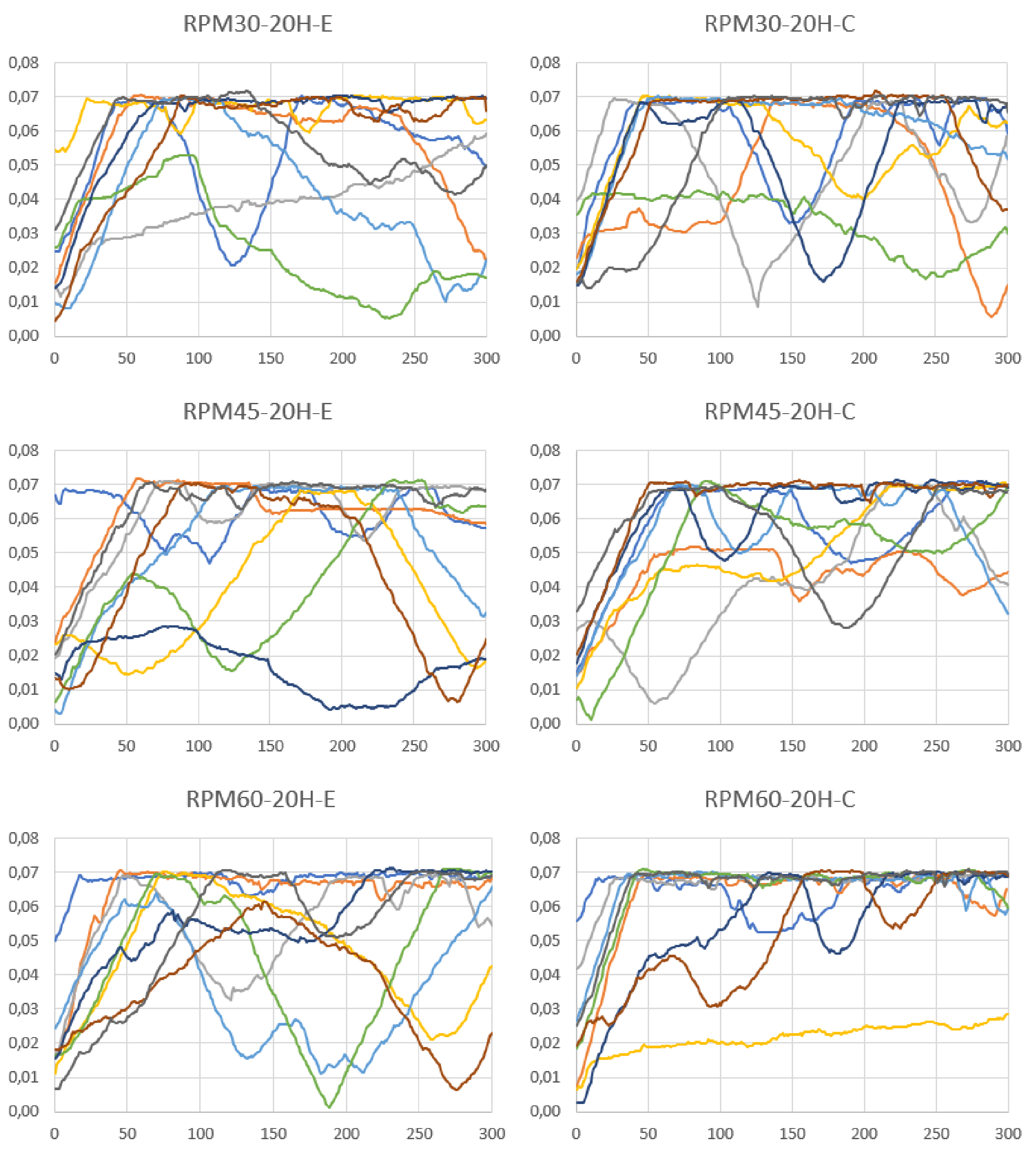


Fig. 5.15 Distance from the center of the arena over time - 20 hours exposure

Table 5.3 Mean time spent on the side - percentage

Condition	Mean time on the side [%]	Standard deviation
RPM0-5h	43	28
RPM30-5h-E	34	23
RPM30-5h-C	37	26
RPM45-5h-E	48	33
RPM45-5h-C	29	36
RPM60-5h-E	26	22
RPM60-5h-C	25	23
RPM0-20h	42	34
RPM30-20h-E	30	26
RPM30-20h-C	35	22
RPM45-20h-E	28	19
RPM45-20h-C	32	25
RPM60-20h-E	28	25
RPM60-20h-C	52	27

Table 5.4 T-test results, comparison with control samples of same duration

Condition	T-test
RPM30-5h-E	0.55
RPM30-5h-C	0.59
RPM45-5h-E	0.78
RPM45-5h-C	0.38
RPM60-5h-E	0.25
RPM60-5h-C	0.25

(a) 5-hours

Condition	T-test
RPM30-20h-E	0.48
RPM30-20h-C	0.65
RPM45-20h-E	0.38
RPM45-20h-C	0.21
RPM60-20h-E	0.40
RPM60-20h-C	0.57

(b) 20-hours

is possible to find out whether different experiments follow a particular trend. Figure 5.16 shows the 5-hours experiments, values are strongly distributed throughout the range, and thus are far from the mean value, as also evident from the table 5.3. Same thing is observed for 20-hours experiments. As can be seen in the Fig. 5.17, however, the pair of experiments characterized by the same velocity have a slight increase in mean value if the tip is moved from the outer to the center of rotation.

Overall, it is possible to observe that the control animals tend to the edge and once reached move along it, the animals subjected to experiments do not show substantial differences between them but compared to the control show more chaotic behavior. In fact, animals subjected to simulated microgravity tend to initially move toward the

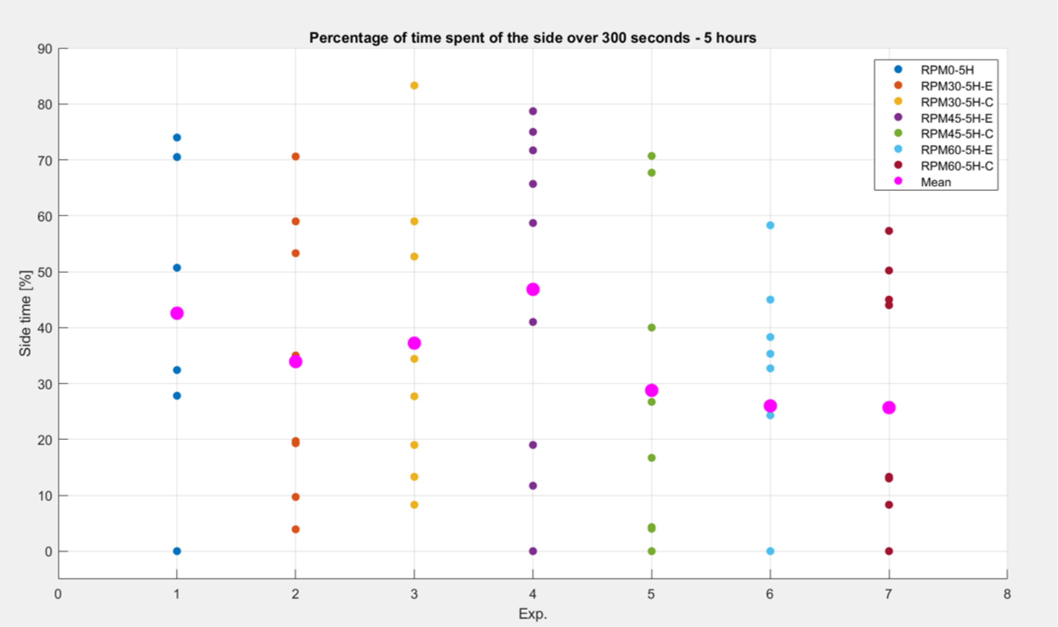


Fig. 5.16 Percentage value of time spent on the side - 5 hours exposure

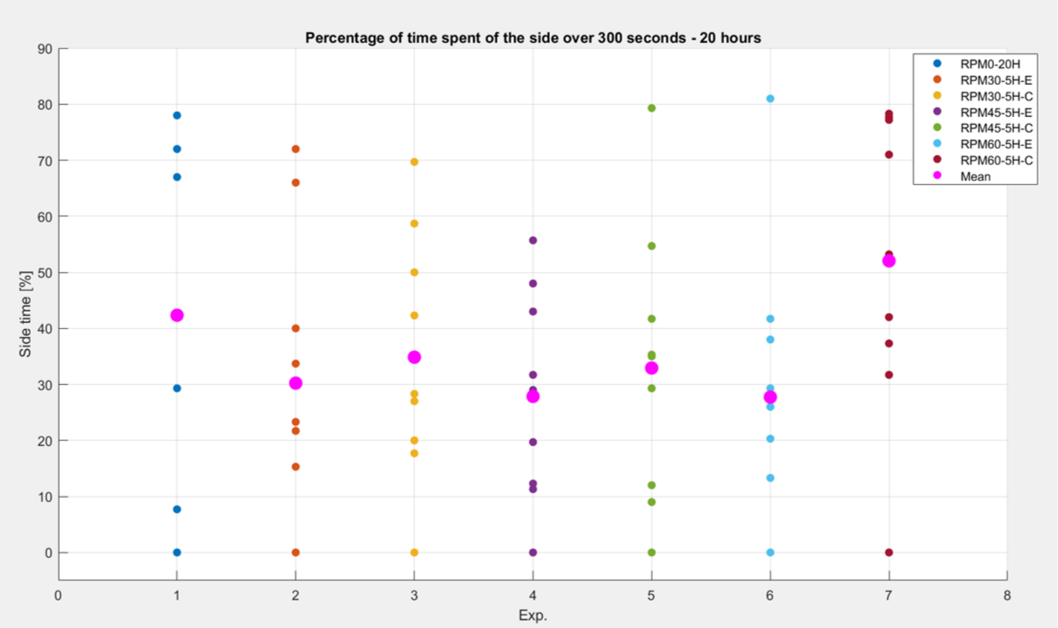


Fig. 5.17 Percentage value of time spent on the side - 20 hours exposure

edge but later return toward the center and thus do not follow a true line within the arena. This could be caused by a feeling of disorientation, perhaps planarians after being on the RPM struggle to orient themselves, and because of this the survival instinct also is missed. It is a natural instinct, indeed, for prey animals to seek areas that are enclosed and protected rather than open areas, such as the center of the plate, due to their natural behavior, in open spaces in fact they become easy prey.

5.5 Behavior

Two main behaviors were measured in the study, gliding and scrunching, all other behaviors were considered as *other*[1]. The percentage values below refer to the mean value for each second, on total 300 seconds of recording for each planarian.

Table 5.5 Average percentage of behaviors recorded for each condition

Condition	Gliding	Scrunching	Other
RPM0-5H	64 %	2 %	34 %
RPM30-5h-E	77 %	3 %	20 %
RPM30-5h-C	84 %	5 %	12 %
RPM45-5h-E	87 %	1 %	12 %
RPM45-5h-C	73 %	2 %	26 %
RPM60-5h-E	66 %	0 %	34 %
RPM60-5h-C	66 %	1 %	33 %
RPM0-20H	82 %	2 %	17 %
RPM30-20h-E	76 %	2 %	22 %
RPM30-20h-C	82 %	1 %	18 %
RPM45-20h-E	77 %	0 %	22 %
RPM45-20h-C	90 %	1 %	10 %
RPM60-20h-E	91 %	2 %	7 %
RPM60-20h-C	83 %	0 %	17 %

The behavior mostly observed is the normal locomotion, gliding, followed by *other* thus not helpful for proper classification. *Scrunching* is the least observed behavior.

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[1] In “other” the most observed behavior is the upside-down position, which to date, however, has not yet been studied and is the reason why we have not considered it separately

Table 5.6 T-test results for **gliding**, comparison with control samples

Condition	T-test
RPM30-5h-E	0.58
RPM30-5h-C	0.40
RPM45-5h-E	0.33
RPM45-5h-C	0.74
RPM60-5h-E	0.95
RPM60-5h-C	0.93

(a) 5-hours

Condition	T-test
RPM30-20h-E	0.80
RPM30-20h-C	0.99
RPM45-20h-E	0.83
RPM45-20h-C	0.67
RPM60-20h-E	0.96
RPM60-20h-C	0.60

(b) 20-hours

Table 5.7 T-test results for **Other**, comparison with control samples

Condition	T-test
RPM30-5h-E	0.56
RPM30-5h-C	0.35
RPM45-5h-E	0.36
RPM45-5h-C	0.36
RPM60-5h-E	0.99
RPM60-5h-C	0.97

(a) 5-hours

Condition	T-test
RPM30-20h-E	0.82
RPM30-20h-C	0.97
RPM45-20h-E	0.80
RPM45-20h-C	0.72
RPM60-20h-E	0.99
RPM60-20h-C	0.99

(b) 20-hours

It is important to remember that the mean value is just a starting information. No significant differences are observed between the control and the results of the various experiments. Furthermore, in Table 5.6 and 5.7 it is possible to observe the all T-tests shows results greater than 0.2.

The normal locomotion of planarians is characterized by a linear displacement on the surface, as explained in section 4.1, in the study called gliding and it is the most observed behavior. Planarians, however, abandon this behavior in favor of others when they are uncomfortable, stressed, feel pain, etc. The behavior here called scrunching is instead characteristic of when animals feel pain and then contract their bodies "like a snake" to move, this behavior however is minimally observed in our study, in fact in only two cases it reaches 6% of the total but the mode is 0%. However, it is interesting to observe that the "other" behavior increases, if only marginally, compared to the control sample, this means that after exposure to simulated microgravity they are disturbed by something. Since in most cases it is observed that the animals find themselves upside-down and therefore try to re-establish themselves in their normal position one possible interpretation is that the planarians lose their sense of orientation and thus of gravity, this means that they don't notice that they are upside down and by not distinguishing up or down when they then start to move they can't reposition themselves correctly to be able to glide.

5.6 Position in the tube after the exposure

At the end of each experiment, including control, the position of the animals in the tubes was evaluated. Cap, Middle and Tip, looking at the overall average tip seems to be the favored position, 46% of cases, but, so far, we don't have enough information. Moreover, the results of the experiment were not significantly different from those of the control samples. Planarians were always sticky to the wall at the end of the exposure; only two animals out of 120 measured were floating at the end of the exposure.

Table 5.8 Animals' position inside the tubes after exposure - Percentage of times - 5 hours exposure

Condition	Cap %	Middle %	Tip %
RPM0-5H	17	33	50
RPM30-5h-E	44	22	33
RPM30-5h-C	44	11	44
RPM45-5h-E	11	22	67
RPM45-5h-C	11	33	56
RPM60-5h-E	33	11	56
RPM60-5h-C	56	22	22

Table 5.9 Animals' position inside the tubes after exposure - Percentage of times - 20 hours exposure

Condition	Cap %	Middle %	Tip %
RPM0-20H	17	0	83
RPM30-20h-E	67	22	11
RPM30-20h-C	56	22	22
RPM45-20h-E	33	11	56
RPM45-20h-C	22	33	44
RPM60-20h-E	22	22	56
RPM60-20h-C	0	44	56

Fig. 5.18 Marks made to track the position of the planarians while on the RPM

Interesting to note, in both cases of 5-hours and 20-hours, the overall percentage of Cap, Middle, Tip remains the same. Cap = 31%, Middle = 22%, Tip = 47%

To find out whether planarians have a preferred position inside the tubes, experiments lasting two hours each, were performed with the RPM at 10 deg/s, 30deg/s and 60 deg/s with the tips both inward and outward. Every 10 minutes the RPM is paused and a mark is placed on each tube on the position of the planarians. Regardless of the RPM or orientation of the tubes, each planarian was always in the tip, unlike the findings at the end of the experiments presented above.

At the beginning of the study it was observed that the animals always tended to position themselves in the tip of the tubes but this control was done for only two hours and by continuously interrupting the RPM. In the rest of the study the position of the aniamals was evaluated only at the end of the exposure, what was obtained was that the animals did not concentrate solely in the tip but also in the center or in the cap area. This tells us that the period of exposure makes a difference in their behavior and that stopping RPM also has consequences for planarians, although to date a real cause-and-effect link has not been identified. Moreover the reason we had put the tubes in two different positions was because we thought they were in the tip all the time and therefore the accelerations might be different depending on the distance from the center, yet in light of the results we got, maybe, this is not necessary but we need a larger sample to actually understand the situation. Also although the reason why we selected the two positions might not persist is done,

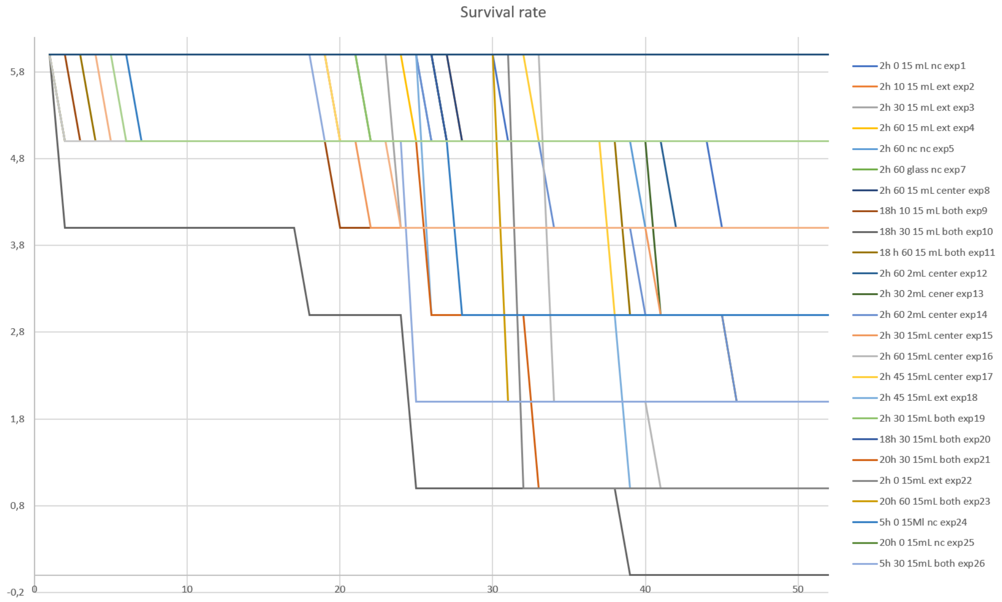

Fig. 5.19 Survival rate - 50 days

maybe it is not there anymore but there might be other things, in fact we notice a slight difference in "time spent on the edge" if we chage the position from External To Center, note that again this is a preliminary observation. During the exposure we observed that the animals remain attached to the wall at all times, so the belly sticks to the surface while the back is in contact with the fluid. The upper part of the body of these animals is not very sensitive to mechanical stimuli, and combined with the fact that they have a very small thickness they are probably little disturbed by fluid motion. Fluid dynamics within the tubes therefore was not evaluated within the study as it was deemed to be of minor interest but needs more study.

5.7 Survival rate

To examine this parameter, all animals used were evaluated, even those belonging to discontinued experiments. In general, high numbers of deaths are not observed in the first 20 days after the experiments, on the other hand, a sharp decrease in the number of surviving animals is observed between 20 and 40 days. As can be seen in figure 5.19 the only experiment for which death of all planarians is recorded within 50 days is the 18-hour experiment at 30 deg/s in the 15-mL tubes.

As far as is known about planarians, we know that they can survive for a very long time and have amazing regenerative abilities. The data collected to date regarding the survival rate of these animals seem to confirm their strong adaptive and survival ability. Only one experiment recorded the death of all six subjects, 18 hours at 30 deg/s, but being an isolated case it is not, at present, to be considered relevant. The rest of the samples did not show interesting mortality or survival rates, and there were no differences when moving from one speed to another or from one duration to another.

5.8 Dismissed experiments

The aim of the entire study is to understand if and how the RPM can be used to simulate microgravity environment for planarians. Because of this the RPM experiments running at 10 deg/s were dismissed due to poor simulated microgravity results, as were the experiments lasting only two hours. This happened because, as noted in the previous chapter, good simulated microgravity values are not achieved before 9000 seconds (2.5 hours) and therefore the results obtained are not useful for our study. Similar reasons led to the exclusion of experiments at rotational speeds greater than 10 degrees per second; at these speeds, the desired G = 0.2g objective is reached more slowly and with greater difficulty.

Working on pilot experiments leads us to make some mistakes, in order to limit the variables as much as possible, we try to always use the same procedure and to do exactly the same thing every time. Experiments lasting 18 hours were performed from 18:00 to 12:50, then recorder from 12:00 to 12:55, all other experiments were recorded between 14:00 and 14:55; 18-hours experiments were not coherent with the procedure and were abandoned on behalf of 20-hour ones. Considering that the 18-hour experiments were not excluded because of their duration, but rather because of practical recording issues, it is possible to introduce them again in the future if the planning is consistent.

15 mL ClearLine Centrifuge Tubes were used for all experiments, but at the very beginning of the study other tubes have been evaluated. We tried polypropylene tubes with rounded tip, without the cap; no significant differences were noted, however they were more difficult to close and use so they have not been used in the future. Glass tubes were evaluated as well but for the same reason above we stopped using

them. 2 mL polypropylene tubes have been tried out, but their dimension wasn't suitable for the study and given the smaller size we realized the fluid-dynamic would be much more complicated due to the bigger intersection of the boundary layer and chaotic motion.

5.9 Observations and General Remarks on the Results

The biological aspect and the mechanical aspect of the study will not be discussed separately in this section, as they are closely related and difficult to distinguish when analyzing the responses of the animals to mechanical stimuli.
The pivotal notion to be mindful of is that this study is a *Pilot study*, this means that it can only provide limited information, the sample is indeed smaller than normal experiments and some logistical issues may not be completely resolved (e.g. rubber band as sample holder). Pilot studies are intended to determine whether an experiment is feasible, that's the reason why the results previously are mostly a summary of statistics of all the data collected.

Chapter 6

Conclusion

The study conducted in this research served the purpose of gathering preliminary data and testing logistical aspects before undertaking a larger-scale study, accordingly, it can be considered a pilot study. Additionally, it revealed valuable insights into potential deficiencies or issues with the experimental design. The findings of this research provide a foundation for further refinement and optimization of future experiments, ensuring the successful implementation of more extensive studies in the field of microgravity simulation using the Random Positioning Machine. The pilot study proved instrumental in identifying areas for improvement and guiding the progression of subsequent research endeavors.
What we did in this study then was to analyze different methods, different conditions, and then different kinds of experiments. By meticulously examining these factors, we aimed to ascertain the suitability and efficacy of the RPM as a reliable tool for simulating microgravity and facilitating experimentation with planarians. Through our rigorous assessment, we sought to determine the potential of the RPM as a valuable platform for future investigations in the field of microgravity research involving planarian organisms.

From a mechanical perspective, the RPM demonstrates its potential as a tool for creating simulated microgravity conditions. Four rotational speeds were evaluated, 10 deg/s, 30 deg/s, 45 deg/s, deg/s, and 60 deg/s. The results 5 show that the lowest velocity is not sufficient to achieve approproiate values of G and was therefore excluded from the study. The remaining 3 velocities, on the other hand, were found to be suitable for microgravity simulation, and after about 10000 seconds their trends asystatically tend to 0g. Given the moderate velocities and small RPM dimesions, the results show that the centrifugal acceleration is of the order of 10e-3g and therefore negligible for the purpose of the study.

In terms of biological validation, we analyzed the behavior of planarians after being exposed to simulated microgravity. In order to do that we evaluated four different parameters, three of them evaluated the behavior during the recording time: distance travelled by planarians during the recording, the time spent on the edge of the recording arena ancd their type of movement, and the other one regards the survival rate. The experimental findings suggest that planarians display remarkable resilience and consistency in their behavioral responses, seemingly unaffected by the diverse conditions imposed upon them. These observations emphasize their adaptive nature and reinforce the notion that planarians exhibit consistent behaviors irrespective of the experimental variables. Exposure greater than 2 hours total is also recommended so that an appropriate G value can be achieved, subsequent experiments should be conducted especially exceeding 20 hours of exposure in order to really understand how simulated microgravity can interfere with animals' behavior and survival.

In summary, the mechanical aspects of the study, including the performance and functionality of the RPM, demonstrated satisfactory outcomes. However, further exploration and experimentation are required to enhance our understanding of the biological responses and implications in the context of microgravity conditions. The data collected in this study demonstrate the feasibility of utilizing our RPM as a space analog for simulating microgravity environments. The observed results provide evidence supporting the effectiveness of the RPM in replicating key aspects of microgravity conditions. These findings open up new possibilities for conducting research in microgravity-like conditions on Earth, allowing for the investigation of biological responses and behaviors that may be relevant to space exploration and human health. Furthermore, the successful implementation of the RPM as a space analog encourages further exploration and refinement of its application in studying other organisms and biological systems in simulated microgravity settings. Despite the fact that more research is required, to date we can state that the direction taken appears to be the right one and shows great potential. The study conducted in this research served the purpose of gathering preliminary data and testing logistical aspects before undertaking a larger-scale study, accordingly, it can be considered a pilot study. Additionally, it revealed valuable insights into potential deficiencies or issues

with the experimental design. The findings of this research provide a foundation for further refinement and optimization of future experiments, ensuring the successful implementation of more extensive studies in the field of microgravity simulation using the Random Positioning Machine. The pilot study proved instrumental in identifying areas for improvement and guiding the progression of subsequent research endeavors.
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Appendix A

This appendix presets the main mathematical and statistical tools used in this study to analyze the results obtained.

T-test

The t-test is a statistical test used to determine if there is a significant difference between the means of two groups. It compares sample means, variances, and sizes to calculate a t-value, which is then compared to a critical value to assess significance. It is commonly used for hypothesis testing and evaluating differences in data.

$$T = \frac{m-\mu}{std/\sqrt{N}}$$

Standard Deviation

Standard deviation measures the dispersion or variability of data points around the mean, it provides a quantitative measure of how spread out the data points are from the average.
A higher standard deviation indicates greater variability in the data, while a lower standard deviation suggests that the data points are closer to the mean, because of this it is often used to assess the variability or consistency of experimental or observational data.

$$std = \sqrt{\frac{1}{N-1}\sum_{i=1}^{N}(x_i-\bar{x})^2}$$

Standard Error

Standard error estimates the variability of sample means around the population mean, it is indeed a measure of the precision or reliability of the sample mean as an estimate of the population mean. It provides an indication of how much the sample means are expected to vary from one sample to another.

$$ste = \frac{std}{\sqrt{(N)}}$$

Appendix B

Appendix B

B.1 Space exploration

Humans are driven to explore the unknown, discover new worlds, push the boundaries of our scientific and technical limits, and then push further [12]. In the not-so-distant future, mankind will return to the Moon, arrive at Mars, and on a daily basis, our astronauts must contend with the environment of space. As written before, space exploration, however, is far from easy, involving numerous difficulties, challenges, and problems. Indeed, NASA [13] considers five main categories of hazards, which in turn obviously involve a wide range of consequences:

- **Radiation**: space radiation consists of three kinds of radiation: particles trapped in the Earth's magnetic field, particles shot into space during solar flares (solar energetic particles), and galactic cosmic rays [14]. Although to date there are no data on astronauts with acute radiation syndrome, even though it is necessary to note that as of now we only have data from LEO missions or lower [15], radiation is a serious health hazard, in fact, could lead to several medical problems, such as developing cancer, CNS decrements, exhibiting degenerative tissue effects, or developing acute radiation syndrome [16]. Besides the problems presented above, there has yet to be a system that allows such a phenomenon to be simulated on Earth.
- **Isolation and confinement**: an essential aspect of the mission plan is the fact that the crew is living a significant distance from "home," in small spaces, and

with other members of the crew. These factors have profound psychological impacts and are a key component of the mission plan, both for missions to the ISS but especially in future missions to the Moon and Mars, they will in fact take humans much farther from Earth and for much longer periods of time [17].

- **Distance from Earth**: in reference to what was mentioned in the previous point, the distance from Earth has a variety of consequences [17], in addition to psychological ones we also have more practical and technical problems, such as delayed communications, long timelines for return in case of emergency situations, inability to receive supplies [13]. It is therefore necessary to implement life-support and self-support systems that can be used without the intervention of personnel or ground-based elements.

- **Gravity difference**: during space missions astronauts experience different gravity forces, 3G/4G during launch and reentry, 0G/10E-6G interplanetary transit and LEO, 0.38G on Mars, and 0.16 G on Moon [17]. Humans, like all other existing creatures, were born, developed, and adapted to live in a 1G environment, remaining for prolonged times in microgravity conditions can, therefore, cause a variety of disorders and health problems such as dysregulation of the immune system, bone loss, muscle atrophy, cardiac problems or impaired wound healing [18].

- **Hostile/closed environment**: the habitat in which the astronauts live for the duration of the mission is itself a challenge: in addition to being a livable environment from a psychological point of view, it must also provide the right conditions of temperature, pressure, and humidity necessary to enable life [19].

Therefore, we might think that the best idea is to pursue space exploration while avoiding the presence of humans on these missions but for now that is not possible. So far the use of robots in space research is not yet widespread enough to replace human versatility and intelligence, no matter how advanced technology has become; furthermore, humankind has always been driven to explore, discover and overcome its limits. Above all the space environment represents a great irreplaceable laboratory in which to study the human body and its ability to adapt and survive[20] [17]. Due

Fig. B.1 ESA astronaut Samantha Cristoforetti on ISS, microgravity condition - closed environment

Fig. B.2 Planet Earth seen from Mars - NASA's Curiosity rover. When Curiosity took the photo, Earth was about 160 million kilometers from Mars. Credit: NASA Jet Propulsion laboratory-Caltech/Malin Space Science Systems/Texas A&M University.

to the above, crewed missions represent a must-have research and exploration tool to date.

In accordance with what written in the fourth point, one of the greatest challenges is the condition in which people or objects appear to be weightless: microgravity [21]. Microgravity affects the human body and all biological systems in several and profounds ways, it is essential to have a thorough understanding of this phenomenon and how it interferes with human life in order to ensure the safety of the astronauts and more chance of survival in space but mostly developing a better understanding of gravity's role in various biological processes [2] is crucial to space exploration and space biology.

However, the study of the effects related to microgravity is extremely challenging and has many limitations since everywhere on earth we are subjected to 1G. The alternative solution, however, would be to conduct space experiments, but these would present great risks and high expenses. It is, therefore, necessary to develop effective devices capable of simulating this condition properly on Earth. In laboratories, Random Positioning Machines are widely used as a tool for simulating microgravity [22]. They are constructed of two frames, thus with two axes of rotation perpendicular to each other, which rotate at a constant speed by randomly altering the rotation direction, the algebraic sum of the gravity vector allows them to reach an average gravity value of about 0.02G. To generate effects comparable to the effects of true microgravity it is crucial that directional changes be made faster than the object’s response time to gravity. [23].

B.2 Microgravity problems on astronauts

Technically speaking, gravity does exist everywhere in the universe, it is defined as the force that attracts two bodies to each other. It is therefore important to emphasize that the condition to which astronauts are subjected on the ISS or during space travel is not the absence of gravity but the sum of the accelerations is zero. In other words, the astronauts do not have a particular direction that they are pulled, so they do not feel any particular acceleration.
If subjected to microgravity for very short periods this does not create problems or decompensation but for longer periods the consequences may be for example bone loss, muscle weakness, and space motion sickness. We can classify these issues into four macro categories indeed, the problems encountered can be related to the following [24]:

- **Neurovestibular system**. The vestibular system consists of the labyrinth and five senses; the integration of inputs from the eyes, the inner ears, and the position sensor stabilizes the body. However, weightlessness alters otolith sensing, vision, semicircular canals, and proprioception in early flight, when the sensory inputs the brain receives inputs (proprioception, vestibular and visual system) that are in conflict with each other, symptoms similar to motion sickness symptoms occur, they are therefore termed “space motion sickness” (SMS).

- **Cardiovascular and respiratory systems**. The force of gravity and the hydrostatic pressure maintain an optimal level of blood pressure in our bodies and a proper level of gas distribution in the alveoli. Under microgravity conditions, blood pressure is distributed at a value of about 100 mmHg throughout the body, this means that the pressure in the brain increases while due to the phenomenon called "fluid shift" the blood pressure in the legs decreases, moreover During spaceflight, the myocardial volume decreases by 8–10%. At the same time, we can observe lung size change (shortening in the vertical and widening in the horizontal directions), homogeneous alveolar gas filling, and reduced functional residual capacity (as in supine position at 1G) due to the absence of abdominal weight and hydrostatic gradients [17]. Related to this issue it is also possible to observe in astronauts postflight orthostatic intolerance.

- **Musculoskeletal system and bone metabolism system**. Microgravity conditions cause muscle loss and atrophy, the two major causes are the disappearance of mechanical constraints and a decrease in muscular activity that follows. The main issue affecting the skeletal system is bone calcium loss during microgravity: the bones become fragile during microgravity exposure and astronauts incur osteoporosis, which can lead to serious health problems even after the astronaut or cosmonaut has returned to Earth.
- **Immunology and hematology**. During spaceflight, we can observe a reduction in cellular components, especially erythrocytes, associated with anemia, at the same time it has been reported in recent studies that humans have dysregulated immune responses and that latent herpes virus reactivates during orbital spaceflight [25].

[26] [27]

Appendix C

Challenge of *random*

The challenge of randomness in computing and calculators remains an unresolved issue within academic discourse. The reliance on deterministic algorithms and finite computational resources poses limitations in generating true randomness. Despite efforts to employ pseudo-random number generators, they exhibit periodic patterns and lack unpredictability.

To generate random numbers, machines typically use algorithms known as random number generators (RNGs). There are two types of RNGs:

- Pseudo-Random Number Generators (PRNGs): These algorithms use a seed value as an input and apply mathematical formulas to produce a sequence of numbers that appear to be random. However, the sequence is deterministic and will repeat after a certain period. PRNGs are widely used due to their efficiency and speed but may not provide true randomness.

- True Random Number Generators (TRNGs): TRNGs generate numbers from inherently random physical processes, such as atmospheric noise, radioactive decay, or thermal noise. These processes are unpredictable and provide a higher level of randomness compared to PRNGs. TRNGs require specialized hardware to capture the random physical phenomena.

Both PRNGs and TRNGs play a role in generating random numbers. PRNGs are suitable for many applications where statistical randomness is sufficient, while TRNGs are preferred for cryptographic purposes or scenarios requiring high levels of unpredictability.