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Assessing black soldier fly pupation and survival in lunar regolith simulant: Implications for sustainable controlled habitats on the Moon

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ABSTRACT

Bioregenerative life-support systems (BLSSs) will be crucial for extended space missions and extraterrestrial habitats. The black soldier fly, *Hermetia illucens*, is recognized for its efficient organic waste consumption, making it well-suited for closed environments like spacecraft. Our study assessed *H. illucens* adaptability to different substrates, including a lunar regolith simulant, pertinent to future lunar colonization. Remarkably resilient, *H. illucens* prepupal larvae successfully pupated in all tested substrates, but pupation timing varied, with no-substrate larvae pupating later. Pupal stage duration also differed, particularly with lunar regolith simulant and sand treatments resulting in longer durations. Substrate treatments significantly influenced the number of emerged adults, with lunar regolith simulant yielding more adults than the no-substrate treatment. Additionally, sand and wood shavings treatments produced more adults, highlighting *H. illucens* adaptability to various substrates, including lunar regolith. These findings are crucial for future BLSSs design. Additionally, *H. illucens* adaptability to lunar regolith provides insights into life's adaptability in space environments, guiding future experiments on celestial bodies. This study provides critical data on how different substrates, including lunar regolith simulants, influence *H. illucens* development and survival, advancing BLSSs and ecological science in both space and terrestrial contexts.

1. Introduction

Bioregenerative life-support systems (BLSSs) represent cutting-edge ecological and space engineering endeavors geared towards maintaining human survival in environments with constrained resource availability, notably during extended space missions or within controlled habitats on the Moon, Mars or other celestial bodies [1–3]. These systems employ a synergistic interplay of diverse biological organisms, including plants, algae, and microorganisms, to facilitate the continuous recycling and replenishment of crucial life-sustaining elements such as oxygen, water, and sustenance [4–6]. By exploiting natural processes like photosynthesis, respiration, and biogeochemical cycling, BLSSs aim at establishing self-contained, sustainable ecosystems that mitigate dependency on resupply missions and curtail the ecological footprint of human habitation in extraterrestrial environments.

Insects have recently attracted significant interest in the context of BLSSs for space exploration due to several compelling reasons [7,8]. Particularly, the black soldier fly *Hermetia illucens* (Diptera: Stratiomyidae) is highly efficient at consuming organic waste during its larval stage [9], making this species valuable in closed environments like spacecraft and space stations, where effective waste management is essential. *H. illucens* larvae reduce the volume and mass of organic waste through digestion [10,11], minimizing the need for storage and disposal. Indeed, spacecraft and space habitats have limited space, and any system designed for life support must be compact and efficient [12]. Black soldier fly larvae can be reared in relatively small containers [13], making them suitable for space environments where space is at a premium. Furthermore, the larvae of this species are rich in protein and fats [14,15], making them a potential source of nutrition for the crew. In space, traditional food supplies can be limited and expensive to transport [16]. Rearing these larvae could provide a sustainable and protein-rich food source for astronauts, reducing the reliance on Earth-sourced supplies. Incorporating black soldier fly larvae into BLSSs can create a circular economy where waste materials are converted into valuable resources. The larvae consume waste, grow in mass, and then can be harvested as a nutritious food source or used as feed for other an-

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imals. This closed-loop system aligns with the principles of sustainability and resource efficiency [17]. In addition to waste reduction, black soldier fly larvae can be used to bioconvert plant-based biomass into valuable compounds [18,19]. This could be particularly useful for using plant material that might be grown as part of a bioregenerative agriculture system in space. Employing these larvae in space provides a practical opportunity to study closed ecological systems, mimicking natural ecosystems and offering insights applicable to sustainability on Earth.

Studying the interaction of *H. illucens* larvae with lunar regolith (the layer of loose, fragmented material covering the solid bedrock on the Moon) is important especially in the context of future lunar colonization and long-duration space missions. In particular, the prepupae, which represent the final immature stage, exhibit a notable tendency to migrate from their food source towards drier substrates to undergo successful pupation and transform into adult flies [10,20]. Prepupae at this stage are at their largest size and have significant protein and fat content to support metamorphosis [21,22]. There are subtle morphological changes, such as a downward-curving labrum that helps them move to a suitable pupation site [23]. Lunar regolith could potentially be used as a growth medium for *H. illucens* at this stage. Understanding if *H. illucens* can pupate and thrive in lunar regolith, after migrating from the food source, could play a vital role in advancing their maintenance on the Moon, thus contributing to the development of sustainable and self-sufficient lunar habitats, reducing the need for continuous resupply missions from Earth. In addition, exploring the way model organisms engages with lunar regolith can provide valuable insights into the adaptability of life in space environments [24]. It may help answer questions about the potential for life to thrive on other celestial bodies, and it could inform future experiments involving other organisms on lunar or martian regoliths.

This study aims at assessing the effects of a lunar regolith simulant on the pupation process and survival of *H. illucens*. Indeed, the pupal phase plays a crucial role in the life cycle of holometabolous insects, as it encompasses roughly half of the total immature developmental period [25,26]. Soil composition is pivotal for many insect species in influencing larval migration within the soil, ultimately affecting the survival of both larvae and pupae [27–29]. However, there are no studies that have investigated the pupation rates and emergence success of insect larvae in lunar regolith. Terrestrial soils are a complex system of organic materials, minerals, gases, liquids, and their interplay [30], which are markedly distinct from lunar soil [31], generally considered an harsh substrate for life [32]. Studying the life cycle of *H. illucens* on lunar regolith not only can advance our understanding of biology and ecology, but also can have practical implications for future lunar exploration, colonization, and sustainability.

2. Materials and methods

2.1. *Hermetia illucens* rearing

Adult flies were reared in laboratory conditions (27 ± 1.0 °C, 60 ± 10 % RH, 12:12 L:D) in nylon mesh fabric cages (90 × 50 × 50 cm) and fed *ad libitum* with water and sugar cubes. Oviposition occurred on corrugated cardboard surfaces that were subsequently collected and placed directly onto the substrate used for rearing the larvae. Larvae were housed in plexiglass boxes measuring 25 × 15 × 10 cm and nourished with standard Gainesville diet [13]. Pupae were transferred in ventilated containers and the newly emerged adults moved to adult cages three times a week.

2.2. Substrates types

The influence of four different substrate types (lunar regolith simulant, sand, wood shavings, no-substrate) was assessed on various life history parameters of *H. illucens*.

Lunar regolith simulants are engineered to closely replicate the characteristics of lunar regoliths, encompassing their chemical compositions, mineralogical constituents, particle size distributions, and key engineering attributes [33]. In this study, we employed the LHS-1 Lunar Highlands Simulant, sourced from Space Resource Technologies co-located at the CLASS Exolith Lab in Orlando, Florida, USA. LHS-1 emulates a representative highlands region on the Moon, accurately integrating a precise blend of mineral compositions and rock fragments while aligning with the particle size distribution patterns commonly observed in Apollo mission lunar regoliths [34–36].

As sand substrate we used a non-toxic natural substrate commonly used to set up the bottom of aquariums (fine ivory white quartz, Amtra).

The third substrate used was wood shavings consisting of virgin hardwood of poplar [37].

In Fig. 1 are reported the pictures of the LHS-1 Lunar Highlands Simulant, sand substrate, and wood shavings.

2.3. Experimental design

Different substrate treatments were added to distinct plastic containers with dimensions of 60 mm in diameter and 20 mm in height, filling each container by 5 mm on a wet piece of filter paper (Whatman Limited, Maidstone, Kent, United Kingdom) to keep insects from drying out, except for the treatment with no-substrate [37]. Prepupal larvae, visibly recognizable by their change in color from cream to black [38], were individually located into containers with the different substrate types. To prevent escape, the containers were covered with transparent chiffon fabric having a mesh size of 0.05 mm. Experiment were conducted with the same laboratory conditions used for rearing the insects

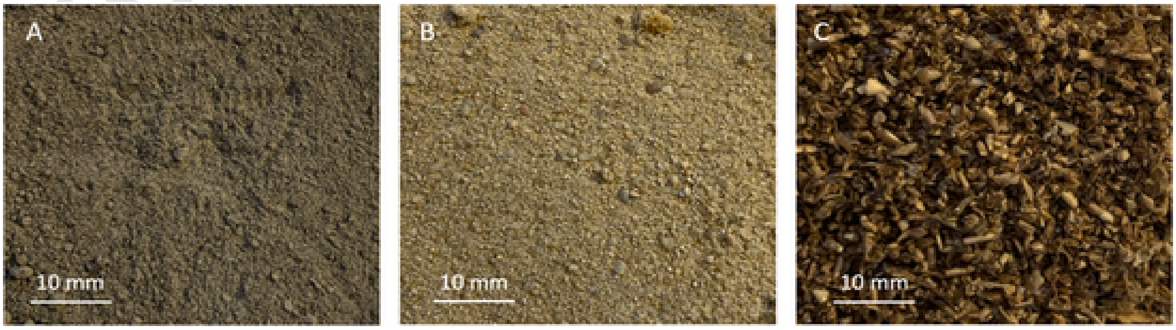

Fig. 1. Pictures of the LHS-1 Lunar Highlands Simulant (A), sand substrate (B), and wood shavings (C) used in the experiments.

to ensure optimal climatic settings. For each substrate treatment we recorded: i) the number of larvae that successfully pupated, defined as when larvae become completely rigid exhibiting no elasticity [37]; ii) time to reach the pupal stage (e.g. from the location of the prepupal larvae in the container until their complete pupation); iii) the duration of pupal stage, (e.g. from pupation until adult emergence); iv) the number of emerged adults.

Fig. 2 shows a fifth instar larva, a prepupal stage, and a pupal stage of *H. illucens*.

Insects were overseen every 12 h to record the pupation and adult emergence time. In Fig. 3 is represented a newly emerged *H. illucens* adult and its old pupa after having spent the pupation period on LHS-1.

For each substrate type 90 insects were tested.

2.4. Statistical analysis

The dataset pertaining to the influence of different substrate treatments on critical developmental stages of *H. illucens* individuals did not conform to the assumptions of normal distribution and homoscedasticity. This was validated through rigorous statistical testing, with results indicating a lack of normality (Shapiro–Wilk test, $p < 0.05$) and heteroscedasticity (Levene's test, $p < 0.05$). Consequently, non-parametric statistical techniques were employed to examine and interpret the data. In particular, the Kruskal-Wallis test was applied, and subsequently, the Steel-Dwass test was employed to assess the influence of distinct substrate treatments on both the time required to reach the pupal stage and the duration of the pupal stage. A probability threshold of $p < 0.05$ was employed to assess the importance of disparities between values.

For evaluating the effect of different substrate treatments on the number of larvae successfully undergoing pupation and the number of emerged adults, a generalized linear model (glm) utilizing a binomial distribution was employed. This model is represented as $y = X\beta + \varepsilon$, where 'y' denotes the vector of observations, which encompass the outcomes of successful and unsuccessful pupation as well as the emergence of individuals. The matrix 'X' signifies the incidence matrix, 'β' stands for the vector of fixed effects, relating to the substrate treatment, and 'ε' represents the vector encompassing random residual effects. In order to determine the significance of disparities between the observed values, a predetermined probability threshold of $p < 0.05$ was employed for assessment. For the significance of differences between values, a probability level of $P < 0.05$ was used.

Fig. 2. From left to right: fifth instar larva, prepupal stage (sixth instar larva), and pupal stage of *Hermetia illucens*.

Fig. 3. An adult *Hermetia illucens* individual just emerged from the pupa exposed to the LHS-1 Lunar Highlands Simulant.

Box plots illustrating the data were generated by using MATLAB R2021b.

The analysis of all data was conducted using R software 4.2.0.

3. Results

Our findings uncovered noteworthy variations in the pupation process and survival of *H. illucens* across the substrates tested, shedding light on the adaptability and responsiveness of this species to environmental factors. This section offers a comprehensive analysis of the pronounced effects observed in the context of substrate-driven developmental alterations, and in particular on the response exhibited in the presence of LHS-1.

All larvae tested successfully pupated regardless of the type of substrate they were exposed. However, the time to reach the pupal stage was significantly affected by different substrate treatments ($\chi^2 = 93.55$, *d.f.* $= 3$, $P < 0.0001$). *H. illucens* prepupal larvae located in the container with no-substrate pupated significantly later than those located in containers containing LHS-1 ($Z = 6.91$; $P < 0.0001$), sand ($Z = 6.79$; $P < 0.0001$), and wood shavings ($Z = 8.86$; $P < 0.0001$). In addition, prepupal larvae exposed to the lunar regolith pupated later than insects exposed to the wood shavings ($Z = 3.2$; $P = 0.0074$), (Fig. 4).

The duration of pupal stage was importantly different based on different substrate treatments ($\chi^2 = 59.75$, *d.f.* $= 3$, $P < 0.0001$). Particularly, adult emergence from pupae occurred significantly earlier in presence of the no-substrate treatment than when pupae were exposed to LHS-1 ($Z = -6.48$; $P < 0.0001$), and sand treatments ($Z = -5.72$; $P < 0.0001$). When insects were exposed to LHS-1, pupal duration was significantly longer than when exposed to the wood shavings ($Z = 4.95$; $P < 0.0001$). Pupal duration lasted significantly more in presence of sand than wood shavings ($Z = 3.86$; $P = 0.0006$), (Fig. 5).

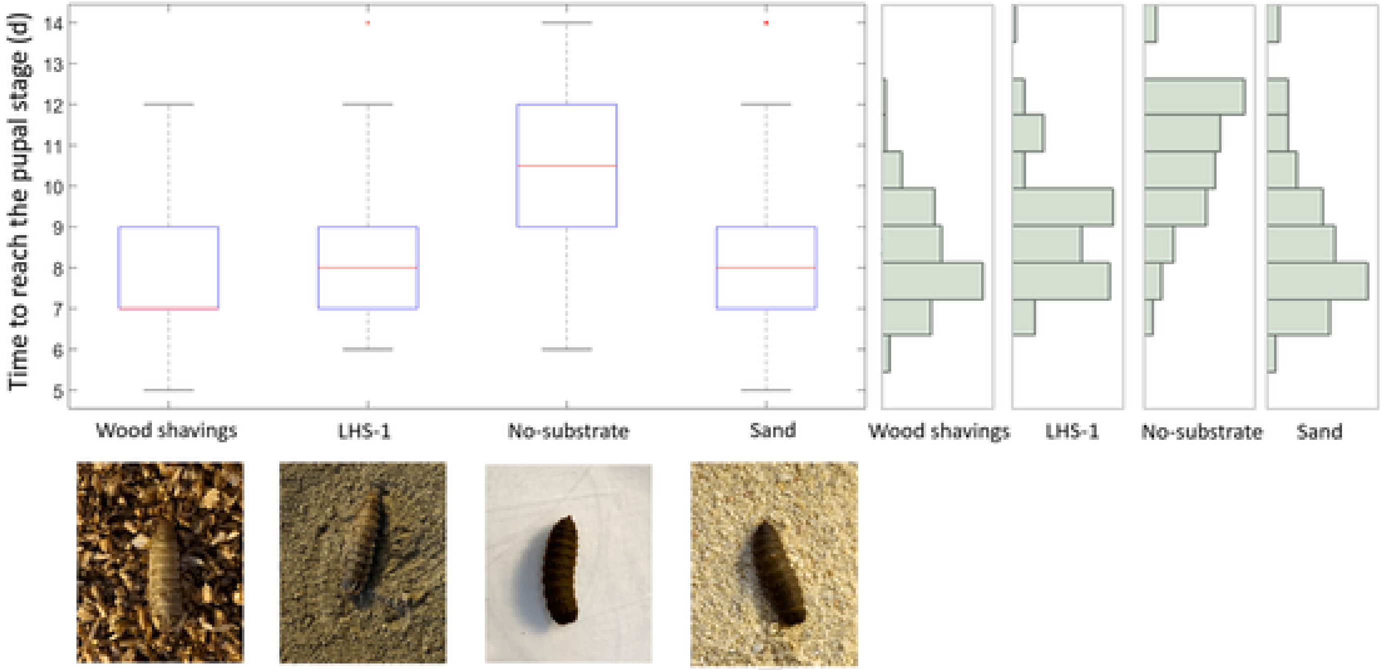

Fig. 4. Time needed to reach the pupal stage in prepupal larvae of *Hermetia illucens* exposed to different substrate treatments (e.g., wood shavings, LHS-1 Lunar Highlands Simulant, sand, and no-substrate). Each box plot displays the median (represented by the red line) along with the range of data dispersion (including lower and upper quartiles, as well as any outliers). Additionally, each box plot is accompanied by adjacent histograms illustrating the distribution of the data. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

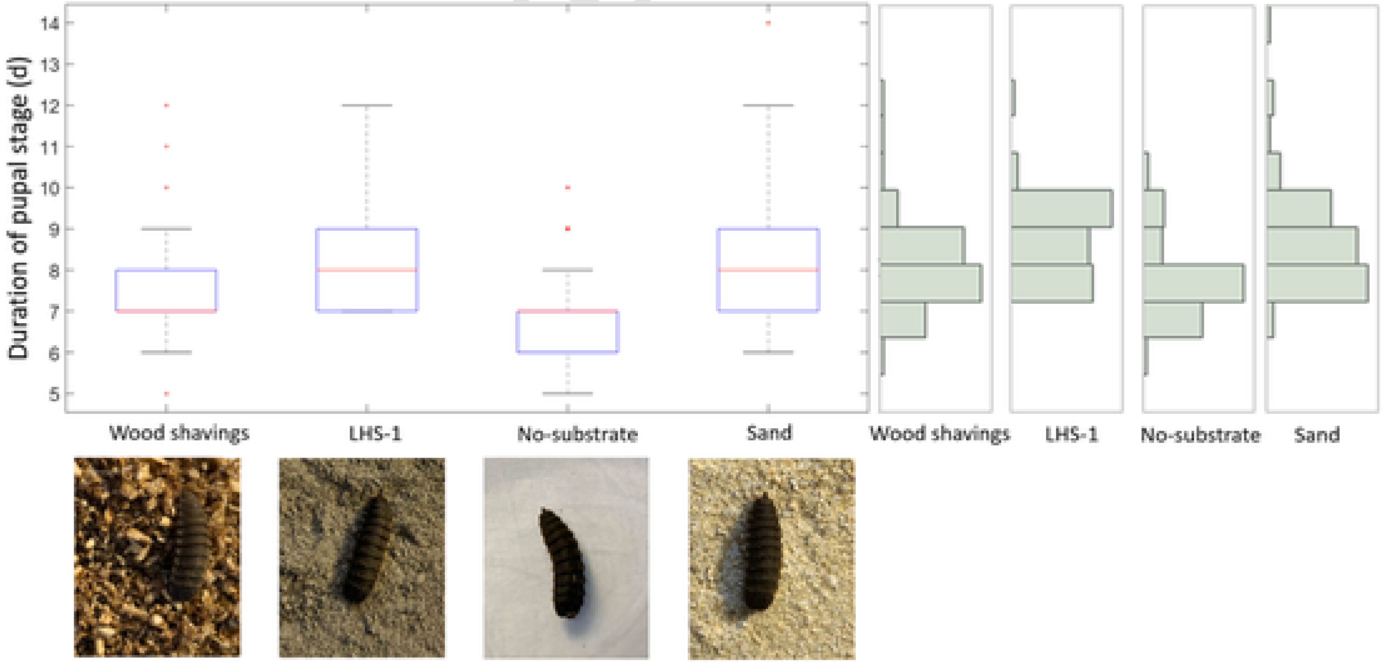

Fig. 5. Pupal stage duration in *Hermetia illucens* exposed to different substrate treatments (e.g., wood shavings, LHS-1 Lunar Highlands Simulant, sand, and no-substrate). Each box plot displays the median (represented by the red line) along with the range of data dispersion (including lower and upper quartiles, as well as any outliers). Additionally, each box plot is accompanied by adjacent histograms illustrating the distribution of the data. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Different substrate treatments had a significant impact on the number of adults that successfully emerged from pupae ($\chi^2 = 8.58$; *d.f.* $= 3$; $P = 0.03$). In particular, the exposure to LHS-1 led to a higher number of emerged adults compared to the exposure to no-substrate ($\chi^2 = 4.58$; *d.f.* $= 1$; $P = 0.03$). A higher number of emerged adults was also observed when pupae were exposed to sand compared to no-substrate ($\chi^2 = 4.57$; *d.f.* $= 1$; $P = 0.03$). When pupae were exposed to wood shavings a higher number of adults emerged compared to no-substrate ($\chi^2 = 6.27$; *d.f.* $= 1$; $P = 0.01$), (Fig. 6).

4. Discussion

The results of this study provide unique insights into the adaptability of *H. illucens* to different substrates and, more specifically, its potential to pupate and thrive on lunar regolith simulants. These findings have significant implications for BLSSs in space exploration and may shed light on the broader adaptability of life in extraterrestrial environments [1,6,39]. This research is pivotal for BLSSs, as it aligns perfectly with the strategic vision of leveraging useful organisms like *H. illucens* to enhance waste management and nutrition [2,40]. Ultimately, this would reduce reliance on Earth resupply missions, paving the way for sustainable lunar habitats and the development of BLSSs.

One of the key findings we found is that *H. illucens* prepupal larvae successfully pupated regardless of the substrate they were exposed to. This resilience of pupation in various substrates aligns with the generalist nature of the species, known for its ability to adapt to different environments and consume a wide range of organic materials [9,41].

The observed differences in the time to reach the pupal stage highlight the potential influence of substrate type on the developmental timeline of *H. illucens*. Prepupal larvae located in containers with no substrate exhibited delayed pupation compared to those in the presence of LHS-1, sand, or dry larval diet. This delay may be attributed to the need for prepupal larvae to search for drier substrates conducive to pupation, as reported by Diener et al. [10,20]. It is worth noting that while pupation time varies, the larvae's ability to adapt and complete their life cycle in different substrates underscores their versatility in closed environments like spacecraft and space stations.

The duration of the pupal stage, a crucial period in the life cycle of holometabolous insects [26], also exhibited variations based on substrate type. Pupae exposed to lunar regolith simulants and sand remained in the pupal stage for a longer period than those in the presence of no substrate, and dry larval diet. These differences may reflect the suitability of each studied substrate for pupal development. The accelerated pupal emergence, resulting from the absence of a suitable substrate, is likely linked to the behavior of postfeeding larvae expending excessive energy in searching for an appropriate pupation site. Consequently, they may have lacked the necessary energy reserves to undergo a robust metamorphosis [37,42]. Furthermore, the longer pupal duration observed in the lunar regolith and sand treatments suggests that these substrates may pose other challenges or provide unique conditions that influence the development of *H. illucens*.

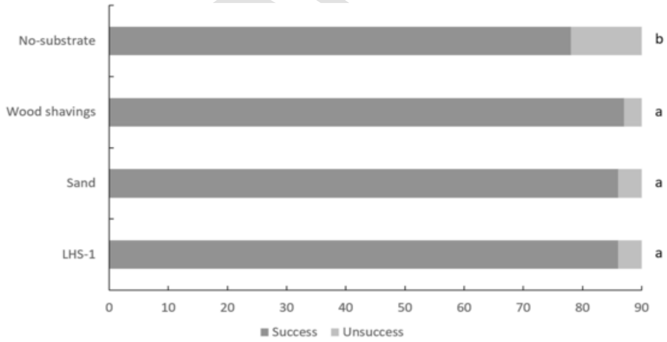

Fig. 6. Impact of different substrate treatments (e.g., wood shavings, LHS-1 Lunar Highlands Simulant, sand, and no-substrate) on the successful emergence of adult *Hermetia illucens* from their pupal stage. Different letters denote statistically significant variations.

The number of emerged adults was significantly affected by substrate treatments, with the presence of LHS-1 leading to a higher number of emerged adults compared to the no-substrate treatment. This result suggests that lunar regolith may provide a favorable environment for the transition from pupae to adults. Moreover, the sand and wood shavings treatments also resulted in a higher number of emerged adults compared to the no-substrate treatment. This implies that various substrates can influence the survival and emergence of adult *H. illucens* differently, confirming previous research [37].

The success of *H. illucens* in pupating and emerging from different substrates underscores their potential as waste management agents and a sustainable source of nutrition for long-duration space missions [40]. Their adaptability to diverse substrates, including lunar regolith simulants, hints at the feasibility of utilizing lunar regolith in the development of BLSSs for the Moon colonization and extended space exploration. These findings could significantly reduce the reliance on resupply missions from Earth and contribute to the establishment of self-sufficient lunar habitat [43,44].

Additionally, this study's implications extend beyond space exploration. Understanding how model organisms like *H. illucens* interact with lunar regolith can offer insights into the adaptability of life in space environments [45,46]. It may also inform future experiments involving other organisms and lunar or martian regolith, addressing broader questions about the potential for life to thrive on celestial bodies beyond Earth [47,48].

Overall, this study provides critical data on the influence of different substrates, including lunar regolith simulants, on the development and survival of *H. illucens*. These findings hold promise for advancing BLSSs in space, reducing waste, and enhancing the sustainability of lunar habitats. Further research into the specific mechanisms underlying these substrate-driven effects could uncover novel insights into the adaptability of life in extraterrestrial environments, supporting the goals of space exploration and advancing ecological science on Earth.

5. Conclusions

Our study demonstrates the adaptability of *H. illucens* to a lunar regolith simulant, shedding light on its potential role in BLSSs for space exploration. The prepupal larvae exhibited resilience by successfully pupating in all tested substrates, emphasizing the versatility of this species in closed environments such as spacecraft. Substrate type influenced pupation timing and pupal duration, with lunar regolith simulant exhibiting notable impacts. Despite variations, the larvae's ability to complete their life cycle in diverse substrates underscores their suitability for waste management and nutrition in space habitats. The high number of emerged adults in lunar regolith simulant suggests its potential as a favorable environment for the transition from pupae to adults. These findings hold significant implications for sustainable lunar colonization, reducing reliance on Earth resupply missions, and advancing BLSSs. Moreover, the adaptability of *H. illucens* to lunar regolith provides insights into life's potential adaptability in space environments, guiding potential future experiments on celestial bodies. This study contributes critical data to enhance our understanding of substrate-driven effects on *H. illucens* development, supporting the goals of space exploration and ecological science in both terrestrial and extraterrestrial contexts.

Uncited reference

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CRediT authorship contribution statement

Donato Romano: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing. **Adriano Di Giovanni:** Data curation, Investigation, Resources, Writing – review & editing. **Cesare Stefanini:** Formal analysis, Investigation, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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