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RESEARCH ARTICLE

EFFECT OF COMPOST SUBSTRATE COMPOSITION ON SPERM CHARACTERISTICS AND REPRODUCTIVE ADAPTABILITY OF *EISENIA FETIDA* AND *PERIONYX EXCAVATUS*

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ABSTRACT

Earthworms play a pivotal role in organic waste management and soil fertility enhancement through their burrowing and feeding activities. Among various parameters influencing their efficiency in vermicomposting, reproductive potentialparticularly sperm characteristics serves as a key indicator of species fitness and adaptability to specific substrate environments. This study investigates the impact of different compost mixtures on sperm concentration, viability, and morphology in two commonly used vermicomposting earthworm species: *Eisenia fetida* and *Perionyx excavatus***.** Three compost treatments were prepared using combinations of cow dung and sugarcane bagasse (T1), Horse dung and sugarcane bagasse (T2), and a mixture of both cow and horse dung with sugarcane bagasse (T3), along with a control group reared in garden soil (T0). Sperm were isolated from mature clitellated individuals after a 60-day rearing period under controlled conditions. Sperm parameters were assessed using standard hemocytometric and viability staining techniques, while morphological abnormalities were evaluated microscopically. Results indicated a significant variation in sperm count and viability across treatments, with the highest sperm concentration and viability observed in the T3 group, followed by T1 and T2. The control group showed the lowest values. Notably**,** *Perionyx excavatus* consistently exhibited higher reproductive indices than *Eisenia fetida* under identical composting conditions. Statistical analysis confirmed that substrate composition significantly influences sperm physiology, suggesting that mixed dung substrates optimize the reproductive environment for earthworms ($p < 0.001$). These findings highlight the relevance of sperm analysis as an early indicator of reproduction and compost efficiency in earthworms. The study contributes to the growing understanding of substrate-dependent reproductive modulation and offers practical implications for optimizing vermiculture systems through tailored compost formulations.

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INTRODUCTION

Earthworms are considered crucial bioengineers in terrestrial ecosystems owing to their vital roles in soil structure formation, organic matter decomposition, and nutrient cycling (Edwards & Bohlen, 1996). Their presence enhances soil aeration, porosity, and fertility, thereby sustaining agricultural productivity. In addition to their ecological significance, earthworms have emerged as essential model organisms for environmental monitoring and waste management, particularly in vermitechnology (Domínguez & Edwards, 2004). Among the numerous aspects of earthworm biology, reproductive physiologyespecially sperm production and qualityis a critical determinant of population dynamics, ecological adaptation, and composting efficiency (Garg *et al.,* 2006; Tripathi & Bhardwaj, 2004). Reproduction in earthworms is a complex physiological process involving the production of hermaphroditic gametes, copulation, cocoon formation, and hatching. Sperm quality and viability directly influence fertilization success, cocoon viability, and the long-term sustainability of earthworm populations in both natural and artificial environments (Reinecke & Viljoen, 1990). Spermatozoa, being highly sensitive to environmental conditions, serve as valuable indicators of physiological stress and environmental toxicity. Parameters such as sperm count, viability, and morphology have been used as biomarkers in various studies evaluating the impact of pollutants, feed quality, and substrate composition (Snook, 2005; Selvan *et al.,* 2012).

Substrate quality and composition significantly affect earthworm reproduction. Organic manures, especially animal dung, serve as nutrient-rich substrates for earthworms, facilitating faster growth and higher reproductive outputs (Yasir & Garg, 2011). Cow dung is widely acknowledged as a superior substrate due to its balanced C:N ratio, rich microbial flora, and moisture-holding capacity (Gajalakshmi & Abbasi, 2004). Similarly, horse dung, though slightly fibrous and high in cellulose, contributes to enhanced aeration and microbial diversity when combined with other substrates. The addition of agro-industrial residues like sugarcane bagasse further improves substrate porosity, water-holding capacity, and microbial colonization, thereby optimizing the microenvironment for earthworm activity (Suthar, 2009).

Previous studies have demonstrated that feed quality influences not only the somatic growth and reproduction rate of earthworms but also gamete physiology (Edwards *et al.,* 1998). However, while cocoon production and hatchling rate have been widely studied as indicators of reproductive success, there is relatively limited focus on **sperm characteristics** such as concentration, viability, and morphology. These microscopic parameters are crucial for early-stage reproductive assessment and provide insights into spermatogenesis efficiency under varying environmental and nutritional conditions. Species-specific responses to substrate quality also play a pivotal role in vermiculture practices. *Eisenia fetida* and *Perionyx excavatus* are two prominent epigeic earthworm species extensively used in composting due to their high consumption rates and rapid reproductionto organic substrates (Ismail *et al.,* 2005). While *E. fetida* has been widely employed in European and temperate regions, *P. excavatus* has shown remarkable adaptability and reproductive success in tropical conditions, making it a preferred species in South Asian composting systems (Sinha *et al.,* 2008). Comparative studies between these two species in response to substrate variations can provide valuable insights into their reproductive strategies and optimization in waste management systems. The quality of spermatozoa, as affected by compost composition, has not yet been extensively investigated in the context of vermicomposting. Studies in related invertebrate models have established that oxidative stress, nutritional imbalance, and exposure to toxins can lead to sperm abnormalities, reduced motility, and diminished fertility (Gage *et al.,* 2002; Lewis & Austad, 1994). Extrapolating from such findings, it can be inferred that substrate conditionsespecially those influencing oxidative balance and energy metabolismplay a critical role in determining sperm health in earthworms.

Moreover, recent interest in biochemical and molecular markers of reproduction in invertebrates has further emphasized the need for detailed studies on reproductive physiology under controlled substrate conditions. Sperm analysis provides a rapid and quantifiable approach to assess the reproductive potential of composting species before reaching the cocooning stage, thus allowing early intervention and optimization of compost inputs. Against this background, the present study aims to evaluate the influence of different compost combinationson sperm concentration, viability, and morphology in selected earthworm species. By examining these parameters, this research seeks to bridge the knowledge gap between compost composition and earthworm reproductive physiology, contributing not only to fundamental ecological understanding but also to practical improvements in vermitechnology and sustainable organic waste management.This work is expected to provide novel insights into the cellular-level responses of earthworms to substrate environments and may contribute to the development of **species-specific substrate protocols** that maximize reproduction and biomass turnover in vermiculture operations.

MATERIALS AND METHODOLOGY

Earthworm Samples: Adult clitellated specimens of *Eisenia fetida* and *Perionyx excavates* was selected for the study due to their known reproductive adaptability and common use in vermicomposting (Reinecke & Viljoen, 1991). Earthworms were sourced from a laboratory-maintained culture and acclimatized to standard laboratory conditions prior to experimentation.

Compost Substrate Treatments: Four compost substrates were prepared for assessing the influence on sperm characteristics: T1 – Cow dung mixed with sugarcane bagasse (CD+SB); T2 – Horse dung mixed with sugarcane bagasse (HD+SB); T3 – A combination of cow dung, horse dung, and sugarcane bagasse (CD+HD+SB); T4 – Control group using plain soil with minimal organic content. Each treatment was prepared in triplicate and replicated five times per species (n = 5), ensuring statistical robustness.

Pre-treatment and Gut Clearance: Sexually mature earthworms were carefully rinsed with distilled water to remove adhering soil or compost particles. They were then transferred onto moist filter paper for 12–24 hours to facilitate gut clearance. This procedure is essential to avoid contamination during dissection (OECD, 2008).

Dissection and Sperm Extraction: Dissections were performed under a dissecting microscope. Earthworms were immobilized in petri dishes containing phosphate-buffered saline (PBS, pH 7.4). A dorsal incision was made between segments 10–15 to expose the seminal vesicles—key reproductive structures containing spermatocytes and mature spermatozoa (Chakrabarty & Bhattacharya, 2012). The seminal vesicles were carefully excised and gently crushed in ~100 µL of PBS within an Eppendorf tube to release sperm. The suspension was filtered through fine mesh or centrifugation briefly to remove tissue debris.

Sperm Concentration Estimation: Sperm concentration was quantified using a hemocytometer (Neubauer chamber). A 10 µL aliquot of sperm suspension was loaded into the chamber, and sperm cells were counted under a compound microscope at 400× magnification. The concentration (in cells/mL) was calculated using the standard hemocytometer formula: Sperm count = Average count per square × dilution factor × 10^4 (cells/mL) (Jamieson, 1981). Sperm Viability Assay: Viability of sperm cells was assessed using Trypan Blue (0.4%) staining. Equal volumes of sperm suspension and dye were mixed and incubated for 2–3 minutes. A 10 µL sample was mounted on a glass slide, covered with a cover slip, and examined under the microscope. Live sperm remained unstained, while dead sperm took up the dye- blue of Trypan Blue(Garg & Kaushik, 2005). Viability percentage was calculated as: Viability (%) = (Live sperm / Total sperm) × 100.

Sperm Morphology Analysis: Morphological analysis of sperm was conducted using Eosin-Nigrosin staining. Sperm smears were air-dried, fixed in methanolfor 5 minutes, and stained for 10 minutes. Slides were examined under oil immersion at 1000× magnification to identify abnormalities in the head, midpieceor tail (Chakrabarty & Bhattacharya, 2012).

Statistical Design and Replication: Each compost treatment was replicated five times per species, resulting in robust datasets for statistical analysis. Parameters such as sperm concentration (×10^6 cells/mL), sperm viability (%), and percentage of abnormal morphology were compared between treatments using ANOVA and t-tests to identify significant differences among substrate compositions using IBM SPSS 21 (Reinecke & Viljoen, 1991; OECD, 2008).

RESULTS

The sperm concentration varied significantly across the different compost treatments in both *Eisenia fetida* and *Perionyx excavates* (Fig.1 and Fig.2). Among the treatments, T3 recorded the highest mean sperm concentration, with *E. fetida* showing a value of 61.20 ± 1.21 ×10^6 cells/mL, and *P. excavatus* showing *65.90 ± 1.42 ×10^6 cells/mL. This was followed by T1, where the mean sperm concentration for *E. fetida* was 58.40 ± 1.52 ×10^6 cells/mL, and *P. excavatus* recorded *62.70 ± 1.83 ×10^6 cells/mL. In T2, slightly lower values were observed: *E. fetida* at 53.60 ± 1.47 ×10^6 cells/mL, and *P. excavatus* at *60.10 ± 1.60 ×10^6 cells/mL. The lowest sperm concentration was recorded in the control group (T4), where only minimal organic matter was provided. In this group, *E. fetida* showed a sperm count of 37.80 ± 1.34 ×10^6 cells/mL, and *P. excavatus* showed 41.60 ± 1.25 ×10^6 cells/mL(Table.1). Sperm viability followed a similar trend to sperm concentration, with the highest percentages observed in the T3 treatment. *Eisenia fetida* in T3 exhibited a sperm viability of 89.80 ± 1.22%, while *P. excavatus* showed 91.50 ± 0.88%. In T1, the viability was slightly lower, with *E. fetida* with 85.60 ± 1.10%, and *P. excavatus* showing 88.70 ± 0.90%. T2 displayed a mean sperm viability of 83.20 ± 0.98% for *E. fetida* and 87.30 ± 1.10% for *P. excavates.* The control treatment (T4) again showed the lowest sperm viability, with *E. fetida* recording 68.40 ± 1.60%, and *P. excavatus* 72.10 ± 1.48% (Table.1). The one-way ANOVA test revealed that the differences in Sperm viability and sperm concentration among the treatments were highly significant ($p < 0.001$) for both species. Post hoc analysis using Tukey's HSD indicated that T3 was significantly superior to all other treatments, while T1 and T2 were statistically similar but better than the control. A reverse trend was observed in the percentage of abnormal sperm morphology (Fig.1 and Fig.2), where T4 (control) recorded the highest proportion of abnormal sperm cells, and T3 had the lowest. In *E. fetida,* the highest abnormal morphology was 12.8 ± 0.72% in T4, followed by 7.1 ± 0.61% in T2, 6.4 ± 0.58% in T1, and 5.6 ± 0.39% in T3. Similarly, *P. excavatus* showed 11.2 ± 0.67% abnormalities in T4, 6.2 ± 0.49% in T2, 5.8 ± 0.43% in T1, and the lowest (4.9 ± 0.37%) in T3 (Table.2). The differences in abnormal sperm morphology were also found to **be** highly significant (p < 0.001) by ANOVA for both species. Tukey's HSD test revealed that the control group (T4) had a significantly higher percentage ofsperm abnormalities compared to the other treatments, while T3 had significantly fewer abnormalities than all other groups. Across all treatments, *Perionyx excavatus* consistently showed higher sperm concentration and viability, and lower abnormal morphology percentages compared to *Eisenia fetida*, although the differences between the species were not statistically analyzed in this experiment. However, observationally, *P. excavatus* appeared to be more responsive to enriched compost environments. The results clearly demonstrate that the quality and nutrient richness of the compost substrate play a crucial role in influencing the reproductive health of earthworms. The combined dung treatment (T3) offered a more balanced and enriched medium, significantly enhancing sperm production and viability while reducing abnormalities. This suggests a synergistic effect of cow and horse dung combined with sugarcane bagasse, likely due to improved microbial activity, moisture retention, and nutrient profile.

Table 1. Sperm Concentration (×10⁶ cells/mL) and Sperm viability (%) in different treatments using *E. fetida* and *P. excavatus* (Mean ± SE) (P value- 0.001)

Treatment (n=5)	Sperm Concentration		Sperm viability	
	E. fetida	**P. excavatus**	*E. fetida*	**P. excavatus**
T1 (CD+SB)	58.40 ± 1.52	62.70 ± 1.83	85.60 ± 1.10	88.70 ± 0.90
T2 (HD+SB)	53.60 ± 1.47	60.10 ± 1.60	83.20 ± 0.98	87.30 ± 1.10
T3 (CD+HD+SB)	61.20 ± 1.21	65.90 ± 1.42	89.80 ± 1.22	91.50 ± 0.88
T4 (Control)	37.80 ± 1.34	41.60 ± 1.25	68.40 ± 1.60	72.10 ± 1.48
F- Value	35.42	42.10	47.92	51.75

Table. 2: Abnormal Sperm Morphology (%) in different treatments using *E. fetida* and *P. excavatus* (Mean ± SE) (P value- 0.001)

Treatment (n=5)	*E. fetida*	*P. excavatus*
T1 (CD+SB)	6.4 ± 0.58	5.8 ± 0.43
T2 (HD+SB)	7.1 ± 0.61	6.2 ± 0.49
T3 (CD+HD+SB)	5.6 ± 0.39	4.9 ± 0.37
T4 (Control)	12.8 ± 0.72	11.2 ± 0.67
F-Value	39.88	43.17

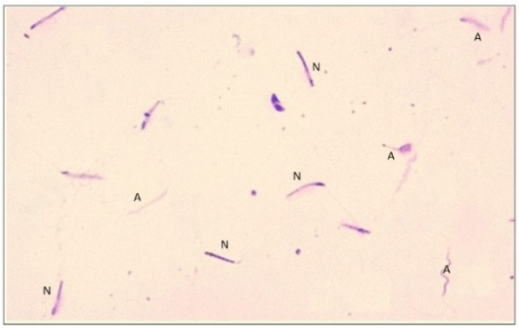

Fig. 1: Sperm morphology of *Eisenia fetida* Normal sperm (N) and Abnormal sperm (A) (100 X)

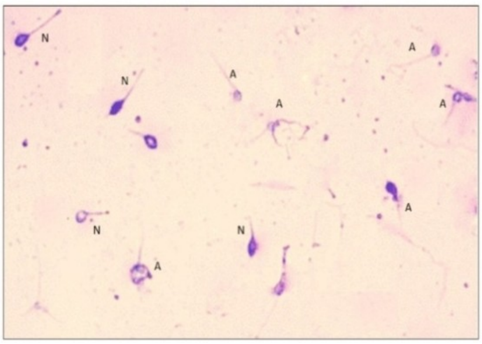

Fig. 2. Sperm morphology of *Perionyx excavates* Normal sperm (N) and Abnormal sperm (A) (100 X)

DISCUSSION

The present study evaluated the impact of different compost compositions—namely cow dung + bagasse (T1), horse dung + bagasse (T2), and a mixture of cow dung + horse dung + bagasse (T3)- on the sperm concentration, viability, and morphology in two epigeic earthworm species, *Eisenia fetida* and *Perionyx excavatus*. The findings revealed significant treatment-dependent variations across all studied parameters, with the T3 treatment consistently producing superior results. This outcome emphasizes the profound influence of organic substrate composition on reproductive performance in earthworms. Sperm concentration was found to be significantly enhanced in worms cultured in T3 compost. The superior sperm count in this group could be attributed to the balanced nutrient availability and synergistic microbial activity provided by the combination of cow and horse dung along with sugarcane bagasse. Cow dung is known to be rich in nitrogen and harbor a diverse microbial population, while horse dung adds fibrous bulk and boosts bacterial diversity, collectively creating an optimal environment for earthworm physiology and reproduction (Edwards & Bohlen, 1996; Bhat *et al.,* 2018). Moreover, sugarcane bagasse likely contributed to improved aeration and moisture retention, both of which are crucial for maintaining a healthy compost microenvironment that supports earthworm activity and gametogenesis (Gajalakshmi & Abbasi, 2004). Higher sperm production under nutrient-enriched conditions aligns with the findings of Garg *et al.* (2006), who observed increased cocoon production and hatchling rates in nutrient-rich substrates, indicating enhanced reproductive fitness.

The significantly higher sperm viability observed in T3-treated earthworms further supports the hypothesis that '*compost composition directly impacts reproductive physiology'*. Viability is a marker of sperm integrity and mitochondrial functionality (Snook, 2005), which is largely dependent on cellular oxidative stress levels. Rich organic matter in T3 could have supported antioxidant enzyme activity within the worms, reducing reactive oxygen species (ROS)-induced sperm damage (Tripathi & Bhardwaj, 2004). Additionally, a higher organic carbon to nitrogen ratio (C:N) in T3 may have helped regulate the metabolic rate and stress response of earthworms, contributing to improved sperm vitality.These results are consistent with observations by Yasir and Garg (2011), who reported a positive correlation between substrate enrichment and reproductive output in *E. fetida*. Their study suggested that optimal carbon and nitrogen availability enhances reproductive organ development, ultimately improving gamete quality. Abnormal sperm morphology was most prevalent in the control group, which lacked organic enrichment. Morphological defects in sperm such as bent tails, irregular heads, or cytoplasmic droplets are commonly associated with poor nutrition, suboptimal environmental conditions, or chemical stress (Gage *et al.,* 2002). The control group's limited nutrient availability likely led to substandard spermatogenesis due to physiological stress, mimicking nutrient deprivation scenarios (Selvan *et al.,* 2012). Conversely, the enriched environments of T1, T2, and especially T3 seemed to alleviate such stresses, thereby promoting the production of morphologically normal sperm. This aligns with previous findings in invertebrates where diet quality significantly impacted sperm structure and fertility (Lewis & Austad, 1994). Notably, studies in annelids and other oligochaetes have demonstrated that enriched diets contribute to increased germinal cell activity and reduced reproductive anomalies (Sinha *et al.,* 2008). Although both species responded positively to organic enrichment, *Perionyx excavatus* consistently outperformed *Eisenia fetida* in sperm concentration and viability across all treatments. This may be due to the faster metabolic rate and higher reproductive turnover of *P. excavatus*, as documented by Ismail *et al.* (2005). Additionally, *P. excavatus* may have a higher tolerance for variable substrate conditions, making it more adaptive to enriched composts (Edwards *et al.,* 1998). However, both species demonstrated significant reproductive enhancement under enriched treatments, highlighting the universal applicability of such composts in vermiculture systems.

From an ecological perspective, improved sperm health reflects the overall well-being of earthworms in a composting system. Sperm metrics are early biomarkers of reproductive success, which translates to better cocoon production, hatching success, and population sustainability (Reinecke & Viljoen, 1990). Therefore, the use of mixed-dung compost such as T3 not only benefits worm reproduction but may also enhance vermicomposting efficiency and organic matter decomposition**,** leading to more productive and sustainable waste management systems. These findings have practical applications in vermiculture and sustainable agriculture. They suggest that the deliberate selection of compost inputs particularly a combination of animal dung and plant residues can maximize reproductive potential and population buildup of composting earthworm species.

CONCLUSION

In conclusion, the study demonstrates that compost composition significantly affects sperm concentration, viability, and morphology in earthworms especially *Perionyx excavatus* consistently outperformed *Eisenia fetida*. The mixed-dung substrate (T3) offered a nutrient-balanced, microbially active, and moisture-retentive environment, which maximized reproductive outcomes. These findings not only enhance our understanding of earthworm reproductive biology but also reinforce the role of tailored composting inputs in optimizing vermicomposting systems.

Authors' Contribution: Shashank K. R. and Ramyashree M. carried out the experimental work, data collection, and analysis. Mahadevaswamy M. designed the research plan, supervised the study, and provided critical guidance at all stages. All the authors contributed to the preparation and revision of the manuscript and all authors have read and approved the final manuscript.

Conflict of Interest: The authors declare that there is no conflict of interest regarding the publication of this research work.

***Ethical Statement*:** This study did not involve any experiments on higher vertebrates, and therefore no ethical approval was required.

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