# Chapter 1: Brood morphometry and digging behaviour

## 1.1 INTRODUCTION

Insects have a remarkable level of diversity, displaying a vast range and possessing a complicated evolutionary lineage (Sahney et al., 2010; Speight, 2017). Insects fulfill significant roles within the realm of agriculture, acting as pests that have a substantial impact, serving as vectors for important diseases, pollinating crops, acting as parasites to other insects, and functioning as bio-indicators of environmental changes (Mandal et al., 2014; Bouchard et al., 2017). According to Stork et al., (2015) and Paulson et al., (2023), the order Coleoptera, which has an estimated 1.5 million distinct species of beetles, accounts for more than 40% of the total number of documented arthropod species.The Scarabaeidae family, also known as dung beetles, is recognised as one of the largest families within the Coleoptera order, encompassing a remarkable diversity of about 30,000 species worldwide (Banerjee, 2014; Cajaiba et al., 2017), with three subfamilies Aphodiinae, Scarabaeinae, and Geotrupidae with approximately 6,850 species worldwide (Chandra and Gupta, 2012; Stone et al., 2021). The first comprehensive account on the Scarabaeinae of India was published in the 'Fauna of British India, including Ceylon and Burma (Arrow, 1931; Paulian, 1945, 1980, 1983; Balthasar, 1963, 1974; Mikšić, 1977; Endrödi, 1985; Chandra, 1986, 1999; Gupta, 1986; Kabakov, 2006; Krikken, 2009; Sabu et al., 2011). Further research related to the variety of dung beetles were carried out by Chandra and Ahirwar, (2007), Chandra and Singh, (2011), and Chandra and Gupta, (2011, 2012a, 2012b, 2012c). These studies together documented a total of 124 species, which were classified into 45 genera across 11 subfamilies. These studies were undertaken in the regions of Madhya Pradesh and Chhattisgarh. Thakkar and Parikh, (2016) as well as Singhal et al., (2018) have documented a total of 24 dung beetle species in the Vadodara region of Gujarat.

Scarabaeinae (dung beetles) are closely related species that rely on organic materials such as dung, carrion, and decaying fruits for food and reproduction (Scholtz et al., 2009; Simmons and Ridsdill-Smith, 2011).Various studies (Kakkar and Gupta, 2009; Brown et al., 2010; Gullan and Cranston, 2010) have demonstrated that dung beetles possess a diverse array of ecological services, along with both morphological and behavioural adaptations, which contribute to their widespread distribution. Dung decomposition is facilitated by the consumption of dung by these insects. According to

Arenallo et al., (2017), these organisms consume mammalian faeces that are abundant in micro-organisms, which serve as a valuable supply of fibrous material for the purpose of nurturing their larvae.

According to previous studies conducted by Frank et al., (2017) and Pandya et al., (2023), it has been observed that dung beetles exhibit a preference for omnivorous dung over herbivorous dung, with carnivore dung being the least favoured. Several studies have documented a heterogeneous pattern of dung consumption and relocation, which is impacted by various factors including soil characteristics and moisture levels (Nichols et al., 2008), the quality of the dung (Braga et al., 2013), and the cooperative behaviour of pairs (De Groot et al., 2002; Slade et al., 2011; Banerjee, 2014; Tarasov and Dimitrov, 2016; Singh et al., 2019). Based on their foraging behaviour and nest construction strategies, dung beetles can be classified into **four distinct groups**: **Telecoprid** (rollers), which roll small portions of dung away before burying and laying eggs in them; **Endocoprid** (burrowers), which directly lay eggs within the dung; **Kleptocoprids** (dwellers), which lay eggs in dung that has been buried by other dung beetles; and **Paracoprid** (tunnelers), which construct nests beneath the dung pad prior to egg-laying (**Fig. 1.1**) (Halffter and Matthews, 1966; Doube, 1990; Hernández et al., 2011; Chao et al., 2013).

Furthermore, previous studies have confirmed that these organisms possess the capacity to enhance soil fertility, enhance soil permeability, stimulate plant growth, promote seed dispersal, govern parasite growth, and reduce greenhouse gas emissions by utilizing dung for nutrition and reproduction (Latha and Sabu, 2018). Despite the multitude of ecological benefits they provide, human activities have been found to cause a decline in the variety of dung beetles in both forested and pasture environments, as evidenced by studies conducted by Nichols et al., (2009), Basto-Estrella et al., (2014), and Kim et al., (2021). The decrease in dung beetle population and the subsequent fall in ecological services they offer is a matter of concern (Nichols et al., 2008). Therefore, it is crucial to do comprehensive research on dung beetles, since they play a vital role in preserving a balanced and thriving ecosystem (Salomão et al., 2020).

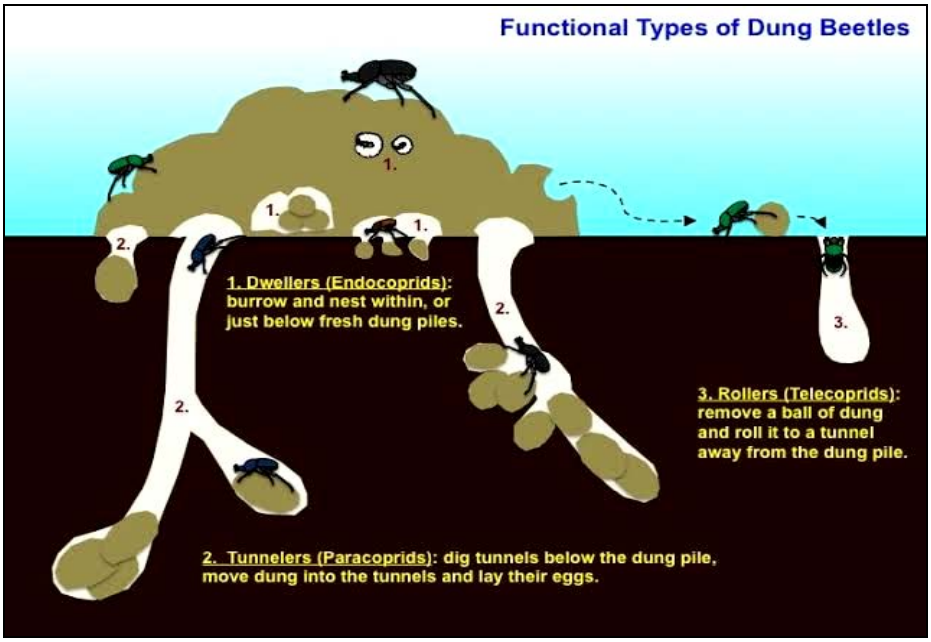



**Figure 1.1:** Dung beetles classified based on their dung relocation strategies (Bertone et al., 2004).

In the life history of dung beetles, the nesting behaviour is a crucial characteristic. Dung beetles exhibit a variety of nesting patterns which ranges from simple ones to highly complex ones (Scholtzet al., 2009; Halffter et al., 2013; Cortez et al., 2021). Further, Heurta et al., (2023) asserted that the Scarabaeinae have seven different nesting patterns (I to VII).

Paracoprids have garnered significant interest as a result of their distinctive nesting strategy. The species under consideration are mostly distributed in forest and agricultural settings worldwide (Andresen, 2005; Sabu et al., 2006; 2007; Venugopal et al., 2012). They construct underground nesting chambers, usually positioned under or adjacent to the food availability, in which they collect and manage food for their offspring. These insects demonstrate nesting patterns categorized as I, II, and III **(Fig. 1.2)**. In the case of simple nests, each gallery is characterized by the presence of a single brood ball. Conversely, compound nests are distinguished by the occurrence of two or more brood balls inside each gallery. Pattern I involve the female creating many brood balls, depositing eggs within them, and afterwards departing from the nest. In contrast, Pattern II entails the female constructing a more substantial brood ball, which includes a layer of dirt put on top of the dung following egg-laying. Following this, the female exits the nest, which now contains two or three brood balls. In pattern III, the female engages

in nest construction, creating many brood balls, and assumes responsibility for the care of the offspring throughout their larval development (Heurta et al., 2023).

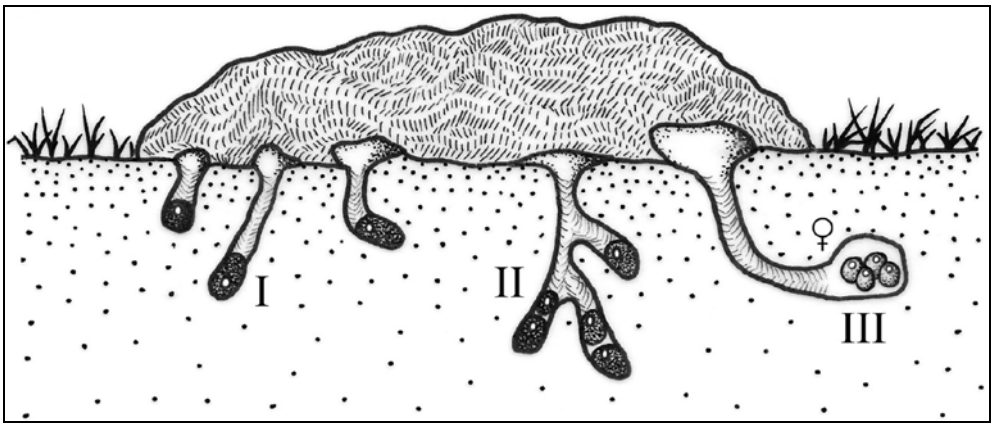



**Figure 1.2:** Nesting patterns I, II, and III (Halffter and Edmonds, 1982).

*Digitonthophagus gazella* (*D. gazella*), a paracoprid species, has a distinct nesting behaviour. The distribution of their presence is evident in numerous countries, such as Africa, America, Australia (Noriega et al., 2020), Arabia, Madagascar, Pakistan, and Sri Lanka (Chandra and Gupta, 2013). Additionally, their occurrence has been documented in various regions of India, including the Vadodara district in Gujarat (Sabu et al., 2011; Chandra et al., 2012; Pawara et al., 2012; Gupta et al., 2014; Thakkar and Parikh, 2016; Singhal et al., 2018). Morphological structure of their homologous legs with their fore tibia has helped them in developing novel ways of resource acquisition, along with burrowing and tunnel making, underneath the dung and soil. The dung beetles engage in excavation of tunnel by digging and supplying dung to their progeny in the shape of brood balls located at the blind end of each tunnel (Pulido and Zunino, 2007; Moczek, 2009; Khadakkar et al., 2019).They construct one or multiple brood balls that are densely packed and covered with soil. The thickness of the soil coating differs among species and may result in various forms such as spheroid, ovoid, or pear (Scholtz et al., 2009; Halffter et al., 2013; Genise, 2017). The coating is believed to serve as a physical barrier, offering defence from predators, parasites, harmful microbes, and desiccation (Scholtz et al., 2009; Halffter et al., 2013; Cantil et al., 2014). Insects commonly employ mechanical or physical protection as a strategy to ensure the safety of their offspring (Hunt and Simmons, 2000; Ayasse and Paxton, 2002; Hilker and Meiners, 2002) with bisexual cooperation between the sexes to some extent. Both, male and female makes the brood ball from the dung source, and roll it together up to a certain distance. Further, males are often seen excavating the tunnel while the females sit on the top of brood ball and rapidly

pat the surface with her front tibiae. Subsequent to the burial of the brood ball, copulation takes place following which the female assumes responsibility for nest maintenance during the larval stage. The male additionally remains within the nest, safeguarding both the brood ball and the female, so preventing other males from engaging in mating behaviour with her (Favila et al., 2005). The reproductive behaviour shown by dung beetles encompass several aspects, including sexual exploration, sexual recognition, competition for mates and resources used for nesting, sperm competition, and parental care (Huerta et al., 2023).

Various kinds of parental care are observed in insects (Tallamy and Wood, 1986; Clutton-Brock, 1991; Trumbo, 2012). Certain species exhibit pre-ovipositional care behaviours prior to the deposition of eggs. These behaviours include a range of activities, involving the provision of food resources, the selection of appropriate sites for egg deposition, the construction of nests, the development of protective structures or chemical defences for eggs and larvae, and the alteration of the surrounding environment (Royle et al., 2012; Smiseth et al., 2014; Machado and Trumbo, 2018). It is not surprising that the majority of dung beetle species allocate a substantial amount of time and energy towards the construction of nests and the safeguarding of their offspring. This involves the deposition of a solitary egg within an egg chamber, which is then sealed (Hunt and Simmons, 2000). The larva resides within the chamber for the duration of its development until it undergoes pupation. Consequently, the preservation of the brood ball's structural integrity is crucial for the larva's survival during its developmental stages. Certain brood balls possess a narrow conduit for aeration, which serves to connect the exterior environment with the egg chamber. In the case of dung beetles, this conduit may be equipped with a filter composed of dung fibres (Cantil et al., 2014). The utilisation of nutrient-rich dung for developing offspring facilitates, distinctive behavioural and physiological adjustments that contribute to sub-sociality and biparental behaviour (Arce et al., 2012; Heurta et al., 2013; Panaitof et al., 2016). This is achieved by offering protection against competition and desiccation (Rauter and Moore, 2002; Kim et al., 2021). Biparental care is a prevalent phenomenon seen in dung beetles belonging to the genus *Onthophagus*, characterised by the presence of a complex network of tunnels (Halffter and Edmonds, 1982). Furthermore, it has been shown that parental provisioning techniques in *D. gazella* may differ depending on parental size, with bigger parents tending to generate larger brood masses (Hunt and Simmons, 2002; Steiger, 2013).

While much is known on the biology of tunnelling dung beetles, only few studies have focused on the nesting and reproductive behaviour of ball-rolling dung beetles, with the account of *D. gazella*. ***Therefore, the present study is an attempt to investigate the nest architecture, understand the brood morphometry and digging behaviour of D. gazella.***

## 1.2 MATERIALS AND METHODOLOGY

### Collection and Identification of Dung Beetle

*Digitonthophagus gazella* (12-14 mm long, 139 mg in weight) were collected from the agricultural fields of Channi (22.363˚N, 73.166˚E), Sindhrot (22.331˚N, 73.063˚E), and Timbi (23.149˚7N, 74.002˚E) of Vadodara city, located in Gujarat (**Fig. 1.3**). Collection of *D. gazella* was carried out during the time of dawn and dusk, in the months of June to November (2020-22). The dung beetles were collected by using the handpick method from the dung pats, and dung heaps, and by digging the soil under the dung pats with the help of shovel/trowel (30 cm) (**Fig. 1.4**), and were brought to laboratory for identification and rearing.

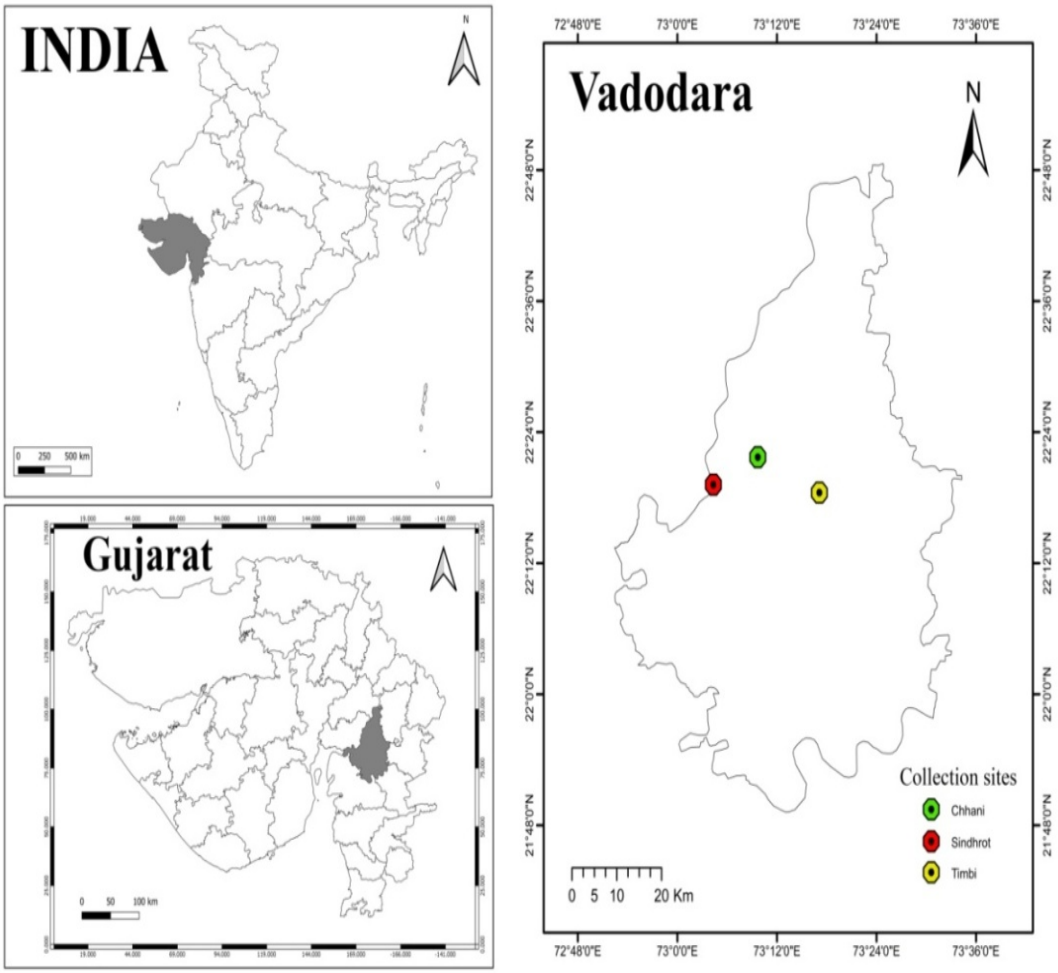



**Figure 1.3:** Collection sites of *D. gazella* from Vadodara district, Gujarat, India. *D. gazella* were collected from the outskirts of Vadodara district, Channi (22.363˚N, 73.166˚E), Sindhrot (22.331˚N, 73.063˚E), and Timbi (23.149˚7N, 74.002˚E).

**Figure 1.4:** Collection of *D. gazella* from the selected sites. Burrow opening indicated their presence. The burrow was carefully dug with the help of shovel to collect the beetles.

Morphological identification was done up to the species level with the help of standard taxonomic keys (Arrow, 1931; Balthasar 1963; Chandra and Gupta, 2013) and by comparing with the specimens in department repository. Molecular identification was done using marker gene- COI, which has been found to be an important gene for species identification and has been the most widely used for DNA barcoding (Mandal et al., 2014). Genomic DNA samples were prepared from fresh insect. Total genomic DNA was extracted from the dissected femoral muscle of dung beetle using the phenol chloroform method (Huang et al., 2006) and DNA quantification was done using the nanodrop and quality was assessed by running agarose gel electrophoresis. Further, the extracted DNA was used for PCR amplification of COI gene using primers (**Table 1.1**). The amplified DNA was assessed by conducting agarose gel electrophoresis followed by Sanger sequencing and Barcoding.

**Table 1.1:** Primers of COI genes

| **DNA marker: Cytochrome *c* oxidase subunit I primers** | **Primer sequence (5' to 3')** | **Reference** |
|---|---|---|
| LCO-1490 | GGTCAACAAATCATAAAGATATTGG | Folmer et al., 1994 |
| HCO-2198 | TAAACTTCAGGGTGACCAAAAAATCA | Folmer et al., 1994 |

**Rearing and Acclimatization of Dung Beetle in Laboratory**

Morphologically and molecularly identified *D. gazella* were (12-14 mm long, 7-8 mm wide) were maintained under laboratory conditions in earthen pots following the method proposed by Gaikwad and Bhawane, (2015) (**Fig. 1.5**). Rearing medium in the pots was the sandy soil (obtained from collection sites, pH-6.8) and fresh dung of the cattle was used as food resource for the dung beetles. Fresh dung of buffalo (rich in carbohydrate content (Pandya et al., 2023)) was obtained from the stable adjacent to the collection site with the help of trowel (30 cm). Fresh dung (250 g) was added to the pot every alternate day. Further, these earthen pots were covered with a black cloth at the top, and placed in a large plastic tray containing moist sand for maintaining the temperature (22° to 26°C), and humidity (70%), with a 10L: 12D light regime (Bang et al., 2004). The rearing of *D. gazella* was maintained throughout experimentation.

**Figure 1.5:** Rearing medium for dung beetle. *D. gazella* were allowed to acclimatize in the convenient size earthen pot placed in the mud tray. Temperature (26°C) and humidity (70%) was maintained within this medium.

**Tunnel pattern**

After acclimatization and rearing, 10 dung beetles (5-males and 5-females of same size and weight) were released in the earthen pots and were monitored for the appearance of the holes on the dung layer confirming the initiation of the tunnel formation (**Fig. 1.6**). On $10^{th}$, $20^{th}$ and $30^{th}$ day of experimentation, hot wax was poured into the tunnel till the burrow opening and allowed to cool for 48 hours. Then after, the tunnel casts were excavated at the end of $10^{th}$, $20^{th}$, and $30^{th}$ day. For morphometry analysis, the

measurements of castings were done by following the method of Sinha, (2014), where following parameters were taken.

- Number of openings
- Length of the tunnel
- Total depth of the tunnel
- Diameter of the tunnel
- Area of burrow opening
- Numbers of branches
- Patterns of the tunnel

Area = where a is the length of the burrow opening, b is the width of burrow opening.

**Figure 1.6:** Observation of tunnel making in rearing medium; arrow points the tunnel formed and the circle represents the branch formed near the blind end of tunnel into which the brood balls are placed.

**Brood ball architecture and morphological traits**

For brood morphometry and life cycle study, five pair of adults (about 14 mm long) was released in earthen pot. Brood balls formed at the end of $10^{th}$, $20^{th}$ and $30^{th}$ days were collected, counted and photographed using a Nikon D5200 camera with 18-5mm Nikon lense. A longitudinal section was made using a scalpel to determine the internal structure and measure the wall thickness and external shield layer. The diameter of the brood balls was measured along an axis that corresponds to the largest width of the protuberance and the provision chamber. The height was measured along an axis that runs through the protuberance and the provision chamber, and the brood balls were cut along this axis.All measurements were performed manually using the digital vernier caliper (Zhart, India). Data were expressed in millimetres (mm).

**Brood Morphometry and Life Cycle**

At 12 hour interval, broodballs were monitored for the development of the individuals, starting from egg up to the adult stage. The opening in the brood balls was immediately sealed after observation with the help of fresh dung. Then after, length, and weight measurements of each stage of development of *D. gazella* were recorded with the help of vernier caliper (Zhart, India) and analytical balance (Wensar, PGB200, India) (Singh et al., 2019).

**Investigation of Digging Genes Involved in Digging Behaviour**

To understand the role of tibial teeth in digging of tunnels, we analyzed the gene expression of two genes that ancestrally function in embryonic patterning and thus entirely outside the spatial and temporal context of leg formation, and which are recruited to help shape the formation of tibial teeth. Therefore, the expression patterns of the two genes; dll and ems were analyzed in both males and females (Linz et al., 2019). For this, the dung beetles' leg tissue was isolated on $10^{th}$, $20^{th}$, and $30^{th}$ days of tunnel formation. Further, the RNA isolation was performed, followed by cDNA synthesis, RT-PCR using the primers of dll and ems.

**Total RNA Extraction (Trizol Method)**

For total RNA extraction, leg tissue was isolated from both male and female dung beetles after 10, 20 and 30 days using PBS (pH-7±0.5). The tissue (50-100 mg) was weighed and homogenized in 500 µL Trizol reagent (Invitrogen). For complete dissociation of nucleoprotein complexes, samples were incubated for 5 minutes at room temperature. The incubation was followed by the addition of 100 µL chloroform and was vigorously shaken for effective mixing of both the solutions. The samples were kept at room temperature for 5 minutes till the aqueous and organic layers were distinct. Thereafter, the tubes were subjected to centrifugation at 12,000x G for 15 minutes at 4°C. The mixture got separated into a lower red phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. An aliquot of upper aqueous phase was then transferred into a new 1.5 mL micro centrifuge tube. Precipitation was done by adding 500µL of isopropanol to the supernatant that was transferred. The samples were kept in room temperature for 10 minutes, centrifuged at 12,000 RPM for 15 minutes at 4°C. After precipitation the supernatant was discarded without disturbing the pellet and was washed in 500 µL of

75% ethanol and then 500µL absolute ethanol was added to the pellet. Effective mixing was done by gentle inversion and was further subjected to centrifugation at 7,500 RPM for 5 minutes at 4°C. The pellet was resuspended by adding 40 µL of DEPC water (Diethylpyrocarbonate), was quantified spectrophotometrically at 260nm using NanodropC and was stored in -20º C.

**cDNA synthesis**

First strand of cDNA was synthesized from each sample using Thermo Scientific Verso cDNA Synthesis Kit (AB-1453/A). Verso Reverse Transcriptase Verso is an RNA-dependent DNA polymerase with a significantly attenuated RNase H activity. Verso can synthesize long cDNA strands, up to 11 kb, at a temperature range of 42°C to 57°C. In reaction, 1 µg RNA was used as a template for cDNA synthesis using oligodT primers. The volume of each component was for a 20µL final reaction. The reaction mix is mentioned in the **Table 1.2**.

**Table 1.2:** PCR Reaction Mixture

| **Components** | **Volume** |
|---|---|
| 5X cDNA synthesis buffer | 4 µL |
| dNTP Mix | 2 µL |
| anchored oligo dT /random hexamers | 1 µL |
| RT Enhancer | 1 µL |
| Verso Enzyme Mix | 1 µL |
| Template (RNA) | 1-5 µL |
| Molecular grade nuclease-free Water | Up to 20µL |
| Total Volume | 20 µL |

After setting up reaction mix, samples were kept in thermo cycler in PCR conditions (**Table 1.3**)

**PCR conditions**

**Table 1.3:** Reverse transcription cycling program for cDNA synthesis

| | **Temperature** | **Time** | **Number of cycles** |
|---|---|---|---|
| cDNA synthesis | 42 ℃ | 30 min | 1 cycle |
| Inactivation | 95 ℃ | 2 min | 1 cycle |

**RT-PCR Amplification**

Quantitative RT-PCR was performed using PowerUp SYBR Green Master Mix (A25741, Applied Biosystems, USA) in Quant Studio 12K (Life technology) FAST real-time PCR machine with primers to detect selected messenger RNA (mRNA) targets (**Table 1.4**). The real time PCR conditions (**Table 1.5**) for the primer sequences of digging genes (**Table 1.6**) were maintained, and the melting curve of each sample was measured to ensure the specificity of the products. Beta Actin was used as an internal control to normalize the variability in the expression levels and data was analyzed using 2-ΔΔCT method (Livak and Schmittgen, 2001).

**Table 1.4:** Real Time PCR mix

| **Components** | **Volume (10 µL/well)** |
|---|---|
| PowerUp SYBR Green Master Mix (2X) | 5 µL |
| Forward Primer (10µM) | 0.5 µL |
| Reverse Primer (10µM) | 0.5 µL |
| DNA Template | 1 µL |
| Molecular grade Nuclease free water | 3 µL |
| Total | 10 µL |

**Table 1.5:** Real Time PCR Conditions

| **Steps** | **Temperature** | **Duration** | **Cycle** |
|---|---|---|---|
| UDG activation | 50ºC | 2 minute | Hold |
| Dual- Lock DNA polymerase | 95ºC | 5 minute | Hold |
| Denature | 95ºC | 45 seconds | 40 cycles |
| Anneal | 59ºC | 30 seconds | |
| Extend | 72ºC | 1 minute | |
| Melt Curve | 72ºC | 8 minute | Hold |

**Table 1.6:** Real time PCR primer sequences of digging genes

| Sr. No. | Accession No. | Gene Name | Primer type | Sequence | $T_m$ |
|---|---|---|---|---|---|
| 1 | NT_033778.4 | dll | Forward | AGCCCGATATACCGTACCCA | 59.35˚C |
| | | | Reverse | AGGAGACTTCGAAAGGGGGA | 59.35˚C |
| 2 | NT_033777.3 | ems | Forward | AGTTTATGCCCAATCCAGGCA | 57.87˚C |
| | | | Reverse | TCCAAAAGATACTTACTTCCAGGG | 59.30˚C |

**Data Analysis**

Each experiment was done in triplicate. Statistical analysis was done using Graphpad Prism 9 software. The data was analyzed using one way and two way ANOVA test followed by multiple comparison test (Tukey's). Results are presented as mean ± SEM. The level of significance was set as $*p<0.05$, $** p<0.01$, $*** p<0.001$.

## 1.3 RESULTS

### Morphological Identification


**Figure 1.7:** Morphological features of *D. gazella*.

Morphological identification (**Fig. 1.7**) was done using standard references (Génier and Moretto, 2017) and the characteristic features of *D. gazella* are noted (**Table 1.7**).

**Table 1.7:** Morphological characters for the identification of *D. gazella.*

| | | |
|---|---|---|
| **Identification marks** | **Size** | 12-14mm |
| | **Colour** | Brown to dark brown |
| | **Elytra** | Yellow to mottled yellowish brown |
| | **Pronotum** | Glossy, blackish brown in colour |
| | **Protibia** | Short, with external teeth more robust |
| | **Male** | Slightely curved acute horns. |
| | **Female** | Strongly elevated ridge extending between eyes |
| | **Foreleg** | Presence of tibial teeth and spurs. Protibial apicointernal tooth enlarged, with dorsal ridge extending to apex |

**Source:** (Génier and Davis, 2017; Génier and Krell, 2017; Pokhrel et al., 2020)

**Molecular Identification:**

**DNA quantification**

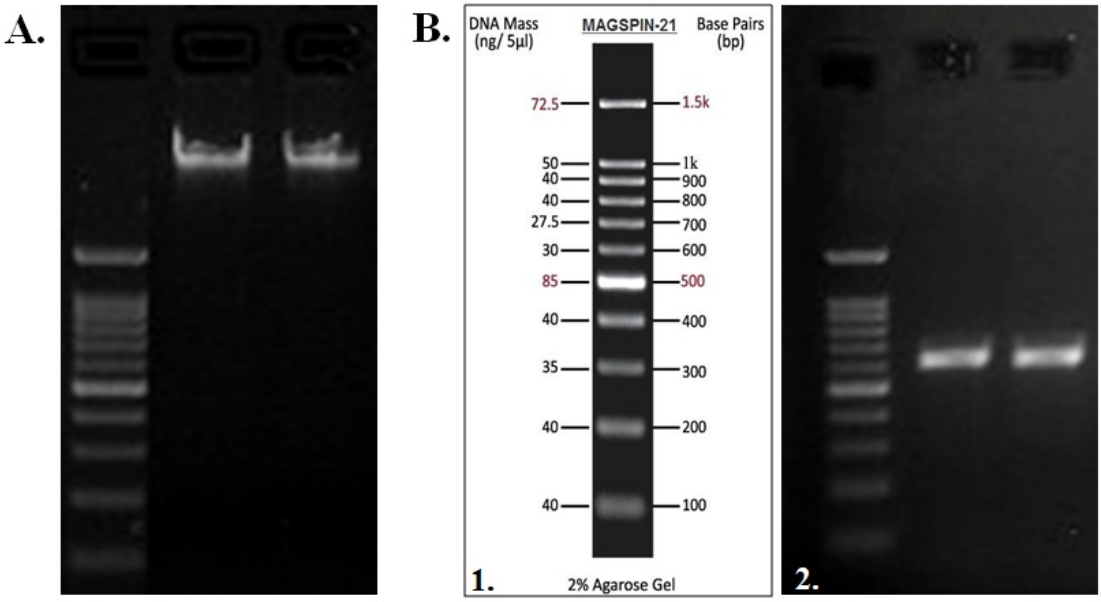



**Figure 1.8:** Results of Agarose Gel Electrophoresis. A. Genomic DNA B. PCR Product 1. Base pair size 2. PCR product of COI.

Bands of Genomic DNA are shown in **Fig. 1.8A** and that for the COI gene in **Fig. 1.8B**. The COI gene consisted of 720 bp, when run on 2% Agarose gel. Further, the barcode (**Fig. 1.9**) and sequence of amplified COI gene was obtained. DNA sequencing of Cytochrome oxidase (COI) was carried out to prove that the selected species of dung beetle was *D. gazella*. Sequence alignment and homology search was performed using MEGA 7 software. The obtained sequence was subjected to NCBI BLAST and was confirmed that there was 99.96% for COI homology with *D. gazella* with E value zero, confirming its identity.

**Barcode and sequence of COI gene**

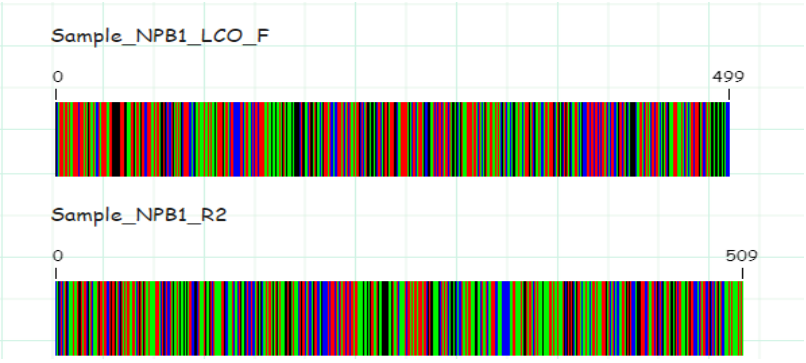



**Figure 1.9:** Barcode of COI gene in *D. gazella*

***D. gazella* Contig:**

GGGTCTCCTCCTCCTATAGGATCAAAGAATGTAGTATTTAAATTTCGATCTGT
AAGAAGTATAGTAATTGCCCCTGCTAGAACTGGAAGTGATAAGAGAAGAAG
AAGGGCTGTAATTGCTACAGCTCATGCAAATAATGGTATTCGATCAAATGTTA
TTCCTGTTGATCGTATATTAATTACTGTAGTAATAAAATTTACTGCTCCTAGAA
TAGAAGAGATTCCGGCTAAATGAAGTCTAAAAATTGCCAAATCAACTGAAGC
TCCTCCATGAGCAATATTAGATGATAAAGGTGGATAAACTGTTCATCCAGTTC
CAGCCCCTCTTTCTACTATTCTTCTTATTAAAAGAAGAGTTAATGAAGGGGGA
AGTAATCAAAATCTTATATTATTTATTCGTGGAAAAGCTATATCAGGAGCACC
TAATATTAAAGGAACTAATCAATTTCCAAACCCCCCAATTAAAATAGGTATTA
CTATAAAGAAAATTATAATAAATGCGTGTGCAGTTACAATAACATTATAAATT
TGATCATCACCAATTAGTGTCCCAGGGTTTCCTAATTCTGCTCGAATTAGGAG
TCTTAAAGATGTTCCCACTATTCCTGCTCATGATCCAAATATAAAATATA

**Nesting Behaviour**

During the period of acclimatization and rearing, the nesting behaviour of *D. gazella* was observed (**Fig. 1.10**). Both (male and female) dung beetles spent most of the time feeding and constructing the nest. On the second day of their release, both male and female dung beetles started constructing the tunnel and carrying dung (brood balls) along the tunnel. Males were observed more frequently on the surface of dung and females were seen occasionally. Eventually, the appearance of holes over the dung layer was the confirmation of tunnel formation and egg-laying. Female sealed the brood ball after ovipositioning and spent an average of 12-24 hours covering it with the layer of soil and which made it appear cylindrical in shape.

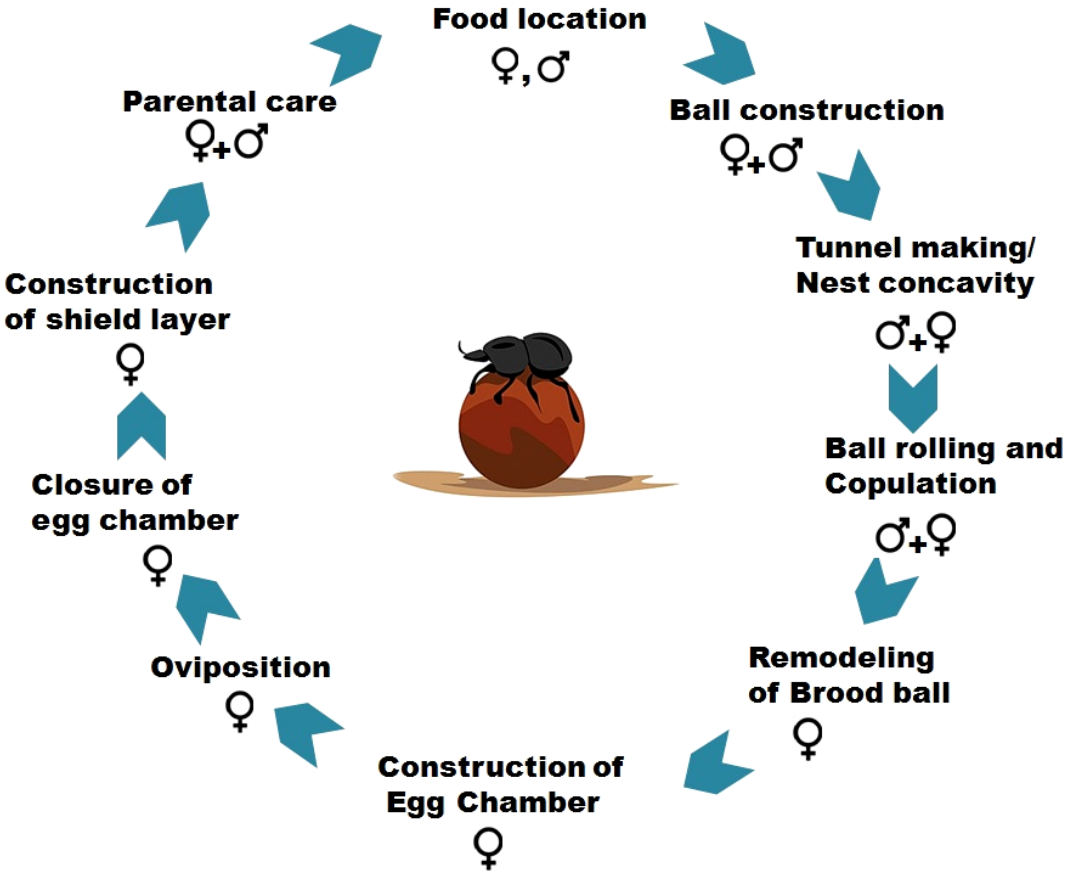



**Figure 1.10:** Nesting behaviour of *D. gazella*. Cycle repeated. Comma (,) indicates that the activity is performed by either female or the male alone; addition symbol (+) between the sexes indicates cooperation required.

### Tunnel pattern

Construction of the tunnel was carried out by both males and females underneath the dung. Observations of the tunnel obtained at three different time points i.e. $10^{th}$, $20^{th}$, and $30^{th}$ day (**Table 1.8)**. Under laboratory conditions, the tunnel pattern studies indicate that *D. gazella* constructs a simple tunnel with type II pattern, over the period of time (**Fig. 1.11a**). The total depth, length, and area of the burrow cast were found to be significantly ($p<0.05$) increasing with increasing period of time (**Fig. 1.11b**).

**Table 1.8:** Observation of tunnel pattern of *D. gazella* on 10th, 20th and 30th day; *Here, NBO= Number of Burrow Openings; L= Length (cm); TD = Total Depth (cm); D = Diameter of burrow (cm); Area ($cm^2$); NOB= Number of Branches of burrows.* Values represent mean±SEM. *(n=3).*

| Serial No. | Observations | 10 days | 20 days | 30 days |
|---|---|---|---|---|
| **1** | **NBO** | 1 | 1 | 1 |
| **2** | **L (cm)** | 14.7±0.057 | 16.9±0.06 | 19.8±0.05 |
| **3** | **TD (cm)** | 9.8±0.061 | 12.9±0.056 | 13.5±0.058 |
| **4** | **DOB (cm)** | 1.11±0.01 | 1.11±0.01 | 1.16±0.001 |
| **5** | **Area ($cm^2$)** | 12.8±0.062 | 14.72±0.057 | 18.02±0.058 |
| **6** | **NOB** | 3 | 4 | 4 |
| **7** | **Pattern** | Simple | Complex | Complex |

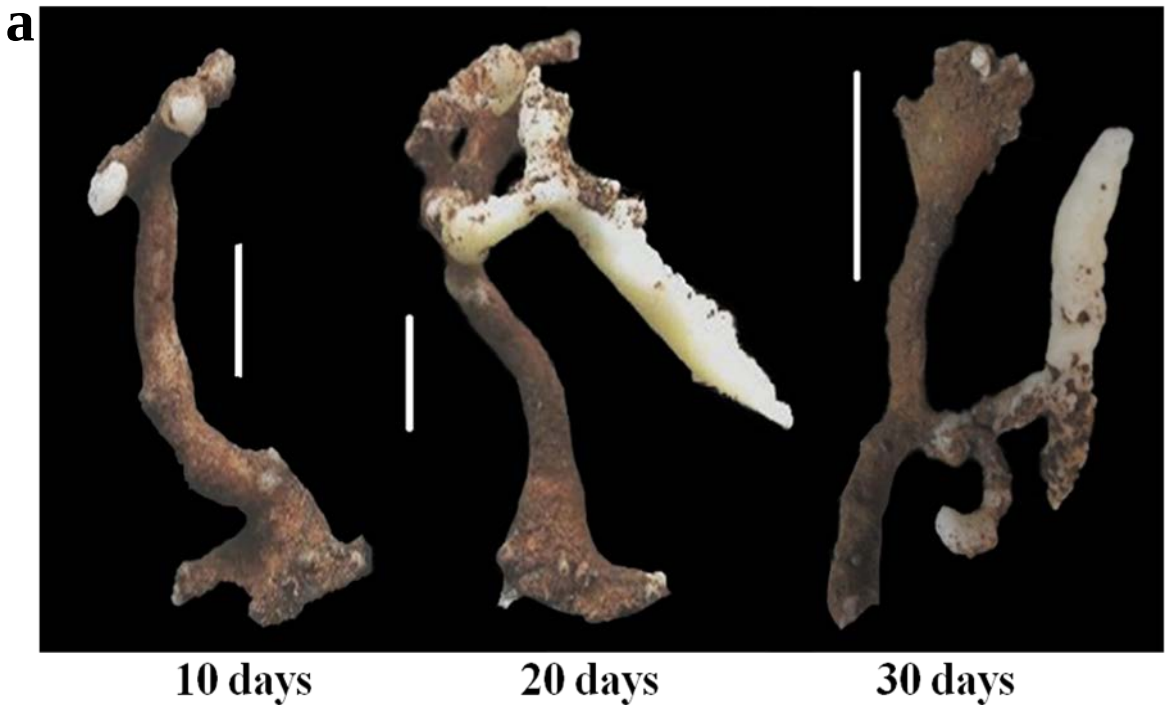



**Figure 1.11:** The tunnel pattern of *D. gazella*. (a) The tunnel formed at the end of the 10th, 20th, and 30th day is shown (scale = 4 cm). (b) The graph represents the key measures (length, depth and diameter) of tunnel formation. The length and depth of the tunnel were observed to increase significantly ($p<0.001$) in a tie dependent manner (10th, 20th, and 30th day). Each value represents Mean±SEM. *Here, $p<0.001$*** (n=3).*

**Brood Ball Architecture and Morphometry**

Tunnels were dug and the brood balls were removed which were spherical to cylindrical, elongated in appearance (**Fig. 1.12a**). The average number (Mean±SEM) of the brood ball was found to be 50±0.76, 139.2±0.46, and 155.6±0.83 after 10, 20 and 30 days respectively (**Fig. 1.12b**). Brood ball morphometry showed that the brood balls were spherical with strongly stacked dung containing a single egg at the centre of the ball. Further, the morphological traits of the brood balls were also measured (**Fig. 1.13**) and are summarized in **Table 1.9**. It was observed that the size of brood balls (38.32±5.89 mm) were 2.74 times larger than the adults (14.67±1.78 mm).

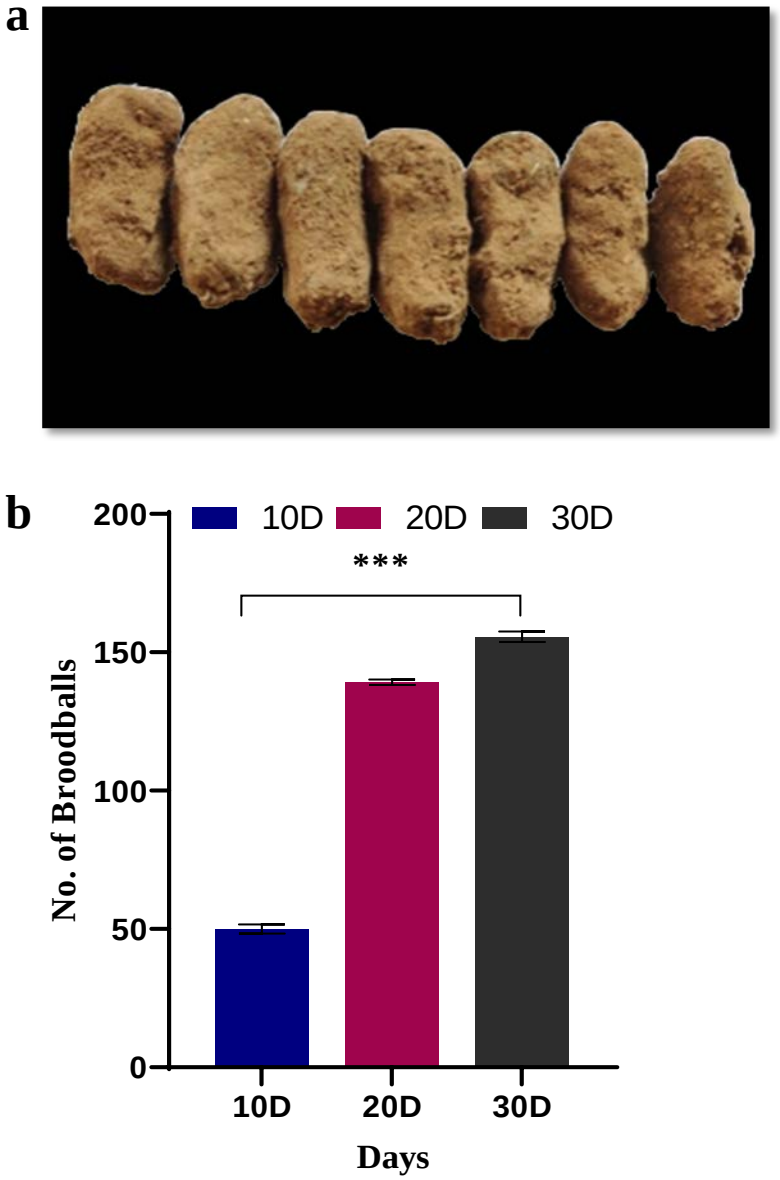



**Figure 1.12:** Brood balls formed by *D. gazella* (a) Spherical shaped brood balls (b) Number of brood balls formed at the end of 10$^{th}$, 20$^{th}$ and 30$^{th}$ day. Each value represents Mean±SEM (n=3).

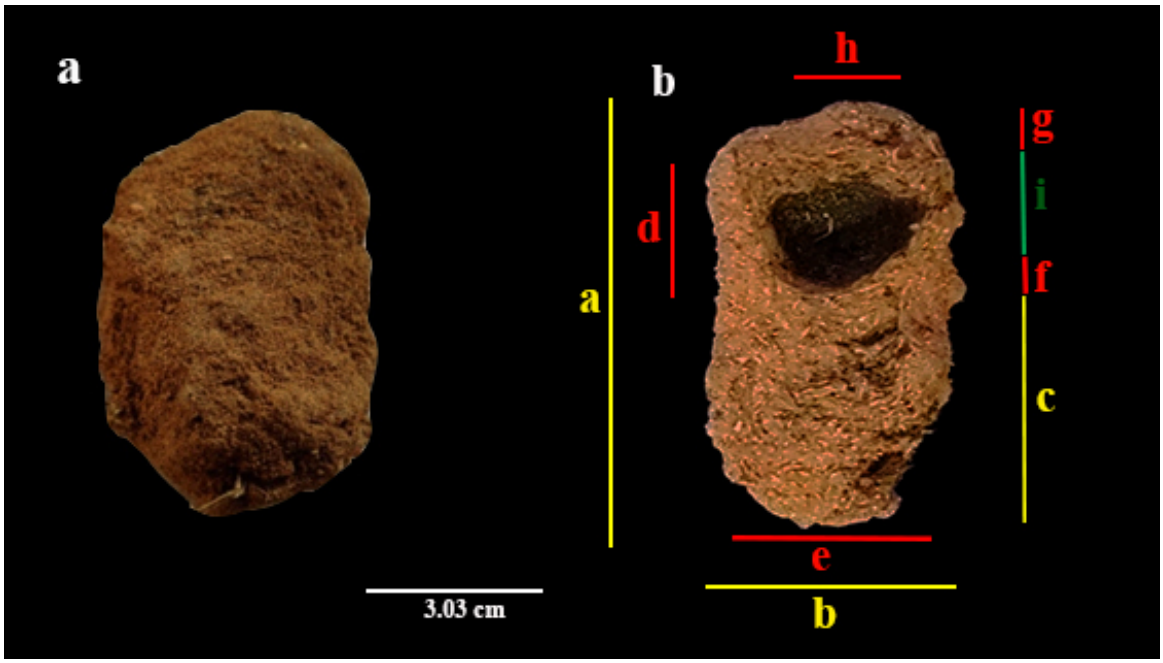



**Figure 1.13:** Morphological traits in brood balls formed by *D. gazella*.

**Table 1.9:** Morphological traits of brood balls and shield layers of *D. gazella* (n=10).

| | **Morphological traits** | **Mean (mm) ± SEM** |
|---|---|---|
| a | Total height | 38.3 ± 3.4 |
| b | Total equatorial diameter | 15.5±0.51 |
| c | Thickness of shield layer | 4±0.29 |
| d | Height of Brood ball | 9.5±0.29 |
| e | Equatorial diameter of brood ball | 10.5±0.36 |
| f | Thickness of brood ball layer | 12±3.13 |
| g | Height of protuberance | 4.4±0.64 |
| h | Diameter of protuberance | 12.2±0.86 |
| i | Diameter of provision chamber | 7.5±0.87 |

**Life cycle**

Mating and fertilization begins after 1-3 days of releasing the beetles in the setup. The brood balls were cylindrical in shape and only one egg was observed in egg chamber of each brood ball. The life cycle of *D. gazella* comprised of 4 stages, *i.e.*, egg, larva ($1^{st}$ instar, $2^{nd}$ instar, and $3^{rd}$ instar), pupa, and adult (**Fig. 1.14a**). The duration of different developmental stages is presented in **Table 1.10 & Fig. 1.14b**.The total development period was found to be of 30 days. Further, the length, diameter, and weight of all the developing stages were recorded (**Table 1.11 & Fig. 1.15**).

**Egg:** Each elongated, cylindrical brood ball showed a single egg laid in the central chamber in a vertical position. The eggs were elongated, cylindrical, and creamy white in colour. The length, diameter, and weight of the egg were recorded as 2.49±0.04 mm, 1.47±0.05 mm and weight 6±0.39 mg of the egg was noted. The egg stage was observed to last for 2 to 4 days, followed by egg hatching and larval emergence.

**Hatching:** Close to the hatching, the eggshell becomes transparent, and the larva was visible. The larva emerged with its abdominal end first. The larva underwent contraction and expansion its body several times until it completely freed itself from the shell.

**Larva:** After 2-4 days, the larva was transparent, with only the tips of the mandibles being dark brown. The first instar larvae had its characteristic hump, which remained transparent for a few days and was used as a pivot when fed on the dung. The second instar larva showed characteristic mandibles. 3$^{rd}$ instar was observed to be largest stage and it comprised of the highest length (20.64±1.14 mm), diameter (2.38±0.32 mm) and weight (326±0.25 mm) as compared to that of 1$^{st}$ and 2$^{nd}$ instar. The development period of three instars were observed to range from 5 to 8 days for 1$^{st}$ instar, 9 to 12 days for 2$^{nd}$ instar, and 13 to 24 days for the 3$^{rd}$ instar. Towards the end of the third instar, the larva started constructing a pupal cell. They excreted a greyish brown paste from the abdominal end and held on the truncated end of the abdomen.

**Pupa:** The newly developed pupa was creamy white and shiny, which later on turned to yellowish brown. Sexual dimorphism was evident in the pupa: pupae of male had two horns on the head and a median projection, whereas the female had only a median projection. The pupal stage lasted for 25 to 29 days, followed by its transformation into adult. The diameter of 6.3±0.48 mm was observed in the pupal stage.

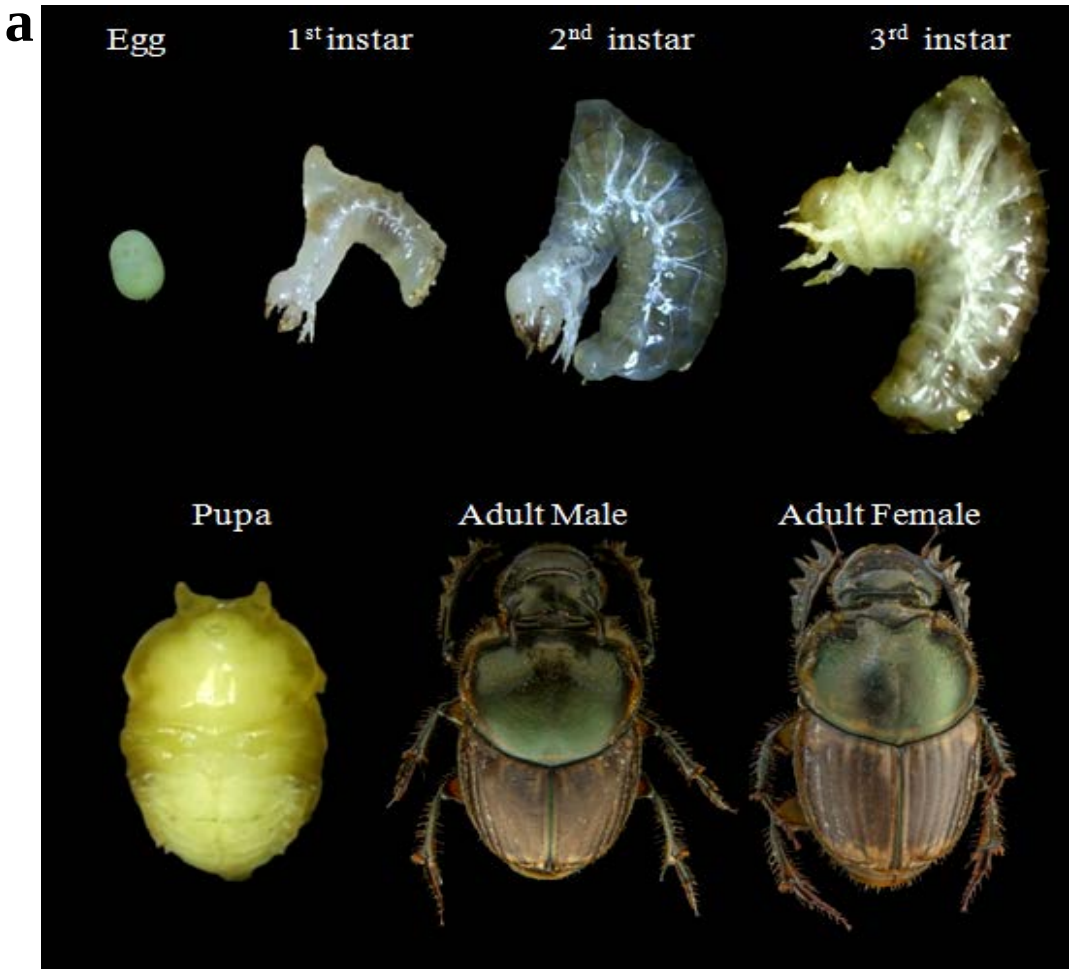
**Adult:** After 28 to 32 days, the adult emerged from the brood mass, which further showed pigmentation and maturation within 2-3 days and its longevity period ranged from 60 to 80 days. Complete sexual dimorphism was observed in adults where in males had vertical, elongated horns between the eyes and protibia was found to be slightly curved medially; females had a strong elevated ridge between eyes on the head with less slightly curved protibia. The measurements of adult's length, diameter and weight were recorded as 14.67±1.03 mm, 6.9±0.86 mm and 139±0.09 mg. Cylindrical shaped brood balls (Mean±SEM; n=15) had a length of 38.3±3.4 mm, a diameter of 15.5±0.51 mm, and weighed 745±0.77 mg (**Table 1.11**).

**Table 1.10:** The developmental period (Mean±SEM) for different stages of life cycle of *D. gazella.*

| Stage | Time (Days) | | Mean±SEM | Development Days |
|---|---|---|---|---|
| | Minimum | Maximum | | |
| Egg | 2 | 4 | 3.42± 0.36 | 3±0.42 |
| First Instar | 5 | 8 | 6.05± 0.62 | 3±0.78 |
| Second Instar | 9 | 12 | 11.28± 0.56 | 5±0.31 |
| Third Instar | 13 | 24 | 23.76± 1.6 | 11±1.42 |
| Pupa | 25 | 29 | 28.98±0.77 | 4±0.7 |
| Adult | 28 | 32 | 31.83± 0.84 | 2±1.06 |

**Table 1.11:** Brood morphometry of different developmental stages (Mean±SEM) of *D. gazella.*

| Stage | Length (mm) | Diameter (mm) | Brood Weight (mg) |
|---|---|---|---|
| Egg | 2.49±0.04 | 1.47±0.05 | 6±0.39 |
| 1st instar | 3.63±0.32 | 1.36±0.03 | 18±0.32 |
| 2nd instar | 5.78±0.54 | 1.75±0.43 | 127±0.48 |
| 3rd instar | 20.64±1.14 | 2.38±0.32 | 326±0.25 |
| Pupa | 11.36±1.38 | 6.3±0.48 | 136±0.34 |
| Adult | 14.67±1.03 | 6.9±0.86 | 139±0.09 |
| Brood ball | 38.3±3.4 | 15.5±0.51 | 745±0.77 |



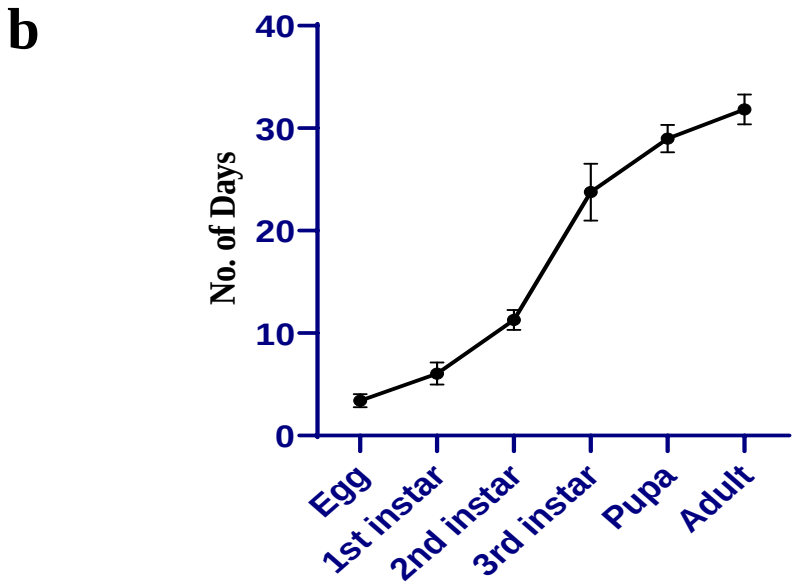




**Figure 1.14:** Study on life cycle of *D. gazella*. **(a)** The stages of the life cycle starting from egg, larva (1st, 2nd, and 3rd instar), pupa and adult are observed. **(b)** Comparative accounts on the duration of the different stages in the life cycle of *D. gazella*. Values represent Mean±SEM (*n=3)*.

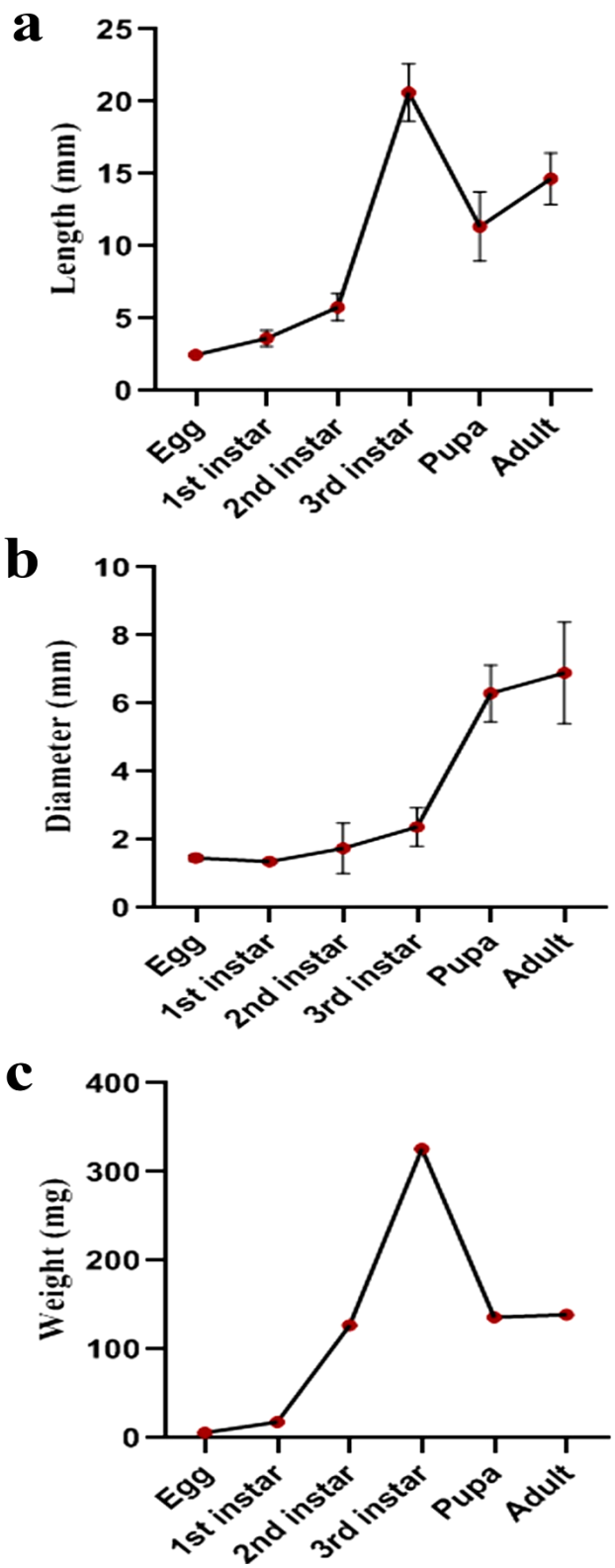



**Figure 1.15:** Comparative account of (a) brood length, (b) brood diameter, and (c) brood weight of different stages of the life cycle of *D. gazella*. Brood length, diameter, and weight of developmental stages increase, and the 3rd instar larva shows the maximum length and weight(*n=3)*.

**Digging genes involved in digging behaviour**

Adult male and female were observed to have a strong digging apparatus and were involved in the extravagant digging activity. They used their fore tibia which had tibial teeth in digging soil underneath the dung. In the present study, we tried to understand the role of digging genes in the digging and hence tunnelling activity by *D. gazella* in the selected time points i.e. $10^{th}$, $20^{th}$ and $30^{th}$ day. In the 30 days of digging the soil and tunnel formation, the maximum amount of digging was observed after 10 days, followed by 20 and 30 days. Thus, with respect to digging and tunnelling, the results of digging genes showed a decline in dll and ems gene expression (**Table 1.12**) with the increasing time, proving its digging behaviour to be maximum after 10 days, followed by 20 and 30 days (**Fig. 1.16**).

**Table 1.12:** The fold change in dll and ems (Mean ± SEM) in the male and female *D. gazella.*

| **Group** | **dll** | | **ems** | |
|---|---|---|---|---|
| | **Males** | **Females** | **Males** | **Females** |
| Control | 1±0.276 | 1±0.165 | 1±0.418 | 1±0.235 |
| 10 days | 7.284±0.499** | 6.988±0.189** | 2.916±0.292** | 3.876±0.189** |
| 20 days | 3.095±0.485* | 2.838±0.182* | 1.793±0.123 | 1.574±0.177 |
| 30 days | 1.55±0.280 | 1.478±0.390 | 1.152±0.513 | 0.559±0.102 |

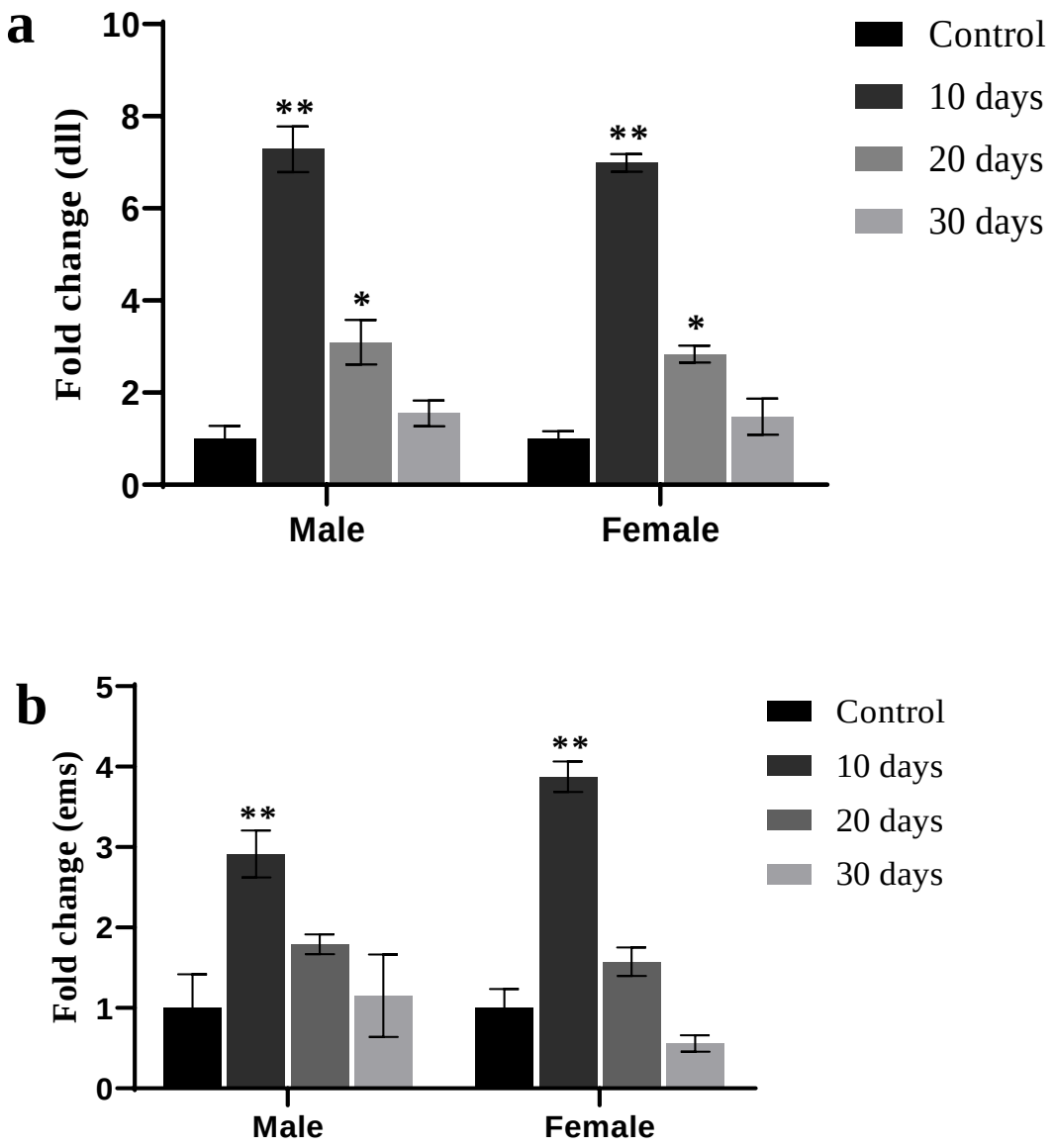



**Figure 1.16:** The fold change in digging genes in male and female dung beetle (a) dll (b) ems. Each value represents Mean±SEM. *p<0.5*, p<0.01**.*

## 1.4 DISCUSSION

Morphological characteristics are indicative of the manner in which organisms engage in physical interactions with their surroundings, therefore enabling and limiting their capacity to perform particular behaviours and activities (Barton et al., 2011; Traugott et al., 2015). Adult Scarabaeidae are distinctive, and *gazella* species are relatively easy to distinguish using taxonomic keys. Male and female *D. gazella* represented a discrete sexual dimorphism where in the males are 8–12 mm and females are 8.0–13.0 mm in size, males comprise a pair of horns which are short, slightly divergent in frontal view, gradually tapering from base to apex and are absent in females; protibial apicointernal tooth are enlarged in males; protibia are short, with external teeth more robust in females (Genier and Moretto, 2017). However, both are yellow to mottled yellow in colour which can be similar to other dung beetle species, this feature call the significance of DNA barcoding to enable their easy, rapid and accurate identification. The rapid identification of unfamiliar specimens is accomplished through the use of DNA barcoding techniques on adult specimens, hence facilitating the identification of any developmental stage (Oba et al., 2015; Wu et al., 2017). Therefore, the current work has provided an unambiguous identification of the taxonomic classification of *D. gazella* by the use of COI gene barcoding and sequencing techniques, which had previously been validated by Singhal et al., (2018).

*D. gazella* is mostly renowned for its distinctive activity of extracting dung from the ground and later on compact it into tunnels as a means of providing sustenance for its offspring. The current study has provided evidence that the nesting behaviour, tunnel formation, brood mass development, and parental care displayed by *D. gazella* are similar to those observed in other species of Onthophagus (Huerta and García-Hernández, 2013; Arellano et al., 2017; Sane et al., 2020). Nevertheless, some disparities have been noted. The observation of cooperation between male and female individuals was evident throughout the activities of ball rolling and first nest construction by *D. gazella*. Based on the initial observations of nesting activity, it is possible to discern that the tunnel follows a type II nesting pattern, as previously documented for some species of tunneler dung beetles. Our findings align with the prior research published by Heurta et al., (2023). Further, a time-dependent complexity in the formation of tunnel was observed. On $10^{th}$ day, the tunnel consisted of only 3 branches which were found to increase on $20^{th}$ and $30^{th}$ day resulting into 4 branches, housing linearly arranged brood masses. A time-dependent

increase in the length and total depth observed in the present study is in accordance with Sane et al., (2020), who have reported structural diversity and behavioural principles on insect architecture and have opined that the dung beetles follow the process of Markovian-building as it helps them to construct a larger and deeper pit lined with steeper walls for the protection of the broods. Earlier, it has been reported that the width of the tunnel is directly proportional to the beetle's body size (Klingenberg and Monteiro, 2005). However, in the present study, similar-sized dung beetles were selected and therefore no difference was observed in the diameter of the tunnel (Bertossa, 2011; Macagno et al., 2016).

Tunnelers make nests and lay spherical, cylindrical brood masses by sexual co-operation. In natural conditions, *D. gazella* digs a simple and deeper nest and forms several brood masses in a single tunnel (Moczek, 2009; Hernández et al., 2011; Hanski and Cambefort, 2014). Moreover, among the limited number of dung beetle species that have been investigated, it has been observed that male aid contributes to the improvement of reproductive success through the augmentation of brood ball quantities. Previous research has indicated that the presence of male help in the formation of burrows resulted in a higher rate of dung supply. This, in turn, led to an increased production of brood balls and thus enhanced female fecundity (Hunt and Simmons, 1998). The studies conducted by Arenallo et al., (2017), Johari et al., (2023), and Kerman et al., (2023) have shown that there is a substantial difference in the average number of brood balls generated by females when they are paired with males compared to when they are unpaired in *O. binoidis* and *O. vaca*. Therefore, in accordance with the previously published research, the current study examines *D. gazella* species exhibited enhanced digging of tunnels and a higher production of brood balls within a range of 50-155, throughout a period of 10-30 days of nest construction. This rise was observed under laboratory conditions and was attributed to the cooperative efforts of both individuals involved.

Furthermore, the brood ball morphometry of *D. gazella* was also observed. The morphological traits (length, diameter, thickness of shield layer, thickness of brood ball, diameter of provision chamber) on which the nesting behaviour is dependent did not show much variation within the brood balls produced. However, in significant alterations in the morphometry of the brood ball, probably due to its larger size, amount of dung was adequate for nursing the broods and thereby did not entail restructuring or modification of the brood ball as observed in other species of dung rollers including *O. lecontei*, *O.*

*incensus*, *O. taurus*, *O. hirculus*, *C. unicolor*, and *C. histrio* (Gaikwad and Bhawane, 2015; Arellano et al., 2017; Cortez et al., 2021; Kishi, 2014; Rohner and Moczek, 2021). However, formation of the additional layer of soil by the females on the top of the provision chamber after laying egg was perceived very distinctly in the present study.The size of the layer (2-4mm) was noted, which is likely helping in assured food supply, maintaining the moisture andproviding protectionagainst desiccation, predators, parasites and pathogenic microorganisms to the developing larvae and pupa (Moczek, 2010; Singh et al., 2019; Cortez et al., 2021). This protection is a form of parental care showcasing defence against the growing offspring and is also reported in other dung beetle species (Biedermann and Nuotclà, 2020; Meunier et al., 2022; Nervo et al., 2022).

In the present study, the life cycle of *D. gazella* was observed to be of 28 to 30 days with distinct 4 stages of development viz. egg, larva (3 instars), pupa and adult. The average developmental period noted in the present study is probably due to ambient temperature which was maintained during the experiment, and shows the similarity with other Scarabaeinae: 34-38 days in *O. incensus* (Heurta et al., 2010), 39 days in *O. lecontei* (Arellano et al., 2017), 30 to 34 days in *Nesosisyphus spp.* (Philips et al., 2004), 30 to 35 days in *O. reticornustus* (Gaikwad and Bhawane, 2015) and 30 days in *O. taurus* (Johari et al., 2022).The morphometry of developing brood throughout the life cycle revealed considerable divergence from egg to adult, the length(2.49±0.08 mm to14.67±1.78 mm), diameter (1.47±0.09 mm to 6.9±1.49 mm), and weight (6 mg to 139 mg)was found to increase with each developing stage, but surprisingly there was no difference in length of the brood ball which is in the agreement with the work done by Arellano et al., (2017) and Singh *et al*., (2019).

As the brood turns into an adult, it comes out of the brood ball and undergoes sexual maturation (Huerta and García-Hernández, 2013).Then after, the adult dung beetles begins its nesting activity which involves digging and tunnelling, brood ball construction, mating and egg laying by female. According to Linz et al., (2019), they exhibit a total of three pairs of legs that are serially homologous. Furthermore, the concept of strict homology may be applied to specific segments of these legs, including the femur, tibia, and tarsal segments. The fore tibia of the dung rollers has a powerful digging apparatus in the form of teeth enabling them to use ecological niche compacted soil and the distal end of tibia possess tibial spur, two-pronged projection which plays a critical role in digging. It is postulated that a tool resembling a shovel, which is larger

than usual for digging purposes, exhibits interactions between male and female individuals. These interactions have been documented to impact various activities, including reproduction, competition, and cooperation. Such activities have a substantial influence on the provisioning of ecosystem functions and services, leading to the emergence of novel life-history strategies such as tunnelling and subterranean reproduction (Fernandes et al., 2011; Nervo et al., 2022). Previous research utilised a combination of behavioural and developmental genetic methodologies to investigate the role and development of the front tibia in dung beetles. These studies showed that the presence of tibial teeth enhances the beetles' digging performance. Furthermore, it has been observed that the development of these teeth is facilitated by the significant repurposing of various genes and pathways that are typically involved in the formation of the beetle's legs (Linz et al., 2019).

According to the analysis of 16 leg genes conducted previously, it has been determined that 13 of these genes are essential for the proper development of tibial teeth. Additionally, a group of 7 genes (dac, lim1, ser, odd, bowl, sob, drm) have been identified as being involved in the patterning of the leg, specifically the tibia. Another set of 6 genes (dll, sp8, ab, dachs, krn, egfr) have been found to have supplementary functions in the context of leg formation. Furthermore, two genes (ems and mex3) have been found to have distinct effects on the size, shape, and spacing of tibial teeth (Angelini et al., 2012). The present research employed a comprehensive methodology encompassing both behavioural and genetic analyses to evaluate the functional significance of the anterior tibia in relation to the manifestation of digging-related genes (dll and ems). A significant ($p<0.5$) decline in the dll and ems in a time dependent manner from 10 to 30 days in both males and females clearly indicates the functional importance of the digging genes in the initial phase of nesting behaviour. Our result is in agreement with the earlier reported work of Linz et al., (2019); Jugovic and Koprivnikar, (2021), wherein they have emphasized the role of the tibial genes in behaviour and ecology of dung beetles.

Therefore, the present study has unravelled the nesting behaviour of *D. gazella* in laboratory condition which was emphasised ontunneling, brood ball-making and parental care. While many of the costs and benefits associated with biparental cooperation have been thoroughly explored, both in the field and in the laboratory (Panitof et al., 2016), we have still an incomplete understanding of the neurophysiology and the underlying molecular mechanism involved in promoting plasticity in nesting behaviours. Factors that

shape this remarkable plasticity are well characterized, however, the neuromodulatory analysis is crucial to discern precise neural and physiological mechanisms that allow these flexible nesting behaviours.

## 1.5 CONCLUSION

This study contributes to the knowledge of fundamental aspects of nesting biology of *D. gazella*. Both male and female are involved in construction of tunnel and rolling down the brood balls. In our study, it was observed that *D. gazella* built a simple nest with type II pattern within the period of 10, 20 and 30 days, post which they carry the brood ball to the blind end of tunnel, reproduce and the female lay egg inside the brood ball chamber. The number of brood balls increased in a time dependent manner, and the overall brood ball morphometry of *D. gazella* is also reported to be distinct from other dung beetle species. The life cycle study has opined the development from egg to adult to range from 28 to 30 days and the overall brood morphometry changes over time and stages with maximum length, and weight were recorded for the $3^{rd}$ instar larva which is observed to be the largest stage in their life cycle. Further, a decline in the expression of digging genes (dll and ems) in a time dependent manner i.e. $10^{th}$, $20^{th}$ and $30^{th}$ day, has also proved its role in digging and is the first study suggesting its functional importance in the nesting behaviour of *D. gazella*.