UNRAVELLING THE NEURAL REGULATION IN NESTING BEHAVIOUR OF DUNG BEETLE ON EXPOSURE TO INSECTICIDE

Research Synopsis for Ph.D.

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Introduction

Scarabaeidae (Dung beetles) is one of the largest family of order Coleoptera, which contains more than 30,000 species in the world (Banerjee, 2014; Cajaiba et al., 2017), including three subfamilies Aphodiinae, Scarabaeinae, and Geotrupidae (Chandra and Gupta, 2012; Stone et al., 2021). The first comprehensive account on the subfamily 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) and Krikken (2009), Sabu et al., (2011)]. Later, comprehensive work on the diversity of dung beetles was conducted by Chandra and Ahirwar, (2007), Chandra and Singh, (2011), and Chandra and Gupta, (2011, 2012a, 2012b, 2012c) and have recorded 124 species belonging to 45 genera in 11 subfamilies from Madhya Pradesh and Chhattisgarh. Thakkar and Parikh, (2016), and Singhal et al., (2018) have also reported 24 species of dung beetles from Vadodara, Gujarat.

Dung beetles exhibit a wide range of ecological functions (Kakkar and Gupta, 2009; Brown et al., 2010; Gullan and Cranston, 2010), morphological as well as behavioural adaptations making them universally distributed. They carry out dung decomposition by feeding on dung. Based on their burial activity and their pasture productivity, Hernández et al., (2011) have classified them into four types: Telecoprid (rollers), Endocoprid (burrowers), Kleptocoprids (dwellers), and Paracoprid (tunellers) (**Fig. 1**). They feed mainly on micro-organism rich mammalian dung as a source of fibrous material to brood their larvae. Mostly dung beetles prefer omnivorous over herbivorous dung and least preferred is carnivore dung (Frank et al., 2017; Pandya et al., 2023). Depending upon soil type, and moisture (Nichols et al., 2008), dung quality (Braga et al., 2013), as well as pair cooperation (Slade et al., 2011), diverse pattern of consumption and relocation of dung has been reported (De Groot et al., 2002; Banerjee, 2014; Tarasov and Dimitrov, 2016; Singh et al., 2019). Further, they are known to enhance soil fertility, soil permeability, plant growth, seed dispersal, control parasitic growth, and reducing the emission of greenhouse gases by utilizing the dung for food and reproduction (Latha and Sabu, 2018). Despite of numerous ecological benefits, a decline in dung beetle diversity due to anthropogenic activities has been observed in forests and pastures (Nichols et al., 2009; Basto-Estrella et al., 2014; Kim et al., 2021); entailing jeopardy of their population and the ecosystem

services they provide (Nichols et al., 2008). Hence, it becomes imperative to carry out intensive studies on dung beetles which are essential in maintaining a healthy ecosystem (Salomão et al., 2020).

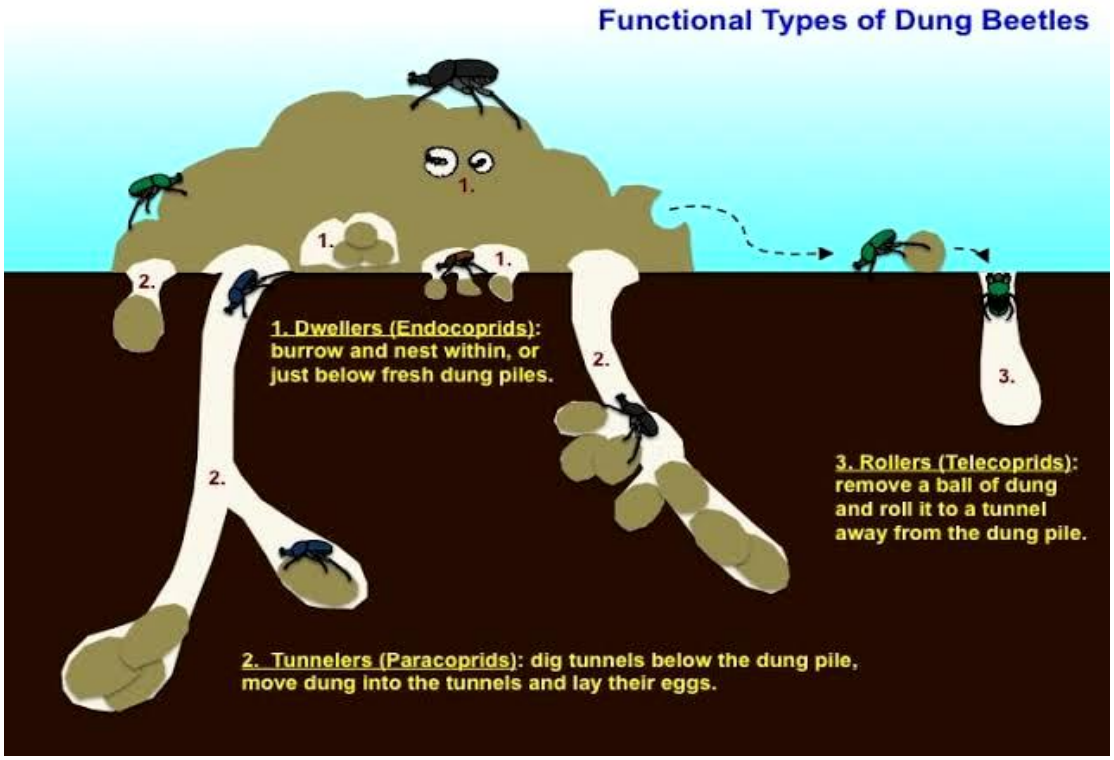
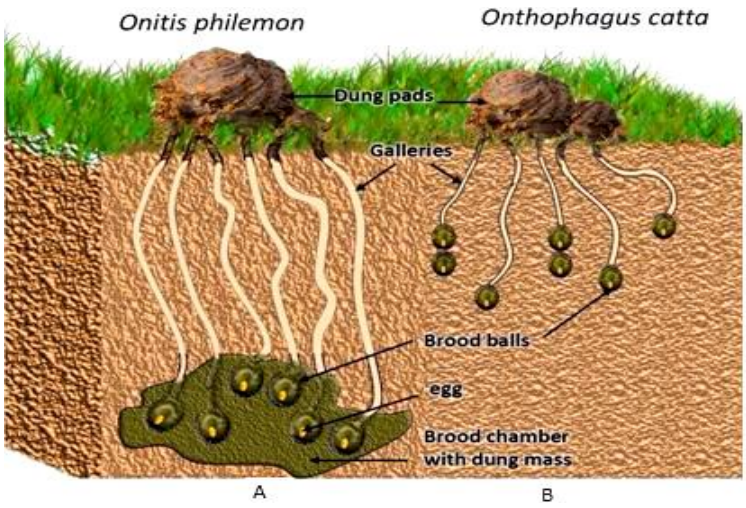


Figure 1: Dung beetles classified based on their dung burial activity

Of all the types of dung beetles, paracoprids are in attention due to their unique pattern of nesting. They are found predominantly in the forest and agri habitats (Sabu et al., 2006; 2007; Venugopal et al., 2012) across the globe (Andresen, 2005). *Digitonthophagus gazella* (Fabricius, 1787), a paracoprid shows a unique behaviour of nesting. Their presence is well documented in many countries including Africa, America, introduced in Australia (Noriega et al., 2020) Arabia, Madagascar, Pakistan and Sri Lanka (Chandra and Gupta, 2013), however it has also been recorded in many parts of India (Sabu et al., 2011; Chandra et al., 2012; Pawara et al., 2012; Gupta et al., 2014; Thakkar and Parikh, 2016) including Vadodara district (Singhal et al., 2018), Gujarat. Adult *D. gazella* are yellow to mottled yellowish brown in colour and show a complete sexual dimorphism. Males have slightly curved and acute horns while the females have a strongly elevated ridge that extends between the eyes (Chandra and Gupta, 2013). They have

three pair of homologous legs and their fore tibia consists of a tooth like structure with a strong burrowing ability which helps them build tunnels underneath dung and soil.



(Pessôa et al., 2017)

Figure 2: Dung burial and tunneling by dung beetles A. *Onitis philemon* B. *Onthophagus catta*

Dung beetles excavate tunnels by digging and provide dung to offspring in the form of brood balls at the blind end of each tunnel (Pulido and Zunino, 2007; Moczek, 2009; Khadakkar et al., 2019) (**Fig. 2**), with only a single egg deposited into an egg chamber and sealed (Hunt and Simmons, 2000). The larva lives inside the chamber throughout its development until pupation. Utilization of rich and ephemeral dung by growing offspring promotes unique behavioural and physiological adaptations leading to sub sociality and biparental behaviour (Arce et al., 2012; Panaitof et al., 2016; Heurta et al., 2013) by providing protection to the offspring from competition and desiccation (Rauter and Moore, 2002; Kim et al., 2021).

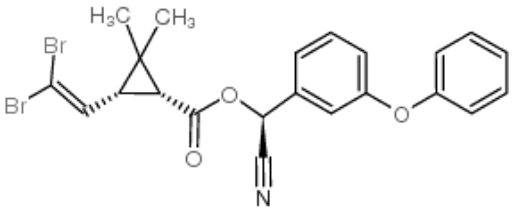
The reproductive behaviours of dung beetles comprise sexual exploration, sexual recognition, competition for mates and food utilized for nesting, sperm competition, and parental care (Huerta et al., 2023). The pervasive role of neurotransmitters in reproductive behaviour have been explored extensively in vertebrates (Adkins-Regan, 2005), however, this aspects are meagerly investigated in insects (Riddiford, 2012). For a very long period, neuromodulation in the central nervous system is considered to be the cause of the plasticity of behavioural responses (Zhukovskaya and Polyanovsky, 2017). Biogenic amines such as Dopamine (DA), serotonin (5-HT), and Octopamine, acts as neurotransmitters, neuromodulators, and neurohormones, and are crucial for controlling a variety of physiological processes in insects (Lange and Orchard, 2021; Sasaki et al., 2021). The catecholamine enzymes are known to participate in decarboxylation step

of neurotransmitter biosynthesis, neurotransmitter metabolism and innate immunity (Hodgetts and O'Keefe, 2006; Lin et al., 2020). The first enzyme in catecholamine biosynthesis pathway to be identified was DOPA decarboxylase (DDC) which catalyzes the decaboxylation of L-DOPA to DA, and later, 5-hydroxydecarboxylase (5-HTPDC) was discovered to catalyze the decarboxylation of 5-HTPDC to 5-HT. Baring a few reports, neurotransmitters like Dopamine, Serotonin, Acetylcholine, and Nitric Oxide are reported in *Drosophila*, *Manduca sexta* (Linnaeus, 1763), *Anopheles gambiae* (Giles, 1902), *Anopheles stephensi* (Liston, 1901) (Muller, 1996; Jacklet, 1997; Charpentier et al., 2000; Davies, 2000; Bicker, 2001; Vleugels et al,. 2015). Biogenic amines, Octapamine, DA, and 5-HT has been extensively explored in the social context-dependent fighting behaviour, and territorial dominance in cricket, *Gryllus bimaculatus* (Dyakonova and Krushinsky, 2013), fruit fly, *Drosophila melanogaster* (Alekseyenko et al., 2014; Zwarts et al., 2012), stalk eyed fly, *Teleopsis dalmanni* (Bubak et al., 2014a, 2014b; Casasa et al., 2017), bumble bee (Watanabe and Sasaki, 2022), oviposition by diamondback moth, *Plutella xylostella* (Li et al., 2020). Further, these biogenic amines have also been accounted to play a vital role in cuticle sclerotization, melanisation, reproduction and social interaction (Beggs and Mercer, 2009; Andersen, 2010; Vleugels et al., 2015; Verlinden, 2018; Singhal et al., 2019). Given the robust links established between 5-HT and DA and the modulation of behavioural state, we hypothesized that the biogenic amines DA and 5-HT, represents the most likely candidates for the neuromodulatory control of nesting behaviour in *D. gazella.*

Paracoprid beetles' tunneling and dung burial activity have made them economically and ecologically important and are now utilized worldwide for pasture improvement and biocontrol of pest flies (Génier and Davis, 2017). However, detrimental effects of insecticide residues on dung fauna has resulted in loss of services they provide (Beynon et al., 2015). Insecticides administered to livestock to control pest and parasite are excreted in the faeces in unmetabolized state, at proportions that becomes toxic to dung fauna (Mann et al., 2015; Slade & Roslin, 2016). The survival or reproductive performance of dung fauna reduces substantially in dung contaminated with insecticides (Vale et al., 2015), thereby altering the metabolic and physiological activities (Sands et. al., 2018). Synthetic products like Pyrethroids are derived from natural pyrethrins, isolated from the flowers, and are highly toxic and expeditious insecticide

exhibiting tremor-type syndrome, allergic reactions, and ataxia (Meunier et al., 2020; Galadima et al., 2021). Pyrethroids has extensive applications in horticulture, agriculture, and pest control, also used in livestock management to reduce a range of ticks, mites, biting flies and lice and are often associated with health and environmental issues if used excessively in the long term (Jacobs and Scholtz, 2015; Vale et al., 2015; Dudley et al. 2017; Sands et al., 2018; Andjani et al., 2019; Serrão et al., 2022).

Table 1: Insecticide (Source: PPDB)

Chemical name	**Deltamethrin**
Mode of Action	It acts by both direct contact and ingestion. It can act on nerve membranes by delaying the closing of the activation gate for the sodium ion channel. (Worthing & Walker, 1987)
CAS RN	52918-63-5
IUPAC name	[(S)-Cyano-(3-phenoxyphenyl)-methyl] (1R,3R)-3-(2,2-dibromoethenyl)-2,2-dimethyl-cyclopropane-1- carboxylate
Chemical formula	$C_{22}H_{19}Br_2NO_3$
Molecular structure	
Molecular weight (g/mol)	505.21
Log P (at 25^{o}C)	1.5
Relative density	1.5g/cm^3
Henry's Law Constant (Pa m^3 mol^{-1})	1.2 x 10^{-4}
Manufactures and suppliers of products	Sigma Aldrich
Example products using this active	

Deltamethrin (**Table 1**) in particular is a type II synthetic pyrethroid which is well known to affect the sodium channel (Meunier et al., 2020). It is one of the most widely used pyrethroid whose sublethal concentrations entail major physiological damages in a multitude of target and non-target insects (Cutler, 2013; Müller, 2018). It induces oxidative stress and leads to

production of reactive oxygen species (ROS) which are negatively damaging entities contributing to oxidative stress, resulting into serious cell damage (Zug and Hammerstein, 2015). Insects have a variety of antioxidant enzymes such as superoxide dismutase, catalase, glutathione transferase, and glutathione reductase that work together to respond to an onslaught of dietary and endogenously produced oxidants (Boardman, 2012). Other than this, Cytochrome P_{450} has been described to play an important role in insecticide metabolism of honey bees (Suchail et al., 2004; Liu and Zhang, 2004) including biosynthesis, breakdown, and detoxification of endobiotics and xenobiotics, cellular metabolism and homeostasis (Hu et al., 2017; Palrasu and Siddavaram, 2018). Thus, for the present study, we hypothesized that insecticides alter the physiological activities, thereby leading to generation of reactive oxygen species in *D. gazella.* Deltamethrin was selected for the present study since little is known currently about its effect on parcoprid and the limited work undertaken suggests that it may cause prolonged impact on dung beetles (Mann et al., 2015). ***Overall, the present study will focus on understanding neural regulation in Nesting Behaviour of Dung Beetle on exposure to insecticide,*** for which following objectives were designed.

Objectives

1) **To understand the brood morphometry and digging behaviour of Dung Beetle**
    a) To carry out the morphological and molecular identification
    b) Rearing and acclimatization of dung beetle in laboratory
    c) To study the life cycle, brood morphometry, and nesting pattern
    d) To study the digging pattern and the associated digging genes
2) **To understand the role of neurotransmitters in the nesting pattern and tunnel pattern of dung beetle**
    a) By evaluating the neurotransmitter levels of: Acetylcholine, Dopamine and Serotonin
    b) To check the enzyme activity of:
        - Dopa-Decarboxylase
        - 5 Hydroxy-tryptophan Decarboxylase
3) **To evaluate the toxicity of insecticide on Nesting behaviour of dung beetle**
    a) Finding out the LC_{50} value
    b) To estimate the level of Neurotransmitters and alteration in Nesting behaviour

Materials and Methodology

Objective 1: To understand the brood morphometry and digging behaviour of Digitonthophagus gazella

a) Collection and identification of dung beetles at morphological and molecular level:

Digitonthophagus gazella 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 Western India (**Fig. 3**). Collection of *D. gazella* was carried out during the time of dawn and dusk, in the months of June to November for three years (2020-23). 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), and were brought to laboratory for identification and rearing.

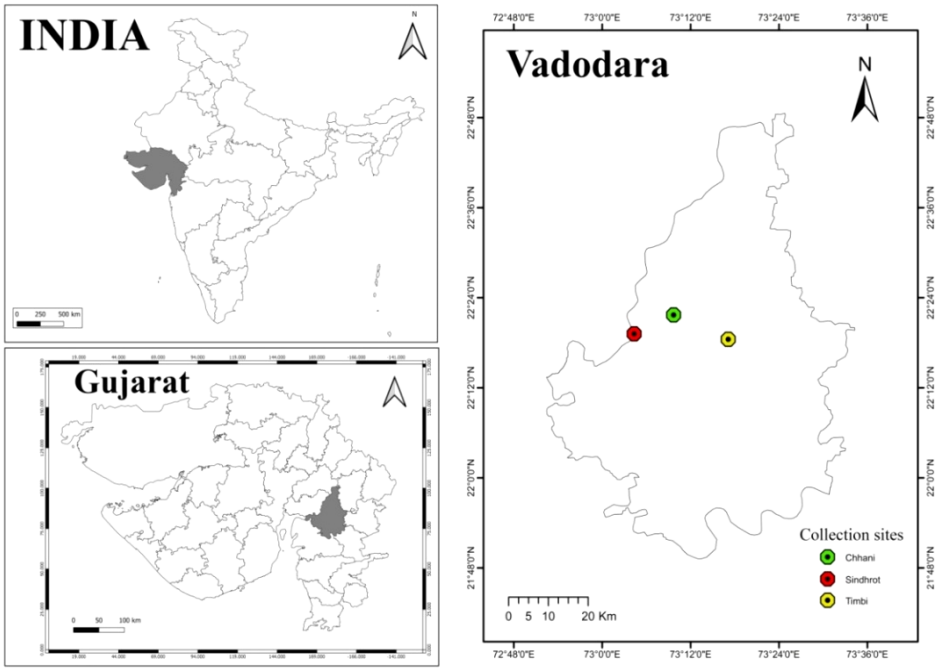


Figure 3: Map represents the collection sites for *D. gazella* from Vadodara district of Gujarat, India. *D. gazella* were collected from the outskirts of Vadodara district such as Channi (22.363˚N, 73.166˚E), Sindhrot (22.331˚N, 73.063˚E), and Timbi (23.149˚7N, 74.002˚E).

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 performed 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). For the present study,

genomic DNA samples were prepared from fresh insect. Total genomic DNA was extracted by dissecting the femoral muscle of dung beetle using the phenol chloroform method (Huang et al., 2006) and the 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 2**). The amplified DNA was assessed by conducting agarose gel electrophoresis followed by Sanger sequencing and Barcoding. The obtained sequence will be further uploaded on NCBI.

Table 2: Primers of COI genes obtained

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

b) Rearing and acclimatization of dung beetle in laboratory.

Dung beetles (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). Rearing medium in the pots was the sandy soil (obtained from collection sites, pH-6±0.5) and fresh dung of the cattle, 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 with the help of trowel (30 cm), near the same agricultural fields of beetles' collection, and a 250 g of dung was added to the pot after every 24 hours. Further, these earthen pots were covered with a black cloth at the top, and placed in a large tray containing moist sand for maintaining the temperature (22° to 26°C), and humidity (70±5%), with a 10L: 12D light regime (Bang et al., 2004).

c) To study the life cycle and Brood morphometry

For life cycle study and brood morphometry, five pair of adults (14 mm long) was released in earthen pot. The tunnel pattern was observed on an equal time period of 10^{th}, 20^{th}, and 30^{th} day, and the brood balls formed were collected for the morphometry (length, diameter, and weight). At 12 hours interval, brood balls were monitored for the development of the individuals, starting from egg up to the adult stage. The opening of 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 were recorded with the help of vernier caliper (Zhart, India) and analytical balance (Wensar, PGB200, India) (Singh et al., 2019).

d) To study the nesting 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. The burrow casts were excavated at the end of 10^{th}, 20^{th}, and 30^{th} day. The casting and measurement of the tunnel was done following the method of Sinha, (2013).

- 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.

e) To study the digging pattern and the associated digging genes

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 done, followed by cDNA synthesis (**Table 3 & 4**) and RT-PCR (**Table 5 & 6**) using the primers of dll and ems (**Table 7**).

Total RNA extraction (Trizol method)

For total RNA extraction, leg tissue in PBS (pH-7) was isolated from both male and female dung beetles after 10^{th}, 20^{th} and 30^{th} day. The tissue (50-100 mg) was weighed and homogenized in 500µL 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 100µL 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 12,000 RPM 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 500µL 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 below.

Table 3: 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 thermocycler in following conditions

PCR conditions

Table 4: Reverse transcription cycling program for cDNA synthesis

	Temperature	**Time**	**Number of cycles**
cDNA synthesis	42 °C	30 min	1 cycle
Inactivation	95 °C	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. 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 5: 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 6: 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 7: 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	TCCAAAAGATACTTACTTCCA GGG	59.30˚C

Objective 2: To understand the role of neurotransmitters in the nesting behaviour of Digitonthophagus gazella

a) To Estimate the neurotransmitter levels- Dopamine, Serotonin, Acetylcholine, Nitric oxide

Preparation of tissue extract to estimate Dopamine and Serotonin (Schlumpf et al., 1974)

After 10th, 20th, and 30th day of tunneling, each pair (of 5 pairs) of male and female *D. gazella* was collected and sacrificed by keeping it in -20ºC for 1 minute and then dissecting out the brain on ice blocks, by using sterile forceps in ice-cold saline (pH-7.4), followed by storage in -20ºC. On the day of experiment, the tissue samples were homogenized in HCl-Butanol, followed by centrifugation at 6000 rpm for 20 minutes. Then, the supernatant was added to the centrifuge tubes containing Heptane and HCl. After vigorous shaking, the tubes were centrifuged at 6000 rpm for 20 minutes to separate the two phases, and the overlaying organic phase was discarded. Then the aqueous phase (0.2 mL) was taken for 5-HT and Dopamine assay. All steps were carried out at 0^{o}C.

Estimation of dopamine

To the 0.2 mL of aqueous phase, 0.05 mL of 0.4 M HCl and 0.1 mL of EDTA / Sodium acetate buffer (pH-6.9) were added, followed by 0.1 mL iodine solution (0.1 M in ethanol) for oxidation. The reaction was stopped by addition of 0.1 mL Na_2SO_3 solution after 2 min followed by heating of the solution to 100°C for 6 min. The samples were then allowed to cool down at room temperature, post which the excitation, and emission spectra were read at 330-375 nm in the spectrofluorimeter.

Estimation of Serotonin

To 0.2 mL aqueous extract, 0.25 mL of OPT reagent was added. The fluorophore was allowed to develop by heating the samples at 100°C for 10 min. Then, the sample was allowed to reach equilibrium with the room temperature, and the readings were taken at 360-470 nm in the spectrofluorimeter.

Estimation of Acetylcholine esterase activity

The rate of AChE activity was measured according to the method described earlier by Ellman et al., (1961). Male and female dung beetles were dissected on ice blocks to avoid denaturation and maintain temperature, and brain tissues were collected after 10^{th}, 20^{th} and 30^{th} day. A 20mg/mL of tissue was homogenized in 0.05M phosphate buffer. This step was followed by addition of 0.5 mL Triton X-100 and 0.2 mL EDTA. The samples were centrifuged at 6000rpm for 20 mins at 4^{o}C. Then, 0.1 mL of supernatant was taken into the cuvette as a source of enzyme, followed by addition of 2.86 mL of phosphate buffer. The sample was incubated for 5 minutes at room temperature, post which, 50 µL DTNB solution was added to the cuvette, followed by addition of 30 µL of AChI (0.075M) to cuvette. The blank for such a run consisted of buffer, substrate, and DTNB solutions. Absorbance was recorded at 412 nm using UV visible spectrophotometer (PerkinElmer Lambda 25, India).

Estimation of Nitric Oxide

NO levels were estimated by following the method of Miranda et al. (2001). In this method, isolated tissues (100 mg) were homogenized in 10 vol ice-cold saline solution using a homogenizer. Upon disruption, absolute ethanol was added (2:1 vol ratio) to precipitate all proteins. After allowing materials to separate over a 15 min period at 25^{o}C, the supernatant was recovered. To 0.5 mL tissue extract, 0.5 mL vanadium chloride (8 mg VCl_3/mL) was added rapidly followed by addition of 0.25 mL of 2% sulfanilamide and 0.25 mL of 0.1% N-(1-naphthyl)-ethylene diamine. The mixture was vortexed and incubated at 37^{o}C for 30 min. Then, the absorbance was measured at 540nm in a UV spectrophotometer (PerkinElmer Lambda 25, India).

Similar procedures were followed for the non-breeding beetles (control), where male and female beetles were kept in separate earthen pots.

b) To estimate the gene expression of the neurotransmitters synthesizing enzymes

Brain tissue were collected from the male and female dung beetles after 10^{th}, 20^{th} and 30^{th} day of tunneling to estimate the level of expressions for the neurotransmitter synthesizing enzymes (**Table 8**) involved in the regulation of neurotransmitters.

Table 8: Real time PCR primer sequences of neurotransmitter synthesizing enzymes

Sr. no.	Accession no.	Gene Name	Primer type	Sequence	T_m (˚C)
1	NM_001102586	Dopa decarboxylase (ddc)	Forward	CAAAAGCCCGACAAATGGG	60.03
			Reverse	AGTTGGCGGTGGGGAAATAG	60.04
2	NW_022587571.1	5-HTPdecarboxylase/ aromatic-L-amino-acid (5-htpdc)	Forward	GCGTGGAATGCTGTCTTAGTT	58.92
			Reverse	GCATTATCTGCCCTTGTTGTGT	59.91
3	NT_033777.3	choline acetyltransferase (chat)	Forward	ATCGAGCCGCATTGTGTGT	60.38
			Reverse	CGGAAAGTTCGTGGGCTCT	60.00
4	NT_033779.5	Nitric oxide synthase (nos)	Forward	TCTCTACGACTGGAGTTGGCT	60.27
			Reverse	AATGACGTCCACGAGTTCTG	57.93

Objective 3: To evaluate the alteration in the Nesting behaviour of dung beetle on exposure to insecticide

This objective was designed to evaluate the effect of test chemical on dung beetles. The test chemical selected was the technical grade of Deltamethrin (Sigma 45423-250MG, Deltamethrin Perstanal 250MG). It was mixed with dung (as per OECD guidelines, Reference no. 207) to which the adult beetle were exposed and assessed under laboratory conditions and observed for the behavioural alteration.

a) Finding out the LC_{50} values

Preparation of Deltamethrin

Deltamethrin was selected for the present study and was procured from Sigma Aldrich (45423, Saint Louis, USA). The IUPAC name is [(S)-Cyano-(3-phenoxyphenyl)-methyl] (1R, 3R)-3-(2,2-dibromoethenyl)-2,2-dimethyl-cyclopropane-1-carboxylate (**Table 1**). Stock solution of

Deltamethrin (0.1mg/L) was prepared by dissolving it in acetone and was stored at room temperature.

Experimental procedure for LC_{50} determination and behavioural studies:

After the completion of acclimatization for a day, dung beetles were exposed with test concentration of 0.005, 0.01, 0.05, 0.5 and 1ppm, there were three replicates in each test concentration and each replicate containing 10 *D. gazella* (5 Male + 5 Female). After every 24 hours, behavioural alterations in *D. gazella* were observed and mortality rate was recorded for every 48 hours. The collected data was computed according to probit analysis method of Finney, (1971).

b) To estimate the level of neurotransmitters and alteration in nesting behaviour

Alteration in neurotransmitters on exposure to Deltamethrin

5 pairs of male and female *D. gazella* were exposed to the dung treated with sub-lethal concentrations (LD, MD, and HD) of Deltamethrin. After 10^{th}, 20^{th}, and 30^{th} day of tunneling, each pair of male and female *D. gazella* for each group (control, LD, MD, and HD) were sacrificed, and the brain was dissected in ice-cold saline (pH-7.4). Further, the rate of AChE activity was measured according to the method described earlier by Ellman et al., (1961) and NO levels were estimated by following the method of Miranda et al., (2001). Biogenic amines *(*DA and 5-HT) estimation was carried out following the method of Schlumpf et al., (1974).

Data Analysis:

- Probit analysis (Finnet, 1971) was used to calculate median lethal concentration and time with their upper and lower confidence limits.
- Statistical analysis was done using Graphpad prism 8 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 $*p<0.05, ** p<0.01, *** p<0.001$.

Results

OBJECTIVE 1

a) Morphological Identification

Figure 4: Morphology of *D. gazella*

Morphological identification (**Fig 4, Table 9**) was done using standard references and the characteristic features of *D. gazella*.

Table 9: 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; Génier and Moretto, 2017; Pokhrel et al., 2020)

b) Molecular Identification:

DNA quantification

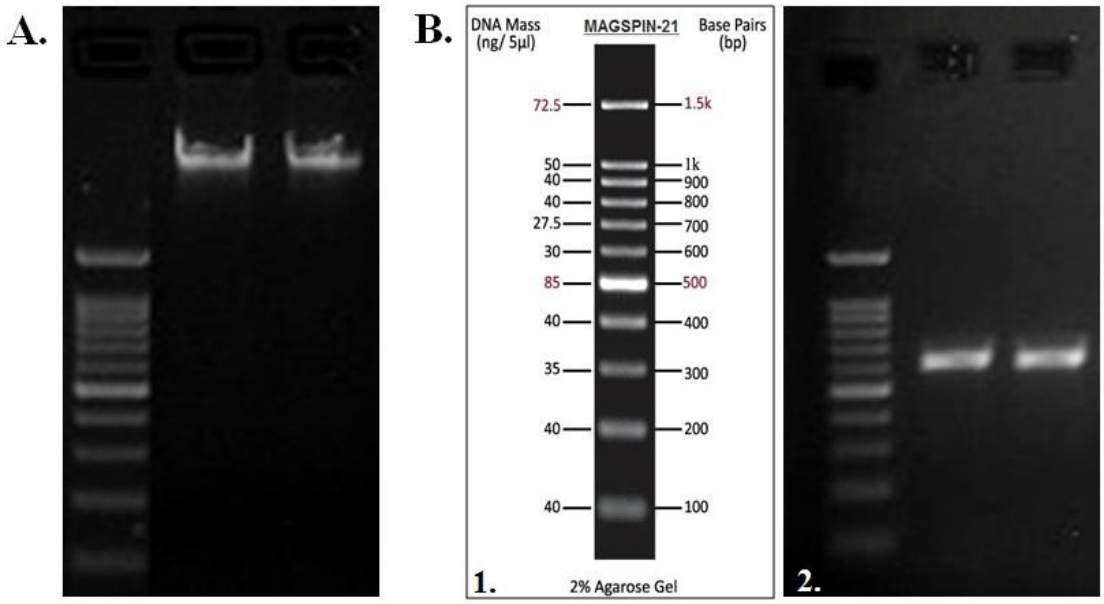


Figure 5: Results of Agarose Gel Electrophoresis. A. Genomic DNA B. PCR Product 1. Base pair size 2. PCR product of COI

Bands of Genomic DNA are shown in **Fig. 5A** and that for the COI gene in **Fig. 5B**. The COI gene consisted of 720bp, when run on 2% Agarose gel. Further, the barcode (**Fig. 6**) and sequence of amplified COI gene was obtained which showed similarity with the dung beetle species, *D. gazella*, on NCBI Blast, confirming its identity.

Barcode and sequence of COI gene

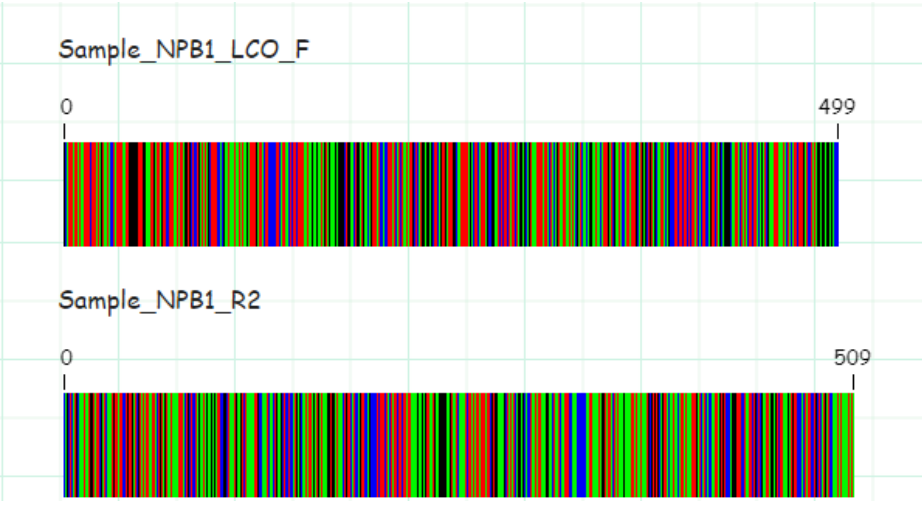


Figure 6: Barcode of COI gene in *D. gazella*

Sequence for LCO_F

GCATTTATTATAATTTTCTTTATAGTAATACCTATTTTAATTGGGGGGTTTGGAAATT
GATTAGTTCCTTTAATATTAGGTGCTCCTGATATAGCTTTTCCACGAATAAATAATAT
AAGATTTTGATTACTTCCCCCTTCATTAACTCTTCTTTTAATAAGAAGAATAGTAGAA
AGAGGGGCTGGAACTGGATGAACAGTTTATCCACCTTTATCATCTAATATTGCTCAT
GGAGGAGCTTCAGTTGATTTGGCAATTTTTAGACTTCATTTAGCCGGAATCTCTTCTA
TTCTAGGAGCAGTAAATTTTATTACTACAGTAATTAATATACGATCAACAGGAATAA
CATTTGATCGAATACCATTATTTGCATGAGCTGTAGCAATTACAGCCCTTCTTCTTCT
CTTATCACTTCCAGTTCTAGCAGGGGCAATTACTATACTTCTTACAGATCGAAATTTA
AATACTACATTCTTTGATCCTATAGGAGGAGGAGACCC

Sequence for R2

CAGCTCATGCAAATAATGGTATTCGATCAAATGTTATTCCTGTTGATCGTATATTAAT
TACTGTAGTAATAAAATTTACTGCTCCTAGAATAGAAGAGATTCCGGCTAAATGAAG
TCTAAAAATTGCCAAATCAACTGAAGCTCCTCCATGAGCAATATTAGATGATAAAGG
TGGATAAACTGTTCATCCAGTTCCAGCCCCTCTTTCTACTATTCTTCTTATTAAAAGA
AGAGTTAATGAAGGGGGAAGTAATCAAAATCTTATATTATTTATTCGTGGAAAAGCT
ATATCAGGAGCACCTAATATTAAAGGAACTAATCAATTTCCAAACCCCCCAATTAAA
ATAGGTATTACTATAAAGAAAATTATAATAAATGCGTGTGCAGTTACAATAACATTA
TAAATTTGATCATCACCAATTAGTGTCCCAGGGTTTCCTAATTCTGCTCGAATTAGGA
GTCTTAAAGATGTTCCCACTATTCCTGCTCATGATCCAAATATAAAATATA

Consensus Sequence

GGGTCTCCTCCTCCTATAGGATCAAAGAATGTAGTATTTAAATTTCGATCTGTAAGA
AGTATAGTAATTGCCCCTGCTAGAACTGGAAGTGATAAGAGAAGAAGAAGGGCTGT
AATTGCTACAGCTCATGCAAATAATGGTATTCGATCAAATGTTATTCCTGTTGATCGT
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CCAATTAAAATAGGTATTACTATAAAGAAAATTATAATAAATGCGTGTGCAGTTACA
ATAACATTATAAATTTGATCATCACCAATTAGTGTCCCAGGGTTTCCTAATTCTGCTC

GAATTAGGAGTCTTAAAGATGTTCCCACTATTCCTGCTCATGATCCAAATATAAAAT
ATA

Nesting behaviour

During the period of acclimatization and rearing, the 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.

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 10)**. Under laboratory conditions, the tunnel pattern studies indicate that *D. gazella* constructs a simple tunnel over the period of time (**Fig. 7A**). 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. 7B**). Conversely, results of digging genes showed a decline in dll and ems gene expression (**Table 11**) with the increasing time, proving its digging behaviour to be maximum after 10 days, followed by 20 and 30 days (**Fig. 8**).

Table 10: Observation of burrow cast of *D. gazella* on 10^{th}, 20^{th} and 30^{th} 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*

Sr. No	Observations	10 days	20 days	30 days
1	NBO	1	1	1
2	L	14.7	16.9	19.8
3	TD	9.8	12.9	13.5
4	DOB	1.11	1.11	1.16
5	Area	12.8	14.72	18.02
6	NOB	3	4	4
7	Pattern	Simple	Simple	Simple

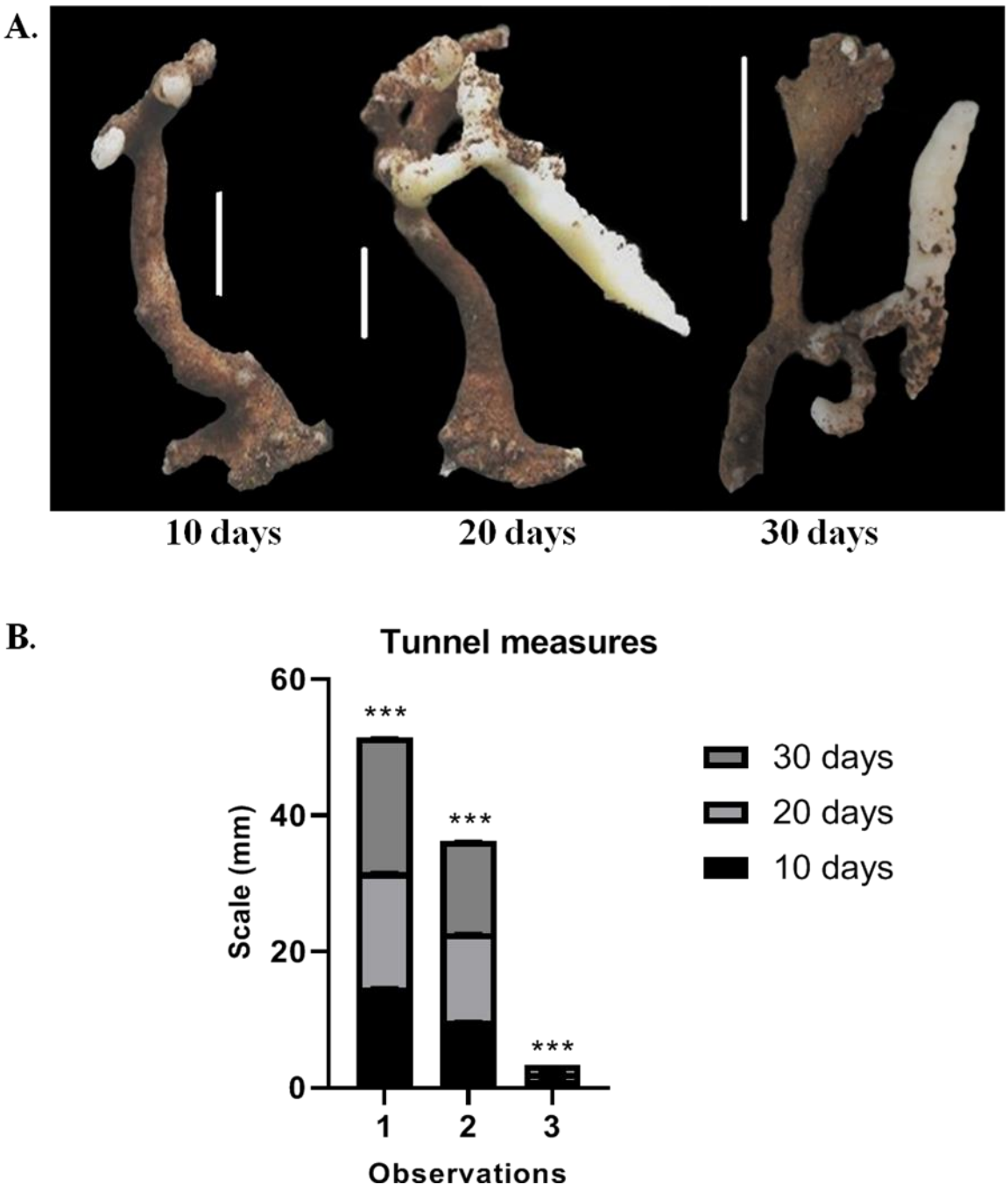
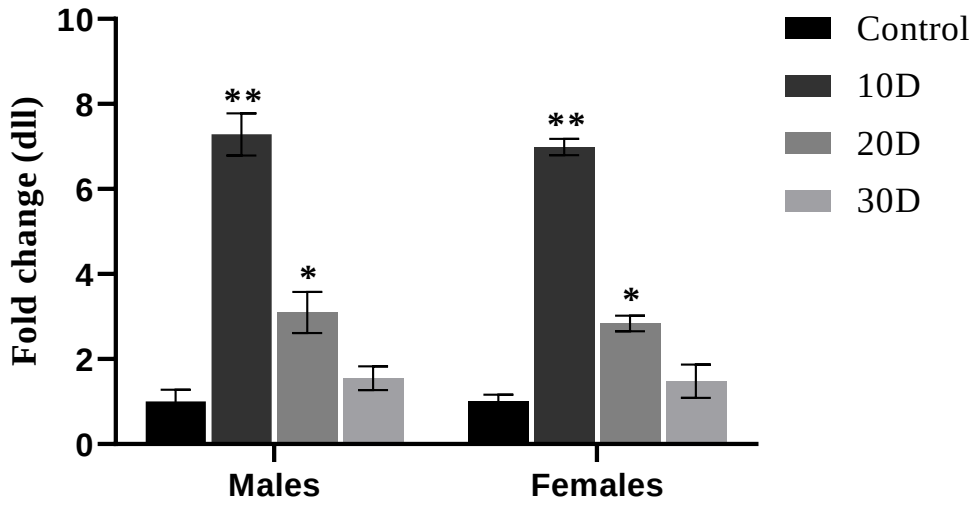


Figure 7: The tunnel pattern of *D. gazella* is shown. A. The tunnel formed at the end of the 10th, 20th, and 30th day is shown (scale = 2 cm). B. The graph represents the key measures of tunnel formation. The length (1), depth (2), and diameter (3) of the tunnel were observed to increase significantly ($p<0.001$) with the increasing number of days (10th, 20th, and 30th day). *Here,* $p<0.001$*** was obtained from statistical analysis done using two-way ANOVA *(n=3)*

Table 11: The fold change in the expression of dll and ems (Mean ± SEM) in the males and females

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

8(A)



8(B)

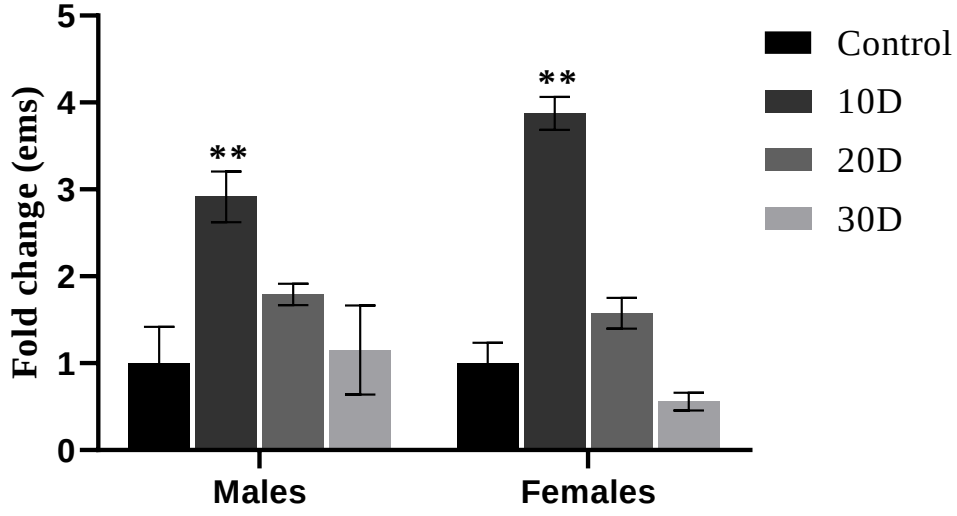


Figure 8: The fold change in digging genes in male and female dung beetle (A) dll (B) ems

Brood morphometry

Tunnels were dug and the brood balls were removed. The average number (Mean±SE) of the brood ball was found to be 50±0.76, 139.2±0.46, and 150.4±0.8 after 10, 20 and 30 days respectively. Brood morphometry showed that the brood balls were spherical with strongly stacked dung containing a single egg at the centre of the ball (**Fig. 9A**). Cylindrical shaped brood masses (Mean±SD; n=15) had a length of 33.72±5.89 mm, a width of 7.8±0.89 mm, and weighed 745±1.34 mg (**Table 12**).

Life cycle

The duration of different developmental stages are presented in **Table 13 & Fig. 9B**. The life cycle of *D. gazella* comprised of 4 stages, *i.e.*, egg, larva (1st instar, 2nd instar, and 3rd instar), pupa, and adult (**Fig. 9C**). The total development period was found to be of 30 days. Further, the length, diameter, and weight of all the developing stages were recorded (**Fig. 9D**). Each elongated, cylindrical brood ball showed a single egg laid in the central chamber in a vertical position. The eggs were elongated, cylindrical, and yellowish white. 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 was used as a pivot when fed on the dung. The second instar larva showed characteristic mandibles. 3rd instar was observed to comprise of the highest length and weight where as the highest diameter was found to be of pupa. The newly developed pupa was creamy white and shiny, sexual dimorphism was evident in the pupa. The 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 25-27 days, followed by its transformation into adult. After the emergence from the brood mass, adults showed pigmentation and maturation within 2-3 days and its longevity period was 60 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.

Table 12: Brood morphometry of different developmental stages (Mean±SD) of *D. gazella*

Stage	Length (mm)	Diameter (mm)	Brood Weight (mg)
Egg	2.49±0.08	1.47±0.09	6±0.67
1st instar	3.63±0.56	1.36±0.06	18±0.56
2nd instar	5.78±0.94	1.75±0.74	127±0.83
3rd instar	20.64±1.98	2.38±0.56	326±0.43
Pupa	11.36±2.39	6.3±0.83	136±0.58
Adult	14.67±1.78	6.9±1.49	139±0.16
Brood ball	33.72 ± 5.89	7.8±0.89	745±1.34

Table 13: The developmental period for various stages of life cycle of *D. gazella*

Serial Number	Stage	Time (Days)		Mean± S.D.	Development Days
		Minimum	Maximum		
1	**Egg**	2	4	3.42± 0.634	3±0.783
2	**First Instar**	5	8	6.05± 1.08	3±1.356
3	**Second Instar**	9	12	11.28± 0.97	5±0.538
4	**Third Instar**	13	24	23.76± 2.78	11±2.456
5	**Pupa**	25	29	28.98±1.34	4±1.23
6	**Adult**	28	32	31.83± 1.45	2±1.894

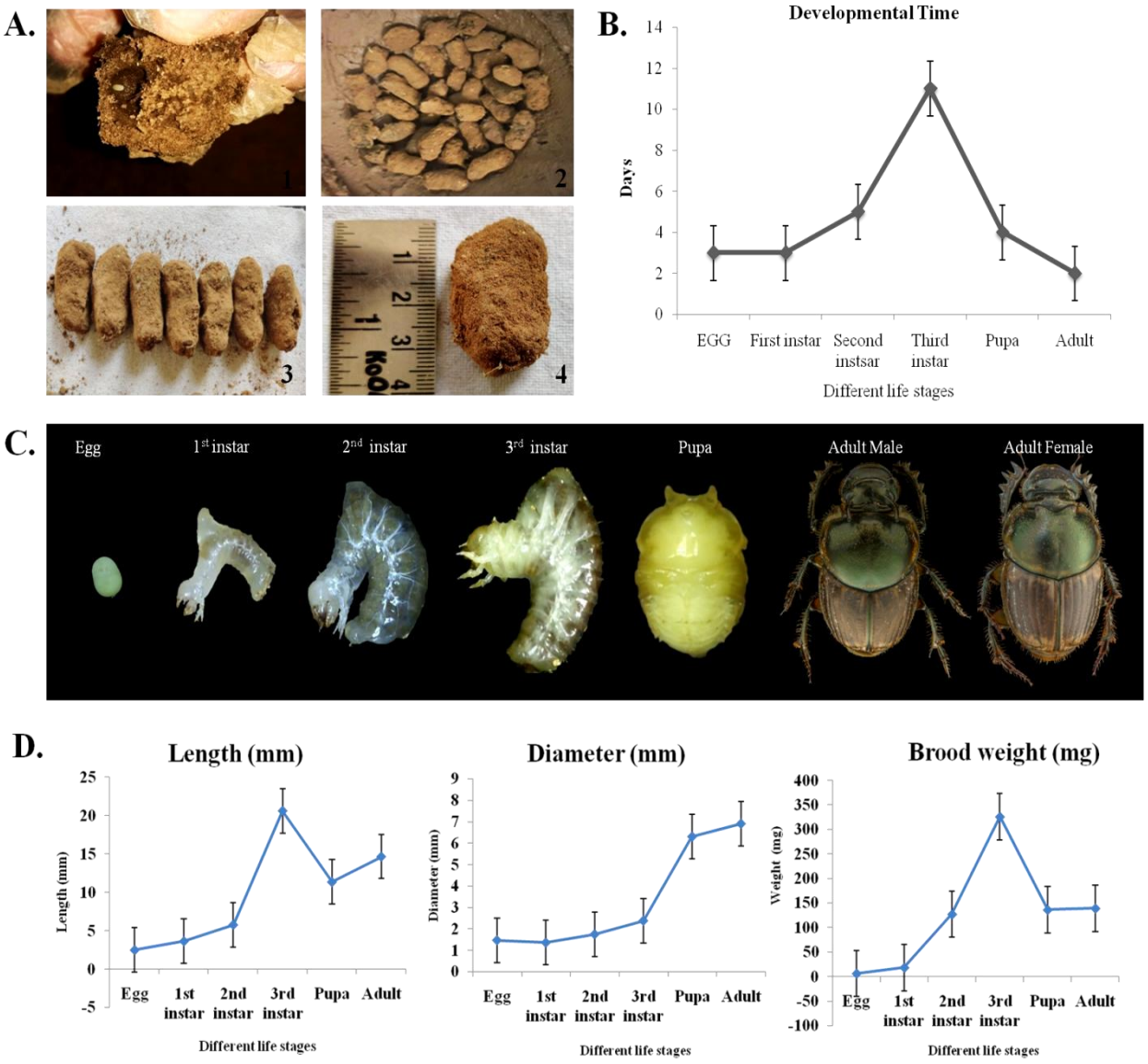


Figure 9: Study on brood masses and life cycle of *D. gazella*. A. Brood morphometry. Brood balls' measures were noted (2, 3, 4) with each brood ball containing one egg (1). B. Comparative account on the duration of the different stages in the life cycle of *D. gazella*. C. The stages of the life cycle starting from egg, larva (1st, 2nd, and 3rd instar), pupa and adult are observed. D. Comparative account of brood length, diameter, and weight of different stages of the life cycle of *D. gazella*. Brood length, diameter, and weight increase with higher developmental stage except that the 3rd instar larva shows the maximum length and weight. Here, *n=5*

OBJECTIVE 2

Neurotransmitter levels during nesting behaviour

To have an insight into whether there is any significant role of the neurotransmitters in the nesting behaviour of *D. gazella*; brain levels of DA, 5-HT, AChE, and NO were biochemically analyzed. A significant time-dependent increase in all the neurotransmitters was observed on the 10th, 20th, and 30th day of introduction of *D. gazella* into the experimental setup as compared to control (**Fig.10.1-10.4**). The lowest level of neurotransmitters was recorded for the control group and the maximum increase in the levels of neurotransmitters was found to be on the 30th day in male and female *D. gazella*. 5-HT was found to be higher in males compared to females, whereas the levels of DA were more in females compared to males.

Table 14.1: The level of Dopamine in the brain of *D. gazella*

Serial No.	Dopamine (Mean ± SE)		
	Group	**Males**	**Females**
1	Control	172.83±0.216	170.687±0.168
2	10 days	199.36±0.153**	175.86±0.585**
3	20 days	211.38±2.042**	218.36±2.04**
4	30 days	206.36±1.655**	219.513±2.98**

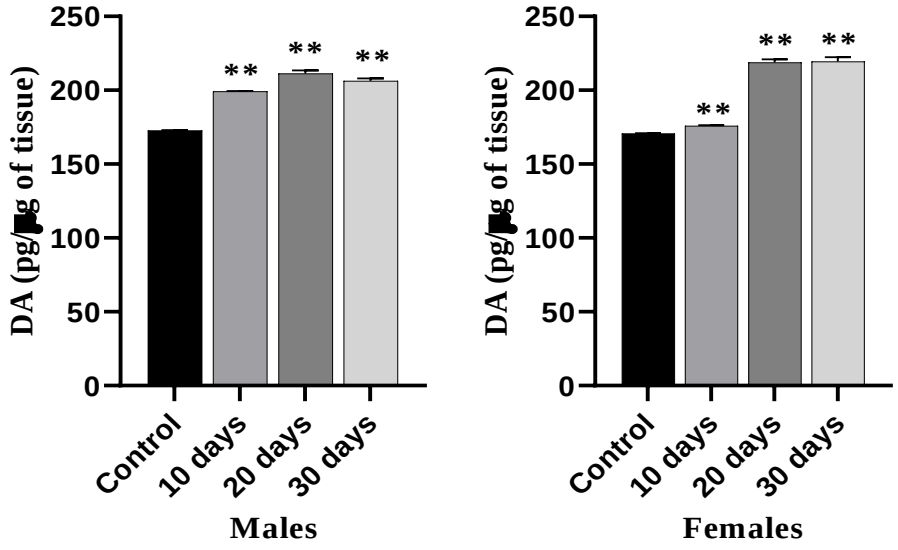


Figure 10.1: Rate of AChE activity (with the unit mmol/mL/min x 10^{-4} per g of tissue) on the 10th, 20th, and 30th day of introduction of males and females into the experimental setup. *The error bars indicate SE with significant values;*p<0.05 **p<0.01*

Table 14.2: The level of Serotonin in the brain of *D. gazella*

Serial No..	Serotonin (Mean ± SE)		
	Group	**Males**	**Females**
1	Control	76.46±0.5	70.79±0.37
2	10 days	93.58±1.44**	83.57±0.67**
3	20 days	99.17±1.5**	84.17±0.96**
4	30 days	101.7±2.66**	85.897±0.61**

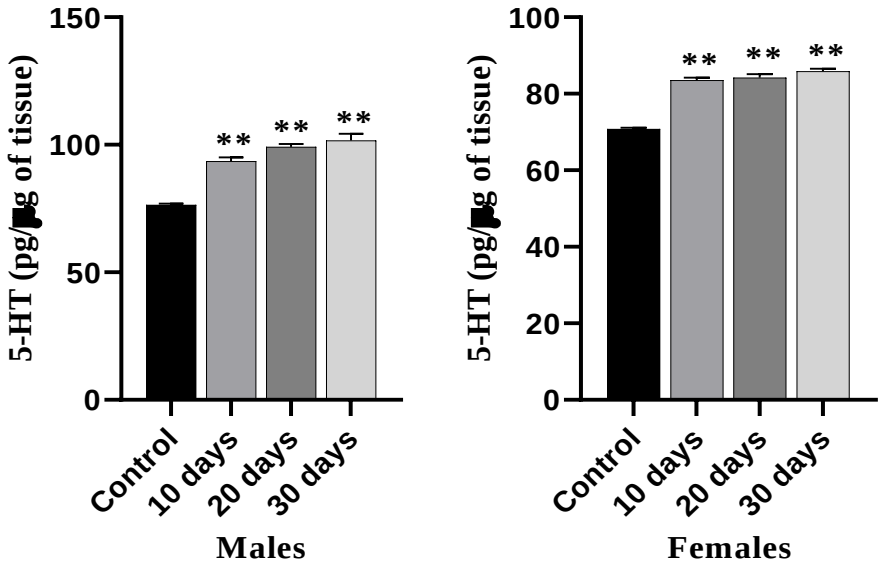


Figure 10.2: Serotonin (5-HT) levels on the 10th, 20th, and 30th day of introduction of males and females into the experimental setup. *The error bars indicate SE with significant values;*p<0.05 **p<0.01*

Table 14.3: Rate of AChE activity in the brain of *D. gazella*

Serial No.	Acetylcholine esterase activity (Mean ± SE)		
	Group	**Males**	**Females**
1	Control	0.0237±0.002	0.031267±0.003
2	10 days	0.0397±0.002	0.0404±0.004
3	20 days	0.0514±0.010*	0.055667±0.001**
4	30 days	0.0578±0.003**	0.061967±0.0006**

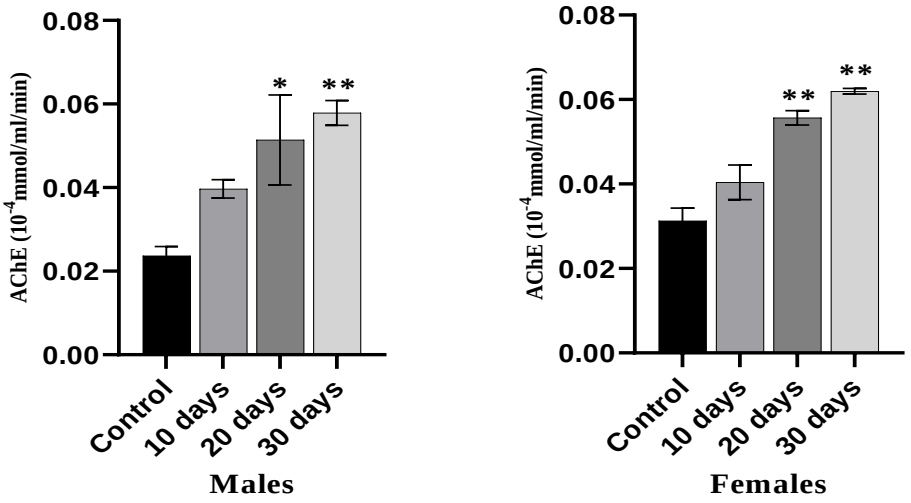


Figure 10.3: Dopamine levels on the 10th, 20th, and 30th day of introduction of males and females into the experimental setup. *The error bars indicate SE with significant values;*p<0.05 **p<0.01*

Table 14.4: The Nitric oxide content in the brain of *D. gazella*

Sr no.	Nitric oxide (Mean ± SE)		
	Group	**Males**	**Females**
1	Control	2.036±0.058	2.234±0.079
2	10 days	4.244±0.120**	4.32±0.026**
3	20 days	5.049±0.023**	5.10433±0.036**
4	30 days	5.268±0.076**	5.335±0.054**

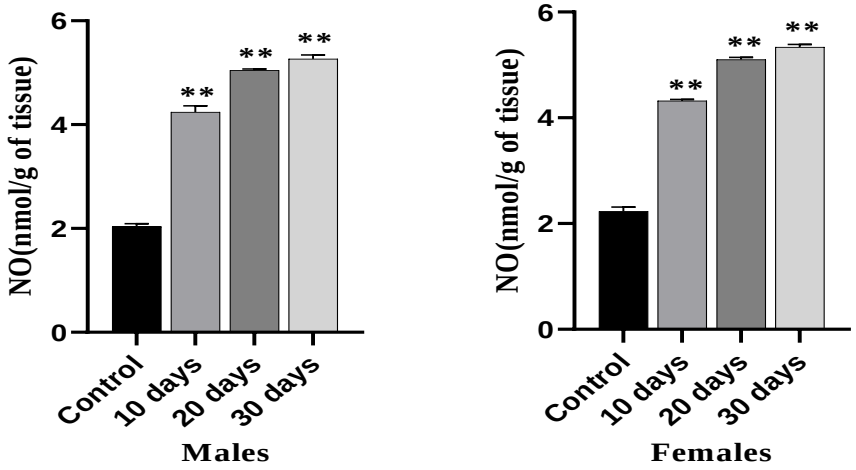


Figure 10.4: Nitric Oxide levels on the 10th, 20th, and 30th day of introduction of males and females into the experimental setup. *The error bars indicate SE with significant values;*p<0.05 **p<0.01*

Neurotransmitter biosynthesizing enzymes in nesting behaviour

The results of gene expression of the neurotransmitters synthesizing enzymes (**Table 15**) revealed a significant ($p<0.01$) increase in the expression of dopa decarboxylase (ddc), 5-hydroxytryptophan decarboxylase (5-htpdc), choline acetyl transferase (chat), and nitric oxide synthase (nos) (**Fig. 11**) after 10^{th}, 20^{th} and 30^{th} day along with the increase in the level of neurotransmitters confirming the role of neurotransmitters in the nesting behaviour of *D. gazella.*

Table 15: The fold change in the expression of ddc and 5-htpdc

Group	**ddc**		**5-htpdc**	
	Males	**Females**	**Males**	**Females**
Control	1 ±0.211	1±0.409	1±0.483	1±0.1024
10 days	3.402±0.527	2.933±0.077	4.401±0.479	2.911±0.281*
20 days	11.929±0.726**	15.542±0.288**	13.798±0.279**	13.319±0.201**
30 days	11.468±0.129*	16.563±0.105**	17.625±0.184**	14.013±0.260**
Group	**chat**		**nos**	
	Males	**Females**	**Males**	**Females**
Control	1±0.232	1±0.460	1±0.614	1±0.009
10 days	3.564±0.320*	5.243±0.033**	5.932±0.308**	7.307±0.464**
20 days	10.163±0.279**	11.439±0.033**	10.280±0.336**	12.978±0.038**
30 days	15.352±0.184**	16.478±0.012**	15.711±0.453**	17.333±0.239**

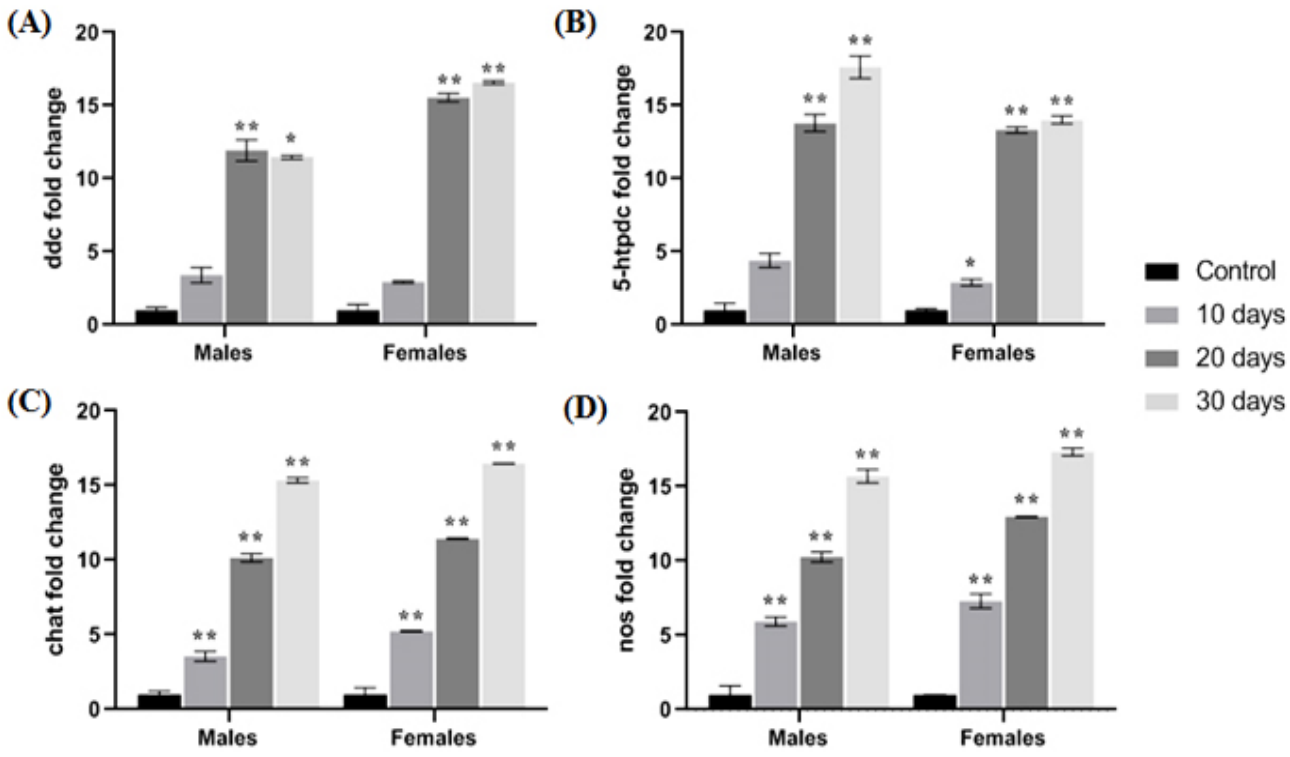


Figure 11: Neurotransmitters synthesizing enzyme gene expressions (A) ddc (B) 5-htpdc (C) chat (D) nos

OBJECTIVE 3

Evaluation of Toxic effects of Deltamethrin in *D. gazella*

a) Determining LC_{50} value of Deltamethrin

LC_{50} value of Deltamethrin was determined using Probit Analysis after 48 hours of exposure of dung beetles. The 50% probit mortality ranged between 0.05 to 0.5 ppm concentrations (**Table 16**). LC_{50} value was obtained as 0.275 ppm from the dose response curve (**Fig. 12**). Further, the sub-lethal concentrations: Low dose (LD)-1/20^{th} of LC_{50}, Medium dose (MD) - 1/10^{th} of LC_{50}, and High dose (HD)-1/5^{th} of LC_{50} (**Table 18)** were used to understand the effects of Deltamethrin in the neurophysiology during nesting behaviour of Dung beetles; *D. gazella*.

Table 17: Probit Mortality obtained after 48 hours of exposure to Deltamethrin

Concentration (0.1mg/L)	log Concentration	% Mortality	Probit Mortality
0.005	-1.30	0	0
0.01	-1.00	0	0
0.05	-0.30	0	0
0.5	0.70	80	5.84
1.0	1.00	100	8.09

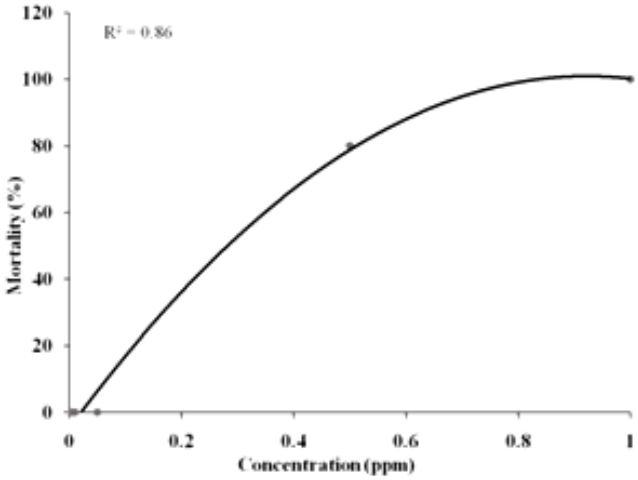


Figure 12: Dose response curve for the LC_{50} determination of Deltamethrin after 48 hours of exposure

Table 17: LC_{50} value obtained and the sub-lethal doses selected for further studies

Serial No.	Doses	Values
1.	LC_{50}	0.275ppm
2.	Low Dose (LD)	0.014ppm
3.	Medium dose (MD)	0.028ppm
4.	High Dose (HD)	0.055ppm

b) Alteration in neurotransmitters on exposure to Deltamethrin in *D. gazella*

A significant time-dependent (10^{th}, 20^{th}, and 30^{th} day) decrease in all the neurotransmitters was observed on exposure to sub lethal concentrations of Deltamethrin as compared to control (**Table 18** and **Fig.13.1-13.4**). The decreasing trend of neurotransmitters was observed in both male and female *D. gazella*.

Table 18: Brain neurotransmitter levels (Mean±SEM) in the male and female *D. gazella* on exposure to Deltamethrin after (a) 10 (b) 20 and (c) 30 days

		Individuals	DA	5-HT	AChE	NO
10 Days	UT	Males	199.36±0.15	93.581±1.44	0.0397±0.002	4.24±0.12
		Females	175.86±1.63	83.57±0.67	0.0404±0.004	4.32±0.03
	LD	Males	182.867±3.78**	89.75±1.06	0.0393±0.002	3.42±0.25*
		Females	166.25±1.3	74.45±2.59*	0.0397±0.002	3.54±0.2*
	MD	Males	143.603±0.585**	71.23±0.76**	0.038±0.002	1.62±0.23**
		Females	144.6±4.826**	61.34±1.88**	0.0386±0.002	2.14±0.3**
	HD	Males	159.33±1.84**	68.9±3.33**	0.0367±0.003	0.62±0.02**
		Females	179.75±2.5	65.13±3.37**	0.0383±0.002	1.01±0.2**
20 Days	UT	Males	211.385±2.04	99.18±1.54	0.0514±0.011	5.04±0.02
		Females	218.84±2.04	84.17±0.97	0.056±0.002	5.104±0.04
	LD	Males	209.8±3.07	99.97±2.877	0.0496±0.001	3.54±0.09**
		Females	214.24±4.42	79.26±0.398	0.053±0.004	3.68±0.103**
	MD	Males	178.343±1.924**	74.89±2.39**	0.0416±0.004	2.04±0.3**
		Females	191.76±6.85**	70.54±0.125**	0.043±0.002	2.68±0.27**
	HD	Males	171.511±3.431**	81.7±2.002**	0.0387±0.003	1.44±0.18**
		Females	187.297±4.161**	71.496±0.95**	0.041±0.003	2.07±0.131**
30 Days	UT	Males	206.36±1.66	101.7±2.66	0.0579±0.003	5.268±0.07
		Females	219.51±2.99	85.897±0.62	0.062±0.001	5.335±0.05
	LD	Males	184.483±3.09**	109.6±4.712	0.052±0.003	3.49±0.18**
		Females	186.93±5.02**	85.53±3.76	0.0577±0.003	3.69±0.23**
	MD	Males	166.757±3.29**	74.103±2.85**	0.0437±0.004*	2.04±0.21**
		Females	174.243±2.84**	69.013±3.102**	0.045±0.002*	2.4±0.39**
	HD	Males	162.273±2.12**	79.76±3.92	0.0397±0.003**	1.13±0.06**
		Females	165.753±2.88**	67.103±5.933*	0.0413±0.003**	1.318±0.05**

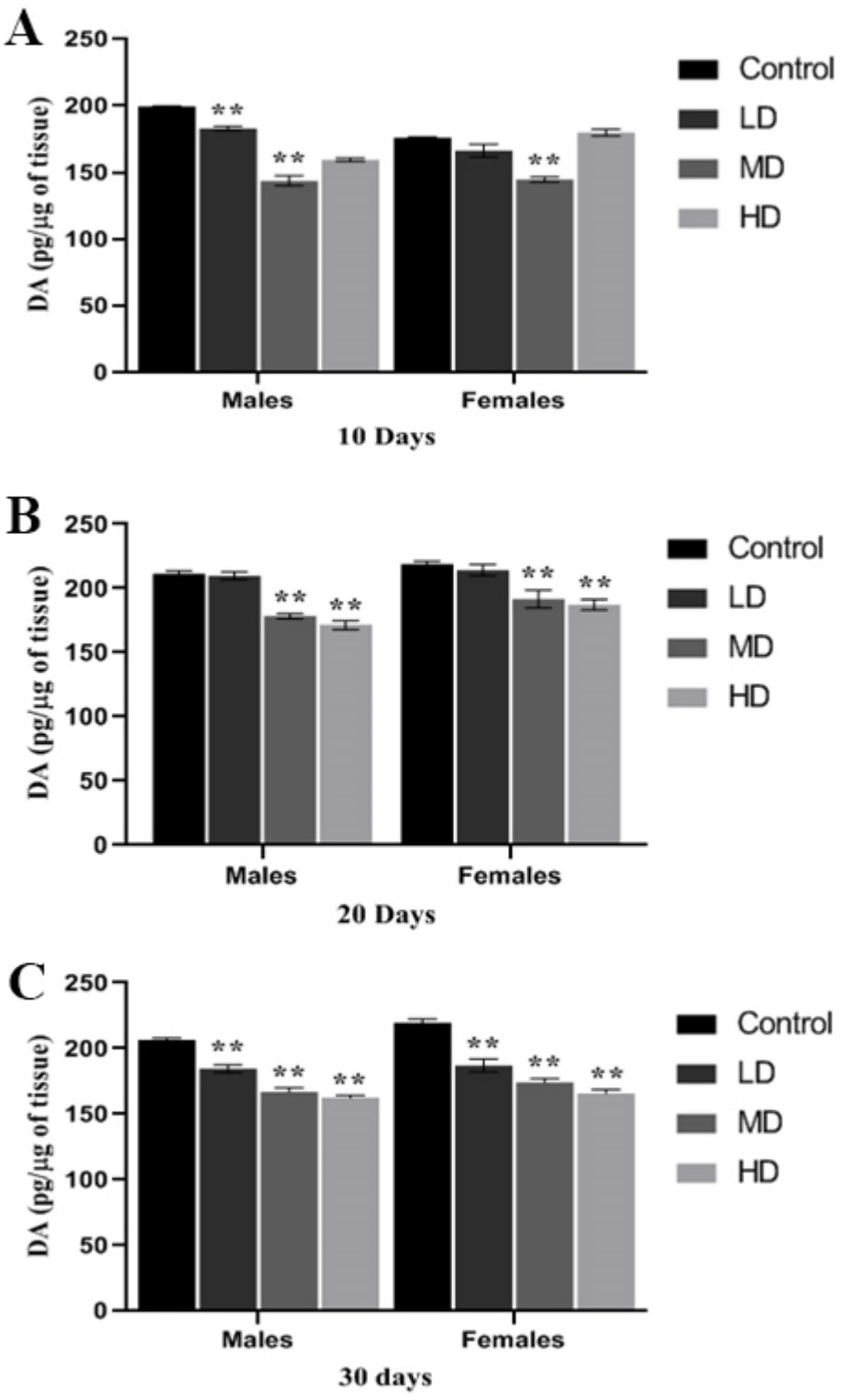


Figure 13.1: Brain DA levels in the male and female *D. gazella* on exposure to Deltamethrin after (A) 10 (B) 20 and (C) 30 days. **p<0.05 **p<0.01*

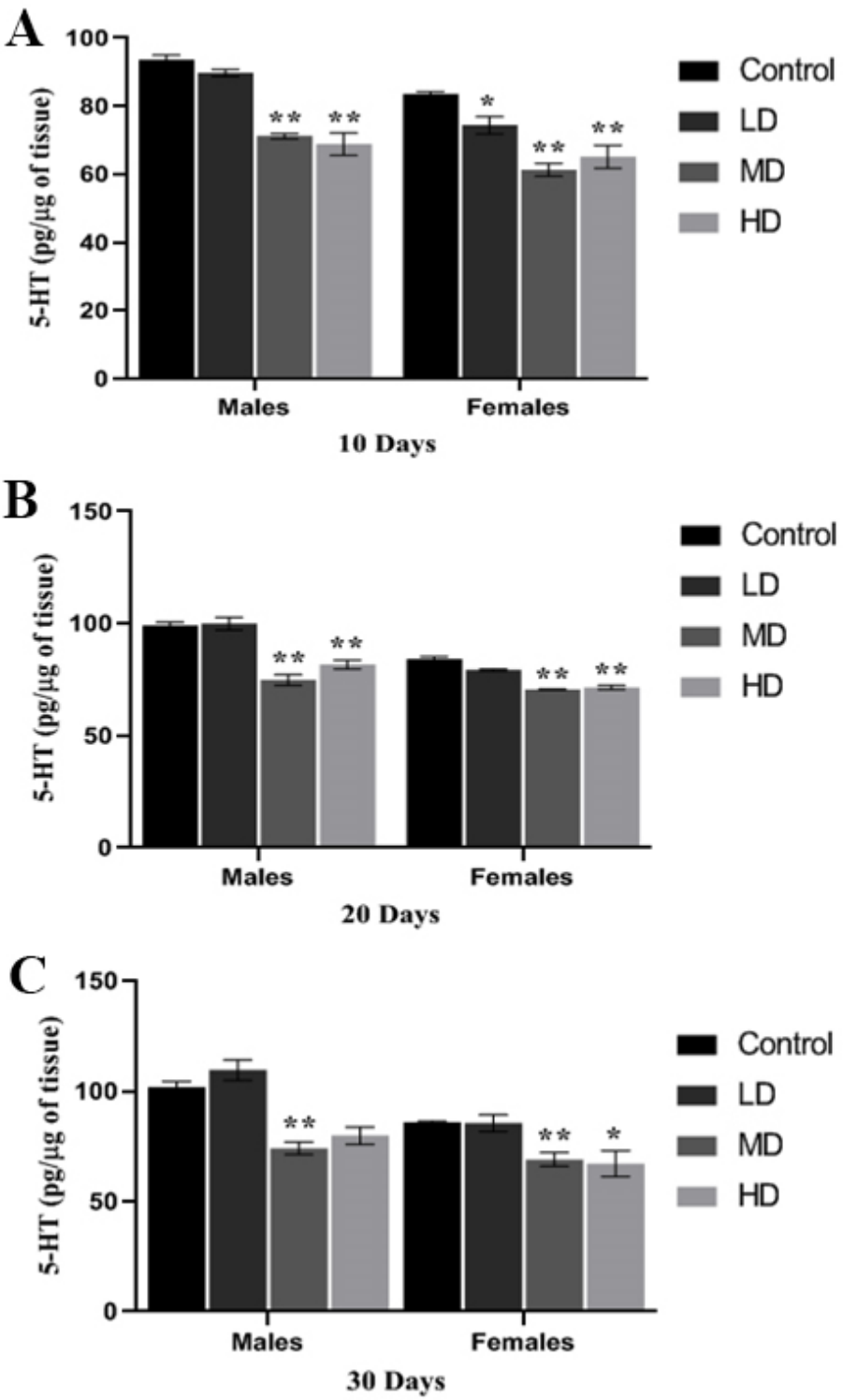


Figure 13.2: Brain 5-HT levels in the male and female *D. gazella* on exposure to Deltamethrin after (A) 10 (B) 20 and (C) 30 days. *$p<0.05$ **$p<0.01$

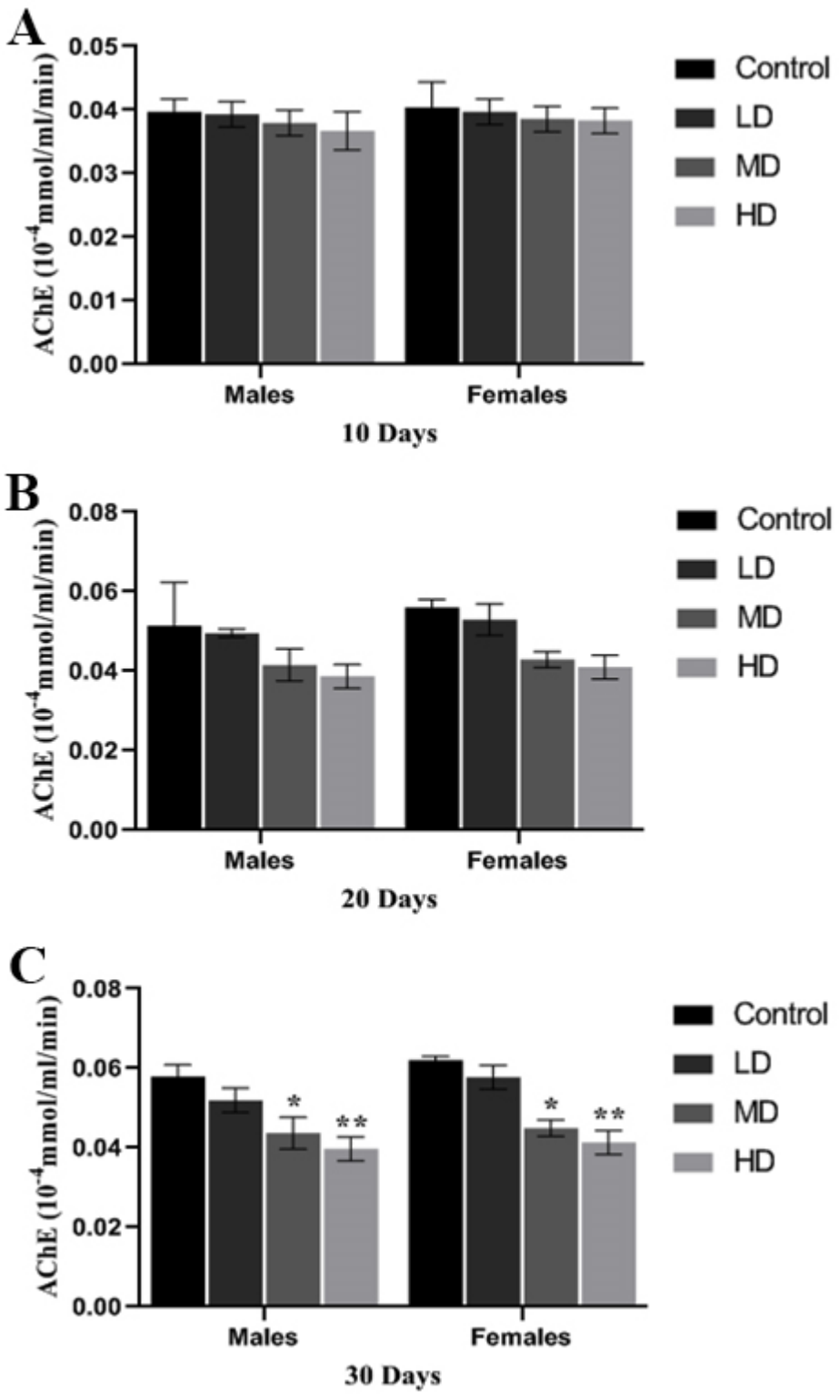


Figure 13.3: Brain AChE activity in the male and female *D. gazella* on exposure to Deltamethrin after (A) 10 (B) 20 and (C) 30 days. *$p<0.05$ **$p<0.01$

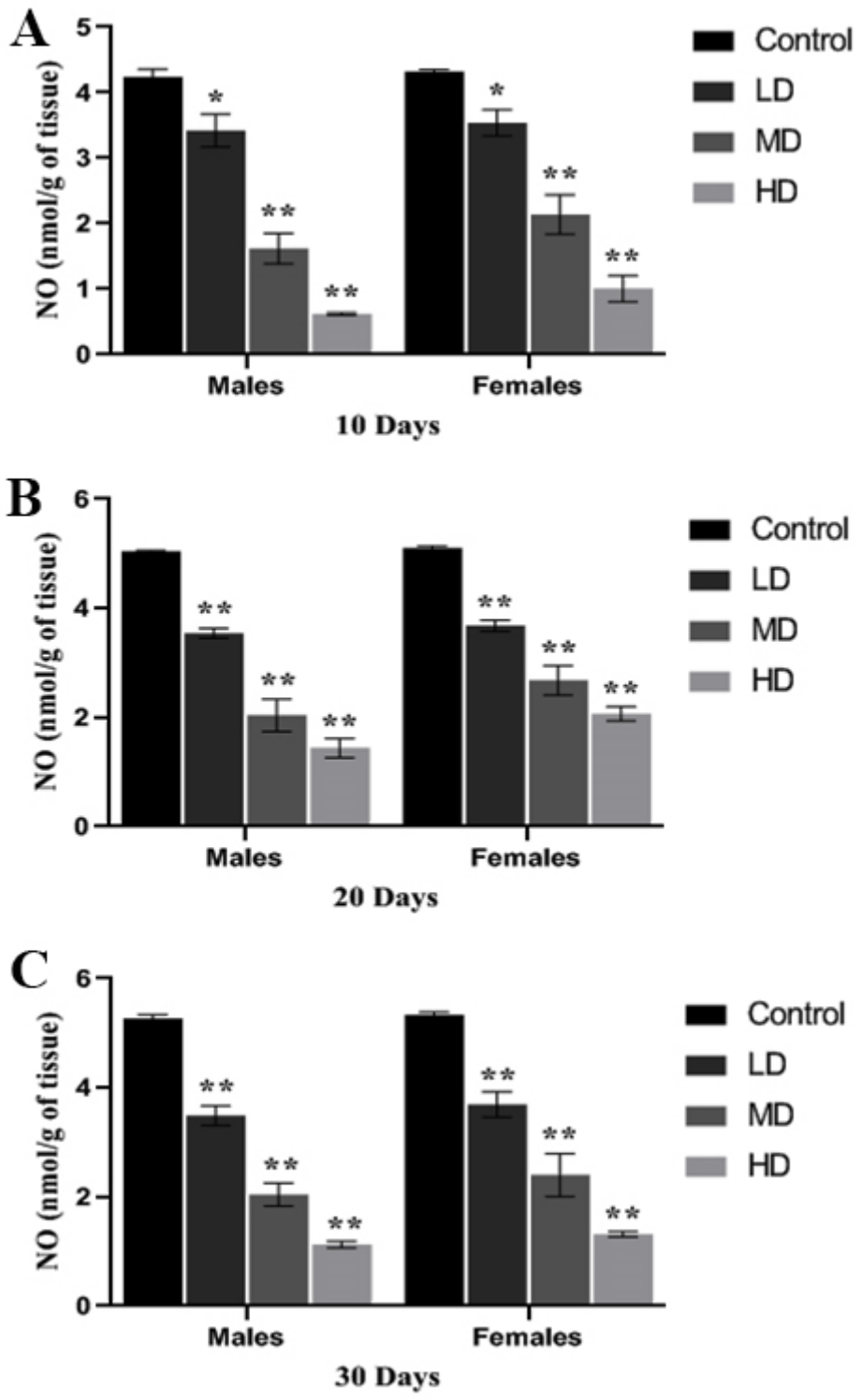


Figure 13.4: Brain NO levels in the male and female *D. gazella* on exposure to Deltamethrin after (A) 10 (B) 20 and (C) 30 days. **p<0.05 **p<0.01*

Discussion:

Diagnoses based on external morphology refer to well-developed individuals. Morphological traits reflect the way in which organisms physically interact with their environment, and can facilitate or constrain the ability of an organism to carry out specific behaviours and tasks (Barton et al., 2011; Traugott et al., 2015). Adult Scarabaeidae are distinctive (Fig. 4), 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 7.5–11.5 mm and females are 8.0–12.0 mm in size, males comprise a pair of horns which are short, slightly divergent in frontal view, gradually tapering from base to apex, while horns are absent in females; protibial apicointernal tooth enlarged in males; protibia 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 calls the significance of DNA barcoding to enable their easy, rapid and accurate identification. Rapid identification of unknown specimens may be achieved through DNA barcoding of adult specimens, ultimately aiding in identification of any life stage in the future (Oba et al., 2015; Wu et al., 2017). Hence, the present study has clarified the identity of *D. gazella*, by COI gene barcoding and sequencing, previously recorded by Singhal et al., (2018).

Digitonthophagus gazella is known for its behaviour of removing the dung from the pat and compacting it in tunnels for provisioning to their offspring. The present study has proved that the nesting behaviour (tunnel formation, broodmass formation, and parental care) by *D. gazella* is similar to other *Onthophagus* species (Huerta and García-Hernández, 2013; Arellano et al., 2017; Sane et al. 2020); however, there are few differences observed. In the present study, 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 was also observed. Sane et al., (2020) in their studies of structural diversity and behavioural principles on insect architecture have reported the tunnel pattern of many insects, proposed the process of nesting architecture by insects, 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 which protect 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 significant difference was observed in the length and diameter of the tunnel (Bertossa, 2011; Macagno et al., 2016).

The number of teeth varies in different species which facilitates more efficient and deeper digging in two behavioral contexts- escape from threats and subterranean reproduction (Linz et al., 2019). Front tibia of dung beetles, a shovel-like enlarged digging tool is presumed to have interactions between male and female and has been reported to influence certain activities or processes, such as reproduction, competition, or cooperation with individuals, which significantly affect the provisioning of ecosystem functions and services, further it also allows them to access soil as a habitat, and to evolve tunnelling and subterranean reproduction as novel life-history strategies (Fernandes et al., 2011; Nervo et al., 2022). Based on the 16 leg genes analyzed earlier, 13 are known to be required for the correct formation of tibial teeth, 7 genes (dac, lim1, Ser, odd, bowl, sob, drm) are associated with patterning the leg including the tibia, a second group of 6 genes (dll, sp8, ab, dachs, Krn, EGFR) have additional roles in context of leg formation, and 2 genes (ems and mex3) uniquely affect size, shape and spacing of tibial teeth (Angelini et al., 2012). In the present study, the digging genes expressed underlying tibial teeth showed a significant ($p<0.5$) decline in the dll and ems in both males and females, proving its tunneling activity in nesting behaviour of *D. gazella* (Macagno et al., 2016; Cómbita-Heredia et al., 2018; Losada et al., 2018). 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.

Tunnelers make nests and lay spherical, cylindrical brood masses by sexual co-operation. Once the egg is laid, female seals the broodball with dung and soil for protecting the growing larvae and pupa. As the brood turns into an adult, it comes out of the brood ball and undergoes sexual maturation (Huerta and García-Hernández, 2013). 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). Conversely, in the present study, although *D. gazella* dug deeper tunnel, the number of brood masses formed were not as high as described in natural conditions. The reduced number of brood masses is perhaps due to the restricted area provided in

the laboratory conditions. However, the brood morphometry did not show any alterations, and our observations are in accordance with the earlier work (Moczek, 2010; Singh et al., 2019).

Dung beetles larvae are known to continuously restructure and physically modify their brood ball environment for the benefit of their growth and subsequent adult fitness. Previous research suggests that the relatively small *Onthophagus taurus* (Schreber, 1759) is considerably more dependent on such brood ball modifying behaviour than the much larger *D. gazella* (Schwab et al., 2017). In the present study, the brood ball morphometry was also observed throughout the life cycle, there was no difference in the morphometry of the brood ball. Thus, our observations are in agreement that *D. gazella* being larger in size compared to *O. taurus* does not restructure or modify the broodball environment (Kishi, 2014; Rohner and Moczek, 2021).

Neurotransmitters play a critical role in regulating many aspects of insect physiology and biochemistry. They also coordinate larval growth and maturation and ensure normal individual development (Di Bari et al., 2016; Trang and Khandar, 2021). Previous studies have revealed that brain activity of AChE increases just after eclosion and remains at this stage throughout life in *Apis mellifera* (Linnaeus, 1758), *Tribolium castenum* (Herbst, 1797), and *Drosophila melanogaster* (Meigen, 1830) (Hao et al., 2021). Further, precedent observations have revealed an apparent correlation between physical activity and levels of AChE in active insects such as houseflies, honeybees, ants, and cockroaches than in less active insects such as lepidopterous larvae (Grünewald and Siefert, 2019). The increased AChE in adult male and female dung beetle accounted in the present study is thus self-explanatory and in agreement with the earlier work done (Palestrini and Rolando, 2001). Also, a significant increase in the levels of AChE along with the enzyme Choline Acetyl Transferase (chat) confirms the role of AChE in nesting behaviour of *D. gazella*. Further, a distinct difference in the level of AChE in females and males in the present study validates more physical activity for tunnel making and broodball formation. Single females generally remove more dung than single males, which suggests that females are more effective than males (Nervo et al., 2022), thereby, supporting our findings of higher level of AChE in females.

Biogenic amines are neuroactive substances which controls responses of sensory neurons, activities of neurons, and movements of muscles, resulting in modification of behaviour

(Watanabe and Sasaki, 2021; Sasaki and Watanabe, 2022). The ubiquitous biogenic amines, 5-HT and DA activate neural circuitry to regulate behaviour (Libersat and Pflueger, 2004; Bergan, 2015). Conserved aminergic circuits (Barron et al., 2010; Perry et al., 2016) and patterns of receptor expression (Blenau and Thamm, 2011) can control behaviour in diverse species across insect orders. The principle biogenic amines (DA and 5-HT) interact with hormone signalling pathways to elicit distinct behavioural and developmental responses (Pfaff and Joels, 2016). Dung beetles show multiple occurrences of the evolution of familial sociality, including biparental care (Costa, 2006; Cunningham et al., 2015; Panaitof et al., 2016). Burying beetles possess a astonishing neuroendocrine control with reference to their reproductive strategies, which includes tunnel pattern, brood-ball making, and parental care of their young ones (Hunt and Simmons, 2002; Harano et al., 2008). In the present study in an attempt to have an insight for the role of biogenic amines, a significant elevation of DA and 5-HT in males and females on the 10^{th}, 20^{th}, and 30^{th} days of tunneling is implying the probable role of these neurotransmitters in nesting behaviour (Misof et al., 2014; Song et al., 2015; Kamhi et al., 2017). A marginal high titer of 5-HT in males compared to females (Trumbo, 2019) can be correlated to the social context-dependent aggression in males during copulation (Stevenson and Schildberger, 2013; Alekseyenko and Kravitz, 2014). Parallel to the increase in 5-HT, an increased level of DA in males and females further reflects and proves the mitigating role of DA and its delicate adjustments in reinforcing the nesting activities (Rillich and Stevenson, 2014; Guerra et al., 2016; Bhatt et al., 2018; Auletta, 2019).

The insect olfactory system has evolved several modulatory systems to maximize foraging efficiency for resources. Foraging behaviour is reported to be maximally sensitive to olfactory cues in many insects (Kloppenburg and Mercer, 2008; Mizunami et al., 2009; Verlinden, 2018; Linn et al., 2020; Chatterjee et al., 2021). In insects, mating triggers changes in the behaviour and physiology of females, such as increasing oviposition and re-mating (Avila et al., 2011; Al-Wathiqui et al., 2016). Oviposition is known to be elicited by peptides and proteins transferred from male accessory glands through mating (Carmel et al., 2016) and by physical stimulation by males during mating (Li et al., 2020). The increased level of DA in the present study is thus in response to the olfactory stimuli as well as mating during the nesting. Parallel to the increase in monamine neurotransmitter (DA and 5-HT) levels, gene expression of enzymes (ddc and 5-

htpdc) involved in neurotransmitter metabolism also increased transcriptionally, thereby establishing the molecular involvement of the genes in its nesting behaviour.

Being a comparatively recently discovered neurotransmitter, the functions of nitric oxide in the nervous system are still only partially known. NO has been proved to promote habituation and has been implicated in modifying diverse neuronal circuits, such as increasing the digging rhythms of ovipositing and sensitivity of the taste receptors to chemicals (Cano et al., 2017). NO has been described to act as a retrograde neurotransmitter and plays an important role in reproduction, learning, and memory (Strauss, 2002; Popov et al., 2005; Wessnitzer and Webb, 2006; Ridgel et al., 2007). NO has a potent role in the signalling mechanism of the insect nervous system and participates by controlling behaviour at various levels such as perception of external stimuli, integration, selection of appropriate action and adaptive performance by neuron-muscular and neurosecretory systems (Heinrich and Ganter, 2007; Weinrich et al., 2008). Foraging behaviour has been well explored in Drosophila larvae and *Bombyx mori* (Linnaeus, 1758) (Seki et al., 2005), experience-dependent fighting in crickets (Aonuma et al. 2004) and for sound production in grasshopper (Wenzel et al., 2005). In the present study, a significant increase in NO was observed in both male and female dung beetles which partially uncover the contribution of NO in above mentioned behaviour. However, a significant increase in the levels of NO along with the enzyme Nitric oxide synthase (nos) confirms the role of NO in nesting behaviour of *D. gazella*.

Deltamethrin, a synthetic pyrethroid affects the sodium channels of nerve filaments, GABA receptors and chloride and calcium channels (Hassanein et al., 2018), thereby delaying the synaptic membrane transmission by interrupting Na+ channel closing time (Ray and Fry, 2006; Parween and Jan, 2019). Earlier studies have detected traces of pyrethroid in the dung and have proved that it is the presence of pyrethroid which is responsible for the mortality of dung beetles in agriculture fields (Vale et al., 2004; Chihiya et al., 2006). In the laboratory also a similar pattern has been reported by Wardhaugh, (1998); Torr et al., (2007); Mohammadi et al., (2021); Sands et al., (2018), where 50% mortality of the paracoprid beetle, *Metacatharsius troglodytes* (Boheman) has been noted. In the present study, 50% mortality was observed at 48 hours' interval at the sub-acute dose (0.275mg/L), which probably has entered through contact (Rehman et al., 2014; Brink and Berg, 2019). Further, at higher doses Deltamethrin exposure also

promoted tremor, immobility, darken body and eventually death in *D. gazella* which is in agreement with earlier reported work (Weaving 2018; Hussain et al., 2021).

Pesticide exposure in general on insect behaviours has been explored (Mazzi and Dorn, 2012; Müller, 2018; Parkinson et al., 2020) and lethal effects (Gutiérrez et al., 2017; Shaw et al., 2019; Vander Pan et al., 2019) and at sublethal concentrations, deltamethrin entails major physiological damages in a multitude of target and non-target insects (Cutler, 2013; Müller, 2018), reduces females' fecundity in honeybees, parasitoid wasps and cockroaches (Dai et al., 2010; Teder and Knapp, 2019), impairs larval development in honeybees and the wasp *Trichogramma achaeae* (Oliveira et al., 2018; Yang et al., 2020) and inhibits molting processes in the stable fly *Stomoxys calcitrans* (Reissert-Oppermann et al., 2019). Previous studies have reported that the effects of deltamethrin on egg development, hatching rate and juvenile weight (Mauduit et al., 2021). In the present study, in an attempt to have an insight into the nesting behaviour, exposure of Deltamethrin resulted into reduced number of broodballs, number of egg laying, hatchings and survival of offsprings after 10, 20 and 30 days, which are in agreement with the earlier reported studies (Skouras et al., 2021).

Earlier research has established that deltamethrin induced neurotoxicity is linked to behavioural alterations (Ogut et al., 2019; Lie et al., 2022), and in turn lead to change in the neurotransmitter levels (Khalil et al., 2022). Given that, the enzyme acetylcholinesterase (AchE) activity, DA and 5-HT levels are strongly inhibited by pyrethroids at low concentrations (Hassanein et al., 2018; Dworzańska et al., 2020). Parallel, in the present study, the sub-lethal exposure of Deltamethrin altered the levels of neurotransmitters (AChE, biogenic amines-DA and 5-HT, and NO) in a time dependent manner (10, 20, and 30 days) and caused a decrease in the nesting behaviour (tunneling, brooding, resproduction and parental care) making our findings consistent with the previous studies (Fouad and Abotaleb, 2021; Pitzer, 2021).

Conclusion

Dung beetles provide several ecosystem services and are ideal bio-indicators making them best suitable for studying the effects of urbanization or anthropogenic activities like habitat destruction, fragmentation, and edge effect on biodiversity. Thus, the conservation of dung beetles is necessary as the quality of these ecosystem services depends solely on their diversity,

abundance and biomass. However, little is known about the reproductive strategies and the underlying neuroendocrine mechanism that individual body contains over such services. Hence, deeper understanding of the physiological basis of ecosystem services provided by dung beetles as well as individual-based perspective of *D. gazella* endows a better understanding on its ecology and biology. Therefore, the present study throws a light on the role of neurotransmitters in the nesting behaviour of dung beetle. It was observed that the reproduction pattern mainly depends on their tunnel pattern, which is habitually lined with dung, and they construct "brood balls" from dung where in they lay egg. The significant elevation of AChE, DA, 5-HT, and NO levels, in the brain of dung beetles on the 10^{th}, 20^{th} day, and 30^{th} day is intriguing to link neurotransmitters with nesting behaviour in *D. gazella*. Furthermore, the molecular mechanism underlying the synthesis of neurotransmitters i.e. the gene expressions of ddc, 5-htpdc, chat and nos has also confirmed its role in the nesting behaviour in a time dependent manner. Thus, our findings suggest that of all the neurotransmitters, DA and 5-HT have a prominent role in the behavioural transitions associated with the initiation of tunnel patterns. To our knowledge, this is the first study exploring the link between neurotransmitters and the nesting behaviour of *D. gazella*. On the other hand, the level of neurotransmitters decreased effectively on exposure to Deltamethrin, thereby, influencing the digging behaviour, which in turn affects the reproduction, brood formation, and parental care in the nesting behaviour of *D. gazella.*

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