Chapter 2: Understanding the nesting behaviour of *Digitonthophagus gazella*: Role of neurohormones

2.1 INTRODUCTION

Dung beetles (Coleoptera: Scarabaeidae) are fascinating creatures known for their ecological role as decomposers and their unique behavioural adaptations (Halffter et al., 2011). These beetles exhibit intricate behaviours, such as dung ball rolling and orientation, which play a crucial role in their survival and reproduction. By digging tunnels, tunnelers supply dung to offspring at the tunnel's blind end in the shape of brood balls (Moczek, 2009; Khadakkar et al., 2019), with only a single egg deposited into an egg chamber and sealed (Pandya et al., 2022). They feed primarily on the dung of wild and domestic mammals (Arellano et al., 2017), use it to provide housing and food for their larvae (Gaikwad and Bhawane, 2015; Pandya et al., 2023). The larva lives inside the chamber throughout its development until pupation. Utilizing rich, transient dung during growing up fosters distinctive behavioural and physiological changes that result in 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 (Kim et al., 2021). Understanding sociality and the mechanics underlying complex behaviours may both be aided by understanding parental care (Cunningham, 2020). Parental care can be uniparental or biparental, often within a species and provide direct or indirect care. A substantial body of research has been done to pinpoint the genetic impacts connected to parental care in a variety of animals, including variations in gene expression (Cunningham, 2020). Parental care involves many distinct, distinctive behaviours, including as protecting the young, building and maintaining a nest or reproductive resource, controlling body temperature, preparing food, and providing food directly to the young (Royle et al., 2012). Even while social behaviour and parental care are collectively used to describe these unique behaviors, the underlying genetic pathways may vary. Behind these behaviours lies a complex interplay of physiological processes, including the action of neurotransmitters (Verlinden et al., 2010; Kannan et al., 2022; Xing et al., 2023).

Neurotransmitters, chemical messengers in the nervous system, are secreted from the neurosecretory cells of insect brain which are located on the mushroom bodies (**Fig. 2.1**). NTs have been found to influence the behaviour of insects in various ways (Nelson and

Trainor, 2007; Kamhi et al., 2017; Watanabe and Sasaki, 2022). They are small molecules that transmit signals across synapses, enabling communication between neurons (Libersat and Pflueger, 2004; Bergan, 2015). Several neurotransmitters have been identified in beetles in general, including serotonin, dopamine, octopamine, and gamma-aminobutyric acid (GABA). Each neurotransmitter serves unique functions within the beetle's nervous system, regulating behaviour and physiological processes (Watanabe and Sasaki, 2022). For a very long period, neuromodulation in the central nervous system is well thought-out to be the cause of plasticity of behavioural responses (Zhukovskaya and Polyanovsky, 2017). The study of nesting behaviour in *D. gazella* offers valuable insights into the intricate dynamics of tunnelling, brood ball formation, reproduction, and parental care. This work sheds light on the complex interplay between ecological, social, and neuroendocrine elements that govern behavioural regulation. Broad research has been conducted on the important roles played by neurohormones in regulating reproductive social behaviour in vertebrates (Ketterson and Nolan, 1992; Buntin, 1996; Adkins-Regan, 2005). Neurotransmitters are of particular importance to social behaviour, as they have a profound and highly conserved influence. Insects, while being extensively studied, still lack comprehensive understanding in some areas of reproductive behaviour and physiology, such as the hormonal mechanisms underlying parental care (Trumbo 2002; Riddiford 2012).

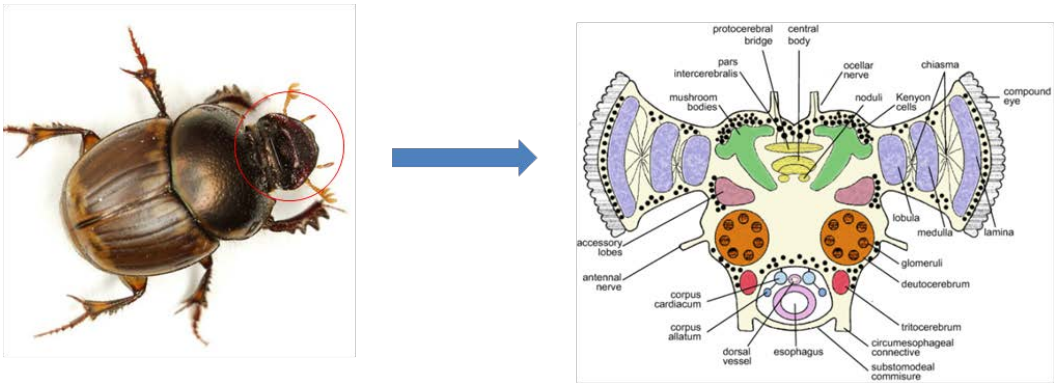


Figure 2.1: Insect brain structure showcasing mushroom bodies with neurosecretory cells on its periphery and neurpiles inside it (Fharbach, 2006).

Biogenic amines, including dopamine (DA), and serotonin (5-hydroxytryptamine, 5-HT) function as neurotransmitters, neuromodulators, and neurohormones and are essential for regulating a number of physiological processes in insects (Lange and Orchard, 2021; Sasaki et al., 2021). Dopamine is a neurotransmitter associated with reward and motivation. It is found widely in both vertebrates and invertebrates and plays critical roles in learning and memory, motor behaviour, sleeping and arousal, and phase change in insects (Mizunami et

al., 2009; Agarwal et al., 2011). On the other hand, serotonin is crucial for controlling insect feeding behaviour, aggressiveness, and rhythmic behaviour (Tomioka et al., 1993; Dacks et al., 2003; Dierick and Greenspan, 2007; French et al., 2014). The serotonin system is intricately connected to the beetle's internal compass, enabling them to utilize celestial cues for navigation (Immonen et al., 2017; Dacke et al., 2019). Biogenic amines also have a connection to oviposition in many insects. Biogenic amines (DA and 5-HT) has been explored extensively in the social context-dependent fighting behaviour, and territorial dominance in insects including 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 strong associations between 5-HT and DA, and the modulation of behavioural state that have been demonstrated, we hypothesized that the biogenic amines DA and 5-HT constitutes the most plausible candidates for the neuromodulatory control of nesting behaviour in *D. gazella*.

In addition to biogenic amines, Acetylcholine esterase (AChE) is critical for controlling insect behaviour, through interactions with other neurotransmitters and neuromodulators. AChE is an enzyme that plays a crucial role in the termination of cholinergic signalling by rapidly hydrolyzing acetylcholine (ACh) into choline and acetate, effectively terminating the transmission of nerve impulses mediated by ACh. It plays a vital role in regulating muscle contraction and motor control in insects. In dung beetles, AChE is involved in the modulation of muscle activity during various behaviours, including flight, digging, rolling dung balls, and navigating the environment. By rapidly hydrolyzing ACh at neuromuscular junctions, AChE helps regulate the timing and coordination of muscle contractions, allowing for precise movement and behavioural responses (Perić-Mataruga et al., 2017; Cabirol and Haase, 2019).

Furthermore, nitric oxide (NO), a gaseous neurotransmitter, too influences insect behaviour beyond its interactions with biogenic amines (Trimmer et al., 2004). NO is a signalling molecule that regulates numerous physiological processes, behaviours and social interactions in insects through mechanisms such as modulation of synaptic plasticity, sensory

processing, and reproductive behaviours (Bicker, 2001; Cayre et al., 2005). It is associated with various behaviours, including feeding, mating, aggression, and olfaction in insects (Rillich and Stevenson, 2019). NO interacts with AChE by modifying its activity and expression, providing a means to regulate cholinergic neurotransmission. In the case of dung beetles, these mechanisms are crucial for their ability to perform complex behaviours related to reproduction, navigation, and resource acquisition. Baring a few reports, Dopamine, Serotonin, Acetylcholine esterase activity, 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). Therefore, the goal of the present work was to investigate if the levels of the biogenic amines DA, 5-HT, AChE, and NO change during the transition from non-parental to parental state in the *D. gazella*.

Further, the role of neurotransmitter-synthesizing enzymes in confirming the activity of neurotransmitters in insects is crucial for understanding the functionality and regulation of these chemical messengers. Dopa decarboxylase (ddc), 5-hydroxytryptophan decarboxylase (5-htpdc), choline acetyltransferase (chAt), and nitric oxide synthase (nos) are important enzymes involved in the synthesis of key neurotransmitters and neuromodulators, namely dopamine, serotonin, acetylcholine, and nitric oxide (Vavricka et al., 2014). The first enzyme in catecholamine biosynthesis pathway to be identified is Dopa decarboxylase (DDC), a catalyst for L-Dopa to dopamine conversion. In the pathway of synthesizing dopamine, L-Dopa is first produced from the amino acid L-tyrosine by the enzyme tyrosine hydroxylase. Dopa decarboxylase then converts L-Dopa into DA (Hodgetts and O'Keefe, 2006; Lin et al., 2020). Direct parental care and aggression are both substantially correlated with the dopaminergic system (Zhao and Li, 2009; Dulac et al., 2014; Rilich and Stevenson, 2014). 5-HTPDC is an enzyme that plays a very significant role in the final step of conversion of 5-hydroxytryptophan (5-HTP) into serotonin (5-HT) (Mora-Villalobos and Zeng, 2018). Moreover, the escalation of aggression (Alekseyenko et al., 2010) and sociality (Antsey et al., 2009), as well as parental care (Zhao and Li, 2009; Dulac et al., 2014), are all related to the serotoninergic system. Choline Acetyl transferase (chAt) is an enzyme responsible for synthesizing acetylcholine (ACh), a neurotransmitter that mediates cholinergic signalling in the nervous system. In the pathway of ACh synthesis, chAt plays an essential role as catalyst by transferring the acetyl group from acetyl-CoA to choline, which leads to the formation of ACh which is involved in regulating the functions of the peripheral nervous system and

neuromuscular junctions in insects. Intensive studies on the cholinergic system have been reported which shows its association with the cholinergic signal transduction essential for olfactory learning and memory formation in insects (Grünewald and Siefert, 2019). Nitric oxide synthase (nos), an enzyme, synthesizes nitric oxide (NO), a gaseous neurotransmitter and signalling molecule involved in regulation of physiological processes and social interactions in insect populations (Muller, 1996; Jaszczak et al., 2015). Through the control of endocrine signals in insects, nitric oxide system regulates NOS activity in the prothoracic gland and transforms the information about the state of separate tissues' growth into coordinated tissue growth (Jaszczak et al., 2015).

Neuropeptides (NPs) are ubiquitous signalling molecules that operate as neurotransmitters, neuromodulators, or hormones, controlling a variety of physiological and behavioural processes. They are by far the most diverse class of signalling molecules from structural and functional aspect. In insects, neuropeptides function as regulating hormones secreted into the bloodstream as well as neuromodulators in the central and peripheral nervous systems. In recent years, research has highlighted the significance of neuropeptides as important regulators of insects' physiological, behavioural and developmental processes, and modulation of neuronal and muscular activity (Muratspahić et al., 2020). Additionally, they play a critical role in controlling a variety of behavioural processes related to eating, courting, sleeping, learning and memory, stress, addiction, and social interactions (Schoofs et al., 2017). These regulations are mainly due to changes in the neural circuits controlling the link between cues and behaviour.

One of the important neuropeptides that has been found in a variety of insect species is the neuropeptide F (npf), a multifunctional neuropeptide that is essential for feeding, metabolism, courtship, reproduction, aggression, ethanol sensitivity, locomotor circadian cycles, learning, and stress reactions (Yue et al., 2017). Npf receptors (npfr) play a major role in carrying out the functions of npf (Cui and Zhao, 2020). Npf is structurally related to neuropeptide Y (NPY) in vertebrates and has been reported in a wide range of insects, first reported in locust, *Locusta migratoria* (Schoofs et al. 1988), then fruit fly *Drosophila melanogaster* (Brown et al. 1999), *Bombyx mori*, *Reticulitermes flavipes*, *Schistocerca grearia*, *Helicoverpa assulta*, *Anopheles gambiae*, *Aedes aegypti*, *Helicoverpa armigera* and *Ostrinia furnacalis* (Clynen et al., 2006; Roller et al., 2008; Nuss et al., 2010; Liu et al., 2013; Yue et al., 2016, 2017). It regulates feeding behaviour in *B. mori*, *H. zea*, *H. armigera*, *O. furnacalis* and *H. assulta* (Roller et al., 2008; Huang et al., 2011; Liu et al., 2013; Yue et

al., 2016, 2017); a sex-specific expression pattern and aggression in *D. melanogaster* (Lee et al., 2006). Further, the study conducted by Cui and Zhao, (2020) examines the regulatory mechanisms of male courting and ovarian maturation in two species, namely the desert locust *S. gregaria* and *Rhodnius prolixus*. The research focuses on the expression of a certain gene in the brain, particularly in clock neurons and the mushroom body, which is associated with the daily circadian rhythm and sleep patterns.

Inotocin (it) is structurally related to oxytocin and vasopressin, which are well-known neuropeptides in vertebrates, but its role in insects is not as extensively studied. It is primarily expressed in the insect brain, specifically in specific regions such as the corpora cardiaca and/or corpora allata, which are neuroendocrine centres involved in regulating various physiological processes (Liutkeviciute et al., 2016). Studies have reported the role inotocin in regulating aspects of social organization was well as behaviour, including queen-worker interactions, nest mate recognition, in reproductive physiology and social interactions related to reproduction such as male/female pair bonding and parental care and aggression (Gruber et al., 2012; Masnjak, 2022). Although common among insects, only a few species have been studied thoroughly with respect to their function and particular behaviours it impacts (Liutkevičiūtė et al., 2018; Muratspahić et al., 2020). It has been detected in various insect species, including locust *Locusta migratoria*, red flour beetle *Tribolium castaneum*, beetle *Lethrus apterus* (Nagy et al., 2021), parasitic wasp *Nasonia vitripennis* (Stafflinger et al., 2008), black garden ant, *Lasius niger* (Chérasse and Aron, 2017; Keov et al., 2018).

In the historical records, naming conventions frequently obscure the links between neuropeptides within different species or phyla. A notable illustration of this is the superfamily of Wamide neuropeptides. Wamides have been termed to as myoinhibitory peptide (MIP), allatostatin B (ASTB), prothoracicostatic peptide (PTSP), WWamide, GLWamide, or metamorphosin A (MMA), depending on the species in which they were studied. The locust myoinhibitory peptide (LOM-MIP) was the first Wamide to be identified. In 1991, Schoofs and colleagues discovered LOM-MIP as a suppressor of visceral muscle activity in the oviduct and hindgut. In crickets, *D. melanogaster*, and *Manduca sexta*, as well as in some insect species, Wamides have been shown to regulate life cycle transitions, larval settlement, and metamorphosis as a part of a peptidergic signalling cascade that is initiated by ecdysis triggering hormone (Davis, 2003; Kim et al., 2006a and 2006b; Santos et al., 2007). Myosin inhibiting peptide (mip) is a neuropeptide which acts by inhibiting the contraction of muscles through the modulation of myosin, a protein involved in muscle contraction (Bullard

and Pastore, 2011). MIP has also been associated with reproductive behaviours in insects, particularly during courtship displays (Lange et al., 2012; Hasebe and Shiga, 2021).

Our previous study has revealed the nesting behaviour of *D. gazella* which forms a simple type II pattern of nest with the brood balls placed at the blind end of the tunnel. Both male and female are involved in nesting and brood ball making, reproduction, sealing of the brood balls most often by females, contributing to parental care. The factors governing agonistic behaviour in the immediate sense can act as the substrate for growth selection since the outcome of aggressive encounters between males frequently influences reproductive success. However, there is a lacuna on the neuroendocrine regulation involved in the nesting behaviour of dung beetle, *D. gazella*. Therefore, the aim of the present study was to investigate the nesting behaviour of *D. gazella* through neuronal regulators. Understanding their role in nesting behaviour of *D. gazella* will enhance our knowledge of the molecular mechanisms that underlies their complex behaviours and adaptations. The level of NT, NT synthesizing enzymes, NPs will fill up the lacunae and will enhance our knowledge on how neurochemistry can be leveraged to investigate the link between behaviour and physiology.

2.2 MATERIALS AND METHODOLOGY

Estimation of the Neurotransmitter (NT) Levels – Dopamine (DA), Serotonin (5-HT), Acetylcholine Esterase (Ache), Nitric Oxide (NO)

DA and 5-HT tissue extract (Schlumpf et al., 1974)

After 10^{th}, 20^{th}, and 30^{th} day of tunnelling, each pair (of 5 pairs) of male and female *D. gazella* was sacrificed, and the brain was dissected using sterile forceps in ice-cold saline (pH-7.4), followed by storage in -20˚C. On the day of experiment, the tissue samples were homogenized in HCl-Butanol solution (0.85:100v/v) for 1 min, followed by centrifugation at 6000 RPM for 20 mins. Then, the supernatant was added to the centrifuge tubes containing 1.25 mL Heptane and 0.15 mL HCl. After 10 min of vigorous shaking, the tubes were centrifuged at 6000 RPM for 20 mins at 4˚C to separate the two phases, and the overlaying organic phase was discarded. Then, the aqueous phase (0.2 mL) was taken for the estimation of 5-HT and Dopamine. All steps were carried out at 0^{o}C.

Estimation of DA

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 0.1mL iodine solution (0.1 M in ethanol) for oxidation. The reaction was stopped by addition of 0.1mL Na_2SO_3 solution after 2 mins and 0.2 mL Acetic acid after 1.5 mins, followed by heating of the solution to 100°C for 6 mins. The samples were then allowed to come to room temperature, post which the excitation and emission spectra was read at 330-375 nm in the spectrofluorimeter.

Estimation of 5-HT

To 0.2 mL aqueous extract, 0.25 mL of O-pthaldehyde (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 ambient temperature, and the readings were taken at 360-470 nm in the spectrofluorimeter.

Estimation of AChE 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 open on ice, and brain tissues were collected after 10, 20 and 30 days. A 20 mg/mL of tissue was homogenized in 0.05 M phosphate buffer. This step was followed by addition of 0.5mL Triton X-100 and 0.2 mL EDTA. The samples were centrifuged at 6000 RPM for 20 min at 4˚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 NO

NO levels were estimated by following the method of Miranda et al., (2001). In this method, isolated tissues (100 mg) were homogenized in 10 volume ice-cold saline solution using a homogenizer. Upon disruption, absolute ethanol was added (2:1 volume 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 (8mg VCl3/mL) was added rapidly followed by addition of 0.25 mL of 2% sulphanilamide and 0.25 mL of 0.1% N-(1-naphthyl)-ethylene diamine. The mixture was then vortexed and incubated at 37°C for 30 min. Then, the absorbance was measured at 540nm in a UV spectrophotometer (PerkinElmer Lambda 25, India).

Estimating the Gene Expressions of the NT Synthesizing Enzymes

To confirm the role of neurotransmitters in the nesting behaviour of *D. gazella*, in the present study, the gene expression of neurotransmitter synthesizing enzymes were estimated as follows.

Total RNA Extraction (Trizol Method)

For total RNA extraction, brain tissue was isolated from both male and female dung beetles in PBS (pH-7) after 10, 20 and 30 days of tunnelling. 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 mins 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 mins till the aqueous and organic layers were distinct. Thereafter, the tubes were subjected to centrifugation at 12,000 RPM for 15 mins 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 500 µL 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 mins at 4°C. After precipitation the supernatant was discarded without disturbing the pellet and was washed in 500 µL of 75% ethanol and then, 500 µL 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 mins 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 -20˚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 (**Table 2.1**). The volume of each component was for a 20 µL final reaction. The reaction mix is mentioned in the table below.

Table 2.1: 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 conditions as given in **Table 2.2**

PCR conditions

Table 2.2: 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 (**Table 2.3, 2.4, 2.5**). 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 2.3: 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 2.4: 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 2.5: Real Time PCR Primer sequences of neurotransmitter synthesizing enzymes

Serial No.	Accession No.	Gene Name	Primer Type	Sequence	T_m (˚C)
1	NM_001102586	dopa	Forward	CAAAAGCCCGACAAATGGG	60.03

		decarboxylase (ddc)	Reverse	AGTTGGCGGTGGGGAAATAG	60.04
2	NW_022587571.1	5-HTP decarboxylase/ 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

Neuropeptides involved in the nesting behaviour

To understand the behaviour patterns such as feeding, reproducing, parental care involved in the nesting behaviour by both male and female *D. gazella*, the gene expressions of the neuropeptides were estimated in the present study. The brain tissue of both male and female *D. gazella* were isolated in PBS (pH-7) after 10^{th}, 20^{th} and 30^{th} day, followed by RNA isolation, cDNA synthesis, followed by RTPCR Amplification as described earlier (**Table 2.3 & 2.4**). The Real Time PCR primer sequence of the selected neuropeptides was as given in the **Table 2.6.**

Table 2.6: Real Time PCR Primer sequences of neuropeptides

Serial No.	**Accession No.**	**Gene Name**	**Primer Type**	**Sequence**	**T_m (˚C)**

1	XM_017921162	Neuropeptide F (npf)	Forward	GACCCCAGTCTCATTCAGGC	61.40
			Reverse	GTTGGTTGTAGTCAGGGCGT	55.00
2	NC_007424.3	Neuropeptide F receptor (npfr)	Forward	GACTCGTATGGGGGCGTAAA	59.35
			Reverse	GGTTCGAATCGGGTCAAGGA	59.35
3	NM_001085362	Inotocin (it)	Forward	CATTTGCTGCGGACCTTTCG	59.35
			Reverse	CGTTCGTGGAAAAAGCCCTC	59.35
4	NC_007422.5	Inotocin receptor (itr)	Forward	AGGGGCTGAGCTTCTTCTTG	59.35
			Reverse	AATGCCGCTGAAAGGAGAGT	57.30
5	NT_037436.4	Myoinhibitory peptide (mip)	Forward	AGTTCTCCGCGTCTTAGTGTG	59.82
			Reverse	GGTCCTTTTTCAGAAGCTTACAC	58.87

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

Data Analysis

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 $*p<0.05$, $**$ $p<0.01$, $***p<0.001$.

2.3 Results

Neurotransmitter Levels during Nesting Behaviour

To have an insight into whether there is any significant role of neurotransmitters in the nesting behaviour of *D. gazella*; brain levels of 5-HT, DA, AChE, and NO were biochemically analyzed. Results bared a significant time-dependent increase in all the neurotransmitters on the 10^{th}, 20^{th}, and 30^{th} day of introduction of *D. gazella* into the experimental setup in comparison to control (**Table 2.7a-d**). The levels of dopamine for control were observed to be 172.8 pg/mg tissue in males and 170.6 pg/mg of tissue in females. A significant rise in the levels of Dopamine was observed on the 10^{th}, 20^{th} and 30^{th} day as 199.3 pg/mg, 211.3 pg/mg, and 206.3 pg/mg of tissues in males and 175.8 pg/mg, 218.3 pg/mg, and 219.5 pg/mg of tissue in females. Of note, our result revealed a trend towards elevated DA levels in females compared to males on 10^{th} day, and an elevated DA level in females on 20^{th} and 30^{th} day indicating its role in successful tunnelling and reproduction (**Fig. 2.2a**).

5-HT estimation in control was observed to be 76.4 pg/mg and 70.7 pg/mg of tissue, in males and females, respectively. Results show a significant increase in the level of 5-HT with the range of 93.5 pg/mg, 99.1 pg/mg, 101.7 pg/mg of tissue in males as compared to 83.5 pg/mg, 84.17 pg/mg, and 85.8 pg/mg of tissue in females on 10^{th}, 20^{th} and 30^{th} day, respectively. The higher level of 5-HT in males is decisive in aggression and mating (**Fig. 2.2b**).

Further, the rate of AChE activity was found to be $0.023x10^{-4}$ mmol/ml/min and $0.031x10^{-4}$ mmol/ml/min in the control males and females. However, the AChE activity enhanced from $0.039x10^{-4}$, $0.051x10^{-4}$, and $0.057x10^{-4}$ mmol/ml/min in males and $0.04x10^{-4}$, $0.055x10^{-4}$, $0.061x10^{-4}$ mmol/ml/min in females on 10^{th}, 20^{th} and 30^{th} day. So, the significant increase in the activity of AChE is probably due to rapid degradation of the Acetylcholine in the nervous system of *Digitonthophagus gazella*, male and female respectively. However, the rate of AChE activity is found to be higher in females in comparison to males. Further, the Nitric oxide content measured in *D. gazella* which ranged from 2.03 nmol/g of tissue in control males, and 2.23 nmol/g of tissue, in control females (**Fig. 2.2c**). The NO content (nmol/g of tissue) was also found to elevate from 4.24, 5.04, 5.26nmol/g of tissue in males and 4.32, 5.1, and 5.33nmol/g of tissues in females on 10^{th}, 20^{th} and 30^{th} day, respectively. The NO content was found to be higher in both males and females on the 20^{th} and 30^{th} days of tunneling which has a probable role in the nesting behaviour of *D. gazella* (**Fig. 2.2d**). Moreover, 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*.

Table 2.7(a): The Level of Dopamine (DA) in the brain of *D. gazella.*

Serial No.	DA (Mean±SEM)		
	Group	**Male**	**Female**
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**

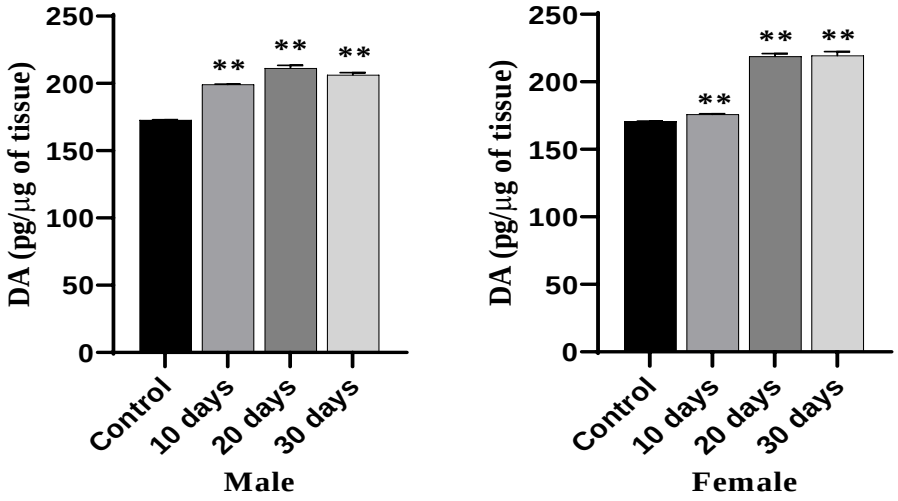


Figure 2.2(a): DA levels on the 10th, 20th, and 30th day of introduction of male and female into the experimental setup. The error bars indicate SEM with significant values;*$p<0.05$ **$p<0.01$.

Table 2.7(b): The level of serotonin (5-HT) in the brain of *D. gazella.*

Serial No.	5-HT (Mean±SEM)		
	Group	**Male**	**Female**
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**

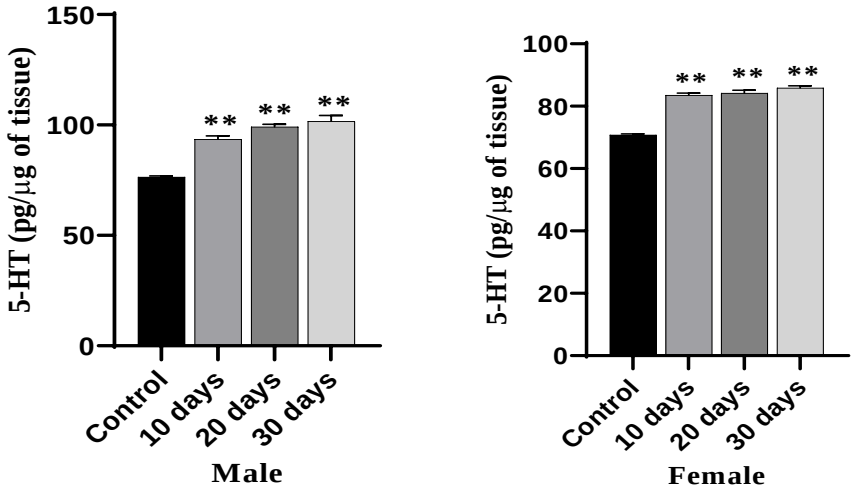


Figure 2.2(b): Serotonin (5-HT) levels on the 10th, 20th, and 30th day of introduction of male and female into the experimental setup. The error bars indicate SEM with significant values;**p<0.05 **p<0.01*.

Table 2.7(c): Rate of AChE Activity in the brain of *D. gazella.*

Serial No.	AChE (Mean±SEM)		
	Group	**Male**	**Female**
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**

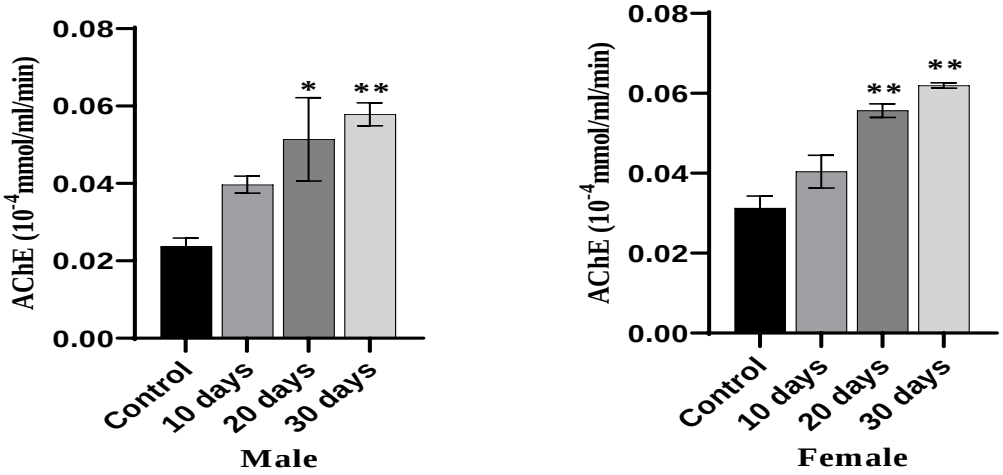


Figure 2.2(c): Rate of AChE activity (with the unit mmol/mL/min x 10^{-4} per g of tissue) on the 10th, 20th, and 30th day of introduction of male and female into the experimental setup. The error bars indicate SEM with significant values*;*p<0.05 **p<0.01*.

Table 2.7(d): The nitric oxide (NO) content in the brain of *D. gazella.*

Serial No.	Nitric Oxide (Mean±SEM)		
	Group	**Male**	**Female**
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**

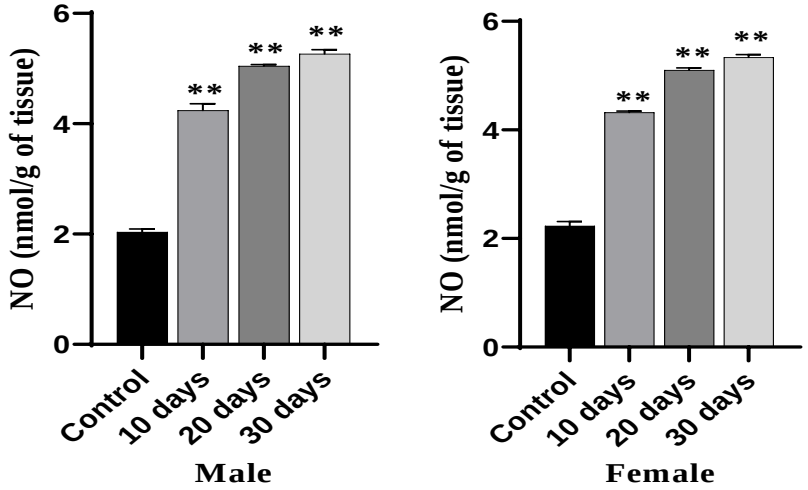


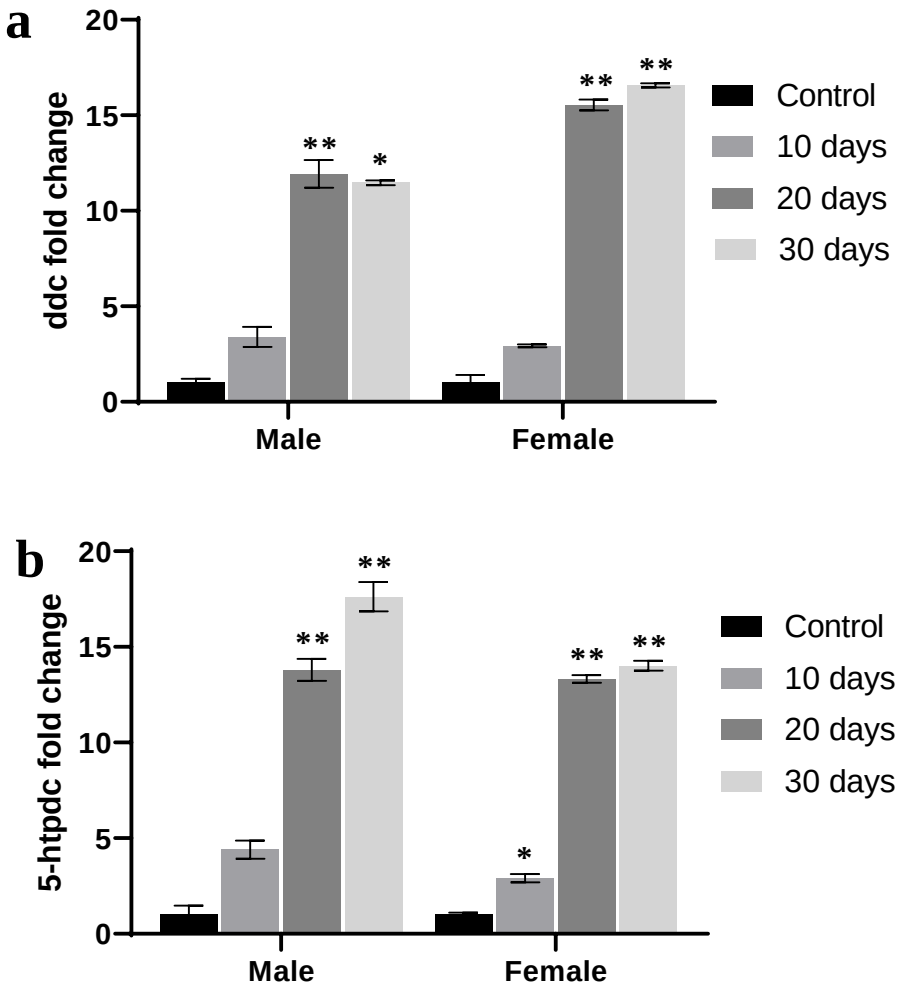
Figure 2.2(d): NO levels on the 10th, 20th, and 30th day of introduction of male and female into the experimental setup. The error bars indicate SEM with significant values*;*p<0.05 **p<0.01.*

Neurotransmitter biosynthesizing enzymes in nesting behaviour

The results of gene expression of the neurotransmitters synthesizing enzymes (**Table 2.8**) 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. 2.3a-d**) after 10th, 20th and 30th day, along with the increase in the level of neurotransmitters signifying their role in the nesting behaviour of *D. gazella.*

Table 2.8: Fold change in the expression of ddc and 5-htpdc

Group	ddc		5-htpdc	
	Male	**Female**	**Male**	**Female**
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**	
	Male	**Female**	**Male**	**Female**
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**



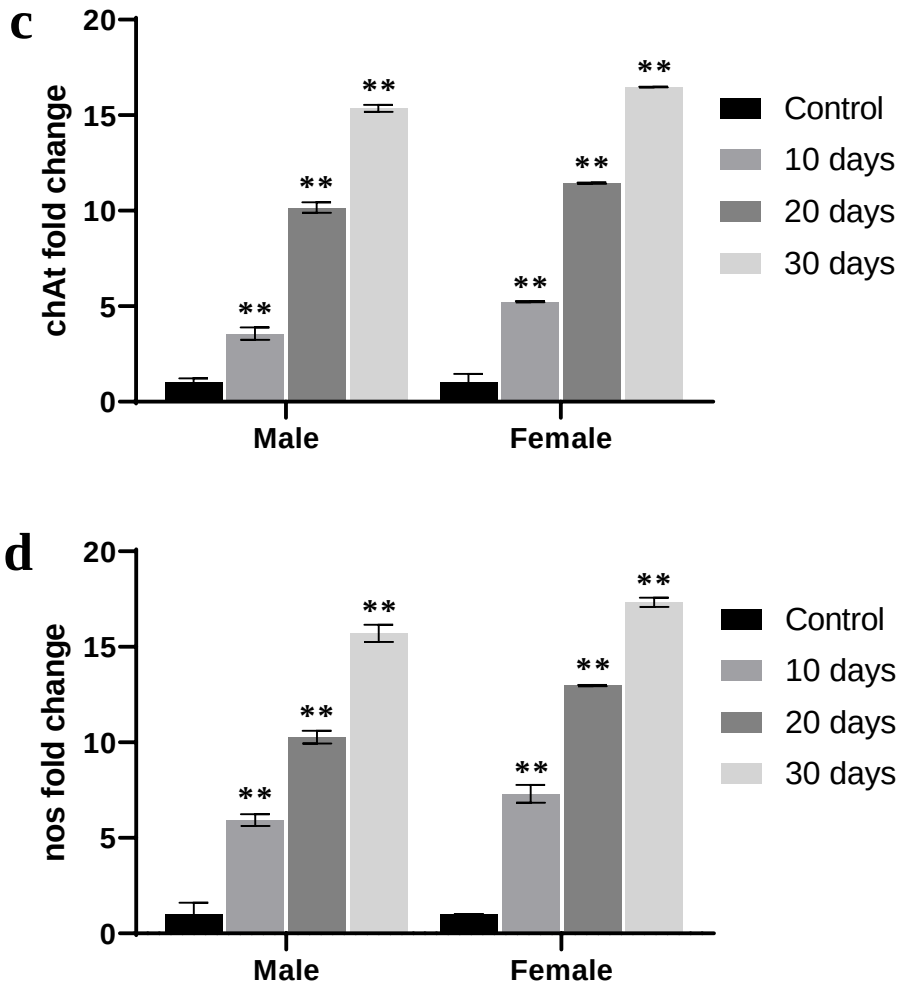


Fig. 2.3: Neurotransmitters synthesizing enzyme gene expressions (a) ddc (b) 5-htpdc (b) chAt (d) nos in the brain of male and female *D. gazella* after 10th, 20th and 30th day. The error bars indicate SEM with significant values*;*p<0.05 **p<0.01*.

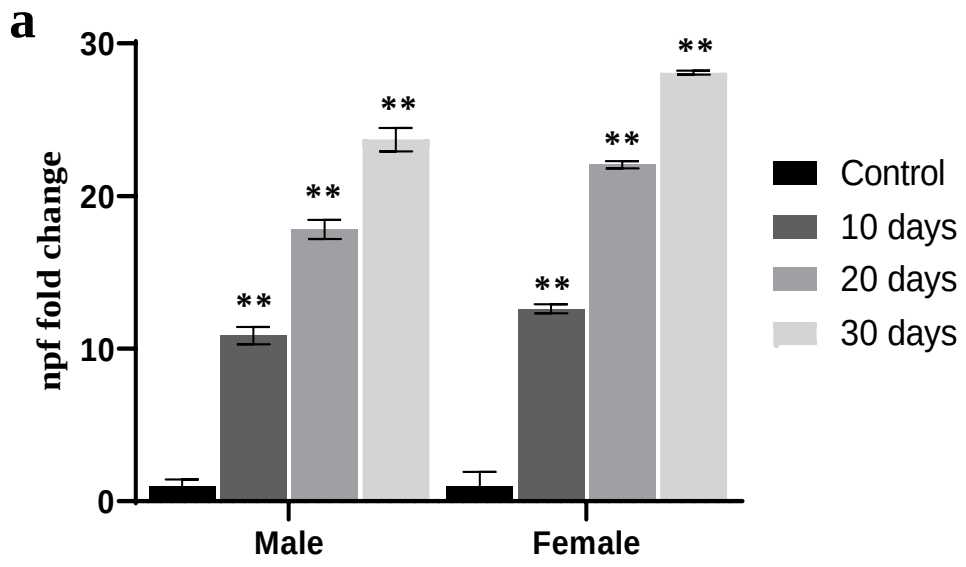
Neuropeptides involved in the nesting behaviour

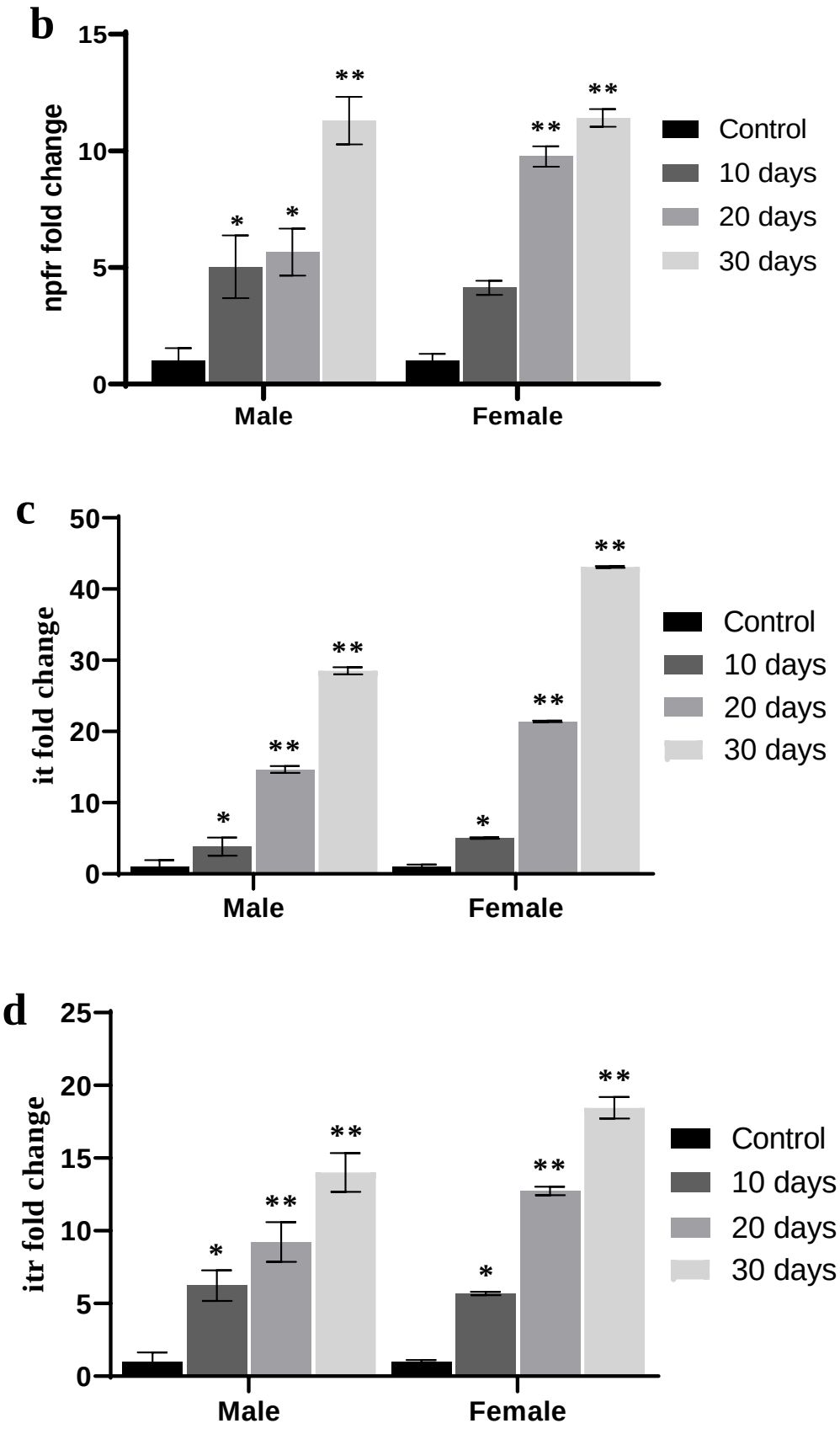
The gene expressions of the neuropeptides revealed a significant increase in the fold change expression of **npf**, **npfr**, **it**, **itr** and **mip**, in both the male and female *D. gazella* after 10th, 20th, and 30th days of nesting (**Table 2.9**). The expression pattern of neuropeptide F (npf) and neuropeptide F receptor (npfr) in male and female *D. gazella* was found to increase in a time dependent manner suggesting its role in the reproduction, feeding and provisioning food in the form of broodballs for the offspring (**Fig. 2.4ab**). Further, in females, expression of inotocin (it) and inotocin receptors (itr) elevated in a time dependent manner suggesting its role in the parental behaviour, during which the females seals the brood ball with soil and stays near the brood ball for around 24-48 hours (**Fig. 2.4cd**). A similar trend of increase was

also noted for the males, however, the fold change expression in them were less as compared to females suggesting their role in parental behaviour. Furthermore, the expression of myosin inhibiting peptide (mip) gene was also found to increase significantly in a time dependent manner (**Fig. 2.4e**).

Table 2.**9**: The fold change in the level of neuropeptides (NPs) in male and female *D. gazella*

	Individual	**npf**	**npfr**	**it**	**itr**	**mip**
Control	**Male**	1±0.436	1±0.436	1±0.918	1±1.112	1±0.499
	Female	1±0.924	1±0.924	1±0.302	1±0.107	1±0.710
10 Days	**Male**	10.853±0.56**	10.853±0.561**	3.823±1.261*	6.234±1.055**	7.815±1.214**
	Female	12.61±0.3**	12.611±0.303**	5.038±0.109**	5.683±0.109**	7.189±0.016**
20 Days	**Male**	17.8±0.63**	17.803±0.625**	14.658±0.483**	9.234±1.360**	10.464±0.709**
	Female	22.05±0.24**	22.051±0.241**	21.404±0.109**	12.737±0.299**	12.872±0.299**
30 Days	**Male**	23.69±0.76**	23.693±0.756**	28.522±0.506**	14.015±1.328**	11.01±0.517**
	Female	28.09±0.13**	28.086±0.124**	43.105±0.109**	18.453±0.743**	17.708±0.109**

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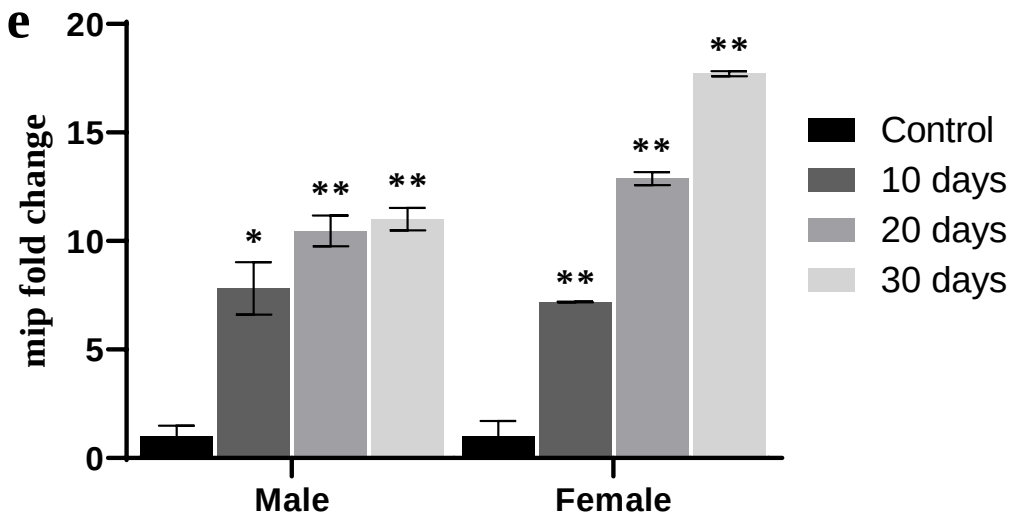

Fig. 2.4: Fold change expression of neuropeptide gene (a) npf (b) npfr (c) it (d) itr (e) mip in *D. gazella.* The error bars indicate SEM with significant values;*$p<0.05$ **$p<0.01$.

2.4 DISCUSSION

Neurotransmitters (NTs) are important regulators of the physiology and biochemistry of many insect species. Coordinating larval growth and maturation, NTs ensures normal development as a part of parental care (Di Bari et al., 2016; Trang and Khandar, 2021). Biogenic amines are neuroactive substances which controls responses and activities of neurons, movements of muscles, resulting in modification of behaviour (Watanabe and Sasaki, 2021; Sasaki and Watanabe, 2022). 5-HT and DA, two ubiquitous biogenic amines, stimulate brain circuits to control behaviour (Libersat and Pflueger, 2004; Bergan, 2015). Conserved aminergic circuits and patterns of receptor expression are diverse in species across insect orders, resulting into a diversified activities (Barron et al., 2010; Blenau and Thamm, 2011). 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 exhibit several examples of the development of familial sociality, such as biparental care (Cunningham et al., 2015; Panaitof et al., 2016). Burying beetles possess an astonishing neuroendocrine control with reference to their reproductive strategies, which includes tunnel pattern, brood-ball making, and parental care (Hunt and Simmons, 2002; Harano et al., 2008). The present research reveals a noteworthy increase in DA and 5-HT levels among both male and female on the 10^{th}, 20^{th}, and 30^{th} day of tunnelling. This finding suggests a potential involvement of these neurotransmitters in the manifestation of nesting behaviour, as previously shown by Misof et al., (2014), Song et al., (2015), and Kamhi et al., (2017). Aggression in males during copulation is linked to higher 5-HT titre in males than in females (Trumbo, 2019; Stevenson and Schildberger, 2013; Alekseyenko and Kravitz, 2014). In addition to reflecting and demonstrating the mitigating effect of DA and its careful modifications in supporting the nesting behaviours, an increasing level of DA in males and females was reported which was parallel to the increase in 5-HT. Our results are thus in agreement with the earlier reported work (Auletta, 2019) where authors have suggested that the vital roles of NTs in different behaviours and other physiological processes. Further studies (Kamhi et al., 2017; Sasaki and Watanabe, 2022) have also opined that the physiological processes and conserved behaviours are controlled by NTs which modulates complex social activities through collective interactions affecting the reproductive strategies. In order to improve resource foraging efficiency, the insect olfactory system has developed a number of modulatory mechanisms, and it is well known to be responsive to these cues (Verlinden, 2018; Linn et al., 2020; Chatterjee et al., 2021). Mating in female triggers

alterations in the physiology and behaviour, such as increasing oviposition and re-mating (Avila et al., 2011; Al-Wathiqui et al., 2016), as well as physical stimulation (Li et al., 2020) and the transfer of peptides and proteins from male accessory glands. The increased level of DA in the present study is probably in response to the olfactory stimuli as well as mating during the nesting.

Previous research on *Apis mellifera* (Linnaeus, 1758), *Tribolium castenum*, and *Drosophila melanogaster* (Meigen, 1830), among other species, has shown that brain activity of AChE increases shortly after eclosion and stays at this level throughout life (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 compared to less active insects (Palestrini and Rolando, 2001; Grünewald and Siefert, 2019), hence, elevated AChE in male and female *D. gazella* in the present study is thus self-explanatory and in agreement with the earlier work done. However, a distinct increase in the levels of AChE in females than males in the present study validates more physical activity of females in tunnel making and brood ball formation (Nervo et al., 2022).

Cano et al., (2017) in their studies have implicated NO to promote habituation, modify diverse neuronal circuits (such as increasing the digging rhythms of oviposition and sensitivity of the taste receptors to chemicals). NO has been described to act as a retrograde neurotransmitter and plays an essential role in reproduction, learning, and memory (Wessnitzer and Webb, 2006; Heinrich and Ganter, 2007; Ridgel et al., 2007; Weinrich et al., 2008), proving its potent role in the signalling mechanism. Foraging behaviour has been well explored in Drosophila larvae and *Bombyx mori* (Linnaeus, 1758), experience-dependent fighting in crickets, and sound production in grasshopper (Aonuma et al., 2004; Seki et al., 2005; Wenzel et al., 2005). However, being a comparatively recently discovered NT, the neuroendocrine role of NO in *D. gazella* are still only partially known. In order to bridge this gap in knowledge, the current study sought to investigate the involvement of NO in the nesting behaviour of *D. gazella*. The significant rise in NO levels among male and female dung beetles provides valuable insights into the role of NO in the aforementioned behaviours.

The pathways of NTs biosynthesis enzymes in insects appear to be similar to those seen in vertebrates (Farooqi et al., 2022). NTs biosynthesizing enzymes- ddc, 5-htpdc, chAt, and NOS enzymes are involved in the multistep conversion and play a critical regulatory role, for the formation of DA, 5-HT, ACh and NO. Role of dopaminergic system in aggression and parental care (Alekseyenko et al., 2013); association of serotinergic system in sociality,

parental care as well as the increase in aggression (Alekseyenko et al., 2010; Dulac et al., 2014); cholinergic signal transduction for olfactory learning and memory formation (Grünewald and Siefert, 2019); role of nos in translating information of growth and regulation of endocrine signals, (Jaszczak et al., 2015) has been well explored. However, to our knowledge, such studies are lacking for *D. gazella*. To fill the knowledge gap, an effort was undertaken to comprehend the significance of NTs synthesising enzymes and link it to changes in the NTs. In our study, parallel to the increase in NT (DA and 5-HT) levels, gene expression of enzymes (ddc and 5-htpdc) involved in neurotransmitter metabolism was also observed to increase transcriptionally, thereby establishing the molecular involvement of the genes in its nesting behaviour and confirming its considerable role in the dung ball rolling, tunnelling, orientation behaviour enhancing the reproductive ability of *D. gazella*. Additionally, the role of AChE in terminating cholinergic signalling, regulating muscle control, sensory processing, and facilitating learning and memory processes accentuate its importance in specific behaviours, such as dung location, navigation, and reproductive strategies by *D. gazella*. Hence, a significant increase in the expressions of choline acetyl transferase (chAt) which forms acetylcholine (ACh) along with the increase in AChE confirms the role of AChE in nesting behaviour of *D. gazella*. Furthermore, a significant increase in the levels of NO along with the enzyme nos confirms the role of NO in nesting behaviour of *D. gazella*.

Insects are capable of incredible intricate behaviour despite having relatively little brains. According to studies till date, NPs, repored in insects' brain, can serve a wide range of functions in the brain, and even a single NP may have multiple functions (Nässel and Homberg, 2006). According to Muratspahić et al., (2020), neuropeptides have a crucial role in the control of several physiological, behavioural, and developmental processes in insects. Within minutes they start to have an impact which can be highly localized effects targeting a small number of very specific neuronal circuits or highly widespread effects that target a large number of diffuse neural circuits. Despite the enormous growth in insect NP research, very few NPs have so far been associated to behavioural outcomes. Npf, for instance, regulate behaviours related to feeding, reproduction, learning, and stress. Role of neuropeptides in regulating the feeding and reproductive behaviour of many insects are just recently known (Cunningham et al., 2017; Fadda et al., 2019; Benowitz et al., 2019) and have opined that the alterations in the expression of neuropeptides are reflected on its abundance that influences mating, feeding, aggression, and social tolerance in different behaviour.

Further, they have also explained how the burying beetle *Nicrophorus vespilloides*, *N. orbicollis*, and honeybee social behaviour differ in their expression of the npf and npfr between parental and non-parental groups. A significant rise in the expression of npf and npfr in *D. gazella*, in the present study, has thus orchestrated important events during development has a modulated the feeding, reproduction and foraging activities, ensuring their nutritional needs for optimum growth and establishing its regulation in feeding and foraging (Nassel et al., 2008; Altstein and Nässel, 2010; Nagata et al., 2012; Carlsson et al., 2013). Hence, our results are in agreement with the earlier work of Ko and coworkers (2015), who have opined that npf enhances attraction to odours during starvation in flies, similarly npf over expression induces continuous feeding in nesting *D. gazella*.

Although the precise functions of these molecules remain little comprehended across several taxa, the neuropeptide group consisting of oxytocin and vasopressin has been identified in various taxonomic classifications. Although OT/VP-like peptides exhibit a significant level of sequence similarity, they are referred to by distinct names across various taxa. The functional role of inotocin signalling in ants, locusts, and beetles has been extensively investigated by several researchers, known to be associated with several processes such as water balance, myoactivity, nesting, offspring care, affiliation, and aggressiveness. (Gruber and Muttenthaler, 2012; Liutkevičiūte et al., 2016; Giglio et al., 2017; Fetter-Pruneda et al., 2021; Caldwell and Young, 2006; Gruber, 2014; Aikins et al., 2008; Stafflinger et al., 2008). Although several functions of inotocin have been identified, there is currently a lack of research on the involvement of inotocin in parental care. Potticary et al., (2022) demonstrated a correlation between inotocin levels in *N. orbicollis* and both non-social and social context in their research. They also observed that the expression of inotocin varies with age in male virgin beetles that were isolated, but no such variation was observed in females. The expression of inotocin in pre-reproductive males was shown to be reduced compared to post-reproductive phases, suggesting a potential involvement of inotocin in preparing males for the reproductive process. Our study shows that inotocin levels in the *D. gazella* increased in both males and females' individuals which can be correlated with the propensity to its foraging activity during nesting period and are in agreement with the earlier work of (Nagy, 2021) where inotocin titre has been related with the parental care behaviour. Parental care in burying beetles encompasses both direct and indirect forms of support. Direct care involves the provision of regurgitated food to offspring, while indirect care entails the maintenance of the carcass during the presence of larvae, without reliance on

social interactions (Potticary et al., 2022). Therefore, based on the findings of the current research, it can be inferred that inotocin probably plays a role in the regulation of reproductive behaviour, including parental care, in *D. gazella*. This inference is supported by the observed increase in expression levels of inotocin (it) and its receptor (itr) during the nesting and breeding period, which coincides with the shift in beetle behaviour from mate searching to pair formation and subsequent parental care.

All insects include myostimulatory neuropeptides, which are hormones that are known to be released from neurohaemal organs, the retrocerebral complex, and the abdominal perisympathetic organs (Urbański et al., 2018). More lately, mip expression has been shown in *D. melanogaster* (Kolodziejcyk and Nässel, 2011), blowfly *Calliphora vomitoria* (Kolodziejcyk and Nässel, 2011), and cockroach *Rhyparobia maderae* (Schulze et al., 2012) in visual neurons and their putative association with neurons of the circadian clock. Additionally, research has shown that mip expression in various regions of brain, including the central complex of *L. migratoria, R. maderae*, and *D. melanogaster* (Kahsai and Winther, 2011). In insects such as fruitfly, mosquito (Yamanaka et al., 2010), tick (Paluzzi, 2016), kissing bug (Peymen et al., 2019), and silkworm (Simo et al., 2013), mip receptors have also been biochemically characterized. Studies by Hensgen et al., (2022) have proved the role of mip in the modulation of feeding-related muscle activity and oviduct contractions in migratory locust, *Locusta migratoria*. It inhibits the contractions of the gut muscles, slowing down digestion and influencing feeding behaviour; contributes to the modulation of circadian behaviours, including locomotor activity and rest-activity patterns (Oh et al., 2014). Moreover, Hussain et al., (2016) have also reported the role of mip in male-specific abdominal movements and pheromone release, influencing mating success and reproductive interactions. Thus, in order to elucidate the roles that this pleiotropic neuropeptide play in the nesting behaviour of *D. gazella*, we analyzed the expression levels of mip. A significant increase in mip, in the present study probably helps *D. gazella* during dung ball rolling, foraging, and reproduction, influencing and coordinating the intensive work during complex behaviour and helping for precise control of muscle contractions, ensuring efficient locomotion and behavioural execution. Our work is in accordance with the work (Poels et al., 2010; Jang et al., 2017; Hussain et al., 2016), where they have reported mip titres with muscular activity. Thus, in insects, the regulation of all physiological processes is governed by two interconnected systems, namely the neurological system and the endocrine system. (Lubawy et al., 2020), which together interplay, communicate and regulates all the

physiological processes that are essential for the survival of the organism in normal as well as stressful conditions (Adamski et al., 2019; Nasssel et al., 2019).

2.5 CONCLUSION

Dung beetles, in particular, rely on their well-developed olfactory systems to locate and navigate dung resources for food and reproduction. By unravelling the intricate relationship between neurotransmitters and nesting behaviour in *D. gazella*, our study provides valuable insights into the neural mechanisms governing complex behaviours. DA and 5-HT, including AChE and NO are involved in the regulation of digging behaviour, brood ball formation, navigational abilities, and reproduction. The role of these neurotransmitters in the nesting behaviour was further proved by the elevation in the neurotransmitter biosynthesizing enzymes- ddc, 5-htpdc, chAt, and nos. The activities of these enzymes provide evidence of the synthesis and release of neurotransmitters, confirming their involvement in nesting behaviour. Moreover, from the present study, it can be concurred that neuropeptides- npf, it, and mip are among the key neuropeptides, influencing various aspects of their reproductive behaviour, aggression, feeding, and motor control.