BACKGROUND

Dung beetles have emerged as valuable ecological indicators for biodiversity assessment due to their taxonomic and ecological knowledge and sensitivity to habitat changes (Nichols et al., 2007; Numa et al., 2009; Otavo et al., 2013; Martínez et al., 2017; Barragán et al., 2021). They are often recognized for rolling dung balls across landscapes; provide essential ecosystem services, referred to as "nature's pooper scooper," assisting farmers worldwide in natural and organic pest control (Sanchez, 2019; Saha et al., 2021). Dung beetles display a wide range of life history strategies and body sizes, reflected in their functional traits and ecological roles, such as dung removal, seed dispersal, and soil bioturbation (Braga et al., 2013; Noriega et al., 2021; Barragán et al., 2022). These traits make them excellent models for studying functional diversity and ecosystem functionality in grazing lands (Louzada and Silva, 2009). Dung beetle diversity is critical for dung degradation, preventing dung accumulation, which can lead to health problems for humans and ecological issues (Pecenka and Lundgren, 2018). Their services reduce the need for livestock health treatments and soil fertilization (Lousey and Vaughan, 2006; Lopez-Collado et al., 2017). Dung beetle introduction programs enhance their ecosystem services and knowledge networks for technology transfer (Forgie et al., 2010; Ryan et al., 2011).

Dung beetles play a pivotal role in pasture nutrient cycling by removing and burying dung, enriching the soil with organic matter and enhancing percolation. Dung beetles can be classified into five main functional groups: tunnellers, rollers, dwellers, non-nesters, and kleptocoprids. Tunnellers, the most beneficial for pasture health, dig tunnels beneath dung and create brood balls containing their offspring (Gomez-Cifuentes et al., 2019; Tonelli, 2021). They also have ecological benefits, disrupting breeding habitats for pests and parasites, reducing the spread of pathogens, and promoting plant growth and seed dispersal (deCastro-Arrazola et al., 2023). Despite their ecological importance, dung beetle diversity is declining due to human activities, posing a threat to ecosystem services (Nichols et al., 2009). Thus, intensive studies on dung beetles are imperative to maintain healthy ecosystems (Salomão et al., 2020).

Insecticide exposure can disrupt chemical communication systems in insects, leading to changes in behaviours like foraging, oviposition site selection, and pheromone communication. These changes are driven by complex physiological mechanisms involving hormones and neurohormones, ultimately affecting reproductive success (Wei et al., 2004).

However, there are lacunae of information as far as the effect of Deltamethrin on *D. gazella* is concerned.

Thus, considering the points mentioned above, the present study was aimed to investigate the neural regulation in the nesting behaviour of dung beetle (D. gazella) on exposure to insecticide (Deltamethrin).

Chapter 1: Brood morphometry and digging behaviour

In the life history of dung beetles, nesting behaviour plays a pivotal role. Dung beetles exhibit a diverse range of nesting patterns, encompassing both simple and highly complex strategies (Scholtz et al. 2009; Halffter et al. 2013; Cortez et al., 2021). Furthermore, Heurta et al., (2023) have identified seven distinct nesting patterns (I to VII) within the Scarabaeinae subfamily. Of particular interest are the paracoprid dung beetles, which have garnered attention due to their unique nesting patterns. These beetles are commonly found in forest and agricultural habitats (Sabu et al., 2006; 2007; Venugopal et al., 2012) across the world (Andresen, 2005). They construct underground nesting galleries, typically situated beneath or adjacent to their food source, where they collect and manipulate food for their offspring. These paracoprid dung beetles primarily exhibit nesting patterns I, II, and III (Fig. 2). In pattern I, the female constructs multiple brood balls, lays eggs within them, and then departs from the nest. In pattern II, the female forms a thicker brood ball and adds a layer of soil over the dung post-oviposition, after which she leaves the nest, containing two or three brood balls. Pattern III involves the construction of nests with several brood balls, with the female providing care for the progeny during the larval development stage (Heurta et al., 2023).

Digitonthophagus gazella, a paracoprid dung beetle, exhibits unique nesting behaviour and is found in various regions, including Africa, America, Australia, Arabia, Madagascar, Pakistan, Sri Lanka, and multiple parts of India. These beetles possess specialized leg structures, particularly the fore tibiae, which aid in resource acquisition, burrowing, and tunnelling within dung and soil. They excavate tunnels to provide dung in the form of compact brood balls at the end of each tunnel. These brood balls can take on different shapes and are coated with soil, serving as a protective barrier against predators, parasites, pathogens, and desiccation. Both male and female dung beetles are involved in constructing and rolling the brood balls, with males often excavating tunnels while females pat the surface of the brood ball rapidly. Copulation typically occurs after burying the brood ball, and females take on the responsibility of nest care during larval development. Males also stay within the nest, guarding the brood ball and the female to prevent other males from mating with her.

Dung beetles' reproductive behaviours encompass various aspects such as sexual exploration, sexual recognition, competition for mates and nesting resources, sperm competition, and parental care. Parental care in dung beetles involves nest construction and brood protection, with a single egg being deposited in an egg chamber and sealed off. Larvae remain inside the chamber until pupation, emphasizing the importance of the brood ball's integrity for their survival. Some brood balls have a narrow aeration conduit connected to the egg chamber, which can include a filter made of dung fibers. Utilizing nutrient-rich but ephemeral dung resources leads to unique behavioural and physiological adaptations, promoting sub-sociality and biparental care. Biparental care is particularly common in dung beetles like *D. gazella*, where larger parents produce larger brood masses.

While much is known about the biology of tunnelling dung beetles, there is limited research on the nesting and reproductive behaviour of ball-rolling dung beetles, including *D. gazella*. Thus, in the present study, we investigated nest architecture, brood morphology, and digging behaviour in *D. gazella*, contributing to our understanding of the nesting behaviour of these fascinating dung beetles.

In the present study, dung beetles 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. 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). Collected beetles were brought to laboratory for identification (morphological and molecular level) and rearing in laboratory conditions. Further to understand the tunnel pattern, tunnel casts were excavated at the end of 10th, 20th, and 30th day. For morphometry analysis, the measurements of castings were done by following the method of Sinha, (2013), where parameters including the number of openings, length of the tunnel, total depth of the tunnel, diameter of the tunnel, area of burrow opening, numbers of branches, and patterns of the tunnel were taken. Further, brood balls formed at the end of 10th, 20th and 30th days were collected, counted and photographed using a Nikon D5200 camera with 18-5mm Nikon lens. At 12 hour interval, brood balls were monitored for the development of the individuals, starting from egg up to the adult stage. Then after, length, and weight measurements of each stage of development of *D. gazella* were recorded with the help of vernier caliper (Zhart, India) and analytical balance. Then, to understand the role of digging genes in tunnel formation by dung beetles, 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 10th, 20th, and 30th days of tunnel formation. Further, the RNA isolation was performed, followed by cDNA synthesis, RT-PCR using the primers of dll and ems. For data analysis, each experiment was done in triplicate. Statistical analysis was done using Graphpad Prism 9 software. The data was analyzed using one way and two way ANOVA test followed by multiple comparison test (Tukey's). The results of the present study are given as follows:

Figure 1: Morphological Features of *D. gazella*

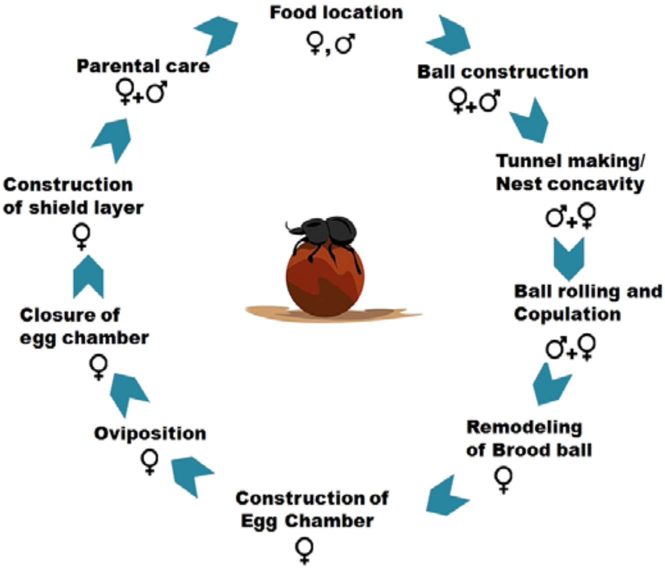


Figure 2: Nesting behaviour of *D. gazella*. Cycle repeated. Comma (,) indicates that the activity is performed by either female or the male alone; addition symbol (+) between the sexes indicates cooperation required.

Table1: Observation of tunnel pattern of *D. gazella* on 10th, 20th and 30th day; *Here, NBO= Number of Burrow Openings; L= Length (cm); TD = Total Depth (cm); D = Diameter of burrow (cm); Area (cm^2); NOB= Number of Branches of burrows*

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	Complex	Complex

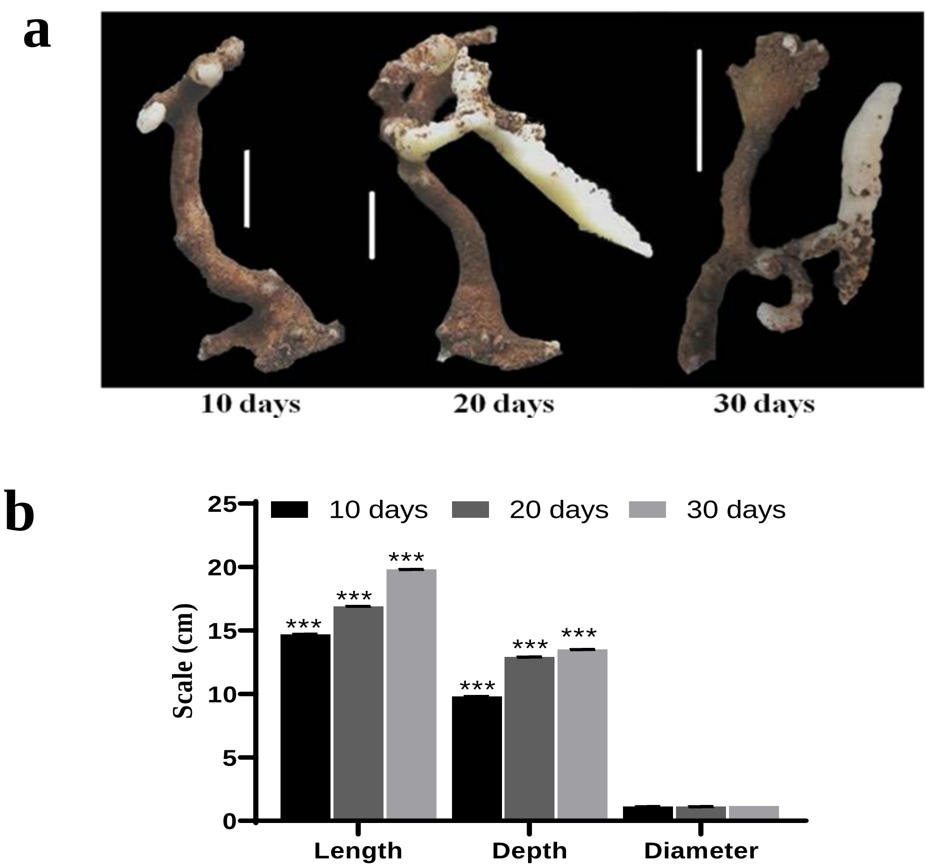


Figure 3: The tunnel pattern of *D. gazella*. (a) The tunnel formed at the end of the 10th, 20th, and 30th day is shown (scale = 2 cm). (b) The graph represents the key measures (length, depth and diameter) of tunnel formation. The length and depth of the tunnel were observed to increase significantly ($p<0.001$) in a tie dependent manner (10th, 20th, and 30th day). *Here,* $p<0.001$*** *(n=3).*

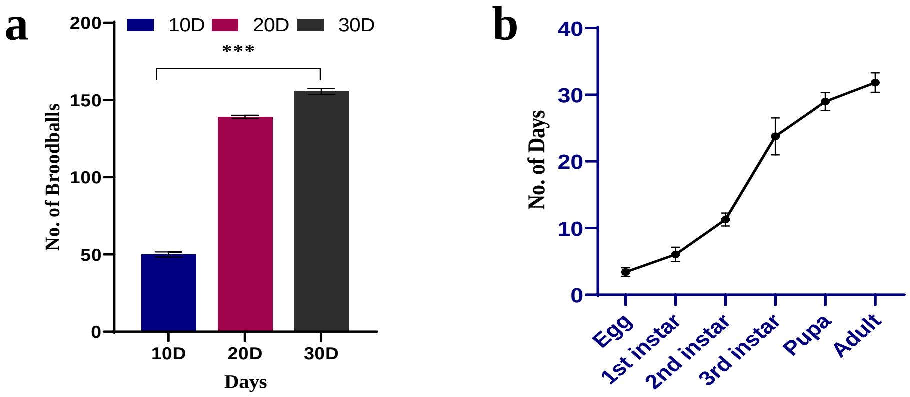


Figure 4: (a) Spherical shaped brood balls formed by *D. gazella* (b) Comparative accounts on the duration of the different stages in the life cycle of *D. gazella*.

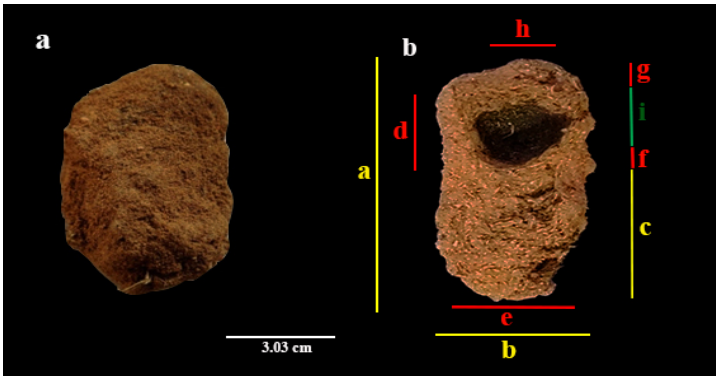


Figure 5: Morphological traits in brood balls formed by *D. gazella.*

Table 2: 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	38.3 ± 5.89	15.5±0.89	745±1.34

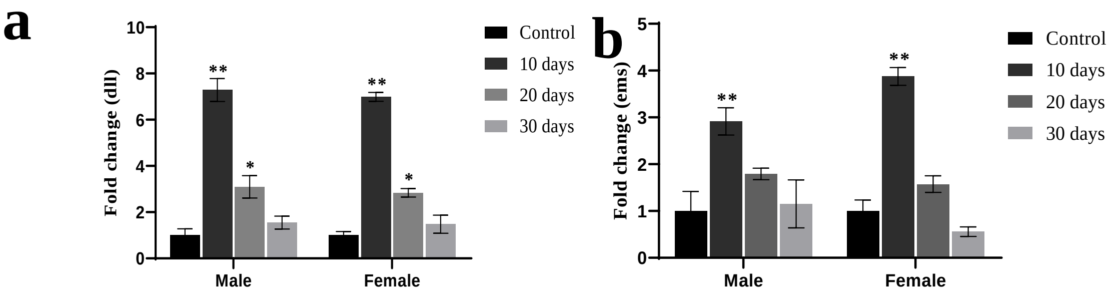


Figure 6: The fold change in digging genes in male and female *D. gazella* (a) dll (b) ems.

Digitonthophagus gazella shares nesting traits with other *Onthophagus* species (Huerta and García-Hernández, 2013; Arellano et al., 2017; Sane et al., 2020). However, our observations revealed distinctions, including cooperative behaviour between sexes during ball rolling and initial nest construction phases. The early-stage nesting pattern identified aligns with type II patterns observed in tunnelling dung beetles, consistent with previous findings (Heurta et al., 2023). Moreover, our study unveiled a time-dependent complexity in tunnel development. By the 10th day, tunnels had three branches, increasing to four branches by the 20th and 30th day, housing linearly arranged brood masses. This corresponds to a Markovian-building process facilitating the construction of larger, deeper pits with steeper walls (Sane et al., 2020).

In our study, the life cycle of *D. gazella* was approximately 28 to 30 days, comprising egg, larva (3 instars), pupa, and adult stages. Significant changes in brood morphometry occurred throughout the life cycle, including length, diameter, and weight, with no variation in brood ball length (Arellano et al., 2017; Singh et al., 2019). Upon reaching adulthood, dung beetles undergo sexual maturation and engage in nesting activities, including tunnel excavation, brood ball construction, mating, and female egg-laying. Dung beetles possess three pairs of legs, with the fore tibia playing a pivotal role, featuring teeth for soil excavation and a tibial spur crucial for digging. In our study, we reported that the digging genes such as dll and ems were found to be significantly expressed during the initial phase of nesting behaviour (Linz et al., 2019; Jugovic and Koprivnikar, 2021).

In conclusion, our study sheds light on the nesting behaviour of *D. gazella* under laboratory conditions, with a focus on tunnelling, brood ball construction, and parental care. While the costs and benefits of biparental cooperation have been extensively studied, there remains an incomplete understanding of the neurophysiological and molecular mechanisms driving plasticity in nesting behaviours. Although we have a good understanding of the factors shaping this plasticity, further investigation into neuromodulatory aspects is crucial to unravel the precise neural and physiological mechanisms enabling these flexible nesting behaviours.

Chapter 2: Understanding the Nesting Behaviour of D. gazella: Role of Neurohormones

The reproductive behaviours of dung beetles encompass a range of activities, including sexual exploration, sexual recognition, and competition for mates and nesting resources, sperm competition, and parental care (Huerta et al., 2023). While the role of neurotransmitters in reproductive behaviour has been extensively studied in vertebrates (Adkins-Regan, 2005), investigations in insects have been relatively limited (Riddiford, 2012). For a considerable period, neuromodulation within the central nervous system has been implicated in shaping the plasticity of behavioural responses (Zhukovskaya and Polyanovsky, 2017).

Biogenic amines, such as Dopamine (DA), serotonin (5-HT), and Octopamine, serve as neurotransmitters, neuromodulators, and neurohormones, playing pivotal roles in regulating various physiological processes in insects (Lange and Orchard, 2021; Sasaki et al., 2021). Enzymes involved in catecholamine biosynthesis are known to participate in decarboxylation, influencing neurotransmitter biosynthesis, metabolism, and innate immunity (Hodgetts and O'Keefe, 2006; Lin et al., 2020). DOPA decarboxylase (DDC), the first enzyme identified in the catecholamine biosynthesis pathway, catalyzes the conversion of L-DOPA to DA. Subsequently, 5-hydroxydecarboxylase (5-HTPDC) was discovered to catalyze the conversion of 5-HTP to 5-HT. Acetylcholine esterase (AChE) is a pivotal enzyme controlling insect behaviour by interacting with neurotransmitters and neuromodulators. Its primary role involves swiftly breaking down acetylcholine (ACh) into choline and acetate, effectively halting nerve impulse transmission mediated by ACh. This enzyme is crucial in governing muscle contraction and motor control in insects. In dung beetles, AChE plays a significant role in regulating muscle activity during various behaviors such as flight, digging, dung ball rolling, and environmental navigation. By rapidly hydrolyzing ACh at neuromuscular junctions, AChE ensures precise timing and coordination of muscle contractions, facilitating accurate movement and behavioural responses (Perić-Mataruga et al., 2017; Cabirol and Haase, 2019). Serving as a signalling molecule, NO plays a role in regulating various physiological processes, behaviours, and social interactions in insects. It achieves this by modulating synaptic plasticity, sensory processing, and reproductive behaviours (Bicker, 2000; Malaterre et al., 2005). NO is associated with a range of insect behaviours, including feeding, mating, aggression, and olfaction (Rillich and Stevenson, 2019). Moreover, NO interacts with acetylcholine esterase (AChE) by affecting its activity and expression, providing a mechanism to regulate cholinergic neurotransmission. While there are a few reports of neurotransmitters like Dopamine, Serotonin, Acetylcholine, and Nitric Oxide in insects such as *Drosophila*, *Manduca sexta*, *Anopheles gambiae*, and *Anopheles stephensi* (Muller, 1996; Jacklet, 1997; Charpentier et al., 2000; Davies, 2000; Bicker, 2001; Vleugels et al,. 2015), their role in reproductive behaviours has not been extensively explored.

Given the established links between neurotransmitters and the modulation of behavioural states, the present study was focused on the neurotransmitters, including, biogenic amines, DA and 5-HT, as well as AChE and NO which represent prime candidates for the neuromodulatory control of nesting behaviour in *D. gazella* dung beetles. For this, the neurotrasnmitters (DA, 5-HT, AChE, and NO) were estimated and the NT synthesizing gene expression, as well as expression of the NPs genes in both males and females was analyzed, by isolating the brain tissue on 10th, 20th, and 30th days of tunnel formation. Further, the RNA isolation was performed, followed by cDNA synthesis, RT-PCR using the primers. For data analysis, each experiment was done in triplicate. Statistical analysis was done using Graphpad Prism 9 software. The data was analyzed using one way and two way ANOVA test followed by multiple comparison test (Tukey’s). The results obtained were as follows:

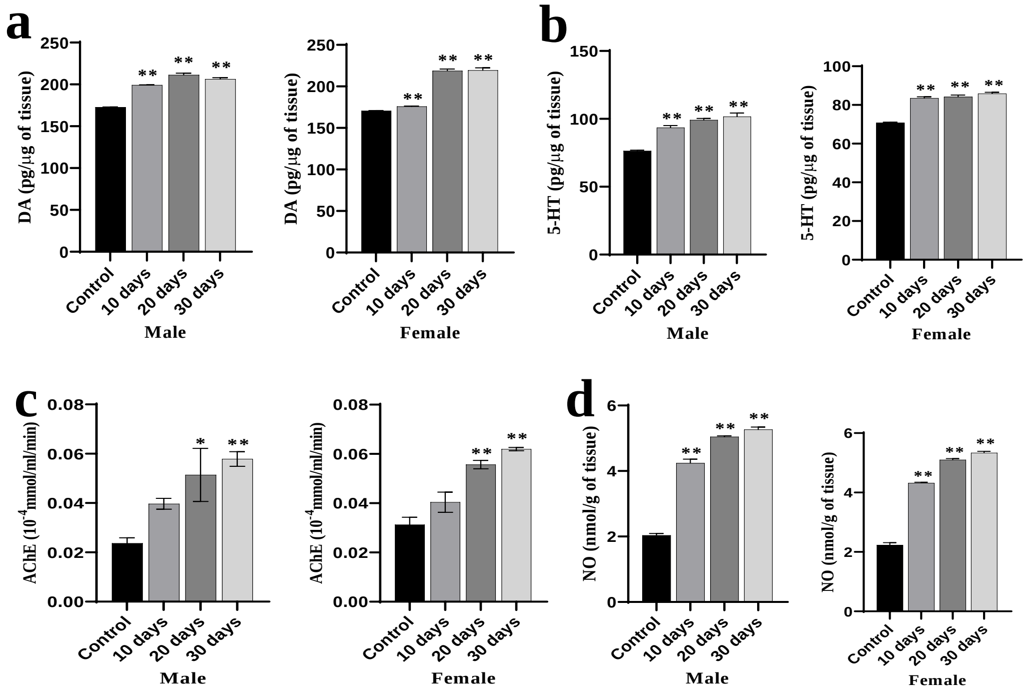


Figure 7: (a) Dopamine (DA) levels (b) Serotonin (5-HT) levels (c) Rate of AChE activity (with the unit mmol/mL/min x 10^{-4} per g of tissue); (d) Nitric Oxide (NO) levels on the 10th, 20th, and 30th day of introduction of male and female into the experimental setup. *The error bars indicate SEM with significant values;*$p<0.05$ **$p<0.01$*

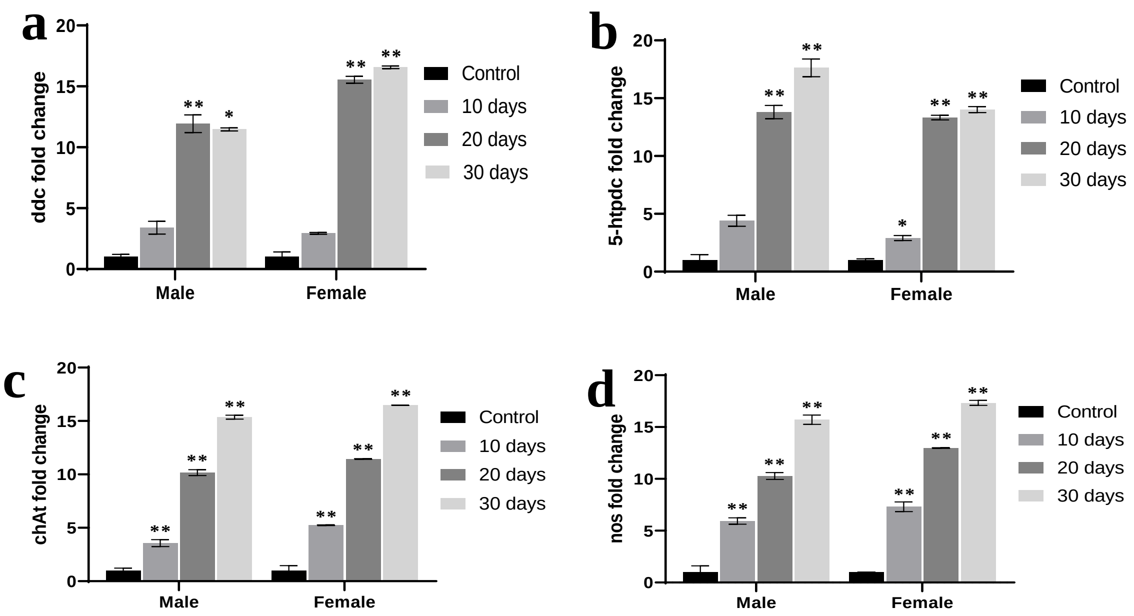


Fig. 8: Neurotransmitters synthesizing enzyme gene expressions (a) ddc (b) 5-htpdc (b) chAt (d) nos. *The error bars indicate SEM with significant values;*p<0.05 **p<0.01*

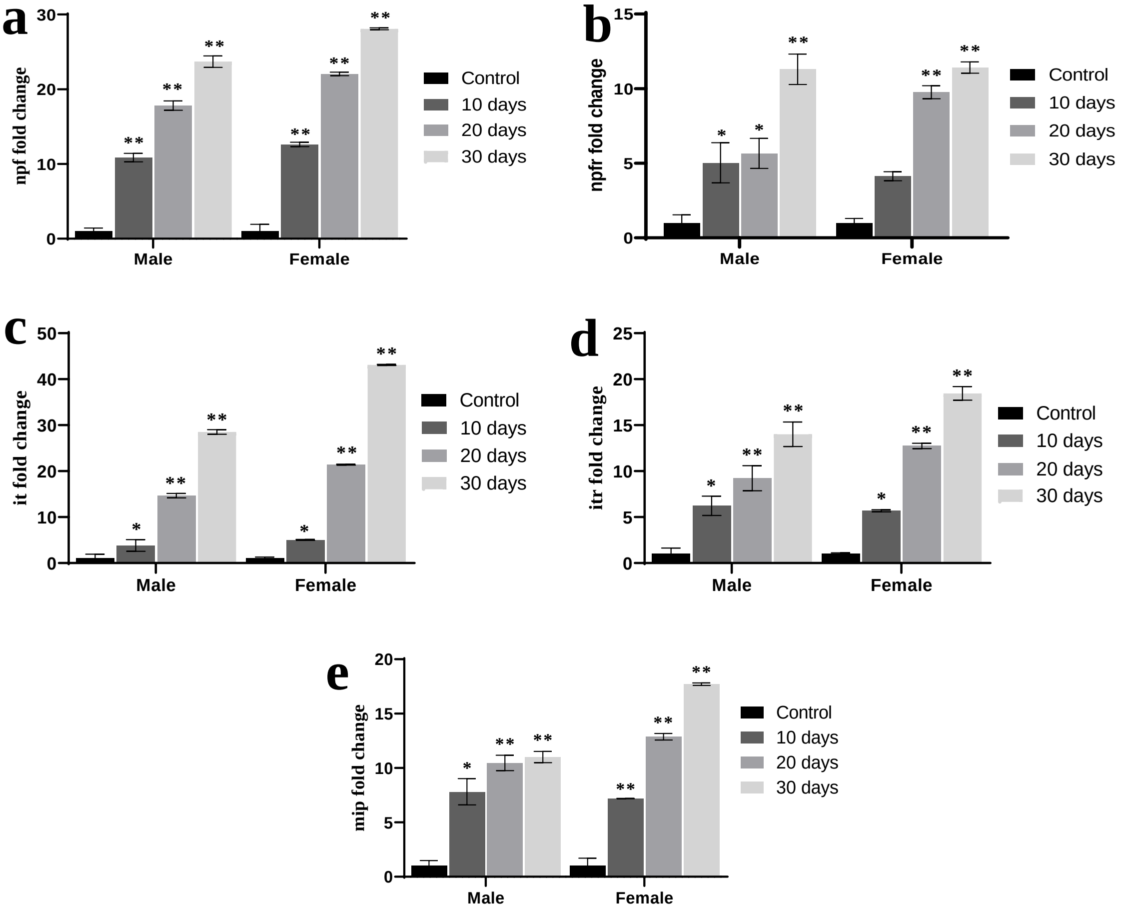


Fig. 9: Fold change expression of neuropeptide gene (a) npf (b) npfr (c) it (d) itr (e) mip in *D. gazella*. *The error bars indicate SEM with significant values;*p<0.05 **p<0.01*

In this study, elevated levels of dopamine (DA) and serotonin (5-HT) on the 10th, 20th, and 30th days of tunnelling indicate their involvement in nesting behaviour (Misof et al., 2014; Song et al., 2015; Kamhi et al., 2017). Males displayed slightly higher 5-HT levels, potentially linked to context-dependent male aggression during copulation (Trumbo, 2019). The increased DA levels in both sexes further support their role in reinforcing nesting activities. These findings align with previous research (Auletta, 2019) highlighting the critical roles of neurotransmitters (NTs) in various behaviours and physiological processes, including complex social activities and reproductive strategies (Kamhi et al., 2017; Sasaki and Watanabe, 2022). The study also explored acetyl cholinesterase (AChE) levels, associated with physical activity in insects (Hao et al., 2021). Females exhibited higher AChE levels, possibly due to their increased physical activity during tunnel construction and brood ball formation (Nervo et al., 2022). NO likely influences signaling mechanisms, affecting foraging behaviour and other complex activities in beetles (Koto et al., 2019).

Enzymes such as DDC and 5-HTPDC, with their gene expression paralleling the increase in NT levels, confirmed their involvement in *D. gazella*'s nesting behaviour. Further increase in Choline Acetyl Transferase (chAt) and acetylcholinesterase (AChE) activity, along with increased NO and nos gene expression is associated with olfactory learning, muscle control, and sensory processing. This study highlights their role in dung location, navigation, and reproductive strategies.

Furthermore, neuropeptides like npf, it, and mip influence various aspects of reproductive behavior, aggression, feeding, and motor control (Nässel and Homberg, 2006; Potticary et al., 2022; Hussain et al., 2016). Inotocin (it) likely plays a role in parental care and reproductive behavior, as its levels increased during nesting. Myosin inhibiting peptide (mip) was implicated in muscle activity, feeding, and oviduct contractions, potentially helping coordinate complex behaviours, such as dung ball rolling and foraging, by regulating muscle contractions in *D. gazella*.

In summary, this study sheds light on the intricate relationship between neurotransmitters, enzymatic processes, and neuropeptides in regulating the nesting behaviour of *D. gazella*. The findings provide valuable insights into the neural mechanisms underlying complex behaviours in dung beetles and potentially other insects.

Chapter 3: Toxic effects of Deltamethrin on biochemical and histological alterations in D. gazella

Paracoprid dung beetles play a crucial role in agriculture and pest control worldwide (Génier and Davis, 2017). However, the detrimental effects of insecticide residues in dung have disrupted their valuable services (Beynon et al., 2015). Insecticides, commonly used to manage livestock pests, are often excreted in toxic quantities in dung, significantly reducing the survival and reproductive performance of dung fauna (Vale et al., 2015). These toxic residues alter the metabolic and physiological activities of these beetles (Sands et al., 2018).

Synthetic insecticides like Pyrethroids, derived from natural pyrethrins, are potent but have raised health and environmental concerns due to their widespread and prolonged use (Jacobs and Scholtz, 2015; Vale et al., 2015; Dudley et al., 2017; Sands et al., 2018; Andjani et al., 2019; Serrão et al., 2022). Deltamethrin, a type II synthetic pyrethroid, is well-known for its impact on sodium channels (Meunier et al., 2020). Even at sub lethal concentrations, this widely used pyrethroid inflicts significant physiological damage on various insects (Cutler, 2013; Müller, 2018). It induces oxidative stress, leading to the production of harmful reactive oxygen species (ROS) that can cause severe cellular damage (Zug and Hammerstein, 2015). Insects have a repertoire of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione transferase (GST), and glutathione reductase (GSH), which collaborate to counteract the effects of oxidants (Boardman, 2012). Furthermore, Cytochrome P450 plays a pivotal role in insecticide metabolism, including honey bees, by mediating biosynthesis, breakdown, detoxification of compounds, and contributing to cellular metabolism and homeostasis (Hu et al., 2017; Palrasu and Siddavaram, 2018). In our study, we selected Deltamethrin for investigation due to limited existing knowledge about its effects on paracoprid dung beetles, *D. gazella* with preliminary evidence suggesting potential lasting impacts (Mann et al., 2015), and the results obtained are as follows:

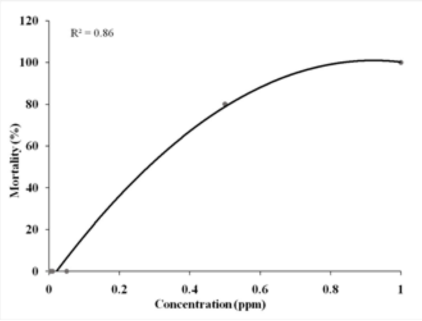


Figure 10: Dose response curve for the LC_{50} determination of Deltamethrin after 48 hours of exposure.

LC_{50} value was obtained as 0.275 ppm from the dose response curve (**Fig.**). Further, the sub-lethal concentrations: Low dose (LD)-1/20th of LC_{50}, Medium dose (MD) - 1/10th of LC_{50}, and High dose (HD)-1/5th of LC_{50} were used to understand the toxic potential of Deltamethrin on *D. gazella*

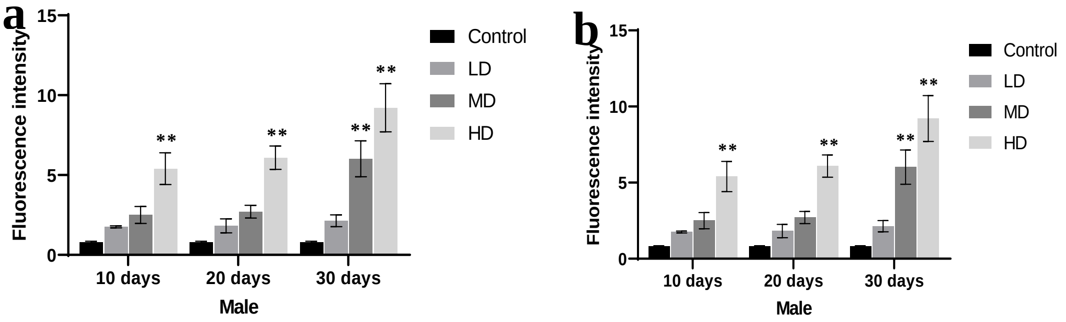
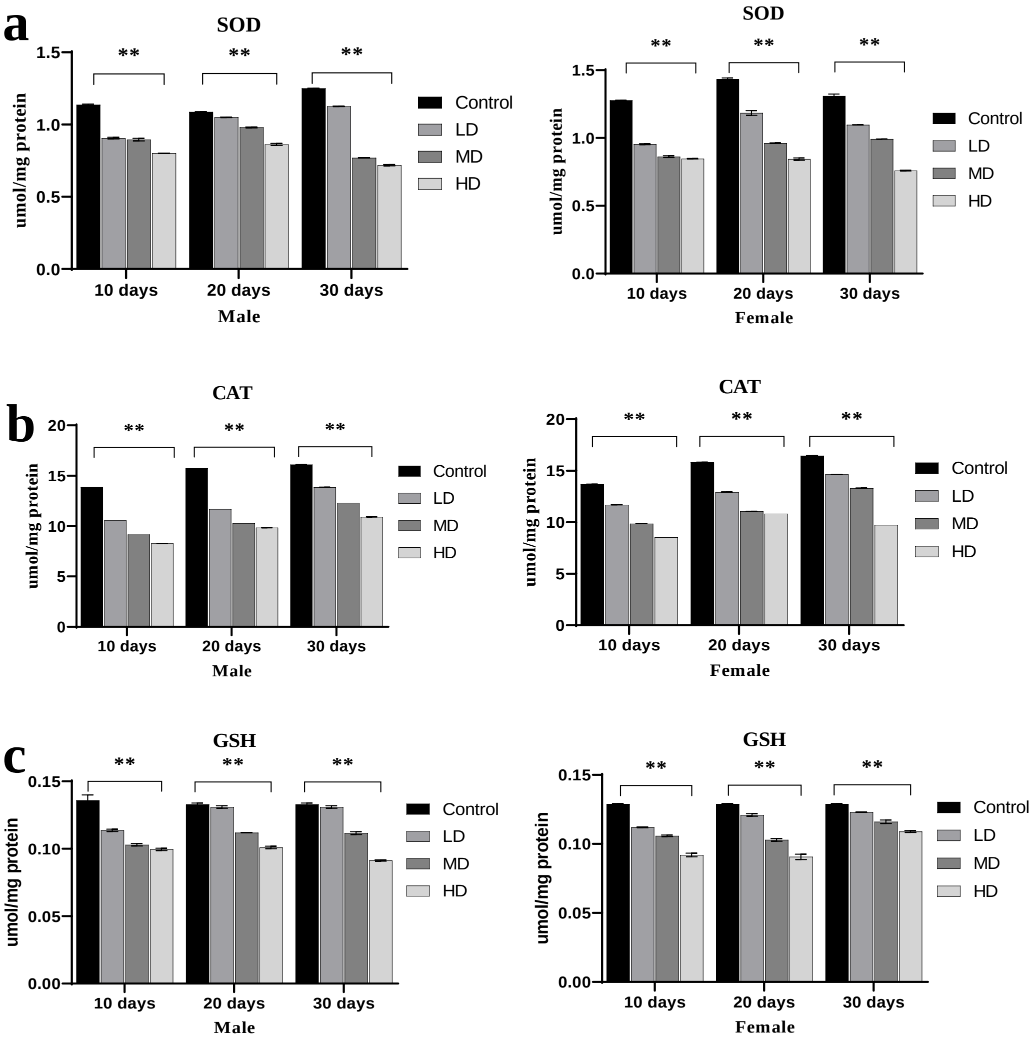


Figure 11: Fluorescence intensity (mean±SEM) of the DCFHDA staining, in the single cell suspension of the brain tissue of (a) male (b) female *D. gazella* after exposure to sub-lethal doses (LD, MD, and HD) of Deltamethrin, in comparison to control. *(p<0.5*, p<0.01**)*



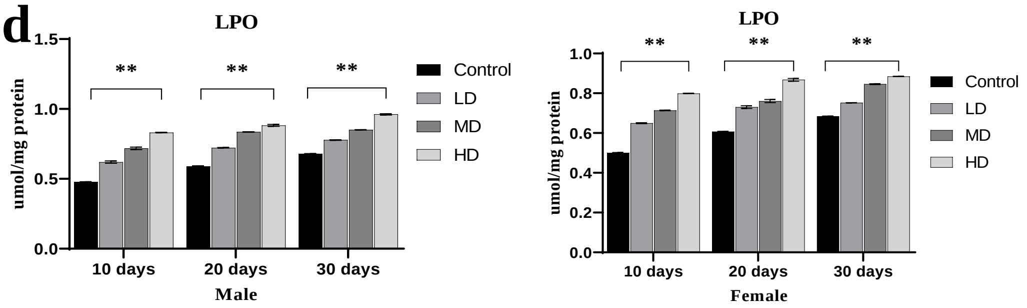


Figure 12: Antioxidant levels after exposure to sub-lethal concentrations of Deltamethrin in comparison to control for 10, 20 and 30 days. **(a)** SOD **(b)** CAT **(C)** GSH **(d)** LPO levels in the brain tissue of male and female *D. gazella*. Each value represents Mean±SEM. *(p<0.5*, p<0.01**)*

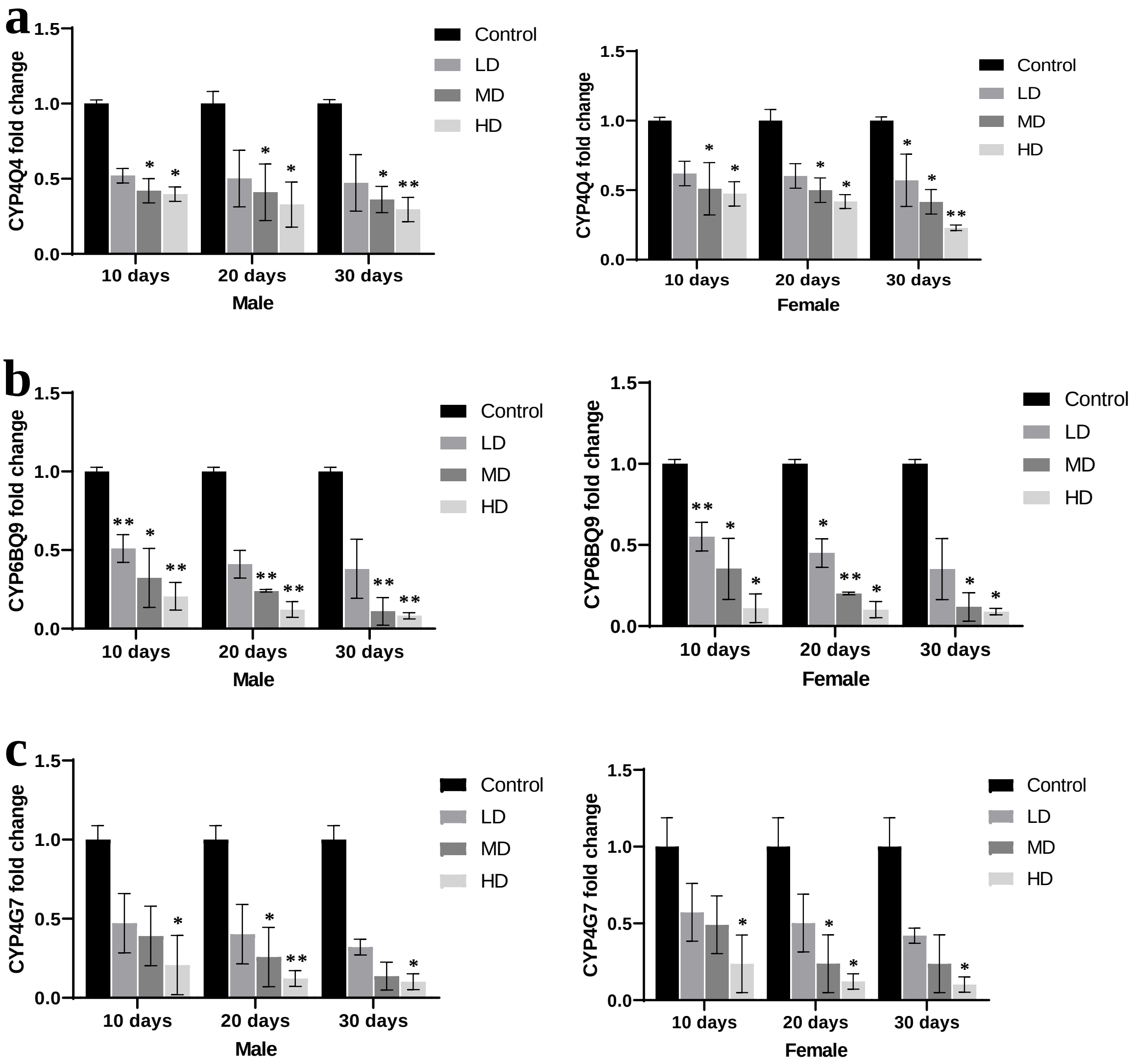


Figure 13: CYP mRNA gene expression after exposure to sub-lethal concentrations of Deltamethrin in comparison to control for 10, 20 and 30 days, in the brain tissue of female *D. gazella* (a) CYP4Q4 fold change (b) CYP6BQ9 fold change (c) CYP4G7 fold change. Each value represents Mean±SEM. *(p<0.5*, p<0.01**).*

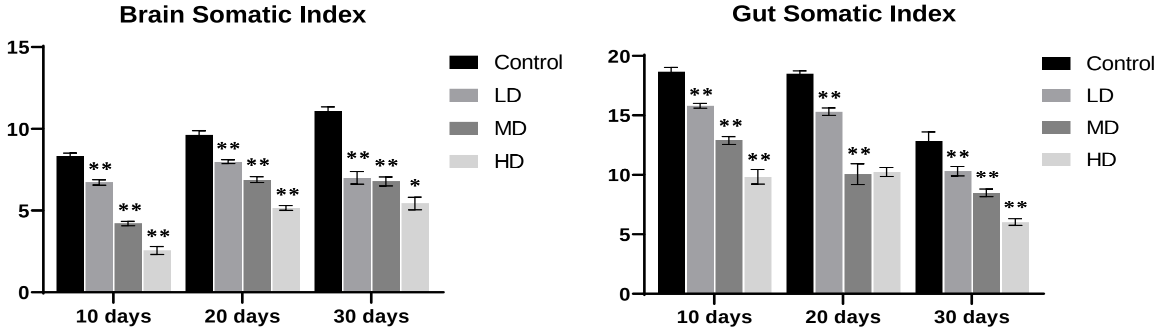


Figure 14: Brain and Gut somatic index of *D. gazella* after exposure to sub-lethal concentrations of Deltamethrin in comparison to control for 10, 20 and 30 days. Each value represents Mean±SEM. *(p<0.5*, p<0.01**).*

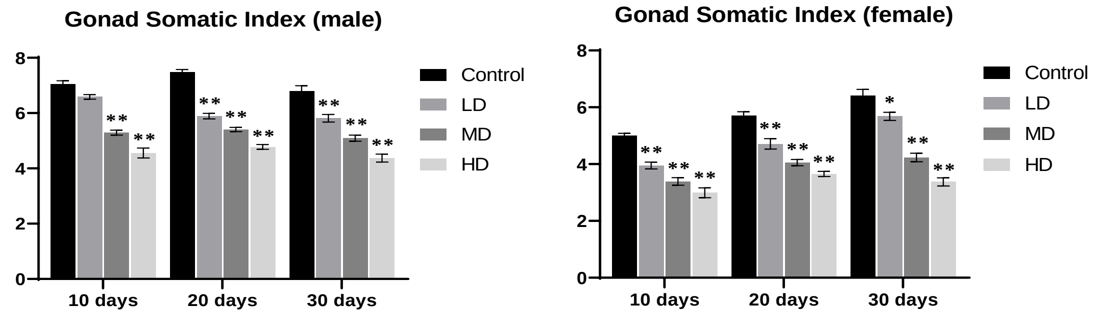


Figure 15: Gonad somatic index male and female after exposure to sub-lethal concentrations of Deltamethrin in comparison to control for 10, 20 and 30 days. Each value represents Mean±SEM. *(p<0.5*, p<0.01**).*

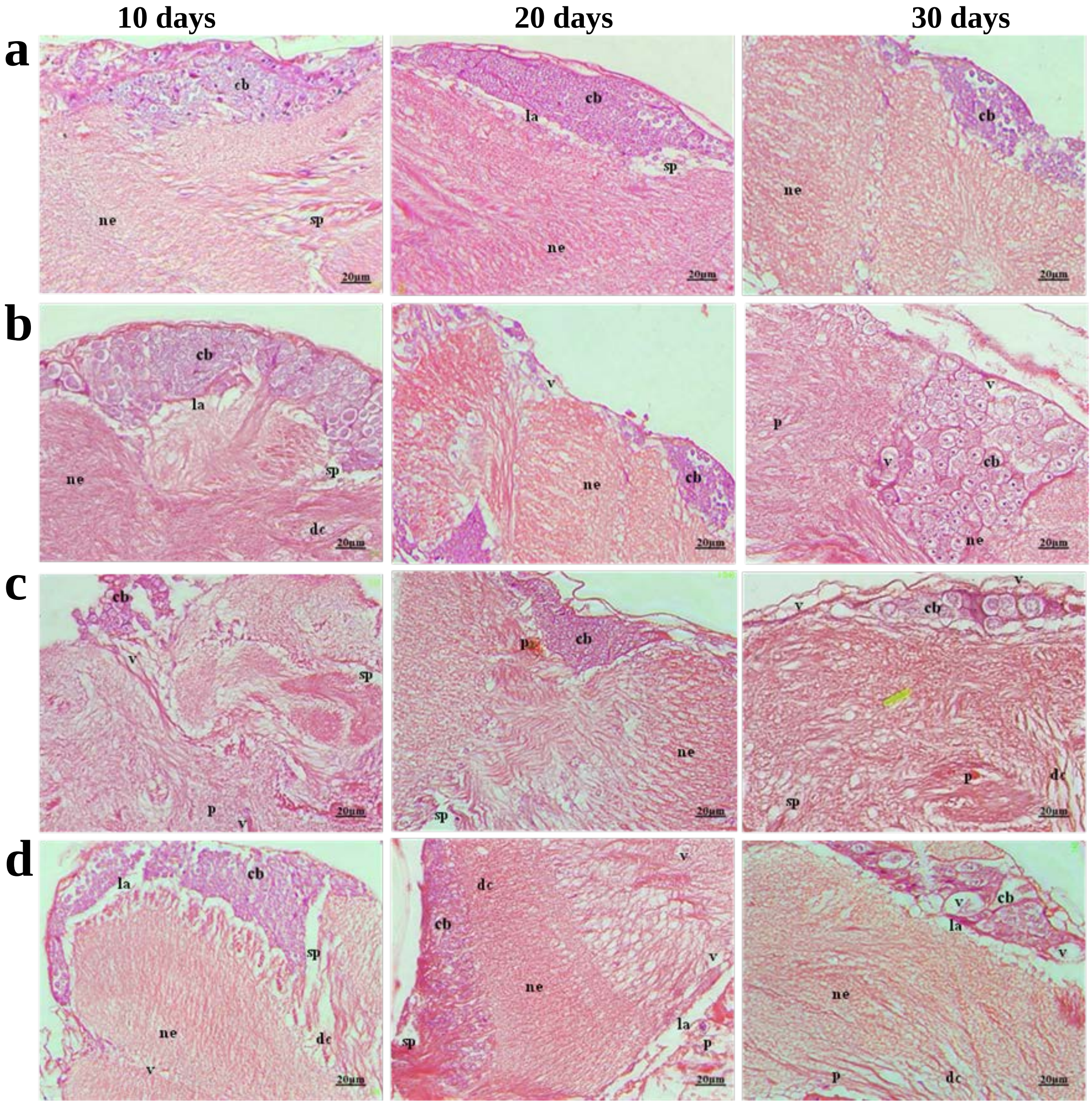


Figure 16: Histological sections of *D. gazella* brain stained with hematoxylin and eosin, after 10, 20 and 30 days of exposure to Deltamethrin. (a) Control treatment with no exposure to insecticide (b) Exposure to LD of Deltamethrin. (c) Exposure to MD of Deltamethrin. (d) Exposure to HD of Deltamethrin. Magnification 40X, scale=20µm. Here, cb: cellbodies; ne: neuropiles; v: vacuoles; sp: spaces; p: pyknotic nucleus; dc: degredative changes; la: lacuna.

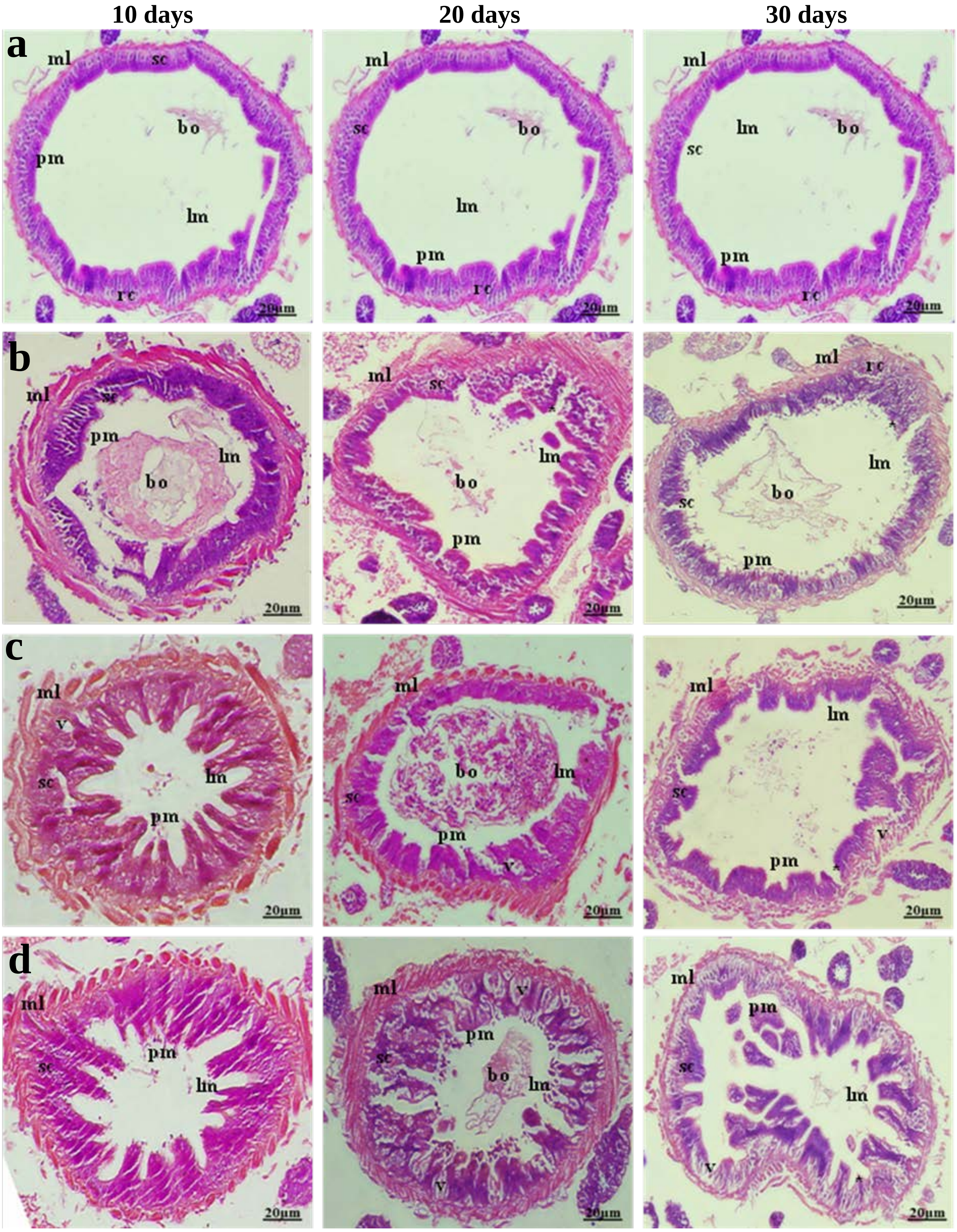


Figure 3.17: Histological sections of *D. gazella* midgut stained with hematoxylin and eosin, after 10, 20 and 30 days of exposure to Deltamethrin. (A) Control treatment with no exposure to insecticide (B) Exposure to LD of Deltamethrin. (C) Exposure to MD of Deltamethrin. (D) Exposure to HD of Deltamethrin. Magnification 40X, scale=20µm. Here, ml: muscles; sc: secretary cells; lm: lumen; bo: bolus; pm: peritrophic membrane; rc: regenerative cells.

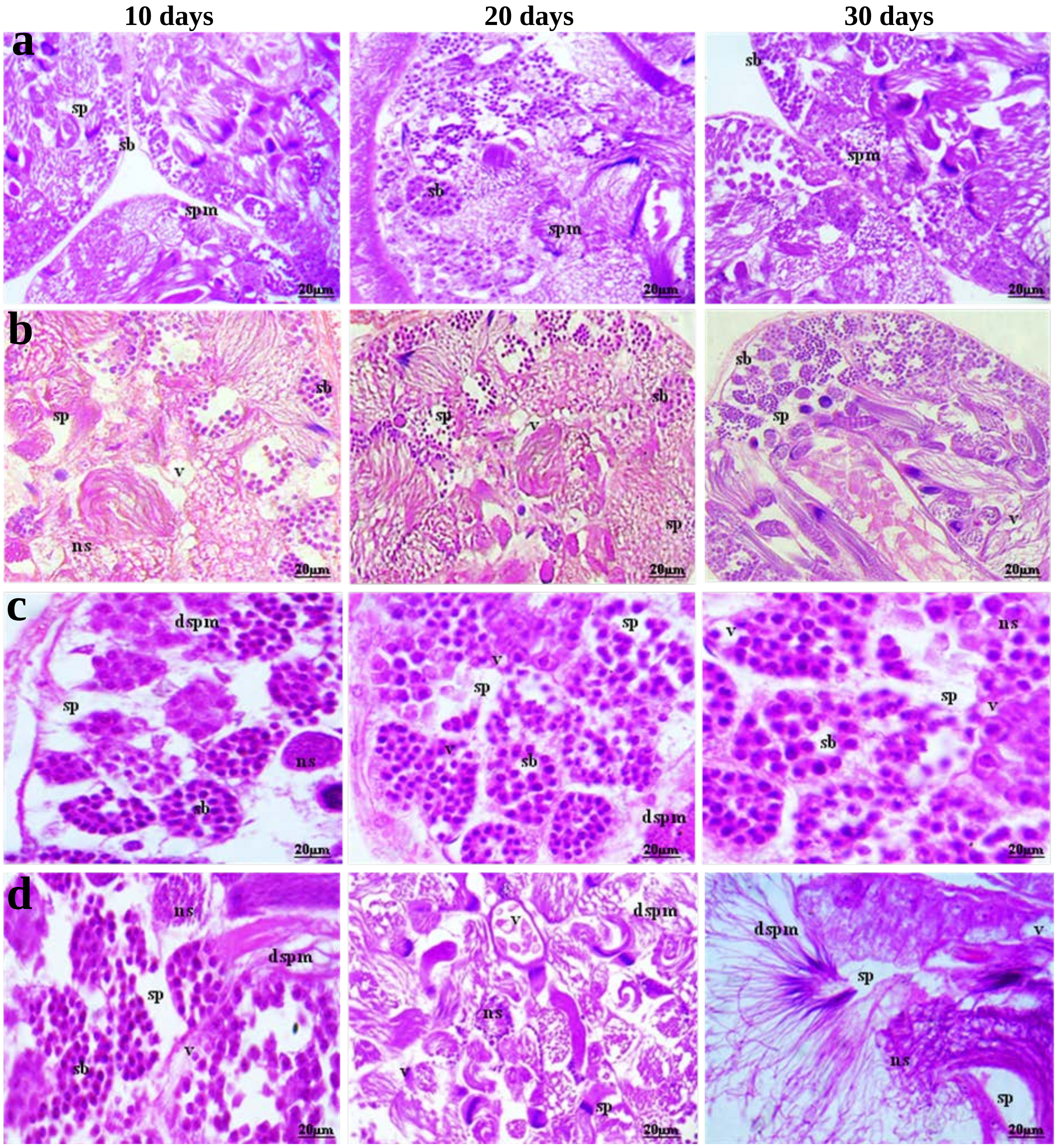


Figure 3.18: Transverse sections of *D. gazella* testis stained with hematoxylin and eosin, after 10, 20 and 30 days of exposure to Deltamethrin (A) Control treatment with no exposure to insecticide (B) Exposure to LD of Deltamethrin. (C) Exposure to MD of Deltamethrin. (D) Exposure to HD of Deltamethrin. Magnification 40X, scale=20µm. Here, sa: shrinkage acini; ns: necrotic sperm (ns); sp: spaces; dspm: disintegrated spermatocytes; v: vacuoles; spm: spermatocytes; sb: sperm bundles.

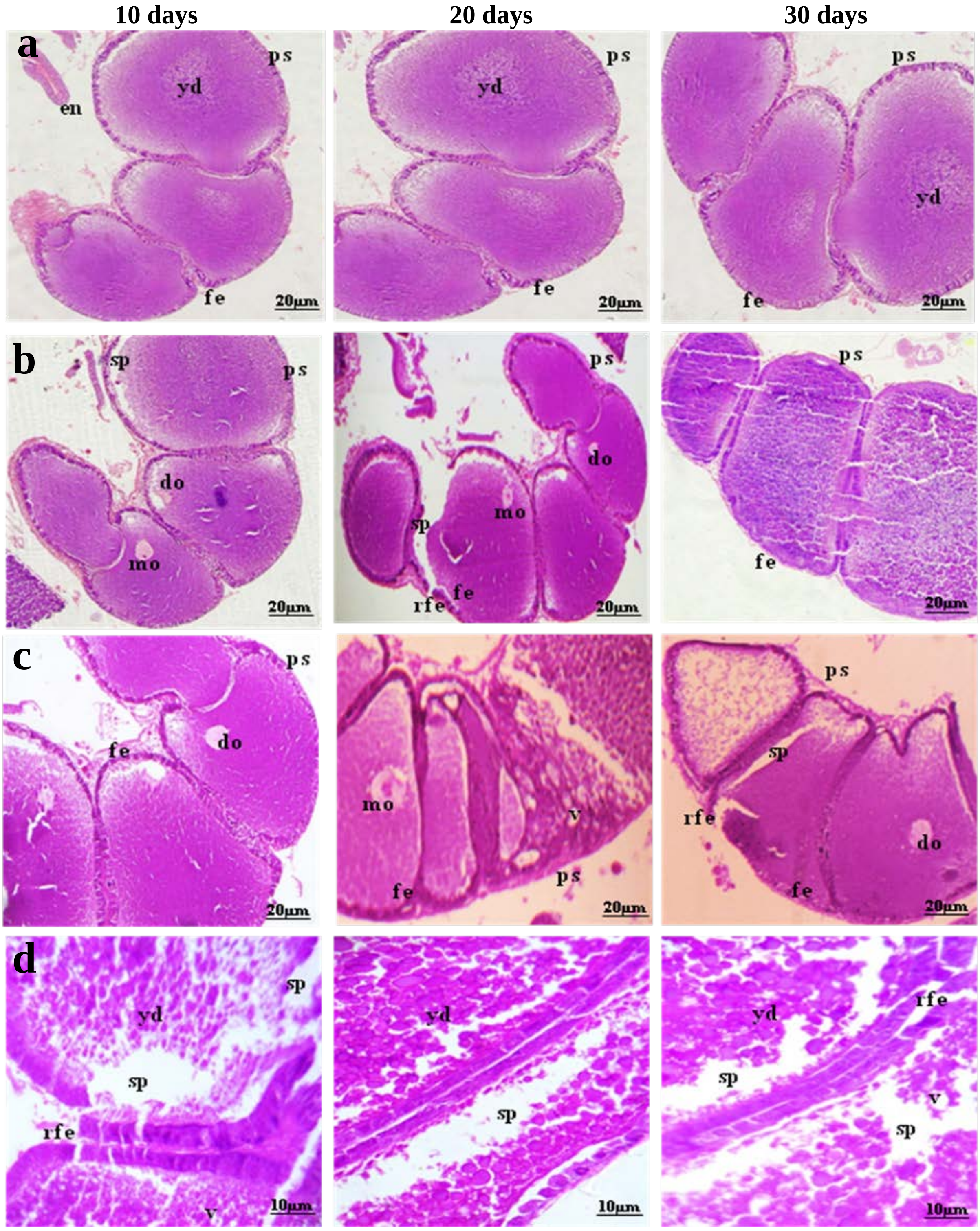


Figure 3.19: Transverse sections of *D. gazella* ovaries stained with hematoxylin and eosin, after 10, 20 and 30 days of exposure to Deltamethrin (A) Control treatment with no exposure to insecticide (B) Exposure to LD of Deltamethrin. (C) Exposure to MD of Deltamethrin. (D) Exposure to HD of Deltamethrin. Magnification: 40X, scale=20µm; 100X, scale=10 µm. Here, ps: peritoneal sheath; fe: follicular epithelium; en: endochorion; Rfe: ruptured follicular epithelium; sp: spaces; mo: mature oocytes; v: vacuoles; do: degenerated oocytes.

In our study, we explored the impact of Deltamethrin, a commonly used pesticide, on *D. gazella*. Deltamethrin exposure induced a range of behavioural changes in *D. gazella*, including jerky movements, loss of equilibrium, tremors, and integument darkening. These effects are attributed to its interference with voltage-gated sodium channels in insect neuronal membranes, disrupting nervous system signalling. Further investigation confirmed a dose and time-dependent increase in ROS production in the brain tissue using DCFH-DA staining. Oxidative stress, mediated by ROS, is a common mechanism of pesticide toxicity.

Moreover, we assessed the response of *D. gazella* to oxidative stress by quantitatively analyzing lipid peroxidation (LPO) and antioxidant mechanisms, including superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH). Deltamethrin exposure resulted in dose and time-dependent changes in LPO and antioxidant mechanisms, indicating tissue damage and a compromised defence system against oxidative stress. The depletion of GSH levels further suggested impaired antioxidant defences. Additionally, we examined the activity of cytochrome P450 (CYP450), an enzyme involved in detoxifying toxicants in insects. Deltamethrin exposure led to a significant down regulation of CYP4G7, CYP6BQ9, and CYP4Q4 expression in a dose and time-dependent manner. This down regulation is noteworthy, as previous studies have shown CYP6BQ9 to be predominantly expressed in the brains of pyrethroid-resistant insects (Nauen et al., 2022). While our study did not focus on insecticide resistance, it highlighted the impact of Deltamethrin on CYP450 activity.

Furthermore, we conducted organosomatic indices and histopathological examinations to evaluate the effects of Deltamethrin on the brain, gut, and gonads of *D. gazella*. Exposure resulted in a reduction in the brain somatic index, accompanied by histological changes indicating potential functional disturbances. In the gut, we observed a dose and time-dependent decrease in the gut somatic index, along with histological alterations suggesting reduced digestion and absorption, impacting energy production and overall physiology. The gonads, ovaries and testes, exhibited reduced gonadosomatic indices and histomorphological changes indicative of disrupted reproductive potential.

In summary, Deltamethrin exposure had profound and irreversible pathological effects on the brain, midgut, and gonads of *D. gazella*. These findings highlight the potential risks posed by Deltamethrin exposure to non-target dung beetles, which play important ecological and economic roles.

Chapter 4: Neurophysiological alterations in the nesting behaviour of D. gazella on exposure to Deltamethrin

Neurotransmitters and neuropeptides play essential roles in regulating physiological and behavioural processes in insects, including responses to stressors like insecticides (Gruntenko et al., 2016; Lubawy et al., 2020). Biogenic amines, such as octopamine (OA), dopamine (DA), and serotonin (5-HT), are known to fluctuate in various insect species under adverse conditions and can influence behaviours like feeding, reproduction, and stress responses (Pflüger and Duch, 2011; Gruntenko et al., 2004). These amines are rapidly released into the hemolymph following stress exposure, contributing to homeostasis restoration (Armstrong and Robertson, 2006).

Pyrethroids can alter neurotransmitter levels and metabolites in the insect brain (Kori et al., 2018). Neuropeptides, on the other hand, regulate various physiological processes in insects, including responses to stress induced by insecticides (Li et al., 2020; Schoofs et al., 2017; Ragionieri et al., 2022). They are released as chemical signals during stressful conditions and govern behavioural responses. Neuropeptide-like peptides such as npf, it, and mip have been linked to stress responses and their regulation (Schoofs et al., 2017; Ragionieri et al., 2022). Studies using molecular genetics techniques have investigated the roles of neuropeptides and their receptors in various insect species, shedding light on their functions in behaviour and physiology (Nässel and Homberg, 2006; Ragionieri et al., 2022; Pandit et al., 2018; Ragionieri and Predel, 2020).

Insecticides, even at sub-lethal concentrations, can have significant impacts on insect biology, affecting fecundity, development, and lifespan (França et al., 2017). These effects often result from behavioural changes during the reproductive phase, and various insecticides have been found to influence reproductive behaviours and success (Storch et al., 2017; Mahmoudvand et al., 2012; Dong et al., 2016). Insecticide exposure can disrupt chemical communication systems in insects, leading to changes in behaviours like foraging, oviposition site selection, and pheromone communication. These changes are driven by complex physiological mechanisms involving hormones and neurohormones, ultimately affecting reproductive success (Wei et al., 2004).

Our study has confirmed Deltamethrin's toxic potential on biochemical and histological parameters which further has shed light on its involvement in altered neurophysiology by investigating the role of neurotransmitters (biogenic amines and nitric oxide) and neuropeptides in the nesting behaviour of *D. gazella*. The results obtained were as follows:

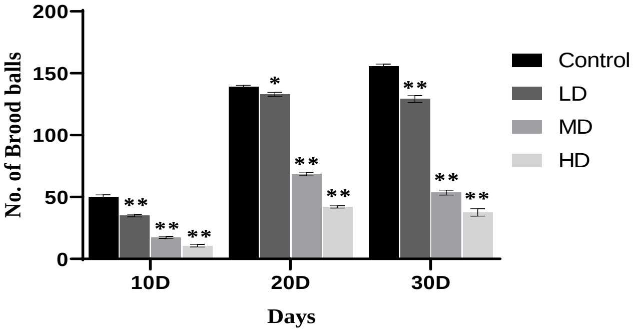


Figure 15: Mean±SEM number of brood balls formed by *D. gazella* after 10th, 20th and 30th day of exposure to Deltamethrin

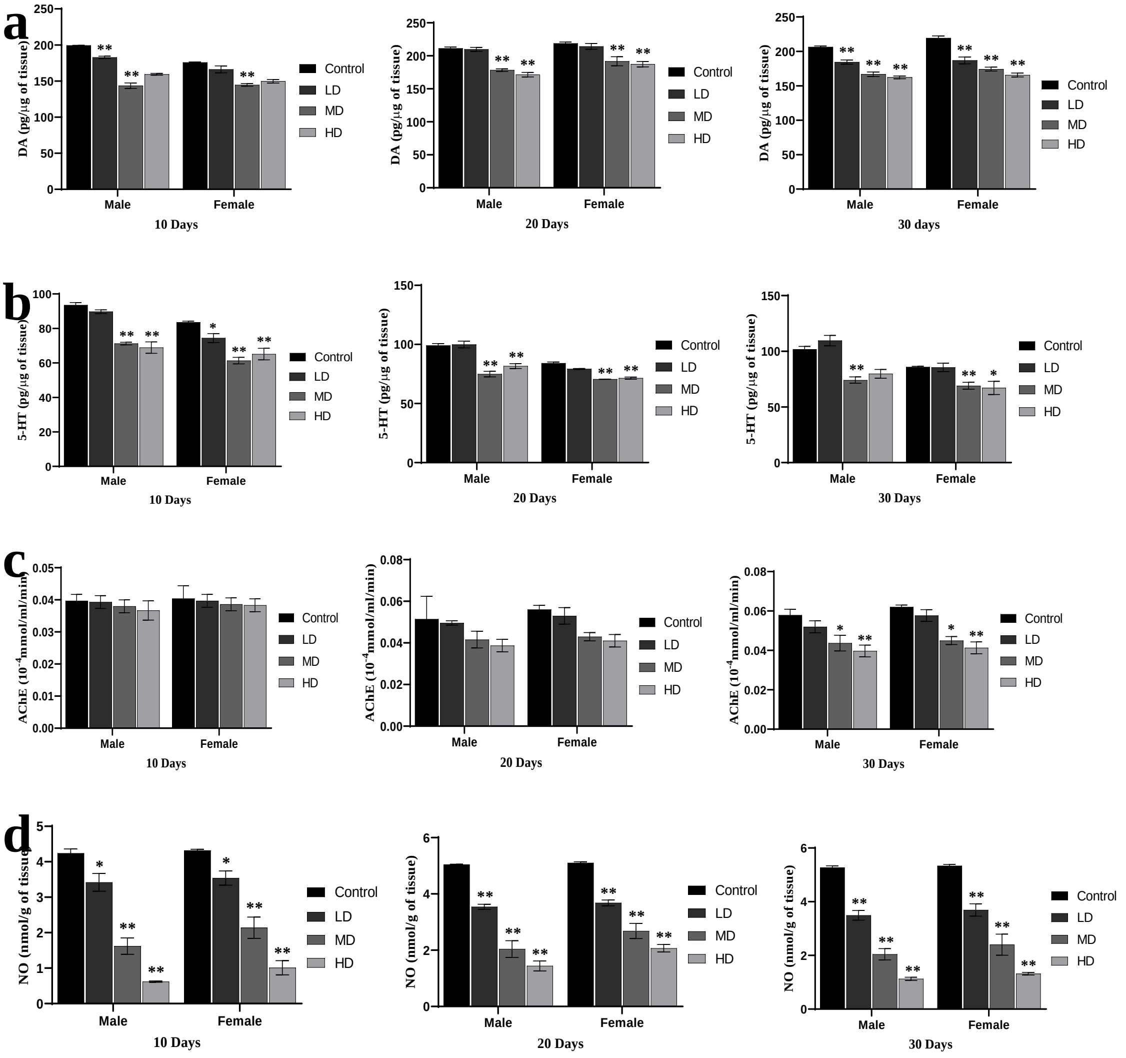


Figure 16: Brain NTs (a) DA levels (b) 5-HT levels (c) AChE activity (d) NO levels in the male and female *D. gazella* on exposure to Deltamethrin after 10th, 20th, and 30th days. **p<0.05 **p<0.01*

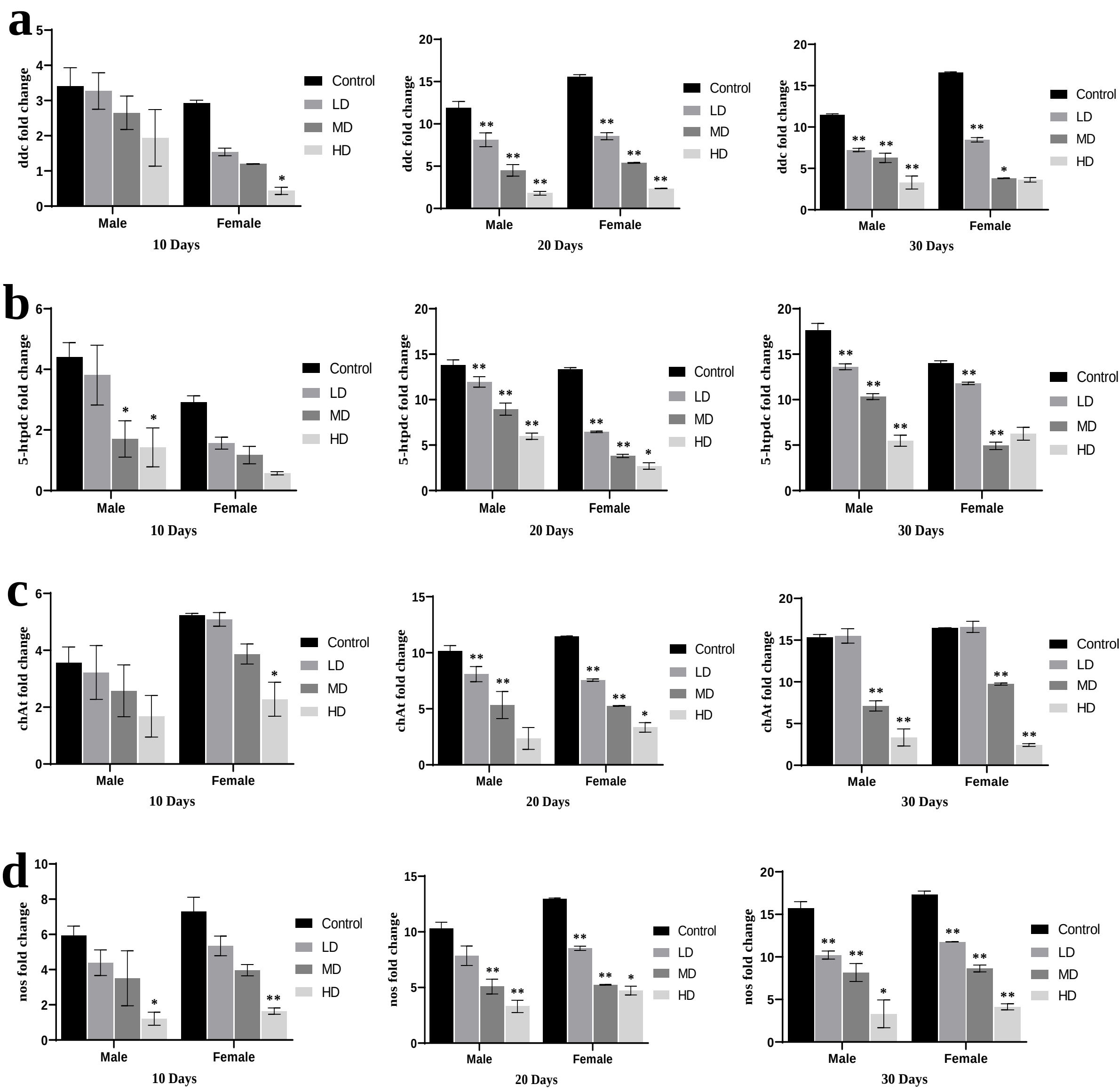


Figure 17: NT synthesising enzyme gene expressions in the brain of male and female *D. gazella* on exposure to Deltamethrin (a) ddc fold change (b) 5-htpdc fold change (c) chat fold change (d) nos fold change after 10th, 20th, and 30th day. *$*p<0.05$ $**p<0.01$*

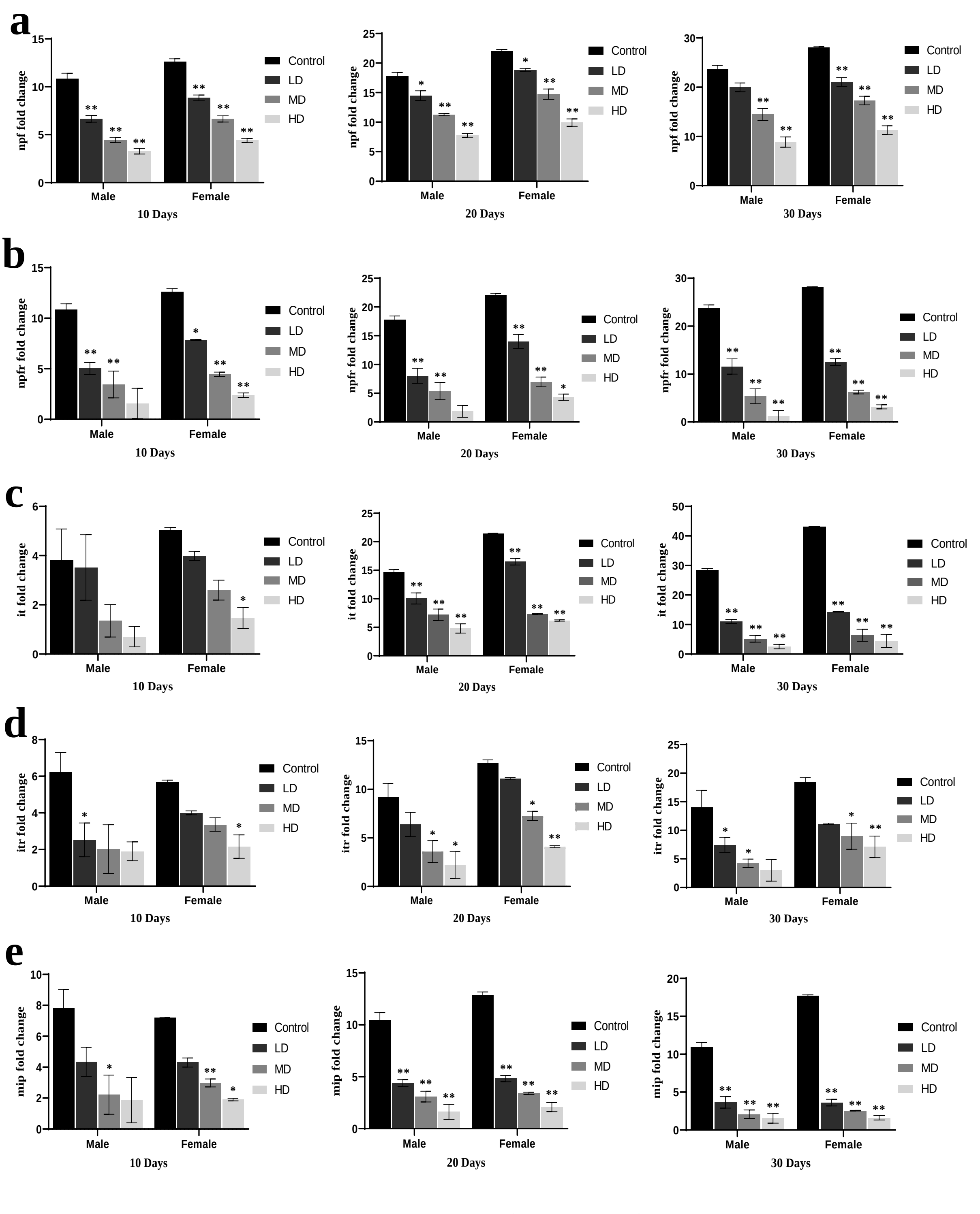


Figure 18: NPs gene expressions in the brain of male and female *D. gazella* on exposure to Deltamethrin (a) npf fold change (b) npfr fold change (c) it fold change (d) itr fold change (e) mip fold change in the brain of male and female *D. gazella* on exposure to Deltamethrin after 10th, 20th, and 30th day. $*p<0.05$ $**p<0.01$

In *D. gazella*, exposure to Deltamethrin resulted in a decline in the number of brood balls over time, indicating decreased functional efficiency and reproductive potential. This reduction in reproductive capacity was accompanied by behavioural changes, including alterations in neurotransmitter levels, notably the inhibition of acetyl cholinesterase (AChE). AChE inhibition leads to the accumulation of acetylcholine, causing prolonged nerve cell depolarization. The interaction between AChE and choline acetyltransferase (ChAT) may also be influenced by insecticide exposure (Grünewald and Siefert, 2019; Bourguet et al., 2020; Johnson et al., 2021).

Deltamethrin exposure induced oxidative stress and impacted dopamine neurotransmission, leading to altered gene expression of dopamine synthesizing enzymes (ddc). These changes could result in modifications in locomotion and other behaviours (Figueira et al., 2017; Xu et al., 2015). Disruptions in dopamine transmission can affect various nesting behaviours of *D. gazella*, including tunneling, reproduction, brood ball formation, and parental care (França et al., 2017). Moreover, a significant reduction in serotonin (5-HT) levels and the expression of the 5-HT synthesizing enzyme (5-htpdc) was observed in *D. gazella* following Deltamethrin exposure, suggesting a role for 5-HT in modulating nesting behaviours (Dillen et al., 2013; Deng et al., 2014). Nitric oxide (NO), an important signalling molecule in insects associated with various physiological processes, was also affected by Deltamethrin exposure. This exposure led to reduced NO levels and the expression of nitric oxide syntheses (NOS), indicating its involvement in altering nesting behaviour of *D. gazella* (Sadekuzzaman et al., 2018).

Additionally, neuropeptides (NPs) like neuropeptide F (npf), inotocin (it), and myoinhibiting peptides (mip) play critical roles in regulating physiological functions in insects, including feeding, metabolism, and reproduction (Yeoh et al., 2017; Liutkeviciute et al., 2016). Deltamethrin exposure resulted in a dose-dependent reduction in the expression of these neuropeptides (npf, it, and mip) and their receptors (npfr, itr), suggesting their involvement in modulating nesting behaviour (Aljedani, 2021; Sakthivel et al., 2022).

In conclusion, Deltamethrin exposure affects the nesting behaviour of *D. gazella* through multiple mechanisms, including alterations in neurotransmitter levels, disruption of dopamine and serotonin signalling, changes in nitric oxide production, and modulation of neuropeptides and their receptors. These complex interactions result in a range of neurological manifestations, ultimately impacting the reproductive efficiency and behaviours of *D.*

gazella. Further research is needed to fully understand the molecular signalling pathways involved in these effects.

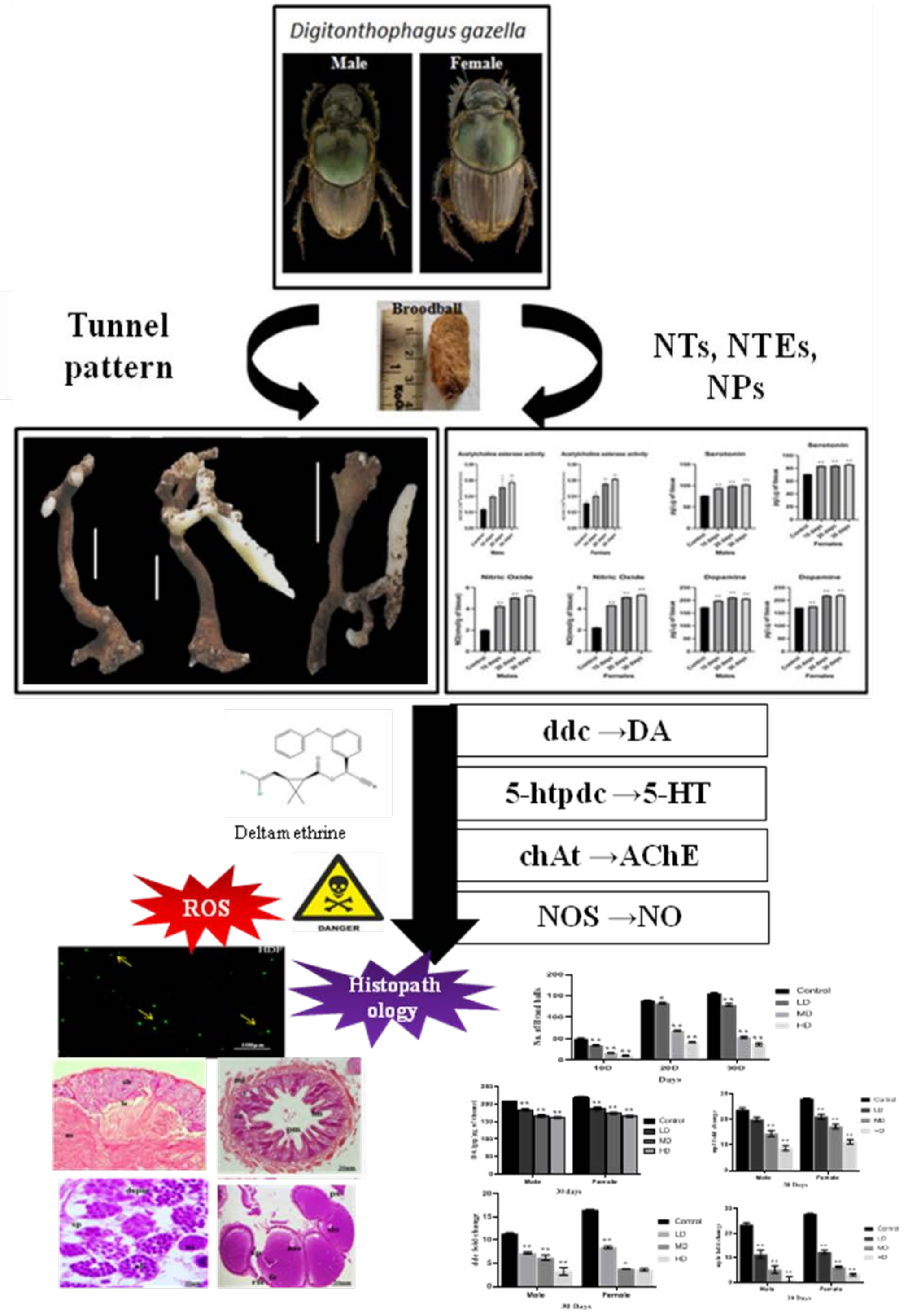


Figure 19: Graphical Abstract of the neural regulation in the nesting behaviour of *D. gazella* on exposure to Deltamethrin.

Study Highlights

The present study contributes the following points:

1) **Nesting Biology Insights:** The study enhances our understanding of the nesting biology of *D. gazella*, highlighting the active involvement of both males and females in tunnel construction and brood ball preparation.
2) **Nest Structure and Pattern:** *D. gazella* was observed to construct a simple nest with a type II pattern within 10, 20, and 30 days. After this period, they transport the brood ball to the tunnel's blind end for reproduction, with females laying eggs inside the brood ball chamber.
3) **Time-Dependent Changes:** The research revealed time-dependent increases in the number of brood balls and morphometric variations in *D. gazella*'s brood balls, setting it apart from other dung beetle species. The life cycle analysis indicated a developmental period of 28 to 30 days, with the third instar larva being the largest stage.
4) **Digging Genes:** The decline in the expression of digging genes (dll and ems) over time (10th, 20th, and 30th days) underscored their functional significance in *D. gazella*'s nesting behaviour.
5) **Neurotransmitter Involvement:** The study elucidated the role of neurotransmitters such as dopamine (DA), serotonin (5-HT), acetylcholinesterase (AChE), and nitric oxide (NO) in regulating various aspects of nesting behaviour, including digging, brood ball formation, navigation, and reproduction. The study also observed increased activity of neurotransmitter biosynthesizing enzymes (ddc, 5-htpdc, chAt, and nos) as evidence of their involvement.
6) **Neuropeptides' Influence:** Neuropeptides, namely npf, it, and mip, were identified as key players influencing reproductive behaviour, aggression, feeding, and motor control in *D. gazella.*
7) **Deltamethrin's Toxic Effects:** Exposure to Deltamethrin, a common insecticide, for 30 days led to disruptions in antioxidant balance, resulting in decreased enzyme activity and elevated production of reactive oxygen species (ROS). This induced oxidative stress, tissue damage, inflammation, and structural abnormalities in organs like the brain, gut, ovaries, and testes.

8) **Neurological Manifestations:** This study is the first to explore the neural regulation in *D. gazella* upon Deltamethrin exposure, revealing the critical role of neural mechanisms in nesting behaviour. Prolonged exposure to Deltamethrin induced various neurological manifestations, affecting tunnelling, mating, and brood care. These changes are likely mediated by the modulation of neurotransmitters, alterations in neuropeptide expression, and the known neurotoxic properties of Deltamethrin, leading to decreased neural conduction.

In summary, this research provides comprehensive insights into the nesting biology and neural regulation of *D. gazella*, highlighting the impact of Deltamethrin exposure on their physiology and behaviour. The findings emphasize the importance of responsible pesticide use and monitoring for toxicity in exposed organisms.