Chapter 3: Toxic effects of deltamethrin on biochemical and histological alterations in *Digitonthophagus gazella*

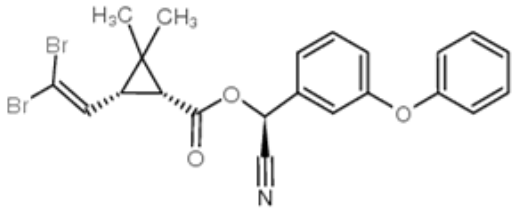
3.1 INTRODUCTION

Paracoprid beetles' tunneling and dung burial activity have made them economically and ecologically important and they are currently employed on a global scale for the purposes of pasture enhancement and the biological control of pest flies (Génier and Davis, 2017). Human-induced stressors are impacting dung beetle populations worldwide, with most monitored populations decreasing (Sánchez-Bayo and Wyckhuys, 2019; Cavallaro et al., 2023). These beetles are essential for ecosystem health, performing various ecological roles. The availability of dung, which is a transient resource to dung fauna, has led to the loss of services when exposed to insecticide (Beynon et al., 2015). Insecticides are chemicals used to kill insects, usually by disrupting vital physiological processes within their body. Several studies have reported pyrethroid insecticides reducing beetle larvae and adult numbers in dung (Mann et al., 2015; Vale et al., 2015; Sands et al., 2018; Bruinenberg et al., 2023; Stanton et al., 2018). Pesticide exposure scenarios, such as seed dust, agricultural runoff, and dusting, threaten dung beetle survival due to their life history strategies and affect the ecosystem functions they provide in agro-ecosystems.

Livestocks are often administered with insecticides to manage pest and parasite infestations. These insecticides are subsequently eliminated from the animals' bodies by faecal excretion, in unmetabolized state with the concentrations toxic to dung-dwelling species (Mann et al., 2015; Slade and Roslin, 2016). The survival or reproductive performance of dung fauna reduces substantially in dung contaminated with insecticides (Vale et al., 2015), thereby altering the metabolic and physiological activities (Sands et. al., 2018). Synthetic products like pyrethroids are developed from naturally occurring pyrethrins, which are extracted from flowers. These products are known for their potent insecticidal properties, but they also pose significant health risks. Exposure to pyrethroids can lead to the development of tremor-type syndrome, allergic reactions, and ataxia (Meunier et al., 2020; Galadima et al., 2021). They are widely utilised in the fields of horticulture, agriculture, and pest control (Vale et al., 2015; Dudley et al., 2017; Sands et al., 2018). Additionally, it is also used in livestock management for the purpose of mitigating the presence of various parasites such as ticks, mites, biting flies, and lice, and are often associated with health and

environmental issues if used excessively in the long term (Jacobs and Scholtz, 2015; Andjani et al., 2019; Serrão et al., 2022).

Table 3.1: Insecticide **(**Source: PPDB)

Chemical name	**Deltamethrin**
Mode of Action	The mechanism of action involves both direct contact and ingestion. The compound has the ability to exert its effects on neuronal membranes through the mechanism of delaying the closure of the activation gate of the sodium ion channel (Worthing and Walker, 1987).
CAS RN	52918-63-5
IUPAC name	[(S)-Cyano-(3-phenoxyphenyl)-methyl] (1R,3R)-3-(2,2-dibromoethenyl)-2,2-dimethyl-cyclopropane-1- carboxylate
Chemical formula	$C_{22}H_{19}Br_2NO_3$
Molecular structure	
Molecular weight (g/mol)	505.21
LogP (at 25^{o}C)	1.5
Relative density	1.5g/cm^3
Henry's Law Constant (Pa m^3 mol^{-1})	1.2 x 10^{-4}
Manufactures and suppliers of products	Sigma Aldrich

Deltamethrin (**Table 3.1**), a specific synthetic pyrethroid of class II, is widely recognised for its capacity to impact the sodium channel (Meunier et al., 2020). It is one of the most widely used pyrethroid whose sub lethal concentrations result in significant physiological damages in both target and non-target insects (Cutler, 2013; Müller, 2018). It induces oxidative stress and leads to production of reactive oxygen species (ROS) which are negatively damaging entities contributing to oxidative stress, resulting into serious cell damage (Zug and Hammerstein, 2015). Several insecticide classes can induce ROS production, including pyrethroids, organophosphates, and neonicotinoids (Krůček et al., 2015). ROS encompasses chemicals such as superoxide radicals (O^{2-}), hydrogen peroxide

(H_2O_2), and hydroxyl radicals (OH^-), which exhibit significant reactivity and have the potential to impair several cellular constituents, including DNA, proteins, and lipids which are mediated by mechanisms such as protein synthesis inhibition and cell membrane permeabilization. This oxidative damage can disrupt essential cellular functions and decrease reproductive performance and lead to cell death or dysfunction (Dickinson and Chang, 2011; Blount et al., 2016). ROS generation has also been reported to alter the electrochemical gradient at the membrane (Chen et al., 2010; Faize et al., 2011; Fatma et al., 2018). However, insects possess an inherent capacity in the form of an antioxidative response system to protect themselves from damage induced by ROS. The antioxidant response system is involved in the detoxification of ROS and consists of low-molecular weight antioxidants and antioxidant enzymes. These components work together to delay and avoid the damaging effects of oxidative agents on bio-molecules (Halliwell and Gutteridge, 2015). These entities fulfill a crucial function in the preservation of homeostasis as they actively engage in cellular signalling networks that regulate programmed cell death, gene expression, and immune defence systems (Gupta et al., 2010; Świętek et al., 2019). According to Lai et al., (2012), there is a direct relationship between the cellular levels of ROS and the enzymatic activity. This suggests that the anti-oxidative mechanism plays a crucial role in mitigating oxidative stress. Besides, the formation and further increase in ROS can occur through different mechanisms, such as inhibiting antioxidant enzymes like superoxide dismutase (SOD) or glutathione peroxidase (GPx) (Yildiztekin et al. 2015). SOD is an enzyme in the first line of defence against ROS, that catalyzes the conversion of the superoxide radical (O_2-) into hydrogen peroxide (H_2O_2) and oxygen (O_2), thereby, preventing the formation of highly reactive hydroxyl radicals. Further, Catalase (CAT) enzyme catalyzes the decomposition of hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2) and prevents the accumulation of H_2O_2, which can lead to the formation of hydroxyl radicals (Chakrabarti et al., 2015). Glutathione-S-transferases (GSTs) exhibit peroxidase activity, have a role in detoxification processes which thereby contributes in ROS defence. Furthermore, Lipid peroxidase (LPO) is an enzymatic antioxidant that plays a crucial role in the detoxification of reactive oxygen species (ROS) and the increased activity indicate its potential involvement in the detoxification of hydrogen peroxide (H_2O_2) during oxidative stress produced by pesticides and is well explored (Qiao et al., 2019). Oxidoreductase enzyme activity indicates pesticide toxicity and tolerance.

Cytochrome P_{450} (CYP_{450}) has been described to have an essential role in insecticide metabolism of insects (Suchail et al., 2004; Liu and Zhang, 2004) including biosynthesis, breakdown, and detoxification of xenobiotics, cellular metabolism and homeostasis (Hu et al., 2017; Manikandan and Nagini, 2018). These are a large family of enzymes found in various organisms, including insects. Among insects, several CYP450 families have been identified and some prominent ones include CYP4, CYP6, CYP9, CYP12, CYP345 (Mao et al., 2009). Furthermore, the identification and characterization of CYP450 enzymes in insects, including beetles, are ongoing research topics. Detoxification enzymes play a crucial role in the metabolism of insecticides and are prominently expressed in insects including pest scarab beetle species. Notably, the activation of CYP450 serves as a vital mechanism for the detoxification of neonicotinoids (Adesanya et al., 2018 and Cavallaro et al., 2022).

Histopathological alterations are linked to intricate biochemical and physiological reactions in response to any stressor. Histopathological parameters possess the potential to serve as biomarkers, particularly in the realm of evaluating health outcomes resulting from exposure to various chemical species. Notably, these parameters exhibit cellular disparities between control animals and those exposed to pesticides, thereby influencing tissue integrity (Dogan et al., 2021). Previous studies have documented the harmful impacts of Spinosad insecticide on non-target insects, including pollinators, predators, and parasitoids (Cisneros et al., 2002; Williams et al., 2003; Biondi et al., 2012). The study conducted by Lakshmi et al., (2010) provided evidence of the adverse impact of spinosad exposure on the survival and reproductive success of *Cyrtorhinus lividipennis* and *Tytthus parcives* (Heteroptera: Miridae). The histological, physiological, and behavioural impacts of imidacloprid in brain tissue are well explored by Martelli et al., (2020) in *Drosophila melanogaster*. Previous studies have documented histological and ultrastructural alterations in the midgut tissue of the ground beetle *Blaps polychresta* following exposure to nickel oxide nanoparticles (NiO-NPs) (El-Ashram et al., 2021). Moreover, it has been documented that permethrin has toxic properties that impede the survival of *Pediocactus nigrispinus*, while also causing histological and cytological changes in the midgut. This suggests that the cellular stress caused by this pesticide has the potential to disturb essential physiological processes, including digestion (Martínez et al., 2018). The study conducted by Vinha et al., (2021) has documented the impacts of deltamethrin on locomotion, respiration, feeding, and histological alterations in the midgut of *Spodoptera frugiperda* larvae.

The binding of the insecticide to receptors may trigger oxidative stress, reduce energy levels, and induce alterations in tissue histology. Studies till date have been focused on histology, biochemical, physiological or molecular mechanism on exposure of insecticides. So, we investigated the impacts of the deltamethrin on *D. gazella* to understand the mechanisms by which it impacts neuroendocrine aspect along with the histological alterations in the structure of brain, gut and gonads. Mode of action of deltamethrin being contact inhibition and ingestion, brain and gut are the major targets, which however affects the reproductive physiology in insects. However, there is a lacuna of studies linking the oxidative stress and alteration in histoarchitecture, particularly with reference to *D. gazella*. Hence, in the present study, an attempt was made to have an insight into toxic potential of deltamethrin, ROS dependent neurotoxicity along with the histoarchitectural alterations in brain, gut and gonads.

3.2 MATERIALS AND METHODOLOGY

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

Determination of LC_{50} Values

Preparation of deltamethrin

Deltamethrin was selected for the present study and was procured from Sigma Aldrich (45423, Saint Louis, USA). The IUPAC name is [(S)-Cyano-(3-phenoxyphenyl)-methyl] (1R, 3R)-3-(2,2-dibromoethenyl)-2,2-dimethyl-cyclopropane-1-carboxylate (**Table 3.1**). Stock solution of deltamethrin (0.1 mg/L) was prepared by dissolving it in acetone and was stored at room temperature.

Experimental Procedure for LC_{50} Determination and Behavioural Studies

The present study was designed to evaluate the effect of technical grade of deltamethrin (Sigma 45423-250MG, Deltamethrin Perstanal 250MG), on the dung beetles under controlled laboratory conditions. For this, stock solution of deltamethrin (1 mg/L) was prepared by dissolving it in acetone (25474, Sulab, India) and was stored at room temperature. A total of 150 dung beetles were used for this experiment (n=3). Following a day of acclimatization, for each test concentration, 250 g of dung was thoroughly mixed with test concentrations of 0.005, 0.01, 0.05, 0.5 and 1.0 ppm which included 10 individuals (5 Male + 5 Female), in contrast to the untreated control. The concentration range was selected from the studies conducted by Sands et al. (2018). For each concentration, a specific amount of deltamethrin was transferred to a 50 mL falcon tube (546041 PP, Tarsons, India) using a micropipette (4652050, Thermo Fisher Scientific, UK) and then diluted with acetone to a final volume of 10 mL. Prior to pouring, the mixture was properly blended and then gently added to the dung while continuously mixing. The dung was further swirled for an additional 2 minutes to ensure that the insecticide was evenly dispersed throughout. For the control batch, a precise amount of 10 mL of acetone was mixed with the dung in the exact same manner. Further, mortality rate was recorded for 48 hours and collected data was computed using probit analysis to find out LC_{50} value.

After obtaining the LC50 value, sub-lethal concentrations (1/20th, 1/10th, and 1/5th) of deltamethrin were selected as low dose (LD), medium dose (MD), and high dose (HD) for the sub acute studies (Tabebordbar et al., 2020). D. gazella were then exposed to the dung treated with sub-lethal concentrations (LD, MD, and HD), up to 10th, 20th, and 30th day of tunnelling to analyze the toxicity with respect to the control for further studies.

D. gazella exposed to the test concentrations (0.005, 0.01, 0.05, 0.5 and 1.0 ppm) for LC_{50} determination were simultaneously observed for the behavioural changes at the intervals of 24 and 48 hours (Dardiotis et al., 2023). Additionally, since the LC50 was obtained for 48 hours, the behavioural changes noted by visual observations were also recorded for the same duration.

Nervous system being the first target of action of insecticides, the brain tissues of D. gazella was dissected out after 10, 20 and 30 days of exposure to deltamethrin and further estimation were done to evaluate its toxic effects on the physiology and behaviour.

H_2DCF-DA Staining (Yu et al., 2016)

Principle

The 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) fluorescent probe reacts with several ROS including hydrogen peroxide, hydrogen radicals and peroxynitrite. The cell-permanent lipophilic H_2DCF-DA passively diffuses into cells and is retained in the intracellular level after cleavage by intracellular esterase. Upon oxidation by ROS, the non-fluorescent H_2DCF-DA is converted to the highly fluorescent hydrophilic 2', 7'-dichlorofluorescence (DCF). The fluorescence intensity of DCF is proportional to the amount of ROS produced by the cells.

Methodology

The production of intracellular ROS was measured by oxidation of DCF-DA. Prior to the use, H_2DCF-DA stock solution was prepared in dimethyl sulfoxide (DMSO) under sterile condition in laminar flow hood. First step was to prepare single cell suspension, for which the brain tissue was dissected out in ice-cold 1X PBS, followed by immediate transfer into a 1.5 mL centrifuge tube. The tissue was washed using 2 mL of antibiotic (streptomycin) mixed PBS solution for three times. After washing, 0.5 mL of Trypsin (0.05%) -EDTA (0.02%) solution was added and incubated for 10 mins. To stop any further enzymatic activity, equal volume of Hank's Balanced Salt Solution (HBSS) was added, and the brain tissue was passed several times through 100 µL pipette tube, followed by centrifugation at 3000 RPM for 3

mins. The supernatant was discarded and the pellet was resuspended in PBS and the solution was then passed through the 100 µm cell strainer (Hi-media, India), to eliminate any clump or undigested tissue. Then, the tubes were centrifuged again at 3000 RPM for 3 mins, followed by resuspension of the cell pellet in 500 µL 1X PBS. This gives a single cell suspension and the cells were then incubated with DCFH-DA dye at 37°C for 30 minutes to allow the diffusion of the fluorescent probe into the cells and its subsequent hydrolysis to non-fluorescence dichlorofluorescein (DCFH) under the action of intracellular esterase. Intracellular ROS generation was measured by fluorescence microscopy with the excitation and emission wavelengths set as 488 and 528 nm, respectively.

Antioxidant enzymes

Superoxide Dismutase (SOD) (Kakkar et al., 1984)

Principle:

In this assay, the formazan formed at the end of reaction which indicates presence of the enzyme. One unit of the enzyme activity is defined as the enzyme concentration required for inhibiting 50% of the optical density of chromogen formed in one minute at 560 nm under assay condition.

Methodology:

For the SOD assay, brain tissues of male and female *D. gazella* were dissected out on 10^{th}, 20^{th}, and 30^{th} day of exposure to sub lethal concentrations (LD, MD, and HD) washed with PBS in the tubes and then homogenized in cold normal saline which were subsequently centrifuged. The supernatant was discarded, 2 mL of 0.01% digitonin was added to the pellet to yield the enzyme extract. The tubes were incubated for 15 minutes, centrifuged at 3000 rpm and the supernatant was used as sample. In this procedure, control solution consisted of 2.4 mL phosphate buffer (0.052 M sodium pyrophosphate buffer at a pH 8.4 adjusted with 0.052 M (Na $H_2PO_4.H_2O$), 0.1 mL of freshly prepared phenazine methosulphate (PMS; 186 µM in double distilled water). Then, 0.3 mL of nitrate tetrazolium (NBT; 300 µM; freshly prepared in double distilled water) and 0.2 mL of enzyme was added prior to the addition of NADH in a total 3 mL of assay system. The reaction was stopped by the adding 1 mL of acetic acid exactly 90 seconds after the addition of NADH. Then 4 mL of n-Butanol was added to the tubes and shaken vigorously to extract the formazon. Subsequently, the tubes were centrifuged for 10 minutes at 2000 rpm and supernatant was used for the measurement of optical density at 560 nm against butanol on a visible spectrophotometer.

Calculation $\frac{OD\ OF\ Control}{O.D\ of\ Sample} \times accuracyfactor \times dilution \div STDEnzymeUnit$

- Where, accuracy factor = 1; dilution = 2.5; standard enzyme unit = 3.0
- Enzyme activity = x/mg protein
- The activity was expressed as units SOD/mg protein

Catalase (CAT) (Sinha, 1972)

Principle:

Dichromate in acetic acid reduces the chromic acetate when heated in the presence of H_2O_2 with the formation of per chromic acid intermediate. The chromium acetate is measured colorimetrically at 620nm.

Methodology:

The incubation mixture had total volume of 2 mL, which contained 0.1 mL of dilute homogenate, 1.0 mL of phosphate buffer and 0.4 mL distilled water to which 0.5 mL of H_2O_2 solution was left out in control tubes. Then, it was incubated for 1 min at 37˚C and the reaction was stopped by the addition of 2.0 mL potassium dichromate acetic acid reagent. The samples were kept in boiling water bath for 15 minutes, finally cooled and then absorbance was measured at 570 nm against control.

Calculation $\frac{Absorbance\ of\ sample\ X\ STD\ concentration(\mu mol)}{Enzyme\ X\ Std\ Absorbance \times protein\ (\frac{mg}{ml})}$

Reduced Glutathione (GSH) (Ellman, 1959)

Principle:

GSH is a non proeinsulfyhydryl compound which contains a free thiol group. 5, 5' – dithiobis- (2- nitrobenzoic acid) (DTNB) is a disulfide compound, which is readily reduced by sulfhydryl compound forming a highly yellow coloured anion, read at 412 nm. The absorbance is proportional to amount of GSH.

Methodology:

Cells were homogenized in 3 mL phosphate buffer and precipitated with 2 mL of 10% TCA. Following to the centrifugation, 1 mL of supernatant was taken to which 0.5 mL of Ellman's reagent (19.8 mg of 5, 5'- dithiobis-(2- nitrobenzoic acid) (DTNB) in 100 mL of 10% sodium citrate) and 3 mL of phosphate buffer was added. Blank was run without homogenate. Mixture was incubated at 37˚C for 30 min and absorbance was measured at 412 nm. GSH standards were treated in a similar way and the colour developed was read at 412 nm to make

a linear plot. Absorbance of unknown samples was plotted against concentration with respect to standards.

Lipid Peroxidation (LPO) Thiobarbituric Acid Reactive Species Assay (TBARS) (Ohkawa et al., (1979)

Principle:

This assay is based on the appearance of a red chromophore that absorbs at 532 nm following the reaction of Thiobarbituric acid (TBA) with malonyl dialdehyde (MDA) and other breakdown products of lipid peroxidase which together called as thiobarbituric acid reactive substances (TBARS).

Methodology:

To make 10% homogenate, brain tissue was washed several times with 0.1 M phosphate buffered saline (PBS) (pH 7.4) and homogenized. Then, in the tubes with mixture of solutions containing 0.2 mL of 8.1% sodium dodecyl sulphate (SDS), 1.5 mL of 20% acetic acid and 1.5 mL of 1% Thiobarbituric acid (TBA), 0.2 mL of 10% homogenate was added. The blank was prepared for each sample by replacing TBA solution with distilled water. The solution was mixed and heated in a water bath at 95˚C for 60 mins. The tubes were then immediately cooled and 2 mL of aliquot was transferred to a centrifuge tube in which an equal volume of 10% TCA was added. The solution was mixed and centrifuged at 1000 RPM for 15 mins. The aliquot of the resulting supernatant fraction was read against blank on Spectrophotometer at wavelength of 532 nm.

Concentration of MDA = $\frac{O.D\ of\ sample \times dilution \times 10^9}{Control\ factor\ (1.56 \times 10^5) \times 10^6 cells}$

Estimating the CYP Gene Expressions

In the present study, the gene expression of CYP was 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 exposure to sub lethal doses of deltamethrin. The tissue (50-100 mg) was weighed and homogenized in 500µL Trizol reagent (Invitrogen). For complete dissociation of nucleoprotein complexes, samples were incubated for 5 minutes at room temperature. The incubation was followed by the addition of 100µL chloroform and was vigorously shaken for effective mixing of both the solutions. The samples were kept at room temperature for 5 minutes till the aqueous and organic layers were distinct. Thereafter,

the tubes were subjected to centrifugation at 12,000 RPM for 15 minutes at 4°C. The mixture got separated into a lower red phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. An aliquot of upper aqueous phase was then transferred into a new 1.5 mL micro centrifuge tube. Precipitation was done by adding 500µL of isopropanol to the supernatant that was transferred. The samples were kept in room temperature for 10 minutes, centrifuged at 12,000 RPM for 15 minutes at 4°C. After precipitation the supernatant was discarded without disturbing the pellet and was washed in 500µL of 75% ethanol and then 500µL absolute ethanol was added to the pellet. Effective mixing was done by gentle inversion and was further subjected to centrifugation at 7,500 RPM for 5 minutes at 4°C. The pellet was resuspended by adding 40 µL of DEPC water (Diethylpyrocarbonate), was quantified spectrophotometrically at 260nm using NanodropC and was stored in -20º C.

cDNA Synthesis

First strand of cDNA was synthesized from each sample using Thermo Scientific Verso cDNA Synthesis Kit (AB-1453/A). Verso Reverse Transcriptase Verso is an RNA-dependent DNA polymerase with a significantly attenuated RNase H activity. Verso can synthesize long cDNA strands, up to 11 kb, at a temperature range of 42°C to 57°C. In reaction, 1 µg RNA was used as a template for cDNA synthesis using oligodT primers. The volume of each component was for a 20 µL final reaction (**Table 3.2**). The reaction mix is mentioned in the table below.

Table 3.2: PCR Reaction Mixture

Components	Volume
5X cDNA Synthesis Buffer	4 µL
dNTP Mix	2 µL
Anchored Oligo dT /Random Hexamers	1 µL
RT Enhancer	1 µL
Verso Enzyme Mix	1 µL
Template (RNA)	1-5 µL
Molecular Grade Nuclease Free Water	Up to 20µL
Total Volume	20 µL

After setting up reaction mix, samples were kept in thermo cycler in following conditions

PCR conditions

Table 3.3: Reverse transcription cycling program for cDNA synthesis

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

RT-PCR Amplification

Quantitative RT-PCR was performed using PowerUp SYBR Green Master Mix (A25741, Applied Biosystems, USA) in Quant Studio 12K (Life technology) FAST real-time PCR. The primers were designed for CYP P40 isoforms of Phase I metabolic reactions - cyp4q4, cyp6bq9, and cyp4g7 (**Table 3.4, 3.5, 3.6**). The melting curve of each sample was measured to ensure the specificity of the products. β-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 3.4: Real Time PCR Mix

Components	**Volume (10 µL/well)**
PowerUp SYBR Green Master Mix (2X)	5 µL
Forward Primer (10µM)	0.5 µL
Reverse Primer (10µM)	0.5 µL
DNA Template	1 µL
Molecular Grade Nuclease Free Water	3 µL
Total	10 µL

Table 3.5: Real Time PCR Conditions

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

Table 3.6: Real Time PCR Primer Sequences of Neurotransmitter Synthesizing Enzymes

Serial No.	Gene Name	Accession No.	Primer type	Sequence	T_m (˚C)
1	cyp4q4	NM_001039440.1	Forward	TCATGCGAACCCCAGAACAA	59.89
			Reverse	GATTAACGCCCCCGTTACCA	60.11
2	cyp6bq9	NM_001190793.1	Forward	CGCGGTTTAGTCGCTGATG	59
			Reverse	GAAAGCTTGTGCTGCGAGTT	59
3	cyp4g7	NM_001114388.1	Forward	CTTCGGGACGATTTGGACGA	60.11
			Reverse	TCGCCTCGACCATGAAATCC	60.18

Oraganosomatic Index and Histology

Organosomatic Indices

Organ somatic indices of brain, gut, and gonads were calculated for 5 individuals. To get the organ weight to the body weight ratios of dung beetles as follows: weight of dung beetle/weight of the organ x100 (Dogan and Can, 2011).

- **BSI**: $\frac{Brain\ weight}{Body\ weight}$ x 100
- **GSI**: $\frac{Gut\ weight}{Body\ weight}$ x 100
- **GoSI**: $\frac{Gonad\ weight}{Body\ weight}$ x 100

Histology

Histology serves as one of the vital parameter to validate the toxic effect of any xenobiotic. So, to evaluate the toxic effect(s) of sub lethal concentrations of deltamethrin on D. gazella, the organs including brain, gut, and gonads of 360 individuals (180 males + 180 females) were dissected out in the insect saline solution (0.13 M NaCl; 0.01 M Na2HPO4; 0.02 M; KH2PO4; pH 7.2), post exposure. Fresh tissues were fixed in 4% paraformaldehyde for 24 hours, dehydrated, embedded in paraffin wax, and sectioned at 5 µm, then stained with hematoxylin and eosin and examined under light microscope (Metzler M, India) (Gutiérrez et al., 2016; Martínez et al., 2018).

Data Analysis

Statistical analysis was done using Graphpad prism 9.0 software by 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 *$p<0.05$, ** $p<0.01$, *** $p<0.001$.

3.3 RESULTS

LC_{50} value of deltamethrin was determined using Probit Analysis after 48 hours of exposure of dung beetles. The 50% probit mortality ranged between 0.05 to 0.5 ppm concentrations (**Table 3.7**). LC_{50} value was obtained as 0.275 ppm (R^2 = 0.86, df = 1, *P < 0.01*) from the dose response curve (**Fig. 3.1**). 95% confidence interval was found between -0.945 to 1.495 ppm. 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} (**Table 3.8)** were used to understand the toxic potential of deltamethrin on *D. gazella*

Table 3.7: Probit Mortality Obtained After 48 Hours of Exposure to deltamethrin.

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

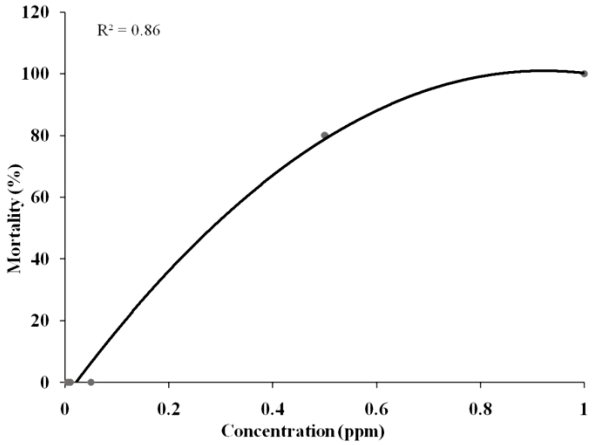


Figure 3.1: Dose response curve for the LC_{50} determination of deltamethrin after 48 hours of exposure.

Table 3.8: LC_{50} Value Obtained and the Sub-Lethal Doses Selected for Further Studies.

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

Behavioural alteration was observed for acute toxic evaluation of deltamethrin where the maximum behavioural alteration was reported at higher concentration (1.0 ppm) and minimum was reported at lowest concentration (0.005 ppm). Among the behavioural alterations, loss of equilibrium and integument darkening was found maximum at highest concentration followed by 0.5 ppm concentration. Together with this, jerky movements, remarkable immobility were also reported in tested male and female dung beetles. Moreover, tremor was recorded to be moderate at highest concentration (1.0 ppm) followed by 0.5 ppm (**Table 3.9**).

Table 3.9: Marked behaviour of *D. gazella* after 48 hours of exposure to Deltamethrin. Here, *(-)→normal movement, (+)→ mild, (++)→ moderate, (+++)→maximum behavioural changes*

Sr. No.	Behaviour	Concentration (ppm)				
		0.005	**0.01**	**0.05**	**0.5**	**1.0**
1	Jerky movements	-	+	+	++	+++
2	Loss of Equilibrium	-	-	-	++	+++
3	Tremor	-	-	+	+	++
4	Immobility	-	-	-	++	+++
5	Integument darkening	-	-	+	++	+++

H_2DCF-DA Staining

The present study suggests that the exposure to test chemical resulted in alteration in the normal cellular activity which was estimated qualitatively as well as quantitatively. A qualitative biochemical assay that was performed using DCFH-DA dye showed significant increase in the level of intracellular ROS in the neurosecretory cells of *D. gazella* by the fluorescent staining, after being exposed to LD, MD, HD of deltamethrin in comparison to that of control (**Fig. 3.2 a & b**). Further, results suggest that deltamethrin emits relatively brighter green fluorescence for HD, which specifies more level of ROS generation (**Fig. 3.3 & 3.4**). This clearly indicates that deltamethrin plays a major role in generating higher cytotoxic impacts. Moreover, to confirm the ROS which plays a key role in initiating apoptosis, the SOD, CAT, GSH and LPO Activity were also measured for *D. gazella* after exposure to test chemical.

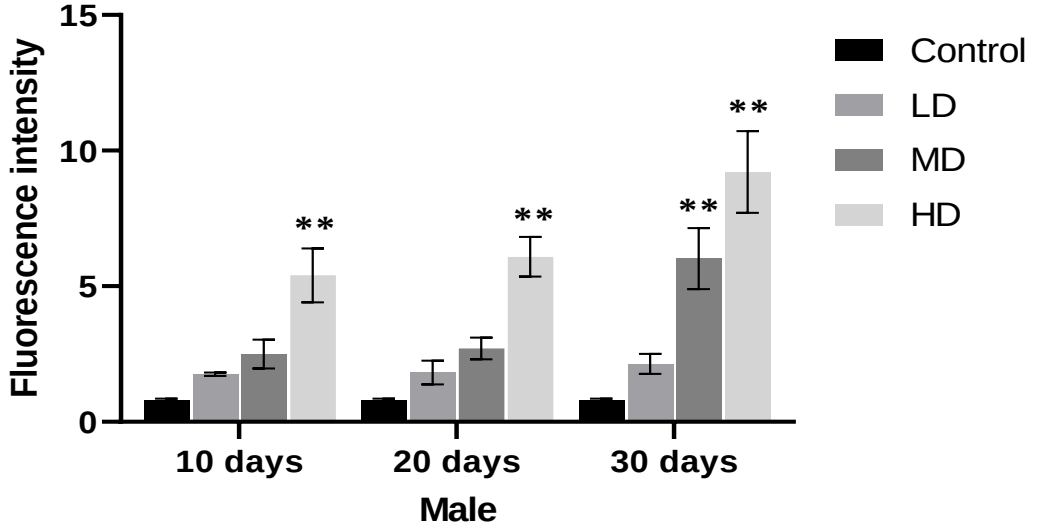


Figure 3.2a: Fluorescence intensity (Mean±SEM) of the DCFHDA staining, in the single cell suspension of the brain tissue of male *D. gazella* after exposure to sub-lethal doses (LD, MD, and HD) of deltamethrin, in comparison to control. *(p<0.5*, p<0.01**).*

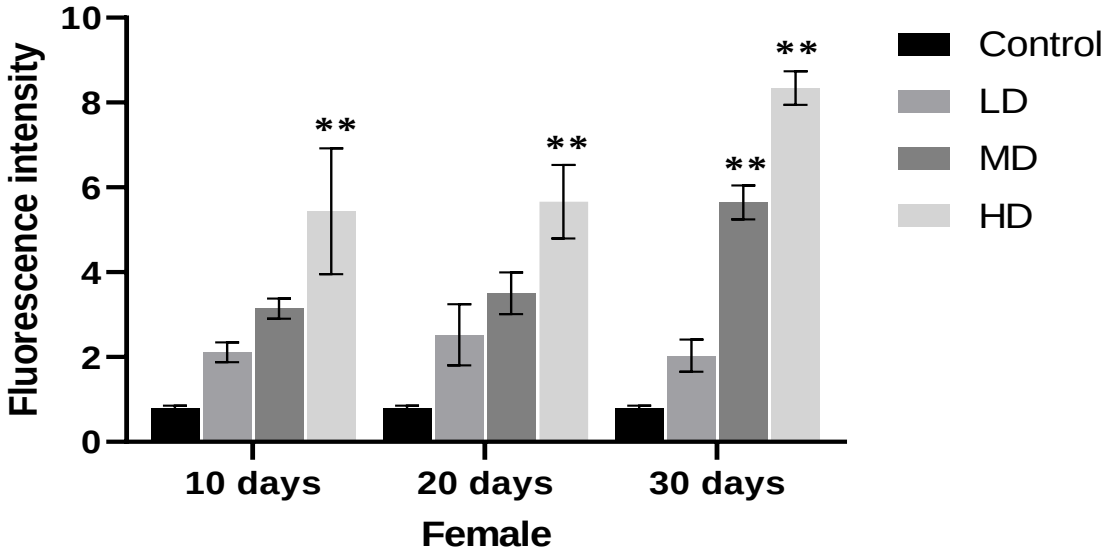


Figure 3.2b: Fluorescence intensity (Mean±SEM) of the DCFHDA staining, in the single cell suspension of the brain tissue of female *D. gazella* after exposure to sub-lethal doses (LD, MD, and HD) of deltamethrin, in comparison to control. *(p<0.5*, p<0.01**).*

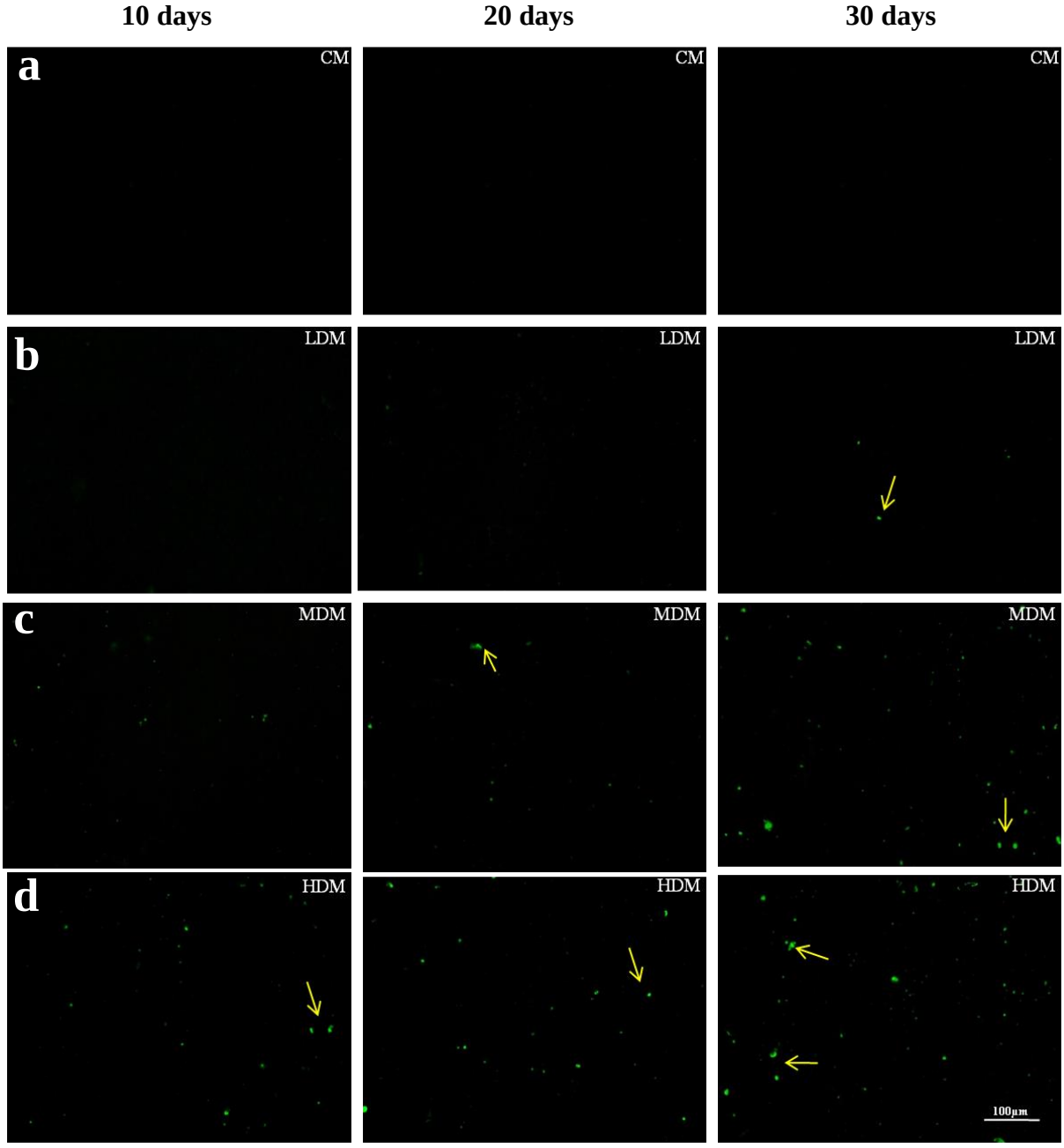


Figure 3.3: Effects of sub-lethal concentrations of deltamethrin in comparison to control, for the generation of ROS in single cell suspension of brain tissue of male *D. gazella* were determined by DCFH-DA staining (10X, scale = 100µm). (a) Control (b) Exposure to LD (0.14 ppm) (c) Exposure to MD (0.28 ppm) (d) Exposure to HD (0.55 ppm), after 10^{th}, 20^{th} and 30^{th} day are given. Green fluorescence represents the intracellular ROS. Here, the yellow arrow indicates a bright green nucleus with increased ROS level, cell membrane blebbing, and nuclear fragmentation, with maximum increase in ROS production, after 30 days of exposure to HD.

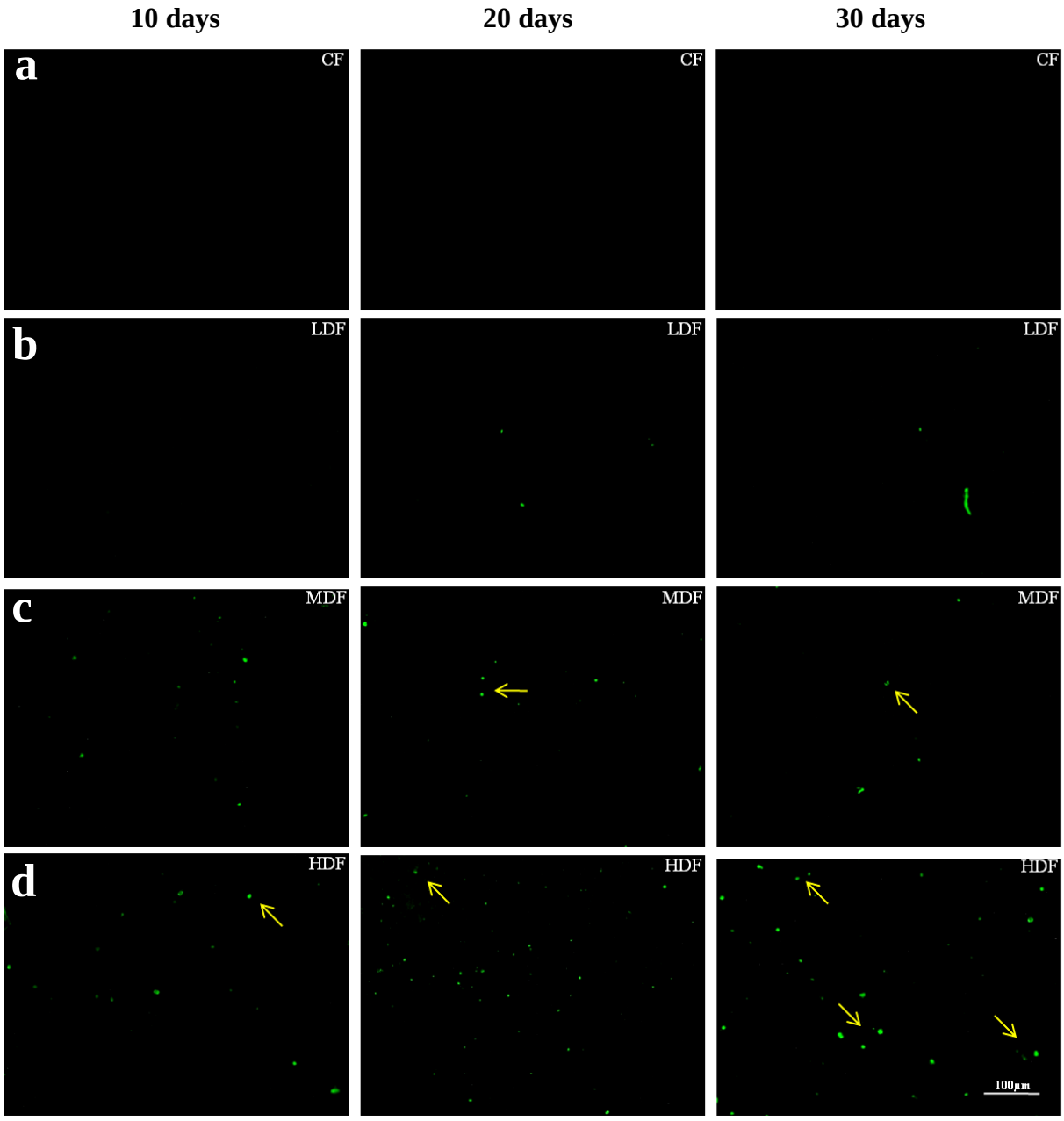


Figure 3.4: Effects of sub-lethal concentrations of deltamethrin in comparison to control, for the generation of ROS in single cell suspension of brain tissue of female *D. gazella* were determined by DCFH-DA staining (10X, scale = 100µm). (a) Control (b) Exposure to LD (0.14ppm) (c) Exposure to MD (0.28ppm) (d) Exposure to HD (0.55ppm), after 10^{th}, 20^{th} and 30^{th} day are given. Green fluorescence represents the intracellular ROS. Here, the yellow arrow indicates a bright green nucleus with increased ROS level, cell membrane blebbing, and nuclear fragmentation, with maximum increase in ROS production, after 30 days of exposure to HD.

Antioxidant Activity

The quantitative estimation of ROS was carried out to measure the amount of cellular damage caused by ROS as well as enhanced antioxidant defence mechanism. As observed in the figure, the antioxidant enzymes were found to decline with the increasing sub-lethal concentrations of deltamethrin. The results of enzymatic (SOD and GSH) and non-enzymatic antioxidant activity (GSH and LPO) are represented in **Table 3.10**. A significant ($p<0.01$) decrease in SOD, CAT, and GSH activity for the beetles exposed to deltamethrin as compared to control after 10^{th}, 20^{th} and 30^{th} day, suggesting amplified oxidative stress and increased ROS production (**Fig. 3.5, 3.6 & 3.7**). Lipid peroxides, product of oxidative damaged lipid resulting from lipid peroxidation are induced by ROS whose quantification of thiobarbituric acid represents the extent of oxidative damage. The amount of LPO was measured for the beetles treated with sub lethal concentrations of deltamethrin (**Fig. 3.8**). The results show significant increase of LPO levels for *D. gazella* exposed to sub-lethal doses of Deltamethrin, as compared to that of control after 10^{th}, 20^{th}, and 30^{th} days, suggesting increased oxidative stress.

Thus, from the results obtained it can be conferred that deltamethrin is responsible for generation of oxidative stress. Moreover, high dose was found to be most toxic dose and is involved in major production of ROS and eventually carry out cellular damage such as LPO and the activation of antioxidant defence mechanisms.

Table 3.10: Values obtained for the level of SOD, CAT, GSH and LPO after exposure to the sub-lethal concentrations (LD, MD, and HD) of deltamethrin in comparison to control, after 10th, 20th, and 30th day in both male and female *D. gazella*. (*p<0.5*, p<0.05***).

Days	Individuals	Doses	SOD (µmol/mg)	CAT (µmol/mg)	GSH (µmol/mg)	LPO (µmol/mg)
10 Days	Male	Control	1.136±0.005	13.874±0.0063	0.136±0.004	0.478±0.001
		LD	0.905±0.005**	10.564±0.0006**	0.113±0.001**	0.619±0.002**
		MD	0.896±0.0088**	9.156±0.0035**	0.103±0.001**	0.717±0.002**
		HD	0.801±0.0010**	8.267±0.0013**	0.099±0.001**	0.830±0.003**
	Female	Control	1.277±0.0019	13.695±0.0083	0.129±0.001	0.5±0.003
		LD	0.954±0.0034**	11.699±0.0006**	0.112±0.002**	0.649±0.004**
		MD	0.862±0.0069**	9.865±0.0020**	0.105±0.0005**	0.713±0.003**
		HD	0.847±0.0019**	8.535±0.0006**	0.092±0.0013**	0.799±0.002**
20 Days	Male	Control	1.0869±0.0018	15.742±0.0034	0.133±0.001	0.589±0.003
		LD	1.0504±0.0018**	11.687±0.0025**	0.131±0.0008**	0.722±0.002**
		MD	0.980±0.0033**	10.286±0.0012**	0.112±0.001**	0.835±0.004**
		HD	0.862±0.0069**	9.834±0.0041**	0.101±0.001**	0.882±0.002**
	Female	Control	1.433±0.0088	15.830±0.0034	0.129±0.002	0.606±0.001
		LD	1.184±0.0017**	12.933±0.0011**	0.121±0.001**	0.730±0.002**
		MD	0.961±0.0023**	11.066±0.0021**	0.103±0.001**	0.760±0.005**
		HD	0.844±0.0083**	10.830±0.0016**	0.090±0.003**	0.867±0.007**
30 Days	Male	Control	1.25±0.0016	16.114±0.0159	0.133±0.001	0.679±0.002
		LD	1.126±0.0018**	13.865±0.0032**	0.131±0.0008**	0.777±0.002**
		MD	0.769±0.0010**	12.310±0.0029**	0.112±0.001**	0.850±0.002**
		HD	0.718±0.0045**	10.915±0.0025**	0.091±0.004**	0.961±0.004**
	Female	Control	1.308±0.0157	16.465±0.0155	0.129±0.006	0.683±0.002
		LD	1.096±0.0012**	14.638±0.0095**	0.123±0.008**	0.752±0.003**
		MD	0.992±0.0012**	13.315±0.0046**	0.116±0.002**	0.846±0.002**
		HD	0.759±0.0031**	9.7413±0.0013**	0.109±0.001**	0.884±0.001**

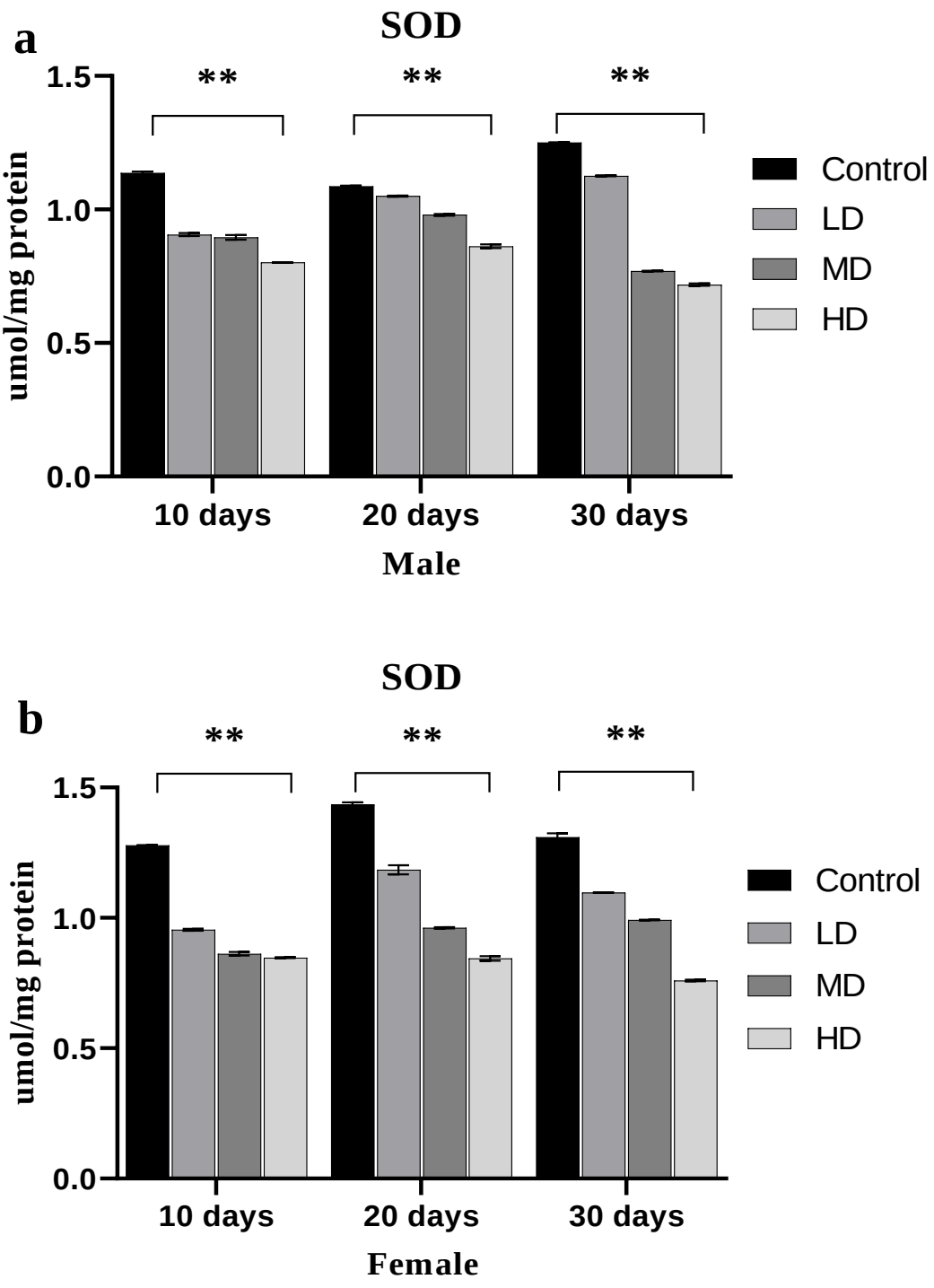


Figure 3.5: Superoxide dismutase (SOD) activity after exposure to sub-lethal concentrations of deltamethrin in comparison to control for 10th, 20th and 30th day (a) SOD activity in the brain tissue of male (b) SOD activity in the brain tissue of female. Each value represents Mean±SEM. *(p<0.5*, p<0.01**).*

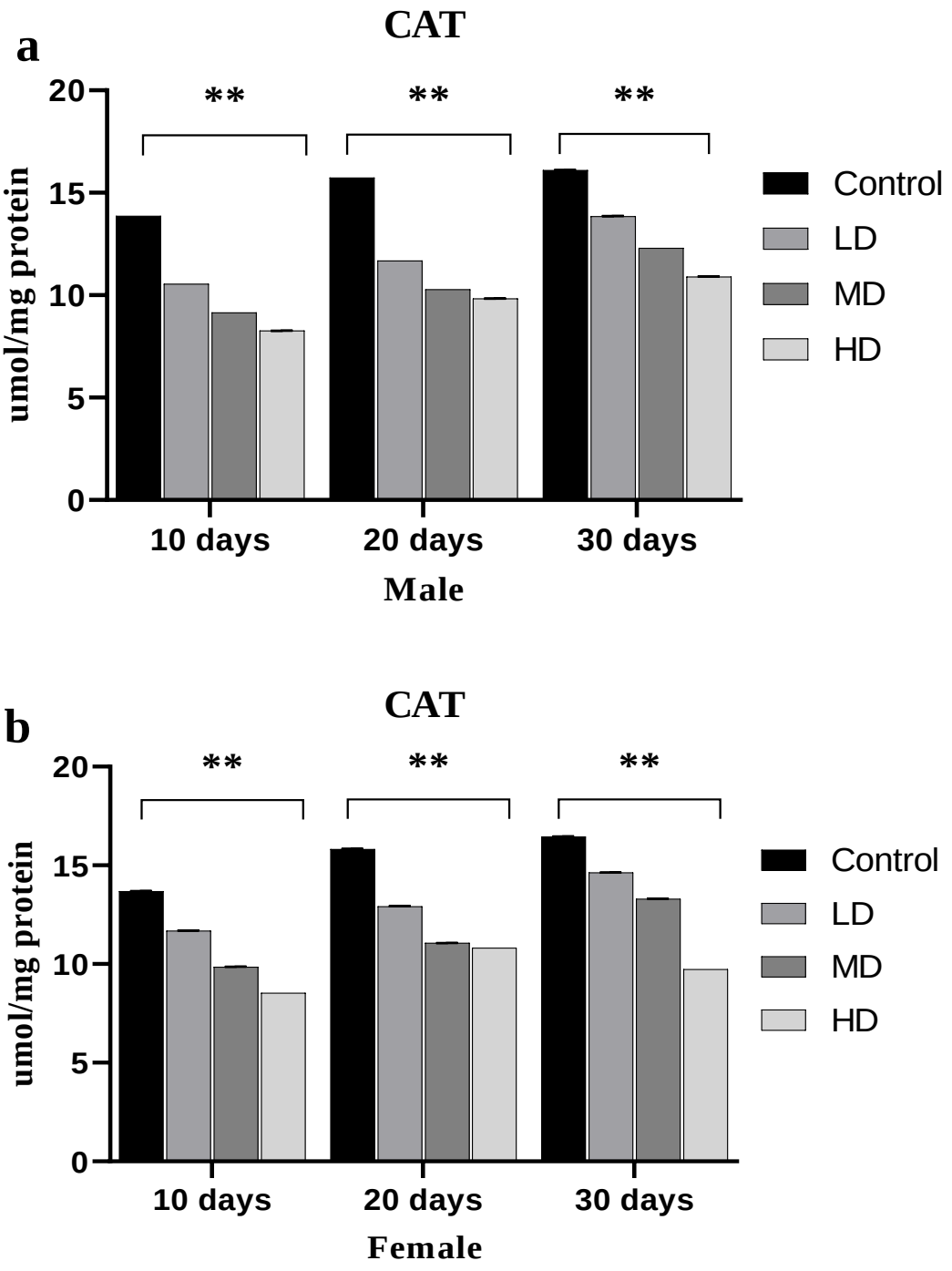


Figure 3.6: Catalase (CAT) activity after exposure to sub-lethal concentrations of deltamethrin in comparison to control for 10th, 20th and 30th days (a) CAT activity in the brain tissue of male (b) CAT activity in the brain tissue of female. Each value represents Mean±SEM. *($p<0.5$*, $p<0.01$**).*

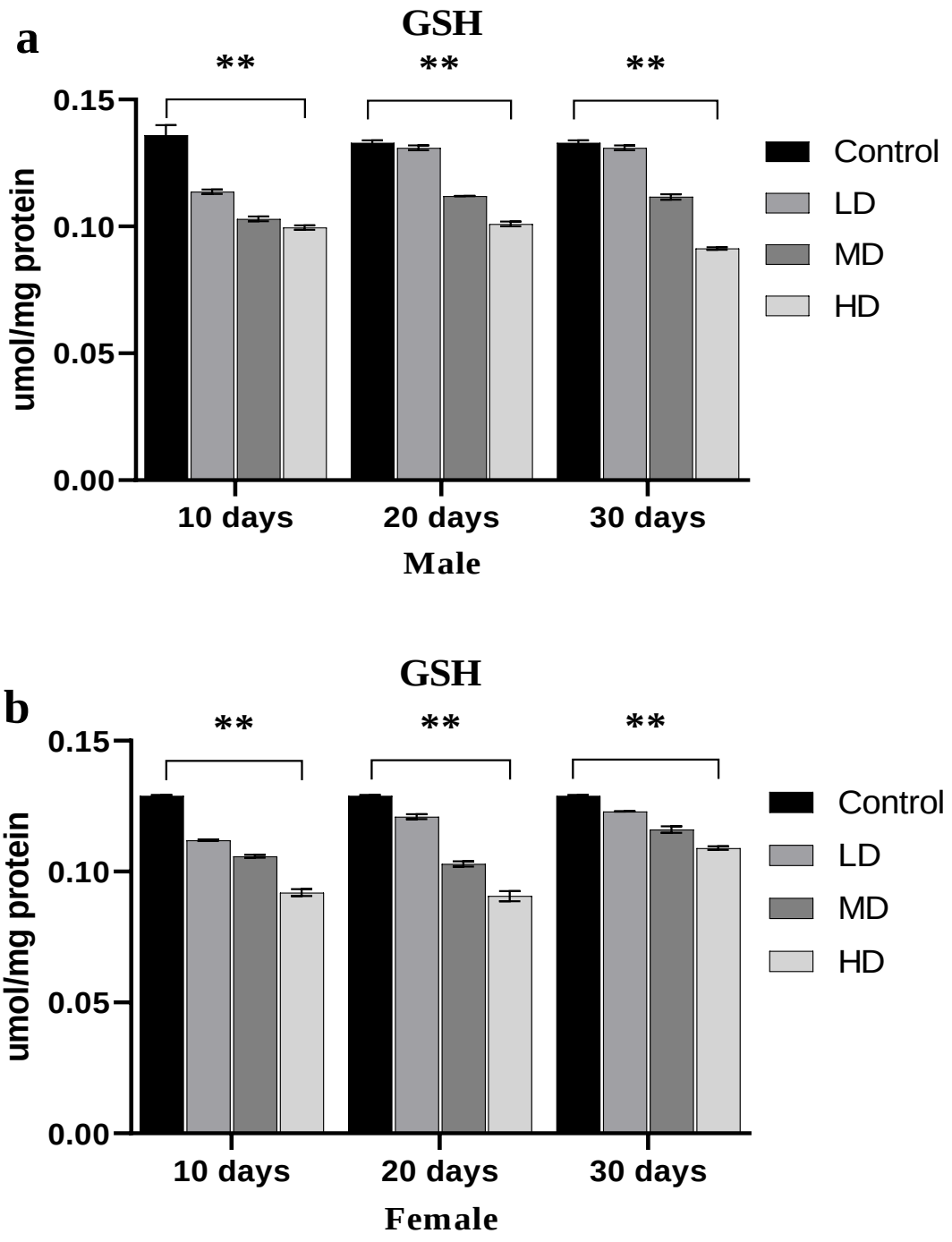


Figure 3.7: Reduced Glutathione (GSH) levels after exposure to sub-lethal concentrations of deltamethrin in comparison to control for 10, 20 and 30 days (a) GSH level in the brain tissue of male (b) GSH level in the brain tissue of female. Each value represents Mean±SEM. *($p<0.5$*, $p<0.01$**).*

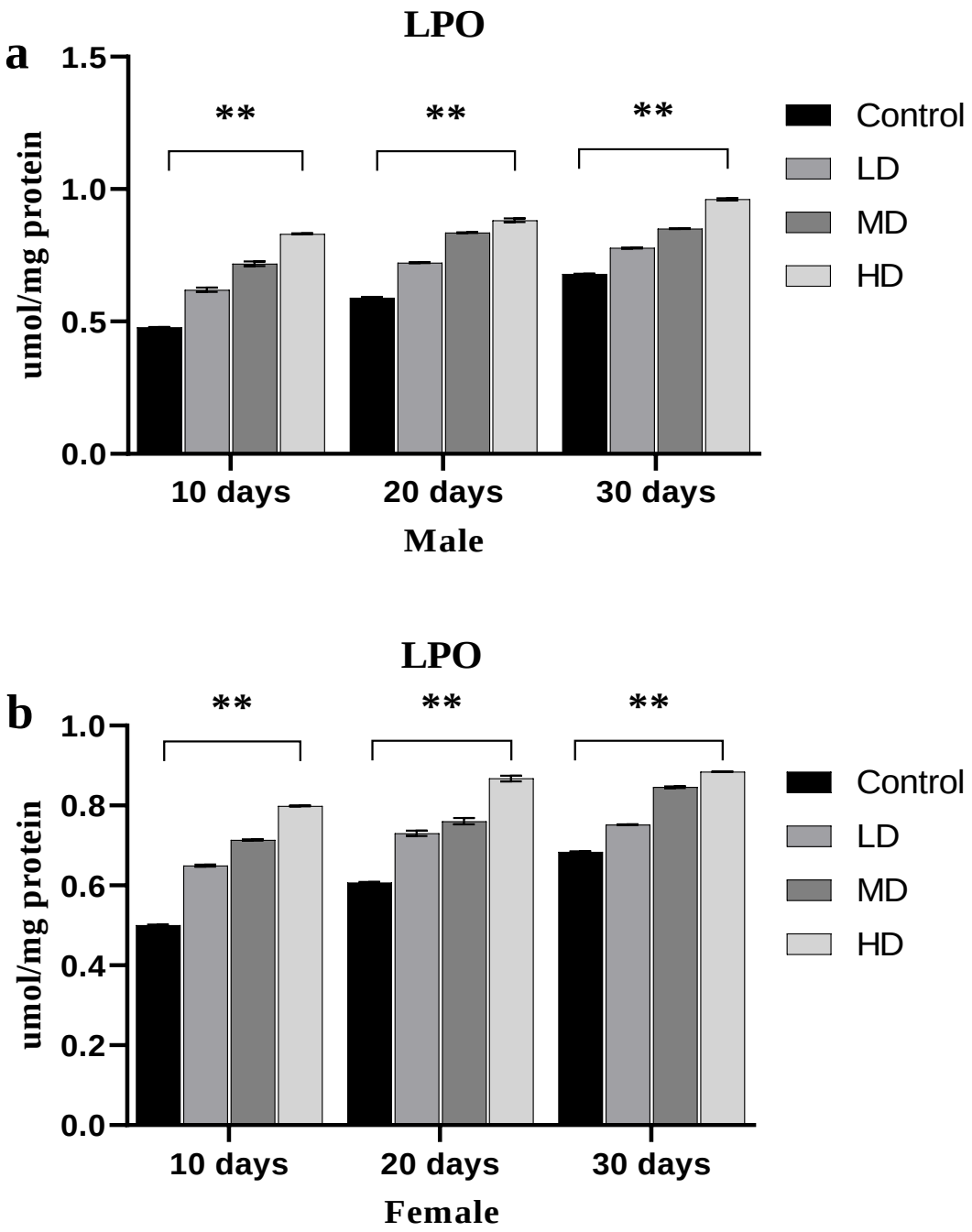


Figure 3.8: Lipid Peroxidase (LPO) levels after exposure to sub-lethal concentrations of deltamethrin in comparison to control for 10, 20 and 30 days. (a) LPO levels in the brain tissue of male (b) LPO levels in the brain tissue of female. Each value represents Mean±SEM. *($p<0.5$*, $p<0.01$**).*

CYP Gene Expression

CYP P_{450} enzymes play an important role in detoxification of xenobiotics induced toxicity. In the present study, three CYP gene expressions were carried out to understand the toxic potential of deltamethrin on *D. gazella*. Results revealed a significant ($p<0.01$) time and dose dependent decrease in the mRNA gene expression of cyp4q4 (**Fig. 3.9**), cyp6bq9 (**Fig. 3.10**), and cyp4g7 (**Fig. 3.11**), in both male and female *D. gazella* (**Table 3.11**), suggesting the increased oxidative stress and loss of ability to detoxify the toxicity induced by deltamethrin.

Table 3.11: CYP gene expression after exposure to the sub-lethal concentrations of deltamethrin in comparison to control, after 10, 20th, and 30th days in both male and female *D. gazella*. (*p<0.5*, p<0.05***)

Days	Individuals	Doses	cyp4q4	cyp6bq9	cyp4g7
10 Days	Male	Control	1±0.025	1±0.027	1±0.088
		LD	0.520±0.048	0.510±0.088**	0.472±0.188
		MD	0.420±0.080*	0.323±0.188*	0.391±0.188
		HD	0.397±0.048*	0.206±0.088**	0.207±0.187*
	Female	Control	1±0.025	1±0.027	1±0.188
		LD	0.620±0.088	0.551±0.088**	0.572±0.188
		MD	0.510±0.188*	0.353±0.188*	0.491±0.188
		HD	0.473±0.088*	0.110±0.088*	0.237±0.187*
20 Days	Male	Control	1±0.080	1±0.027	1±0.088
		LD	0.501±0.188	0.410±0.088	0.402±0.188
		MD	0.410±0.188*	0.241±0.008**	0.258±0.188*
		HD	0.328±0.150*	0.122±0.050**	0.122±0.050**
	Female	Control	1±0.080	1±0.027	1±0.188
		LD	0.602±0.088	0.450±0.088*	0.502±0.188
		MD	0.5±0.088*	0.201±0.088**	0.238±0.188*
		HD	0.418±0.050*	0.102±0.050*	0.122±0.050**
30 Days	Male	Control	1±0.027	1±0.027	1±0.088
		LD	0.473±0.188	0.381±0.188	0.320±0.050
		MD	0.362±0.088*	0.110±0.088**	0.137±0.088
		HD	0.295±0.080**	0.082±0.020**	0.102±0.050*
	Female	Control	1±0.027	1±0.027	1±0.188
		LD	0.571±0.188*	0.351±0.188	0.420±0.050
		MD	0.416±0.088*	0.118±0.088*	0.237±0.188
		HD	0.229±0.020**	0.089±0.020*	0.102±0.050*

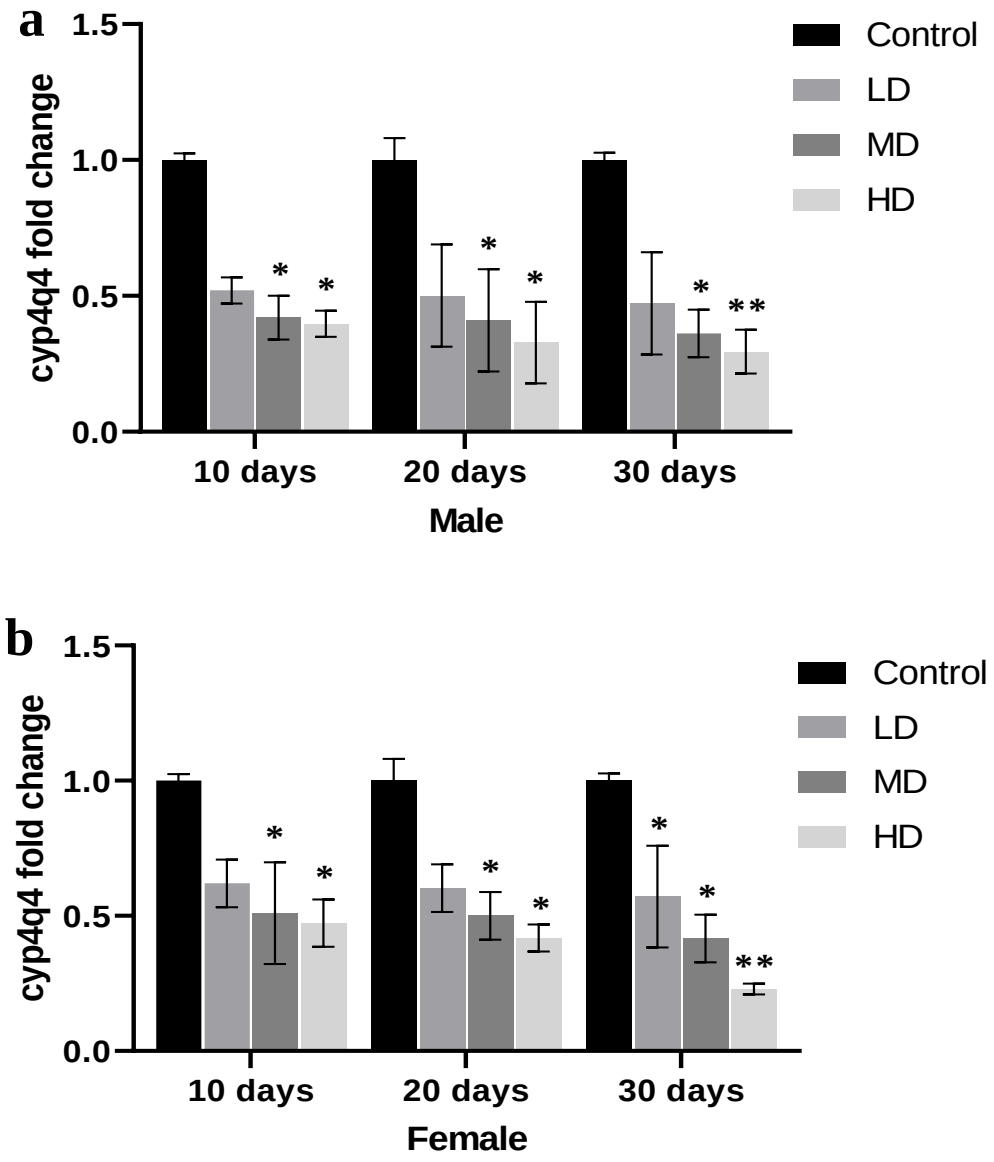


Figure 3.9: cyp4q4 mRNA gene expression after exposure of *D. gazella* to sub-lethal concentrations of deltamethrin in comparison to control for 10th, 20th and 30th day (a) cyp4q4 fold change in the brain tissue of male (b) cyp4q4 fold change in the brain tissue of female. Each value represents Mean±SEM. *(p<0.5*, p<0.01**).*

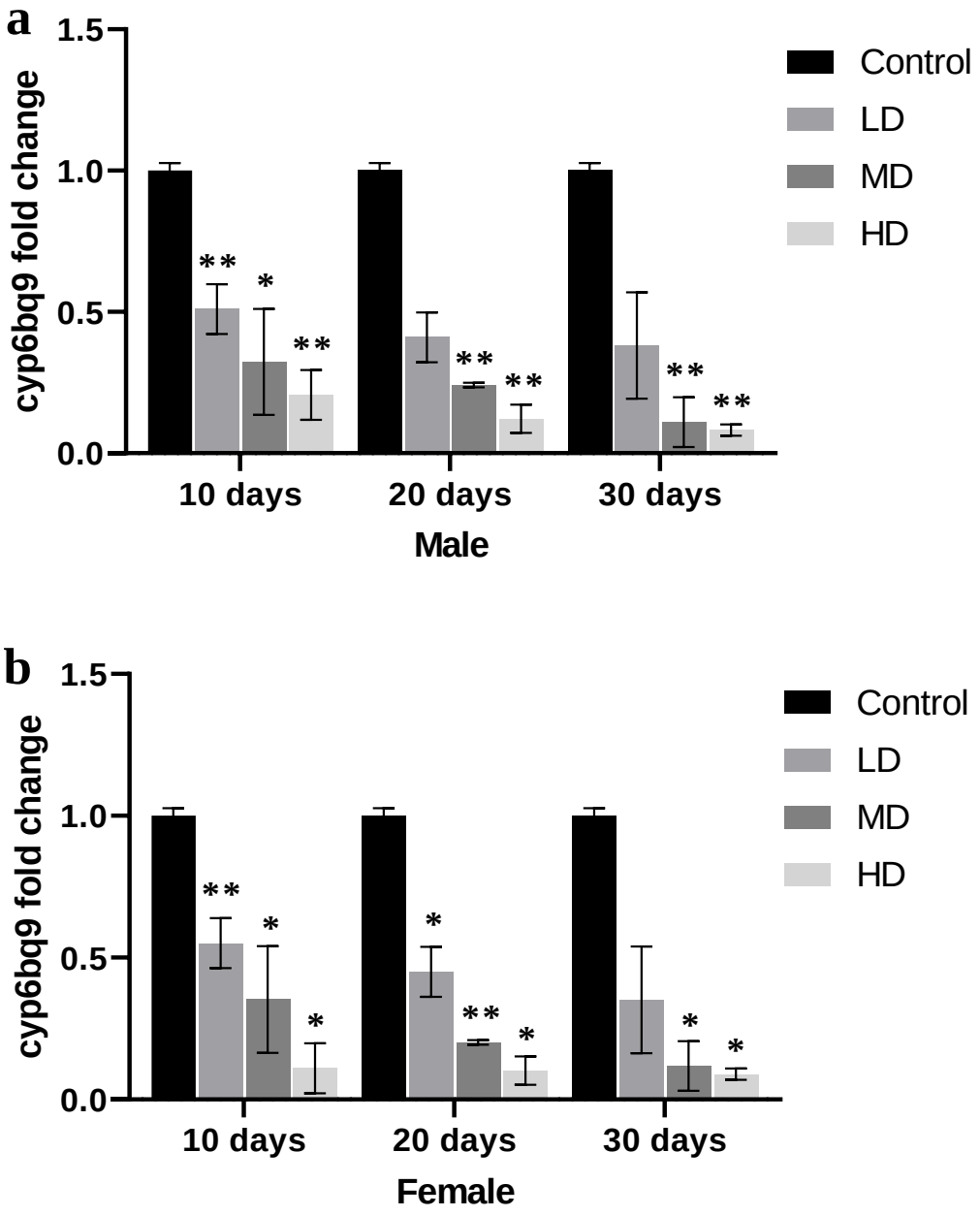


Figure 3.10: cyp6bq9 mRNA gene expression after exposure to sub-lethal concentrations of deltamethrin in comparison to control for 10, 20 and 30 days (a) cyp6bq9 fold change in the brain tissue of male (b) cyp6bq9 fold change in the brain tissue of female. Each value represents Mean±SEM. *(p<0.5*, p<0.01**).*

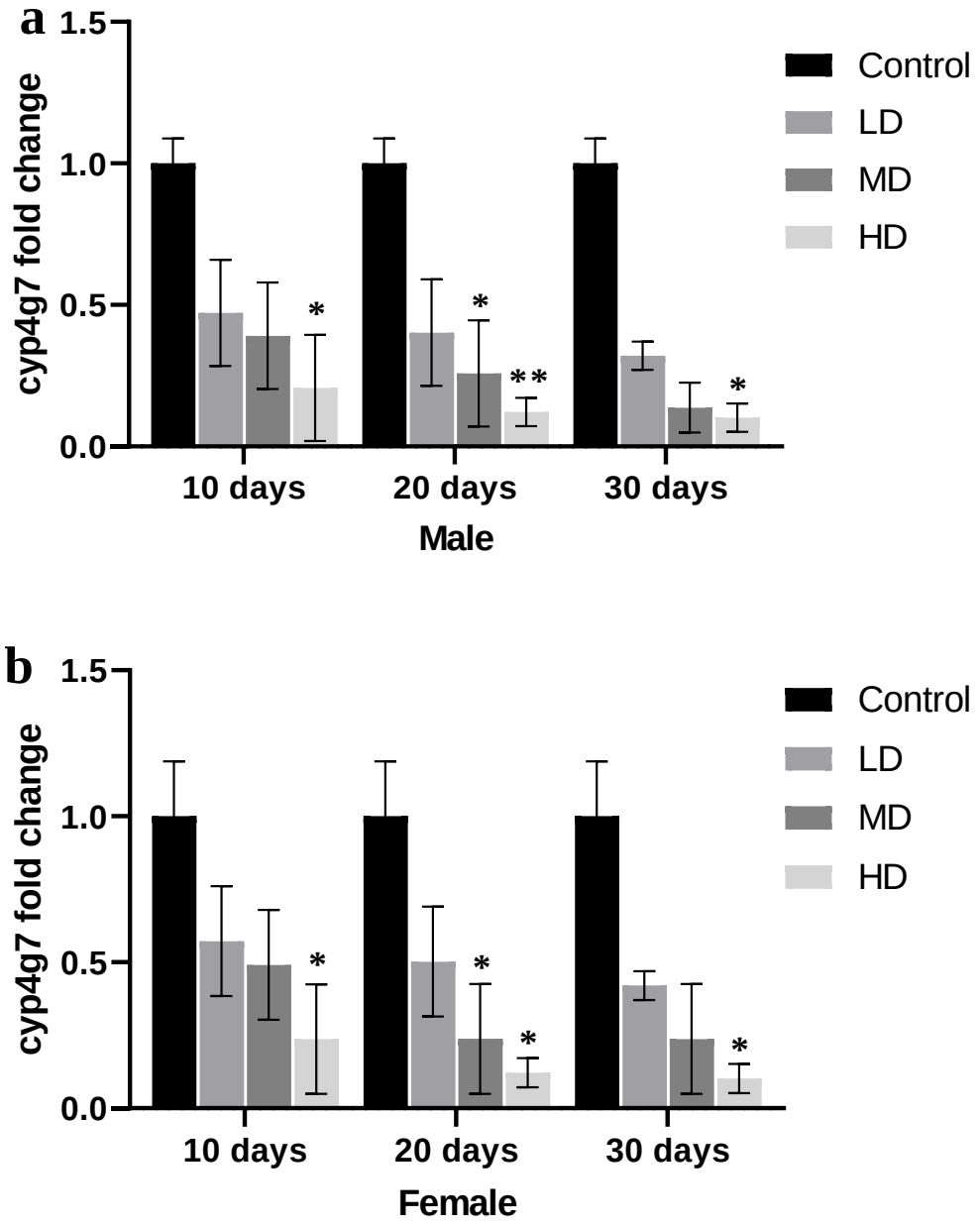


Figure 3.11: cyp4g7 mRNA gene expression after exposure to sub-lethal concentrations of deltamethrin in comparison to control for 10, 20 and 30 days (a) cyp4g7 fold change in the brain tissue of male (b) cyp4g7 fold change in the brain tissue of female. Each value represents Mean±SEM. *(p<0.5*, p<0.01**).*

Organosomatic Index

Organosomatic index gives an indication to the degree of damage. The results of brain somatic index (**Fig. 3.12a**) were found to reduce three times for HD in comparison to control after 10^{th}, 20^{th} and 30^{th} days of exposure. Similarly, the gut somatic index (**Fig. 13.2b**) was also observed to decline 10 times as compared to control. Moreover, the gonad somatic

index, for both male (testis) (**Fig. 3.12c**) and (ovary) (**Fig. 3.12d**) was also found to decrease 2 times as compared to control. Overall, a significant ($p<0.01$) decline in the organo-somatic indices of brain, gut, gonads, in a time and dose dependent manner are suggestive of the deltamethrin induced toxicity even at the sub-lethal concentrations.

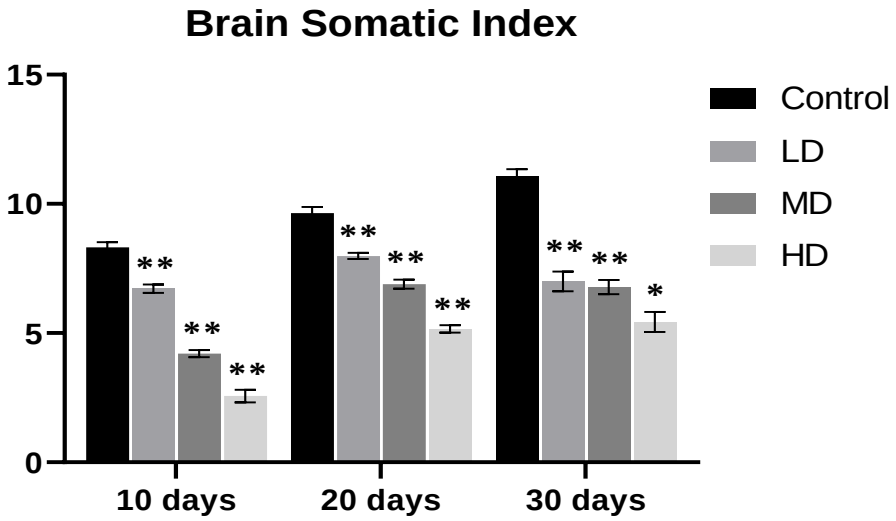


Figure 3.12a: Brain somatic index of *D. gazella* after exposure to sub-lethal concentrations of deltamethrin in comparison to control for 10, 20 and 30 days. Each value represents Mean±SEM. *(p<0.5*, p<0.01**).*

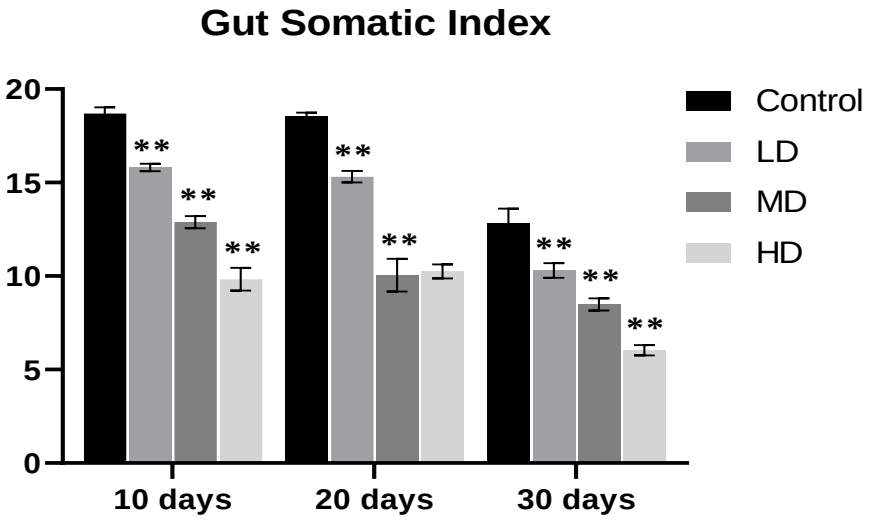


Figure 3.12b: Gut somatic index of *D. gazella* after exposure to sub-lethal concentrations of deltamethrin in comparison to control for 10, 20 and 30 days. Each value represents Mean±SEM. *(p<0.5*, p<0.01**).*

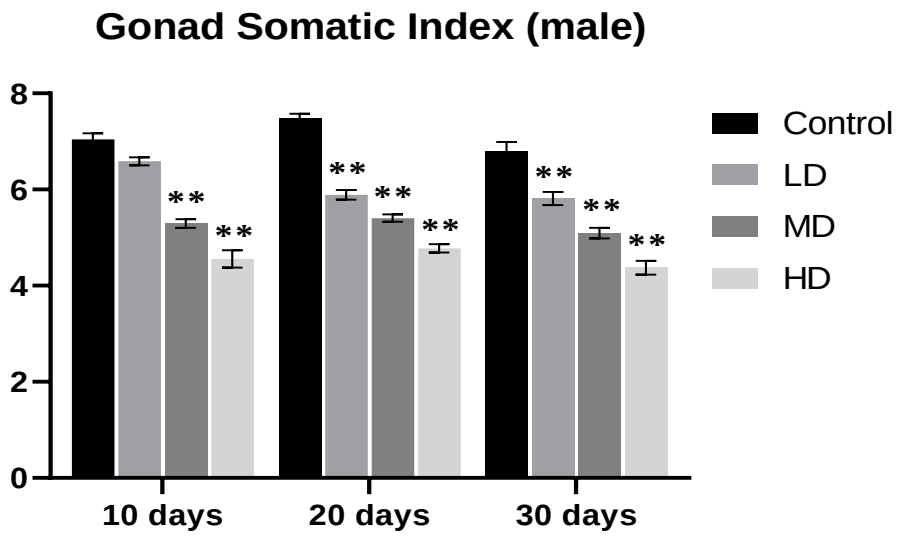


Figure 3.12c: Gonad somatic index (male) of *D. gazella* after exposure to sub-lethal concentrations of deltamethrin in comparison to control for 10, 20 and 30 days. Each value represents Mean±SEM. *(p<0.5*, p<0.01**).*

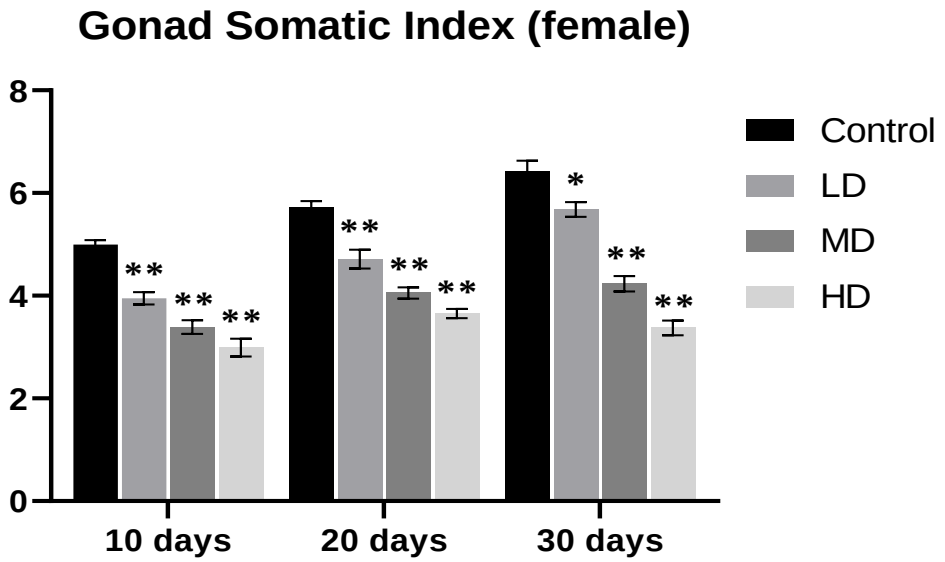


Figure 3.12d: Gonad somatic index (female) of *D. gazella* after exposure to sub-lethal concentrations of deltamethrin in comparison to control for 10, 20 and 30 days. Each value represents Mean±SEM. *(p<0.5*, p<0.01**).*

Histological Evaluation

To confirm the alterations in the organosomatic indices, the histological studies of brain, gut, and gonads of both the sexes of *D. gazella* was done after 10^{th}, 20^{th} and 30^{th} days of exposure to deltamethrin.

The brain of *D. gazella* consisted of well-defined regions, showing neuronal cell bodies (cb) in the periphery and condensed neuropiles (ne) in the central region of the brain (**Fig. 3.13a**). The presence of several pyknotic nuclei (p) was the only difference found in the brains of *D. gazella* exposed to deltamethrin (**Fig 3.13 b, c, d**). However, vacuolation (v) in the neuronal cell bodies was observed in the beetles exposed to LD, MD, and HD of deltamethrin, which increase after 10^{th}, 20^{th} and 30^{th} days of exposure (**Table 3.12**).

The alimentary canal of insects comprises structurally of three distinct regions: the foregut, midgut, and hindgut, the midgut possesses no cuticular intima (Chapman, 1998). Histologically, the normal midgut consists of a unicellular layer (epithelium) resting upon a basement membrane. This membrane is surrounded externally by circular and then by longitudinal muscle fibers. The epithelium consists of secretory (columnar) (sc), clusters of small regenerative cells (rc), each of which contains a relatively large nucleus and strongly basophilic cytoplasm. The epithelium is also protected from the food particles by a detached sheath, peritrophic membrane (pm), surrounding lumen (lm). Also, the normal midgut appears with luminal surface of the epithelium, which is provided with a striated border constituting long microvillus (**Fig. 3.14a**). deltamethrin exposed *D. gazella* showed several histological modifications in a dose dependent manner in the gut, after 10^{th}, 20^{th}, and 30^{th} day (**Fig. 13.4 b, c, d**). Alterations such as the disruption of epithelial cells causing its break off, ruptured peritrophic membrane (pm) and separation of the muscular layer (ml), leading to formation of spaces (sp) between the epithelial layer and the muscle layer was observed for LD, MD, and HD (**Table 3.13**). Further, elongation of secretory cells was observed for the beetles exposed to MD and HD after 10^{th} days of exposure. Moreover, microvilli of the secretory cells were curled and ruptured in addition to appearance of vacuoles due to disruption of the epithelial cells and muscle layer for the beetles exposed to MD and HD, after 20^{th} and 30^{th} days (**Fig. 13.4 c, d**).

The testis is composed of numbers of testicular follicles. Histologically, each follicle is composed of several acini, each of which is externally connected with the peritoneal membrane. The normal follicles are completely filled by cysts at different phases of spermatogenesis (**Fig. 13.5a**). The apical region contains numerous primordial germ cells.

The lumen contains many primary spermatocytes and secondary spermatocytes. The secondary spermatocytes are smaller in size and greater in number than the primary ones due to mitotic and meiotic divisions. The sperms are elongated, hair-like structures arranged in the form of bundles in the lumen of the follicle. *D. gazella* exposed to deltamethrin showed a dose dependent alteration in the histoarchitecture of testicular follicle such as shrinkage acini (sa), vacuolation (v), disintegrated spermatocytes (dspm), reduced spermatocytes (spm) and sperm bundles (sb) (**Table 3.14**; **Fig. 13.5 b, c, d**). Due to cytoplasmic degradation, formation of spaces (sp) increased in the lumen of the follicle in addition to appearance of necrotic sperms (**Fig. 13.5d**) in the beetles exposed to HD after 10^{th}, 20^{th} and 30^{th} days.

The female *D. gazella* has paired ovaries which are composed of numbers of ovarioles. Histologically, the normal ovariole is differentiated into a strand of cells containing prominent nuclei. Oogonia are scattered on the periphery followed by young oocytes where various stages of oocyte development occurs. Mature oocytes develop as a final stage of oogenesis, which also increases in size when oocytes grow (**Fig. 13.6a**). However, on exposure to deltamethrin, a time and dose dependent alterations were observed in the ovarian histology. A significant increase in the alterations of ovaries was observed for the beetles exposed to LD, MD, and HD, where in degenerated oocyes (do), increased vacuolation (v), ruptured follicular epithelium (Rfe), and ruptured peritonial sheath (ps), and increased spaces (sp) appeared in a time dependent manner (**Table 3.15**; **Fig. 13.6 b, c, d**). Moreover, the ovary of the beetles exposed to HD appeared as immature with atrophied ovarioles, ruptured follicular epithelium, and degenerated oocytes with their contents depleted off. Additional damages to the follicular epithelium including detachment and shrinkage of external sheath are recorded. Vacuolation and spaces appeared in different degrees inside the ovarioles (**Fig. 13.6d**).

Table 3.12: Representation of histological alteration in brain of *D. gazella* on exposure to deltamethrin

	10 Days				20 Days				30 Days			
	C	LD	MD	HD	C	LD	MD	HD	C	LD	MD	HD
Cell bodies (cb)	++	+	+	+	++	+	+	+	+	+	+	+
Vacuoles (v)	-	+	++	+++	-	-	+	+++	+	+	++	+++
Spaces (sp)	-	+	+	+	-	+	+	+++	-	+	++	+++
Pyknotic Nucleus	-	+	+	+	-	+	+	+++	-	+	++	+++
Degradative Changes (dc)	-	+	+	+	-	+	+	++	-	+	++	++

Table 3.13: Representation of histological alteration in gut of *D. gazella* on exposure to deltamethrin

	10 Days				20 Days				30 Days			
	C	LD	MD	HD	C	LD	MD	HD	C	LD	MD	HD
Muscles (ml) Thickness	++	+	+	+	++	+	+	-	+	+	+	+
secretory Cells (sc)	+	+	++	++	+	+	+	+	+	+	+	+
Lumen (lm)	+	+	+	+	+	+	+	+	+	+	+	+
Peritrophic Membrane (pm)	+	+	+	+	+	+	+	+	+	+	+	+
Regenerative Cells (rc)	-	+	++	+	-	+	+	++	-	+	+	++

Table 3.14: Representation of histological alteration in testis of *D. Gazella* on exposure to deltamethrin

	10 Days				20 Days				30 Days			
	C	LD	MD	HD	C	LD	MD	HD	C	LD	MD	HD
Shrinkage acini	-	-	++	++	-	++	++	+++	-	+	++	+++
Necrotic sperm	-	-	++	++	-	++	++	+++	-	+	++	+++
Spaces (sp)	-	+	+	+	-	+	+	+++	-	+	++	+++
Disintegrated spermatocytes (dps)	-	+	++	++	-	++	++	+++	-	+	++	+++
Vacuoles (v)	-	+	++	+++	-	-	+	+++	+	+	++	+++

Table 1.15: Representation of histological alteration in ovarian follicles of *D. Gazella* on exposure to deltamethrin

	10 Days				20 Days				30 Days			
	C	LD	MD	HD	C	LD	MD	HD	C	LD	MD	HD
Intact peritoneal sheath(ps)	+++	++	++	+	+++	+++	+++	+++	+++	++	+	+
Rupture epithelium (re)	-	-	++	++	-	++	++	+++	-	+	++	+++
Spaces (sp)	-	+	+	+	-	+	+	+++	-	+	++	+++
mature oocytes (mo)	+	+	+	+	+	+	+	+	+	+	+	+
Vacuoles (v)	-	+	++	+++	-	-	+	+++	+	+	++	+++
degenerated oocyte (ds)			++	++				++				++

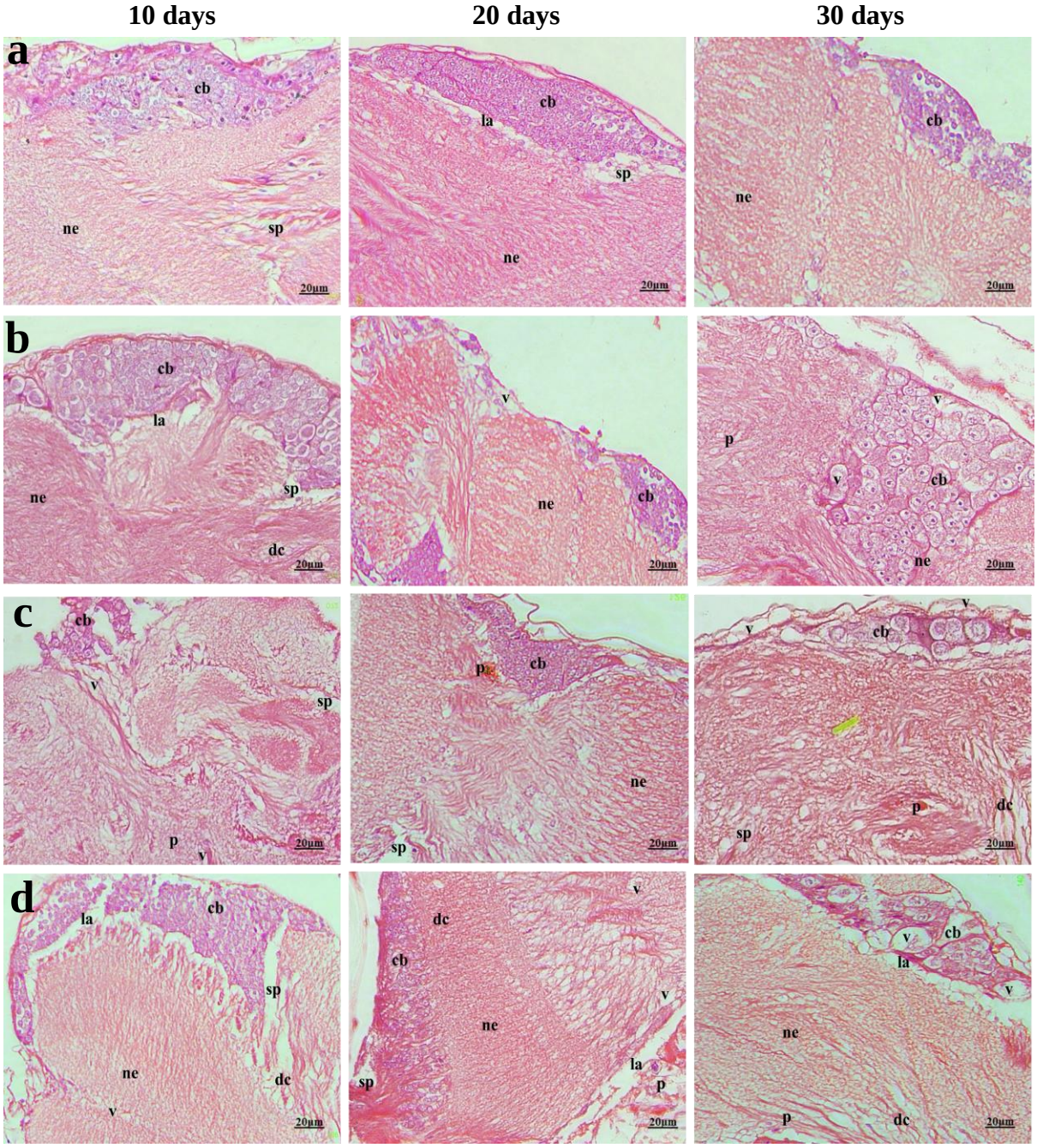


Figure 3.13: Histological sections of *D. gazella* brain stained with hematoxylin and eosin, after 10th, 20th and 30th day of exposure to deltamethrin. (a) Control treatment with no exposure (b) Exposure to LD of deltamethrin. (c) Exposure to MD of deltamethrin. (d) Exposure to HD of deltamethrin. Magnification 40X, scale = 20µm. Here, cb: cellbodies; ne: neuropiles; v: vacuoles; sp: spaces; p: pyknotic nucleus; dc: degredative changes; la: lacuna.

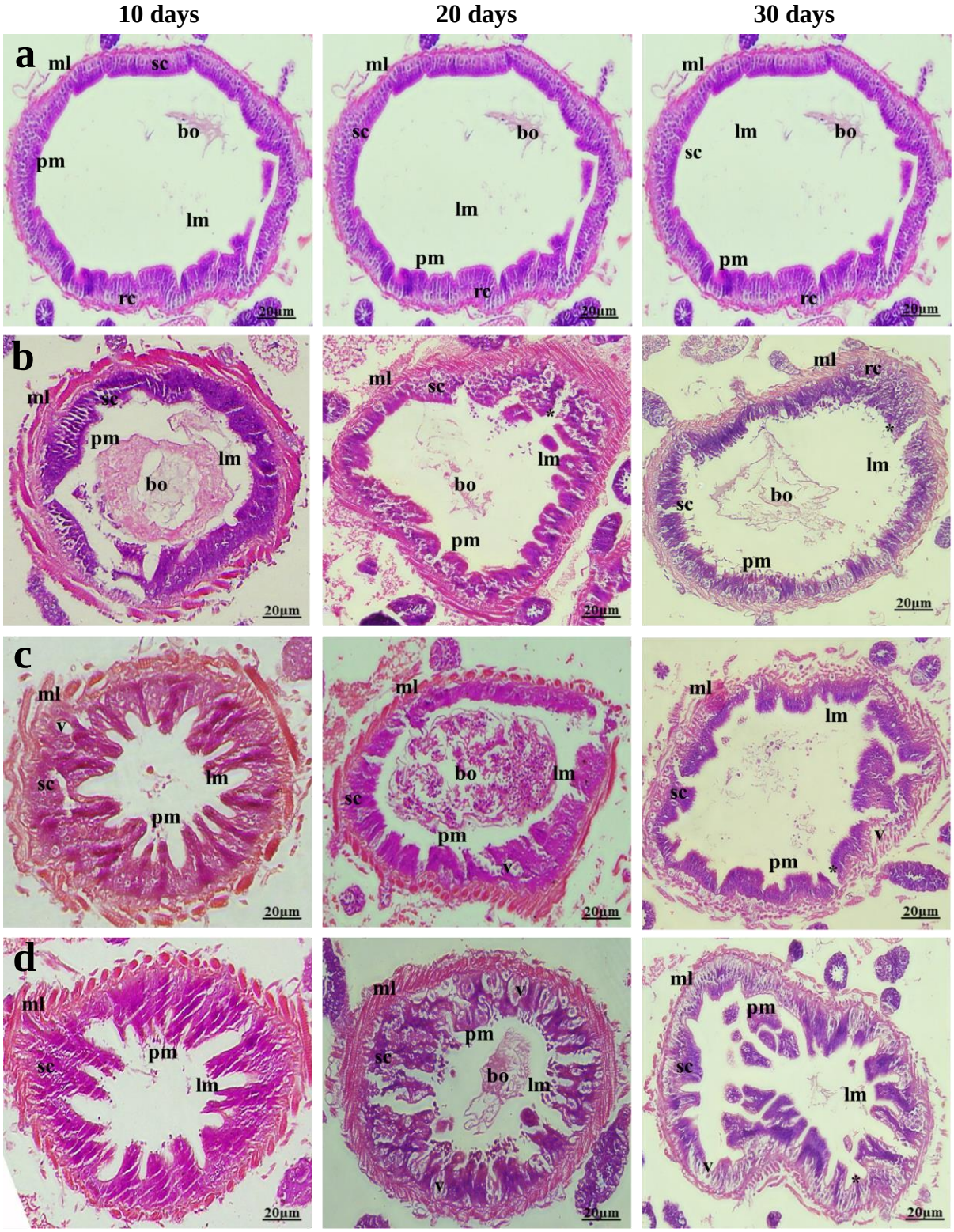


Figure 3.14: Histological sections of *D. gazella* midgut stained with hematoxylin and eosin, after 10th, 20th, and 30th day of exposure to deltamethrin. (a) Control treatment with no exposure to insecticide (b) Exposure to LD of deltamethrin. (c) Exposure to MD of deltamethrin. (d) Exposure to HD of deltamethrin. Magnification 40X, scale = 20µm. Here, ml: muscles; sc: secretary cells; lm: lumen; bo: bolus; pm: peritrophic membrane; rc: regenerative cells.

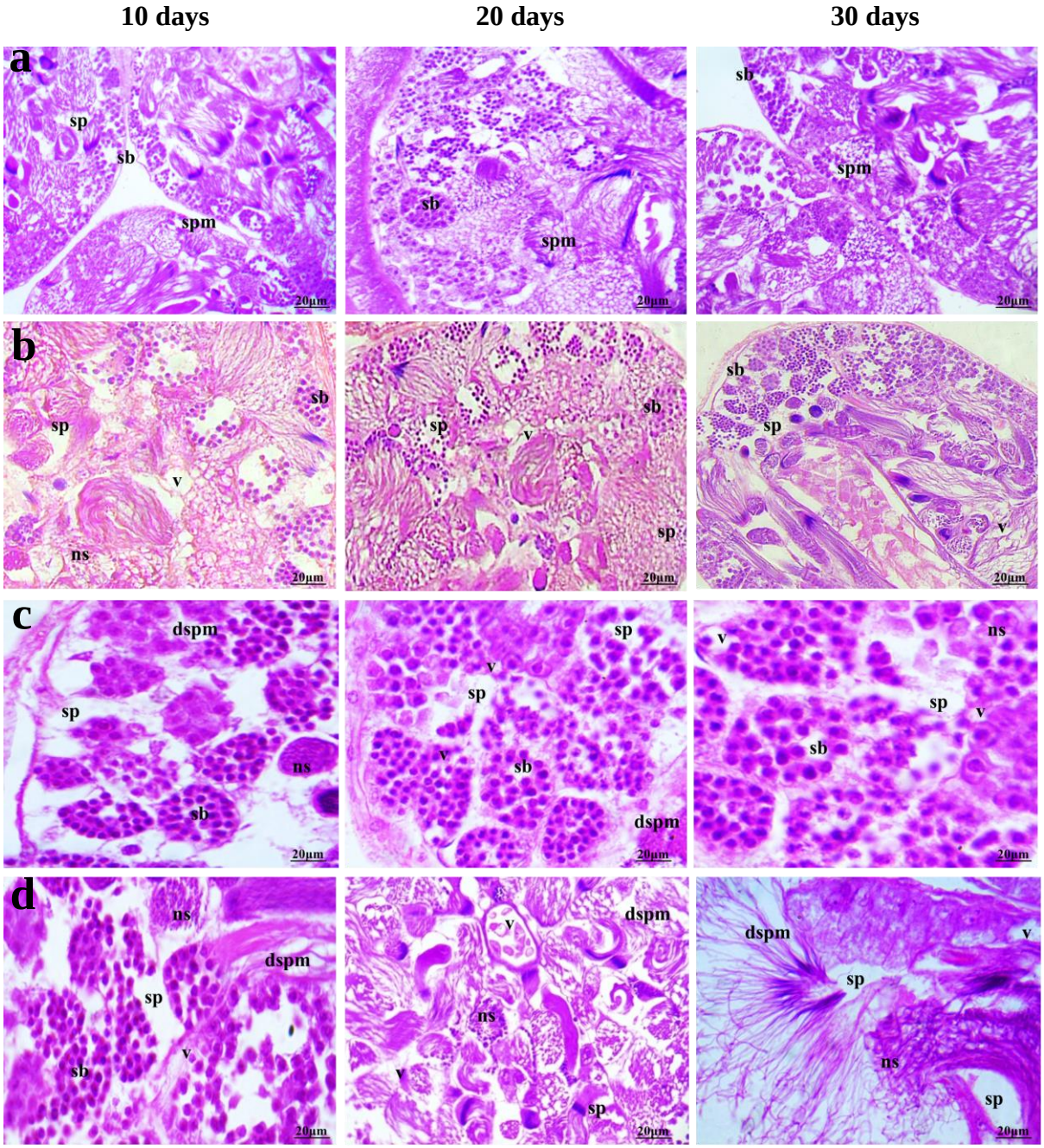


Figure 3.15: Transverse sections of *D. gazella* testis stained with hematoxylin and eosin, after 10th, 20th, and 30th day of exposure to deltamethrin (a) Control treatment with no exposure to insecticide (b) Exposure to LD of deltamethrin. (c) Exposure to MD of deltamethrin. (d) Exposure to HD of deltamethrin. Magnification 40X, scale = 20µm. Here, sa: shrinkage acini; ns: necrotic sperm (ns); sp: spaces; dspm: disintegrated spermatocytes; v: vacuoles; spm: spermatocytes; sb: sperm bundles.

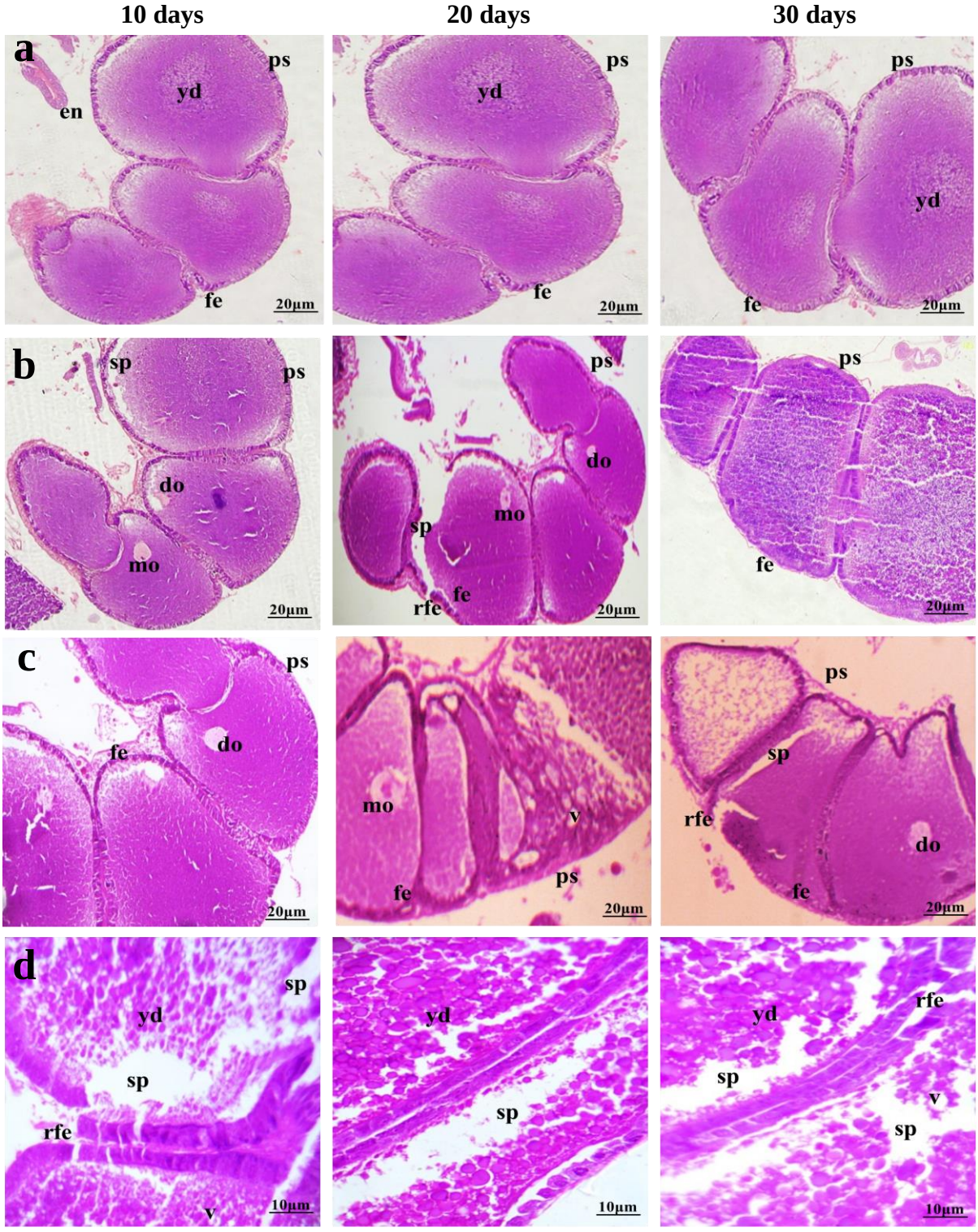


Figure 3.16: Transverse sections of *D. gazella* ovaries stained with hematoxylin and eosin, after 10th, 20th and 30th day of exposure to deltamethrin (A) Control treatment with no exposure to insecticide (B) Exposure to LD of deltamethrin. (C) Exposure to MD of deltamethrin. (D) Exposure to HD of deltamethrin. 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.

3.4 DISCUSSION

The impact of sub lethal doses of deltamethrin on a wide range of insect behaviour has been extensively documented in previous studies (Mazzi and Dorn, 2012; Müller, 2018; Parkinson et al., 2020). However, the specific consequences of these levels on *D. gazella* remain largely unexplored. So, this study aimed to fill a gap in current knowledge by examining the harmful effects of deltamethrin, a widely utilised insecticide in pasturelands, at various concentrations (LD, MD, HD) corresponding to the LC_{50} value, throughout a sub-acute exposure period (Mann et al., 2015; Sands et al., 2018). In the present study, the LC_{50} value of deltamethrin was obtained as 0.275ppm at 48 hours, on the basis of which sub-acute study dose $1/20^{th}LC_{50}$ (Low dose), $1/10^{th}LC_{50}$ (Medium dose), $1/5^{th}$ LC_{50} (High dose) were chosen for further studies. Deltamethrin exposure exhibited jerky movement, followed by loss of equilibrium, resulting into tremors, which probably has entered through contact as well as ingestion (Rehman et al., 2014; Brink and Berg, 2019). Further, at higher doses the intensity of tremor and immobility was more, along with darkening of the integument (Weaving, 2018; Hussain et al., 2021). The observed changes in behaviour and impaired balance may be attributed to a deficiency in the coordination between the nervous and muscular systems. This deficiency is likely caused by a delay in synaptic transmission, which can be attributed to the ability of certain substances to modify the functioning of voltage-gated sodium channels in the neuronal membranes of insects. As a result, the normal electrical signalling within the nervous system is disrupted (Soderlund, 2010). Our results are in agreement with the earlier reported work in insects including paracoprid beetle, *Metacatharsius troglodytes* (Boheman) (Torr et al., 2007; Sands et al., 2018; Weaving, 2018; Brink and Berg, 2019; Hussain et al., 2021; Mohammadi et al., 2021).

Deltamethrin induces significant toxicity in both vertebrates and invertebrates, potentially attributable to oxidative stress, the formation of reactive oxygen species (ROS), and perturbations in metabolic processes (Rehman et al., 2006; Muller et al., 2008; Banji et al., 2013; Romero et al., 2015). In their studies, Zorov et al., (2014), observed that the elevation in reactive oxygen species (ROS) generation leads to the onset of mitochondrial dysfunction through the induction of autocatalytic processes. These events result in the conversion of molecules into free radicals, thereby initiating a detrimental signalling cascade. DCFH-DA dye is a colorimetric and fluorimetric probe which detects oxidative species. In the present study, to demonstrate the production of ROS in brain tissue of *D. gazella*, DCFH-DA staining was done, where, a consistent dose and time dependent increase in the

production of ROS indicating and confirming the generation of ROS. The findings of our study align with other research that has established the role of oxidative stress in triggering the production of reactive oxygen species (ROS) in the Sf9 cell line (Yang et al., 2017; Pandya et al., 2021). Moreover, the toxicity of numerous pesticides has been linked to oxidative stress, which is characterised by an augmented generation of reactive oxygen species (El-Demerdash, 2011). Till date the *in vivo* as well as *in vitro* studies on the effects of xenobiotics have been focussed on either for discovery of novel pesticides or its resistance, however, there are a very few studies reporting the toxic effects of pesticides on the non-target insects like dung beetles which are ecologically and economically important species (Sands et al., 2018).

Generally, on exposure to pesticides, the oxidative stress is generated in the body as a result of imbalance between ROS and antioxidants (Wang et al., 2016), body starts responding by different mechanisms which include lipid peroxidation (LPO) and expression of various antioxidants mechanism-SOD, CAT and GSH. Expression of these two mechanisms is the clear indication of pesticide toxicity and counter mechanisms exhibited by the organism (Chen et al., 2012; Nathan and Cunningham-Bussel, 2013; Martínez, et al., 2016; Yang et al., 2017). Hence, in the present study, to confirm how *D. gazella* responds to the production of ROS, quantitative analysis of lipid peroxidation (LPO) and antioxidants mechanism-SOD, CAT and GSH was performed as they are considered as biomarkers of tissue damage. A significant dose and time dependent alteration in LPO ($p<0.01$) and antioxidant enzymes mechanism ($p<0.01$) was obtained.

Glutathione (GSH) plays a crucial role as an antioxidant, participating in both enzymatic and non-enzymatic mechanisms to counteract the detrimental effects of reactive oxygen species (ROS). Additionally, GSH is involved in the process of detoxifying pesticides by reacting with electrophilic chemicals and substituting hydrogen, chlorine, or nitro-groups (Lushchak, 2012). The alteration in the concentrations of various glutathione forms signifies a modification in the equilibrium between prooxidants and antioxidants, a phenomenon frequently observed in situations of stress generated by pesticides (Atamaniuk et al., 2013). The cellular redox state is regulated by the redox component known as glutathione (GSH). The reduced form of glutathione (GSH) serves as a primary cellular thiol component within the antioxidative system. The current investigation demonstrates a significant time and dose dependent reduction in glutathione (GSH) levels suggesting a compromised defence mechanism against oxidative stress generated by deltamethrin (Lushchak et al., 2018). The

oxidation of GSH during the scavenging of ROS results in the formation of a mixed disulfide with glutathione protein. Therefore, the cell's capacity to reduce or synthesise GSH plays a crucial role in its ability to successfully regulate oxidative stress (Khare et al., 2019). The second cellular mechanism to defend the ROS is through antioxidant enzymes (SOD and CAT) (Rezg et al., 2008). Superoxide dismutase (SOD) is a crucial antioxidant enzyme that plays a key part in scavenging superoxide radicals within living organisms. Its primary job is to maintain normal physiological conditions and aid in the adaptation to stressors (Song et al., 2017). Catalase (CAT) is an enzyme that contains hematin and is mostly located within the peroxisomes of cells. It plays a crucial role in the intracellular defence mechanism and serves as a vital component of the antioxidant system (Ndonwi et al., 2019). In this study, a notable decline in the activity of superoxide dismutase (SOD) and catalase (CAT) was detected as a result of the excessive formation of reactive oxygen species (ROS) induced by deltamethrin and our finding aligns with previous results published by Awad and Ghazawy, (2016). The likely mechanism via which deltamethrin exhibits its activity involves the inhibition of Sodium/Potassium ATPases, resulting in impaired cellular respiration and an increased production of oxygen free radicals (Adamski et al., 2005). The impact of Phosalone on lipid peroxidation (LPO) has been documented, leading to a notable reduction in the *in vitro* activity of catalase (CAT) (Altuntas et al., 2003).

The excessive generation of reactive oxygen species (ROS) has been established as a causative factor in the development of oxidative stress, unless it is effectively eliminated by the action of endogenous antioxidants (Amin et al., 2006). Therefore, an excessive generation of reactive oxygen species (ROS) could result in the depletion of antioxidants or the direct impact of deltamethrin on the peroxidation process. Pesticides, including chlorpyrifos and diazinon, have been demonstrated to elevate intracellular levels of reactive oxygen species (ROS) and LPO, a process that is regulated by intracellular GSH (Giordano et al., 2007). In addition, the level of LPO estimated in *D. gazella* brain, a significant ($p<0.05$) increase in the LPO level, directly proportional to the increase in ROS (Cortés-Iza and Rodríguez, 2018). Earlier studies by Basu, (2003) have also found carbon tetrachloride (CCL4) to enhance Lipid peroxidation by ROS generation. LPO is well recognised as a crucial marker for oxidative harm to cellular constituents caused by an over abundance of ROS. This process can result in many biological consequences, encompassing modifications in signal transduction, gene expression, and programmed cell death (Kannan and Jain, 2004). Our results are in line with those reported by Meunier et al., (2020), where sublethal exposure to deltamethrin impairs

maternal egg care in the European earwig *Forficula Auricularia*; Dai et al., (2010) have proved the toxic potential of sub lethal concentrations of bifenthrin and deltamethrin on fecundity, growth, and development of the honey bee *Apis mellifera ligustica*; Gutiérrez et al., (2017) have reported the deltamethrin exposure impairs swimming behaviour of two backswimmer species *Bueno atarsalis* and *Martarega bentoi*; Tooming et al., (2014) documented the sub-lethal impacts of pyrethroid pesticide on the motor and locomotor behaviours of the non-targeted beneficial carabid beetle *Platynus assimilis*.

Summing up the overall oxidative stress response by way of physiological defence mechanisms composed of detoxification and antioxidant systems proves the toxic potential of deltamethrin, which further confirmed by performing the CYP activity due to its involvement in the metabolism and detoxification of toxicants in insects (Lu et al., 2021). Cytochrome P_{450} proteins exhibit similarities to antioxidant enzymes, since the uncoupled catalytic cycle of cytochrome P_{450} has traits that bear resemblance to peroxidase enzymes (Matteis et al., 2012). The enzymes discussed in this context are of significant importance in the metabolic system due to their role in controlling the levels of endogenous chemicals. Additionally, they play a crucial role in the metabolism of xenobiotics, including medicines, pesticides, and plant poisons, by facilitating oxidation processes (Zhu et al., 2016). The surge in scientific investigations concerning the comprehensive assemblage of P_{450} genes, commonly referred to as the CYPome, in arthropods, can be attributed to the notable escalation in the quantity of invertebrate genomes that have been subjected to sequencing (Rane et al., 2019). Moreover, the improvement of advanced protein and genetic methodologies for the purpose of functionally expressing P_{450}s have provided researchers with unique prospects to investigate their regulation, molecular physiology, and significance in xenobiotic metabolism. The phenomenon of metabolic resistance has been traditionally linked to heightened levels of enzyme activity. However, in recent times, there has been a better understanding of the various mechanisms that contribute to the quantitative alterations in P_{450} expression (Nauen et al., 2022). Numerous P_{450} enzymes that are increased in resistant phenotypes have been functionally characterised as directly metabolising pesticides, hence imparting insecticide resistance. On the other hand, there is a lower prevalence of P_{450}-mediated insecticide resistance associated with decreased levels of P_{450} expression and indirect processes driven by P_{450} that impact the penetration of pesticides. Although other insecticides have been demonstrated to activate P_{450} genes, particularly at sub lethal concentrations, the response to deltamethrin remains insufficient. Therefore, in the present

study, an attempt was made to look into the CYP_{450} activity on exposure to deltamethrin with respect to *D. gazella*. A significant ($p<0.01$) dose and time dependent down regulation in the expression of cyp6bq9 was observed, which is in contrast to earlier reported work, where the expression of cyp6bq9 was primarily observed in the brain of *T. castaneum* which exhibited resistance to pyrethroids. This brain tissue has been shown to possess a high concentration of the target protein for this group of insecticides, namely the voltage gated sodium channel (Zhu and Snodgrass, 2003; Zhu et al., 2010) as well as in *Apis cerana cerana* (Shi et al., 2013; Zhang et al., 2019). The functional validation of the involvement of P450s in resistance in vivo has been successfully accomplished by many methods including RNAi-mediated suppression, transgenic overexpression, and targeted ablation of P_{450} genes. However, a detailed biochemical and genomic approach will facilitate the further understanding by which P_{450} mediates insecticide metabolism (Zhang et al., 2018), since the present was not focused on insecticide resistance.

Breakthroughs in the field of ecotoxicology have facilitated the development of a wide range of biomarkers that can effectively assess exposure to pesticides or their associated impacts. Somatic indices have been used to estimate of individual fitness (Norrdahl et al., 2004). Organosomatic indices refer to the ratios of organ weight to body weight, with the organ weight being measured relative to the body mass (Ronald and Bruce, 1990). These indices are crucial for assessing the harmful effects of chemicals on target organs (Giullo and Hinton, 2008). They also indicate the physiological condition of the specimens based on fat accumulation, gonad development, general well-being, or changes in response to the toxic effects of pesticides (Upadhyay, 2019). By understanding the alterations in the organ weight to body mass in relation to the histomorphological alterations of the tissue will help us in understanding the toxic effect of deltamethrin. With the increase in pesticide exposure periods, tissues weight of body decreases (Bhartia and Rasool, 2021). Alterations in the organosomatic index (quantitative) along with the histopathology (qualitative) performed in the present study are the first of its kind reported in insects. Moreover, there are lacunae on the oraganosomatic and histopathological effects on the key organs in dung beetles exposed to pyrethroids including Deltamethrin. Thus, in the present study an attempt was made to evaluate the organosomatic index and histopathological alterations in the key organs including brain, gut, gonads of *D. gazella* exposed to deltamethrin.

Traces of insecticides have been reported to be present in the soil and dung in pasture lands (Mann et al., 2015). Pointing to the fact dung beetles are also inadvertently being

exposed as non-target organisms. The possible impact of this particular mode of exposure to neurotoxic substances on the malleability of the central nervous system, particularly the growth of the brain, is a matter of concern. The brain is particularly susceptible to damage caused by free radicals due to its elevated metabolic rate and limited regenerative ability, relative to other organs (Anderson, 2004). Oxidative stress is widely recognised as a primary etiological component contributing to the decline in cognitive function (Praticò, 2008). In the present study, a significant dose and time dependent decrease in the brain somatic index was obtained. Parallel to this histological alterations were also observed, where an intact structure of the brain with condensed neuropiles in the centre and neuronal cell bodies on the periphery were noted in control brain tissue. Exposure to insecticides caused swelling of neuronal cell bodies (cb) and the formation of vacuoles (v), fluid-filled spaces (sp) and neuronal cells with pyknotic nucleus (p) were clearly observed within the brain tissue, suggesting an overall disturbed cytomorphology of the brain, leading to potential functional disturbances. In contrast to organochlorine and other persistent chemicals, it has been observed that insecticides like deltamethrin undergo quick degradation upon exposure to sunlight (Wheelock et al., 2005; Lawler et al., 2008) and that it does not have any impact on the water and sediments of aquatic ecosystems (Amweg et al., 2006; Feo et al., 2010). However, it has been suggested that this phenomenon may enhance the risk of deltamethrin poisoning for benthic organisms (Pawlisz et al., 1998). *D. gazella* being nocturnal in its habit, chances of sunlight reaching the deeper layer of soil cannot be expected. Hence, the pathological alterations in the brain tissues are self-explanatory. it has been proposed that deltamethrin has the potential to interfere with ion transport mechanisms in the epithelial tissues of both aquatic and terrestrial insects (Alves et al., 2010). In concurrence with the earlier reported toxic effect of deltamethrin (Gutiérrez et al., 2016), the histomorphological modification in the brain tissue has also led to change in the physiology of transmission of *D. gazella* which can probably lead to the negative impact on the nesting activities *i.e.* tunnelling and brood ball formations.

One of the modes of action of deltamethrin is ingestion by which it passes through the food and reaches gut. A dose and time dependent decrease in the gut somatic index in deltamethrin exposed gut of *D. gazella* was observed in comparison to control. Parallel to gut somatic index, histological alterations in the midgut was also observed in the present study. The midgut of *D. gazella*, when not subjected to deltamethrin, exhibited a monolayered epithelium consisting of columnar digestive cells that abutted the basal lamina. Additionally,

there were intermittent clusters of regenerative cells encircled by circular and longitudinal muscles. The phenomenon of cytomorphological modifications in the insect midgut resulting from exposure to insecticides has been documented across several insect species (Alves et al., 2010; Konus et al., 2013; Fu et al., 2014; Xu et al., 2015; Li et al., 2016). In the present study, a time and dose dependent histological changes in the structure of midgut of *D. gazella* was observed, which was characterized by cell degeneration (dc), such as cytoplasmic vacuolization (v) and widening of the space (sp) between the epithelium and the basal membrane of the insect, disruption of peritrophic membrane (pm) and secretory cells (sc). The absorptive epithelial cells exhibited a noticeable reduction in size and deformation, accompanied by changes in the morphology and organisation of goblet cells and regenerative cells (rc). Although the distinct visibility of these cells was not observed, it was observed that the cohesive layer of epithelial cells remained in close proximity to the basement membrane (bm). The higher concentration of the deltamethrin resulted in further decreased absorptive surface area and increased gut lumen (lm). The detachment of the epithelial layer from the basement membrane led to the formation of substantial spaces (sp) that were detached. The intensity of alterations was dose and time dependent and seems to be supplanted by deltamethrin exposure. Cytomorphological alterations in the midgut are illustrated in non-target insects such as *Apis mellifera* in response to exposure to thiamethoxam (Catae et al., 2014), *Calliabaetis radiates* exposed to deltamethrin (Gutierrez et al., 2016), the predator *Ceraeochrysa claveri* exposed to azadirachthin (Scudeler and dos Santos, 2013), and *Chironomus calligraphus* exposed to cypermethrin (Lavarías et al., 2017). Further, cytoplasmic vacuolation observed in the secretory cells of midgut at higher dose is due to the induced chemical stress (Lockshin and Zakeri, 2004), and quantitative alterations in ROS parameters- enzymatic (SOD, CAT), non-enzymatic (GSH) as well as CYP P_{450} (Feyereisen, 1999; Yurchenko and Morozove, 2022) gene further substantiates our result and that deltamethrin exposure in *D. gazella* has decreased the secretory activity for detoxification. Loss in the integrity of peritrophic membrane (pm) as well as reduction in regenerative cells is suggestive of reduced protection and absorption and loss of ability in replacing the damaged epithelium (Terra and Ferreira, 2012). Summing up the alterations in the gut of *D. gazella* on exposure of deltamethrin, thus suggests overall reduction in digestion and absorption through midgut cells, disturb the body metabolism and lead to reduced energy production, which in turn impacts the normal physiology and thereby affects its nesting activities.

Exposure of deltamethrin resulted into reduced gonado-somatic index (ovary and testis) indicative of the histomorphological alterations in a time and dose dependent manner (Huang et al., 2016). Histopathological alterations in the ovary has been reported earlier by Rai et al., (2011) in *Poekilocerus pictus*; El Naggar et al., (2010) in red palm weevil; Osman et al., (2015) in *Blapspolycresta* on exposure of various chemicals/stressors. In the present study, the ovary of the exposed beetles showed an immature atrophied ovarioles along with the ruptured follicular epithelium, vacuolation (v) and spaces (sp) in different degrees, degenerated oocytes (do) with their depleted contents. Furthermore, observations were made of supplementary impairments to the follicular epithelium (fe), specifically the detachment and shrinking of the exterior peritoneal sheath (ps), within the ovarioles. Appearance of degenerated oocytes (do) was indicative of general necrosis. Additionally, the researchers conducted studies on additional abnormalities affecting the follicular epithelium (fe) in the ovarioles; noted the detachment and shrinking of the external peritoneal sheath (ps). (Shakeet and Saroj, 2009; Rai et al., 2011; Al-Dhafar and Sharaby, 2012). Histopathological alterations in the ovaries can result in complete ovarian destruction, subsequently leading to either the absence of egg production or sterility of the generated eggs (Samira et al. 2010). Overall vacuolation and reduced size of oocytes is indicative of inhibition of ovarian development (Osman et al., 2015). Histopathological alterations in the ovaries further results into ovarian destruction, subsequently leading to a failure of oocyte production or sterility of the oocytes produced (Hami et al., 2005; Samira et al., 2010; Amaral et al., 2018). Summing up together, deltamethrin exposure has resulted into distinct histomorphy of the ovary in *D. gazella* and is in agreement with the earlier reported work on histomorphological alterations (Martano et al., 2023). Further, studies by Sharaby et al. (2012); Eldin et al., (2016) have also reported abnormalities in the developing oocytes on exposure to pesticides.

A time and dose dependent reduction in the gonad somatic index for testis of *D. gazella* was observed in the present study along with it the histomorphy also gets altered. Earlier studies have reported the histological structures of testes by İzzetoğlu and Gülmez, (2017) in three different *Tentyria* species belonging to Tenebrionidae family; Gaikwad and Patil, (2017) in Cerambycidae; Çağlar et al., (2020) in *Capnodis tenebrionis*; Erbey et al., (2021) in Curculionidae, under normal conditions. However, there is lacuna as far as histomorphological alterations on exposure of pesticide on testis are concerned. So, in an attempt to understand the toxic effects on the histological modifications in the male gonads of *D. gazella*, a time and dose dependent alterations were observed. The testicular follicles of

the insects that were subjected to deltamethrin exhibited a reduction in size of the acini (sa), deterioration of the peritoneal membrane (pm), disintegration of both primary and secondary spermatocytes, and disorganisation of sperm with a decrease in their quantity. The lumen (lu) within the follicle demonstrated increases spaces (sp), as well as the appearance of disintegrated sperm cells (dspm) and necrotic sperms (nspm), as a result of cytoplasmic deterioration. Further, appearance of vacuoles (v) and spaces (sp) may arise as a result of cytoplasmic breakdown, aggregation, and shrinkage, hence collapse of spermatogenesis which is in accordance with Ambika and Selvisabhanayakam (2012) who have reported alterations in testis on exposure of neem oil and those by Emerald and Rameshkumar (2012) where they have reported altered testis structure on exposure of heavy metals. Overall, the deleterious effect of deltamethrin on the gonads of both sexes may affect the reproductive potential which probably leads to reduced reproduction and overall nesting activities and leading to decrease in the population of ecologically and economically important dung beetles. Furthermore, summing up together, all the histological results (brain, midgut and gonads) of deltamethrin exposed *D. gazella* exhibited substantial and permanent pathological abnormalities.

3.5 CONCLUSION

Deltamethrin, a commonly used insecticide, can have negative effects on dung beetles when exposed to sub-lethal doses for 30 days compared to control which includes disruption in antioxidant balance, reducing the activity of enzymes that protect against oxidative damage. It also leads to the production of reactive oxygen species (ROS), causing cellular damage and oxidative stress due to an imbalance between ROS production and antioxidant defence mechanism. Moreover, prolonged exposure resulted in tissue damage, inflammation, and structural abnormalities leading to histological alterations in organs like the brain, gut, ovaries and testis. These effects underscore the importance of responsible pesticide used and monitoring for signs of toxicity in exposed animals. Thus, from the present study, it can be concluded that deltamethrin leads to increased ROS, reduced antioxidant enzyme activity, reduced organosomatic indices and histomorphological alterations proving its toxic potential, which further induces altered physiology and behaviour in *D. gazella*.