Chapter 4: Neurophysiological alterations in the nesting behaviour of *Digitonthophagus gazella* on exposure to deltamethrin

4.1 INTRODUCTION

Insect physiology and behaviour flexibility that allows for responses to a variety of environmental obstacles is essential for insect survival and reproduction (Nässel and Zandawala, 2019). Optimal nutrient and energy balance, successful reproduction, and coordination between the internal and external environments are all ensured by these adjustments, which are primarily coordinated by the nervous and endocrine systems (Owusu-Ansah and Perrimon, 2015; Yapici et al., 2014; Anderson, 2016). According to several studies (Nässel, 2009 and 2018; Marder, 2012; Taghert and Nitabach, 2012; van den Pol, 2012; Kim et al., 2017; Nusbaum et al., 2017), these systems employ signals characterized by varying spatial and temporal resolutions. Synaptic transmission frequently entails the use of small-molecule neurotransmitters or electrical conduction across gap junctions. This process is characterized by rapid temporal dynamics, occurring within milliseconds, and precise spatial localization at synaptic junctions. Different kinds of neuromodulators, neurohormones, or hormones carry out signalling at a slower timeframe (seconds to hours). These are frequently produced at locations far from real synapses or receptors. In addition to nitric oxide, this sort of signalling also makes use of monoamines, neuropeptides, peptide hormones, steroids, fatty acids, and other substances. Additionally, under both routine and stressful circumstances, the neuroendocrine system interacts to control a number of physiological processes and preserve system-wide homeostasis (Hartenstein, 2006; Adamski et al., 2019). The two main groups of neurosecretory chemicals, biogenic amines and neuropeptides, mediate a range of physiological activities (Hartenstein, 2006; Chowanski et al., 2016, 2017). According to Chowanski et al., (2017) neuropeptides have a crucial role in regulating metabolic processes, maintaining ion balance, and facilitating muscle contractions, such as the cardiac rhythm. These bioactive molecules are predominantly synthesized inside the central nervous system (CNS). Neuropeptides with homologous structures frequently have comparable roles across insect species (Bendena, 2010).

We can anticipate that in response to insecticide exposure, molecules responsible for neural regulation in dung rollers' nesting behaviour, such as neurotransmitters and neuropeptides, can be important players. These chemicals are pivotal in physiological and

behavioural processes, exerting direct influence on the ability to withstand harsh environmental circumstances. Recent studies have indicated that biogenic amines, including octopamine (OA), dopamine (DA), and serotonin (5-HT), play a significant role in the physiological response to stress. It has been shown that the biogenic amine levels of certain insect species undergo changes in response to unfavourable conditions (Hirashima et al., 2000).

The current understanding of biogenic amines' functions in insects' points to a broad range of potential outcomes. They take involvement in the control of a variety of behaviours, including social interactions, eating, and reproduction (Pflüger and Duch, 2011). Biogenic amines also cause the body to react negatively to certain environmental stresses, such as those brought on by insecticides (Adamo, 2008). OA and DA are released into insect haemolymph in the first several minutes after exposure. According to research by Gruntenko et al., (2004), this starts a chain of events that are meant to bring about equilibrium. It is interesting, as reported by Armstrong and Robertson, (2006) and further reinforced by Gruntenko and Rauschenbach, (2018) that the production of these biogenic amines in response to stress circumstances does not appear to be particular to the kind of stressor. According to research by Kori et al., (2018), pyrethroids have been discovered to change the concentrations of neurotransmitters and monoamine neurotransmitter metabolites in the brain. Additionally, NPs are essential for controlling a number of physiological and behavioural processes in insects, such as growth, lifespan, metabolic equilibrium, reproduction, and stress response (Lubawy et al., 2020). Multiple NPs are synthesized and released by beetles under intricate regulation (Yeoh et al., 2017). The mechanism is closely controlled and likely vulnerable to unfavourable situations like low temperature, extreme cold, or stress brought on by insecticides (Li et al., 2020). Numerous chemical signals are generated in reaction to stress, either directly between cells or systemically. These are signals that control behavioural responses, such as peptides similar to npf, it, and mip that are implicated in the stress response and may help to control it (Schoofs et al., 2017; Ragionieri et al., 2022).

Through the use of molecular genetics, the functional characteristics of a few neuropeptides and their receptors have been studied in the *D. melanogaster*. Neuropeptide functions in the cockroach *Leucophaea maderae* have also been studied using behavioural and electrophysiological experiments (Nässel and Homberg, 2006). Furthermore, Nässel and Zandawala, (2019) have uncovered how peptides function in CNS circuits to affect behaviour

and physiology in their investigations, which also emphasized new neuropeptides and the increased application of cutting-edge genetic approaches. The whole gene set for the neuropeptides and neuropeptide-like peptides typical of insects in *Schistocerca* has undergone significant conservation, according to Ragionieri et al., (2022). In order to better understand how neuropeptides affect the physiology and behaviour of *H. abietis* and to create specific neuropeptide-based tools for *H. abietis* control, Pandit et al., (2018) identified neuropeptide F in the model beetle *T. castaneum* as well as 24 putative neuropeptide and 9 leucine-rich repeat containing G proteins coupled receptor-encoding transcripts. Additionally, Ragionieri and Predel, (2020) have thoroughly investigated the possible neuropeptide antecedents of the carabid beetle *Pogonus chalceus*.

The consequences of insecticides on insect biology manifest themselves through diminished oviposition, prolonged immature stage development, or shortened life span. Previous research conducted by França et al., (2017) has demonstrated that some neurotoxic insecticides can affect the reproductive capacity and fertility of insects through behavioural changes, particularly during their reproductive phase, even when administered at sub lethal doses. According to Storch et al., (2017), the insecticides lufenuron, methoxyfenozide, spinosad, endosulfan, novaluron, and tebufenozide have sub lethal effects on *Anticarsia gemmatalis*. These effects manifest as reductions in pupal weight, adult lifespan, and fertility. In a study conducted by Mahmoudvand et al., (2012), it was observed that the insecticide hexaflumuron exhibited significant effects on various life stages of *Plutella xylostella*. Specifically, the application of hexaflumuron resulted in a decrease in the overall egg count, duration of oviposition, pupation, and emergence of adult individuals. Similarly, *Helicoverpa assulta* was affected by cyantraniliprole in a sub lethal manner, which resulted in a reduction in the adult fertility and pupal weight of the parental generation (Dong et al., 2017).

Exposure to insecticides alters the chemical communication system, changing a number of insect behavioural traits like food seeking, oviposition site selection, pheromone communication, and others that depend on intricate physiological mechanisms involving hormones and neurohormones, ultimately reducing the likelihood of insect reproduction (Price et al., 2011; Candolin and Wong, 2012; Schoonhoven, 2018). Certain insecticides that affect the endocrine system may also have an impact on reproductive behaviour. The study conducted by Wei et al., (2004) investigated the impact of Deltamethrin on the calling behaviour and sex pheromone production in *Ostrinia furnacalis*. The findings of the study

indicated that *O. furnacalis* exhibited a compensatory mechanism in response to exposure to the insecticide. Specifically, male individuals that were able to survive the contact with the insecticide displayed a reduced response to pheromone, whereas the surviving female individuals exhibited an increased production and release of pheromone.

Thus, from the literature reviewed, it can be summarized that largely the studies with reference to insecticide and neuroendocrine system is focused on pest species. But to our knowledge, there is currently little understanding about how Deltamethrin impacts nesting behaviour of *D. gazella*. Hence, in the present study, after confirming the toxic potential of Deltamethrin on biochemical and histological parameters, our next goal was to have an insight into its involvement in altered neurophysiology by confirming the role of neurotransmitters-biogenic amines and nitric oxide, as well as neuropeptides, in the nesting behaviour of *D. gazella* (**Fig. 4.1**).

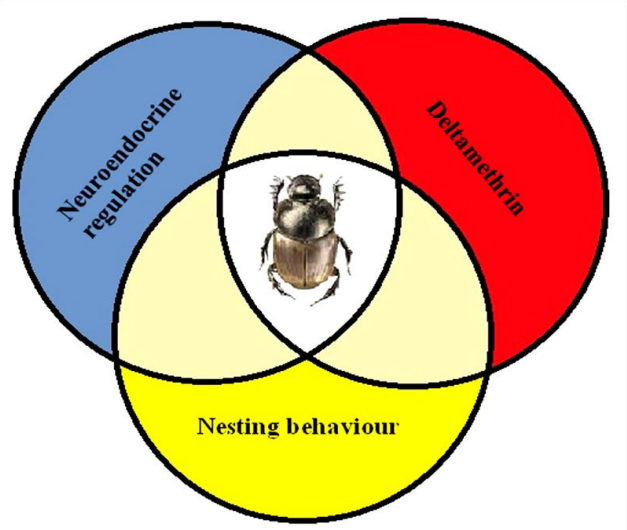

Figure 4.1: The association of neuroendocrine regulation in the nesting behaviour of *D. gazella* on exposure to Deltamethrin.

4.2 MATERIALS AND METHODOLOGY

To evaluate toxicity potential of Deltamethrin, further assays were performed to check whether it leads to alteration in the neuroendocrine regulation and physiology. For this, 5 pairs of male and female *D. gazella* were exposed to the dung treated with 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} of Deltamethrin, in comparison to control, for all- 10th, 20th, and 30th days of tunnelling, after which the levels of NTs, NT synthesizing enzymes, and NP's expression were assessed, to understand the effects of Deltamethrin in the neurophysiology during nesting behaviour of Dung beetles; *D. gazella*.

Estimation of the Neurotransmitters

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

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

Estimation of DA

To the 0.2 mL of aqueous phase, 0.05 mL of 0.4 M HCl and 0.1 mL of EDTA / Sodium acetate buffer (pH-6.9) were added, followed by 0.1 mL iodine solution (0.1 M in ethanol) for oxidation. The reaction was stopped by addition of 0.1 mL Na_2SO_3 solution after 2 mins and 0.2 mL acetic acid after 1.5 mins, followed by heating of the solution to 100°C for 6 mins. The samples were then allowed to come to room temperature, post which the excitation and emission spectra was read at 330-375 nm in the Spectrofluorimeter.

Estimation of 5-HT

To 0.2 mL aqueous extract, 0.25 mL of OPT reagent was added. The fluorophore was allowed to develop by heating the samples at 100°C for 10 min. Then, the sample was

allowed to reach equilibrium with the ambient temperature, and the readings were taken at 360-470 nm in the spectrofluorimeter.

Estimation of AChE

The rate of AChE activity was measured according to the method described earlier by Ellman et al., (1961). Male and female dung beetles were dissected open on ice, and brain tissues were collected after 10, 20 and 30 days. A 20 mg/mL of tissue was homogenized in 0.05 M phosphate buffer. This step was followed by addition of 0.5 mL Triton X-100 and 0.2 mL EDTA. The samples were centrifuged at 6000 RPM for 20 min at 4°C. Then, 0.1 mL of supernatant was taken into the cuvette as a source of enzyme, followed by addition of 2.86 mL of phosphate buffer. The sample was incubated for 5 minutes at room temperature, post which, 50 µL 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) solution was added to the cuvette, followed by addition of 30 µL of Acetylcholine Iodide (AChI) (0.075 M) to cuvette. The blank for such a run consisted of buffer, substrate, and DTNB solutions. Absorbance was recorded at 412 nm using UV visible spectrophotometer (PerkinElmer Lambda 25, India).

Estimation of NO

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

Estimating the Gene Expressions of the Neurotransmitters synthesizing enzymes

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

Total RNA Extraction (Trizol Method)

For total RNA extraction, brain tissue was isolated from both male and female dung beetles in PBS (pH-7) after 10, 20 and 30 days of tunnelling. The tissue (50-100 mg) was weighed and homogenized in 500 µL Trizol reagent (Invitrogen). For complete dissociation of

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

cDNA Synthesis

First strand of cDNA was synthesized from each sample using Thermo Scientific Verso cDNA Synthesis Kit (AB-1453/A). Verso Reverse Transcriptase Verso is an RNA-dependent DNA polymerase with a significantly attenuated RNaseH 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 4.1**). The reaction mix is mentioned in the table below.

Table 4.1: PCR Reaction Mixture

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

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

PCR Conditions

Table 4.2: Reverse transcription cycling program for cDNA synthesis

	Temperature	**Time**	**Number of cycles**
cDNA synthesis	42 °C	30 min	1 cycle
Inactivation	95 °C	2 min	1 cycle

RT-PCR Amplification

Quantitative RT-PCR was performed using PowerUp SYBR Green Master Mix (A25741, Applied Biosystems, USA) in Quant Studio 12K (Life technology) FAST real-time PCR machine with primers to detect selected messenger RNA targets (**Table 4.3, 4.4, 4.5**). The melting curve of each sample was measured to ensure the specificity of the products. Beta Actin was used as an internal control to normalize the variability in the expression levels and data was analyzed using 2-ΔΔCT method (Livak and Schmittgen, 2001).

Table 4.3: Real Time PCR mix

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

Table 4.4: Real time PCR conditions

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

Table 4.5: Real time PCR primer sequences of neurotransmitter synthesizing enzymes.

Serial No.	Gene Name	Primer type	Sequence	T_m (˚C)
1	dopa decarboxylase (ddc)	Forward	CAAAAGCCCGACAAATGGG	60.03
		Reverse	AGTTGGCGGTGGGGAAATAG	60.04
2	5-HTP decarboxylase/ aromatic-L-amino-acid (5-htpdc)	Forward	GCGTGGAATGCTGTCTTAGTT	58.92
		Reverse	GCATTATCTGCCCTTGTTGTGT	59.91
3	choline acetyltransferase (chat)	Forward	ATCGAGCCGCATTGTGTGT	60.38
		Reverse	CGGAAAGTTCGTGGGCTCT	60.00
4	nitric oxide synthase (nos)	Forward	TCTCTACGACTGGAGTTGGCT	60.27
		Reverse	AATGACGTCCACGAGTTCTG	57.93

Neuropeptides involved in the nesting behaviour

To understand the behaviour patterns such as feeding, reproducing, parental care involved in the nesting behaviour by both male and female *D. gazella*, the gene expressions of the neuropeptides were estimated in the present study. The brain tissue of both male and female D. gazella were isolated in PBS (pH-7) after 10^{th}, 20^{th} and 30^{th} day, followed by RNA isolation, cDNA synthesis, followed by RTPC Amplification as described earlier. The Real Time PCR primer sequence of the selected neuropeptides was as given in the **Table 4.6.**

Table 4.6: Real Time PCR Primer Sequences of neuropeptides.

Serial No.	Gene Name	Primer type	Sequence	T_m (˚C)
1	Neuropeptide F (npf)	Forward	GACCCCAGTCTCATTCAGGC	61.40
		Reverse	GTTGGTTGTAGTCAGGGCGT	55.00
2	Neuropeptide F (npfr)	Forward	GACTCGTATGGGGGCGTAAA	59.35
		Reverse	GGTTCGAATCGGGTCAAGGA	59.35
3	Inotocin (it)	Forward	CATTTGCTGCGGACCTTTCG	59.35
		Reverse	CGTTCGTGGAAAAAGCCCTC	59.35
4	Inotocin receptor (ir)	Forward	AGGGGCTGAGCTTCTTCTTG	59.35
		Reverse	AATGCCGCTGAAAGGAGAGT	57.30
5	Myoinhibitory peptide (mip)	Forward	AGTTCTCCGCGTCTTAGTGTG	59.82
		Reverse	GGTCCTTTTTCAGAAGCTTACAC	58.87

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

Data Analysis

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

4.3 RESULTS

During the period of exposure to deltamethrin (LD, MD, HD), a marked distinction in the nesting behaviour of *D. gazella* was observed. Both male and female spent less time in feeding and constructing nest and an overall reduced activity of tunnelling and brood ball making.

Number of Brood balls formed after exposure to deltamethrin

During the period of nesting, the appearance of hole was less frequent for the deltamethrin treated group in comparison to control. An altered nesting activity was noted where a relatively less number of brood balls were observed in a time and dose dependent manner (**Fig. 4.2**). Relative to the number of brood balls produced by dung beetles that were not exposed to deltamethrin (50±0.76), *D. gazella* exposed to LD buried 3 fold (35±0.5) less brood balls, and those exposed to MD buried 6 fold (17.4±0.39) less brood balls where as beetles exposed to HD buried the 7 fold (10.6±0.48) less number of brood balls, after 10 days. A similar trend was observed for the beetles after 20 days, where beetles exposed to LD buried 4 fold (133±0.73) less brood balls, those exposed MD buried 5 fold (68.6±0.64) less brood balls, followed by HD with 7 fold (42±0.42) less brood balls buried in comparison to control beetles which buried 139.2±0.45 brood balls. After 30 days of exposure, *D. gazella* buried 155.6±0.83 brood balls, however, after exposure to LD they buried 2 fold (129.2±1.2) less brood balls, beetles exposed to MD buried 6 fold (53.6±0.87) less brood ball, where as those exposed to HD buried 8 fold (37.6±1.34) less number of brood balls. Across all levels of dose and days of exposure, *D. gazella* buried least number of brood balls on exposure to HD for 30 days.

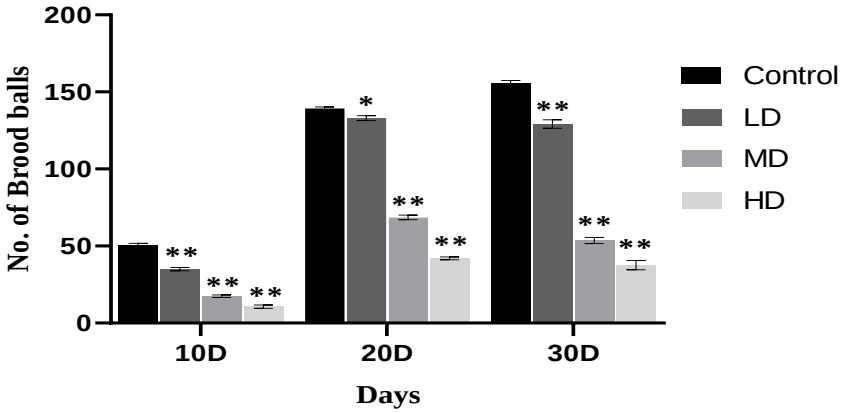

Figure 4.2: Mean±SEM number of brood balls formed by *D. gazella* after 10th, 20th and 30th day of exposure to Deltamethrin. (**p<0.5, **p<0.01*)

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

While *D. gazella* sensitivity to deltamethrin was affected by altered nesting and brood ball making, results of neurotransmitters also showed a time and dose dependent significant reduction in the level of biogenic amines (DA and 5-HT), along with a reduced AChE activity as well as reduced NO content (**Table 4.7**) in the brain of both-male and female. The results showed that exposure to LD, MD, and HD, reduced DA levels to 5-8%, 19-27% and 18-21% in comparison to control after 10 days in *D. gazella*. Similar trend was observed after 20 days, where the beetles showed 2-4%, 14-17%, and 15-20% reduced DA compared to control, whereas after 30 days, DA levels reduced to 12-16%, 20-22%, and 23-26% reduced DA in comparison to beetles not exposed to deltamethrin (**Fig. 4.3**). Thus, HD shows the most reduced level of DA in both male and female after 30 days. Similarly, a decreased level of 5-HT was also observed for all 10, 20 and 30 days of exposure to deltamethrin, and the HD caused 22% reduced level of 5-HT in the brain of both the male and female after 30 days of being exposed (**Fig. 4.4**). Further, toxic potential of deltamethrin was also proved from the results of AChE activity in *D. gazella*. Results bared a significant decrease of 31-33% after 30 days of exposure to HD (**Fig. 4.5**). Two-way analysis of variance showed that the deltamethrin treatment caused a time dependent inhibition of AChE. Furthermore, the NO level was also measured and was found to decrease significantly in a time and dose dependent manner. The beetles exposed to HD after 10 days showed 77-85% decline, and that after 20 days showed 66-75% decline, whereas after 30 days, 75-79% decline in the level of NO was obtained from the brain, in comparison to the control beetles (**Fig. 4.6**). Overall, reduction in NTs even at low concentration is suggestive of deltamethrin induced neurotoxicity and is linked to behavioural alterations.

Table 4.7: Brain neurotransmitter levels (Mean±SEM) in the male and female *D. gazella* on exposure to deltamethrin after 10th, 20th, and 30th day.

Days	Individuals	Doses	DA	5-HT	AChE	NO
10 Days	Male	Control	199.36±0.15	93.581±1.44	0.0397±0.002	4.24±0.12
		LD	182.867±3.78**	89.75±1.06	0.0393±0.002	3.42±0.25*
		MD	143.603±0.585**	71.23±0.76**	0.038±0.002	1.62±0.23**
		HD	159.33±1.84**	68.9±3.33**	0.0367±0.003	0.62±0.02**
	Female	Control	175.86±1.63	83.57±0.67	0.0404±0.004	4.32±0.03
		LD	166.25±1.3	74.45±2.59*	0.0397±0.002	3.54±0.2*
		MD	144.6±4.826**	61.34±1.88**	0.0386±0.002	2.14±0.3**
		HD	149.75±2.5	65.13±3.37**	0.0383±0.002	1.01±0.2**
20 Days	Male	Control	211.385±2.04	99.18±1.54	0.0514±0.011	5.04±0.02
		LD	209.8±3.07	99.97±2.877	0.0496±0.001	3.54±0.09**
		MD	178.343±1.924**	74.89±2.39**	0.0416±0.004	2.04±0.3**
		HD	171.511±3.431**	81.7±2.002**	0.0387±0.003	1.44±0.18**
	Female	Control	218.84±2.04	84.17±0.97	0.056±0.002	5.104±0.04
		LD	214.24±4.42	79.26±0.398	0.053±0.004	3.68±0.103**
		MD	191.76±6.85**	70.54±0.125**	0.043±0.002	2.68±0.27**
		HD	187.297±4.161**	71.496±0.95**	0.041±0.003	2.07±0.131**
30 Days	Male	Control	206.36±1.66	101.7±2.66	0.0579±0.003	5.268±0.07
		LD	184.483±3.09**	109.6±4.712	0.052±0.003	3.49±0.18**
		MD	166.757±3.29**	74.103±2.85**	0.0437±0.004*	2.04±0.21**
		HD	162.273±2.12**	79.76±3.92	0.0397±0.003**	1.13±0.06**
	Female	Control	219.51±2.99	85.897±0.62	0.062±0.001	5.335±0.05
		LD	186.93±5.02**	85.53±3.76	0.0577±0.003	3.69±0.23**
		MD	174.243±2.84**	69.013±3.102**	0.045±0.002*	2.4±0.39**
		HD	165.753±2.88**	67.103±5.933*	0.0413±0.003**	1.318±0.05**

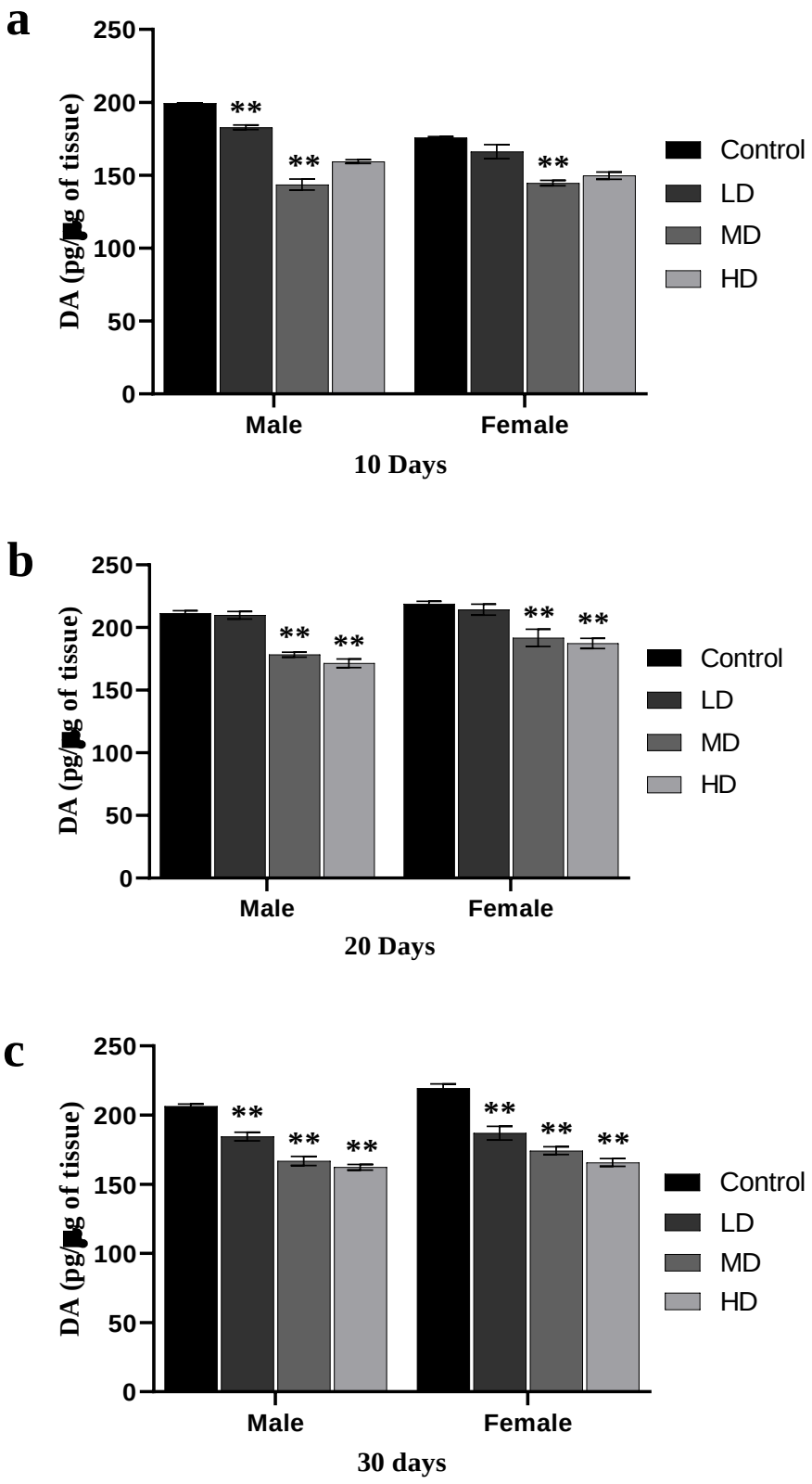

Figure 4.3: Brain DA levels in the male and female *D. gazella* on exposure to deltamethrin after (a) 10 days (b) 20 days and (c) 30 days. (**p<0.05 **p<0.01)*

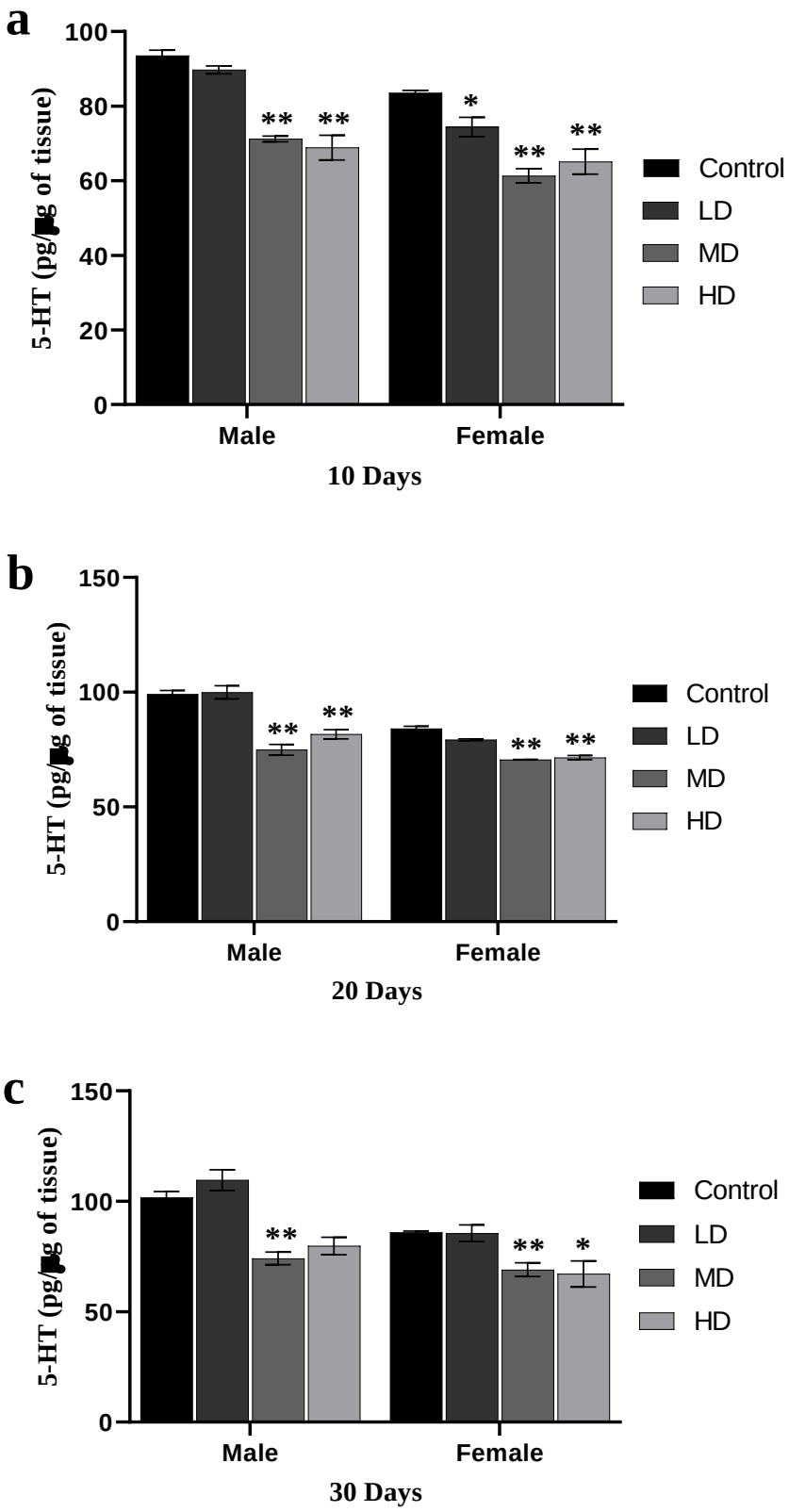

Figure 4.4: Brain 5-HT levels in the male and female *D. gazella* on exposure to deltamethrin after (a) 10 days (b) 20 days and (c) 30 days. (**p<0.05 **p<0.01)*

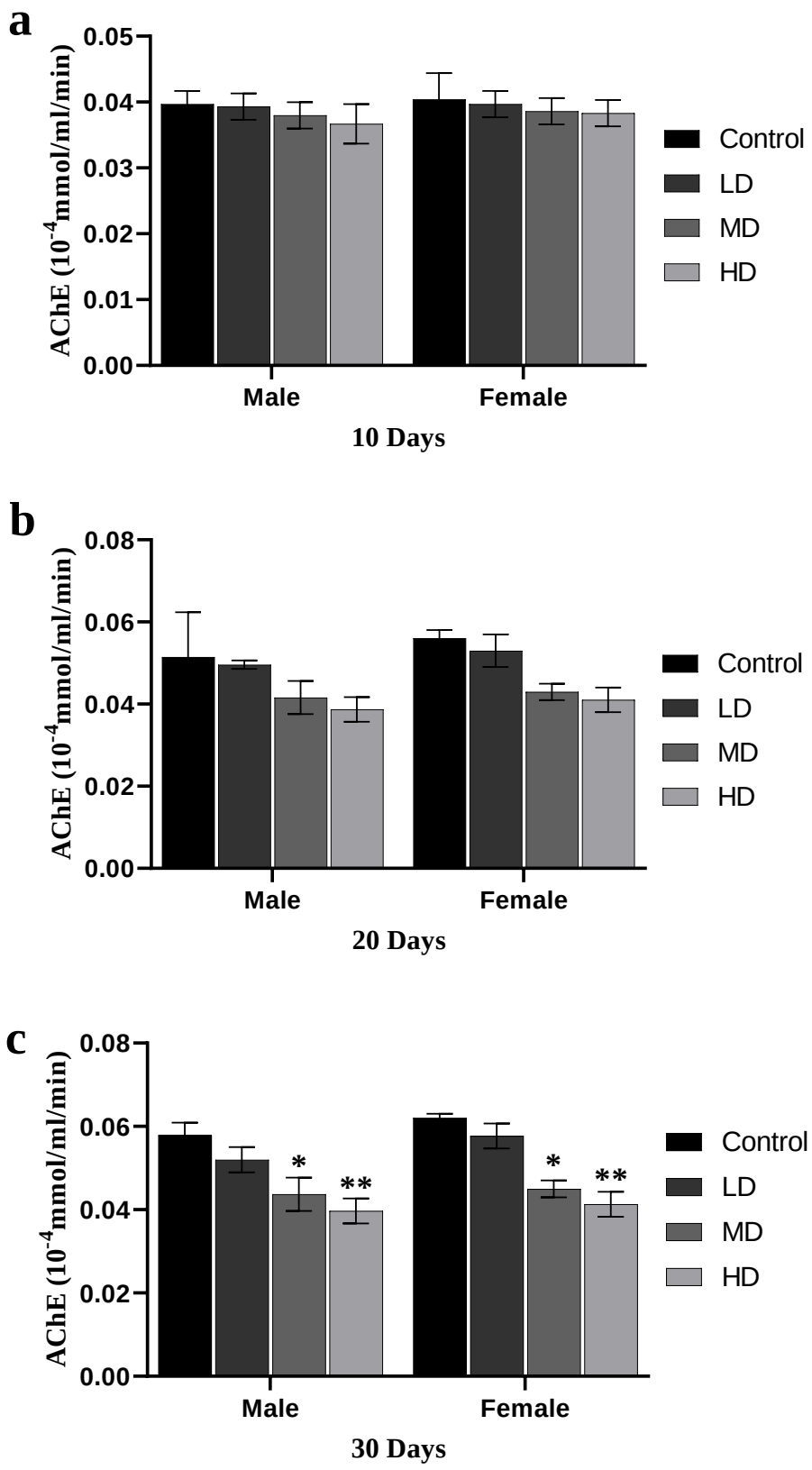

Figure 4.5: Brain AChE activity in the male and female *D. gazella* on exposure to deltamethrin after (a) 10 days (b) 20 days and (c) 30 days. (*$p<0.05$ **$p<0.01$)

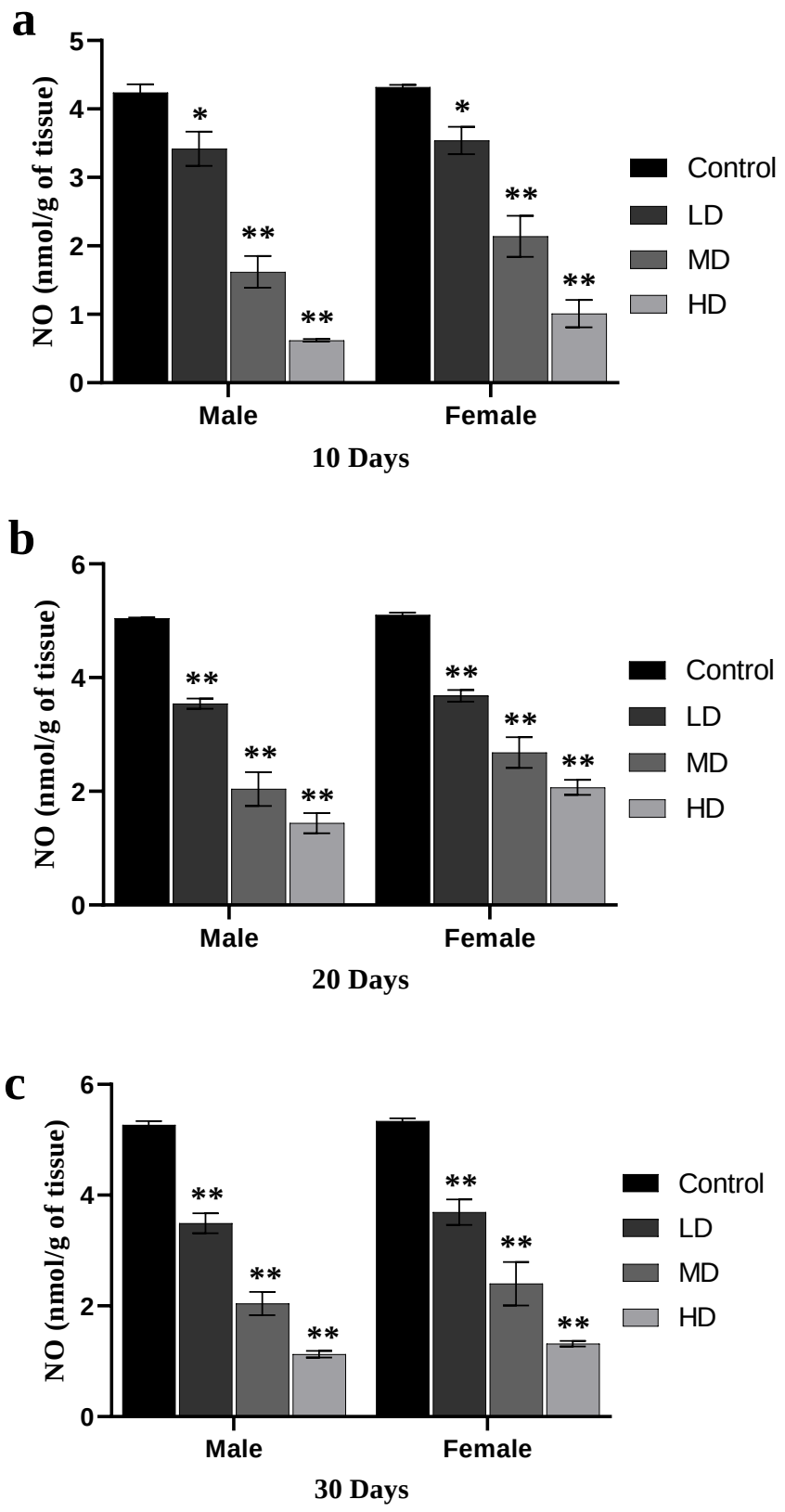

Figure 4.6: Brain NO levels in the male and female *D. gazella* on exposure to deltamethrin after (a) 10 days (b) 20 days and (c) 30 days. *(*p<0.05 **p<0.01)*

Alteration in Neurotransmitter Biosynthesizing Enzymes on Exposure to deltamethrin

After assessing the toxic potential of deltamethrin in altering the NTs synthesis, mRNA gene expressions of NT synthesizing enzymes (ddc, 5-htpdc, chAt, and nos) were assessed since they play an important role in synthesis of the NTs. The results of NT synthesizing enzymes showed a significant ($p<0.01$) time and dose dependent decline in both male and female *D. gazella* (**Table 4.8; Fig. 4.7-4.10**), suggesting reduced synthesis of neurotransmitters in response to the toxicity generated by deltamethrin even at lower concentrations, which thereby altered the neural regulation in *D. gazella*.

Table 4.8: Fold change expression of Neurotransmitter synthesizing enzymes (Mean±SEM) in the male and female *D. gazella*, after exposure to deltamethrin on 10th, 20th and 30th day.

Days	Individuals	Doses	ddc	5-htpdc	chat	nos
10 Days	Male	Control	3.402±0.527	4.401±0.479	3.564±0.555	5.932±0.535
		LD	3.272±0.520	3.808±0.988	3.218±0.948	4.390±0.729
		MD	2.651±0.476	1.702±0.599*	2.576±0.912	3.509±1.562
		HD	1.939±0.805	1.425±0.641*	1.681±0.732	1.209±0.368*
	Female	Control	2.933±0.077457	2.911±0.218	5.243±0.057	7.307±0.804
		LD	1.540±0.109	1.563±0.198	5.089±0.239	5.346±0.560
		MD	1.197±0.009	1.169±0.287	3.869±0.352	3.965±0.321
		HD	0.434±0.103*	0.569±0.051	2.281±0.599*	1.641±0.181**
20 Days	Male	Control	11.929±0.726	13.798±0.576	10.163±0.484	10.280±0.583
		LD	8.128±0.818**	11.944±0.577**	8.093±0.675**	7.857±0.876
		MD	4.505±0.687**	8.955±0.665**	5.337±1.209**	5.078±0.664**
		HD	1.804±0.227**	5.974±0.344**	2.357±0.976	3.303±0.554**
	Female	Control	15.542±0.288	13.319±0.201	11.439±0.057	12.978±0.065
		LD	8.544±0.412**	6.459±0.088**	7.565±0.109**	8.528±0.192**
		MD	5.319±0.045**	3.807±0.185**	5.262±0.026**	5.249±0.037**
		HD	2.362±0.025**	2.699±0.354*	3.346±0.427*	4.723±0.395*
30 Days	Male	Control	11.468±0.129	17.625±0.766	15.352±0.318	15.711±0.786
		LD	7.226±0.198**	13.615±0.321**	15.508±0.860	10.212±0.472**
		MD	6.275±0.567**	10.335±0.319**	7.115±0.611**	8.160±1.060**
		HD	3.291±0.777**	5.483±0.615**	3.333±1.018**	3.308±1.632*
	Female	Control	16.563±0.105	14.013±0.260	16.478±0.021	17.333±0.414
		LD	8.461±0.272**	11.789±0.129**	16.595±0.668	11.773±0.013**
		MD	3.812±0.036*	4.920±0.405**	9.733±0.126**	8.645±0.397**
		HD	3.623±0.273	6.248±0.705	2.424±0.161**	4.133±0.357**

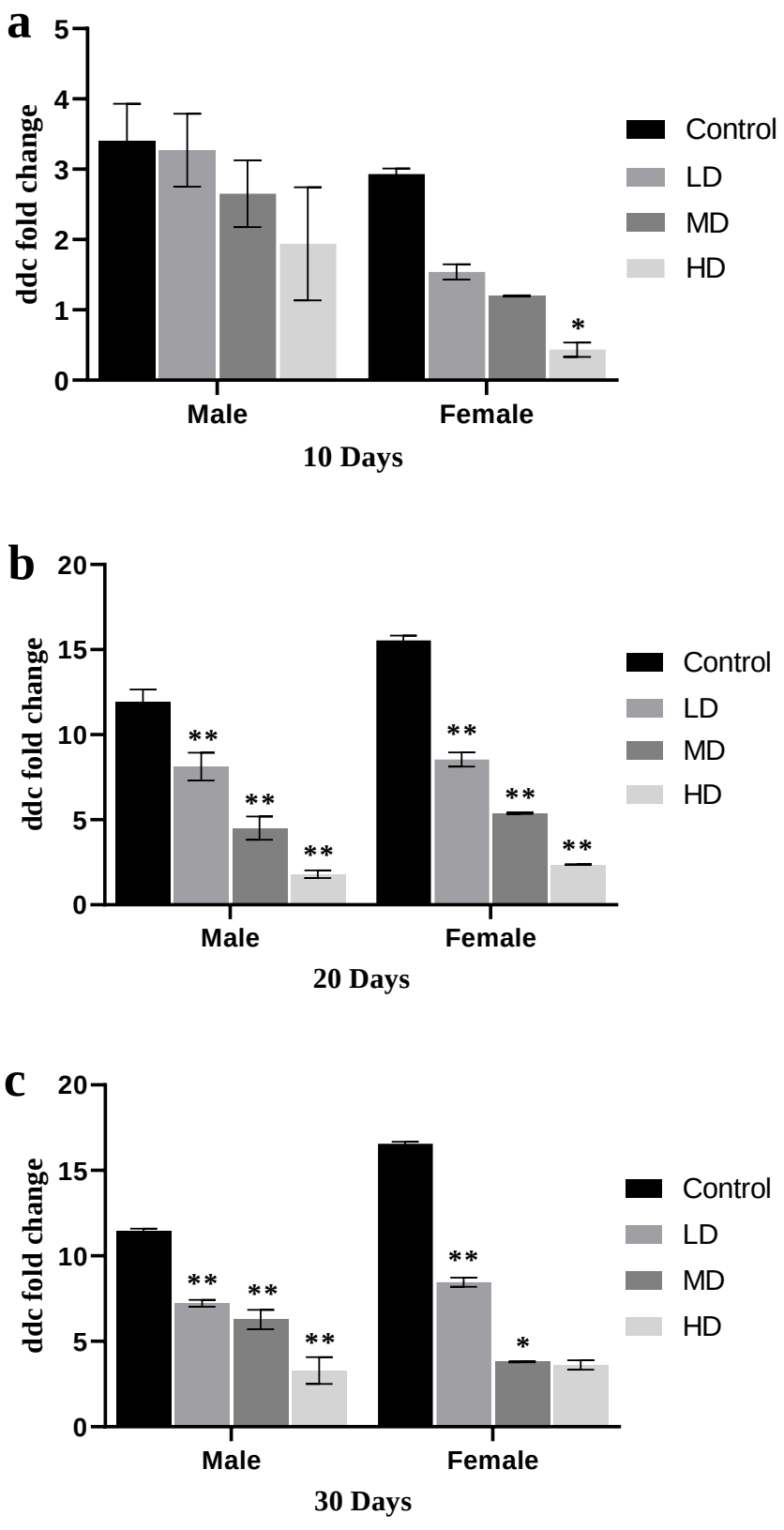

Figure 4.7: ddc fold change in the brain of male and female *D. gazella* on exposure to deltamethrin after (a) 10 days (b) 20 days and (c) 30 days. *(*p<0.05 **p<0.01)*

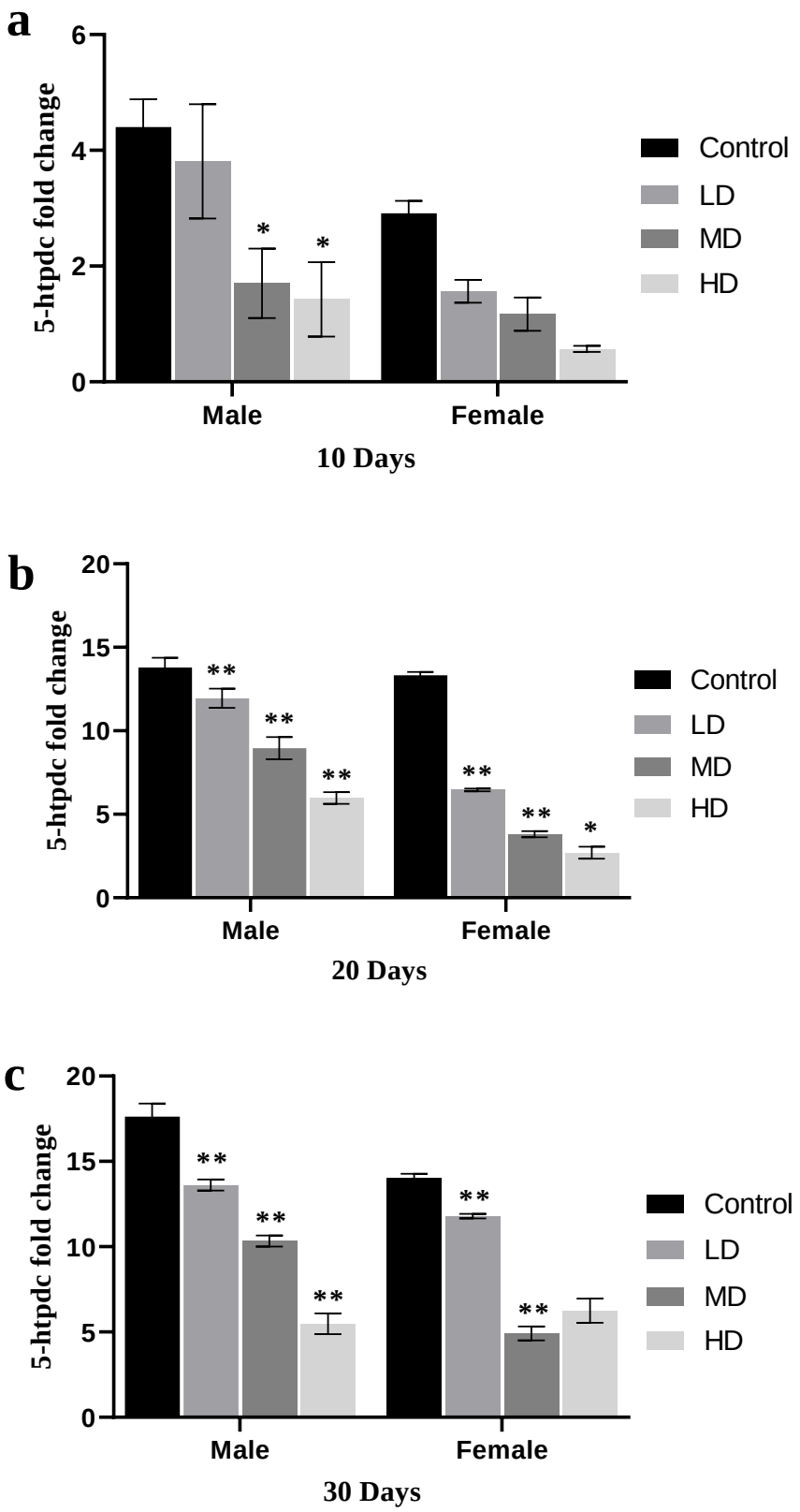

Figure 4.8: 5-htpdc fold change in the brain of male and female *D. gazella* on exposure to deltamethrin after (a) 10 days (b) 20 days and (c) 30 days. (**p<0.05 **p<0.01)*

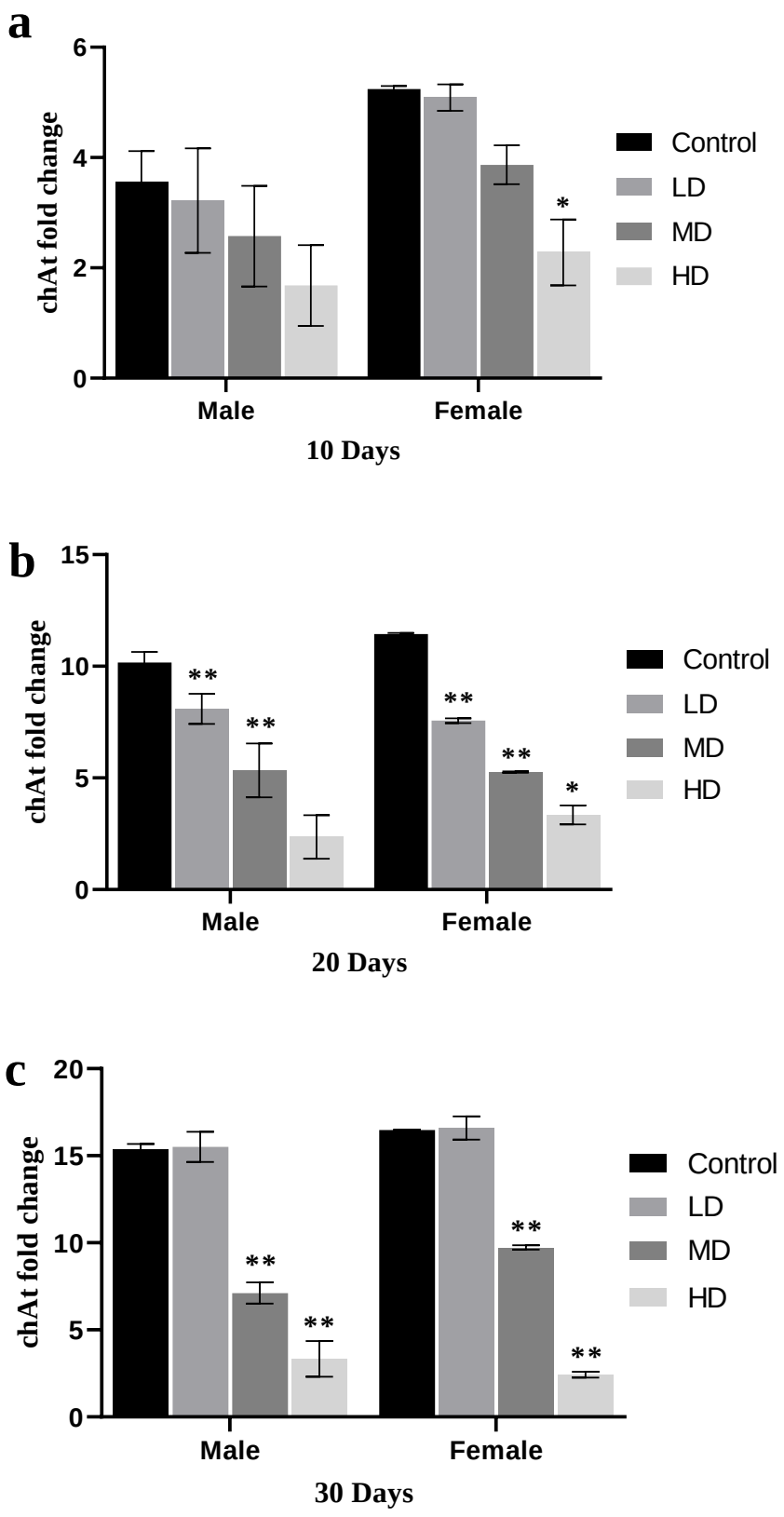

Figure 4.9: chAt fold change in the brain of male and female *D. gazella* on exposure to deltamethrin after (a) 10 days (b) 20 days and (c) 30 days. (**p<0.05 **p<0.01)*

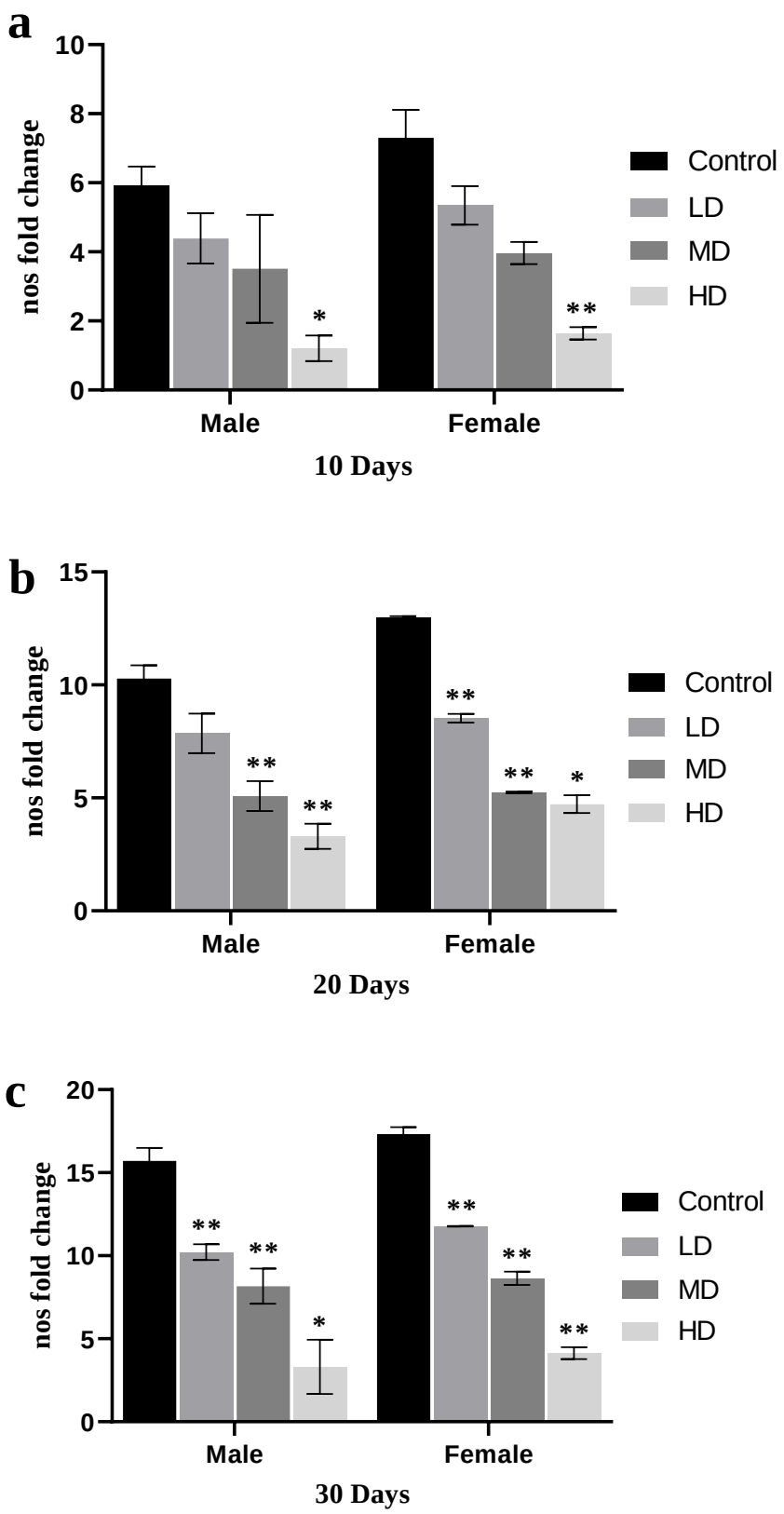

Figure 4.10: nos fold change in the brain of male and female *D. gazella* on exposure to deltamethrin after (a) 10 days (b) 20 days and (c) 30 days. (**p<0.05 **p<0.01)*

Alteration in Expression of Neuropeptides on Exposure to Deltamethrin

The expression profiles of NPs (npf, npfr, it, itr, mip) in brain of *D. gazella* was determined by qRT-PCR. Deltamethrin exposure resulted into an altered expression of NPs (**Table 4.9**). Results of mRNA gene expression revealed a significant down regulation in NPs-npf, it, mip, along with a decline in the expression patterns of its receptors- npfr, itr, in a time and dose dependent manner, with a maximum decline up to 9-fold change, on exposure to HD after 30 days was observed (**Fig. 4.11-4.15**). Thus, deltamethrin, upon exposure, triggered a substantial reduction in neuropeptide expression levels in *D. gazella*, is thereby suggestive of disturbing their usual physiological processes and influencing nesting behaviour.

Table 4.9: Fold change expression of neuropeptides (Mean±SEM) in the male and female *D. gazella* on exposure to deltamethrin after 10^{th}, 20^{th}, and 30^{th} day.

Days	Individuals	Doses	npf	npfr	it	itr	mip
10 Days	Male	Control	10.85±0.56	10.85±0.560	3.8±1.3	6.2±1.1	7.8±1.2
		LD	6.65±0.35**	5.02±0.6**	3.5±1.3	2.5±1**	4.5±1
		MD	4.46±0.27**	3.45±1.3**	1.3±0.7	2.16±1.3	2.22±1.3*
		HD	3.28±0.31**	1.57±1.5	0.7±0.5	1.89±0.5	1.8±1.5
	Female	Control	12.61±0.3	12.61±0.3	5.1±0.2	5.68±0.1	7.2±0.1
		LD	8.85±0.3**	7.83±0.1*	3.9±0.2	4.004±0.109	4.3±0.3
		MD	6.64±0.33**	4.44±0.23**	2.6±0.5	3.4±0.4	2.979±0.3**
		HD	4.40±0.21**	2.4±0.218**	1.5±0.4*	2.16±0.6**	1.9±0.1*
20 Days	Male	Control	17.8±0.63	17.8±0.6	14.7±0.5	9.23±1.4	10.5±0.8
		LD	14.48±0.8*	8.035±1.3**	10.1±1**	6.4±1.3	4.4±0.3**
		MD	11.28±0.2**	5.38±1.5**	7.2±1**	3.6±1.3*	3.9±0.6**
		HD	7.78±0.34**	1.85±1.1	4.8±0.8**	2.2±1.4*	1.6±0.7**
	Female	Control	22.05±0.24	22.051±0.2	21.40±0.1	12.7±0.3	12.8±0.3
		LD	18.83±0.22*	13.97±1.2**	16.5±0.6**	11.1±0.2	4.8±0.3**
		MD	14.74±0.9**	6.9±0.9**	7.3±0.11**	7.3±0.5*	3.7±0.1**
		HD	9.93±0.6**	4.317±0.6*	6.2±0.2**	4.1±0.2**	2.1±0.5**
30 Days	Male	Control	23.69±0.8	23.693±0.756	28.5±0.6	14±3	11±0.6
		LD	19.97±0.9	11.57±1.6**	11.2±0.7**	7.5±1.3*	3.7±0.8**
		MD	14.46±1.2**	5.367±1.5**	5.2±1.2**	4.2±0.7*	2.1±0.6**
		HD	8.84±1.1**	1.27±1.1**	2.53±0.8**	2.9±1.9	1.6±0.7**
	Female	Control	28±0.1	28.086±0.124	43.1±0.2	18.3±0.7	17.7±0.1
		LD	21.05±0.9**	12.6±0.7**	14.3±0.3**	11.8±0.4	3.7±0.5**
		MD	17.3±0.9**	6.8±0.8**	6.3±2.02**	8.9±2.3*	2.6±0.1**
		HD	11.26±0.9**	3.2±0.4**	4.45±2.4**	7.2±1.91**	1.6±0.3**

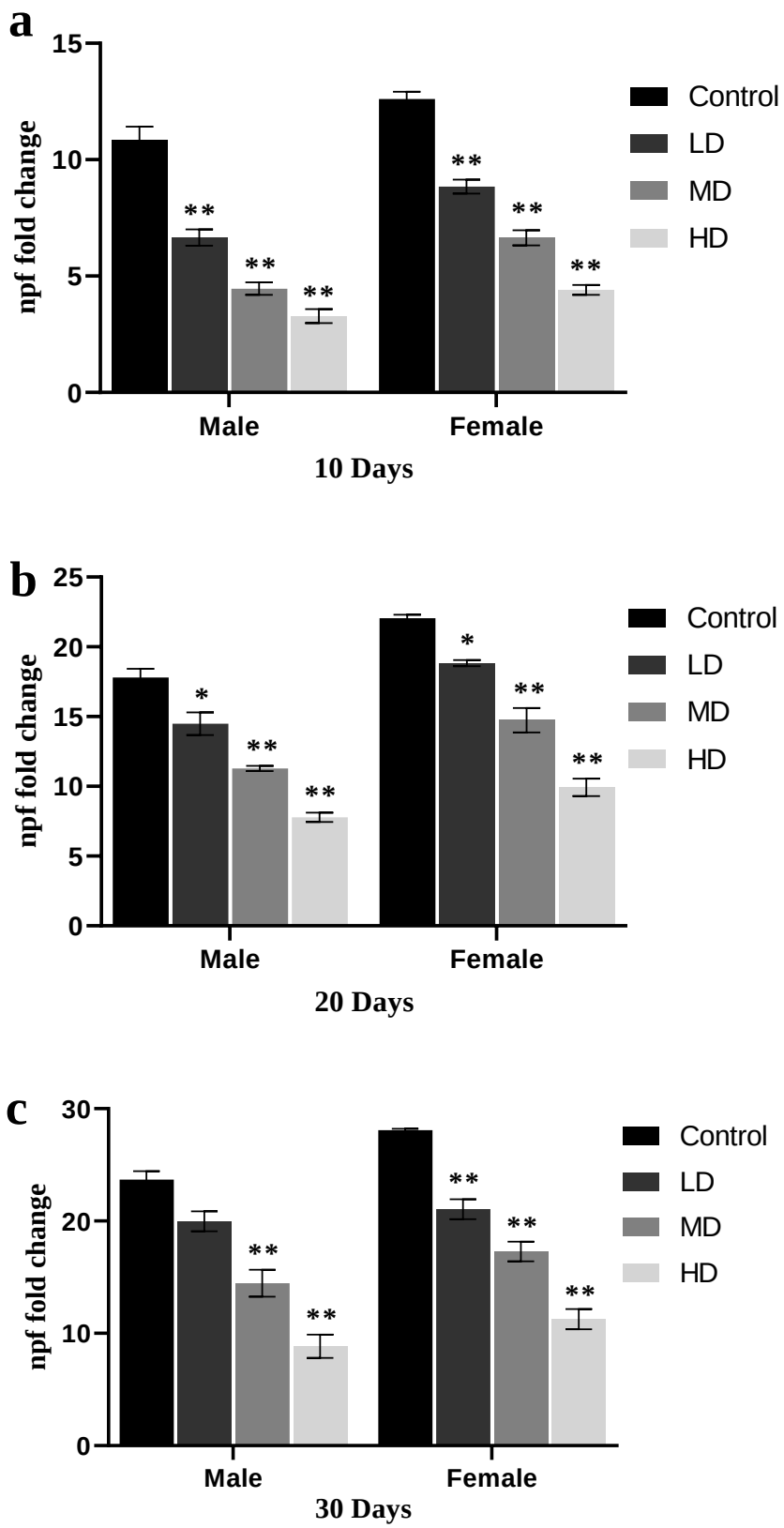

Figure 4.11: npf fold change in the brain of male and female *D. gazella* on exposure to deltamethrin after (a) 10 days (b) 20 days and (c) 30 days. (**p<0.05 **p<0.01)*

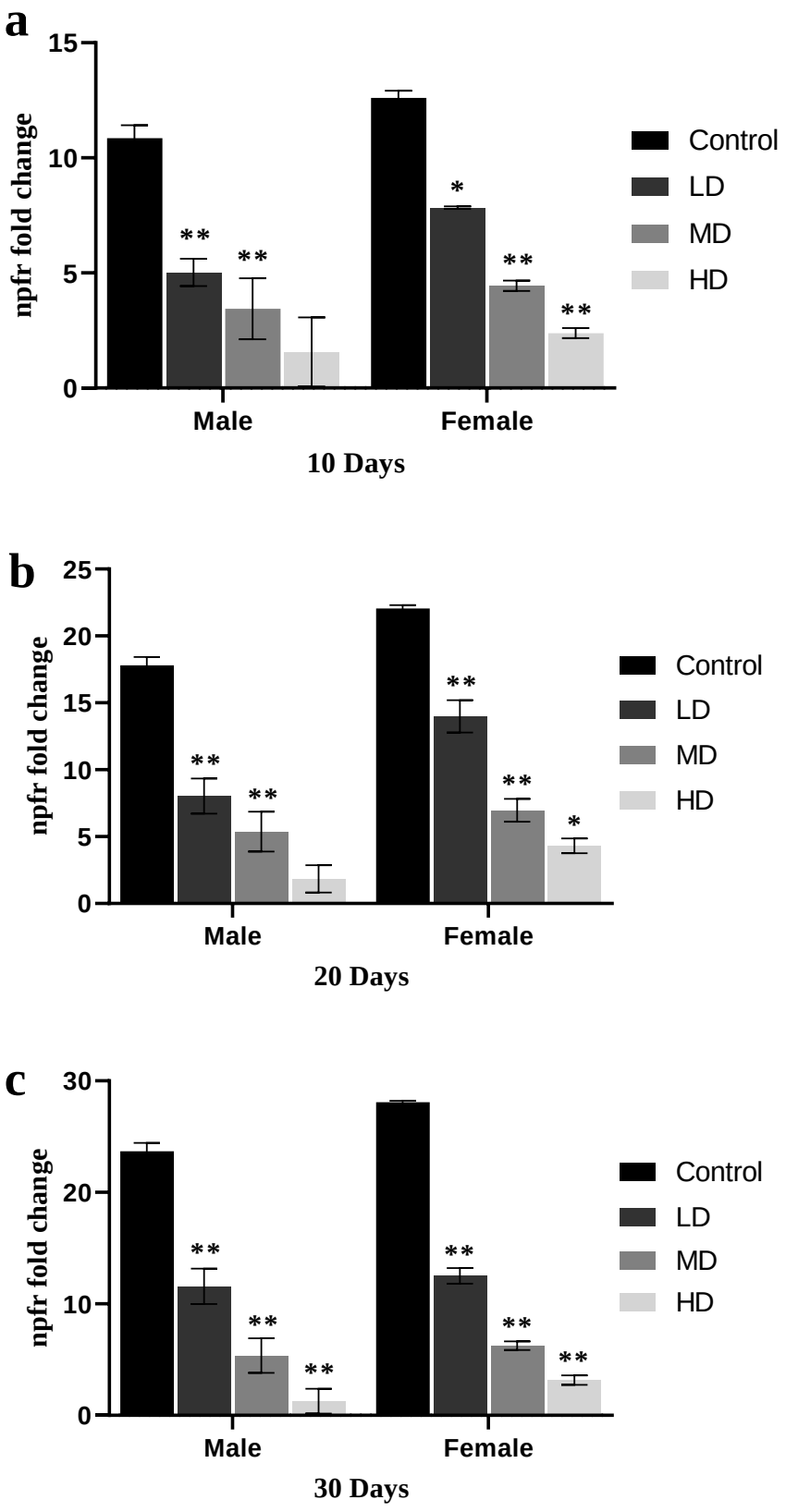

Figure 4.12: npfr fold change in the brain of male and female *D. gazella* on exposure to deltamethrin after (a) 10 days (b) 20 days and (c) 30 days. (**p<0.05 **p<0.01)*

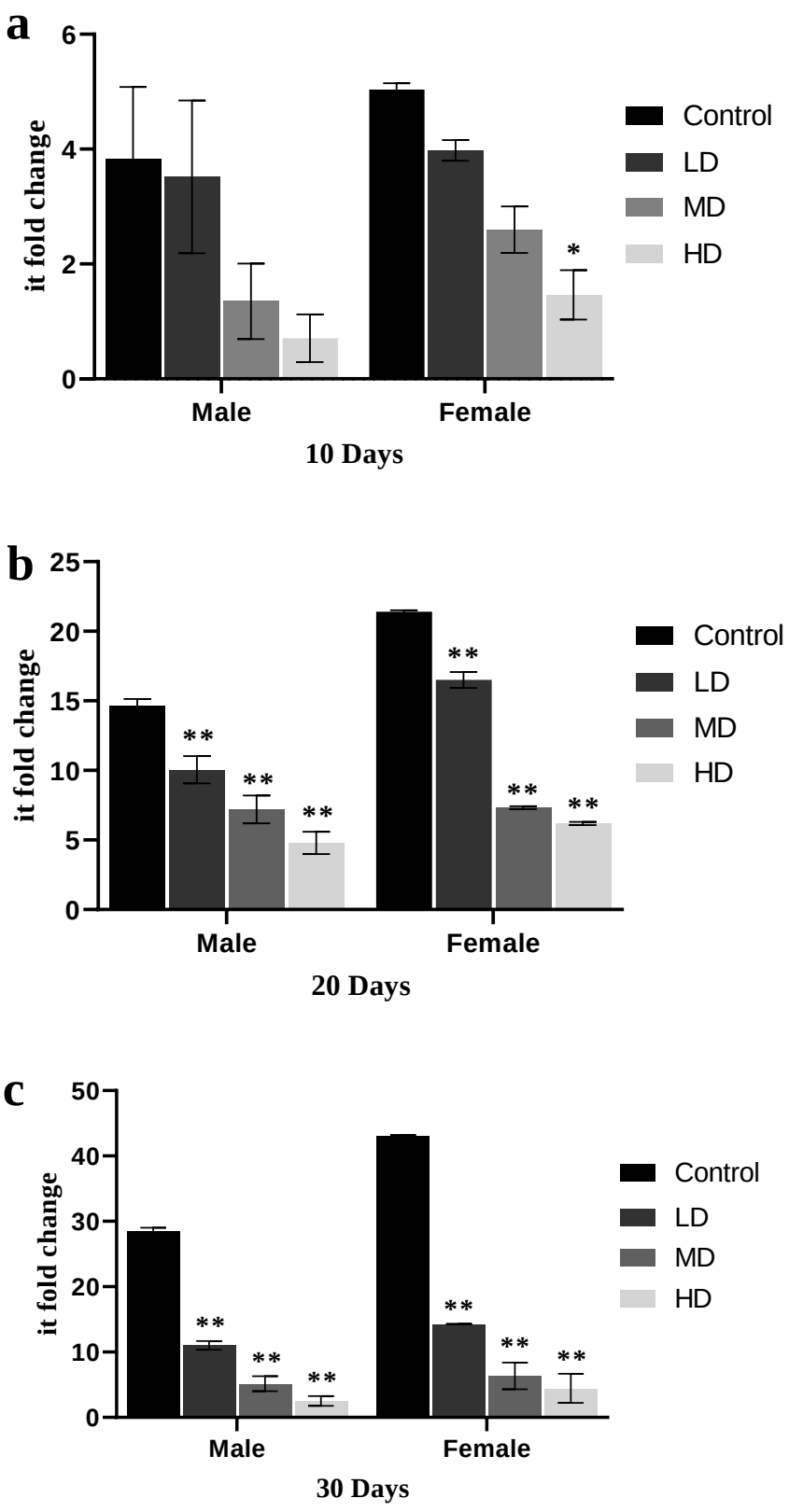

Figure 4.13: it fold change in the brain of male and female *D. gazella* on exposure to deltamethrin after (a) 10 days (b) 20 days and (c) 30 days. *(*p<0.05 **p<0.01)*

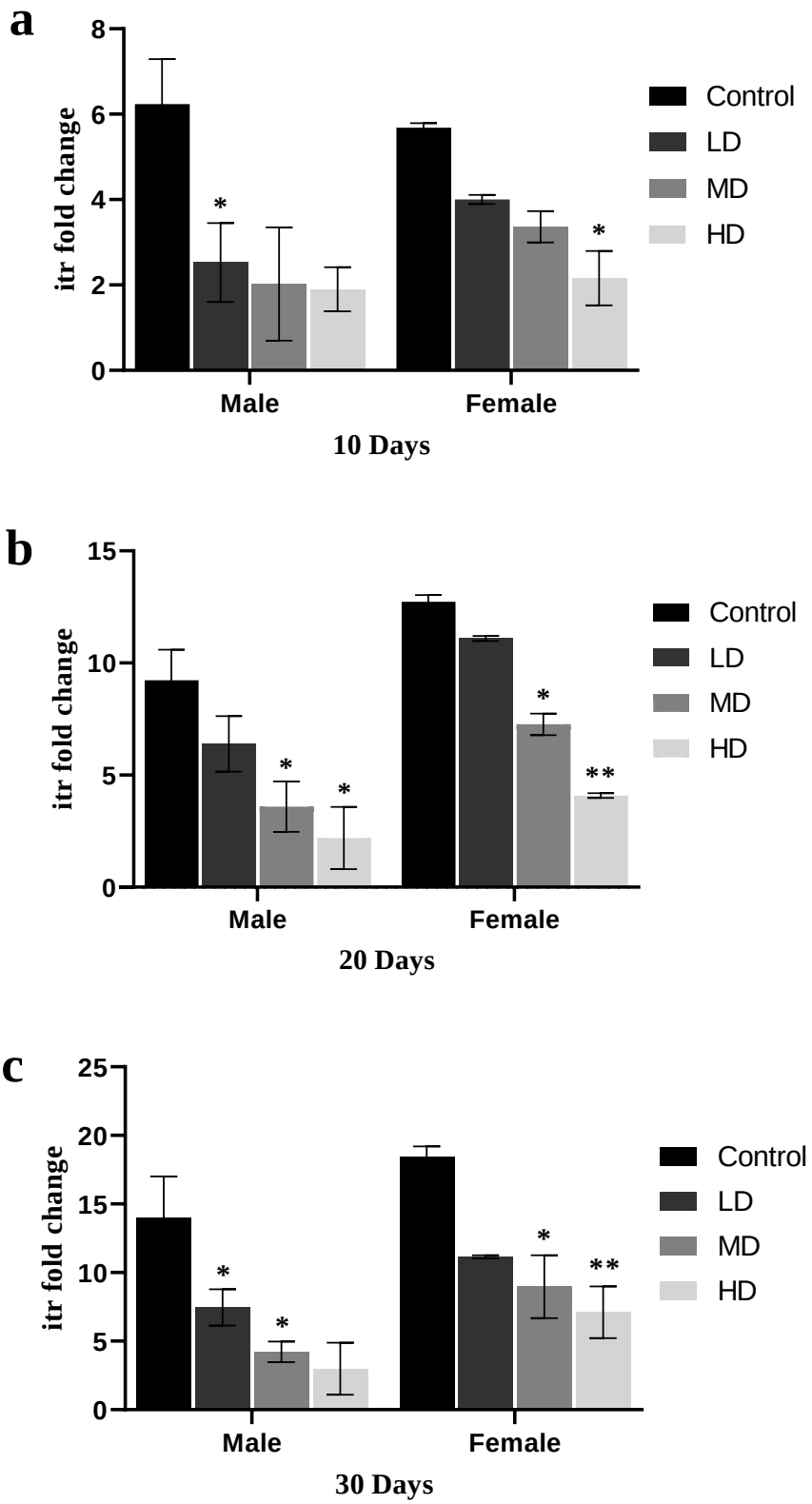

Figure 4.14: itr fold change in the brain of male and female *D. gazella* on exposure to deltamethrin after (a) 10 days (b) 20 days and (c) 30 days. (**p<0.05 **p<0.01)*

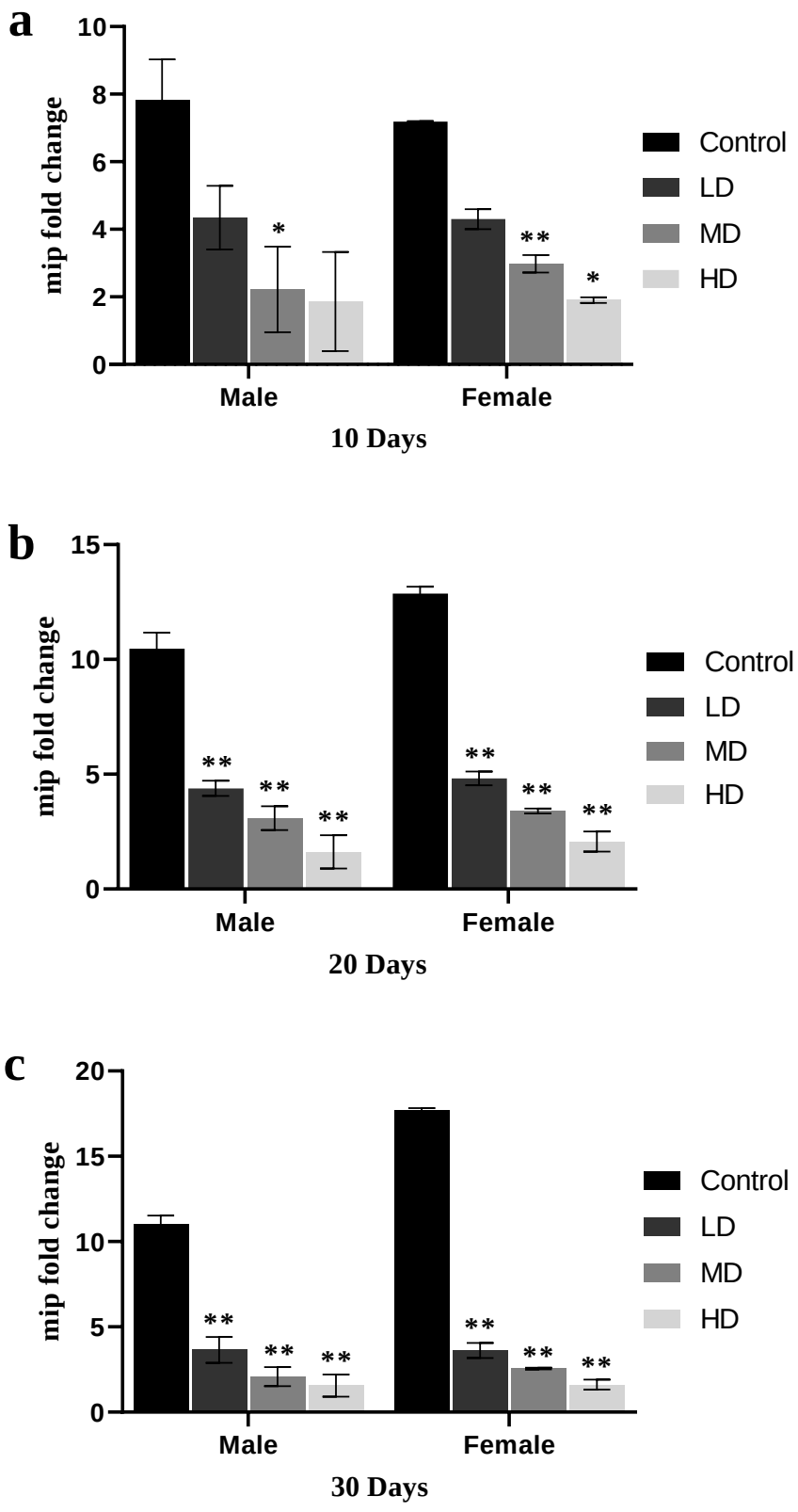

Figure 4.15: mip fold change in the brain of male and female *D. gazella* on exposure to deltamethrin after (a) 10 days (b) 20 days and (c) 30 days. (**p<0.05 **p<0.01)*

4.4 DISCUSSION

The selective toxicity of most insecticides, whereby they exhibit greater toxicity towards insects compared to mammals, can be attributed to their distinct mechanisms of action on target receptors and channels. Pyrethroids have the ability to interfere with the regular functioning of nerve cells by specifically targeting the axon, a crucial area involved in nerve signal transmission. The mechanism by which they exert their effects involves the inhibition of sodium channels, which serve as the regulatory switch for nerve cells. This inhibition is achieved by impeding the closure or deactivation of these channels, as described in studies conducted by Costa et al., (2008), Seralini and Jungers, (2021), and Toprak et al., (2021). The mechanism of action of the insecticide involves specifically affecting the nervous system of insects, which comprises a network of interconnected cells known as neurons. Within this network, electrical charges known as impulses propagate along the neurons, transmitting signals from one nerve cell to another across the synapse, a gap between neurons. This transmission is facilitated by a chemical messenger called a neurotransmitter (Suiter and Scharf, 2009). The mushroom bodies of the brain of insects have been identified as potential regulators and coordinators of locomotor activity (Mizunami et al., 1998; Besson and Martin, 2005; Blenau and Thamm, 2011). Previous studies have investigated the impact of insecticide exposure on insect behaviours, examining both lethal and sub lethal dosages (Mazzi and Dorn, 2012; Gutiérrez et al., 2017; Müller, 2018; Shaw et al., 2019; Vander Pan et al., 2019 Parkinson et al., 2020).

Deltamethrin is a synthetic pyrethroid insecticide that is frequently employed in fields to manage pest populations. Deltamethrin has been found to cause significant physiological harm to both target and non-target insects, as evidenced by studies conducted by Cutler, (2013), Rehman et al., (2014), and Müller, (2018). According to Dai et al., (2010) and Teder and Knapp, (2019), there have been reports indicating a reduction in the fecundity of female honeybees, parasitoid wasps, and cockroaches. Additionally, studies by Oliveira et al., (2018) and Yang et al., (2020) have shown that this phenomenon also impairs larval development in honeybees and the wasp species *Trichogramma achaeae*. Furthermore, Reissert-Oppermann et al., (2019) have observed that it inhibits moulting processes in the stable fly *Stomoxys calcitrans*. In the present study, in an attempt to have an insight into the nesting behaviour, exposure of deltamethrin was reported to reduce the number of brood balls in a time and dose dependent manner, indicating considerable loss of functional efficiency and reducing

reproductive potential over time (Manning and Cutler, 2020). Effects of the insect growth regulator, Methoprene, on *Onthophagus taurus* by Niño et al., (2009), was reported to reduce progeny survival, however, there was no reduction in number of brood balls formed, and have opined that reduction in the progeny survival was due to altered food quality on exposure. Furthermore, Ishikawa and Iwasa, (2019) in their studies have reported a significant reduction in the numbers of brood balls on exposure of ivermectin in species of Scarabaeidae and Geotrupidae. Thus, our results are in agreement to the earlier reported studies (Dadour et al., 2000; Errouissi et al., 2001; Iwasa et al., 2007; Gonzalez-Tokman et al., 2023, 2017; Martínez et al., 2017; Mauduit et al., 2021).

Pyrethroid compounds in insects provoke consecutive impulses which impede the nerve physiology and ultimately results in the death (Ju et al., 2023). Deltamethrin exposure on insects like dung beetles are complex and can vary based on factors such as dosage, exposure duration, and species-specific sensitivity and in turn alters the levels of neurotransmitters and lead to changed physiology and behaviour (Wardhaugh et al., 1998; Cavallaro et al., 2023). Neurotransmitters, which serve as signalling molecules, have the ability to modify the functioning of the nervous system. They have ability to induce alterations in cellular physiology, such as adjustments in the electrical potential of neurons and regulation of ion channels. The aforementioned alterations might potentially provide enduring effects, extending over a significant duration following the original encounter, particularly within the context of the growing nervous system. This assertion is substantiated by research undertaken by Slikker et al., (2005), Grandjean and Landrigan, (2006, 2014), and Smirnova et al., (2014). The significance of neurotransmitter signalling in both the developing and adult nervous system has been emphasized by Fritsche et al., (2018). These neurotransmitters are synthesized within nerve cells from precursor molecules through specific enzymatic pathways. These enzymes play a critical role in converting precursor molecules into active neurotransmitters. Sub lethal concentrations of deltamethrin are known to impact the activity of enzymes responsible for synthesizing neurotransmitters. The exposure can lead to decreased enzyme activity, thereby reducing the production of neurotransmitters. Alterations in neurotransmitter levels and the enzymes involved in their synthesis have a significant effect on a dung beetles behaviour, coordination, and physiological functions.

Prior studies have demonstrated a correlation between deltamethrin-induced neurotoxicity and changes in behaviour (Ogut et al., 2019). Furthermore, these behavioural modifications have been found to be associated with variations in neurotransmitter levels (Khalil et al., 2022). According to Soderlund, (2012), the interaction between the Deltamethrin molecule and the α subunit of the channel results in a state of continuous openness, hence inhibiting its ability to undergo closure. Consequently, the entry of sodium ions into the nerve cells leads to a state of prolonged depolarization. Among the several neurotransmitters, acetyl cholinesterase (AChE) is regarded as a significant molecular target for numerous pesticides (Gomes et al., 2022), such as deltamethrin, which effectively decreases the enzymatic activity of AChE by altering the active binding site of the substrate (Iwan and Golec, 2020). Williamson et al., (2013) found coumaphos, aldicarb, chlorpyrifos, and donepezil to be toxic and AChE inhibitors in honey bees; Badiou and Belzunces, (2008) have suggested AChE to be a pertinent biomarker for detecting exposure of pyrethroids. Moreover, the inhibition of this enzyme results in the prevention of the metabolic breakdown of the neurotransmitter acetylcholine, hence impedes the polarization process of the post-synaptic membrane. Choline acetyltransferase (ChAT), the enzymatic catalyst responsible for the synthesis of acetylcholine, plays a significant role either as an alternative target site for pesticides or as a component that promotes insect survival following exposure to insecticides. Hence, the correlation between the activities of acetyl cholinesterase (AChE) and choline acetyltransferase (chAt) might elucidate the remarkable toxicological phenomena, as evidenced by the studies conducted by Grünewald and Siefert, (2019), and Johnson et al., (2021). Therefore, the current study aimed to comprehend this unusual interaction. The sub-lethal exposure of deltamethrin resulted into a decreased activity of AChE along with the expression of chat gene, in a time and dose dependent manner in *D. gazella*, probably inducing, co-inhibition of both the enzyme and chat gene. Interaction of AChE and chat gene till date have been well explored for the resistance mechanism in number of insects (Menozzi et al., 2004; Li et al., 2013; Bu et al., 2015; Aljedani, 2021; Sakthivel et al., 2022), however, its role in other physiological functions is not well investigated. Ours is the first study which suggests the co-inhibition of both AChE and chat gene having a role in altered neurophysiology of *D. gazella* probably causing behavioural impairments leading to reduced reproductive efficiency.

The neurotoxicity caused by pyrethroids is attributed to their capacity to trigger the production of free radicals, which plays a crucial role in the degeneration of dopaminergic neurons. According to previous research, it has been observed that deltamethrin has an initial binding affinity towards the Phe1519 residue. However, it does not induce the required conformational modifications that are essential for the development of the binding site (Tan et al., 2005). As a result, this leads to a deceleration in the rate of channel activation and a subsequent alteration in the membrane potential (Singh et al., 2012). The binding of deltamethrin leads to the prolonged opening of sodium channels, facilitating an increased influx of sodium ions and subsequent depolarization of the neuronal membrane. This process induces a state of hyper excitability, ultimately preventing the production of action potentials. Dopamine transporters play a crucial role in the proper functioning of dopamine and are important for the efficient termination of dopamine neurotransmission by the quick absorption of dopamine into the presynaptic terminal (Beggs et al., 2005). Numerous researches has provided evidence indicating that changes in the expression of Dopamine transporters can significantly impact the susceptibility of dopamine neurons to pyrethroids, such as deltamethrin. Hence, the exposure to deltamethrin has the potential to enhance the vulnerability of dopamine neurons to toxic damage by augmenting their absorption via Dopamine transporters (Elwan et al., 2006). The specific method by which pyrethroids affect dopaminergic sensitivity in *D. gazella* remains unclear. Therefore, comprehending the mechanism via which deltamethrin affects dopamine (DA) transmission might offer valuable insights into its correlation with exposure and parental behaviour in *D. gazella*.

Dopamine (DA) is a neurotransmitter that is generated by the enzyme DDC from the aromatic amino acid tyrosine in insects. It is highly prevalent in the central nervous system (Daubner et al., 2011). The central nervous system of insects is responsible for integrating locomotion, cognition, and endocrine secretion, while also serving a crucial role in the regulation of movement, motivation, reward, and several other significant processes (Coban and Filipov, 2007; Draper et al., 2007; Wicker-Thomas and Hamann, 2008). Studies have suggested that exposure to insecticides can lead to changes in dopamine levels in insects, affecting their behaviour and physiology. For example, altered dopamine levels might lead to impaired movement, foraging, mating, and other essential behaviours (Dasari and Cooper, 2004; Mustard et al., 2010). The role of dopamine in modulating behaviour in honeybees is characterized by Mustard et al., (2010), and has confirmed perturbations in dopamine

signaling. Additionally, Figueira et al., (2017) conducted a study examining the impact of atrazine on the behaviour and dopaminergic neurotransmission of *Drosophila melanogaster*, specifically focusing on the impacts during embryonic and larval development. The researchers found that exposure to atrazine resulted in a reduction in both dopamine (DA) content and mRNA gene expression of the ddc gene. In a study conducted by Xu et al. (2015), a comprehensive analysis was performed to identify genes that may have a role in neurotransmitter production and transport in the *Chilo suppressalis*, a species of rice striped stem borer.

In the present study, deltamethrin exposure on *D. gazella* has resulted in a significant reduction in DA content as well as mRNA gene expression of DA synthesizing enzyme i.e., ddc in both male and female. Previous research by Xiang et al., (2012) has documented a correlation between diminished dopaminergic transmission and a decline in ddc gene expression within the central nervous system of animals. Consequently, it may be inferred that the exposure to deltamethrin leads to a decrease in dopamine (DA) levels, possibly due to alterations in dopaminergic neurotransmission. This reduction in DA levels has been previously seen to result in decreased locomotor activity in both mutant larval and adult *D. melanogaster* (Majeed et al., 2016). Moreover, deltamethrin is generally a good "knockdown" agent due to its ability to induce repetitive firing in axons, resulting in restlessness, un-coordination and hyperactivity followed by prostration and paralysis (Draper et al., 2007). Thus, exposure to deltamethrin has altered the nesting behaviour of *D. gazella*, which involves tunnelling, reproduction, brood ball formation, as well as biparental care and is in agreement with the earlier reported studies in various insects (França et al., 2017).

The effect of 5-HT and its synthesizing enzyme 5-htpdc on various aspects of insect physiology and behaviour has been extensively studied. Previous research has demonstrated its involvement in circadian behaviour, sleep regulation, feeding patterns (Gasque et al., 2013, Haselton et al., 2009), learning and memory processes (Sitaraman et al., 2008, Sitaraman et al., 2012), gregarious and swarming behaviour (Anstey et al., 2009), aggression levels (Dierick and Greenspan, 2007), olfactory responses (Zhang and Gaudry, 2016), haemocyte phagocytosis (Qi et al., 2016), and other physiological processes. In the current study, an investigation was conducted to gain understanding of the involvement of 5-HT and its synthesizing enzyme 5-htpdc in the nesting behaviour of *D. gazella* when exposed to Deltamethrin. The findings revealed a noteworthy decrease in the levels of both 5-HT and 5-

htpdc. In a recent study conducted by Li et al. (2022), the researchers investigated the effects of CRISPR-Cas9 technology on mutant *Aedes aegypti*. The findings of their research revealed notable alterations in growth and development, including body contraction, decreased mobility, and diminished stress response to external stimuli. Additionally, Liu et al., (2013) conducted a study on the impact of reserpine on the reproductive capabilities of the stable fly species *Stomoxys calcitrans* (L.). Their findings provide evidence that biogenic amines have a role in the mating behaviour of this species by affecting the transfer of sperm. The significance of the salivary gland in salivation, a crucial component of host-seeking behaviour, blood feeding, duration of probing, and oviposition in *Anopheles gambiae* and *Aedes aegypti*, can be deduced by observing the elevated expression levels of 5-HT receptors and the existence of 5-HT immunoreactive nerves within the gland (Lee et al., 2003; Ngai et al., 2019). As the objective of this study was to examine the altered behavioural patterns in *D. gazella* resulting from exposure to deltamethrin, the findings indicate a notable decrease in 5-HT and 5-htpdc levels, which supports the hypothesis that deltamethrin lead to disruption of nesting behaviour which is linked to its depletion and is contingent upon both time and dosage. The findings of this study are consistent with other research conducted on different insect species (Price and Berry, 2006), and contribute to our understanding of the significant influence of serotonin on nesting behaviours and mating in *D. gazella*.

Nitric oxide (NO) is a ubiquitous signalling molecule that serves pivotal functions in several biological processes. The production of nitric oxide (NO) occurs through the l-arginine-nitric oxide route, facilitated by the enzyme nitric oxide synthase (NOS), which utilizes the amino acid l-arginine, as a precursor (Vanin, 2016). Previous research has indicated that nitric oxide (NO) is linked to the neural processing and long-term memory formation of chemical and visual cues in insects, as demonstrated by Trimmer et al. (2001). Nappi et al., (2000) and Foley et al., (2003) have demonstrated that NO may elicit cellular and humoral immunological responses in *Drosophila* by activating Toll/IMD signal pathways. Additionally, the relationship between the production of nitric oxide synthase (NOS) and the immunological response in *Manduca sexta* has been established by Eleftherianos et al., (2009). The study conducted by Ishii et al., (2013) demonstrates that in *Bombyx mori*, a species of silk moth, the ENF-family cytokine stimulates the expression of NOS, leading to an increase in the concentration of nitric oxide (NO). This increase in NO concentration subsequently activates the expression of genes responsible for antimicrobial

peptides (AMPs). Furthermore, the study conducted by Sadekuzzaman et al., (2018) has provided evidence supporting the presence of cross talk mechanism between nitric oxide (NO) and eicosanoid signaling in *Spodoptera exigua*. This was achieved by implementing RNA interference (RNAi) techniques to inhibit the expression of the NOS gene, leading to diminished levels of NO and consequently a decline in the immune response. Furthermore, Elzaki et al., (2020) have documented that the stimulation of the nitric oxide (NO) cycle through the utilization of citrulline and arginine has the potential to reinstate the vulnerability of brown plant hoppers to the pesticide imidacloprid. *In vitro* studies by (Yan et al., 2022) have reported the inhibitory effect of multiple insecticides on nos activity. In an attempt to fill the gap of our understanding for the role of NO in nesting behaviour, we obtained a significant reduction in nos and its synthesizing enzymes nos in both the sexes of *D. gazella*, on exposure to deltamethrin. Thus, we infer that, along with the associated alteration in other biogenic amines, NO too has an important role in modifying the parental behaviour in *D. gazella*, thereby altering its nesting behaviour (tunneling, brooding, reproduction and parental care). However, the mechanism by which it operates can be confirmed only after looking into the detailed molecular signalling.

In 1922, Stefan Kopeĉ, a Polish scientist, postulated the involvement of brain chemicals in regulating the physiological phenomena of moulting and metamorphosis. Currently, it is well acknowledged that a multitude of physiological processes in insects are regulated by tiny, bioactive peptides. Neuropeptides are denoted as such due to their synthesis occurring within modified neurons. Neuropeptides are a class of chemical messengers that are secreted by neurons and travel via the haemolymph of insects to reach target organs. They play a crucial role in coordinating many physiological tasks like as mating, oviposition, moulting, water balance, and fat mobilization (Yeoh et al., 2017). Neuropeptides play a crucial role in regulating vital biological processes in insects and possess significant potential due to their remarkable specificity. The literature has shown the presence of NPs in membrane preparations derived from the heads of *Drosophila melanogaster*, as well as brain preparations obtained from *L. dispar*. Additionally, it has been established that the activity of NPs in Drosophila is susceptible to inhibition by phosphoramidon and retrothiorphan, as described by Waye and Trudeau in (2011). NPS have been documented to exhibit agonistic, antagonistic, or modulatory properties, leading to subsequent modifications in a wide range of physiological, behavioural, and hormonal

functions. These alterations have implications for reproduction, development, growth, and the management of various stressors and challenges (Cui and Zhao 2020). The neuropeptide F (npf) is a multifunctional neuropeptide that has been found in several insect species. It serves crucial functions in a range of physiological processes including feeding, metabolism, courtship, reproduction, aggressiveness, ethanol sensitivity, locomotor circadian cycles, learning, and stress responses. The aforementioned functions of npf are executed via the activation of npf receptors (npfr), which have been extensively investigated in several organisms including *Drosophila melanogaster, Solenopsis invicta, Locusta migratoria, Schistocerca gregaria* and *Bombyx mori*. Several studies have been conducted on this topic (Chen and Pietrantonio, 2006; Clynen et al., 2006; Chen et al., 2013; Dillen et al., 2013; Deng et al., 2014). Down regulation of npf and npfr expression has been reported cessation of feeding in cotton bollworm larvae Li et al., (2023). The excessive intake of dietary sugar in *Drosophila* (Malita et al., 2022) and *Ostrinia furnacalis* larvae (Yu et al., 2022) is seen as a result of the suppression of gut signalling mediated by npf. Moreover, previous studies have identified inotocin receptors, which are considered insect counterparts of oxytocin/vasopressin-like receptors, exclusively in *T. castaneum, Nasonia vitripennis, Camponotus floridanus* and *Harpegnathos saltator* (Stafflinger et al., 2008; Bai and Palli, 2013; Birgül et al., 2021). The order Coleoptera, which has the distinction of being the biggest insect order in terms of the number of described species, currently has little understanding of neuropeptides, particularly in relation to inotocin and its receptor. Research has focused on about 20 species within this order, which have been observed to possess an inotocin signalling system (Liutkeviciute et al., 2016). Inotocin has been shown to influence social behaviour associated with reproduction in burying beetle *Nicrophorus vespillodes* with reference to parental care, however, the effect of insecticide in general and deltamethrin in particular has not yet been investigated. Moreover, the study of the myoinhibiting action of MIP, a neuropeptide, in visceral muscle preparations has been conducted in many species including *Gryllus bimaculatus, Drosophila melanogaster* and *Rhodnius prolixus* (Caers et al., 2012; Lange et al., 2012). The inhibition of juvenile hormone (JH) biosynthesis has been demonstrated in *G. bimaculatus* through in vitro studies (Williamson et al., 2001). In the case of *D. melanogaster* and *M. sexta*, it has been reported that MIP can suppress the activity of neurons involved in the ecdysis program (Kim et al., 2006; Yamanaka et al., 2010). However, there are gaps in our understanding about the involvement of dung beetles in nesting

behaviour. In this study, an investigation was conducted to examine the involvement of neuropeptides (npf, it, mip) and their corresponding receptors (npfr, itr) in the nesting behaviour of *D. gazella*. The findings of this study revealed a notable decrease in the mRNA gene expressions of these neuropeptides in a dose-dependent manner following exposure to deltamethrin. The altered expression of neuropeptides (NPs) and their corresponding receptors provides evidence of their participation in nesting behaviour. This altered expression is associated with changes in tunnelling activity, decreased reproductive capacity, resulting in a lower quantity of brood balls and parental care. Consequently, these factors collectively impede the nesting process.

4.5 CONCLUSION

Our study is the first reported interaction of neural regulation in *D. gazella* on exposure of deltamethrin and explains their critical role in nesting behavioural processes. Altogether, the complex, multilevel investigations on the physiology and behaviour induces a wide variety of neurological manifestations that include alterations in tunnelling, mating and brood care. These changes are probably mediated by: i) direct modulation of neurotransmitters- biogenic amines (DA and 5-HT), AChE, and NO; and their synthesizing genes (ddc, 5-htpdc, chat, and nos) ii) alterations in the expression and/or activity of NPs (npf, it, mip) and receptors (npfr, itr). Additionally, as deltamethrin exposure has a known neurotoxic property, has resulted into a decreased conduction velocity along the axon, resulting in decreased neural conduction.