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The Fungicide Thiram may Disrupt Reproductive Cycle of Domestic Male Pigeon (*Columba livia domestica*) Subjected to a Long Photoperiod

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SS, SH, SS and CS carried out the experimental work. LD performed the histological study. SS wrote discussion. CA corrected the langusge of the article.

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ABSTRACT

Fungicides are toxic chemicals, very much used in agriculture, but they are associated with the appearance of certain metabolic, carcinogenic, neurotoxic and fertility disorders. The objective of this work is to study the toxic effect of the dithiocarbamate "thiram 80% purity" on seasonal reproduction of male domestic pigeons *Columba livia domestica*, subjected to a long photoperiod (19L: 05D). The fungicide was orally administered at 5 and 10 mg/Kg body weight/day for 10 consecutive weeks.Testicular volume and weights were measured weekly, whereas semen quality, and histopathological profile were investigated at week 10.The obtained results reveal that under a long photoperiodthe sexual activity of the control lasted only 04 weeks, characterized by significant increase in the testicular volume, followed by spontaneous gonadal regressionup to week 10. Consequently, an azospermia and lack of germ cells in control birds was noticed,which confirm the testicular regression.In the treated groups, thiram delayed the refractory phase along the experimental period, but testicular weight were superior in the treated pigeons compared to the control during the last weeks. Treated pigeons had more dead spermatozoa compared to the control. Remarkably, abnormal spermatozoa were much higher in the group received 5mg than that of 10mg.The histological profile revealed degenerative changes in testes of treated pigeons with elongated and irregular diameter of seminiferous tubules, degenerative of Sertoli cells, severe atrophy of Leydig cells and pronounced decrease in the interstitial space. However, in the treated groups the stages of spermatogenesis appear unaffected, accompanied withimmature and malformed spermatozoa in the lumen.To conclude, oral administration of thiram may affect the seasonal reproduction of pigeon by disturbing the histo-architecture of testes and sperm quality under long photoperiod. Such changes may be responsible for delayed refractory phase.The intact stages of spermatogenesis indicates that the thiram-exposed pigeons were not in the refractoriness period.

INTRODUCTION

Thiram (tetramethyl thiuram disulfide, TMTD) belongs to the dithiocarbamate family of pesticide. It is widely used as a foliar treatment on fruits, vegetables, ornamentals and turf crops (Kunkur *et al*., 2007), and as a seed treatment to control seedling blights and a number of fungi that cause "damping off" in seedlings (Lohse *et al*., 2015). Also, it is used by humans in the treatment of scabies and as a bactericide for skin disorders (Ceresera *et al*., 2001) and is used in rubber industry as an accelerator and a vulcanization agent (Grosicka *et al*., 2005).

In spite of its benefits, thiram poses a potential threat to people and to the environment (Lohse *et al*., 2015). Thiram is slightly soluble in water (30 ppm at 25°C), (Aulakh *et al*., 2005), this makes its elimination from the natural environment very difficult (Gupta *et al*., 2012). It is being a toxic substance for aquatic life; with an LC50 of less than 5 ppm for most of fish species (Sharma *et al*., 2003).

In the human body, Thiram is metabolized to

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give carbon disulfide, causing liver damage. Its group dithiocarbamate (DTC) reacts with sulfur- containing enzymes and co-enzymes, leading to blockage of their catalytic activity and causing cellular metabolism perturbation (Sharma *et al*., 2003). Also, due to their DTC group, thiram forms easily complexes with metals and exhibits a specific redox behavior at mercury and gold electrodes (Hernandez-Olmos, 2000). Thiram is also, known as an inducer of allergic dermatitis and as an inhibitor of angiogenis, a stimulator of the eyes, skin, and respiratory tract. The dermal LD_{50} in rats is more than 2000g. Rakitsku *et al*. (2016) have revealed that workers in the rubber industry and hospital surgical staff who use rubber gloves showed skin lesions as hand eczema or dermatitis. However, Hakama and Kilpikari (1980) have reported an increased risk of stomach, lung, bladder and blood cancer in people working in the rubber industry. In addition to this, thiram has been found to be a mutagenic (Agrawal *et al*., 1997; Ardito *et al*., 1997) and a teratogenic agent (Robens, 1969).

Chronic exposures have been found to affect adversely reproductive function in both female rodents (Short *et al*., 1976) and birds (Weppelman *et al*., 1980; Wedig *et al*., 1968). Stoker *et al*. (1993) have reported that thiram disrupts the hormonal control of ovulation in female rats. It has been demonstrated that Thiram was found to inhibit dopamine B-hydroxylase (DBH), thereby affecting norepinephrine (NE) synthesis which plays an important role in the hypothalamic regulation of pituitary function (Lippmann and Lioyd, 1971). Zdzienicka *et al*. (1982) have confirmed that thiram induced sperm head abnormalities in mice. A remarkable increase in the frequency of chromosomal aberrations and abnormal sperms was observed in treated mice exposed to thiram (Hema-Prasad *et al*., 1987). Another study performed on germ cells of Swiss albino males had showed by 80-200- 320 mg/Kg body of thiram. Though, pregnant mice given oral doses of thiram10-30 mg/animal from day 5 to 15 of during pregnancy had delivered fetuses and pups malformed, characterized by cleft palates, micrognathia, wavy ribs and distorted bones (Short *et al*., 1976).

Furthermore, some farmers used seeds treated with pesticides to avoid the need for spraying after plantation (Prosser *et al*., 2006). But, treated seeds may put granivors farmland birds at risk. In consequence, birds' mortality related to treated seed ingestion in several cases was reported (Stanley and Bunyan, 1979).

In view of the lack of information concerning the effect of thiram on pigeon reproduction, the current study has been undertaken to investigate the possible effects of thiram on reproductive cycle of domestic male pigeon (*Columba livia domestica*).

MATERIALS AND METHODS

Chemicals and dose selection

Thiram (tetramethylthiuram disulfide, CAS 137-26-8) chemical purity 80%, 5.5% surface additives and about 14.5% kaolin, was supplied by Sigma-Aldrich. Although in the literature the bird exposure dose were variable from 670 to 2800 mg/Kg, a dose of 5 and 10 mg/kg/day, have been chosen in this experiment.

Animals

Male pigeon (*Columba livia domesica*), with average body weight of 200–250 g were acquired from Skikda (North-East of Algeria) at the end of February. Pigeons were kept in metal cages measuring 100x100x100 cm, with six birds per cage. The cages were placed inside light-controlled rooms. Food (chick crumbs) and water were provided *ad libitium*. Birds were divided into three groups of 6 individuals each, where the first group was used as a control, but the second one has received orally 5 mg/Kg/day of thiram. However, third group was given 10 mg/Kg/day of thiram. All groups were held under artificial photoperiod of (19L:5D) by using electrical clock of 72 watts.

Laparotomy and blood sampling

Gonadal development was assessed by laparotomy at intervals of approximately 15 days. The gonads were examined through a small incision in the body wall between the last two ribs, after anesthetizing the incision with viscous lidocain. The dimensions of the left testis was measured to the nearest 0.5 mm. Testicular volume was calculated as V= 4/3 π a2 b; where, a is half the width and b is half the length (long axis).

Semen quality

After 10 weeks of experiment, pigeons were sacrificed; their testes were dissected out and weighed. The epididymis was carefully separated from the testis. 1 µl of sperm was added to 49µl of physiological water NaCl0.9%. The sperm suspension was examined within 5 min after their isolation from epididymis. Both motile and immotile spermatozoa were then counted. Results were finally expressed as percent

For the evaluation of the sperm morphology, the diluted sperm was stained with 1% eosin after 2 min as explained (Narayana *et al*., 2002). Briefly, the sperms in the smears were visualized under 40× or oil immersion objectives and any abnormalities of either heads or tails were noted.

Histology

After decapitation, testes were immediately fixed

in Bouin's fluid for 24 h, hydrated in alcohol grades and cleared in toluene prior to embedding in paraffin wax. Sections of 5µm thick were cut by microtome, stained with haematoxylin and Eosin and mounted on diesterase phthalate xylene.

Statistical analysis

Data wes expressed as mean±SEM. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Student's t test to assess significant differences among treatment groups. All statistical analyses were performed using Minitab version 16.

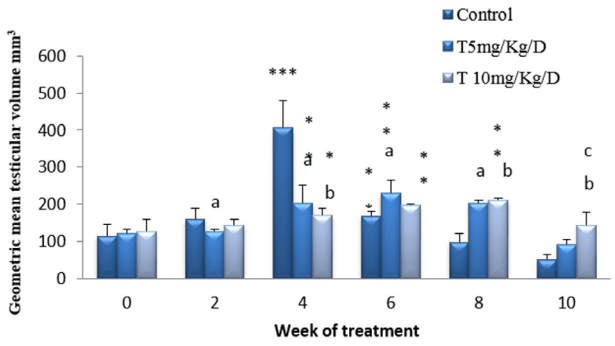

Fig. 1. Variation of the mean testicular volume (m±SE) of male pigeons (*Columba livia domestica*) subjected to a long photoperiod (19L:05D) and exposed to thiram (n=6). a, difference between control and D1; b, difference between control and D2; c, difference between D1 and D2.

RESULTS

General toxicity

No mortality was observed during the experimental period. However at dose of 10mg/Kg, certain symptoms of intoxication as weakness and diarrhea were observed in most birds.

Changes in testicular volume

Changes in gonadal size measured during the present study are shown in Figure 1. At the beginning of the experiment, birds had a mean testicular size of 120.16 ± 6.10 mm^3. Control birds that were kept at long photoperiod throughout the experiment (19L:5D), had maintained fully reproductive cycle, characterized by significant ($p \leq 0.05$) increase in the testicular volume up to the fourth week, followed by spontaneous gonadal regression, with testes reaching a minimal size of 49.48 ± 14.19 mm^3 ($p \leq 0.01$) by week 10 of the experiment. Treated groups had not showed any increase in testicular size along the experimental period. However, mean testes size were superior in the treated pigeons compared to the control at the end of the experiment;in which testes' volumes were 90.20± 15.99 mm^3 and 144.06± 15.99 mm^3 in treated pigeon at 5 mg and 10 mg, respectively.

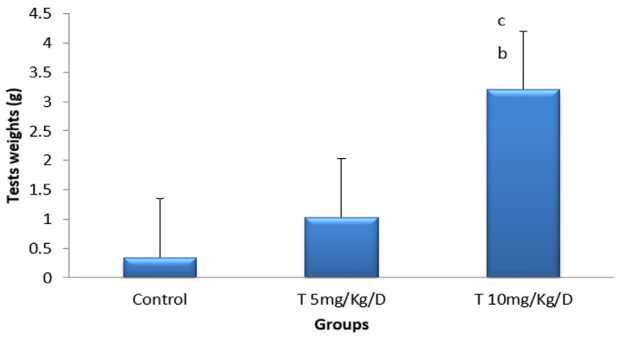

Fig. 2. Variation of the mean testicular weight at week 10 (m±SE) of male pigeons (*Columba liviadomestica*) subjected to a long photoperiod (19L: 05D) and exposed to thiram (n=6). a, difference between control and D1; b, difference between control and D2; c, difference between D1 and D2.

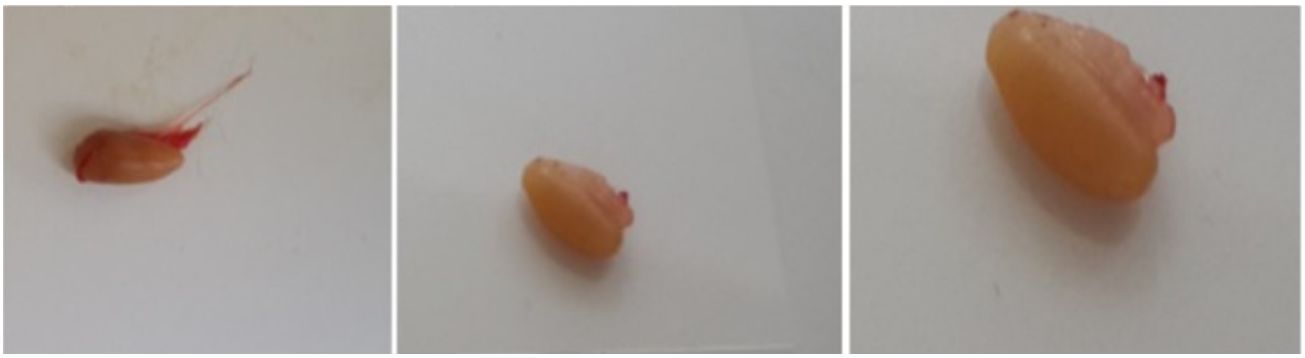

Fig. 3. Selected pictures of testes of male pigeons (*Columba liviadomestica*) at week 10 (C: control; D1, 5mg/Kg/D; D2, 10mg/Kg/D) subjected to a long photoperiod (19L: 05D) and exposed to thiram (n=6).

Testicular weight

Figure 3 shows selected pictures of testes of male pigeons *Columba livia domestica* from the three groups (C, control; D1, dose 1; D2, dose2) exposed to long photoperiod.

The mean testicular weight of pigeons is shown in Figure 2, where control individuals had fully regressed gonads. There were a significant (P>0.01) differences between the mean testicular weight of the two treated groups of birds, but they were all, of course, considerably greater (P<0.01) than the testicular weight of the control.

Sperm quality

The sperm quality is shown in Table I. The obtained results revealed an azospermia in control pigeons after 10 weeks of experiment. However, the study showed a significant increase (P<0.001) of the dead spermatozoa in the treated pigeons. An increase in sperm abnormalities (P<0.05) in treated pigeon at 5 mg/Kg/day and (P<0.01) in treated pigeon at 10 mg/Kg/day (Table I). The abnormal spermatozoawas inversely related to the dose, where the percentage of abnormal sperm was 74.6% and 33.6% at 5 mg and 10 mg, respectively (Table I). The abnormalities were in the head, neck and tail region of the spermatozoa. Thus, tail abnormalities are superior thanthat of the head in both treated pigeons.

Histopathological studies

The testis of control pigeon (Fig. 4) exhibited regular seminiferous tubules with a lack of successive stages of spermatogenesis and the absence of spermatozoa in the lumen of somniferous tubules marking thus, gonads regression and a refractoriness period. Figure 5 shows histology feature in the testis of pigeons received 5 mg/Kg/day of thiram. The somniferous tubules begin to have an irregular diameter; lumen is more or less extended. The somniferous tubule showed successive stages of transformation of spermatogonia into spermatozoa which appear malformed. A slight decrease in Sertoli cells, and an atrophy of Leydig cells.

Table I.- Effects of Thiram on sperm quality in male pigeons (*Columba livia domestica*) subjected to long photoperiod (19L:5D) and treated for 70 days.

Groups	Sperm vitality (%)		Sperm abnormalities (%)		Abnormalities (%)	
	Motile	Immotile	Normal	Abnormal	Head	Tail
Control	0	0	0	0	0	0
Thiram 5mg/Kg/D	22±10.1^{a*}	78±6.47^{a***}	74.66 ±6.4^{a***}	25.3±6.4^{a*}	5±3^{a}	95±5^{a***}
Thiram 10mg/Kg/D	20±6.32^{b*,c}	80±6.3^{b***,c}	65.4±15.9^{b***,c}	33.6±17.5^{b**,c}	11.2± 13.2^{b,c}	88.2±13.2^{b***,c}

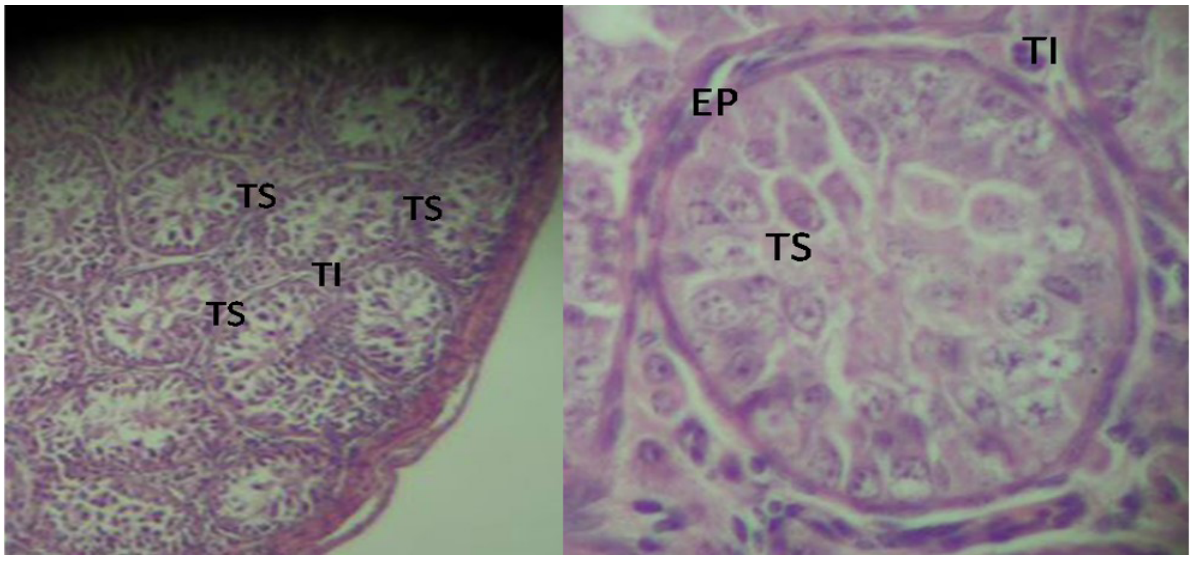

Fig. 4. The testis of control pigeons (*Columba livia domestica*) subjected to long photoperiod (19L:5D) for 10 weeks H&E. Magnification x10 and x40. The histological profile showing azospermia and lack of spermatogenesis stage. TS, somniferous tubules; TI, interstitial tissue; EP, epithelium.

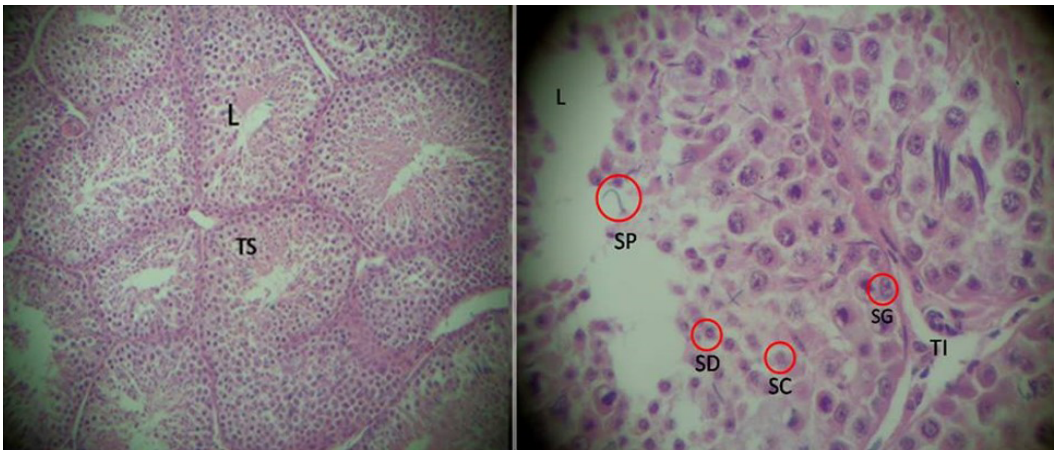

Fig. 5. Testis of pigeon (*Columba livia domestica*) exposed to Thiram 5 mg/Kg/day for 10 weeks and subjected to long photoperiod (19L:5D), H&E. Magnification x10 and x40. L, lumen; Sp, spermatozoa; SG, spermatogonia; SC, spermatocyte; SD, spermatide. Degenerative in the histoarchitecture of testis are seen. Irregular somniferous tubules with all successive stages of spermatogenesis, some malformed spermatozoa in the lumen, slight decrease in Sertoli cell counts and a decrease in the interstitial spaces.

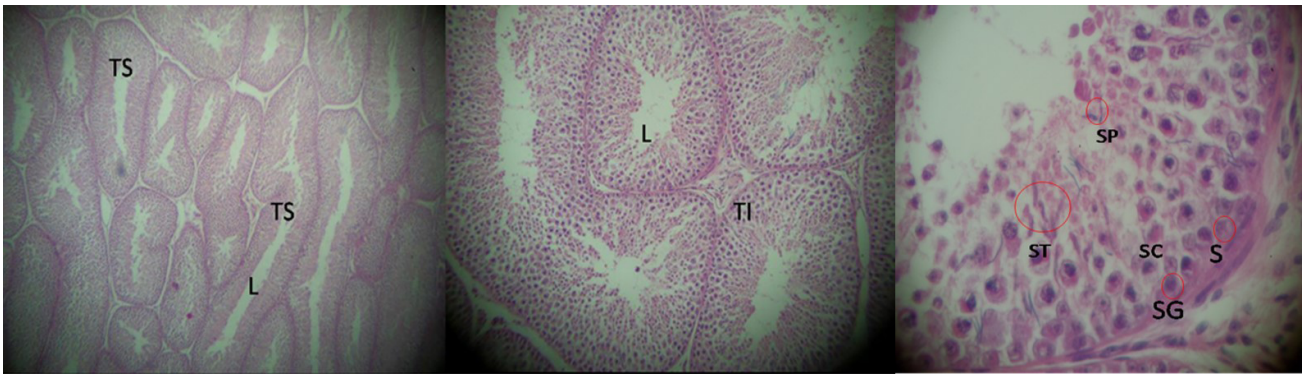

Fig. 6. Testis of pigeon (*Columba livia domestica*) exposed to Thiram at 10 mg/Kg/day after 10 weeks (H&E. Magnification x10 and x40). L, lumen; Sp, spermatozoa; SG, spermatogonia; SC, spermatocyte; SD, spermatide; S, Sertoli cells. Acute degenerative in the histoarchitecture of testis characterized by elongated and irregular diameter of seminiferous tubules with all the successive stages of spermatogenesis. Deteriorating of Sertoli cells, a pronounced decrease in interstitial space and severe atrophy of Leydig cells.

Histological examination of the testes from 10 mg/Kg/day treated pigeons (Fig. 6) showed marked degenerative changes which include elongated and irregular diameter of seminiferous tubules with degenerative of Sertoli cells. The study showed that the stages of spermatogenesis appear unaffected. However, spermatozoa seem immature and malformed. Furthermore, a severe decrease in interstitial space and severe atrophy of Leydig cells was observed.

DISCUSSION

The data reported in this study demonstrate that in vivo exposure to the fungicide thiram dose-dependently impairs pigeon's reproductive functions, by altering gonadal growth. In birds, gonadal growth and regression are highly seasonal and relate to environmental factors such as food availability and the photoperiod length (Budki *et al*., 2008). Therefore, the day length has been well defined as the regulator of different metabolic and reproductive activities in many avian species (Hahn and Shackleton, 2008; Dixit and Singh, 2011). Since the work of Rowan (1929), it has been clear that the primary environmental factor used by birds to time reproduction is the annual changes in photoperiod. Birds have extra-retinal photoreceptors which they use, in conjunction with a circadian clock, to measure photoperiod (Dawson *et al*., 2001; Kumar *et al*., 2004). Findings from this work indicate that under artificial photoperiod (19L:5D),

birds maintained a fully reproductive cycle characterized by full mature testes at the 4th week, followed by spontaneous gonadal regression. The physiological mechanism underlying the photo-stimulation is that an increase in photoperiod elevates the rate of secretion of gonadotrophin-releasing hormone (GnRH), leading to elevated gonadotrophin secretion, and hence gonadal maturation such as luteinizing hormone (LH) and follicule hormone (FSH), which in turn induce gonad growth and steroid hormone production (Wingfield and Farner, 1993). However, the administration of thiram at a rate of 5 mg/Kg/day and 10 mg/Kg/day to male pigeons under long days for 10 weeks, has inhibited the development of testes, and disturb their reproductive cycle. It has been recorded a lower means in testes sizes during the experiment in treated birds compared to the control. At the physiological level, it is difficult to discuss the correlation between the inhibiting effect of pesticides and the reproductive cycle of birds, but it is possible is attributed to the mechanism of measure of photoperiod. Therefore they hadn't estimated the true photoperiod, and consequently all photoperiod would be regarded as being short (Wilson and Reinert, 1993). Thirame like dithiocarbamates have a potential to disrupt the endocrine system and may affect growth, metabolism, reproduction and behavior etc. disrupting gonadal and adrenal axes and thyroid function are documented by many authors (Diamanti-Kandarakis *et al*., 2009; Fraites *et al*., 2009; Pandey *et al*., 2017). In birds mixture of mancozeb and imidacloprid had distrupt the pituitary-thyroid axis (Pandey and Mohanty, 2015). It is possible that thiram have interfered with testis function and indirectly acted at the level of hypothalamus or pituitary gland, or also directly on the testis as number of pesticides has showed testicular toxicity (Recio *et al*., 2005). Moreover, Goldman *et al*. (1990) have reported that the insecticide chlorodimeform may destroy endocrinologic homeostasis by suppressing GnRH release. It has also been reported that xenobiotics may affect reproductive function by direct insult to the cell populations within the gonads resulting in a feedback mechanism impairment of the hypothalamus and the pituitary (Pasqualini *et al*., 1990). Stoker *et al*. (1993) had reported that thiram is able to block the LH surge and inhibit subsequent ovulation if administered during a sensitive period prior to the initiation of the surge. In other study, Stoker *et al*. (1996) found that a single exposure to the fungicide thiram (50 mg/kg) during the critical period of proestrus prior to mating, resulted in a decrease in the rate of fetal development, as well as a reduction in the number of live fetuses and an increase in the number of resorptions on GD 20. Epidemiological and experimental studies support the hypothesis that high exposure to thiram provokes a significant fertility decrease and the qualitative impairment of offspring (Dănulescu *et al*., 2004).

The thyroid gland plays an essential role in the etiology of seasonal reproduction in birds (Boulakoud and Goldsmith, 1991). Thus, the active thyroid function is essential for this process, leading to the occurrence of refractoriness under long days. It was known that thiram and other xenobiotics causes tyroid dysfunction in animals and disrupt hypothalamo-pituitary axis (Pandey and Mohant, 2017).

Furthermore, the decreased sperm motility indicates the cytotoxic damage caused by thiram on germ cells, which was proportional to the dose-level. Sperm morphology also has an important relationship to sperm motility, where the reduced motility observed in this study is probably due to the morphological aberrations reported earlier (Kasker *et al*., 1994). Induced sperm abnormalities indicate point mutations in germ cells (Narayana *et al*., 2002), which should have triggered structural changes in cell organelles involved in head and tail formation, leading to sperm malformation. It has been reported that thiram induced sperm head abnormalities in mice (Zdzienicka *et al*., 1982). Furthermore, Hema-Prasad *et al*. (1987) have reported the mutagenic effects of thiram on germ cells of Swiss albino male. Pinar (2013) revealed that propineb may be a mutagen agent due to the observed rise in the frequency of mouse sperm abnormalities.

The weight of testes is largely dependent on the mass of differentiated spermatogenic cells, but the reduction of their weights, was consistent with the elimination of germ cells (Chapin and Lamb, 1994). In this study results have shown a decreased weight of control testes due to the refractoriness (Boulakoud and Goldsmith, 1991). However this study revealed, significant increaseof testicular weight with increasing thiram concentrations, which may be owed to elongated and enlarged lumen and interstitial spaces of the semineferous tubules. Other study revealed a testicular atrophy with damaged germinal epithelium, accompanied with reduced sperm motility and viability in male adult rats exposed to maneb and zineb (Lucier *et al*., 1977). It has been showed that the carbamate insecticide carbaryl has affected spermatogenic cells and caused leydig cells degeneration and altered serum testosterone and gonadotrophin levels (Shrivastava and Shrivastava, 1998). Slimani *et al*. (2014) have revealed that treatment of pigeons with propineb induced severe testicular lesion in which the simininferous tubules were elongated and having slought germ cells at the level of their lumens with detachment of the seminiferous epithelium.

The presence of spermatozoa and theintact stages of spermatogenesis in the semineferous tubules of treated birds by 5 and 10 mg/Kg/day indicated that these pigeons were not in the refractoriness period.

CONCLUSION

In conclusion, our results indicate that under a long daily photoperiod of (19L:5D), male domestic pigeon (*Columba livia domestica*) maintained a fully reproductive cycle characterized by full mature testes at the 4th week, followed by spontaneous gonadal regression. Semen quality and the histopathological profile indicate an azospermia in control birds and confirm the refractoriness period in these birds. However, the administration of thiram at a rate of 5 and 10 mg/Kg/day to male pigeons under long days, has inhibited the development of testes, and disturbs their reproductive cycle. Treated pigeons had more dead and abnormal spermatozoa. The histological profile revealed degenerative changes in testes of treated pigeons with elongated and irregular diameter of seminiferous tubules, degenerative of Sertoli cells, severe atrophy of Leydig cells and pronounced decrease in the interstitial space. The intact stages of spermatogenesis indicates that the thiram-exposed pigeons were not in the refractoriness period.

Statement of conflict of interest

Authors have declared no conflict of interest.

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