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# Impact of Methoprene on Spermatogenesis of the Freshwater Crab *Travancoriana schirnerae*

**Latha Nadkandi Padmanabhan, Sudha Devi Arath Raghavan and Aswani Ayanath**

**Abstract**
This study investigated the impact of methoprene, a juvenile hormone analog, on spermatogenesis of the freshwater crab *Travancoriana schirnerae*, abundant in the wetlands of Wayanad. Administration of sublethal dose of methoprene demonstrated reduced gonadosomatic indices and considerable degree of changes in the morphology and histoanatomy of the testis leading to partial or total suppression of spermatogenesis during active phase. Histopathological changes include shrinkage and vacuolization of acini, disintegration of basal lamina and germinal epithelium, deformation, disarray and lower chromatin content of primary gonia and a reduction in number of germ cells. Decline in mature sperm concentration, abnormal morphology and irregular arrangement of sperms was also observed in experimental crabs, in contrast to the fully laden acini with mature spermatozoa arranged in circles of control crabs.

**Keywords:** Freshwater crab, histology, juvenile hormone analog, methoprene, *Travancoriana schirnerae*

## 1. Introduction
The potential of juvenile hormone analogs (JHAs) as pest control agents has gained momentum in the last few years. Unlike the environmentally burdensome chemical insecticides which cause immediate death of target insects, JHAs interfere with growth and development, making it impossible for the immature insects to become adults. In adult insects, JHAs hinder the reproductive processes resulting in sterile individuals [1, 2]. One of the extensively used JHAs is methoprene which has desirable features such as short environmental half-life, low toxicity and minimal impact on non-target organisms (NTOs) [3, 4]. It is used in the management of both agricultural and non-agricultural pests especially mosquitoes [5, 6].
Methoprene enters the freshwater environments either by direct application for controlling aquatic pests or indirectly through land drainage or erosion from adjacent methoprene treated agricultural lands [1, 6]. The biological activity of aqueous solutions of methoprene in the field is affected by sunlight, temperature and microbial action. Repeated application of methoprene in shallow and poorly flushed waters is considered to be hazardous to aquatic biota and the environment. Studies by Kikuchi et al. [7] and Hershey et al. [8] have demonstrated barely minor adverse effects of methoprene on aquatic biota while others have shown that methoprene can be quite toxic to non-target aquatic organisms, including insects [9] and crustaceans [10].
In non-target crustaceans, studies have indicated that application of JHAs affects growth [11], survival [12], metamorphosis [13], limb regeneration and moulting [14, 15], energy metabolism and development [16]. Much concern has been raised in the past regarding the potential for methoprene use in larvicidal activities which may cause huge impact on crustaceans such as shrimps, crabs and lobsters. Celestial and McKenney [17] showed a significant reduction in the survival of larval stages of the mud crab *Rhithropanopeus harrisii* exposed to methoprene. Horst and Walker [18] found that methoprene inhibited morphogenesis and shell formation in the postmoult blue crab *Callinectes sapidus*. In the same species, exposure to methoprene at environmental concentrations 2-10 µM resulted in morbidity, mortality, reduction in the number of successful hatchlings and lethargic behaviour in surviving zoeae [19].
Methoprene exposure caused a significant reduction in larval metamorphosis in the estuarine grass shrimp *Palaemonetes pugio* [20] and lethality in the juvenile mysid shrimp *Mysidopsis bahia* [21]. Methoprene significantly affected energy and testosterone metabolism [22],

moulting [23] and survival and longevity [24] in non-target crustaceans. Gradoni [25] found that juvenile amphipods are more sensitive to methoprene than adults.

Studies regarding the impact of methoprene on reproductive aspects of non-target macrocrustaceans are limited [11]; majority of the documented works were directed to acute toxicity [26] and most toxicological studies on crustaceans have not addressed cellular effects [27]. Payen and Costlow [28] observed disturbed spermatogenesis in methoprene (Altosid) treated *R. harrisii*. So far there has been no comprehensive study on the impact of methoprene on reproduction in freshwater crabs. Hence, in the present study, an attempt has been made to investigate the histopathological changes induced in the testis of the freshwater crab *Travancoriana schirnerae* on administration of sublethal dose of methoprene. Histological changes not only give an early indication of pollution hazard but also provide useful data on nature and degree of damage to cells and tissues [29].

## 2. Materials and methods

### 2.1 Test chemical

Methoprene is a long chain hydrocarbon ester, characterized as an amber or pale yellow liquid with a faint fruity odour [30]. The commercial product, Diacon II® containing 33.6% (S)-methoprene/L as active ingredient (Wellmark International, IL, USA) was used as the source of methoprene in this study. The test solutions were prepared in distilled water just before administration.

### 2.2 Determination of $LD_{50}$ for 48 hours

Adult male intermoult crabs (carapace width 4.0-5.0 cm, body weight 22.39-47.56 g; 33.52±7.56 g) were collected from the paddy fields of Ondayangadi, about 5 km northeast of Mananthavady (11.82° N and 76.02° E, altitude 767 m) in Wayanad district of Kerala, India. Fifty crabs were distributed into five groups of ten each. Group I individuals received injections of 50 ppm (S)-methoprene (in 100 µl distilled water) into the body cavity through the arthrodial membrane of the third walking leg. Similarly, individuals of Group II, III, IV and V received injections of 100, 150, 200 and 250 ppm (S)-methoprene/100 µl distilled water, respectively. The crabs were kept separately in plastic tubs and observed for 48 h. The mortality in each group was recorded. The $LD_{50}$ (155 ppm for 48 h) was determined using Probit analysis [31] (Randhawa, 2009). One tenth of the $LD_{50}$ (15 ppm) was used as the test dose in the present study.

### 2.3 Experimental design

Adult male intermoult crabs were collected for a period of one year (March 2014-February 2015) from the same paddy fields mentioned above and acclimatized to laboratory conditions for three days. They were fed with boiled egg and pulses during the acclimation and experimental period.

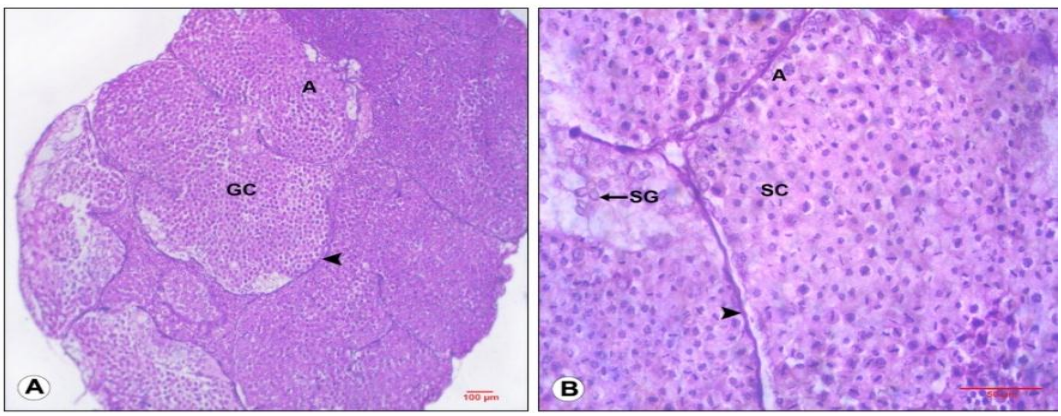
Every month, ten untreated crabs were maintained as controls and another ten as experimentals. To each experimental crab, 100 µl 15 ppm (S)-methoprene was carefully injected into the body cavity through the arthrodial membrane of the third walking leg on days 1, 8, 15 and 22. A week after administration of the fourth dose (on day 30), the animals were sacrificed. Testes from experimental and control crabs were carefully dissected out, weighed and fixed in Bouin's solution for histological analyses. The gonadosomatic index (GSI) was calculated using the formula: wet weight of gonad (g)/wet weight of whole body (g) × 100. The tissues were dehydrated in graded series of ethanol and embedded in paraffin wax. Sections 5-6 µm thickness were stained with haematoxylin-eosin and examined under a Leica DM 500 Research Microscope. Measurements were recorded using an image analysis system of Biowizard software. Photomicrographs were taken with a DG 330/210 camera attached to the microscope. Student's t-test was applied in analyzing the data.

## 3. Results

### 3.1 Histopathology of testis of methoprene treated crabs during active phase (May-June)

Histological observations of normal, untreated crabs during active phase revealed normal testicular architecture with regular course of spermatogenesis. Acinar walls appeared well stretched as the acini were fully packed with germ cells (Figure 1A, B). The most prominent feature of this phase was that majority of the acini were packed with mature spermatozoa (Figure 1C).

The testes of treated crabs showed abnormal testicular architecture. There occurred a significant reduction in GSI value and acinar diameter when compared to control crabs (Tables 1, 2). Acinar boundaries appeared indistinct in some areas of the testis and degenerative changes were perceptible in the germ cells. Methoprene injection affected the regular, round or oval shape of gonial cells (Table 3). Their nuclei were seen enlarged and vacuolated and chromatin marginated along the nuclear membrane was frequently noticed (Figure 1D). In some acini, arrest of division of gonia was observed. Primary and secondary spermatocytes were found reduced in size and exhibited nuclear pycnosis preceding degeneration (Table 3). Damaged, elongate spermatids could be seen in a small number of acini.



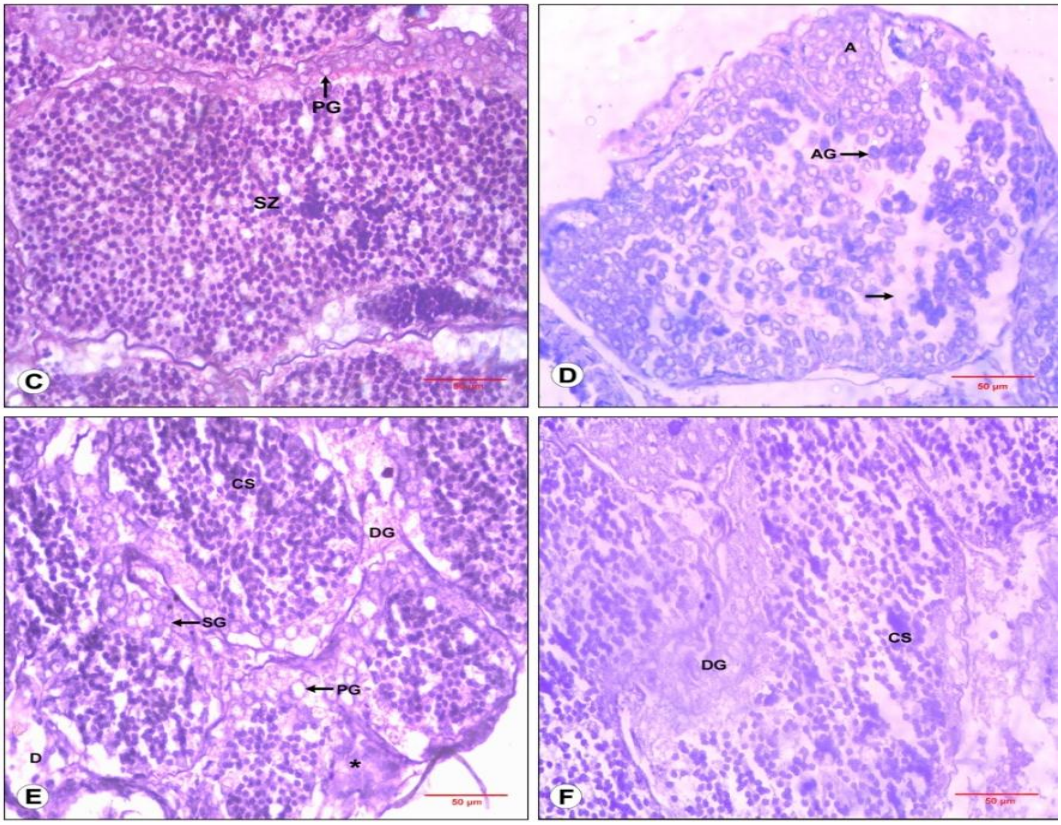



**Fig 1:** Histological features of the testis of control (A-C) and methoprene treated (D-F) *T. schirnerae* during active phase (May-June) of spermatogenesis.

(A) Acini fully packed with active germ cells, (B) Acini with dividing spermatocytes, (C) Mature spermatozoa arranged in circles in acini, (D) Acinus with abnormal gonia and cell free spaces, (E) Acini with clumped sperms and gonia showing signs of degeneration and dissolution, (F) Degenerative changes in the gonial cells and spermatozoa.

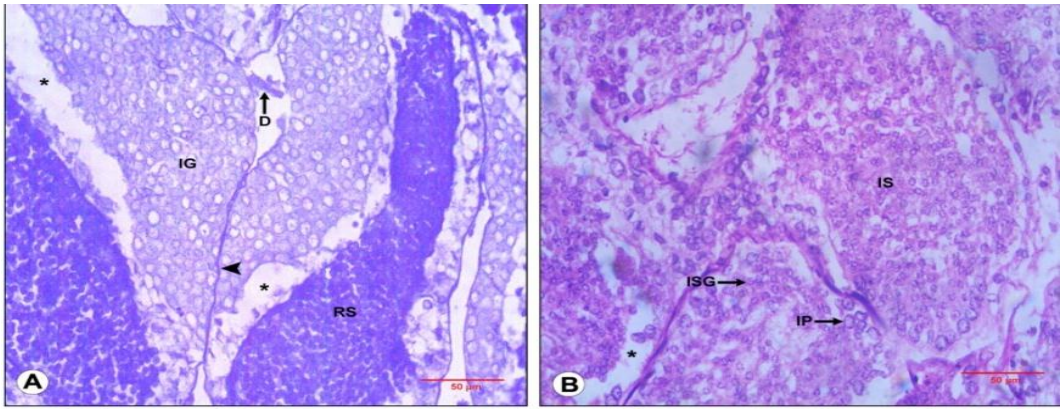
A: Acinus; GC: Germ cells; SG: Secondary gonia; SC: Spermatocytes; PG: Primary gonia; SZ: Spermatozoa; AG: Abnormal gonia; CS: Clumped sperms; DG: Dissoluted gonia; D: Debris; Arrow head indicates acinar wall; Arrow indicates cell free spaces; Asterix indicates sperm dissolution.

Methoprene treatment appeared to have hampered the development of sperms as indicated by their reduced size (Table 3). Acini packed with sperms arranged in circles (not pycnotic) were still perceptible in some areas of the testis. Many acini carried clumped sperms arranged in the centre and gonia towards the periphery showing signs of degeneration and dissolution (Figure 1E, F). Methoprene injection during this phase caused a significant decrease in sperm count when compared to controls.

### 3.2 Histopathology of testis of methoprene treated crabs during inactive phase (July-February)

The annual spermatogenic activity began to decline after mating in July-August. The testis remained inactive till February as evidenced by a progressive fall in GSI value and acinar diameter (Tables 1, 2) (Figure 2A-C).

Methoprene treatment hastened the disintegration and degeneration of the germ cells during the inactive phase. When compared to the untreated control crabs, GSI values and acinar diameter of treated crabs reduced drastically (Tables 1, 2). Light microscopy revealed disorganization of acinar elements, extensive inter and intra acinar spaces and cell debris. In some areas of the testis, acinar boundaries were indistinct or broken and the cell types could not be distinguished. The acinar contents appeared inconsistent, clumped, inconspicuous and detached from the wall (Figure 2D). Furthermore, atrophied nature of acini and changes in the basal lamina including irregularity and indentations were observed. Acini showing degenerative changes and appearance of vacuolated germ cells increased.



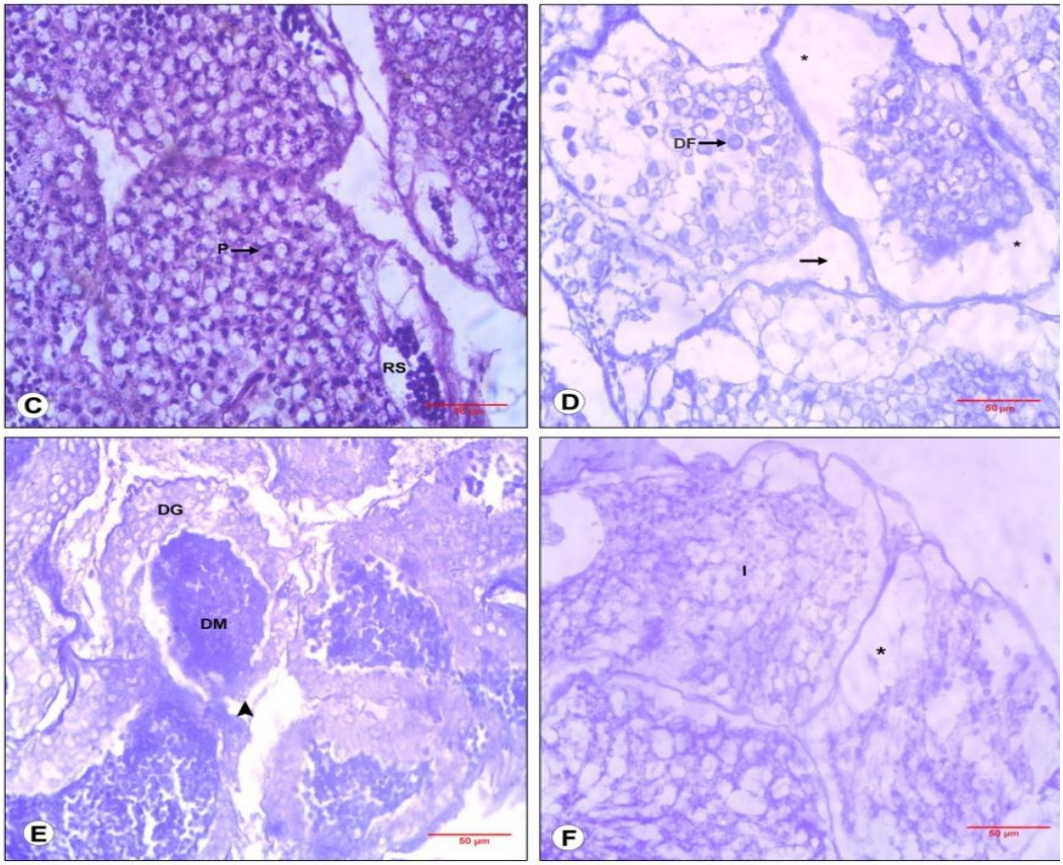



**Fig 2:** Histology of testis of control (A-C) and treated (D-F) crabs during inactive phase (July-February) of spermatogenesis.

(A) Highly pycnotic residual sperms and inactive gonia, (B) Cell free spaces, inactive secondary gonia and primary spermatocytes, (C) Acini with pycnotic primary gonia and highly condensed residual sperm masses towards the periphery, (D) Detached acinar contents and deformed gonia, (E) Degeneration and dissolution of gonia and spermatozoa, (F) Dissolved appearance of germ cells in acini.

D: Debris; IG: Inactive gonia; RS: Residual sperms; IS: Inactive primary spermatocytes; ISG: Inactive secondary gonia; IP: Inactive primary gonia; P: Pycnotic gonia; DF: Deformed gonia; DG: Dissoluting gonia; DM: Dissolved sperm mass; I: Indistinct germ cells; Arrow head indicates acinar wall; Arrow indicates inter acinar space; Asterix indicates intra acinar space.

**Table 1:** Gonadosomatic index of control and methoprene treated crabs during different phases of spermatogenesis.

| Phase | Months | Control | Experimental |
|---|---|---|---|
| Active | May-Jun | 0.181±0.01 | 0.129±0.01* |
| Inactive | July-Aug | 0.159±0.02 | 0.117±0.02* |
| | Sep-Oct | 0.138±0.01 | 0.108±0.01* |
| | Nov-Dec | 0.101±0.02 | 0.085±0.001* |
| | Jan-Feb | 0.077±0.01 | 0.069±0.01$^{\#}$ |
| Revival | Mar-April | 0.120±0.03 | 0.103±0.02$^{\#}$ |

The values are represented as Mean±SD. *$P$<0.05, $^{\#}P$>0.05.

Histological examination revealed distorted and loosely arranged germ cells giving a dissolved appearance to the germ cells in many acini. Germ cell count was significantly decreased when compared to the control group. Arrested division stages in primary gonial cells, deformation with enlarged and vacuolated nuclei were frequently noticed (Figure 2D). Acini with loosely packed and degenerated gonial cells were abundant. Arrest of division stages and chromatin condensation and necrosis of spermatocytes was noticed in many acini. Spermatids reduced in size, showing nuclear pycnosis and signs of degeneration were frequently encountered in the lumen of some acini. Acini with clumped sperms arranged in the middle or periphery were noticed. A few acini carried degenerating sperms and deformed gonia in the periphery showing signs of degeneration and dissolution (Figure 2E). Shrunken acini enclosing clumped and dissolved sperm masses and reduced intra luminal sperm concentration was perceptible. Pycnotic residual sperm masses undergoing degeneration and dissolution were observed. In some acini, germ cells appeared dissolved and could not be identified (Figure 2F).

**Table 2:** Changes in the acinar diameter of control and methoprene treated crabs during different phases of spermatogenesis.

| Phase | Months | Control (µm) | Experimental (µm) |
|---|---|---|---|
| Active | May-Jun | 364.81±12.21 | 295.10±12.92* |
| Inactive | July-Aug | 241.37±16.38 | 203.68±3.59* |
| | Sep-Oct | 218.28±2.62 | 202.40±1.93* |
| | Nov-Dec | 191.68±4.70 | 172.09±4.33* |
| | Jan-Feb | 173.63±3.06 | 160.70±6.00* |
| Revival | Mar-April | 194.74±8.54 | 173.71±12.82* |

The values are represented as Mean±S.D. *$P$<0.05

### 3.3 Histopathology of testis of treated crabs during revival phase (March-April)

The annual spermatogenic activity was found revived by March-April, perceptible by the proliferation of gonial cells from the germinal epithelium and pronounced increase in the GSI value and acinar diameter (Tables 1, 2).

Histological observations revealed that methoprene treatment during the revival phase affected the activities of germ cells leading to partial arrest of the testicular processes when compared to the untreated control crabs (Figure 3A-C). The GSI value and the average size of acini decreased significantly (Tables 1, 2).

Methoprene affected the proliferation of gonia cells as their distribution was very much reduced in treated crabs. The regular, round to oval shape of gonial cells was lost and their uniformity was disturbed. Their nuclei appeared shrunken, distorted and chromatin contents were clumped (Figure 3D). Shrunken primary and secondary spermatocytes were noticed. In some acini, dissolved sperm masses were noticed towards the interior while arrested gonia cells were seen towards the periphery (Figure 3E). A few acini carried pycnotic spermatids. Phagocytes were often encountered among the degenerating germ cells. Prominent cell free zones were noticed within and outside the acini (Figure 3F).

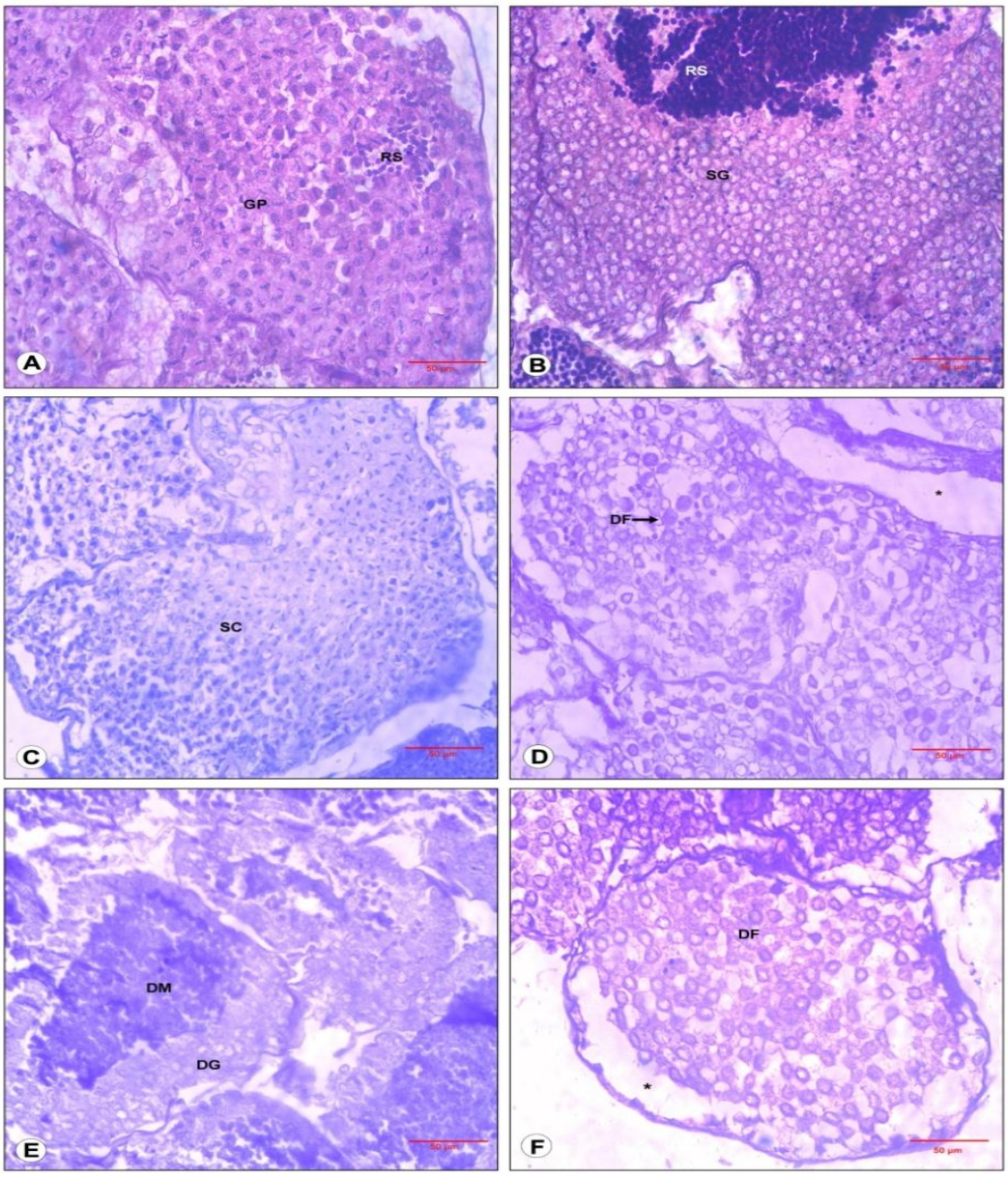



**Fig 3:** Histology of testis of control (A-C) and methoprene treated (D-F) crabs during revival phase (March-April) of spermatogenesis.

(A) Gonial proliferation, (B) Acini fully packed with secondary gonia, (C) Acini with dividing spermatocytes, (D) Deformed gonia with shrunken nuclei and clumped chromatin content, (E) Acini with dissoluted sperm masses and degenerating gonia, (F) Acinus showing deformed gonia and cell free spaces.

RS: Residual sperms; GP: Gonial proliferation; SG: Secondary gonia; SC: Dividing spermatocytes; DF: Deformed gonia; DM: Dissolved sperm masses; DG: Degenerating gonia; Asterix indicates cell free space.

**Table 3:** Impact of methoprene on the size of germ cells of *T. schirnerae* during active phase of spermatogenesis.

| Germ cells | Control (µm) | Experimental (µm) |
|---|---|---|
| Primary gonia | 9.1-12.0 | 8.2-11.5 |
| Secondary gonia | 6.1-9.0 | 4.5-8.2 |
| Primary spermatocytes | 5.1-6.0 | 3.8-4.5 |
| Secondary spermatocytes | 3.1-4.0 | 2.8-4.0 |
| Spermatids | 2.0-3.5 | 2.0-3.0 |
| Spermatozoa | 4.0-5.0 | 3.3-3.8 |

## 4. Discussion

The present investigation on acute exposure of sublethal dose of methoprene during different phases of spermatogenesis demonstrated reduced GSI values and considerable degree of changes in the morphology and histoanatomy of the testis leading to partial or total suppression of spermatogenesis.

A reduction in the GSI of methoprene treated *T. schirnerae* was perceptible in all the phases of spermatogenesis. Similar observations on decrease in GSI values were reported in other non-target crustaceans exposed to JHAs, pesticides, heavy metals and pollutants. Significantly low GSI values were recorded in male *Somanniathelphusa pax* collected from arsenic contaminated sites of Hanoi [32, 33]. Wang et al. [34] observed reduced testis weight in the freshwater crab *Sinopotamon henanense* exposed to cadmium. Tributyltin (TBT) exposure exhibited decreased GSI values in the estuarine mud crab *Scylla serrata* and the giant freshwater prawn *Macrobrachium rosenbergii* [35, 36]. On the contrary, oral administration of low and high doses of arsenic did not induce any change in the GSI of male freshwater crab *Geothelphusa dehaani* [33]. Methoprene induced reduction in GSI observed in the present study possibly suggest a reduction in testicular weight caused by degeneration of spermatogenic cells which is an indication of testicular atrophy. Gonadosomatic index reaches its maximum at the peak period of activity and has been considered as a reliable estimate for gonadal maturity of a species [37].

Our results clearly demonstrated that methoprene induced histopathological changes in the testis, such as shrinkage and vacuolization of acini, disintegration of basal lamina and germinal epithelium, deformation, disarray and lower chromatin content of gonia and reduction in the number of germ cells, were clear indications of atrophic conditions. Our findings were in agreement with the results documented in several non-target crustaceans treated with JHAs, pesticides, heavy metals and pollutants [29, 33, 34]. In *R. harrisii*, long exposure to Altosid caused considerable reduction in spermatogenesis: disappearance of gonia, stoppage of gonial mitosis and spermatocyte meiosis, presence of many spermatocytes and few spermatids [28]. In the freshwater crab *Paratelphusa hydrodromous*, sublethal concentration of cadmium brought about prominent histological variations in the testicular tissue as indicated by reduction in the number of germ cells, increased size of lumen of seminiferous tubules, disorganization of tubules and lower chromatin content of the proliferating cells and germinal epithelial cells [38].

Light microscopic examination of the testes of control *S. olivacea* showed normal testicular architecture while cadmium treatment resulted in vacuolar degeneration of spermatogenic epithelium, atrophy of seminiferous tubules, edema in the interstitial space as well as extensive necrosis associated with impaired spermatogenesis [39]. Treatment with different concentrations of cadmium induced major histopathological defects such as lesioned germ cells, edema in the interstitial space and extensive germinal layer necrosis in the testis of *S. henanense* [34]. Oral administration of high dose of arsenic in *G. dehaani* illustrated only spermatogonia and spermatocytes while spermatogenesis progressed till spermatozoa stage in the untreated control crabs [33].

Treatment with organophosphate and organochlorine pesticides caused swollen and degenerated testicular layer, vacuolization of acinar contents, reduction in spermatogenic material and disappearance of gonial cells leading to total disintegration of testicular processes in *S. serrata* [40]. Histopathological changes observed in the testicular tissue in *M. lamerrii* and *M. kistensis* include breakdown of germinal epithelium, deformed tubules, hindered gonial proliferation, vacuolization, degeneration of germ cells, damaged proliferation zone and reduced spermatogenic mass on exposure to fenitrothion and phenol respectively [41, 42, 43]. On the contrary, Economopoulos and Gordon [44] did not find any modification of spermatocyte differentiation in the nymphs of the large milkweed bug *Oncopeltus fasciatus* treated with JHAs. Landa and Metwally [45] observed normal spermatogenesis in pupae of Coleoptera treated with a JHA. Szollosi [46] did not observe direct effects on spermatogenesis in late larvae of acridids treated with JHAs like synthetic Cecropia juvenile hormone or hydroprene.

The current study described a decline in mature sperm concentration and irregular arrangement of sperms in acini of treated crabs during the active phase in contrast to acini fully laden with mature spermatozoa arranged in circles of control crabs. Concomitant findings have been reported in other non-target crustaceans exposed to pesticides and pollutants. In *S. henanense*, treatment with different concentrations of cadmium reduced sperm counts adversely affecting male fertility [34]. Different concentrations of TBT yielded abundance of immature sperm cells and a decline in the number of mature spermatozoa in *M. rosenbergii* [47]. Yang et al. [48] found that sperm counts were 20% lower in the amphipod *Echinogammarus marinus* in sites polluted with poly-chlorinated biphenyls, metals and hydrocarbons.

The present investigation also described abnormal sperm morphology as a consequence of methoprene administration during the active phase. Wang et al. [49] found ultrastructural changes in sperm cells with damaged acrosome, mitochondria, endoplasmic reticulum and cell membranes in cadmium injected *S. yangtsekiense*. Decreased sperm length in *M. rosenbergii* exposed to TBT was documented by Revathi et al. [47]. A decline in the concentration, irregular arrangement and abnormal morphology of mature spermatozoa in acini of treated crabs of the present study can be ascribed to the deformation and degeneration of germ cells induced by methoprene. McLachlan et al. [50] have commented that the reduction in sperm quantity or quality may be caused by alterations in testicular development, intrinsic defects in the ability of germ cells to divide and differentiate or impacts on the hormonal regulatory pathway.

In *T. schirnerae*, sensitivity of the testis to methoprene changed depending on the phase of spermatogenesis, i.e. the testis was more sensitive to methoprene during the active and revival phases and less sensitive during the inactive phase. The reduction in GSI values in addition to histopathological changes was more evident in the active and revival phases when compared to the inactive phase. Yamaguchi et al. [33] reported that the sensitivity of the testicular tissue to arsenic changes depending on the season or the developmental stage of the testis in *S. pax*. On the other hand, Gangotri and Matkar [51] revealed damaged testicular layer and vacuolations in testicular tubules of the freshwater crab *Barytelphusa guerini* subjected to acute and chronic treatment of sugar industry effluents during breeding and non-breeding seasons.

The exact mechanism of methoprene induced histopathological changes in the testis of *T. schirnerae* is unclear. There are two possibilities: one is direct toxicity of methoprene on germ cells which include gonia, spermatocytes, spermatids and spermatozoa, thus negatively impacting spermatogenesis and male fertility in turn. Another

possibility is that methoprene disrupts the endocrine system, continuously inhibiting spermatogenesis by causing a reduction in the circulating gonad stimulating hormone (GSH) titres which in turn inhibits the secretion of androgenic gland hormone (AGH). Spermatogenesis in crustaceans is regulated by GSH from the brain and thoracic ganglion [52, 53] and AGH from the androgenic gland (AG). The AGH has been shown to play a role in the regulation of male sex differentiation and spermatogenesis in crustaceans [54, 55]. In the present study, methoprene injections might have hindered the activity of the AG eventually causing malfunctioning of the testicular cells.

According to Payen and Costlow [28], inhibition of testicular processes by Altosid in *R. harrisii* indicates a chemosterilizing action of methoprene, either working with methyl farnesoate or outcompeting it for the same active site. Several authors reported the adverse effects of insecticides on gonad development and maturation and steroidogenesis in NTOs [43, 56, 57]. Sastry and Miller [58] reported methoprene induced destabilization of intracellular lysosomes which result in cellular damage. Mercier et al. [59] suggested that the histopathological changes in the gonad is due to the probable loss of biochemical reserves reflected by thinning of germinal epithelium as well as poor development of gonad.

## Conclusion

Acute exposure of sublethal dose of methoprene during different phases of spermatogenesis of *T. schirnerae*, a non-target crustacean, demonstrated reduced GSI values and considerable degree of changes in the morphology and histoanatomy of the testis leading to partial or total suppression of spermatogenesis. Results of this study provide evidence that the reproductive physiology of non-target organisms is at risk, in spite of methoprene being a comparatively safer option in pest control.

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