בית ספר- "מעלה שחרות"

סמל מוסד- 770602

טלפון: 08-6355930

The influence of hormone-like substances on male mice sexual behavior and fertility.

מגישה:

לנה צ'יריפנוב

קיבוץ אילות

ת"ז: 1556511731

טלפון: 0547378031

תאריך הגשה: 22.03.2016

מנחה:

דר' גבי בנט

קיבוץ יהל, 88850

מרכז מדע ים המלח והערבה

בית ספר "מעלה שחרות" יטבתה

052-8835997

1. Table of contents

1. Abstract

One of the problems associated with modern industry is the release of toxic chemicals to the environment. This happens due to inappropriate treatment in industrial wastes. Among these chemicals, one may find biologically active substances, some with hormonal activity. These Hormone-like substances, or endocrine disruptors, affect sexual behavior and fertility of exposed animals. The chemical 4 tert-octylphenol (4TOP) serves in the chemical industry as a precursor for glues, pesticides, medicines, emulsifiers, paints, oils and others, and vinclozolin serves as a fungicide. These two chemicals are classified as endocrine disruptors. The aim of this work was to test the influence of these two chemicals on the sexual behavior and fertility of male mice as well their weight gain, in order to demonstrate the danger of endocrine disruptors to the environment. The experiment consisted of 36 mice in the age of one month (just recently weaned), 12 mice served as a control group and drank only water, 12 mice drank water with 4TOP and 12 mice drank water with vinclozolin. Chemical concentration was 100 ppm in both substances.The mice were grown under these conditions for 66 days during which they were weighted every 2 weeks. At the end of that period they were introduced to adult fertile females from outside the experiment and the first 3 minutes of the encounter were filmed in order to document their mating behavior. The films were analyzed for the times and total duration of the various mating gestures performed by the male. The male were then left with 2 males for another 10 days and then separated. If the females gave birth it was a sigh that the male was fertile. It was found that both 4TOP and vinclozolin caused a slower growth of the mice, male and especially females. After 66 days, treated mice were significantly smaller than control mice. It was also found that both 4TOP and vinclozolin disrupted to some extent male sexual behavior and caused some reduction in both ano-genital olfactory inspection and mounting. Moreover, both chemicals were found to reduce male fertility to some extent. This study, thus, demonstrate further the effect of these two chemicals on growth and fertility of exposed mice and most probably other mammals, and further demonstrate the danger of the release of endocrine disruptors to the environment.

2. Introduction

2.1- The biology of the house mouse (based on Berry, 1970).

2.1.1- Taxonomy

The house mouse (Fig. 1) is a member of the myomorpha taxon, which is a sub-order of the rodents and includes the mice, voles, jerboas, etc. It belongs to the family of *Muridae* (mice and rats) which have three molars on each side of each jaw, each having a basic pattern of nine cusps. The rodents are an order in the class *Mammalia* class.

Fig 1: The house mouse

2.1.2- Anatomy and morphology

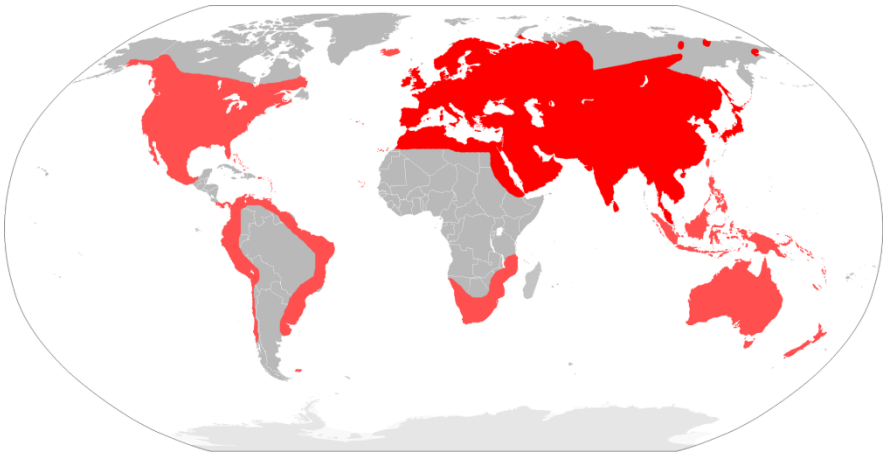
The house mice have an adult body length (nose to base of tail) of 7.5-10 cm and a tail length of 5-10 cm. The weight is typically 10-25g, while males are heavier than females. The length of the tail is the most sensitive indicator for the temperature of the environment the mice live in.it was found that tails of mice's that live in a colder environment are shorter than those of mice living in hotter environments. It is assumed that shorter tails are beneficiary in a colder environment since the tail, that isn’t covered by coat, is responsible for substantial heat loss from the mouse to the environment. Of course shorter tails are non-adaptive in the sense that they reduce the ability of the mouse to maneuver while running. Both over-all body-length and tail-length continue to increase slowly throughout the life of the mouse. There is a considerable variation in size and weight among like-aged individuals in a population, even among older adults.

Dorsal coat color ranged from yellow through brown to black, whereas ventral coat color ranged from white to black. Dark coat color was observed in more humid and closed habitats (darker background color) and pale coat color in drier, more open habitats (lighter background color). This characteristic might imply for the role of general environment color as a selective force for dorsal coat color of the house mice. Other selective forces that could affect mouse's coat color are thermoregulation- darker color absorbs more sun light and turns it into heat and the opposite for lighter coat color.

2.1.3- Habitat

House mice can be found mostly in the temperate part of the world, wherever humans are found. World range of the house mouse (Fig. 2) does not include frozen parts of the world, most Africa and inner south America.

Fig. 2: world range of the house mouse



The most common habitats of mice are buildings, barns, and free-living areas next to human settlements. These habitats offer very different physical conditions for the mice, affecting the population organization. For example, the range of free-living animals is greater than that of mice living in buildings. Range size affects interactions with other individuals, intensity of territoriality, food gathering strategy, mating availability and more. Small ranges are apparently the reason why mice living in buildings become isolated from the populations in other buildings or in fields. Barn populations are intermediate in this respect: the barns are colonized by individuals from field populations immediately after their filling in the autumn and thereafter the resulting

population remain fairly isolated for the time the barn is full. In buildings, mice may colonize any area where food is available. Food availability in houses is usually low. It is generally uncommon to see house mice living independently of humans.

2.1.4- Reproduction and life-history

Female mice become sexually mature at around six weeks, although later under crowded or cold conditions. Females born in the wild in the autumn may not come into breeding condition until the following spring. Males attain puberty somewhat later than females, but they are less affected by environmental fluctuations. Wild-living mice have a definite breeding season. In Britain this is approximately from April to September.
Fertilization is possible for about 10-12 hours after ovulation; the peak time of day for ovulation is 03.00; and most copulation takes place in laboratory mice between 22.00 and 01.00.

The young weigh approximately one g. at birth. They are hairless and helpless. The rate of post-natal growth depends on the amount of milk available, and this in turn depends on litter size. Generally speaking, mice are close to 10 g. when they are weaned at the age of 3 weeks.

In a female mouse, the number of ova shed is probably most closely determined by the size of the mother—the larger the female, the more ova are released.
Litter size in laboratory mice increases for the first two or three litters of any female, and decreases in high parities. Ovulation rate increases for the first few litters but thereafter remains constant. In wild-living mice, mean litter size increases to a maximum in June and July, then decreases again.

2.1.5- Male sexual/mating behavior (based on Burns-Cusato et al, 2004).

During mating, male mouse performs a series of characteristic gestures that usually come in a continual order. The male mouse begins an encounter by general investigation the female, by usual nose to nose sniffing. Later, the male investigates the female’s ano-genital region, often lifting or pushing her with his nose. Ano-genital olfactory investigation is also termed "rooting". In the next step, the male will try to mount the female from behind. If achieved,

the male then presses his front legs against the female's side and makes rapid, shallow pelvic thrusts. Following this, come deep and slow pelvic thrusts, intromission and ejaculation while freezing. During the course of mating, both male and female produce ultrasonic voices by which they signal to each other. Steroid hormones and olfactory cues, released by both the male and female, are also an important part of the mating.

2.1.6- Diseases

Pathological conditions are rarely found in wild mice. Most reports which refer to disease describe the situation during the decline of house mice "plagues". For example, pneumonia seems to have been an important cause of death in a plague in Kern County, California. Large numbers of mice dead from disease were reported during two Russian outbreaks.

2.1.7-Predation

The clearest evidence of mortality is, of course, provided by predation. House mice fall prey to owls, hawks, cats, dogs, skunks and snakes. Barn owls are particularly efficient mice predators. A single family of these owls can consume more than a dozen mice in one night. House mice usually live only one year in the wild due to predators and exposure to unfriendly environments. In captivity, mice may live up to three years. However, humans, through the use of pest control strategies, ranging from traps to exclusion, are also formidable house mouse enemies.

2.1.8-Social Relationships

Within a family group (of one male, several females, and youngs) there is normally no aggressive behavior, but strange mice of either sex are attacked, even by quite young mice. Usually all individual in the family that are big enough are participating in attack. When two residents meet, there is a momentary pause for identification by sniffing. If the stranger flees when approached, the retreat stimulates attack by the resident; if it freezes, sniffing is followed by a direct attack. Lactating females are particularly aggressive towards strangers, and, when excited, will attack any mouse that approaches the nest, including the resident male. The young males leave the company of the females and young when they begin developing aggressive behavior.

When two males are confined together, they fight savagely and persistently until one establishes dominance (the dominant is almost always the larger animal). Thereafter the dominant continues actively to seek and pursue the subordinate. When the dominant is active, the subordinate avoids confrontation or remains within its nest box; when the dominant is inactive, the subordinate roams freely, although avoiding the dominant's nest. If a new mouse is introduced at this time its initial retreat from the subordinate produces an immediate response of dominant behavior. When a number of males are introduced, one becomes a despotic dominant, and social hierarchies are set up among the subordinates.

3.2 Hormones (Based on Neave, 2008)

Hormones are organic substances secreted by both plants and animals that function in the regulation of physiological activities and in maintaining homeostasis. They carry out their functions by causing responses in specific organs or tissues that are adapted to react to minute quantities of them. Hormones are secreted from the endocrine glands in the body. These glands are ductless, so hormones are secreted directly into the blood stream rather than by way of ducts. The major endocrine glands in the body include: pituitary, pineal, thymus, thyroid, adrenal glands, and pancreas. In addition, men produce hormones in their testes and women produce them in their ovaries.

3.2.1 Classification of the major hormones by their function and origin.

Hypothalamic hormones: thyrotropin releasing hormone (TRH), growth hormone releasing hormone (GHRH), dopamine, prolactin (PRL), oxytocin, arginine and vasopressin.

Anterior pituitary hormones: thyroid-stimulating hormone (TSH), prolactin (PRL), follicle-stimulating hormone (FSH).

Pancreatic hormones- Involved in glucose balance in the blood and …. (for the somatostatin): insulin, glucagon, somatostatin, all peptides.

Male reproductive hormones: peptide- inhibin; steroids- testosterone, dihydrotestosterone

Female reproductive hormones: peptides - inhibin, oxytocin, human chronic somatotropin; steroids- progesterone.

Pineal hormones: amino acid or fatty acid derived- melatonin, serotonin.

3.2.2 Sex hormones.

Both classes of male and female hormones are present in both males and females alike, but in vastly different amounts. Most men produce 6-8 mg of the male hormone testosterone (an androgen) per day, compared to most women who produce 0.5 mg daily. Female hormones, estrogens, are also present in both sexes, but in larger amounts for women.

3.2.3 Female.

Estrogens are the sex hormones produced primarily by a female's ovaries that stimulate the growth of a girl's sex organs, as well as her breasts and pubic hair, known as secondary sex characteristics. The ovaries, which produce woman's eggs, are the main source of estrogen from female body. The adrenal glands, located at the top of each kidney, make small amounts of this hormone too and so do fat tissues. Estrogen moves through blood and acts everywhere in the body.

Estradiol is the most potent estrogen that is found naturally in women. It is produced by the ovaries, adrenal gland and also by the placenta during pregnancy. In females, estradiol acts primarily as a growth hormone for the reproductive organs including the vagina, the fallopian tubes, the endometrium and the cervical glands. Estradiol also enhances growth of the womb’s muscle layer, the myometrium. In addition, the hormone maintains oocytes (eggs in the ovary) and triggers a series of events that lead to ovulation. The changes that begin around puberty are driven by estradiol. These changes are enhanced during the reproductive age and then become less pronounced after the menopause, as estradiol levels decline. Estradiol is required for normal breast development, alteration of body shape, skin changes, and the fat distribution profile that is typical of females. Estradiol in males is produced by the Sertoli cells of the testes.

Progesterone, another steroid hormone, The hormone is produced in the ovaries, the placenta (when a woman gets pregnant) and the adrenal glands. It helps prepare women's body for conception and pregnancy,

maintains pregnancy and regulates the monthly menstrual cycle. It also plays a role in sexual desire.
One of progesterone's most important functions is to cause the endometrium to secrete special proteins during the second half of the menstrual cycle, preparing it to receive and nourish an implanted fertilized egg. If implantation does not occur, estrogen and progesterone levels drop, the endometrium breaks down and menstruation occurs.

If a pregnancy occurs, progesterone is produced in the placenta, and levels remain elevated throughout the pregnancy. The combination of high estrogen and progesterone levels suppress further ovulation during pregnancy. Progesterone also encourages the growth of milk-producing glands in the breast during pregnancy.

High progesterone levels are believed to be partly responsible for symptoms of premenstrual syndrome (PMS), such as breast tenderness, feeling bloated and mood swings. When you skip a period, it could be because of failure to ovulate and subsequent low progesterone levels. (Leon-Olea et al 2015).

3.2.4 Male.

Androgens are a group of hormones that primarily influence the growth and development of the male reproductive system. The predominant and most active androgen is testosterone, which is produced by the male testes. The other androgens, which support the functions of testosterone, are produced mainly by the adrenal cortex—the outer portion of the adrenal glands—and only in relatively small quantities. Androgens help trigger the development of the testes and penis in the male fetus. They trigger the process of puberty and influence the development of facial, body and pubic hair, deepening of the voice, and muscle development, the male secondary sex characteristics. After puberty, androgens, specifically testosterone, play a role in the regulation of the sex drive.

Only a very small amount of androgen is secreted before puberty. Androgens also are needed for the development of the male reproductive system. Androgens also are necessary for the formation of sperm cells and for the maintenance of sexual interest and behaviour. Also, the regression of

scalp hair, or baldness, are influenced by androgens. Androgens enhance bone growth and increase the number and thickness of muscle fibres in the male body. Other growth patterns that androgens stimulate are kidney weight and size, the increase of protein in bone tissue, the regeneration of red blood cells (erythrocytes), the presence of pigments in the skin, and the increased activity of sweat and sebaceous (oil-producing) glands.

Testosterone, the main steroid male hormone, is produced by the male testis and is responsible for development of the male sex organs and secondary sex characteristics. The pituitary gland controls the level of testosterone in the body. When the testosterone level is low, the pituitary gland releases a hormone called luteinizing hormone (LH) that tells the testicles to make more testosterone. Before puberty, the testosterone level in boys is normally low. The level of testosterone is the highest around age 40, then gradually becomes less in older men.

3.3- Hormone-like substances or endocrine disruptors (Based on NIEHS, 2010).

Hormone-like substances are chemicals with totally unrelated structure to any of the hormones, either proteins or steroids in animal or any of the plant hormones. Moreover, these chemicals belong to different classes of chemicals, so that only their activity as hormones or hormone disruptors is the common denominator. Hormone-like substances act in various ways:

1. They attach to hormone specific receptors instead of the hormone itself and invoke its activity.
2. As disruptors, they act through the blockage or disruption of hormonal activity. Their blockage activity in done through various mechanisms, either by blocking the target receptors of the hormone, and by that neutralizing the hormone effect , or by directly interacting with the hormone and neutralize it.
3. Sometimes, through the interaction with the hormones, the invove another, sometime unrelated activity.

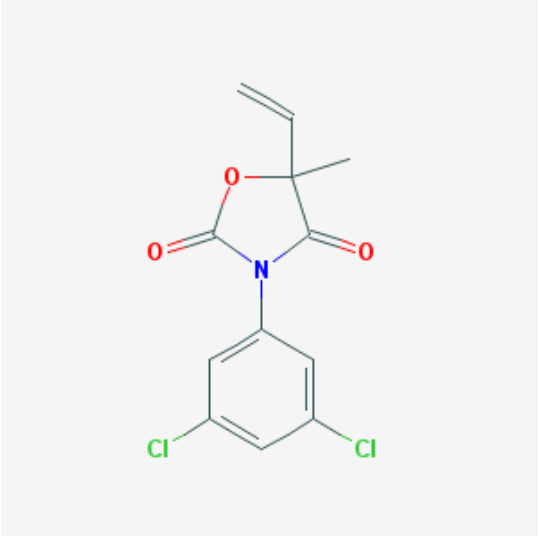
Hormone like substances, acting in both animal and plants, can be either produced biologically by microorganisms mainly or chemically produced manmade. In some instances, the chemicals are intermediators in industrial production of certain products, released untreated from factories and in some cases, the chemicals are end products with certain activity, with a side effect as hormone-like. The dispersion of these chemicals into the environment is also very variable- sometimes they are dispersed through untreated wastes, either aqueous, gaseous or solid and sometimes they are dispersed in purpose, like pesticides (see below).

Hormone-like substances have sometimes severe environmental effects, not via actual killing of the animals rather than reducing their propagation and environmental fitness, for instance, exposure to female hormone-like substances will reduce male aggression and its ability to reach competent females. Nowadays there is a great concern regarding the environmental damage of hormone-like substances.

3.3.1- Vinclozolin

Vinclozolin is a systemic dicarboximide fungicide that is used on fruits, vegetables, ornamental plants, and turf grass. It is a colorless crystal with slight aromatic order. It was first introduced by BASF (the largest chemical producer in the world) in Germany in 1976. The chemical formula of vinclozolin is $C_{12}H_9Cl_2NO_3$- Fig. 3.

Fig. 3: Chemical structure of vinclozolin



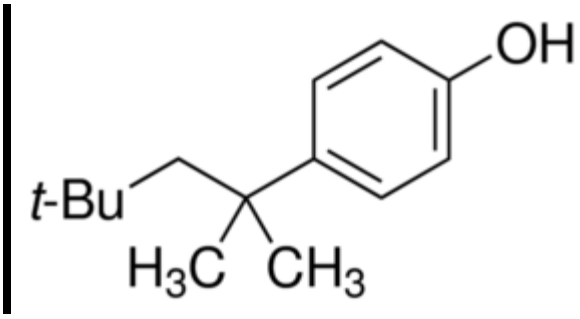
Vinclozolin and its metabolites are known to be endocrine disruptors and act as androgen receptor antagonists. The main effect induced by vinclozolin is related to its antiandrogen activity and its ability to act as a competitive antagonist of the androgen receptor. Vinclozolin can mimic male hormones, like testosterone, and bind to androgen receptors, while not necessarily activating those receptors properly. There is evidence that vinclozolin itself binds weakly to the androgen receptor but at least two of its metabolites are responsible for much of the antiandrogenic activity (EPA, 2016).

When male rats were given low dose (>3 mg/kg/day) of vinclozolin, effects such as decreased prostate weight, weight reduction in sex organs, nipple development, and decreased ano-genital distance were noted. At higher doses, male sex organ weight decreased further, and sex organ malformations were seen, such as reduced penis size, the appearance of vaginal pouches and hypospadias (urine/sperm tube opens at the middle of the penis) (EPA, 2016). In the rat model, it has been shown that the antiandorgenic effects of vinclozolin are most prominent during the developmental stages (EPA, 2016). In utero, this sensitive period of fetal development occurs between gestation days 16-17 (Gray et al, 2001). Embryonic exposure to vinclozolin can influence sexual differentiation, gonadal formation, and reproductive functions (Anway et al, 2006). In bird models, vinclozolin and its metabolites were shown in vitro and in vivo to inhibit androgen receptor binding and gene expression. Vinclozolin caused reduced egg laying, reduced fertility rate, and a reduction in successful hatches (EPA, 2016). Androgens also play a role in puberty, and it has been shown that antiandrogen like vinclozolin can delay pubertal maturation (Gray et al, 2001). Antiandrogenic toxins are also known to alter sexual differentiation and reproduction in the rabbit model. Male rabbits exposed to vinclozolin in utero or during infancy did not show a sexual interest in females or did not ejaculate (Gray et al, 2001). Since the androgen receptor is widely conserved across species lines, antiandrogenic effects would be expected in humans (EPA, 2016). In vertebrates, vinclozolin also acts as a neuroendocrine disruptor, affecting behaviors tied to locomotion, cognition, and anxiety (leon-Olea, 2015).

3.3.2- 4-Tert-Octylphenol (4TOP).

4-tert-Octylphenol (henceforth- 4TOP) - Fig. 4, belongs to a group of related substances called alkylphenols. Chemically, these chemicals are the result of alkylation of phenols. The octyl group is a chain of eight carbon atoms, which may be branched or linear. It is a solid, which is usually marketed in the form of a powder, flakes, or briquettes. It is also available in molten form. 4TOP is a chemical intermediate, and is mainly used to make phenolic resins (98%) that are converted into ethoxylates to produce surfactants. The phenolic resins are also used in rubber processing to make tyres (82%). Minor uses include being a component in printing inks and electrical insulation varnishes, and in the production of ethoxylated resin for offshore oil recovery. Octylphenol ethoxylates are mainly used in emulsion polymerisation, textile processing, water-based paints, pesticide and veterinary medicine formulations (Environment agency UK, 2005). Only two european suppliers are known, and further market information is commercially sensitive. 4TOP is expected to biodegrade relatively quickly in the environment, it is fairly soluble in water, and its octanol–water partition coefficient implies a moderate bioaccumulation potential in fish. The substance is expected to partition mainly to soil and sediment when it is released to the environment. In general, no reliable environmental monitoring data are available, which means that most of the exposure assessment is based on generic industry information and a number of assumptions. It is acutely toxic to aquatic organisms and may cause long-term adverse effects in the aquatic environment. Potential risks to wastewater treatment plant and soil are also identified for one specific life cycle step. 4TOP is suspected of damaging fertility of unborn child and can cause damage to reproductive organs through prolonged or repeated exposure (Environment agency UK, 2005). 4TOP is a prevalent environmental pollutant which binds to estrogen receptors and exerts estrogenic actions in vitro. This chemical is of concern because it may cause effects on endocrine systems in wildlife and people. The effect of 4TOP on reproductive systems and reproduction was shown in rainbow trout, carp, salmon and medaka fish (Knorr and Brawnback, 2002) and in rats (Laws et al, 2000).

Fig. 4: Structure of 4TOP



3.4- Hypothesis

One of the problems associated with modern industry is the release of toxic chemicals to the environment. This happens when there is an inappropriate treatment in industrial wastes. Among these chemicals, one may find biologically active substances, some with hormonal activity. These Hormone-like substances or endocrine disruptors affect sexual behavior and fertility of exposed animals. The chemical 4TOP serves in the chemical industry as a precursor for glues, pesticides, medicines, emulsifiers, paints and oils and vinclozolin is a chemical that serves as a fungicide. These two chemicals are known as endocrine disruptors able to affect endocrine system of exposed animals. The aim of this work was to test the influence of these two chemicals on the sexual behavior and fertility of male mice as well their weight gain, in order to demonstrate the danger of endocrine disruptors to the environment. The hypothesis was that these two chemicals would affect both, weight gaining of the mice, males and females, and both, sexual behavior and fertility, of male mice.

4. Materials and methods

4.1- Animals and growth condition.

Each mouse involved in the experiment was born and grown in the educational greenhouse at " Maale Shaharut" school. The mice were kept in plastic cages whose dimensions were 37x30x15 centimeters. A 20x10 centimeter hole was made in the cage cover and a metal net was placed instead to allow air supply. Five cm wide sawdust layer was placed on the cage's flour to serve as a lining and to absorb mice's waste. Each cage contained an up-side down plastic box which served as a shelter, on top of which, a small metal can, which served as a water container, was placed. Another can which was placed on the sawdust layer, served as a food container. The mice food (rabbit feed of Ambar Ltd.) contained 17% of protein, 4% of fat, 44.5% of carbohydrates, 15% of cellulose, 7.5 of ash (minerals) and 12% of moisture. Water/feed/lining exchange/addition were done at the most every other day. The room in which the mice were grown was lit by natural daylight and was air-conditioned to give temperatures fluctuating between 24^{o}C-29^{o}C.

4.2- Experimental setup.

In this experiment, 36 mouse participated, about half of them males. The mice were 2 months +/- 1 week of age, all of them about a week after weaning. At the beginning of the experiment (7.10.15) mice were divided into three groups: water imbibing, water+vinclozolin imbibing and water+4TOP imbibing. Each group contained 12 mice which were divided into 2 cages, 6 individuals in each. At this stage, sex distinction wasn't possible. Males and females were separated from each other 2 weeks later (on 21.10.15), when sexual signs were clearer. At this stage, since male and female number weren't equal, another cage was added into each group. The exposure period for the chemicals was halted on 16.12.15, after 66 days. At this stage, the males were encountered with females from outside of the experiment to record their mating behavior (see section 4.4). Following this stage, each male that participated in the experiment was tested for fertility (section 4.5).

From 16.12.15 (the end of exposure period) and on, all mice were provided with water only.

During the course of the exposure period, water (+/- the chemicals) was provided to the mice to satisfaction. In addition, the amount of water imbibed by the mice was measured on November 15-21, in the middle of the experiment, in order to calculate the amount of 4TOP and vinclozolin taken daily by the mice and to be able to provide the mouse with water to satisfaction, without too much wasting the chemicals.

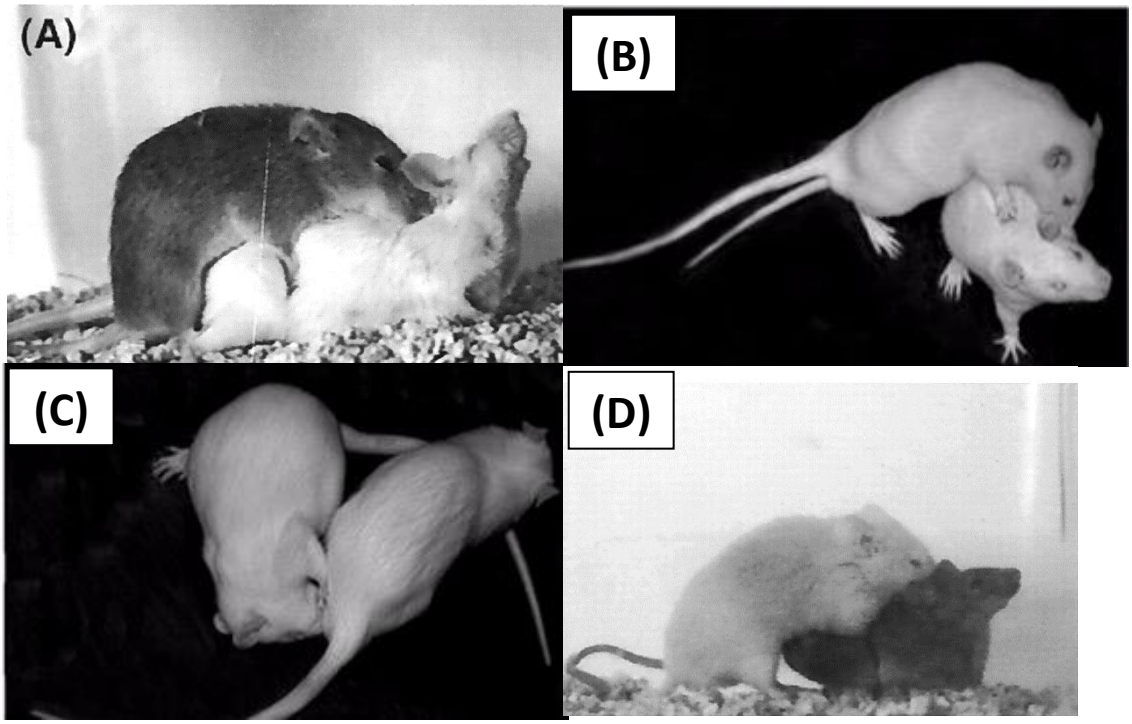
4.3- Preparation of vinclozolin and 4TOP.

Two hundred and fifty mg of each chemical were solubilized in 25 ml of 100% ethanol to give 10 mg/ml concentrate. The chemicals were completely dissolved by a thorough vortexing and kept in a sealed polypropylene tube in darkness at room temperature. Keeping them in 4^{o}C caused precipitation of the chemicals. Upon distribution, the chemicals were diluted 1/100 in desalinated water and mixed thoroughly, to give final concentration of 100 ppm of the chemicals (and 1% alcohol) in the mice drinking water.

4.4- Recording of mating behavior.

Right at the end of the exposure period, each male was placed individually in a clean and unused cage, along with an adult female. The first 3 minutes of the encounter were recorded by a video camera to capture male mating behavior- nose sniffing, ano-genital olfactory inspection, mounting, vocalization, biting and pelvic thrusts (Fig. 5). Females were replaced in each encounter. For each treatment (water, water+vinclozolin and water+4TOP), 3 mice were taken. The videos were analized for the times and and total duration of each gesture during each encounter.

Fig 5: Male mouse mating gestures: Pelvic thrust; (A), biting; (B), ano-genital olfactory inspection; (C) and mounting; (D).



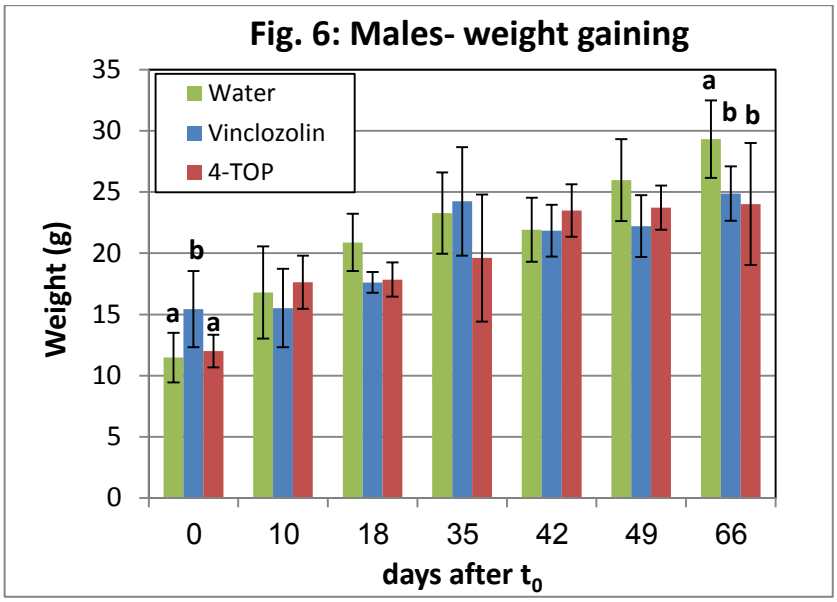
4.5- Male fertility test.

In order to follow male fertility problems associated with their exposure to the chemicals (time and concentration), each male was transferred into a clean cage and left for ten days with an adult female. After 4 days, another female was introduced into the cage in order to rule out female infertility. 10 days following encounter onset, males were taken out of the cages and the females were left to give birth (or not). If the female gave birth, it was a sign that the male is fertile.

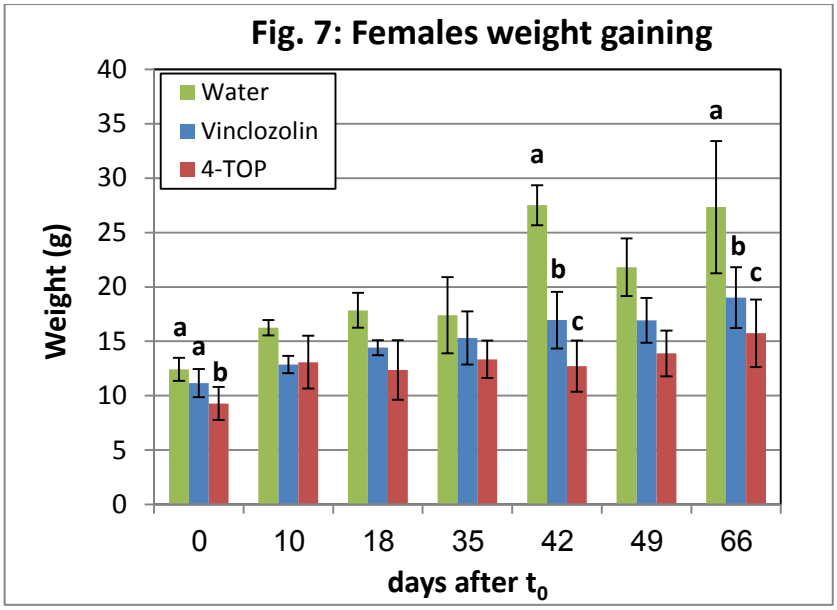
4. Results

In this experiment we tested the influence of hormone-like substances that are released to the environment on mice body weight gaining and mating behavior and fertility of male mice. To that end we grew mice, male and females, for 66 days, and provided them water only as a control, and with the addition of 2 hormone-like substances, vinclozolin or 4TOP, to 100 ppm concentration each in drinking water. During the exposure period, we measured body weight gaining and at the end of it we measured the mating behavior of the male mice (see materials and methods), against adult females taken out of the experiment. Subsequently, we tested male fertility by leaving each of the male mice with two females for 10 days and tested the present or absence of pregnancy.

Body weight gaining of the mice, male and female, along the exposure period, is presented in Fig. 6 and 7, and in tables 1 and 2.



*- for each day, columns bearing different letters have a significant difference of p<0.1 between them.



*- for each day, columns bearing different letters have a significant difference of p<01 between them.

Table 1: predicted linear growth rate ($g_{*}day^{-1}$)- **a**, and the correlation coefficient between the predicted line and the actual measurements- $\mathbf{R^2}$, of males and females mice, imbibed by either water, water+ vinclozolin and water+ 4TOP.

Males			
	Water	Vinclozolin	4TOP
a	0.24	0.175	0.158
R^2	0.91	0.86	0.84

Females			
	Water	Vinclozolin	4TOP
a	0.216	0.113	0.071
R^2	0.773	0.971	0.7

Table 2: percent reduction in body weight on day 66 (end of exposure period) in water+vinclozolin and water+4TOP imbibed males and females mice, related to those imbibed with water only.

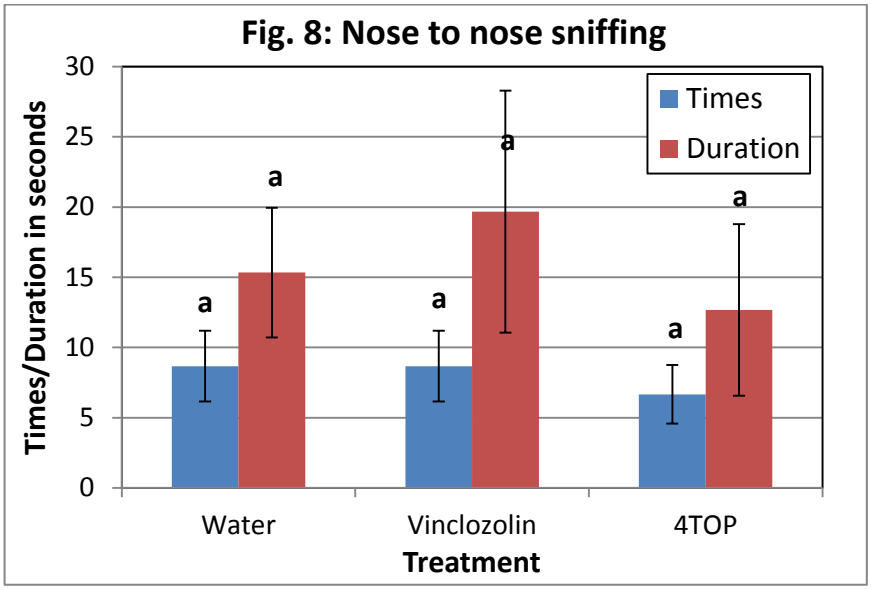
	% reduction	
	Vinclozolin	4TOP
Males	15	18
Females	30	42

From Figs 6 and 7 and tables 1and 2, one can see that:

1. At the end of the exposure period (day 66), there is a significant difference between the weight of control male mice (drinking only water) and treatment male groups (those that drank water+vinclozolin or water+4TOP).
2. In male mice, this significant difference is evident only at the end the exposure period.
3. In female mice, this significant difference between control (drinking only water) and treatment groups can be seen as early as on 10th day.
4. In both female and male mice, the predicted linear growth rate of treatments groups is lower than that of control groups.
5. The predicted linear growth rate of water+4TOP imbibed mice in both females and males is lower than that of water+vinclozolin imbibed mice.
6. In water+vinclozolin, as well as in water+4TOP imbibed mice, the predicted linear growth rate of females in lower than that of males.
7. At the end of the experiment, the percentage of body weight loss (treatment groups vs. control groups) of female mice is greater than that of male mice.

As already mentioned, at the end of the exposure period, the mating behavior of the male mice was recorded in order to ascertain the influence of vinclozolin or 4TOP on the former. The influence of both vinclozolin and 4TOP on the various gestures associated with male mating behavior- nose to nose sniffing , ano-genital olfactory investigation (sniffing of the behinds), mounting, biting, vocalization and pelvic thrusts, were recorded (see materials and methods). The gestures that were observed in this experiment were nose

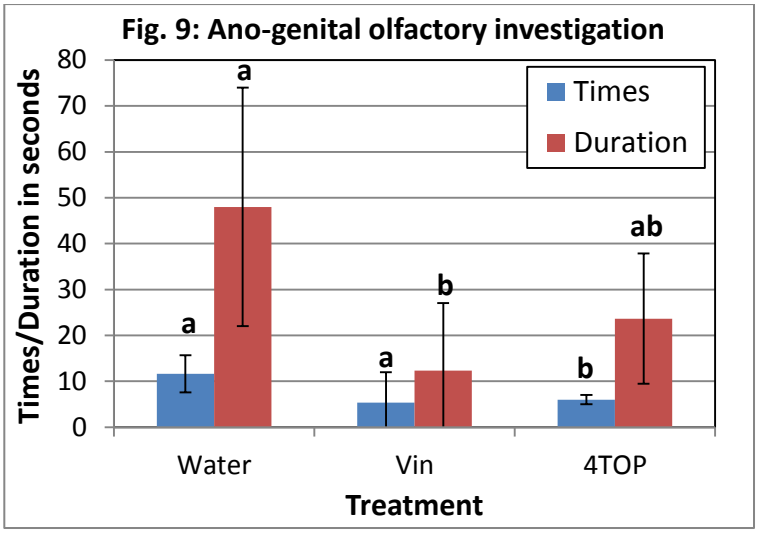
sniffing (Fig. 8), ano-genital olfactory investigation (sniffing of the behinds) made by male (Fig. 9) or mutually (Fig. 10) and mounting (Fig. 11).



*- columns of the same parameter, either times or duration, bearing different letters have a significant difference of $p<0.1$ between them.

From Fig. 8 it can be seen that:

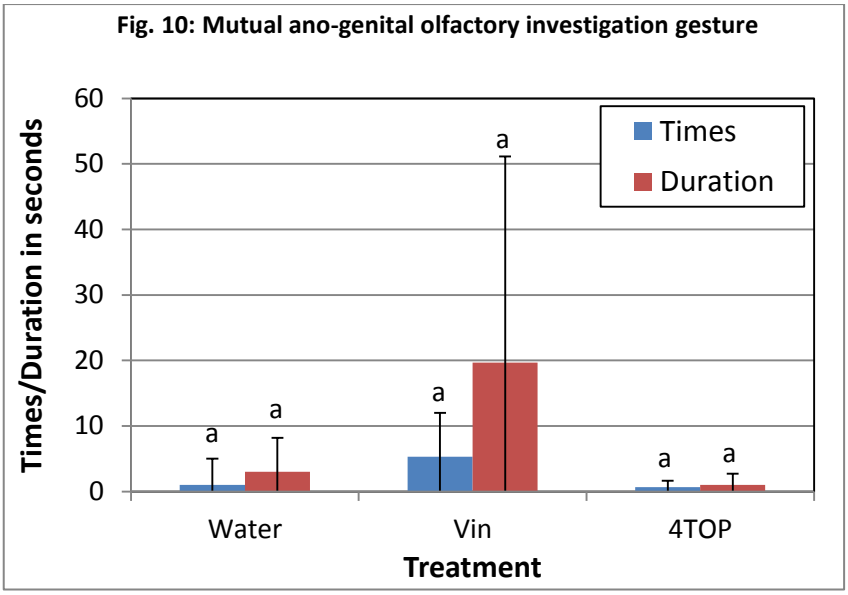
1. There is no significant difference between treatments and control groups in the amount of nose to nose gesture in the first 3 minutes of encounter between the males and females (see materials and methods).
2. There is no significant difference between treatments and control groups in the total duration of nose to nose gesture in the first 3 minutes of encounter males and females (see materials and methods).



*- columns of the same parameter, either times or duration, bearing different letters have a significant difference of $p<0.1$ between them.

From figure 9: it can be seen that:

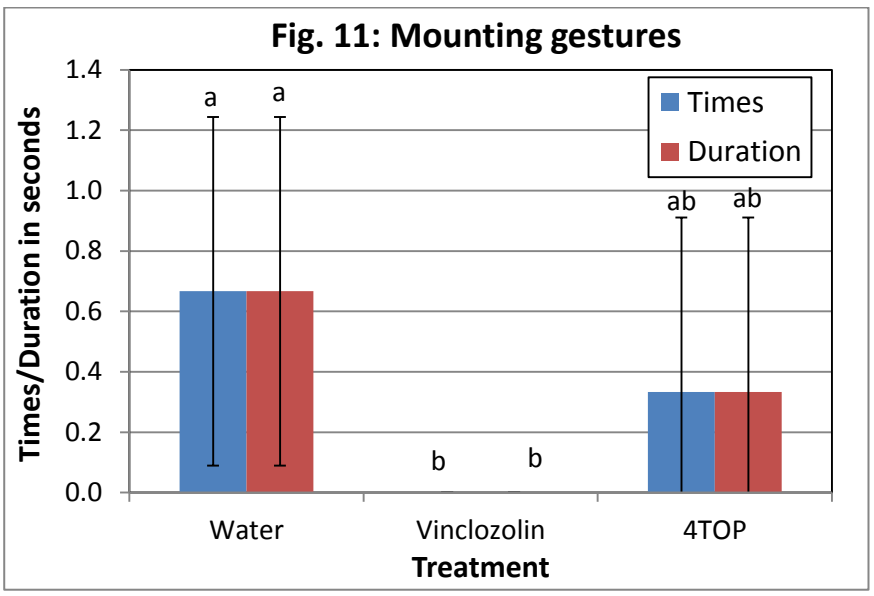
1. There is no significant difference between the amount of ano-genital olfactory investigation gestures (sniffing of the behinds) of control group and that of water+vinclozolin treatment, but there is a significant difference between the former and water+4TOP.
2. There is a significant difference in the total duration of ano-genital olfactory investigation gestures between control group and water+vinclozolin treatment.
3. As to water+4TOP treatment, the total duration of ano-genital olfactory investigation gestures is not significantly different from the control group on the one hand and nor from the water+vinclozolin treatment on the other.



*- columns of the same parameter, either times or duration, bearing different letters have a significant difference of p<0.1 between them.

From Fig. 10 it can be seen that:

1. There is not a significant difference in the amount and total duration of mutual ano-genital olfactory investigation gestures (sniffing of the behinds) (see materials and factors) between control and treatment groups.



*- columns of the same parameter, either times or duration, bearing different letters have a significant difference of p<0.1 between them.

From Fig. 11 it can be seen that:

1. There is a significant difference in both, the amount and total duration, of mounting gestures (see materials and factors) between control group and the water+vinclozolin treatment.
2. As to the water+4TOP treatment, the total amount and total duration of mounting gestures is not significantly different from the control group on the one hand and nor from the water+vinclozolin treatment on the other.

As already mention in materials and methods, following the first encounter between the males from the experiment with females outside of it, we left each of these males with two females for 10-14 days to mate with, to record male fertility. If the females gave birth it was a sigh that the male is fertile. The results of that stage are presented in table 3.

Table 3: fertility test: the percentage of females that gave birth after two weeks encounter with males from either control group (mice imbibed with water only), vinclozolin (mice imbibed with water+vinclozolin) or 4TOP (mice imbibed with water+4TOP). n=3

Control	Vinclozolin	4TOP
100%	67%	67%

From table 3 it can be seen that:

1. Hundred percent of the females that were left to mate with males from control group gave birth.
2. Only 67% of the females that were left to mated with males from both 4TOP and vinclozolin group gave birth.

6. Discussion

In this experiment we tested the influence of hormone-like substances that are released to the environment on mice body weight gaining and mating behavior and fertility of male mice. To that end we grew mice, male and females, for 66 days, and provided them water only as a control, and with the addition of 2 hormone-like substances, vinclozolin or 4TOP, to 100 ppm concentration each in drinking water. During the exposure period, we measured body weight gaining and at the end of it we measured the mating behavior of the male mice (see materials and methods), against adult females taken out of the experiment. Subsequently, we tested male fertility by leaving each of the male mice with two females for 10 days and tested the present or absence of pregnancy.

The results we have found in this study were:

1. Both 4TOP and vinclozolin caused a slower growth of the mice, male and especially females. After 66 days, treated mice were significantly smaller than control mice.
2. Both 4TOP and vinclozolin disrupted to some extent male sexual behavior and caused some reduction in both ano-genital olfactory inspection and mounting.
3. Both chemicals were found to reduce male fertility to some extent.

As can be seen in fig. 6 and 7, and in tables 1and 2, both vinclozolin and 4TOP brought about a reduction in mice body weight gaining, so that at the end of the exposure period, treated, male and especially females, were significantly smaller than control mice. In contrast to our findings, Blake et al (2004) found no significant difference in body weight between water or water+4TOP imbibed rats. They used two-month old Fischer 344 rats and exposed them for 4 month to 10^{-5}M, 10^{-7}M, 10^{-9}M 4TOP in drinking water. There are 3 differences between ours and Blake et al (2004) experiment: 1. In our experiment, we provided the mice with 100ppm of 4TOP which is $5X10^{-4}$M, 50 times higher than the highest concentration used by Blake et al (2004). 2. Our mice weighted between 10-25g and the rats used in Blake et al (2004) experiment weighted 350-360 g, 14 times higher. 3. The exposure period in

our experiment was 66 days and in Blake et al (2004) the exposure period was 110-125 days, twice as much as ours. All in all, the amount of 4TOP to body weight per time in our experiment was much higher than in Blake et al (2004) experiment. These differences may explain why 4TOP in our experiment brought about a significant reduction in body weight gaining of treated mice, that was absent in Blake et al (2004). In another experiment (Gray et al, 1994) that reached results similar to ours, administration of vinclozolin (100-200 mg/kg body weight/day) to pregnant rats (day 14 and on), and 3 days post-natal, caused a significant reduction in the adult bodyweight of the offspring. In the work of Knorr and Braunbeck (2002), Japanese Medaka fish were exposed to concentrations of 0, 2, 20 and 50 μg/l of 4TOP from 4 h post fertilization of the parents until the maturity of the offspring. Exposure to concentrations above 2 μg/l octylphenol caused a 20-30% increase in mortality of fish, both before and after hatching. In addition growth of the fish was significantly reduced in the treatment of 50 μg/l octylphenol. The cited experiment thus demonstrates, like in our experiment, the ability of octylphenol at least in the used protocols, to reduce the growth of exposed animals. The fact that very low 4TOP concentration (2,000 times lower than ours) were able to affect the fish is because the fish are circulating the water plus the chemical all the time through their gill system so the actual concentration in their body in much higher than in the water. In addition, not only the offspring were exposed to the chemical rather the parents too.

As can be seen in both figures 9 and 11, both vinclozolin and 4TOP, under our experimental conditions, caused a significant reduction in two gestures associated with male mating behavior (ano-genital olfactory inspection and mounting). In their work, Colbert et al (2005) have shown that oral administration of vinclozolin to pregnant long-evans rat at a concentration of 12 mg/kg/day, caused a significant change in androgen-mediated behavioral functions of the males, such as erections and emissions. In addition, Gray et al (1994) found that administration of vinclozolin (100-200 mg/kg body weight/day) to pregnant rats (day 14 and on), and 3 days post-natal, caused number of reproductive malfunctions of the offspring such as the inability to ejaculate normally due to hypospadias. In another work, Gray

et al (1999) show that the exposure of Medeka fish to 10-50 μg 4TOP for 6 month post hatching caused reduction is some courtship activities such as number of approaches to the female, number of cyrcles around her and number of copulations, and reproductive success. In line with these findings, Toft and Baatrup (2001) found that 30 or 60 days exposure of adult guppy fish to 4TOP at nominal concentrations of 100-900 μg/l changed important sexual characteristics in the adult males such as reduction in body color intensity and testes development. All these results, together with ours, demonstrate that both 4TOP and vinclozolin are potent disruptors of sexual/mating/courtship behavior of both fish and rodent males.

In our experiment we show a 23% reduction in male fertility in either 4TOP or vinclozoine exposed males (table 3). In their experiment, Knorr and Braunbeck (2002) were able to show that Japanese Medaka fish, exposed to concentrations of 0, 2, 20 and 50 μg/l 4TOP for 10 days were 11% less fertile than control males. Along with these findings, Gray et al (1999) shoed a decreased reproductive success (lesser offspring) in males Medaka fish that were exposed to to 10-50 μg 4TOP for 6 month post hatching. Again, vinclzolin concentration employed in these experiments were much lower that ours, but since the fish are concentrating the chemical while passing the water plus the chemical through their gills, the actual 4TOP and vinclozolin concentration in their body must have been much higher.

To summarize, cited articled and the results of we have obtained in our study demonstrate the potential of both vinclozoine and 4TOP to disrupt growth and sexual behavior and fertility of males of animals exposed to these chemicals. The concentrations used in our study and the cited ones they broadly varied and made comparison between the results challenging. Moreover, environmental concentration of these chemicals also varying and the concentration potential in target animals also varying (fish are able to concentrate chemicals in the water to much higher extent comparing to mammals for instance). Nevertheless any release of these chemicals to the environment is dangerous since long exposure for even low concentrations of these chemicals may lead to disruptions in growth, sexual behavior and

fertility of exposed. We assume that fish may be more sensitive to the exposure to these chemicals than mammals.

7. Bibliography

Anway, M.D, Leathers, C. and Skinner M.K. (2006). Endocrine disruptor vinclozolin induced epigenetic transgenerational adult-onset disease. Endocrinology, 147: 5515-23.

Berry, R.J. (1970). The natural history of the house mouse. Field Studies, 3:219-262.

Blake C.A., Boockfor F.R., Nair-Menon J.U., Millette C.F., Raychoudhury S.S. and McCoy G.L. (2004). Effects of 4-tert-octylphenol given in drinking water for 4 months on the male reproductive system of Fischer 344 rats. Reproductive Toxicology, 18:43-51.

Burns-Cusato, M., Scordalakes, E.M. and Rissman, E.F. (2004). Of mice and missing data: what we know (and need to learn) about male sexual behavior. Physiology and Behavior, 83:217–232.

Colbert, N.K.W., Pelletier, N.C., Cote, J.M., Concannon, J.B., Jurdak, N.A., Minott S.B. and Markowski. V.P. (2005). Perinatal Exposure to Low Levels of the Environmental Antiandrogen Vinclozolin Alters Sex-Differentiated Social Play and Sexual Behaviors in the Rat. Environmental Health Perspectives, 113:700-707.

Environment agency UK, (2005). Environmental risk evaluation report:

4-tert-pentylphenol (CAS no. 80-46-6).
https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/290845/scho0208bnqr-e-e.pdf

EPA. 2016. Registration eligibility decision: vinclozolin.

EPA. (2016). R.E.D Facts Vinclozolin.

Gray L.E., Ostby J., Furr J., Wolf C.J., Lambright C., Parks L., Veeramachaneni D.N., Wilson V., Price M., Hotchkiss A., Orlando E. and Guillette L. (2001). Effects of environmental antiandrogens on reproductive development in experimental animals. Human Reproduction Update. 7:248-6

Gray M.A., Ostby, J.S. and Kelce, W.R. (1994). Developmental effects of an environmental antiandrogen: the fungicide vinclozolin alters sex differentiation of the male rat. Toxicology and applied pharmacology, 129:46-52.

Gray M.A., Kevin L.T. and Metcalfe C.D. (1999). Reproductive success and behavior of Japanese Medaka (*Oryzias latipes*) exposed to 4-tert-octylphenol. Environmental Toxicology and Chemistry, 18:2587–2594.

Knorr, S. and Braunbeck, T. (2002). Decline in reproductive success, sex reversal and developmental alterations in Japanese medaka (Oryzias latipes) after continuous exposure to octylphenol. Ecotoxicology Environmental Safety, 51:187-96.

Laws, S.C., Carey, P.A., Ferrell, J.M., Bodman, G.J. and and Cooper, R.L. (2000). Estrogenic Activity of Octylphenol, Nonylphenol, Bisphenol A and

Methoxychlor in Rats. Toxicological sciences, 54:154–167.

León-Olea, M., Martyniuk, C.J., Orlando, E.F., Ottinger, M.A., Rosenfeld, C.S., Wolstenholme, J.T. and Trudeau, V.L. (2015). Current concepts in neuroendocrine disruption. General and Comparative Endocrinology 203:158–173.

Neave, N. (2008). Hormones and behavior a psychological approach. Cambridge University Press.

National Institute for Environmental Health Sciences- NIEHS (2010). Endocrine disruptors. https://www.niehs.nih.gov/health/materials/endocrine_disruptors_508.pdf

Toft G. and Baatrup E. (2005). Sexual Characteristics Are Altered by 4-tert-Octylphenol and 17b-Estradiol in the Adult Male Guppy (*Poecilia reticulata*). Ecotoxicology and Environmental Safety 48:76–84.