UNIVERSITY OF NAMIBIA

DEPARTMENT OF FISHERIES AND AQUATIC SCIENCES

THE EFFECTS OF ENDOCRINE DISRUPTING CHEMICALS ON THE HEPATOSOMATIC INDEX AND GONADOSOMATIC INDEX OF *Oreochromis mossambicus*

DONE BY: KALWENYA LESSYN N

December 2012

THE EFFECT OF ENDOCRINE DISRUPTING CHEMICALS ON THE HEPATOSOMATIC INDEX AND GONADOSOMATIC INDEX OF

(*Oreochromis mossambicus)*

DONE BY:

KALWENYA LESSYN

(200846523)

SUPERVISORS:

Fisheries department: Mr. Martin Tjipute

Biology department: DR.E JULIES & MR.A FAUL

Submitted to the department of Fisheries and Aquatic sciences, Faculty of Agriculture and Natural Resources University of Namibia, in partial fulfillment of the requirement for the award of the degree of Bachelor of Science in Fisheries and Aquatic Sciences at the University of Namibia.

CERTIFICATION

This is to certify that this report has been examined and approved for an award of degree of Bachelor of Science in Fisheries and Aquatic Sciences at the University of Namibia

Supervisors:

X

Mr. Martin Tjipute
Lecturer

X

Dr. E. Julies
supervisor

X

Mr. A. Faul
supervisor

X

HEAD OF DEPARTMENT

ACKNOWLEDGEMENTS

First and foremost I would like to give thanks to the Lord God Almighty for taking me through this season and making it possible for me to carry out this project as it was quiet tough to finally get a sensible topic and have all the equipment in place and available, thus a give praise to God for His grace and for strength to carry out my project. I would also like to show my sincere gratitude to the department of Fisheries and Aquatic Sciences for their continued support throughout this project. Special thanks go to Mr. Martin Tjipute for helping me collect my samples of fish from the Hardap Dam together with Mr. Akawa T. and Mr. Nyandoto, and Mr. Tjipute for allowing me to be supervised by Dr. E.Julies and Mr. A.Faul from the biology department in his absence, Mr. Twalinohamba Akawa for his effort in helping me get all the equipment I needed to keep my project running and for his aid in collecting water samples from Gammams, Goreangab dam and WINGOC. My deepest gratitude has to go to the Biology Department, specifically to Dr. Julies for availing herself and sacrificing her time to supervise my project despite her busy schedule and for allowing me to use their laboratory and lab equipment (glass aquariums, pumps, air pipes and stones) may God bless you for your continued help and supervision from the very beginning, without your assistance my project would not have been possible; Mr. Faul for also taking time out to supervise and assist me, for taking time to collect samples from Hardap dam, for collecting water samples from Gammams, Goreangab dam and WINGOC, for your lab assistance towards the end of my project and for helping me run the necessary tests on my sample for sensible results, thank you so much for your continued help. I would also like to show my appreciation to Mr. Hofmann for his effort in making sure that the technical part of my project was intact. And for everyone else that helped and encouraged me and made my project possible, I give my gratitude, may the good Lord bless you.

DECLARATION

I hereby declare that this work is the product of my own research efforts, undertaken under the supervision of Dr. E. Julies and Mr. A.Faul and has not been presented elsewhere for the award of the degree. All the sources have been duly and appropriately acknowledged.

.....

1. Abstract

EDCs (endocrine-disrupting chemicals) have been defined as exogenous substances or mixtures that alter the functions of endocrine systems and consequently cause adverse effects in an intact organism or its progeny, sub-population. EDCs are structurally similar to the endogenous steroid hormone 17β-estradiol, while others may not be seen structurally related to naturally occurring steroids. (Diniz *et al*. 2005) Thus, many non-steroidal compounds, such as flavonoids, lignins, sterols and fungal metabolites, and synthetic chemicals can interact with sex hormone receptors or can modulate their metabolism and biosynthesis. In this study we investigated the effects of the presence of EDCs in two different water systems namely the Gammams outlet and Goreangab dam on the liver and gonads of the tilapia fish. The focus was on the *Oreochromis mossambicus* species.72 Juvenile fish were exposed to different water systems to determine the effect of possible endocrine disrupting chemicals present in the waters on the gonadosomatic and hepatosomatic indices of the fish. An ELISA test was performed to detect Vitellogenin and a two sample t-test was performed to test for significant differences. A comparative study to assess the estrogenic potency of treated domestic sewage effluent from a sewage treatment plant in Lisbon (Diniz *et al* 2005) showed an increase in vitellogenin induction in the exposed fish. The results obtained from the t-test showed that there were no significant differences in the HSI of (a, b, c, & d) except the test between the positive control and negative control in the tank in (a) which showed a significant difference, for the GSI the significant differences were in (a) the positive control against the negative control, in (b) Gammams against Goreangab dam, and in (c) negative control against Goreangab dam. The rest of the treatments in (a, b, c, & d) showed no significant differences in GSI. Detection of VTG in exposed fish was low at 450nm and for

this reason could not reveal the presence of the estrogen-inducible protein in the treatment groups. Nonetheless the irregularity seen in this study is less evidence of no effect than a consequence of the difficulty in studying, especially with the lack of histological tests

TABLE OF CONTENTS:

PAGE:

CHAPTER ONE

1. Introduction

Tilapia is one of the most important aquaculture species and part of its characteristics is that it is "extremely" resistant to viral, bacteriological and fungal diseases than other aquaculture species. Its tolerance to a wide range of salinity, and its ability to relish in "warm" water at temperatures between 29°C and 31°C is a major attribute. Growth rates decline rapidly at temperatures below 20°C. Thus it is to the consumers benefit to know the health condition of their food source and the factors that affect the growth, function and health of the specific fish and it is to a countries economical benefit to be alert on the possible effects that certain chemicals present or being released in water bodies have on fish. As a fish scientist, fishery manager, quality controller, it should be ones aim to provide the necessary information that renders a product to be safe and fit for human consumption. This can only be ascertained if studies are done on the environment of the fish, the factors influencing water quality of that specific water body, the different chemical compounds such as endocrine disruptive chemicals (EDCs) that are present in that water body and their sources (sewage effluents) and how all these components bring about changes in the growth of the fish, its physiology, pathology and internal organs in particular the liver and gonads as well as the blood of the fish. This in turn helps State and local Government agencies to assess sewage treatment plant efficacies in removing EDCs and potential impacts to receiving waters. Thus there is a need for fishery biologists to research on the effects of EDC and Sewage effluents on the liver and gonads of fish.

African countries such as Côte d'Ivoire, Egypt, Ghana, Malawi, Nigeria and Zambia are successfully involved in integrated aquaculture whereas private investment in commercial

aquaculture production and growth of this sector have been reported in Egypt, Kenya, Namibia, Nigeria, Malawi, South Africa, and Zimbabwe. (NAGA, 2003). Aquaculture in Africa has come a long way since it was first introduced. Nonetheless, aquaculture in Africa is going through an exciting phase of evolution and growth after numerous false developments. This lack of development exists against a backdrop of conditions that would benefit greatly from the rapid development of aquaculture on the continent, namely high incidence of poverty, malnutrition, and unemployment. (Hecht 2000) The development of domestic and export markets for fish, changing macro-economic environments and the stagnation of inland capture fisheries in sub-Saharan Africa has made investment in aquaculture production. (Jamu. and Ayinla, 2003). Although *tilapia* is indigenous to Africa, the continent has been lagging behind other countries in aquaculture production of the fish. In the past few years however, tilapia aquaculture has grown significantly in Africa and more projects are in the pipeline (infofish.org). Africa is an enormous continent, the second largest in the world. It consists of 54 million nation states with an estimated population of about 800million. There is a very long coastline and large inland water bodies that are suitable for aquaculture. Though African aquaculture production is less than impressive as it only accounts for about 0.9 percent (404 571 tons) on global scale, the continent may be opening up for development and tilapia is the prime producer. (E.Hempel & B. Mapfumo 2011)

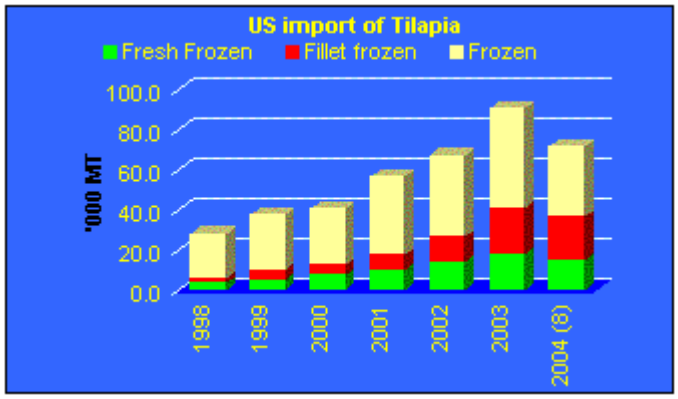

Figure 1: Graph depicting the ever growing import trend of tilapia for the United States of America.

Source: (http://www.worldseafoodmarket.com)

With an increase in population size and an increase demand for water, various aquaculture ventures in the world are now using reclaimed water or semi-purified water. Countries such as Japan, China, Vietnam, USA, Tunisia and California use reclaimed water as a source for this water demanding activity. (Vigneswaran S, *et al*., 2004). The East Calcutta sewage fisheries are the largest single wastewater use system involving aqua-culture in the world. The fish ponds receive raw sewage from Calcutta on a batch of basis and fishermen have developed appropriate operation techniques. Initially raw sewage is allowed to flow into the ponds and after 12 days the contents is disturbed by repeated netting and manual agitation with split bamboos for oxidation, mixing and quick recovery of water quality. After 25 days from initial filling with sewage, the ponds are ready to be stocked with fish. Estimates of total production and yield of fish from the Calcutta fisheries vary from 4156 tonnes of fish from 6993 ha of fisheries in 1948 to 4-9 tonnes/ha in 1984. The fisheries supply the city markets with 10-20 tonnes of fish per day, providing 10-20 percent of the total demands. (http://www.fao.org)

EDCs (endocrine-disrupting chemicals) have been defined as exogenous substances or mixtures that alter the functions of endocrine systems and consequently cause adverse effects in an intact

organism or its progeny, sub-population. EDCs are structurally similar to the endogenous steroid hormone 17β-estradiol, while others may not be seen structurally related to naturally occurring steroids. (Diniz *et al*. 2005) Thus, many non-steroidal compounds, such as flavonoids, lignins, sterols and fungal metabolites, and synthetic chemicals can interact with sex hormone receptors or can modulate their metabolism and biosynthesis. EDCs exert their effects by mimicking endogenous hormones antagonizing normal hormones, altering the natural pattern of hormone synthesis or metabolism, or modifying hormone receptor levels. Because of these actions, EDCs have the potential to interfere with normal reproduction and development, which are controlled by an array of hormonal signals. (Kosai *et al.* 2011). The aquatic environment has been termed the ultimate sink for natural and man-made chemicals and EDCs have been found in freshwater, estuarine, and marine environments, raising the possibility that EDCs impact organisms living in those aquatic environments. Some studies detected endocrine disruption in wild life in general (Tyler *et al*., 1998; Taylor and Harrison, 1999; Vos *et al,* 2000) and specifically in marine and estuarine organisms (Oberdorster and Cheek, 2000). EDCs encompasses a wide variety of chemicals including natural and synthetic hormones, plant constituents, pesticides, compounds used in the plastic industry and consumer products and other industrial by-products and pollutants.(Gadd *et al* 2005) Common EDCs include phthalate acid esters, DDT, DDE, PCBs, dioxins, and other tributyl tin. These chemicals can exert profound and adverse effects on aquatic animals by interfering with the endocrine system, potentially resulting in reduced fertility and population declines. (Oberdorster *et al* 2000).

According to Laws (2000), the discharge of sewage, whether treated or not, can create serious water pollution problems in the receiving water body. The high concentrations of suspended solids and nutrients, as well as BOD of raw sewage, create a great potential for causing cultural

eutrophication problems. The fact that raw sewage also contains a high concentration of pathogens is cause for concern from a public health stand point as well. Sewage plants have previously been identified as an important source of environmental estrogens to aquatic environments (Gadd *et al*., 2005).Sewage effluent from human sources is the most prominent source of hormone pollution which is characterized by the presence of natural hormones including estrone, estradiol, testosterone, estriol and athinylestradiol. Estrogen and testosterone can come from animals in the form of runoff from cattle pasture, fishpond effluent, fields fertilized with chicken manure and effluent from coops and barn (Barel-Cohen *et al*., 2005). Pollution may damage organisms directly by increasing their mortality, or interfering with the processes of food acquisition and uptake, and reducing their growth and reproduction rates. Growth represents the integration of feeding, assimilation and energy expenditure over a period of time. Poor growth means less energy is available for reproduction, which will in turn reduce the species fitness and lead to a decline in population. Growth and reproduction therefore can serve as a time-integrated indicator of the general wellbeing of the organism. Endocrine disruption occurs when exogenous chemicals interact with internal endocrine signaling pathways in an organism, endocrine disrupting chemicals exert their effects by mimicking endogenous hormones, antagonizing normal hormones, altering the natural pattern of hormone synthesis or metabolism, or modifying hormone receptor levels. Thus EDCs have the potential to interfere with normal reproduction and development, which are controlled by a series of hormonal signals (Mills *et al* 2005).

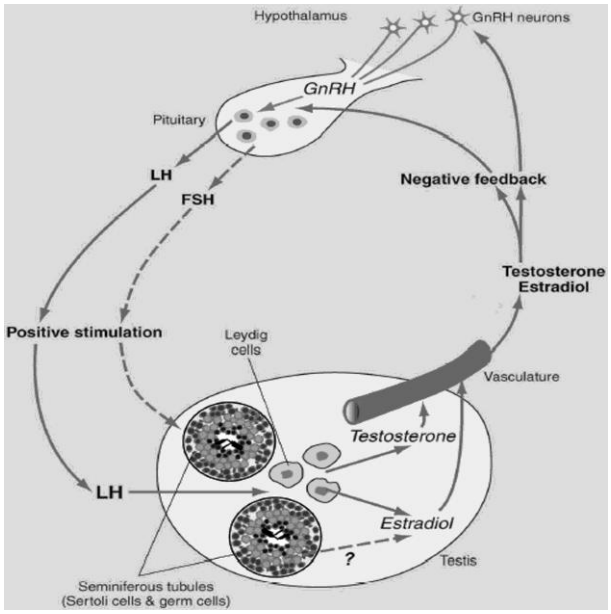

Figure 2: Overview of the teleost hypothalamic-pituitary-gonadal axis

Source: ILAR Journal Vol 45(4)

The cells of the testis are diagramed; however, cells with similar roles are present in the ovary. The linkages between components of the axis simplistically illustrate how the system maintains a dynamic equilibrium. HPG axis functions as a dynamic system throughout each life-stage of the organism, early in development, through gonadal development, and finally into adult life-stages. As illustrated in Figure 2, each component is linked via positive and/or negative feedback loops into a dynamic but stable control system. This system is capable of maintaining the organism so that when the proper external cues are received, a new dynamic state resulting in the development of viable gametes, reproductive behavior, and finally reproduction is achieved. However, exogenous chemical signals (xenobiotic molecules) are capable of interfering with the dynamic equilibrium of the HPG axis, either by distressing it into a new state when inappropriate or by rendering the system incapable of responding properly to normal environmental cues. In general, organisms are most susceptible to these perturbations during the developmental phases

of the life-cycle, or during activational phases leading to reproduction in mature animals. (Gerald *et al* 2011)

In this study we investigated the effects of the presence of EDCs in two different water systems namely the Gammams outlet and Goreangab dam on the liver and gonads of the tilapia fish. The focus was on the *Oreochromis mossambicus* species.72 Juvenile fish were exposed to different water systems to determine the effect of possible endocrine disrupting chemicals present in the waters on the gonadosomatic and hepatosomatic indices of the fish. Previous studies indicated that EDCs are present in the water used. Effluents were obtained from the Gammams wastewater treatment plant outlet and from Goreangab dam.

1.1 Literature Review

McLachlan (2011) The term "endocrine disrupting chemicals" is commonly used to describe environmental agents that alter the endocrine system. These agents include environmentally persistent organochlorines, pentachlorophenol, herbicides, fungicides, insecticides, pharmaceuticals and heavy metals such as cadmium, lead and mercury to name but a few of the chemicals.

Environmental endocrine research has looked at chemicals that mimic or block endogenous vertebrate steroid hormones by interacting with the hormone's receptors. Environmental chemicals known to do this do so most often with receptors derived from the steroid/thyroid, retinoid gene family. There are numerous reports of reproductive and developmental abnormalities in species ranging from snails to humans that have been associated with exposure to environmental hormones (primarily estrogens).

Table 1: Examples of reproductive and developmental abnormalities attributed to endocrine disruption

Species	**Observation**	**Contaminant**
Mammals		
1.Seals	Impaired reproductive functions	PCBs
2.Gulls	Abnormal development of ovarion tissue and oviducts in male embryos	o,p'-DDT
3.Waterbirds	Egg shell thinning, mortality, developmental abnormalities, growth retardation	DD, PCBs, AhR agonists
FISH		
1.Mosquito fish	Abnormal expression of secondary sex characters, masculinization	Androstenedione
2.Roach	Heramaphroditism, vitellogenin in males, altered testes development	Sewage effluent mixture
3.Flatfish	Decreased hormone levels, reduced ovarion development, reduced egg/larvae viability	PAHs
Invertebrates		
1.Snails	Masculinization, imposex, formation of additional female organs, malformed oviducts, increased oocyte production	Tributylin, bisphenol A, octylphenol
2.Marine copepods	Stimulate sexual maturation and egg production	Bisphenol A
3.*Daphnia magna*	Delayed molting time	PCB29, arochlor1242, diethyl phthalate

Abbreviations: AhR, aryl hydrocarbon receptor; DDE, dichlorodiphenyldichloroethylene; DDT, dichlorodiphenyltrichloroethane; PAH, polyaromatic hydrocarbons; PCB, polychlorinated biphenyl.

Source: Environmental Signaling and endocrine disruption, June 2001.

Mills and Chichester (2005).reviewed evidence that addressed two questions: Firstly, whether EDCs in the aquatic environment has the potential to impact the reproductive health and survival of various fish species, and secondly, whether EDCs in the aquatic environment actually impacted the reproductive health and sustainability of indigenous populations of fish. They found that the hypothesis that EDCs in the aquatic environment can impact the reproductive health and sustainability of indigenous fish populations is not so likely. Furthermore, they pointed out that the scarcity of evidence linking impacts of environmental EDCs with changes in reproductive success of indigenous fish populations may reflect a critical need for a dependable method or indicator to assess reproduction of fish in situ. There is a need for more studies that investigate whether fish populations routinely exposed to EDCs in situ are experiencing changes in population structure. Therefore, linking endocrine disruption and reproductive impairment with an ecologically relevant impact on the sustainability of real fish populations remains, with few exceptions an open challenge.

Lye et al (1997) exposed Flounder *Platichthys flesus* to effluent from a sewage treatment works to analyze abnormalities in the reproductive health of the fish. Fish were obtained from three sites in northern England; the Solway firth, which receives only low levels of sewage effluent and two sites in the Tyne Estuary which receives effluent from major sewage treatment works as well as a number of industrial discharges. In the study four lines of evidence suggested that the reproductive health of flounder is being influenced by exposure to oestrogenic substances: 1) Male fish with serum containing VTG, a reliable bio-indicator of oestrogen exposure, were recorded from all the sites studied. The frequency of occurrence was lowest at (20%) in the Solway population and reached (60%) at one of the sites in the Tyne. 2) Serum concentrations of VTG were also highest in fish from the Tyne stations. 3) Male fish from Tyne also displayed

high levels of testicular abnormalities (up to 53% of fish) compared to the Solway population (no abnormalities recorded) and, 4) the HSI of male flounder from the Tyne were significantly greater than for males from the Solway site.

A comparative study to assess the estrogenic potency of treated domestic sewage effluent from a sewage treatment plant located in Lisbon (Chelas): Sixty mirror carp (*Cyprinus carpio*) were exposed to different concentrations of the sewage effluent (0%, 25%, 50% and 100%) for two periods of 28 days in two different seasons (winter/spring). Vitellogenin induction in males was a biomarker of exposure to xenoestrogens. At the end of the experiment, blood samples were taken for vitellogenin analysis and the fish were sacrificed and dissected. Gonad samples were taken for histological evaluation of the sewage effects. The results showed an increase in vitellogenin induction in exposed fish, both males and females depending on the different dilution of the sewage effluent. In comparison with controls, the gonadosomatic index decreased significantly ($p<0.05$) in fish exposed to 100% treated effluent. Although statistically not significant, the hepatosomatic index was high in all exposed fish. Histological abnormalities in fish gonads were evaluated and related to the different percentages of sewage effluent. Seasonal variations found in estrogenic responses were attributed to weather influences on sewage dilution. (Diniz *et al*. 2005)

1.2 Research question:

Do Endocrine Disruptive Chemicals and Sewage effluents in our native waters have an effect on the liver and gonads of *Oreochromis mossambicus*?

1.2.1 Research objectives:

(a) General objective:

To compare the Hepatosomatic and Gonadosomatic indices of *Oreochromis mossambicus* exposed to different water qualities from four different sites.

(b) Specific objective:

To assess the effect of water quality on the hepatosomatic and gonadosomatic indices of the *Oreochromis mossambicus.*

The primary aim of the study is to find out if the EDCs present in our waters are at levels high enough to cause any changes in the hepatosomatic index as well as the gonadosomatic indices of the Mozambique Tilapia *(Oreochromis mossambicus)* exposed to water from a natural water body and water with sewage effluents as compared to treated reclaimed water. The objectives of the research experiment is mainly to give more insight into the adverse physiological effects that endocrine disrupters in our native water may have on freshwater fish in particular the tilapia fish, and potential effects on other aquatic species as well as the aquatic environment as a whole.

1.3 Hypotheses:

H_{01}: There is no significant difference in the GSI of fish exposed to various concentrations of EDCs

H_{11}: There is a significant difference in the GSI of fish exposed to various concentrations of EDCs

H_{02}: There is no significant difference in the HSI of fish exposed to various concentrations of EDCs

H_{12}: There is a significant difference in the HSI of fish exposed to various concentrations of EDCs

1.4 Data Analysis:

A statistical t-test was done to test for significant difference in mean gonadosomatic and hepatosomatic indices of the total fish in each treatment level respectively, with the level of significance set at 0.05 and the ELISA method was used for quantitative measurements of vitellogenin.

CHAPTER TWO:

2. Materials and Methods:

Study Sites:

The criteria for selection of the water sources used for the various treatments depended on the water quality based on the level of contamination and the different contaminants present in the water source. Water samples were collected from three areas 1.Gammams-outlet pond & 2. Goreangab dam, both with steroid hormones of which only three (Estradiol, Estrone and testosterone) were considered for the exposure as they have the potential to cause estrogenic effects on the fish, 3. Water for the negative and positive control was collected from Windhoek Goreangab Operating Company (WINGOC) which was free from EDCs. The positive control consisted of water from WINGOC to which a tablet of Estrofem with a 2mg estradiol concentration was added.

Table 2: table showing the three hormones and their detected levels for Gammams (inlet and outlet ponds) and Goreangab dam.

Water system	Estradiol levels	Estrone levels	Testosterone levels
Gammams inlet	78pg/ml	79pg/ml	259pg/ml
Gammams outlet	8pg/ml	9pg/ml	10pg/ml
Goreangab dam	Below detection level	Below detection level	Below detection level

Source: (AK Faul, E Julies, and EJ Pool (Unpublished)

Eight 20litre buckets were used during the experiment and labeled according to the locations in which the water samples were collected for the different treatments. Two buckets were used for each sample. The first collection of water was done on the 4th of October 2011 and the mode in which the samples were collected was by using containers; two 25litre containers were used for collecting water from the Gammams maturation pond, two 20litre containers were used to get water from WINGOC, and two 25litre containers were used to collect water from the dam itself. The containers were brought into the lab on the same day and left for about half an hour or so just to level with the temperature of the lab which was set at room temperature, after the 30 minutes the different samples were measured into the 20litre buckets at volumes of 15litres each, the water was aerated using air stones and left to stand overnight. On the 5th of October 2011 the actual experiment started whereby a specimen of 9 male fish was placed in each bucket. Throughout the experiment, no form of water filtering system was used, and water was collected every second week in the same containers to fill the buckets for each treatment respectively.

Fish collection and acclimatization in laboratory:

Juvenile fish were collected in August and September 2011 from the Hardap dam. The first samples collected consisted of 200 Tilapia juveniles weighing approximately between 10-25g of both sexes by means of a small meshed size sampling net and was transported to the University of Namibia. The second batch collected had 150 juvenile fish also in the same weight range as the first batch and of both sexes. The samples were then kept in the Biology laboratory for further exposure and study.

Figure 3: the Biology laboratory at the University of Namibia where the study was carried out.

The plastic bags containing the fish were placed in a tank containing tap water for half an hour for pre-acclimatization and after an hour, the fish were transferred into the tank with tap water and acclimatized for about a week at a constant temperature of 24˚C.

Figure 4: the tank in which the fish were brought in from the Hardap dam and left to acclimatize for two weeks.

Treatments:

The tanks were divided into various treatments namely the positive control, Gammams maturation pond, Goreangab dam, and two negative controls and each treatment had two replicates and each replicate had a total number of 9 fish which were placed in at random from the big tank but only the male fish were used for this exposure which lasted for about 5 weeks. They were fed fish flakes throughout the experiment three times per day; in the morning between 7:30 and 8:00, midday at 12:00pm and later in the evening at about 18:00pm and no measurement of the portions was done, that is the flakes were distributed just as much as my fingers could hold.

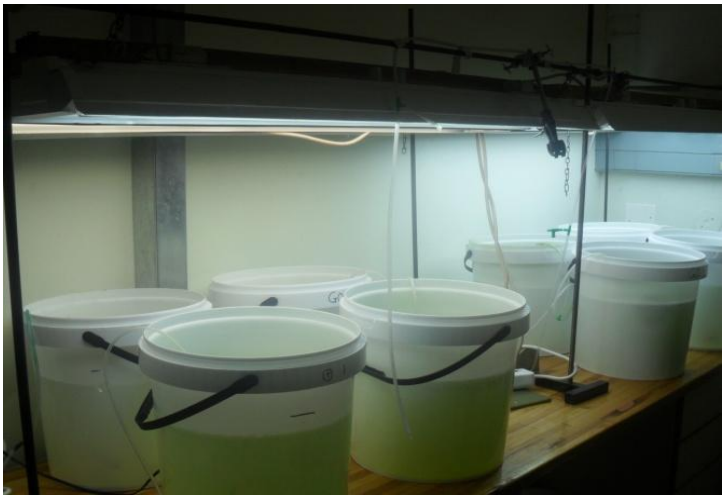

Figure 5: The eight tanks in which the fish were transferred to for further exposure of EDCs

Determination of HSI:

The hepatosomatic index was determined from each fish by first weighing the whole life fish to obtain the total body mass. The fish was then dissected using instruments from a dissecting kit (scissors, blades, dividers) by first removing the caudal fin, anal fin and pelvic fin by using scissors after which the fish was cut open beginning at the vent using the blade and made sure

that the cutting was not too deep to avoid damaging the internal organs. Then the liver was dissected out of the individual fish and its weight was obtained using a sensitive weight balance

HSI = (weight of liver/total body weight of fish)*100. (Aquatic Toxicology 104:2011)

Determination of GSI:

The Gonad was dissected out of the individual fish using a pair of tweezers/dividers which made it easier to extract the gonads and put them aside in order to take the individual weights which was obtained by placing the gonads cautiously on the sensitive balance scale. The results were then obtained and recorded on the data collection sheet. In this project only the male fish where considered simply because the males are the ones that are prone to sex reversals. (http://www.biosense.com) Estrogens and estrogen-like compounds released into the aquatic environment have been shown to interact with the hormonal system of wildlife and induce female specific responses in male and juvenile organisms. Such endocrine disruption can result in adverse effects on sex ratio, fertility and behavior of the fish. Based on the measurements and results obtained, the individual weight of the gonads were relatively smaller compared to the individual body weight as well as looking at the overall gonadosomatic index averages of all five treatments it showed low weight results as compared to the hepatosomatic index – Weight of gonad. Only males were considered in this study due to the fact that such changes,(sex reversal changes) were only prominent in the males as it is much easier to determine the changes in the quantity and quality of sperm than of eggs. (Ebrahimi M. 2005.9:65-70)

GSI = (Weight of Gonad/total body weight of fish) *100 (Aquatic Toxicology 104,2011)

Vitellogenin analysis:

After weighing the fish, blood was collected with a syringe from just in front of the caudal fin. The blood was centrifuged and the plasma in the supernatant was collected with a clean syringe and stored in glass vials at -80°C until analysis.

The Somatic Indices: The HSI and GSI of the different treatments were tested against each other in the following way:

Two sample t-test for the HSI between:

a) Positive control against Gammams, Goreangab Dam, negative control and negative control in tank.

b) Goreangab dam against Gammams and the negative control

c) Negative control against Gammams

d) Negative control in tank against Gammams

Two sample t-test for the GSI between:

a) Positive control against Gammams, Goreangab dam, negative control and negative control in tank

b) Gammams against Goreangab dam, negative control and negative control in tank

c) Negative control against Goreangab

d) Negative control in tank against Goreangab dam

CHAPTER THREE

3. RESULTS and DISCUSSION:

Table 3: Calculated HIS

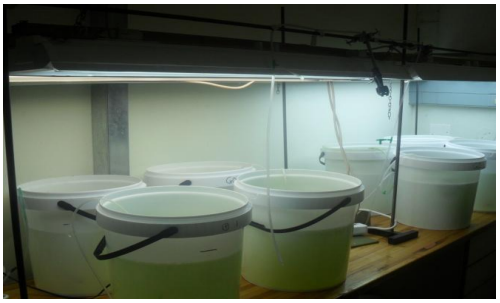

Table 4: Determination of GSI

WATER SYSTEM	SEX	BODY WEIGHT(g)	GONAD WEIGHT(g)	GSI
positive control A2	male	17		
positive control A3	male	12.78	0.03	0.23
positive control A4	male	22.3	0.06	0.27
positive control A5	male	10.65	0.03	0.28
positive control B1	male	37.17	0.1658	0.45
positive control B3	male	11.59		
positive control B4	male	21.77	0.0244	0.11
positive control B5	male	20.94	0.1159	0.55
positive control B6	male	23.67	0.0474	0.2
positive control B7	male	8.43	0.0048	0.06
positive control B8	male	20.12	0.019	0.09
positive control B9	male	15.92	0.0449	0.28
GAMMAMS OUTLET A4	male	20.63	0.0881	0.43
GAMMAMS OUTLET A5	male	12.28	0.0291	0.24
GAMMAMS OUTLET A6	male	27.03	0.1414	0.61
GOREANGAB DAM A2	male	8.82	0.0126	0.14
GOREANGAB DAM A3	male	9.11	0.0141	0.15
GOREANGAB DAM A4	male	7.62	0.0085	0.11
GOREANGAB DAM A5	male	11.6	0.0295	0.25
GOREANGAB DAM A6	male	12.36	0.0181	0.15
GOREANGAB DAM A7	male	13.45	0.0761	0.57
GOREANGAB DAM B1	male	16.04	0.036	0.22
GOREANGAB DAM B2	male	14.38	0.0278	0.19
GWRP A1	male	16.66	0.0688	0.41
GWRP A2	male	10.76	0.0281	0.26
GWRP A3	male	30.61	0.1491	0.49
GWRP A4	male	35.81	0.3232	0.9
GWRP A5	male	17.36	0.0721	0.42
Negative control (tank)	male	8.53	0.0048	0.056272
Negative control (tank)	male	10.22	0.0775	0.758317
Negative control (tank)	male	19.14	0.0811	0.42372
Negative control (tank)	male	12.03	0.0193	0.160432
Negative control (tank)	male	14.23	0.0419	0.294448
Negative control (tank)	male	22.62	0.0837	0.370027

A two sample t-test was performed to test for statistical differences in the GSI & HSI values calculated for the different treatments; the p-value was set at 0.05%. The results obtained from the t-test showed that there were no significant differences in the HSI of (a, b, c, & d) except the test between the positive control and negative control in the tank in (a) which showed a significant difference, for the GSI the significant differences were in (a) the positive control against the negative control, in (b) Gammams against Goreangab dam, and in (c) negative control against Goreangab dam. The rest of the treatments in (a, b, c, & d) showed no significant differences in GSI. Detection of VTG in exposed fish was low at 450nm and for this reason could not reveal the presence of the estrogen-inducible protein in the treatment groups. The calculated means shows that the controlled fish had a much higher liver mean weight of 0.54g and Gammams with a mean liver weight of 0.37g as compared to the other treatments, however Gammams showed a decreased mean gonadosomatic index of 0.03g as compared to the two controls (GWRP 0.49g and negative control 0.34g).

Table 5: Calculated averages of the total fish body, liver and gonad weights for each treatment.

	Body weight	Liver weight	Gonad weight
Positive control	18.52833333	0.058783333	0.05422
Gammams outlet	19.98	0.0681	0.0862
Goreangab dam	11.6725	0.0412125	0.0278375
GWRP	22.24	0.07732	0.12826
Negative control	14.46166667	0.0696	0.051383333

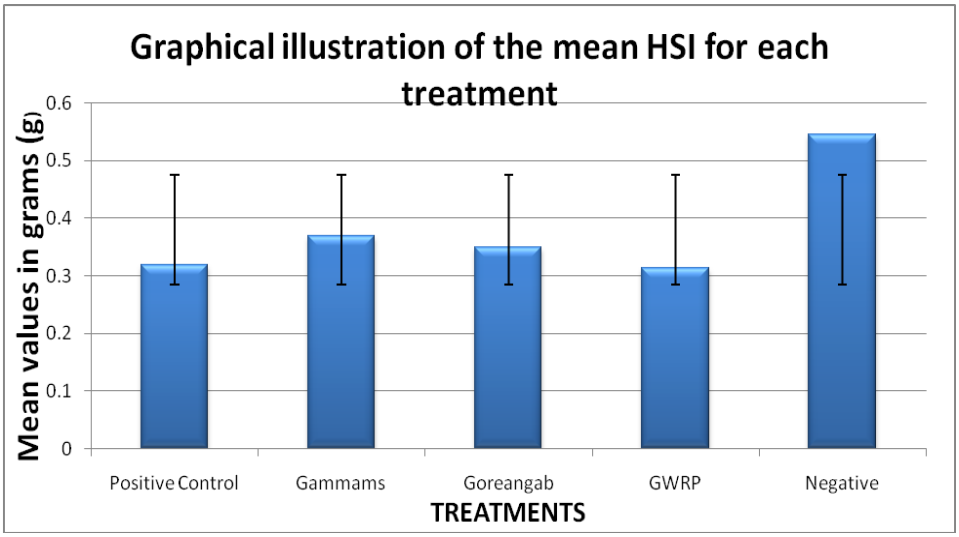

Figure 6: Graph depicting the difference in mean values of HSI between the treatments

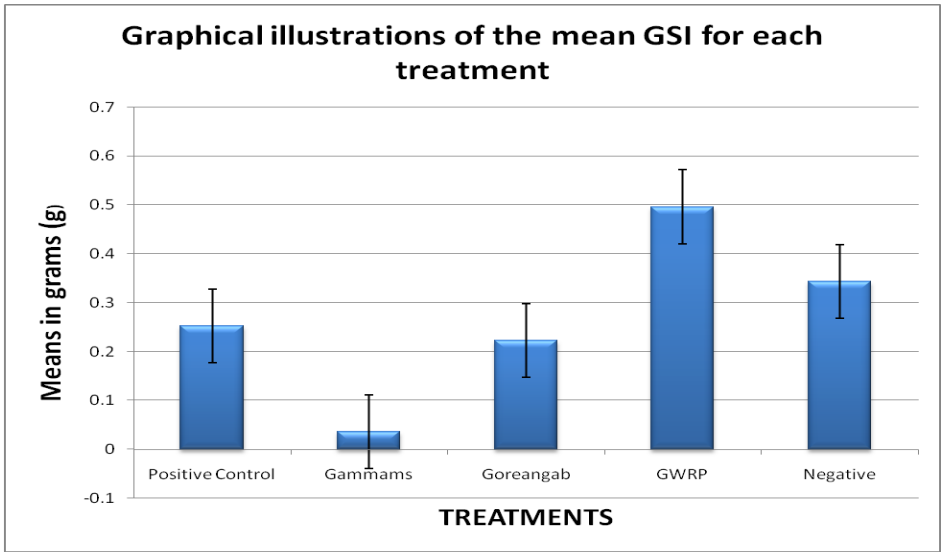

Figure 7: Graph depicting the differences in mean values if GSI between the treatments

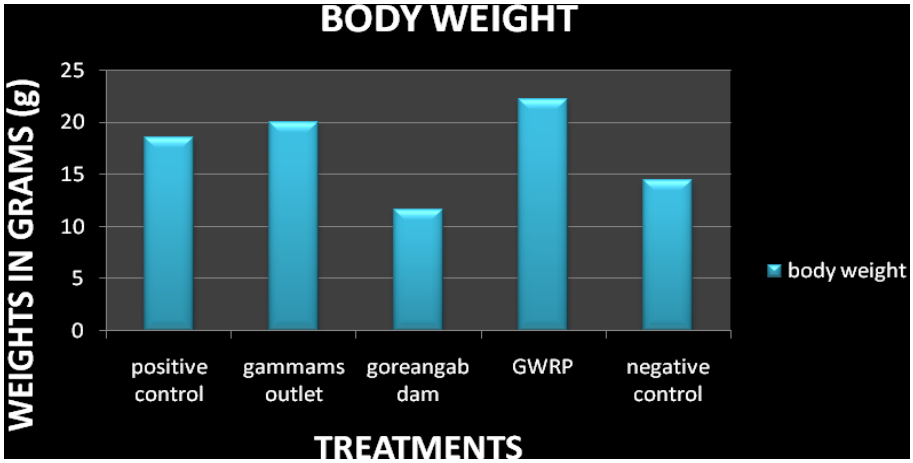

Figure 8: graphical illustrations for the average fish body weights of the total fish in each treatment

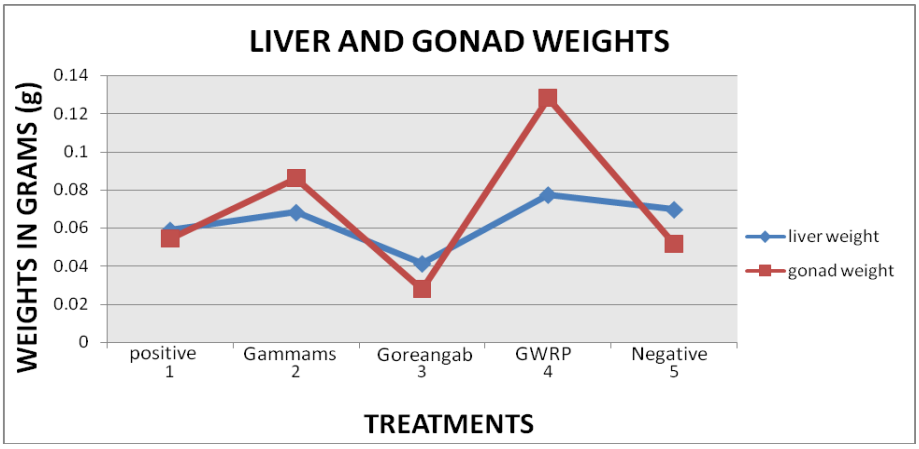

Figure 9: graphical illustrations of the average liver and gonad weights for the treatments

Table 6: ***table of means and standard deviation for GSI and HSI***

	HIS	Stdev	GSI	Stdev
Positive Control	0.318333	0.118616	0.252	0.155048
Gammams	0.37	0.426667	0.036056	0.185023
Goreangab	0.35	0.387225	0.2225	0.147624
GWRP	0.314	0.093167	0.496	0.240894
Negative	0.544885	0.297426	0.343869	0.24398

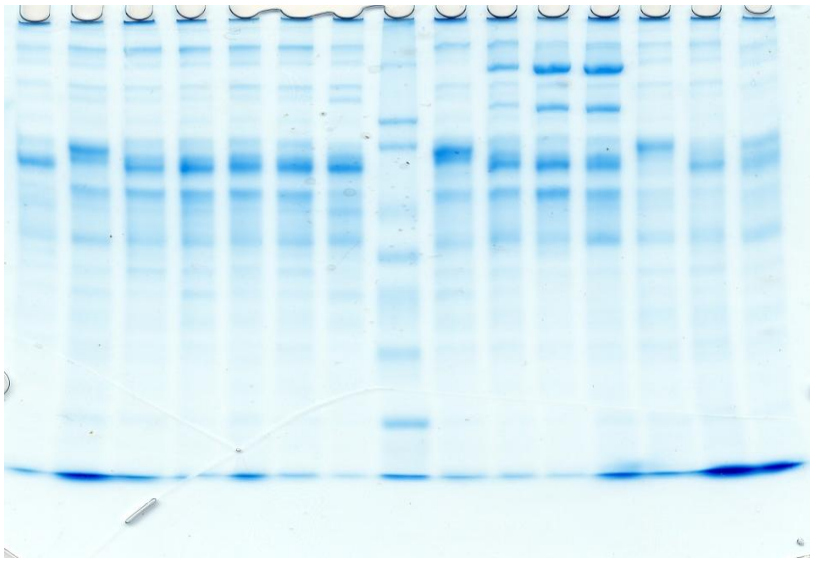

Figure 10: Electrophoresis gel showing the Vitellogenin fractions in the plasma

Much of attention has been focused on the endocrine disrupting chemicals (EDCs) on fish species (Abdel-Hameid 2007) The present study was aimed at determining whether or not the levels of EDCs present in our native water systems are high enough to have estrogenic effects on the tilapia fish, in the study male fish were subjected to water with presence of EDC, water with Estrofem tablet and reclaimed water. From the vitellogenin test the absorbance obtained was 450nm, which concludes that

there were no estrogenic effects on the fish. Considering the experimental design and the set up of the laboratory, the differences in the HSI and GSI was brought about by factors other than the presence of EDCs in the distinct treatments. Stress levels of the fish during the exposure period played a major role in the difference in mean HSI and GSI values firstly because the set up of the "tanks" did not allow for a conductive environment for the fish, in the sense that the tanks were quiet small and taking in account the food intake and the absence of a filtering system as the water was only renewed every second week which resulted in high levels of excretion, and fish not properly feeding due to poor water quality in the treatments as the tanks were only cleaned every second week. All these factors played a role in the significant differences in mean HSI and GSI, and because of these stress levels the results were clouded as to whether the levels of EDCs in our native waters are at levels high enough cause any adverse effects or changes in the liver and gonads of the fish in question. Even though vitellogenin synthesis was detected at very low levels of 450nm, we cannot conclude that the detected levels of EDCs had no effect on the HSI and GSI of the tilapia fish, or whether the changes in HSI and GSI were due to the absence or presence of EDCs in the individual treatments considering the stress factors in the treatments. However, studies have shown that fish exposed to high levels of EDCs had increased vitellogenin synthesis, decreased growth performance, and suffered reproductive impairment.

Nonetheless the irregularity seen in this study is less evidence of no effect than a consequence of the difficulty in studying, especially with the lack of histological tests.

CHAPTER FOUR

4.1 Recommendations:

Future research should look at comparing similar water systems e.g. comparisons between Goreangab dam and Avis dam or von Bach dam.

Larger fish may have been used and the experiment or period of exposure could be extended to give more sensible results.

A different hormone could be used in the positive control or the same hormone but at a higher concentration equal to the levels in the waters being studied.

More fish could be used in each treatment and each treatment could have more replicates to account for the high mortality rate. .

Histological tests could be performed to give more accurate results

5. References

1. Abdel-Hameid N.A.H. (2007). Physiological and Histopathological Alterations Induced by Phenol Exposure in (*Oreochromis aureus)* Juveniles. *Turkish Journal of Fisheries and Aquatic Sciences 7: 131-138*

2. Ankley G. T, Johnson R.J. (2011). Small Fish Models for Ide.jpgying and Assessing the Effects of Endocrine-disrupting Chemicals. *ILAR Journal* 45:4

3. Diniz M.S, Peres I, Pihan J.C. (2005). Comparative study of the estrogenic responses of mirror carp (*Cyprinus carpio*) exposed to treated municipal sewage effluent (Lisbon) during two periods in different seasons. *Science of the Total Environment 349:129– 139*

4. Dong- Huyk, Soon-Ae Lee, Gi Soo Kang, Jinwon Seo, Sung-kyu Lee. (2007) Stressor identification and health assessment of fish exposed to wastewater effluents in Miho stream, South Korea. *Chemosphere 67 (2282-2292)*

5. Ebrahimi M. (2005). Vitellogenin Assay by enzymes-linked immunosorbent assay as a biomarker of endocrine disrupter chemicals pollutions. *Indian J. Occup Environ Med, 9:65-70*

6. Gadd J, Stewart C, and Sikes E. (2005). Estrogenic activity and known environmental estrogens in sewage effluent. *Australian Journal of Ecotoxicology, 11:149-154*

7. Gronen S, Denslow N, Manning S, Barnes S, Barnes D, and Marius B.1999. Serum Vitellogenin Levels and Reproductive Impairment of Male Japanese Medaka (*Oryzias latipes*) Exposed to 4-tert-Octylphenol. *Environmental Health Perspective, 107(5) 385-389*

8. Hempel E, Mapfumo B. (2011). Tilapia Aquaculture in Africa. *INFOFISH International, 6:27l*

9. Jamu D.M, Ayinla O.A. (2003). Potential for the development of Aquaculture in African Countries. NAGA, WorldFish Center Quarterly Vol. 26 No. 3 Jul-Sep

10. Kosai P, Jiraungkoorskul W, Sachamahithinant C, Jiraungkoorskul K. (2011). Induction of testis-ova in Nile tilapia (Oreochromis niloticus) exposed to 17β-estradiol. Natural Science, III (3: 227-233)

11. Lesley J. Mills, Clinton Chichester. Review of evidence: Are endocrine-disrupting chemicals in the aquatic environment impacting fish populations? Science of the Total Environment 2005;343:1-34

12. Lye C.M, Frid C.I.J, Gill M.E, McCormick D. (1997). Abnormalities in the Reproductive Health of Flounder (Platichthys flesu) Exposed to Effluents from a Sewage Treatment Works. Marine Pollution Bulletin 34 (1:34-41)

13. McLachlan A.J. (2001). Environmental Signaling: What Embryos and Evolution Teach Us About Endocrine Disrupting Chemicals. *Endocrine Reviews, 22:319-341*

14. Mills L.J, Chichester C. (2005). Review of evidence: Are endocrine-disrupting chemicals in the aquatic environment impacting fish populations? *Science of the Total Environment, 343:1-34*

15. Rogan W.J, Ragan B.N. (2007). Some evidence of effects of environmental chemicals on the endocrine system in children

16. Vigneswaran S., Sundaravadivel M. (2004) Wastewater Recycle, Reuse and Reclamation- Recycle and Reuse of Domestic wastewater, Encyclopedia of life support systems. EOLSS Publishers, Oxford, UK.

6. Appendix:

Abbreviations:

EDCs – Endocrine Disrupting Chemicals

GSI – Gonadosomatic Index
GWRP – Gammams Water Reclamation Plant
HSI – Hepatosomatic Index
HPG – Hypothalamus-Pituitary-Gland
VTG – Vitellogenin

TWO SAMPLE T—TEST:

```
ttest  hispostivcontrol ==    hisgamma, unpaired

Two-sample t test with equal variances
------------------------------------------------------------------------------
Variable |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
hispos~l |      12    .3183333    .0342414    .1186158    .2429685    .3936982
hisgamma |       3        .37    .0208167    .0360555    .2804332    .4595669
---------+--------------------------------------------------------------------
combined |      15    .3286667    .0279262    .1081577    .2687709    .3885624
---------+--------------------------------------------------------------------
    diff |          -.0516667    .0710197               -.2050955    .1017621
------------------------------------------------------------------------------
    diff = mean(hispostivcontrol) - mean(hisgamma)                t =  -0.7275
Ho: diff = 0                                     degrees of freedom =       13

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.2399         Pr(|T| > |t|) = 0.4798          Pr(T > t) = 0.7601

.
ttest  hispostivcontrol ==     hisgorean, unpaired

Two-sample t test with equal variances
------------------------------------------------------------------------------
Variable |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
hispos~l |      12    .3183333    .0342414    .1186158    .2429685    .3936982
hisgor~n |       8        .35    .1369045    .3872245    .0262722    .6737278
---------+--------------------------------------------------------------------
combined |      20        .331    .0564097    .2522718    .2129332    .4490668
---------+--------------------------------------------------------------------
    diff |          -.0316667    .1180654               -.2797128    .2163794
```

```
------------------------------------------------------------------------------
   diff = mean(hispostivcontrol) - mean(hisgorean)               t =  -0.2682
Ho: diff = 0                                     degrees of freedom =       18

   Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.3958         Pr(|T| > |t|) = 0.7916          Pr(T > t) = 0.6042


ttest  hispostivcontrol ==   hisnegativecont, unpaired

Two-sample t test with equal variances
------------------------------------------------------------------------------
Variable |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
hispos~l |      12    .3183333    .0342414    .1186158    .2429685    .3936982
hisneg~t |       5       .314    .0416653    .0931665    .1983185    .4296815
---------+--------------------------------------------------------------------
combined |      17    .3170588    .0263986    .1088442    .2610963    .3730213
---------+--------------------------------------------------------------------
   diff |           .0043333    .0598264               -.1231835    .1318502
------------------------------------------------------------------------------
   diff = mean(hispostivcontrol) - mean(hisnegativecont)         t =   0.0724
Ho: diff = 0                                     degrees of freedom =       15

   Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.5284         Pr(|T| > |t|) = 0.9432          Pr(T > t) = 0.4716

.


ttest  hispostivcontrol ==   hisnegativconttank, unpaired

Two-sample t test with equal variances
------------------------------------------------------------------------------
Variable |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
hispos~l |      12    .3183333    .0342414    .1186158    .2429685    .3936982
hisneg~k |       6    .5448848    .1214237    .2974262    .2327552    .8570144
---------+--------------------------------------------------------------------
combined |      18    .3938505     .051207     .217253    .2858131    .5018878
---------+--------------------------------------------------------------------
   diff |          -.2265514    .0965886               -.43131   -.0217929
------------------------------------------------------------------------------
   diff = mean(hispostivcontrol) - mean(hisnegativcont~k)        t =  -2.3455
Ho: diff = 0                                     degrees of freedom =       16
```

```
   Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0161         Pr(|T| > |t|) = 0.0322          Pr(T > t) = 0.9839

.
ttest   hisgorean ==  hisgamma, unpaired

Two-sample t test with equal variances
------------------------------------------------------------------------------
Variable |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
hisgor~n |       8         .35    .1369045    .3872245    .0262722    .6737278
hisgamma |       3         .37    .0208167    .0360555    .2804332    .4595669
---------+--------------------------------------------------------------------
combined |      11    .3554545    .0978437    .3245108    .1374452    .5734639
---------+--------------------------------------------------------------------
    diff |              -.02    .2314827               -.5436503    .5036503
------------------------------------------------------------------------------
    diff = mean(hisgorean) - mean(hisgamma)                       t =  -0.0864
Ho: diff = 0                                     degrees of freedom =        9

   Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.4665         Pr(|T| > |t|) = 0.9330          Pr(T > t) = 0.5335

.
ttest    hisgamma ==   hisgorean, unpaired

Two-sample t test with equal variances
------------------------------------------------------------------------------
Variable |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
hisgamma |       3         .37    .0208167    .0360555    .2804332    .4595669
hisgor~n |       8         .35    .1369045    .3872245    .0262722    .6737278
---------+--------------------------------------------------------------------
combined |      11    .3554545    .0978437    .3245108    .1374452    .5734639
---------+--------------------------------------------------------------------
    diff |               .02    .2314827               -.5036503    .5436503
------------------------------------------------------------------------------
    diff = mean(hisgamma) - mean(hisgorean)                       t =   0.0864
Ho: diff = 0                                     degrees of freedom =        9

   Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.5335         Pr(|T| > |t|) = 0.9330          Pr(T > t) = 0.4665

.
```

```
ttest    hisnegativecont ==  hisgamma, unpaired

Two-sample t test with equal variances
------------------------------------------------------------------------------
Variable |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
hisneg~t |       5        .314    .0416653    .0931665    .1983185    .4296815
hisgamma |       3         .37    .0208167    .0360555    .2804332    .4595669
---------+--------------------------------------------------------------------
combined |       8        .335    .0277746    .0785584    .2693235    .4006765
---------+--------------------------------------------------------------------
    diff |             -.056    .0575963               -.1969331    .0849331
------------------------------------------------------------------------------
    diff = mean(hisnegativecont) - mean(hisgamma)                 t =  -0.9723
Ho: diff = 0                                     degrees of freedom =        6

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.1842         Pr(|T| > |t|) = 0.3685          Pr(T > t) = 0.8158

.
ttest    hisnegativconttank ==  hisgamma, unpaired

Two-sample t test with equal variances
------------------------------------------------------------------------------
Variable |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
hisneg~k |       6    .5448848    .1214237    .2974262    .2327552    .8570144
hisgamma |       3         .37    .0208167    .0360555    .2804332    .4595669
---------+--------------------------------------------------------------------
combined |       9    .4865899    .0838386    .2515157    .2932578    .6799219
---------+--------------------------------------------------------------------
    diff |          .1748848    .1782678               -.2466516    .5964211
------------------------------------------------------------------------------
    diff = mean(hisnegativcont~k) - mean(hisgamma)                t =   0.9810
Ho: diff = 0                                     degrees of freedom =        7

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.8204         Pr(|T| > |t|) = 0.3593          Pr(T > t) = 0.1796

.
ttest    hisgorean ==   hisnegativecont, unpaired

Two-sample t test with equal variances
------------------------------------------------------------------------------
Variable |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
```

```
hisgor~n |       8        .35    .1369045    .3872245    .0262722    .6737278
hisneg~t |       5       .314    .0416653    .0931665    .1983185    .4296815
---------+--------------------------------------------------------------------
combined |      13    .3361538    .0835244    .3011516    .1541697     .518138
---------+--------------------------------------------------------------------
    diff |              .036    .1789881               -.3579501    .4299501
------------------------------------------------------------------------------
    diff = mean(hisgorean) - mean(hisnegativecont)                t =   0.2011
Ho: diff = 0                                     degrees of freedom =       11

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.5779         Pr(|T| > |t|) = 0.8443          Pr(T > t) = 0.4221

.


ttest    hisgorean ==    hisnegativconttank, unpaired

Two-sample t test with equal variances
------------------------------------------------------------------------------
Variable |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
hisgor~n |       8        .35    .1369045    .3872245    .0262722    .6737278
hisneg~k |       6    .5448848    .1214237    .2974262    .2327552    .8570144
---------+--------------------------------------------------------------------
combined |      14     .433522    .0944076    .3532409    .2295668    .6374773
---------+--------------------------------------------------------------------
    diff |          -.1948848    .1904252               -.6097856     .220016
------------------------------------------------------------------------------
    diff = mean(hisgorean) - mean(hisnegativcont~k)               t =  -1.0234
Ho: diff = 0                                     degrees of freedom =       12

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.1631         Pr(|T| > |t|) = 0.3263          Pr(T > t) = 0.8369

.
ttest     hisnegativecone ==    hisnegativconttank, unpaired

Two-sample t test with equal variances
------------------------------------------------------------------------------
Variable |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
hisneg~t |       5       .314    .0416653    .0931665    .1983185    .4296815
hisneg~k |       6    .5448848    .1214237    .2974262    .2327552    .8570144
---------+--------------------------------------------------------------------
combined |      11    .4399372    .0752219    .2494828    .2723324     .607542
```

```
---------+--------------------------------------------------------------------
    diff |          -.2308848    .1394081               -.5462478    .0844783
------------------------------------------------------------------------------
    diff = mean(hisnegativecont) - mean(hisnegativcont~k)         t =  -1.6562
Ho: diff = 0                                     degrees of freedom =        9

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0660         Pr(|T| > |t|) = 0.1321          Pr(T > t) = 0.9340

.
 ttest     gsipositivecontrol ==     gsigamma, unpaired

Two-sample t test with equal variances
------------------------------------------------------------------------------
Variable |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
gsipos~l |      10        .252    .0490306    .1550484    .1410851    .3629149
gsigamma |       3    .4266667    .1068228    .1850225   -.0329548    .8862881
---------+--------------------------------------------------------------------
combined |      13    .2923077    .0477192     .172054    .1883365    .3962789
---------+--------------------------------------------------------------------
    diff |          -.1746667    .1059266               -.4078095    .0584762
------------------------------------------------------------------------------
    diff = mean(gsipositivecon~l) - mean(gsigamma)                t =  -1.6489
Ho: diff = 0                                     degrees of freedom =       11

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0637         Pr(|T| > |t|) = 0.1274          Pr(T > t) = 0.9363

.


ttest     gsipositivecontrol ==     gisgorean, unpaired

Two-sample t test with equal variances
------------------------------------------------------------------------------
Variable |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
gsipos~l |      10        .252    .0490306    .1550484    .1410851    .3629149
gisgor~n |       8       .2225     .052193     .147624    .0990832    .3459168
---------+--------------------------------------------------------------------
combined |      18    .2388889    .0349031    .1480814    .1652497     .312528
---------+--------------------------------------------------------------------
    diff |              .0295    .0720264               -.1231891    .1821891
------------------------------------------------------------------------------
    diff = mean(gsipositivecon~l) - mean(gisgorean)               t =   0.4096
```

```
Ho: diff = 0                     degrees of freedom =       16

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.6562         Pr(|T| > |t|) = 0.6876          Pr(T > t) = 0.3438

.
ttest     gsipositivecontrol ==   gsinegativecont, unpaired

Two-sample t test with equal variances
------------------------------------------------------------------------------
Variable |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
gsipos~l |      10        .252    .0490306    .1550484    .1410851    .3629149
gsineg~t |       5        .496    .1077311    .2408942    .1968904    .7951096
---------+--------------------------------------------------------------------
combined |      15    .3333333    .0555035     .214964    .2142902    .4523764
---------+--------------------------------------------------------------------
    diff |            -.244    .1017327               -.4637801   -.0242199
------------------------------------------------------------------------------
    diff = mean(gsipositivecon~l) - mean(gsinegativecont)         t =  -2.3984
Ho: diff = 0                     degrees of freedom =       13

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0161         Pr(|T| > |t|) = 0.0322          Pr(T > t) = 0.9839

.
ttest     gsipositivecontrol ==     gsinegativconttank, unpaired

Two-sample t test with equal variances
------------------------------------------------------------------------------
Variable |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
gsipos~l |      10        .252    .0490306    .1550484    .1410851    .3629149
gsineg~k |       6    .3438694    .0996044    .2439799    .0878281    .5999106
---------+--------------------------------------------------------------------
combined |      16    .286451     .0476813    .1907251    .1848208    .3880812
---------+--------------------------------------------------------------------
    diff |          -.0918694     .098946               -.3040874    .1203487
------------------------------------------------------------------------------
    diff = mean(gsipositivecon~l) - mean(gsinegativcont~k)        t =  -0.9285
Ho: diff = 0                     degrees of freedom =       14

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.1844         Pr(|T| > |t|) = 0.3689          Pr(T > t) = 0.8156

.
```

```
 ttest      gsigamma ==      gisgorean, unpaired

Two-sample t test with equal variances
------------------------------------------------------------------------------
Variable |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
gsigamma |       3    .4266667    .1068228    .1850225   -.0329548    .8862881
gisgor~n |       8      .2225     .052193     .147624    .0990832    .3459168
---------+--------------------------------------------------------------------
combined |      11    .2781818    .0532544    .1766249    .1595236    .3968401
---------+--------------------------------------------------------------------
    diff |           .2041667    .1060918               -.0358297    .4441631
------------------------------------------------------------------------------
    diff = mean(gsigamma) - mean(gisgorean)                       t =   1.9244
Ho: diff = 0                                     degrees of freedom =        9

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.9568         Pr(|T| > |t|) = 0.0864          Pr(T > t) = 0.0432

.
ttest      gsigamma == gsinegativecont, unpaired

Two-sample t test with equal variances
------------------------------------------------------------------------------
Variable |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
gsigamma |       3    .4266667    .1068228    .1850225   -.0329548    .8862881
gsineg~t |       5       .496    .1077311    .2408942    .1968904    .7951096
---------+--------------------------------------------------------------------
combined |       8        .47    .0743544    .2103059    .2941799    .6458201
---------+--------------------------------------------------------------------
    diff |          -.0693333    .1634589               -.4693029    .3306362
------------------------------------------------------------------------------
    diff = mean(gsigamma) - mean(gsinegativecont)                 t =  -0.4242
Ho: diff = 0                                     degrees of freedom =        6

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.3431         Pr(|T| > |t|) = 0.6862          Pr(T > t) = 0.6569

.
 ttest      gsigamma ==      gsinegativconttank, unpaired

Two-sample t test with equal variances
------------------------------------------------------------------------------
```

```
Variable |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
gsigamma |       3    .4266667    .1068228    .1850225   -.0329548    .8862881
gsineg~k |       6    .3438694    .0996044    .2439799    .0878281    .5999106
---------+--------------------------------------------------------------------
combined |       9    .3714685     .07263     .21789    .2039834    .5389536
---------+--------------------------------------------------------------------
    diff |            .0827973    .1617091               -.299584    .4651786
------------------------------------------------------------------------------
    diff = mean(gsigamma) - mean(gsinegativcont~k)                t =   0.5120
Ho: diff = 0                                     degrees of freedom =        7

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.6878         Pr(|T| > |t|) = 0.6244          Pr(T > t) = 0.3122

.


ttest   gsinegativecont == gisgorean, unpaired

Two-sample t test with equal variances
------------------------------------------------------------------------------
Variable |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
gsineg~t |       5       .496    .1077311    .2408942    .1968904    .7951096
gisgor~n |       8      .2225     .052193     .147624    .0990832    .3459168
---------+--------------------------------------------------------------------
combined |      13    .3276923    .0627792    .2263535    .1909083    .4644763
---------+--------------------------------------------------------------------
    diff |             .2735    .1066079               .0388575    .5081425
------------------------------------------------------------------------------
    diff = mean(gsinegativecont) - mean(gisgorean)                t =   2.5655
Ho: diff = 0                                     degrees of freedom =       11

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.9869         Pr(|T| > |t|) = 0.0263          Pr(T > t) = 0.0131

.
ttest       gsinegativconttank ==      gisgorean, unpaired

Two-sample t test with equal variances
------------------------------------------------------------------------------
Variable |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
gsineg~k |       6    .3438694    .0996044    .2439799    .0878281    .5999106
gisgor~n |       8      .2225     .052193     .147624    .0990832    .3459168
```

```
---------+--------------------------------------------------------------------
combined |      14    .2745154    .0524502    .1962506    .1612037    .3878271
---------+--------------------------------------------------------------------
    diff |           .1213694    .1046035               -.1065422    .3492809
------------------------------------------------------------------------------
    diff = mean(gsinegativcont~k) - mean(gisgorean)               t =   1.1603
Ho: diff = 0                                     degrees of freedom =       12

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.8658         Pr(|T| > |t|) = 0.2685          Pr(T > t) = 0.1342

.
```