EFFECT OF PHYTOGENIC FEED ADDITIVES ON GONADAL DEVELOPMENT IN MOZAMBIQUE TILAPIA (*OREOCHROMIS MOSSAMBICUS*)

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circulation (Blazer, 2002; Pait and Nelson, 2002; Manning, 2005; Mills and Chichester, 2005; Evans *et al*, 2011). Although hormones reach all parts of the organism's (e.g. fish) body, only target cells with compatible receptors are able to respond. Binding of the receptor and a hormone could alter the cell's existing proteins or activate genes that will build a new protein (Moutsatsou, 2007). Results indicate that exposure of an organism to levels of EDCs or natural hormones which can interfere with the proper functioning of the endocrine system could have severe effect on the reproductive endocrine function leading to alteration in reproductive development (Jobling *et al*, 1998; Damstra *et al*, 2002; Manning, 2005; Mills and Chichester, 2005; Cheshenko *et a*l, 2008; Sassi-Messai *et al;* 2009; Blazer *et al*, 2012). The structural changes as seen in Plate 5.2 shows that Pawpaw or Moringa interacted with the endogenous hormones to elicit the observed effects of *O. mossambicus* gonad integrity.

5.4.3 Management of reproduction in tilapia-Puberty control

The wild Mozambique tilapia (*Oreochromis mossambicus*) reaches maturity at a size that ranges from 110mm to 285mm. In a fast growing culture environment *O. mossambicus* could spawn at an age of three months, weighing between 60 and 100g, but in a poorly fertilised pond fish might weight as low as 15g (Shelton and Popma, 2006).

Puberty is "the developmental period comprising the transition from an immature juvenile to a mature adult state of the reproductive system, i.e. the stage of development during which an individual becomes capable of reproducing sexually, implying functional competence of the brain–pituitary–gonad (BPG) axis" (Okuzawa, 2002). During puberty the two main functions of the gonad become active, namely, the production of germ cells and the synthesis of reproductive (steroid) hormones. These hormones regulate germ cell development in both females (e.g. oestrogens and vitellogenesis) and males (e.g. androgens and spermatogenesis). Puberty is considered completed with the first batch of fertile gametes (Okuzawa, 2002; Munakata and Kobayashi, 2010; Taranger *et al*., 2010)

In the natural environment the effect of EDCs would depend on various factors including: the potency or efficacy of the EDC; its concentration; duration of exposure; bioconcentration potential; presence of other EDCs; life stage; season; other environmental stressors present (e.g., temperature, photoperiod, salinity, and other contaminants) and mobility of the individual (Pait and Nelson, 2002). In aquarium setting, the space available for the fish appears to be in more proximity to the fish in comparison to the wild. The phytoestrogen provided or incorporated as part of Pawpaw and Moringa seed meal, can be manipulated in such a way that the feed can fit or depict the criteria described by Pait and Nelson (2002).

The endogenous hormone, estrogen that controls sexual differentiation, maturation and reproduction (Jobling *et al*, 1998), is biosynthesised through the steroidogenic enzyme cytochrome P450 aromatase, which converts androgens to oestrogens (Afonso *et al*., 2001). So any compound or chemical that has the capacity to replace an estrogen in its activity in the cell, will eventually affect the functional activity exerted by

the estrogen. Generally, the assumption is that phytoestrogens inhibit steroidogenic enzymes by competitive inhibition with natural substrates for a particular enzyme.

In fish, the enzyme aromatase CYP19 is often the target of EDCs, which modifies its expression and function, changing the level of estrogen production, disrupting estrogen biological processes including malfunctioning of the reproductive system (Cheshenko *et al*, 2008; Zhao and Mu, 2011). And phytoestrogens, such as daidzein, genistein, quercetin, kaempferol, β-sitosterols saponins and the triterpenes which appear to be potent modulators of the activity of the aromatase enzyme and reported to exist in Pawpaw and Moringa, can be used to manipulate puberty in tilapia to control the precocious breeding behaviour in production systems.

According to Zhao and Mu (2011) the regulation of reproduction by the brain is through the brain-pituitary-gonad (BPG) axis, which controls the pituitary gonadotropin hormone including the gonadotropin releasing hormone (GnRH), follicle stimulating hormone (FSH) and luteinizing hormone (LH). The FSH and LH are the hormones which induce the synthesis of the sex hormones such as 17β-estradiol (E2) and testosterone (T), which control ovulation and sper development. The E2 operates on a positive and negative feedback roles on the gonadotropin or its upstream hormone synthesis and secretion, through direct interaction with estrogen receptors. In this way phytoestrogen plays a role in mimicking or antagonizing the E2 function. It is believed that the BPG aixs is activated at the onset of puberty, which is also depended on several external and internal factors such as photoperiod, water temperature, somatic growth and gonadal status (Okuzawa, 2002; Taranger *et al*, 2010). For instance, photoperiod has been manipulated to delay the initiation of puberty in salmonids (Taranger *et al*, 2010). Both endogenous and exogenous steroid hormones are considered to have positive effect on pubertal development (Schulz and Goos, 1999. It has been proposed that sex steroids are the natural inducers of onset of puberty in male African catfish, and that a long-term treatment of 11ketotestosterone or its precursor stimulated testicular growth and spermatogenesis, probably through direct effect on the testes (Cavaco *et al*, 1998; Schulz and Goos, 1999).

The Pawpaw and Moringa seed powder used in this experiment have been found to contain some phytoestrogens β-Sitosterol; genistein, diadzein, equol, quercetin and kaempferol (Moutsatsou, 2007; Zhao and Mu, 2011). These phytoestrogens that have the ability to interfere with the activity of BPG axis (known to control puberty and reproduction) could affect puberty in fish. Judging by the disturbances as observed in the gonads of fish fed meals containing P5.0, P10.0 and M10.0g/kg BD (Figures 5.2 and 5.3), Pawpaw and Moringa have the capacity to effect changes in the reproductive system of fish. The possible ways by which Pawpaw and Moringa which contains multiple forms of phytochemicals with proven estrogenic or androgenic activity can elicit the effects shown above could be through two or more routes described above by (Blazer, 2002; Pait and Nelson, 2002; Manning, 2005; Mills and Chichester, 2005; Evans *et al*, 2011).

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5.5 Conclusions

It can be concluded that Pawpaw and Moringa Seed powder have the capacity to delay puberty, and restrain the precocious breeding in tilapias, thus could be used successfully to control reproduction in tilapia. Further studies need to be conducted on the appropriate inclusion levels, duration of application and size of the fish for commercial or practical application.

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Chapter 6

The Effect of Pawpaw Seed meal and Moringa Seed Meal on The Sexual Differentiation of Mozambique Tilapia Fry (*Oreochromis mossambicus*)

Abstract

The use of all-male populations is the prefered option to control reproduction in tilapia aquaculture and to utilise the superior growth characteristics of males compared to females. The most commonly used method to produce all-male tilapia populations is the use of the synthetic steroid, 17α-methyltestosterone (MT) to induce sex-reversal. However due to the perceived health and environmental hazards, the use of natural reproductive inhibitors should be explored for use in aquaculture. Phytoestrogens are phytochemicals that induce biological responses in vertebrates and mimic or modulate the actions of endogenous oestrogens (such as 17β-estradiol), usually by binding to estrogen receptors. This study evaluated the effect of Papaw or Moringa seed as potential endocrine modulators that could affect sexual differentiation in juvenile Mozambique tilapia, *Oreochromis mossambicus*. Experimental treatments were respectively Pawpaw seed powder (P) during the 1st and Moringa seed powder (M) during the 2nd trial, into a commercial tilapia starter feed (15g/kg Basal Diet), together with a Negative Control (NC) comprising of the Basal Diet, and Positive Control containing 17α-Methyltestosterone (MT), fed to first exogenous feeding *O. mossambicus* fry for a period of 30 days. The sex ratio and survival rates were recorded at age 90 days. Phenotypic sex was determined by macro-examination of gonads. Results of the 1st trial indicate that Pawpaw Seed (P) had a significant effect on the sexual differentiation of *O mossambicus* fry, whilst neither the Positive Control (MT) nor the Negative Control (NC) induced any significant deviation from the expected 1:1 ratio. In the 2nd trial both the Moringa Seed and the Positive Control treatments showed a significant effect on the sex ratio of tilapia fry. None of the treatments in any of the trials had any significant effect on survival rate. The inclusion of Pawpaw and Moringa seed in the diets of first exogenous feeding of mixed sex *O. mossambicus* fry showed some phytoestrogenic effects on the gonadal development without any effect on survival.

6.1 Introduction

Tilapia have become one of the most important food fishes of the world with a significant increase in production from 383,654 metric tonnes (mt) in 1990 to 3,497,391 mt in 2010 (FAO, 2012). Much of the growth can be ascribed to the increased efficiency of monosex (i.e. all-male) populations obtained through hormonal manipulation of early sexual differentiation of fry (El-Sayed *et al.,* 2012). In Sub-Saharan Africa (SSA), tilapia culture is still hampered by uncontrolled reproduction in ponds, which result in the harvest of stunted fish with low nutritional and commercial value. Most of the advanced biotechnologies developed to manage or manipulate the precocious breeding strategies of tilapia in culture systems are to a large extent not accessible to farmers in rural and peri-urban SSA. Studies over the last two decades have indicated only monosex tilapia production systems in SSA to be financially viable in terms of commercial small-scale culture, together with polyculture systems of the African catfish (*Clarias* species) in Ghana and Nigeria (Stadtlander *et al.,* 2008).

The critical developmental period of differentiation of fish sex into male or female is plastic and is controlled by genetic, physiological and/or environmental factors (Devlin and Nagahama, 2002; Rougeot *et al.,* 2008). Sexual differentiation can therefore be manipulated towards the preferred gender, such as all-male in the

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case of tilapias (Pandian and Varadaraj, 1990; Popma and Lovshin, 1995; Phelps, 2006; El-Sayed, 2006) that contribute significantly towards the expansion of tilapia culture.

Among the four major ways of producing all-male tilapia, i.e. manual/hand sorting, hybridization, genetic manipulation, and hormonal sex reversal, the technique of using androgenic hormones to sexually reverse to an all-male stock has become the most common practice in commercial culture. In spite of its laborious nature manual sorting remains the most common practice in rural SSA culture. Hybridization and genetic manipulation remain unavailable to SSA farmers due to technical constraints and cost.

The use of hormones for sex reversal is however, under increasing public scrutiny due to perceived potential health risks, environmental impacts, and social constraints (El-Sayed, 2006; Lückstadt *et al.,* 2006). In most developing countries, including SSA there is no effective regulation on the use of androgenic hormones while it also remains difficult to acquire due to cumbersome import and export regulations.

Considering the problems associated with the use of androgenous hormonal treatment, the shortcomings of the existing methods and techniques enumerated by Mair and Little (1991) and the unavailability to rural and peri-urban SSA, an alternative approach is worth investigating.

Phytoestrogens as Endocrine Disrupting Chemicals

Natural reproduction inhibitors found in medicinal plants provide an alternative for the control of sexual differentiation in fish. Studies have shown that some phytochemicals are natural steroid-like substances with estrogen-like biological activity, called phytoestrogens (i.e. estrogenic compounds found in plants). A phytoestrogen may be defined "as any substance or metabolite that induces biological responses in vertebrates and mimic or modulate the actions of endogenous oestrogens (such as 17β-estradiol), usually by binding to estrogen receptors" (Patisaul and Jefferson, 2010).

Medicinal plants have been used successfully to induce sterility in laboratory animals (Bodharkar *et al*., 1974; Das, 1980). It raises the question whether such plant bioactive chemicals which act as the natural reproductive inhibitory agents could be used to control tilapia recruitment in ponds. The effect of phytoestrogens on fish was first reported in trout and sturgeon (Pelissero *et al.*, 1991; Pelissero and Sumpter, 1992; Pelissero *et al*., 1996). Estrogens are steroid hormones involved in the control of important reproduction- related processes, including sexual differentiation, maturation and exhibition of secondary sex characteristics, which indicates that a proper function of the fish body requires a balance of estrogens. Nakamura *et al.* (1998) emphasized that, in the teleost fish ovarian differentiation begins at an earlier stage than testicular differentiation, with biosynthesis of estradiol -17β from cholesterol, through enzymatic activity of aromatase. In the biosynthesis of estrogen, at the final step cytochrome P450 aromatase, encoded by the cyp19 gene, converts androgens into estrogens. Modulation of aromatase CYP19 expression and function can cause alteration in the rate of estrogen production, disturbing the local and systemic levels of estrogens (Nagahama, 1994; Nakamura *et al*., 1998; Strüssmann and Nakamura, 2002).

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There are some reports on the effect of phytochemical extracts such as quercetin, genistein and diadzein (Dabrowski *et al.,* 2004; 2005; de Oca, 2005), which did not significantly affect the sex ratio of Nile tilapia. El-Sayed *et al.* (2012) evaluated the action of daidzein and genistein in soyabean meal used as protein source on 17α–methyltestosterone sex reversal treatment of Nile tilapia larvae. After 28-day trial these authors reported that the fish fed high inclusion levels of diadzein and genistein (10 to 30mg/kg) had an increased percentage of female with increasing elevated level of the phytoestrogen from 24.5 to 47.8% and from 17.5 to 42.5% respectively. They concluded that diadzein and genistein have a significant estrogenic effect on sexual differentiation of Nile tilapia larvae, and thus weakened methyltestesterone masculinization activity.

Ruksana *et al.* (2010) tested the efficacy of an aluminium laden compound, exemestane (EM), which is considered to be an aromatase inhibitor, on sex differentiation in genetically female Nile tilapia. These authors concluded that all females of the Nile tilapia treated with high levels of EM (1000 and 2000 μg/g of feed) during the critical developmental period for sex differentiation, developed testes. In addition they reported that the sex reversed males appeared to be sexually functional, because they had testes with efferent ducts and with all the stages of spermatogenesis (i.e. from primary spermatogonia to spermatozoa).

This study therefore, hypothesized that a diet containing natural estrogenic / androgenic compounds will affect the sexual differentiation and gonadal activity of sexually undifferentiated tilapia fry and disturb the sex ratio in favour of a particular sex (i.e. male or female).

6.2 Materials and Methods

The trial was conducted in a recirculating aquaria system (RAS) at the Welgevallen Experiment Farm, Stellenbosch University, South Africa (see Chapter 3, for more detail on the facilities and management). The Geographical Position System (GPS) coordinates are 33°56' 33.95" S and 18°51'56.15". The facility consist of 72 glass tanks, each tank with a dimension of 58 x 57 x 40 cm with water holding capacity of 94 litres.

The study was conducted between June and October 2011 consisting of two trials, the first for which Pawpaw Seed powder (P) was included in a commercial tilapia starter feed (Basal Diet, BD), and fed to first exogenous feeding Mozambique tilapia (*Oreochromis mossambicus*) fry, after the absorption of yolk sac.

During the second trial Moringa Seed powder (M) was included in the Basal Diet, and fed to first exogenous feeding *O. mossambicus* fry. The experimental design for trial 1 and 2 is presented in Tables 6.1 and 6.2 respectively. A negative control (NC) treatment comprising of the Basal Diet, and Positive Control containing 17α-Methyltestosterone (MT), were included in both parts of the study. The details of the preparation of the Pawpaw seed powder and Moringa seed powder and the experimental diets are presented in Chapter 3.

Table 6.1 The experimental design (3 x 4 = 12 factorial) for the assessment of the effect of 17α-Methyltestosterone and Pawpaw Seed meal on sexual differentiation in sexually undifferentiated post hatch tilapia fry, *Oreochromis mossambicus*.

Treatments	Designation	Number of fish	Dosage (g/kg of Basal Diet[1])
Negative Control	$NC_{(I-IV)}$	4x200	No dosage
Positive Control	$MT_{(I-IV)}$	4x200	60mg 17α-Methyltestosterone
Pawpaw Seed	$P_{(I-IV)}$	4x200	15g Pawpaw Seed powder

[1] Basal Diet: AQUANUTRO Tilapia starter diet (Nutroscience (Pty) Ltd, SA). Replicates per treatment = 4

Table 6.2 The experimental design (3 x4 = 12 factorial) for the assessment of the effect 17α-Methyltestosterone and Moringa Seed meal on sexual differentiation in sexually undifferentiated fry (9-12 day post hatch) of the tilapia, *Oreochromis mossambicus*.

Treatments	Designation	Number of fish	Dosage (g/kg of Basal Diet[1])
Negative Control	$NC_{(I-IV)}$	4x200	No dosage
Positive Control	$MT_{(I-IV)}$	4x200	60mg 17α-Methyltestosterone
Moringa Seed	$M_{(I-IV)}$	4x200	15g Moringa Seed powder

[1] Basal Diet: AQUANUTRO Tilapia starter diet (Nutroscience (Pty) Ltd, SA). Replicates per treatment = 4

A detail description of the experimental procedures and data recording has been provided in Chapter 3, including selection of breeders and fry production, stocking of fry (sexually undifferentiated fish 9 – 12 day-post-hatch), feeding, water quality and maintenance and data collection (i.e. survivorship and sex ratio).

In this experiment III, after the 30 trial, fish were fed on basal diet for another 60 days. At the end of the 90 day experimental period (i.e. 30 days treatment and 60 days control diet) gender could be comfortably determined in the fish; so), 50 fish were randomly sampled from each of the 4, replicates equal to 200 fish per treatment and sex determined.

Gender was determined by the presence/absence of specific external morphological characteristics, i.e. genital papillae, and the length and shape of the dorsal and anal fins. Gender was confirmed by gonadal examination, through dissection. The total number of males and females identified after dissection were recorded. Also, 4 fish (2males and 2 females) were randomly taken from each group of 50 fish, and the total length, body depth and body weight measured, and their gonads fixed in Bouin's solution.

Mortality was monitored and recorded for all the experiments and the data was used to calculate survival rates. Dead fish were removed from the tank on daily basis as a standard management procedure

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Data recorded during the study were captured and analysed in Microsoft Excel 2010. A Chi-square analysis was performed to test for the significance of deviation from the expected 1:1, male: female sex ratio (Bhujel, 2008) at a 5% probability level for each replicate group as well as for the pooled data for each treatment. Using the statistical programme, SAS Version 9.1, a one-way analysis (ANOVA) was performed to test for the significance of variance in the deviation from the 1:1 ratio, at $P<0.05$.

Survival rate was determined for each treatment group, as well as the pooled data for each treatment, expressed as the mean ± sd according to the equation below:

$$\text{Survival Rate (\%)} = \frac{\text{Final Number of Fish}}{\text{Initial Number of Fish}} \times 100$$

(Source: Huang and Chin, 1997, modified)

6.3 Results

6.3.1 Effect of treatment on sex ratio

The observed sex ratio of the *O. mossambicus* fingerlings fed Pawpaw Seed meal at a concentration of 15g/kg Basal Diet over 90-days is presented in Table 6.3 and Figure 6.1. Out of 200 fish examined per replicate fed the Pawpaw Seed treatment a total of 65.0 ± 4.58 were classified as males on the basis of gonadal assessment (Figure 6.1). Pawpaw Seed (P) had a significant effect on the sexual differentiation of *O mossambicus* fry with the chi-square value against the expected 1:1 sex ratio of 18.0 ($P_{(<0.05)} = 3.841$).. Neither the Positive Control (MT) nor the Negative Control (NC) induced any significant deviation, as confirmed by the respective Chi-square values. In the Positive Control group a total of 10 fish from the 200 sampled were found to possess both testicular and ovarian tissues, whilst two such cases were found amongst 200 fish sampled in the Negative Control group, and none from the Pawpaw Seed treatment group.

Table 6.3 The male:female sex ratio in a mixed sex population of tilapia (*Oreochromis mossambicus*), after a 90 day treatment as Negative Control (CT), Positive Control (MT) and Pawpaw Seed (P).

Treatment	Male	Female	Intersex	Total Fish Examined	Sex Ratio	Calculated χ^2	$P_{(0.05;\,1)}$ $\chi^2=3.841$
NC	107	91	2	200	1.18:1	1.30	NS
MT	104	86	10	200	1.08:1	0.32	NS
P	130	70	0	200	1.88:1	18.00	S

The observed sex ratio of the *O. mossambicus* fingerlings fed Moringa Seed meal at a concentration of 15g/kg Basal Diet, over a 90-day trial period, is presented in Table 6.4 and Figure 6.2. In this trial both the Moringa Seed (M) and the Positive Control (MT) treatments showed a significant effect on the sex ratio in developing tilapia *O mossambicus*. From the 200 fish examined from fish fed M treatment group an average

of 65.50 ± 4.12 were classified as males (Figure 6.2), confirming a significant deviation from the expected 1:1 sex ratio against the Chi-square value of $P_{(<0.05;\ 1)}$ =3.841, and p-value < 0.001.

Table 6.4 The male:female sex ratio in a mixed sex population of tilapia (*Oreochromis mossambicus*), after a 90 day treatment as Negative Control (NC), Positive Control (MT) and Moringa Seed (M).

Treatment	Male	Female	Intersex	Total Fish Examined	Sex Ratio	Calculated χ^2	$P_{(0.05;\ 1)}$ χ^2=3.841
NC	90	110	0	200	0.82:1	2.00	NS
MT	143	57	0	200	2.51:1	36.98	S
M	131	69	0	200	1.90:1	19.22	S

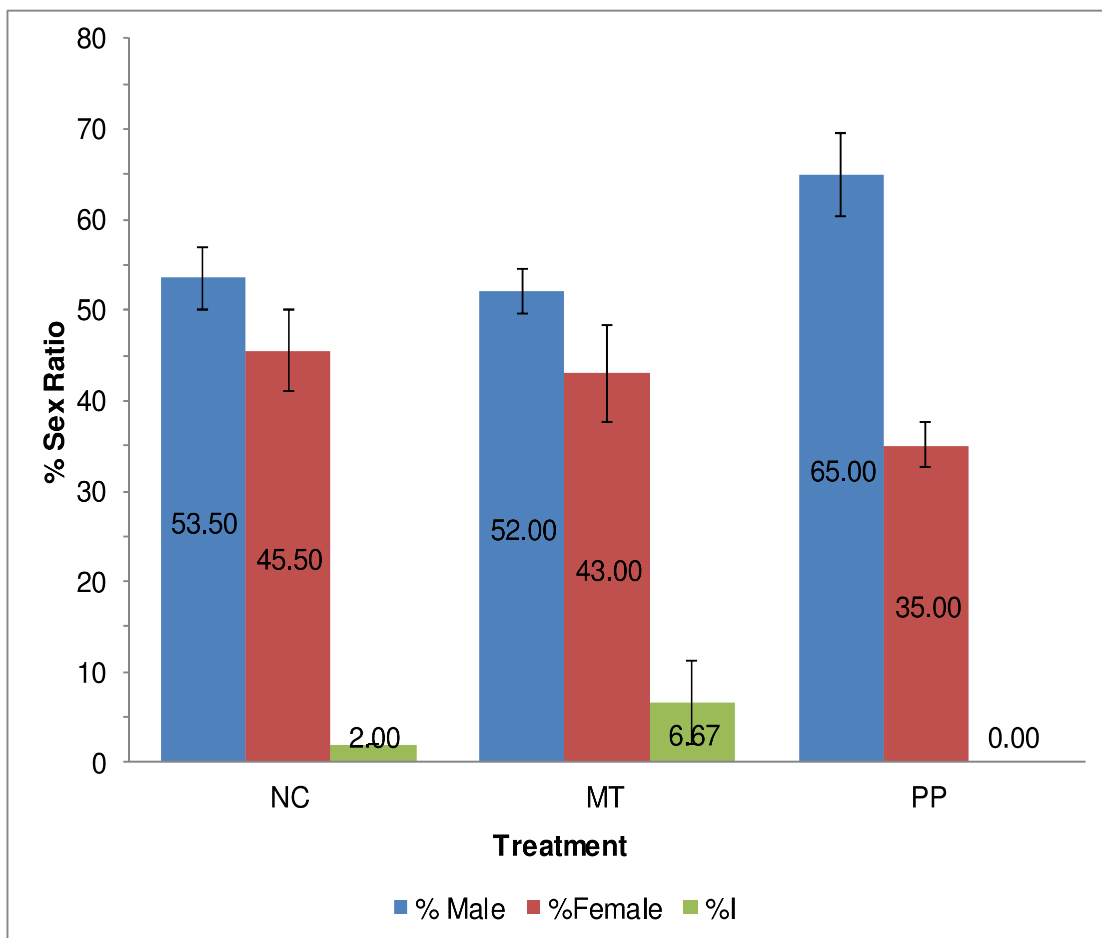

Figure 6.1 The Sex ratio (percentage) of Tilapia *O.mossabicus* at age 90 days over 4 replicates and 3 treatments Negative Control (NC), Positive Control (MT) and Pawpaw Seed (PP)

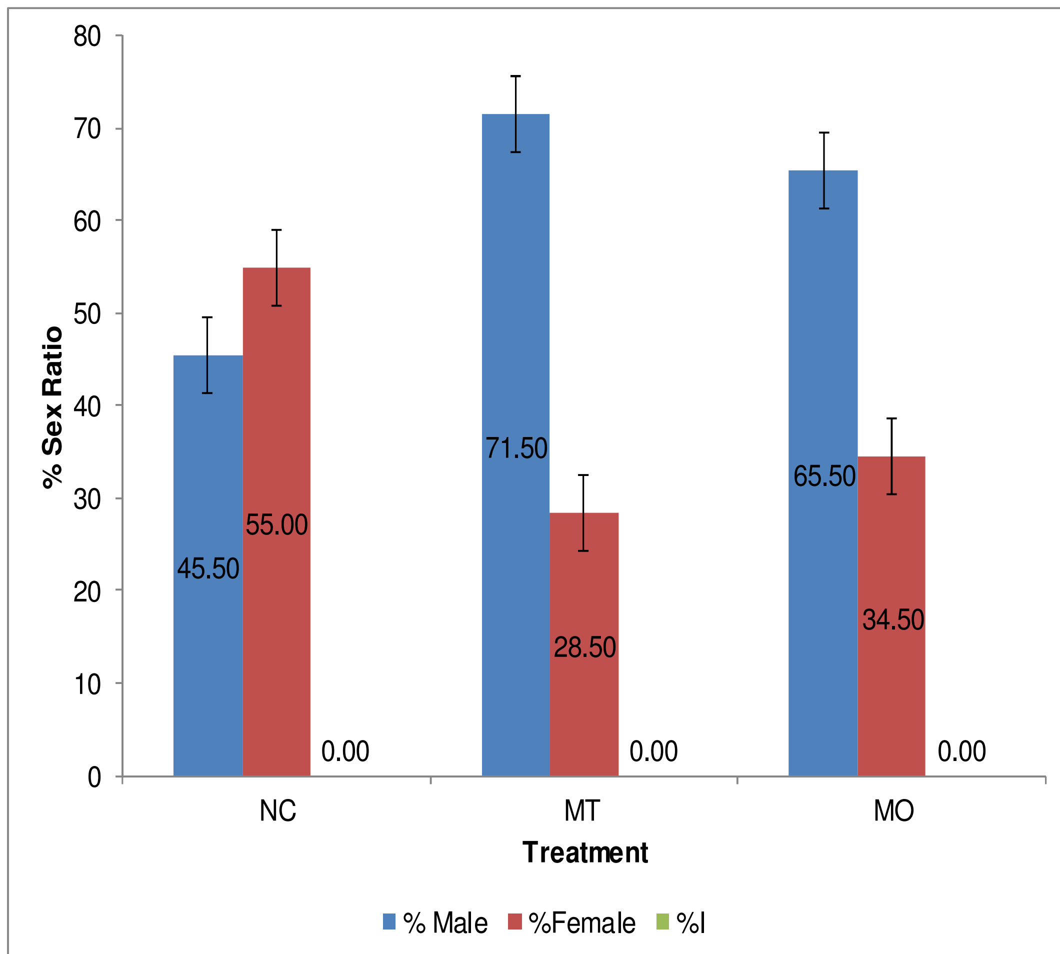

Figure 6.2 The sex ratio (percentage) of Tilapia (O. *mossambicus*) at age 90 days over 4 replicates and 3 treatments: Negative Control (NC), Positive Control (MT) and Moringa Seed (MO).

None of the gonads from sampled fish amongst any of the treatments displayed both testicular and ovarian tissue as in the case of the first trial.

6.3.3 Effect of treatment on survival rate

The effect of the treatments on survival during the first trial is shown in Table 6.5. The highest percentage survival was found in the Negative Control group at 97.88 ± 2.29, although not significantly different from any of the other treatments at $P<0.05$.

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Table 6.5 Survival rates of Tilapia (*O. mossambicus*) over 90-day treatment as Negative Control (NC), Positive Control, (MT) and Papaw Seed (P), with 4 replicates per treatment.

Treatment (Diet)	Initial Number	Final Number	Survival (%± sd)
Negative Control (NC)	4x200	783	97.88 ± 2.29[a]
Positive Control (PC)	4x200	774	95.75± 3.28[a]
Pawpaw Seed (P)	4x200	772	96.50 ± 2.12[a]

[a] Values with similar superscript are not significant, Chi-square p-value, at P<0.05, 1df

Significant differences in survival rate were recorded during the 2nd trial in relation to the M treatment groups with an average of 91.00 ± 6.70, compared to that of the Negative and Positive Control groups, as reported in Table 6.6.

Table 6.6 Survival rates of Tilapia (*O. mossambicus*) over 90-day trial treatment as Negative Control (NC), Positive Control, (MT) and Moringa Seed (M), with 4 replicates per treatment

Treatment (Diet)	Initial Number	Final Number	Survival (%± s.d)
Negative Control (NC)	4x200	698	87.25 ± 5.32[a]
Positive Control (PC)	4x200	697	87.13± 5.51[a]
Moringa Seed (M)	4x200	728	91.00 ±6.70[b]

[a ,b] Values with different superscript are significant and same not significant, Chi-square p-value, at P<0.05, 1df

6.4 Discussion

Pawpaw and Moringa seed contain a broad spectrum of phytochemicals including polysaccharides, vitamins, minerals, enzymes, proteins, alkaloids, glycosides, fats and oils, lectins, saponins, flavonoids, sterols and several phenolics (Krishna *et al*., 2008, Kumar *et al*., 2010). Both plant species are highly valued food crops in SSA, with additional application in several traditional medicines (Raji *et al*., 2005; Anwar *et al.,* 2007; Bose, 2007; Krishna *et al*., 2008). The choice of Pawpaw and Moringa seeds for experimental treatments of tilapia stem from their known effect as a natural reproduction inhibitor, and it is readily available year round in Sub-Saharan Africa (SSA). Pawpaw and Moringa have been tested on animal models, such as mice and rats, showing antifertility properties (Krishna *et al.,* 2008; Kumar *et al.,* 2012). Some attempts have been made to use crude Pawpaw Seed as antifertility agent on sexually matured Nile tilapia to control its reproduction, with results which indicate that Pawpaw can interfere with breeding in tilapia (Ekanem and Okoronkwo, 2003; Jegede and Fagbenro, 2008; Abbas and Abbas, 2011).

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Exogenous first feeding of tilapias starts at 9-12 days post hatching following depletion of the yolk (Rana, 1988) with no differentiation of the gonads. Sexual differentiation is known to occur in the genus *Oreochromis* fry at or around 17-19 days after hatching (Yamazaki, 1983; Mair and Little, 1991; El-Sayed, 2006). It is therefore, possible to intervene at this early stage of development in the fish to direct gonadal development to produce monosex populations. Pandian and Varadaraj (1987) reported that in the Mozambique tilapia, *O. mossambicus* the period where the fry will be most sensitive for such manipulation is in the period 10 to 20 days (i.e. at about 15 - 30mm length) of the post hatching period. The morphological parameters (i.e. length and weight) and age used in this study conform to the assertion that a successful sex change or reversal requires newly hatched fry of length with a range 9-12mm, presumed to have sexually undifferentiated gonads (Green and Teichert-Coddington,1993), and a body mass in the range of 10mg to 30mg (i.e. 0.0010g -0.0030) (Phelps, 2006). According to Nakamura *et al.* (1998) gonadal sex differentiation in teleost fish is affected by several exogenous factors such as sex hormones, temperature, and pH. Therefore, applying any of these parameters mentioned above during the critical period of sexual differentiation will affect the direction in which the phenotypic sex of the fish could be expressed.

The use of phytochemicals as a possible alternative to 17MT has received much attention since the estrogenic activity of flavonoids in fish diet was reported by Pelissero *et al.* (1991). Notable attempts on the use of phytochemicals to induce sex change of tilapias have been reported by Dabrowski *et al.* (2004; 2005) and; de Oca (2005). These authors used the refined phytochemicals that included quercetin, genistein and diadzein (Pelissero *et al.*, 1996), and reported that the phytochemicals did not significantly affect the masculinization of tilapia. De Oca (2005) indicated that pure genistein and quercertin did not affect the sex ratio of genetically all-female Nile tilapia, by obtaining equal female to male ratio compared to control group of 97% female. Green and Kelly (2009) investigated the potential of genistein to alter phynotypic sex during sexual differentiation in channel catfish and reported an increase in the proportion of phenotypical male after long exposure to genistein. These studies provided the preliminary insight on the *in vivo* response to pure phytochemicals when dietary administration was used in sexually undifferentiated fish.

This study used crude Pawpaw and Moringa Seed powder to investigate the potential of these biochemical plant components, acting as endocrine disrupters, to influence the direction gonadal sex differentiation in Mozambique tilapia (*Oreochromis mossambicus*). For each of the treatments, a significant increase was obtained in the percentage males of 65.0 ± 4.58 for Pawpaw and 65.50 ± 4.12 for Moringa.

Shelton (2006) intimated that the extensive use and success of sex reversal is largely based on the following assumptions (a) treatment must proceed during a critical period of gonadal differentiation, (b) steroids (androgens/estrogens) mimic natural induction by genetic sex-determining factors so as to alter development of the phenotypic or gonadal sex, (c) the exogenous steroid must be efficacious, adequately concentrated, and efficiently delivered so as to provide the physiological or pharmacological effect; (d) steroid-induced development of gonadal sex does not spontaneously revert, and, (e) genotypic sex is not affected by the phenotypic alteration treatment. The reliability of any chemical to induce effect on fish gonad will depend largely on the amount available for the fish to ingest and present at the site of estrogenic activity (Phelps,

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2006; Shelton, 2006). The significant increase in the observed percentage of males is an indication of the presence of phytoestrogens in Pawpaw and Moringa seed and its effect on sexual differentiation in relation to the postulations of Shelton (2006).

The reported phytoestrogens identified in Pawpaw plant parts include β-sitosterol, saponins, and flavonoids (Krishna *et al*., 2008) and that of Moringa plant parts include saponins, oleanolic acid-3-glucoside and the flavonoids, quercetin and kaempferol (Anwar *et al*., 2007; Kumar *et al.,* 2010). The interaction of these phytoestrogens with the endogenous hormones of the fish could be seen as the cause of change in sex ratio and indicates that the dietary administration of Pawpaw or Moringa in the crude form could affect gonadal sex differentiation in juvenile tilapia.

The exact mechanism through which Pawpaw and Moringa seed could act at the cellular or molecular level have not been established, however two possible scenarios have been adduced for expression of phytoestrogens in endocrine modulation. Phytoestrogens that show close structural similarity to androgens and estrogens, have been shown under experimental conditions in *in vitro* studies to block the biosynthesis and action of estrogens by (1) inhibition of aromatase activity and other steroid metabolism related enzymes, or (2) by competition for the estrogenic nuclear receptors (α and β ER), that could possibly mimic the sex-reversal effects of androgen treatments in fish (Dabrowski *et al*., 2005; de Oca, 2005; Moutsatsou, 2007).

In the teleost fish ovarian differentiation commences at an earlier stage than as in testicular tissue. Sexual differentiation into ovarian cells occurs with biosynthesis of estradiol -17β from cholesterol, through enzymatic activity of aromatase (Nagahama, 1994; Nakamura *et al.,* 1998). Thus estrogenenic hormones are a product of aromatase, and thus any chemical compounds, such as the phytoestrogens (β-sitosterol, saponins, oleanolic acid-3-glucoside, genistein, quercetin and kaempferol) that can block the activity of aromatase, suppressing estrogen biosynthesis in cells (Pelissero *et al*., 1996; Dabrowski *et al*., 2004), which could switch ovarian development to testicular development. The studies of Dabrowski *et al.* (2005), de Oca (2005) and El-Sayed *et al.* (2012) give an indication that phytoestrogens could exert influences on gonadal differentiation of fish. It has not been established that, Pawpaw and Moringa are aromatase inhibitors, however, considering the fact that both plants contain the above known phytoestrogens with known aromatization activity, it can be postulated that both Pawpaw and Moringa are potential aromatizing agents.

In relation to the second scenario, *in vitro* studies have indicated that some phytoestrogens have structures similar to that of natural steroid hormones, thereby it can mimic, compete and displace endogenous estrogens from binding sites on estrogen receptors (Moutsautsou, 2007), consequently, they can act as antiestrogens or weak estrogens thereby eliciting their biological activity. According to Matthews *et al.* (2000) activity of estrogen in target cells manifest itself through binding to estrogen receptors. Based on the chemical structure phytoestrogens such as quercetin, genistein and diadzein, have a capacity to compete for binding sites and displace estradiol at the estrogen receptors (ERs) binding sites, thereby modulating the genetic expression of sexual orientation (Miksick, 1995; Morito *et al*., 2001; de Oca, 2005; Moutsautsou,

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2007). Therefore phytoestrogens present in Pawpaw and Moringa, can elicit the observed effects in fish gonads.

The manifestation of phytoestrogen effect depends on factors which include introduction of the chemical to the fry that allow for interaction to occur during the critical period of gonadal differentiation; the capacity of phytoestrogens to mimic natural estrogen that can induce the genetic sex-determining factors to alter development of the phenotypic or gonadal sex and more importantly it must be efficacious, adequately concentrated, and efficiently delivered so as to provide the physiological or pharmacological effect (Shelton, 2006). The most important criteria to determine whether a natural or synthetic compound can successfully induce sex reversal, are a) the time of onset of treatment, b) the duration of treatment, c) the dose and type of hormones used, and d) the quantity of the hormone, or bioactive chemicals (Yamazaki, 1983; Nagahama, 1994; Nakamura *et al.*, 1998).

The success of sex-reversal techniques in tilapias to produce all-male populations has been found to depend on the intake of feed containing hormones which is in turn influenced by many factors (Mair and Little, 1991; El-Sayed, 2006). tTese including a) the quality of treated feed, composition of the raw ingredients, method of preparation and storage; b) palatability and particle size (both affect actual intake of first-feeding fry); c) feeding frequency and mode (e.g. ad libitum or, percentage of body weight and number of times feed is offered) and availability of treated food to all fry.

The two most recent studies involving the use of refined phytoestrogens- genistein and diadzein to induce sex reversal presented conflicting results. De Oca (2005) reported that genistein and diadzein could not masculinize genetically all-female Nile tilapia fish, whilst El-Sayed *et al.* (2012), indicates that genistein and diadzein decreased the masculinization effect of 17α-methyltesterone in genetically all-female Nile tilapia fish.

The results from both experiments indicated that the inclusion of Pawpaw and Moringa Seed had no negative impact on the survival rate of tilapia fry compared to Positive and Negative Control diets ($P<0.05$, Tables 6.1and 6.2). In fact the Moringa seed treatment yielded a significantly higher survival rate compared to the Positive and Negative Control diets ($P<0.05$).

El-Sayed *et al.* (2012) pointed to the importance of maintaining a good rate of survival during the production and supply of tilapia fry and fingerlings. The critical period of sexual differentiation in *Oreochromis* fry at 17-19 days after hatching (Yamazaki, 1983; Mair and Little, 1991; El-Sayed, 2006), is also the period where the fish is very fragile and susceptible to environmental factors, stress and disease.

6.5 Conclusions

The inclusion of the crude form of Pawpaw and Moringa seed, containing phytoestrogens such as genistein, quercetin, kaempferol, diadzein and, β-sisterol, resulted in up to 65% masculinization in a mixed sex

population of Mozambique tilapia fry. From these results it can be concluded that phytoestrogens in Pawpaw and Moringa seed affected sexual differentiation in first feeding fry. Further studies are, however, required to understand the exact mechanism through which the Pawpaw and Moringa seed exert their effect. It could be postulated, that the results are a manifestation of multiple actions of the various phytoestrogen compounds present in Pawpaw and Moringa seed, and not based on the single activity of one particular phytochemical. The biochemical composition of Pawpaw and Moringa Seed should be investigated further to establish the relative quantities and range of the phytoestrogens.

The results obtained provide an indication of the use of Pawpaw and Moringa as a possible alternative to induce sex reversal in mixed sex tilapia populations. This could be of particular importance to aquaculture development in rural areas of Sub-Saharan African countries, given the abundant year round availability of these compounds. Further studies are required to optimize dosage levels for Pawpaw and Moringa Seed, in terms of concentration, duration and methods of application, as well as the interaction of the treatments with key environmental factors such as water temperature.

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Chapter 7

Determination of the Triterpene Acids (Oleanolic Acid and Ursolic Acid) in Pawpaw Seed Meal, Moringa Seed Meal and Fish Tissues

Abstract

The study attempted to quantitify the levels of triterpene acids, oleanolic acid (OA) and ursolic acid (UA), in Pawpaw and Moringa seed powder through the use of ultra-high performance liquid chromatography with electrospray ionization mass spectrometer detection (UHPLC-ESI-MS/MS). The oleanolic acid and ursolic acid are isomeric compounds that are difficult to separate. Studies have shown that both oleanolic acid and ursolic acid possess antifertility property. Only OA was found in Moringa seed. None of the triterpene acid was found in the Pawpaw seed. The precence of oleanolic acid in the Moringa seed powder was confirmed at a concentration level of 0.508µg/g ± 0.032. Analysis need to be extended to include Moringa seed from various parts of Sub-Saharan Africa to establish the range of oleanolic acid and ursolic acid composition. The presence of oleanolic acid explains the observed effect of Moringa Seed powder on sexual development and differentiation in tilapia with the potential for application in controlling the problem of precocious breeding in tilapia culture in small scale farming systems in Sub-Saharan Africa.

7.1 Introductions

7.1.1 Rationale for Use of Phytochemicals

The early maturation and habitual spawning associated with mixed tilapia culture remains a challenge to the expansion of tilapia production in Sub-Saharan African (SSA) countries such as Ghana. For commercial and profitable culture to be fully embraced by resourced-poor rural farmers in SSA, the problem of prolific breeding and stunted growth associated with tilapia production has to be addressed.

The monsex culture i.e. maintaining only male tilapia in a culture system is a potential way to address the above mentioned problems, and is presently the preferred cultivation system on commercial farms (Popma, and Lovshin, 1995). The use of hormones to manipulate the sexual differentiation of tilapia to yield all male populations is the common technology that is used today, but the technology is not readily available to majority of SSA countries. Despite elimination of such hormones from fish on cessation of treatment to non-detectable levels after 90 days, and in fish harvested after 6 months (Green and Teichert-Coddington, 2000; Fitzsimmons, 2007), the use of such hormones is still perceived as a potential health risk by fish consumers in developed countries (Lückstädt *et al.*, 2006). Considering the potential health and environmental risks associated with the use of hormones (Dabrowski *et al.*, 2005; Lückstädt *et al.,* 2006), and the shortcomings of existing methods and techniques discussed by Mair and Little (1991), alternative methods to control

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undesirable tilapia recruitment in ponds needs to be investigated, such as the use of natural reproduction inhibitors found in plants .

Medicinal plants have the potential to be used for the manipulation of gender differentiation in tilapia, due to the fact that they contain bioactive components that have an antifertility action in animals. Some of these plants have been used successfully to induce sterility in laboratory animals (Bodharkar *et al.*, 1974; Das, 1980), and also as human antifertility agents in traditional medicine treatments in various countries (Kumar *et al.*, 2012). Pawpaw (*Carica papaya*) seed, administered orally to male albino rats, effectively controlled their reproduction (Udoh and Kehinde 1999) and reduced sperm motility (Pathak *et al.*, 2005). Aqueous extracts of the root and bark of *Moringa oleifera* are effective in preventing implantation in rats (Shukla *et al.*, 1989) with the ethanolic extract of *M. oleifera* leaves indicating 100% abortifacient activity in female albino rats (Charles Foster strain) (Nath *et al.*, 1992). Anwar *et al.* (2007) in a review reported that the root of *M. oleifera* has antifertility properties, with the root and flowers containing the abortifacient compounds.

There is therefore some merit in the assessment of similar effects on reproduction of tilapia. These studies suggest that a contraceptive efficacy of dietary plant nutrients with antifertility or abortifacient activity could be used by resourced-poor producers to combat problems of tilapia precocious breeding in production ponds.

7.1.2 Bioactive compounds with abortifacient / antifertllity properties

Kitts (1994) defined bioactive compounds as “extranutritional constituents that typically are naturally occurring in small quantities in plant products and lipid rich foods”. However, according to Bernhoft (2010), the classical definition of bioactive compound is that “it is secondary plant metabolites that have pharmacological or toxicological effects in man and animals”. The author further states that bioactive compounds can be either beneficial or detrimental to animals depending on the quantity eaten and the content of intake. The secondary metabolites that are produced after ingestion of plants that contain antifertility compounds, have been found to have important functions in the living plants which produce them. For example, flavonoids can protect the original plant against free radicals generated during photosynthesis. Terpenoids may attract pollinators or seed dispersers, or inhibit competing plants. Alkaloids usually ward off herbivorous animals or insect attacks (phytoalexins). Plants produce astounding varieties of these secondary plant metabolites, which are now being exploited for their potential inclusion as feed additives in livestock and aquaculture feeds (Francis *et al.*, 2002; Makkar *et al.*, 2007).

Phytogenic feed additives are plant-derived products or compounds incorporated into animal feeds to improve productivity of livestock, swine and poultry through amelioration of feed properties and food quality, and the optimization of overall production performance. Such additives are claimed to exert antioxidative, antimicrobial, and growth-promoting effects in livestock, which can potentially be associated with improved feed consumption, supposedly because of improved palatability of the diet (Windisch *et al.*, 2007; Scheurmann *et al.*, 2009). Steiner (2009) summed “phytogenics as a term that refers to group of natural

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growth promoters (NGPs) or non-antibiotic growth promoters (NAGPs) derived from herbs, spices and other plants". For instance, saponins, are considered as a special class of phytogenic substances, because they are able to reduce intestinal ammonia (NH_3) and hence alleviate an important stress factor to animal health (Francis *et al.*, 2002a; 2002b). Phytogenics are also being tried as a replacement for fish meal in aquaculture feeds (Francis *et al.*, 2002b; Makkar *et al.*, 2007).

Phytochemicals known as phytoestrogens (such as isoflavonoids, flavonoids and saponins) are natural estrogenic or estrogenic compounds found in plants such as soy, tea, fruits and vegetables that present an anti-estrogenic or estrogenic activity (Pelissori *et. al*, 1991a; 1991b; Pelissori and Sumpter, 1992; Dabrowski *et al.,* 2004). Phytoestrogens are structurally or functionally similar to or mimic mammalian estrogens and can thus have an effect on the sexual differentiation of fish.

Studies have identified some phytochemicals such as the triterpene acids- oleanolic acid (OA), ursolic acid (UA) (Rajasekaran *et.al*, 1988), and their derivatives e.g. glycoside (Das, 1980), oleanolic acid-3 β-glucoside and oleanolic acid 3-β-D-glucuronide (Das *et al.*, 2011), saponin (steroidal or triterpenoid glycoside) (Makker *et. al.*, 2007; Souad *et al.*, 2007) isolated from plants as possessing abortifacient properties and thus have the potential to be used as an antifertility treatment. Oleanolic acid and ursolic acid are hydroxyl pentacyclic triterpenoic acids (HPTAs), considered as ubiquitous triterpenoids in plant kingdom (Liu, 2005). Oleanolic acid and UA are isomers with similar chemical structures and often exist simultaneously in the same plant as aglycones of saponin and as free acids (Janicsák *et al.*, 2006). These isomeric triterpenic acids occur mostly in medicinal herbs and plants and naturally in a large variety of vegetarian foods forming an integral part of the human diet (Liu, 2005; Furtado *et al.*, 2008). According to Zhou *et al.* (2011) the distribution of oleanolic acid and ursolic acid in plants vary greatly between different families, genera and species.

Rajasekaran *et al.* (1988) tested antifertility activity of oleanolic acid isolated from the flowers of *Eugenia jambolana* in male albino rats. They reported that oleanolic acid arrested spermatogenesis but did not cause any abnormality to spermatogenic cells, Leydig cells and Sertoli cells in the rats. In a review of oleanolic acid, Liu (2005) reported an antifertility effect in rats with oleanolic acid also inhibiting testosterone 5α-reductase activity. In a study by Mdhuli and Van der Horst (2002), oleanolic acid was administered orally to a male Wister rat, for 30-days followed by a 14 day withdrawal period. Their study indicated that oleanolic acid induced reversible sterility in the male Wistar rats, without any adverse effects on *libido*.

Ursolic acid has been found to be the most abundant triterpenic acid in the leaves of the plant *Ocimum sanctum* (Rajasekaran *et al.*, 1980; Raja, 2012). Ursolic acid has been shown to have the potential of inhibiting sperm motility (Chattopadhyay *et al.*, 2005), and also possess antifertility activity in rats and mice (Raja, 2012).

Das *et al.* (2011) evaluated the spermicidal activity of oleanolic acid 3-β-D-glucuronide (OAG), an active principle isolated from root extracts of *Sesbania sesban*, which showed some significant spermicidal activity.

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The antifertility effect of these triterpene acids is attributed to their antiestrogenic effect which may be responsible for arresting spermatogenesis (Das *et al*., 2011; Raja 2012) in the tested animal models.

7.1.3 Methods for determination of triterpene acids

Bioactive compounds such as oleanolic acid and ursolic acid are usually present as a minor component of a plant extract. Because oleanolic acid and ursolic acid are isomers with similar chemical structures (see Chapter 2, section 2.6.3.7, Figure 2.12), they are very difficult to separate. According to Liang *et al.* (2009) a number of techniques and methods have attempted to quantitatively separate the tritepene acids in different plant materials over the past decades. Some of the methods include gas chromatography (GC), high-performance liquid chromatography (HPLC), cyclodextrin-modified micellar electrokinetic chromatography (CD-MEKC), micellar electrokinetic capillary chromatography (MECC) and nonaqueous capillary electrophoresis (NACE). These authors emphasised that each of the method have bottlenecks, for insntance in GC, sample preparation is time consuming and difficult, and a need for pre-column treatment. In the CD-MEKC, MECC and NACE methods, there is a complicated buffer system with low reproducibility. They concluded that HPLC, using a mobile phase consisting of an aqueous acid buffer is preferably the method used to determine oleanolic and ursolic acid.

7.1.4 High Performance Liquid Chromatography (HPLC)

According to Kellner *et al.* (2004) the high performance liquid chromatography (HPLC) is the method used to separate unipolar and isomeric compound. The HPLC technique is a unique, which is highly adptable in its usage, and now used in isolation of natural products. Current, it is the method of choice in the study of quality of herbal plant products (Fan *et al.,* 2006; Sasidharan *et al*., 2011). The HPLC method allows for expeditious separation of multicomponent samples on both preparartuve and analytica scles.

The basic bench top HPLC instrument design consist of a solvent delivery pump, a sample introduction device which could be an auto-sampler or manual injection valve, an analytical column, a guard column, detector and a recorder or a printer (Kellner *et al*., 2004; Swartz, 2005; Sasidharan *et al*., 2011). Separation of chemicals by means of HPLC is based on the principle that in a particular column and mobile phase different compounds have different migration rates. Kellner *et al.* (2004) emphasised that the magnitude of separation depend mostly on the choice buffer solutions used for the stationary phase and mobile phase. In general, the mobile phase system in HPLC use a single unchanged solution (i.e. isocratic system), for identification and separation of phytochemicals (Kellner *et al*., 2004; Swartz, 2005; Sasidharan *et al*., 2011).

Identification of compounds using HPLC involve test running a HPLC assay. A proper HPLC that can be used to separate closely related samples must have the right mobile phase system, constant flow rate, suitable detectors and appropriate column. Each compound should have a characteristic peak under certain chromatographic conditions. Developing a separation assay involves selecting a detector and setting it at optimal detection levels. In separation assay, a recognised peak of the known sample is observed on the

chromatograph, with its own retention time and separated from other peaks at the detection levels on which the assay is being performed (Kellner *et al.*, 2004; Swartz, 2005).

One of the established rapid, convenient and reliable method to separate and quantify oleanolic acid and ursolic acid is reversed-phase high performance liquid chromatographic (RP-HPLC). Reverse-phase has been used to separate and quantify oleanolic acid and ursolic acid in the following plants which include: *Macrocarpium officinalis* (Wang *et al.*, 2008); *Oldenlandia diffusa* (Liang *et al.*, 2009); leaves of *Olea europaea*, *Salvia officinalis*, *Thymus vulgaris, Origanum majorana* (Kontogianni *et al.*, 2009) and *Eriobotrya japonica* Lindl (Xu *et al.*, 2012).

Some of the proven detectors which have been used in phytochemical analysis include ultra-violet (UV), diode array detector (DAD) coupled with mass spectrometer (MS) (Tsao and Deng, 2004; Kellner *et al.*, 2004). The suitability of a UV detector is determined by the fact that the majority of naturally occurring compounds have some UV absorbance at low wavelengths (190-210 nm) and UV has a high sensitivity capable of detecting minute amounts of bioactive chemicals within a sample (Lia *et al.*, 2004).

7.1.5 Current Forms of High Performance Liquid Chromatography

The high performance liquid chromatography has proven to be a useful technique in analytical science. According to He (2000), liquid chromatography coupled with mass spectrometry (LC/MS) is one of the powerful techniques for the analysis of complex botanical extracts. It is able to provide relevant information making it possible to identify structurally related compounds. Therefore, a combined HPLC and MS technique ensure rapid assessment and a reliable identification of bioactive compunds in medicinal herbs, particularly, where there is no pure standard (Cai *et al.*, 2002; Ye *et al.*, 2007).

A new category of HPLCs are the high performance liquid chromatography-electrospray ionization-mass spectrometer detection (HPLC–ESI-MS) and ultra-high performance liquid chromatography-electrospray ionization- tandem mass spectrometry (UHPLC-ESI-MS/MS). According to Swartz (2005) UPLC-ESI-MS/MS retains the basic principles and the practicality of HPLC, whilst increasing the overall interlaced attributes of speed, sensitivity and resolution. The latter method is now considered to be the most powerful method for the separation, quantification and structural determination of position isomers with very similar structures, such as, oleanolic acid and ursolic acid in a variety of botanical raw materials (Makarov *et al.*, 2009). According to Voutquenne *et al.* (2003) oleanolic acid gives a quasi -molecular ion at *m/z* 455, [M-H]- in ESI negative mode. The UPLC-ESI-MS/MS technique has been utilised by several investigators such as Wang, *et al.* (2008), Liang *et al.* (2009), Scheidner *et al.* (2009), Csuk and Siewert (2011), Pollier and Goossens (2012), and Zhang *et al.*, (2012) for separation and quantification of oleanolic acid and ursolic acid.

Owing to the difficulty associated with the separation of the steromeric isomers, the study used UPLC-ESI-MS/MS to rapidly and accurately determine the levels of oleanolic acid and ursolic acid in Pawpaw and

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Moringa seed powder, which has been used to separate dyes (Li *et al*., 2010), and also in pharmacokinetic studies (Han *et al*., 2010; Liu *et al*., 2012).

7.2 Material and Methods

7.2.1 Methodology and Location

Pawpaw and Moringa seed powder samples were analysed at the Central Analytical Facilities, Mass Spectrometry Unit (CAF-MSU), in Stellenbosch University. In addition, fish meat tissue samples were also analysed to determine whether and how much of the active compounds were incorporated in the carcasses of the fish fed diets containing the Pawpaw and Moringa seed powder.

7.2.2. Pawpaw and Moringa Seed Powder

The processing and preparation of the respective samples (i.e. Pawpaw and Moringa seed powder) analysed in this part of the study is discussed in detail in Chapter 3, section 3.3.1.1 and 3.3.1.2.

7.2.3 Fish Tissues Samples

Two fish were randomly sampled from the treatment groups that were fed 5.0, 10.0 and 15.0 g/kg base diet (BD) of Pawpaw seed powder and Moringa seed powder. Each fish sampled was placed in sealed plastic bag, labelled and stored in a -20°C fridge. Problems were experienced with the cold storage of the samples beyond project control, in that the freezing unit defrosted causing the unrecorded thawing of the samples. Only the head region and the fillets components could therefore, be used for the analysis for the presence of the triterpene acids. Prior to being analysed, each fish was descaled, and the carcass separated into the head and the rest of the body. The skin and internal organs were removed, where after the head and fillet components were ground using a blender (RUSSELL HOBBS, 1000W Satin Blender with 1.5 litre jug capacity). For ease of grinding, 100mL of distilled water was added gradually in the process. Wet samples were freeze dried at -80°C, and stored at -20 ^{o}C until further analysis.

7.2.4 Reagents and Chemicals

The purified forms of oleanolic acid and ursolic acid were obtained from Sigma-Aldrich (Gmbh, D-91625 Schnelldorf, Germany). The other media required for analysis (ethanol, methanol, acetonitrile and chloroform) were also from (Sigma-Aldrich, Pty). Purified water which was filtered from Milipore water purification system was used throughout the experiment.

(a) Preparation of Standard Solutions

A stock solution of oleanolic acid and ursolic acid was prepared in 950 µL methanol and 50 µL dichloromethane for easy and complete dissolution, to obtain 150mg/L or ppm and stored at 4 ^{o}C. Between 6 and 10 sets of the stock solution were prepared beforehand, and used as required.

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Calibration standards (0.0012, 0.0024, 0.012, 0.06, 0.12, 0.06 mg/L) were prepared from the stock solution by serial dilution of methanol (Wang *et al.*, 2008). A standard solution for recovery was prepared through methanol serial dilution with final concentration of 12.0mg/L. From this solution 50 µL with a final concentration of 0.15mg/L after a 10 times concentration of the sample was used to spike the extracting materials for the efficiency of extraction procedure.

(b) Selection of Solvents for Extraction

Factors determining analyte recovery include the affinity of the compound to be analysed for the solvent, the solvent to sample volume ratio, and the number of extraction steps. As this was a first attempt to determine oleanolic acid and ursolic acid in Pawpaw and Moringa seed, and fish tissues, a number of solvents were tested for effective extraction. More so, it is known that oleanolic acid and UA in nature coexist in micro quantities, yields are usually very low (Rajasekaran *et al.*, 1988), and show almost the same solubility in different solvents, depending on the accuracy of the measurements (Schneider *et al.*, 2009). Owing to their similar molecular structure differing only in the position of two methyl groups (see Chapter 2), their extraction and separation always pose a challenge (Cheng *et al.*, 2011).

The following extraction solvents were attempted which included:

a) Acetonitrile (modified from Sharma *et al.*, 1997)
b) Mixture made up of 50% Acetonitrile and 50% Chloroform (modified from Wang *et al.*, 2008)
c) Mixture of 95% Ethanol and 5% Pure water (modified from Xia *et al.*, 2012)
d) Mixture of 50% Acetonitrile, 45 % Methanol and 5% Chloroform (modified from Wang *et al.*, 2008 and Xia *et al.*, 2012)

Each solution was tested twice, for its efficacy as an extraction solvent following modified procedures as used in Sharma *et al.* (1997); Wang *et al.* (2008); and Liang *et al.* (2009). The extractants were analysed following standard procedures as described below, with the solvent consisting of 50% Acetonitrile, 45 % Methanol and 5% Chloroform giving the best recovery rate. This solvent was therefore used for all subsequent extractions.

(c) Selection of the Mobile Phase

To find the optimal elution conditions, two mobile phase systems composed of simple buffers were tested. The buffers were chosen on the basis of conformity and usage with respect to calibration and specifications of mobile phase in CAF-MSU UPLC-MS instrumentation. The first consisted of 0.1% formic acid-acetonitrile (25:75, v/v). This could not separate the standard oleanolic and ursolic acids. Another mobile phase made up of a buffer of 10mM ammonium acetate and 100% methanol at a ratio of 25:75, v/v was tested. The second buffer combination was able to give clear separation of the two triterpene acids (Figure 7.1). Thus the mobile phase used in this experiment for the LC-MS analysis consisted of 10mM ammonium acetate and 100% methanol, at a ratio of 25:75, v/v.

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7.2.5 Extraction Procedure

The dried powder (1.0 g) of the respective specimen (Pawpaw or Moringa seed powder; fish tissues: head region and fillet) was accurately weighed using a RADWAG 2007 scale (Model WLC1/A1, RADWAG®). Each 1.0g of powdered sample was placed in a capped 50mL centrifuge plastic tube and 20mL of extraction solvent (i.e. 50% Acetonitrile, 45 % Methanol and 1% Chloroform, v/v/v) was added at a liquid to material ratio of 20:1 (Xia *et al.*, 2012). The mixture was mixed thoroughly for 50-60 seconds by a Vortex mixer (VORTEX GENIE 2, Model G-560 E, Bohemia, NY, USA). The mixed samples were then transferred to 500mL flat-bottom flask (Erlenmeyer) and immersed in the water bath component of the sonication device (Sonication Bath, Lasec Lab & Scientific Equipment). Sonication was done for 30minutes whereafter the samples were centrifuged at 7500rpm, 4°C for 20 minutes (Eppendorf Centrifuge 5430R, Eppendorf AG 22331, Hamburg, Germany). After extraction and centrifugation, the supernatant was aspirated. The extraction process was repeated to obtain a solution of 35-40 mL before being dried (Wang *et al.*, 2008). For the drying out of the samples about 5-7 mL of the collected supernatant was repeatedly transferred into 14mL centrifuge plastic bottle and exposed to a stream of nitrogen (N_2) gas until completely dried. The dried sample was stored at 4°C, until later analysis. The samples were resuspended in 4mL methanol before UPLC-MS analysis.

7.2.6 UPLC-ESI- MS/MS analysis

Oleanolic acid and ursolic acid were analysed using ultra performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS/MS). The separation was carried out with a Waters ACQUITY Bridged Ethyl Hybrid, (BEH) C18 1.7µm, 2.1 x 100 mm column. OA and UA were separated using an isocratic elution mobile phase consisting of 10 mM ammonium acetate-methanol (25:75, v/v) at a flow rate of 0.38 mL/min and at a column temperature of 50 ^{o}C.

The Xevo TQ (Waters Corporation, Milford, MA, USA) was operated in ESI negative mode and the m/z 455 was monitored (the $[M–H]^-$ ion for both OA and UA). Although oleanolic acid and ursolic acid showed no significant collision-induced dissociation (CID), the MS was still operated in multiple reaction monitoring (MRM) mode, monitoring the transition from m/z 455 but to 455 to minimize non-specific peaks. The cone voltage and collision energy were x and y respectively.

The peak area was used to calculate the amount of oleanolic acid and ursolic acid from the standard curve. Detection of the pure chemicals of oleanolic acid and ursolic acid from the spike samples as well as in the samples was determined by using the analytical software called MassLynx, Version 4.0 (Micromass 2002). The chromatograms of oleanolic acid and ursolic acid in standard solution and in the sample are shown in Figure 7.2 (i and ii).

7.2.7 Recovery

To test the extraction recovery, to the 1.0g dried plant seed powder 50 µL of the standard solution (made up of oleanolic acid and ursolic acid, concentration of 12.0mg/l), was added, before and extraction was done

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through the same procedure as described above (7.2.5). The amount of solvent used for a single extraction was 40ml (which had been concentrated 10 times), so a spiked sample extractant has a concentration of the stocked solution 0.15ppm (or mg/L). Each final extractant dried for analysis was designated as either spiked or non-spiked (e.g. extract from Moringa powder spiked with pure chemical was designated as Spiked Moringa, MS and vice versa- Moringa Non-Spiked, MNS).

Therefore, for every sample there were two sets of solutions, spiked (S) and non-spiked (NS). Out of these, UPLC-MS analysis was conducted and data evaluated using the MassLynx Version 4.0 software (Micromass, 2002). UPLC-MS analyses were accomplished as detailed above.

The recovery of oleanolic acid and ursolic was determined as follows

$$\text{Recovery (\%)} = \frac{(A - B)}{C} \times 100\%$$

Where, A is the result after addition, B is the amount of sample without adding standards, C is the added amount of the standards.

Recovery was studied in three replicates of the samples (both spiked and non-spiked), and the average or mean values ± SD were recorded (i.e. standard deviation, as recovery or relative standard deviation, RSD). The results are presented as a chromatogram and in tables.

7.2.8 Quantification of Oleanolic Acid and Linearity in Moringa Seed Powder

Quantification

The content was computed from the relation below.

$$\text{Amount of Chemical in Sample } (\mu g/g) = \frac{C \text{ (ppm)}}{(1g)} \times \frac{(40ml \text{ solvent})}{(10 \times \text{concentration})}$$

Where C is concentration of injected sample (non-spiked)

Linearity

A calibration standard of 0.0012, 0.0024, 0.012, 0.06, 0.12, 0.06 mg/mL were prepared for oleanolic acid from the stock solution by the serial dilution of methanol and corresponding peak area from the chromatograph generated. Using Microsoft Excel (Office 2010), these values were used to construct calibration curve in the range of 0.0012–0.6 (ppm or mg/ mL) for oleanolic acid. A regression curve was used to calibrate the linearity relationship between the peak area of the chromatograph and concentration of oleanolic acid estimation. The calibration curve was prepared by determining the best fit of the peak area ratio against concentration (Devaraj *et al.*, 2011).

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7.3. Results

7.3.1 Separation of Oleanolic acid and Ursolic acid in Standard solution

Oleanolic acid and ursolic acid mixed in the standard solution could be successfully separated using the mobile phase 10mM ammonium acetate as solvent A and 100% methanol as solvent B at a ratio of 25:75 (v/v) from the standard solution. The chromatograph of oleanolic acid and ursolic acid is shown in Figure 7.1, and the separation of the two compounds was approaching the baseline (Wang *et al.*, 2008).

Retention time for oleanolic acid and ursolic acid was 7.0min and 7.4min respectively. Both compounds were detected as a quasi-molecular ion peak at *m/z* 455 $[M-H]^-$, with oleanolic acid being represented by the higher peak, and ursolic acid the slower peak.

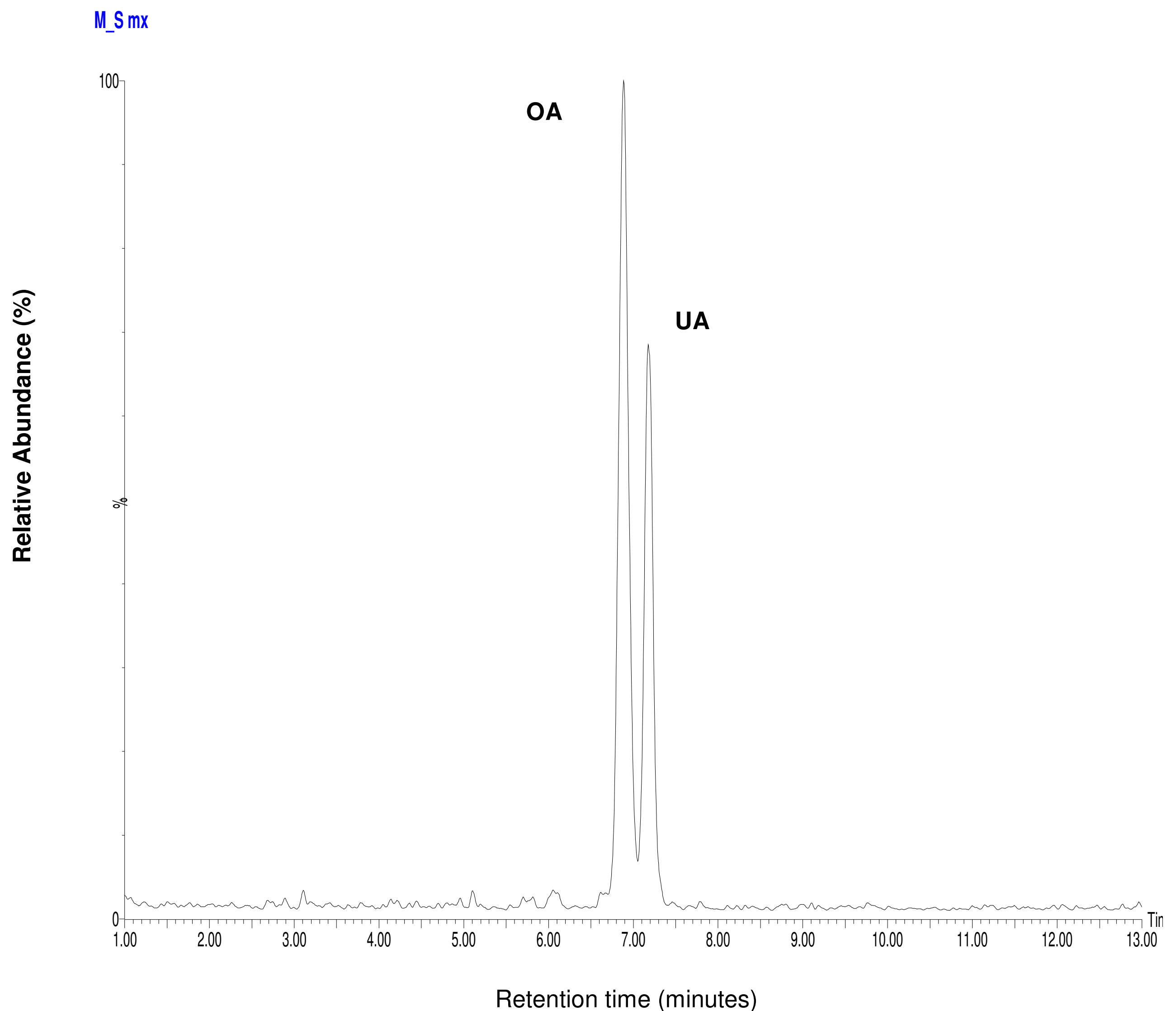

Figure 7.1 Chromatogram of oleanolic acid and ursolic acid in standard solution OA, oleanolic acid; UA, ursolic acid

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7.3.2 Recovery of Oleanolic acid and Ursolic acid in the Spike Specimens

Recovery of oleanolic acid and ursolic acid in the spiked samples was obtained in both the Moringa and Pawpaw seed powder but not in the fish tissues. The recovery of oleanolic acid and ursolic acid in the spiked Moringa seed sample is presented in Tables 7.1 and 2.2. Mean recovery of oleanolic acid and ursolic acid in Moringa seed was 96.467% (n=3); %RSD = 9.8 and 90.3% (n=3) %RSD = 5.7 respectively.

Table 7.1 UPLC Instrument detection of concentration for Oleanolic Acid Recovery based on Spiked (0.15mg/L of standard solution) and Non-Spiked Samples of Moringa Seed Powder.

Quantity Injected (µg/l)	Non Spikes (NS ppm)	Spikes (S+0.15 ppm)	% Recovery µg/g	% RSD
0.124	0.133	0.272	92.600	
0.119	0.129	0.264	89.600	
0.127	0.118	0.279	107.200	
		Mean (± s.d) (n=3)	96.467 ± 9.416	9.760

Table 7.2 UPLC Instrument detection of concentration for Ursolic Acid Recovery based on Spiked (0.15mg/L of standard solution) and Non-Spiked Samples of Moringa Seed Powder.

Quantity Injected (µg/l)	Non Spikes (NS ppm)	Spikes (S+0.15 ppm)	% Recovery µg/g	% RSD
0.123	-	0.143	95.500	
0.127	-	0.128	90.200	
0.124	-	0.135	85.200	
		Mean (±s.d) (n=3)	90.300±5.151	5.704

7.3.3 Detection and Quantification of Oleanolic Acid in Moringa Seed

From the UPLC analysis only oleanolic acid was detected in the non-spiked Moringa seed powder (MNS) but not in the other non - spiked samples (i.e. Pawpaw seed or fish tissues). UA was not detected or found in any of the non-spiked samples (i.e. Pawpaw seed, Moringa seed or fish tissues). Results obtained are shown Table 7.3. The average content of oleanolic acid in Moringa Seed Powder was 0.508µg/g ±0.032. The chromatograph of oleanolic acid from the Moringa seed is shown in Figure 7.2.

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Table 7.3 UPLC Instrument detection of concentration Oleanolic Acid in Moringa Seed Powder based on Non-Spiked Samples

Quantity Injected (µg/l)	Non Spikes (NS ppm)	Recovery µg/g
0.127	0.118	0.472
0.124	0.133	0.533
0.119	0.129	0.517
	Average (n=3)	0.508 ± 0.032

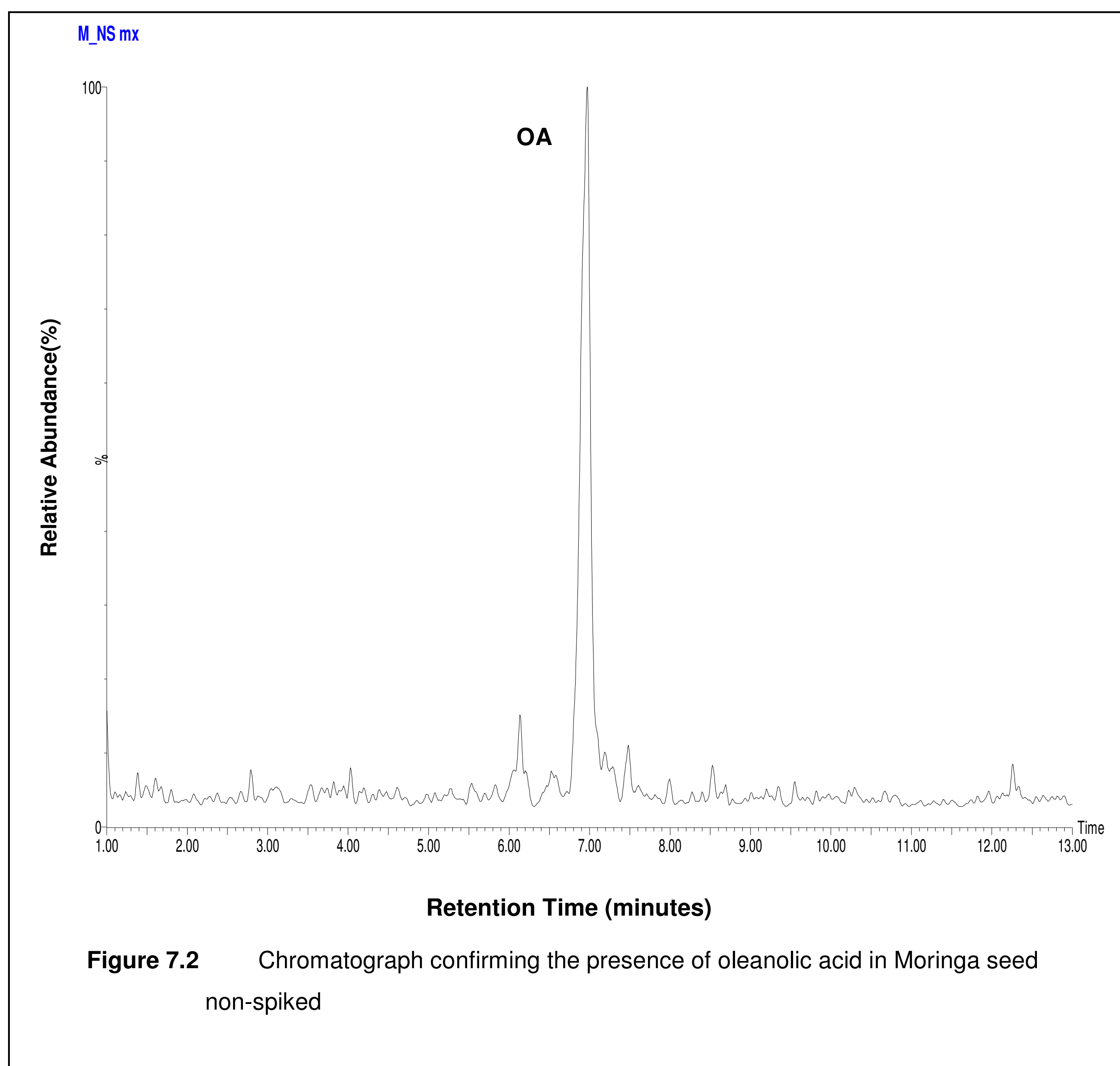

Figure 7.2 Chromatograph confirming the presence of oleanolic acid in Moringa seed non-spiked

7.3.4 Linearity of Oleanolic acid to Detection in Moringa Seed

The relationship between the estimated amount and detection is presented in Table 7.4 and Figure 7.3. The calculated regression equations between the peak area (y) against concentration (x) and their coefficients of determination (R^2) were as follows: oleanolic acid, y= -1074.5 x^2 + 5760.9 x + 3.5 and (R^2 = 0.9993).

Table 7.3 TargetLynx Calibrated concentrations of oleanolic acid expected in Moringa Seed

Concentration(ppm), (x)	Detection Peak area of OA (y)
0.0012	12.1
0.0024	19.3
0.012	92.9
0.06	402.1
0.12	644.8
0.60	3074.0

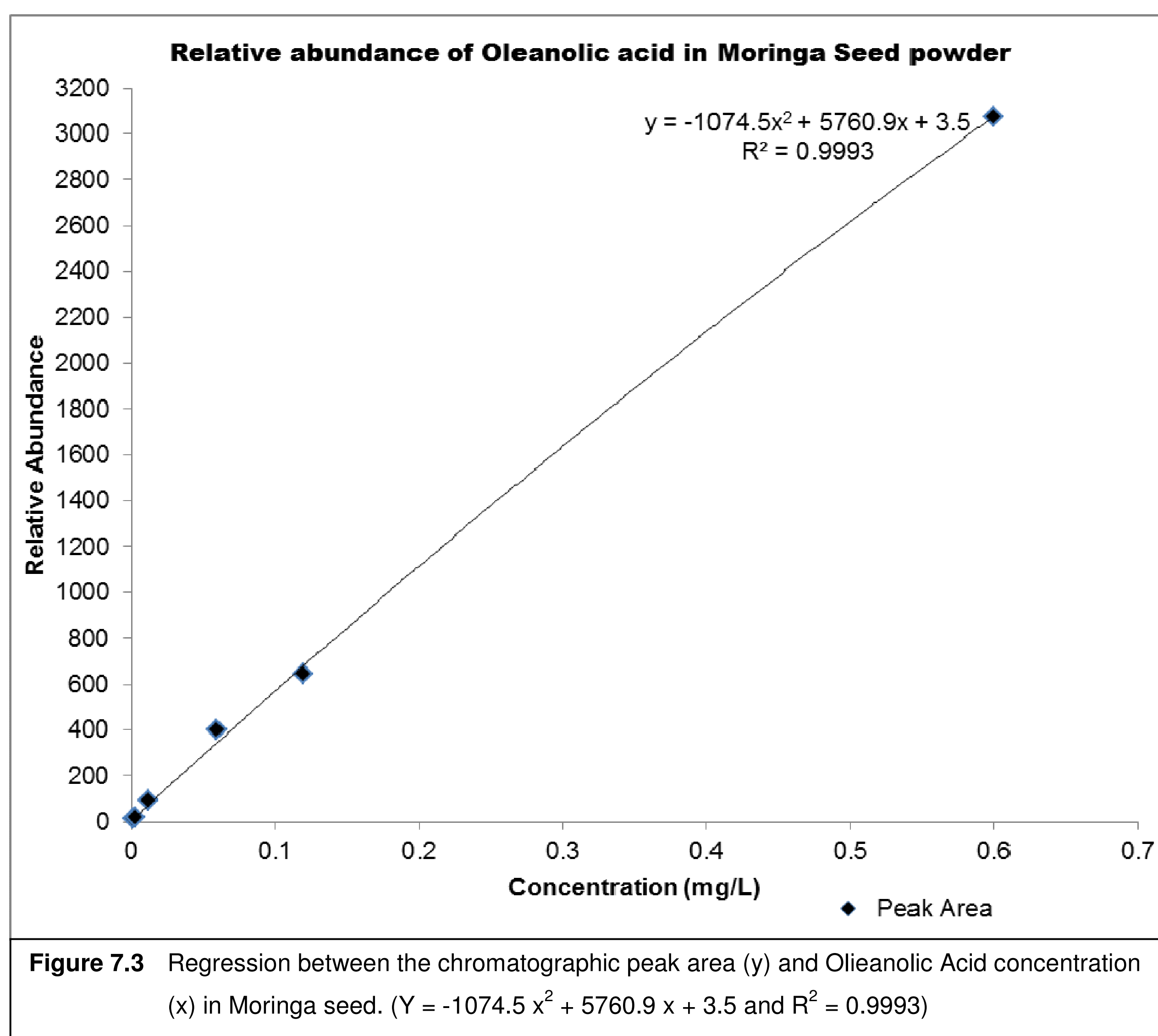

Figure 7.3 Regression between the chromatographic peak area (y) and Olieanolic Acid concentration (x) in Moringa seed. (Y = -1074.5 x^2 + 5760.9 x + 3.5 and R^2 = 0.9993)

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7.4 Discussion

7.4.1 Recovery

Oleanolic acid and ursolic acid are isomeric triterpenic acids with similar chemical structure, that commonly existing in the same plant. Therefore, it is very difficult to separate them when determining a complex mixture with high performance liquid chromatography (HPLC). However, an ultra-performance liquid chromatography (UPLC), which runs on chromatographic principles designed to have a column packed with very small particles with higher flow rates for increased speed, with superior sensitivity and high resolution enables rapid separation and quantification (Swartz, 2005; Zhang *et al.*, 2012). Using an UPLC-ESI-MS/MS, oleanolic acid and ursolic acid could be recovered and separated when added to a plant extract. In this study, the mean recovery (MR) of oleanolic acid was 96.5% (n=3) with relative standard deviations (%RSD) of 9.8, whiles that of ursolic acid was 90.3% (n=3) and %RSD was 5.7 was achieved. The limit of detection (LOD) was similar at 0.0006ppm (0.6ppb).

Studies have shown that several factors determine the effective recovery of a compound of a complex mixture and such factors may include the affinity of the compound for the solvent, the solvent to sample volume ratio, and type and concentration of extracting solvent (Liang *et al.*, 2009; Tiwari *et al.* 2011; Xia *et al.*, 2012; Xu *et al.*., 2012).The recovery of the spiked oleanolic acid and ursolic acid from the samples show a clear affinity between the solvents used and the respective compounds, with ≥90% recovery.

7.4.2 Separation of Oleanolic Acid and Ursolic Acid in Standard Solution

Oleanolic acid and ursolic acids were conveniently separated using the mobile phase 10mM ammonium acetate as solution [A] and 100% methanol as solution [B] in a ratio of 25:75, (v/v), from the standard solution using UPLC-ESI-MS/MS, on retention time of 7.0min, and 7.4min for O LEANOLIC ACID and ursolic acid respectively.

Several forms of HPLC methods such as reversed-phase high performance liquid chromatography (RP-HPLC) and HPLC in tandem with mass spectrometry (HPLC- MS/MS) have been used to separate oleanolic acid and ursolic acid in complex mixtures found in medicinal plants and herbs. However, the retention time tends to be long. For instance using RP-HPLC, Wang *et al.* (2008) reported a retention time between 10 and 20 minutes for oleanolic acid and UA respectively. In the study of Xu *et al.*, (2012), retention time for oleanolic acid was 20.58 minutes, whiles that reported for UA was 21.57 minutes. Liang *et al.*, (2009) used HPLC, with a mobile phase made up of methanol-0.2% ammonium acetate in water (83:17), to completely separate oleanolic acid and ursolic acid from the herb O*ldenlandia diffusa,* at a retention time between 25 and 30. In all of these studies oleanolic acid appeared earlier on the chromatogram than the ursolic acid. In this study the retention time for oleanolic acid was 7.0 minutes, whiles that for ursolic acid was 7.4 minutes. Thus, from the chromatograph (Figure 7.1), in this study, oleanolic acid is the one with the higher peak, and ursolic acid the shortest. This therefore indicates that oleanolic acid and ursolic acid can be separated.

Using the mobile phase 10mM ammonium acetate as A and 100% methanol as B in a ratio of (25:75, v/v), a complete separation of oleanolic acid and ursolic acid was successfully achieved (Figure 7.1), from the standard solution. Liang *et al.* (2009) satisfactorily obtained a chromatographic separation of oleanolilc acid and ursolic acid using a mobile phase of methanol-0.2% ammonium acetate in water (83:17), confirming that the mobile phase selected was adequate to separate oleanolic acid and ursolic acid.

Oleanolic acid and ursolic acid are isomeric compounds with similar chemical structure and therefore difficult to separate. Makarov *et al.* (2009) reported that using, UPLHC with an ACQUITY BEH C18 (1.7µm, 2.1 x 100mm) columns with isocratic mobile phase, at temperature of 45°C, they could separate 3 structurally related nitramine compounds. Similarly, Zhang *et al.*, (2012) used ultra-high performance liquid chromatography / quadrupole time-of-flight mass spectrometry (UHPLC/QTOF-MS) with an ACQUITY BEH C18 (1.7µm, 2.1 x 100mm) columns with isocratic mobile phase to identify flavonols and completely separate complex triterpene glycosides in *Luo-Han-Guo* extract, using a mobile phase consisting of (A) 0.1% formic acid in water and (B) acetonitrile. The authors also stated that triterpene glucosides could be fully ionized in negative ESI mode, thus enhancing their separation. This indicates that compounds with similar chemical structures that could be ionized may be separated. The separation column for this study was Waters ACQUITY BEH C18 (1.7µm, 2.1 x 100mm), with isocratic mobile phase which could separate the two isomeric triterpene acids. Thus, under the right conditions UPLC-ESI-MS/MS is capable of separating isometric compounds like oleanolic acid and ursolic acid, and be clearly identified.

7.4.3 Detection of Oleanolic Acid and Ursolic Acid in the Specimens

According to Tiwari *et al.* (2011) the type of solvent and extraction procedure influences the successful determination of biologically active compounds from plant materials. Some of the merits of a proper plant solvent include the ease of evaporation at low heat, and promotion of rapid physiological absorption of the extract. Xia *et al.* (2012) found that maximum extraction of oleanolic acid and ursolic acid could be achieved from plants using a mixed solvent of 95% ethanol and 5% water at a liquid to solid ratio of 20:1.

In this study a mixture of 50% acetonitrile, 45 % methanol and 1% chloroform, as extracting solvent was used in liquid to solid ratio of 20:1(v/v) The use of combinations of solvents conforms to studies by Liang *et al.* (2009), Tiwari *et al.* (2011), Xia *et al.* (2012) and Xu *et al.* (2012). The triterpenic acids, oleanolic acid and ursolic acid often exist simultaneously in the same plants. From three sets of samples of all the specimen (extracts of Moringa, Pawpaw seed powder, Fish tissues) were subjected to the same test described above. The chromatogram (Figure 7.1 & 7.2) obtained were used to identify oleanolic acid and ursolic acid using the retention times and peak height in the two figures. In Figure 1, two peaks were observed with oleanolic acid at 7.0 minutes, whiles ursolic acid was 7.4 minutes. In Figure 7.2 only one peak was observed which came out of the Moringa extract. By comparison of retention time and chromatographic peak height, with that of the standard it was authenticated that oleanolic acid was present in Moringa seed powder, but not in the other specimen (i. e. Pawpaw seed, fish tissues). Thus the presence of oleanolic acid could only be detected in the Moringa seed powder.

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According to Swartz (2005), UPLC technology is an improved form of HPLC, to provide increased resolution, speed and sensitivity to accurately detect micro-contents of complex matrix efficiently. In this study only oleanolic acid was detected in the non-spike Moringa seed with a clear distinct chromatographic peak (Figure 7.2). Ursolic acid could not give a distinctive chromatographic peak as seen in Figure 7.2. The over 90% of recovery for oleanolic acid and ursolic acid from the spiked samples, clearly demonstrates the efficiency of the extraction procedure and solvent. Several investigators such as Liang *et al.* (2009); Tiwari *et al.* (2011); Xia *et al.* (2012) and Xu *et al.* (2012) have used UPLC to successfully separate and quantify oleanolic acid and ursolic acid in plant samples.

The chromatograph in Figure 7.2 showed a wave at the base which demonstrates the presence of some bioactive compounds but in small quantities. Non detection of UA in Moringa could mean that it was present but in small quantity. Pollier and Goosens (2012) have reported that oleanolic acid occurs both as a free acid and as an aglycone precursor for triterpenoid saponins in which it is linked to one or more sugar chains in plants. It also accumulates at very low concentrations in plants, this could explain the reason why both oleanolic acid and ursolic acid were not detected in Pawpaw. The absence of oleanolic acid and ursolic acid in Pawpaw seed powder could also mean they were present in their derivatives such as oleanolic glycoside (Das, 1980), oleanolic acid-3 β-glucoside and oleanolic acid 3-β-D-glucuronide (Das *et al.*, 2011).

7.4.4 Quantification of Oleanolic Acid and Linearity in Moringa Seed Powder

The average content of Oleanolic acid in Moringa Seed Powder obtained was 0.5079µg/g ±0.032, (n=3), with a correlation coefficient of linear regression analysis (i.e. coefficient of determination, r^2) of 0.9993. It has been established that the Moringa plant parts contain pyhtoestrogens including β-sitosterol, caffeoylquinic acid, quercetin and kaempferol (Anwar *et al.*, 2007; Amaglo *et al.,* 2010; Devaraj *et al.*, 2011); saponin in the seed powder (Kawo *et al.*, 2009); steroids, triterpenoids, flavonoids, saponins, and anthraquinones in the leaves (Kasole *et al.,* 2010); steroids, triterponoids, saponins in the bark (Lambole and Kumar (2011), and saponins, flavonoids and steroids in the leaves (Bamishaiye *et al.*, 2011).

7.5 Conclusion

The extraction method adopted followed standard procedure, however, ursolic acid could not be quantified in Moringa. As to why both oleanolic acid and ursolic acid could not be recovered in Pawpaw seed can also not be explained. The plausible explanation is that triterpines usually are present in plants in micounits and also exist in their glycoside forms. New extraction procedures need to be explored in future studies, since establishing their existence is critical to their usage. It could be concluded from available literature that the findings from this study is the first report of the identification of oleanolic acid in Moringa seeds indicating that oleanolic acid is present in Moringa seeds. The presence of oleanolic acid in Moringa seeds adds more credence to its potential use as antifertility agent, which could be exploited in the control of tilapia precocious breeding. Further studies need to be conducted on Moringa seed from other parts of Sub-Saharan Africa (other than Zambia) to find out the extent of oleanolic acid and ursolic acid composition.

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Chapter 8

Conclusions and recommendations

Tilapia production plays an important role in global food security, in particular amongst the rural communities of developing countries. The viability of commercial and small-scale Mozambique tilapia (*Oreochromis mossambicus*) culture is complicated by an early onset of sexual maturity, which results in uncontrolled reproduction, overpopulation and retarded growth in aquaculture systems. Various techniques have been developed to control the precocious breeding pattern of tilapia in culture systems, but they all have drawbacks. For example the most effective techniques (hormonal sex reversal) is not suitable for use by rural small scale farmers due to its complexity and limited access to the required resources and substances, e.g. steroid hormones and alcohol.

Natural reproduction inhibitors (phytochemicals) found in traditional medicinal plants such as Pawpaw (*Carica papaya*) and Moringa (*Moringa oleifera*), provide potential alternative for the control of sexual differentiation in fish. Previous studies have indicated that phytochemicals that behave like natural steroid-like substances, particularly with an estrogen-like biological activity, occur in these plants. Such phytochemicals are referred to as phytoestrogens, and can be defined as substances or metabolites that induce biological responses in vertebrates by mimicking or modulating the actions of endogenous oestrogens (such as 17β-estradiol), usually by binding to estrogen receptors.

The hypothesis was set that a diet containing natural phytogenic compounds will affect the reproductive potential of Mozambique tilapia (*Oreochromis mossambicus*; MT) in aquaculture systems. The study therefore investigated the potential of Pawpaw seed meal (P) and Moringa seed meal (M), as part of a commercial tilapia diet, to be used as endocrine disrupting compounds (EDC's) to control the reproduction of sexually mature Mozambique tilapia (MT; 20-45g), to inhibit the attainment of sexual maturity in immature MT (2-8g), and to influence sexual differentiation of MT fry (9-12 days post-hatch) to produce all-male populations.

The effect of PSM and MSM as endocrine disrupting compounds to control reproduction in sexually mature Mozambique tilapia (20-45g)

This first part of the study investigated the effect of PSM and MSM included at levels of 0.5, 1.0, 5.0, 10.0 and 15.0g/kg of a commercial tilapia feed (BD), on the gonad integrity and function of sexually mature Mozambique tilapia. A total of 550 fish were allowed to acclimatize for 7 days, and were then fed the respective treatment diets for a period of 60 days. Histological examination of gonad tissue indicated that diets containing levels of 5.0, 10.0, & 15.0 g/kg BD of Pawpaw and Moringa respectively, severely affected gonad integrity. The most pronounced effects observed in the male fish included degeneration of the seminiferous tubules, disintegration of the spermatozoa, and the presence of testes-ova. In the female fish, atretic oocytes and depletion of yolk particles were observed.

The synergistic effect of PSM and MSM to control reproduction in sexually mature Mozambique tilapia (20-45g)

This part of the study investigated the potential interaction of the phytochemicals present in the Pawpaw and Moringa seeds in affecting gonad integrity and function of sexually mature Mozambique tilapia. A total of 150 fish were allowed to acclimatize for 7 days, and were then fed the respective treatment diets for a period of 60 days. The combination of Pawpaw and Moringa did not prove to have a more pronounced effect on gonad integrity than what was observed when the individual experimental compounds were included in the respective experimental diets.

The effect of PSM and MSM as endocrine disrupting compounds to inhibit the attainment of sexual maturity in sexually immature Mozambique tilapia (2-8g)

The second part of the study investigated the potential of Pawpaw and Moringa included at levels of 0.5, 1.0, 2.0, 5.0, 10.0g/kg of a commercial tilapia feed (BD), to inhibit sexually immature Mozambique tilapia te attain sexual maturity. A total of 1650 fish were acclimatized for 7 days, and were then fed the respective treatment diets for a period of 60 days. Eggs were observed in the ovaries of the sexually immature fish, but spawning did not occur. Testis integrity and function was also affected, and degeneration of the gonad tissue was observed. Ova-testes were observed in fish that received 10.0g of Pawpaw and/or Moringa/kg basal diet. Both Pawpaw and Moringa also were effective in skewing the sex ratio to yield 71% male and 29% female fish respectively.

The effect of PSM and MSM as endocrine disrupting compounds to influence sexual differentiation in Mozambique tilapia fry (9-12 days post-hatch)

This component of the study investigated the effect of Pawpaw and Moringa included at a levels of 15g/kg of a commercial tilapia feed (basal diet, BD), on the sexual differentiation of Mozambique tilapia fry. A total of 2400 fish were acclimatized for 7 days, and were then fed the respective treatment diets for a period of 60 days.

The inclusion of the crude form of Pawpaw and Moringa seeds believed to contain phytogenic chemicals such as genistein, quercetin, kaempferol, diadzein and, β-sisterol, resulted in significant degree of masculinization (up to 65%) in a mixed sex population of Mozambique tilapia fry. It can thus be concluded that the sexual differentiation of the fry could be successfully manipulated to obtain populations with a predominant male gender.

Biochemical analysis of the phytogenic components of Pawpaw and Moringa

The standard method of extraction allowed for the isolation of only oleanolic acid, but not ursolic acid in Moringa. The reason for the isolation of oleanolic acid and ursolic acid could not be recovered in Pawpaw seed can also not be explained. The plausible explanation is the triterpenes usually are present in plants in microunits and also exist in their glycoside forms, which can make extraction difficult. New extraction

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procedure needs to be explored in future studies, since establishing their existence is critical to their usage. It could be concluded from available literature that the findings from this study are the first report of the identification of oleanolic acid in Moringa seeds. This study indicated that oleanolic acid is present in Moringa seed. The presence of oleanolic acid in Moringa seeds add more credence to its viability as antifertility agent, hence could be exploited in the control of tilapia precocious breeding. Further studies need to be conducted on Moringa seed from other parts of Sub-Saharan Africa (other than Zambia) to find out the extent of oleanolic acid and ursolic acid composition. This needs to be investigated to ensure that the availability of the plant seeds is not a limiting factor for its potential use to control tilapia breeding.

Summary

Pawpaw and Moringa seeds can be considered as a possible alternative to induce sex reversal in mixed sex tilapia populations. This could be of particular importance to aquaculture development in rural areas of Sub-Saharan African countries, given the abundant year round availability of these compounds. The study indicated that both Pawpaw and Moringa seeds contain bio-active chemicals that are capable of disrupting the gonad function, differentiation and sexual maturation of Mozambique tilapia. Oleanolic acid was successfully isolated in Moringa seeds, and this study is the first report on the successful isolation in Moringa seeds. It has also been proven to have antifertility effects in vertebrates.

It was proved that both Pawpaw and Moringa seeds have antifertility properties that can be exploited to control or prevent reproduction of Mozambique tilapia in aquaculture systems. Sperm production was affected, as evident in the degeneration of the testicular tissue samples. Egg production, ovulation and spawning were all affected, as evident in the difference in colour of the degrading eggs, as well as the absence of spawning. Ovo-testes were observed in cases where diets containing 10.0g Pawpaw and/or Moringa/kg basal diet were fed.

Further studies are required to optimise the preparation of the experimental compounds; to determine the optimal age at which to commence administration of the experimental compounds; to determine the optimal duration of administration; to determine the optimal inclusion levels and potential interaction with environmental factors such as water temperature and pH.

Further studies are, however, required to understand the exact mechanism through which the Pawpaw and Moringa seeds exert their effect. It is possible that the results observed in the present study can be ascribed to the action of not just one but multiple phytogenic compounds present in both the Pawpaw and Moringa seeds. The biochemical composition of Pawpaw and Moringa seeds needs to be studied to determine the different phytogenic compounds present in these seeds, as well as the relative concentrations with regards to each other.

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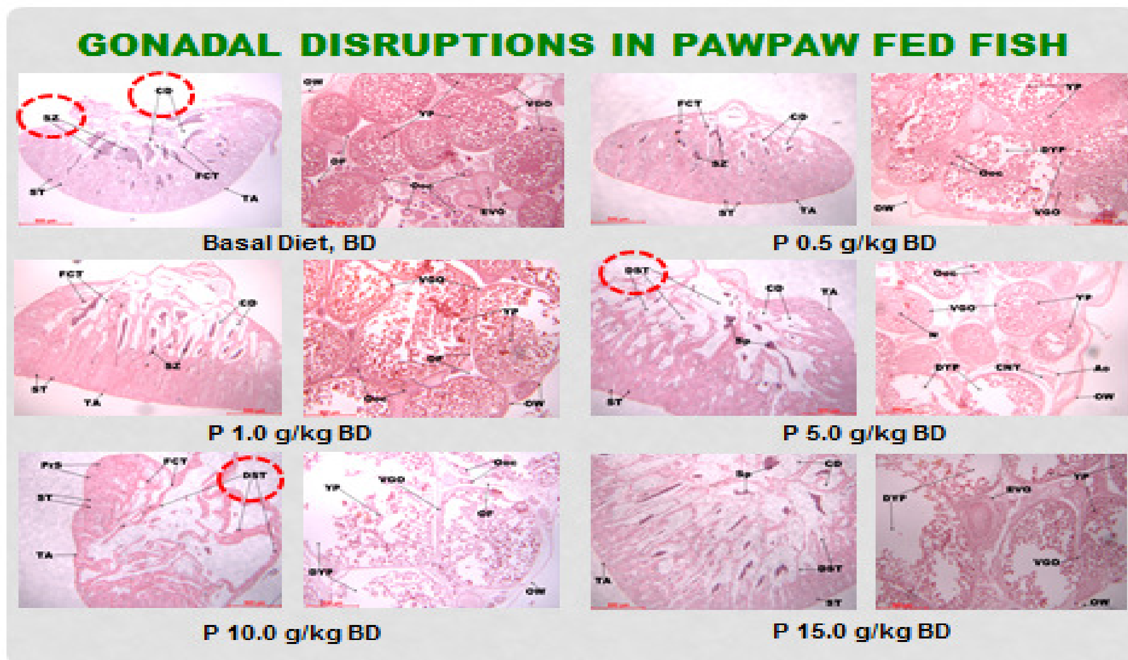
Additional studies need to determine the optimal inclusion levels of the phytogenic compounds, as well as how their efficacy to manipulate the reproductive potential and ability of Mozambique tilapia are influenced by environmental factors such as water temperature.

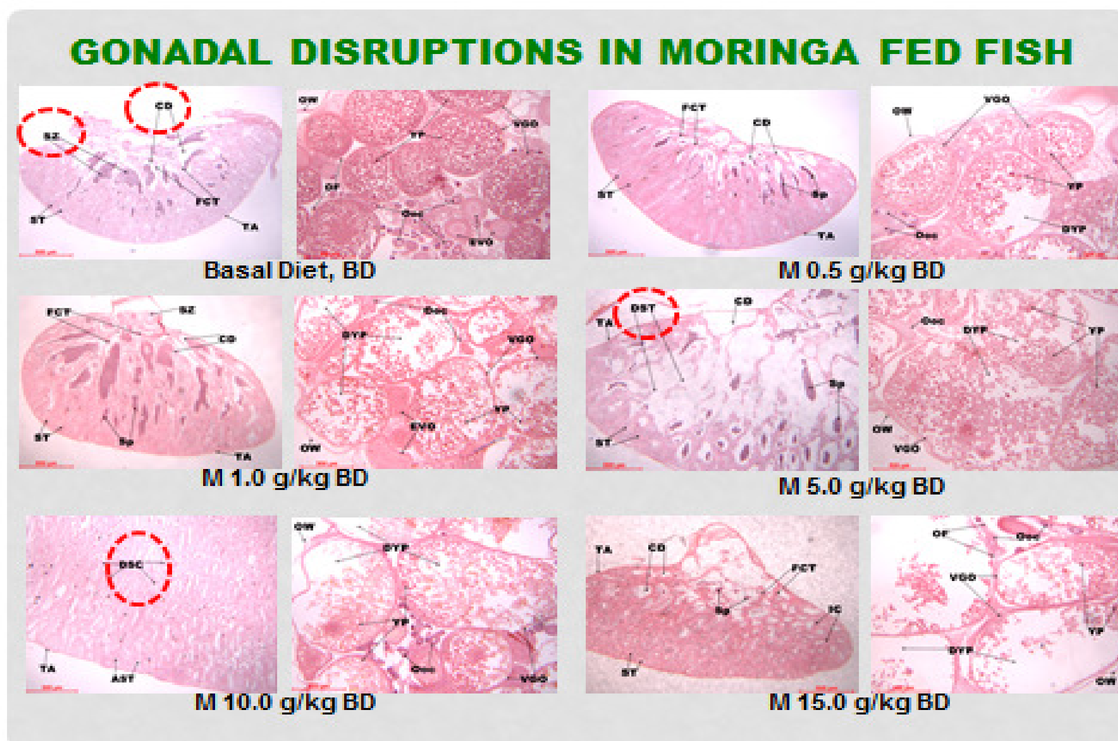
Eggs were observed in the ovaries of sexually immature fish, but spawning did not occur. Future studies should investigate the influence of the EDCs present in Pawpaw and Moringa seeds on egg formation, maturation and eventual release. The potential interaction of the phytochemicals present in the Pawpaw and Moringa seeds with environmental factors to influence egg production and ovulation, and sperm production also warrant further investigation.

APPENDICES

A] Pictorial information of gonadal integrity (in sexually mature and immature Mozambique tilapia, *Oreochromis mossambicus,* fed Pawpaw and Moringa seed powder meal and different Inclusion levels)

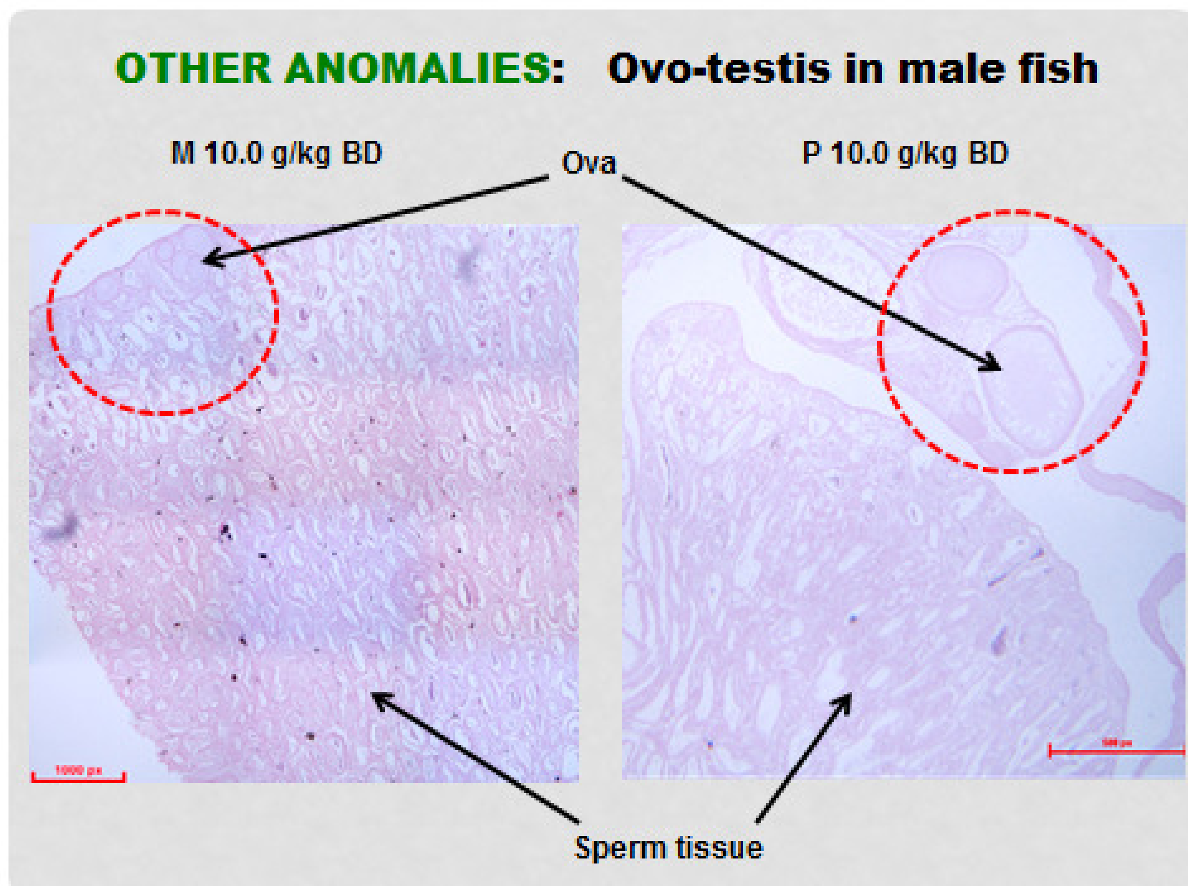
(i) Gonad integrity in sexually mature *Oreochromis mossambicus*





Key

AST = atrophied seminiferous tubules;
CD = collecting duct**;**
DSC = disintegrating sperm cells;
DST = degenerating seminiferous tubules;
DYP = depleting yolk particles;
EVO = early developing oocytes;
FCT = fibrous connective tissue;
OF = ovigerous follicle;
Ooc = developing oocytes;
OW = ovarian wall;
PrS = Primary spermatogonia;
Sp = spermatids;
ST = seminiferous tubules;
SZ = spermatozoa;
TA = tunica albuginea;
VGO = vitellogenic oocytes;
YP = yolk particles.
Bar = 500µm



(ii) Gonad integrity in sexually immature *Oreochromis mossambicus*

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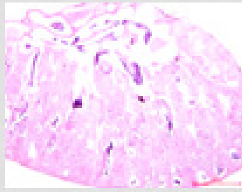
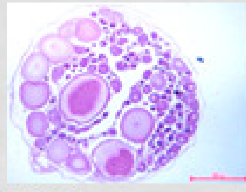
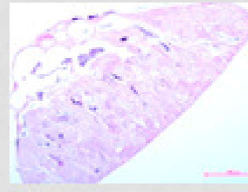
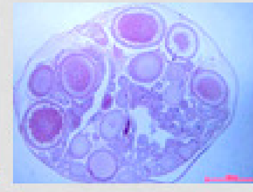
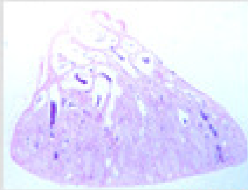
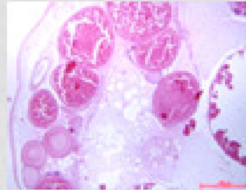
GONADAL DISRUPTIONS IN PAWPAW FED FISH

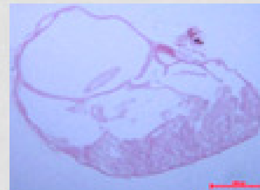
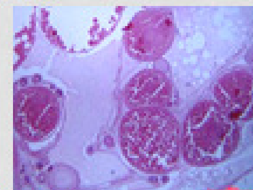
Basal Diet, BD

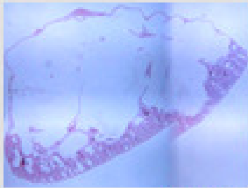
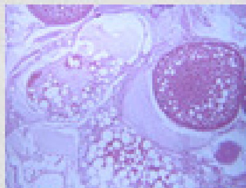
P 0.5 g/kg BD

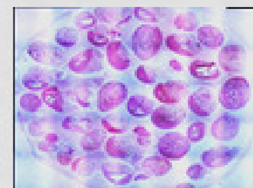
P 1.0 g/kg BD

P 2.0 g/kg BD

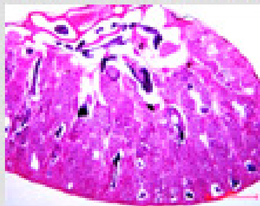
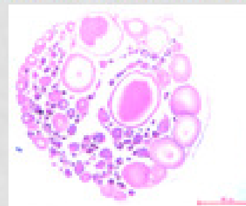



P 5.0 g/kg BD

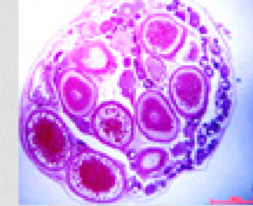


P 10.0 g/kg BD

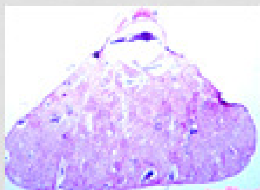
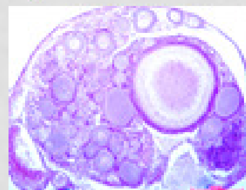
GONADAL DISRUPTIONS IN MORINGA FED FISH

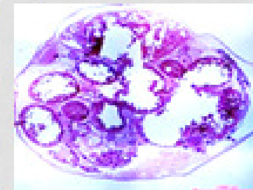
Basal Diet, BD



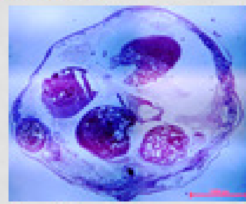
M 0.5 g/kg BD

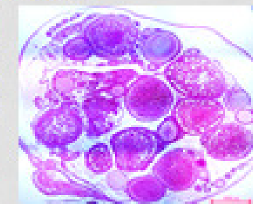
M 1.0 g/kg BD



M 2.0 g/kg BD



M 5.0 g/kg BD



M 10.0 g/kg BD

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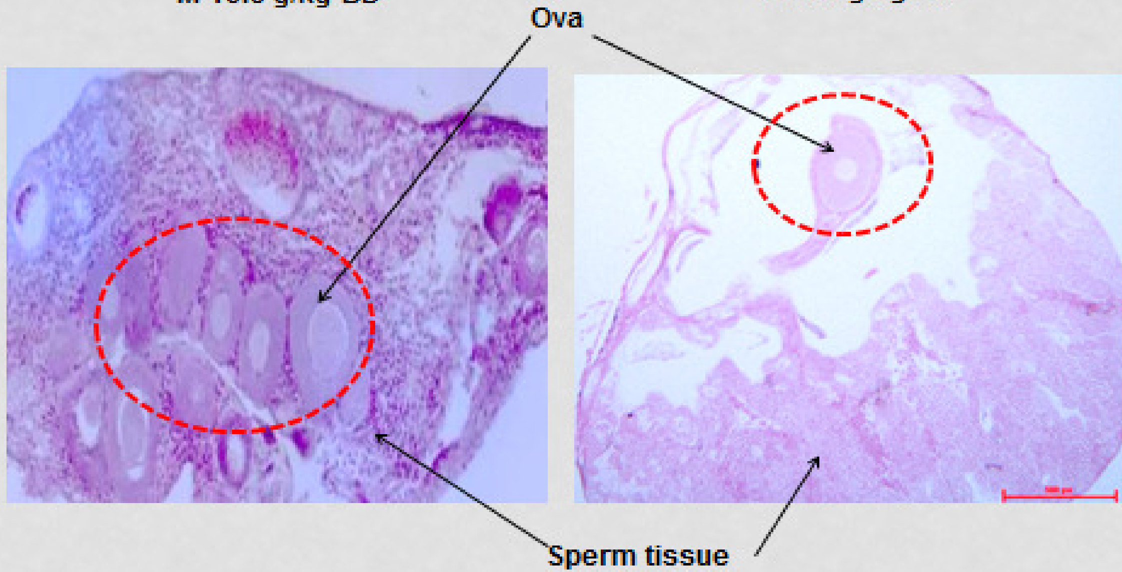
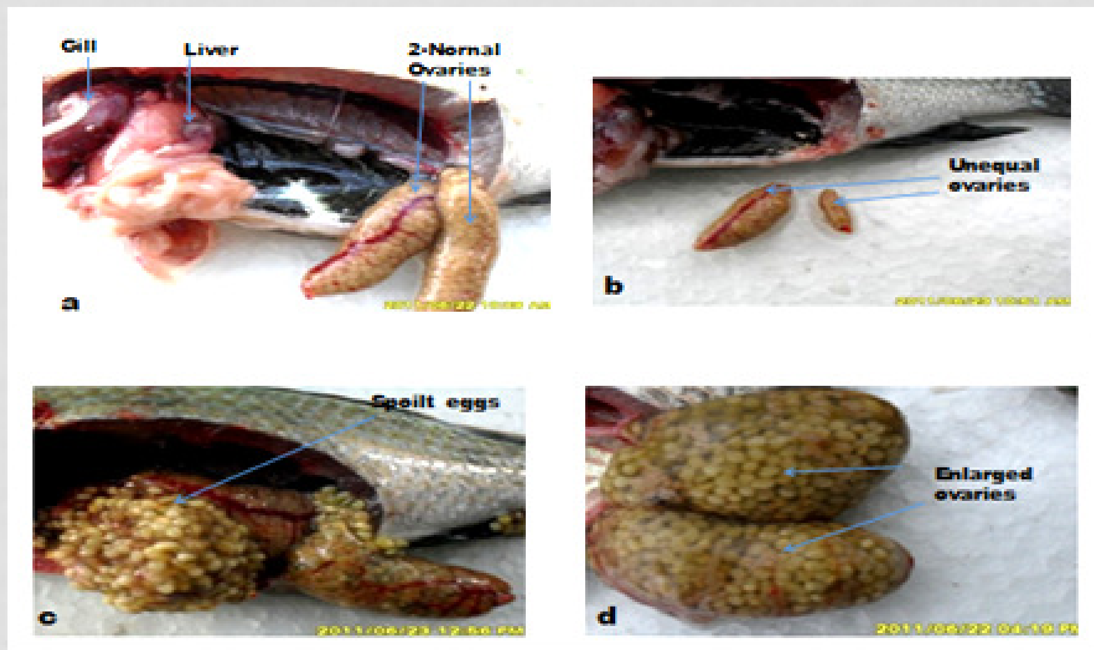
OTHER ANOMALIES: Ovo-testis in male fish



INFLUENCE OF PAWPAW AND MORINGA ON GONAD DEVELOPMENT



a) Normal ovaries, similar in size and shape, b) unequal sized gonads, c) enlarged ovaries with degenerated or deformed eggs (abnormal colour), and d) enlarged ovaries

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B] List of Chemicals used in the experiment

1. 17-α methyltestosterone
2. Alcohol (70-100%, absolute)
3. Bouin's solution (acetic acid 5%, formaldehyde 9%, picric acid 0.9%)
4. DPX mountant (a mixture of distyrene, plasticizer, and xylene)
5. Purified forms of oleanolic acid and ursolic acid
6. Ethanol
7. Methanol
8. Acetonitrile
9. Chloroform
10. Dichloromethane
11. Formic acid
12. Ammonium acetate

C] Histological preparation of slides (as used in the Laboratory of Histology Department, Faculty of Health Sciences, Tygerberg Campus, Stellenbosch University)

i) Tissue processing, using Shanddon Elliot Duplex Processor

The tissue passes through the following chemicals for 17 24 hours

1. 10% Formalin	(7-10 hours)
2. 70% Alcohol	(2 hours)
3. 96% Alcohol	(1.5 hours)
4. 96% Alcohol	(1.5 hours)
5. 99% Alcohol	(1 hour)
6. Absolute Alcohol	(1 hour)
7. Absolute Alcohol	(1 hour)
8. Absolute Alcohol	(1 hour)
9. Xylene	(1.5 hours)
10. Xylene	(1 hour)
11. Wax I	(1 hour)
12. Wax II	(1 hour)

ii) Embedding (instrument: LEICA EG 1160)

The tissues were embedded in paraffin wax at 60 ^{o}C, in an embedding instrument

iii) Sectioning cutting (instrument Microtome, Model, Bright 5040)

Using a microtome, 5μm -8μm thickness sections were cut.

All tissues were oriented on the wax block in such a way to produce transverse sections (TS).

However, few tissues of the testis were cut in longitudinal sections due to the size of the organ.

For each specimen at least 3 sections were placed on a glass slide in warm water.

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iv) Staining procedure (LEICA AUTO STAINER, XL)

All the slides were stained for 25 minutes using with Ehrlich's haematoxylin and counterstained with eosin, through the following steps: Haemtoxylin and Eosin (H&E) staining procedure

STEP 1 **Incubation**

a) Incubate the slides for (at least 60 minutes) in an autoclave with a constant temperature of 60 ^{o}C

STEP 2 **Hydration**

a) Submerged the slides in Xylene-solution for 2 minutes (repeat 2x)

b) Submerged the slides in pure alcohol (100%) for 1 minute (repeat 2x)

c) Submerged the slides 96% alcohol for 1 minute (repeat 2x)

d) Submerged the slides 70% alcohol for 1 minute (repeat 2x)

e) Wash with distilled water (dH_2O) for 2 minutes

STEP 3 **Staining**

a) For 4 minutes, stain with Haematoxylin

b) Wash for 3 minutes with distilled water (dH_2O)

c) For 2.30 seconds, stain with Eosin

d) Wash for 2 minutes with distilled water (dH_2O)

STEP 4 **Dehydration**

a) Submerged the slides in 70% alcohol for 0.20 seconds

b) Submerged the slides in 96% alcohol for 0.15 seconds (repeat 2x

c) Submerged the slides pure alcohol (100%) for 0.15 seconds (repeat 2x

d) Submerged the slides Xylene-solution for 0.30 seconds (repeat 2x

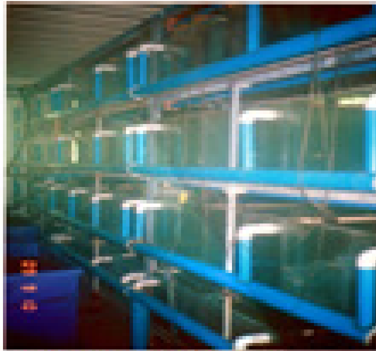
e) Submerged the slides in another round for 1 minute in Xylene-solution

STEP5 **Mounting**

a) Mount the slides with the use of DPX (DPX Mountant for histology is a mixture of distyrene, a plasticizer, and xylene used as a synthetic resin mounting media, that replaces xylene-balsam. It is a popular **all purpose** mounting medium that allows slides to be cleaned easily after setting and will not cause fading when used with a wide variety of stains) medium and cover slides. DPX Mountant is suitable for HE-(Hematoxylin-Eosin)

Cover slips were attached to the slides with DPX mounting solution. DPX mountant for histology is a mixture of distyrene, plasticizer, and xylene

D] Construction of Experimental Facility

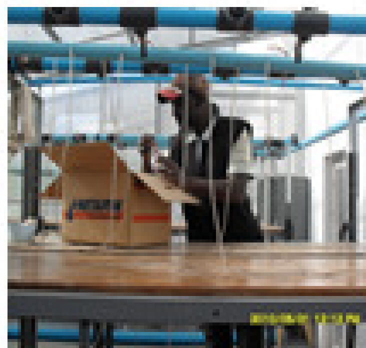


Old glass tanks used to construct experiment unit

Dismantled glass tanks (72), used for the experiment

Steel Platforms that will hold the glass tanks

Steel platforms with wooden planks & inlet pipe lines



Fixing of flexible air and water tubes to inlet pipe lines

Inlet system & air lines

Construction of 3meter Biofilter

Completed Biofilter

Water pumps connected to sumps & biofilter

Mechanical filter connected to sumps & biofilter

Completed system with pumps, sumps, biofilter & glass tanks

Complete functioning system with fish & experimental diets

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E] Tables of Statistical Analysis

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa- 1
12:20 Friday, February 15, 2013
The GLM Procedure - Class Level Information

Class	Levels	Values
Tmt	3	C M P
Conc	6	0 0.5 1 5 10 15
Sex	2	F M

Data for Analysis of Length Weight Liver HSI

Number of Observations Read	108
Number of Observations Used	108

Data for Analysis of Depth

Number of Observations Read	108
Number of Observations Used	107

Data for Analysis of Gonad GSI

Number of Observations Read	108
Number of Observations Used	107

NOTE: Variables in each group are consistent with respect to the presence or absence of missing values.

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 2
12:20 Friday, February 15, 2013
The GLM Procedure - Dependent Variable: Length

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	21	13707.85741	652.75511	12.63	<.0001
Error	86	4444.55000	51.68081		
Corrected Total	107	18152.40741			

R-Square	Coeff Var	Root MSE	Length Mean
0.755154	4.838621	7.188937	148.5741

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Sex	1	12353.15115	12353.15115	239.03	<.0001
Tmt	2	15.18684	7.59342	0.15	0.8636
Conc	4	380.08673	95.02168	1.84	0.1288
Tmt*Conc	4	295.56001	73.89000	1.43	0.2310
Tmt*Sex	2	44.60408	22.30204	0.43	0.6509
Conc*Sex	4	382.06332	95.51583	1.85	0.1270
Tmt*Conc*Sex	4	237.20528	59.30132	1.15	0.3398

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 3
12:20 Friday, February 15, 2013
The GLM Procedure - Dependent Variable: Weight

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	21	11274.68935	536.88997	7.82	<.0001
Error	86	5901.72820	68.62475		
Corrected Total	107	17176.41754			

R-Square	Coeff Var	Root MSE	Weight Mean
0.656405	15.58337	8.284005	53.15926

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Sex	1	10318.34352	10318.34352	150.36	<.0001
Tmt	2	8.02188	4.01094	0.06	0.9433
Conc	4	229.14363	57.28591	0.83	0.5068
Tmt*Conc	4	336.20120	84.05030	1.22	0.3063
Tmt*Sex	2	1.47464	0.73732	0.01	0.9893
Conc*Sex	4	261.04988	65.26247	0.95	0.4387
Tmt*Conc*Sex	4	120.45460	30.11365	0.44	0.7802

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 4
12:20 Friday, February 15, 2013

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The GLM Procedure - Dependent Variable: Liver

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	21	3.74642796	0.17840133	2.01	0.0129
Error	86	7.61976000	0.08860186		
Corrected Total	107	11.36618796			

R-Square	Coeff Var	Root MSE	Liver Mean
0.329612	24.18003	0.297661	1.231019

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Sex	1	1.84877602	1.84877602	20.87	<.0001
Tmt	2	0.50025692	0.25012846	2.82	0.0649
Conc	4	0.34177816	0.08544454	0.96	0.4314
Tmt*Conc	4	0.22875722	0.05718931	0.65	0.6316
Tmt*Sex	2	0.03352920	0.01676460	0.19	0.8280
Conc*Sex	4	0.58410162	0.14602540	1.65	0.1696
Tmt*Conc*Sex	4	0.20922881	0.05230720	0.59	0.6705

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 5
12:20 Friday, February 15, 2013

The GLM Procedure - Dependent Variable: HSI

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	21	8.55528237	0.40739440	1.89	0.0220
Error	86	18.58626230	0.21611933		
Corrected Total	107	27.14154467			

R-Square	Coeff Var	Root MSE	HSI Mean
0.315210	19.76184	0.464886	2.352444

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Sex	1	3.30474557	3.30474557	15.29	0.0002
Tmt	2	1.62926646	0.81463323	3.77	0.0270
Conc	4	0.41457075	0.10364269	0.48	0.7506
Tmt*Conc	4	0.36481111	0.09120278	0.42	0.7924
Tmt*Sex	2	0.28322404	0.14161202	0.66	0.5219
Conc*Sex	4	1.68538019	0.42134505	1.95	0.1095
Tmt*Conc*Sex	4	0.87328425	0.21832106	1.01	0.4068

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 6
12:20 Friday, February 15, 2013

The GLM Procedure - t Tests (LSD) for Length

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	86
Error Mean Square	51.68081
Critical Value of t	1.98793
Least Significant Difference	2.7508
Harmonic Mean of Cell Sizes	53.98148

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Sex
A	159.073	55	M
B	137.679	53	F

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa -7
12:20 Friday, February 15, 2013

The GLM Procedure - t Tests (LSD) for Weight

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	86
Error Mean Square	68.62475
Critical Value of t	1.98793
Least Significant Difference	3.1698

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Harmonic Mean of Cell Sizes 53.98148

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Sex
A	62.754	55	M
B	43.202	53	F

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 8
12:20 Friday, February 15, 2013

The GLM Procedure - t Tests (LSD) for Liver

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	86
Error Mean Square	0.088602
Critical Value of t	1.98793
Least Significant Difference	0.1139
Harmonic Mean of Cell Sizes	53.98148

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Sex
A	1.35945	55	M
B	1.09774	53	F

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 9
12:20 Friday, February 15, 2013

The GLM Procedure - t Tests (LSD) for HSI

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	86
Error Mean Square	0.216119
Critical Value of t	1.98793
Least Significant Difference	0.1779
Harmonic Mean of Cell Sizes	53.98148

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Sex
A	2.53064	53	F
B	2.18073	55	M

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 10
12:20 Friday, February 15, 2013

The GLM Procedure - t Tests (LSD) for Length

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	86
Error Mean Square	51.68081
Critical Value of t	1.98793
Least Significant Difference	4.379
Harmonic Mean of Cell Sizes	21.30178

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Tmt
A	148.771	48	P
A	148.640	50	M
A	147.300	10	C

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 11
12:20 Friday, February 15, 2013

The GLM Procedure - t Tests (LSD) for Weight

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NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	86
Error Mean Square	68.62475
Critical Value of t	1.98793
Least Significant Difference	5.046
Harmonic Mean of Cell Sizes	21.30178

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Tmt
A	53.689	48	P
A	52.752	50	M
A	52.656	10	C

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa -12
12:20 Friday, February 15, 2013
The GLM Procedure - t Tests (LSD) for Liver

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	86
Error Mean Square	0.088602
Critical Value of t	1.98793
Least Significant Difference	0.1813
Harmonic Mean of Cell Sizes	21.30178

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping		Mean	N	Tmt
	A	1.37100	10	C
B	A	1.27375	48	P
B		1.16200	50	M

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 13
12:20 Friday, February 15, 2013
The GLM Procedure - t Tests (LSD) for HSI

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	86
Error Mean Square	0.216119
Critical Value of t	1.98793
Least Significant Difference	0.2832
Harmonic Mean of Cell Sizes	21.30178

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping		Mean	N	Tmt
	A	2.6580	10	C
B	A	2.3989	48	P
B		2.2468	50	M

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 14
12:20 Friday, February 15, 2013
The GLM Procedure - Tests (LSD) for Length

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	86
Error Mean Square	51.68081
Critical Value of t	1.98793
Least Significant Difference	4.9179
Harmonic Mean of Cell Sizes	16.88889

NOTE: Cell sizes are not equal.

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Means with the same letter are not significantly different.

t Grouping	Mean	N	Conc
A	150.900	20	0.5
A	149.850	20	1
A	149.200	20	15
A	147.300	10	0
A	146.895	19	10
A	146.474	19	5

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 15
12:20 Friday, February 15, 2013
The GLM Procedure - t Tests (LSD) for Weight
NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	86
Error Mean Square	68.62475
Critical Value of t	1.98793
Least Significant Difference	5.667
Harmonic Mean of Cell Sizes	16.88889

NOTE: Cell sizes are not equal.
Means with the same letter are not significantly different.

t Grouping	Mean	N	Conc
A	55.035	20	0.5
A	54.588	20	1
A	52.656	10	0
A	52.337	19	5
A	52.224	20	15
A	51.753	19	10

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa -16
12:20 Friday, February 15, 2013
The GLM Procedure - t Tests (LSD) for Liver
NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	86
Error Mean Square	0.088602
Critical Value of t	1.98793
Least Significant Difference	0.2036
Harmonic Mean of Cell Sizes	16.88889

NOTE: Cell sizes are not equal.
Means with the same letter are not significantly different.

t Grouping		Mean	N	Conc
	A	1.3710	10	0
B	A	1.3205	20	0.5
B	A	1.2200	20	1
B	A	1.1995	19	10
B	A	1.1935	20	15
B		1.1458	19	5

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 17
12:20 Friday, February 15, 2013
The GLM Procedure - t Tests (LSD) for HSI
NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	86
Error Mean Square	0.216119
Critical Value of t	1.98793
Least Significant Difference	0.318
Harmonic Mean of Cell Sizes	16.88889

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NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping		Mean	N	Conc
	A	2.6580	10	0
B	A	2.4452	20	0.5
B		2.3240	19	10
B		2.2974	20	1
B		2.2914	20	15
B		2.2447	19	5

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 18

12:20 Friday, February 15, 2013

The GLM Procedure - Dependent Variable: Depth

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	21	1224.344081	58.302099	6.19	<.0001
Error	85	800.216667	9.414314		
Corrected Total	106	2024.560748			

R-Square	Coeff Var	Root MSE	Depth Mean
0.604746	7.960850	3.068275	38.54206

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Sex	1	1058.283825	1058.283825	112.41	<.0001
Tmt	2	5.443935	2.721968	0.29	0.7496
Conc	4	91.175814	22.793954	2.42	0.0545
Tmt*Conc	4	30.391899	7.597975	0.81	0.5241
Tmt*Sex	2	1.442759	0.721380	0.08	0.9263
Conc*Sex	4	9.846158	2.461539	0.26	0.9019
Tmt*Conc*Sex	4	27.759691	6.939923	0.74	0.5692

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 19

12:20 Friday, February 15, 2013

The GLM Procedure - t Tests (LSD) for Depth

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	85
Error Mean Square	9.414314
Critical Value of t	1.98827
Least Significant Difference	1.18
Harmonic Mean of Cell Sizes	53.45794

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Sex
A	41.6000	55	M
B	35.3077	52	F

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 20

12:20 Friday, February 15, 2013

The GLM Procedure - t Tests (LSD) for Depth

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	85
Error Mean Square	9.414314
Critical Value of t	1.98827
Least Significant Difference	1.8722
Harmonic Mean of Cell Sizes	21.23494

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Tmt
A	38.8936	47	P
A	38.5000	10	C

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A	38.2200	50	M

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 21
12:20 Friday, February 15, 2013
The GLM Procedure - t Tests (LSD) for Depth
NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	85
Error Mean Square	9.414314
Critical Value of t	1.98827
Least Significant Difference	2.108
Harmonic Mean of Cell Sizes	16.75102

NOTE: Cell sizes are not equal.
Means with the same letter are not significantly different.

t Grouping	Mean	N	Conc
A	39.500	20	0.5
A	39.450	20	1
A	38.944	18	5
A	38.500	10	0
A	37.421	19	10
A	37.400	20	15

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 22
12:20 Friday, February 15, 2013
The GLM Procedure - Dependent Variable: Gonad

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	21	67.19928512	3.19996596	18.91	<.0001
Error	85	14.38472980	0.16923212		
Corrected Total	106	81.58401491			

R-Square	Coeff Var	Root MSE	Gonad Mean
0.823682	-557.1516	0.411378	-0.073836

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Sex	1	61.42309752	61.42309752	362.95	<.0001
Tmt	2	0.84820180	0.42410090	2.51	0.0876
Conc	4	0.59345610	0.14836402	0.88	0.4815
Tmt*Conc	4	0.86697747	0.21674437	1.28	0.2840
Tmt*Sex	2	0.01842660	0.00921330	0.05	0.9470
Conc*Sex	4	1.77530804	0.44382701	2.62	0.0403
Tmt*Conc*Sex	4	1.67381758	0.41845440	2.47	0.0505

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 23
12:20 Friday, February 15, 2013
The GLM Procedure - Dependent Variable: GSI

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	21	100.8539930	4.8025711	33.52	<.0001
Error	85	12.1781042	0.1432718		
Corrected Total	106	113.0320972			

R-Square	Coeff Var	Root MSE	GSI Mean
0.892260	64.44374	0.378513	0.587354

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Sex	1	95.89686517	95.89686517	669.34	<.0001
Tmt	2	0.91188776	0.45594388	3.18	0.0465
Conc	4	0.60540740	0.15135185	1.06	0.3832
Tmt*Conc	4	0.60177042	0.15044260	1.05	0.3864
Tmt*Sex	2	0.01367062	0.00683531	0.05	0.9534
Conc*Sex	4	1.13382156	0.28345539	1.98	0.1051
Tmt*Conc*Sex	4	1.69057008	0.42264252	2.95	0.0246

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 24
12:20 Friday, February 15, 2013

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The GLM Procedure - t Tests (LSD) for Gonad

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	85
Error Mean Square	0.169232
Critical Value of t	1.98827
Least Significant Difference	0.1582
Harmonic Mean of Cell Sizes	53.45794

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Sex
A	0.70537	52	F
B	-0.81054	55	M

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 25
12:20 Friday, February 15, 2013

The GLM Procedure - t Tests (LSD) for GSI

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	85
Error Mean Square	0.143272
Critical Value of t	1.98827
Least Significant Difference	0.1456
Harmonic Mean of Cell Sizes	53.45794

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Sex
A	1.56097	52	F
B	-0.33316	55	M

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa -26
12:20 Friday, February 15, 2013

The GLM Procedure - t Tests (LSD) for Gonad

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	85
Error Mean Square	0.169232
Critical Value of t	1.98827
Least Significant Difference	0.251
Harmonic Mean of Cell Sizes	21.23494

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping		Mean	N	Tmt
	A	0.0350	50	M
B	A	-0.1560	47	P
B		-0.2320	10	C

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 27
12:20 Friday, February 15, 2013

The GLM Procedure - t Tests (LSD) for GSI

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	85
Error Mean Square	0.143272
Critical Value of t	1.98827
Least Significant Difference	0.231
Harmonic Mean of Cell Sizes	21.23494

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping		Mean	N	Tmt
	A	0.7064	50	M
B	A	0.4925	47	P
B		0.4382	10	C

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 28
12:20 Friday, February 15, 2013
The GLM Procedure - t Tests (LSD) for Gonad
NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	85
Error Mean Square	0.169232
Critical Value of t	1.98827
Least Significant Difference	0.2825
Harmonic Mean of Cell Sizes	16.76471

NOTE: Cell sizes are not equal.
Means with the same letter are not significantly different.

t Grouping	Mean	N	Conc
A	0.0376	19	15
A	0.0243	20	0.5
A	-0.0696	19	5
A	-0.1270	20	1
A	-0.1536	19	10
A	-0.2320	10	0

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 29
12:20 Friday, February 15, 2013
The GLM Procedure - t Tests (LSD) for GSI
NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	85
Error Mean Square	0.143272
Critical Value of t	1.98827
Least Significant Difference	0.2599
Harmonic Mean of Cell Sizes	16.76471

NOTE: Cell sizes are not equal.
Means with the same letter are not significantly different.

t Grouping		Mean	N	Conc
	A	0.7074	19	15
B	A	0.6478	20	0.5
B	A	0.6062	19	5
B	A	0.5395	19	10
B	A	0.5151	20	1
B		0.4382	10	0

Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa Interaction 147. 15:50 Wednesday, August 15, 2012
The GLM Procedure - Class Level Information

Class	Levels	Values
Treat	3	17MT P10M5 P5M10
Sex	2	F M

Number of Observations Read	28
Number of Observations Used	28

Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa Interaction 148 15:50 Wednesday, August 15, 2012
The GLM Procedure - Dependent Variable: Length

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	1799.211905	359.842381	6.22	0.0010
Error	22	1273.466667	57.884848		
Corrected Total	27	3072.678571			

R-Square	Coeff Var	Root MSE	Length Mean
0.585552	4.743484	7.608209	160.3929

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Sex	1	1497.047802	1497.047802	25.86	<.0001
Treat	2	287.412587	143.706294	2.48	0.1066
Treat*Sex	2	14.751515	7.375758	0.13	0.8810

Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa Interaction 149 15:50 Wednesday, August 15, 2012

The GLM Procedure - Dependent Variable: Weight

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	1698.017944	339.603589	5.13	0.0029
Error	22	1457.197867	66.236267		
Corrected Total	27	3155.215811			

R-Square	Coeff Var	Root MSE	Weight Mean
0.538162	12.59903	8.138567	64.59679

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Sex	1	1114.695425	1114.695425	16.83	0.0005
Treat	2	542.628114	271.314057	4.10	0.0307
Treat*Sex	2	40.694405	20.347203	0.31	0.7386

Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa Interacti 150 15:50 Wednesday, August 15, 2012

The GLM Procedure - Dependent Variable: Depth

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	261.4404762	52.2880952	8.30	0.0002
Error	22	138.6666667	6.3030303		
Corrected Total	27	400.1071429			

R-Square	Coeff Var	Root MSE	Depth Mean
0.653426	6.075743	2.510584	41.32143

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Sex	1	148.6815018	148.6815018	23.59	<.0001
Treat	2	93.4347319	46.7173660	7.41	0.0035
Treat*Sex	2	19.3242424	9.6621212	1.53	0.2381

Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa Interacti 151 15:50 Wednesday, August 15, 2012

The GLM Procedure - Dependent Variable: Gonad

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	16.49572760	3.29914552	6.97	0.0005
Error	22	10.40648879	0.47302222		
Corrected Total	27	26.90221639			

R-Square	Coeff Var	Root MSE	Gonad Mean
0.613174	-3971.694	0.687766	-0.017317

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Sex	1	13.85150264	13.85150264	29.28	<.0001
Treat	2	1.52392469	0.76196234	1.61	0.2224
Treat*Sex	2	1.12030028	0.56015014	1.18	0.3248

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Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa Interacti 152
15:50 Wednesday, August 15, 2012

The GLM Procedure - Dependent Variable: GSI

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	21.50400478	4.30080096	9.63	<.0001
Error	22	9.82161301	0.44643695		
Corrected Total	27	31.32561778			

R-Square	Coeff Var	Root MSE	GSI Mean
0.686467	154.0844	0.668159	0.433632

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Sex	1	17.97800621	17.97800621	40.27	<.0001
Treat	2	2.53594917	1.26797459	2.84	0.0799
Treat*Sex	2	0.99004939	0.49502469	1.11	0.3477

Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa Interacti 153
15:50 Wednesday, August 15, 2012

The GLM Procedure - Dependent Variable: Liver

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	4.29871143	0.85974229	5.82	0.0014
Error	22	3.25096000	0.14777091		
Corrected Total	27	7.54967143			

R-Square	Coeff Var	Root MSE	Liver Mean
0.569391	24.99646	0.384410	1.537857

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Sex	1	0.03766220	0.03766220	0.25	0.6187
Treat	2	4.25478378	2.12739189	14.40	0.0001
Treat*Sex	2	0.00626545	0.00313273	0.02	0.9790

Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa Interaction154
15:50 Wednesday, August 15, 2012

The GLM Procedure - Dependent Variable: HSI

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	8.08931776	1.61786355	7.22	0.0004
Error	22	4.92956520	0.22407115		
Corrected Total	27	13.01888296			

R-Square	Coeff Var	Root MSE	HSI Mean
0.621353	19.77726	0.473362	2.393464

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Sex	1	1.24955949	1.24955949	5.58	0.0275
Treat	2	6.62590286	3.31295143	14.79	<.0001
Treat*Sex	2	0.21385542	0.10692771	0.48	0.6268

Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa Interaction 155
15:50 Wednesday, August 15, 2012

The GLM Procedure - t Tests (LSD) for Length

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	22
Error Mean Square	57.88485
Critical Value of t	2.07387
Least Significant Difference	5.979

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Harmonic Mean of Cell Sizes 13.92857

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Sex
A	167.200	15	M
B	152.538	13	F

Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa Interaction 156 15:50 Wednesday, August 15, 2012

The GLM Procedure - t Tests (LSD) for Weight

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	22
Error Mean Square	66.23627
Critical Value of t	2.07387
Least Significant Difference	6.3958
Harmonic Mean of Cell Sizes	13.92857

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Sex
A	70.471	15	M
B	57.819	13	F

Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa Interaction 157 15:50 Wednesday, August 15, 2012

The GLM Procedure - t Tests (LSD) for Depth

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	22
Error Mean Square	6.30303
Critical Value of t	2.07387
Least Significant Difference	1.973
Harmonic Mean of Cell Sizes	13.92857

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Sex
A	43.4667	15	M
B	38.8462	13	F

Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa Interaction 158 15:50 Wednesday, August 15, 2012

The GLM Procedure - t Tests (LSD) for Gonad

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	22
Error Mean Square	0.473022
Critical Value of t	2.07387
Least Significant Difference	0.5405
Harmonic Mean of Cell Sizes	13.92857

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Sex

A	0.7382	13	F
B	-0.6721	15	M

Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa
Interaction 159 15:50 Wednesday, August 15, 2012

The GLM Procedure - t Tests (LSD) for GSI

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	22
Error Mean Square	0.446437
Critical Value of t	2.07387
Least Significant Difference	0.5251
Harmonic Mean of Cell Sizes	13.92857

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Sex
A	1.2944	13	F
B	-0.3123	15	M

Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa
Interaction160 15:50 Wednesday, August 15, 2012

The GLM Procedure - t Tests (LSD) for Liver

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	22
Error Mean Square	0.147771
Critical Value of t	2.07387
Least Significant Difference	0.3021
Harmonic Mean of Cell Sizes	13.92857

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Sex
A	1.5720	15	M
A	1.4985	13	F

Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa
Interaction 161 15:50 Wednesday, August 15, 2012

The GLM Procedure - t Tests (LSD) for HSI

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	22
Error Mean Square	0.224071
Critical Value of t	2.07387
Least Significant Difference	0.372
Harmonic Mean of Cell Sizes	13.92857

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Sex
A	2.6204	13	F
B	2.1968	15	M

Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa

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Interaction 162 15:50 Wednesday, August 15, 2012

The GLM Procedure - t Tests (LSD) for Length

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	22
Error Mean Square	57.88485
Critical Value of t	2.07387
Least Significant Difference	7.3445
Harmonic Mean of Cell Sizes	9.230769

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Treat
A	164.100	10	17MT
A	158.625	8	P10M5
A	158.100	10	P5M10

Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa
Interaction 163 15:50 Wednesday, August 15, 2012

The GLM Procedure - t Tests (LSD) for Weight

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	22
Error Mean Square	66.23627
Critical Value of t	2.07387
Least Significant Difference	7.8564
Harmonic Mean of Cell Sizes	9.230769

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Treat
A	69.921	10	17MT
B	61.898	10	P5M10
B	61.315	8	P10M5

Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa
Interaction 164 15:50 Wednesday, August 15, 2012

The GLM Procedure - t Tests (LSD) for Depth

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	22
Error Mean Square	6.30303
Critical Value of t	2.07387
Least Significant Difference	2.4236
Harmonic Mean of Cell Sizes	9.230769

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Treat
A	43.600	10	17MT
B	40.250	8	P10M5
B	39.900	10	P5M10

Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa
Interaction 165 15:50 Wednesday, August 15, 2012

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The GLM Procedure - t Tests (LSD) for Gonad

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	22
Error Mean Square	0.473022
Critical Value of t	2.07387
Least Significant Difference	0.6639
Harmonic Mean of Cell Sizes	9.230769

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Treat
A	0.2286	10	P5M10
A	0.0017	8	P10M5
A	-0.2785	10	17MT

Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa Interaction 166 15:50 Wednesday, August 15, 2012

The GLM Procedure - t Tests (LSD) for GSI

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	22
Error Mean Square	0.446437
Critical Value of t	2.07387
Least Significant Difference	0.645
Harmonic Mean of Cell Sizes	9.230769

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Treat
A	0.7282	10	P5M10
A	0.4974	8	P10M5
A	0.0881	10	17MT

Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa Interaction 167 15:50 Wednesday, August 15, 2012

The GLM Procedure - t Tests (LSD) for Liver

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	22
Error Mean Square	0.147771
Critical Value of t	2.07387
Least Significant Difference	0.3711
Harmonic Mean of Cell Sizes	9.230769

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Treat
A	2.0500	10	17MT
B	1.3525	8	P10M5
B	1.1740	10	P5M10

Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa Interaction 168 15:50 Wednesday, August 15, 2012

The GLM Procedure - t Tests (LSD) for HSI

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NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	22
Error Mean Square	0.224071
Critical Value of t	2.07387
Least Significant Difference	0.457
Harmonic Mean of Cell Sizes	9.230769

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Treat
A	3.0377	10	17MT
B	2.1859	8	P10M5
B	1.9153	10	P5M10

Experiment II 60 Day Sampling (Raw Measurements Jun 20, 2011) Males 188
15:37 Thursday, August 16, 2012

The GLM Procedure - Dependent Variable: mLength

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	583.078535	58.307854	2.78	0.0095
Error	44	923.669444	20.992487		
Corrected Total	54	1506.747980			

R-Square	Coeff Var	Root MSE	mLength Mean
0.386978	3.598409	4.581756	127.3273

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treat	2	110.8618687	55.4309343	2.64	0.0826
Conc	4	150.4063889	37.6015972	1.79	0.1477
Treat*Conc	4	321.8102778	80.4525694	3.83	0.0093

The GLM Procedure - Dependent Variable: mWeight

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	431.171408	43.117141	1.82	0.0851
Error	44	1042.743181	23.698709		
Corrected Total	54	1473.914588			

R-Square	Coeff Var	Root MSE	mWeight Mean
0.292535	11.75608	4.868132	41.40948

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treat	2	70.0419346	35.0209673	1.48	0.2393
Conc	4	157.3155389	39.3288847	1.66	0.1765
Treat*Conc	4	203.8139341	50.9534835	2.15	0.0905

The GLM Procedure - Dependent Variable: mDepth

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	75.6093434	7.5609343	1.42	0.2036
Error	44	234.3583333	5.3263258		
Corrected Total	54	309.9676768			

R-Square	Coeff Var	Root MSE	mDepth Mean
0.243927	6.109675	2.307883	37.77424

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treat	2	19.88767677	9.94383838	1.87	0.1666
Conc	4	6.89527778	1.72381944	0.32	0.8606
Treat*Conc	4	48.82638889	12.20659722	2.29	0.0746

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Experiment II 60 Day Sampling (Raw Measurements Jun 20, 2011) males 192
15:37 Thursday, August 16, 2012

The GLM Procedure - Dependent Variable: mGonad

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	3.92755600	0.39275560	1.83	0.0823
Error	44	9.42146536	0.21412421		
Corrected Total	54	13.34902137			

R-Square	Coeff Var	Root MSE	mGonad Mean
0.294221	-18.28968	0.462736	-2.530036

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treat	2	1.03898147	0.51949073	2.43	0.1001
Conc	4	0.57820776	0.14455194	0.68	0.6128
Treat*Conc	4	2.31036677	0.57759169	2.70	0.0428

The GLM Procedure - Dependent Variable: mGSI

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	3.82133766	0.38213377	1.66	0.1203
Error	44	10.10497887	0.22965861		
Corrected Total	54	13.92631653			

R-Square	Coeff Var	Root MSE	mGSI Mean
0.274397	-29.37253	0.479227	-1.631549

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treat	2	0.69390818	0.34695409	1.51	0.2320
Conc	4	0.52544967	0.13136242	0.57	0.6843
Treat*Conc	4	2.60197981	0.65049495	2.83	0.0356

The GLM Procedure - Dependent Variable: mLiver

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	0.94661035	0.09466104	1.67	0.1192
Error	44	2.49682000	0.05674591		
Corrected Total	54	3.44343035			

R-Square	Coeff Var	Root MSE	mLiver Mean
0.274903	18.77515	0.238214	1.268773

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treat	2	0.24937091	0.12468545	2.20	0.1232
Conc	4	0.49239328	0.12309832	2.17	0.0882
Treat*Conc	4	0.20484617	0.05121154	0.90	0.4708

Experiment II 60 Day Sampling (Raw Measurements Jun 20, 2011) males 195
15:37 Thursday, August 16, 2012

The GLM Procedure - Dependent Variable: mHSI

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	6.76530744	0.67653074	3.32	0.0027
Error	44	8.95756632	0.20358105		
Corrected Total	54	15.72287376			

R-Square	Coeff Var	Root MSE	mHSI Mean
0.430284	14.69437	0.451200	3.070561

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treat	2	2.18757189	1.09378595	5.37	0.0082
Conc	4	2.24120854	0.56030214	2.75	0.0397
Treat*Conc	4	2.33652700	0.58413175	2.87	0.0339

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The GLM Procedure - Least Squares Means

Treat	Conc	mLength LSMEAN	LSMEAN Number
C	0	123.716667	1
M	0.5	124.316667	2
M	1	126.633333	3
M	2	126.116667	4
M	5	129.833333	5
M	10	135.966667	6
P	0.5	127.633333	7
P	1	127.633333	8
P	2	125.750000	9
P	5	128.450000	10
P	10	124.550000	11

Least Squares Means for effect Treat*Conc
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: mLength

i/j	1	2	3	4	5	6
1		0.8369	0.3197	0.4120	0.0405	0.0001
2	0.8369		0.4283	0.5377	0.0635	0.0002
3	0.3197	0.4283		0.8593	0.2755	0.0024
4	0.4120	0.5377	0.8593		0.2063	0.0014
5	0.0405	0.0635	0.2755	0.2063		0.0400
6	0.0001	0.0002	0.0024	0.0014	0.0400	
7	0.1834	0.2586	0.7317	0.6033	0.4518	0.0062
8	0.1834	0.2586	0.7317	0.6033	0.4518	0.0062
9	0.4866	0.6233	0.7619	0.8999	0.1658	0.0010
10	0.1095	0.1608	0.5340	0.4250	0.6355	0.0128
11	0.7750	0.9362	0.4760	0.5915	0.0751	0.0003

Dependent Variable: mLength

i/j	7	8	9	10	11
1	0.1834	0.1834	0.4866	0.1095	0.7750
2	0.2586	0.2586	0.6233	0.1608	0.9362
3	0.7317	0.7317	0.7619	0.5340	0.4760
4	0.6033	0.6033	0.8999	0.4250	0.5915
5	0.4518	0.4518	0.1658	0.6355	0.0751
6	0.0062	0.0062	0.0010	0.0128	0.0003
7		1.0000	0.5191	0.7794	0.2931
8	1.0000		0.5191	0.7794	0.2931
9	0.5191	0.5191		0.3565	0.6808
10	0.7794	0.7794	0.3565		0.1852
11	0.2931	0.2931	0.6808	0.1852	

Treat	Conc	mWeight LSMEAN	LSMEAN Number
C	0	38.9841667	1
M	0.5	39.2835000	2
M	1	41.6083333	3
M	2	40.5620000	4
M	5	42.7173333	5
M	10	48.4300000	6
P	0.5	40.0855000	7

P	1	40.4673333	8
P	2	39.4943333	9
P	5	44.8696667	10
P	10	39.0021667	11

Least Squares Means for effect Treat*Conc
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: mWeight

i/j	1	2	3	4	5	6
1		0.9230	0.3987	0.6109	0.2318	0.0037
2	0.9230		0.4542	0.6800	0.2708	0.0048
3	0.3987	0.4542		0.7356	0.7204	0.0319
4	0.6109	0.6800	0.7356		0.4876	0.0141
5	0.2318	0.2708	0.7204	0.4876		0.0702
6	0.0037	0.0048	0.0319	0.0141	0.0702	
7	0.7223	0.7957	0.6233	0.8777	0.3973	0.0095
8	0.6324	0.7025	0.7127	0.9756	0.4688	0.0131
9	0.8692	0.9457	0.4959	0.7304	0.3009	0.0058
10	0.0625	0.0764	0.2953	0.1688	0.4882	0.2538
11	0.9954	0.9276	0.4019	0.6149	0.2340	0.0037

Dependent Variable: mWeight

i/j	7	8	9	10	11
1	0.7223	0.6324	0.8692	0.0625	0.9954
2	0.7957	0.7025	0.9457	0.0764	0.9276
3	0.6233	0.7127	0.4959	0.2953	0.4019
4	0.8777	0.9756	0.7304	0.1688	0.6149
5	0.3973	0.4688	0.3009	0.4882	0.2340
6	0.0095	0.0131	0.0058	0.2538	0.0037
7		0.9019	0.8486	0.1274	0.7266
8	0.9019		0.7535	0.1598	0.6365
9	0.8486	0.7535		0.0878	0.8737
10	0.1274	0.1598	0.0878		0.0632
11	0.7266	0.6365	0.8737	0.0632	

		mDepth LSMEAN	LSMEAN
Treat	Conc	LSMEAN	Number
C	0	5.8833333	1
M	0.5	37.7500000	2
M	1	38.0666667	3
M	2	39.1666667	4
M	5	35.8666667	5
M	10	38.6333333	6
P	0.5	37.6500000	7
P	1	36.9166667	8
P	2	37.8833333	9
P	5	39.8666667	10
P	10	37.8333333	11

Least Squares Means for effect Treat*Conc
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: mDepth

i/j	1	2	3	4	5	6
1		0.2077	0.1418	0.0295	0.9909	0.0662

2	0.2077		0.8293	0.3371	0.2037	0.5482
3	0.1418	0.8293		0.4551	0.1389	0.6997
4	0.0295	0.3371	0.4551		0.0288	0.7166
5	0.9909	0.2037	0.1389	0.0288		0.0646
6	0.0662	0.5482	0.6997	0.7166	0.0646	
7	0.2326	0.9457	0.7766	0.3044	0.2283	0.5040
8	0.4827	0.5710	0.4350	0.1304	0.4757	0.2459
9	0.1776	0.9276	0.9006	0.3841	0.1741	0.6099
10	0.0091	0.1541	0.2241	0.6339	0.0088	0.4027
11	0.1884	0.9547	0.8737	0.3660	0.1848	0.5864

Dependent Variable: mDepth

i/j	7	8	9	10	11
1	0.2326	0.4827	0.1776	0.0091	0.1884
2	0.9457	0.5710	0.9276	0.1541	0.9547
3	0.7766	0.4350	0.9006	0.2241	0.8737
4	0.3044	0.1304	0.3841	0.6339	0.3660
5	0.2283	0.4757	0.1741	0.0088	0.1848
6	0.5040	0.2459	0.6099	0.4027	0.5864
7		0.6179	0.8737	0.1360	0.9006
8	0.6179		0.5113	0.0494	0.5332
9	0.8737	0.5113		0.1811	0.9728
10	0.1360	0.0494	0.1811		0.1706
11	0.9006	0.5332	0.9728	0.1706	

Treat	Conc	mGonad LSMEAN	LSMEAN Number
C	0	-2.90440142	1
M	0.5	-2.34066359	2
M	1	-2.91855222	3
M	2	-2.17154859	4
M	5	-2.10114156	5
M	10	-2.56492372	6
P	0.5	-2.65046697	7
P	1	-2.27811686	8
P	2	-2.76953152	9
P	5	-2.50624058	10
P	10	-2.62481443	11

Least Squares Means for effect Treat*Conc
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: mGonad

i/j	1	2	3	4	5	6
1		0.0605	0.9617	0.0161	0.0087	0.2523
2	0.0605		0.0546	0.5663	0.4175	0.4476
3	0.9617	0.0546		0.0142	0.0077	0.2334
4	0.0161	0.5663	0.0142		0.8110	0.1858
5	0.0087	0.4175	0.0077	0.8110		0.1202
6	0.2523	0.4476	0.2334	0.1858	0.1202	
7	0.3903	0.2956	0.3646	0.1089	0.0672	0.7714
8	0.0379	0.8318	0.0340	0.7175	0.5485	0.3324
9	0.6472	0.1499	0.6132	0.0470	0.0273	0.4881
10	0.1806	0.5744	0.1659	0.2590	0.1733	0.8420

11	0.3446	0.3369	0.3210	0.1286	0.0804	0.8388

Dependent Variable: mGonad

i/j	7	8	9	10	11
1	0.3903	0.0379	0.6472	0.1806	0.3446
2	0.2956	0.8318	0.1499	0.5744	0.3369
3	0.3646	0.0340	0.6132	0.1659	0.3210
4	0.1089	0.7175	0.0470	0.2590	0.1286
5	0.0672	0.5485	0.0273	0.1733	0.0804
6	0.7714	0.3324	0.4881	0.8420	0.8388
7		0.2100	0.6861	0.6246	0.9306
8	0.2100		0.1002	0.4399	0.2425
9	0.6861	0.1002		0.3732	0.6234
10	0.6246	0.4399	0.3732		0.6873
11	0.9306	0.2425	0.6234	0.6873	

The GLM Procedure - Least Squares Means

LSMEAN

Treat	Conc	mGSI LSMEAN	Number
C	0	-1.94967616	1
M	0.5	-1.39594806	2
M	1	-2.01879010	3
M	2	-1.25061283	4
M	5	-1.24498002	5
M	10	-1.82635587	6
P	0.5	-1.72486140	7
P	1	-1.36164981	8
P	2	-1.83212612	9
P	5	-1.68106979	10
P	10	-1.66096399	11

Least Squares Means for effect Treat*Conc

Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: mGSI

i/j	1	2	3	4	5	6
1		0.0745	0.8207	0.0259	0.0247	0.6861
2	0.0745		0.0458	0.6339	0.6209	0.1626
3	0.8207	0.0458		0.0149	0.0142	0.5288
4	0.0259	0.6339	0.0149		0.9853	0.0641
5	0.0247	0.6209	0.0142	0.9853		0.0616
6	0.6861	0.1626	0.5288	0.0641	0.0616	
7	0.4622	0.2837	0.3375	0.1248	0.1205	0.7393
8	0.0588	0.9104	0.0356	0.7159	0.7021	0.1324
9	0.7000	0.1572	0.5412	0.0615	0.0592	0.9849
10	0.3803	0.3520	0.2712	0.1626	0.1573	0.6341
11	0.3460	0.3867	0.2441	0.1827	0.1769	0.5880

Dependent Variable: mGSI

i/j	7	8	9	10	11
1	0.4622	0.0588	0.7000	0.3803	0.3460
2	0.2837	0.9104	0.1572	0.3520	0.3867
3	0.3375	0.0356	0.5412	0.2712	0.2441
4	0.1248	0.7159	0.0615	0.1626	0.1827
5	0.1205	0.7021	0.0592	0.1573	0.1769
6	0.7393	0.1324	0.9849	0.6341	0.5880

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7		0.2372	0.7251	0.8858	0.8340
8	0.2372		0.1278	0.2977	0.3288
9	0.7251	0.1278		0.6207	0.5751
10	0.8858	0.2977	0.6207		0.9474
11	0.8340	0.3288	0.5751	0.9474	

Treat	Conc	mLiver LSMEAN	LSMEAN Number
C	0	1.45950000	1
M	0.5	1.25050000	2
M	1	1.26166667	3
M	2	1.38016667	4
M	5	1.44033333	5
M	10	1.07283333	6
P	0.5	1.23183333	7
P	1	1.11616667	8
P	2	1.12700000	9
P	5	1.41883333	10
P	10	1.19766667	11

Least Squares Means for effect Treat*Conc
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: mLiver

i/j	1	2	3	4	5	6
1		0.1724	0.1960	0.6011	0.8993	0.0138
2	0.1724		0.9413	0.3941	0.2143	0.2446
3	0.1960	0.9413		0.4358	0.2420	0.2167
4	0.6011	0.3941	0.4358		0.6916	0.0474
5	0.8993	0.2143	0.2420	0.6916		0.0188
6	0.0138	0.2446	0.2167	0.0474	0.0188	
7	0.1379	0.9020	0.8439	0.3302	0.1734	0.2970
8	0.0276	0.3774	0.3394	0.0867	0.0370	0.7750
9	0.0326	0.4168	0.3763	0.1000	0.0434	0.7209
10	0.7885	0.2699	0.3026	0.7986	0.8872	0.0265
11	0.0892	0.7275	0.6731	0.2322	0.1144	0.4118

Dependent Variable: mLiver

i/j	7	8	9	10	11
1	0.1379	0.0276	0.0326	0.7885	0.0892
2	0.9020	0.3774	0.4168	0.2699	0.7275
3	0.8439	0.3394	0.3763	0.3026	0.6731
4	0.3302	0.0867	0.1000	0.7986	0.2322
5	0.1734	0.0370	0.0434	0.8872	0.1144
6	0.2970	0.7750	0.7209	0.0265	0.4118
7		0.4467	0.4902	0.2211	0.8216
8	0.4467		0.9430	0.0507	0.5913
9	0.4902	0.9430		0.0592	0.6414
10	0.2211	0.0507	0.0592		0.1492
11	0.8216	0.5913	0.6414	0.1492	

Treat	Conc	mHSI LSMEAN	LSMEAN Number
C	0	3.69981667	1
M	0.5	3.16635000	2
M	1	3.00810000	3

M	2	3.33438333	4
M	5	3.36780000	5
M	10	2.23145000	6
P	0.5	3.02970000	7
P	1	2.88181667	8
P	2	2.87640000	9
P	5	3.14585000	10
P	10	3.03450000	11

The GLM Procedure - Least Squares Means

Least Squares Means for effect Treat*Conc

Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: mHSI

i/j	1	2	3	4	5	6
1		0.0682	0.0195	0.2070	0.2509	<.0001
2	0.0682		0.5820	0.5590	0.4839	0.0021
3	0.0195	0.5820		0.2591	0.2141	0.0093
4	0.2070	0.5590	0.2591		0.9073	0.0004
5	0.2509	0.4839	0.2141	0.9073		0.0003
6	<.0001	0.0021	0.0093	0.0004	0.0003	
7	0.0234	0.6344	0.9400	0.2915	0.2425	0.0076
8	0.0063	0.3242	0.6603	0.1199	0.0956	0.0276
9	0.0060	0.3151	0.6467	0.1157	0.0921	0.0288
10	0.0586	0.9431	0.6317	0.5123	0.4409	0.0025
11	0.0244	0.6463	0.9267	0.2991	0.2491	0.0073

Dependent Variable: mHSI

i/j	7	8	9	10	11
1	0.0234	0.0063	0.0060	0.0586	0.0244
2	0.6344	0.3242	0.3151	0.9431	0.6463
3	0.9400	0.6603	0.6467	0.6317	0.9267
4	0.2915	0.1199	0.1157	0.5123	0.2991
5	0.2425	0.0956	0.0921	0.4409	0.2491
6	0.0076	0.0276	0.0288	0.0025	0.0073
7		0.6069	0.5938	0.6860	0.9867
8	0.6069		0.9849	0.3599	0.5953
9	0.5938	0.9849		0.3502	0.5824
10	0.6860	0.3599	0.3502		0.6983
11	0.9867	0.5953	0.5824	0.6983	

Experiment II -60 Day Sampling (Raw Measurements Jun 20, 2011) Females 60

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The GLM Procedure - Class Level Information

Class	Levels	Values
Treat	3	C M P
Conc	6	0 0.5 1 2 5 10

Number of Observations Read 42

Number of Observations Used 42

The GLM Procedure - Dependent Variable: mLength

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	315.207870	31.520787	0.70	0.7133
Error	31	1387.384722	44.754346		
Corrected Total	41	1702.592593			

R-Square Coeff Var Root MSE mLength Mean

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0.185134	5.488498	6.689869	121.8889	

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treat	2	80.6695326	40.3347663	0.90	0.4164
Conc	4	47.0467693	11.7616923	0.26	0.8995
Treat*Conc	4	187.4915684	46.8728921	1.05	0.3989

The GLM Procedure - Dependent Variable: mWeight

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	256.459746	25.645975	0.64	0.7673
Error	31	1239.711405	39.990690		
Corrected Total	41	1496.171151			

R-Square	Coeff Var	Root MSE	mWeight Mean
0.171411	17.43069	6.323819	36.27980

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treat	2	161.3567352	80.6783676	2.02	0.1501
Conc	4	24.6119831	6.1529958	0.15	0.9598
Treat*Conc	4	70.4910275	17.6227569	0.44	0.7782

The GLM Procedure - Dependent Variable: mDepth

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	118.9029101	11.8902910	0.98	0.4809
Error	31	376.4972222	12.1450717		
Corrected Total	41	495.4001323			

R-Square	Coeff Var	Root MSE	mDepth Mean
0.240014	9.935677	3.484978	35.07540

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treat	2	54.59678131	27.29839065	2.25	0.1226
Conc	4	34.09047754	8.52261939	0.70	0.5967
Treat*Conc	4	30.21565121	7.55391280	0.62	0.6503

The GLM Procedure - Dependent Variable: mGonad

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	4.47329324	0.44732932	0.25	0.9874
Error	31	55.30612950	1.78406869		
Corrected Total	41	59.77942274			

R-Square	Coeff Var	Root MSE	mGonad Mean
0.074830	-247.8558	1.335690	-0.538898

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treat	2	0.05987355	0.02993678	0.02	0.9834
Conc	4	3.65911412	0.91477853	0.51	0.7268
Treat*Conc	4	0.75430557	0.18857639	0.11	0.9797

The GLM Procedure - Dependent Variable: mGSI

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	3.84426170	0.38442617	0.21	0.9931
Error	31	55.61031278	1.79388106		
Corrected Total	41	59.45457448			

R-Square	Coeff Var	Root MSE	mGSI Mean
0.064659	283.5544	1.339358	0.472346

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treat	2	0.14989916	0.07494958	0.04	0.9591
Conc	4	3.23895254	0.80973814	0.45	0.7706
Treat*Conc	4	0.45540999	0.11385250	0.06	0.9922

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Experiment II -60 Day Sampling (Raw Measurements Jun 20, 2011) Female 66
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The GLM Procedure - Dependent Variable: mLiver

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	0.49552188	0.04955219	0.43	0.9186
Error	31	3.54465722	0.11434378		
Corrected Total	41	4.04017910			

R-Square	Coeff Var	Root MSE	mLiver Mean
0.122648	33.84430	0.338148	0.999127

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treat	2	0.00478309	0.00239155	0.02	0.9793
Conc	4	0.20234457	0.05058614	0.44	0.7770
Treat*Conc	4	0.28839422	0.07209856	0.63	0.6444

The GLM Procedure - Dependent Variable: mHSI

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	4.45035498	0.44503550	1.00	0.4650
Error	31	13.80049674	0.44517731		
Corrected Total	41	18.25085172			

R-Square	Coeff Var	Root MSE	mHSI Mean
0.243844	24.42070	0.667216	2.732175

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treat	2	1.28911617	0.64455809	1.45	0.2505
Conc	4	1.40929015	0.35232254	0.79	0.5397
Treat*Conc	4	1.75194866	0.43798717	0.98	0.4307

New Experiment II -60 Day Sampling of Growth Study Ia

Obs	Conc	Type	TYPE	FREQ	mLength	mWeight	mDepth	llength	lweight	ldepth
1	0.0	C	3	18	217.611	199.797	68.3889	122.75	38.2450	36.40
2	0.5	M	3	16	209.688	179.666	64.5625	120.35	35.0275	36.45
3	0.5	P	3	19	213.105	180.045	65.0526	125.60	37.8325	37.45
4	1.0	M	3	20	207.150	166.228	63.7000	118.50	35.1850	36.15
5	1.0	P	3	18	213.278	178.839	65.3333	124.80	38.5640	38.45
6	2.0	M	3	18	206.444	165.389	63.1111	120.45	35.4185	36.00
7	2.0	P	3	19	207.000	159.179	61.8421	124.35	40.7700	38.80
8	5.0	M	3	17	206.647	177.506	66.1765	127.75	42.8235	37.55
9	5.0	P	3	16	214.563	184.547	65.3125	121.70	38.1470	5.80
10	10.0	M	3	18	206.278	166.711	63.1111	126.00	39.9190	36.25
11	10.0	P	3	17	213.529	188.168	67.6471	120.35	35.5730	35.90

The GLM Procedure - Class Level Information

Class	Levels	Values
Conc	6	0 0.5 1 2 5 10
Type	3	C M P

Number of Observations Read 11
Number of Observations Used 11

The GLM Procedure - Dependent Variable: mLength

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	145.4593346	24.2432224	5.23	0.0656
Error	4	18.5418561	4.6354640		
Corrected Total	10	164.0011907			

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R-Square	Coeff Var	Root MSE	mLength Mean
0.886941	1.022900	2.153013	210.4812

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Conc	5	81.61128476	16.32225695	3.52	0.1232
Type	1	63.84804988	63.84804988	13.77	0.0206

The GLM Procedure - Dependent Variable: mWeight

Source	DF	Sum Squares	Mean Square	F Value	Pr > F
Model	6	1166.238157	194.373026	3.39	0.1288
Error	4	229.410032	57.352508		
Corrected Total	10	1395.648189			

R-Square	Coeff Var	Root MSE	mWeight Mean
0.835625	4.280650	7.573144	176.9158

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Conc	5	1041.784039	208.356808	3.63	0.1177
Type	1	124.454117	124.454117	2.17	0.2147

The GLM Procedure - Dependent Variable: mDepth

Source	DF	Sum Squares	Mean Square	F Value	Pr > F
Model	6	28.15282854	4.69213809	1.73	0.3107
Error	4	10.87096225	2.71774056		
Corrected Total	10	39.02379079			

R-Square	Coeff Var	Root MSE	mDepth Mean
0.721427	2.538949	1.648557	64.93070

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Conc	5	26.10396608	5.22079322	1.92	0.2734
Type	1	2.04886246	2.04886246	0.75	0.4342

The GLM Procedure - t Tests (LSD) for mLength

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	4.635464
Critical Value of t	2.77645
Least Significant Difference	6.4567
Harmonic Mean of Cell Sizes	1.714286

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping		Mean	N	Conc
	A	217.611	1	0
B	A	211.396	2	0.5
B		210.605	2	5
B		210.214	2	1
B		209.904	2	10
B		206.722	2	2

The GLM Procedure - t Tests (LSD) for mWeight

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	57.35251
Critical Value of t	2.77645

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Least Significant Difference	22.711
Harmonic Mean of Cell Sizes	1.714286

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping		Mean	N	Conc
	A	199.797	1	0
B	A	181.027	2	5
B	A	179.855	2	0.5
B	A	177.439	2	10
B		172.533	2	1
B		162.284	2	2

The GLM Procedure - t Tests (LSD) for mDepth

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	2.717741
Critical Value of t	2.77645
Least Significant Difference	4.9439
Harmonic Mean of Cell Sizes	1.714286

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping		Mean	N	Conc
	A	68.389	1	0
B	A	65.744	2	5
B	A	65.379	2	10
B	A	64.808	2	0.5
B	A	64.517	2	1
B		62.477	2	2

The GLM Procedure - t Tests (LSD) for mLength

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	4.635464
Critical Value of t	2.77645
Least Significant Difference	5.775
Harmonic Mean of Cell Sizes	2.142857

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping		Mean	N	Type
	A	217.611	1	C
B	A	212.295	5	P
B		207.241	5	M

The GLM Procedure - t Tests (LSD) for mWeight

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	57.35251
Critical Value of t	2.77645
Least Significant Difference	20.313

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Harmonic Mean of Cell Sizes	2.142857

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Type
A	199.797	1	C
B	178.155	5	P
B	171.100	5	M

The GLM Procedure - t Tests (LSD) for mDepth

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	2.717741
Critical Value of t	2.77645
Least Significant Difference	4.4219
Harmonic Mean of Cell Sizes	2.142857

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Type
A	68.389	1	C
A	65.038	5	P
A	64.132	5	M

The GLM Procedure - Class Level Information

Class	Levels	Values
Conc	5	0.5 1 2 5 10
Type	2	M P

Number of Observations Read	10
Number of Observations Used	10

The GLM Procedure - Dependent Variable: mLength

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	97.6734345	16.2789058	4.69	0.1162
Error	3	10.4080541	3.4693514		
Corrected Total	9	108.0814886			

R-Square	Coeff Var	Root MSE	mLength Mean
0.903702	0.887942	1.862619	209.7682

Source	DF	Type I SS	Mean Square	F Value	Pr > F
mIlength	1	1.11128202	1.11128202	0.32	0.6110
Conc	4	27.58960524	6.89740131	1.99	0.2995
Type	1	68.97254729	68.97254729	19.88	0.0210

The GLM Procedure - Least Squares Means

Conc	mLength LSMEAN	LSMEAN Number
0.5	211.393068	1
1	209.771550	2
2	206.528388	3
5	211.181312	4
10	209.966549	5

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Least Squares Means for effect Conc
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: mLength

i/j	1	2	3	4	5
1		0.4528	0.0800	0.9183	0.4996
2	0.4528		0.1812	0.5274	0.9244
3	0.0800	0.1812		0.0949	0.1633
4	0.9183	0.5274	0.0949		0.5666
5	0.4996	0.9244	0.1633	0.5666	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

Type	mLength LSMEAN	H0:LSMean1= LSMean2 Pr > |t|
M	207.117104	0.0210
P	212.419243	

The GLM Procedure - Class Level Information

Class	Levels	Values
Conc	5	0.5 1 2 5 10
Type	2	M P

Number of Observations Read 10
Number of Observations Used 10

The GLM Procedure - Dependent Variable: mWeight

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	692.2004130	115.3667355	2.71	0.2211
Error	3	127.5303192	42.5101064		
Corrected Total	9	819.7307322			

R-Square	Coeff Var	Root MSE	mWeight Mean
0.844424	3.733646	6.519977	174.6276

Source	DF	Type I SS	Mean Square	F Value	Pr > F
mIweight	1	26.8247180	26.8247180	0.63	0.4850
Conc	4	514.5588816	128.6397204	3.03	0.1949
Type	1	150.8168134	150.8168134	3.55	0.1561

The GLM Procedure - Least Squares Means

Conc	mWeight LSMEAN	LSMEAN Number
0.5	177.571220	1
1	170.927857	2
2	162.540787	3
5	184.933910	4
10	177.164293	5

Least Squares Means for effect Conc
Pr > |t| for H0: LSMean(i)=LSMean(j) -Dependent Variable: mWeight

i/j	1	2	3	4	5
1		0.3842	0.1114	0.4068	0.9550
2	0.3842		0.2952	0.1559	0.4129
3	0.1114	0.2952		0.0482	0.1110
4	0.4068	0.1559	0.0482		0.3513

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5	0.9550	0.4129	0.1110	0.3513

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

H0:LSMean1= mWeight LSMean2

Type	LSMEAN	Pr > |t|
M	170.716145	0.1561
P	178.539082	

The GLM Procedure - Class Level Information

Class	Levels	Values
Conc	5	0.5 1 2 5 10
Type	2	M P

Number of Observations Read 10
Number of Observations Used 10

The GLM Procedure - Dependent Variable: mDepth

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	15.31734883	2.55289147	0.73	0.6631
Error	3	10.55147193	3.51715731		
Corrected Total	9	25.86882076			

R-Square	Coeff Var	Root MSE	mDepth Mean
0.592116	2.903789	1.875409	64.58488

Source	DF	Type I SS	Mean Square	F Value	Pr > F
mIdepth	1	1.22693244	1.22693244	0.35	0.5963
Conc	4	11.73309048	2.93327262	0.83	0.5831
Type	1	2.35732590	2.35732590	0.67	0.4729

The GLM Procedure - Least Squares Means

mDepth LSMEAN

Conc	LSMEAN	Number
0.5	64.8224594	1
1	64.6060286	2
2	62.5872468	3
5	65.7008681	4
10	65.2078079	5

Least Squares Means for effect Conc
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: mDepth

i/j	1	2	3	4	5
1		0.9161	0.3247	0.6730	0.8577
2	0.9161		0.3609	0.6096	0.7897
3	0.3247	0.3609		0.2075	0.2998
4	0.6730	0.6096	0.2075		0.8142
5	0.8577	0.7897	0.2998	0.8142	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

H0:LSMean1= mDepth LSMean2

Type	LSMEAN	Pr > |t|
M	64.0471319	0.4729
P	65.1226324	

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Growth Experiment II - Female

The GLM Procedure - Class Level Information

Class	Levels	Values
Conc	6	0 0.5 1 2 5 10
Type	3	C M P

Number of Observations Read 196

Number of Observations Used 196

The GLM Procedure - Dependent Variable: Length

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	2933.74895	293.37489	0.68	0.7420
Error	185	79793.77657	431.31771		
Corrected Total	195	82727.52551			

R-Square	Coeff Var	Root MSE	Length Mean
0.035463	9.870190	20.76819	210.4133

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Conc	5	1497.527560	299.505512	0.69	0.6283
Type	1	1105.465019	1105.465019	2.56	0.1111
Conc*Type	4	330.756366	82.689092	0.19	0.9425

The GLM Procedure - Dependent Variable: Weight

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	25391.9827	2539.1983	1.19	0.2973
Error	185	393363.6455	2126.2900		
Corrected Total	195	418755.6282			

R-Square	Coeff Var	Root MSE	Weight Mean
0.060637	26.11588	46.11171	176.5658

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Conc	5	19093.69608	3818.73922	1.80	0.1156
Type	1	2178.92995	2178.92995	1.02	0.3127
Conc*Type	4	4119.35667	1029.83917	0.48	0.7472

The GLM Procedure - Dependent Variable: Depth

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	705.06606	70.50661	1.19	0.2973
Error	185	10923.23496	59.04451		
Corrected Total	95	11628.30102			

R-Square	Coeff Var	Root MSE	Depth Mean
0.060634	11.84298	7.684043	64.88265

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Conc	5	476.7854775	95.3570955	1.62	0.1580
Type	1	37.0202192	37.0202192	0.63	0.4295
Conc*Type	4	191.2603650	47.8150913	0.81	0.5203

The GLM Procedure - t Tests (LSD) for Length

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	185
Error Mean Square	431.3177
Critical Value of t	1.97287
Least Significant Difference	10.482
Harmonic Mean of Cell Sizes	30.55857

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NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping		Mean	N	Conc
	A	217.611	18	0
B	A	211.543	35	0.5
B	A	210.485	33	5
B	A	210.053	38	1
B	A	209.800	35	10
B		206.730	37	2

The GLM Procedure - t Tests (LSD) for Weight

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	185
Error Mean Square	2126.29
Critical Value of t	1.97287
Least Significant Difference	23.273
Harmonic Mean of Cell Sizes	30.55857

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping		Mean	N	Conc
	A	199.80	18	0
B	A	180.92	33	5
B	A	179.87	35	0.5
B	A	177.13	35	10
B		172.20	38	1
B		162.20	37	2

The GLM Procedure - t Tests (LSD) for Depth

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	185
Error Mean Square	59.04451
Critical Value of t	1.97287
Least Significant Difference	3.8783
Harmonic Mean of Cell Sizes	30.55857

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping		Mean	N	Conc
	A	68.389	18	0
B	A	65.758	33	5
B	A	65.314	35	10
B	A	64.829	35	0.5
B		64.474	38	1
B		62.459	37	2

The GLM Procedure - t Tests (LSD) for Length

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	185
Error Mean Square	431.3177
Critical Value of t	1.97287

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Least Significant Difference	9.3449
Harmonic Mean of Cell Sizes	38.448

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping		Mean	N	Type
	A	217.611	18	C
B	A	212.180	89	P
B		207.191	89	M

The GLM Procedure - t Tests (LSD) for Weight

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	185
Error Mean Square	2126.29
Critical Value of t	1.97287
Least Significant Difference	20.749
Harmonic Mean of Cell Sizes	38.448

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Type
A	199.80	18	C
B	177.71	89	P
B	170.73	89	M

The GLM Procedure - t Tests (LSD) for Depth

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	185
Error Mean Square	59.04451
Critical Value of t	1.97287
Least Significant Difference	3.4575
Harmonic Mean of Cell Sizes	38.448

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping		Mean	N	Type
	A	68.389	18	C
B	A	64.966	89	P
B		64.090	89	M

F] Limitations and Constraints to the Experiment

1 LIMITATIONS TO THE WORK

-Unavailability of fish at the start of the experiment

-Size Range

-Age and conditions of rearing fish

-Operational difficulties

-Preparation of feed fortnightly

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2 CONSTRAINTS TO THE EXPERIMENT

Fish Selection

The experiment faced a number of challenges which nearly marred its smooth take off, which include:

-By the time the experiment commenced available fish produced in December 2009 which would have provided uniform size for the study had been sold out to farmers by the Technical Manager citing lack of space in the existing facilities, and also overcrowding stretching the carrying capacity of the available space in the systems (grey system and raceway)

-The rest were sent to the tunnel (pond system made from tarpaulin, another holding facility for rejected fish and those not being used for experiments) to mix with stunting and diseased fish, suffering massive mortality

-Similarly fish produced for experiment II suffered the same fate of limited holding facility and desire to disposed them off by the Technical Manager

-This actually placed a strain on selection of fish for the experiment.

Experiment I

-It took more than 8months (September 2009 - July 2010) to complete experimental unit

-When it became fully operational in August 2010 there were no or very small fish for the experiment, particularly those specifically breed for the experiment either disposed off or dead

- Fish for stocking were not enough, so had to rely on those from the tunnel for stoking, hence the wide size range, 20-40g

-It took 20days to obtain sexually mature fish even within that wide size range to stock 70 tanks

-Because fish kept in the tunnel were overcrowded, they appeared stunted. So when placed in more spacious glass tanks, the fish quickly showed reproductive behavior (i.e. dark bodied fish developed within 3-days of stocking trying to establish territory)

-Most fish had poorly developed urogenital (or urinogenital) papilla and fins (dorsal and anal) which were the main external morphological characteristics used to differentiate and select male and female, and that led to wrong sexing of some fishes. This thus, increased number of sexes in some tanks by 1 or 2. So instead of having 5♂:5♀, some tanks had 4 or 3♂:6 or 7♀, and vice versa (6 or 7♂:4 or 3♀).

-In replicate tanks where 5-8 fishes died it was difficult to get a specimen at the 60-day sampling for analysis.

Experiment II

-Available fish weighing 5 -10g appeared old, because after 3days in glass tanks some started breeding (i.e. a fish weighing 8g had eggs in the mouth so had to be removed)

-Most of the fish were stunted, produced in November-December 2010, and due to limited space available were over crowded (Fry nursed at high densities for considerable periods of time, prior to stocking may be stunted, but sexually matured and will thus spawn at smaller size, Mair & Little, 1991). This is the case of fish in grey system being reared, which I had to use for the experiment, thus the long stocking period.

-Fishes showing signs of maturation and spawning behavior had to be replaced with sexually immature ones, hence the wide size range, 2-8g.

-It took 20days to get sexually immature fish to stock the 55 tanks

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3 CHALLENGES

Some Challenges on the construction

The whole layout of the experimental unit was constructed between March and June 2010. The materials (glass tanks, steel stands and blue tanks) were parts of an old aquaria system in Block 2 of DA offices. It was dismantled and sent to a greenhouse attached to Block 3 (DA-Buildings) in September 2009. The layout and arrangement of the platforms commenced in October 2009, however, actual construction began in April 2009 and completed in July 2010. Water pumps and heaters were installed and tested in August 2010. The whole experimental unit became fully operational in September 2010, and actual experiment commencing in October 2010. The major challenges encountered in the construction include:

- slow pace in ordering (by technical manager of the DA-WEF) and delivery of materials by suppliers e.g. it took over 2-months for PVC pipes to be delivered (order placed in September 2009 arrived in December 2009)
- non-committal attitude of the technical manager who was to provide technical guidance.
- delay in delivery of components constructed by external contractors e.g. the carrier of inlet-pipes, parts of broken glass tanks
- delay in installation of water pump and heaters
- continuous alterations in the design, particularly, the filtration component

The whole system was built based on my own design adapted from existing recirculating systems on the farm and construction done alone.

The experiment could not commence in February 2011 as was planned in 2010, due to repairs of the experimental unit described above. The main challenge was unavailability of fingerlings in right size 2g. Available fish weighing 5-10g were old, after 3days in glass tanks some started breeding. Capacity of available facilities do not permit production of my enough fingerlings, hence have to take samples from the general pool of fingerlings produced in the farm.

Most were stunted, produced in November-December 2010 (fry nursed at high densities for considerable periods of time, prior to stocking may be stunted, but sexually matured and will thus spawn at smaller size, Mair & Little, 1991). This is the case of fish in grey system (Welgevallen Experimental Farm DA-SU), which I had to use for the experiment, thus taking a long stocking period. Fishes showing signs of maturation and spawning behavior had to be replaced with sexually immature ones, hence the wide size range, 2-8g. It took 20days to get sexually immature fish to stock the 60tanks.

Fish Storage

Samples of fish kept in Department of Genetics central cold room from the first experiment showed signs of deterioration when I went for them in October 2011 for the HPLC analysis. I was informed by the manager of the cold room that the system broke down twice within the year 2011 and they could not transfer my samples to other department cold rooms. The study wanted to do analysis on the head, flesh and internal organs. However, due to the level of deterioration of internal organs, particularly, the gonads could not be used for the analysis that was done.