UNIVERSITI PUTRA MALAYSIA

SEMINAL CHARACTERISTICS OF GENETICALLY IMPROVED FARMED TILAPIA (GIFT) STRAIN INDUCED WITH DIFFERENT DOSES OF SPAWNING AGENT

NUR SYAFIQAH BINTI ABDUL AZIZ

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NUR SYAFIQAH BINTI ABDUL AZIZ

A project paper submitted to the
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CERTIFICATION

It is hereby certified that we have read this project paper entitled "Seminal Characteristics of Genetically Improved Farmed Tilapia (GIFT) Strain Induced with Different Doses of Spawning Agent", by Nur Syafiqah binti Abdul Aziz and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD 4999 – Project

ASSOC. PROF. DR ROSNINA BINTI HJ. YUSOFF
DVM (UPM), MSc (UPM), PhD (Guelph)
Department of Veterinary Clinical Studies
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Supervisor)

ASSOC. PROF. DR HASSAN HJ. MOHD DAUD
DVM (UPM), MSc (Scotland), PhD (England)
Department of Veterinary Clinical Studies
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Co-Supervisor)

PROF. DR MOHD ARIFF OMAR
BSc (LSU), MSc (OSU), PhD (Texas A&M)
Department of Veterinary Pre Clinical Science
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Co-Supervisor)

DEDICATIONS

This project paper is dedicated to Allah S.W.T., who had created me and made all things possible,

To my family,

Father

Mother

Brothers & Sisters

And to all my lecturers who have committed themselves towards the noble cause of education.

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LIST OF ABBREVIATIONS

%	Percent
L	Liter
ml	Milliliter
µl	Microliter
µm	Micrometer
g	Gram
cm	Centimeter
mm	Millimeter
ml/kg	Milliliter per Kilogram
mOsm/kg	Milliosmoles per Kilogram
g/L	Gram per Liter
mg/L	Milligram per Liter
GIFT	Genetically Improved Farmed Tilapia
SGnRH	Salmon Gonadotropin-releasing hormone
GnRH	Gonadotropin-releasing hormone
PG	Pituitary Gland
hCG	Human Chorionic Gonadotropin
LRHa	Luteinizing Releasing Hormones Analogues

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 - Projek

CIRI – CIRI SEMINAL DALAM STRAIN GENETICALLY IMPROVED FARM TILAPIA (GIFT) DIBAWAH PENGARUH AGEN PENELURAN DENGAN MENGGUNAKAN DOS BERBEZA

Oleh

Nur Syafiqah binti Abdul Aziz

2016

Penyelia: Prof. Madya Dr. Rosnina binti Hj. Yusoff

Penyelia Bersama:

Prof. Madya Dr. Hassan Hj. Mohd Daud

Prof. Dr. Mohd Ariff Omar

Strain Genetically Improved Farm Tilapia (GIFT) telah dihasilkan dari tilapia *Nile* melalui pembiakan selektif dan telah diternak secara meluas kerana mempunyai daya tahan yang tinggi terhadap pelbagai keadaan air. Hasil kajian ke atas kesan agen peneluran (untuk merangsang kematangan dan meninggikan pengeluaran sperma) terhadap ciri – ciri sperma yang dihasilkan oleh strain GIFT adalah sangat terhad. Kajian ini dilaksanakan untuk mengetahui kesan dos berbeza bagi agen peneluran (Ovaprim®) ke atas ciri – ciri sperma strain GIFT serta untuk menentukan dos optimum bagi agen peneluran. Terdapat

empat kumpulan rawatan yang terdiri daripada 0 (kawalan), 0.1, 0.3 dan 0.5 ml/kg dan berat badan adalah di antara 200 hingga 360 gram. Suntikan intraotot dilakukan terhadap ikan tersebut dan sperma diambil selepas 12 jam (tempoh pengeraman) melalui tekanan lembut pada abdomen. Hasil sperma perlu dicatit dan parameter sperma seperti tumpatan, pergerakan, kebolehhidupan dan morfometri sperma perlu dinilai. Terdapat perubahan ketara untuk pergerakan sperma tetapi tiada perubahan ketara untuk parameter lain. Kumpulan rawatan 0.5 ml/kg mempunyai purata yang tertinggi bagi tumpatan, pergerakan dan kebolehhidupan sperma, yang mana berupaya untuk menjadi dos optimum bagi Ovaprim®. Oleh yang demikian, semua parameter yang diuji perlu dinilai untuk mengenalpasti kuantiti dan kualiti sperma.

Kata kunci: *strain GIFT, agen peneluran, sperma, dos optimum*

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine, UPM in partial requirement for the course of VPD 4999 – Project.

SEMINAL CHARACTERISTICS IN GENETICALLY IMPROVED FARM TILAPIA (GIFT) STRAIN INDUCED WITH DIFFERENT DOSES OF SPAWNING AGENT

By

Nur Syafiqah binti Abdul Aziz

2016

Supervisor: Assoc. Prof. Dr. Rosnina binti Hj. Yusoff

Co-Supervisor:

Assoc. Prof. Dr. Hassan Hj. Mohd Daud

Prof. Dr. Mohd Ariff Omar

Genetically Improved Farm Tilapia (GIFT) strain was developed through selective breeding of Nile tilapia and was cultured worldwide due to its ability to tolerate wide range of water conditions. There were limited studies on the effects of spawning agent (to induce maturation and increase milt production) on seminal characteristics of GIFT strain. This study was carried out to determine the effects of different doses of a spawning agent (Ovaprim®) on seminal characteristics of GIFT strain as well as to determine the optimum dose of the spawning agent. There were four treatment groups which consisted of 0

(control), 0.1, 0.3 and 0.5ml/kg and the body weight was between the range of 200 to 360 grams. The fish was injected intramuscularly and the milt was collected after 12 hours (latency period) through gentle abdominal pressure. The volume of milt was recorded and sperm parameters such as sperm density, motility, viability and morphometry were evaluated. There was significant difference for sperm motility but other parameters were not significance. Treatment group 0.5 ml/kg has the highest mean for sperm density, motility and viability, which made it as the optimum dose for Ovaprim®. Thus, these parameters are usually being used to determine the sperm quantity and quality.

Keywords: *GIFT strain, spawning agent, milt, optimum dose*

1.0 INTRODUCTION

Tilapia is a freshwater species of the family, Cichlidae and is cultured worldwide for its hardiness as it is able to tolerate a wide range of temperature and salinity (Asian Development Bank, 2005). Nile tilapia was chosen for the breeding program of Genetically Improved Farmed Tilapia (GIFT) strain due to its short generation time of about six months (Asian Development Bank, 2005) compared with other tilapia species including Mozambique, red and black tilapia.

There are several hormonal agents that are commercially available for induction of spawning. These include pituitary gland (PG), human chorionic gonadotropin (hCG), and luteinizing releasing hormone analogues (LRHa). In two recent studies, African catfish has shown success in increasing its sperm quality and quantity when spawning is induced with the synthetic agent, Ovaprim® (Kasi *et al.,* 2015; Gbemisola and Adebayo, 2014). In addition, Ovaprim® was also effective in inducing final maturation, increasing milt production and improving the fecundity in African catfish (Kasi *et al.,* 2015; Achionye-Nzeh and Obaroh, 2012), fresh water Angelfish (Chatterjee *et al.,* 2013) and Rohu (Naeem *et al.*, 2013). However, from our literature search, Ovaprim® has not been used to induce spawning in GIFT strain. Therefore, due to the limited study on the effects of Ovaprim® on the seminal characteristics of GIFT strain, this project was conducted. GIFT strain was chosen due to its availability at the Department of Aquaculture, Faculty of Agriculture, UPM. It is hardy and has a faster growth rate (Asian Development Bank, 2005) compared with other tilapia strains or species.

This study was initiated with the objectives to compare the seminal characteristics of GIFT strain induced with different doses of Ovaprim® and to establish the optimum dose of Ovaprim® in GIFT strain in order to significantly increase tilapia production in meeting the demand for freshwater fish in Malaysia. The justifications for this study includes, to induce and synchronize spawning at any time convenient to the fish farmer as well as to increase freshwater aquaculture production due to decreasing marine fish production.

1.1. HYPOTHESIS

Null hypothesis (H_0) : Whole milt density and individual sperm morphometry, motility and viability are similar in GIFT strain tilapia induced with different doses of spawning agent.

Alternative hypothesis (H_A) : Whole milt density and individual sperm morphometry, motility and viability are different in GIFT strain tilapia induced with different doses of spawning agent.

2.0 LITERATURE REVIEW

2.1 Genetically Improved Farmed Tilapia (GIFT) Strain

Nile tilapia was introduced in Malaysia in 1944 (Ang *et al.,* 1989) for freshwater farming due to the continuous decrement in sustaining captured fishery production to meet high global demand (Subasinghe *et al.,* 2009). However, one of the problems encountered in the farming of Nile tilapia is delayed maturation, which led to a longer period for the broodstock to start spawning (Asian Development Bank, 2005). Thus, the purpose of breeding GIFT strain is to overcome the mentioned problem. GIFT strain has a faster growth rate and can be aquacultured under diverse farm environment so that they can become broodstocks (Asian Development Bank, 2005) with higher harvest growth rate (Santos *et al.,* 2013). Selective breeding of Nile tilapia was used instead of crossbreeding to improve the genetic of Nile tilapia as it is the least expensive way to increase the aquaculture production, where individual genotypes are directly heritable from parents to offspring (Lutz, 2006) as well as to successfully produce a faster growing generations of Nile tilapia (Asian Development Bank, 2005).

Philippines had started selective breeding of GIFT in 1989, where the synthetic base population was established to ensure high genetic variability before selection, using four African wild strains and four existing Asian strains of Nile tilapia farmed in the Philippines (Asian Development Bank, 2005). In Malaysia, GIFT strain is under the care of the World Fish Centre, located in Penang, in collaboration with the Department of Fisheries, Malaysia. GIFT strain was transferred to Malaysia from the Philippines

between end of 2000 and beginning of 2001 (Khaw *et al.,* 2012). The World Fish Centre disseminate GIFT strain to other countries such as Brazil in 2005 (Santos *et al.,* 2013) as well as to the government and private hatcheries in Malaysia (Khaw *et al.,* 2012). GIFT helps to compensate the high demand for protein in both rural and urban areas (Asian Development Bank, 2005). It has been shown that the body weight of GIFT strain is 18%-58% higher compared with non-GIFT strains farmed in Asia (Ansah *et al.,* 2014).

2.2 Sexual Characteristics

The body shape and position of genital papilla are used to differentiate between a male and a female tilapia. In males, the genital opening is found behind the genital papilla (Diagram 1), which can be seen clearly in mature tilapia whereas in females, the genital opening is situated above the genital papilla (Diagram 2) (Chhorn, 2006). The genital papilla should be checked carefully to ensure that they are ready to spawn before hormonal induction. Mature females tend to have a reddish genital papilla and its vaginal opening is clearly seen by the naked eyes (Chhorn, 2006) whereas mature males will release milt (semen) under slight abdominal pressure (Mohd Yusof, 2008).

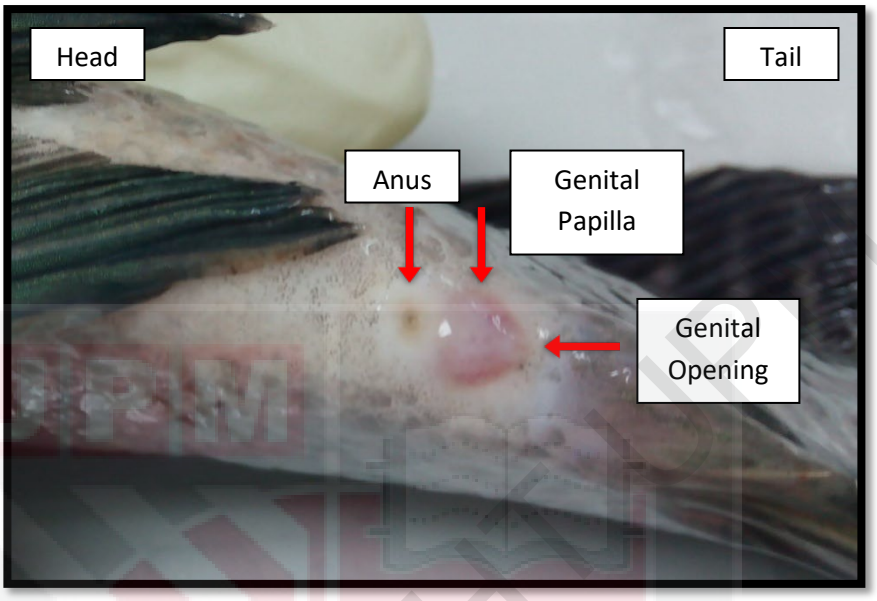


Diagram 1: A male GIFT strain (Genital opening behind genital papilla)

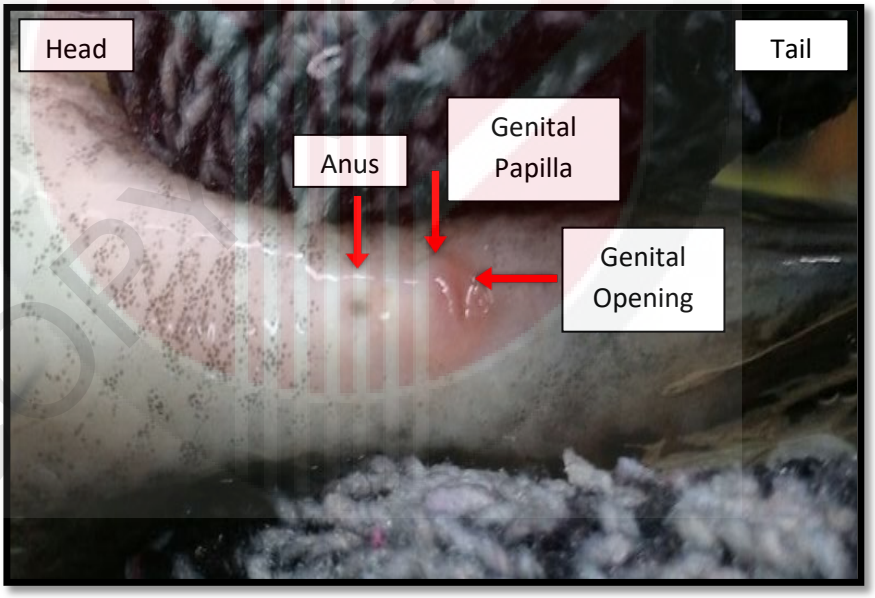


Diagram 2: A female GIFT strain (Genital opening above genital papilla)

According to Dupenchelle and Legendre (2000), there are six stages that represent maturity status. Stage l - immature or virgin fish, Stage ll – early maturation or beginning of maturation and Stage lll – developing stage. Stage lV is the pre-spawning stage, where

the eggs appear yellow in color and the ovaries occupy almost the entire peritoneal cavity in females; males will have whitish colour testicles. Spawning stage is known as stage V, where the eggs are loosened from the ovary and in males, slight pressure on the abdomen will cause the milt to flow out and stage Vl is post spawning stage.

The age and size of tilapia as well as the environmental conditions can affect their sexual maturity. Under a good environmental condition in farm ponds, it can reach sexual maturity at 5-6 months with body weight of 150-200 grams (Popma and Masser, 1999). Sexual maturity can be delayed for several months if the tilapia has a slow growth rate and it may spawn at a weight less than 20 grams in cases of stunted growth (Popma and Masser, 1999). Unfavorable ambience tends to accelerate maturation in tilapia of genus *Oreochromis* (Nyakuni, 2009). Under stressful conditions, earlier maturation can also be induced for the fish to maximize its reproductive success (Charlesworth and Leon, 1976).

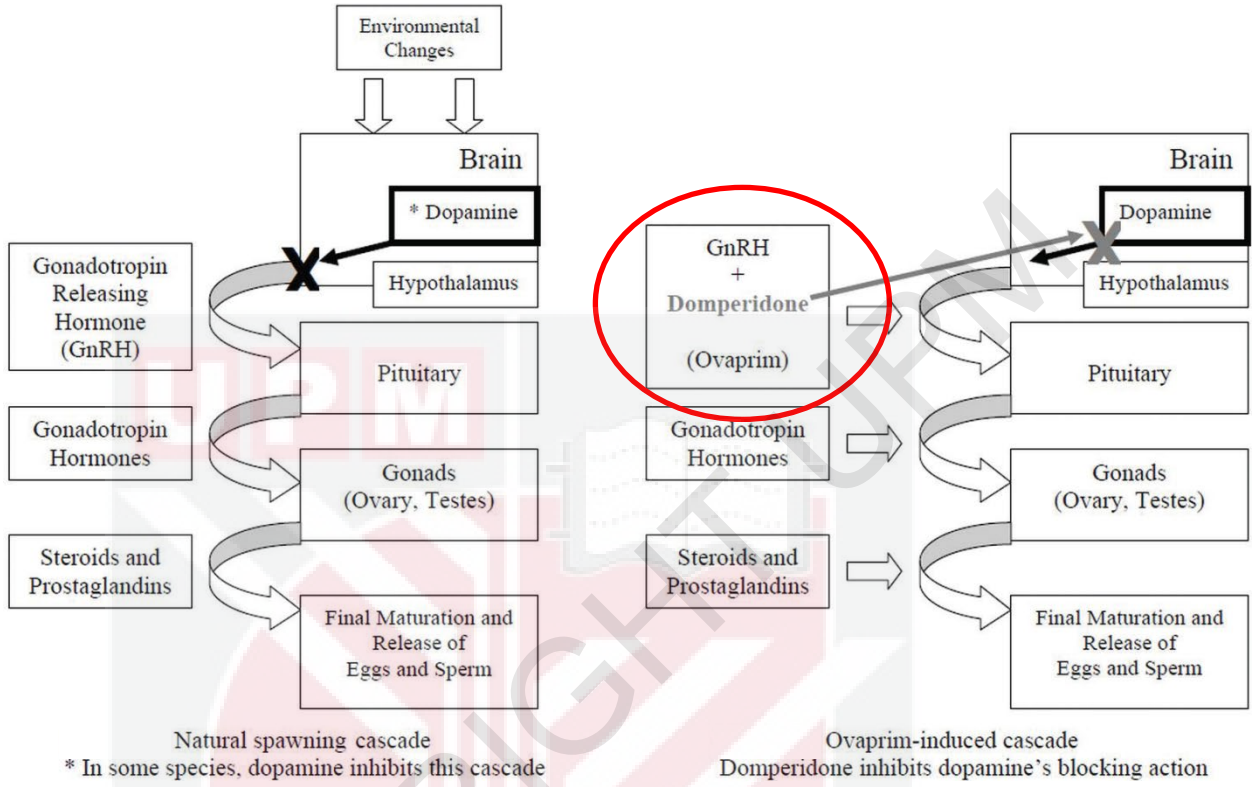
2.3 Physiology of Ovaprim® Induced Reproductive System

According to Olumuji and Mustapha (2012), the aquaculture industry can be sustained and boosted through artificial propagation of the fish population in order to obtain a good quality fish seed all year round. Artificial propagation, through exogenous hormonal induction using synthetic hormones such as Ovaprim® can increase tilapia production as it helps to speed up the spawning event (Mohd Yusof, 2008). Ovaprim® is widely available as a commercial product, which has been proven effective and safe to be used in numerous finfish species and it contains salmon gonadotropin-releasing hormone

analogue (SGnRH) and domperidone (dopamine antagonist) (Naeem *et al*., 2013; Sahoo *et al*., 2007).

Egg maturation and release in females as well as sperm maturation and release in males are dependent on the environmental and internal factors of fish, which will subsequently trigger a hormonal cascade. Generally, the hypothalamus-pituitary-gonadal axis regulates the reproductive processes in fish (Peter *et al.,* 1988). Under natural spawning (Figure 1), hypothalamus releases gonadotropin-releasing hormone (GnRH) to act on the pituitary gland to release gonadotropic hormones (especially GtH-ll). Gonads (ovaries and testes) are stimulated by gonadotropic hormones to synthesize steroids and prostaglandins, which act directly to induce final maturation and release of oocytes in females or sperm in males (Rottmann *et al.,* 1991a; Evans and Claiborne, 2006).

In Ovaprim®-induced spawning (Figure 1), this synthetic GnRH administered into the body cavity or muscle of fish, will enter the blood circulation and acts on the pituitary gland, which will then initiate the hormonal cascade of reproductive system. Natural trigger is no more needed to activate the hormonal cascade (Yanong *et al*., 2009). Under certain conditions such as stress, fish tends to produce a compound called dopamine, which naturally blocks the hormonal cascade by preventing the GnRH to function properly (Rottmann *et al.,* 1991a). Thus, domperidone helps to overcome this problem by inhibiting the release of dopamine (Sridhar and Haniffa, 2002; Pao *et al.,* 1999). Two basic environmental factors that need to be at the optimum level are water temperature and dissolved oxygen content (Sridhar and Haniffa, 2002).



(Yanong *et al.*, 2009)

Figure 2.1: Natural spawning cascade and Ovaprim®-induced cascade

2.4 Spermatogenesis in Fish

Testis in the fish is an elongated structure located in the peritoneal cavity and it elongates posteriorly by a sperm duct and vas deferens, which terminates at the genital papilla. Most teleosts (ray-finned fishes) have a paired testes (Nagahama, 1983) and there are two main types of testicular structure: lobular and tubular. Teleosts have lobular testes, which comprised a number of separated lobules, where primary spermatogonia are found. Sertoli cells enclosed the primary spermatogonium to form spermatogenic cysts that

contained several spermatogonial cells or germ cells through numerous mitotic divisions. The cysts will rupture when all the germ cells are matured and the Sertoli cells will release the sperm into the lobular lumen, which is connected to the sperm duct and this is known as spermiation (Pudney, 1993 and Nagahama, 1983). The primary factor that is responsible for testicular growth is Sertoli cell proliferation and it helps to increase sperm production (Vilela *et al*., 2003).

Spermatocytogenesis is a process where spermatogonia are transformed into primary spermatocytes after they undergo mitotic divisions. Then, they proceed to undergo a meiotic division to produce secondary spermatocytes (two daughter cells), which will transform into haploid spermatids after second meiotic division. A maturation process of spermatids will take place to become functional male gametes, which are known as spermatozoa (Musa, 2010). Spermatogenesis in Nile tilapia depends on water temperature, where the process will be dramatically accelerated at 30°C (Vilela *et al.,* 2003).

2.5 Sperm Motility

Estimation of sperm motility is carried out to measure sperm cell quality and viability (Stoss and Holtz, 1983) and it is usually measured as a percentage of sperm motility or total duration of sperm motility or both (Stoss, 1983). Sperm motility can be scored from 0 to 5 (Cosson, 2004). Sperm motility varies among different species of fish and last about 20 seconds even though some sperm may show agitation with low beat

frequency and with very limited displacement (Billard and Cosson, 1992). It is quite challenging to score the motility by using conventional method due to the presence of variables and is also difficult to make comparisons (Musa, 2010). Thus, computer-aided sperm analysis (CASA) was introduced to measure sperm motility accurately, rapidly and is repeatable (Musa, 2010).

There are several factors that affect sperm motility and it includes temperature. Low temperature will prolong the duration of motility but at the same time it reduces the sperm velocity (Hines and Yashouv, 1971). Sperm are immotile in the reproductive organ and seminal plasma and their motility can only be activated once they are out into the aqueous environment (Stoss, 1983). This is because factors such as ions, pH and osmotic pressure affect motility (Cosson *et al.,* 1999). Spermatozoa of freshwater fish experienced hypoosmotic change when they enter freshwater whereas spermatozoa of marine fish experienced hyperosmotic change due to high osmolality of sea water (Billard, 1978; Morisawa *et al.,* 1983).

3.0 MATERIALS AND METHODS

3.1 Fish Management and Maintenance

About 30 GIFT strains with body weights between 200g and 360g were procured from Puchong Hatchery Unit, Faculty of Agriculture, UPM. These fish were reared in a 40 tonne tank and supplied with underground water. The fish were transported to the Aquatic Laboratory, Faculty of Veterinary Medicine, UPM and each fish was maintained in a 10 L tank. They were acclimatized for two days before injection of Ovaprim® and fed twice daily with commercial pellets (28% crude protein, 5% crude fat, 5% crude fiber and 12% moisture). The feed was given at 5% of their estimated body weight (Kasi *et al*., 2015). Air pumps connected with air stones were placed in each tank for aeration and the fishes were treated with sodium chloride (1.0 g/L) to kill any ectoparasites that present on the body surface as well as to reduce transportation stress.

3.2 Hormonal Injection Technique and Ovaprim® Doses

There were four treatment groups (control, 0.1 ml/kg, 0.3 ml/kg, 0.5 ml/kg) and each group comprised six male GIFT strain. Immobilization of fish was conducted by using an anesthetic agent, tricaine methane sulfonate (MS-222) (Yanong *et al*., 2009) with concentration of 200mg/L (Musa, 2010) for easy administration of drug as well as to prevent stress during handling. Ovaprim® was administered intramuscularly (IM) on the dorsal muscle of the fish above the lateral line but below the anterior part of the dorsal fin (Pao *et al.,* 1999). The needle was slid under the scale towards the head at an angle of 45^{o} to the body's longitudinal axis, to a depth of about 1.5 cm and the plunger was pushed slowly to deliver the hormone into the body muscle (Musa, 2010; Naeem *et al.,* 2013). To

prevent the Ovaprim® from backflow, the area of injection was massaged slowly and left for a few seconds before the needle was withdrawn.

The volume of Ovaprim® was calculated by taking into consideration the drug concentration, body weight and the desired dose of injection. The formula for the calculation of Ovaprim® was according to Syndel Laboratory (2003), the manufacturing company of Ovaprim®. The following is the formula:

Volume of injection (ml) = Body weight x Ovaprim® dosage

A single injection of Ovaprim® was administered to each fish from the treatment groups to induce final maturation (Pao *et al.,* 1999) with fixed latency period of 12 hours.

3.3 Milt Collection

The fish was anaesthesized with MS 222 (200 mg/L) prior to milt collection. The abdomen and urogenital papilla were dried with a clean paper towel to avoid any contamination of sperm with water, urine and body mucus. The abdomen was pressed gently and massaged using the forefinger and thumb from the direction of the head caudally to the tail (Musa, 2010). Milt was collected into a sterile test tube and immediately stored in crushed ice at 4°C (Muchlisin *et al.*, 2015).

3.4 Sperm Density

Sperm count was conducted using a haemocytometer (depth: 0.1mm) (Fauvel *et al.,* 2010). Quick observation of milt was performed by placing 10µl aliquot of milt onto a glass slide and observed under a light microscope at 100x magnification to determine the desired dilution needed. Sperm was activated by adding normal saline or freshwater in the ratio of 1:4 (Navarro *et al.,* 2014). Basically, milts were diluted 100 folds in this study. Sperm count was estimated as described by Tvedt *et al*. (2001). Briefly, 10 µl of diluted sample was pipetted to the underneath of cover slip of a haemocytometer and left to stand for a few minutes. The number of spermatozoa in each of the five larger squares that contained 16 smaller squares, was counted with a tally counter and the sperm density was determined using the following formula (Musa, 2010; Memon *et al*., 2012):

Sperm Density (sperms/ml)= Average cells count x Dilution factor x Conversion factor

The conversion factor is calculated by using the following formula:

Volume = Depth × Area × Number of small squares

$$= \frac{1}{10}\ \text{mm} \times \frac{1}{400}\ \text{mm}^2 \times (5 \times 16)$$

$$= \frac{1}{50}\ \text{mm}^3$$, covert to 1 cm^3 (1 ml). Conversion factor is 50000×

3.5 Sperm Motility

Five µl of diluted sample was pipetted onto a glass slide and observed at 100x magnification with a cover slip for motility score by using Nikon Eclipse E200 microscope. Sperm was scored according to Fauvel *et al*., (2010) based on estimated

percentage of motile cells and the time of progressive movement cessation is considered as the motility duration of sperm (Table 3.1).

Table 3.1: Scoring of sperm motility based on the percentage of motile cells

Score	Percentage of Motile Cells (%)
0	0
1	>0-20
2	>20-40
3	>40-60
4	>60-80
5	>80-100

3.6 Sperm Viability

Sperm viability was determined by using modified eosin-nigrosin staining method (Nimrat *et al.,* 2005; Memon *et al.,* 2012). The stain was prepared by dissolving 1.67g of eosin, 10g nigrosin and 2.9g sodium nitrate in 100ml of distilled water. 10µl of diluted sperm suspension with dilution of 1:100 was pipetted onto a clean glass slide and mixed with 10µl of eosin-nigrosin stain. Five µl of the mixture was pipetted into another clean glass slide to stain the smear. Three duplicates were prepared and allowed to air dry before examination. Each slide was observed under 100x magnification with oil immersion.

Dead sperm were stained purple either completely or partially, whereas live sperm were unstained but rather having a translucent appearance (Evans and Maxwell, 1987). The survival average percentage was obtained by counting a minimum of 100 sperms per glass slide and the sperm viability was calculated by using the following equation (Noor-Hidayati *et al.,* 2014):

$$\text{Live sperm (\%)} = \frac{\text{Total number of live sperms observed}}{\text{Total number of overall sperms observed (live + dead)}} \times 100$$

3.7 Sperm Morphology and Morphometry

Sperm morphology was evaluated using an image analyzer (Moticam Pro 285A). Any sperm abnormalities were identified. Spermatozoa of teleosts are characterized by the absence of acrosome and have a variable shape of head and nucleus (Billard and Cosson, 1992; Billard *et al.,* 1982), present of mitochondria in the midpiece and have a simple flagellum (Billard *et al.,* 1995a). The individual morphometry of sperm was done for each treatment group to find for any significant different in the measurements of sperm length. The sperm was observed under 100x magnification and the image was captured by Moticam Pro 285A, attached to Motic BA410 microscope. Motic Images Plus 2.0 Software was used to measure each parameter.

3.8 Statistical Analysis

All data and measurements were subjected to descriptive analysis and one way analysis of variance (ANOVA) to determine differences among treatments and followed by Tukey HSD post hoc test for parametric data and Kruskal-Wallis for non-parametric data to determine which treatments were significantly different from one another. The significant level of the results was set at $p < 0.05$ and data was reported as Mean+SD. All the statistical analyses were performed using the computerized statistical package, SPSS Statistics 22.

4.0 RESULTS

4.1 Sperm Parameters

The results of induced breeding of GIFT strain with the synthetic hormone, Ovaprim® are tabulated. The mean initial body weight of each GIFT strain used in each

treatment group was recorded (Table 4.1) to determine the volume of Ovaprim® that needs to be injected. Treatment group 0.1 ml/kg showed the highest mean volume of milt that was collected after 12 hours of latency period (Figure 4.1). However, 0.5 ml/kg treatment group gave the highest mean sperm density (Figure 4.2), instead of 0.1 ml/kg group. From these results, the volume of milt was not directly proportional to the sperm density. Volume of milt and sperm density did not fit the normal distribution so, Kruskal-Wallis test was used for non-parametric data and it showed that there were no significant difference ($p>0.05$) among treatment groups for both parameters. The standard deviation especially for Figure 4.2 had larger values compared to the mean and this is due to the variables that are wide apart in each treatment group. To eliminate this problem, the outliers need to be removed and sample size should be larger (more than six).

Table 4.1: Mean initial body weight of GIFT strain used in each treatment group

Treatment Groups	Body Weight (g)
Control	262.17 ± 19.58
0.1 ml/kg	221.67 ± 13.90

0.3 ml/kg	294.67 ± 53.57
0.5 ml/kg	249.50 ± 40.32

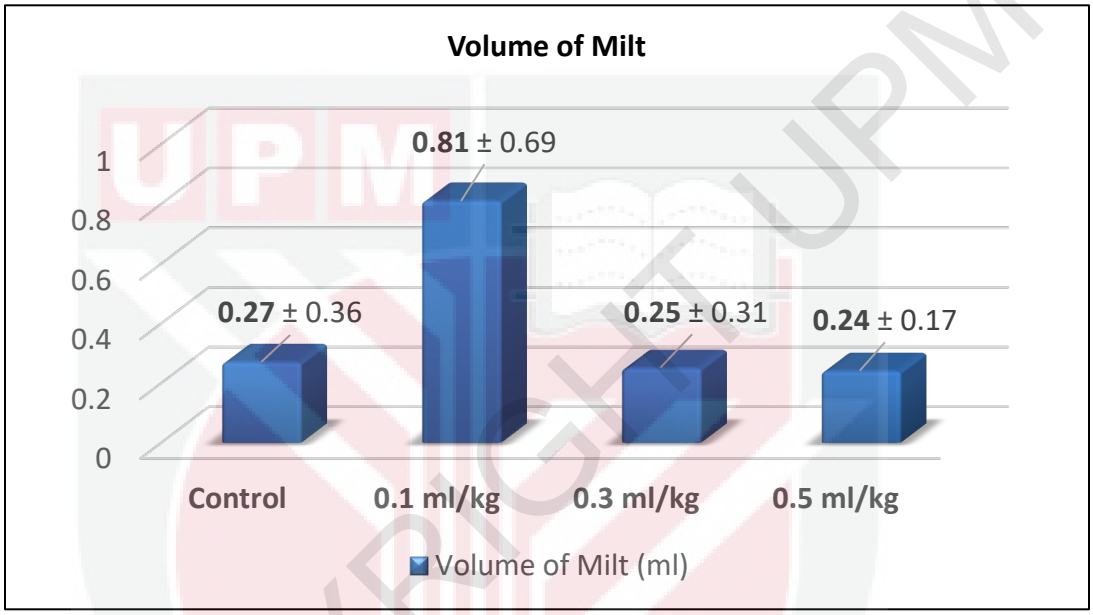


Figure 4.1: Mean volume of milt produced in each treatment group

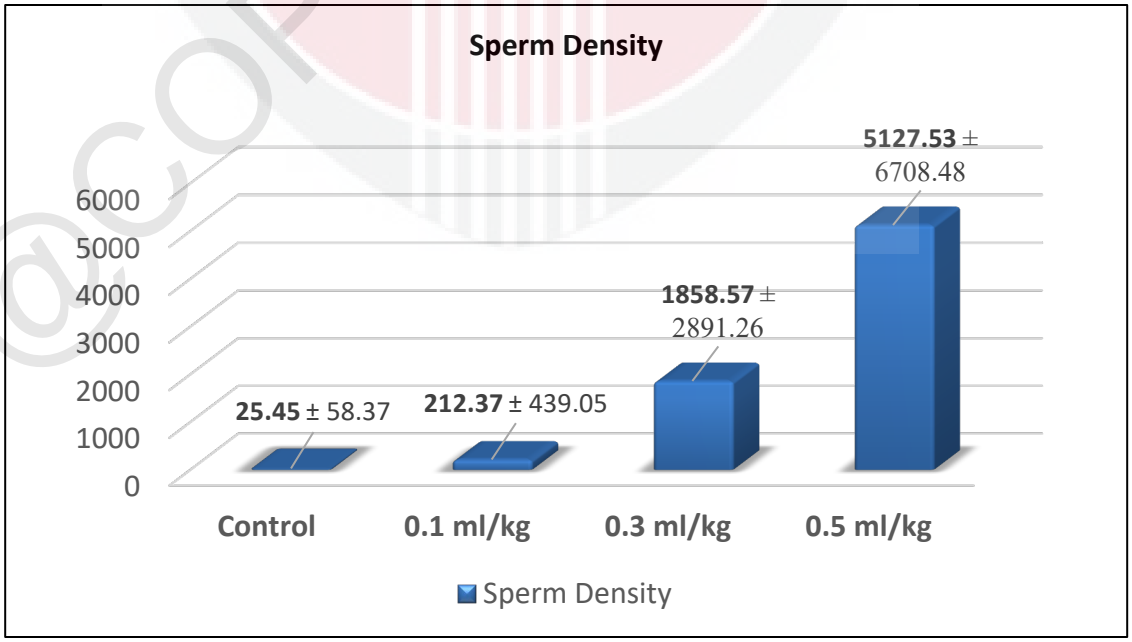


Figure 4.2: Mean sperm density in each treatment group

Sperm parameters (sperm density, motility and viability) are tabulated in Table 4.2. Treatment group 0.5 ml/kg showed the highest mean sperm density, motility and viability compared with the other groups. However, there were no significance difference ($p>0.05$) among the four treatments for sperm density and viability but there was significant difference ($p<0.05$) for the sperm motility.

Table 4.2: Mean sperm density, motility and viability for each treatment group

Sperm Parameters	Control	0.1 ml/kg	0.3 ml/kg	0.5 ml/kg
Density (x 10^6 sperms/ml)	25.45 ± 58.37	212.37 ± 439.05	1858.57 ± 2891.26	5127.53 ± 6708.48
Motility (%)	33.33 ± 27.87	35.83 ± 29.23	41.67 ± 31.89	75.50 ± 15.60
Viability (%)	55.47 ± 6.74	60.96 ± 15.28	69.67 ± 15.76	74.89 ± 13.10

4.2. Sperm morphology and morphometry

Sperm length was tabulated in Table 4.3, where treatment group 0.1 ml/kg showed the highest mean sperm length from head to tail compared to the other treatment groups. However, there was no significant difference ($p>0.05$) among the groups. Figure 4.3 shows spermatozoa, measured by Motic Image Plus 2.0 Software, whereas Figure 4.4 shows sperm morphology of GIFT strain, which consists of head, midpiece and tail.

Table 4.3: Mean sperm length for each treatment group

Treatment Groups	Sperm Length
Control	281.13 ± 144.56

0.1 ml/kg	398.19 ± 50.00
0.3 ml/kg	356.87 ± 9.49
0.5 ml/kg	346.39 ± 28.77

Figure 4.3: Sperm morphometry (L1 – length of midpiece, L2 – length of head, L3 – width of head, L4 – length of tail)

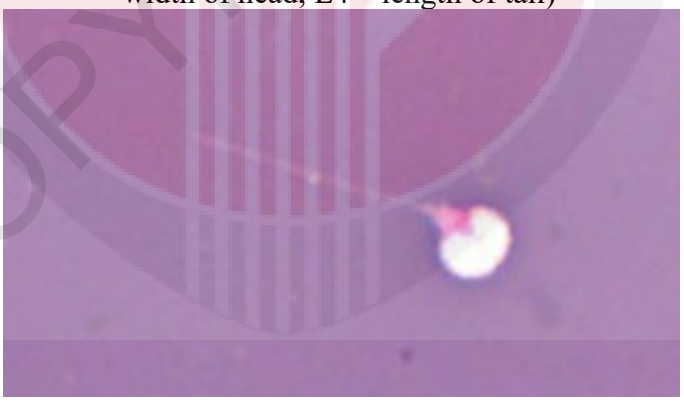


Figure 4.4: Sperm morphology of GIFT strain

Several abnormalities were seen, which were tailless, coiled tail, macrocephaly and amorphous (Figure 4.5).

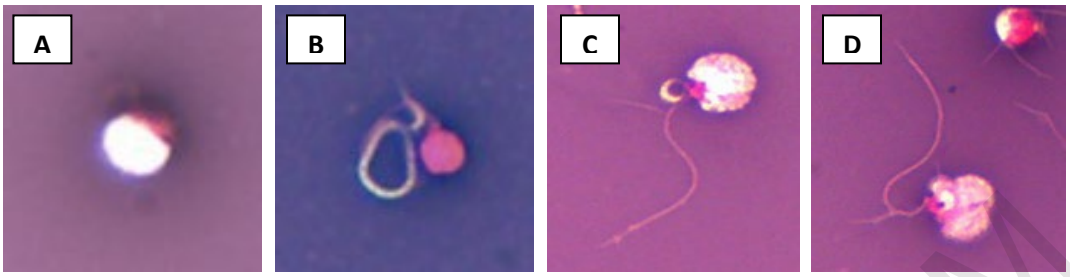


Figure 4.5: Several abnormalities of sperm (A-Tailless, B-Coiled tail, C-Macrocephaly, D-Amorphous)

5.0 DISCUSSION

Based on Popma and Masser, 1999, fish reach sexual maturity at 5 to 6 months old or with body weights of 150 to 200 grams. In this study, all the fish had reached sexual maturity as all of them were between the age of 6 and 7 months old with body weights between 221 grams and 294 grams. The mean volume of milt of group 0.1 ml/kg gave the highest (0.81 ± 0.69 ml) volume compared with the other three groups. However, the volume of milt did not indicate that the sperm density is high in group 0.1 ml/kg but treatment group 0.5 ml/kg showed the highest mean sperm density at 5127.53 ± 6708.48

x 10^6 sperm/ml. Thus, we can say that high volume of milt is not directly proportional to the high sperm density in GIFT strain. This result is supported by Clemens and Grant, (1965) and Garcia, (1991). These authors suggested that the first injection of treatment often induced a transient elevation of seminal plasma production with a much smaller increase in spermatozoa production. This is known as "thinning effect", where there is an increase in milt production but decrease or no increase in sperm density (Zohar and Mylonas, 2001).

The main parameters which are usually used to assess sperm quality and success of fertilization are percentage of sperm motility and duration of motility (Gbemisola and Adebayo, 2014). Treatment group 0.5 ml/kg showed the highest mean of sperm motility and sperm viability with the value of 75.50 ± 15.60% and 74.89 ± 13.10% respectively. However, sperm viability did not have any significance difference among treatment groups but there was significant diference for sperm motility. In this study, the percentage of sperm motility was estimated subjectively. True sperm motility should be assessed by using Computed Assisted Sperm Analysis (CASA), connected to the Hobson Sperm tracker to analyze different parameters of sperm motility such as straight line velocity (μms^{-1}), curvilinear velocity (μms^{-1}) and linearity (%) due to the short duration of sperm motility. The Hobson Sperm tracker is used to record sperm motion (Cosson, 2004). Normal saline (hypoosmotic solution) was used to initiate the motility of sperm of freshwater species and this has been reported by Cosson *et al.,* (1999) and Musa, (2010). This is because the sperm are immotile in the seminal fluid due to osmolality and it needs hypoosmotic shock to trigger its motility (Cosson *et al.,* 1999). The sperm motility was

very short and lasted less than one minute and this finding has been supported by Rurangwa *et al.,* (2004) and Billard *et al.,* (1995a). They reported that spermatozoa moved for less than two minutes and were highly active for less than 30 seconds in most cases, where the motility of sperm decreases progressively (Cosson *et al*., 1999).

Even though sperm viability did not show any significant difference, treatment group 0.5 ml/kg showed the highest mean sperm viability. Spermatozoa that absorb the eosin-nigrosin stain appeared purple in color and it indicates that the spermatozoa are dead whereas live spermatozoa did not take up the stain and remained translucent or white in color. Sperm parameters that did not show any significant difference may be due to stress conditions, contributed during transportation and during acclimatization in a new environment (tank). In addition, the tanks were relatively small. Stress can affect milt production and cause poor reproductive performance in tilapia (Mohd Yusof, 2008). True viability of sperm should be based on their capacity to move and fertilise an egg. Eosin-nigrosin stain method is only a method, which based on the integrity of the sperm membranes (Rurangwa *et al.,* 2004).

For sperm morphology, the sperm of GIFT strain consisted of head, midpiece and tail, which is similar to the mammalian sperm. However, the head of fish is rounded. Treatment group 0.1 ml/kg has the longest sperm length of 398.19 ± 50.00 µm. However, there was no significant difference among the treatment groups. There were several sperm abnormalities that were observed in this study: tailless, coiled tail, macrocephaly and amorphous. Coiled tail occurred due to the usage of normal saline as the diluting and

activating agent for the sperm. Normal saline (osmolality < 160 mOsm/kg) caused the tail of sperm to gradually coiled due to the swelling of tail plasma membrane. Then, it caused the flagellum to shorten with the occurrence of tight bending at the distal part of the flagellum. This leads to the formation of a full loop sperm and eventually causing the sperm velocity to decrease and stop when the flagellum is totally coiled (Saad and Billard, 1987; Perchec *et al.,* 1996). This process is known as osmotic trauma (Fauvel *et al.,* 2010). Thus, water from where the fish belongs needs to be used instead of normal saline as this water has osmolality of approximately 10 mOms/kg, which will not causing osmotic trauma (Perchec *et al.,* 1996).

The optimum or recommended dose of Ovaprim® can be developed based on this study, where the treatment group 0.5 ml/kg showed the highest mean of sperm density, motility and viability. Based on the study carried out by Mohd Yusof, (2008), the female tilapia was induced successfully with ovaprim at the dose of 1.0 ml/kg and males were given half the dose of females. Males of other freshwater species such as Chinese major carps and Indian major carps also had been reported to be induced at half dose given to the female (Bondad-Reantaso, 2007). According to Basavaraja, (2007), the males of all species of carps and catfish were induced with half dose given to the females.

6.0 CONCLUSION

This study shows that the volume of milt produced cannot be used as an indicator of a good sperm quality. This is because high volume of milt did not produce high sperm density when compared to fish that produced a much lower volume of milt. Normal saline can lead to the occurrence of osmotic trauma and gradually causing the tail of sperm to coil. The optimum or recommended dose of Ovaprim® is 0.5 ml/kg as this dose appeared to have the highest mean of sperm density, motility and viability.

7.0 RECOMMENDATIONS

For future study, the sperm motility should be assessed by using CASA because it is more reliable compared with estimation of percentage of motility, which is questionable and depends on personnel view. Transportation and environmental factors such as size of tank must not be neglected as these factors can affect the results directly. Besides than having a latency period of 12 hours, future study can be done to determine the effects of variable latency periods of spawning agent on seminal characteristics as it can also affects the milt production of fish.

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8.0 APPENDICES

8.1 Modified Eosin – Nigrosin Stain Preparation

85ml of distilled water and 10.0 g nigrosin were added into 250ml beaker. Magnetic bar was dropped into the beaker and placed in a hot plate cum stirrer. The mixture was boiled at 70 – 80^{o}C with continuous stirring (overnight) until all granules dissolved. Aluminium foil can be placed to cover the beaker to prevent evaporation but not air tight. Then, eosin (1.67 g) and sodium citrate (2.90 g) were added after the heating stopped and continuous stirring need to be done until the mixture cool down. The mixture was filtered and placed

into a measuring cylinder to make up the volume to 100 ml with distilled water. The stain was stored with a screw cap attached.

8.2 Dosages of Spawning Agent for Freshwater Fish

Table 8.1: The dosage of spawning agent given to males of all species of carp and catfish are half or less than half the dosage given to the females

Females of the following species	Dosages of spawning agent
Catla	0.4 – 0.5 ml/kg
Rohu	0.3 – 0.4 ml/kg
Mrigal	0.25 – 0.3 ml/kg
Fringe-lipped carp	0.3 – 0.4 ml/kg
Silver carp	0.4 – 0.7 ml/kg
Grass carp	0.4 – 0.8 ml/kg
Bighead carp	0.4 – 0.5 ml/kg
Catfish	0.6 – 0.8 ml/kg
Males of all species of carp	**0.1 – 0.3 ml/kg**
Males of catfish	**0.15 – 0.4 ml/kg**

(Basavaraja, 2007)