EFFECT OF SPIRULINA AND FOLIC ACID INTAKE ON THE SECONDARY SEX RATIO OF MOUSE OFFSPRING

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EFFECT OF SPIRULINA AND FOLIC ACID INTAKE ON THE SECONDARY SEX RATIO OF MOUSE OFFSPRING

By

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ABSTRACT

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The association of maternal diet towards the offspring's gender is no longer part of some old wives tale as it has been proven by various researches. However, the detailed information about the effect of nutritious supplements towards the gender of offspring is still lacking. Thus, the main aim of this study was to determine the effect of spirulina and folic acid intake on the secondary sex ratio of mouse offspring. Additionally, the effect of the designed diet on the weight of parental mice and its offspring, the fertilization rate and the *in vitro* development of embryos were also evaluated. The designed dietary groups included control (both dam and sire fed with normal diet), treatment A (dam fed with both spirulina and folic acid while sire only fed with spirulina), treatment B (dam fed with spirulina while sire fed with both spirulina and folic acid), treatment C (both dam and sire fed with spirulina together with folic acid) and treatment D (both dam and sire fed with only spirulina). The mice used were of ICR strain and they were randomly assigned to each treatment groups. The findings of this study revealed the consumption of spirulina by dams will give an offspring gender ratio that is skewed towards male. However, when

additional folic acid is given, the gender ratio of offspring will be drawn back towards the ratio 1:1. Also, a diet with both spirulina and folic acid will cause a significant increase in the weight of both parental mice and its offspring. Additionally, spirulina and folic acid will increase the fertilization rate of mouse oocytes and the blastulation rate of embryos significantly when they are supplemented to parental mice.

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JENNY TEH

DECLARATION

I hereby declare that the project is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

_____

JENNY TEH

APPROVAL SHEET

This project report entitled "**EFFECT OF SPIRULINA AND FOLIC ACID INTAKE ON THE SECONDARY SEX RATIO OF MOUSE OFFSPRING**" was prepared by JENNY TEH and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Agricultural Science at Universiti Tunku Abdul Rahman.

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PERMISSION SHEET

It is hereby certified that **JENNY TEH** (ID No: **13ADB03391**) has completed this final year project/ dissertation/ thesis* entitled "EFFECT OF SPIRULINA AND FOLIC ACID INTAKE ON THE SECONDARY SEX RATIO OF MOUSE OFFSPRING" under the supervision of Dr. Kwong Phek Jin (Supervisor) from the Department of Agricultural and Food Science, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my thesis in PDF format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,

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(JENNY TEH)

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LIST OF ABBREVIATIONS

BPA	Bisphenol A
BSA	Bovine Serum Albumin
CO_2	Carbon Dioxide
DPBS	Dulbecco's Phosphate Buffer Saline
ERM	Embryos Retrieval Medium
♀	Female
hCG	Human Chorionic Gonadotrophin
HIV	Human Immunodeficiency Virus
IU	International Unit
IVC	*In Vitro* Culture
ICR	Institute of Cancer Research
IgA	Immunoglobulin-A
IL-4	Interleukin 4
LDL	Low-Density Lipoprotein
♂	Male
PGD	Pre-implantation Genetic Diagnosis
PMSG	Pregnant Mare's Serum Gonadotrophin

PS	Penicillin and Streptomycin
PVP-360	Polyvinylpyrrolidone-360
SD	Standard Deviation
SERC	Science and Ethical Review Committee

CHAPTER 1

INTRODUCTION

1.1 Background

The perception from the genetic point of view indicates that the sex of offspring for human could never be determined by the mother's oocytes during fertilization as oocyte only contains X-chromosome. Instead, it could be determined by the X- or Y- chromosomes (Bachtrog, et al., 2014) bearing sperms of man that successfully fertilize the oocyte after copulation. Several researches showed that the male reproduction system produced spermatozoa that bear X- and Y-bearing sperm at the same ratio of 1:1, which means that mothers should have an equal chance in conceiving a boy or a girl post-coital, Even though genetic explanation revealed that the chance of conceiving a boy or girl is equal, various efforts in search of methods that may help in controlling the sex of offspring existed since ancient time till present.

Since ancient times, it was believed that having sexual intercourse with sturdy men will give a higher chance in expecting a baby boy. The position of intercourse was also known to influence the gender of the baby. Thus, if a couple wishes to have a son, they should have sexual intercourse in a position such that they are sleeping on their left side (Jones, 1992). A traditional method that has been passed down until the current generation is the addition of certain supplements to the maternal diet (Nager, et al., 1999). It is said that if the maternal diet is added with alkaline supplements, there is a higher chance in

conceiving a baby boy whereas if the maternal diet is added with acidifying supplements, there is a higher chance in conceiving a baby girl. There are also recent medical techniques (both pre-conceptual methods and post-conceptual methods) that assured successful gender selection of babies (Giorgetti, et al., 1995). However, these medical techniques are considered to be much more expensive which means that not everyone can afford this method of sex selection.

In order to make sex selection affordable to everyone, research efforts to validate traditional methods were done and it has been proven that the association of maternal diet towards the offspring's gender is no longer part of some old wives' tales. Now, there are many types of supplements available in the market to ameliorate any nutrient deficiency in human body. Spirulina is a rich source of nutrients that is suitable to be consumed by human regardless of age and gender. Hence, spirulina has been recommended by physicians to couples that are hoping to conceive a baby as it will help in preparing a good paternal or maternal body condition that encourages pregnancy. Besides that, there is another crucial supplement known as folic acid that is generally recommended to be taken by women during the pregnancy period and at times even before conceiving. This supplement is crucial in lowering the risks of birth defects in babies (Juriloff and Harris, 2000). Even though there are many researches done on these individual supplements to study the benefits they provide, there is no research to date that has been conducted to evaluate the effect of folic acid intake consumed together with other nutritious supplements such as spirulina towards the sex ratio of offspring.

1.2 Statement of Problems

The abnormal sex ratio in human populations was seen to be a trend in South Korea, Taiwan, China and other parts of Asia in recent years. This imbalance is mostly caused by the preferences for having son in Asian families. Based on latest research, in Malaysia alone, there are 15,709,654 men and 15,271,217 women which made up the country's total population of 30,980,868 in the year 2016. This shows that the male population in Malaysia (50.7%) is relatively higher than the female population (49.3%) (Department of Statistic Malaysia Official Portal, 2016). Such imbalance of the male and female ratio is believed to be the result of sex selective abortions (Hesketh and Xing, 2006) that has been increasing in Malaysia at an alarming rate. In addition, in some parts of India, if a girl is born in a family that only favours male child, she may receive an unjust or prejudicial treatment as compared to her brothers (Borooah, 2004). Social problems such as criminal cases (including raping, kidnapping and women trafficking) will also increase tremendously if this gap between the male and female ratio keeps getting wider.

On the other hand, male animals in general, are preferred in meat industry whereas female animals are favoured in milk industry. Therefore, when there is an abnormal sex ratio in domestic animals, especially in sheep, goats, cattle and pig, these industries will be impacted on both their milk and meat productions. Hence, if farmers are able to control the sex ratio of these domestic animals effectively, it would be an advantage to them. Nowadays, sperm sorting may be done via flow cytometry (JimÉnez, et al., 2003) to

produce the desired gender of domestic animals. However, this sex selection method requires high investment of expensive equipment. Thus, it can only be afforded by a few farmers who are in commercial scale farming.

1.3 Justification of Study

In order to allow the livestock industry to control the sex ratio of their domesticated animals and parents in selecting the gender of their offspring at an affordable price, the adaptation of natural sex selection through manipulating the maternal diet would be the best way. The supplements chosen for this study (spirulina and folic acid) have been studied individually by many researchers on their individual nutritional content and health benefits. Although so, no research has been done on the effect of consuming both spirulina and folic acid supplements on the secondary sex ratio of offspring and the quality of embryos produced. Thus, this study was done to determine if the consumption of two different supplements at the same time will cause a shift in the secondary sex ratio of offspring. The final findings of this study may be used as a reference to both the livestock industry and parents in controlling the skewness of the secondary sex ratio of offspring. Besides, the manipulation of maternal diet for sex selection is much more ethical as compared to medical techniques that destroy unwanted sexed embryos and this technique is commonly known to us as pre-implantation genetic diagnosis (PGD).

In this study, Institute of Cancer Research (ICR) strain mice (*Mus musculus*) were used as a model because of its motherly instincts and because the ICR

strain mice are considered to be much more docile than mice of other strains. Besides that, mice were chosen instead of other domesticated animals such as cattle or goats because it can reproduce at a fast rate (19 to 21 days) and in large populations (8 to 12 pups per gestation) as compared to other domesticated animals. Thus, mice can be used as a preliminary screening test for farm animals in which their gestation period may take up to nine months. In addition, mice are closely related to humans and other domesticated animals in terms of the symptoms it shows, its behaviour and also its biological characteristics.

1.4 Objectives

The aims of this study were:

1. To determine the secondary sex ratio skewness of offspring derived from parental group fed with different combinations of spirulina and folic acid supplements.
2. To evaluate the weight of both parental mice and its offspring (at four weeks old) derived from all the treatment groups.
3. To investigate the fertilization rate of mouse oocyte derived from all the treatment groups.
4. To investigate the *in vitro* development of embryos derived from parental group fed with different combinations of spirulina and folic acid supplements.

CHAPTER 2

LITERATURE REVIEW

2.1 Sex Determination

2.1.1 Sex Determination Systems

The curiosity on how the two sexes (male and female) are determined in living organisms has long been sparked in the scientific community. Back in 1889, Geddes and Thomson estimated that by the 1800s, there were already more than five hundred theories that were based on sex determination alone (Maienschein, 1984). To put it simple, the determination of sex in an organism is based on the assortment of gametes by both parents as the patterns formed by different combinations of gametes are the ones that decide the male and female character of the offspring (Bachtrog, et al., 2011).

In general, there are 3 main types of sex determination systems which include the XX-XY sex determination system found in humans, the XX-XO sex determination system used by insects and the ZZ-ZW sex determination system found in birds and snakes. For the XX-XY sex determination system, the offspring that inherit one X chromosome from each parent (XX) will be a female and the offspring that inherit one X chromosome from the mother and one Y chromosome from the father (XY) is a male. Thus, when meiosis occurs, females may only produce gametes with an X chromosome whereas the sperms of males may contain either an X chromosome or a Y chromosome. This means that maleness is determined by Y-bearing sperm in humans (Bachtrog,

et al., 2011). Instead, for insects such as crickets and grasshoppers, maleness is determined by the lack of an X chromosome (referred as O) in a sperm cell. This is because females, like humans, inherits one X chromosome from each parent (XX) which allows them to fill all their gametes with an X chromosome whereas for males, instead of inheriting two chromosomes like humans, they only have one X chromosome (XO). Thus, sperm cells produced by male individual may or may not have sex chromosomes (Heimpel and de Boer, 2008). On the other hand, female birds (e.g. hens) are heterozygous (contains one Z chromosome and one W chromosome) and male birds (e.g. rooster) are homozygous (contains two Z chromosomes) (Smith and Sinclair, 2004). Unlike the previous systems, the mismatched chromosome pair is not inherited by male offspring. Still, the offspring produced by these living organisms under the ZZ-ZW sex determination system (birds and snakes) has a ratio that is nearly 1:1 which is similar to that with the sex ratio of offspring produced by both humans and insects (Griffin, et al., 1996).

2.2 Sex Selection

2.2.1 Medical Techniques

An attempt to acquire offspring with the desired gender is known as sex selection. In fact, the practice of sex selection has been done widely across the globe over the years. To date, there are various techniques whereby sex selection can be accomplished and these techniques are done either *in vivo* or *in vitro* and during pre- or post- conception. The pre-conceptual methods involve monitoring and manipulating the sex of offspring before the

fertilization of both the oocyte and sperm. On the other hand, post- conceptual methods involves the monitoring and manipulation of the sex of offspring after fertilization whereby the fertilized oocyte has developed into a foetus.

The pre-conceptual medical techniques is divided into two different procedures: pre-implantation genetic diagnosis (PGD) and sperm sorting. Overall, the pre-implantation genetic diagnosis (PGD) is started off by creating embryos by fertilizing the gametes of parents. Then, these fertilized embryos are allowed to develop *in vitro* before a further biopsy procedure (chromosomal analysis is done by harvesting one cell from each embryo) takes place. Finally, only the fertilized embryo with the desired gender will be transferred into the mother's uterus when it reached the eight-cells stage (Aghajanova and Valdes, 2012). However, sperm sorting is done before any fertilization takes place. Although there are many types of sperm sorting methods such as flow cytometric separation, swim-up procedure, migration-sedimentation, trans-membrane-migration, density gradient centrifugation, glass wool filtration, the glass bead method and the Sephadex columns method (Henkel and Schill, 2003), the ultimate goal of all these sperm separation techniques is to separate the Y-bearing sperms from the X- bearing sperms based on the motility of sperm (Y-bearing sperm is more mobile than X- bearing sperm) and the weight of the sperm's genetic material (X- bearing sperm has more genetic material thus it is heavier than Y- bearing sperm) (Garner, 2006). Eventually, these sex sorted sperms (either bearing the X- chromosome or the Y- chromosome) will be artificially inseminated or will undergo *in vitro* fertilization processes to

produce an offspring with the desired gender (male or female) (Garner, Evans and Seidel, 2013).

Post- conceptual medical techniques such as amniocentesis, chorionic villi sampling and ultrasound are also used in sex selection (Jones, 1992). For amniocentesis, the mother's abdominal wall will be inserted by a hollow needle in order to obtain the sample needed (amniotic fluid that surrounds the foetus) to conduct a DNA analysis. Chorionic villi sampling is also done by analysing the DNA of collected cells to determine the gender of the foetus. These collected cells are sloughed cells obtained by using a suction tube which is inserted either trans-abdominally or trans-cervically during the first trimester of gestation. Last but not least, the gender of foetus can be determined by ultrasound because a high frequency sound source will be placed on the abdomen of the mother to generate a visual image that allows the genital development of foetus to be reviewed. However, this medical technique is only recommended after the 9^{th} week of pregnancy because it is only by this time that the genital organs can be clearly differentiated (Jones, 1992). Thus, by using these medical techniques mentioned above to determine the sex of embryo, the sex of offspring can be selected.

2.2.2 Manipulation of Maternal Diet

According to Johnson (1995), medical techniques such as sperm sorting does provide an approximate of 85% to 90% accuracy on the determination of the gender of offspring. However, other medical techniques such as the pre-

implantation genetic diagnosis (PGD), is considered to be un-ethical because embryos of the undesired gender are aborted or discarded. Henceforth, other techniques that are considered to be more ethical were discovered and these techniques are generally known as natural methods. One of the proven natural methods that can control the sex of the offspring is the manipulation of maternal diet. According to Trivars and Willard (1973) hypothesis, when the maternal condition is at optimum, male offspring is favoured.

Various studies were later done to prove the Trivars and Willard hypothesis and it was found that maternal condition is at optimum when mothers have a well-balanced diet. In 1979, Skjervold and James proved that the ratio of male offspring is significantly higher than the female offspring when mother cattle were fed with a well-balanced diet. Similarly in 1992, William and Gloster found that maternal diet with a low caloric intake tend to produce more male offspring as compared to mothers with a high caloric diet. A recent study done by Mathews, Johnson and Neil in 2008 also confirms that human mothers with a nutritious diet (having cereals for breakfast) have higher chances in getting a son. Other studies which were done on nutrient intake (Table 2.1) by the mother prior gestation also strongly indicated that maternal diet does influence the gender of offspring.

Table 2.1: Timeline of studies done on maternal diet

Year	Study Model	Authors	Findings
1982	Sow	Bolet, et al.	Nutrient intake affects the sex ratio of offspring.
1987	Humans	Crawford, Doyle and Meadows	Diet high in essential fatty acids content favours male offspring.
2001	Mice	Meikle and Westberg	Male offspring are favoured when dams were fed *ad libitum*.
2004	Mice	Rosenfeld and Roberts	Male offspring are favoured when dams were fed with high fat diet.
2008	Humans	Mathews, Johnson and Neil	Mothers with well-balanced diet has higher chance to produce male offspring.
2013	Rats	Gray, et al.	Maternal diet high in fructose will give rise to male offspring.

2.2.3 Other Natural Methods

Other than the manipulation of maternal diet, other natural methods that contribute in sex selection include the conceiving age of mother and the timing of intercourse. In 2012, a study was carried out on humans by Rueness, Vatten and Eskild to relate maternal age with the gender of offspring produced. It was found that there was an increment in the proportion of female offspring when maternal age increased. This is because high maternal age is constantly associated with high stress conditions. Thus, to increase the chances in obtaining male offspring, women are suggested to conceive at an early age.

In addition, studies also showed that the timing of intercourse may influence the gender of offspring born. In 1993, McSweeny suggested that having sexual intercourse in relation to the time of ovulation will have an impact on the probability in conceiving a son or a daughter. It was said that in order to conceive a son, sexual intercourse should not take place until 2 days after 'peak'. In contrast, if sexual intercourse took place 2 days prior the 'peak' period, there is a higher chance in conceiving a girl. This 'peak' period is actually the timing whereby there is a presence of raw-egg white coloured mucus which has a slippery sensation. This mucus is an indication that the woman is fertile. Another research done by Noorlander, Geraedts and Melissen in 2010 supported this finding because their study also showed that the probability of getting a female offspring is higher when sexual intercourse was preceded before ovulation.

2.3 Spirulina

2.3.1 Background of Spirulina

Spirulina, a multicellular, filamentous (diameter of 3 to 12 μm) and microscopic blue-green algae (cyanobacterium) that is helical or spiral in shape is actually the dry biomass of *Arthrospira platensis*. It can be naturally found in the ocean, lakes and also in fresh water. In general, spirulina is actually a prokaryotic organism made up of a pluri-stratified cell wall, inclusions, ribosomes, capsule, a photosynthetic system and the basic fibrils of DNA region. Spirulina is safe to be consumed by humans because the cell wall is easily digested as it is made from both lipopolysaccharides and protein (Ali and Saleh, 2012).

The 3 stages: the fragmentation of trichomes, the enlargement and maturation of hormogonia cells and the elongation of trichomes are the overall life cycle of spirulina. After the trichomes are elongated, they will be divided into filaments where binary fission would take place to increase the number of cells. Usually, spirulina are found in alkaline waters up to a pH of 11 because the high bicarbonate content in these environments enhances the growth of spirulina (Ali and Saleh, 2012). However, spirulina is also known to be highly adaptable in different environments such as brackish water, marshes, sand and soil (Ciferri and Tiboni, 1985). Thus, it can be widely commercialized as supplements, food and feed for the consumption of both humans and animals in large quantities because it can be found almost everywhere. To date, the major companies that commercialized spirulina as one of their products include

Solarium Biotechnology (Chile), Earthrise Farms (USA), Genix (Cuba), Marugappa Chettir Research Center (India) and Hainan DIC Microalgae Co., Ltd (China) (Karkos, et al., 2011).

2.3.2 Nutritional Content of Spirulina

Spirulina is known as the natural superfood because it is highly nutritious when compared to other food. Based on Becker (1984), spirulina contains nutrients such as phytonutrients, essential fatty acids, amino acids, proteins, vitamins and minerals. Thus, spirulina is one of the most recommended supplements to cure malnutrition in adults and children (Masuda, et al., 2014).

Basically, spirulina contains a wide array of pigments and some of them include canthaxanthin, zeaxanthin, xanthophylls, beta-carotene and chlorophyll a. It also contains essential fatty acids such as linolenic and γ-linolenic acid (GLA), omega-3 fatty acids and omega-6 fatty acids and the total fatty acid content in spirulina is about 5% to 6%. Besides, spiruina is rich in amino acids such as valine, leucine and isoleucine. The protein concentration in spirulina is also estimated to be around 55% to 70% of its dry weight. In addition, vitamins found in spirulina include vitamin A, B1 to B12, C, D and E whereas minerals such as sodium, magnesium, iron, phosphorus, calcium and potassium are also found in spirulina (Hoseini, Khosravi-Darani and Mozafari, 2013).

Table 2.2: Nutrient composition of spirulina

Composition	Quantity (per 100 g dry weight)
Potassium	1.83 g
Sodium	1.09 g
Magnesium	25 000 mg
Calcium	16 800 mg
Iron	53.60 mg
Chlorophyll-a	1.30 g
Carotenoids	45 600 mg
Fat (Lipids)	9.50 g
Pro-Vitamin A	21 300 mg
Protein	63.50 mg

(Adapted from Edis Koru, 2012)

2.3.3 Benefits of Spirulina

As early as 1988, studies have showed that the consumption of spirulina helps to reduce the low-density lipoprotein (LDL) cholesterol level in humans (Nakaya, Homa and Goto, 1988). In 1999, the consumption of spirulina was found to enhance the production rate of immunoglobulin-A (IgA) antibody while improving the mucosal immunity in humans (Ishii, et al., 1999). Another study done in 2011 also supported the findings of this study whereby it was further proven that spirulina can enhance the immune system of humans (Karkos, et al., 2011). In addition, the intake of spirulina supplementation will decrease the production of interleukin-4 (IL4) (Cingi, et al., 2008). Hence, it

can aid the human body to fight against allergic diseases such as rhinitis since this allergy is caused by the overproduction of IL-4 in the body (Hershey, et al., 1997). In recent years, researchers have also found that a well-balanced diet supplemented with spirulina helps reduce the viral load significantly while increasing the T-helper cells (CD4 cells) in antiretroviral-naïve (ART-naïve) patients. It was also proven that spirulina may aid in slowing down the progress of HIV disease (Ngo-Matip, et al., 2015).

Besides humans, spirulina is also widely used in poultry and livestock industries to enhance the production, growth and health of farm animals. Many researches have showed that spirulina supplementation in the diet of farm animals will give them positive effects such as having the ability to fight against diseases because spirulina increases the activity of natural killer cells (Qureshi, Garlich and Kidd, 1996); the enhancement in lactation production that eventually results in higher milk production (Kulpys, et al., 2009) and the better growth performance of weaned farm animals (Grinstead, et al., 1998).

Spirulina is also used as a substitute of antibiotics in aquaculture because of its high level of antiviral and antibacterial properties. In 2005, Hemtanon, Direkbusarakom and Bunyaviwat proved that shrimps, both in larval and juvenile stages, when administered with spirulina were found to have successfully fought off the infection of white spot virus. Besides preventing infection of shrimps, spirulina is shown to decrease the level of P53 protein in fish. P53 protein is a natural tumour suppressor that responds to cellular stress

such as oxidative stress, damaged DNA and the depletion of nucleotides. Thus, such decrease of P53 in fish indicates that spirulina has antioxidant effects (Ibrahem and Ibrahim, 2014). According to Ragap, Khalil and Mutawie (2012), an optimal dosage of spirulina for four continuous weeks is able to enhance the immune system of tilapia because unlike the tilapia without the supplementation of spirulina, treated tilapias were not infected by the bacteria *Aeromonas hydrophila*.

2.4 Folic Acid

2.4.1 General Background of Folic Acid

Folate, more commonly known as vitamin B9, can be found naturally in a variety of foods such as breakfast cereals and white breads (Subar, Block and James, 1989) in relatively low concentrations. However, green leafy vegetables and legumes such as green salad and dried beans tend to have abundant folate content (Rossi, Amaretti and Raimondi, 2011). This micronutrient is vital for growth and in maintaining a good health because folate aids in cell division and the growth of cells. At the same time, it is an essential nutrient for metabolism (Weggemans, Schaafsma and Kromhout, 2009). Since folate is a B-vitamin, it is water soluble (Czeizel, et al., 2013). Hence, folate is not stored by the body but instead it is excreted by the body via bile, sweat from skin and urine. Thus, it is important to replenish the body with an adequate amount of folate through staple foods or by consuming folic acid supplementation, which in this case is fortified folate (Rossi, Amaretti and Raimondi, 2011). According to Cuskelly, McNulty and Scott (1996), folate in the form of folic acid is much

more readily absorbed by the body. The recommended intake of folic acid daily is 400 mcg for both men and women (Wong, et al., 2002).

2.4.2 Benefits of Folic Acid

Folic acid is known to reduce the risk of various birth defects (some may be genetically inherited) in newborn babies. However, the main defect that is reduced by folic acid consumption is the neural tube defect. Based on a study done by Shin and Shiota (1999), when there is a supplementation of folic acid to the maternal diet prior conception and during early conception, the incidence of neural tube defects in offspring is greatly reduced. On the other hand, the administration of folic acid in females may enhance the quality of oocytes produced, improve the maturation of oocytes and also aid in the development of embryos (Ebisch, et al., 2007). Additionally, a research done by Wong, et al. (2002) suggested that the percentage of abnormal sperm in men who consumed large amounts of folic acid (> 700 mcg/day) is significantly lower than the percentage of abnormal sperm in men without such supplementation. Folic acid is also known to boost the sperm count of sub-fertile men. According to Ebisch, et al. (2007), sub-fertile men supplemented with folic acid showed a 74% increase in sperm count. The reason behind such increment in sperm count is because folic acid plays an important role in spermatogenesis and the increment of this nutrient enhances the process. A study done in 2003 also reported that folic acid has the ability to reduce the risk of cancer and cardiovascular diseases (Sahin, et al., 2003).

Table 2.3: Timeline on major studies related to folic acid

Year	Study Model	Authors	Findings
1989	Humans	Subar, Block and James	Major food source of folate include cereals, dried beans and white breads.
1999	Mice	Shin and Shiota	Recommended dosage of folic acid for mice is 3 mg/kg body weight.
2000	Mice	Juriloff and Harris	Supplementation of folic acid reduces risk of foetal neural tube defects.
2002	Humans	Wong, et al.	Total normal sperm count increased in sub-fertile men.
2003	Birds	Sahin, et al.	Folic acid reduces heat/oxidative stress.
2007	Humans	Ebisch, et al.	Folic acid promotes better quality oocytes and higher degree of mature oocytes.
2013	Humans	Czeizel, et al.	Folic acid, also known as folate of vitamin B9 is water soluble.

2.5 Fertilization Rate and Development of Embryos

2.5.1 Effect of Nutrients on the Fertilization Rate and Development of Embryos

Nutrition can be associated with the reproductive performance of both humans and farm animals because without nutrition, there is no source of energy for the fertilization of oocytes or for the development of the embryo and foetus. Hence, energy in either adequate, excessive or insufficient will have an impact on reproductive activities. Varghese, et al. (2011) reported that the maternal diet prior pregnancy or in the early stages of pregnancy will determine if the mother will have an optimum or insufficient amount of energy. Other studies have also been carried out to associate nutrition with the rate of fertilization and the development of embryos.

For fertilization rate, dairy cattle were found to have a significantly lower fertilization rate when they were fed with excess protein. This is because when the protein level is high, it will tend to suppress progesterone concentrations while increasing the concentration of plasma urea nitrogen. This change in plasma urea nitrogen concentration eventually causes detrimental effects to the embryos thus reducing the fertilization rate (Butler, 1998). Another study also showed that maternal diet which has high lipid content will cause the rate of fertilization to be low. In addition to the rate of fertilization, the quality of embryos will also be influenced because the high lipid content will cause some adverse modifications in both follicles and oocytes (Leroy, et al., 2010). It was

also reported by Paffoni, et al. (2014) that vitamin D deficiency will alter the ovarian and endometrial level, thus affecting the fertilization rate in women.

Aside from the rate of fertilization, the amount of nutrition consumed has also been proven to affect the embryos' growth and development. In 2006, Grazul-Biska, et al. did a study to test the effect of over-nutrition and malnutrition on the embryos' *in vitro* development. For this research, the ewes that were supposed to be over-nutritious were fed with twice its normal diet whereas the ewes that were supposed to be malnourished were only fed with half the amount of the normal diet. It was seen that the rate of fertilization and the number of morulae and blastocyst formed from two-cell embryos were significantly lower when compared against the control group (ewes were fed with normal diet). Additionally, maternal diet with high fiber intake was shown to improve the growth and development of embryos thereby enhancing the embryos' survival rate (Ferguson, et al., 2007). It was also found that embryos will be degenerated at an early stage when there is an excess consumption of crude proteins (Blanchard, et al., 1990). According to Thangavelu, et al. (2007), when cattle were fed with sunflower seeds which are known for its high monounsaturated fatty acids content (omega-3 fatty acids and omega-6 fatty acids), the number of blastocyst formed from two-cell embryos increased significantly. This indicates that a diet high in unsaturated fatty acids can improve the development of embryos.

2.5.2 Environment Exposed to and Lifestyle Practised by Mother on the Fertilization Rate and Development of Embryos

Researches have shown that the lifestyle of a mother and the environment she lives in during her pre-conceptual and periconceptional periods will have an impact not only on the fertilization rate but also on the development of embryo (Calleja-Agius, 2009).

Among all unhealthy lifestyles, smoking or the continuous inhalation of cigarette smoke is the one lifestyle that has a severe impact on fertilization rate and also the development of embryo. This is because tobacco cigarettes are made up by cotinine, cadmium, nicotine and other carcinogens and toxicants. Based on a research performed by Huang, et al. (2009) on mice, it was found that dams exposed with four weeks of cigarette smoke showed a reduction in the fertilization rate, the quality and the development of embryos. The reason behind this reduced fertilization rate is because the quality of oocyte is affected by the lack of expansion of the cumulus oocyte complex (Cooper and Moley, 2008) which is due to the inhibition of oestrogen and steroid hormone production by the composition of tobacco cigarettes (Younglai, Holloway and Foster, 2005). Besides, smoke produced by tobacco cigarettes will cause impairment in the development of embryos. This is because tobacco smoke will caused the expression of genes to be altered and the zone pellucida to thicken during embryonic development (Cooper and Moley, 2008).

Environmental pollutants such as plasticizers, heavy metals and chemical pesticides may also have an impact on the rate of fertilization and also development of embryo. A study done on mice showed that dams exposed to bisphenol A (BPA) have a low fertilization rate because they had fewer number of oocytes and in this small amount of oocytes, most of them were abnormal (Susiarjo, et al., 2007). In 2005, a research done by Younglai, Holloway and Foster further suggested that the chemical pesticides which are widely used in agriculture fields will cause detrimental effects to oocytes before and after fertilization. It was also reported that the rate of fertilization and the development of embryos are affected by environmental temperatures. According to Paula-Lopes, et al. (2012), the increasing temperature during summer caused the fertility of dairy cattle to reduce. The reason is because when there is an elevated environmental temperature, the nuclear and cytoplasmic compartments of oocytes will be affected. Thus, the maturation of oocytes, the fertilization rate and the development of embryos are disrupted.

CHAPTER 3

MATERIALS AND METHODS

This study was performed in both Agricultural Park and Agricultural Laboratory II under Faculty of Science, Universiti Tunku Abdul Rahman (UTAR), Kampar campus, and was completed within a span of five months (from May 2016 to September 2016). This research gained the approval from University Tunku Abdul Rahman (UTAR) Science and Ethical Review Committee (SERC) since the model used for this study is live mice. The list of brands for the materials and chemicals used in this study are shown in Appendix 1.

3.1 Animal Husbandry

The experimental animals used for this research were mice (*Mus Musculus*) of the Institute Cancer Research (ICR) strain. Both female and male ICR mice were at the age of 10 to 13 weeks old with weights ranging from 15 g to 40 g.

Throughout this research, all mice were kept in an animal holding cabin which is located in Agricultural Park, UTAR Kampar. The temperature of the cabin was regulated with air-conditioners within the range of 18 ℃ to 22 ℃. These mice were allocated to specific cages (Figure 3.1) according to gender and treatment group. Such separation was done to avoid any copulation prior to the treatment conducted. For each cage there is a 530 cm^3 floor size and it can accommodate up to a maximum of 8 mice. Clean water and Gold Coin

commercial feed pellets (500 g/cage/week) (refer Appendix 2 for composition and ingredients of Gold Coin mouse pellet feed) were provided *ad libitum* to all mice. Additionally, in order to reduce the foul odour emitted by wastes excreted by the mice, the beddings of all cages were changed twice a week.

Figure 3.1: Mouse cages arranged according to gender and treatment groups.

3.2 Preparation and Maintenance of Apparatus and Medium

3.2.1 Cleaning and Sterilization of Apparatus and Equipment

Before the commencement of each laboratory work, the apparatus and the work surface were sterilized as an aseptic precaution. For heat tolerant apparatus, such as the tools used for dissection (tweezers, forceps and scissors), centrifuge tubes, mouthpiece, pipette tips and glassware, they were autoclaved at 121^{o}C with 15 psi for 30 to 45 minutes. Then, these apparatus were kept overnight in the oven at 70^{o}C to dry. Before dissection, the surface of the laboratory bench was wiped with 70% ethanol whereas prior to the preparation of hormones and

the *in vitro* culture of embryos, the surface of the laminar flow was first exposed to ultra-violet radiation before it was swabbed by 70% ethanol.

3.2.2 Preparation of Liquefied Spirulina

The recommended dosage of spirulina in mouse is 285 mg/kg body weight (Vidé, et al., 2015). Thus, for this study, liquefied spirulina with a concentration of 8.55 mg/0.1 ml was prepared since the average body weight of mouse was 30 g. This liquefied spirulina was prepared every week to preserve the freshness and quality. In one week, a total of 21 ml liquefied spirulina is needed. To prepare the required concentration and volume of liquefied spirulina, nine spirulina tablets (200 mg/tablet) were first ground into powder form by using pestle and mortar. Then, 21.6 ml (2.4 ml/tablet) of Milli-Q water was added onto the mortar containing spirulina powder and the solution was mixed evenly by using the pestle. The pH of spirulina solution was measured prior storing in a 50 ml conical base centrifuge tube at 4^{o}C.

3.2.3 Preparation of Liquefied Folic Acid

The recommended dosage of folic acid in mouse is 3 mg/kg body weight (Czeizel, et al., 2013). Thus, in this study, liquefied folic acid with a concentration of 0.09 mg/0.1 ml was prepared since the average body weight of mouse was 30 g. In one week, a total of 14 ml liquefied folic acid is needed. To prepare the required concentration and volume of liquefied folic acid, thirty-two folic acid tablets (0.4 mg/tablet) were first ground into powder form by

using pestle and mortar. Then, 14.1 ml (0.44 ml/tablet) of Milli-Q water was added onto the mortar containing folic acid powder and the solution was ground evenly by using the pestle. The pH of folic acid solution was taken prior storing in a 15 ml conical base centrifuge tube.

3.2.4 Preparation of Superovulation Hormones (Pregnant Mare's Serum Gonadotrophin (PMSG) and Human Chorionic Gonadotrophin (hCG))

Pregnant Mare's Serum Gonadotrophin (PMSG) was prepared by adding its freeze-dried crystalline powder (1000 IU/vial) into 20 ml of filtered saline (0.9%) to obtain a concentration of 50 IU/ml. This mixture was later aliquoted into twenty syringes (1 ml each) and was kept refrigerated at -20^{o}C for future use.

For the preparation of human chorionic gonadotrophin (hCG), its freeze-dried crystalline powder form (5000 IU/vial) was dissolved with 100 ml of filtered saline (0.9%) to obtain a concentration of 50 IU/ml. This mixture was later aliquoted into syringes (1 ml each) and was kept refrigerated at -20^{o}C for future use.

3.2.5 Preparation of Dulbecco's Phosphate Buffer Saline (DBPS) Stock Solution

Dulbecco's phosphate buffer saline (DBPS) stock solution was prepared by dissolving 10 DPBS tablets in 1000 ml of Milli-Q water that has been autoclaved. Then, 1 ml of penicillin streptomycin (PS) stock was added into the 1000 ml of Milli-Q water and this DPBS stock solution was mixed evenly by using a magnetic stirrer. The DPBS stock solution was kept refrigerated at 4^{o}C for future embryo retrieval medium (ERM) preparations.

3.2.6 Preparation of Embryo Retrieval Medium (ERM)

Embryo retrieval medium (ERM) was prepared by dissolving 0.025 g of polyvinylpyrrolidone-360 (PVP-360) into 25 ml of DPBS solution. The preparation of ERM was done a day before use due to the low solubility of PVP-360. The prepared medium can be stored for one week when refrigerated at 4^{o}C. ERM must be warmed to 37^{o}C before use by placing it into the water bath.

3.2.7 Preparation of *In Vitro* Culture (IVC) Medium

The frozen stock solution of commercial potassium simplex optimization medium supplemented with amino acid (KSOMaa) and phenol red was first thawed at room temperature. After thawing, 1 ml of KSOMaa solution was transferred into an autoclaved micro-centrifuge tube by using a micropipette (100 – 1000 µl). Then, 0.03 g of bovine serum albumin (BSA) was weighed by

using a digital analytical balance and added into the micro-centrifuge tube containing the KSOMaa solution. The micro-centrifuge tube was gently inverted so that BSA dissolves completely in KSOMaa solution. Then, the mixture was filtered-sterilized into another autoclaved micro-centrifuge tube by using a syringe attached to a syringe filter. This tube was labelled as IVC (*in vitro* culture) media and was sealed with parafilm. The prepared IVC (*in vitro* culture) media can be kept for two days when refrigerated at 4^{o}C.

3.2.8 Preparation of Micro-droplets in Petri Dish

The medium used (for the retrieval and flushing of embryos, holding embryos while transferring and for the IVC of embryos) were all prepared in the form of micro-droplets.

The IVC was prepared one day before any embryo flushing is done. First, a tissue culture dish was labelled as IVC together with the date of preparation and the type of treatment of the dissected mouse. Then, five micro-droplets (60 µl / droplet) of IVC medium were pipetted onto the tissue culture dish (35 mm diameter). Each micro-droplet was positioned as shown in Figure 3.2. Mineral oil was later added until it overlays all the IVC medium droplets. This was done to sustain the conformation of the droplet and to prevent water from evaporating from the medium during incubation. This culture was then equilibrated in the incubator overnight at 37 ℃ with 5% of carbon dioxide.

On the day of embryo flushing, dishes that contain the embryo holding medium and the embryo retrieval medium were prepared individually. For the preparation of the dish containing embryo holding medium, a tissue culture dish was labelled as ERM together with the date of preparation and the type of treatment of the dissected mouse. ERM that was prepared previously was first warmed at 37 ℃ in the water bath. Then, five micro-droplets (60 µl / droplet) of ERM were pipetted onto the tissue culture dish (35 mm diameter). Each micro-droplet was positioned as shown in Figure 3.2. Mineral oil was later added until it overlays all the ERM droplets.

As for the dish used for the retrieval and flushing of embryos, a petri dish was labelled as flushing medium together with the date of preparation and the type of treatment of the dissected mouse. Subsequently, the warmed ERM was pipetted onto the petri dish (60 mm diameter) at 60 µl per droplet repetitively. Each micro-droplet was positioned as shown in Figure 3.2.

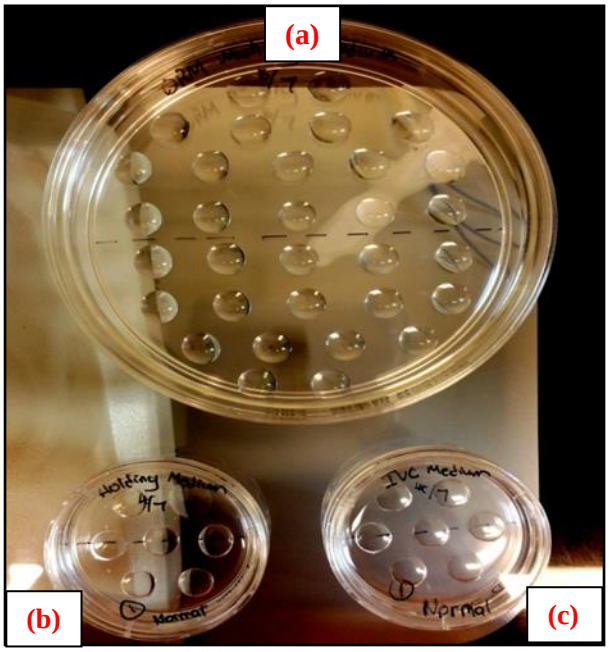


Figure 3.2: Micro droplets arrangement for (a) 60 mm petri dish containing flushing medium; (b) 35 mm embryos holding dish; (c) 35 mm tissue culture dish containing IVC medium.

3.2.9 Preparation of Mouthpiece-controlled Pipette for Embryo Pick-up

The apparatus and material needed during the preparation of a mouthpiece-controlled pipette were metal forceps, Bunsen burner, diamond stone and glass Pasteur pipette. First, the Bunsen burner was lighted up and adjusted until a small blue frame was obtained. Then, one glass Pasteur pipette was removed from the packaging containing all the autoclaved glass Pasteur pipettes. Subsequently, one end of the glass Pasteur pipette was held with the thumb and index finger while the other end (tapered end) was held with forceps. The tapered end of glass Pasteur pipette was then located over the flame and rolled gently until it softens. Once the tapered end becomes softened, it was immediately removed from the flame followed by pulling both ends apart from each other. Both ends were held steadily for a few seconds for the formed capillary segment to cool and harden. The diamond stone was then used to cut

at the point of the desired diameter which is about 150 µm. This tip was viewed under the stereomicroscope to make sure that it is neither jagged nor sharp ended which may cause injury to the embryos during the course of transferring them to IVC for further observations. The prepared mouthpiece-controlled pipette was placed onto the metal block (Figure 3.3) and was sterilised again before usage by exposing it to ultra-violet radiation in the laminar flow.

The preparation of mouthpiece-controlled pipette requires skill and has to be done prior every flushing of embryos. This is because the pipette prepared can only be used once to prevent contamination.

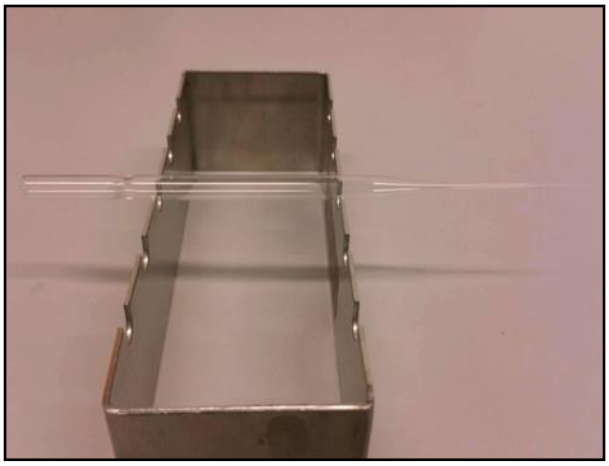


Figure 3.3: Prepared mouthpiece-controlled pipette (glass Pasteur pipette).

3.3 Administration of Liquefied Spirulina and Folic Acid by Oral Gavage

Oral gavage was performed by attaching a 1.5 inches, 20-gauge, curved and stainless steel mouthpiece onto a 1 ml syringe containing either liquefied spirulina or folic acid. This method was done so that the mice will consume a precise volume and dose of spirulina and folic acid which will avoid the under-dosage or over-dosage of either supplement.

To perform the administration of liquefied spirulina and folic acid, the mouse was first held by grasping over the loose skin of its neck by one hand and by curling its tail around the little finger. To avoid being bitten, the head of the mouse was made sure to be held in a vertical and constrict position. Then, the gavage needle was slowly inserted from mouth to the oesophagus then to the last rib of mice (Figure 3.4). After insertion, the syringe plunger was pressed gently to introduce 1 ml of supplement (spirulina or folic acid) into the stomach of the mouse. This procedure requires skill in order to insert the mouthpiece deep enough without choking the mouse. Subsequently, the gavage mouthpiece was removed gently after dosing.

The feeding of both spirulina and/or folic acid was based on treatment groups. The feeding of liquefied spirulina was done daily for four consecutive weeks whereas the feeding of liquefied folic acid was done daily before and after the mating of mice. Both of these feedings were done between 8 am to 10 am in the morning.

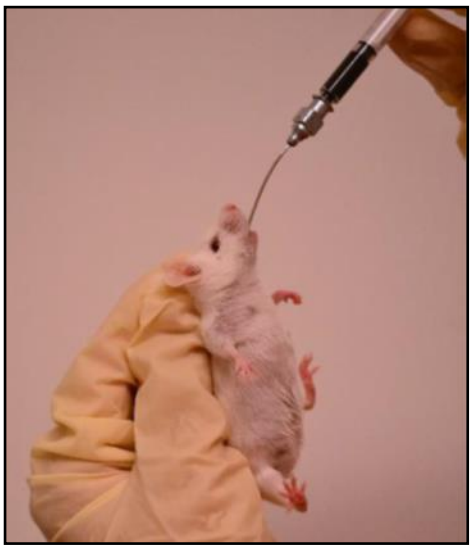


Figure 3.4: Administration of liquefied spirulina and/or liquefied folic acid by oral gavage.

3.4 Measurement on the Weight of Parental Mice after Treatment

After the mice were all fed with supplements according to treatment groups (liquefied spirulina for four consecutive weeks and/or liquefied folic acid before and after mating), the weight of both male and female mice were measured with a digital weighing scale. An empty beaker (250 ml) was first placed onto the digital weighing scale and the reading was tared. Then, each of these mice (both male and female) was placed into the beaker (Figure 3.5) and the weight was recorded according to sex and treatment group.

Figure 3.5: Mouse weighing method.

3.5 Mating (*In Vivo*)

After the mice were fed with supplements (liquefied spirulina for four consecutive weeks and/or liquefied folic acid for one week), both treated fertile male and female were allowed to mate based on their treatment groups by placing one of each gender into the same cage (labelled with treatment group and date of mating). Such placement of male and female mice (ratio of 1:1) was to increase the rate of successful copulation. Once the female mouse showed signs of gestation at the third week post-mating, the male mouse was taken out from the cage, leaving the female mice to give birth naturally (Figure 3.6) in that individual cage.

Figure 3.6: Dam with newborn pups.

3.6 Measurement on the Weight of Mouse Offspring

The measurement on the weight of mouse offspring for all treatment groups were taken four weeks after birth. The procedures to measure the weight of mice offspring are the same with the procedures discussed in sub-chapter 3.5.

3.7 Sex Determination of Mouse Offspring

For all treatment groups, the sex determination of mouse offspring was done four weeks after birth. The sex of mice was determined by the presence or absence of nipple, the presence and absence of scrotum and the length between the genitalia and anus (Figure 3.7). When the offspring is a female, nipple will be present and the length between its genitalia and anus will be shorter. In contrast, when the offspring is a male, scrotum will be present and the length between the genitalia and anus will be longer when compared to a female. The number of both male and female offspring was also recorded for all replicates in different treatment groups.

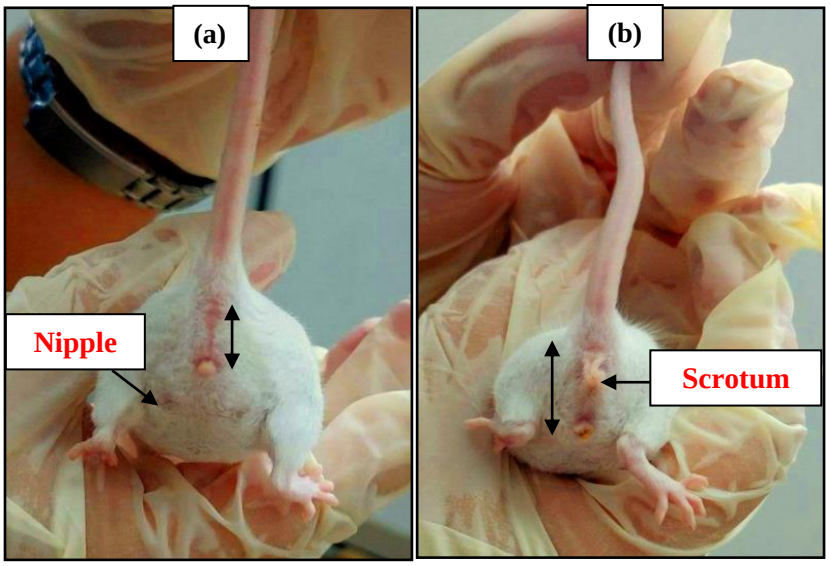


Figure 3.7: Sex of mouse. (a) Female mouse with presence of nipple and shorter length of genitalia and anus distance (refer to arrow); (b) Male mouse with presence of scrotum and longer length of genitalia and anus distance (refer to arrow).

3.8 Superovulation Method and Mating (*In Vitro*)

3.8.1 Superovulation Regime

Gonadotrophins were administered to matured female mice from each treatment group to induce superovulation (more production of matured eggs compared to normal). This superovulation regime was a 5-day process. On the fifth day before dissection was done, the female mouse was injected with 10 IU of pregnant mare's serum gonadotrophin (PMSG) at five in the evening for follicular development. This mouse was allowed to rest for one day before it was injected with another 10 IU of human chorionic gonadotrophin (hCG) on the third day before dissection, also at five in the evening. The injection of hCG was to synchronize oestrous and to induce the ovulation of oocytes. Both the injections were done intraperitoneally (Figure 3.8) while the mice were restrained using the method mentioned in sub-chapter 3.4.

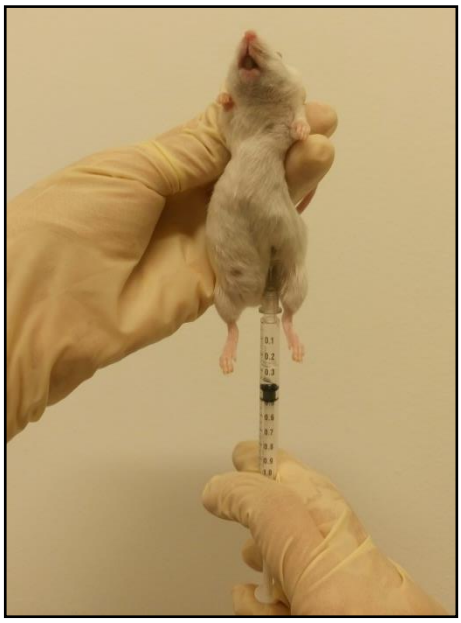


Figure 3.8: Both the injection of PMSG and hCG were done intraperitoneally.

3.8.2 Mating

During the 5-day process (superovulation regime), on the third day before dissection, a fertile male mouse was chosen and placed in a separate cage together with the superovulated female mouse based on treatment groups. Successful copulation was determined before the female mouse was chosen for dissection by observing the presence of vaginal plug at its genitalia.

3.9 Retrieval of Mouse Embryos

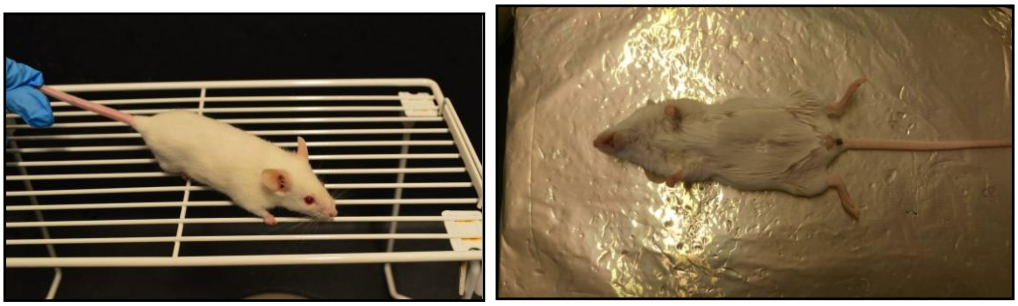
The successfully copulated female mouse was dissected at nine in the morning on the last day of the five-day process (superovulation regime) and two-cell embryos were retrieved. For each treatment group, a total of five female mice were subjected for dissection.

3.9.1 Mouse Dissection

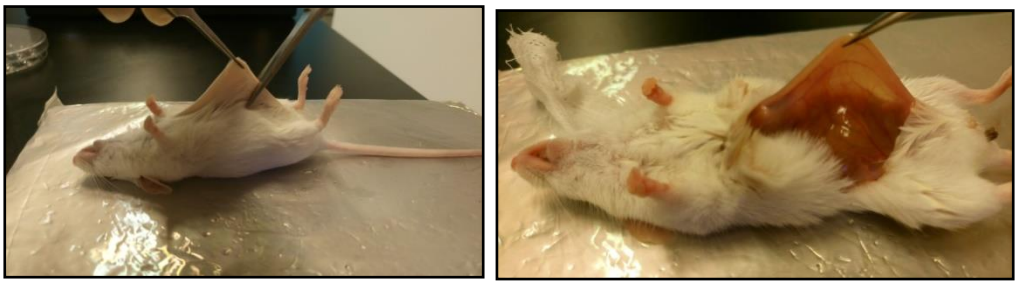
The dissection of mouse was carried out on a sterilized bench. The dissection tools needed were surgical scissors, tweezers, forceps, dissection board and gauze soaked in 70% ethanol. All the dissection tools were autoclaved and further sterilized with 70% ethanol before used to avoid contamination during collection of embryo from the oviduct of female mouse. The entire procedure of mouse dissection was shown in Figure 3.9. Before dissection, the female mouse was sacrificed via cervical dislocation to provide a humane, fast and painless death. In order to perform cervical dislocation, the female mouse was first placed on the metal rack (with rails) so that it may grip firmly on the rails with its forefeet. The tail was then gripped firmly with one hand while the other hand was used to truss up the head of mouse with thumb and index finger. After stabilizing the head of the mouse, the tail was immediately pulled firmly towards the back until the spinal column was dislocated from the skull. The change of colour in the mouse's eyes (from bright red to dull black) indicates the complete death of the mouse.

Subsequently, the dead mouse was placed facing upwards on the sterilized dissecting board. Then, gauze soaked with 70% of ethanol was used to clean the abdomen of mouse by swiping it in a downward direction. The skin on the lower abdominal area was pinched by using a pair of forceps and a small lateral incision was made using a pair of surgical scissors to expose the abdomen. A second incision was made again (with a clean forceps) with the same method on the peritoneum to expose the organs. The gastrointestinal tract covering the

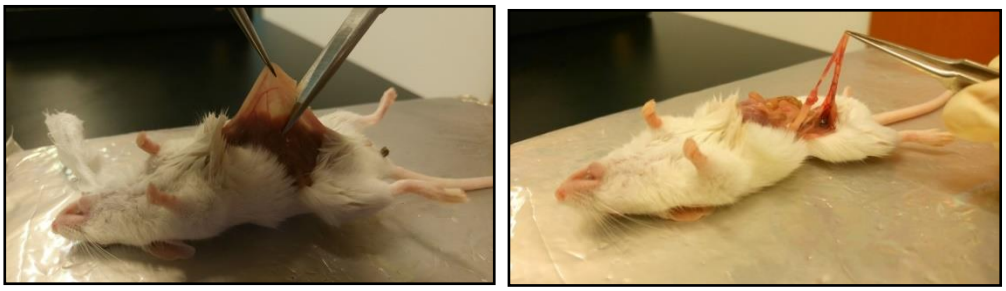
oviducts, ovaries and uterus was then removed by using the same forceps. The uterine horn was later located, grasped and the fat pads together with the excessive blood vessels attached was removed by using tweezers to avoid accidental rupture of both the wanted (oviduct, ovary and uterus) and unwanted (excessive blood vessels) parts. Then, the oviduct was cut from both the left and the right uterine horn and placed into the dish labelled flushing medium which contained ERM droplets for washing and embryo retrieval.



(a) (b)



(c) (d)



(e) (f)

(g)

Figure 3.9: Mouse dissection procedure. (a) Mouse placed on metal rack for cervical dislocation; (b) Place the mouse on the dissecting board facing upwards; (c) First incision on skin; (d) Exposure of peritoneum; (e) Second incision on peritoneum; (f) Exposure of female reproductive organ; (g) Retrieval of ovary and oviduct.

3.9.2 Flushing of Mouse Embryos from Infundibulum of Oviduct

The apparatus and materials needed for this procedure involves stereomicroscope, thermo plate stage warmer, syringe assembled with needle, mouthpiece-controlled pipette, tweezers, flushing medium and holding medium.

The thermo plate stage warmer was first switched on and adjusted to 37^{o}C. The stereomicroscope was also turned on after the dish containing the collected oviduct was placed onto the warm plate under the stereomicroscope in order to locate the infundibulum. Once the infundibulum was located, the opening of infundibulum was stabilized with a pair of tweezers and a syringe (1 ml) filled with warmed ERM mentioned in sub-chapter 3.3.8 assembled with a hypodermic needle of 27-gauge was inserted into the infundibulum. The ERM was flushed into the oviduct repetitively until no embryos were left in the oviduct. These flushed embryos were transferred to another droplet of ERM in the same dish by using mouth-piece assemble pipette to undergo washing before they were transferred again to another dish labelled holding medium. The total number of fragmented cells, one cells and fertilized embryos (two cells) were calculated and recorded during transfer.

3.9.3 *In Vitro* Culture of Mouse Embryos

By using the mouthpiece-controlled pipette, the fertilized embryos were transferred from the holding medium to the IVC medium. For each droplet of IVC medium (60 µl/ droplet), a maximum of eight embryos can only be

cultured in order to ensure optimal development of embryos. Once the embryos were transferred into the IVC medium, the culture dish was immediately placed back into the incubator (37℃ supplemented with 5% of CO_2) so that two-cell arrest of embryos may be avoided. The development of embryos were observed and recorded for five consecutive days until they become blastocyst.

3.10 Experimental Design

An overview of the experimental design is presented in Figure 3.10

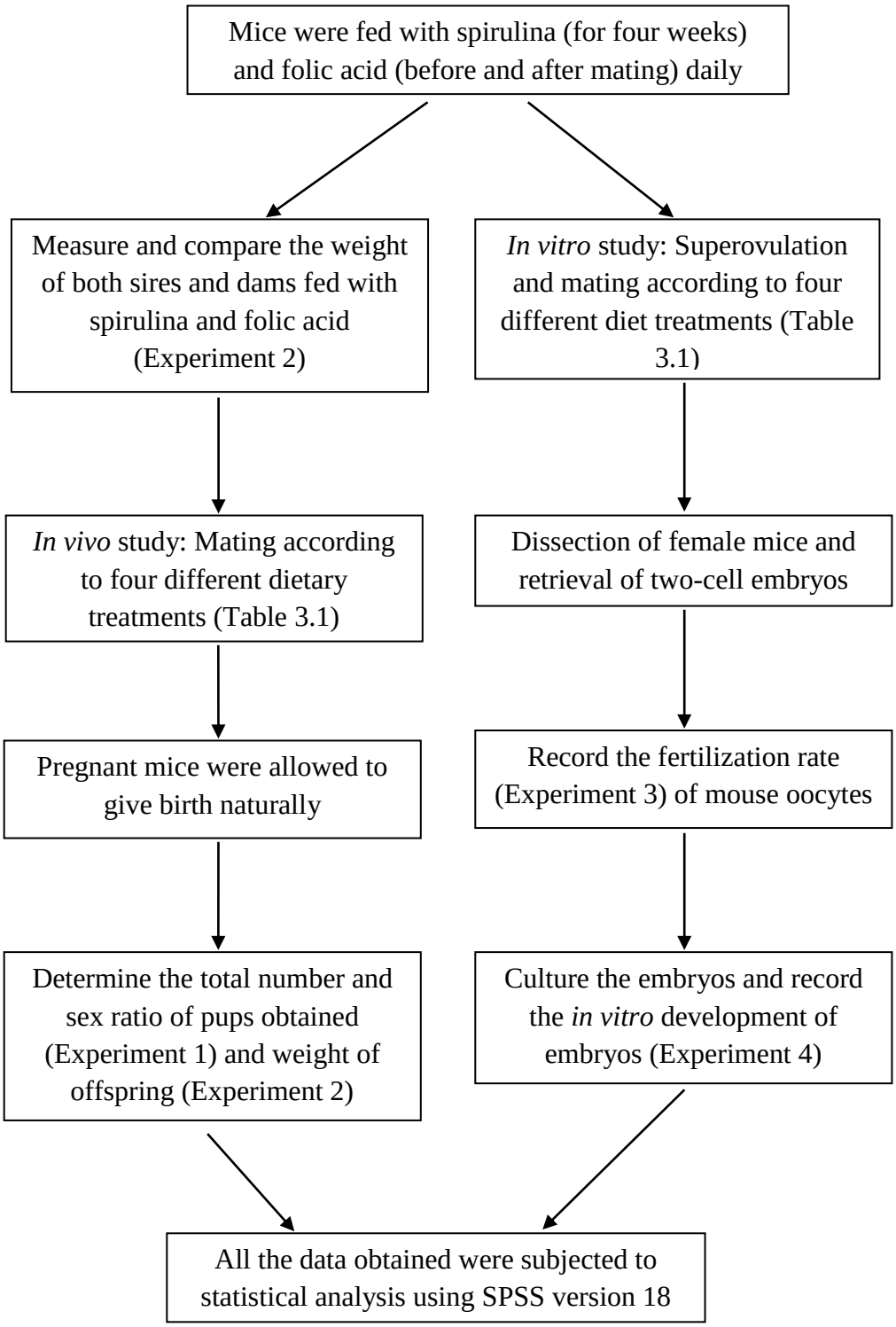


Figure 3.10: An overview of the experimental design

3.10.1 Effect of Spirulina and Folic Acid Intake by Parental Mice on the Offspring's Secondary Sex Ratio Skewness (Experiment 1)

For this experiment, the objective was to study the effect of spirulina and folic acid intake by parental mice on the offspring's secondary sex ratio skewness. In this experiment, a total of five dietary treatments were tested on mice. For each treatment, four male mice (sires) and four female mice (dams) were used. All the mice were mated after being treated with spirulina (four weeks before mating) and folic acid (one week before mating). Folic acid was continued to be fed to the female mice (dams) under this treatment daily for one week post-mating. Then, the pregnant mice were allowed to give birth naturally.

Table 3.1: Five different dietary treatment groups

Treatment	Female Mouse (Dam)	Male Mouse (Sire)
Control	Normal diet	Normal diet
A	Spirulina and Folic acid	Spirulina
B	Spirulina	Spirulina and Folic acid
C	Spirulina and Folic acid	Spirulina and Folic acid
D	Spirulina	Spirulina

Sex determination was done four weeks after the birth of the pups and the method used was discussed in chapter 3.8. Then, the secondary sex ratios for each treatment group were calculated by using the formula below:

$$\text{Secondary sex ratio} = \frac{\text{Number of male offspring}}{\text{Total number offspring}}$$

3.10.2 Effect of Spirulina and Folic Acid Intake on the Weight of Parental Mice and their Offspring (Experiment 2)

For this experiment, the weight (in grams) of both sire and dam before and after treatment (normal diet, diet of only spirulina, diet inclusive of both spirulina and folic acid) were taken down in order to determine if the intake of spirulina and folic acid increases or decreases the overall weight of mice. The weight differences were calculated by using the formula below:

$$\text{Weight of parental mice after treatment} - \text{Weight of parental mice before treatment}$$

In addition, the weight of 15 random offspring from dams of all five dietary treatment groups (refer Table 3.1) was also measured. The method to measure the weight of mice was discussed in sub-chapter 3.5.

3.10.3 Effect of Spirulina and Folic Acid Intake on the Fertilization Rate of Mouse Oocyte (Experiment 3)

In this experiment, a total of twenty dams and twenty sires were involved. After sacrificing and dissecting the dams from five different dietary treatment groups (Table 3.1), oocytes were collected and the fertilization rate was

determined. This was done by recording the total number of one-cell oocytes and two cells embryos for each and every flushing done for all treatment groups. The formula used was as below:

$$\text{Fertilization rate} = \frac{\text{Two cells embryos}}{\text{One cell + Two cells embryos}}$$

3.10.4 Effect of Spirulina and Folic Acid Intake by Parental Mice on the *In Vitro* Development of Embryos (Experiment 4)

For this experiment, the goal was to study the effect of spirulina and folic acid intake by parental mice on the *in vitro* development of embryos. The treatment groups designed for this experiment were the same with the treatment groups mentioned in Experiment 1 (Table 3.1). A total of twenty sires and twenty dams were used in this experiment. After flushing was done, the quality of the embryos were observed and recorded. Subsequently, the *in vitro* development of cultured embryos in IVC medium (located in the incubator at 37℃ and supplemented with 5% of CO_2) were observed and recorded for each treatment until they developed into blastocyst stage.

3.11 Statistical Analysis

Data on the sex ratio of mouse offspring obtained from Experiment 1 was statistically analysed by using one sample t-test whereas the total number of pups and the percentage of male and female offspring which is also obtained from the same experiment were analysed by using one-way analysis of variance (ANOVA) test. Subsequently, data from Experiment 2, Experiment 3 and Experiment 4 were analysed by using ANOVA via SPSS statistical software version 18. Means comparison was also accomplished by using Duncan's multiple range. The p value of < 0.05 is considered statistically significance in this study.

CHAPTER 4

RESULTS

4.1 Effect of Spirulina and Folic Acid Intake by Parental Mice on the Offspring's Secondary Sex Ratio Skewness (Experiment 1)

In this study, the dams from five different treatment groups (control, treatment A, treatment B, treatment C and treatment D) gave birth to a total of 215 pups. From these 215 newborn pups, 44 pups were from control group, 33 pups were from treatment A, 47 pups were from treatment B, 42 pups were from treatment C and the remaining 49 pups were from treatment D.

According to Table 4.1, statistical analysis showed that there was no significant difference in the average number of pups for control (11.00 ±1.63), treatment A (8.25 ±0.50), treatment B (11.75 ±1.71), treatment C (10.50 ±1.91) and treatment D (12.25 ±2.63). This means that when dams were fed with normal feed, with an additional supplementation of spirulina or with additional of both spirulina and folic acid, they will still give birth to similar number of pups.

Table 4.1: Average number of pups obtained from five different dietary treatment groups

Treatment	No. of replicates	Average number of pups (Mean ±SD)
Control	4	11.00 ± 1.63^{ab}
A (♀ Spi+FA x ♂ Spi)	4	8.25 ± 0.50^{a}
B (♀ Spi x ♂ Spi+FA)	4	11.75 ± 1.71^{b}
C (♀ Spi+FA x ♂ Spi+FA)	4	10.50 ± 1.91^{ab}
D (♀ Spi x ♂ Spi)	4	12.25 ± 2.63^{b}

[a,b,] means with different superscripts within a column were not significantly different ($p \geq 0.05$).
Spi means fed with spirulina; Spi+FA means fed with spirulina and folic acid.
♀ means female; ♂ means male; x means 'mated with'.

The sex of pups for each treatment groups were determined and shown in Figure 4.1. For control group, there were 23 male pups and 21 female pups. In treatment A, the number of male and female pups obtained was 17 and 16, respectively. While for treatment B, 32 male pups and 15 female pups were delivered. In treatment C, the dams gave birth to 23 male pups and 19 female pups respectively. Similar trend were observed in treatment D as numerically, the number of male pups were relatively higher compared to female pups (29 and 20, respectively).

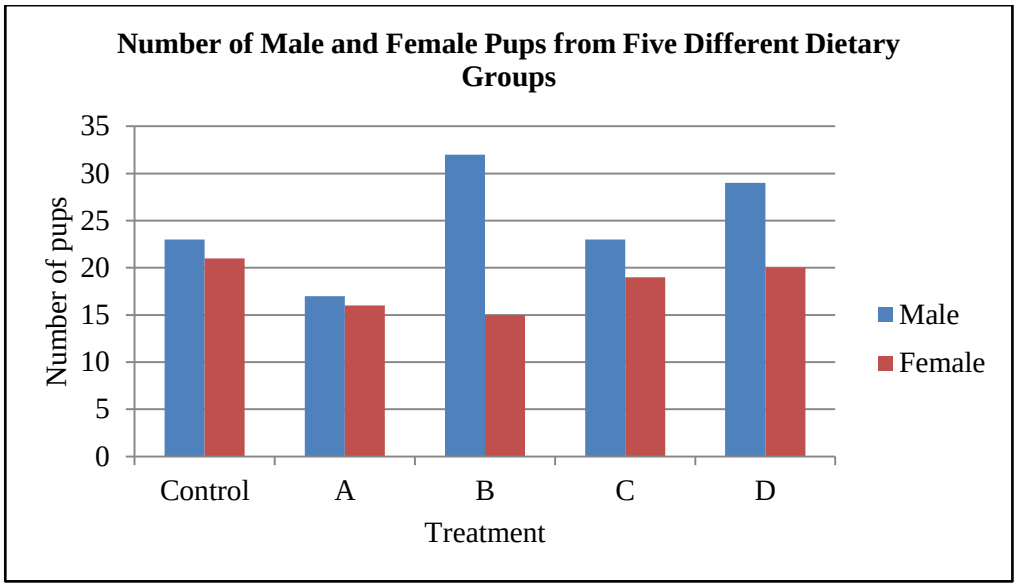


Figure 4.1: Number of male and female pups from five different dietary groups.

Evaluation on the secondary sex ratio of mice offspring were conducted for each dietary treatment groups by using one sample t-test. The initial t-test was conducted for control group by evaluating the sex ratio of offspring against the expected value of 0.5 (derived from the standard sex ratio of 1:1) Result obtained showed that the sex ratio of offspring from the control group (*p* value = 0.30) deviated non-significantly ($p \geq 0.05$) from 0.5 (Table 4.2). Therefore, the following treatments were tested against the expected value of 0.5. The sex ratio for both treatment B (0.68±0.11) and treatment D (0.69±0.10) were significantly higher ($p < 0.05$) from the sex ratio of control group (0.53±0.04), treatment A (0.51±0.11) and treatment C (0.54±0.07).

Table 4.2: Secondary sex ratio of mice offspring and *p* value for five different dietary treatment groups

Treatment	No. of replicates	Sex ratio (Mean ±SD)	Significant *p* value
Control	4	0.53 ± 0.04^{a}	0.30
A (♀ Spi+FA x ♂ Spi)	4	0.51 ± 0.11^{a}	0.81
B (♀ Spi x ♂ Spi+FA)	4	0.68 ± 0.11^{b}	0.04
C (♀ Spi+FA x ♂ Spi+FA)	4	0.54 ± 0.07^{a}	0.34
D (♀ Spi x ♂ Spi)	4	0.69 ± 0.10^{b}	0.04

^{a,b} means with different superscripts within a column were significantly different ($p < 0.05$).
Spi means fed with spirulina; Spi+FA means fed with spirulina and folic acid.
♀ means female; ♂ means male; x means 'mated with'.

Besides analysing the significance of the offspring's secondary sex ratio skewness, the percentage of both male and female offspring in the five different dietary groups was also analysed using one-way analysis of variance (ANOVA) test. The percentage of male offspring from treatment B (68.15 ±10.64) and treatment D (68.43 ±10.10) were significantly higher ($p <$ 0.05) than the percentage of male offspring from other treatment groups (Table 4.3). In contrast, the percentage of female offspring from treatment B (31.85 ±10.64) and treatment D (31.58 ±10.10) were significantly lower ($p <$ 0.05) than the percentage of female offspring from control group (47.30 ±4.36), treatment A (48.60 ±10.58) and treatment C (46.00 ±7.10). This shows that when dams were supplemented with spirulina alone, they will produce more male offspring.

Table 4.3: Percentage of male and female offspring of five different dietary treatment groups

Treatment	No. of replicates	Percentage of offspring according to sex, % (Mean ±SD)	
		Male	Female
Control	4	52.70 ±4.36[a]	47.30 ±4.36[b]
A (♀ Spi+FA x ♂ Spi)	4	51.40 ±10.58[a]	48.60 ±10.58[b]
B (♀ Spi x ♂ Spi+FA)	4	68.15 ±10.64[b]	31.85 ±10.64[a]
C (♀ Spi+FA x ♂ Spi+FA)	4	54.00 ±7.10[a]	46.00 ±7.10[b]
D (♀ Spi x ♂ Spi)	4	68.43 ±10.10[b]	31.58 ±10.10[a]

[a,b] means with different superscripts within a column were significantly different ($p < 0.05$).
Spi means fed with spirulina; Spi+FA means fed with spirulina and folic acid.
♀ means female; ♂ means male; x means 'mated with'.

4.2 Effect of Spirulina and Folic Acid Intake on the Weight of Parental Mice and their Offspring (Experiment 2)

Based on the outcome of the test (Table 4.4), the weight for both sire and dam fed with spirulina and folic acid (13.00 ±3.37 and 9.15 ±1.71, respectively) increased significantly ($p < 0.05$) compared to the weight of sire and dam fed with normal diet (4.70 ±1.29 and 2.20 ±1.15, respectively) and the weight of sire and dam with a diet solely supplemented with spirulina (5.55 ±3.10 and 2.41 ±0.69, respectively). Generally, the weights of sires were significantly heavier ($p < 0.05$) than the weight of dams across all treatment groups.

Table 4.4: Weight differences of parental mice fed with three different diets

Treatment	No. of replicates	Weight differences in parents, g (Mean ±SD)	
		Male (Sire)	Female (Dam)
Normal	4	$4.70 \pm 1.29^{a,y}$	$2.20 \pm 1.15^{a,x}$
Spirulina	4	$5.55 \pm 3.10^{a,y}$	$2.41 \pm 0.69^{a,x}$
Spirulina + Folic acid	4	$13.00 \pm 3.37^{b,y}$	$9.15 \pm 1.71^{b,x}$

^{a,b,} means with different superscripts within a column were significantly different ($p < 0.05$).

^{x,y} means with different superscripts within a row were significantly different ($p < 0.05$).

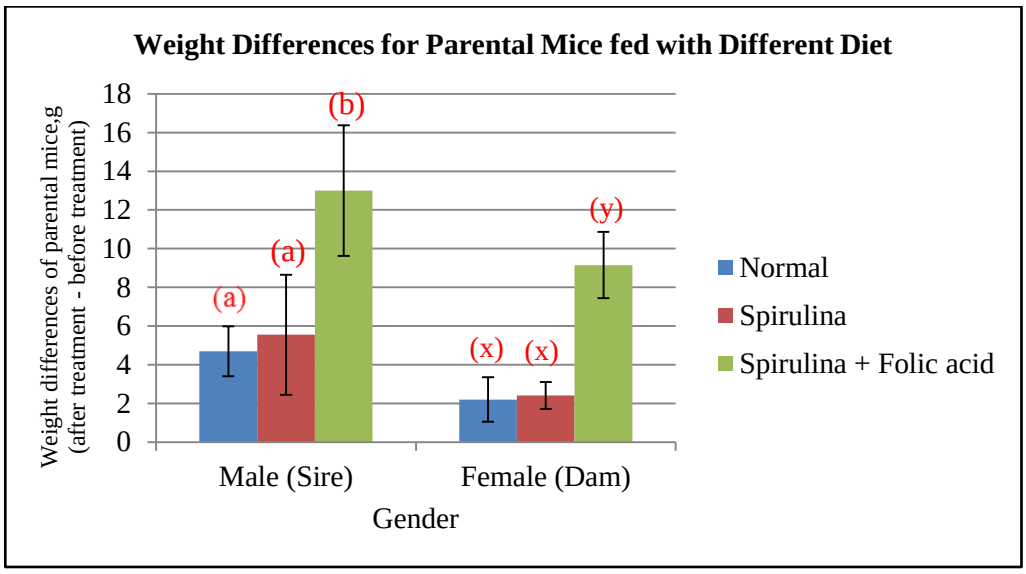


Figure 4.2: Weight differences of parental mice fed with three different diets. Bars with different letters (a, b) within male group and (x, y) within female group are significantly different ($p < 0.05$).

Other than analyzing the weight of parental mice, the weight of offspring were also measured based on the treatment groups of their parents and data obtained analyzed using one-way analysis of variance (ANOVA) test. Result showed that the mean weight of male offspring from dams of treatment A, treatment B and treatment C (26.33 ± 5.98, 21.47 ± 3.80 and 26.80 ± 0.77, respectively) were

significantly higher ($p < 0.05$) than the mean weight of male offspring from dams of control group (17.87 ±3.46). The mean weight of male offspring from treatment D (20.27 ±1.91) showed numerical increase in mean weight. However, this increment was not significant ($p \geq 0.05$) when compared to control group. Similarly, the weight of female offspring from dams of treatment A and treatment C (19.93 ±1.28 and 21.60 ±1.59, respectively) were also significantly higher than the mean weight of female offspring from dams of control (17.07 ±4.20). In contrast, the mean weight of female offspring from treatment B (10.60 ±3.11) and treatment D (8.73 ±2.15) were significantly lower ($p < 0.05$) than the mean weight of female offspring from control (Table 4.5). Generally, the weight of male offspring was significantly heavier ($p < 0.05$) than the weight of female offspring across all treatment groups.

Table 4.5: Weight of offspring born from parental mice treated with five different dietary treatment groups

Treatment	Weight of offspring, g (Mean ±SD)	
	Male	Female
Control	$17.87 \pm 3.46^{a,y}$	$17.07 \pm 4.20^{b,x}$
A (♀ Spi+FA x ♂ Spi)	$26.33 \pm 5.98^{c,y}$	$19.93 \pm 1.28^{c,x}$
B (♀ Spi x ♂ Spi+FA)	$21.47 \pm 3.80^{b,y}$	$10.60 \pm 3.11^{a,x}$
C (♀ Spi+FA x ♂ Spi+FA)	$26.80 \pm 0.77^{c,y}$	$21.60 \pm 1.59^{c,x}$
D (♀ Spi x ♂ Spi)	$20.27 \pm 1.91^{ab,y}$	$8.73 \pm 2.15^{a,x}$

^{a,b,c} means with different superscripts within a column were significantly different ($p < 0.05$).
^{x,y} means with different superscripts within a row were significantly different ($p < 0.05$).
Spi means fed with spirulina; Spi+FA means fed with spirulina and folic acid.
♀ means female; ♂ means male; x means 'mated with'.

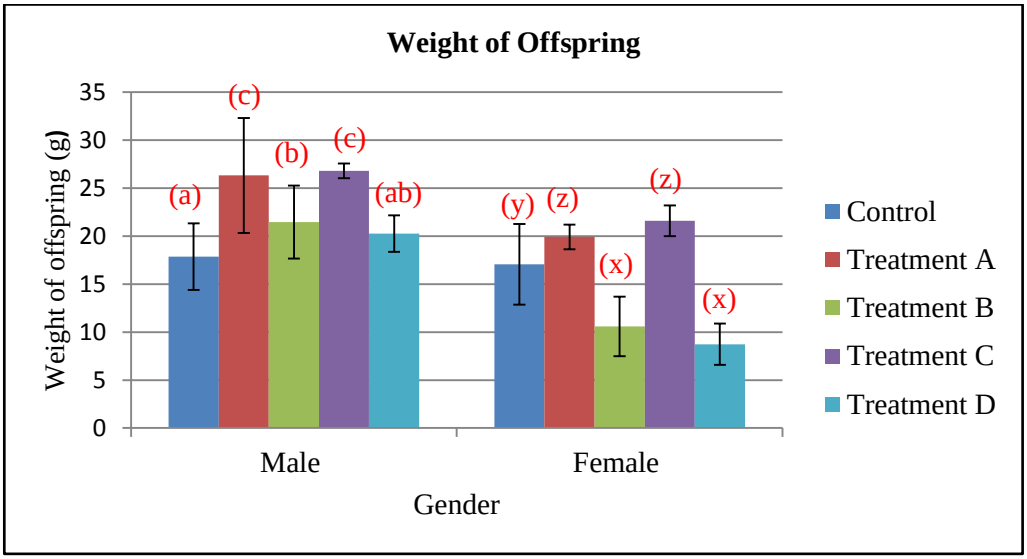


Figure 4.3: Weight of offspring born from parental mice treated with five different dietary treatment groups. Bars with different letters (a, b, c) within male group and (x, y, z) within female group are significantly different ($p < 0.05$).

4.3 Effect of Spirulina and Folic Acid Intake on the Fertilization Rate of Mouse Oocyte (Experiment 3)

The analysis on the fertilization rate of mouse oocyte was performed by calculating the percentage of fertilized and unfertilized oocytes for the five different dietary treatment groups. In this experiment, a total of 484 oocytes were fertilized and formed embryos at two-cell stage whereas 72 oocytes were unfertilized (Figure 4.4) on the second day of post-copulation.

Based on the result shown in Table 4.6, the percentage of fertilized oocytes (two-cell embryos) in treatment D (87.79±7.90) was significantly higher ($p < 0.05$) than the percentage of fertilized oocytes (two-cell embryos) in control.

However, there was no significant difference ($p \geq 0.05$) in the percentage of fertilized oocytes (two-cell embryos) in treatment A (77.89±7.90), treatment B (77.28±5.41) and treatment C (76.2±14.59) when compared to control (67.83±7.94) although the percentage of fertilized embryos for all these groups were numerically higher than control group.

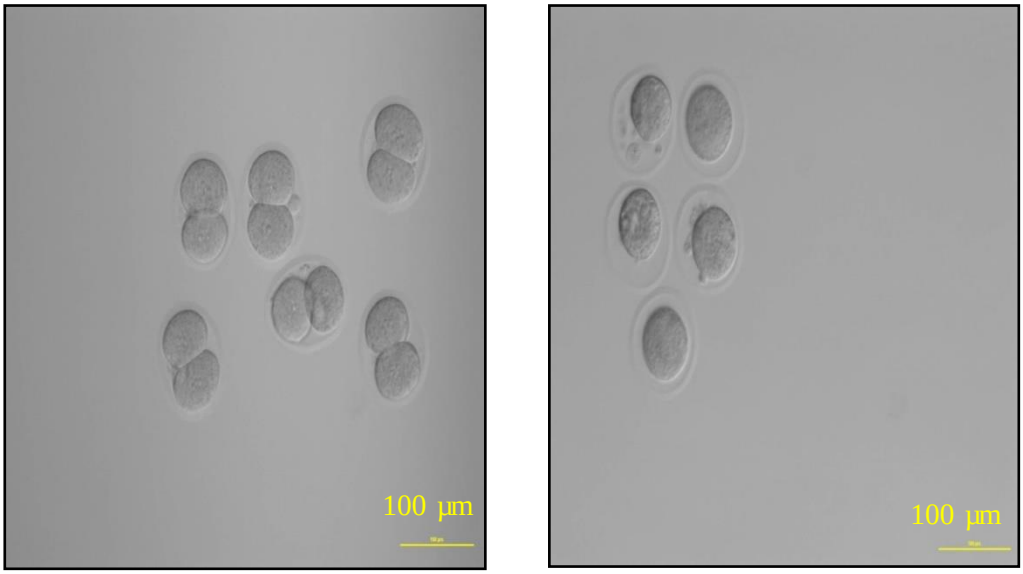
On the other hand, the percentage of unfertilized oocytes was significantly lower ($p < 0.05$) in treatment D (12.21±7.90) as compared to control group (32.16±7.94). Similarly, there was no significant difference ($p \geq 0.05$) in the percentage of unfertilized oocytes in treatment A (22.11±7.90), treatment B (22.73±5.41) and treatment C (23.13±14.59) when compared to control (32.16±7.94) although the percentage of unfertilized embryos for all these groups were numerically lesser than control group.

Table 4.6: Percentage of fertilized and unfertilized oocytes retrieved from five different dietary treatment groups

Treatment	Replicates	Percentage of embryos (Mean%±SD)	
		Fertilized	Unfertilized
Control	4	67.83 ± 7.94^{a}	32.16 ± 7.94^{b}
A (♀ Spi+FA x ♂ Spi)	4	77.89 ± 7.90^{ab}	22.11 ± 7.90^{ab}
B (♀ Spi x ♂ Spi+FA)	4	77.28 ± 5.41^{ab}	22.73 ± 5.41^{ab}
C (♀ Spi+FA x ♂ Spi+FA)	4	76.2 ± 14.59^{ab}	23.13 ± 14.59^{ab}
D (♀ Spi x ♂ Spi)	4	87.79 ± 7.90^{b}	12.21 ± 7.90^{a}

[a,b] means with different superscripts within a column were significantly different ($p < 0.05$).

Spi means fed with spirulina; Spi+FA means fed with spirulina and folic acid. ♀ means female; ♂ means male; X means 'mated with'.



(a) (b)

Figure 4.4: Mouse embryos retrieved. (a) Fertilized embryos at 2-cells stage; (b) Unfertilized oocytes under 100x magnification.

4.4 Effect of Spirulina and Folic Acid Intake by Parental Mice on the *In Vitro* Development of Embryos (Experiment 4)

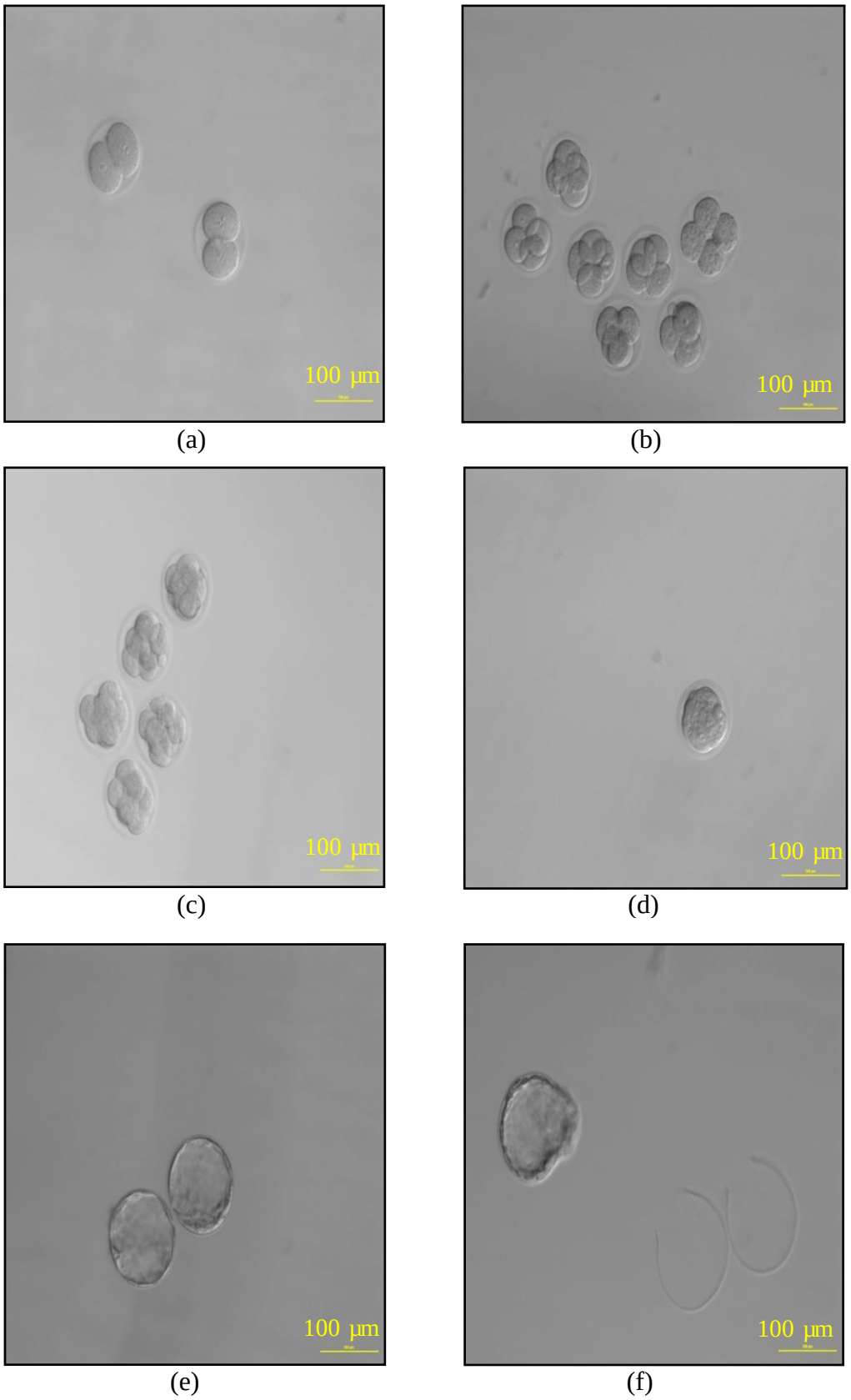
The *in vitro* development of embryos (from two-cells to four-cells, eight-cells, morula and blastocyst stages) from all five different dietary treatment group were also investigated. For this study, a total of 484 two-cell embryos retrieved from dams of all treatment groups in Experiment 3 were cultured in the incubator. The *in vitro* developmental rate of these embryos from two-cell stage to blastocyst was recorded daily from day-2 post-copulation until day-5.

Results in Table 4.7 showed that there is a significant increment ($p < 0.05$) on the percentage of two-cells embryos that successfully developed into blastocyst stage for all treatment groups (treatment A, treatment B treatment C and treatment D) when they are compared against the control group. This means that when dams were supplemented with spirulina alone or with both spirulina and folic acid, they will have a tendency to produce two-cell embryos that have a significantly higher chance in developing into blastocyst stage. The percentage of embryos that reached blastocyst stage for treatment A (97.53 ± 2.83), treatment B (97.38 ± 2.27), treatment C (99.28 ± 1.25) and treatment D (97.98 ± 3.50) were significantly higher ($p < 0.05$) than control (84.82 ± 2.58). In other words, the percentage of blastocyst for all the treatment groups ranged from 97% to 100% which is considered a high percentage. The photomicrographs of embryos from two-cells to blastocyst stage were shown in Figure 4.5.

Table 4.7: *In vitro* development of embryos retrieved from five different dietary treatment groups

Treatment	*In vitro* development of embryos (Mean%±SD)				
	Two-cells	Four-cells	Eight-cells	Morula	Blastocyst
Control	100.00±0.00[a] (46*)	93.39±0.96[a] (43/46)	93.39±0.96[a] (43/46)	93.39±0.96[a] (43/46)	84.82±2.58[a] (39/46)
A (♀ Spi+FA x ♂ Spi)	100.00±0.00[a] (123*)	97.53±2.83[b] (121/123)	97.53±2.83[b] (121/123)	97.53±2.83[b] (121/123)	97.53±2.83[b] (121/123)
B (♀ Spi x ♂ Spi+FA)	100.00±0.00[a] (129*)	100.00±0.00[b] (129)	100.00±0.00[b] (129)	100.00±0.00[b] (129)	97.38±2.27[b] (125/129)
C (♀ Spi+FA x ♂ Spi+FA)	100.00±0.00[a] (82*)	99.28±1.25[b] (81/82)	99.28±1.25[b] (81/82)	99.28±1.25[b] (81/82)	99.28±1.25[b] (81/82)
D (♀ Spi x ♂ Spi)	100.00±0.00[a] (104*)	100.00±0.00[b] (104)	100.00±0.00[b] (104)	100.00±0.00[b] (104)	97.98±3.50[b] (102/104)

[a,b,] means with different superscripts within a column were significantly different ($p < 0.05$).
Spi means fed with spirulina; Spi+FA means fed with spirulina and folic acid.
♀ means female; ♂ means male; x means 'mated with'.
* means total number of cells



(a) (b) (c) (d) (e) (f)

Figure 4.5: Photomicrograph of *in vitro* development of mouse embryos. (a) 2-cells embryos; (b) 4-cells embryos; (c) 8-cells embryos; (d) Compacted morula; (e) Blastocyst; (f) Hatched blastocyst under 100x magnification.

CHAPTER 5

DISCUSSION

5.1 Effect of Spirulina and Folic Acid Intake by Parental Mice on the Offspring's Secondary Sex Ratio Skewness (Experiment 1)

For this experiment, the analysis showed that the average number of offspring did not differ significantly ($p \geq 0.05$) regardless whether the dams were fed with normal diet (control), a diet with spirulina (treatment B and D) or a diet with both spirulina and folic acid (treatment A and C). In other words, the supplementation of either spirulina or folic acid into the dams' diet did not influence the average number of offspring produced. However, when dams were fed with spirulina alone, the offspring produced have a significantly higher percentage of male offspring compared to dams fed with normal diet or with both spirulina and folic acid. Such result indicates that the sex ratio of offspring is significantly skewed towards male when dams were fed solely with spirulina.

According to Huang, et al. (2014), the number of offspring produced by dams fed with different amounts of folic acid (5 mg/kg body weight and 40 mg/kg body weight) did not differ significantly with the control group where dams were fed with normal diet. In addition, based on a study done by Chamorro, et al. (1997), the different concentrations of spirulina (0%, 10%, 20% and 30%) fed to parental mice did not affect the number of offspring produced. This finding was also supported by a research done by Low (2016) which stated that the supplementation of spirulina to dams does not significantly increase the

average number of offspring produced when compared to the dams fed with normal diet. The result obtained from this experiment was consistent with the findings of the above studies and thus confirmed that the supplementation of spirulina and folic acid in different combinations will not give a significant increment in average number of offspring produced.

Generally, spirulina is known as one of the many superfoods because it is high in antioxidants, proteins, fatty acids, vitamins and minerals (Belay, et al., 1993). The fatty acids in spirulina can be further divided into two groups which are omega-3 fatty acid and omega-6 fatty acid (Ötleş and Pire, 2001). Based on a recent study done by Mousavi, et al. (2016), it was recognized that dams with a diet rich in omega-6 fatty acid tend to produce offspring with a sex ratio that is significantly skewed towards male. Besides, sow with a diet rich in sodium and potassium was also seen to favour the birth of male offspring (Bolet, et al., 1982) and spirulina has been proven to contain both sodium and potassium in high concentrations (Tang and Suter, 2011). Therefore, these may be some of the reasons that contributed to the significant skewness of the secondary sex ratio of offspring towards male when dams were fed solely with spirulina supplement.

In contrast, the supplementation of folic acid alone to maternal diet has been found to favour the birth of female offspring. According to Czeizel, Dudas and Metneki (1994), dams fed with folic acid supplementation gave birth to more female offspring compared to dams supplemented with trace minerals (50.1%

against 48.1%). Additionally, it was also suggested that dams fed with folic acid-containing vitamins during peri-conception will result in a slight shift (Scholl and Johnson, 2000) on the secondary sex ratio offspring which was initially at a ratio of 1:1 (neutral) towards more female offspring.

Unlike the above studies done, folic acid was not solely supplemented to dams in this experiment. Instead, folic acid was an additional supplement combined with the feeding of spirulina. Thus, based on the result obtained, the current study has discovered that when folic acid is supplemented with spirulina, it will have an impact on the secondary sex ratio of offspring by drawing the skewness of the offspring's secondary sex ratio towards 1:1 (neutral). In other words, the dams fed with spirulina and folic acid supplementation will produce a similar number of both male and female offspring.

5.2 Effect of Spirulina and Folic Acid Intake on the Weight of Parental Mice and their Offspring (Experiment 2)

In this sub-experiment, parental mice fed with supplementation of only spirulina did not show any significant increment in body weight when compared to the weight of parental mice in control group. Instead, when there was an additional supplementation of folic acid, the weight of parental mice increased significantly. Similar trend were observed on the weight of offspring as the offspring for both male and female produced by dams fed with both spirulina and folic acid showed a significantly heavier weight than other offspring.

The result on the weight of parental mice or its offspring can be supported by previous studies done by Mousavi, et al. (2016) that both omega-3 and omega-6 fatty acids that are also found abundantly in spirulina do not give any significant increment in weight when they are supplemented towards mice. However, when additional folic acid was supplemented to the initial iron diet provided by spirulina, there will be a significant increase in weight because the iron-folic acid diet has shown to enhance appetite (Kanani and Poojara, 2000).

Another study done by Janczyk, et al. (2006) also showed that dams fed with a diet of *Chlorella* sp. which is a microalgae source rich in omega fatty acids produced offspring that do have any significant increment in birth weight. However, according to Krumdieck, et al. (1977), it has been proven that folic

acid increased the formation of haemoglobin during embryo development which in turn gave a significantly heavier offspring.

It has been found that the health of offspring is associated with the offspring's birth weight (Wilcox, 2001). Thus, since folic acid is known to lower birth defects (e.g. neural tube defects) (Juriloff and Harris, 2000) and increase the chances of a healthy offspring, it seems fair that the birth weight of offspring produced by dams with additional folic acid supplementation to be significantly heavier than other offspring produced by dams without any additional folic acid supplementation.

In general, the weight of sires and male offspring were significantly higher than the weight of dams and female offspring. This is due to the effect of hormones in the bodies of both male and female. It has been reported that both androgens and testosterones found in the male body enhances food consumption (Earley and Leonard, 1979) and increases the amount of weight gained (Asarian and Geary, 2006) respectively. On the other hand, oestrogen in females is found to reduce food consumption and also the amount of weight gained (Acosta-Martinez, Horton and Levine, 2007). Another study that supported this finding was done by Burdge, et al. (2008), where it states that female offspring tends to have a slower weight gain as compared to male offspring.

5.3 Effect of Spirulina and Folic Acid Intake on the Fertilization Rate of Mouse Oocyte (Experiment 3)

Based on the result obtained for this experiment (Table 4.6), it seemed that the percentage of fertilized embryos is significantly higher when both parental mice were supplemented with spirulina only. Besides that, the percentage of fertilized embryos from parental mice fed with different combinations of additional folic acid also increased numerically when compared to control group.

In recent years, spirulina has been known to improve the health of both the oocyte and sperm (Argüelles-Velázquez, et al., 2013) due to its antioxidant properties provided by vitamins A and E (Ali and Saleh, 2012) which protects these gametes from being damaged by free-radicals. According to Kazemi, Ramezanzadeh and Nasr-Esfahani in 2015, dietary antioxidants are proven to promote the oocyte's developmental and maturation rate. Similarly, it was also reported that dietary antioxidants improves the motility of sperm while reducing oxidative damage towards sperm (Collins and Rossi, 2015). Theoretically, when there is an improvement in the health of both oocyte and sperm, there will also be and increment in the fertilization rate of oocytes.

Besides, spirulina also contains selenium. Based on the study done by Kacheva, et al. (2008), when the organic compound of selenium (selenopyran) is added to the diet of sows, the number of pre-ovulatory follicles increased significantly in its ovaries. This is because selenopyran stimulates the ovaries to have a

better response towards hormonal stimulation (superovulation). Such response, in turn, will give rise to the outcome of embryos (Kistanova, et al., 2009). Therefore, the significant increment in the percentage of fertilized embryos from dams fed with spirulina only is consistent and can be supported with the above studies.

The supplementation of folic acid was also proven by Ebisch, et al. (2007) to provide a better oocyte quality and to give more mature oocytes. Thus, when dams in this experiment were supplemented with an addition of folic acid, there was a numerical increase in the percentage of fertilized embryos compared to the control group.

5.4 Effect of Spirulina and Folic Acid Intake by Parental Mice on the *In Vitro* Development of Embryos (Experiment 4)

In this experiment, result showed that the mean percentage of blastocyst formation from two-cell embryos obtained from dams supplemented with different combinations of spirulina and folic acid was significantly higher when compared to the mean percentage of blastocyst formation from two-cell embryos obtained from dams in control group.

According to Ashworth, Toma and Hunter in 2009, maternal conditions played a vital role in the growth and development of embryos and the intake of nutrients is one of the factors that contributed to a good maternal condition.

Thus, when dams were fed with a basic supplementation of spirulina (treatment A, treatment B, treatment C and treatment D) which is a rich source of nutrients (Belay, et al, 1993), there was a higher mean percentage of blastocyst formation from 2-cell embryos. To be specific, the high percentage of blastocyst formation may be caused by the high concentrations of copper found in spirulina (Johnson and Shubert, 1986). According to the research done by Gao, et al. (2011), the rate of morulae and blastocysts can be greatly increased when there is a sufficient concentration of copper in the maternal diet. In addition, the fatty acids found in spirulina may have aided the formation of blastocysts from two-cell embryos because it has been reported that two-cell embryos obtained from cows fed with omega-3 and omega-6 fatty acids has a significantly higher percentage of blastocyst formation (Cerri, et al., 2009). It was also reported that the presence of folic acid is vital during pre-implantation because it is an essential nutrient that aids in early embryo development (from two-cell embryo to blastocyst). According to Fong and Bongso (1999), high blastulation rates will give rise to the rate of successful implantation.

5.5 General Discussion

Based on the findings of this study, dams fed solely with spirulina supplementation gave a secondary sex ratio of offspring that is significantly skewed towards male. However, when there is an additional supplementation of folic acid to the diet, the secondary sex ratio of offspring will be drawn back towards neutral. Such findings will allow the effective control of sex ratio in the agriculture industry whereby two contradicting supplements will not be fed

together. For an example, in order to breed more male calf in the meat industry, the cow will be fed with a diet solely with a supplementation of spirulina without addition of folic acid prior copulation (Low, 2016). In contrast, when more female calf is needed in the milk industry, the cow should only be fed with a diet solely with a supplementation of folic acid without addition of spirulina. The same concept can be applied by human parents so that the rate of sex-selective abortion may be reduced worldwide and there will also be lesser discrimination in care practices that is initially implemented because the parents did not get their desired son or daughter (Hesketh and Xing, 2006). Still, if there is a family history of heredity diseases and the parents do not mind having a son or a daughter, the mother is suggested to take in both spirulina and folic acid supplements as it will give a higher chance to have a healthy baby.

5.5.1 Suggestion for Future Study

In this study, the supplementation of additional folic acid was done peri-conceptual on dams with spirulina diet and it showed that folic acid did give an impact on the secondary sex ratio of offspring produced (drawn back towards ratio 1:1). Therefore, in near future, a study on the influence of folic acid on dams with spirulina diet fed either before or after mating towards the secondary sex ratio of offspring may be done. The purpose of the proposed study is to determine whether dams fed with supplementation of only spirulina peri-conception and addition of folic acid post-conception could still retain the sex ratio skewness towards male.

CHAPTER 6

CONCLUSIONS

In conclusion, the main findings of this study proposed that dams fed solely with spirulina will produce offspring with a secondary sex ratio that is skewed towards male. However, the feeding of spirulina with folic acid to dams will skew the secondary sex ratio of offspring back towards the ratio 1:1 (neutral). Although so, the average number of pups produced will not be influenced by the supplementation of either spirulina or folic acid. The sub-findings of this study also showed that dams and sires fed with a diet inclusive of additional folic acid supplement will have significant weight increments whereas offspring from dams fed with additional folic acid supplement will have a heavier mean weight. In addition, the fertilization rate of oocytes from dams fed with spirulina alone will be significantly higher and the fertilization rate of oocytes from dams fed with spirulina and folic acid will be numerically higher when compared to control group. Moreover, the feeding of spirulina and folic acid to one or both parental mice will give a significant increase on the blastulation rate of embryos.

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APPENDICES

Appendix 1 (a) (List of materials, equipment and facilities):

Item / Product		Description / Content	Model / Catalogue Number	Manufacturer
No.	Name			
1	Stereomicroscope	-	SM2161	Motic
2	Micro warm plate	-	-	Tokai Hit
3	Inverted microscope	-	Eclipse Ti	Nikon
4	Water bath	-	FCE 20	Memmert
5	Micropipette	2.5 µl, 10 µl, 100 µl, 1000 µl	-	Eppendorff
6	Diamond stone	-	-	-
7	Forceps	4 inches	-	-
8	Dissecting scissors	5 inches	-	-
9	Oral gavage mouthpiece	1.5 inches, 20-gauge, curved	-	-
10	Laminar flow	-	AC2-4E1	ESCO
11	Autoclave	-	HVE-50	HIRAYAMA
12	Weighing balance	-	CP224S	Sartorius

Appendix 1 (b) (Disposables):

Item / Product		Description / Content	Model / Catalogue Number	Manufacturer
No.	Name			
1	Disposable gloves	-	-	Hang Care
2	Syringe	1 ml	SS+01T	Terumo
3	Hypodermic needle	-	NN2613R	Terumo
4	Gauze	-	-	-
5	Micropipette tips	White, yellow, blue	-	Axygen
6	Plastic Petri dish	35 mm, 60 mm	-	NUNC IVF Product Line
7	Aluminium foil	-	-	Reynolds Foil Inc.
8	Parafilm	-	PM-996	Bemis Flexible Packaging
9	Microcentrifuge tube	1.5 ml	Watson	131-8155C
10	Sawdust	-	-	-
11	Masks	-	-	-

Appendix 1 (c) (List of chemicals):

Item / Product		Description / Content	Model / Catalogue Number	Manufacturer
No.	Name			
1	PCV	-	360	Sigma-Aldrich
2	Ethanol	-	-	-
3	Phosphate buffered saline (PBS)	-	PD0435	Bio Basic Canado Inc.
4	Penicilin streptomycin (PS)	-	15140-122	Gibco Life Technologies Corporation
5	Pregnant mare's serum gonadotrophin (PMSG)	-	-	Internet International BV., Boxmeer, Holland
6	Human chorionic gonadotrophin (hCG)	-	-	Internet International BV., Boxmeer, Holland
7	Mineral oil	-	M8410-1L	Sigma-Aldrich
8	Spirulina	Tablets	-	Elken
9	Hydrochloric acid	-	-	-

Appendix 2 (Gold Coin (702-P) mouse pellet feed composition and ingredients):

Composition:

a) Crude Protein (21%)	c) Crude Fat (3%)	e) Ash (8%)	g) Phosphorus (0.4%)
b) Crude Fiber (5%)	d) Moisture (13%)	f) Calcium (0.8%)	

Ingredients:

a) Corn	f) Molasses	k) Trace Minerals and Enzymes
b) Soybean Meal	g) Calcium Carbonate	l) Approved Antimicrobials
c) Other Grains and Grain-by-products	h) Dicalcium Phosphate	m) Additives
d) Animal Protein	i) Amino Acids	
e) Vegetable Oil	j) Vitamins	

Appendix 3: Appendix for Chapter 4 (Results)

Appendix 3 (Table 1): ANOVA result for total number of pups from different treatment groups

One way-ANOVA

Descriptives

Total Number of Pups

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	4	11.0000	1.63299	.81650	8.4015	13.5985	9.00	13.00
Treatment A	4	8.2500	.50000	.25000	7.4544	9.0456	8.00	9.00
Treatment B	4	11.7500	1.70783	.85391	9.0325	14.4675	10.00	14.00
Treatment C	4	10.5000	1.91485	.95743	7.4530	13.5470	9.00	13.00
Treatment D	4	12.2500	2.62996	1.31498	8.0652	16.4348	10.00	16.00
Total	20	10.7500	2.14905	.48054	9.7442	11.7558	8.00	16.00

ANOVA

Total Number of Pups

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	38.500	4	9.625	2.931	.056
Within Groups	49.250	15	3.283		
Total	87.750	19			

Post Hoc Tests

Homogeneous Subsets

Total Number of Pups

Duncan

Treatment	N	Subset for alpha = .05	
		1	2
Treatment A	4	8.2500	
Treatment C	4	10.5000	10.5000
Control	4	11.0000	11.0000
Treatment B	4		11.7500
Treatment D	4		12.2500
Sig.		.059	.227

Means for groups in homogeneous subsets are displayed.

a Uses Harmonic Mean Sample Size = 4.000.

Appendix 3 (Table 2): One-sample t-test results for sex ratio of offspring from different treatment groups

One-sample t-test

Control

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
Control	4	.5270	.04364	.02182

One-Sample Test

	Test Value = 0.5					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
Control	1.237	3	.304	.02700	-.0424	.0964

Treatment A

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
Treatment A	4	.5140	.10583	.05292

One-Sample Test

	Test Value = 0.5					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
Treatment A	.265	3	.808	.01400	-.1544	.1824

Treatment B

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
Treatment B	4	.6823	.10624	.05312

One-Sample Test

	Test Value = 0.5					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
Treatment B	3.431	3	.042	.18225	.0132	.3513

Treatment C

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
Treatment C	4	.5400	.07100	.03550

One-Sample Test

	Test Value = 0.5					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
Treatment C	1.127	3	.342	.04000	-.0730	.1530

Treatment D

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
Treatment D	4	.6850	.10083	.05041

One-Sample Test

	Test Value = 0.5					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
Treatment D	3.670	3	.035	.18500	.0246	.3454

Appendix 3 (Table 3): ANOVA results for percentages of male and female offspring from different treatment groups

One way-ANOVA

Descriptives

Percentage of Male Pups

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	4	52.7000	4.36425	2.18212	45.7555	59.6445	46.20	55.60
Treatment A	4	51.4000	10.58332	5.29166	34.5596	68.2404	37.50	62.50
Treatment B	4	68.1500	10.63657	5.31829	51.2248	85.0752	54.50	80.00
Treatment C	4	54.0000	7.09977	3.54988	42.7027	65.2973	44.40	61.50
Treatment D	4	68.4250	10.09897	5.04948	52.3553	84.4947	60.00	83.00
Total	20	58.9350	11.16782	2.49720	53.7083	64.1617	37.50	83.00

ANOVA

Percentage of Male Pups

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1179.928	4	294.982	3.719	.027
Within Groups	1189.758	15	79.317		
Total	2369.686	19			

Post Hoc Tests

Homogeneous Subsets

Percentage of Male Pups

Duncan

Treatment	N	Subset for alpha = .05	
		1	2
Treatment A	4	51.4000	
Control	4	52.7000	
Treatment C	4	54.0000	
Treatment B	4		68.1500
Treatment D	4		68.4250
Sig.		.701	.966

Means for groups in homogeneous subsets are displayed.

a Uses Harmonic Mean Sample Size = 4.000.

Appendix 3 (Table 4): ANOVA results for weight differences of parental mice fed with three different diets

One way-ANOVA

Descriptives

Weight Differences of Sire

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Normal	4	4.7000	1.29099	.64550	2.6457	6.7543	3.20	6.20
Spirulina	4	5.5500	3.09570	1.54785	.6241	10.4759	2.80	9.80
Spirulina + Folic acid	4	13.0000	3.36650	1.68325	7.6431	18.3569	8.00	15.00
Total	12	7.7500	4.61785	1.33306	4.8160	10.6840	2.80	15.00

ANOVA

Weight Differences of Sire

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	166.820	2	83.410	11.080	.004
Within Groups	67.750	9	7.528		
Total	234.570	11			

Appendix 2 (Table 5): ANOVA results for weight of offspring from different treatment groups

One way-ANOVA

Descriptives

Weight of Male Pups

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	15	17.8667	3.46135	.89372	15.9498	19.7835	13.00	23.00
Treatment A	15	26.3333	5.98411	1.54509	23.0194	29.6472	19.00	35.00
Treatment B	15	21.4667	3.79599	.98012	19.3645	23.5688	18.00	30.00
Treatment D	15	20.2667	1.90738	.49248	19.2104	21.3229	18.00	25.00
Treatment C	15	26.8000	.77460	.20000	26.3710	27.2290	26.00	28.00
Total	75	22.5467	4.98726	.57588	21.3992	23.6941	13.00	35.00

ANOVA

Weight of Male Pups

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	910.453	4	227.613	17.130	.000
Within Groups	930.133	70	13.288		
Total	1840.587	74			

Post Hoc Tests

Homogeneous Subsets

Weight of Male Pups

Duncan

Treatment	N	Subset for alpha = .05		
		1	2	3
Control	15	17.8667		
Treatment D	15	20.2667	20.2667	
Treatment B	15		21.4667	
Treatment A	15			26.3333
Treatment C	15			26.8000
Sig.		.076	.370	.727

Means for groups in homogeneous subsets are displayed.

a Uses Harmonic Mean Sample Size = 15.000.

Appendix 3 (Table 6): ANOVA results for fertilization rate of oocytes retrieved from different treatment groups

One way-ANOVA

Descriptives

Fertilized Oocytes

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	4	67.8325	7.93551	3.96776	55.2053	80.4597	56.52	73.91
Treatment A	4	77.8925	7.89835	3.94917	65.3245	90.4605	70.13	85.71
Treatment B	4	77.2750	5.41057	2.70529	68.6656	85.8844	70.27	81.82
Treatment C	4	76.2000	14.58946	7.29473	52.9849	99.4151	57.69	88.89
Treatment D	4	87.7900	7.90006	3.95003	75.2192	100.3608	77.97	96.15
Total	20	77.3980	10.49920	2.34769	72.4842	82.3118	56.52	96.15

ANOVA

Fertilized Oocytes

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	804.749	4	201.187	2.340	.102
Within Groups	1289.682	15	85.979		
Total	2094.431	19			

Post Hoc Tests

Homogeneous Subsets

Fertilized Oocytes

Duncan

Treatment	N	Subset for alpha = .05	
		1	2
Control	4	67.8325	
Treatment C	4	76.2000	76.2000
Treatment B	4	77.2750	77.2750
Treatment A	4	77.8925	77.8925
Treatment D	4		87.7900
Sig.		.177	.123

Means for groups in homogeneous subsets are displayed.

a Uses Harmonic Mean Sample Size = 4.000.

Appendix 3 (Table 6): ANOVA results for *in vitro* development of embryos retrieved from different treatment groups

One way-ANOVA

Descriptives

Blastocyst Stage

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	3	84.8233	2.58101	1.49015	78.4117	91.2349	82.35	87.50
Treatment A	3	97.5300	2.83138	1.63470	90.4965	104.5635	94.44	100.00
Treatment B	3	97.3833	2.26734	1.30905	91.7509	103.0157	96.00	100.00
Treatment C	3	99.2767	1.25285	.72333	96.1644	102.3889	97.83	100.00
Treatment D	3	97.9800	3.49874	2.02000	89.2886	106.6714	93.94	100.00
Total	15	95.3987	5.93611	1.53270	92.1114	98.6860	82.35	100.00

ANOVA

Blastocyst Stage

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	426.064	4	106.516	15.836	.000
Within Groups	67.260	10	6.726		
Total	493.324	14			

Post Hoc Tests

Homogeneous Subsets

Blastocyst Stage

Duncan

Treatment	N	Subset for alpha = .05	
		1	2
Control	3	84.8233	
Treatment B	3		97.3833
Treatment A	3		97.5300
Treatment D	3		97.9800
Treatment C	3		99.2767
Sig.		1.000	.423

Means for groups in homogeneous subsets are displayed.

a Uses Harmonic Mean Sample Size = 3.000.