REPRODUCTIVE FUNCTIONS IN HEAT ADAPTED FEMALE WISTAR RATS

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CERTIFICATION

This is to certify that this research work on "**Reproductive functions in heat adapted female Wistar rats**" was carried out by Omogiade Glory Uyi with matriculation number **PG/BMS1818373** in partial fulfillment of the award of masters of science (M.Sc) degree in Human Physiology in the Department of Physiology, School of Basic Medical Sciences, University of Benin, Benin City.

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DEDICATION

This work is dedicated to Almighty God, the creator and giver of life for His faithfulness, mercy and intentionality towards me.

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I would like to express my sincere gratitude to my supervisor Prof. O. I. Ajayi for the continuous support throughout this study, for his wisdom, motivation, encouragement and time. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better supervisor for this study.

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TABLE OF CONTENT

LIST OF FIGURES

LIST OF PLATES

ABSTRACT

Heat adaptation is the physiologic adaptation that occur in response to recurrent elevations in core and skin temperatures from high ambient temperatures over the course of 7-14 days or more, prompting the negative responses of heat stress. Heat adaptation plays a large role in the physical response and overall ability of the body to cope with heat exposure. The current climate change with attendant heat waves served as the basis to study the adaptive mechanism to the increase in environmental temperature. This study was therefore aimed at investigating the effect of heat-adaptation on the reproductive functions of female rats. A total of 48 (36 adult and 12 immature) virgin female Wistar rats and 6 male Wistar rats were used for this study. Experiment was carried out in different phases, with all phases having 6 animals each in control and heat adapted groups. The rats in heat adapted groups were housed in a special heated chamber 5 hours per day, maintaining a constant ambient temperature of 34-39°C for the different study phases, the control group animals were kept in an ambient temperature of 25-30°C. Baseline parameters and samples collected were used for estrous cycle classification, uterine contractility, hormonal, histological and gene expression assays. Statistical analysis was done using the GraphPad Prism version 8.1. It was observed in this study that heat adaptation had no significant effect on estrus cyclicity, uterine and ovarian weight, uterine and ovarian morphology, female reproductive hormonal levels, and contractility of both pregnant and non-pregnant isolated uterus as well as litter size and weight. There was a significant increase in both surface and core body temperature of the heat adapted group compared to the control group. Heat shock protein-70 (HSP-70) was significantly ($P<0.05$) upregulated in the heat adapted group. In conclusion relatively stable reproductive indices are associated with heat adapted female rats while significantly up-regulated Heat shock protein-70 (HSP-70) gene could be the regulatory adaptive mechanism to prevent any negative consequences.

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Heat adaptation (also known as heat acclimatization when naturally induced such as seasonal heat adaptation or heat acclimation when heat is induced via artificial means) is the physiologic adaptation that occur in response to recurrent elevations in core and skin temperatures from either exercise or high ambient temperatures, or a combination of both over the course of 7-14 days or more, prompting the negative responses of heat stress (Casa and Csillan, 2009; Periard *et al.,* 2015). The magnitudes of adaptations are determined by the intensity, duration, frequency, and number of heat exposures, as well as the environmental conditions such as temperature, humidity and solar radiation and physical work rate (metabolic heat production) (Epstein and Moran, 2008; Periard *et al.,* 2015).

Heat adaptation plays a large role in the physical response and overall ability of the body to cope with heat exposure. Almost every aspect of an organism's life, beginning with the formation of gametes and progressing through conception, prenatal and postnatal development, aging, morbidity, and death, is affected in several ways by temperature. It has been discovered that acute heat exposure leads to changes in the cardiovascular, circulatory, nervous, endocrine and reproductive system (Hutter *et al.,* 1996; Hall *et al.,* 2000; Ilievska *et al.,* 2016). When exposed to uncomfortably elevated temperature, some mechanisms are activated to maintain thermal balance of the body in a state known as heat stress (Jia *et al.,* 2012), the oxidative capacities of major heat-producing organs such as the liver, kidney, and brown adipose tissue (BAT) are reduced during heat acclimation as their core temperature also increases (Gordon, 1993). High environmental temperatures impair the female reproductive process at various stages of pubertal development, affects conception and can be a cause of embryonic mortality

(Krishnan *et al.,* 2017). At present, most of the studies on heat stress and heat injury models have focused on economic animals such as dairy cow, buffalo, ewe, and pig (Zheng *et al.*, 2019). The few studies on heat stress in female rats, different modeling conditions and inconsistent conclusions has not made it suitable for further study of the human menstrual cycle

Temperature is the degree of average quantity of heat in a place or the body, it is measured using a thermometer in Celsius or Fahrenheit scale. The average body temperature is 98.6^0F (37 °C). Weather which is the atmospheric condition of a place over a period of time is strongly affected by temperature (Abdullahi *et al.,* 2019). The major cause of heat adaptation in this part of the world (Nigeria) is as result of the hot weather condition. Nigeria has a unique tropical climate with two distinct seasons; the wet and the dry season. Dry season spans from November to march with a very hot temperature especially in the southern and northern region, while the wet season spans from end of March to October with a dry break in August. The temperature varies between 22°C and 31°C depending on the region and season, it can go as high as 43°C which is mostly the case in most part of the country (Chikezie *et al.,* 2016; Falaju, 2020). Body temperatures between 40°C and 41°C can cause hyperthermia lethal effect (Jardine, 2007). The choice of animal model has been proven to have great influence on the outcome of results (Elvis-Offiah *et al.,* 2016). Humans share physiological similarities with rodents and this help to extrapolate discoveries to human. Rats and mice are mostly used because they are easy and flexible to manage (Elvis-Offiah *et al.,* 2016).

1.2 Problem Statement

It was hypothesized that high temperature can have a negative effect on female reproductive functions, hence this study. One of the causes of infertility might be heat-adaptation related issues. This study will help ascertain if the female reproductive functions are affected by heat adaptation and also to know if there are mutation in genes as a result of heat stress.

1.3 Aim of the Study

To investigate the effect of heat-adaptation on the reproductive functions of female rats.

1.4 Research Questions

1. Does heat adaptation delay or facilitate puberty?
2. How does heat adaptation alter female reproductive cycle?
3. Does heat adaptation affect female's receptivity to male during mating?
4. Does heat adaptation alter reproductive hormones such as estrogen and progesterone?
5. Does heat adaptation affect contractility of both pregnant and non-pregnant uterus?
6. Does heat adaptation cause gene mutation?
7. What happens to the reproductive organs of heat-adapted females in terms of tissue morphology?

1.5 Specific Objectives

1. To investigate the effect of heat adaptation in uterotrophic assay such as onset of vagina opening using immature female rats.
2. To determine the effect of heat adaptation on estrus cycle.
3. To investigate the effect of heat adaptation on fertility.
4. To investigate the effect of heat adaptation on reproductive hormones (estrogen, progesterone, luteinizing hormone, and follicle stimulating hormone.
5. To investigate the effect of heat adaptation on uterine contractility in pregnant and non-pregnant uterus.
6. To determine the effect of heat adaptation on specific genes expression.
7. To determine the effect of heat adaptation on the morphology of reproductive organs

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

The reproductive functions of some female rodents such as rats have been affected strongly when they are exposed to heat above or between an ambient temperature of 20-24°C and core body temperature of 37.0-37.9°C (Gordon, 1993). There is increased estrous-cycle duration and reduced food intake in rats following acclimation to 35°C, (Hamid and Zakaria, 2013), increased production of reactive oxygen species (ROS) (Ozawa *et al.,* 2002), increased lipid peroxidation, protein oxidation and inflammation, increased progesterone levels during diestrus (Kurowicka *et al.,* 2007; Ilievska *et al.,* 2016), lowered ovarian activity, reduced percentages of fertilized and implanted ova as well as reduced number of metrial glands. Rats reared at ambient temperatures of 30-32°C have also been reported to have smaller litters and early weaning times compared to those that were not (Yamauchi *et al.,* 1981; Ilievska *et al.,* 2016).

2.2 The Reproductive System

The reproductive system is the system by which mammals produce offspring (Harrison, 2020). The reproductive system have four major functions which are; to produce egg or sperm cells, to transport and sustain the cells, to nurture developing offspring and to produce sex hormones. The organs are divided into primary organs consisting of the ovaries and testes and the secondary or accessory organs consisting of penis, epididymis, vas deferens, ejaculatory ducts, fallopian tubes, uterus and vagina. The ova or egg is produced by the ovary located in the pelvic cavity and the spermatozoa from the testes housed in the scrotum, a skin sac suspended behind the penis. The ovaries and testes are also responsible for the production of sex hormones that are needed for the development of secondary sexual characteristics and the proper functioning of the reproductive tract. Spermatozoa are conveyed from the testis to the ducts for storage and

released through the penis alongside with seminal fluid during ejaculation. The fallopian tube functions by conveying ovum for fertilization and then to the uterus for implantation and foetal development.

2.2.1 Anatomy and Physiology of the Female Reproductive System

The organs of the female reproductive system are involved in carrying out several functions ranging from production of ova or oocytes to conception and post conception. The female reproductive system comprises of both internal and external genitalia. The external genitalia consist of the vagina, the minor and major labia, and the clitoris, the internal genitalia consists of the cervix, the fallopian tubes, ovaries and uterus.

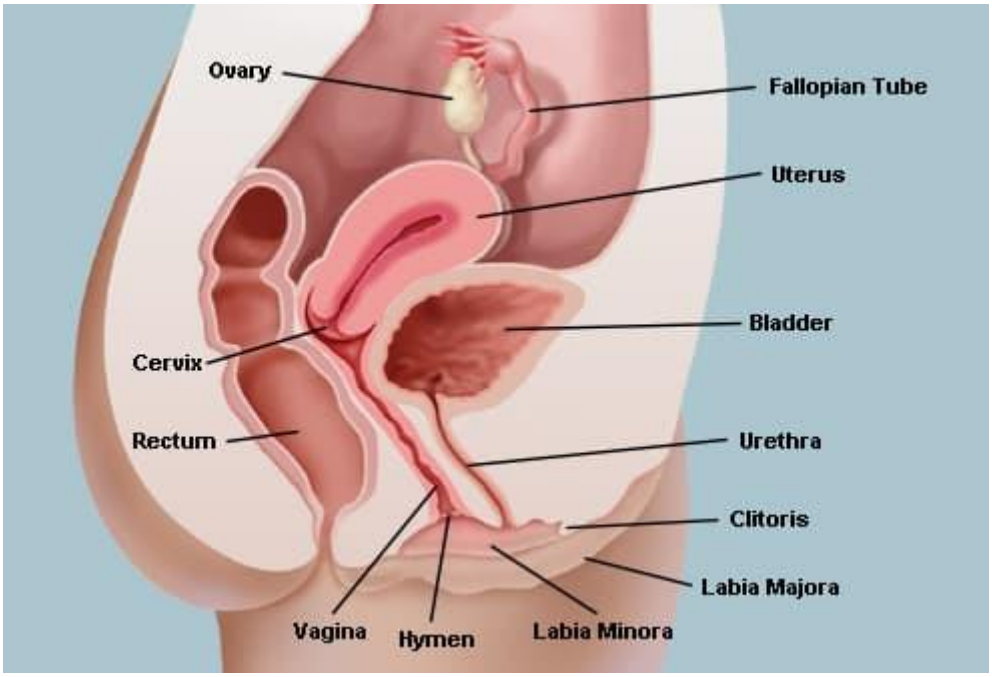


Figure 1: The female reproductive organs (Rodriguez *et al.*, 2020)

The vagina

It is an elastic, soft flexible and muscular canal that extends from the vulva to the cervix. It serves a site for penile penetration during sexual intercourse, a birth canal that baby passes through during childbirth and as a conduit for menstrual flow from the uterus. The opening of

the vagina lies in the urogenital triangle and it is protected by the labia. Blood is supplied to the vagina by vaginal artery. The upper part of the vagina is innervated by pelvic plexus while the lower part is innervated by pudendal nerve (Dutta, 2014).

The uterus

The uterus is located in the pelvic posterior to the bladder and anterior to the rectum. It is a hollow, pear shaped organ responsible for different functions, labour and delivery. It is divided anatomically into four segment; the fundus which is the point that connects the uterus to the fallopian tubes, the body or corpus, isthmus (the neck of the uterus) and the cervix extending downwards opening into the vagina. The uterus lies in different positions such as anteflexed or retroverted, anteverted or retroverted and rotated (occurs during pregnancy). The most common position occurring in 50% of women is anteflexed and anterverted positions.

Uterine and ovarian arteries supplies blood to the uterus. The arteries branches off as the arcuate arteries that supplies the endometrium and then as radial and spiral arteries. Uterine changes in shape and size depends on the reproductive phase and response to sex hormones. In pre-pubertal age it is small, in reproductive age the body of the uterus is bigger than the cervix while in postmenopausal it becomes atrophic with a body smaller than the cervix (Al-Qattan and Al-Qattan, 2018).

The uterus functions by nurturing the fertilized ovum. The fertilized ovum implants into the endometrium to be nourished, as the embryo develops the uterus expands for proper accommodation and it contracts as the cervix dilates during labour to expel the fetus (Ameer, *et al.,* 2017). Some of the gynecological disorder that affect the uterus are polyps, leiomyoma (fibroid), endometriosis and uterine cancer (Paul *et al.,* 2018).

Uterine structure

Three tissue layers makes up the uterus; they are endometrium, myometrium and perimetrium or serosa. The endometrium is the inner lining of the uterus composed of the functional layer after it sheds during menstruation resulting in menstrual bleeding. The basal layer becomes the new functional layer. The myometrium is the middle layer of the uterus and it is composed of smooth muscle while the perimetrium is the outer layer composed of epithelial cells.

The endometrium in the menstrual cycle

The uterine endometrium functions by preparing the uterus for implantation, maintains pregnancy if implantation occurs, and menstruates in the absence of pregnancy. All these shows that the endometrium plays a fundamental role in reproduction and the mechanisms (such as the interaction between the endocrine system and the central nervous system) behind the different roles are steroid regulated because the endometrium composing of stromal, epithelial, vascular, and immune cells; is a complex multicellular target for steroids. A rapid endometrial repair occurs involving inflammation resolution, angiogenesis, tissue remodeling, and formation of new tissue without residual scarring or loss of function. The menstruating endometrium can be considered as a wonderful physiological example of an injured surface that is required to rapidly repair each month (Critchley *et al.*, 2020).

During the human menstrual cycle, the endometrium is exposed each month to a progressive pattern of circulating ovarian sex hormones that are crucial for growth regulation and endometrial differentiation. The major hormones are ovarian 17β-estradiol (E2) and pregn-4-ene-3, 20-dione (progesterone; P4), and their concentrations fluctuates in a patterned manner throughout the menstrual cycle.

Menstrual cycle phases

The orderly cyclic sloughing of the endometrium in response to hormonal interplay by the hypothalamus, pituitary gland and the ovaries id known as menstruation. The first day of menstrual bleeding which lasts between 3 to 5 days marks the beginning of the cycle and its last for 28 days for an average cycle duration. When the cycle length is less than 21 days it is termed polymenorrhea, if more than 35 days it is called oligomenorrhea and if does not occur during the reproductive age it is called amenorrhea. The average volume of blood shed during menstruation is about 30 mL. During the extremes of reproductive age that is menarche and menopause the menstrual cycle is usually most irregular as a result of anovulation and inadequate follicular development (Reed and Carr, 2015). The different phases of menstrual cycle are;

Proliferative phase: this phase lasts between 14 and 18 days for an average 28 days cycle. In this phase estradiol which is at its peak causes endometrial tissues and vessels to proliferate and develop. The proliferative phase occurs concomitantly with the follicular phase of ovarian cycle, and after ovulation the corpus luteum secrets progesterone marking the end of this phase. The proliferative phase is subdivided into early, mid-, and late proliferative phases. The early proliferative phase occurs right after menses, usually around day 4 to day 7 when there is formation of thin linear layer on the surface of the regenerating endometrium. In the early phase mitotic activity is exhibited by clustered stroma and there is enlargement of cellular nuclei with little surrounding cytoplasm. From the early phase the endometrium progresses to the mid-proliferative phase, occurring around day 8 to day 10 of the cycle, the glands becomes curved and elongated as against the spindle shape in the early phase. The late proliferative phase finally occurs between day 11 and 14, the glands becomes coiled and clustered undergoing active mitosis and nuclear pseudostratification. The stratum functionalis layer that is the inner lining of the endometrium gets to a maximum thickness of about 0.5 to 5mm forming a trilaminar

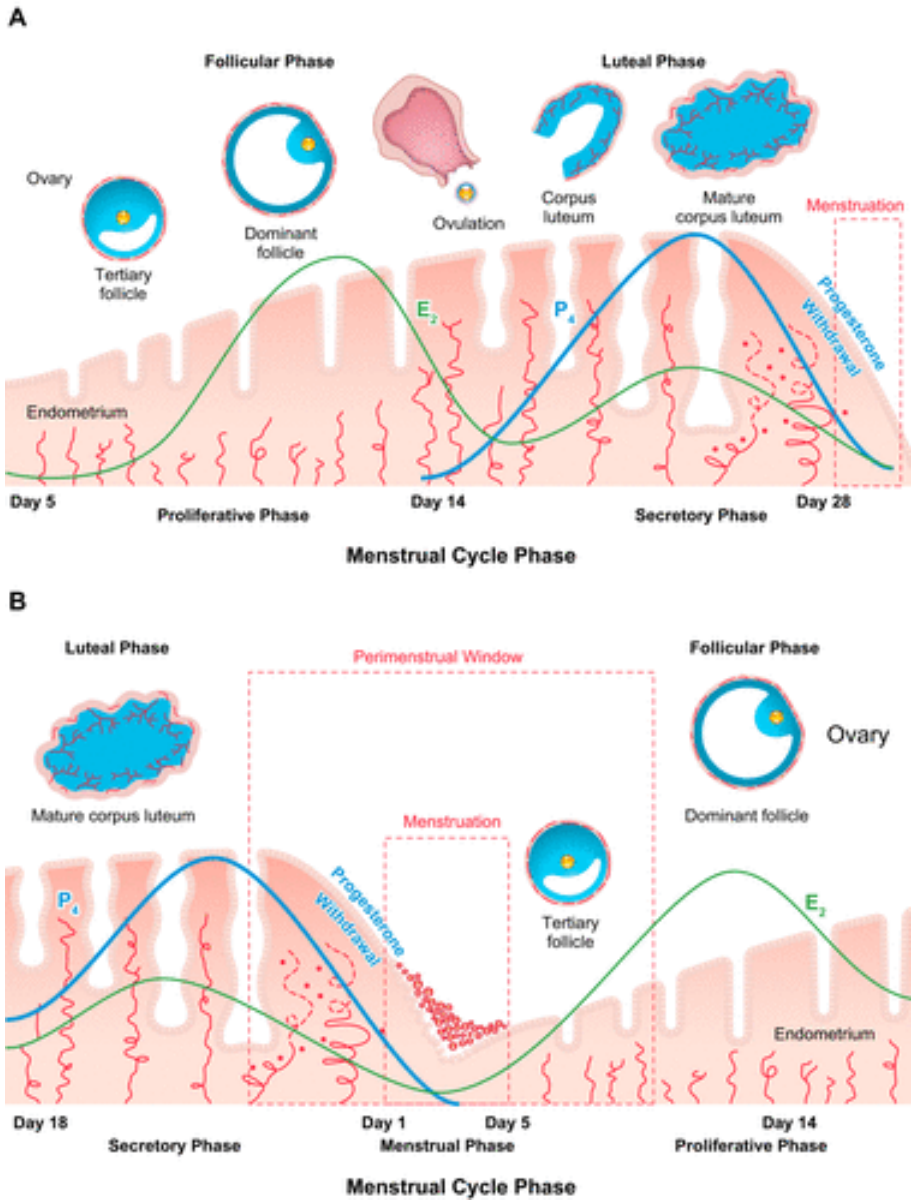
appearance. The trilaminar endometrium consists of a thin inner line and an outer basil layer, both echogenic with a dark boarder middle layer. There is elongation of spiral arteries to supply enough blood to the endometrium (Monis and Tetrokalashvili, 2020).

Changes also occur in the cervix during the proliferative phase as a result of the increased level of estradiol. The cervix becomes dilated and there is production of mucous like discharge, thin and watery in nature by the cervix to naturalize the acidic pH of the vagina, creating a conducive environment for sperm entering (Chaudhari *et al.,* 2014)

The secretory phase: this phase occur concurrently with luteal phase of ovarian cycle, progesterone is the dominant hormone in this phase preparing the uterus for possible pregnancy. (Gargett and Masuda 2010). The endometrium consists of a simple columnar epithelium histologically, covering a multicellular stroma which contains connective tissue cellular components with luminal surface, spiral arteries and innate immune cells as well as an element endometrial adult stem cells. These populations of endometrial progenitor stem cells differentiates into stromal cells and epithelial cells, contributing to the efficient replacement and maintenance of the endometrium that is required to restore endometrial integrity with menstruation. In human, these progenitor stem cells are confined to the endometrial basal layer. The process of Endometrial stromal cells (a target for progesterone) transforming into specific secretory “decidual cells” which provides both a nourishing and receptive cell essential for embryo implantation and onward placental development is known as decidualization (Santamaria *et al.,* 2018). Cyclic adenosine triphosphate (cAMP) is an initiator of the endometrial decidualization, starting initially in perivascular endometrial stromal cells before spreading throughout the endometrial stroma as well as the luminal epithelium useful for embryo implantation (Gellersen and Brosens, 2014). Though in humans, decidualization is independent of embryo-endometrial relationship and it occurs rapidly in the presence of progesterone. A recent study described rapid decidualization in menstruating rodent known as

spiny mouse that was discovered (Bellofiore *et al.*, 2018). This phase ends with the first day of menstrual flow.

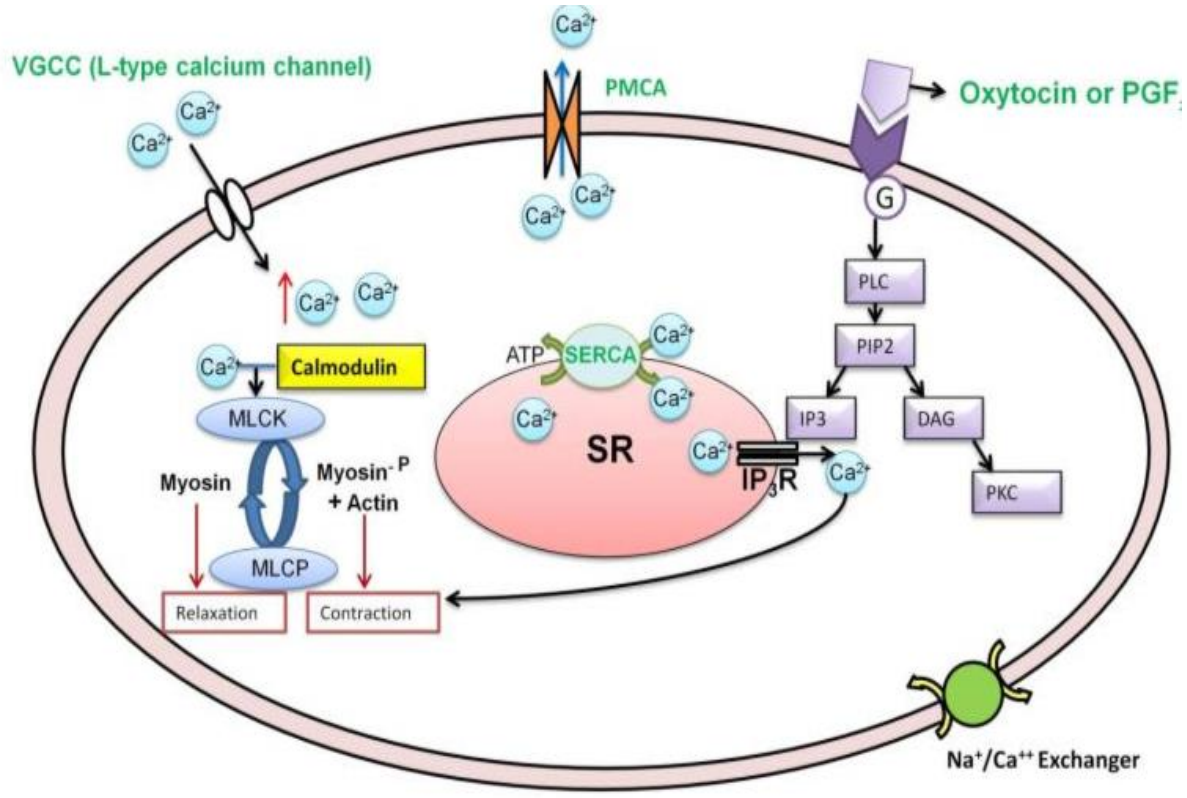
Menstruation: in the absence of fertilization there is regression of corpus luteum which forms a scar-like structure known as corpus albicans in the ovary as a result of an abrupt decline in progesterone and estradiol level initiating menstruation onset. At this point the endometrial functional lining known as the functionalis begin to shed alongside with other endometrial morphological changes such as tissue edema, increased numbers of leukocytes, increased blood flow, and the vessels become fragile and permeable (Evans and Salamonsen 2012) Garry *et al*., 2010. Matrix metalloproteinases (MMPs) focal activation in menstrual lysis regions, enhanced prostaglandin synthesis and expression of inflammatory mediators such as cytokines or chemokines (interleukin-8), cyclooxygenase-2 (COX-2). Withdrawal of progesterone modulates nuclear factor (NF)κB pathway and E series of prostaglandin receptors and associated signaling pathways and its implicated in the regulation of menstruation. Evidence of perturbation of these signaling pathways has been discovered in women with aberrant menstruation, which usually presents as heavy menstrual bleeding (HMB) (Gaide *et al*., 2009).



Reed and Carr (2015).

Figure 2: The human menstrual cycle. *A*: estradiol is the dominant hormone acting on the endometrium during the proliferative phase (ovarian equivalent = follicular phase). The secretory phase occurs subsequent to ovulation, when the corpus luteum secretes progesterone (ovarian equivalent = luteal phase). *B*: peri-menstrual window (luteo-follicular transition): rearrangement of the traditional menstrual cycle to focus on the significant endocrine and endometrial changes that occur during menstrual breakdown and repair.

Uterine contractility



The smooth muscle of the uterus contracts with or without nervous or hormonal impulse making the uterus a myogenic organ. Though there are agonist that act on the cells of the uterine smooth muscle to modulate uterine contractility (Wray *et al.*, 2017).

It was reported that non-pregnant uterine contraction are uncoordinated and that it originates and starts from the lowest part and isthmus of the uterus with different intensity but greatest at the lowest part of the uterus having higher frequency than that of the isthmus. The contraction of the pregnant uterus on the other hand are more coordinated during and before labour (Koutras *et al.*, 2021). Autonomous nervous system, neuromuscular stimulation and/or hormonal changes are involved in processing the stimuli released by the myometrium even though stimuli is produced solely by individual myometrial muscle fiber (Hill-Eubanks *et al.*, 2011).

The phosphorylation of the regulatory light chains is responsible for myosin and actin interaction in the smooth muscle of the uterus. Phosphorylation and activation of myosin light chain kinase (MLCK) is as a result of the calcium ion binding to calmodulin. Calcium ion is released into the intracellular through the L-type voltage gated channels and from the sarcoplasmic reticulum. The uterus has a phasic smooth muscle with the occurrence of action potential which causes depolarization and the opening of L-Type calcium channels thereby causing the contraction of the uterus. Blocking of this channel inhibits uterine contractility. Some studies suggested that other channels such as T-type calcium channels contributes to the entrance of calcium into the human myometrium (Longbottom *et al.,* 2000).

The intensity of the resting potential depends on the difference between the intracellular and extracellular concentration of Na^+ and K^+ ions, this mechanism ultimately regulates the total potential of myometrial contractility. The electrical stimulating action of myometrial cell's electrolyte is referred to as "electromechanical coupling" (Goldsztejn and Nehorai, 2020). The resting potential of -60 to -90 mV, after electrical stimulation of the muscle can reduce below a limit value results in a massive influx of Na+ ions inside the cell thereby resulting in a change in ion concentration on both sides of the membrane (Chappell and Payne, 2020).

The sarcoplasmic reticulum (SR)-calcium ATPase (SERCA) is responsible for the transport of calcium into the SR. Inositol triphosphate production and agonist (such as oxytocin) stimulation will not modulate uterine contraction if there is no calcium entry into the intracellular or if calcium ion entry is inhibited. Oxytocin stimulates calcium entry and decreases calcium ion efflux to prolong and increase calcium ion transient bringing about uterine contraction (Wray, 2007).

Vascular and other smooth muscle studies proposed that calcium ion activated potassium ion (BK) channels on plasma membrane is the target for calcium ion released from the

sarcoplasmic reticulum. RyR opening causes the occurrence of SR calcium release causing the calcium sparks that happens sufficiently around BK channels.

Relaxation of uterine contraction hyperpolarization is caused by BK channel opening leading to decreased calcium ion as a result of decreased L-type calcium channels opening. The BK channels found, expressed and distributed in the uterus as reported in studies, are regulated gestionally suggesting that they might be responsible for the uterine quiescence maintenance that occurs before term.

The fallopian tube

The fallopian tubes also known as uterine tubes or oviducts are two-sided serous muscular conduits found between the ovaries and the uterus in the female pelvis. They originates from the uterine horns extending laterally within the mesosalpinx superior end of the broad ligament to the ipsilateral ovary where they terminate. They have a diameter lesser than 1mm and a length between 11 and 12cm (Thurmond *et al*., 2009). Anatomically, the fallopian tube comprises of four sections; the uterine (comprising of uterine ostium and a short section closest to the horn of the uterus), isthmus (the site for possible fertilization likely), ampulla (lateral to the isthmus), and infundibulum (opens into fimbriae and peritoneal cavity, connecting to the nearby ovary) (Han and Sadiq, 2019). The fallopian tubes acts as a passageway transporting the ovum or gamete from the ovary to the uterus as a result of the muscular layer contractions of the fallopian tube, cilia movements and tubal secretory fluids work together. Short and frequent contractions mixes tubal fluid while the continuous tonic contractions contributes to anterograde transport of ovum or gamete and it allows the entrance of an embryo across the utero-tubal junction at the most hormonally optimal point during the menstrual cycle (Ezzati *et al*., 2014).

The Ovary

The ovary is a pair of organ attached on both sides of the uterus within the peritoneal cavity that produces an ovum and also act as an endocrine gland by producing sex hormones that regulates the menstrual cycle and fertility (Colvin and Abdullatif, 2013). The Mesovarium, fallopian tube, ovarian ligament and blood vessels makes up the ovarian pedicle (Baskett *et al.,* 2014). The ovary consists of different layers; the ovarian cortex which is the outer layer consists of stroma, ovarian follicles and corpus luteum emerging from the follicles. The follicle is composed of the granulosa cells, membrane granulosa, cumulus oophorous, zona pellucida, corona radiata, primary oocyte, theca cells, antrum and liquor folliculi. The innermost layer of the ovary is the ovarian medulla, the follicles are what distinguished the cortex from the medullar (Brown and Russell, 2013).

The ovary controlled by the hypothalamus and pituitary gland secretes high level of hormones at puberty and in response there is development of secondary sex characteristics and its role in pregnancy and fertility. The hormones secreted are progesterone, estrogen, inhibin and androgen (Richards and Pangas, 2010).

As women advances in age, there reduction of ovarian follicles leading to a decleine in reproductive performance and eventually menopause at about 40 to 52 years of age. The difference in menopausal age maybe as a result of environmental factors, lifestyle habits or genetic factors. Different testing methods are used for fertility determination based on maternal age. Follicle stimulating hormone (FSH) and gonadotropin releasing hormone (GnrH) are measured in these tests. Anti-mullerian (AMH) hormone level and antral follicule count (AFC) are measured to determine ovarian aging. Ovarian follicle quality is determined by the level of AMH (Titus *et al.,* 2013).

Ovarian cycle

The cyclic complex event that occurs within the ovary that bring about ovarian follicle's formation, growth, ovulation and transition to corpora lutea is known as ovarian cycle (Richards and Pangas, 2010). The different phases of the ovarian cycle are;

Follicular phase: this phase commences on the first day of menstruation and ends with the commencement of ovulation on day 14 in an average cycle of 28 days. Low levels of estrogen, progesterone and inhibin A sends a negative feedback to the anterior pituitary and hypothalamus bringing about the release of gonadotropin releasing hormone (GnRH) and follicule stimulating hormone (FSH), FSH in turn stimulates ovarian granulosa cells to recruits and cause the maturation of ovarian follicles which eventually leads to the ovulation of one graffian follicle (Monis and Tetrokalashvili, 2020). The other follicles known as atretic follicles undergo atresia while the graafian follicle undergoing development secretes estrogen which is responsible for uterine cycle proliferative phase (Haroun, 2016).

Ovulation

Ovulation is the release of matured oocyte from the ruptured follicle as a result of luteinizing hormone surge secreted by the pituitary gland as a result of the positive feedback of estrogen and inhibition of follicle stimulating hormone by inhibin B. LH increases the liquor folliculi salt concentration causing an increase in osmotic pressure and graafian follicle rupture. After release the follicle remains active and it becomes a corpus luteum secreting progesterone which prepares the uterus for possible embryo implantation (Melmed *et al.*, 2011). This process occurs once in every cycle in the absence of pregnancy, usually on the 14^{th} day for an average 28 days cycle. About 500 oocyte out of the millions of oocytes present at birth in the ovary are ovulated and others degenerates (Haroun, 2016).

Luteal phase: in this phase the ovarian follicles undergoes different stages before it becomes a corpus luteum which secretes progesterone. Immediately after ovulation the ovarian follicle's wall collapses, folds and bleeds because of the abrupt reduction of pressure. At this point the ovarian follicle becomes corpus hemorrhagium because of the blood clot formed from the bleeding and it last about three days before it finally become corpus luteum. The granulose cells becomes enlarged, polyhedral in shape, and develops lutein, a yellowish carotenoid pigment. The cells becomes granulose luteal cells with capillaries growing along it absorbing blood clot while the theca interna cells becomes caluteal cells. Progesterone and little concentration of estrogen are been secreted by corpus luteum, this function is induced by the human chorionic gonadotropin if fertilization eventually occurs until the placenta takes over but if there no fertilization the corpus luteum degenerates after 9 to 14 days (Haroun, 2016)

Hormonal control of the ovarian cycle

Anterior pituitary gland in the first half of the cycle secrets FSH that is responsible for the development of the mature graafian follicle from the primary ovarian follicle. The graafian follicle secretes estrogen that inhibits FSH and stimulates luteinizing hormone (LH) surge as well as the secretion of luteotrophic hormone (LTH) that causes ovulation during the mid-cycle. As the cycle ends, corpus hemorrhagicum becomes corpus luteum with the help of LH and corpus luteum, stimulated by LTH, secretes progesterone and a little concentration of estrogen resulting in corpus luteum regression if there is no fertilization. The low lwvwl of estrogen stimulates the secretion of FSH and this marks the beginning of a new cycle (Haroun, 2016)

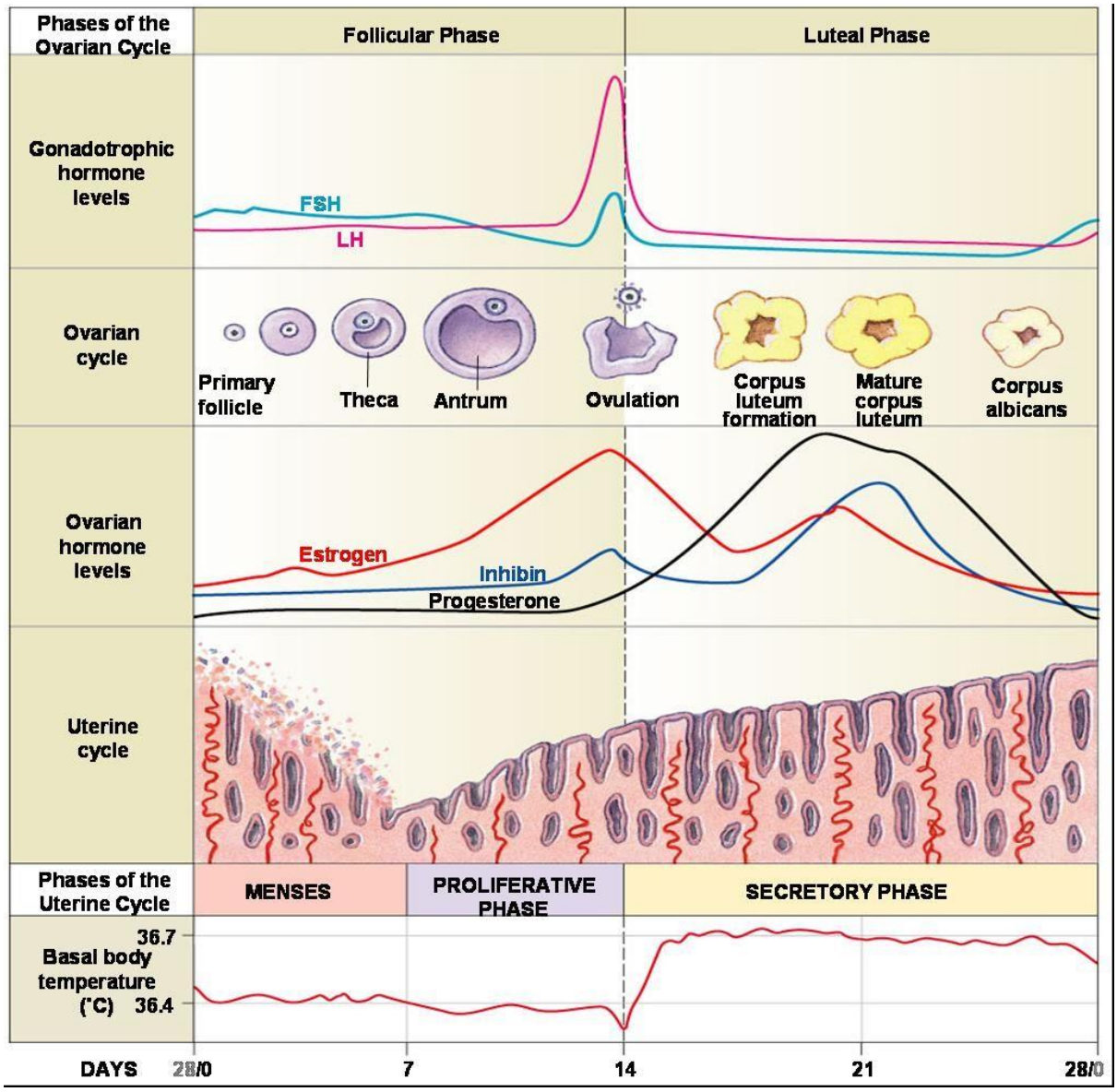


Figure 3: Ovarian and menstrual cycle (Reed and Carr, 2015).

Female Reproductive Hormones

In women before menopause, 50% of testosterone is produced by the ovaries and released directly into the blood stream. The other 50% of testosterone in the blood stream is made from conversion of the adrenal pre-androgens (DHEA and androstenedione) to testosterone in other parts of the body. Estrogen is responsible for the appearance of secondary sex characteristics for females at puberty and for the maturation and maintenance of the reproductive organs in their mature functional state. Progesterone prepares the uterus for

pregnancy, and the mammary glands for lactation. Progesterone functions with estrogen by promoting menstrual cycle changes in the endometrium (Marieb, 2013).

Gestation

Gestation is the period of fertilization to parturition, that is, the period between conception and delivery. Conception occurs when the matured oocyte is fertilized by a male sperm cell, at this point the oocyte become a zygote. After fertilization the zygote travels to the uterus through the oviduct, on the 6-8 day the outer layers of the zygote or blastocyst implants itself into the endometrium by engulfing the cells of the endometrium while the chorion another layer of the blastocyst continuously release human beta chorionic gonadotropin **(β-HCG) that stimulates the corpus luteum to secrete progesterone until the placenta is ready to take over**. The present of **HCG** hormone in urine or serum confirms pregnancy.

The length varies between animals and that of human is 266 days, with the period divided into three equal trimesters. In the first trimester, endometrial lining through diffusion is responsible for nutrient and waste exchange and the placenta forms as the embryo's outer layer merges with the endometrium. The placenta eventually takes over the responsibility of exchanging nutrient and waste between the embryo or fetus and the mother through the blood and passive immunity is also provided as some immunoglobins of the mother pass through the placenta. During this first trimester the development of internal organs such begins, at the fifth week the heart, limb buds, liver and eyes are formed but non-functional, the body forms at the eighth week and the term fetus is used. Exposing the fetus at this stage to any toxins can be severe on the fetus.

The growth of the fetus estimates to about 30cm during the second trimester, the mother feels the first movement because as the fetus because active and there is progression in the development of all organs and structures. The function of the placenta continues until delivery.

The third trimester is the period where the most rapid growth occurs, the fetus grows to about 50cm weighing about 3-4 kg. The weight of the fetus puts pressure on the bladder making the mother to always urinate and this is the period that the mother is most uncomfortable. Organs such as the liver and the nervous system continues to develop till birth.

Labour and Parturition

Labour occurs at the end of the third trimester. It is the process of expelling the fetus and the placenta out of the uterus through the vagina or surgically as cesarean operation. As labour approaches, estrogen hormone stimulates the binding of oxytocin to its receptors on the uterine wall causing the reorientation of the head of fetus towards cervix as the uterus contracts. During labour the diameter of cervix continues to increase sending nerve impulses to the hypothalamus to stimulate the secretion of more oxytocin from the posterior pituitary which then causes the contraction of the uterine wall. Simultaneously, prostaglandin is secreted by the placenta to augment and intensify uterine contractions.

Labour occurs in three stages, in the first stage, the cervix dilates to about 10cm and becomes thin to enable easy expulsion of the fetus and the placenta. In the second stage, as the uterus contract and the mother pushes compressing the abdominal muscle to aid expulsion or delivery of the baby. The last stage is for the delivery of the placenta. Synthetic oxytocin is used clinically to manage dysfunctional labour.

After delivery, the baby to feeds from the mothers breast that has been prepared during the third trimester of pregnancy. As the baby suckles the breast, prolactin and oxytocin are secreted as result of the signals sent to the hypothalamus, prolactin stimulates milk production while oxytocin promote the release of breast milk.

The Estrus Cycle

The use of experimental animals in research base studies can never be over emphasized. Some of the advantages of using animal models for studies are experimental subject availability, safe subjects handling, and easy elimination of study alteration except in cases of death, disease severity standardization, prophylaxis possibility, and ability to perform invasive tests, wide range of tissue sampling and the overall ability to control experimental conditions (Ajayi and Akhigbe, 2020).

Some of the rodents used are mice, rats, rabbits and guinea pigs. They are mostly used for reproductive function research because of the easy mode of handling as well as regular and short estrous cycle of mice and rats. Estrous assessment in experimental animals is valuable in hypothalamic-pituitary-ovarian axis integrity, reproductive status of the reproductive system and the environmental effect of chemical and drugs on reproductive health. It also essential in the selection of a female to be mated with a male to achieve a timed pregnancy (Auta and Hassan, 2016).

Rodent's or animal's reproductive cycle is referred to as estrus cycle. Mice reproductive cycle begins with vaginal opening at about 26 days after birth and before vaginal cornification which occurs about 10 days after. The most essential characteristics in mice used as pubertal predictor is apoptosis-mediated vaginal opening associated with increased level of estradiol. Vaginal opening in rats occurs alongside the first ovulation and their pubertal onset is characterized by pulsatile release of luteinizing hormone when they are about 30 days old (Cora *et al.*, 2015).

The estrous cycle is similar to human menstrual cycle but unlike the menstrual cycle, the estrous cycle consists of four phases, which are; the proestrus, estrus, metestrus and dietrus, all lasting for 5 to 6 days (Auta and Hassan, 2016).

Proestrus: this is the preparatory period before the heat period. It is characterized by a rise in the level of circulating estradiol and a little rise in prolactin level leading to the release of LH and FSH (McLean *et al.,* 2012). Assessing the visual display of the vagina during this phase, the vaginal tissues appears reddish-pink in colour and moist. Microscopically, the dominant cells in the vaginal smear is nucleated epithethial cells round and uniform in shape and size (Ajayi and Akhigbe, 2020).

Estrus: this phase correlates with human ovulation. It is associated with abrupt decline in estradiol level and a peak in the level of FSH and LH especially. The visual appearance of the vagina in this phase is similar to that seen in the proestrus phase but with a lighter pink colour and more prominent striation as well as less moisture. Vaginal cytology in this phase shows numerous anucleated cornified epithelial cells with irregular shape and a granulated cytoplasm (Ajayi and Akhigbe, 2020).

Metestrus: metestrus phase occurs in the absence of conception, it is correlated with the early secretory phase of human reproductive cycle having high concentration of progesterone. Morphologically the vaginal tissues are pale and dried. The dorsal lip is not oedematous in early metestrus (Ajayi and Akhigbe, 2020).

Diestrus: this phase correlates with late secretory phase of human reproductive cycle and just like the metestrus phase there is increase in progesterone level. There is small vaginal opening, no moisture and the colour of the vaginal is bluish purple when viewed morphologically (Ajayi and Akhigbe, 2020).

Estrous cycle assessment

Different techniques are used to classify or assess the different phases of estrous cycle. They are morphological techniques, vaginal cytology, and histological techniques (Byers *et al.*, 2012).

Morphological technique: this is a non-invasive, cheap, less-stressful and simple method of estrous cycle assessment. The microscopic changes observed in the estrus phase are swelling of the vulva, vaginal secretion and uterine congestion. To carry-out this technique, the mouse or rat is held on retraining surface and the tail is carefully lifted in other to gain visual assess of the vagina and the examination is done non-hastily to avoid misinterpretation. It is the fastest method used to classify the estrus phase (Ekambaram *et al.*, 2017).

Vaginal cytology: just like the morphological technique, vaginal cytology is accurate, reliable, non-invasive, inexpensive and widely accepted. It is the commonest technique applied in estrous cycle determination. This method requires microscopic skills to examine vaginal secretion cells and it can be tedious and time consuming (McLean *et al.*, 2012). In carrying out this technique, the animal is held restraining its forepaws and tail. Using the pipette or sterile latex bulb filled with about 100ul of distilled water, the vagina is flushed out gently by slowly releasing the fluid into the vagina and drawing it back into the pipette that is placed in the entrance of the vaginal canal. The flushing is done four to five times from the same pipette. The smear is withdrawn to a glass slide, dried, fixed with methanol, and stained with either 0.1% crystal violet or methylene blue or Romanowsky-type stains. The stain is washed off after 3 minutes and the dried slide is placed under the microscope to ascertain the dominant cells present in other to classify the phase of the estrous cycle. Vaginal smear consists of three types of cells, they are leucocytes, cornified epithelial cells and nucleated epithelial cells (Auta and Hassan, 2016).

Histological technique: this method is accurate and reliable but some of its disadvantages are that it is invasive, and cannot be used to determine the estrus cycle of life laboratory animals, it also expensive, time consuming and requires high level of skill. In this technique, the animal is humanely sacrificed and the cervix, uterus and ovaries are carefully isolated. 10% formalin is used to fix the organs and a longitudinal sections of the organs is prepared and stained with

hematoxylin and eosin. The slide is viewed under a light microscope. The morphology of the corpus luteum can be used to determine estrous cycle phases (Westwood, 2008).

GENES REGULATING HEAT STRESS

High ambient temperature is a critical challenge, there are different parameters used in evaluating the levels of heat stress, they include growth rates, hormones and blood metabolites. In recent times, with the advancement of biotechnology, profiling of genes expressed during heat stress has been listed as one of the parameters evaluated. Gene expression evaluation gives an insight of cell response to heat stress (Saleh and Al-Zghoul, 2019). One of the various activities stimulated to protect cells from heat stress is gene responses, and the gene expressed incudes; heat shock protein (HSP), neuronal nitric oxide synthase (nNOS), brain-derived neutrophic factor (BDNF), c-FOS like protein, some antioxidant enzyme genes such as catalase (CAT), superoxide dismutase (SOD) and nicotinamide adenine dinucleotide phosphate oxidase (NOX4) and the immune-related genes such as toll-like receptors (TLRs) and cytokines (Goel *et al.,* 2021). Using gene expression profiling in the case of temperature variation, gives better evaluation accuracy in the case. Defense mechanism of the immune system can also be evaluated as they are affected by heat stress as well.

Heat shock proteins: stress factors such as hypoxia, viral infection, oxidative stress and high temperature activates heat shock (HS) genes also referred to as stress genes. Heat shock proteins (HSPs) are known to be responsible for the different protective functions that occurs in cells especially the prevention of denatured protein aggregation to enable their folding and stimulation of irreversibly damaged proteins' degradation, making them molecular chaperones. Most importantly they protect cells from stress especially heat induced stress, making them a suitable parameter to be evaluated during heat stress. According to their molecular weight they are classified into different families, they include;

1. HSPs with small molecular weight of 10-30 kDa tagged sHSPs
2. Hsp40 with 40kDa molecular weight
3. Hsp60 also known as the chaperonines
4. Hsp70 with molecular weight between 68 and 78kDa
5. Hsp90 with molecular weight between 82 and 96kDa
6. Hsp100 belonging to the heterogeneous proteins group having molecular weight of between 78 and 104kDa
7. Hsp110 having molecular weights between 80 and 170kDa (Kampinga *et al.,* 2009).

Hsps are also classified as inducible and constitutive. The inducible Hsps genes are expressed to extremely low levels and their transcription intensity increases with stress way above the baseline, an example of this class is Hsp70. The constitutive Hsps are expressed to high relative levels even during normal temperatures and their transcriptions increases in several folds but unlike the inducible Hsps that increases dramatically (akhotia and Prasanth, 2002). The most known and studied Hsps are Hsp70 and Hsp90 genes. Hsp70 protects the body against oxidative stress induced by heat, while Hsp90 plays its role by interacting with client proteins at folding later stages and in configuration modification (Xie *et al.,* 2014). The expression of Hsp70 gene is easily estimated in liver, heart, brain, and lungs tissues and each of them responds differently to changes in temperature (Wegele *et al.,* 2004), this might be as a result of the influence of heat shock factors activating the upstream promoter sequences for genes encoding heat shock protein. It has been reported that body temperature increases above normal during hyperthermia as a result of overheating which causes thermoregulation failure that eventually activates cellular defense mechanism through nuclear factor (NF)-kB mediated by proinflammatory cytokine induction (Pockley, 2003).

OTHER GENES: BDNF and c-FOS genes helps cells to adapt to hostile environment. They are expressed in the hypothalamus. One of the major issue that emerge as a result of heat stress

is oxidative stress. The Reactive oxygen species (ROS) is generated by cells as a result of heat damage, is induced by NOX4 gene. NOX4 gene is controlled by SOD and CAT antioxidant enzymes as well as GPX (gluthathione peroxidase). Free radicals are broken down by SOD to hydrogen peroxide which is further broken to water and molecular oxygen by CAT and GPX. The role of cytokines on the other hand, is to maintain homeostasis and control pathogen while TLRs protects the cell against attacks of pathogens that arise from increased membrane permeability (Goel *et al.,* 2021).

2.2 Review on the Effect of Heat-adaptation on female reproductive functions

As far back as 1930, rats were used for adaptation studies by Sundstroem, they were exposed to graded levels of reduced cooling powers and biochemical and physiological analysis were done. In 1960, Brobeck studied the relationship between food consumption and temperature, and it was discovered that high temperature caused a decrease in body weight as a result of poor eating habit. Similar study was done by Sod-Moriah and Pollack (1970) and recovery was recorded when the animals were taken to a different environment confirming the previous report, a longer estrus period by 24% and lowered mating rate as well as distorted fertilized ova development in heat-adapted rats were also recorded when compared to the control group. Sod-Moriah (1971) did another heat-adaptation study on female rats and it was recorded that there was no negative effect on the corpora lutea, implantation and gestation time.

Heat acclimation was used to induce bradycardia in sand rats (Psammomys obesus), partial sympathetic withdrawal generated decreased intrinsic heart rate after the administration of propranolol and atropine (Horowitz and Meiri, 1993).

Jia and his teammates in 2012 did a review similar to that of Hafez (1964), on reproductive performance of both male and female mammals exposed to heat stress. In their reports; delayed young animals' sexual maturity, low libido, low volume of ejaculate, decrease sperm count and

sperm density as a result of poor or no spermatogenesis because there is lack of heat regulation by the testis and scrotum during heat stress in males was recorded. Estrous cycle, mating time, pregnancy rate, implantation, embryonic development and litter size was also affected in females exposed to heat stress. A review by Hansen in 2009 recorded similar findings alongside some genetic changes of tissue resistance to high temperature.

Aging and acute heat exposure effect on protein oxidation, lipid peroxidation and inflammation in rats have been studied, significant increase in malondialdehyde concentration and higher plasma concentration of Tumor Necrosis Factor alpha (TNF-α) in both old and young heat exposed rats was reported (Ilievska *et al.,* 2016). This report is similar to that of Mladenov *et al.,* (2006) on the effect of vitamin C on lipid hydroperoxides and carbonyl groups content of rat plasma depending on age and acute heat exposure.

Effect of early thermal experience on pituitary-gonadal axis in female rats (Kurowicka *et al.,* 2007), is a study done on both heat-acclimated and heat reared female rats. Reduction in weight, puberty delay, decreased prolactin plasma concentration in proestrus phase, increased prolactin plasma concentration in metestrus phase and high progesterone plasma concentration in proestrus phase was recorded in heat exposed animals. There was no effect on estrous cycle as males were kept in cages adjacent to the females' cages and no change in the estrogen, LH, and FSH plasma concentration was observed in the study. The same study was carried out on male rats a year later (Kurowicka *et al.,* 2007).

Observed in the study of "developmental competence and oxidative state of mouse zygotes heat-stressed maternally and in vitro", was alteration of oviduct physiological condition and oxidative stress causing embryonic death in hyperthermic animals. The percentage of developed embryo was lower in maternal heat stressed group but not in direct zygote heat stressed group (Ozawa *et al.,* 2002). A similar study done on susceptibility of ovarian oocyte,

preimplantation embryos' developmental ability, and off-spring quality to maternal hyperthemia, published reduced percentage of zygote to blastocyst stage, altered embryos' development and low litter size in heat stressed group compared to control group. Pups of both groups had the same ability to development normally (Aroyo *et al.,* 2006).

Heat acclimation or acclimatization studies using human models have also been published, effect of heat adaptation in relation to exercise and occupation such as sports has been reported by different scientist in recent times (Periard *et al.,* 2015).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Animals

A total of 48 (36 adult and 12 immature) virgin female Wistar rats and 6 male Wistar rats was used for this study. They were purchased and housed in the Animal unit of the Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Benin. The animals were kept under controlled conditions, with access to pelletized rodent feed and water. Ethical approval was obtained from the Faculty of Pharmacy Animal Ethics Committee on use of animals, University of Benin.

3.2 Experimental design.

Experiment was carried out in different phases, with all phases having 6 animals in all groups as follows;

Group 1: control group (CG; controlled temperature)

Group 2: heat adapted group (HAG)

3.3 Heat adaptation protocol

Rats was housed in a special heated chamber 5 hours per day, maintaining a constant ambient temperature of 30-40°C for the different study duration; 21 days for estrous cycle phase, 21 and 18 days for gestation phases, 50 days for uterotrophic assay. This is a modified protocol of Magal *et al.*, (1981) and Ilievska *et al.*, (2016).

3.4 Measurement of Ambient temperature

Animals in control group was kept in a cage with an ambient temperature of 25-30°C. Ambient temperature was measured and monitored using a thermometer placed on the floor of the cage away from any source of heat.

3.5 Baseline Measurements

Body temperature was assessed by measuring core temperature using a probe thermometer and Surface Temperature was measured from the skin using an infrared thermometer with thermal sensitivity of <0.05°C as described by Karvinen, *et al.,* (2016).

3.6 Estrous Cyclicity

Estrous cycle samples was collected (as smear) from the vagina using a pipette containing about 0.2mL of normal saline solution, between 10am and 12 noon. Smear was stained with methylene blue and observed under a digital light microscope. Each sample was categorized to one of the four oestrus cycle phases: proestrus, estrus, diestrus, or metestrus. This was done for 21 days, animals on the diestrus phase after the 21st day was humanely sacrificed via cervical dislocation (Bafor *et al.,* 2020).

The uterus, ovaries and blood samples were isolated. Uterine horn was divided into segments, a segment of the uterine horn was stored in a sample bottle containing Bouin's fluid for histopathological assay, another segment of uterine horn was stored in -80°C for HSP70 gene expression analysis (RNA Extraction and Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR) using RNA Extraction Kit) and the remaining uterine segment was kept in aerated physiological salt solution for ex-vivo assay. The ovaries was kept in a sample bottle containing 10% formal-saline for histopathological assay. Blood samples was withdrawn from the abdominal aorta and used to assess serum level of estrogen, progesterone, luteinizing hormone (LH) and follicle stimulating hormone (FSH) using ELISA kits.

3.7 Mating of Animals

Female rats on proestrus phase of the estrus cycle was mated overnight with male rats in a ratio of 2 (females):1(male). The female rats were observed early in the morning following mating

for the presence of vaginal plug to ascertain mating, that day was regarded as gestational day 0. Female receptivity to male during mating was observed.

3.8 Gestational phase one

On gestational day 18 pregnant animals was sacrificed to check for implantation sites. The fetuses were carefully expressed from the uterus and the uterine tissue was isolated and kept in an aerated physiological salt solution for ex-vivo assay.

3.9 Gestational phase two

Another set of pregnant rats was left to litter, preterm labour or preterm birth was looked out for. Birth weight of offspring and litter size in each group was determined on the day of parturition till day 50.

3.10 Ex vivo uterine contractility for both pregnant and non-pregnant

A segment of the uterine strip (of the above mentioned isolated uteri) kept in aerated physiological salt solution (PSS) was mounted in a 10mL organ bath containing PSS (NaCl- 154.00M, NaHCO3- 5.95M, D-glucose- 2.78M, KCl- 5.63M, and CaCl·2H2O- 2.05M) maintained at 37 ^{0}C with continuous aeration. A tension of 0.5g was applied to the tissue as it equilibrates for 30 minutes, after which spontaneous contraction was recorded for 30 minutes using an isolated organ bath connected to a transducer and a powerlab. This was done for both pregnant and non-pregnant uterine strip as described by Bafor *et al.,* (2019).

3.11 Prepubertal phase

Immature female rats (day 0 to day 50) was used for this assay. The animals was observed daily for vaginal opening, body weight and temperature was measured. On day 50, animals were sacrificed and samples was collected for the various analyses as described by Bafor *et al*., (2020). Vaginal opening or vaginal opening onset (slight canalization) was regarded as an indication of estrogenic activity.

Blood samples was collected from the abdominal aorta and reproductive organs (uterus and ovaries) were isolated from the animals under chloroform anesthesia. The collected blood samples was placed in plain tubes for the assessment of serum estrogen levels. The uterus was cleaned of connective tissues and fats, the wet and blotted weight was recorded, and they were stored in a sample bottle containing Bouin's fluid for histopathological examination after Hematoxylin and Eosin-staining. The ovaries were also weighed, they were kept separately in sample bottles containing 10% formal-saline for same examination as the uterine tissue.

3.12 Histological analysis

Paraffin technique was used, the tissues were sliced into thin paraffin segments, carefully placed on a slide and stained using routine hematoxylin and eosin method as described by Kluwe (1981). The slides were viewed using a digital microscope.

3.13 Data Analysis

Statistical responses was analysed using the GraphPad Prism version 8.1. All results was presented as mean ± S.E.M. (n=6 rats per group). Students T-test and Analysis of Variance (ANOVA) will be used to compare the means obtained and p-value of less than 0.05 was considered as statistically significant.

CHAPTER FOUR

RESULTS

Female Wistar rats were grouped into control and heat adapted groups to study the effect of heat adaptation on female reproductive functions. Animals in the heat adapted groups were kept in a heated cage between the temperatures of 34-39°C. The control group animals were kept in an ambient temperature of 25-30°C. Baseline parameters and samples collected were used for estrous cycle classification, uterine contractility, hormonal, histological and gene expression assays. Statistical analysis was done using the GraphPad Prism version 8.1. It was observed in this study that heat adaptation does not cause any significant change in the reproductive functions of female Wistar rats in the areas of estrus cycle, prepubertal assays, hormonal assay, uterine and ovarian histological assays.

4.1 effect of heat adaptation on prepubertal phase

Figure 4.1.1 shows that there is a significant increase in body temperature of the animals in the heat adapted group compared to those in the control group. This is because heat adapted was exposed to a higher temperature of 35^0C– 40^0C while the control group was kept in an ambient temperature of 30^0C– 35^0C.

Figure 4.1.2 shows the uterine contractility results. There was no difference between the spontaneous uterine contraction of the control and heat adapted group as shown by the original tracings (a). The analyzed results showed an increase in both uterine mean frequency (b) and amplitude (c) of the control group compared to the heat adapted group, though not significantly/

Figure 4.1.3 shows the relative uterine (wet and dried) and ovarian weight of both the control and heat adapted group. As observed in the graphs organ weight of the control group was higher than that of the heat adapted group but not significantly.

Figure 4.1.4 shows a non-significant increase in the level of estrogen and progesterone of the control more than that of the heat adapted group.

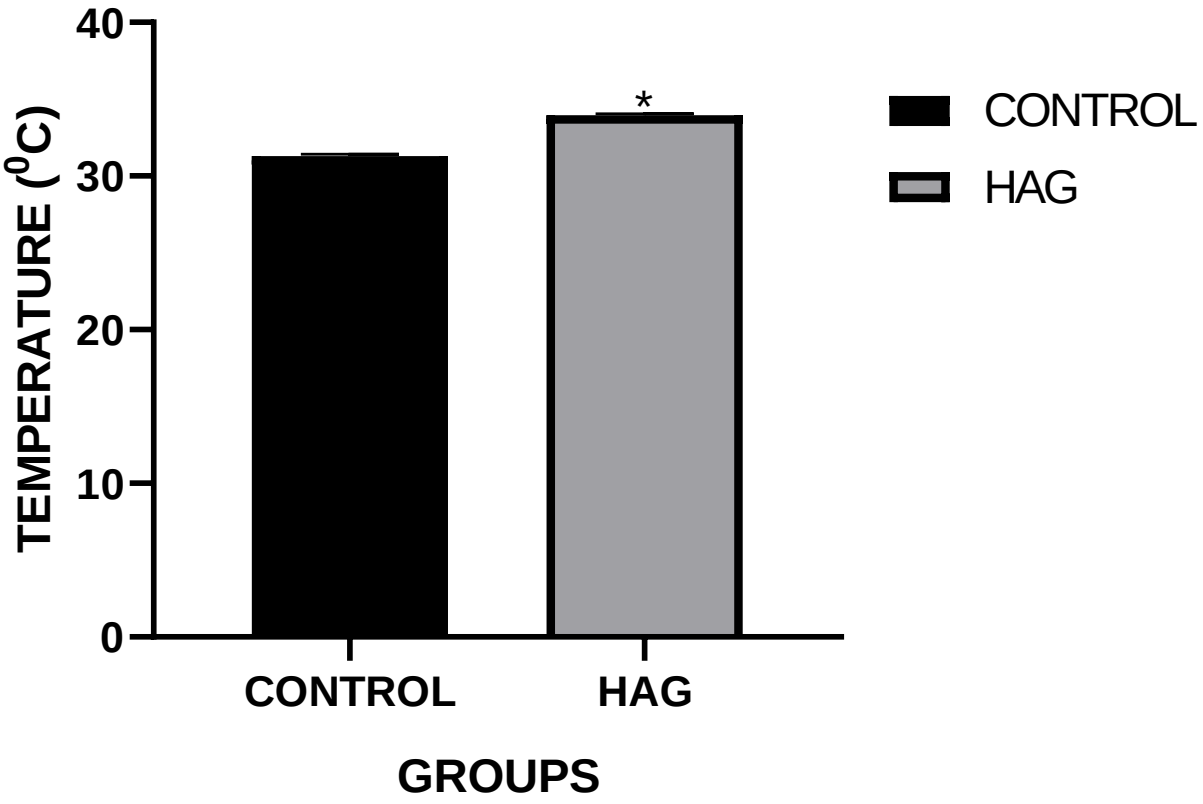


Figure 4.1.1 Body temperature of rats in both control and heat adapted group. Values are means ± SEM (n = 6 animals). * P<0.05. HAG- Heat Adapted Group

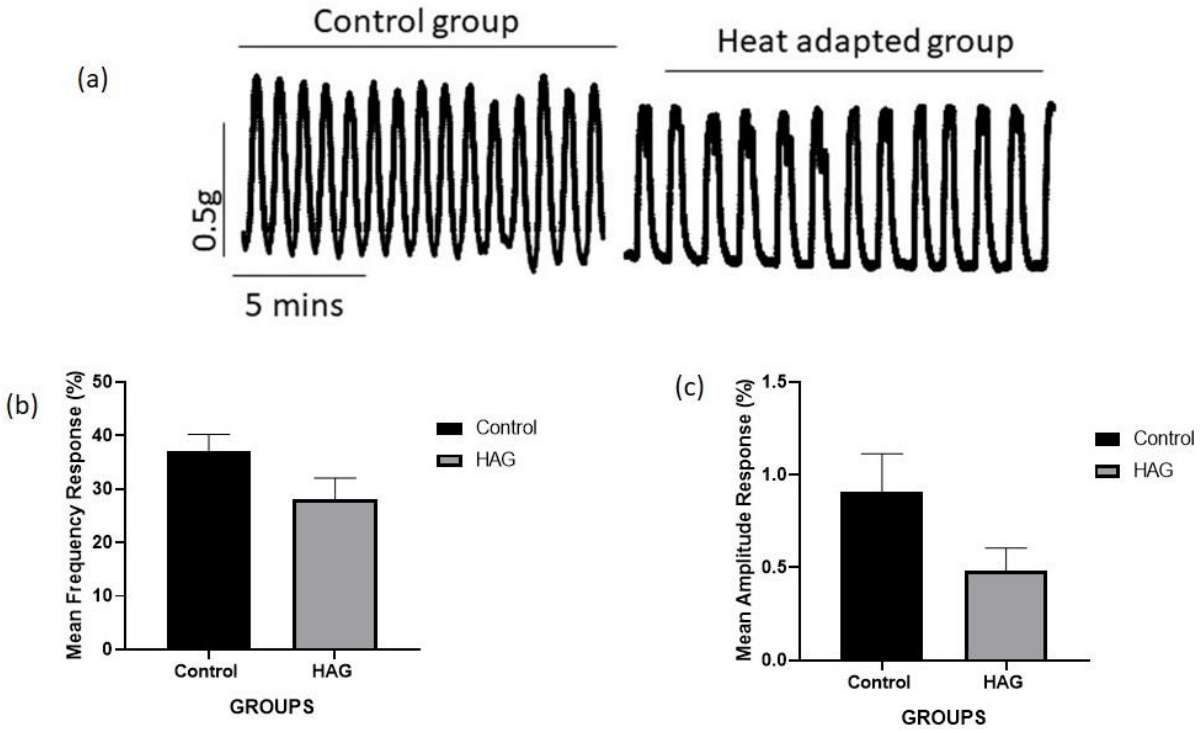


Figure 4.1.2 original tracing showing prepubertal phase spontaneous uterine contractions of both control and heat adapted groups (a), uterine mean frequency response (b) and uterine mean Amplitude response (c). Values are means ± SEM (n = 6 animals). HAG- Heat Adapted Group

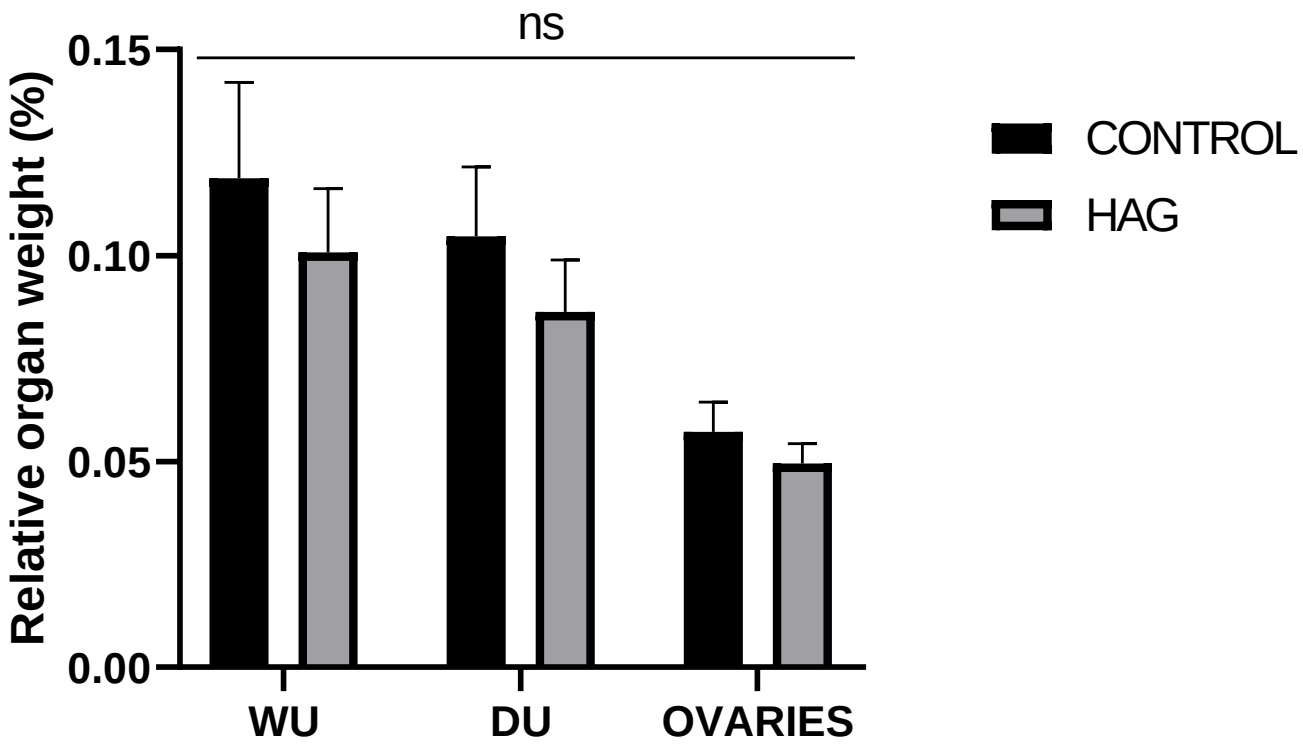


Figure 4.1.3 prepubertal phase relative organ weight (WU-weight uterus, DU-dried uterus) Data represent the mean ±SEM for 6 rats in each group. ns- Not significant. HAG- Heat Adapted Group

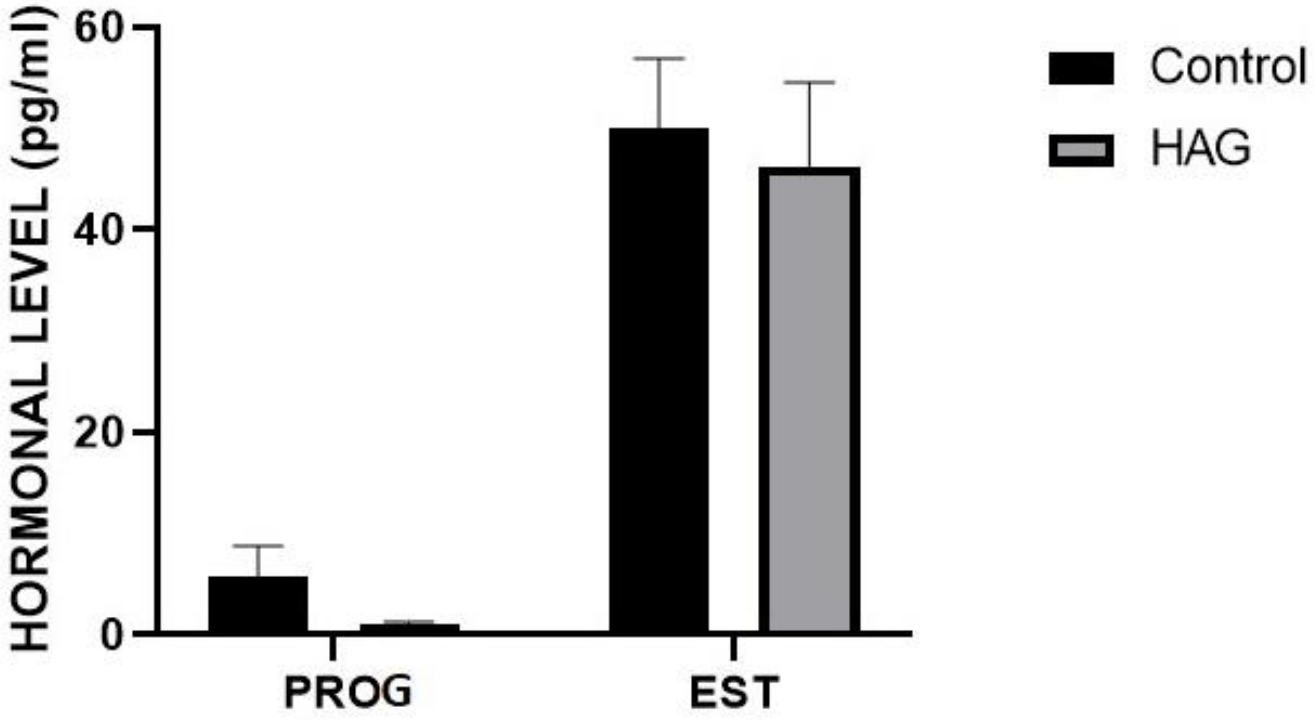


Figure 4.1.4 prepubertal phase hormonal level. Data represent the mean ±SEM for 6 rats in each group ns- Not significant. PROG-progesterone. EST-estrogen. HAG- Heat Adapted Group

Figure 4.1.5 shows a non-significant increase in the level of follicle stimulating hormone of the control group and an equal level of luteinizing hormone in both control and heat adapted group.

Plate 4.1 shows a representative histological section of uterus of both control and heat adapted group. The control uterus revealed a prominent endometrial glands embedded with a visible close up of simple columnar epithelium and lamina propria, while that of the heat adapted group reveals endometrial glands embedded in the lamina propria, also with visible close up of simple columnar epithelium and lamina propria undergoing regenerative changes.

Plate 4.2 shows a representative histological section of the ovaries of both control and heat adapted groups. That of the control group reveals numerous corpus luteum with visible primary

unilaminar and secondary multilaminar follicle as well as eccentrically located oocyte, while that of the heat adapted group reveals a large corpus luteum with secondary follicles seen within the parenchyma.

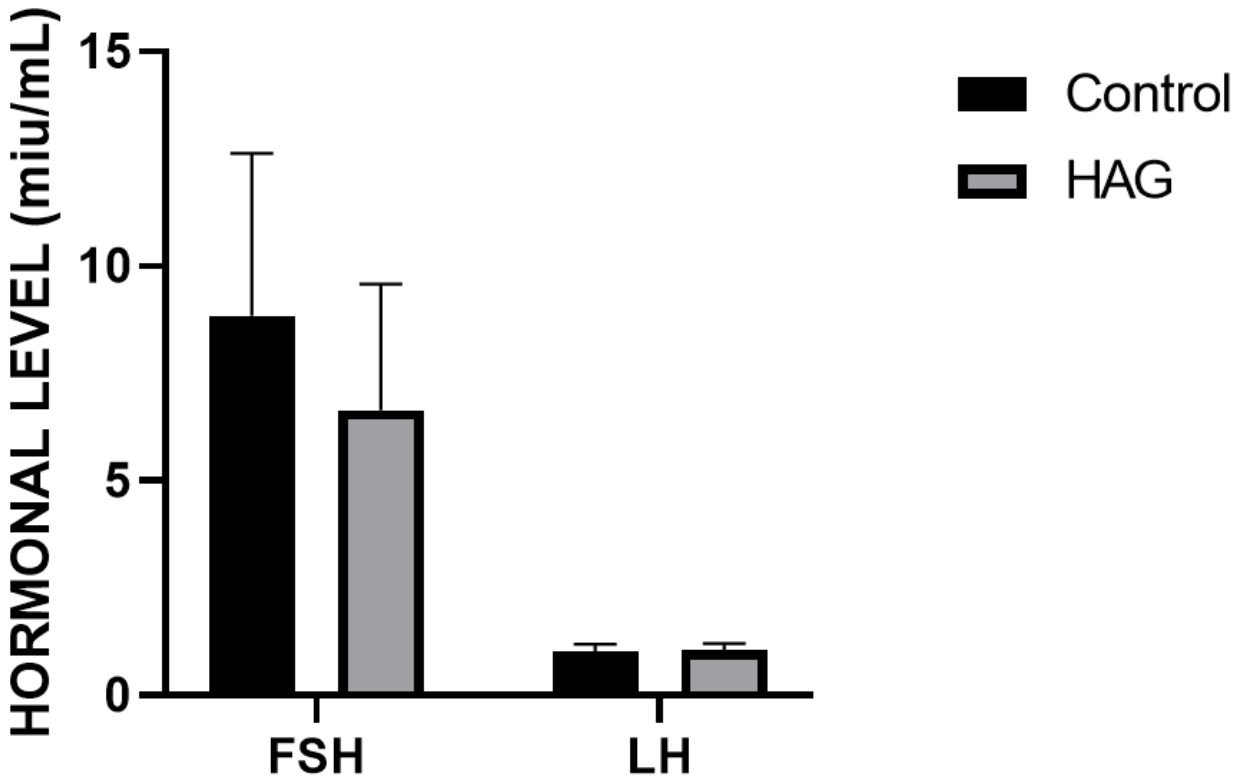


Figure 4.1.5 prepubertal phase hormonal level. Data represent the mean ±SEM for 6 rats in each group. FSH-follicle stimulating hormone. LH-luteinizing hormone. HAG- Heat Adapted Group

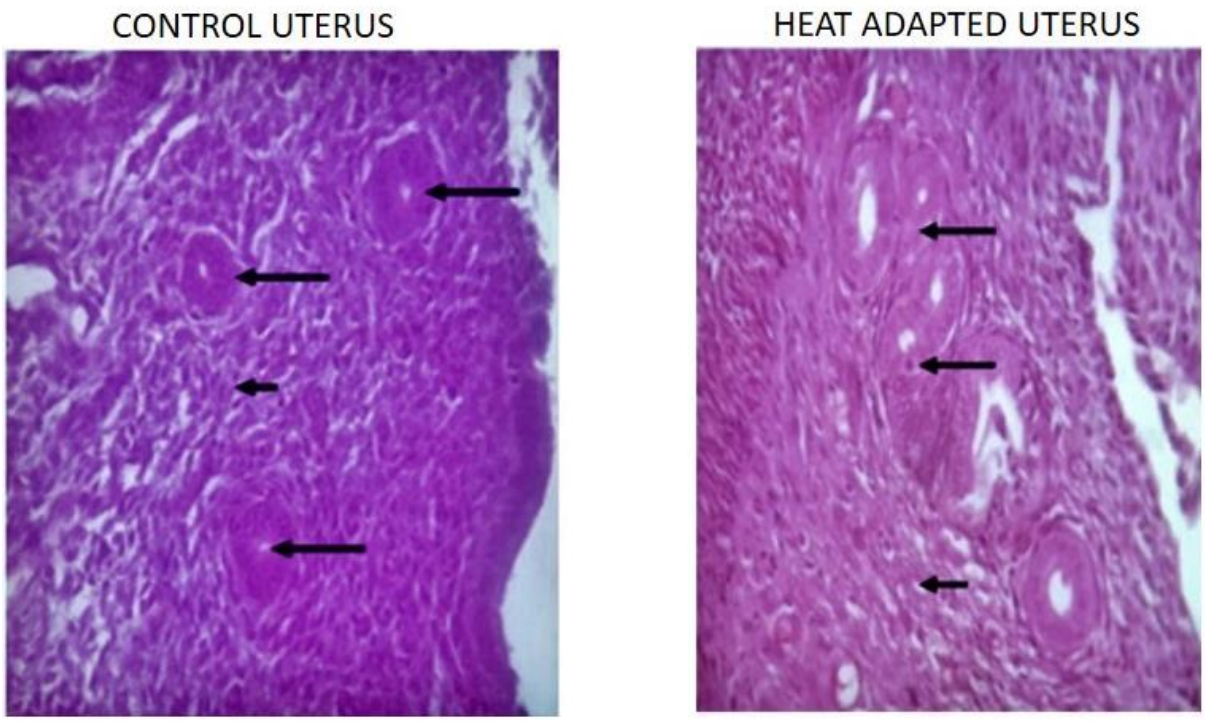


Plate 4.1 section of uterine histology of both control and heat adapted group revealing prominent endometrial glands (long arrows) embedded in the lamina propria (short arrows), there is visible close up of simple columnar epithelium and lamina propria.

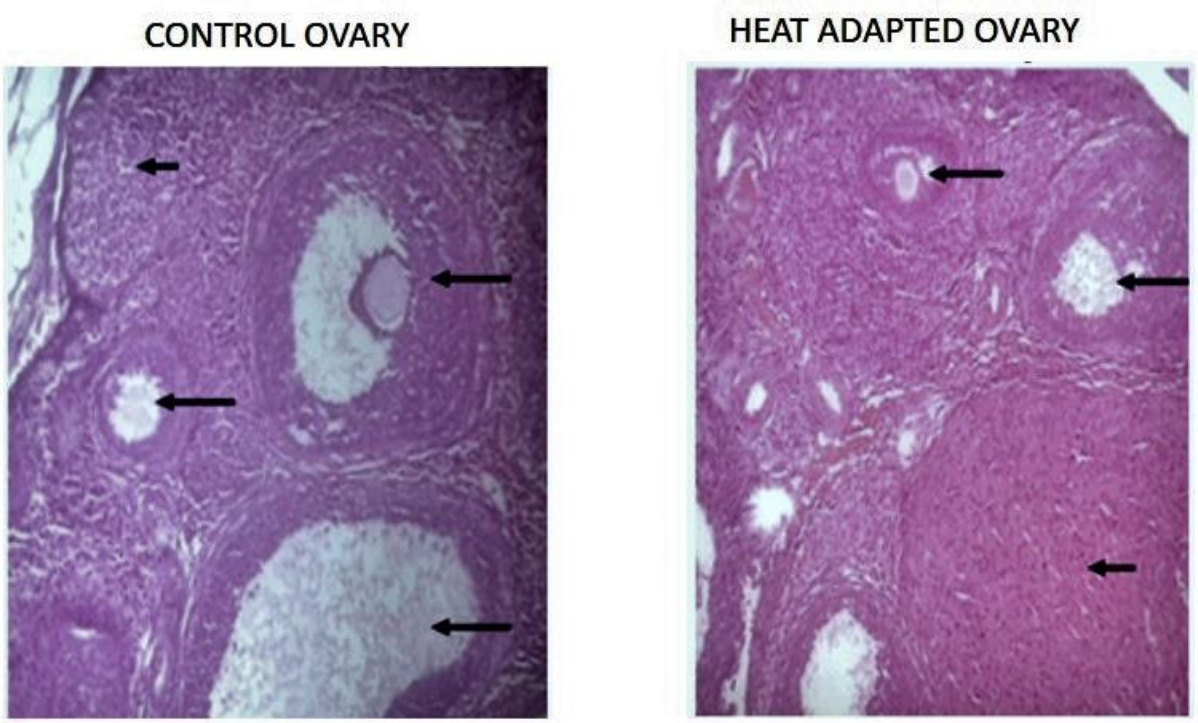


Plate 4.2 ovarian histology section of both the control and the heat adapted group. Control ovary reveals numerous corpus luteum (long arrow) with visible primary unilaminar and secondary multilaminar follicle and eccentrically located oocyte (long black). There is thick germinal epithelium and zona granulosa with corona radiata and reduced corpus luteum (short arrow). Heat adapted ovary reveals large corpus luteum (long black arrow) with secondary follicles seen within the parenchyma (short arrow).

4.2 Effect of Heat adaptation on the estrus cycle

Figure 4.2.1 shows surface and core body temperature of rats in both control and heat adapted group of the estrus cycle phase. There was a significant difference in both surface and core body temperature of the heat adapted group compared to that of the control group.

Figure 4.2.2 shows the estrous cycle pattern of animals in both control (black) and heat adapted (red) group of estrus cycle phase. The control group maintained a regular cycle pattern whilere there was an irregular cycling pattern for the heat adapted group; they maintained the metestrus

phase for first 6 days and as from the 7th day there was changes in their cycling pattern that eventually regularized from day 16 to day 21.

Figure 4.2.3 shows the relative weight of the wet uterus (WU), dried uterus (DU) and ovaries. There was no significant difference between control and heat adapted group.

Figure 4.2.4 shows original tracing of the uterine contractility of both control and heat adapted groups (a). There was no difference between the mean frequency response of the control and heat adapted group (b) but the mean amplitude response (c) of the control group was slightly higher than that of the heat adapted group.

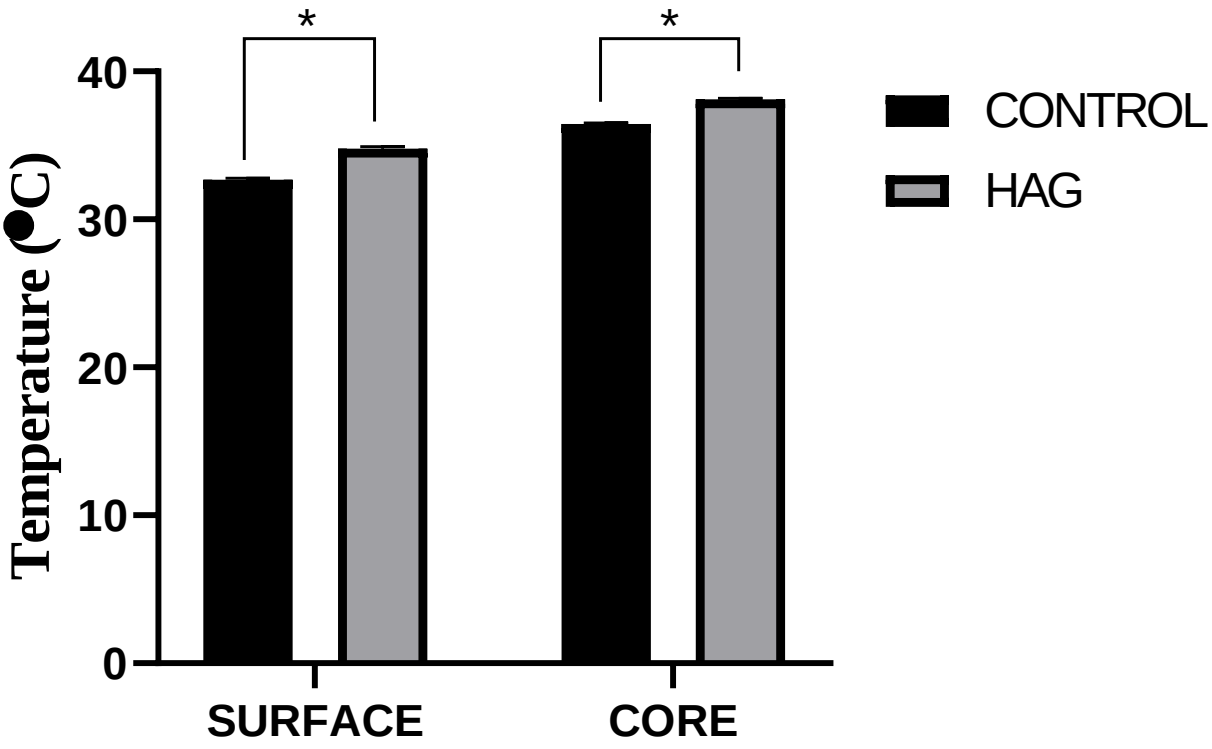


Figure 4.2.1 surface and core body temperature of rats in both control and heat adapted group of the estrus cycle phase. Values are means ± SEM (n = 6 animals). * P<0.05. HAG- Heat Adapted Group

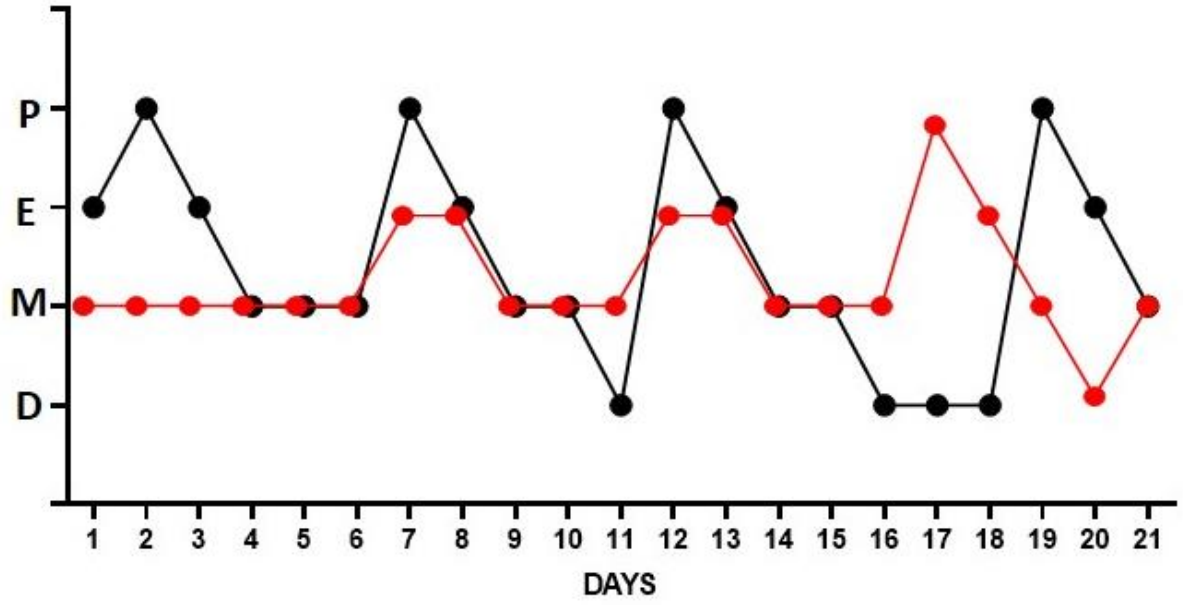


Figure 4.2.2 Estrous cycle pattern of animals in both control (black) and heat adapted (red) group of estrus cycle phase. No significant changes were observed, animals maintained almost the same cycling pattern. P-proestrus. E-estrus. M-metestrus. D-diestrus.

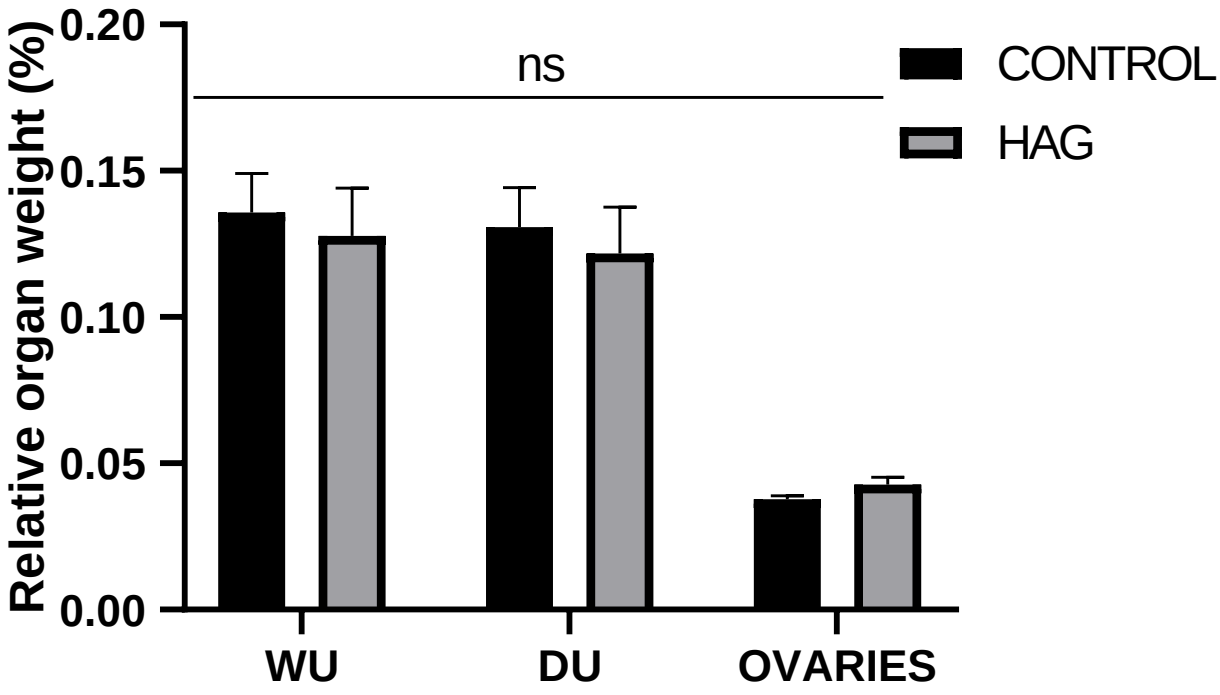


Figure 4.2.3 Wet uterus (WU), dried uterus (DU) and ovarian relative weight (Estrus cycle phase). Data represent the mean ±SEM for 6 rats in each group. ns- Not significant. HAG- Heat Adapted Group

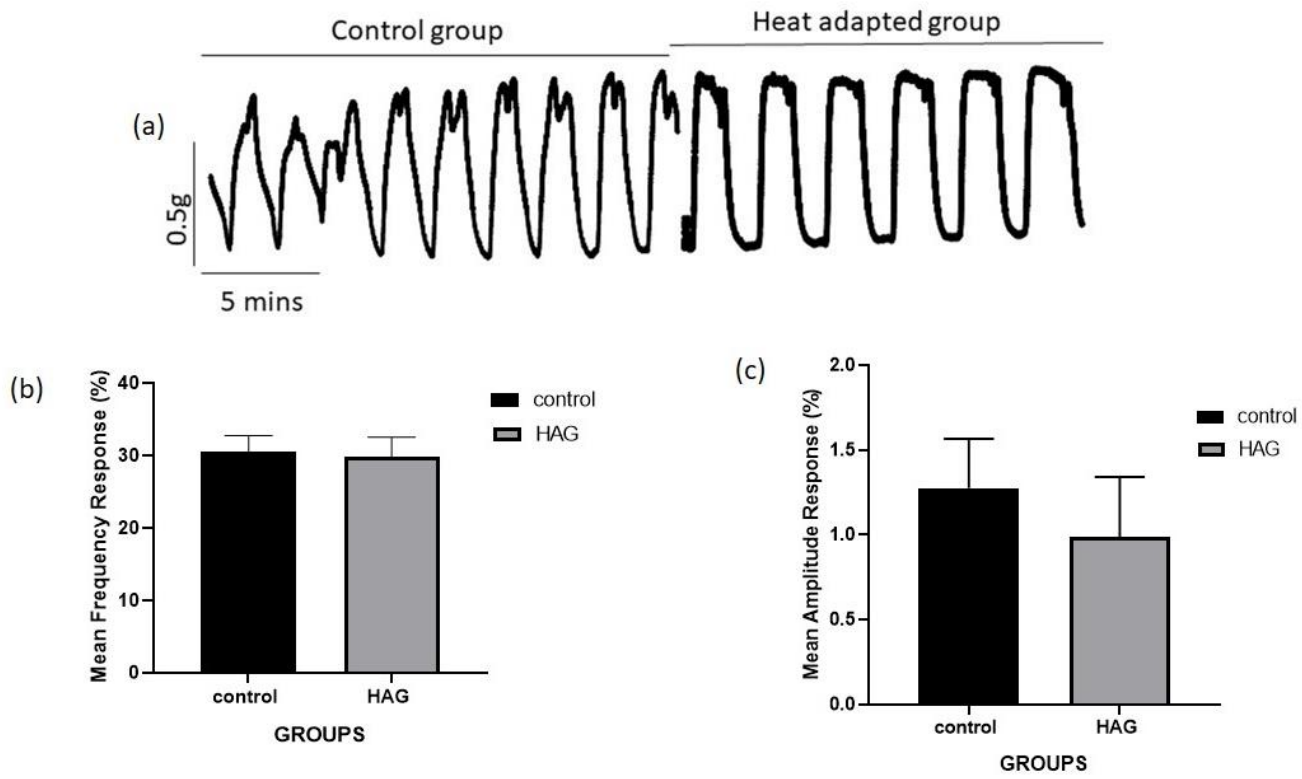


Figure 4.2.4 Original tracing showing uterine contractility of both control and heat adapted groups (a). Estrus cycle phase uterine contractility (mean response of frequency (b) and amplitude (c). Data represent the mean ±SEM for 6 rats in each group. ns- Not significant. HAG- Heat Adapted Group

Figure 4.2.5 shows the level of estrogen and progesterone for both control and heat adapted group. There was a significant increase in estrogen level of the heat adapted group while there was no change in the progesterone level of both groups

Figure 4.2.6 shows the level of follicle stimulating hormone luteinizing hormone for both control and heat adapted group. The levels of both hormones of the control group was higher than that of the heat adapted group but the difference was not significant

Figure 4.2.7 shows the relative mRNA HSP-70 gene expression for both control and heat adapted groups. HSP-70 was significantly expressed in the heat adapted group compared to the control group

Plate 4.3 shows histology section of uterus for both control and heat adapted groups. The control uterus shows normal endometrial epithelium and stroma, having underlying myometrium composed of mature smooth muscles admixed with some endometrial glands close to the endometrium. That of the heat adapted group also shows a normal endometrial epithelium and stroma.

Plate 4.4 shows histology section of the ovary for both control and heat adapted group. The control ovary shows presence of a primary follicle containing a primary oocyte surrounded by theca and granulosa cells as well as other follicles at different stages of maturation within an ovarian stroma that consist of cells with normochromic spindle-like nuclei and pale to eosinophilic cytoplasm. For the heat adapted ovary, there is presence of a graffian follicle containing a primary oocyte surrounded by theca and granulosa cells.

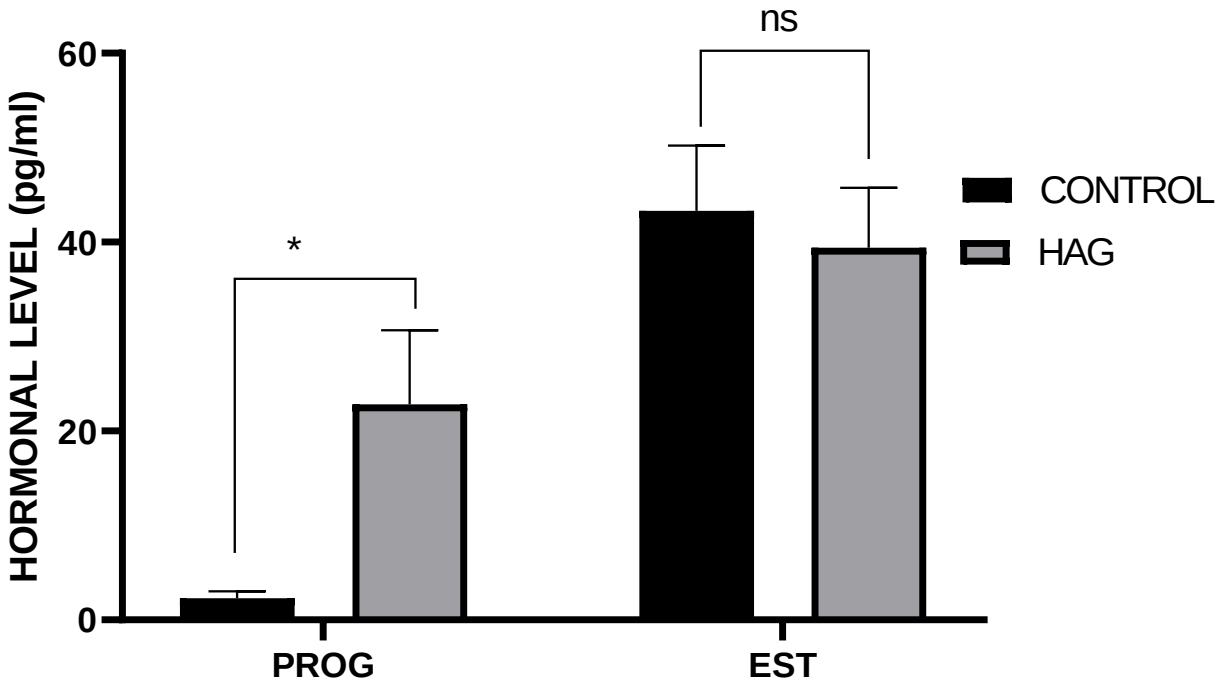


Figure 4.2.5 Estrus cycle hormonal level. PROG-progesterone and EST-estrogen. Data represent the mean ±SEM for 6 rats in each group ***** P<0.05. ns- Not significant. HAG- Heat Adapted Group

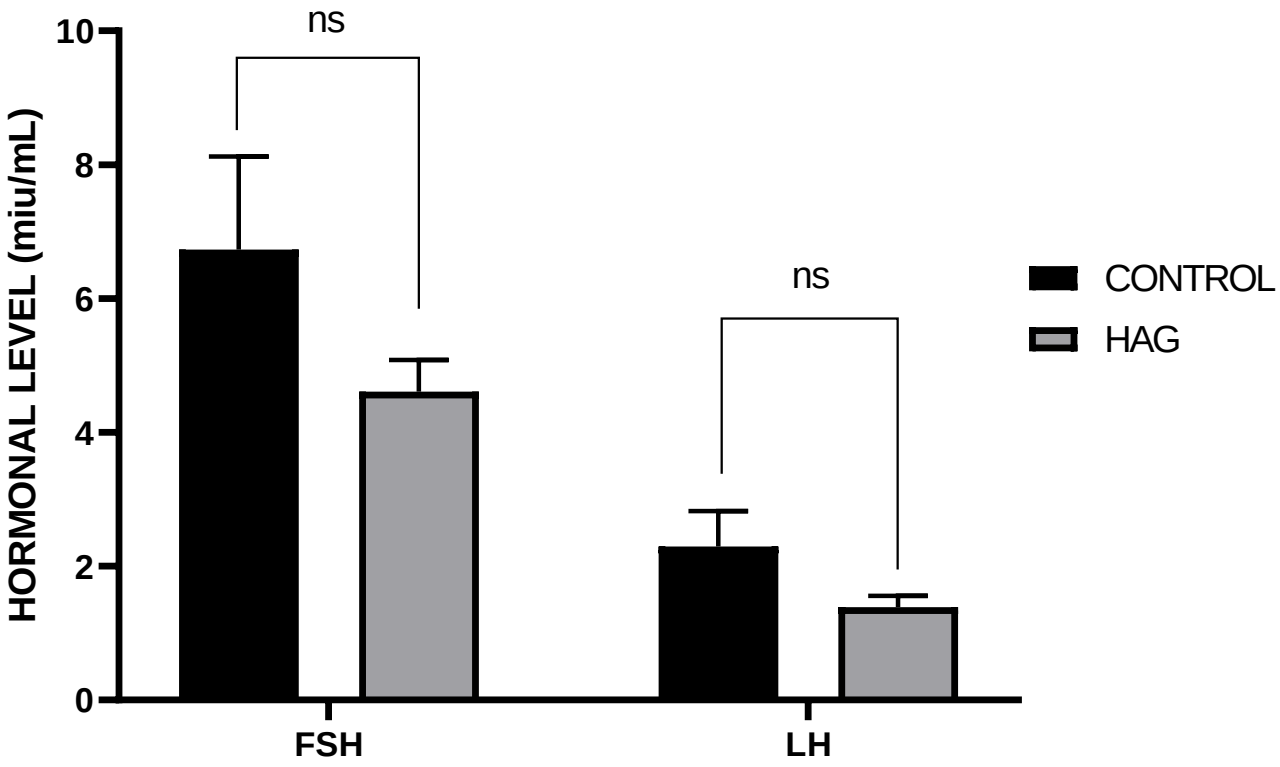


Figure 4.2.6 Estrus cycle hormonal level. FSH-follicle stimulating hormone and LH-luteinizing hormone. Data represent the mean ±SEM for 6 rats in each group. ns- Not significant. HAG- Heat Adapted Group

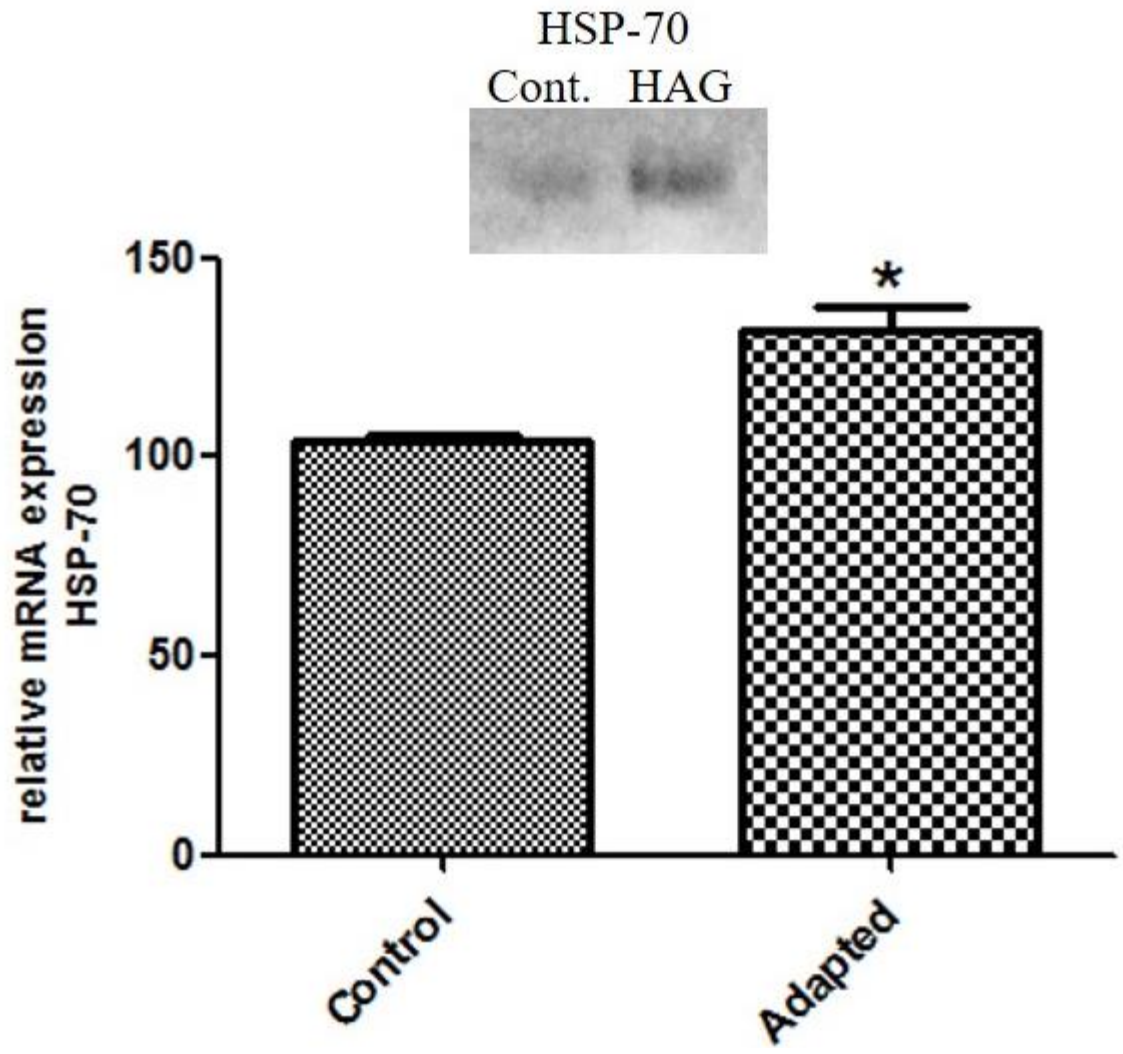


Figure 4.2.7 Relative mRNA HSP-70 gene expression for both control and heat adapted groups of the estrus cycle phase. Values are means ± SEM (n = 4 animals). ***** P<0.05. HAG- Heat Adapted Group

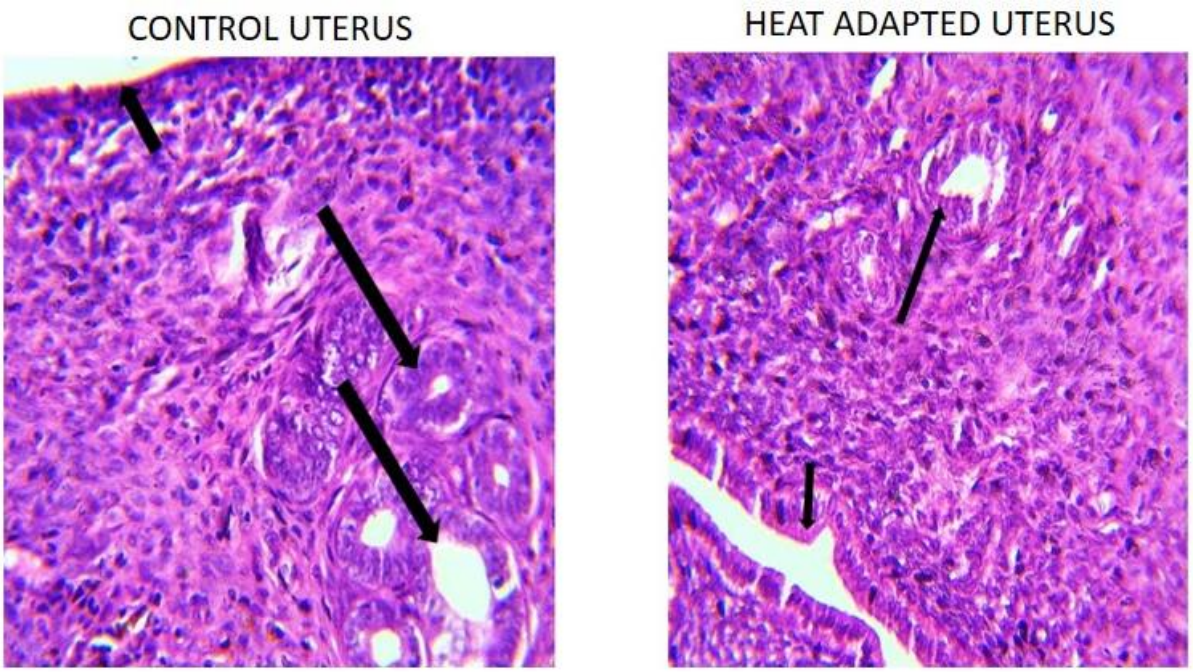


Plate 4.3 Section of uterine corpus showing normal endometrial epithelium and stroma (long arrows). The underlying myometrium is composed of mature smooth muscles admixed with some endometrial glands close to the endometrium. Short arrow shows endometrial lining.

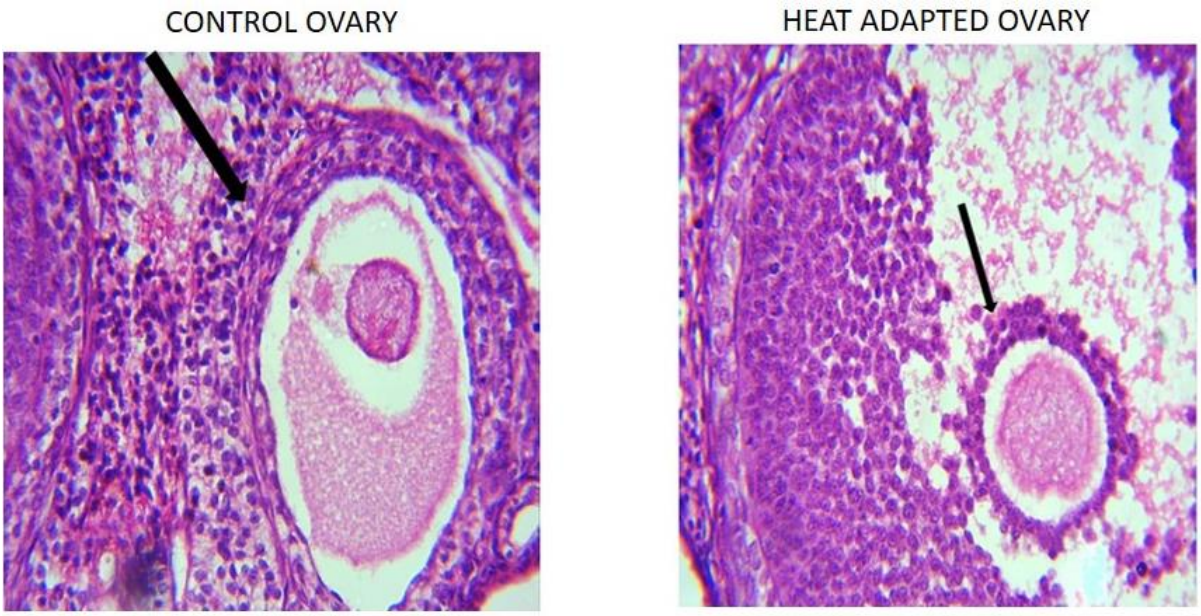


Plate 4.4 Section of the ovary showing presence of a primary follicle (arrows) containing a primary oocyte surrounded by theca and granulosa cells. Also present are other follicles at different stages of maturation within an ovarian stroma that consist of cells with normochromic spindle-like nuclei and pale to eosinophilic cytoplasm.

4.3 Hormonal level comparison between estrus phase and prepubertal phase

Figure 4.3.1 shows hormonal level comparison between estrus cycle phase and prepubertal phase for follicle stimulating hormone and luteinizing hormone. The follicle stimulating hormone level of the prepubertal phase was significantly higher than that of the estrus cycle phase in both control and heat adapted group. The level of luteinizing hormone for estrus cycle phase was higher than that of the prepubertal phase in both control and heat adapted group, but the difference was not significant.

Figure 4.3.2 shows hormonal level comparison between estrus cycle phase and prepubertal phase for progesterone and estrogen hormones. The level of progesterone in the control group of the prepubertal phase was higher than that of the estrous cycle phase, while there was a significant increase in the level of progesterone in the estrus cycle phase more than that of the prepubertal phase. For estrogen level, the prepubertal phase has a higher concentration in both control and heat adapted group.

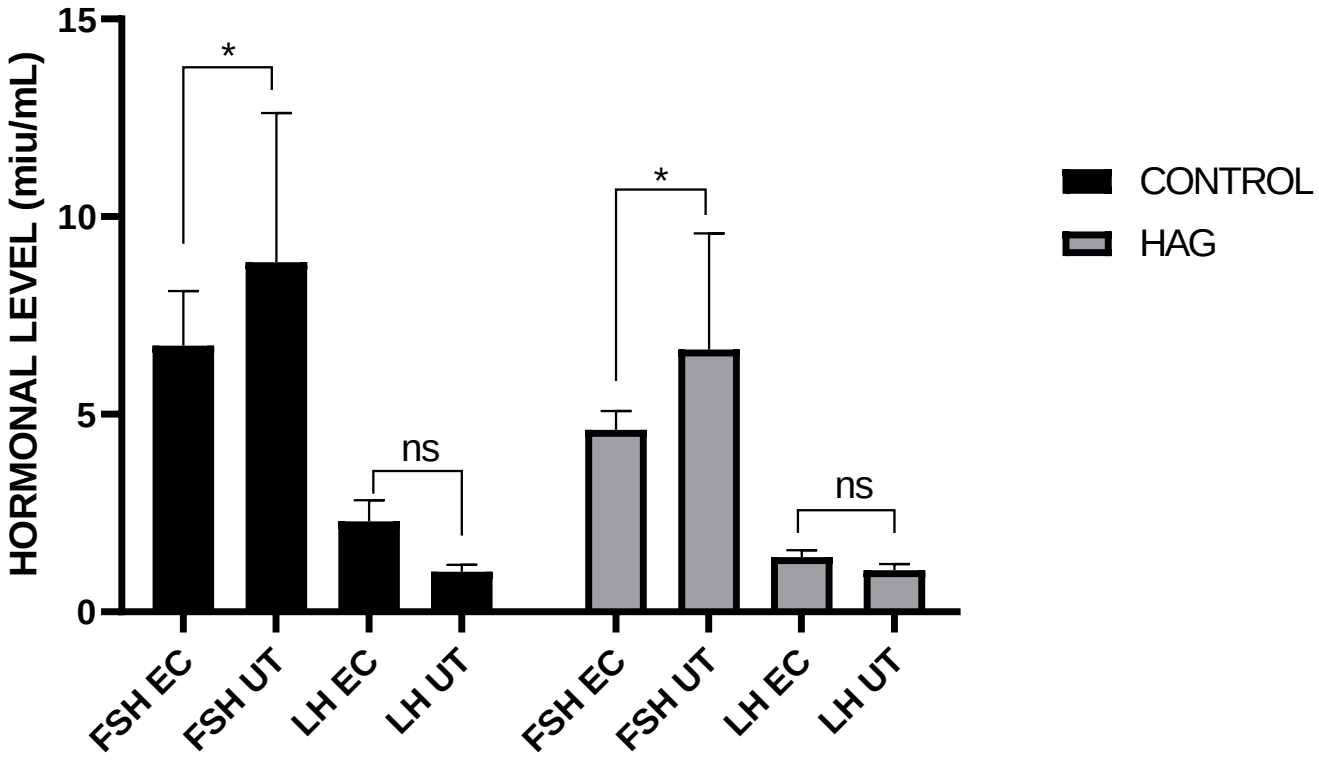


Figure 4.3.1 hormonal level comparison between estrus cycle phase and prepubertal phase. Data represent the mean ±SEM for 6 rats in each group. * P<0.05, ns- Not significant. FSH EC-follicle stimulating hormone for estrus cycle phase. FSH UT-follicle stimulating hormone for prepubertal phase. LH EC-luteinizing hormone for estrus cycle phase. LH UT-luteinizing hormone for prepubertal phase. HAG- Heat Adapted Group

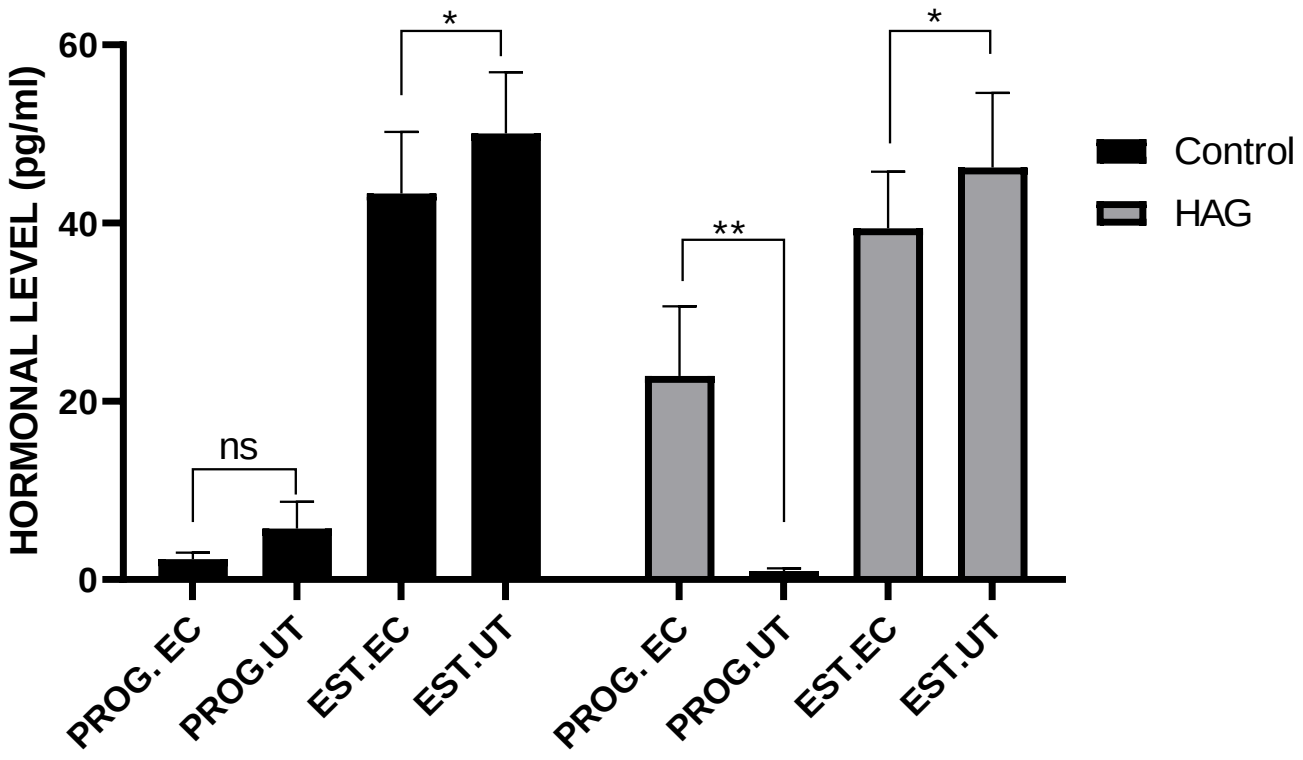


Figure 4.3.2 hormonal level comparison between estrus cycle phase and prepubertal phase. Data represent the mean ±SEM for 6 rats in each group. * P<0.05, ** P<0.01, ns- Not significant. PROG EC-progesterone for estrus cycle phase. PROG UT- progesterone for prepubertal phase. EST EC-estrogen for estrus cycle phase. EST UT-estrogen prepubertal phase. HAG- Heat Adapted Group

4.4 Results on the effect of heat adaptation on gestational phase 1

Figure 4.4.1 shows surface and core body temperature for both control and heat adapted group. There was a significant increase in both surface and core body temperature of the heat adapted group.

Figure 4.4.2 shows original tracing of spontaneous uterine contractions of both control and heat adapted groups (a). Mean frequency response (b) of the control group was higher than the heat adapted group meanwhile mean amplitude response (c) of the heat adapted group increased more than the control group. The difference in both responses was not significant.

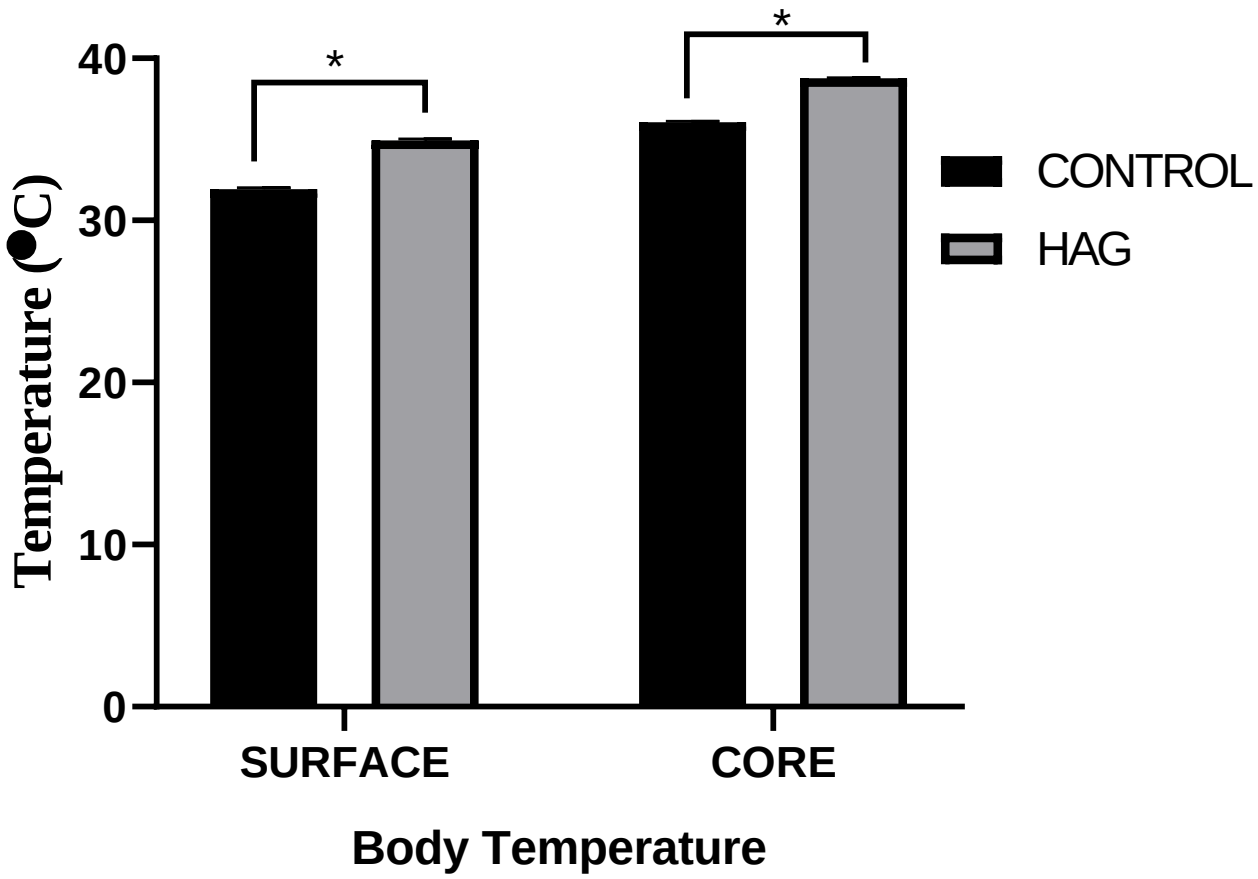


Figure 4.4.1 Surface and core body temperature measurement for both control and heat adapted group (gestational phase 1). Values are means ± SEM (n = 6 animals). * P<0.05. HAG- Heat Adapted Group

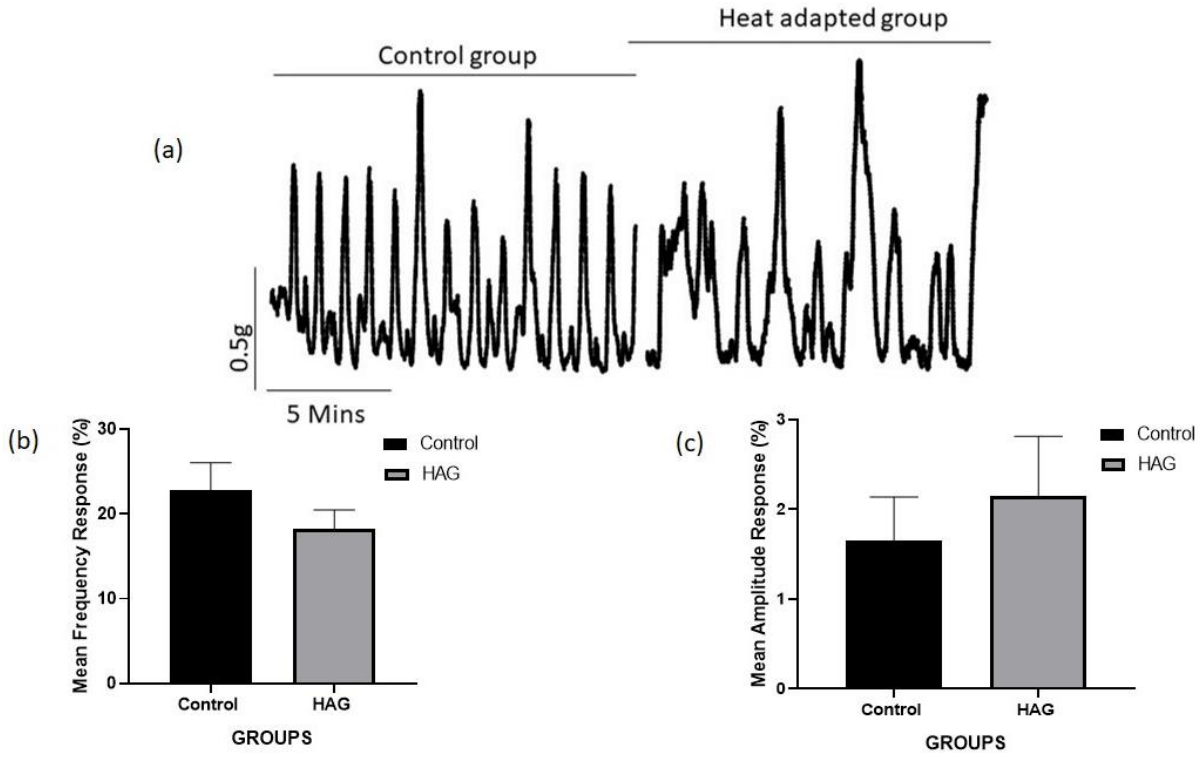


Figure 4.4.2 original tracing showing spontaneous uterine contractions of both control and heat adapted groups (a) uterine mean frequency response (b) mean amplitude response (c) of gestational phase 1. Values are expressed as means ± SEM (n = 6 animals). HAG- Heat Adapted Group

4.5 Results on the effect of heat adaptation on gestational phase 2

Figure 4.5.1 shows body temperature for both control and heat adapted group. There was a significant increase in the body temperature of the heat adapted group.

Figure 4.5.2 litter size of control and heat adapted group. The litter size of the heat adapted group was higher than that of the control group but not significantly.

Figure 4.5.3 percentage of dead litters of both control and heat adapted. The percentage of death was higher in the control group but not significantly

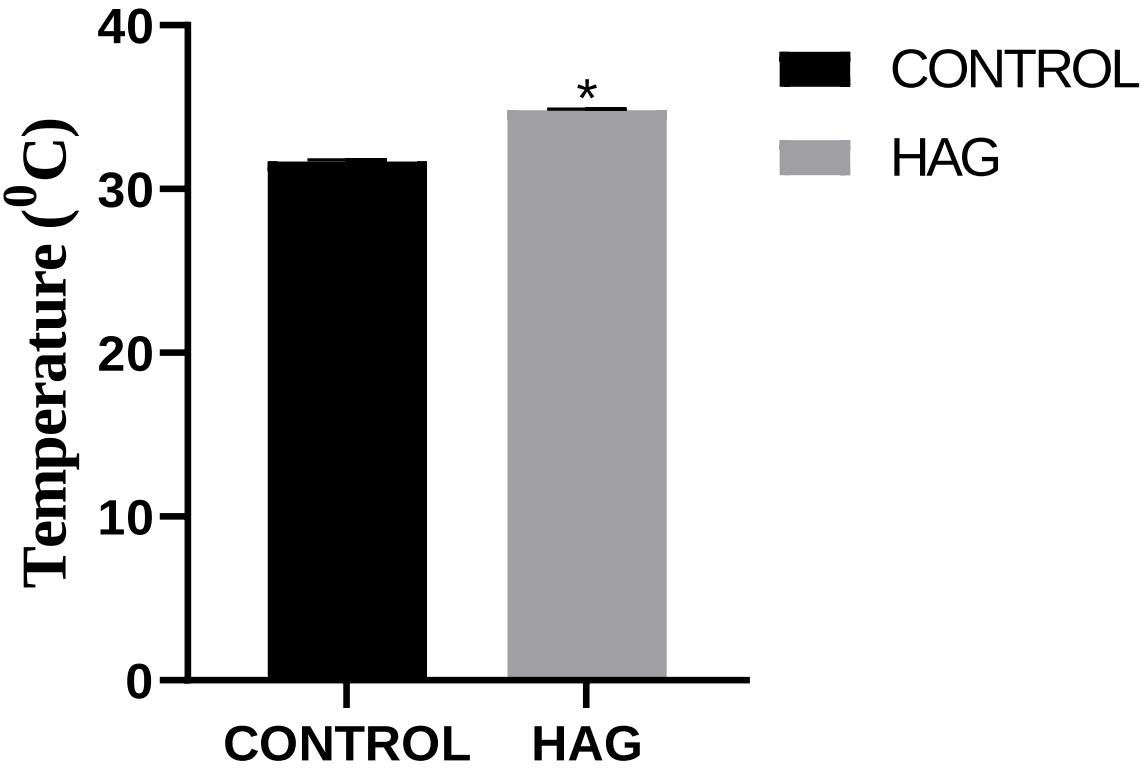


Figure 4.5.1 Surface body temperature measurement for both control and heat adapted group (gestational phase 2). Values are means ± SEM (n = 6 animals). * P<0.05. HAG- Heat Adapted Group

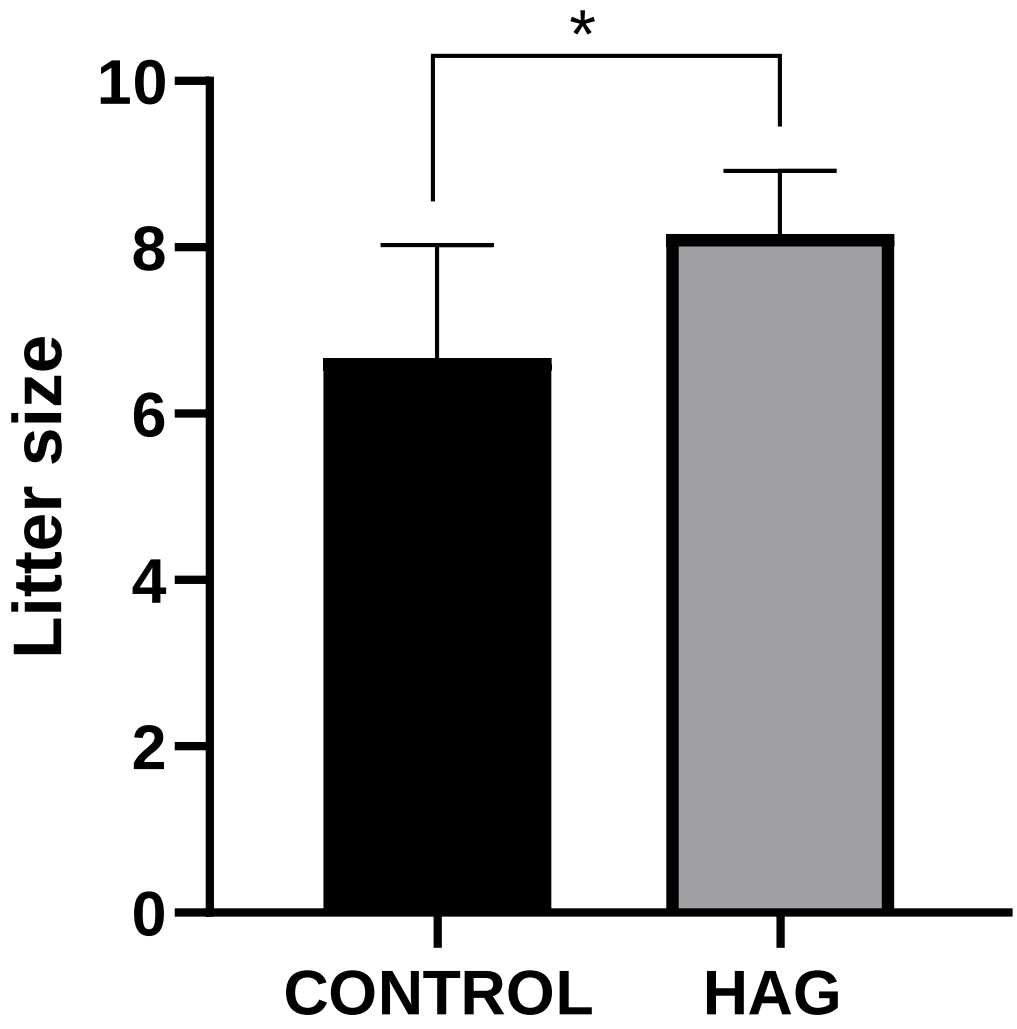


Figure 4.5.2 litter size of control and heat adapted group in gestational phase 2. Values are means ± SEM (n = 6 animals). ***** P <0.05. HAG- Heat Adapted Group

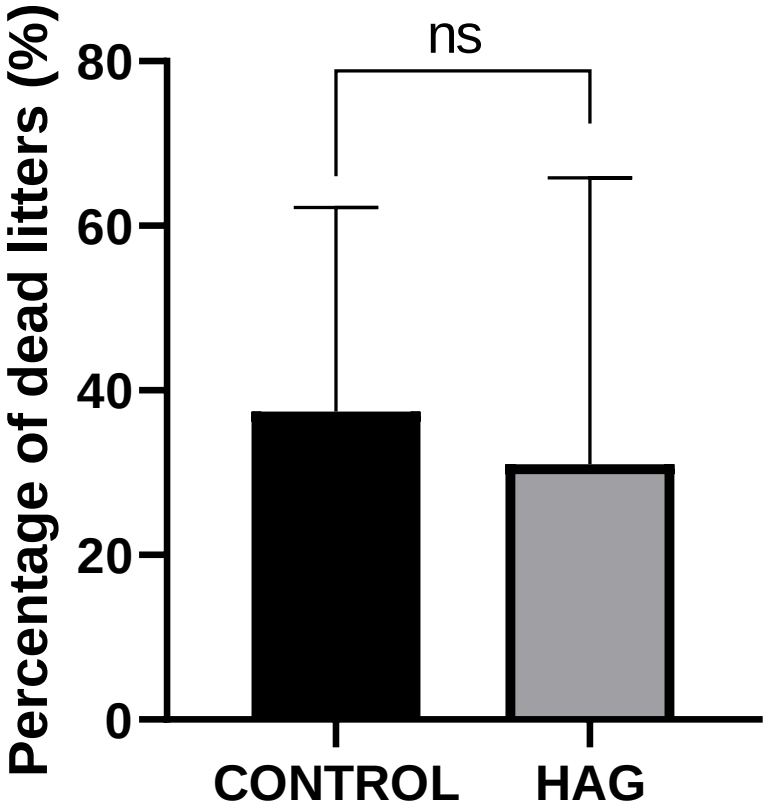


Figure 4.5.3 percentage of dead litters of both control and heat adapted group in gestational phase 2. Values are means ± SEM (n = 6 animals). Ns - Not significant. HAG- Heat Adapted Group

CHAPTER FIVE

DISCUSSION AND CONCLUSION

5.1 Discussion

The impact of temperature on the reproductive functions of women, especially in this era of global warming, has drawn attention because of the intricacy of the female reproductive system. However, it was observed in this study that heat adaptation does not cause any significant change in the reproductive functions of female Wistar rats in the areas of estrus cyclicity, uterine and ovarian weight, uterine and ovarian morphology, female reproductive hormonal levels, and contractility of both pregnant and non-pregnant isolated uterus as well as litter size and weight.

In all the phases of this study, there was a significant change in both surface and core temperature (T_{core}) of the control and heat adapted group (figures 4.1.1, 4.2.1, 4.4.1 and 4.5.1). According to previous studies (Wang *et al.,* 2016; GaiHong *et al.,* 2020), T_{core} indicates the efficiency of the body thermoregulatory system during the heat exposure. Dysfunction of the thermoregulatory center occurs when T_{core} gets to 42° C ± 0:5° C. In this current study, the temperature was limited to 39° C ± 0:5° C and less than 40.0° C, which is "compensable heat stress" (Cheung, *et al.,* 2000) and there was no abnormal behavior or death in the heat adapted group.

The results also showed that the relative wet and dried weight of the uterus as well as the ovarian weight in the heat adapted group was lower than that of the control group in both prepubertal phase and estrous cycle phase (figures 4.1.3 and 4.2.3). However, no significant difference was detected between both groups. It can be speculated that the weight of wet uterine pressure and partial edema might be induced by the proliferation of uterine glands, as well as the increase in the estrogen-triggered uterine microvascular permeability. Other study reported

a significant increase in uterine weight of experimental group exposed to heat, it was stated that uterine function of the female rats in the heat stress group was damaged as a result of hyperthermia and edema or hypertrophy of the tissue (GaiHong *et al.,* 2020). In this study, it is safe to mention that such damage did not occur to both the uterus and ovaries.

Uterine contractility results in this study showed that there was no significant difference between the control group and heat adapted group of the prepubertal phase (figure 4.1.2), estrus cycle phase (figure 4.2.4) and gestational phase 1 (figures 4.4.2). The uterus which is composed of three layers one of them is the myometrium (smooth muscle cells of the uterus), brings about the spontaneous uterine contraction (pehlivanoglu *et al.,* 2013). Contraction in the non-pregnant uterus functions to expel parts of the endometrial layer during the initial follicular phase and in the pregnant uterus it aids the expulsion of the fetus during paturition (Wray *et al*., 2003). Non-pregnant uterus contracts more when the animal is in its late follicular phase (late proestrus) or in the ovulation phase (estrus). The frequency of uterine contractions increases at the follicular phase of the menstrual cycle and this is decreased at the luteal phase and the influx of calcium has also been reported to play a primary role in spontaneous uterine contraction (Wray *et al*., 2003; Floyd and Wray 2007). It may therefore be that heat adaptation did not inhibit calcium influx in both the non-pregnant and pregnant uterus. The increased uterine mean frequency response (figure 4.4.2 (b)) of the pregnant uterus associated with decreased amplitude (figure 4.4.2 (c)) that occurred in the gestational phase 1 are not well understood considering the complexity of the uterus, however, Dominic and Reinke (1968) suggested that differences in the levels of estrogen may cause inverse relationship between frequency and force of contraction and progesterone which is high during pregnancy has been reported to cause asynchronous electrical activity in the uterus (Csapo and Takeda, 1965) resulting in a decrease in amplitude and increase in frequency.

In the estrous cycle phase, estrus cycle result showed that there was no significant difference between the heat-adapted group and control group but the animals in the control group maintained a normal cycling pattern for 21 days while those in the heat adapted group stayed more on the metestrus phase and normalized towards the end of the 21 days study as shown in figure 4.2.2. Previous studies have shown that short-term heat stress does not affect estrous cycling (Sils *et al.,* 2002; Ayodeji and Roland 2020), and the disorder of the estrous cycle induced by long-term heat stress was only reported by a Russian group in 1975 (Bao, 1975). Estrous phase determination is necessary when selecting a female animal that will be mated with a male for pregnancy or for estrous tracking as a parameter that may alter research results. Hypothalamic-pituitary-ovarian axis integrity and the proper functioning of the female reproductive system is measured by assessing estrous cycle in experimental animals (Auta and Hassan, 2016). This assessment can be used to evaluate drugs and chemicals effects on reproductive function which are often seen as reproductive organs morphology, histology and cytology disruption as well as duration alteration of particular phases of the estrous cycle (Byers *et al.,* 2012). According to Ayodeji and Roland (2020), the proestrus phase correlates with human follicular stage, which is linked with an increase in the concentration of circulating estradiol and little surge in prolactin, leading to an increase in luteinizing hormone (LH) and Follicle Stimulating Hormone (FSH). The increase in the concentration of FSH as well as a swift decline in the level of estradiol correlates with ovulation and estrus phase. Metestrus and diestrus respectively correlates with early and late secretory stages of the menstrual cycle having high progesterone level. This might explain the increase in progesterone level of the heat adapted group in the estrus cycle phase of this study when compared to the control group as shown in figure 4.2.6, the animals in the heat adapted groups stayed more in the metestrus phase which is linked to an increased progesterone level as explained by Ayodeji and Roland

(2020). Progesterone and estrogen have a direct effect on the response to stress, which varied at different stages of the estrous cycle.

This study also showed no significant difference in the level of follicle stimulating hormone (FSH) and luteinizing hormone (LH), Progesterone and oestrogenof the heat adapted group and the control in both the prepubertal phase and estrous cycle phase (figures 4.1.5 and 4.2.6). (figure 4.1.4) the same results were obtained in a study done by GaiHong *et al.,* (2020). In a normal physiological state, the body can maintain FSH/LH within the normal range through self-regulation. However, under stress, the function of the hypothalamic-pituitary-gonadal (HPG) axis is affected, this disrupts the feedback regulation of the hypothalamus-pituitary-ovarian (HPO) axis, which in turn, might cause a decline in the ovarian reserve function. In women, the synergistic effect of LH and FSH on ovarian follicles and granulosa cells leads to an increase of estradiol in the blood, which is essential for the production of estrogen and progesterone (González-Alonso and Calbet, 2003). This might explain the reason for the low hormonal concentration of the heat adapted group when compared with the control group. Comparing the hormonal level of the estrous cycle phase and the prepubertal phase (figures 4.3.2 and 4.3.3) it was observed that the hormonal level of the prepubertal phase was higher than that of the estrous cycle phase except for progesterone level that was significantly higher in the estrous cycle phase.

Heat shock protein 70 (Hsp70) protects the body against oxidative stress induced by heat, (Xie *et al.,* 2014), this might be as a result of the influence of heat shock factors activating the upstream promoter sequences for genes encoding heat shock protein. It has been reported that body temperature increases above normal during hyperthermia as a result of overheating which causes thermoregulation failure that eventually activates cellular defense mechanism through nuclear factor (NF)-kB mediated by proinflammatory cytokine induction (Pockley, 2003). Hsp70, as a classic stress marker, is a kind of protein that responds to long-term or chronic

stress and can slow down the magnification of stress-related response, thereby having a certain protective effect on the body (Wegele *et al.,* 2004). In this study, the uterine Hsp70 gene of female rats (figure 4.2.7) exposed to heat was upregulated significantly, helping to explain rats' adaptation to heat for the period of exposure.

The histology of the uterine and ovarian tissues in the heat-adapted group did not differ from that of the control group in the prepubertal phase (figures 4.1.6 and 4.1.7) and estrus cycle phase (figures 4.2.8 and 4.2.9). The uterine and ovarian structures (columnar epithelium, endometrium, fibrous cell stroma, corpus luteum and zona granulosa) were intact without any abnormality. In the study done by GaiHong *et al.,* (2020) it was reported that in the group exposed to heat, uterine cavity of the female rats was narrow and the luminal epithelial cells of the endometrium had an irregular morphology with epithelial cytoplasmic vacuolization and local cell proliferation as well as uterine glands dilation and few number of lamina propria neutrophils as a result of thermal damage. In mice, as reported in a study done by Li *et al.,* (2016), chronic heat stress exposure causes an increase in the number of antral follicles with severe apoptotic signals. It will be safe to claim that the experimental animals did not experience thermal damage.

Edwards *et al.,* (2001) reported that mammalian preimplantation embryos are sensitive to high temperatures. Maternal heat stress or the resultant hyperthermia leads to an increased loss of early stage embryos. Heat adaptation as observed in this study did not affect mating, fertilization and gestation. Litter size of heat adapted group was significantly higher than that of the control group (figure 4.5.2) but the percentage of death was higher in control group than in heat adapted group (figure 4.5.6) though not significantly. This findings can be attributed to the ability of heat adapted groups to produce HSP70 that protect cells from stress for thermotolerance. Previous finding deepened the understanding of cellular mechanism that results in the vulnerability of early embryos to heat stress (Rivera and Hansen, 2001), but it

remained unclear why exposure of embryos to fluctuating temperatures causing heat stress did not decrease embryonic development even when the heat stress continued throughout gestation. In mice, in vivo and in vitro experiments showed that embryo development is significantly inhibited by heat stress approximately 48–72 h after fertilization, the stage that is most sensitive to heat stress is approximately the time of zygotic genome activation (ZGA), which occurs at the 2-cell stage in mice, after this stage, heat stress exposure has less effect on the rate of development and cell proliferation (Sakatani *et al.*, 2004). It was also observed that there was no difference in pup weight both at time of birth and at weaning. It seems that a selection process takes place throughout the course of development, eliminating embryos/fetuses that do not possess the complete developmental features needed to become viable offspring. Previous studies in bovine embryos indicated that the apoptotic status of blastocysts derived from oocytes heat-shocked during early stages of maturation did not differ from controls (Roth and Hansen 2004; Aroyo et al., 2007). It was also observed that the developmental potential (vaginal opening precisely) of litters of the heat adapted groups was similar to that of the control group. This is similar to the findings of Galletly *et al.*, (2001) that observed that the developmental potential of pups that developed from heat-stressed mice was similar to that of the control group. The hypothalamic-pituitary-gonadal (HPG) axis is important for reproductive function (couse *et al.,* 2003). The hypothalamus releases gonadotropin releasing hormone (GnRH) which stimulates the anterior pituitary to secrete follicle stimulating hormone (FSH) and luteinizing hormone (LH). FSH and LH act on the ovary, stimulating folliculogenesis and estradiol synthesis which is responsible for vaginal opening. In this study heat adaptation did not alter the HPG axis.

5.2 Conclusion

This study has shown that heat adaptation does not affect reproductive functions of female rats in terms of estrous cycle, reproductive organ morphology, female reproductive hormones, fertility and pubertal development. The relatively stable reproductive indices associated with heat adapted female rats are significantly up-regulated while Heat shock (HSP-70) gene protein could be the regulatory adaptive mechanism that prevented any negative consequence.

R**EFERENCES**

Abdullahi, A., Yahuza, Y., Amodu, U. S., Imhanfidon, O. J., Ogobor, E. A., Abimaje. O. S., Adejoh, J. and Obasi R. C. (2019). A Study of the Daily Weather Variation of Abuja, North Central, Nigeria: A Case Study of the Month of April. *International Journal of Engineering and Applied Sciences* **6(8):** 76-79.

Ajayi, A. F., and Akhigbe, R. E. (2020). Staging of the estrous cycle and induction of estrus in experimental rodents: an update. *Fertility research and practice*, ***6***(1): 1-15.

akhotia S.C. and Prasanth K.V. (2002). Tissue- and development-specific induction and turnover of hsp70 transcripts from loci 87A and 87C after heat shock and during recovery in Drosophila melanogaster. *J. Exp. Biol*. 205, 345–358.

Al-Qattan MM, Al-Qattan AM. Fibromodulin: Structure, Physiological Functions, and an Emphasis on its Potential Clinical Applications in Various Diseases. J Coll Physicians Surg Pak. 2018 Oct;28(10):783-790.

Ameer, M. A., Fagan, S. E., Sosa-Stanley, J. N., & Peterson, D. C. (2017). Anatomy, abdomen and pelvis, uterus.

Aroyo, A. Yavin, S., Arav, A. and Roth, Z. (2007). Maternal hyperthermia disrupts developmental competence of follicle-enclosed oocytes: In vivo and ex vivo studies in mice. *Theriogenology* **67**:1013–1021

Aroyo, A., Yavin, S., Arav, A. and Roth, Z. (2007). Maternal hyperthermia disrupts developmental competence of follicle-enclosed oocytes: In vivo and ex vivo studies in mice. *Theriogenology* **67**:1013–1021

Auta, T. and Hassan, A. T. (2016). Alteration in oestrus cycle and implantation in Mus musculus administered aqueous wood ash extract of Azadirachta indica (neem). *Asian Pacific J Reproduction*. 5(3):188–192.

Auta, T. and Hassan, A. T. (2019). Alteration in oestrus cycle and implantation in Mus musculus administered aqueous wood ash extract of Azadirachta indica (neem). *Asian Pacific J Reproduction.* **5**(3):188–192.

Ayodeji F, A. and Roland E. A. (2020). Staging of the estrous cycle and induction of estrus in experimental rodents: an update. *Ajayi and Akhigbe Fertility Research and Practice*. 6:5-25

Bafor, E. E., Ukpebor, F., Elvis-offiah, U, Uchendu, A., Omoruyi, O. and Omogiade, U. G. (2020). Justicia flava Leaves Exert Mild Estrogenic Activity in Mouse Models of Uterotrophic and Reproductive Cycle Investigations. *J Med Food* **23**(4):395-408.

Bafor, E. E., Ukpebor, F., Omoruyi, O., Ochoyama, E., Omogiade, G., Ekufu, J., and Edrada-Ebel, R. (2019). Tocolytic activity assessment of the methanol leaf extract of Justicia flava Vahl (Acanthaceae) on mouse myometrial contractility and preliminary mass spectrometric determination of secondary metabolites. *J Ethnopharmacol* **243**:112087.

Bao, Y. S. (1979). Changes of female sexual cycle under the chronic effect of high temperature. *Foreign Medical Reference Materials (Hygiene fascicles)* 1:42.

Barney, C. C., Schanhals, M. E., Grobe, J. L., Andresen, B. T. and Traver, M. (2015). Heat acclimation and thirst in rats. *Physiol Rep* **3**(12):1-14.

Baskett, T. F., Calder, A. A. and Arulkumaran, S. (2014). Munro Kerr's Operative Obstetrics E-Book. Elsevier Health Sciences. p. 268

Bedrak, E., K. Fried, V. Samoiloff, and U. A. Sod-Moriah (1977). The effect of acclimation to elevated ambient temperature on progesterone levels in blood and corpora lutea of cycling and four-day pregnant rats. *J. Therm. Biol.* **2**:83-87.

Bellofiore N, Rana S, Dickinson H, Temple-Smith P, Evans J. Characterization of human-like menstruation in the spiny mouse: comparative studies with the human and induced mouse model. *Hum Reprod* 33: 1715–1726, 2018.

Brobeck, J. R. (1960). Food and temperature. In: Recent Progress in Hormone Research. G. Pincus, Academic Press, New York. **16**:439-459.

Bronikowski, A., Carter, P., Swallow, J., Girard, I., Rhodes, J. and Garland, T. (2001). Open-field behavior of house mice selectively bred for high voluntary wheel-running. *Behav Genet* **31**:309–16.

Brown, H. M. and Russell, D. L. (2013). "Blood and lymphatic vasculature in the ovary: Development, function and disease". *Human Reproduction Update*. **20** (1): 29–39.

Byers, S. L., Wiles, M. V., Dunn, S. L. and Taft, R. A. (2012) Mouse estrous cycle identification tool and images. *PLoS One*. 7(4):e35538.

Byers, S. L., Wiles, M. V., Dunn, S. L. and Taft, R. A. (2012). Mouse estrous cycle identification tool and images. *PLoS One*. 7(4):e35538.

Casa, D. and Csillan, D. (2009). Preseason heat-acclimatization guildelines for secondary school athletics. *J Athl Train.* **44**(3):332-333.

Chalmers, S., Esterman, A., Eston, R., Bowering, K. J. and Norton, K. (2014). Short-term heat acclimation training improves physical performance: a systematic review, and exploration of physiological adaptations and application for team sports. *Sports Med* **44**: 971–988.

Chappell M and Payne S: The action potential. biosystems and biorobotics. In: Physiology for Engineers. Guglielmelli E (ed.). Rome,Springer Nature, pp 36-38, 2020.

Chaudhari UK, Metkari SM, Manjaramkar DD, Sachdeva G, Katkam R, Bandivdekar AH, Mahajan A, Thakur MH, Kholkute SD. Echography of the cervix and uterus during the proliferative and secretory phases of the menstrual cycle in bonnet monkeys (Macaca radiata). J Am Assoc Lab Anim Sci. 2014 Jan;53(1):18-23

Chen, G. H., Wang, Y. J., Zhang, L. Q. and Zhou, J. N. (2004). Age- and sex-related disturbance in a battery of sensorimotor and cognitive tasks in Kunming mice. *Physiol Behav* **83**:531–41.

Cheung, S. S., McLellan, T. M. and Tenaglia, S. (2000). The thermophysiology of uncompensable heat stress: Physiological manipulations and individual characteristics. *Sports Medicine*. **29**(5):329–359.

Colvin, C. W. and Abdullatif, H. (2013). "Anatomy of female puberty: The clinical relevance of developmental changes in the reproductive system". *Clinical Anatomy*. **26** (1): 115–129

Cora, M. C., Kooistra, L. and Travlos, G. (2015). Vaginal cytology of the laboratory rat and mouse: review and criteria for the staging of the estrous cycle using stained vaginal smears. *Toxicol Pathol*. **43**:776–93.

Cora, M. C., Kooistra, L. and Travlos, G. (2015). Vaginal cytology of the laboratory rat and mouse: review and criteria for the staging of the estrous cycle using stained vaginal smears. *Toxicol Pathol*. 43:776–793.

Critchley, H. O., Maybin, J. A., Armstrong, G. M., & Williams, A. R. (2020). Physiology of the endometrium and regulation of menstruation. *Physiological reviews*. 100(3):1149-1179

Dutta DC (2014). DC Dutta's Textbook of Gynecology. JP Medical Ltd. pp. 2–7

Ekambaram, G., SKS, K. and Joseph, L. D. (2017). Comparative Study on the Estimation of Estrous Cycle in Mice by Visual and Vaginal Lavage Method. *J Clin Diagnostic Res*. 11(1):AC05–7.

Elvis-Offiah, U. B., Bafor, E. E., Eze, G. I., Igbinumwen, O., Viegelmann, C. and Edrada-Ebel, R. (2016). In vivo investigation of female reproductive functions and parameters in nonpregnant mice models and mass spectrometric analysis of the methanol leaf extract of Emilia Coccinea (Sims) G Dons. *Physiol Rep* **4**(23):1-17.

Epstein, Y., and Moran, D. S. (2008). Extremes of temperature and hydration. In: Keystone JS, Kozarsky PE, Freedman DO, Nothdurft HD, Connor BA, editors. Travel medicine. 2nd ed. Philadelphia: Mosby. Pp. 413–422.

Evans J, Salamonsen LA. Inflammation, leukocytes and menstruation. *Rev Endocr Metab Disord* 13: 277–288, 2012

Ezzati M, Djahanbakhch O, Arian S, Carr BR. Tubal transport of gametes and embryos: a review of physiology and pathophysiology. J Assist Reprod Genet. 2014 Oct;31(10):1337-47

Falaju, J. (2020). NIMET explains reasons for harsh weather conditions. Guardian Nigeria news.

Foitzik, T., Hotz, H. G., Eibl, G., Buhr, H. J. (2000). Experimental models of acute pancreatitis: are they suitable for evaluating therapy? *Int J Color Dis*. 15:127–135

Gaide Chevronnay HP, Galant C, Lemoine P, Courtoy PJ, Marbaix E, Henriet P. Spatiotemporal coupling of focal extracellular matrix degradation and reconstruction in the menstrual human endometrium. ***Endocrinology*** 150: 5094–5105, 2009

GaiHong A., XueWei C., Chao L., Li Z., MengFan W., JiaJun C., Qiang M., DanFeng Y. and Jing, W. (2020). Pathophysiological Changes in Female Rats with Estrous Cycle Disorder Induced by Long-Term Heat Stress. *BioMed Research International* **1**:1-10

Galletly, C., Clark, C. R., McFarlane, A. C. and Weber, D. L. (2001). Working memory in posttraumatic stress disorder—an event-related potential study. *J Trauma Stress* **14**:295–309

Gargett CE, Masuda H. Adult stem cells in the endometrium. *Mol Hum Reprod* 16: 818–834, 2010

Garrett, A. T., Creasy, R., Rehrer, N. J., Patterson, M. J. and Cotter, J. D. (2012). Effectiveness of short-term heat acclimation for highly trained athletes. *Eur J Appl Physiol* **112**: 1827–1837.

Garrett, A. T., Goosens, N. G., Rehrer, N. J., Patterson, M. J. and Cotter, J. D. (2009). Induction and decay of short-term heat acclimation. *Eur J Appl Physiol* **107**: 659–670.

Garrett, A. T., Rehrer, N. J. and Patterson, M. J. (2011). Induction and decay of short-term heat acclimation in moderately and highly trained athletes. *Sports Med* **41**: 757–771.

Garry R, Hart R, Karthigasu KA, Burke C. Structural changes in endometrial basal glands during menstruation. *BJOG* 117: 1175–1185, 2010.

Gellersen B, Brosens JJ. Cyclic decidualization of the human endometrium in reproductive health and failure. *Endocr Rev* 35: 851–905, 2014.

Gill, N. and Sleivert, G. (2001). Effect of daily vs intermittent exposure on heat acclimation. *Aviat Space Environ Med* **72**: 385–390.

Goel, A., Ncho, C. M. and Choi, Y. H. (2021). Regulation of gene expression in chickens by heat stress. *J Anim Sci Biotechnol*. **12**(1):11-23

Goldsztejn U and Nehorai A: A myofibre model for the study of uterine excitation-contraction dynamics. Scientific Reports 10(1): 2020.

González-Alonso, J. and Calbet, J. A. L. (2003) Reductions in systemic and skeletal muscle blood flow and oxygen delivery limit maximal aerobic capacity in humans. *Circulation* **107**(6):824–830.

Gordon, C. J. (1993). Temperature regulation in laboratory rodents, Research Triangle Park, North Carolina, Cambridge University Press, New York. Pp 163-180.

Hamid, Y. M. and Zakaria, A.B. (2013). Reproductive characteristics of the female laboratory rat. *African Journal of Biotechnology* **12**(19): 2510-2514.

Han, J. and Sadiq, N.M., 2019. Anatomy, Abdomen and Pelvis, Fallopian Tube.

Hansen, P. J. (2009). Effects of heat stress on mammalian reproduction. *Phil. Trans. R. Soc. B* **364**: 3341–3350.

Haroun, H.S.W. (2016). Reproductive cycles in females. *MOJ Womens Health*, *2*(2), pp.62-64.

Harrison, R. J. (2020). *Human reproductive system. Encyclopedia Britannica.* https://www.britannica.com/science/human-reproductive-system

Hill-Eubanks DC, Werner ME, Heppner TJ and Nelson MT: Calcium signaling in smooth muscle. Cold Spring Harb Perspect Biol 3(9): a004549, 2011.

Horowitz, M. and Meiri, U. (1993). Mechanism of heat acclimation induced bradycardia in the sand rat. *Journal of Basic & Clinical Physiology & Pharmacology* **4**(1-2): 37-46.

Ilievska, J., Cicimov, V., Antova, E., Gjorgoski, I., Hadzy-Petrushev, N. and Mladenov, M. (2016). Heat-induced oxidative stress and inflammation in rats in relation to age. *Research in Physical Education, Sport and Health* **5** (2): 123-130.

Jardine, D. S. (2007). Heat illness and heat stroke. *Pediatr. Rev* **28**:249–258.

Jia, T., Jia, X. and Zhong, C. (2012). Effects of Heat Stress on the Reproductive Performance of Mammalians. *Biomedical Engineering and Biotechnology* **181**:1408-1411.

Jin, S., Yang, L. and Zang, H. (2019). Influence of free-range days on growth performance, carcass traits, meat quality, lymphoid organ indices, and blood biochemistry of Wannan yellow chickens. *Poultry Science*, **98**(12):6602–6610.

Kampinga H. H., Hageman J., Vos M. J., Kubota H., Tanguay R. M., Bruford E. A., Cheetham M.E., Chen B. and Hightower L. E. (2009). Guidelines for the nomenclature of the human heat shock proteins. Cell Stress Chaperones. 14:105–111

Karna, K. K., Choi, B. R., Kim, M. J., Kim, H. K. and Park, J. K. (2019). The effect of Schisandra chinensis Baillon on cross-talk between oxidative stress, endoplasmic reticulum stress, and mitochondrial signaling pathway in testes of varicocele-induced SD rat. *International Journal of Molecular Sciences*. **20**(22):5785.

Karvinen, S.M., Silvennoinen, M., Ma, H., Törmäkangas, T., Rantalainen, T., Rinnankoski-Tuikka, R., Lensu, S., Koch, L. G., Britton, S. L. and Kainulainen, H. (2016). Voluntary Running Aids to Maintain High Body Temperature in Rats Bred for High Aerobic Capacity. *Front Physiol* **7**:311.

Kluwe, W. V. (1981). Renal function tests as indicators of kidney injury in subacute toxicity studies. *Toxicol Appl Pharmacol* **57**(3):414-424.

Koutras, A., Fasoulakis, Z., Syllaios, A., Garmpis, N., Diakosavvas, M., Pagkalos, A., Ntounis, T. and Kontomanolis, E.N., 2021. Physiology and Pathology of Contractility of the Myometrium. *in vivo*, *35*(3), pp.1401-1408.

Krishnan, G., Bagath, M., Pragna, P., Vidya, M. K., Aleena, J., Archana, P. R., Sejian, V. and Bhatta, R. (2017). Mitigation of the Heat Stress Impact in Livestock Reproduction. In: Theriogenology. Pp 64-86.

Kurowicka, B., Gajewska, A. and Franczak, A. (2006). Effect of early thermal experience on pituitary-gonadal axis in female rats. *Reproductive Biology* **6**(1): 63–77.

Kurowicka, B., Gajewska, A. and Franczak, A. (2007). Effect of warm-rearing and heat acclimation on pituitary gonadal axis in male rats. *International Journal of Andrology* **31**: 579–587.

Li, L., Wu, J., Luo, M., Sun, Y., and Wang, G. (2016). The effect of heat stress on gene expression, synthesis of steroids, and apoptosis in bovine granulosa cells. Cell Stress and Chaperones, 21(3), 467–475.

Macfarlane, W. V., Pennycuik, R. P. and Thrift, E. (1957). Resorption and loss of foetuses in rats living at 35°C. *J. Physiol., Lond*. **135**:451-459.

Magal, E., Kaplanski, J., Sod-Moriah, U. A., Hirschmann, N. & Nir, I. (1981). Role of pineal gland in male rats chronically exposed to increased temperature. Journal of Neural Transmission 50, 267–273.

Marieb, E. (2013). *Anatomy & physiology*. Benjamin-Cummings. p. 903.

McLean, A. C., Valenzuela, N., Fai, S. and Bennet, S. A. (2012). Performing vaginal lavage, crystal violet staining, and vaginal cytological evaluation or mouse estrous cycle staging identification. *J Vis Exp*. **15**(67):e4389.

Melmed, S., Polonsky, K. S., Larsen, P. R. and Kronenberg, H. M. (2011). *Williams Textbook of Endocrinology* (12th ed.). Saunders. p. 595

Mladenov M, Gjorgoski I, Stafilov T, Duridanova D. (2006). Effect of vitamin C on lipid hydroperoxides and carbonyl groups content of rat plasma depending on age and acute heat exposure. *Journal of Thermal Biology* **31** (8): 588-593.

Monis, C. N., & Tetrokalashvili, M. (2020). Menstrual cycle proliferative and follicular phase. *StatPearls [Internet]*.

Ozawa, M., Hirabayashi, M. and Kanai, Y. (2002). Developmental competence and oxidative state of mouse zygotes heat-stressed maternally or in vitro. *Reproduction* **124**:683–689.

Ozawa, M., Tabayashi, D., Latief, T. A., Shimizu, T., Oshima, I., and Kanai, Y. (2005). Alterations in follicular dynamics and steroidogenic abilities induced by heat stress during follicular recruitment in goats. Reproduction, 129(5), 621–630.

Paul, P. G., Gulati, G., Shintre, H., Mannur, S., Paul, G. and Mehta, S. (2018). Extrauterine adenomyoma: a review of the literature. Eur J Obstet Gynecol Reprod Biol. 228:130-136.

Pennycuik, R. P. (1964). The effect on rats of chronic exposure to 34°C. IV. Reproduction. *Aust. J. biol. Sci*. **17**: 245-251.

Periard, J. D., Racinais, S. and Sawka, M. N. (2015). Adaptation and mechanisms of human heat acclimation: Applications for competitive athletes and sports. *Scand J Med Sci Sports*. **25**(1):20-38.

Pockley, A. G. (2003). Heat shock proteins as regulators of the immune response. *Lancet*. **362**:469–76.

Reed, B. G., & Carr, B. R. (2015). The normal menstrual cycle and the control of ovulation. Reed BG, Carr BR. The Normal Menstrual Cycle and the Control of Ovulation. In: Endotext. MDText.com, Inc., South Dartmouth (MA); 2000. PMID: 25905282.

Reed, B.G. and Carr, B. R. (2015). The normal menstrual cycle and the control of ovulation. Harrison's Principles of Internal Medicine. 11th ed. New York: McGraw-Hill, 1987: 1818-1837.

Richards, J. S and Pangas, S. A. (2010). The ovary: basic biology and clinical implications . *The Journal of Clinical Investigation*. **120** (4): 963–972.

Rivera, R. M. and Hansen, P. J. (2001). Development of cultured bovine embryos after exposure to high temperatures in the physiological range. *Reproduction*. **121**:107–115.

Rockwell, L. C., Pillai, S., Olson, C. E. and Koos, R. D. (2002). Inhibition of vascular endothelial growth factor/vascular permeability factor action blocks estrogen-induced uterine edema and implantation in rodents. *Biology of Reproduction*. **67**(6):1804–1810.

Rodriguez, F., Camacho, A., Bordes, S., Gardner, B., Levin, R. and Tubbs, R. (2020). Female ejaculation: An update on anatomy, history, and controversies. *Clinical Anatomy*. **34**(1):103-107.

Roth, Z. and Hansen, P. J. (2004). Involvement of apoptosis in disruption of developmental competence of bovine oocytes by heat shock during maturation. *Biol Reprod* **71**:1898–906.

Sakatani, M., Kobayashi, S. and Takahashi, M. (2004). Effects of heat shock on in vitro development and intracellular oxidative state of bovine preimplantation embryos. *Mol Reprod Dev*. **67**:77–82.

Saleh, K. M. M. and Al-Zghoul, M. B. (2019). Effect of acute heat stress on the mRNA levels of cytokines in broiler chickens subjected to embryonic thermal manipulation. *Animals (Basel)*. **9**(8):499

Santamaria X, Mas A, Cervelló I, Taylor H, Simon C. Uterine stem cells: from basic research to advanced cell therapies. *Hum Reprod Update* 24: 673–693, 2018

Sawka, M. N., Latzka, W. A., Montain, S. J., Cadarette, B. S., Kolka, M. A., Kraning, K. K. II. and Gonzalez, R. R. (2001). Physiologic tolerance to uncompensable heat: intermittent exercise, field vs laboratory. *Med Sci Sports Exerc* **33**: 422–430.

Sawka, M. N., Leon, L. R., Montain, S. J., Sonna, L. A. (2011). Integrated physiological mechanisms of exercise performance, adaptation, and maladaptation to heat stress. *Compr Physiol* **1**:1883–1928.

Sejian, V., Maurya, V. P., and Naqvi, S. M. K. (2011). Effect of thermal stress, restricted feeding and combined stresses (thermal stress and 16 | BONI restricted feeding) on growth and plasma reproductive hormone levels of Malpura ewes under semi-arid tropical environment. Journal of Animal Physiology and Animal Nutrition, 95(2), 252–258.

Sils, I. V., Matthew, C. B. and Bastille, A. M. (2002). Estrus related differences in response to a hot environment in telemetry equipped female rats. *Journal of Thermal Biology*. 27(4): 279–284.

Sod-Moriah, U. A. (1971). Reproduction in the heat-acclimatized female rat as affected by high ambient temperature. *J. Reprod. Fertil.* **26**:209-218.

Sod-Moriah, U. A. and Pollack, E. (1970). Food and water consumption and the reproductive performance of the heat-acclimated female rat. *Int. J. Bwchim. Biomet.* **14**:293-300.

Sundtroem, E. S. (1960). Contribution to tropical biochemistry and physiology; supplementary experiments on rats adapted to graded levels of reduced cooling power. *Univ. Cali. Publ. Physiol*. **7**:103-195.

Taylor, N. A. S. (2000). Principles and practices of heat adaptation. *J Hum Environ Sys* **4**:11–22.

Taylor, N. A. S. (2014). Human heat adaptation. *Compr Physiol* **4**:325–365.

Taylor, N. A. S. and Cotter, J. D. (2006). Heat adaptation: guidelines for the optimization of human performance. *Int Sport Med J* **7**:1–37.

Thurmond AS, Machan LS, Maubon AJ, Rouanet JP, Hovsepian DM, Moore A, Zagoria RJ, Dickey KW, Bass JC. A review of selective salpingography and fallopian tube catheterization. Radiographics. 2000 Nov-Dec;20(6):1759-68.

Titus, S; Li, F; Stobezki, R; Akula, K; Unsal, E; Jeong, K; Dickler, M; Robson, M; Moy, F; Goswami, S; Oktay, K (2013). "Impairment of BRCA1-related DNA double-strand break repair leads to ovarian aging in mice and humans". *Sci Transl Med*. **5** (172).

Wang, J., Wang, S. and Zhang, W. (2016). Proteomic profiling of heat acclimation in cerebrospinal fluid of rabbit. *Journal of Proteomics* **144**:113–122.

Wegele H, Muller L and Buchner J. (2004). Hsp70 and Hsp90--a relay team for protein folding. *Rev Physiol Biochem Pharmacol*. **151**:1–44

Westwood, F. R. (2008). The female rat reproductive cycle: a practical histological guide to staging. *Toxicol Pathol*. **36**:375 –84.

Wray S, Kupittayanant S, Shmygol A, Smith R and Burdyga T: The physiological basis of uterine contractility: A short review. Experimental Physiology 86(2): 239-246, 2017.

Xie J, Tang L, Lu L, Zhang L, Xi L, Liu HC. (2014). Differential expression of heat shock transcription factors and heat shock proteins after acute and chronic heat stress in laying chickens (Gallus gallus). *PLoS One*. **9**: e102204

Yamauchi, C, S. Fujita, T. Obara, and T. Ueda (1981). Effects of room temperature on reproduction, body and organ weights, food and water intake, and hematology in rats. *Lab. Anim. Sci.* **31**:251-258.

Zheng, M. Nagaoka, K. and Watanabe, G. (2019). "Pre-pubertal exposure to high temperature impairs ovarian and adrenal gland function in female rats. *The Journal of Veterinary Medical Science*, **81**(2):279–286.