THE EFFECTS OF MELATONIN ON THE TESTIS, EPIDIDYMIS AND SPERM PHYSIOLOGY OF THE WISTAR RAT

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DECLARATION OF THESIS STATUS

This thesis is my own independent research work and is being submitted in fulfilment of the requirements for the degree of Master of Science in Zoology in the Department of Zoology and Entomology, Rhodes University, Grahamstown, South Africa. It has not been previously submitted in whole or in part for any degree or examination at any other university.

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ABSTRACT

Melatonin is a product of the pineal gland and is postulated to play an antigonadotropic role in the reproductive system of mammals. The reproductive system of non-seasonally breeding mammals is believed to be not as responsive to melatonin treatment as that of seasonally breeding mammals. Recently, there has been increasing support from *in vivo* and *in vitro* studies, for the hypothesis that melatonin has negative effects on sperm physiology, especially on sperm motility. High and/or low seminal concentrations of melatonin have been associated with abnormalities in human sperm motility and concentration.

In this study, I examined the effects of melatonin on the testis, epididymis and sperm physiology, using *in vivo* and *in vitro* experiments, in a non-seasonally breeding mammal.

Treatment, *in vivo*, with exogenous melatonin for six weeks did not inhibit testosterone production or spermatogenesis, nor did it affect the mass of the testes and epididymides at dissection, the concentration the morphology of spermatozoa. However, melatonin *in vivo* had a small, but significant negative effect on sperm motility and sperm motility index. *In vitro* incubation of spermatozoa with melatonin reduced the percentage (%) of forward progressive movement (fpm), increased the % reduction in fpm, reduced the vigor or quality of sperm motility, reduced the sperm motility index, and delayed and/or prolonged the transition of one pattern of sperm motility to the subsequent patterns. Melatonin increased the pH of the culture medium, and the increased pH, and the ethanol utilized as a solvent for melatonin, both negatively affected all the sperm motility parameters that were assessed in my study. The effects of ethanol

increased with time, and the effects of pH increased with both time and increasing pH. Melatonin *in vitro* did not inhibit capacitation and the acrosome reaction, but it delayed the onset and the progression of capacitation and the acrosome reaction.

These results suggest that while melatonin did not inhibit spermatogenesis in the Wistar rat, it may influence sperm motility. Therefore, the presence of high concentrations of melatonin in the reproductive fluids may inhibit sperm motility. With further detailed research, melatonin may have a potential use as a contraceptive drug.

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CHAPTER ONE
INTRODUCTION

Melatonin (N-Acetyl-5-methoxytryptamine) was first extracted and isolated from a mammalian pineal gland and chemically identified by Lerner, Case and Takashe in 1959 (in Scharrer and Scharrer, 1963; Meyer and Theron, 1988). Melatonin is produced in the pineal gland, which is located between the cerebral hemispheres, deep in the centre of the brain of most mammals (Reiter and Robinson, 1995). Melatonin is secreted particularly at night as the true hormone of the pineal gland (Stetson and Tay, 1983; Meyer and Theron, 1988; Reiter and Robinson, 1995).

Melatonin is synthesized from the amino acid tryptophan, serotonin being an intermediate step in its biosynthesis (Pevet, 1981; Meyer and Theron, 1988; Dryer, 1999). Its production in the pineal gland of all mammalian species, including humans, is controlled by the photoperiodic environment (Reiter, 1980; Reiter, 1986; Reiter and Robinson, 1995). The content of melatonin in the peripheral blood exhibits a daily fluctuation related to the rhythm of light and darkness, it being higher at night than during the day, with melatonin peaks between 02:00h and 03:00h. Melatonin is released in pulses, the duration and amplitude of which are positively correlated with the occurrence of darkness, thus the rhythm of day and night is represented by the melatonin pulses (Reiter, 1980; Reiter, 1986; Reiter and Robinson, 1995).

Specific mechanisms regulating the release of melatonin from the pineal gland remain unknown (Reiter, 1991), although several mechanisms have been suggested. As melatonin synthesis increases, and the levels of pineal melatonin rise, melatonin is not stored, because of its small size and its lipophilic and hydrophilic properties (Reiter, 1991; Cagnacci, 1996), but released from the

pinealocytes into the circulatory system via passive diffusion down a concentration gradient (Rojansky *et al.*, 1992). Once in the circulatory system, melatonin can be taken up by peripheral nerves, the target organ, the brain, or concentrated in the pituitary gland (Wuurtman *et al.*, 1963), thus its effect on the reproductive organs could result from an action at any of these sites.

In male Wistar rats, approximately one third of the melatonin that is normally released by the pineal or extrapineal sources during night-time is removed from circulation during night-time. Removal is not by hepatic metabolism but by another, yet unknown additional mechanism (Huether *et al.*, 1998). Melatonin has no known toxicity, is readily absorbed by the cells when administered via any route, traverses morphophysiological barriers (Reiter, 1991; 1997) by simple diffusion across the plasma membrane, and its rate of secretion is determined by its rate of synthesis (Nelson, 1999).

Since the production of melatonin has a diurnal cycle, melatonin can influence physiological processes such as body temperature and reproduction, and adjust them to diurnal or seasonal changes in ambient light levels (Reiter, 1980; Meyer and Theron, 1988). It has also been suggested that the melatonin cycle underlies the ability of animals to adjust to different daily rhythms and their ability to perceive and respond appropriately to changes in photoperiod that occur during the course of the year (Reiter, 1980; Meyer and Theron, 1988; Bornman *et al.*, 1992).

Mammalian reproduction is shaped by interactions between a range of environmental, physiological and social factors (Neal, 1986; Bronson, 1989), and fertility is dependent on a complex set of events, involving both male and female components. An important environmental factor that affects reproduction is the day length, and thus melatonin may play a fundamental role in controlling reproduction. The effects of exogenous melatonin seem to differ between

seasonally and non-seasonally breeding mammals. It is well documented that melatonin is as effective as short photoperiod in inhibiting spermatogenesis in seasonally breeding mammals with a pronounced annual reproductive cycle (Vaughan *et al.*, 1988) such as the sheep and the hamster (Stetson and Tay, 1983; Amador *et al.*, 1986; Meyer and Theron, 1988). However, in non-seasonally breeding mammals such as the laboratory rat, exogenous melatonin has either no significant antigonadal effect (Vaughan *et al.*, 1988; Lewinski *et al.*, 1993), or elicits a weak but statistically significant antigonadal response (Lang *et al.*, 1983). It is suggested that in non-seasonally breeding mammals, administering melatonin at the right time of the day (i.e. in late afternoon) would result in effective treatment (Meyer and Theron, 1988).

Several methods have been utilized to administer melatonin to rodents. These methods include subcutaneous constant-release implants, daily injections, daily infusions, and a combination of any of the three methods. Variations in response to treatment with melatonin have been attributed to numerous exogenous and endogenous factors. These include factors such as the method of administering, melatonin, the time of the day at which the dose was administered, the length of melatonin treatment, the photoperiod under which melatonin was administered, the age, sex, and species of animals, and individual variation within a species (Wuurtman *et al.*, 1963; Reiter, 1978; Chen *et al.*, 1980; Chen and Reiter, 1980; Lang *et al.*, 1983; Anderson *et al.*, 1988; Edmonds and Stetson, 1994; Niklowitz *et al.*, 1996).

Despite widely published information on the effects of melatonin on mammalian reproduction, knowledge of the mechanism(s) and specific site(s) of action of melatonin remain speculative (Knotts *et al.*, 1988). Like other hormones, melatonin is thought to exert its actions through binding to membrane receptor proteins in target organs (Cohen *et al.*, 1978). The presence of specific receptors indicates a target for melatonin action, but the lack of receptors does not imply

a lack of effect of melatonin because melatonin traverses morphophysiological barriers (Reiter 1991; 1997) by simple diffusion across the plasma membrane. Melatonin may also have intracellular actions, which are both receptor independent and receptor dependent (Bettahi *et al.*, 1998).

Melatonin receptors or putative receptors and sites of action are extremely widespread, exist in both neural and extraneural tissues, and vary widely in distribution among species (Reiter, 1991). Thus melatonin may act at a variety of peripheral and/or central sites. These receptors and sites of action have been identified and characterized in ovarian tissue of hamster, rat and human, rat and hamster uterus, hamster testes (Cohen *et al.*, 1978); mouse brain (Glass and Lynch, 1981); rat corpus epididymis (Li *et al.*, 1998); rat brain and pituitary (Williams *et al.*, 1995), rat corpus and cauda epididymides (Yu *et al.* 1994), and in human spermatozoa (Pitout *et al.*, 1991; van Vuuren *et al.*, 1992).

The hypothalamo-pituitary- gonadal axis (Cohen *et al.*, 1978; Li *et al.*, 1998) seems to be the physiological site for the action of pineal melatonin. In the brain, the sites for the antigonadal action of melatonin are located in the rostral brainstem, particularly in the supra- and retrochiasmatic areas (Glass and Lynch, 1981). Melatonin may alter the steroid negative feedback mechanisms in the hypothalamus and the pituitary gland, and this may be one of the mechanisms by which melatonin affects reproduction (Amador *et al.*, 1986; Nelson, 1999). Melatonin may intervene in the control of the secretion of gonadotropins, possibly by acting on specific receptors localized in the median eminence and in the midbrain (Fraschini *et al.*, 1968a, b; Reiter, 1991, 1993), and thereby could regulate the functional status of the gonads and control the seasonal reproductive capability of an animal (Reiter, 1993). In addition, the reported inhibitory effect of melatonin on steroidogenesis in gonadal tissue in vitro (Tamura *et al.*, 1998; Persengiev and

Kehajova, 1991), and the ability of gonads to effectively take up circulating melatonin (Rojansky *et al.*, 1992), supports the hypothesis that a direct regulatory action of melatonin on the gonads appears to be in existence.

Whether the physiology of the pineal gland is regulated by feed-back mechanisms via either the anterior pituitary gland (Fraschini *et al.*, 1968a, b; Cohen *et al.*, 1978; Glass and Lynch, 1981; Amador *et al.*, 1986; Li *et al.*, 1998; Reiter, 1991, 1993; Nelson, 1999), or the primary or secondary sex organs alone or in combination (Tamura *et al.*, 1988; Vaughan *et al.*, 1986; Persengiev and Kejahova, 1991), is not yet clear (Knotts *et al.*, 1988).

Many clinical problems including complex infertility treatments and contraception hinge on a full understanding of the cellular and the molecular events determining the fertility of an animal. Spermatogenesis and sperm physiology provide prognostic information for the reproductive potential and fertility of an animal.

There have been reports that melatonin negatively affects the physiology of human spermatozoa, with high semen melatonin levels associated with abnormal sperm forward motility rates (Oosthuizen *et al.*, 1986; van Vuuren *et al.*, 1988; Yie *et al.*, 1991; Irez *et al.*, 1992), or complete abolishment of movement (Oosthuizen *et al.*, 1986), and low semen melatonin levels associated with a higher incidence of abnormal sperm progression (van Vuuren *et al.*, 1988). In addition, high melatonin levels have been associated with mild oligospermy (lower sperm concentration) and azoospermy (no spermatozoa in seminal fluid), in patients (Yie *et al.*, 1991).

However, there are conflicting views about whether melatonin affects sperm motility or not, and contradictory results have been produced from the same laboratory. Oosthuizen *et al.* (1986) supports the hypothesis that melatonin negatively affects sperm motility, while Bornman *et al.*

(1989; 1992) oppose the hypothesis.

A variety of factors are involved in the initiation, maintenance and regulation of sperm motility (Gibbons, 1983; Cunningham, 1983; Eddy, 1988; Millete, 1999), but a unifying hypothesis on how all these factors interact to accomplish this, is lacking. Since melatonin receptors have been identified in the epididymis (Li *et al.*, 1998; Yu *et al.* 1994), and on spermatozoa (Pitout *et al.*, 1991; van Vuuren *et al.*, 1992), a role for melatonin in controlling sperm motility as spermatozoa transit through the epididymis may be possible. The reported abnormalities in sperm motility at both high (Oosthuizen *et al.*, 1986; van Vuuren *et al.*, 1988; Yie *et al.*, 1991; Irez *et al.*, 1992) and low (van Vuuren *et al.*, 1988) concentrations of seminal melatonin, may be an indication that a certain critical concentration of melatonin is required for normal sperm physiology.

Sperm motility, capacitation and the acrosome reaction are consecutive steps depicting normal sperm physiology (Ohl and Menge, 1996). Performing specific tests for the assessment of sperm physiology is essential for the prognosis of the fertility of an animal, and these specific tests form important parameters in assessing sperm function (Oosthuizen *et al.*, 1986; Acosta *et al.*, 1990; Keel and Webster, 1990; Ohl and Menge, 1996). Such specific tests include semen analysis, including sperm concentration and morphology; detailed sperm motility assessment; motility longevity tests; hypo-osmotic swelling test; mucus penetration assays; tests of capacitation: hyperactivation, acrosome reaction, and acrosin assays; antisperm antibody assays; sperm penetration assays; hemizona binding assays; artificial insemination (AI), and *in vitro* fertilization (IVF). There is no single assay available to assess the *in vivo* or *in vitro* fertilizing potential of spermatozoa as there are numerous steps, which together represent normal sperm functioning (Ohl and Menge, 1996).

Capacitation characteristics that are mostly easily quantified, for example, the acrosome reaction, hyperactivated motility and fertilizing ability, all present the terminal events or stages of capacitation and thus make it difficult to assess and provide little or no indication of transition to the capacitated state (Fraser and Herod, 1990). The changes that occur during capacitation, prior to the acrosome reaction, are much more difficult to assess (Perez *et al.*, 1996). The chlortetracycline (CTC) fluorescence assay has proved useful for assessing and monitoring sperm capacitation and the acrosome reaction in spermatozoa from different species such as mouse (Saling and Storey, 1979; Lee and Storey, 1986; Fraser and Herod, 1990; Kholkute *et al.*, 1995), ram (Perez *et al.*, 1996), and human (Mortimer, 1994). In the mouse, 3 patterns of CTC fluorescence, (F, B, and AR) have been observed and correlated with the progression of capacitation and the acrosome reaction (Fraser and Herod, 1990). Spermatozoa exhibiting hyperactivated type of motility which resembles a figure-8 pattern have also been considered to be capacitated (Fraser, 1977).

A complete understanding of the effects of melatonin on spermatogenesis and sperm physiology of a non-seasonally-breeding mammal, can allow researchers to make predictions about its effects on spermatogenesis and sperm physiology of similar mammals, and to utilise such knowledge to better manipulate reproduction, whether for the conservation of wild species or for commercial purposes, or to control reproduction or alleviate the trauma of individuals who are subfertile.

The primary aim of my study was to investigate the effects of melatonin on the testis, epididymis and sperm physiology using a non-seasonally breeding mammal as a model, and to clarify the conflicting views about whether melatonin affects sperm physiology or not. The laboratory rat was selected as an ideal research animal as it starts breeding at approximately 35 days of age, breeds readily throughout the year in the laboratory (Asdell, 1946), and is easily available in South

Africa.

CHAPTER TWO

THE EFFECTS OF MELATONIN ON SPERMATOGENESIS AND SPERM MOTILITY IN WISTAR RATS

The aim of these experiments was to investigate and assess the effects of melatonin *in vivo* on spermatogenesis, the position of the testes, body mass, plasma testosterone concentration, sperm concentration, the presence of spermatozoa in the epididymal fluid and sperm motility of a non-seasonal breeding rodent.

2.1 MATERIALS AND METHODS:

2.1.1 Animal husbandry and acclimation:

The Rhodes University Animal Ethical Standards Committee approved the housing and the handling of the Wistar rats, and all the experiments that were undertaken in this study.

Adult male laboratory rats (255-367g) of the Wistar strain were utilized for the experiments and were supplied by the South African Vaccine Producers, Johannesburg. Animals were kept in a controlled environment (CE) room under long photoperiod conditions (14L:10D-lights on 06:00, lights off 20:00), at approximately 25^0C.

The animals were maintained in groups of five males per clear plastic cage (38.5 x 38.5 x 22.5cm) with wire mesh bases and covers. The male rats were not housed in the same room as female rats. The animals were provided with commercial nutri-chunks dog food (Phoenix Roller Mills, Grahamstown) and water *ad libitum (ad lib.)*. The cages were cleaned twice a week (every

Monday and Friday) prior to feeding the animals.

The animals were marked individually across their tails by drawing line(s) with a water-resistant black marker pen. For example, an animal allocated as animal number 2 in a particular cage had two lines on its tail. The animals were marked to allow identification and differentiation of the animals during handling, injection, weighing and at sacrifice.

The animals were acclimatized for a period of two weeks and were weighed at the end of the acclimation period before the start of the experiment. The position of the testes was determined by palpation each time the animals were weighed and before they were sacrificed.

2.1.2 Experimental protocol:

2.1.2.1 Trial experiment:

A trial experiment extending over a period of four weeks and utilizing 15 animals divided randomly into three groups of five each was undertaken. The three animal groups were allocated as 1: control, 2: saline, and 3: melatonin group. Animals in group 1 did not receive any treatment, group 2 received a daily 0.1millilitre (ml) saline injection per animal, and, group 3 received a daily injection of 25μg melatonin per 0.1ml saline per animal. The injections were administered between 16:00h and 18:00h. Vaughan et al. (1988), has used a similar dose in male rats.

2.1.2.2 Experiment:

Two experimental runs, each lasting six weeks were undertaken. The two experiments were independent of each other with regards to the time of the year at which they were run and the animals utilized for each experiment. For each experiment, 30 animals were utilized and allocated into groups as in the trial experiment but each group was composed of ten animals. The animals

received the same treatment as in the trial experiment, but the daily dose of melatonin and the duration of treatment were increased to 50μg melatonin per 0.1ml saline per animal and six weeks, respectively.

The animals were weighed once every week (every Monday) throughout the experiment and weight gain or loss was monitored. For the first experiment vernier calipers were used to determine the testis size as measured by the lengths and widths of the testes within the scrotal sac. For each testis, the length was multiplied by the width and the mean of the two values was then taken as the actual measure of testis size for each animal. Testes were not measured in the second experiment as the results from the first experiment revealed that the external measures of testis size did not accurately reflect the size of the testes as determined at autopsy.

2.1.3 Preparation and administering of injections:

The glassware used in the experiment was sterilized with dry heat for one hour at 150°C. Sterile saline served as a control solution and as a diluent for making up the melatonin injections. Melatonin injections were prepared as a stock solution every two weeks, by dissolving 3.5mg melatonin powder (Sigma Chemical Co., St. Louis, MO) in absolute ethanol, and making up with saline to yield a solution for injection containing 50μg of melatonin per 0.1ml saline, and of an ethanol to saline ratio of 1:9v/v. Melatonin may be prepared as stock solutions, stored in sterile conditions at 4^0C until use, and is stable in aqueous solution (Cavallo and Hassan, 1995).

1ml Syringes (Terumo Corporation, Tokyo, Japan) were filled up to 0.1ml with saline and melatonin solutions and stored at 4^0C. 26G Injection needles (Terumo Corporation, Tokyo, Japan) were attached to the syringes and used to inject the animals. The needles were used once for each animal and the injections were administered subcutaneously at the nape of the neck.

2.1.4 Laboratory conditions and sterile techniques:

The laboratory used for dissection and for the other experimental procedures was 3.3 x 2.3m. The air ventilators were sealed to allow minimal flow-through of air into the laboratory. Sterile methods were used throughout the experiments. Every two weeks, the laboratory was cleaned and sterilized by first dusting the walls, bench tops and floor which were then washed with water. The bench tops, floor and all the technical equipment were sterilized with 70% alcohol.

A water-jacketed incubator (Forma Scientific, Ohio) connected to a medical CO_2 cylinder (Messer Fedgas, (Pty) Ltd , Port Elizabeth) was utilized for constant ideal conditions for the equilibration of medium and paraffin oil, and for sperm incubation. The incubator was set at 36^0C, 95% air, 5% CO_2. A Bacharach Fyrite gas analyser (Pittsburg, USA) was utilized to determine and monitor the CO_2 content in the incubator. Two pyrex bowls filled with distilled water were placed at the bottom of the incubator to maintain a high level of humidity inside the incubator.

The night prior to any experimental procedure, the laboratory and equipment to be used were sterilized. The dissecting equipment was dipped in 70% alcohol and then flamed. The microscopes and other equipment, bench tops and the floor were sterilized by wiping with a paper towel soaked with 70% alcohol.

A slide warmer was used to warm the dissecting equipment, heamocytometer, slides, autopipette tips, medium and sperm suspensions whilst they were temporarily out of the incubator. An oil heater was switched on overnight to maintain the laboratory air temperature at approximately 36^0C. This was done to minimize the differences in air temperature between the laboratory and the incubator. The microscope light was switched on for approximately 30 minutes to warm the microscope stage prior to the use of a microscope during an experimental procedure.

2.1.5 Medium

The culture medium used for all the experiments was Dulbeco's Eagle medium 12-7077 (DMEM; Biowittaker, Maryland). Immediately prior to the addition of spermatozoa to DMEM, DMEM was supplemented (hereafter referred to as SDMEM) with 3mg/ml of fatty acid-free bovine serum albumin (BSA; Boehringer Mannheim, (Pty) Ltd, Germany), 100 IU/ml penicillin (Novopen, Novo Nordisk, Johannesburg), (Bavister and Andrews, 1988; Stewart-Savage, 1993; Perez *et al.*, 1996), and pipetted (1ml/well) into four-microwell plates (Nunclon, AEC-Amersham, Denmark).

A BSA concentration of 3mg/ml was utilized for supplementing the culture medium as concentrations of BSA as high as 9mg/ml and greater have been shown to decrease the fertile life of spermatozoa (Stewart-Savage, 1993). The addition of BSA and penicillin to the sperm culture medium confers specific advantages. Penicillin and BSA protect spermatozoa and stimulate, improve and maintain sperm motility (Bavister; 1974; Harrison *et al.*, 1982; Bavister and Andrews, 1988; Boatman and Robbins, 1991).

Paraffin oil was used to overlay sperm suspensions. Prior to an experimental procedure, paraffin oil and DMEM were equilibrated in the incubator for approximately 12 hours and 60 minutes, respectively. An overlay of equilibrated paraffin oil as part of the sperm culture system confers specific advantages (Dale and Edler, 1997):

1. the oil acts as a physical barrier, separating droplets of medium from an atmosphere of airborne particles or pathogens;
2. oil prevents evaporation and delays gas diffusion, thereby keeping pH, temperature, and osmolality of the medium stable during sperm handling or manipulations, protecting the spermatozoa from significant fluctuations in their micro-environment. The physical properties of oil result in very slow diffusion of gas;

3. oil prevents evaporation: humidified and pre-equilibrated oil allows the use of non-humidified incubators, which are easier to clean and maintain.

2.1.6 Sacrifice

At the end of the experimental period, the animals were sacrificed by administering an intra-peritoneal (i.p.) injection of Euthanase (1ml/kg) per animal, (Premier Pharmaceuticals Company, South Africa). The animals were sacrificed in the morning between 8:00h and 10:00h over a period of ten days. One animal was sacrificed from each experimental group per day, dissected rapidly and blood and spermatozoa immediately collected and processed.

2.1.7 Blood collection and processing:

The sternum region was sterilized by wiping with a paper towel soaked with 70% alcohol. It was then cut open so that the heart was exposed. Blood was immediately collected from the heart using a 22G needle connected to a 5ml syringe (Terumo Corporation, Tokyo, Japan) and transferred into eppendorf tubes. The needle and syringe were used once for each animal. The blood was centrifuged (10 minutes at 1600***g***), the plasma aspirated into other eppendorf tubes and then frozen at -20^0C until assayed.

2.1.8 Collection, processing and assessment of epididymal spermatozoa:

2.1.8.1 Collection and processing:

The lower abdomen and testis region was sterilized by wiping with a paper towel soaked with 70% alcohol. A small incision exposing the testes and epididymides was made using a pair of dissecting scissors. The testes and epididymides were excised, dissected free of fat, weighed and their mass recorded.

The cauda epididymides were excised from the testes-epididymides complex and put into SDMEM (0.5ml) in a four-microwell plate lid on a slide warmer where they were cleaned of any blood or hair. The cauda epididymides were punctured and gently pressed using fine forceps to extrude spermatozoa into SDMEM (1ml) in one of the micro-wells. Spermatozoa from the cauda epididymis are mature and exhibit greater vigor of motility than spermatozoa from the caput epididymis, with the nature of sperm motility changing from being sluggish and circular to rapid forward progressive movement (Harper, 1982; Gibbons, 1983; Mohri *et al.*, 1983; Eddy, 1988; Yanagimachi, 1988; Biegler, 1994; Cooper, 1999). The sperm suspension was mixed using an autopipette fitted with a pre-warmed autopipette tip and assessed for the presence of spermatozoa, sperm concentration and motility.

2.1.8.2 Assessment of spermatozoa:

The white cell chambers of the superior improved bright-lined Neubauer haemocytometer counting chamber (VWR Scientific, Piscataway, New Jersey) was utilized for the assessment. Accurate assessment of sperm concentration can be made using a haemocytometer to count a sample of immobilized spermatozoa. However, a more practical method which allows simultaneous judgement of normal, motile and immotile concentrations, as well as the type of sperm motility exhibited (Dale and Edler, 1997), was more appropriate for this study and is described below. Assessment was done in quadruplicate for each animal and the mean was then taken as the actual value for a particular time, for each sperm suspension.

2.1.8.2.1 Spermatozoa and sperm concentration:

A mixed sperm suspension (105µl) was pipetted under the cover slip of the warmed haemocytometer, which was then examined using a transmitted light compound microscope (Nikon, Japan), under 400x magnification. An intact motile spermatozoon without any

observable structural defects under the light microscope, was considered to be normal. The number of spermatozoa in 10 squares was counted, expressed as concentration by converting data to a number of spermatozoa per ml of solution (sperm/ml) value and results recorded:

Sperm concentration in millions/ml = the mean number of spermatozoa in 10 squares x 250000

2.1.8.2.2 Motility:

Three parameters of sperm motility [the percentage (%) of forward progressively motile spermatozoa, the vigor or quality, and the pattern of motility shown] were assessed at intervals of one hour over a period of six consecutive hours.

The % of forward progressively motile spermatozoa was expressed as:

$$\% \text{ Motility} = \frac{\text{Number of motile spermatozoa in 10 squares}}{\text{Total number of spermatozoa in 10 squares}} \times 100$$

The vigor or quality of sperm motility was expressed on a standard 6-unit sperm motility scale: (Bavister, 1974; Bavister and Adrews, 1988)

0 = no movement

1.0 = twitching, no forward progressive movement (fpm)

2.0 = slow fpm

3.0 = good, purposeful fpm

4.0 = rapid fpm without hyperactivation

5.0 = hyperactivation

Since the vigor or quality of sperm motility is regarded as being more significant than the % of fpm for capacitation studies (Bavister, 1974), a sperm motility index (SMI) was calculated by

combining two parameters of sperm motility that were assessed :

SMI = % of fpm x (vigor or quality category)^2 (Bavister, 1974)

Changes in the pattern of sperm motility (the third sperm parameter) were generally from, A: unclear movement of spermatozoa without any specific but generally a forward direction accompanied with a good beating of the tail (relatively inflexible middlepiece) and the frequent rotation of the head from side to side ("uncapacitated"), B: vigorous beating of the tail of low amplitude (less flexible principal piece) with a linear direction of movement without hyperactivation of spermatozoa ("half-capacitated") to, C: very vigorous beating of the tail of high amplitude (entire tail length flexible) with hyperactivation of spermatozoa and the direction of motility forming or resulting in a figure-8 pattern ("capacitated") to, D: good beating of the tail of low amplitude with a linear direction of movement without hyperactivation of spermatozoa ("undergone capacitation") (Yanagimachi, 1970; Fraser, 1977). The sperm motility patterns were correlated with time and with the Bavister (1974), and Bavister and Andrews's (1988) standard 6-unit sperm motility scoring scale (Bavister scale).

The initial assessment of all the sperm motility parameters was done simultaneously with the assessment of sperm concentration utilizing the haemocytometer. (Sub-section 2.1.8.2.1.), whereas pre-warmed microscope slides were utilized for the consecutive assessment of the sperm motility parameters.

Immediately after the initial assessment, spermatozoa (approximately 50 000 per well) were introduced into the SDMEM (1ml/well) in each well of a four-microwell plate, overlaid with paraffin oil and immediately put into the incubator. Hourly, over a period of six consecutive hours, a four-microwell plate was temporarily withdrawn from the incubator onto a pre-warmed

slide warmer, and a microdroplet (1μl) from each well pipetted off the top layer onto a pre-warmed slide for the assessment of all sperm motility parameters. The top layer of a sperm suspension contains the most motile spermatozoa as the spermatozoa swim up the medium as the experiment progresses.

The % of fpm for each droplet was determined on a randomly selected region using a transmitted light microscope under medium magnification (1000x). The data for the four micro-wells of each plate were averaged and the mean % of fpm was regarded as the actual % of fpm for a particular hour. The % reduction in fpm was calculated as:

% Reduction in sperm fpm = 100 – (actual % fpm / initial % fpm) x 100

2.1.9 Hormone analysis:

Plasma testosterone from five animals per experimental group was assayed in duplicate utilizing a commercial kit (Coat-A-Count Total Testosterone; Diagnostic Products Corp., Los Angeles, California) that has a broad range of sensitivity of 4 to 16ng/ml and 30-40% maximum binding. Inter- and intra-assay coefficients of variation were less than <12%. Assayed recovery of added testosterone was 98% and serial dilutions of two serum samples produced displacement curves parallel to the standard.

2.1.10 Statistical analysis:

Data are presented as means and ± 1 SD. Statistical analyses were conducted using SigmaStat (1994). Changes in body mass over time, between experimental groups were compared using a repeated measures two way analysis of variance (two way RM ANOVA). Measurements made at dissection were compared with a one way analysis of variance (one way ANOVA), or a Kruskal-Wallis one way analysis of variance (Kruskal-Wallis one way ANOVA). Differences within a

group were compared using a repeated measures one way analysis of variance (one way RM ANOVA), and the relationship between the testis size (measured within the scrotal sac) and the testis mass (at autopsy) was tested using a linear regression. Data in percentages were arcsine transformed for statistical analyses. Differences were considered significant at $P<0.05$.

2.2 RESULTS:

The results from the first and the second experiment were similar and data are presented for the second experiment only, because the testosterone assay was done on blood from these animals. All the males (n=30) had scrotal testes before the start of the experiment.

2.2.1 Changes over the experimental period in the position of the testes and the body mass of experimental animals:

Daily injections of 50μg melatonin per 0.1ml saline and 0.1ml saline did not affect the position of the testes throughout the experiment, and at the end of the experimental period, all the animals in all the experimental groups were scrotal.

Within each experimental group, there was a gradual, statistically significant increase in mass from the start to the end of the experimental period [Figure (Fig.). 2.1; one way RM ANOVA, $P<0.05$ for all experimental groups, Table 2.1]. Statistical analyses of data for the changes in body mass between the experimental groups over the experimental period revealed that while time had a significant effect, treatment did not (two way RM ANOVA, $P<0.05$; $P>0.05$, Table 2.1), and the daily 50μg melatonin per 0.1ml saline and 0.1ml saline injections for six weeks did not affect the body mass.

2.2.2 Dissection data:

At dissection, the differences in mean body mass between all experimental groups were not statistically significant (one way ANOVA, $P>0.05$, Table 2.1). The testis size as measured by the length and width of the testis within the scrotal sac did not accurately reflect the size of the testis as determined at autopsy, and statistical analyses of these data revealed that the relationship between the testis size and the testis weight was not significant (linear regression, $P>0.05$, Fig. 2.2).

Unexpectedly, at dissection the mean mass of the testes and epididymides of the animals from the melatonin group was slightly heavier compared to those of the animals from the saline and the control groups (Table 2.2), however these differences were not statistically significant (Kruskal-Wallis one way ANOVA, $P>0.05$, Table 2.1). The daily 50μg melatonin per 0.1ml saline and 0.1ml saline injections for six weeks did not affect the body mass, or the mass of the epididymides and testes at dissection.

Table 2.1: Results of the statistical analyses of the effects of melatonin, and saline on body mass, mass of the testes and epididymides, and sperm concentration of Wistar rats. Sperm concentration refers to the number of spermatozoa expressed in millions/ml.

Dependent variable	**Between all groups**		**Within groups (time effect)**		
			Control	**Saline**	**Melatonin**
	P value				
Body mass over experimental period	**Treatment effect**	**Time effect**	$1.4x10^{-7*}$ (one way RM ANOVA)	$1.9x10^{-7*}$ (one way RM ANOVA)	$9.6x10^{-8*}$ (one way RM ANOVA)
	0.9 (two way RM ANOVA)	$6.2x10^{-23*}$ (two way RM ANOVA)			
Body mass at dissection	**Treatment effect**		/	/	/
	0.6 (One way ANOVA)				
Mass of the testes and epididymides	0.7 (Kruskal-Wallis one way ANOVA)		/	/	/
Sperm concentration	0.9 (Kruskal- Wallis one way ANOVA)		/	/	/

* significant difference at $P< 0.05$ level.

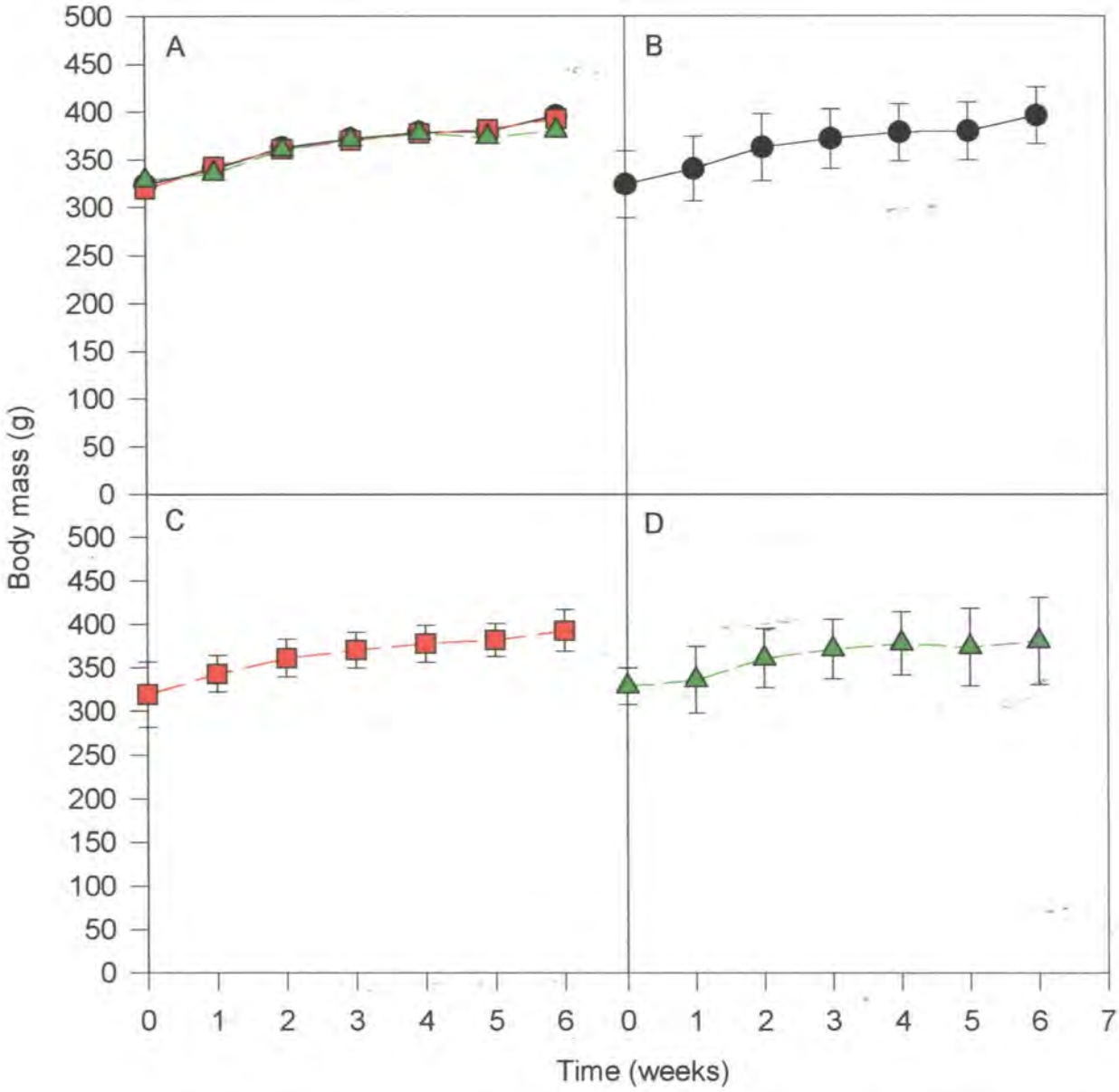

Fig.2.1: The effects of melatonin on body mass from the start to the end of the experiment. In 2.1.A. data are presented as means for all three experimental groups. In B, C and D, data are separated for clarity, and the mean ± 1 SD is shown.

Control (C), saline (S), melatonin (M).

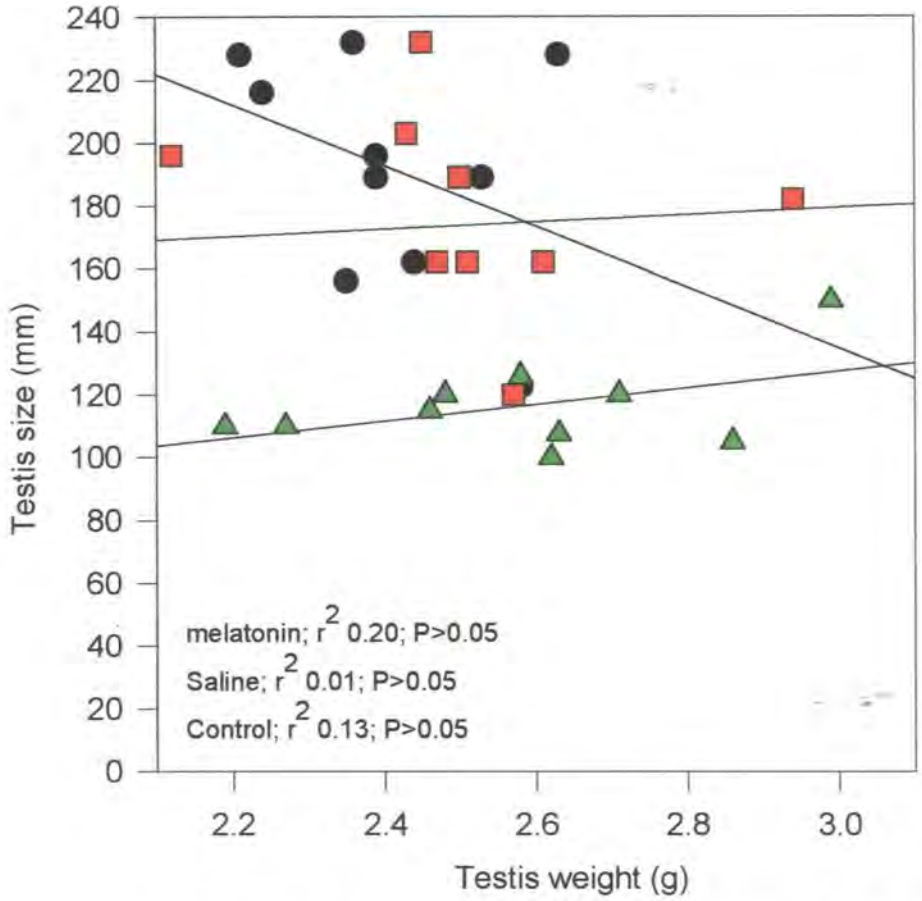

Fig.2.2: A correlation of testes size and testes weight at autopsy of Wistar rats treated with melatonin or saline.

● Control (C), ■ saline (S), ▲ melatonin (M).

2.2.3 Assessment of spermatozoa:

2.2.3.1 Sperm concentration:

At time zero (t_0), all the spermatozoa were intact, motile and without any observable structural defects and were regarded as normal. The sperm concentration of the experimental animals ranged from 5.4 to 5.7 x 10^6sp/ml (Table 2.2), and the differences in sperm concentration between the experimental groups were not statistically significant (Kruskal-Wallis one way ANOVA, P>0.05, Table 2.1).

Table 2.2: The effects of melatonin and saline on body mass, mass of the testes and epididymides, and sperm concentration at dissection of the Wistar rats in different experimental groups. Data are presented as means and ± 1 SD.

Experimental group	Body mass (g)	Mass of the testes and epididymides (g)	Sperm concentration (sperm/ml)
Control	395.9±29.6	2.37±0.1	$5.4x10^{6}$±0.5
Saline	392.59±23.9	2.3±0.2	$5.5x10^{6}$±1.4
Melatonin	380.4±50.4	2.47±0.3	$5.8x10^{-6}$±1.4

2.2.3.2 Motility:

Three parameters of sperm motility: the percent of forward progressive movement (% of fpm), the vigor or quality, and the pattern of motility shown were assessed. At t_0, there was no obvious difference observed in the sperm suspensions of the different experimental groups in the three parameters of the sperm motility that were assessed. In all the sperm suspensions of the different experimental groups, the initial % of fpm (at t_0) ranged between 98.7 to 100, and the initial vigor or quality of sperm motility was 3 on the Bavister scale. Sperm head to head agglutination in the first hour of incubation was generally observed in all the sperm suspensions of the different experimental groups, but disappeared at t_2 when single freely swimming spermatozoa were most common.

In all sperm suspensions from three experimental groups, there was a gradual decline in % fpm throughout the experiment (Table 2.3). Statistical analyses of these data revealed that while time had a significant effect, treatment did not (one way RM ANOVA; two way RM ANOVA, $P<0.05$; $P>0.05$ respectively, Table 2.4).

The % reduction in fpm mirrors the % of fpm, and for all sperm suspensions from three

experimental groups, the % reduction in fpm increased gradually throughout the experiment (Fig. 2.3). Statistical analyses of these data revealed that while time had a significant effect, treatment did not (one way RM ANOVA; two way RM ANOVA, $P<0.05$; $P>0.05$ respectively, Table 2.4). Daily afternoon injections with 50µg melatonin per 0.1ml saline and 0.1ml saline for six weeks did not affect the % fpm and % reduction in fpm of sperm suspensions of the different experimental groups.

In all sperm suspensions from three experimental groups, the scores of the vigor or quality of sperm motility ranged from 3 to 4.5 and increased with time, with the highest scores observed at the fourth and the fifth hour (Table 2.3). A score of 5 was observed in some individual micro-wells but not as a mean score representing all the 4 micro-wells. Statistical analyses of these data revealed that both time and treatment had significant effects (one way RM ANOVA; two way RM ANOVA, $P<0.05$ for both, Table 2.4) on the quality or vigor of sperm motility.

As the experiment progressed, a clearer pattern of sperm motility emerged. Spermatozoa started to swim more freely, more vigorously and a linear direction of fpm was observed. The sperm motility pattern A was observed between t_0 and t_1, B, between t_2 and t_3, but more pronounced at t_3, C, between t_3 and t_5, but more pronounced at t_4, and D, between t_5 and t_6. The sperm motility pattern A was correlated to 3, B to 4-4.5 and C to 4.5-5, on the Bavister scale (Table 2.3). D could not be accommodated on the Bavister scale as such a type of motility would normally be observed only after the score 5 on the Bavister scale.

Between and within experimental groups, SMI was similar in the first two hours (Fig. 2.4). From the second to the third hour there was a gradual increase in SMI in some sperm suspensions followed by a dramatic increase from the third to the fourth hour (Fig. 2.4). The acceptable SMI

values (> 1400, Bavister and Andrews, 1988) were observed from the fourth to the fifth hour (Fig. 2.4). After the fifth hour there was a decline in SMI (Fig. 2.4). Statistical analyses of these data revealed that both time and treatment had significant effects (one way RM ANOVA; two way RM ANOVA, $P<0.05$ for both, Table 2.4) on the SMI. The significant difference in SMI between the groups was found to be between the control and the saline group, and the control and the melatonin group. In summary, daily injections with 50μg melatonin per 0.1ml saline and 0.1ml saline for six weeks affected the vigor or quality of sperm motility and the SMI of sperm suspensions of the different experimental groups.

Table 2.3: The effects of time, melatonin, and saline on the % of sperm forward progressive movement and the vigor or quality of motility of spermatozoa of Wistar rats. Data are presented as means ± 1 SD.

Experimental group	Time (hr)	% Of sperm forward progressive movement	Vigor or quality of sperm motility (Bavister scale)
Control	0	99.85±0.2	3.0±0.0
	1	94.1±1.8	3.0±0.0
	2	92.2±1.5	3.0±0.0
	3	90.9±2.4	3.3±0.36
	4	88.9±1.6	4.3±0.3
	5	87.8±2.8	4.5±0.2
	6	84.5±2.9	3.9±0.2
Saline	0	99.5±0.5	3.0±0.0
	1	93.87±2.23	3.0±0.0
	2	91.6±2.9	3.0±0.0
	3	89.1±2.5	3.2±0.2
	4	88.7±2.9	4.1±0.2
	5	86.9±3.1	4.4±0.21
	6	83.7±4.4	3.9±0.2
Melatonin	0	99.6±0.5	3.0±0.0
	1	93.8±2.2	2.9±0.2
	2	90.7±2.7	3.0±0.0
	3	89.9±4.1	3.1±0.2
	4	88.5±2.7	4.1±0.2
	5	85.9±5.7	4.3±0.3
	6	85.1±2.9	3.9±0.2

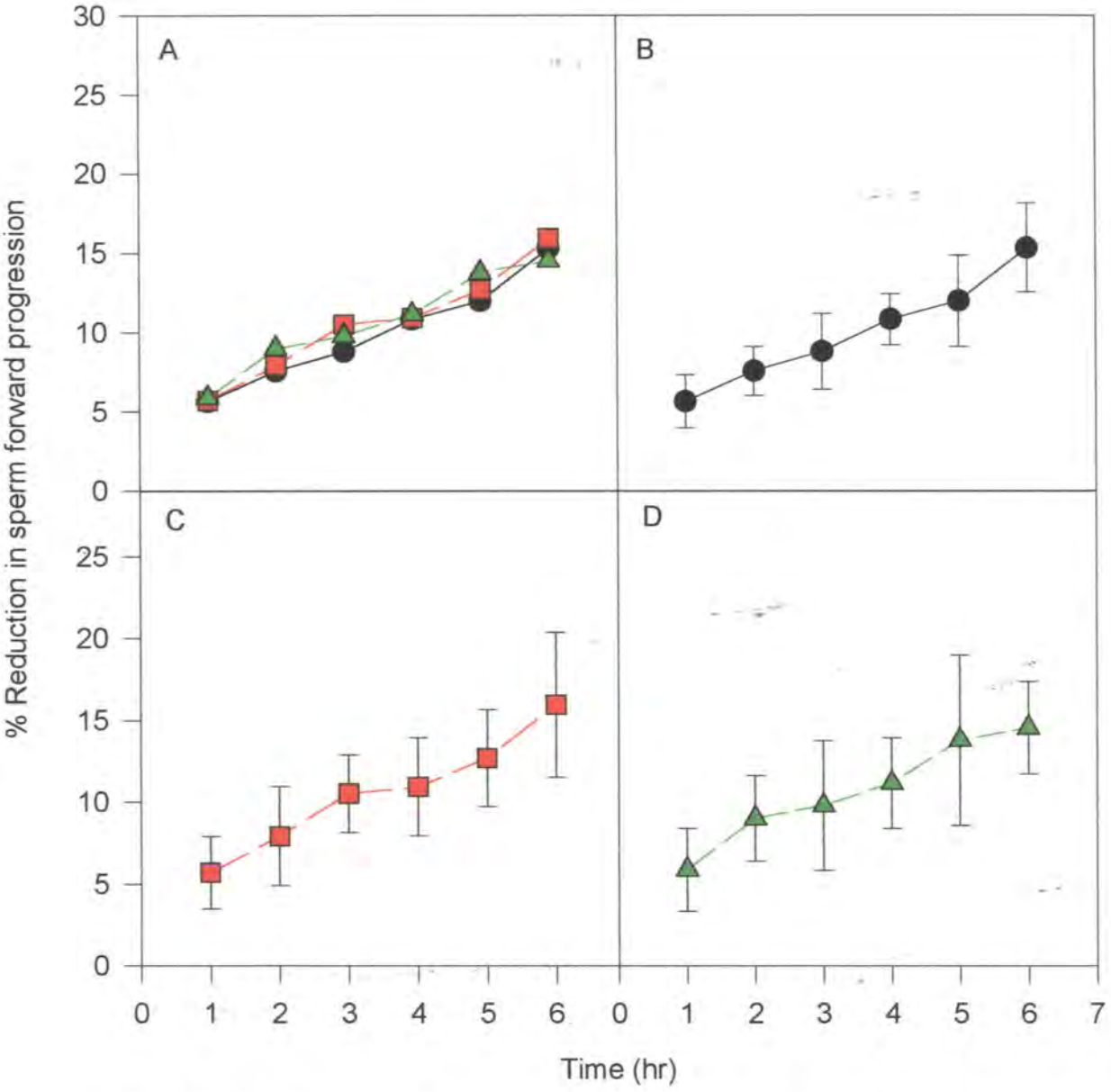

Fig. 2.3.: The effects of melatonin on % reduction in sperm forward progression. In 2.3.A data are presented as means for all three experimental groups are presented. In B, C and D, data are separated for clarity, and the mean ± 1 SD is shown.

Control (C), saline (S), melatonin (M).

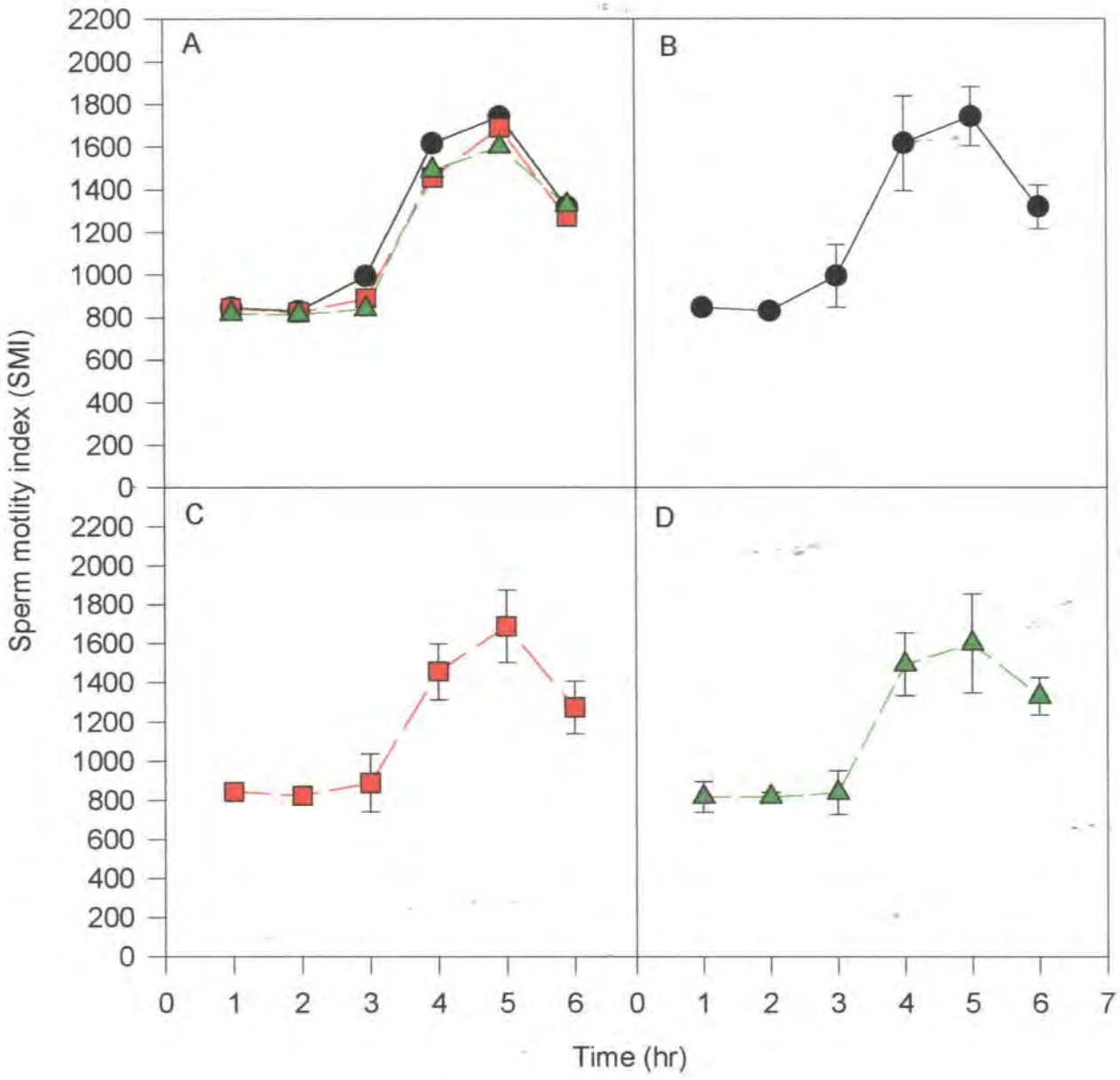

Fig 2.4.: The effects of melatonin on sperm motility index. In 2.4.A, data are presented as means for all three experimental groups. In B, C and D, data are separated for clarity, and the mean ± 1SD is shown.

Control (C), saline (S), melatonin (M).

Table 2.4: Results of the statistical analyses (two way RM ANOVA and one way RM ANOVA) of the effects of time, melatonin and saline on the % of sperm forward progressive movement, % reduction in sperm forward progressive movement, vigor or quality of motility of spermatozoa, and sperm motility index of Wistar rats.

Dependent variable	Between all groups		Within groups (time effect)		
			Control	Saline	Melatonin
	P value				
	Treatment effect	Time effect			
% fpm	0.4	$3.4x10^{-17*}$	$4.8x10^{-13*}$	$5.2x10^{-7*}$	$2.2x10^{-6*}$
% Reduction in fpm	0.6	$3.7x10^{-17*}$	$4.8x10^{-13*}$	$5.2x10^{-7*}$	$2.2x10^{-6*}$
Vigor or quality of sperm motility	$9.5x10^{-3*}$	$4.3x10^{-30*}$	$6.3x10^{-9*}$	$4.0x10^{-10*}$	$3.8x10^{-9*}$
Sperm motility index (SMI)	$7.3x10^{-3}$	$1.5x10^{-26*}$	$2.3x10^{-8*}$	$8.1x10^{-8*}$	$2.9x10^{-21*}$

* significant difference at $P< 0.05$ level.

2.2.4 Hormone analysis:

The mean plasma testosterone concentration values in all experimental groups ranged from 25.17 to 34.43ng/ml (Fig. 2.5). Statistical analyses of these data revealed that there was no significant difference in the concentration of plasma testosterone (one way ANOVA, $P>0.05$) and the daily afternoon injections of 50µg melatonin per 0.1ml saline and 0.1ml saline for six weeks did not affect the concentration of plasma testosterone of the different experimental groups.

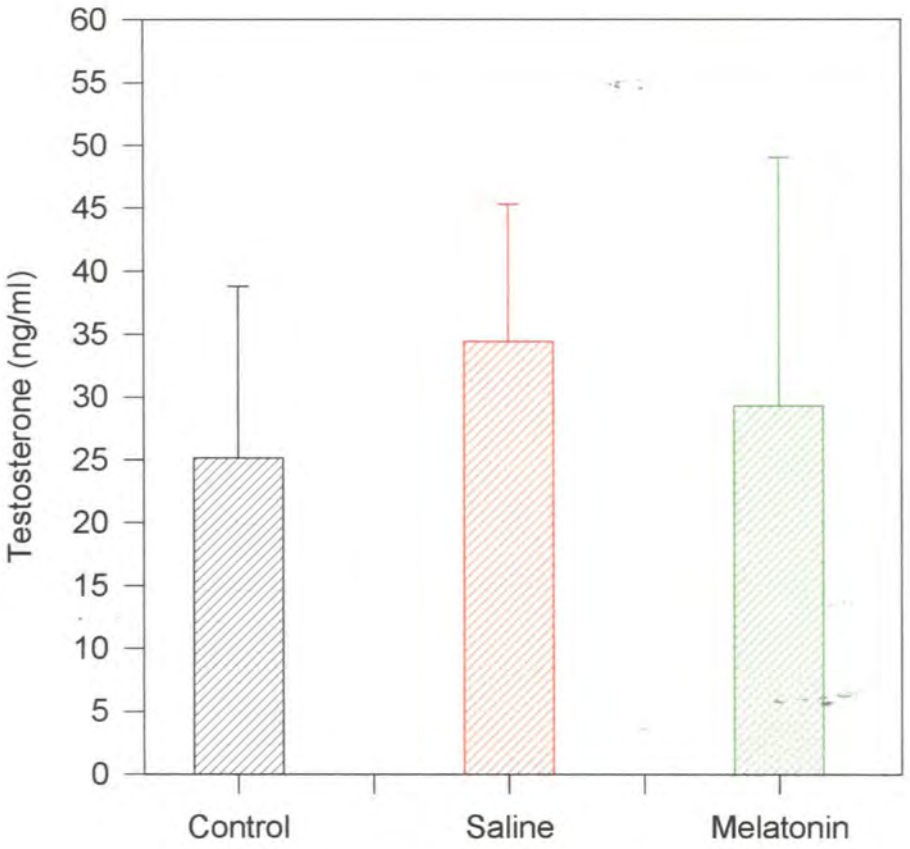

Fig.2.5: The effects of melatonin, and saline on plasma testosterone concentration in Wistar rats. Data are presented as means and ±1 SD.

CHAPTER THREE

THE EFFECTS OF MELATONIN ON SPERM MOTILITY *IN VITRO* IN WISTAR RATS

The aim of these experiments was to investigate and assess the effects of melatonin on motility of rodent spermatozoa *in vitro*. These experiments were designed to mimic the effects of melatonin in the epididymal fluid on sperm motility.

3.1 MATERIALS AND METHODS:

3.1.1 Animal husbandry:

Adult male laboratory rats (316-383g) of the Wistar strain were utilized for the experiments and were supplied by the South African Vaccine Producers, Johannesburg. Animals were kept in a controlled environment (CE) room under long photoperiod conditions (14L:10D-lights on 06:00, lights off 20:00) at approximately 25^0C.

The animals did not receive any treatment and were maintained in groups of five males per clear plastic cage (38.5 x 38.5 x 22.5cm) with wire mesh bases and covers. The male rats were not housed in the same room as female rats. The animals were provided with commercial nutri-chunks dog food and water *ad lib.*. The cages were cleaned twice a week (every Monday and Friday) prior to feeding the animals. The position of the testes was determined by palpation when the animals arrived and before they were sacrificed.

3.1.2 Experimental protocols:

The laboratory conditions and sterile techniques were as described in Section 2.1.4.

3.1.2.1 Trial experiment:

A trial experiment extending over a period of ten days and utilizing 10 animals was run. On each experimental day one animal was weighed and then sacrificed as described in Section 2.1.6. Spermatozoa were collected as described in Section 2.1.8.1 and incubated in one of six different media: SDMEM as used in Section 2.1.5, and five melatonin solutions of different concentrations: 1×10^{-3}M, 1×10^{-5}M, 1×10^{-6}M, 1×10^{-9}M, 1×10^{-12}M. Paraffin oil was used to overlay sperm suspensions. Spermatozoa were processed and assessed hourly for six consecutive hours for the three parameters of sperm motility (the % of fpm, the vigor or quality, and the pattern of motility exhibited) as described in Section 2.1.8.2.

3.1.2.2 Experiment:

3.1.2.2.1 Experiment A: (The effect of melatonin on sperm motility)

Two experimental runs, each lasting a period of ten days and utilizing ten animals each were undertaken. The two experiments were independent of each other with regards to the time of the year at which they were run and the animals utilized for each experiment. This experiment was run in the same way as the trial experiment except that ethanolic DMEM, hereafter referred to as EDMEM, was included as a seventh medium. The inclusion of EDMEM was done to separate any effect of the ethanol used to dissolve melatonin, from the effect of the melatonin on sperm motility *in vitro*.

3.1.2.2.2 Experiment B: (The effect of paraffin oil overlay on motility of spermatozoa incubated in melatonin)

An overlay of equilibrated paraffin oil as part of the sperm culture system confers specific advantages as described in Section 2.1.5, but the highly lipophilic and hydrophilic (Reiter, 1991) nature of melatonin results in the absorption of dissolved melatonin from the solution into the oil

overlay. This results in a change in the concentration of melatonin in the solution as the experiment progresses so that it is difficult to know at any given time during the experiment what the concentration of melatonin in the solution, is.

This experiment was designed and run in a similar way as experiment A, but only six animals and three types of media were utilized to incubate spermatozoa: DMEM, 1 x 10^{-3}M, 1 x 10^{-12}M. The different media utilized for the incubation of spermatozoa were in duplicate and one of each type of medium was overlaid with paraffin oil after the spermatozoa were added into it. This was done so that any effect of an overlay of paraffin oil could be assessed.

3.1.3 Media

EDMEM and the five different melatonin solutions were prepared as stock solutions of 50 and 200ml, respectively. These solutions were stored at 4^0C in aliquots in sterile 20ml bottles and used over a period of three days. The most concentrated melatonin solution was prepared by dissolving melatonin powder (0.0464g) in absolute ethanol (1ml), then making up with DMEM. All the other melatonin solutions of different concentrations were prepared by serial dilution.

The melatonin solutions will hereafter be referred to as MelDMEM (M), and their concentrations will be specified whenever necessary, for example a solution of a concentration of 1x10^{-12}M will hereafter be referred to as -12M. In experiment B, to distinguish between the medium with an oil overlay and the one without an oil overlay, for example –12M with an oil overlay will hereafter be referred to as –12M-O, whereas –12M without an oil overlay will remain being referred to as –12M.

EDMEM was prepared by adding ethanol to DMEM. In the trial experiment and in the first run

of experiment A, the percentage of ethanol in EDMEM and MelDMEM was 10%, whereas in the second run of experiment A and in experiment B it was 0.5%. A lower percentage of ethanol in EDMEM and MelDMEM was preferred over a higher one as the initial experiment revealed that the high percentage greatly reduced sperm motility. Furthermore a lower percentage of 0.4 of ethanol has been used to dissolve melatonin in an experiment assessing the effects of melatonin on sperm motility *in vitro* by Irez *et al.* (1992).

DMEM and EDMEM served as controls. Prior to an experimental procedure, DMEM, EDMEM and the MelDMEM solutions were equilibrated and supplemented (hereafter referred to as SDMEM, SEDMEM, and SMelDMEM) as described for SDMEM in Section 2.1.5. Paraffin oil was equilibrated as described in Section 2.1.5.

3.1.4 Statistical analysis:

Data are presented as means and ± 1 SD. Statistical analyses were conducted using SigmaStat. The effects of the different media and time were compared using a repeated measures two way analysis of variance (two way RM ANOVA) and differences within a medium were compared using a repeated measures one way analysis of variance (one way RM ANOVA) and a repeated measures one way analysis of variance on ranks (one way RM ANOVA on Ranks). Data in percentages were arcsine transformed for statistical analyses. Differences were considered significant at $P<0.05$.

3.2 RESULTS:

3.2.1 Experiment A: (The effect of melatonin on sperm motility)

The results from the first and the second run were similar in terms of their effect but not in the extent of their effect on sperm motility. The high percentage of ethanol (10%) in the medium

utilized for the incubation of spermatozoa in the trial run, greatly reduced sperm motility, and for this reason, only results from the second run are reported here.

All the males had scrotal testes before being sacrificed.

3.2.1.1 Assessment of spermatozoa:

3.2.1.1.1 Sperm concentration:

At t_0, all the spermatozoa were intact, motile and without any observable structural defects and were regarded as normal. The sperm concentration of the experimental animals ranged from 6 to 10.7 x 10^6 sp/ml.

3.2.1.1.2 Motility:

Three parameters of the sperm motility: the percent of forward progressive movement (% fpm), the vigor or quality, and the pattern of motility shown were assessed. At t_0, the initial % of fpm ranged between 98.7 and 100, whilst the initial vigor or quality ranged between 2.5 and 3 (on the Bavister scale), and the sperm motility pattern A was observed. Sperm head to head agglutination in the first hour of incubation was generally observed in all spermatozoa of the different media, but disappeared at t_2 when single freely swimming spermatozoa were most common.

In all media, the % of fpm decreased significantly during the experiment (Table 3.1; $P<0.05$, Table 3.2). The extent to which the % of fpm decreased was significantly affected by both time and medium (two way RM ANOVA; one way RM ANOVA, $P<0.05$ for both, Table 3.2), and increased with increasing melatonin concentration (Table 3.1). Multiple range tests showed that there was a statistically significant difference in the % of fpm between all the different combinations of media except between -6M and –12M, and, -12M and –9M.

The % reduction in fpm mirrors the % fpm, and for all the different incubation solutions, the % reduction in fpm increased gradually with time, and with increasing melatonin concentration (Fig. 3.1). Statistical analyses of these data revealed that both time and medium significantly affected the % reduction in fpm (two way RM ANOVA; one way RM ANOVA, $P<0.05$ for both, Table 3.2). Multiple range tests showed that there was a statistically significant difference in the % reduction in fpm between all the different combinations of media except between -9M and –12M, and, -12M and –6M.

The vigor or quality of sperm motility in all the different media varied significantly throughout the experiment (Table 3.1). In all the media except in –5M and –3M, there was a general increase in the vigor or quality of sperm motility with the highest scores observed at t_4. In –5M and –3M media, a small increase in the vigor or quality was observed with the highest scores observed between t_3 and t_4. Statistical analyses of these data revealed that both time and medium had significant effects on the vigor or quality of sperm motility (two way RM ANOVA; one way RM ANOVA, $P<0.05$ for both, Table 3.2). Multiple range tests showed that there was a statistically significant difference in the vigor or quality of sperm motility between all the other different combinations of media except between EDMEM and -12M, EDMEM and -9M, –6M and –9M, -6M and –12M, -12M and –9M, and, -5M and –3M.

As the experiment progressed, spermatozoa incubated in SDMEM started to swim more freely, more vigorously and a linear direction of fpm was observed. The sperm motility pattern A was observed between t_0 and t_1, B, between t_2 and t_3, but more pronounced at t_3, C, between t_3 and t_5, but more pronounced at t_4, and D, between t_5 and t_6. The sperm motility pattern A was correlated to 3, B, to 4-4.5 and C, to 4.5-5, on the Bavister scale (Table 3.1). In spermatozoa incubated in SEDMEM, -12M, -9M, and –6M a similar trend of change in sperm motility pattern with time

was observed, but the pattern was not as prominent. Progression from one pattern of motility to the next was delayed and/or prolonged, and from the third hour of the experiment, the values on the Bavister scale were lower than the equivalent scores for spermatozoa incubated in SDMEM (Table 3.1)

Spermatozoa incubated in -5M and -3M showed a pattern of sperm motility that was different from that observed in spermatozoa incubated in SDMEM and SEDMEM, and there was no progression from the observed sperm motility pattern A (3 on Bavister scale) to subsequent patterns of sperm motility (Table 3.1).

Both medium and time had significant effects on SMI (Fig. 3.2; two way RM ANOVA; one way RM ANOVA, $P<0.05$ for both, Table 3.2). The acceptable SMI values (>1400, Bavister and Andrews, 1988) were generally observed from the fourth to the sixth hour in spermatozoa incubated in SDMEM. Multiple range tests showed that there was a statistically significant difference in the SMI between all the other different combinations of media except between EDMEM and -6M, –6M and –9M, -6M and –12M, -12M and –9M, and, -5M and –3M.

Table 3.1: The effects of time, melatonin, and ethanol on the % of sperm forward progressive movement and the vigor or quality of motility of spermatozoa of Wistar rats. Data are presented in means and ± 1 SD.

Medium	Time(hr)	% Of sperm forward progressive movement	Vigor or quality of sperm motility (Bavister scale)
SDMEM	1	97.5± 2.0	3.0±0.0
	2	94.6±1.7	3.0±0.0
	3	93.5±1.9	3.6±0.4
	4	91.9±1.3	4.2±0.3
	5	90.5±2.2	4.6±0.2
	6	89.3±3.2	4.4±0.3
SEDMEM	1	92.9±1.9	3.0±0.0
	2	89.4±2.7	2.9±0.2
	3	85.7±4.4	3.1±0.2
	4	84.4±2.3	3.8±0.4
	5	83.6±3.9	3.9±0.5
	6	76.8±4.4	3.7±0.5
-12M	1	91.3±3.1	3.0±0.0
	2	86.1±3.6	2.9±0.2
	3	80.7±4.2	3.1±0.2
	4	78.4±2.8	3.3±0.4
	5	77.9±1.8	3.6±0.6
	6	73.6±4.8	3.3±0.4
-9M	1	89.7±3.9	3.0±0.0
	2	84.3±5.2	2.9±0.3
	3	81.6±4.2	3.0±0.0
	4	77.1±2.7	3.2±0.4
	5	73.9±3.3	3.3±0.4
	6	70.6±6.3	3.2±0.6
-6M	1	88.9±3.6	3.0±0.0
	2	85.9±3.9	2.9±0.3
	3	85.9±3.8	3.1±0.2
	4	79.6±4.9	3.6±0.5
	5	79.9±3.9	3.7±0.5
	6	75.7±6.7	3.4±0.6
-5M	1	86.3±5.6	2.8±0.3
	2	81.2±4.9	2.8±0.4
	3	76.4±6.4	2.7±0.4
	4	69.1±6.7	2.8±0.6
	5	66.4±6.3	3.0±0.6
	6	58.4±9.4	2.5±0.5
-3M	1	85.4±6.3	2.9±0.2
	2	78.6±4.9	2.7±0.3
	3	73.3±4.2	2.6±0.4
	4	65.9±5.9	2.5±0.6
	5	62.6±6.8	2.5±0.6
	6	52.6±7.6	2.2±0.5

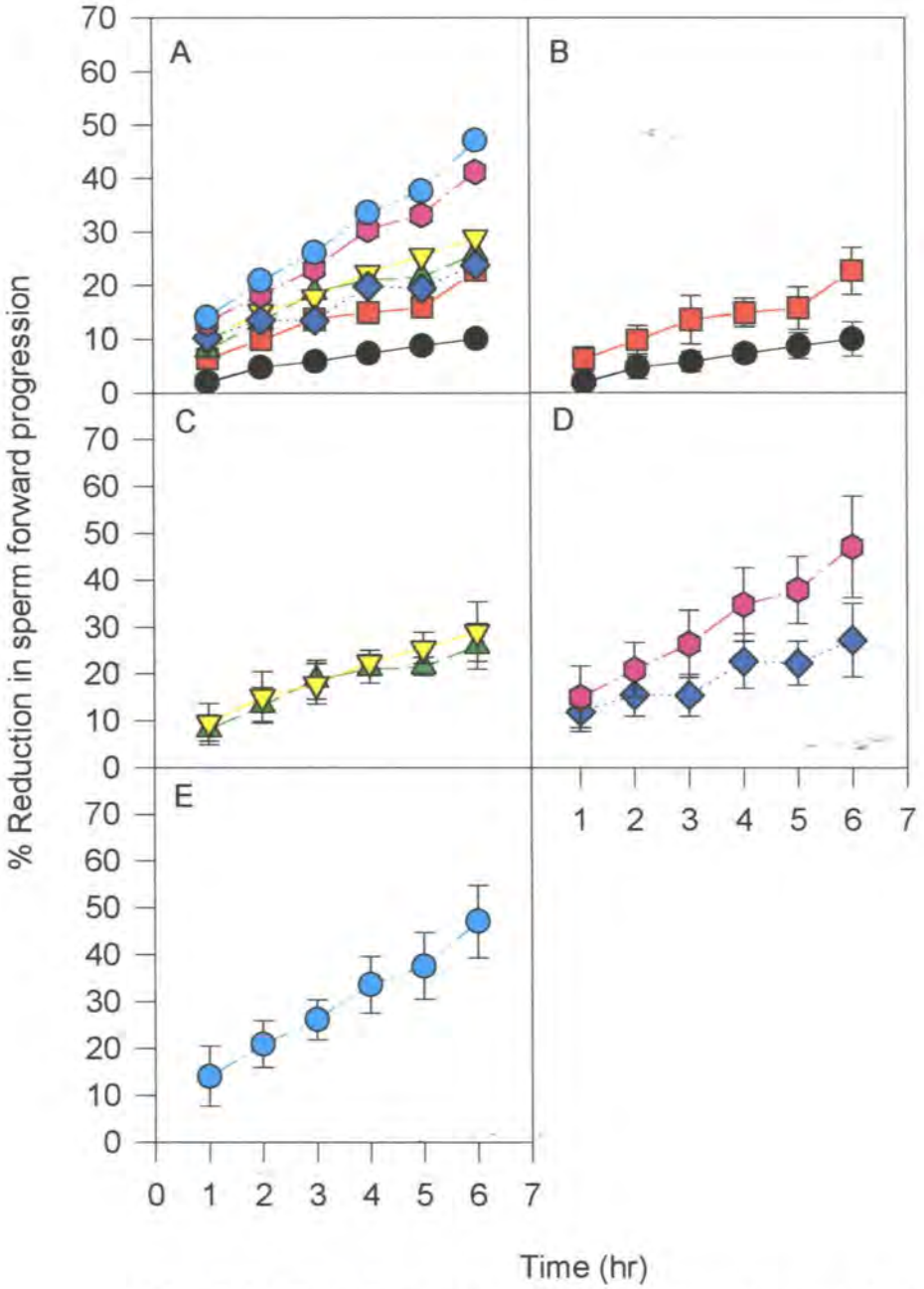

Fig. 3.1.: The effects of melatonin on % reduction of sperm forward progression. In 3.1.A., data are presented as means for all seven media. In B,C, D and E, data are separated for clarity, and the mean ± 1 SD is shown.

SDMEM, SEDMEM, -12M, -9M, -6M, -5M, -3M.

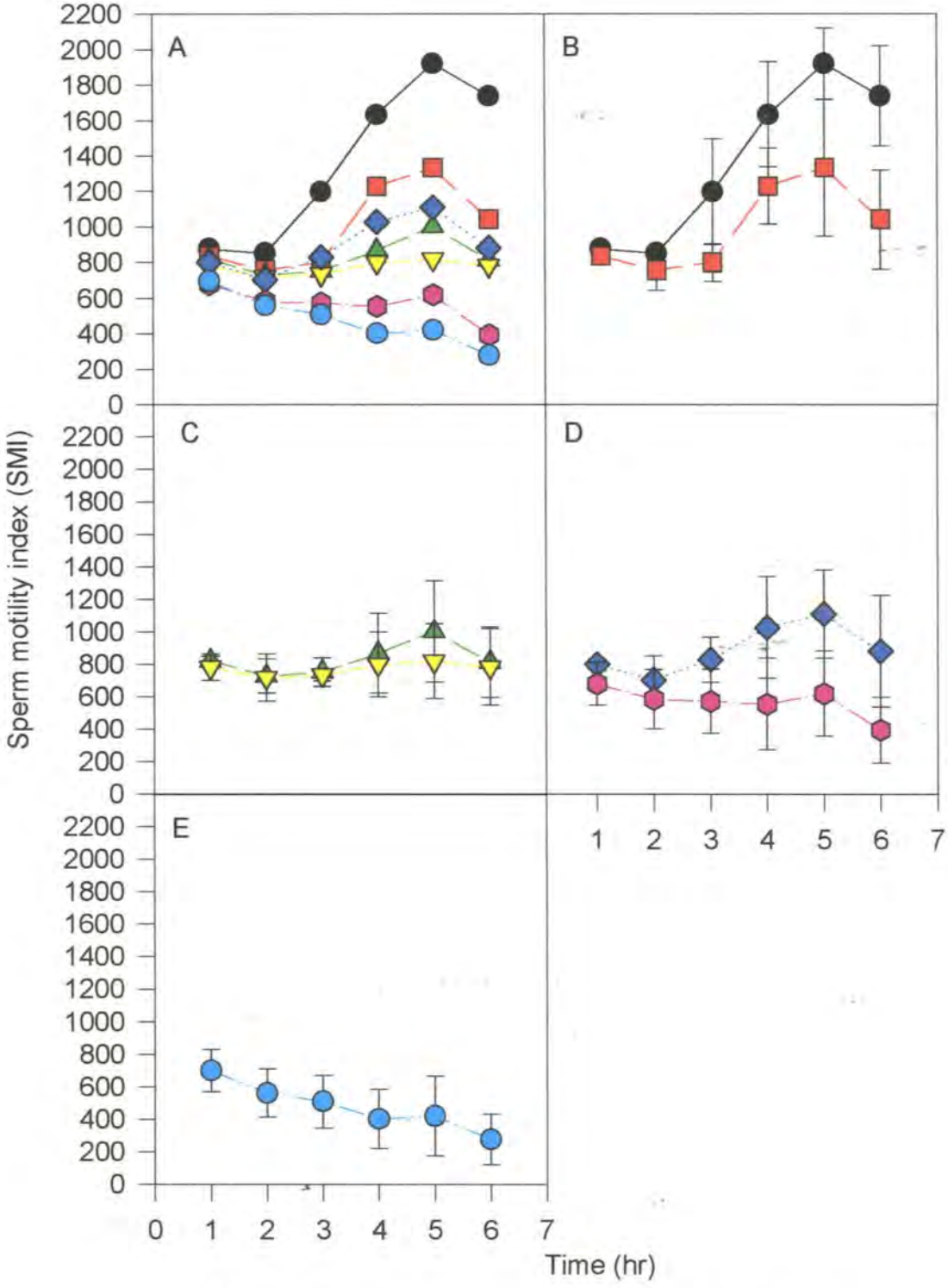

Fig. 3.2.: The effects of melatonin on sperm motility index. In 3.2.A., data are presented for all seven media. In B, C, D and E, data are separated for clarity, and the mean ± 1 SD is shown.

Table 3.2: Results of the statistical analyses (two way RM ANOVA and one way RM ANOVA) of the effects of time, melatonin, and ethanol on the % of sperm forward progressive movement, % reduction in sperm forward progressive movement, vigor or quality of motility of spermatozoa, and sperm motility index of Wistar rats.

Media	Dependant variable							
	% Of sperm forward progressive movement		**% Reduction in sperm forward progressive movement**		**Vigor or quality of sperm motility**		**Sperm motility index**	
	P value							
Between all media	**Treatment effect**	**Time effect**	**Treatment effect**	**Time effect**	**Treatment effect**	**Time effect**	**Treatment effect**	**Time effect**
	$2.5x10^{-27*}$	$1.1x10^{-21*}$	$3.4x10^{-27*}$	$3.1x10^{-22*}$	$1.9x10^{-15*}$	$1.9x10^{-10*}$	$1.9x10^{-20*}$	$2.9x10^{-9*}$
Within media	**Time effect**		**Time effect**		**Time effect**		**Time effect**	
SDMEM	$1.3x10^{-7*}$		$6.9x10^{-11*}$		$7.7x10^{-9*}$		$7.3x10^{-7*}$	
SEDMEM	$1.5x10^{-6*}$		$4.0x10^{-12*}$		$1.7x10^{-6*}$		$4.7x10^{-4*}$	
-12M	$8.8x10^{-15*}$		$9.8x10^{-15*}$		$1.8x10^{-3*}$		0.2	
-9M	$1.3x10^{-14*}$		1.6^{-14*}		$1.8x10^{-2*}$		0.3	
-6M	$1.2x10^{-8*}$		$1.2x10^{-8*}$		$2.5x10^{-6*}$		0.1	
-5M	$6.9x10^{-17*}$		$7.2x10^{-17*}$		$1.5x10^{-2*}$		$5.4x10^{-4*}$	
-3M	$1.8x10^{-20*}$		$1.3x10^{-20*}$		$3.8x10^{-4*}$		$1.9x10^{-12*}$	

* significant difference at $P<0.05$ level.

In summary, melatonin, and melatonin and ethanol had negative effects on sperm motility, *in vitro*.

3.2.2 Experiment B: (The effect of paraffin oil overlay on motility of spermatozoa incubated in melatonin):

3.2.2.1 Assessment of spermatozoa

3.2.2.1.1 Sperm concentration

At t_0, all spermatozoa were intact, motile and without any observable structural defects and were regarded as normal. The sperm concentration of the experimental animals ranged from 3.4 to 12.4 x 10^6sp/ml.

3.2.2.1.2 Motility

Three parameters of the sperm motility: the percent of forward progressive movement (% fpm), the vigor or quality, and the pattern of motility shown were assessed. At t_0, the initial % of fpm ranged between 98.1 and 100, whilst the initial vigor or quality was 2.5 (on the Bavister scale), and sperm motility pattern A was observed. Sperm head to head agglutination in the first hour of incubation was generally observed in all spermatozoa of the different media, but disappeared at t_2 when single freely swimming spermatozoa were most common.

As the experiment progressed, a difference in the pattern of sperm motility was observed between the spermatozoa overlaid with paraffin oil and those without an oil overlay. In spermatozoa incubated in the same type of medium, the exclusion of an oil overlay significantly reduced all parameters of sperm motility (Fig. 3.3, Fig. 3.4; Table 3.3, Table 3.4).

The acceptable SMI values (>1400, Bavister and Andrews, 1988) were observed from the fourth to the sixth hour of the experiment and only in spermatozoa incubated in SDMEM (Fig. 3.4). Multiple range tests showed that there was a statistical significant difference in the vigor or quality between all combinations of media except between SDMEM and –12M-O, and, –12M and

–3M-O, whilst there was no significant difference in the SMI between -12M and –3M-O.

Table 3.3: The effects of time, melatonin, and an oil overlay on the % of sperm forward progressive movement and the vigor or quality of motility of spermatozoa of Wistar rats. Data are presented as means and ± 1 SD.

Medium	Time(hr)	% Of sperm forward progressive movement	Vigor or quality of motility (Bavister scale)
SDMEM-O	1	96.8± 2.6	2.5±0.0
	2	95.6±2.9	3.3±0.3
	3	94.2±1.8	3.9±0.4
	4	93.2±3.1	4.7±0.3
	5	91.3±2.9	4.5±0.0
	6	88.4±1.9	4.1±0.2
SDMEM	1	96.2±2.0	2.5±0.0
	2	92.8±2.0	3.0±0.2
	3	89.13±3.3	3.6±0.5
	4	89.5±3.7	4.3±0.3
	5	83.7±5.3	4.3±0.3
	6	79.0±4.6	3.6±0.4
-12M-O	1	93.8±2.1	2.5±0.0
	2	90.3±3.2	3.2±0.3
	3	86.2±3.6	3.7±0.4
	4	83.0±4.6	4.1±0.2
	5	80.9±6.3	3.8±0.4
	6	77.8±4.6	3.2±0.4
-12M	1	92.3±1.9	2.8±0.6
	2	86.4±3.5	2.8±0.3
	3	81.6±4.7	2.9±0.2
	4	75.0±7.5	2.9±0.5
	5	68.7±3.9	2.7±0.5
	6	66.6±8.2	2.0±0.0
-3M-O	1	91.5±2.0	2.5±0.0
	2	85.2±4.2	2.8±0.3
	3	77.2±5.2	2.9±0.2
	4	70.8±2.1	2.9±0.5
	5	57.3±9.9	2.5±0.5
	6	52.9±4.4	2.2±0.4
-3M	1	86.2±3.1	2.4±0.2
	2	80.8±5.4	2.5±0.3
	3	63.9±9.5	2.1±0.2
	4	50.8±9.5	1.8±0.3
	5	38.1±9.5	1.6±0.4
	6	34.7±6.5	1.3±0.3

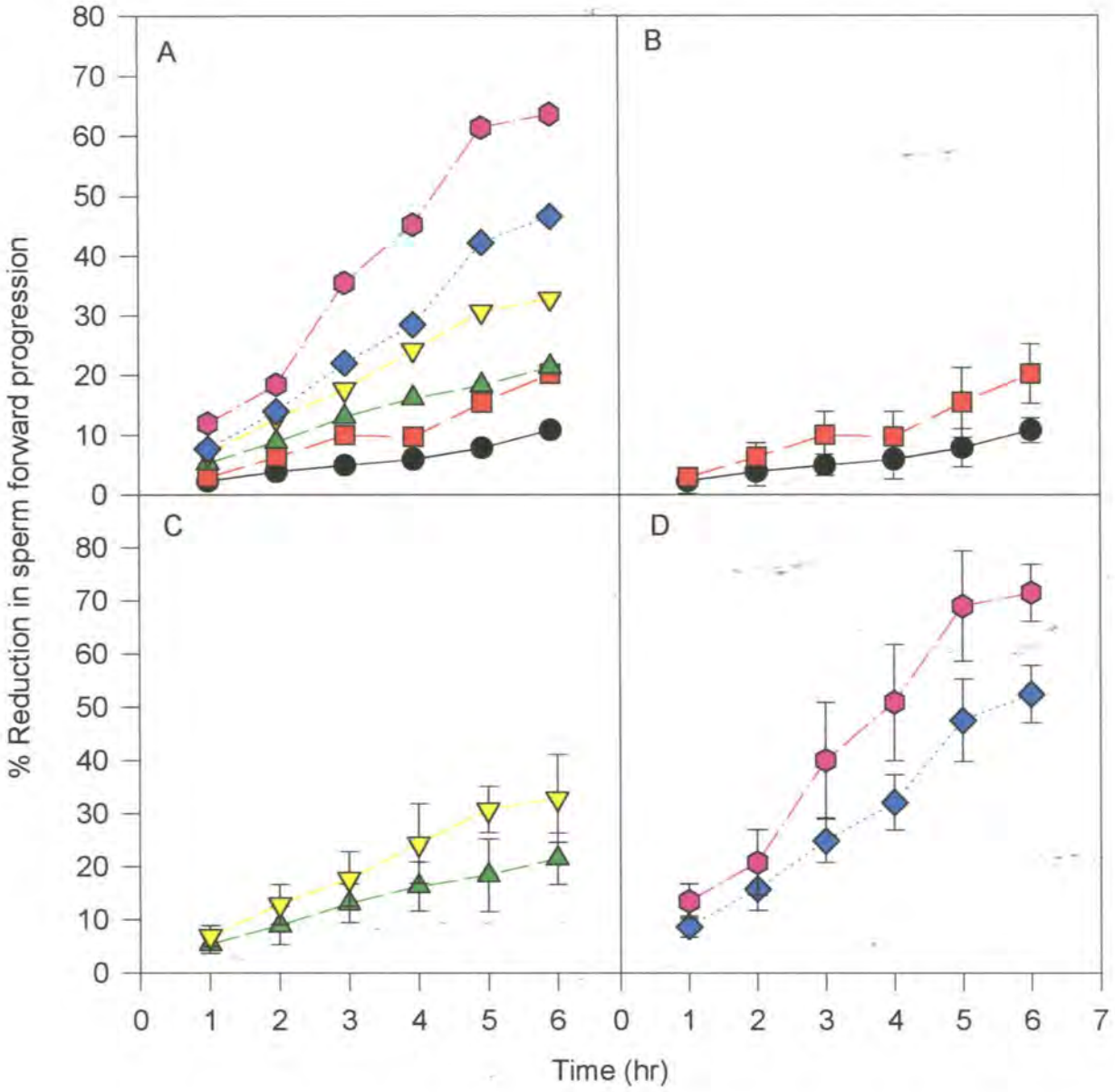

Fig.3.3.: The effects of melatonin and an oil overlay on the % reduction in sperm forward progression. In 3.3.A., data are presented as means for all six media. In B, C, and D, data are separated for clarity, and the mean ± 1 SD is shown.

SDMEM-O, SDMEM, -12M-O, -12M, -3M-O, -3M.

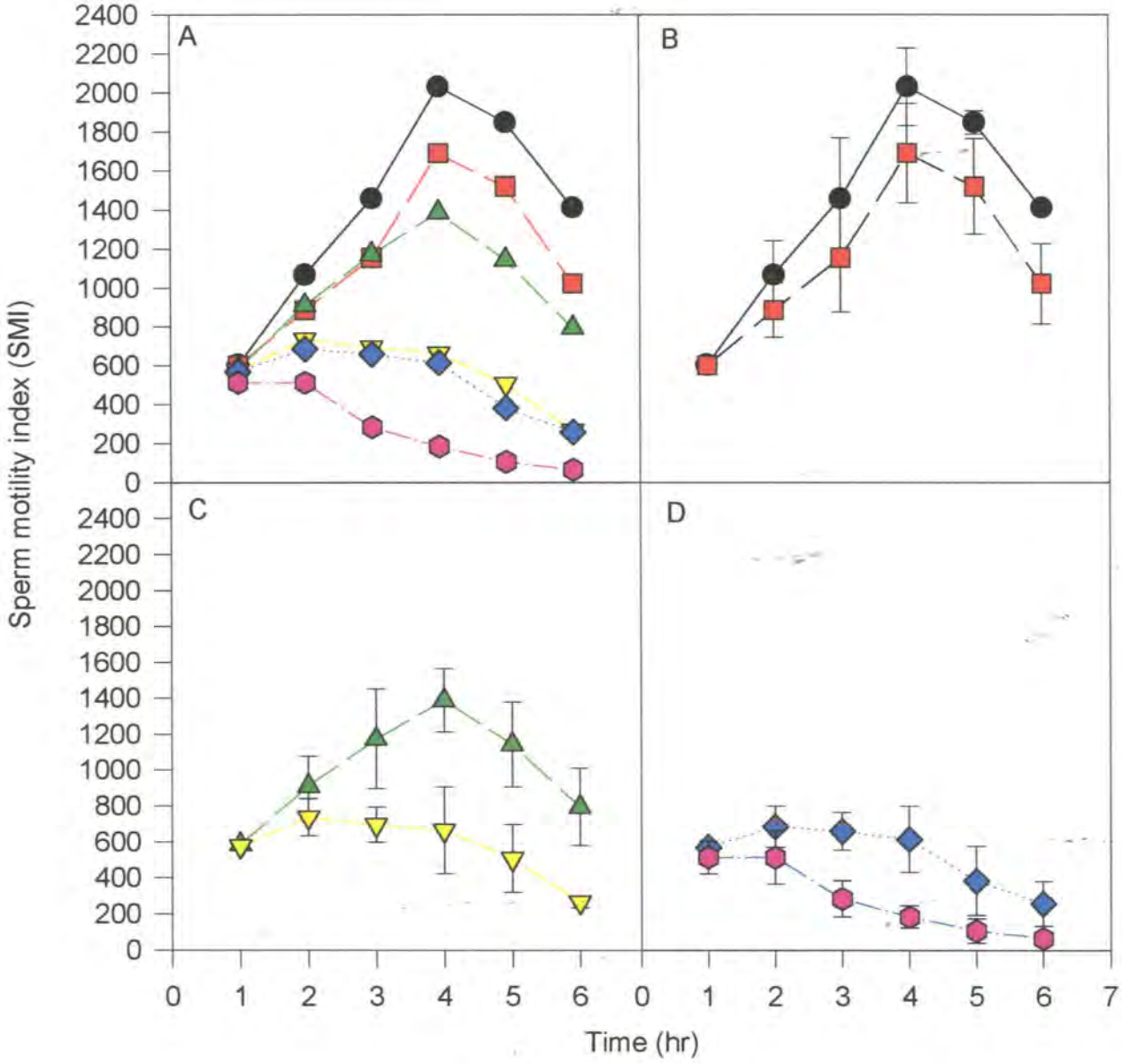

Fig. 3.4: The effects of melatonin and an oil overlay on sperm motility index. In 3.4.A., data are presented for all six media. In B, C, and D, data are separated for clarity, and the mean ± SD is shown.

Table 3.4: Results of the statistical analyses (two way RM ANOVA and one way RM ANOVA) of the effect of time, melatonin, and an oil overlay on the % of forward progressive movement, vigor or quality of motility of spermatozoa, and sperm motility index of Wistar rats.

Media	Dependant variable							
	% Of sperm forward progressive movement		% Reduction in sperm forward progressive movement		Vigor or quality of sperm motility		Sperm motility index	
	P value							
Between all media	**Medium effect**	**Time effect**	**Medium effect**	**Time effect**	**Medium effect**	**Time effect**	**Medium effect**	**Time effect**
	$1.08x10^{-15*}$	$4.33x10^{-17*}$	$8.53x10^{-17*}$	$1.32x10^{-17*}$	$9.89x10^{-17*}$	$3.63x10^{-11*}$	$1.25x10^{-18*}$	$1.05x10^{-11*}$
Between pairs of media	**Medium effect**	**Time effect**	**Medium effect**	**Time effect**	**Medium effect**	**Time effect**	**Medium effect**	**Time effect**
SDMEM and SDMEM-O	$2.71x10^{-3*}$	$1.4x10^{-9*}$	$1.78x10^{-3*}$	$8.8x10^{-11*}$	$1.88x10^{-3*}$	$1.2x10^{-13*}$	$2.19x10^{-3*}$	$6.1x10^{-13*}$
-12M and –12M-O	$2.28x10^{-4*}$	$6.5x10^{-12*}$	$2.64x10^{-4*}$	$3.9x10^{-12*}$	$9.78x10^{-4*}$	$4.5x10^{-7*}$	$1.6x10^{-4*}$	$4.3x10^{-8*}$
-3M and –3M-O	$2.23x10^{-3*}$	$3.7x10^{-14*}$	$8.66x10^{-4*}$	$3.7x10^{-14*}$	$2.34x10^{-4*}$	$1.2x10^{-6*}$	$5.13x10^{-4*}$	$6.1x10^{-10*}$
Within media	**Time effect**		**Time effect**		**Time effect**		**Time effect**	
SDMEM	$9.02x10^{-9*}$		$5.39x10^{-9*}$		$8.321x10^{-5*}$		$1.25x10^{-4*}$	
SDMEM-O	$2.49x10^{-6*}$		$7.83x10^{-8*}$		$3.57x10^{-5*}$		$1.63x10^{-14*}$	
-12M	$3.99x10^{-10*}$		$1.55x10^{-4*}$		$2.19x10^{-3*}$		$1.1x10^{-3*}$	
-12M-O	$1.5x10^{-8*}$		1.58^{-14*}		$1.79x10^{-2*}$		$1.15x10^{-4*}$	
-3M	$3.49x10^{-5*}$		$4.62x10^{-13*}$		$1.72x10^{-7*}$		$3.04x10^{-10*}$	
-3M-O	$3.07x10^{-5*}$		$3.07x10^{-5*}$		$3.53x10^{-3*}$		$9.21x10^{-8*}$	

* significant difference at $P<0.05$ level.

To summarize, within a single type of medium, the addition of an oil overlay increased sperm motility, enhanced the pattern of motility shown, and increased the SMI.

The results from experiments A and B have shown that increasing melatonin concentration resulted in an increase in the % reduction in fpm, reduction in the quality or vigor of motility, and reduction in SMI. Some of these effects can be attributed to the ethanol that was used to dissolve melatonin. These effects of melatonin were ameliorated by the addition of an oil overlay in the sperm culture medium (Experiment B).

CHAPTER FOUR
THE EFFECTS OF MELATONIN ON CAPACITATION AND THE ACROSOME REACTION OF SPERMATOZOA OF WISTAR RATS

The aim of this experiment was to investigate and assess the effects of melatonin on capacitation and the acrosome reaction of rodent spermatozoa *in vitro*.

4.1 MATERIALS AND METHODS:

4.1.1 Animal husbandry:

Adult male laboratory rats (318-430g) of the Wistar strain were utilized for the experiment and were supplied by the South African Vaccine Producers, Johannesburg. Animals were kept in a controlled environment (CE) room under long photoperiod conditions (14L:10D-lights on 06:00, lights off 20:00) at approximately 25^0C.

The animals did not receive any treatment and were maintained in groups of five males per clear plastic cage (38.5 x 38.5 x 22.5cm) with wire mesh bases and covers. The male rats were not housed in the same room as female rats. The animals were provided with commercial nutri-chunks dog food and water *ad lib.*. The cages were cleaned twice a week (every Monday and Friday) prior to feeding the animals. The position of the testes was determined by palpation when the animals arrived and before they were sacrificed.

4.1.2 Experimental protocol:

The laboratory conditions and sterile techniques were as described in Section 2.1.4.

4.1.2.1 Trial experiment:

A trial experiment extending over a period of five days and utilizing five animals was run. On each experimental day one animal was weighed and then sacrificed as described in Section 2.1.6. Spermatozoa were collected as described in Section 2.1.8.1, assessed for motility at t_0, and incubated in equilibrated SDMEM (as was used in Section 2.1.5). Only sperm samples with motility greater than 80% were used in the experiment. The sperm suspensions was overlaid with equilibrated paraffin oil (as was used in Section 2.1.5). The CTC fluorescence assay of Saling and Storey (1979) which has been modified by Fraser and Herod (1990) and whose protocol has been used in other species by Mortimer (1994), was utilized to assess and monitor the stages and progression of capacitation and the acrosome reaction in spermatozoa.

The CTC fluorescence assay has been widely utilized to assess and monitor the stages and progression of capacitation and the acrosome reaction in spermatozoa of various species such as the mouse (Saling and Storey, 1979; Lee and Storey, 1985, 1986; Fraser and Herod, 1990; Kholkute *et al.*, 1995), the guinea pig (Saling and Storey, 1979), in humans (Mortimer, 1994), and the ram (Perez *et al.*, 1996). CTC is light sensitive, thus during an experimental run, the lights of the laboratory were switched off and only dim light coming through the laboratory door window illuminated the laboratory.

4.1.2.2 Experiment:

One experiment lasting a period of ten days and utilizing ten animals was undertaken. The experiment was divided into two runs, each utilizing five animals: the control experimental run, which used SDMEM was done over the first five days; and the treatment experiment run, which used –3M and –5M was done over the last five days of the experimental period.

4.1.3 Chlortetracycline fluorescence assay:

4.1.3.1 Chemicals and reagents:

Chlortetracycline (CTC), Tris- (hydroxymethylamino)-methane (Tris), L-cysteine, NaCl, KCl and glutaraldehyde (Sigma Chemical Company, St Louis, MO, USA) were utilized for the assay. The CTC buffer was prepared by dissolving in distilled water [1litre(L)] 2.24g Tris, 0.606g cysteine and 7.59g NaCl, and was kept at 4^0C at all times. The CTC aqueous stock solution was prepared by dissolving in distilled water (10ml) 2.58g CTC powder. This was aliquoted into 10 eppendorf tubes (1ml), covered with foil and kept in a light proof container at -20^0C at all times.

The Tris buffer for the glutaraldehyde fixative was prepared by dissolving in distilled water (1L) 4.48g Tris, 7.89 cysteine, and 0.373 KCl. The pH of the Tris buffer was adjusted with an HCl: distilled water (1:1 v/v) solution to 8.0 and kept at 4^0C at all times.

On the day of an experimental run, the glutaraldehyde fixative was prepared by pipetting 125µl stock (25%) glutaraldehyde and 875µl Tris buffer into an eppendorf tube. The pH of the glutaraldehyde fixative was adjusted with HCl: distilled water (1:1, v/v) solution to 7.8±0.5. The final CTC solution was prepared by pipetting 3960µl of CTC buffer and 40µl of CTC aqueous stock solution into a volumetric flask (25ml), which were then mixed and their pH adjusted with an HCl: distilled water (1:1, v/v) solution to 7.8±0.5.

On the day of an experimental run, the final CTC solution and the glutaraldehyde fixative were kept on crushed ice in a light proof plastic dish and only used on the day of preparation. A pH meter (300 Series, Beckman Instruments, Inc, CA, USA) was used to assess and monitor the pH of the different solutions that were utilized for the experiment.

4.1.3.2 Method:

Hourly, for six consecutive hours, four clean microscope slides were prepared for the assessment of the fluorescence patterns shown by the spermatozoa. An Olympus microscope using ultraviolet illumination (BX 60, Tokyo, Japan) under oil immersion at 1000x magnification was utilized for assessment. The slides were prepared by pipetting 2.5µl off the top layer of the sperm suspension on to a pre-warmed slide and then adding 2.5µl of CTC final solution. Within approximately ten seconds, 0.25µl glutaraldehyde fixative was pipetted, added to the sperm-CTC suspensions on the slide and then mixed quickly using an autopippette tip. A coverslip was then placed over the sperm-CTC-glutaraldehyde solution. The coverslip was gently compressed using a paper towel, to remove excess fluid so that accurate assessment could be made.

The prepared slides were immediately put in a lightproof plastic container and then later examined utilizing an Olympus microscope (BX 60, Tokyo, Japan) equipped with phase-contrast and epifluorescence optics. The excitation beam was passed through a 330-385 nm exciter filter and CTC fluorescence was observed through a DM 400 dichroic mirror and BA 420 barrier filter. The lights in the microscopy room were switched off and only dim light coming through the laboratory door window illuminated the laboratory.

3 CTC fluorescence patterns are exhibited by capacitating mouse spermatozoa (Fraser and Herod, 1990):

F pattern – with uniform fluorescence over the entire head, characteristic of uncapacitated, acrosome-intact spermatozoa (UCAI);

B pattern – with a fluorescence-free band in the post-acrosomal region, characteristic of capacitated, acrosome-intact spermatozoa (CAI);

AR pattern – with no fluorescence on the head, characteristic of capacitated, acrosome-reacted

spermatozoa (CAR).

In all three CTC patterns, the midpiece also shows bright fluorescence.

In each aliquot, 100 sperm heads (50 randomly selected sperm heads on each of two of the four slides) were classified as having one of the three CTC fluorescence patterns. The percentage occurrence of each pattern was calculated and recorded. Light micrographs were taken, and to produce the best images and light micrographs, the microscope was arranged as follows: the ND 25 filter was in position, the aperture iris diaphragm was open, the field iris diaphragm was open, the light excluding shutter was open, the beam splitting prism was set at 50:50, the WU cube was utilized for ultraviolet (UV) epifluorescence, and the exposure time was set at 16 seconds.

4.1.4 Statistical analysis:

Data are presented as means and ± 1 SD. Statistical analyses were conducted using STATISTICA for windows (1999). The effects of media and time on the occurrence of CTC patterns, were compared using a proper repeated measures Multifactor analysis of variance (RM MANOVA). Data in percentages were arcsine transformed for statistical analyses. Differences were considered significant at $P<0.05$.

4.2 RESULTS:

All the animals were scrotal at sacrifice, and at t_0 the motility of spermatozoa utilized for the experiment was greater than 80%. Data are presented for the experiment only, because the trial experiment used only one medium (SDMEM, which served as the control in the experiment), and did not yield satisfactory images of the sperm heads.

4.2.1 Chlortetracycline fluorescence assay:

As the experiment progressed, spermatozoa incubated in all the three media (SDMEM, -5M, and –3M) showed a similar progression from the F pattern to the B pattern, and finally to the AR pattern (Fig. 4.1).

At t_1, more than 70% of the sperm heads showed the F pattern and after three hours this had decreased to approximately 50% (Fig. 4.1.A and D). Over the same period (t_1-t_3) the occurrence of the B pattern increased from 0% to approximately 50% (Fig. 4.1.B and E) while there was no change in the occurrence of the AR pattern (Fig. 4.1.C and F). At t_4 there were very few sperm heads showing the F pattern (approximately 10%), most sperm heads showed the B pattern (approximately 65%) and approximately 25% showed the AR pattern (Fig. 4.1.). Between t_4 and t_6, the occurrence of the B pattern declined while the occurrence of the AR pattern increased to reach about 65% at t_6 (Fig. 4.1.A and D; C and F).

The F pattern, which is characteristic of uncapacitated, acrosome-intact (UCAI) spermatozoa, was characterized by the uniform appearance of CTC fluorescence over the entire head (Fig. 4.2). The B pattern, that is characteristic of capacitated, acrosome-intact (CAI) spermatozoa, was characterized by a fluorescence–free band in the post-acrosomal region (Fig. 4.3). And the AR pattern, which is characteristic of capacitated, acrosome-reacted (CAR) spermatozoa, was characterized by the lack of fluorescence on the head (Fig. 4.4). In all the three CTC fluorescence patterns, the midpiece also showed a bright fluorescence (Fig. 4.2; 4.3; 4.4). At any given time of the experiment, the sum of the percentages of spermatozoa showing the 3 different CTC patterns yielded 100 percent.

The statistical analyses of these data revealed that there was a significant difference between all

the experimental parameters except between time and medium and within all the different experimental parameters (proper repeated measures MANOVA, Table 4.1). While the incubation medium significantly affected the number of capacitating spermatozoa, multiple range tests revealed that only 3M and the SDMEM were significantly different.

Table 4.1: Results of the statistical analyses (proper repeated measures MANOVA) of the effects of time and melatonin on the CTC fluorescence patterns shown by spermatozoa of Wistar rats.

Experimental variables:	
Between all experimental variables	**P value**
Time	0.00^{*}
Media	$2.3x10^{-2*}$
Pattern	$<0.01^{*}$
Time, Media and Pattern	$8.75x10^{-3*}$
Between pairs of experimental variables	**P value**
Time and Media	0.57
Time and Pattern	$<0.01^{*}$
Media and Pattern	$2.0x10^{-6*}$

*significant difference at $P<0.05$ level.

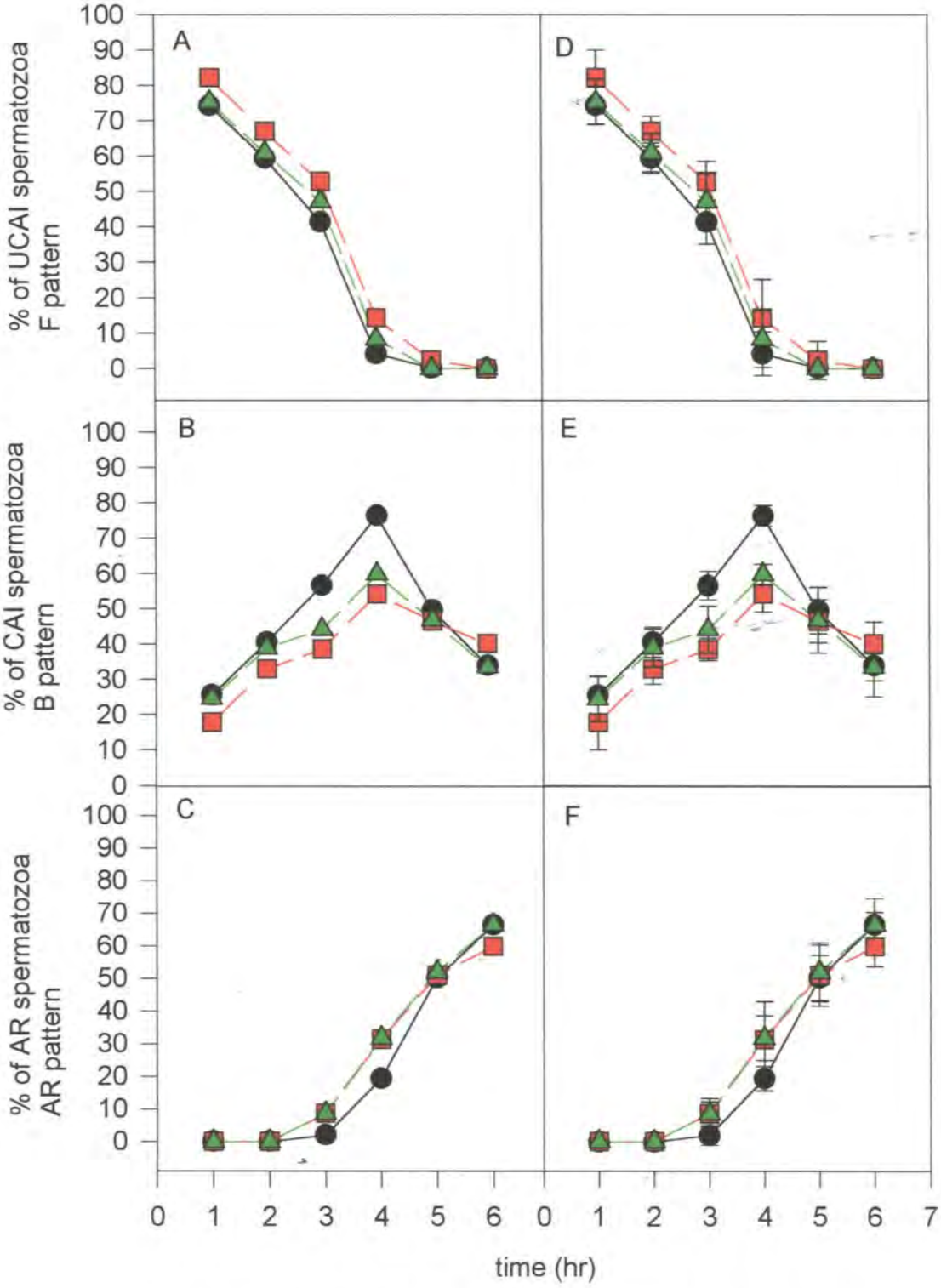

Fig. 4.1.: The effects of melatonin on CTC fluorescence patterns of spermatozoa. In 4.1.A, B, and C, data are presented as means for all three media and in D, E and F the mean ± 1 SD is shown.

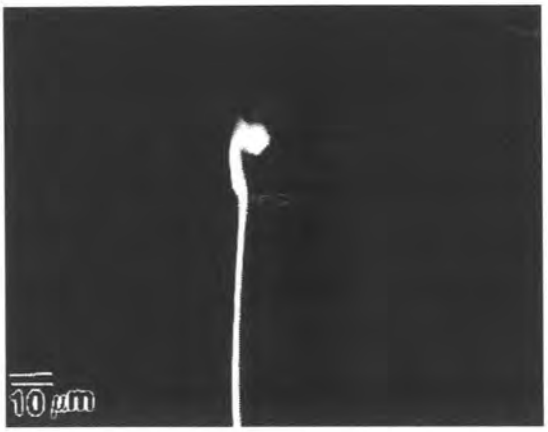

Fig. 4.2.: An epifluorescence micrograph of the F pattern of a CTC-treated Wistar rat spermatozoon: fluorescence over the entire head.

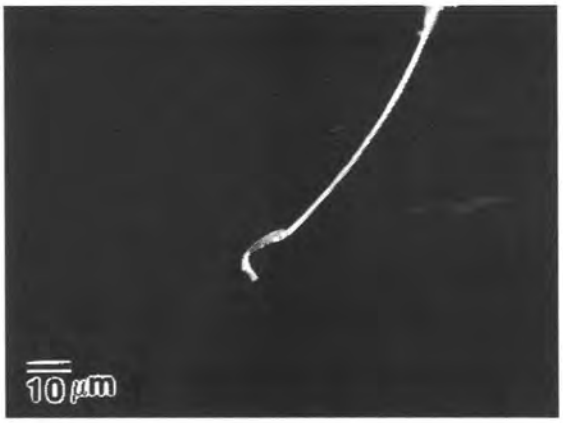

Fig. 4.3.: An epifluorescence micrograph of the B pattern of a CTC-treated Wistar rat spermatozoon: fluorescence-free post acrosomal

Fig. 4.4.: An epifluorescence micrograph of the AR pattern of a CTC- treated Wistar rat spermatozoon: fluorescence free head.

In summary, melatonin negatively affected the time course of onset and the progression of capacitation and the acrosome reaction, as revealed by the CTC fluorescence assay.

CHAPTER FIVE
THE EFFECTS OF pH ON SPERM MOTLITY IN WISTAR RATS

The aim of this experiment was to investigate and assess the effects of pH on rodent sperm motility *in vitro*. The media used for the incubation of spermatozoa in Chapter Three were of different pHs, with DMEM having a pH of 7.6, whilst EDMEM and MelDMEM had a pH range of 7.8 to 8.3.). This experiment was an attempt to separate the effects of melatonin from the effects of pH.

5.1 MATERIALS AND METHODS:

5.1.1 Animal husbandry:

Adult male laboratory rats (297-393g) of the Wistar strain were utilized for the experiment and were supplied by the South African Vaccine Producers, Johannesburg. Animals were kept in a controlled environment (CE) room under long photoperiod conditions (14L:10D-lights on 06:00, lights off 20:00) at approximately 25^0C.

The animals did not receive any treatment and were maintained in groups of five males per clear plastic cage (38.5 x 38.5 x 22.5cm) with wire mesh bases and covers. The male rats were not housed in the same room as female rats. The animals were provided with commercial nutri-chunks dog food and water *ad lib.*. The cages were cleaned twice a week (every Monday and Friday) prior to feeding the animals. The position of the testes was determined by palpation when the animals arrived and before they were sacrificed.

5.1.2 Experimental protocol:

The laboratory conditions and sterile techniques were as described in Section 2.1.4.

5.1.2.1 Experiment:

One experiment lasting a period of five days and utilizing five animals was undertaken. On each experimental day one animal was weighed and then sacrificed as described in Section 2.1.6. Spermatozoa were collected as described in Section 2.1.8.1 and incubated in one of three different media: DMEM (pH of 7.6) and two DMEM media with pH adjusted to 8.0 and 8.2. Paraffin oil was used to overlay sperm suspensions. Spermatozoa were processed and assessed for sperm concentration, and then, hourly for six consecutive hours, assessed for the three parameters of sperm motility (the % of fpm, the vigor or quality, and the pattern of motility shown) as described in Section 2.1.8.2.

5.1.3 Media:

On each experimental day, DMEM was aliquoted into two sterile 20ml bottles. The pH of the DMEM in the two bottles was adjusted with an HCl: distilled water (1:1, v/v) solution to 8.0 and 8.2, and the media will hereafter be referred to as 8.0P and 8.2P. DMEM served as a control. Prior to an experimental procedure, DMEM, 8.0P and 8.2P solutions were equilibrated and supplemented (hereafter referred to as SDMEM, S8P, and S8.2P) as described for SDMEM in Section 2.1.5. Paraffin oil was equilibrated as described in Section 2.1.5.

5.1.4 Statistical analysis:

Data are presented as means and ± 1 SD. Statistical analyses were conducted using SigmaStat. The effects of media and time were assessed using a repeated measures two way analysis of variance (two way RM ANOVA), and differences within a medium were compared using a

repeated measures one way analysis of variance (one way RM ANOVA) and a repeated measures one way analysis of variance on ranks (one way RM ANOVA on Ranks). Data in percentages were arcsine transformed for statistical analyses. Differences were considered significant at $P<0.05$.

5.2 RESULTS:

All the males (n=5) had scrotal testes before being sacrificed.

5.2.1 Assessment of spermatozoa:

5.2.1.1.1 Sperm concentration:

At t_0, all the spermatozoa were intact, motile and without any observable structural defects and were regarded as normal. The sperm concentration of the experimental animals ranged from 4 to 8.6 x 10^6 sperm/ml.

5.2.1.1.2 Motility:

Three parameters of the sperm motility: the percent of forward progressive movement (% fpm), the vigor or quality, and the patterns of motility shown were assessed. At t_0, the initial % of fpm ranged between 96.4 and 100, whilst the initial vigor or quality was 2.5 (on the Bavister scale), and the sperm motility pattern A was observed. Sperm head to head agglutination in the first hour of incubation was generally observed in all spermatozoa of the different media, but disappeared at t_2 when single freely swimming spermatozoa were most common.

In all media, the % of fpm decreased significantly during the experiment (Table 5.1; $P<0.05$, Table 5.2). The extent to which % fpm decreased was significantly affected by both time and medium (two way RM ANOVA; one way RM ANOVA, $P<0.05$ for both, Table 5.2), and increased with

increasing pH.

The % reduction in fpm mirrors the % fpm, and for all spermatozoa from the different culture media, the % reduction in fpm increased gradually with time, and with increasing pH (Fig. 5.1). Statistical analyses of these data revealed that both time and medium significantly affected the % reduction in fpm (two way RM ANOVA; one way RM ANOVA, $P<0.05$ for both, Table 5.2). The vigor or quality of sperm motility in all the different media varied significantly as the experiment progressed (Table 5.1). In SDMEM there was an increase in the vigor or quality of sperm motility with the highest scores observed at t_4. A score of 5 was only observed in some individual SDMEM micro-wells but not as a mean score representing all the 4 micro-wells. In S8.0P and S8.2P media, there was a smaller increase in the vigor or quality with the highest scores observed at t_4. The maximum scores for the vigor or quality of spermatozoa incubated in S8.0P and in S8.2P were lower than the equivalent scores for SDMEM (Table 5.1). Statistical analyses of these data revealed that both time and medium had significant effects on the vigor or quality of sperm motility (two way RM ANOVA; one way RM ANOVA, $P<0.05$ for both, Table 5.2).

As the experiment progressed, spermatozoa incubated in SDMEM started to swim more freely, more vigorously and a linear direction of fpm was observed. The sperm motility pattern A was observed between t_0 and t_1, B, between t_2 and t_3, but more pronounced at t_3, C, between t_3 and t_5, but more pronounced at t_4, D, between t_5 and t_6. The sperm motility pattern A was correlated to 3, B, to 4-4.5 and C, to 4.5-5, on the Bavister scale (Table 5.1). D could not be accommodated on the Bavister scale.

Spermatozoa incubated in S8.0P and S8.2P showed a pattern of sperm motility that was different from that observed in spermatozoa incubated in SDMEM. Progression of spermatozoa from one pattern to the subsequent patterns of motility of sperm motility was delayed and/or prolonged. From the third hour of the experiment, the values on the Bavister scale were lower than the equivalent scores for spermatozoa incubated in SDMEM (Table 5.1), and there was no progression from the observed sperm motility pattern A (3 on the Bavister scale) to subsequent patterns. In spermatozoa incubated in S8.0P, a progression to the B pattern (4-4.5 on the Bavister scale) of sperm motility, was observed.

Sperm incubation in SDMEM with an oil overlay showed the usual (see Chapters two and three) changes in SMI with time. SMI values reached a maximum of 1600 and 1800 at t_4 and t_5 (Fig. 5.2.B). In spermatozoa incubated in S8.0P, there was an initial increase in SMI to values of 1000 at t_4, followed by a decline to SMI values of approximately 400 at t_6 (Fig. 5.2.C). With S8.2P, there was no change in SMI at t_4 after which the SMI declined to approximately 300 at t_6 (Fig. 5.2.D).

Both medium and time had significant effects on SMI (Fig. 5.2.; two way RM ANOVA; one way RM ANOVA, $P<0.05$ for both, Table 5.2).

Table 5.1: The effects of time and pH on the % of sperm forward progressive movement and the vigor or quality of motility of spermatozoa of Wistar rats. Data are presented as means and ± 1 SD.

Medium	Time(hr)	% Of sperm forward progressive movement	Vigor or quality of sperm motility (Bavister scale)
SDMEM	1	96.0± 1.4	2.5±0.0
	2	92.9±1.6	3.1±0.2
	3	92.3±1.7	3.5±0.0
	4	87.1±5.1	4.5±0.0
	5	84.7±4.3	4.5±0.0
	6	85.9±1.0	4.1±0.2
S8.0P	1	90.5±1.4	2.4±0.0
	2	84.4±5.3	3.0±0.0
	3	82.6±5.7	3.0±0.0
	4	75.9±6.5	3.8±0.3
	5	71.9±10.9	3.3±0.5
	6	60.4±16.4	2.7±0.5
S8.2P	1	91.3±2.4	2.5±0.0
	2	81.2±6.9	2.6±0.2
	3	74.1±7.5	2.8±0.3
	4	67.5±7.1	3.1±0.7
	5	58.3±9.6	2.5±0.5
	6	48.4±8.7	2.5±0.5

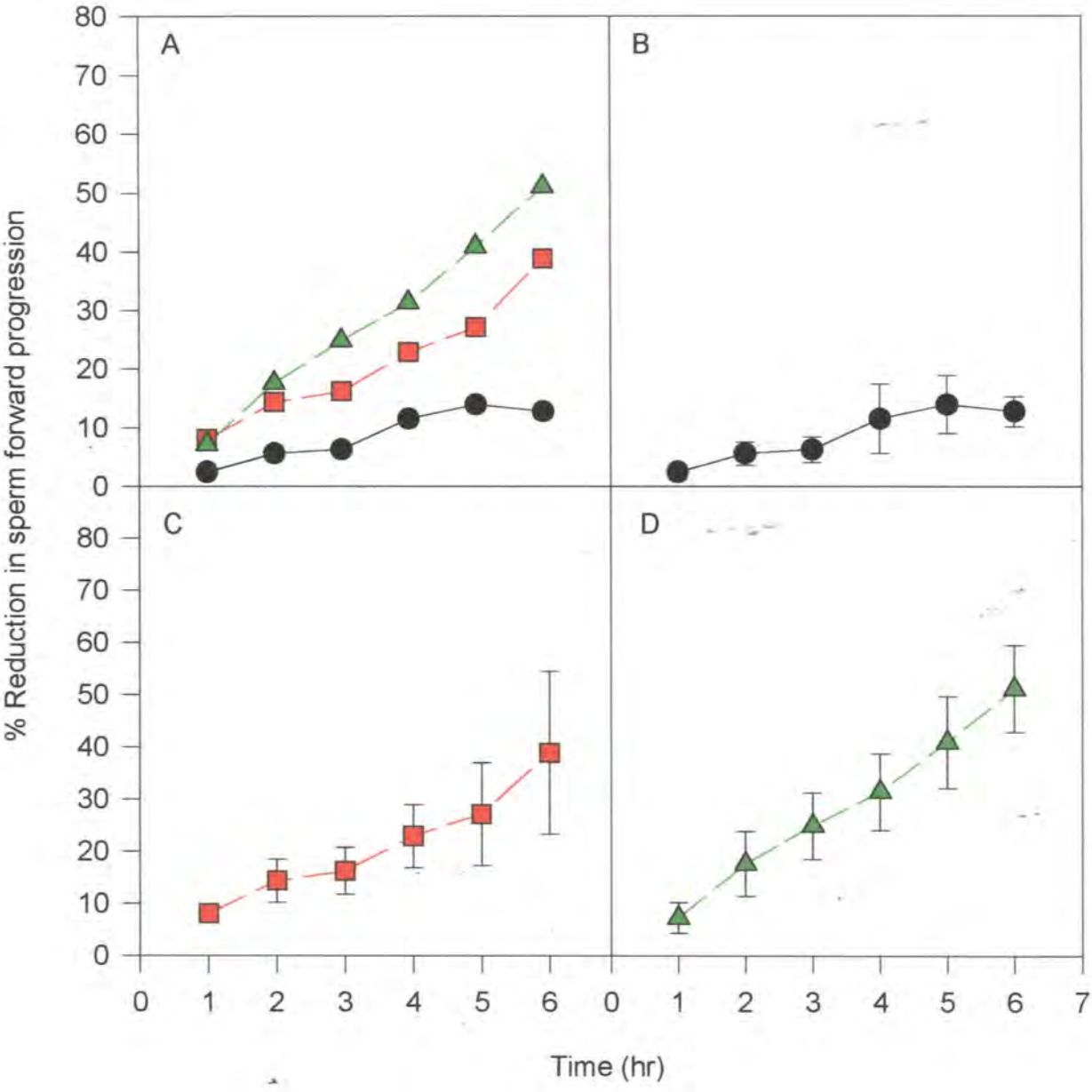

Fig. 5.1.: The effects of pH on % reduction in sperm forward progression. In 5.1.A., data are presented as means for all three media. In B, C, and D data are separated for clarity, and the mean ± 1 SD is shown.

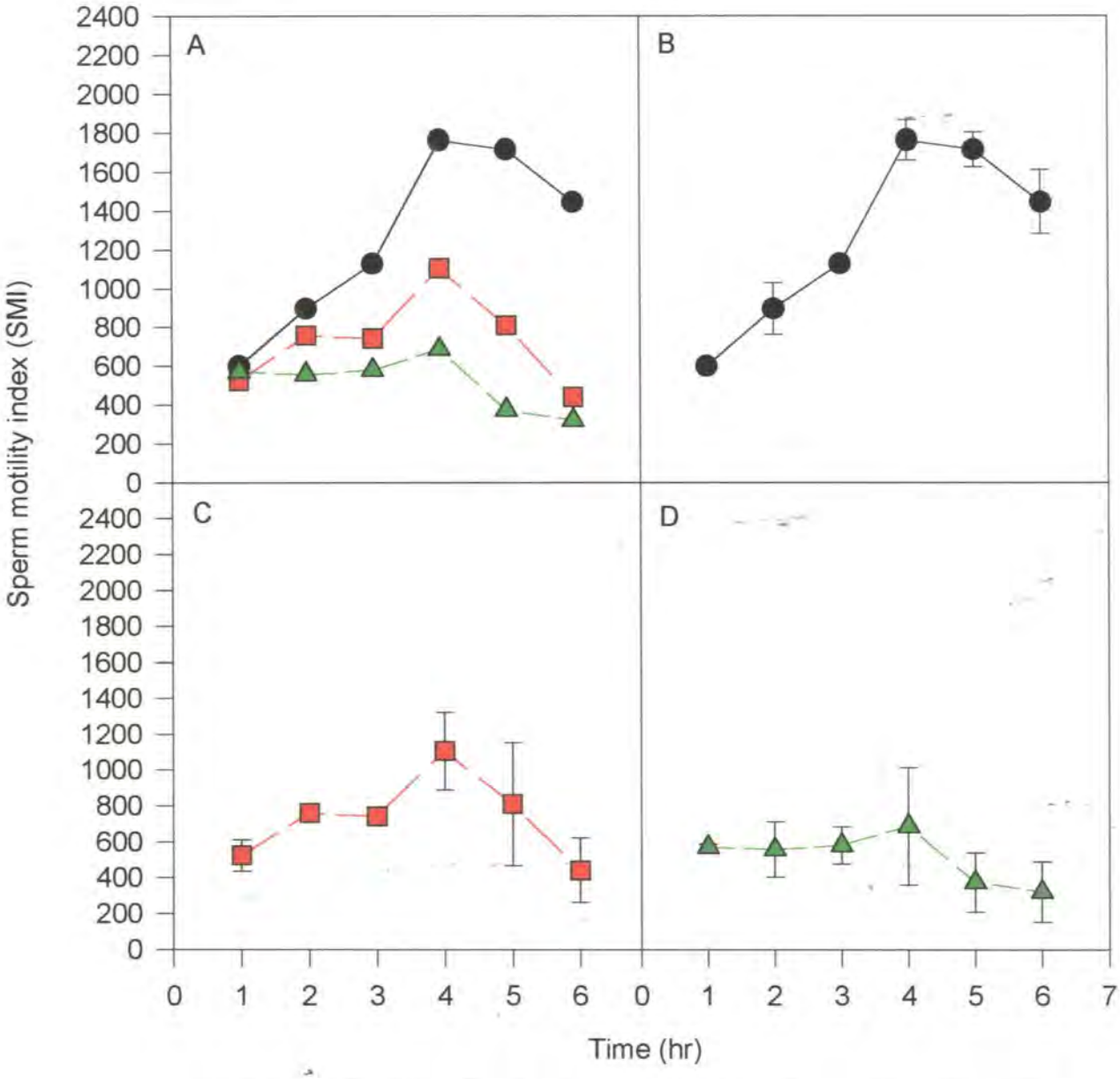

Fig. 5.2.: The effects of pH on sperm motility index. In 5.1.A., data are presented as means for all the three media. In B, C, and D, data are separated for clarity, and the mean ± 1 SD, is shown.

SDMEM, S8P, S8.2P.

Table 5.2: Results of the statistical analyses (two way RM ANOVA and one way RM ANOVA) of the effects of time and pH on the % of sperm forward progressive movement, % reduction in sperm forward progressive movement, vigor or quality of motility of spermatozoa, and sperm motility index of Wistar rats.

Media	Dependant variable							
	% Of sperm forward progressive movement		% Reduction in sperm forward progressive movement		Vigor or quality of sperm motility		Sperm motility index	
	P value							
Between all media	**Treatment effect**	**Time effect**	**Treatment effect**	**Time effect**	**Treatment effect**	**Time effect**	**Treatment effect**	**Time effect**
	$4.8x10^{-5*}$	$9.0x10^{-11*}$	$1.3x10^{-4*}$	$2.5x10^{-10*}$	$4.3x10^{-5*}$	$3.6x10^{-9*}$	$3.9x10^{-7*}$	$2.9x10^{-8*}$
Within media	**Time effect**		**Time effect**		**Time effect**		**Time effect**	
SDMEM	$5.1x10^{-6*}$		$1.6x10^{-6*}$		$1.7x10^{-4*}$		2.210^{-4*}	
S8.0P	$1.3x10^{-6*}$		$1.9x10^{-6*}$		$7.1x10^{-6*}$		$9.4x10^{-4*}$	
S8.2P	$1.5x10^{-10*}$		$4.6x10^{-10*}$		$3.5x10^{-2*}$		$6.0x10^{-3*}$	

* significant difference at $P<0.05$ level.

In summary, increasing pH had a negative effect on sperm motility, *in vitro*.

CHAPTER SIX
GENERAL DISCUSSION

The effects of melatonin on mammalian reproduction are complex, varied and variable, and not fully understood (Wuurtman *et al.*, 1963; Reiter 1978; Lang *et al.*, 1983; Stetson and Tay, 1983, Vaughan *et al.*, 1986; Anderson *et al.*, 1988; Lewinski *et al.*, 1993; Edmonds and Stetson, 1994; Niklowitz *et al.*, 1996).

Different species respond to melatonin treatment in different ways. Whilst male golden hamsters show a complete antigonadal response to melatonin treatment (Stetson and Tay, 1983), male rats do not show such a response at all, even when higher doses of melatonin are administered (Lang *et al.*, 1983). In women, the production of melatonin drops noticeably around the period of ovulation (Reiter and Robinson, 1995), and exogenous melatonin causes an antigonadal response (Voordouw *et al.*, 1992).

Melatonin has quite different effects in rats depending on the age of the animals. In immature female rats, melatonin elicits an antigonadal response, whereas in adults, melatonin elicits a far weaker antigonadal response and for only the first day after treatment (Wuurtman *et al.*, 1963).

In the djungarian hamster, males and females respond differently, with all treated females but only 15% of the treated males showing an antigonadal response (Niklowitz *et al.*, 1996), whereas in syrian hamsters (Vaughan *et al.*, 1986) and golden hamsters (Anderson *et al.*, 1988), males and females both show a similar antigonadal response to melatonin treatment. In adult male rats, melatonin elicits a weak but statistically significant antigonadal response in only one of the

reproductive parameters assessed (Lang *et al.*, 1983) and in females, there is no significant antigonadal response (Lewinski *et al.*, 1993).

There are other factors determining the response of animals to the melatonin treatment: the dose of melatonin administered, the mode in which melatonin is administered to animals, the time of the day at which melatonin is administered, the length of the period of melatonin treatment, and the photoperiod under which melatonin is administered (Reiter, 1978; Anderson *et al.*, 1988; Edmonds and Stetson, 1994). Melatonin may be an antigonadal or a counter- antigonadal agent in animals, and the desired melatonin effect can be achieved by an appropriately timed and/or well chosen mode of administering melatonin to animals (Reiter *et al.*, 1975; Reiter, 1978).

In juvenile male Wistar rats, melatonin exerts a dose-dependent inhibitory action on sexual maturation with chronic doses causing a highly significant decrease in all the reproductive parameters studied (Lang *et al.*, 1983). Similar treatment during pre-pubertal period has no significant effect (Lang *et al.*, 1983).

Whilst late afternoon daily melatonin injections are antigonadal or antigonadotropic in their effect (Stetson and Tay, 1983; Amador *et al.*, 1986), morning injections (Chen *et al.*, 1980), and implanted melatonin pellets (Chen and Reiter, 1980; Vaughan *et al.*, 1988), counteract the antigonadal or antigonadotropic effect of late afternoon daily injections or blinding. The best time for melatonin injections to elicit an antigonadal response is late afternoon (Reiter, 1978; Stetson and Tay, 1983; Anderson *et al.*, 1988; Vaughan *et al.*, 1988), which was the time at which melatonin injections were administered to the animals in my study.

A very lengthy period of melatonin treatment has been shown to result in animals loosing their

already attained antigonadal response to the melatonin treatment and hence start to show a response that is counter-antigonadal (Stetson and Tay, 1983). A possible explanation for the loss of the already attained antigonadal response of animals to melatonin treatment, is the saturation of the presumed receptors by the continued supply of melatonin, which would keep the melatonin receptors in a 'down-regulated' state (Vaughan *et al.*, 1986).

Several *in vitro* experiments demonstrate and support the hypothesis for a direct effect of melatonin on gonadal function. Melatonin, significantly inhibits oxytocin release, reduces progesterone, vasopressin, and calcium adenose monophosphate (cAMP) levels, significantly stimulates calcium guanine monophosphate (cGMP), and stimulates estradiol secretion by granulosa cells isolated from porcine ovaries (Sirotkin, 1994). Melatonin significantly reduces cAMP production, decreases progesterone and estradiol production only in the presence of hCG in preovulatory follicles in the cyclic hamster (Tamura *et al.*, 1998), and significantly stimulates progesterone production by human and bovine granulosa cells (Webley and Luck, 1986). Melatonin decreases testosterone production by mouse Leydig cells (Persengiev and Kehajova, 1991), and significantly reduces the luteinizing hormone-releasing hormone (LHRH)-stimulates release of LH by neonatal pituitary gland cells (Martin *et al.*, 1977).

In a mouse testis *in vitro* system, melatonin at physiological (10^{-10} to 10^{-9} M) and at supra-physiological (10^{-7} M) concentrations fails to suppress hCG or dibutyrl-calcium adenose monophosphate (dbcAMP)-stimulated secretion of testosterone, and such a failure is evidence that melatonin does not interact either with receptor-related or intracellular steriodogenic pathways in the testes to inhibit steriodogenesis (Knotts *et al.*, 1988).

In my study, the animals were kept in environmental conditions that were conducive for spermatogenesis and all the animals were scrotal at the start of the experiment and were scrotal

and producing normal spermatozoa after six weeks of treatment with melatonin. The plasma testosterone concentrations of all three experimental groups were similar and were within the normal physiological range for spermatogenetically active rodents (Sharpe *et al.*, 1988; Perheentupa *et al.*, 1995). The mass of the epididymides and testes of the animals from all three experimental groups were not statistically significantly different. Thus, melatonin did not inhibit spermatogenesis and these results support the hypothesis that the reproductive system of a laboratory bred mammal is not as responsive to exogenous melatonin as that of seasonally breeding mammals like the hamsters (Lang *et al.*, 1983; Reiter *et al.*, 1985). Although it has been suggested that correctly timed exogenous melatonin injections in non-seasonally breeding mammals, may have an antigonadal effect (Meyer and Theron, 1988), Vaughan *et al.* (1988) and Lewinski *et al.* (1993), observed a similar lack of an antigonadal response to melatonin treatment in male and female rats, respectively.

It is established that the dose of melatonin is important, and similar doses of melatonin have been used in previous experiments: (25μg,Vaughan *et al.*, 1988 and Lewinski *et al.*, 1993, 5-100μg, Lang *et al.*, 1983, in rats; 25μg, Vaughan *et al.*, 1986 and Anderson *et al.*, 1988 in syrian and golden hamsters respectively, and 50μg, Niklowitz *et al.*, 1996, in djungarian hamsters). In all the studies reported here on rats (Vaughan *et al.*, 1988; Lewinski *et al.*, 1993), melatonin, either has no significant antigonadal effect, or elicits a weak but statistically significant antigonadal response (Lang *et al.*, 1983). In contrast, in all the studies reported here on hamsters (Vaughan *et al.*, 1986; Anderson *et al.*, 1988; Niklowitz *et al.*, 1996), melatonin elicits a significant antigonadal effect on the reproduction of animals. Since the concentration of melatonin used in my study is similar to that used on hamsters, it is unlikely that the failure to obtain an antigonadal response to melatonin treatment in my study was due to the dose.

The failure of melatonin to inhibit spermatogenesis in my study, even with doses of melatonin as high as 100μg (Lang *et al.*, 1983), could be attributed to the fact that the laboratory rat has been highly inbred for a long period in the laboratory, where there are constant photoperiodic conditions. This has an effect of selecting for those animals that will reproduce under captive conditions and selecting against individuals that are photoresponsive. Consequently, reproduction in the laboratory rat is no longer responsive to the factors such as melatonin associated with natural photoperiodic cues (Vaughan *et al.*, 1988).

Although in my study melatonin treatment *in vivo* did not inhibit testosterone production or spermatogenesis, it did have a small but significant negative effect on sperm motility and SMI, both *in vivo* and *in vitro*. This suggests that the effects of melatonin may be directly on the spermatozoa.

Several studies have been undertaken in an effort to elucidate the effects of melatonin on sperm motility. Various concentrations of melatonin ($6.46x10^{-10}$M, $1.94x10^{-9}$M, and $1.29x10^{-9}$M) have been utilized to investigate the effects of melatonin on human sperm motility *in vitro* (Irez *et al.*, 1992). van Vuuren *et al.* (1988) reported the presence of melatonin in the seminal fluid with levels that were highly significantly positively correlated to the plasma melatonin levels. In addition, it was observed that high seminal melatonin levels (>33.3pg/ml) were associated with abnormal sperm forward motility rates in 13% of the patients, while low seminal melatonin levels (<17.7pg/ml) were associated with a high incidence (63%) of abnormal sperm forward motility rates in patients.

In another study, melatonin reduced motility in human spermatozoa *in vitro* and, remarkably, at high concentrations that were still within physiological levels, completely abolished movement

(Oosthuizen *et al.*, 1986). Furthermore, the authors detected a higher concentration of seminal melatonin in patients with asthenozoospermia (40% progressively motile spermatozoa) than in patients with spermatozoa with normal motility.

Other findings supporting the hypothesis that melatonin has negative effects on sperm motility were reported by Yie *et al.* (1991). A negative correlation was detected between seminal melatonin concentration and the rate of sperm forward motility, with melatonin concentrations in patients with abnormal sperm progression being higher than in the normal group of patients. The authors reported higher levels of melatonin in mild oligospermic (lower sperm concentration) and azoospermic (no spermatozoa in seminal fluid) patients but not in moderate and severe oligospermic patients than in normal groups, although no significant correlation was detected between melatonin and sperm concentration.

In contrast to other authors who have reported results that support the hypothesis that melatonin has negative effects on sperm motility, Bornman *et al.* (1989, 1992), who used radio-immunoassay kits that were different from each other and different from those used by the other authors (reported here) detected no correlation between the human seminal melatonin concentration and any of the semen parameters that were assessed. There was also no statistical difference between the mean plasma and the seminal melatonin levels of patients with good or impaired sperm motility and forward progression, thereby suggesting no influence or role of melatonin on sperm motility.

Throughout my study and in all the experiments, all the animals from the different experimental groups produced spermatozoa that were intact and did not exhibit any observable structural defects and were regarded as normal. In the *in vitro* experiments (chapter three; chapter five), in

spermatozoa incubated in SDMEM, the changes observed in the parameters of sperm motility that were assessed were similar to the changes observed *in vitro*, in spermatozoa from the *in vivo* experiment (chapter two). In my study, spermatozoa incubated in SDMEM (control culture medium) showed a sequence of changes in the pattern of sperm motility that was similar to that reported *in vitro* in hamster (Yanagimachi, 1970; Bavister, 1974; Yanagimachi, 1982; Bavister and Andrews, 1988; Boatman and Robbins, 1991), rat (Shalgi and Phillips, 1988), and human spermatozoa (Irez *et al.*, 1992).

In spermatozoa incubated in SMelDMEM and SEDMEM, a difference in the parameters of sperm motility compared to spermatozoa incubated in SDMEM, was observed. The SMelDMEM concentrations ($1x10^{-3}$M, $1x10^{-5}$M, $1x10^{-6}$M, $1x10^{-9}$M, and $1x10^{-12}$M) that were utilized in this study were within and on either side of the normal physiological range of plasma melatonin concentration in rodents (Knotts *et al.*, 1988). Melatonin negatively affected all the parameters of sperm motility that were assessed: reduced the % of fpm thereby increasing the % reduction in fpm, reduced the vigor or quality of motility, delayed and/or prolonged the transition of one motility pattern to the subsequent pattern, and reduced the SMI. These negative effects were statistically significant and increased with increasing melatonin concentration and with time. However, they were ameliorated by the addition of an oil overlay in the sperm culture medium. How much of this amelioration was due to the extremely lipophilic and hydrophilic nature of melatonin, which would have been taken up by the oil overlay, or to the specific advantages conferred upon the spermatozoa by the use of equilibrated paraffin as an overlay in the sperm culture system (Dale and Edler, 1997), is not certain.

As in my study, melatonin elicits a similar statistically significant reduction of the mean % of fpm of spermatozoa that is both time- and dose-dependent, in humans (Irez *et al.*, 1992).

Furthermore, melatonin reduces the mean sperm velocity (Irez *et al.*, 1992). The difference in my study and that of Irez *et al.* (1992), concerning the dose dependent reduction effect of melatonin on sperm motility is that in my study the highest melatonin concentration was more effective than the lowest concentration in its effects, whereas in the study by Irez *et al.* (1992), the lowest concentration of melatonin was more effective than the highest concentration in its effect. In my study, a concentration range wider than and including the one used by Irez *et al.* (1992), was utilized.

Results from my study (both *in vivo* and *in vitro)* indicate that increasing melatonin concentrations have a negative effect on sperm motility and thus support the hypothesis that melatonin may influence or control sperm motility in mammals.

Sperm motility is possibly under the control of an unknown hormone acting through the receptor/adenylate cyclase/cyclic AMP/protein phosphorylation cascade (Guraya, 1987), and melatonin may be this unknown hormone (Pitout *et al.*, 1991). Several theories attempt to explain the mechanism of action of melatonin on spermatozoa.

1. Sialation of putative melatonin binding sites or receptors.

Sperm immotility is possibly associated with a high seminal sialic acid content and sialic acid may mask the spermatozoal melatonin binding sites (Pitout *et al.*, 1991; van Vuuren *et al.*, 1992). In addition, consistent low-affinity binding sites for melatonin have been detected on the human sperm midpiece only after the removal of sialic acid. Therefore sialic acid may influence any effect that melatonin might have on sperm motility (Pitout *et al.*, 1991; van Vuuren *et al.*, 1992).

There are two possible explanations supporting this theory. Firstly, du Toit *et al.* (1994) detected

a positive correlation between the spermatozoal adenosine triphosphate (ATP) concentrations and the sperm-bound sialic acid concentration. In addition, since sialic acid has been associated with the masking of melatonin binding sites in spermatozoa (Pitout *et al.*, 1991; van Vuuren *et al.*, 1992), sialic acid may mask receptors on the sperm membrane that may affect the metabolism of the spermatozoon (du Toit *et al.*, 1994) thus inhibiting the breakdown of ATP to its lower nucleotides thereby resulting in poor sperm motility.

Secondly, melatonin decreases cyclic AMP (cAMP) production in the rat medial basal hypothalamus (Bettahi *et al.*, 1998), in granulosa cells isolated from porcine ovaries (Sirotkin, 1994), in preovulatory follicles in the cyclic hamster (Tamura *et al.*, 1998), and in the rat corpus epididymis (Li *et al.*, 1998), *in vitro*. Ca^{2+} and cAMP have both been proposed as the intracellular messengers that regulate mammalian sperm motility, with Ca^{2+} stimulating the flagellar apparatus and increased cAMP levels stimulating sperm motility (Shapiro and Eddy, 1980; Yanagimachi, 1988). Therefore, melatonin bound to the sperm midpiece membrane may reduce cAMP levels thereby decreasing sperm motility (Irez *et al.*, 1992). Furthermore, a study supporting this possible mechanism of action of melatonin was undertaken by Li *et al.* (1998), who reported an action of melatonin via its receptors in the regulation of the rat corpus epididymal epithelial cell functions. This regulation was shown to also involve the inhibition of cAMP accumulation.

2. The interaction of melatonin with the microtubular assembly.

It has been suggested that melatonin may interact with the microtubules (Huerto-Delgadillo *et al.*, 1994) and may thus influence sperm motility (Bornman *et al.*, 1989; 1992). Melatonin has been shown to affect the microtubular assembly, and the effects at low concentration (10^{-9}M) were mediated by Ca^{2+}/CaM whereas at higher concentration (10^{-5}M) were related to non-specific

binding of melatonin to tubulin (Huerto-Delgadillo *et al.*, 1994). Intracellular Ca^{2+} and cAMP are both important regulators of the movement of the sperm flagellum (Shapiro and Eddy, 1980; Yanagimachi, 1988). In addition, melatonin may have a colchicine-like inhibitory effect on microtubules (Bornman *et al.*, 1989) and might bind specifically to the protein tubulin at the colchicine-binding site (Irez *et al.*, 1992).

The structure of the flagellum is associated with the movement of the spermatozoa (Bornman *et al.*, 1989). The flagellum contains the energy sources, and the axonemal microtubular complex is responsible for the production of locomotive force for the spermatozoon (Gibbons, 1983; Cunningham, 1983; Eddy, 1988; Millete, 1999). Furthermore, the membrane covering the middle piece contains proteins that may influence the mitochondrial production of ATP necessary for the modulation of microtubule sliding, and numerous axonemal proteins that may be involved in the stimulation of sperm motility (Gibbons, 1983; Millete, 1999).

According to Millete (1999), the sperm microtubules are made up of α- and β-tubulin; kinesin attaches to the surface of microtubles and stimulates motility; dynein which is a protein with ATPase activity is found in the arms extending from microtubular doublets and may generate the force needed for the sliding axonemal elements resulting in motion, and nexin forms the links between the outer microtubular doublets and may be necessary for mammalian sperm flagella motility. Melatonin may therefore execute its action by binding to tubulin of the sperm flagellum and prevent the assembly of the 6S tubulin dimer into the microtubules, thereby decreasing sperm motility (Irez *et al.*, 1992).

The results obtained by Huerto-Delgadillo *et al.* (1994) on how melatonin interacts with the microtubular assembly may also explain why in my study the reduction in sperm motility *in vitro*

was prominent at higher concentrations (10^{-5}M;10^{-3}M) than at lower concentrations (10^{-6}M; 10^{-9}M; 10^{-12}M) of melatonin, as the binding of melatonin to tubulin has been suggested to decrease the sperm motility (Irez *et al.*, 1992).

This theory (the interaction of melatonin with the microtubular assembly) has been refuted by some researchers suggesting that either the colchicine-like inhibitory effects of melatonin on microtubules are applicable only to newly-formed microtubular protein, for example during cell division (Bornman *et al.*, 1989), and not to the already existing sperm flagellar microtubular assembly, or that melatonin does not act like a colchicine-like mitotic or microtubular assembly inhibitor (Poffenbarger and Fuller, 1976).

3. The cellular effects of melatonin and steroid receptors.

This theory suggests that since the cellular effects of melatonin are mainly observed in areas containing steroid receptors, and spermatozoa also have steroid receptors, then the steroid receptors may be a site of action for melatonin in spermatozoa (Irez *et al.*, 1992).

From the previous discussion, it is clear that there are conflicting views on the effect of melatonin on sperm physiology [for example, compare Yie *et al.* (1991) and Irez *et al.* (1992), and Bornman *et al.* (1989, 1992) and Oosthuizen *et al.* (1986)]. These results, both from different laboratories and from the same laboratory, might be due to differences in the techniques used (for example, radio-immunoassay kits) by different researchers.

There is a major flaw with the suggestion that melatonin has no influence on sperm physiology (Bornman *et al.*, 1989; 1992). Firstly, Bornman *et al.* (1989) simply added melatonin powder to spermatozoa and rotated the sperm suspension for 15 minutes before the evaluation of the sperm

motility parameters. It is possible that the melatonin powder did not dissolve completely or at all in the sperm suspension due to its well known extremely lipophilic and hydrophilic nature. With melatonin not completely dissolved in the sperm suspension, the effects of melatonin would not have been as marked, and hence no correlation was found between melatonin and all the sperm motility parameters that were assessed.

Bornman *et al.* (1989) attributes the observed reduction of sperm motility in the earlier study (Oosthuizen *et al.*, 1986) to the solvent they utilized for dissolving melatonin. Although the solvent is not specified in either paper, my results have shown that ethanol does indeed reduce sperm motility. However, this does not necessarily mean that the reduction in sperm motility can be attributed solely to the effect of ethanol.

While my results suggest that melatonin may negatively affect sperm motility, it is important to emphasize that the apparent effect of melatonin may be exaggerated by, in addition to melatonin and ethanol, the resulting changes in pH of the solution, as spermatozoa are sensitive to what constitutes their microenvironment and to changes that occur therein (Bavister and Andrews, 1988).

In my study, it was revealed that ethanol negatively affected all the parameters of sperm motility that were assessed: reduced the % of fpm thereby increasing the % reduction in fpm, reduced the vigor or quality of motility, delayed and/or prolonged the transition of one motility pattern to the subsequent pattern, and reduced the SMI. These negative effects on sperm motility were statistically significant and were pronounced with increasing melatonin concentration and with time.

Ethanol increased the pH of the culture medium as it was realized that EDMEM and the MelDMEM had different pH values compared to DMEM, with DMEM having a pH of 7.6, whilst EDMEM and MelDMEM had a pH range of 7.8 to 8.3. The pHs of 8.0 and 8.2 that were utilized in this study were within the pH range of EDMEM and MelDMEM and they negatively affected all the parameters of sperm motility that were assessed. They reduced the % of fpm thereby increasing the % reduction in fpm, reduced the vigor or quality of motility, delayed and/or prolonged the transition of one motility pattern to the subsequent pattern, and reduced the SMI. A similar observation was made in hamster epididymal spermatozoa incubated in a culture medium of a pH range of 7.2 to 8.4 (Yanagimachi, 1970).

In rodents, the pH in different parts of the reproductive tract is different, with the testes and the epididymis having a pH range of 7.2 to 7.4 and 6.5 to 6.6 (Asdell, 1946). In the different parts of female reproductive tract, a difference in the pH at different times of the reproductive cycle has been reported, with the vagina having a pH range of 4.2 to 6.1 and the uterus having a pH range of 7.3 to 7.7 (Asdell, 1946). In my study, the pH of the control culture medium (DMEM) in which hyperactivation of spermatozoa was observed, was 7.6. Hyperactivated motility was observed in hamster spermatozoa incubated in culture medium droplets having a pH range of 7.4 to 7.6 (Bavister, 1974).

In my study, the hypothesis that melatonin may influence sperm physiology was further supported by the observation made on the effects of melatonin on capacitation and the acrosome reaction. Melatonin did not inhibit capacitation and the acrosome reaction, but it negatively affected the time course of these physiological processes. Melatonin delayed the onset and progression of capacitation and the acrosome reaction, and thereby reduced the percentage of capacitated and acrosome-reacted spermatozoa at any given time during the experiment compared to spermatozoa

incubated in SDMEM. The negative effects of melatonin on capacitation and the acrosome reaction were statistically significant and increased with increasing melatonin concentration.

According to Saling and Storey (1979), the variability in the time of appearance of the different CTC patterns between different sperm suspensions is a reflection of individual differences within a species. Similarly in my study as reflected by the standard deviation bars, a small variability between experimental runs in the percentage of spermatozoa, incubated in the same type of medium, showing a certain pattern of the 3 CTC patterns at a particular time of the experiment, was observed.

In my study, capacitation and the acrosome reaction occurred in a time-dependent manner as shown by the time-dependent change in the distribution of the 3 CTC fluorescence patterns, thereby confirming similar observations made by other researchers (Saling and Storey, 1979; Lee and Storey, 1985; Fraser and Herod, 1990).

In the rat spermatozoa, capacitation is achieved in two to three hours (Bedford, 1970) and the acrosome reaction has begun at about three hours after incubation with the percentage of freely swimming acrosome-reacted spermatozoa with activated motility increasing with time (Yanagimachi, 1982). In my study, as assessed by the observation of hyperativated motility and confirmed by the CTC fluorescence assay, the highest percentage of capacitated spermatozoa was at t_4, and was observed in spermatozoa incubated in SDMEM. Incubation in SEDMEM, and MelEDMEM resulted in lower percentages of capacitated spermatozoa. Although the acrosome reaction, as assessed by the CTC fluorescence assay, had already begun at t_3, the majority of acrosome-reacted spermatozoa were observed at t_6. The difference in the time at which capacitation is achieved by the majority of spermatozoa in my study and that reported by Bedford

(1970) could possibly be attributed to differences between spermatozoa from different animals (Yanagimachi, 1982).

Saling and Storey (1979) made two proposals for the mechanism of action of CTC fluorescence in indicating the progression and the stages of capacitation and the acrosome reaction in mouse spermatozoa.

1. As CTC shows the location of membrane-bound Ca^{2+}, its loss as the process of capacitation and the acrosome reaction progress, could be an indication of the loss of membrane-bound Ca^{2+} from the sperm head, attributed to the loss of specific Ca^{2+} binding groups on the sperm membrane.
2. The CTC- Ca^{2+} complex binds specifically to the plasma membrane overlying the acrosomal region and probably to the close acrosomal membrane, but not to the postacrosomal region. As the acrosomal membrane and the overlying plasma membrane are lost during the acrosome reaction, so is the CTC fluorescence.

The CTC assay, unlike the conventional methods, allows differentiation between uncapacitated and capacitated acrosome-intact spermatozoa (Fraser and Herod, 1990), but does not differentiation between spontaneous and induced acrosome reaction or that caused by degeneration or sperm death (Kholkute *et al.*, 1995). However in this study, spermatozoa utilized for the CTC assay were always autopippeted off the top layer of the sperm suspension as freely swimming spermatozoa swim to the top of the sperm suspension.

According to Bedford (1999) and Yanagimachi (1988), increased adenyl cyclase activity may increase the availability of cAMP which plays an important role in tyrosine phosphorylation of some sperm membrane proteins thereby resulting in a change in the physiological properties of the

membrane. For example, membrane permeability to ions that increases the rate of capacitation, and increased intracellular levels of Ca^{2+} are essential for the onset and progression of the acrosome reaction. Melatonin has been shown to decrease cAMP levels (Sirotkin, 1994; Bettahi *et al.*, 1998; Li *et al.*, 1998; Tamura *et al.*, 1998) and interact with Ca^{2+} through the modulation of Ca^{2+}-activated calmodulin (Ca^{2+}/CaM), (Huerto-Delgadillo *et al.*, 1994). Therefore melatonin may delay the onset and progression of capacitation and the acrosome reaction by reducing the necessary levels of both cAMP and Ca^{2+}.

In conclusion, an overwhelming amount of evidence, including the results of my study, has been presented in support for the hypothesis that melatonin negatively affects sperm physiology, with either high (in this and other studies) and/or low (in other studies) levels of melatonin reducing sperm motility, resulting in abnormal forward sperm motility, associated with abnormal sperm morphology, and with low sperm concentration.

Ethanol seems to be the only solvent that will effectively dissolve melatonin, and in a sperm culture medium it negatively affected sperm motility. Ethanol also increased the pH of the solutions and pH negatively affected sperm motility. Melatonin, ethanol, and the resulting increased pH, all negatively affected the physiology of spermatozoa in a time-, concentration-, and strength-dependent manner, and their negative effects were ameliorated by the inclusion of an oil overlay in the culture medium.

Experiments were undertaken to try and separate the effects of melatonin, ethanol and pH. Although these experiments showed that the negative effects of melatonin on sperm motility increased with both increased pH and ethanol concentration, it remains difficult to separate the effects of these three factors. Similarly, the ameliorating effect of the oil overlay may have been

because the oil overlay absorbs the melatonin from the solution, thus lowering the melatonin concentration, or it may have been because the oil overlay enhances the maintenance of the conditions in the medium, thus protecting the spermatozoa from the fluctuations in their micro-environment.

The results from my study, thus add support to the suggestion that high seminal and/or plasma melatonin levels may be causally linked to reduced sperm motility (Oosthuizen *et al.*, 1986; Yie *et al.*, 1991; Irez *et al.*, 1992).

There is overwhelming evidence in the literature, and in my study, that melatonin does not influence spermatogenesis in non-seasonal-breeding mammals but does have an effect in other seasonal-breeding mammals. There is also evidence supporting the hypothesis that melatonin has negative effects on sperm physiology. In the light of this, it is suggested that, in addition to influencing and/or controlling spermatogenesis in other mammals, melatonin may also influence the physiology of spermatozoa in mammals.

The greatest challenge facing researchers is to understand at the cellular and molecular level how melatonin influences the hypothalamus-pituitary-gonadal axis, and to evaluate critically such information before extrapolating it to humans.

Further experiments on the effects of melatonin on the reproduction of non-seasonal breeding mammals like the human and laboratory rats, could bring about more insight on the potential use of melatonin as a contraceptive agent in both males and females, and as a means of controlling reproduction in other mammals.

REFERENCES

Acosta, A.A., Swanson, R.J., Ackerman, S.B., Kruger, T.F., van Zyl, J.A. & Menveld, R. (1990), (Eds) Human Spermatozoa in Assisted Reproduction. Williams & Wilkins, Baltimore.

Amador, A.G., Richardson, B.A., Klemcke, H.G., Vaughan, M.K., Steger, R.W., Bartke, A. & Reiter, R.J. (1986) Effects of long-term treatment with melatonin or melatonin plus ectopic pituitary transplants on testicular LH/hCG and prolactin receptors in juvenile Syrian hamsters (Mesocricetus auratus). **Journal of Reproduction and Fertility, 78: 557-564.**

Anderson, K.D.; Nichimen, RJ & Turk, FW (1988) Effects of melatonin and 6-Methoxybenzoxalinone on photoperiodic control of testis size in adult male Golden hamsters. **Journal of Pineal Research, 5: 351-365.**

Asdell, S.A. (1946) Patterns of Mammalian Reproduction. Comstock Publishing Co., Inc., New York. pp. 282-283.

Bavister, B.D. (1974) The effect of variations in culture conditions on the motility of hamster spermatozoa. **Journal of Reproduction and Fertility, 38: 431-440.**

Bavister, B.D. & Andrews, J.C. (1988) A rapid sperm motility bioassay procedure for quality-control testing of water and culture media. **Journal of in Vitro Fertilization and Embryo Transfer, 5(2): 67-75.**

Bedford, J.M. (1970) Sperm capacitation and fertilization in mammals. **Biology of Reproduction, 2: 128-158.**

Bedford, J.M. (1999) Sperm capacitation. In: Knobil, E. & Neill, J.D. (Eds.). The Physiology of Reproduction, vol. 4, Academy Press, London, pp. 597-602.

Bettahi, I., Guerrero, J.M., Reiter, R.J., & Osuna, C. (1998) Physiological concentrations of melatonin inhibit the norepinephrine-induced activation of prostaglandin E_2 and cyclic AMP production in rat hypothalamus: A mechanism involving inhibition of nitric oxide synthase. **Journal of Pineal Research, 25: 34-40.**

Biegler, B.E., Aaron's, D.J, George, B.C & Poirier, G.R. (1994) Induction of physiological acrosome reactions in caput epididymal spermatozoa of mice. **Journal of Reproduction and Fertility, 100: 219-224.**

Boatman, D.E. & Robbins, R.S. (1991) Bicarbonate: carbon-dioxide regulation of sperm capacitation, hyperactivated motility, and acrosome reactions. **Biology of Reproduction, 44: 806-813.**

Bornman, M.S., Oothuizen, J.M.C., Barnard, H.C., Schulenburg, G.W., Boomker, D. & Reif, S. (1989) Melatonin and sperm motility. **Andrologia, 21(5): 483-485.**

Bornman, M.S., Schulenburg, G.W., Reif, S., Oosthuizen, J.M.C., DeWet, E.H. & Luus, H.G. (1992) Seminal plasma melatonin and semen parameters. **South African Medical Journal, 81: 485-486.**

Bronson, F.H. (1989) Mammalian Reproductive Biology. Univ. Chicago Press, USA.

Cagnacci, A. (1996) Melatonin in relation to physiology in adult humans. **Journal of Pineal Research, 21: 200-213.**

Cavallo, A., Hassan, M. (1995) Stability of melatonin in aqueous solution. **Journal of Pineal Research, 18: 90-92.**

Chen, H.J., Brainard, G.C., III & Reiter, R.J. (1980) Melatonin given in the morning prevents the suppressive action on the reproductive system of melatonin given in late afternoon. **Neuroendocrinology, 31: 129-132.**

Chen, H.J. & Reiter, R.J. (1980) Influence of subcutaneous deposits of melatonin on the antigonadotropic effects of blinding and anosmia in male rats: A dose dependent study. **Neuroendocrinology, 30: 169-173.**

Cohen, M., Roselle, D. & Chabner, B. (1978) Evidence for cytoplasmic melatonin receptor. **Nature, 274: 894-895.**

Cooper, T.G. (1999) Epididymis. In: Knobil, E. & Neill, J.D. (Eds.). Encyclopedia of Reproduction, vol. 2, Academy Press, London, pp. 1-17.

Cunningham, G.R. (1983) Medical Treatment of the Subfertile Male. In: Lipshultz, L.I. & Howards, S.S. (Eds.). Infertility in the Male, Churchill Livingstone INC., New York, pp. 367-380.

Dale, B. & Edler, K. (1997) *In vitro* Fertilization. Cambridge University Press, Cambridge.

Dryer, S.E. (1999) Pineal Gland, Melatonin Biosynthesis and Secretion. In: Knobil, E. & Neill, J.D. (Eds.). Encyclopedia of Reproduction, vol. 3, Academy Press, London, pp. 800-801.

du Toit, D., Bornman, M.S., van Aswegen, C.H., & du Plessis, D.J. (1994). Sialic acid concentration and sperm motility. **Archives of Andrology, 32: 21-23.**

Eddy, E.M., 1988. The Spermatozoon. In: Knobil, E. & Neill, J.D. (Eds.). The Physiology of Reproduction, Vol. 1. Raven Press, New York, pp. 27-68.

Edmonds, K.E. & Stetson, M.H. (1994) Photoperiod and melatonin affect testicular growth in marsh rice rat (*Oryzomys palustris*). **Journal of Pineal Research, 17: 86-93.**

Fraschini, F., Mess, B., Piva, F. & Martini, L. (1968a) Brain receptors sensitive to indole compounds: Function in control of luteinizing hormone secretion. **Science 159: 1104-1105.**

Fraschini, F., Mess, B. & Martini, L. (1968b) Pineal gland, melatonin and the control of luteinizing hormone secretion. **Endocrinology, 82: 919-924.**

Fraser, L.R. (1977) Motility patterns in mouse spermatozoa before and after capacitation. **Journal of Experimental Zoology, 202: 439-444.**

Fraser, L.R. & Herod, J.E. (1990) Expression of capacitation-dependant changes in chlortetracycline fluorescence patterns in mouse spermatozoa requires a glycolysable substrate. **Journal of Reproduction, 88: 611-621.**

Gibbons, I.R. (1983) Sperm Motility: Mechanisms and Control. In: Andre, J.(Ed.). The Sperm Cell: Fertilizing Power, Surface Properties, Motility, Nucleus and Acrosome, Evolutionary Aspects, Proceedings of the Fourth International Symposium on Spermatology, held in Seillac, France, Martinus Nijhoff Publishers, London, pp. 304-314.

Glass, D.J. & Lynch, G.R. (1981) Melatonin: Identification of sites of antigonadal action in mouse brain. **Science, 214: 821-823**

Guraya, S.S. (1987) Biology of Spermatogenesis and Spermatozoa in Mammals. Springer-Verlag, Berlin Heidelberg.

Harper, M.J.K. (1982) Sperm and Egg Transport. In: Austin, C.R. & Short, R.V. (Eds.). Reproduction in Mammals 1: Germ Cells and Fertilization. Cambridge University Press, Cambridge, pp. 102-127.

Harrison, R.A.P., Dott, H.M. & Foster, G.C. (1982) Bovine serum albumin, sperm motility, and the "dilution effect". **The Journal of Experimental Zoology, 222: 81-88.**

Huether, G., Messner, M., Rodenbeck, A. & Hardeland, R. (1998) Effect of continuous melatonin infusions on steady-state plasma melatonin levels in rats under near physiological conditions. **Journal of Pineal Research, 24: 146-151.**

Huerto-Delgadillo, L., Anton-Tay, F. & Benitez-King, K. (1994). Effects of melatonin on microtubule assembly depend on hormone concentration:Role of melatonin as a calmodulin antagonist. **Journal of Pineal Research, 17: 55-62.**

Irez, T.O., Senol, H., Alagoz, M., Basmaciogullari, C., Turan, F., Kuri, D. & Ertungealp, E. (1992) Effects of indoleamines on sperm motility *in vitro*. **Human Reproduction, 7(7): 987-990.**

Keel, B.A. & Webster, B.W. (1990) CRC Handbook of the Laboratory Diagnosis and Treatment of Infertility. CRC Press, Boca Raton, pp. 136-147.

Kholkute, S.D., Rodriquez, J. & Dukelow, W.R. (1995) *In vitro* fertilization and the effect of progesterone and 17∝-hydroxyprogesterone on acrosome reaction of mouse epididymal spermatozoa. **International Journal of Andrology, 18: 146-150.**

Knotts, L.K., Bruot, B.C. & Glass J.D. (1988) Melatonin does not affect *in vitro* secretion of testosterone in White-footed mouse testis. **Journal of Pineal Research, 5: 521-526.**

Lang, U., Aubert, M.L., Conne, B.S., Bradtke, J.C. & Sizoneko, P.C. (1983) Influence of exogenous melatonin on melatonin secretion and the neuroendocrine reproductive axis of intact male rats during sexual maturation. **Endocrinology, 112(5): 1578-1584.**

Lee, M.A. & Storey, B.Y. (1985) Evidence for plasma membrane impermeability to small ions in acrosome-intact mouse zonae pellucidae, using an aminoacridine fluorescent pH probe: Time course of the zona-induced acrosome reaction monitored by both chlortetracycline

and pH probe fluorescence. **Biology of Reproduction, 33: 235-246.**

Lee, M.A. & Storey, B.Y. (1986) Bicarbonate is essential for fertilization of mouse eggs: Mouse sperm require it to undergo the acrosome reaction. **Biology of Reproduction, 34: 349-356.**

Lewinski, A, Szymczykiewicz, P., Sewerynek, E. & Wajs, E. (1993) Effects of pinealectomy and melatonin administration on certain indices of ovarian hyperplasia and/or hypertrophy in rats with both ovaries intact or after unilateral ovariectomy. **Journal of Pineal Research, 14: 117-127.**

Li, L., Xu, J.N., Wong, Y.H., Wong, J.T.Y., Pang, S.F.& Shiu, Y.W. (1998) Molecular and cellular analyses of melatonin receptor-mediated cAMP signalling in rat corpus epididymis. **Journal of Pineal Research, 25: 219-228 .**

Martin, J.E., Engel, J.N. & Klein, D.C. (1977) Inhibition of the *in vitro* pituitary response to luteinizing hormone-releasing hormone by melatonin, serotonin, and 5-methoxytryptamine. **Endocrinology, 100(3): 675-680.**

Meyer, B.J. & Theron, J.J. (1988) The pineal organ in man: An endocrine gland awaiting recognition. **South African Medical Journal, 73: 300-302.**

Millete, C.F. (1999) Spermatozoa. In: Knobil, E. & Neill, J.D. (Eds.). Encyclopedia of Reproduction, vol. 4, Academy Press, London, pp. 586-596.

Mohri, H., Ishijima, S., & Hiramoto, Y. (1983) Movement Characteristics of Hamster Spermatozoa. In: Andre, J.(Ed.). The Sperm Cell: Fertilizing Power, Surface Properties, Motility, Nucleus and Acrosome, Evolutionary Aspects, Proceedings of the Fourth International Symposium on Spermatology, held in Seillac, France, Martinus Nijhoff Publishers, London, pp. 345-348.

Mortimer, D. (1994) Practical Laboratory Andrology, Oxford University Press, New York, pp. 202-205.

Neal, B.R. (1986) Reproductive characteristics of African small mammals. **Cimbebasia, 8: 113-127.**

Nelson, R.J. (1999) Photoperiodism, vertebrates. In: Knobil, E. & Neill, J.D. (Eds.). Encyclopedia of Reproduction, vol. 3, Academy Press, London, pp. 779-788.

Niklowitz, P., Bockers, T.M. & Lerchl, A. (1996) Afternoon injections of melatonin in the Djungarian hamster *Phodopus sungorus*: Long lasting sex-specific effects and influence of acute treatment on the endogenous pineal melatonin rhythm. **Journal of Pineal Research, 21: 231-238.**

Ohl, D.A. & Menge, A.C. (1996) Assessment of sperm function and clinical aspects of IMP sperm function. **Frontiers of Bioscience, 1: e96-e108.**

Oosthuizen, J.M.C., Bornman, M.S. & Schulenburg, G.W. (1986) Melatonin impairs sperm motility - a novel finding. **South African Medical Journal, 70: 566.**

Perheentupa, A., Bergendahl, M. & Huhtaniemi, I. (1995) Modulation of gonadotropin secretion at the pituitary level by testosterone in gonadotropin-releasing hormone-treated male rats during food deprivation. **Biology of Reproduction, 52: 808-813.**

Perez, L.J., Valcarcel, A., de las Heras, M.A. Moses, D.F. & Baldassarre, H. (1996) *In vitro* capacitation and the induction of acrosomal exocytosis in ram spermatozoa as assessed by the chlortetracycline assay. **Theriogenology, 45: 1037-1046.**

Persengiev, S. & Kehajova, J. (1991) Inhibitory action of melatonin and structurally related compounds on testosterone production by mouse Leydig cells *in vitro*. **Cell Biochemistry and Function, 9: 281-286.**

Pevet, P. (1981) Cytological Aspects of Indoleamine Secretion in the Pineal Gland. In: Birau, N., & Schloot, W. (Eds.). Melatonin: Current Status and Perspectives. Proceedings of an international Symposium on Melatonin, held in Bremen, Federal Republic of Germany, Pergamon Press, Oxford, pp. 23-33.

Pitout, M.J., van Vuuren, R.J.J., van Aswegen, C.H. & Theron, J.J. (1991) Melatonin and sperm motility. **South African Medical Journal, 79: 683.**

Poffenbarger, M. & Fuller, G.M. (1976) Is melatonin a microtubule inhibitor? **Experimental Cell Research, 103: 135-141.**

Reiter, R.J., Vaughan, M.K. & Waring, P.J. (1975) Studies on the minimal dosage of melatonin required to inhibit pineal antigonadotrophic activity in male golden hamsters. **Hormone**

Research, 6: 256-267.

Reiter, R.J. (1978) The pineal gland: A regulator of regulators. **Progress in Psychology and Physiological Psychology, 9: 323-356.**

Reiter, R.J. (1980) The pineal gland and its hormones in the control of reproduction in mammals. **Endocrine Reviews, 1: 109-131.**

Rieter, R.J., Vaughan, M.K., King, T.S., and Karasek, M. (1985) The Mammalian Pineal Gland: Pharmacologic Regulation and Physiologic Consequences. In: Steger, R.W. and Johns, A., (Eds), Handbook of Pharmacological Methodologies for the Study of the Neuroendocrine System, CRC Press, Boca Raton, pp. 331-384.

Reiter, R.J. (1986) Pineal function in the human: implications for reproductive physiology. **Journal of Obstetrics and Gynaecology, 6(2): S77-S81.**

Reiter, R.J. (1991) Pineal melatonin: Cell biology of its synthesis and of its physiological interactions. **The Endocrine Reviews,12(2): 151-180.**

Reiter, R.J., (1993) The melatonin rhythm: both clock and a calendar. **Experentia, 49: 654-664.**

Reiter, R.J. & Robinson, J.O. (1995) Melatonin, your body's natural wonder drug. Bantam Books, New York.

Reiter, R.J. (1997) Antioxidant actions of melatonin. **Advances in Pharmacology, 38: 103-117.**

Reiter, R.J., Vaughan, M.K., & Waring, P.J. (1975) Studies on the minimal dose of melatonin required to inhibit pineal antigonadotrophic activity in male golden hamsters. **Hormone Research, 6: 256-267.**

Rojansky, N., Brzezinski, A. & Schenker, J.G. (1992) Seasonality in human reproduction:an update. **Human reproduction, 7 (6): 735-745.**

Saling, P.M. & Storey, B.T. (1979) Mouse gamete interactions during fertilization *in vitro*. Chlortetracycline as a fluorescent probe for the mouse sperm acrosome reaction. **Journal of Cell Biology, 83: 544-555.**

Scharrer, E. & Scharrer, B. (1963) Neuroendocrinology. Colombia University Press, New York.

Shalgi, R. & Phillips, D. (1988) Motility of rat spermatozoa at the site of fertilization. **Biology of Reproduction, 39: 1207-1213.**

Sharpe, R.M., Donachie, K. & Cooper, I. (1988) Re-evaluation of the intratesticular level of testosterone required for quantitative maintenance of spermatogenesis in the rat. **Journal of Endocrinology, 117: 19-26.**

Shapiro, B.M. & Eddy, E.M. (1980) When sperm meets egg: Biochemical mechanisms of gamete interaction. **International Review of Cytology, 66: 257-302.**

SigmaStat (1994). Jandel Scientific SigmaSuite, P.O. Box 7005, San Rafael, CA.

Sirotkin, A.V. (1994) Direct influence of melatonin on steroid, nonapeptide hormones, and cyclic nucleotide secretion by granulosa cells isolated from porcine ovaries. **Journal of Pineal Research, 17: 112 -117.**

STATISTICA for Windows (1999) StatSoft, Inc., Tulsa, OK.

Stetson, M.H. & Tay, D.E. (1983) Time course of sensitivity of Golden hamsters to melatonin injections throughout the day. **Biology of Reproduction, 29: 432-438.**

Stewart-Savage, J. (1993) Effect of bovine serum albumin concentration and the source on sperm capacitation in the Golden Hamster. **Biology of Reproduction, 49: 74-81.**

Tamura, H., Nakamura, Y., Takiguchi, S., Kashida, S., Yamagata, Y., Sugino, N. & Kato, H. (1998) Melatonin directly suppresses steroid production by preovulatory follicles in the cyclic hamster. **Journal of Pineal Research, 25: 135-141.**

van Vuuren, R.J., du Plessis, D.J. & Theron, J.J. (1988) Melatonin in human semen. **South African Medical Journal, 73: 375-376.**

van Vuuren, R.J., Pitout, M.J., van Aswegen, C.H., & Theron, J.J. (1992) Putative melatonin receptor in human spermatozoa. **Clinical Biochemistry, 25: 125-127.**

Vaughan, M.K., Richardson, B.A., Petterborg, L.J., Vaughan G.M. & Reiter, R.J. (1986) Reproductive effects of 6-chloromelatonin implants and/or injections in male and female Syrian hamsters (*Mesocricetus auratus*). **Journal of Reproduction and Fertility, 78:**

381-387.

Vaughan, M.K., Oaknin, S., Cozzi, B., Li, K. & Reiter, R.J. (1988) Influence of melatonin on testicular regression induced by subcutaneous testosterone pellets in male rats kept in long or short photoperiod. **Journal of Reproduction and Fertility, 82: 277-284.**

Voordouw, B.C.G., Euser, R., Verdonk, R.E.R., Alberda, B.TH., De Jong, F.H., Drogendijk, A. C., Fauser, B.C.M.J., Cohen, M. (1992) Melatonin and melatonin-progesterone combinations alter pituitary-ovarian function in women and can inhibit ovulation. **Journal of Clinical Endocrinology and Metabolism, 74(1): 108-117.**

Webley, G.E. & Luck, M.R. (1986) Melatonin directly stimulates the secretion of progesterone by human and bovine granulosa cells *in vitro*. **Journal of Reproduction and Fertility, 78: 711-717.**

Williams, L.M., Hannah, L.T., Hastings, M.H. & Maywood, E.S. (1995) Melatonin receptors in the rat brain and pituitary. **Journal of Pineal Research, 19: 173-177.**

Wuurtman, J.R., Axelrod, J, & Chu, E.W. (1963). Melatonin, a pineal substance: Effect on the rat ovary. **Science, 141: 277-278.**

Yanagimachi, R. (1970) The movement of Golden hamster spermatozoa before and after capacitation. **Journal of Reproduction and Fertility, 23: 193-196.**

Yanagimachi, R. (1982) *In vitro* sperm capacitation and fertilization of golden-hamster eggs in a

chemically defined medium. In: Hafez, E.S.E. & Semm, K. (Eds). *In vitro* fertilization and embryo transfer. MTP Press, Lancaster, pp. 65-76.

Yanagimachi, R. (1988) Mammalian fertilization. In: Knobil, E. & Neill, J.D. (Eds.). The Physiology of Reproduction, vol.1, Raven Press Ltd., New York, pp. 135-185.

Yie, S.M., Daya, S., Brown, G.M., Deys, L. & YoungLai, E.V. (1991) Melatonin and aromatase stimulating activity of human seminal plasma. **Andrologia,23: 227-231.**

Yu, Z.H., Chow, P.H., and Pang, S.F. (1994) Identification and characterization of 2[^{125}I]-iodomelatonin binding sites in rat epididymis. **Journal of Pineal Research, 17: 195-201.**