CARDIFF UNIVERSITY

A two-part investigation of the biopsychosocial model in male reproductive health

A cross-sectional investigation of the association between infertility diagnosis and emotional distress in men (Part I)
and
a prospective controlled investigation of the effect of psychosocial stress on corticosterone, testosterone and sperm parameters in male rats (Part II)

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Summary

This thesis explored the interactions between psychological well-being and male fertility using the biopsychosocial model. The biopsychosocial model proposes that biological, psychological, and social processes interact and impact on health. These interrelationships were investigated in a sample of men undergoing fertility treatment and in a set of experiments using an animal model of stress.

It is commonly thought that men with male factor infertility suffer more compared to men in couples with other infertility diagnoses, mainly due to the social stigma attached to being a man unable to father. The inter-relationships among diagnosis, psychological stress, and social environment were examined in men during a twelve month period of fertility treatment. It was found that men, regardless of diagnosis, showed signs of suffering over time and perceived some deterioration in their social environment that was at least partly caused by their psychological well-being at the start of treatment.

To better understand how stress and reproductive processes interact, an animal stressor paradigm was developed. Male rats were exposed to a psychosocial cage change stressor (PCCS) where housing alternated every day between being alone, or in a new combination of two or three rats per cage for either 12 or 24 days. The four experiments showed that exposure to PCCS induced a mild physical stress response and consistent effects on reproductive parameters. It was concluded that the psychological and social aspects of the PCCS each have an impact on reproduction.

This thesis has provided evidence of biopsychosocial links in the reproductive context supporting a biopsychosocial model of male fertility.

Publications: Articles, presentations, and posters either incorporated or related to this thesis

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Peronace, L. A., Boivin, J., Schmidt, L. (2006). *Psychological predictors of IVF treatment dropout at five year follow-up.* American Society for Reproductive Medicine. (Poster).

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Abbreviations

µg	micrograms
ABP	androgen-binding protein
ACTH	adrenocorticotropic hormone
ANCOVA	analysis of covariance
ANOVA	analysis of variance
AVP	arginine vasopressin
cm	centimetre
CMS	chronic mild stress paradigm
COMPI	Copenhagen Multi-Centre Psychosocial Infertility research program
CRH	corticotropin-releasing hormone
CS12	PCCS 12 day condition
CS24	PCCS 24 day condition
CTRL	control conditions
DNA	deoxyribonucleic acid
EIA	enzymeimmunoassay
ELISA	enzyme-linked immunosorbent assay
F	F statistic
FP	Fertility Problem Stress Inventory
FSH	follicle-stimulating hormone
g	grams
GnRH	gonadotropin-releasing hormone
HPA	hypothalamic-pituitary adrenal axis
HPG	hypothalamic-pituitary gonadal axis
ICSI	intracytoplasmic sperm injection
IVF	in vitro fertilization
kg	kilograms
LH	luteinizing hormone
M	mean
ml	millilitres
ng	nanograms
p	statistical probability
PCCS	psychosocial cage change stressor
PVN	paraventricular nucleus
r	Pearson product-moment correlation coefficient
R^2	squared multiple correlation
rpms	revolutions per minute
SD, St.Dev	standard deviation
sr^2	semi-partial correlation
St.Err	standard error
T	Wilcoxon Signed Rank test statistic
t	t statistic

T1	Time 1, Baseline
T2	Time 2, 12 month follow-up
TMB	tetramethylbenzidine
VBS	visible burrow system
WHO	World Health Organization
WOC	Ways of coping
B	Beta Weight
χ^2	chi squared

Chapter 1

General introduction

1.1 Introduction

A definition of disease is "an impairment of health or a condition of abnormal functioning" (" WordNet: An Electronic Lexical Database. http://wordnet.princeton.edu/perl/webwn?s=disease.," Retrieved September 7, 2007). Throughout history every civilization has made some attempt to deal with disease and illness. For example, cave drawings found in France, dating back as early as 25000 BC have been found, depicting the use of plants as healing agents (Porter, 1999). Around 112-250 BC Chinese doctors linked physical health to moral and spiritual well-being, which led to cosmic harmony (Porter, 1999). Similarly, Ancient Egyptian medicine believed that illness could be stopped if balance in the spiritual world was restored through prayer, magic, and extensive rituals (Porter, 1999).

Supernatural caused of disease dominated many ancient health and healing cultures, however around the time of ancient Greece divine and natural causes of disease finally began to be distinguished, the ancient Greeks believed in divine causes of illness but also put into practice the use of quarantines, for examples, to prevent the spread of illness through physical contact (Porter, 1999). However, it is only several centuries later that a formal model of natural physiological causes of disease was developed; the biomedical model.

1.2 The Biomedical model

Today, the dominant model used to conceptualise disease is the biomedical model. The biomedical model proposes that, first, all illness and symptoms arise from underlying abnormalities within the body, second, all diseases lead to symptoms and signs, third, mental well-being is separate and unrelated to bodily function, and fourth, the person with whom the disease lies bears no responsibility for its presence (Ludwig, 1975). The biomedical model has been very useful because it formalises illness as

having biological causes and has been incredibly effective in the development of diagnostic techniques and treatments for disease (Alonso, 2004). The biomedical model of disease remains the most common model used to conceptualise disease in medicine, however there is a growing body of evidence indicating that in fact, disease can impact upon psychosocial variables, which in turn can impact upon health (Wade & Halligan, 2004).

Thirty years ago, Engel (1977) recommended that a new model to conceptualise disease be adopted, one that included psychosocial factors. A scientific model is a theoretical construct representing a set of variables and their inter-relationships. A model attempts to explain concepts and enables reasoning and even predictions about those variables, typically, a scientific model is revised or abandoned when one can no longer adequately explain the concept it seeks to explain (Alonso, 2004). The biomedical model cannot readily account for data opposing its basic principles, and yet it continues to be used and some have indicated that the model has now become "dogma", something people *blindly* believe to be true (Engel, 1977). For example, there is a plethora of evidence suggesting that cancer and heart disease impact on well-being and that mental health can impede or facilitate treatment success for these conditions (Kop, 1999; McBride, Clipp, Peterson, Lipkus, & Demark-Wahnefried, 2000), an effect which cannot be explained by the biomedical model.

1.3 The Biopsychosocial model

A broader perspective on disease is proposed in he biopsychosocial model of disease, which states that biological, psychological, and social processes interact with health and disease (Engel, 1977). The biopsychosocial model seeks to treat the person and context in which they live as whole. It addresses questions such as "why" and "what for", as well, "how" (Engel, 1977). While the biomedical model is only

concerned with somatic pathology the biopsychosocial model is a more holistic approach to disease (Suls & Rothman, 2004), that predicts that social situations and events, and psychological states can impact on health (Lipowski, 1977). For example, the biomedical model seeks to understand and explain why up to 93% of patients fail to comply with some aspect of treatment, but also why people who suffer from negative mood, anxiety, and depression are at increased risk of physical illness such as headaches, asthma, and heart disease (Taylor, 1990). The model seeks to explain not only why people with poor mental health are more at risk of illness, but also, why and how poor mental health results in pathological physiological changes (Taylor, 1990). This model recognises the possibility of a bidirectional relationship between well-being and physical health and therefore aims to treat both.

Cancer has a devastating effect on the patient, as well as, friends and family of the person diagnosed. Research has shown that severe acute stress arises at the time of the initial diagnosis (McBride et al., 2000), and that distress and other negative emotional effects can persist throughout or even after treatment. Chronic stress (Cordova et al., 1995) as well as emotional distress, loss of energy, loss of stamina, and fatigue have all been reported in cancer survivors months or even years after the termination of treatment (Broeckel, Jacobsen, Balducci, Horton, & Lyman, 2000). There is also evidence to suggest that emotions can impact on cancer progression. Patients with breast cancer have lower circulating stress hormones in their blood after being exposed to stress reducing interventions (Cruess et al., 2000), and reductions in stress hormones have been associated with an increased immune response, specifically an increase in the number of Natural Killer (NK) cells known to target cancer cells leading to reduced rates of relapse (Larson, Duberstein, Talbot, Caldwell, & Moynihan, 2000; McBride et al., 2000).

Similar effects have also been observed in other diseases. For example, stress provokes coronary heart disease, a condition that is more common in men than in women (Kop, 1999) and the common cold, where it has been found that people who reported having higher levels of negative life event stress were more likely to catch a common cold when exposed to the virus experimentally (Hamrick, Cohen, & Rodriguez, 2002). In summary, research suggests there are bidirectional links between psychological and emotional states on the one hand and physical health on the other.

Some evidence supports the proposal that negative emotions (e.g., anxiety, tension, distress) have a negative impact on sperm quality. Reduced sperm quality was found after negative life event stress (e.g., bereavement) (Fenster et al., 1997), high levels of self-report stress in students during the time of exams (Giblin, Poland, Moghissi, Ager, & Olson, 1988), and occupational stress (Gerhard, Lenhard, Eggert-Kruse, & Runnebaum, 1992). However, little research has been done on distress and reproductive function, and the majority of research that does exist focuses on associations in women. In the present research the biopsychosocial model is used to examine interactions among psychosocial and reproductive health variables in men. Before reviewing the literature on distress and sperm quality, the spermatogenetic cycle will be described.

1.4 Male fertility and spermatogenesis

Fertilisation requires the contribution of both female and male gametes (Johnson, B. J. & Everityy, M.H. (Eds). 2000). For conception to occur there are two distinct components required from the male in order for him to fulfil his part; spermatogenesis, and sexual arousal including ejaculation. Sexual arousal, which includes erection and ejaculation, is required. Erection is highly dependent on the hormone luteinizing hormone (LH) that stimulates the production of testosterone, and

research has shown that men with low levels of LH and testosterone are generally impotent (Flaherty, 1995). During male sexual arousal and erection, contractions of the smooth muscles surrounding the penis lead to an ejaculation (Johnson, B. J. & Everityy, M.H. (Eds). 2000). The development of sperm is a lengthy process which takes many steps over a period of approximately 70 to 80 days in humans (Johnson, B. J. & Everityy, M.H. (Eds). 2000).

Although sexual arousal is important to male fertility before emission can take place an ejaculate must be produced. Semen contains fully developed sperm and seminal fluid, which is added to the sperm from accessory sex glands as the sperm travels through the reproductive tract during emission.

Figure 1.1 is a schematic of the spermatogenetic process. Spermatogenesis takes place mainly in the seminiferous tubules contained within the testes, where the Leydig and Sertoli cells are held. The pituitary gland secretes LH that stimulates the Leydig cells to produce testosterone. The pituitary also secretes follicle-stimulating hormone (FSH), which stimulates the Sertoli cells to produce androgen binding protein (ABP). Although the hormonal control of spermatogenesis is not fully understood it is known that an optimal interaction between LH, FSH, testosterone, and ABP are needed for normal spermatogenesis to take place (Flaherty, 1995). For example, low levels of LH will lead to impotence but normal spermatogenesis (Smals, Kloppenborg, van Haelst, Lequin, & Benraad, 1978), whereas normal LH and deficient FSH leads normal sexual performance and a halt in spermatogenesis (Matsumoto, Karpas, Paulsen, & Bremner, 1983). However, in men with low levels of LH and FSH exogenous LH will restore spermatogenesis to some extent (Matsumoto & Bremner, 1985), as will exogenous FSH (Matsumoto et al., 1983), but only when both LH and FSH are replaced does spermatogenesis return to normal levels (Matsumoto et al., 1983).

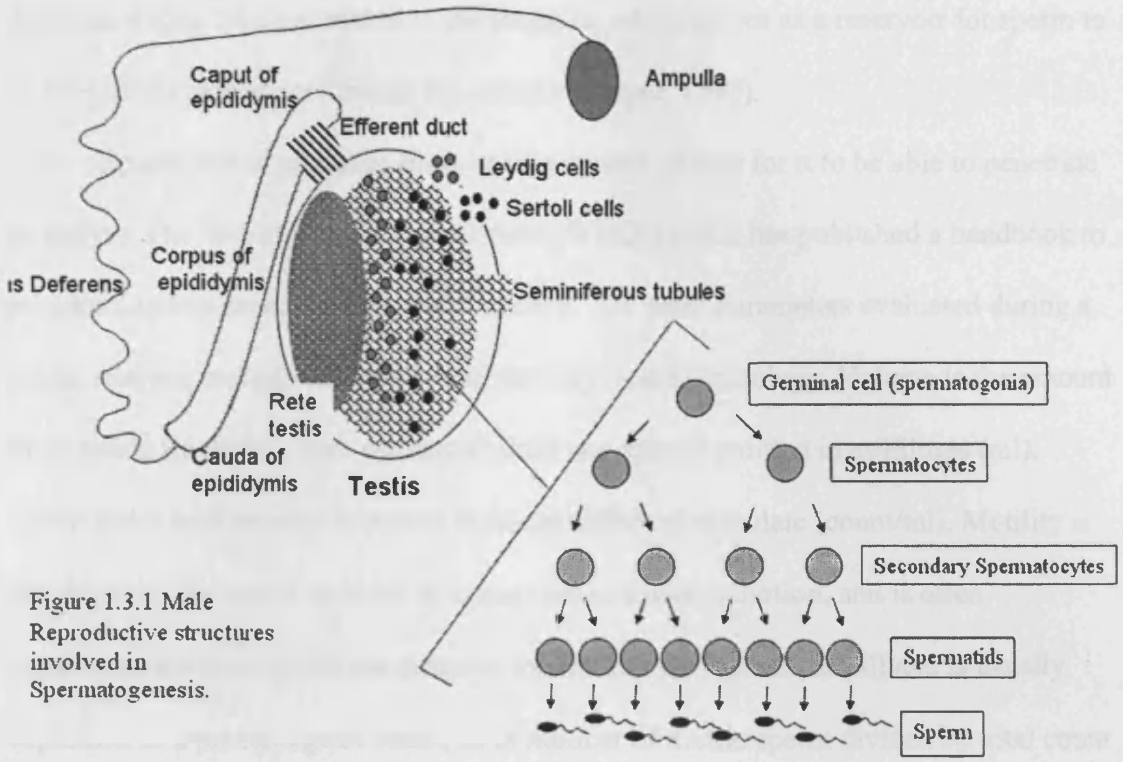

Figure 1.3.1 Male Reproductive structures involved in Spermatogenesis.

Similarly, supra-physiological levels of exogenous testosterone will reinstate, to some extent, spermatogenesis in the absence of LH and FSH (Boccabella, 1963).

In the seminiferous tubules, the original germinal cell divides three times before developing into fully formed sperm (Nussey, 2001). From the seminiferous tubules the sperm is washed into the rete testes, however they are not yet fertile (Nussey, 2001). Sperm recovered from the rete testes show only weak vibrating movement of the tail and swim in a slow circular movement. The sperm then move into the epididymis. From the caput (head) of the epididymis the sperm move into the corpus (body) of the epididymis and gain vigorous unidirectional progression with longitudinal rotation of the head, but are still not capable of fertilizing an egg. In the cauda (tail) of the epididymis the sperm acquire the ability to fertilize and are then transported to the vas deferens (Jones, 1997). The fully developed sperm are then moved through the vas

deferens, a long tube connected to the ampulla, which serves as a reservoir for sperm to be held until ejaculation through the urethra (Harper, 1993).

Sperm that is produced must be of a certain quality for it to be able to penetrate an oocyte. The World Health Organization (WHO) (1992) has published a handbook to set guidelines to assess human sperm quality. The basic parameters evaluated during a sperm analysis include volume, count, motility, and morphology. Volume is the amount of ejaculate (including both semeninal fluid and sperm) emitted in millilitres (ml). Count is the total number of sperm in one millilitre of ejaculate (count/ml). Motility is the ability of the sperm to swim in a progressive forward motion, and is often considered the most important measure for fertility (Jeyendran, 2000) and is usually expressed as a percentage of count, as in number of motile sperm divided by total count times 100. Morphology refers to the shape of the sperm which must include specific features (e.g., oval head, straight tail) to penetrate an egg. Table 1.2 shows the normal range of human sperm quality according to the WHO.

Table 1.2 Parameters and normal range of human sperm and ejaculate according to WHO guidelines.

Parameter	**Normal range**
Volume	2-6 ml
pH	7.2-8.9
Motility	More than 25% in category 'a', or More than 50% in categories 'a+b'
Count	More than 20 x 10^6/ml, less than 250 x 10^6/ml
Morphology	Oval shaped head, long straight tail

When sperm quality falls outside of the normal ranges a man may be diagnosed as having male factor infertility. Infertility is diagnosed after regular unprotected sex for one year without achieving a pregnancy (Sandlow, 2000). Approximately 9% of couples will experience infertility (Boivin, Bunting, Collins, & Nygren, 2007). It has been estimated that infertility is 35% due to a female factor (tubal obstruction, ovarian/

hormonal disturbances), 35% due to male factor (sub-fertile sperm quality) (Speroff, 2005), 20% a combined female and male factor, and 10% unexplained (Speroff, 2005). Although male factor infertility is implicated in over 50% of cases of infertility, little research has been conducted on the well-being of infertile men and the impact of their well-being on their fertility.

Within the body the system that regulate stress and reproduction overlap, it is therefore biologically plausible that that these systems interact.

1.5 The hypothalamic-pituitary adrenal axis

The hypothalamic-pituitary adrenal axis (HPA) regulates stress in the body, this is a complex system of afferent and efferent pathways both excitatory and inhibitory. Briefly, when a stressor is perceived the paraventricular nucleus (PVN) of the hypothalamus secretes corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) into the pituitary portal blood. This stimulates the pituitary secretion of adrenocorticotropic hormone (ACTH) which regulates the glucocorticoid secretion from the adrenal glands (Johansen, Jordan, & Breedlove, 2004). The adrenal glands lie above the kidneys and are composed of two parts, the interior adrenal medulla and the outer adrenal cortex. The adrenal cortex secretes glucocorticoids, a class of steroid hormones including stress hormones such as cortisol and corticosterone, the dominant stress hormones in rodents. Corticosterone has a negative feedback pathway back to the hypothalamus which then reduces the output of ACTH (Ferin, 1999).

1.6 The hypothalamic-pituitary gonadal axis

The hypothalamic-pituitary gonadal axis (HPG) regulates reproduction in the body including the development of the gametes and sexual arousal. The hypothalamus releases gonadotropin-releasing hormone (GnRH) into the anterior pituitary gland which then secretes beta-endorphin, LH, and FSH. LH and FSH control the testes and

stimulate them to produce the androgens testosterone and ABP (Ferin, 1999). As noted previously, LH, FSH, testosterone, and ABP are all involved in the production of sperm.

1.7 HPA and HPG interactions

There is clear overlap between HPA and HPG: the hypothalamus and the pituitary gland are both involved in the control of glucocorticoids and androgens (Ferin, 1999). Furthermore, the hormones secreted also interact (Tilbrook, Turner, & Clarke, 2000). For example chronic injections of glucocorticoids leads to a decrease in LH production which then interferes with normal testosterone production (Tilbrook et al., 2000). This result is thought to be mediated by a reduction in GnRH secretion from the hypothalamus at the time of stress (Tilbrook et al., 2000). Conversely, removing the testicles potentiates the effects of stress, and this potentiation is diminished with testosterone replacement suggesting that testosterone plays an inhibitory role in the HPA axis (Handa, Burgess, Kerr, & O'Keefe, 1994). It has also been hypothesised that the inhibitory effects of testosterone work at the level of the hypothalamus because there is no effect on glucocorticoid receptors in the pituitary gland as a function of orchidectomy or androgen replacement (Viau, Lee, Sampson, & Wu, 2003).

The interaction between the HPA and HPG are not well defined. Many of the more complex pathways are hypothetical at best. More research is therefore required to better understand these interactions and how they may affect human fertility. However, to adequately investigate and test interactions between HPA and HPG rigorous experimental control, not feasible in humans are required.

1.8 Animal models

A major benefit of animal research is that animals can be monitored in carefully controlled environments. Lighting, temperature, humidity, diet, and living conditions (i.e. cage size) are controlled from the moment the animal is born. Even statistical error,

due to individual differences, for example, is minimised because animals are genetically similar to each other through the use of in-bred strains of animals.

Causal experiments can be designed for research into interactions between the HPA and the HPG axes; animals are randomly assigned to groups and the group of animals exposed to stress is compared to the group of animals that are not exposed to stress while all other variables remain the same.

Several dependent variables can be compared between stress and control groups that would not otherwise be possible in humans. For example, post-mortem examination of hypothalamic tissue, the pituitary gland, the adrenal glands, reproductive organs can be assessed in animals but not in humans. Also repeated blood sampling would be needed to assess glucocorticoid levels, as well as, reproductive hormone levels. Clearly, post-mortem examination of tissue is not possible in humans. And although it is possible to obtain regular blood samples and semen samples from men, the procedure is time consuming, uncomfortable, and it is often difficult to recruit volunteers.

In summary, manipulation of animals and control over environmental variables is more feasible in non-human animals so that animal models that lead to testing of causal relationships are more suitable for the investigation of HPA/HPG interactions. Furthermore the ability to utilise measures that would not be possible in humans is essential to establishing where and how the HPA and HPG axes interact.

1.9 The present studies

This thesis takes a translational approach to understanding the interactions between stress and reproduction, by utilising and combining information obtained both from humans and from rats. First, data from a prospective study was analysed to examine the consequences of persistent infertility and treatment failure on emotional well-being of men in fertility treatment. A population of Danish men were asked to fill

out an extensive questionnaire upon entering fertility treatment and then again after one year. Demographic variables, as well, as, mental and physical well being were compared between men in couples with different infertility diagnoses. The aim of this study was to better understand men's experience of infertility and, the extend of their suffering based on their diagnosis.

Second, an animal model of the effects of stress on male reproduction was developed and evaluated in a series of experiments. The goal of these studies was to develop on appropriate animal model of stress and male fertility. These studies sought to model human psychosocial stress, and to assess the impact this stressor had on biological functions such as stress measures (e.g., corticosterone, adrenal glands). In the development of the model the stressor was changed to make the stressor more severe so that stress hormones were sufficiently elevated in experiment conditions compared to controls. Also, reproductive measures were extensively examined, including reproductive organs, and sperm quality. New techniques to assess sperm quality in the rat were developed so that more accurate measures could be taken.

Through the use of human patient populations, as well as, the development of an animal model of stress and reproduction the interactions between mental well-being and reproduction can be investigated. It is important that these interactions be better understood so that suffering in infertile populations can be reduced and so that the negative impact that suffering can have on fertility be minimized.

Chapter 2

Patterns of suffering and support in infertile men:
12 months after unsuccessful treatment.

2.1 Introduction

Male factor infertility is involved in up to 50% of all cases of infertility but there is a limited amount of research that examines the effect of a male factor diagnosis on a man's physical and psychological well-being. The research that does exist has generated two main propositions with regards to men and male infertility. First, men with male factor infertility are proposed to suffer more compared to men with other infertility diagnoses (Connolly, Edelmann, Cooke, & Robson, 1992; Kedem, Mikulincer, Nathanson, & Bartoov, 1990; Lee, Sun, & Chao, 2001; Nachtigall, Becker, & Wozny, 1992). Secondly, male factor infertility is proposed to have such a social stigma that it produces much negative social stress, and a culture of secrecy and protectiveness to the extent that women sometimes even take the blame for the couple's childlessness (Carmeli, 1994; Rowland, 1985; van Balen, Trimbos-Kemper, & Verdurmen, 1996).

It has been reported that men's experience of male factor infertility was more intense then that of men with female factor infertility (Nachtigall et al., 1992), in that the former reported higher levels of infertility distress (Lee et al., 2001), and increased anxiety over time (Connolly et al., 1992). Kedem (1990) found that men with suspected male factor infertility reported increased self-blame, lower self-esteem and increased social isolation compared to men without suspected male infertility. Other studies do not find a difference according to diagnosis in male well-being. For example, Lee (2001) found that distress, marital and sexual satisfaction did not differ according to diagnosis. Connolly (1992) reported an increase rather than a decrease in overall physical health in men with male factor infertility. Van Balen (1996) did not find any differences among diagnostic groups with respect to how their social network reacted to their diagnosis, and most infertile men reported positive reactions when they disclosed to others.

These variations in research findings may be due to methodological issues, in particular to the fact that studies tend to be cross-sectional and make assessments at different time points in the medical experience, for example at the time of diagnosis (Hubert, Hellhammer, & Freischem, 1985; Kedem et al., 1990; Verhaak et al., 2001), during treatment (Berg & Wilson, 1991; Lee et al., 2001; Nachtigall et al., 1992) or at other time points (Daniluk, 1988; Dyer, Abrahams, Mokoena, & van der Spuy, 2004) making it difficult to delineate the source of the stress. These findings highlight the need for longitudinal evaluations. Given the considerable amount of time couples now spend in treatment, it would be important to assess the effect of diagnosis on male well-being, especially in couples who are unsuccessful with treatment.

Many studies have shown that couples report significant distress when treatment fails (Baram, Tourtelot, Muechler, & Huang, 1988; Boivin, Takefman, Tulandi, & Brender, 1995; Leiblum, Kemmann, Colburn, Pasquale, & DeLisi, 1987; Menning, 1980; Newton, Hearn, & Yuzpe, 1990). For example treatment failure has been associated with high levels of stress (Newton et al., 1990), and anger (Leiblum et al., 1987). Boivin et al. (1995) found that women with a moderate amount of treatment failure showed high levels of personal and marital distress. Similarly, Van Balen (1993) reported that couples with long-term infertility, who have faced much treatment failure, report higher levels of depression, low satisfaction with their sex lives, and low levels of well being. The present study examined whether men with a male factor diagnosis experienced a greater increase in distress in a 12 month period of unsuccessful treatment than did men with other diagnoses.

Van Balen proposed that spouses could be motivated to protect each other from the stigma of infertility and this could be particularly true when the infertility was due to a male factor (van Balen et al., 1996). One of the key features of social stigma described

by Goffman (1963) is a discredited moral status. Couples have reported feeling morally judged with regards to their infertility and decision to use assisted reproductive technologies (Ellison & Hall, 2003). Negative comments have included for example, that the couple's infertility was an act or even punishment from God (Ellison & Hall, 2003). In support of Goffman's theory Carmeli (1994) reported that it was common for couples to report secrecy and misdirection with regards to their infertility diagnosis. For example, women reported telling family and friends that the source of the couple's infertility was hers when, in fact, the diagnosis was male factor. It has also been reported that men perceive infertility to be more socially acceptable for a women than for a man (Rowland, 1985). In addition, men reported feeling that there was social support for infertile women whereas they felt ridiculed (Carmeli, 1994). The present study examined the stigma of male infertility in a number of ways. First, if couples were more motivated to protect each other, one would expect higher disagreement to exist between self-reported and spouse reported diagnosis in couples with a male factor versus other diagnosis. Also, differences would be seen between self-reported diagnosis and actual diagnosis reported in the medical records. Van Balen (1996) found that 38% of couples disagreed with medical records with regards to their diagnosis. Secondly, one would expect men with male factor infertility to be less likely to talk to other people regarding their infertility and reasons for their infertility as compared to men with any other infertility. Thirdly, if stigma is an issue one would also expect men with male factor infertility to receive less understanding and more negative comments from those around them than men with any other infertility diagnosis.

It is well accepted that social environment and psychological well-being interact (DeLongis, Folkman, & Lazarus, 1988). The most widespread view is that general social support predicts psychological well-being and health (Walen & Lachman, 2000)

or even buffers the effects of stress on physical and mental health (Cohen & Wills, 1985). Cohen and Wills (1985) have described the "Buffering Hypothesis" where there is positive associations between social support and well-being when social support is perceived to be useful with specific aspects of the stressful event (as compared to general social support). Yet, others have found the reverse in that chronic personal stress has a negative impact on the social environment (Lin, 1989). Thoits (1995) argued that acute stress may mobilize social support in the short term but chronic stress may lead to too great a cost to social networks which breakdown over time. For example 96 mothers experiencing the chronic stress of a disabled child (deafness) reported lower levels of support compared to 118 control mothers (Quittner, Glueckauf, & Jackson, 1990). Moreover, the mothers of deaf children resort to using professionals (i.e. speech therapists, psychologists) in lieu of friends and family who were perceived to be unsupportive (Quittner et al., 1990). To address this issue the extent to which personal and social stress at the start of treatment predicted mental health and perceptions of the social environment 12 months later was examined in this study.

Understanding the psychological effect of fertility problems is also important because there is now converging evidence that psychological distress may affect reproductive function. It is well-established that there is interaction between the hormonal axes that regulates the stress (HPA) and reproductive (HPG) systems. Whilst much of this data is focused on female physiology, a few reports demonstrate that an association between emotional distress and gonadal function may also exist in men (Clarke, Klock, Geoghegan, & Travassos, 1999; Fenster et al., 1997; Kentenich, Schmiady, Radke, Stief, & Blankau, 1992; Pook, Tuschen-Caffier, & Krause, 2004). For example, men who reported a stress life event in the past three months (i.e. death in the family) had lower sperm quality, specifically increased morphological changes,

compared to men who did not experience a stressful life event (Fenster et al., 1997). Pook (2004) found that high scores on the Infertility Distress Scale negatively correlated with decreased sperm quality in men from an andrology clinic. Finally, Clarke (1999) and Kentenich (1992) analysed sperm quality of men at the start of fertility treatment and then again on the day of oocyte retrieval, using a questionnaire on state stress. It was found that stress was significantly lower at the start of fertility treatment as compared to the day of oocyte retrieval. It was also found that at the time of higher stress (oocyte retrieval) sperm quality (concentration, volume, count, and motility) had significantly reduced in quality compared to sample taken prior to the start of treatment when stress was lower (Clarke et al., 1999). The present study also examined whether psychological distress manifested in physical stress reactions known to be indicators of HPA activation.

The aims of the present longitudinal study were to investigate the issues of suffering and the negative social environment experienced by men with male factor infertility in a sample of men who were unsuccessful with treatment. Archival data was extracted for men participating in the Copenhagen Multi-Centre Psychosocial Infertility (COMPI) research program, which is a longitudinal psychosocial investigation of infertile couples in Denmark (Schmidt et al., 2003). Mental health, fertility problem stress, coping and social support were assessed at the onset of treatment and 12 months later, and compared in men across four diagnostic groups. Based on the research reviewed it was hypothesised that the stigma of male factor infertility would manifest in greater reporting inaccuracies with respect to diagnosis and more suffering and social stress in men with male factor infertility compared to men with other diagnoses. Further, it was predicted that pre-treatment psychological and social stress would predict these same variables at 12 months follow-up.

2.2 Methods

2.2.1 Sample

The final sample consisted of N=256 Danish men whose partners had not become pregnant during a 12 month period of treatment and who had participated in the first (Time 1 [T1]) and 12-month follow-up assessment (Time 2 [T2]) in the COMPI research program.

In total, 2, 812 questionnaires were distributed to couples at clinics during the data collection at the first assessment, of which 2, 250 (80.0%) were returned, comprising 1,070 couples (see (Schmidt et al., 2003) for detailed analysis of distribution and response rates for Time 1 and Time 2 assessment). Time 1 couples were mailed the follow-up questionnaire 12 months later and 888 (83.0%) returned questionnaires for both spouses. To make the sample as homogeneous as possible at T1, couples that had had a child with fertility treatment at study entry were excluded (1.8%, N=16, including 5 couples who did not answer this question). Similarly, to ensure homogeneity in the treatment experience during the 12-month study period couples that became parents through adoption rather than treatment (2.0%, N=18, including 7 who did not answer this question) and 14 couples (1.6%) that did not have treatment were excluded. An additional couple were excluded because the number of treatments reported (i.e., 17) was an outlier relative to the group median (i.e., 2), 25 couples (2.8%) were excluded because the Time 2 questionnaire was not completed within the requested time period (i.e., 12 months). Seventy-four men (9.0%) did not respond to the question about diagnosis at T1 and were excluded. With these exclusion the sample consisted of 744 men (70% of T1 1, 070 couples). However, as the study was focused on the effect of treatment failure only couples that were unsuccessful with treatment (N=256) were retained for final analyses, all other couples had achieved their personal goal in the

sense that they either voluntarily ended treatment, treatment was successful, or they adopted, and so on.

These stringent exclusion criteria lead to a very homogenous sample. This design approach was deemed necessary to better understand the impact of biopsychosocial events on male fertility because it is important to examine the issue in a context where inferences can be made that are not complicated by variations in demographics, medical and reproductive factors in the data. For example, in a heterogeneous sample that includes much individual variation, results can be skewed, or obscured. Once initial findings have been investigated in a homogenous sample, it then becomes important for the results to be replicated and examined in different contexts, with different population.

Men in the final sample had been infertile longer than men in pregnant couples in the COMPI sample (n=488) ($t(1,659)=5.67$ $p<.05$: M=4.37, SD = 2.37; M=3.97, SD = 1.86, respectively) and had attempted more treatment cycles ($t(1,659)=50.18$ $p<.001$), M=2.59, SD=1.34; M=1.85, SD=1.28, respectively) in the 12 month duration of this study. No other differences were found between men in subsequently pregnant versus non-pregnant couples.

2.2.2 Materials

The COMPI questionnaire booklet contains numerous questions about health, support, stress, and coping with infertility (Schmidt et al., 2003). Only those questions relevant to the present study are described. All English-language questionnaires were translated into Danish and then back translated into English unless validated Danish versions already existed. Translations were done by two people independently, back-translated into English by two other people, and finally compared for conceptual

correspondence. Any differences which may have occurred were resolved by a third native English speaker.

2.2.2.1 Sociodemographic and medical information.

These questions were used to obtain demographic (e.g., age, years married, children, focus on being a parent, social position) and medical (e.g., diagnosis, years infertile, previous treatment experience, treatment cycles in the last 12 months) information, as well as a measure of focus on being a parent.

2.2.2.2 Fertility problem stress.

The Fertility Problem (FP) Stress Inventory (Abbey, Andrews, & Halman, 1991; Schmidt, 1996), was designed to assess overall disruption and stress created by fertility problems in relation to specific life domains. Subscales included "Personal stress", "Social stress", and "Marital stress" (Boivin & Schmidt, 2005). "Personal stress" contained six items that reflected fertility problem stress for the person (e.g. "stressful for me to deal with this fertility problem," "my life has been disrupted because of this fertility problem"). The "Social stress" subscale contained four items that reflected FP stress in relationships including parents, in-laws, friends, and colleagues (e.g. "stress on the relationship with family", "stress on the relationship with family-in-law"). Finally, the "Marital stress" factor contained four items that reflected the stress of infertility on the marital and sexual relationship (e.g., "caused thoughts about divorce," "stress on our partnership"). Items were rated on two different likert-scales, five-point and four point respectively (i.e., either "strongly disagree (1) to strongly agree (5)" or "none at all (1) to a great deal (4)"). For all variables, higher scores indicated greater fertility problem stress. The range was 6–26 for the personal subscale, 4–16 for the social subscale, and 4–18 for the marital subscale. The Cronbach alpha coefficient for personal stress was .86, social stress was .85, and marital stress was .81.

2.2.2.3 Mental and physical health

General mental health was assessed using the nine item mental health and energy-vitality subscale of the Short-Form-36 Inventory (SF-36: Bjørner, 1997; Ware, 1993), the short-form was used both to reduce the burden on the participants as the entire questionnaire was already long, but also because the short-form has been shown to be reliable at assessing general mental health (Bjørner, 1997). Participants were asked about their mental health and energy levels over the past four weeks and rated each statement (e.g., "Have you been a very nervous person") on a six-point response key ranging from "all of the time" to "none of the time". The validity of the SF-36 subscales has been established in numerous medical populations (Stewart, 1992). Scores were reversed so that a higher score on this measure indicated better mental health (possible range 9-54). The Cronbach alpha coefficient was .93.

Physical health was assessed using nine items from the "Physical stress reactions" subscale of the Stress Profile questionnaire (Setterlind, 1995) and an additional two items from the Pennebaker PILL inventory (Pennebaker, 1982). Items on this subscale assessed a variety of physical reactions (e.g., "racing heart", "muscle tension") which could occur as a result of stressful situations. The measure has shown good psychometric properties: it has high internal reliability and can discriminate between groups experiencing high and low work stress (Setterlind, 1995). Participants indicated how frequently they experienced each symptom in the past four weeks using a six-point response key ranging from "all of the time" to "none of the time". The total score was used for analyses with higher scores indicating more physical stress reactions (possible range 1-45). The Cronbach alpha coefficient was .87

2.2.2.4 Coping

The 19-items used to measure coping strategies in relation to the specific stressor infertility were adapted from the 66-item Ways of Coping (WOC) Checklist, a process-oriented measure of coping derived from Lazarus and Folkman's transactional model of stress (Folkman, 1997; Folkman & Lazarus, 1988; Lazarus, 1984). Several coping strategies exist, however, since any coping strategy is considered neither inherently positive nor negative it was felt that an overall coping effort score would be sufficient for the study aims. Higher scores on this factor indicated more coping effort; with a possible range from 5-20. Participants were requested to rate each item according to how they coped with infertility using a four-point response key ranging "not used" to used a "great deal". The Cronbach alpha coefficients was moderate at .70.

2.2.2.5 Social environment

Social relations were assessed using the theoretical model of support and strain derived in the Danish Longitudinal Health Behaviour Study (Due, Holstein, Lund, Modvig, & Avlund, 1999). Social relations were examined using two 3-item variables; support and understanding from those around you (i.e., spouse, family, friends), and negative reactions and comments from those around you. Both were assessed using a five-point likert scale ranging from "always" to "never" with higher scores indicating greater understanding or more negative comments, respectively. The Cronbach alpha coefficients for understanding was .86, and for .76 negative comments.

2.2.2.6 Openness about infertility

Men's willingness to discuss their infertility with people around them was assessed for three types of information: not being able to have children, reasons why they could not have children, and emotional feelings about being infertile. Responses

were scored on a three-point categorical scale "(1) not to other people, (2) only to close people, (3) to most people I know".

2.2.2.7 Treatment cycles and outcome

At the 12-month follow-up, participants were asked to detail their treatment experiences since completing the first questionnaire. First, participants were asked to indicate the types of treatments they had received and the number of treatment cycles they had undergone. Second, participants were asked to indicate whether they had achieved a pregnancy or not and if they had, whether their partner was currently pregnant or had delivered.

2.2.2.8 Diagnostic information

Data about diagnosis was collected from three sources. First, men were asked to indicate the cause of their fertility problems by checking one or more of the following: my partner's tube(s) is/are blocked, my partner has irregular ovulation or anovulation, I have decreased sperm quality, no cause was proven, other. Four categories were derived from this information, and which determined to which diagnostic group the couple was assigned for the purposes of analysis. The unexplained group was assigned if only "no cause was proven" was indicated, the female factor group was assigned if "my partner's tube(s) is/are blocked" and/or "my partner has irregular ovulation or anovulation" was indicated, the male factor group was assigned if "I have decreased sperm quality" was indicated, the mixed group was assigned if a combination of female factor and male factor were indicated. Second, diagnostic information was also obtained from the spouse who separately completed this questionnaire item. Finally, for a subset of men, (n=226, 88.28%), the factual diagnosis as recorded by medical doctors was extracted from patients' medical charts at the end of treatment.

2.3 Procedure

Four public clinics (Brædstrup, Herlev, Odense, Rigshospitalet) and one private fertility clinic (Fertility Clinic Trianglen) were contacted to enlist their participation in the COMPI project. These clinics were selected because they were large and they covered different geographical regions (large cities including the capital city and more rural areas). Clinics were given a presentation that detailed what would be required of clinic staff and patients. All clinics (n=5) agreed to distribute questionnaires. Clinics were provided with all necessary materials including questionnaire booklets for men and women as well as pre-addressed, stamped envelopes for the return of completed questionnaires.

Couples received questionnaires approximately two weeks prior to treatment (T1) and again 12 months later (T2). Spouses were instructed to complete questionnaires separately, within 10 days of receipt and to post the completed questionnaires in the envelopes provided. Participants who did not wish to participate returned an enclosed non participating form. If the questionnaires or non participating forms were not received, participants were sent a maximum of two reminders at 10-day intervals. Data for this study were collected between January 2000 and August 2001 (T1) and between January 2001 and August 2002 (T2).

The Scientific Ethical Committee of Copenhagen and Frederiksberg Municipalities assessed the study; the study complied with ethical standards according to the Helsinki II declaration. The Danish Data Protection Agency also approved the study.

2.3.1 Data Analysis

Analysis of Variance (ANOVA) was used to assess differences between diagnostic groups in age, years married, years infertile, focus on being a parent,

previous treatment experience, number of treatment cycles in the 12 month study period. For the categorical or ordinal variables whether you have children or not, and social position, chi-square was computed (x^2). To assess changes over time (T1 to T2) in the ordinal variable "do you talk to other people regarding your infertility" the Wilcoxon Signed rank test (T) was used; it was then used to test differences between the four diagnostic groups with Bonferroni correction was (new p-value: $p<0.0125$). To assess change over time, a 4 (Diagnosis: Unexplained, Female, Male, Mixed) X 2 (Time: T1, T2) mixed model ANOVA was computed with time as the repeated measure. In the case of a significant main effect of group Tukey HSD post hoc follow-up tests were used. Significant interactions were followed up using simple effects tests and simple comparisons. The dependent variables for these analyses were: mental health (SF-36), physical stress symptoms, negative comments, understanding, coping, and personal, social and marital fertility problem stress. Regression was used to assess the relationship between mental well being (i.e., SF-36) and social environment (i.e., support and understanding, negative comments). Time 2 social environment was predicted from Time 1 mental health, after controlling for Time 1 social environment, female age and years infertile. Time 2 mental health was predicted from Time 1 social environment controlling for Time 1 mental health, female age and years infertile. The alpha level was set at $p<0.05$. The standardised beta weight (±standard error), semi-partial correlation (sr^2), and t-value are presented for each predictor to indicate the unique contribution of each variable and its significance in predicting the outcome.

2.4 Results

2.4.1 Demographic and medical characteristics

Table 2.1 shows summary statistics for sample sociodemographic and medical characteristics. There were no differences between diagnostic groups on any of these variables.

Overall men were in their mid thirties, (M=34.0 years old, SD=5.0 years). Couples had been married almost 8 years (M=7.6 years, SD=3.7 years), the majority of couples had no children either together or from previous relationships (77.7%, n=199). Most men (93.4%, n=239) were employed and were employed in skilled or white-collar occupations (60.9%, n=156).

There was no significant difference between diagnostic groups according to medical characteristics. The average duration of infertility was (M=4.3, SD=2.4) years, with a range of <1 year to 16 years. Prior to the start of the present study couples had had previous treatment mainly consisting of intrauterine insemination (86.7% of the treated sample, n=222) but also in vitro fertilization/intracytoplasmic sperm injection (IVF/ICSI) (13.3% of treated sample, n=34) or both. In the male factor infertility group, 5.3% (n=4) had used insemination with donor sperm. In terms of treatment during the 12-month study period, the majority of couples, 91.8% (n=235) underwent IVF or ICSI treatment. The average number of treatment cycles was (M=2.6, SD=1.3) and ranged from 1 to 8, and was not statistically different between diagnostic groups.

2.4.2 Concordance about diagnosis among men, their partner and doctors

Men were categorised into four groups based on their self-reported diagnosis on the questionnaire, as described previously. The distribution according to self-reported

Table 2.1 Summary statistics for sociodemographic, fertility and treatment characteristics by diagnostic group

Variables		Unexplained n=81		Female n=79		Male n=75		Mixed n=21		Statistics F or χ^2
Demographic										
Male age[a]		34.3	(4.74)	34.2	(5.09)	33.4	(4.52)	33.5	(6.72)	F=.548
Years married[a]		8.30	(4.10)	7.12	(3.64)	7.49	(3.35)	7.67	(3.51)	F=1.424
Previous children (n,% yes)		21	25.93	21	26.58	13	17.33	2	9.52	χ^2=1.329
Social position (n, %)	Unemployed	5	6.17	4	5.06	6	8.00	2	9.52	
	Unskilled	13	16.05	22	27.85	16	21.33	5	23.81	
	Skilled	56	69.14	45	56.96	43	57.33	12	57.14	
	Professional-Executive	7	8.64	8	10.13	10	13.33	2	9.52	χ^2=2.487
Fertility and Treatment										
Years infertile[a]		4.46	(1.95)	4.31	(2.32)	4.01	(2.54)	5.48	(3.16)	χ^2=2.168
Treatment experience prior to the study (n, %)	Conventional	74	91.36	68	86.08	62	82.67	18	85.71	
	IVF/ICSI	7	8.64	11	13.92	13	17.33	3	14.29	χ^2=1.799
Treatment cycles in the 12 month study period (n, %)	Conventional	8	9.88	5	6.33	7	9.33	1	4.80	
	IVF/ICSI	73	90.12	74	93.67	68	90.67	20	95.20	χ^2=4.502

[a]Mean (standard deviation)

diagnosis was: unexplained (n=81, 31.6%); female (n=79, 30.9%); male (n=75, 29.3%), and mixed (n=21, 8.2%). The diagnosis reported by men was not significantly different (x^2=5.71, df=3, p=.126) from the diagnosis given by their partner. Overall there was 87.9% (n=225) agreement between spouses. In the 12.1% of couples who disagreed, 2.7% were disagreeing about unexplained diagnosis, 3.5% were disagreeing about female factor diagnosis, 5.1% were disagreeing about female factor diagnosis, and 0.8% were disagreeing about mixed diagnosis. In the sub-sample where medical records could be obtained (n=226) men's self-reported diagnosis did not differ significantly from the diagnosis reported in the medical records (x^2=3.27, df=3, p=.352) with agreement in 77.4% of the cases. Of the 22.6% of men who disagreed with the medical records, 7.1% were disagreeing about unexplained diagnosis, 5.3% were disagreeing about female factor diagnosis, 4.9% were disagreeing about female factor diagnosis, and 5.3% were disagreeing about mixed diagnosis.

2.4.3 Personal, social, and marital stress

Figure 2.2 shows personal, social, and marital stress over time by diagnostic category. For personal stress there was no significant main effect of time (F(1, 245)=.32 p=.57) or diagnostic category (F(3, 245)=1.02 p=.38), nor was there an interaction (F(3, 245)=.54 p=.65). Social stress significantly increased over time (F(1, 245)=17.79 p<.01) but diagnosis (F(3, 245)=1.25 p=.16) and the interaction (F(3, 245)=.716 p=.54) were not significant. Marital stress significantly increased over time (F(1, 245)=20.56 p<.001) but the main effect of diagnosis (F(3, 245)=1.18 p=.32) and the interaction (F(3, 245)=.42 p=.74) were not significant.

2.4.4 Mental health, physical stress symptoms

The results revealed that for mental health there was a main effect of time (F(1, 247)=16.45, p<.001) where mental health decreased after 12 months of treatment

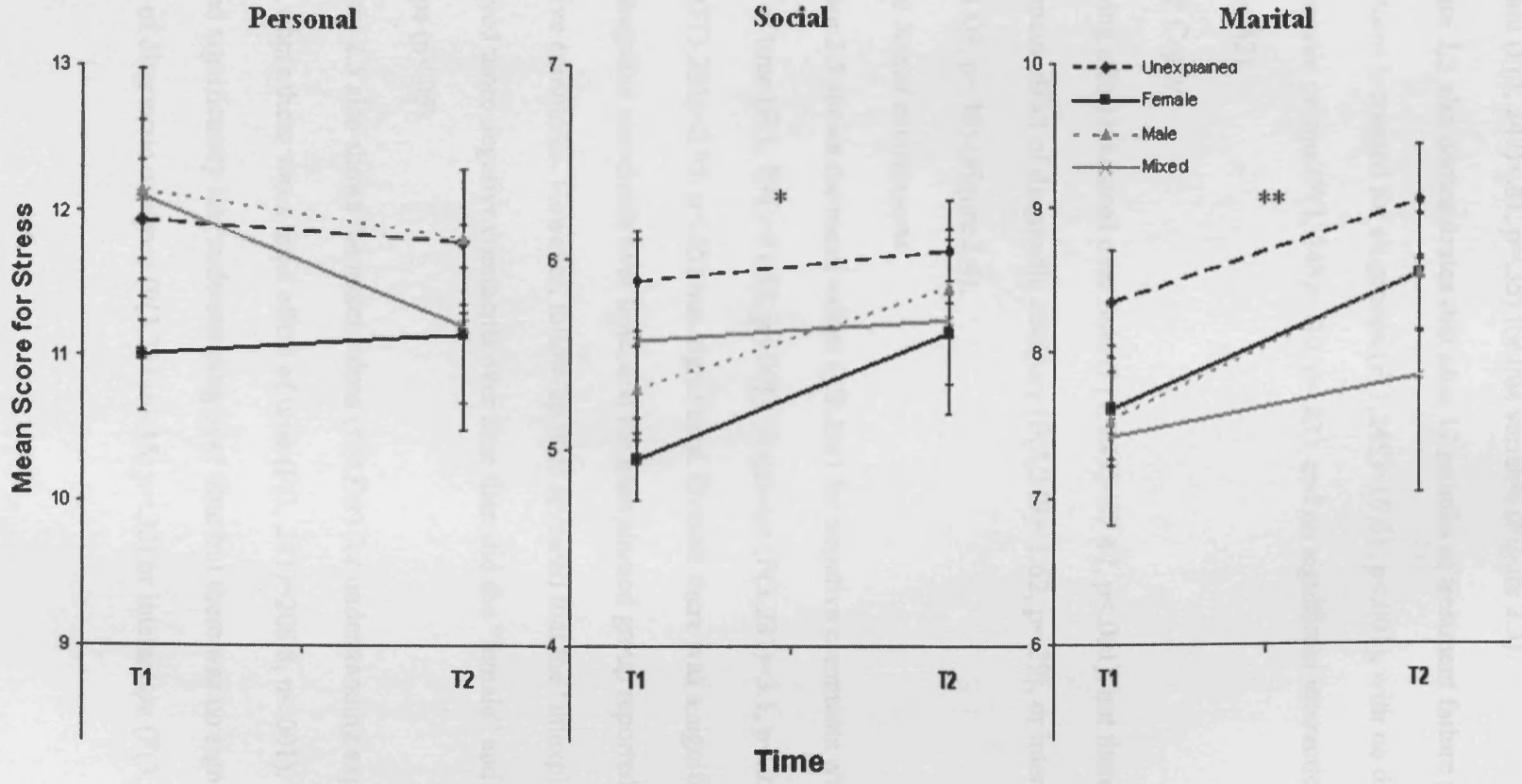

Figure 2.2 Mean scores (±St.Err) for personal, social, and marital stress at Time 1 (T1) and after 12 months of treatment (Time 2, T2) by infertility diagnosis. Asterisk denotes a statistical significant difference between T1 and T2 across all diagnostic groups (*p<.01; **p<.001)

failure. The main effect of diagnosis ($F(3, 247)= .81$, $p=.49$) and the interaction were not significant ($F(3, 247)=.81$, $p=.35$) for this variable (Figure 2.3).

Figure 2.3 also demonstrates that after 12 months of treatment failure physical stress symptoms increased for all groups ($F(1,248)=10.61$, $p<.001$), with no differences among diagnostic groups ($F(3, 248)= .30$, $p=.83$), and no significant interaction ($F(3, 248)=.94$, $p=.42$).

2.4.5 *Coping*

Coping effort increased over time ($F(1, 249)=57.47$, $p<.001$), but there was no significant main effect of diagnostic category ($F(3,249)=1.62$, $p=.29$), or interaction ($F(3,249)=1.03$, $p=.38$) (Figure 2.4).

2.4.6 Social environment

Figure 2.5 shows the mean values (±St.Err) for negative comments where the main effect of time ($F(1, 231)=21.53$, $p<.001$), diagnosis ($F(3,231)=3.1$, $p<.05$) and the interaction ($F(3,231)=2.93$, $p<.05$) was significant. Overall there was a significant increase in negative comments over time, and the unexplained group reported overall more negative comments. However, follow-up tests revealed that the "unexplained" group received more negative comments over time then did the "female" and "male" factor groups ($p<.05$).

Figure 2.5 also shows the mean values (±St.Err) for understanding experienced by the men where there was a main effect of time ($F(1, 231)=205.8$, $p<.001$). Overall men reported significantly less understanding over time but there was no significant main effect of diagnostic category ($F(3,231)=1.18$, $p=.32$) or interaction ($F(3,231)=1.57$, $p=.20$).

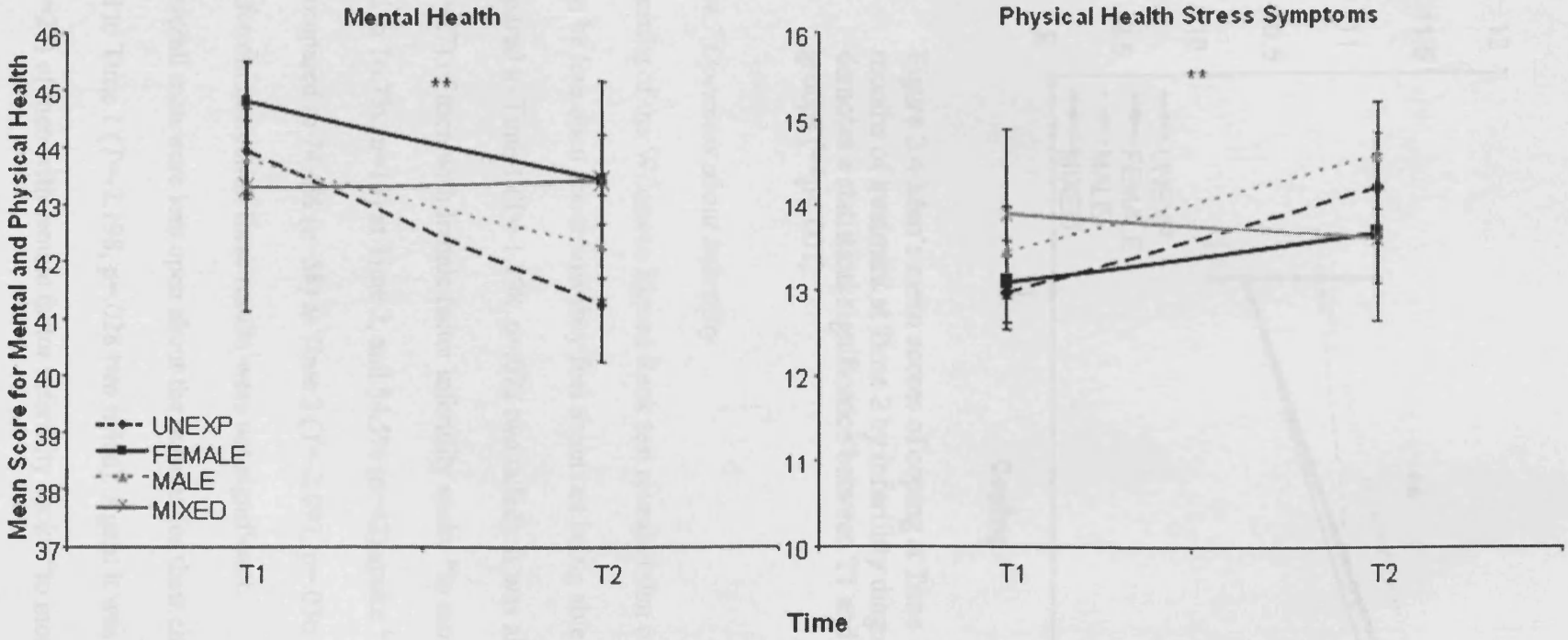

Figure 2.3 Men's mean scores of mental and physical health at Time 1 and after 12 months of treatment at Time 2 by infertility diagnosis. Asterisk demotes a statistical significance between T1 and T2 across all groups (**p<.001).

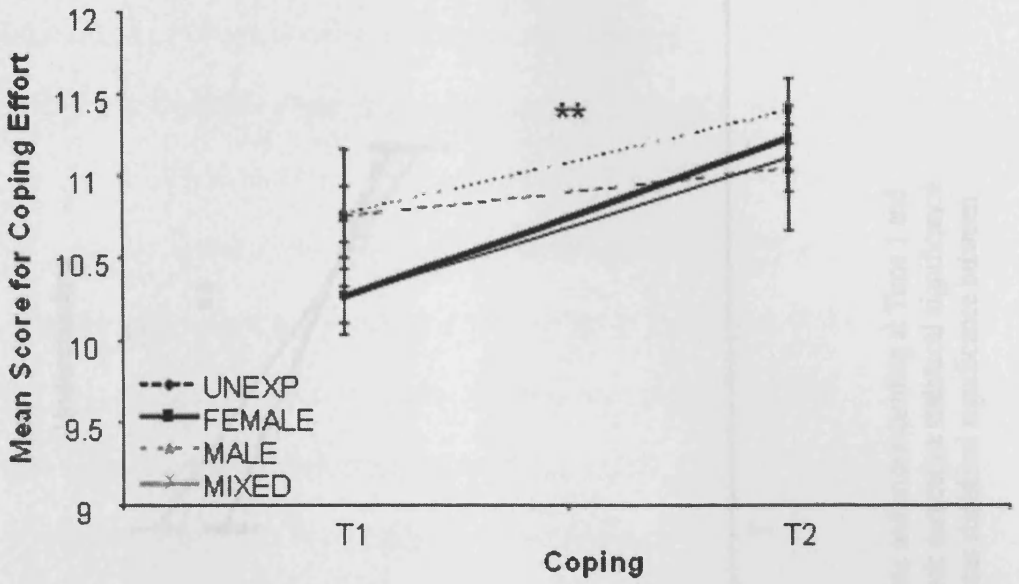

Figure 2.4 Men's mean scores of coping at Time 1 and after 12 months of treatment at Time 2 by infertility diagnosis. Asterisk demotes a statistical significance between T1 and T2 across all groups (**p<.001).

2.4.7 Openness about infertility

Results of the Wilcoxon Signed Rank test revealed that overall there was a trend for men to be less open about how they feel about not being able to have a child at Time 2 as compared to Time 1 (T=-1.798, p=.072 two tailed). It was also revealed at Time 1 35.1% (n=27) of men with female factor infertility spoke "to most people I know" compared to 16.7% (n=13) at Time 2, and 54.5% (n=42) spoke "only to close people" at Time 1 compared to 74.4% (n=58) at Time 2 (T=-2.097, p=.036 two tailed), however, with Bonferroni correction these results were not significant.

Overall men were less open about the reasons for their childlessness at Time 2 compared to Time 1 (T=-2.198, p=.028 two tailed). Again it was revealed that at Time 1 27.3% (n=21) of men with female factor infertility spoke "to most people I know" compared to 14.3% (n=11) at Time 2, and 61.0% (n=47) spoke to "only to close people"

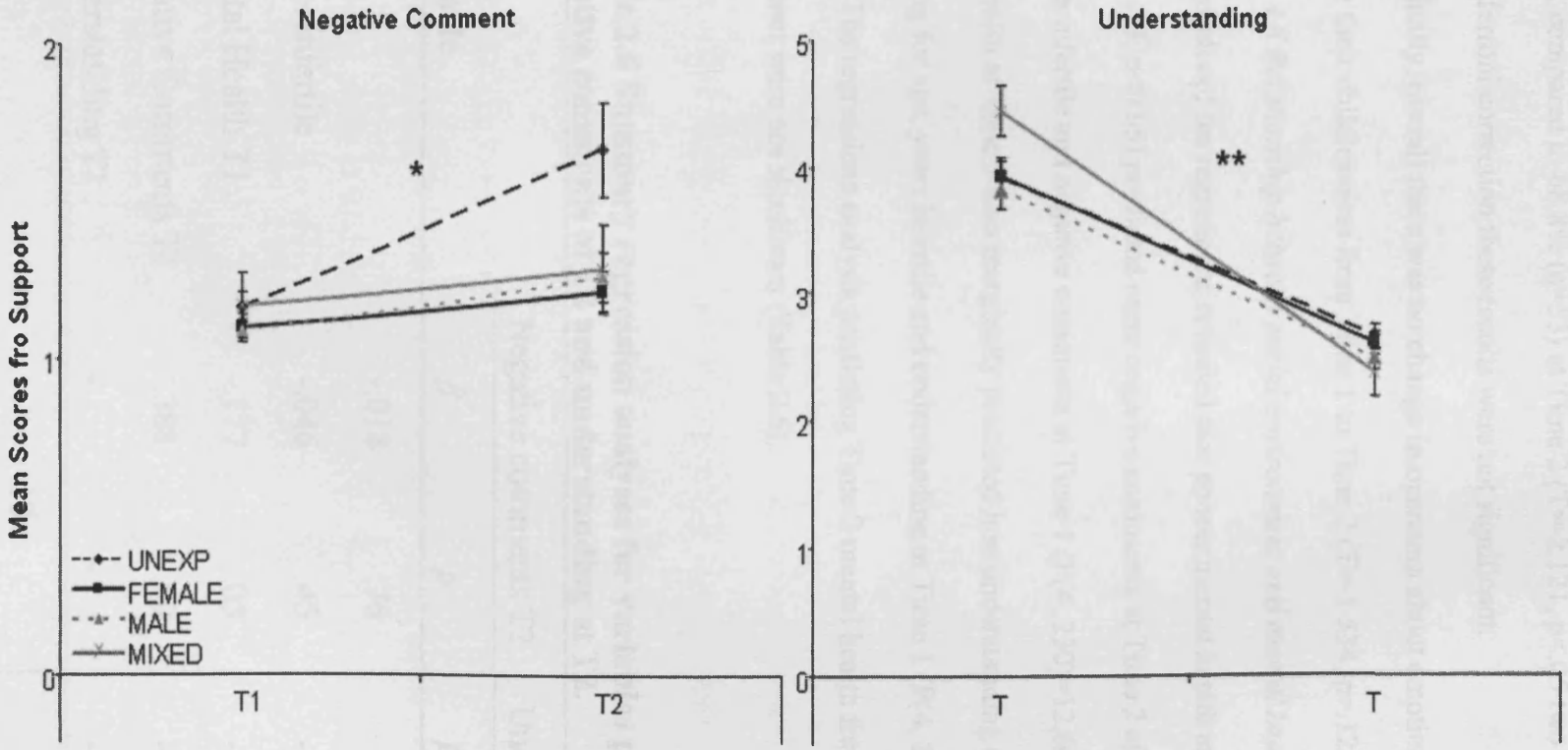

Figure 2.5 Men's mean scores of support variables, negative comments and understanding at Time 1 and after 12 months of treatment at Time 2 by infertility diagnosis. Asterisk denotes a statistical significance between T1 and T2 across all groups (*p<.05, **p<.001). Cross denotes statistical significance between unexplained group, and male and female groups (†p<.05).

at Time 1 compared to 68.8% (n=53) at Time 2 (T=-2.121, p<.05 two tailed), however, with Bonferroni correction these results were not significant.

Finally, overall there was no change in openness about emotional feelings regarding their childlessness from Time 1 to Time 2 (T=-1.534, p=.125 two tailed).

2.4.8 Relationship between social environment and mental health

Results of the regressions revealed that poorer mental health at Time 1 (ß= -.177 t(227)= 1.93, p<0.05) predicted more negative comments at Time 2 after controlling for age, years infertile and negative comments at Time 1 (F(4, 230)=12.66 p<0.01). Poorer mental health at Time 1 also marginally predicted less understanding at Time 2 after controlling for age, years infertile and understanding at Time 1 (F(4, 230)=2.09, p=.083). The regressions analysis predicting Time 2 mental health from Time 1 social environment were not significant (Table 2.6).

Table 2.6 Summary regression analyses for variables predicting negative comments at T2 and understanding at T2.

	Negative comments T2		Understanding T2	
Variable	*β*	*p*	*β*	*p*
Age	-.018	.76	.029	.66
Years infertile	-.046	.45	-.015	.83
Mental Health T1	-.177	.05	-.043	.51
Negative Comments T1	.388	.00	-	-
Understanding T1	-	-	-.171	.01
R^2	.18		.035	
F	12.658***		2.09	

***p<.001

2.5 Discussion

Two principal findings emerged from this investigation. First, there was no evidence to support the idea that there was more stigma or protectiveness among men with male factor infertility. Second, men with male factor infertility did not suffer more than men with other diagnoses, and all men showed increased suffering over time when treatment was not successful. These findings indicate that being in a couple infertile and failing treatment is challenge and emotionally demanding for most men in the present study.

One main finding was that most men were open about their fertility problems. Past research has shown poor agreement between husbands and wives' report of infertility medical information (Berg & Wilson, 1991; van Balen et al., 1996) particularly for recall of information related to male factor infertility (Carmeli, 1994). One cause of this discrepancy may be the desire of couples to protect the man from the stigma of infertility (van Balen et al., 1996). In the present investigation agreement was high between spouses and between men and physician reports. In the 10-20% or so of cases where disagreement existed there was no evidence of greater dispute in the group of men with male factor infertility. It was also found that men across diagnostic groups reported equal levels of talking to other people (e.g., close friends, family) about their infertility, its causes and its emotional impact on their lives at Time 1 when social environments were reported as being supportive. Together these results would suggest that most men, including those with male factor infertility, were open about their fertility problems.

These findings diverge from those of previous research, and this may be due to several factors. First, a discrepancy between spouses or between patient and medical records may be due to miscommunication rather than to secrecy. For example, Van

Balen (1996) found that 38% of couples could not recall the correct diagnosis after speaking with their physicians. Today couples may be more informed about fertility issues compared to a decade ago, for example through greater use of the internet to access fertility information therefore leading to more accurate knowledge among patients (Haagen et al., 2003). Second, if the use of donor sperm and the subsequent withholding of that information from potential offspring was the reason for secrecy in past work, then the low incidence of treatment with donor sperm in our study (i.e., 5%) may explain why there is more openness about diagnostic information in our work compared to before the use of therapies to circumvent male infertility (i.e., intracytoplasmic sperm injection) became more widespread.

A second main finding of the study was evidence that men's social network became more negative and less supportive over time, with greater overall social stress. It has previously been found that depending on the social group investigated (family and close friends) social support is overall positive (van Balen et al., 1996). This study finds evidence in line with these findings however, due to the longitudinal nature of this design it was also demonstrated that social support was not stable and in fact deteriorated over time for all men. Overall men reported that fertility problems caused more stress with in-laws, friends and colleagues and also reported receiving more negative comments and less support. Although data on the nature of negative comments was not available, other studies have found that the most frequent negative comments directed to infertile couples were that they were selfish, they would be lonely in their old age (or after their spouse died) and that they would forever feel unfulfilled (Callan, 1983).

The deterioration in social environment was more pronounced in men with unexplained infertility. It has previously been reported that personality, social

behaviour, and anxiety levels change in men with unexplained infertility (Dhaliwal, Gupta, Gopalan, & Kulhara, 2004), and that women with unexplained infertility are more depressed and more anxious than controls (O'Moore, O'Moore, Harrison, Murphy, & Carruthers, 1983). However, other studies report no differences between unexplained infertility versus other infertility diagnoses in couples (Adler & Russell, 1985; Wischmann, Stammer, Scherg, Gerhard, & Verres, 2001), which is more consistent with the majority of results found here. Overall our results show that men perceive their social environment to be less supportive over time, regardless of diagnosis.

Another finding was that across all diagnostic groups, suffering increased over time when treatment was not successful indicating that suffering was not specific to male factor diagnosis or disproportionate for this group. Suffering included psychological deterioration as evidenced by increased general mental anguish (e.g., despondency, irritation) and physical stress reactions (e.g., muscle weakness, chest pain) and a concomitant increase in the coping effort required to manage fertility problems. This pattern of results indicates that it is involuntary childlessness that is taxing and leads to suffering and not simply the self-blame of being the cause of the couples' infertility, as would be suggested if men with male factor infertility suffered more then men with other diagnoses.

An important issue to address given the parallel deterioration in psychological suffering and social environment is to what extent these two life aspects impact on each other. The link between psychological and social suffering is likely to be bi-directional. DeLongis (1988) found that people with unsupportive social environments were more likely to experience somatic problems. Also, Cohen and Wills (1985) found that social support attenuated or "buffered" the effects of stressful events and others have found that support was predictive of well-being (Walen & Lachman, 2000). However, Thoits

(1995) reported that some stressful events could result in a loss of social support. Specifically chronic stress (e.g. depressive symptoms, death of a close family member) can lead to increased cost for the social network eventually leading to reduced social support. In the present study, it was found that the causal path was stronger for the influence of mental health on perceptions of the surrounding environment (e.g., negative comments and understanding). A persistent lack of treatment success and the increased emotional suffering that it causes may act as a chronic stressor that places a great burden on social networks. Further, it may become increasingly difficult for friends and family, particularly those with children, to know how to act with the emotionally vulnerable infertile couple, and this uncertainty could lead to avoidance of that couple with a secondary effect then being lost opportunities to provide support. Thus it may not be a direct withholding of support that is perceived by the infertile man but a by-product of greater isolation (or withdrawal) of the infertile couple from the wider social network of (fertile) family friends. Indeed several studies show that couples do avoid fertile friends and family as a way of coping with fertility problems (Peterson, Newton, Rosen, & Skaggs, 2006). This burden may cause a breakdown in social support eventually contributing to further increased stress over time.

The results also show that emotional suffering may have physical consequences that could perpetuate the fertility problem. Results showed a significant increase in physical stress reactions over time such as muscle weakness and chest pain. These symptoms are connected to HPA activation. For example, Setterlind (1995) showed that high scores on the Stress Profile measure (from which most items were drawn) were associated with elevated cortisol levels. A few prospective evaluations have shown an association between emotional distress and sperm quality (Clarke et al., 1999; Fenster et al., 1997; Kentenich et al., 1992; Pook et al., 2004). Our results suggest that the

emotional consequences of persistent infertility may, paradoxically, contribute to maintenance of fertility problems. However the causal strength of this association needs confirmation in experiments that can adopt rigorous experimental controls.

The clinical implications of these results depend mainly on what men need in order to manage the strains of persistent infertility. Past research suggests that men might be interested in using psychological counselling to deal with issues such as anxiety, depression, masculinity, and selfhood (Hunt & McHale, 2007; Verhaak et al., 2001), but in practice few men actually take up offers of counselling. One study showed that $\leq 11\%$ of men used counselling and fewer still support groups (Boivin, Scanlan, & Walker, 1999). However, in light of the increasing demands fertility problems pose on coping resources and potential negative effects on physical reactions, it seems imperative that some sort of needs assessment be made to identify what interventions might better suit men. In other areas of health research such as cancer, anonymous online chat groups are used more often then face-to face groups (Campbell et al., 2001). Research is needed to investigate whether men would use infertility related internet support groups. It seems likely that online support groups would be accepted and used by men is shown in other areas of health (Campbell et al., 2001) also the anonymity provided by the internet may also encourage men to openly discuss their feelings.

The results of this prospective study are important because they are the first to demonstrate in a homogeneous cohort of men undergoing fertility treatment that diagnosis is not a critical factor in predicting psychosocial reactions to fertility problems or consequences of failed fertility treatment. Stringent inclusion criteria were applied to ensure the sample was as homogenous as possible with respect to factors that could have impacted on reactions at T1and T2. Furthermore, diagnosis was obtained from three perspectives that of the man, his partner and their physician in 88.3% of the

sample. The multivariate approach enabled the detection of causal effects between psychological and social environment, and these will underpin future research. Finally, this sample was similar to other psychological research indicating that our users were representative of the cohort of people likely to seek out these types of interventions. Together, these strengths ensure that the associations observed in the present study are reliable and valid and can be generalised to patient populations in other fertility clinics.

The limitations of the study were that sufficient information to give precision to deterioration in psychological functioning and the social environment was lacking. The kinds of comments men were receiving was not known nor in what way they perceived their social environment to be less supportive. Similarly, it is not known whether there was an actual or only perceived decrease in social support. Answers to such questions might better be obtained using qualitative analysis. Finally, it is not clear to what extent our findings would replicate across cultures and ethnic groups. Although information about ethnicity was not available in the present study, the ethnic make-up in Denmark is mainly Caucasian, who do not associate with any particular religious group. These results may not generalize to be people from different cultures and/or with strong religious affiliations. There is evidence to suggest, for example, that male factor infertility is very problematic in South Africa, a strongly patriarchical society. A sample of infertile men from South Africa reported suffering from considerable verbal abuse, as well as, a loss in social status (Dyer et al., 2004). It would, therefore, be of interest to replicate this study in different cultures.

In conclusion the findings contribute to much needed research on men's reaction to infertility and infertility diagnosis. The results showed that men in this study, with male factor infertility did not suffer more then men in a couple with infertility due to other causes. When treatment was not successful men's physical and mental health

deteriorated as well as their supportive social environments. There was no compelling evidence to suggest that secrecy existed with regard to diagnosis. Furthermore, the increased strain on coping resources can potentially have physical consequences that could paradoxically impact on fertility. Together the findings suggest that it is important to conduct further investigation regarding interventions to help minimise suffering and improve quality of life for men with fertility problems.

Chapter 3

Testing and developing an animal model of the effects of stress on reproductive outcomes.

3.1 Introduction

As early as the 17th century Rene Descartes dissected animals to gain insight into human anatomy. Since then the development of animal models of anatomy, disease, and drug testing have continued. Animal models provide several advantages over clinical research: easily controlled environment, a greater variety of interventions, as well as, the ability to take measurement that would otherwise be impossible to obtain from humans. Today many animal models of disease exist that continue to help us improve our understanding of many conditions affecting society. Epilepsy (Murphree, Rundhaugen, Kelly, & Ramsay, 2007), glaucoma (Rasmussen & Kaufman, 2005), Alzheimer's disease (Gotz et al., 2007), and even cancer (Bachmann & Lock, 2007; Gonzalez, 2007; Varady & Hellerstein, 2007), are among the many medical conditions for which there are animal models in use. Many psychiatric conditions also utilise animal models, for example, schizophrenia (Nabeshima, Mouri, Murai, & Noda, 2006), autism (Lewis, Tanimura, Lee, & Bodfish, 2007), and post-traumatic stress disorder (Stam, 2007). All these models have the same goal; to better understand disease, and to develop treatments and even cures.

Current knowledge is limited with regards to the impact of psychosocial stress on human male fertility, and an appropriate animal model of stress and infertility would be an important step towards a better understanding of this issue. The rat is a small rodent which is obtained from breeders, housed and cared for. Unlike other rodents, such as the mouse for example, the rat is not as a aggressive an animal and its social patterns also make it well suited as the basis for a model of human social interactions (Koolhaas, de Boer, & Buwalda, 2006).

An ideal animal model, however, should be similar to the disorder it models, in terms of etiology, biochemistry, symptomatology, and treatment (Terje, Vivienne,

Heidi, Espen Borgå, & Mehdi, 2005). Animal models are based on the premise that humans and the animals in question are biologically very similar, and this is indeed the case between human and rats for many conditions, including many aspects of reproductive health. Both humans and rats have HPA and HPG axes which control stress and reproduction, respectively. In fact, the concept of stress and its interaction with the HPA axis was first described in rodents by Hans Selye (1936). Both humans and rats secrete cortisol and corticosterone in response to stress, however the dominant glucocorticoid in humans is cortisol whereas it is corticosterone in the rat.

There are numerous ways to assess the effects of stress in rats. One method in which hormonal responsiveness to stress can be measured is through decapitation and trunk blood collection and analysis of circulating plasma corticosterone levels. It is also possible to collect repeated blood samples from rats allowing for multiple hormonal profiles to be obtained over time with minimal discomfort to the animal. Also, through necropsy at the end of the experiment, weight of the adrenal glands can be recorded. This is an important measure as excessive secretion of glucocorticoids leads to hypertrophy of the adrenal glands (Dallman et al., 1992). And finally, stress has been shown to reduce hedonic responsiveness in rats which leads to a decrease in food consumption (Willner, Moreau, Nielsen, Papp, & Sluzewska, 1996). It is therefore possible to record body weight as measurement of stress (Harris et al., 1998; Marti, Marti, & Armario, 1994; Rybkin et al., 1997; Willner et al., 1996). Although this effect is more commonly seen in moderate to severe stress, body weight can also be examined as a measure of the overall health and well-being of the animal (Marti et al., 1994).

Within the HPG axis there are two notable differences between humans and rats, first, the human spermatogenetic cycle takes approximately 70 to 80 days, whilst it takes approximately 12 days in the rat (Aslam et al., 1999). Second, human sperm heads

are oval in shape whereas they have a hook shape in the rat. In the rat, standard sperm analysis can be carried out in conjunction with other reproductive hormonal assessments such as testosterone. In the rat, sperm analysis is most often analysed from sperm extracted from the epididymis (Ernst, 1989) due to the difficulty in obtaining an ejaculate from rats. Ideally an ejaculated sample of semen would be obtained as contributions from the accessory glands can have an impact on sperm quality (Diamandis et al., 1999), however this technique has not been successfully developed in rodents at this time (see Discussion 3.7).

Other reproductive measures obtained in rats include the dissection and examination of internal reproductive organs, including testicular weight, epididymal weight, and seminal vesicle weight (Almeida, Petenusci, Anselmo-Franci, Rosa-e-Silva, & Lamano-Carvalho, 1998; Gronli et al., 2005; Retana-Marquez, Salazar, & Velazquez-Moctezuma, 1996). The weight of internal organs can give us an idea of the overall functioning of the reproductive system. Testicular and/or seminal vesicle atrophy, for example, is often followed by reduction in function and unstable circulating testosterone levels, known as hypoadrongenism (Glass, Beach, & Vigersky, 1985).

Sperm quality and organ examination are only one level of male fertility, and in rats sexual behaviour is also commonly assessed as part of overall reproduction. Sexual behaviour, including time until ejaculation and number of ejaculations for example, can also be affected by stress and it can have an impact on reproductive measures such as offspring (Agmo, 1997). After sexual interaction in rats the number and quality of copulatory plugs can also be examined. Copulatory plugs form in the vagina of females and consists of constituents of male reproductive accessory glands (i.e., vas deferens, seminal vesicles), and are thought to prevent the outflow of sperm (Voipio, 1998). In rats, copulatory plugs are essential for determining pregnancy, and are known to be very

important for transporting sperm from the cervix to the uterus, however this mechanism is not well understood (Cukierski, Sina, Prahalada, & Robertson, 1991; Sofikitis et al., 1990; Toner, Attas, & Adler, 1987).

There are other important measures of the effects of stress on male reproduction including number of offspring born, or pre- and post-foetal implantation in a female who has mated with a male rat (Almeida, Kempinas, & Lamano Carvalho, 2000). However, the purpose of the present study was to assess the psychosocial influence of stress on male fertility potential which cannot be address by live births since this also requires a female contribution. Although implantation and live birth outcomes can be considered "true" markers of fertility (while other parameters are markers of reproductive potential) they are more complicated to assess. For example, a female colony of rats is required for this type of research and for this reason alone these outcomes are not commonly assessed. Also the presence of another animal, namely the female that carries and delivers the pups, adds numerous uncontrolled influences. Under these conditions it becomes more difficult to discern the source of the problem if a negative reproductive effect is found (e.g., small litter size). For example, stress could influence semen quality in the male, or male sexual arousal, stress could also affect male sexual behaviour which could then affect stress levels of the female which may carry through to fertilisation, pregnancy and even birth. It is therefore important that the effects of stress on male reproductive parameters be examined first before other types of fertility outcomes which rely on the female rat.

Animal models of stress and fertility already exist utilising several different stress paradigms. These can be grouped into five broad stressor categories: physiological, physical, psychological, multiple stressors and social stressor paradigms.

3.1.1Physiological stressors

A physiological stressor is defined by the introduction of exogenous stress hormones into the animal, injections of corticosterone, for example.

When attempting to understand the effects of stress on reproduction in a rodent animal model it is a typical place to start. After injecting different doses of corticosterone directly into the rat, effects on reproductive outcomes can then be assessed. Lerman (1997) injected male rats daily with 0, 10 or 25 mg/kg of corticosterone for a period of six weeks. It was found that injections of either dose of corticosterone resulted in a reduction in body weight, as well as a reduction in the number of copulatory plugs as compared to controls. After mating, females paired with male rats that were injected with the higher dose of corticosterone showed a lower number of pre and post implantation sites as well as fewer live births, as compared to controls. No differences were found in epididymal sperm count or motility.

These results indicate that corticosterone played an important role in the ability of sperm to fertilise (i.e., implantation and live births) but not its quality. It is possible that the reduced rate of pre and post implantation sites was related to the reduced number of copulatory plugs, and the stress effects on reproductive accessory glands, however these measurements were not examined in this experiment. Also the lack of effect on sperm quality could be due to the fact that sperm quality was measured after the rats were exposed to and copulated with female rats, which is known to increase sperm quality (Taylor, Weiss, & Komitowski, 1983; Toner & Adler, 1986), and arousal effects may have obscured the effects of physiological stress. The doses used in this experiment were relatively high, as a dose of 25mg/kg would be considered supra-physiological in rats (Lerman et al., 1997). Although these data are an important indicators of the potential role of corticosterone it is important to note that other studies

that injected rats with lower doses of corticosterone (0.5, 1, 2, 4 mg/kg), more similar to levels that would be seen in response to a mild stressor, showed a lack of effect, and no differences in sexual behaviour (e.g., number of ejacualtions) (Retana-Marquez, Bonilla-Jaime, & Velazquez-Moctezuma, 1998).

One limitation of the physiological stressor paradigm, is that exogenous corticosterone only mimics the final phase of the stress response cascade, and effects on reproductive outcome may arise only (or principally) when the entire HPA axis has been activated.

3.1.2 Physical stressor paradigms

A number of physical stressor paradigms have also been developed. A physical stressor is defined as a stressor that induces a physical effect on the body, pain for example. Stressors that belong to this category may also produce psychological or social stress but as these are considered to be secondary to the physical stressor the paradigms are therefore placed into the physical stressor category.

Although the focus here will be on rodent animal models (rat, mice) it was felt that the study conducted by Cui (1996b) was sufficiently relevant to be included. Cui (1996a) exposed marmoset monkeys to social isolation stress for two years, as well as the physical stress of having blood withdrawn once a week for a two year period. This combination of stressors had detrimental consequences on all sperm parameters (decreased volume, count, motility, and impaired morphology) compared to monkeys housed in pairs that did not have blood taken regularly. It is important to note, however, that a measure of stress, such as cortisol, the dominant glucocorticoid in marmoset monkeys was not taken (Johnson, Brady, Gold, & Chrousos, 1996). Also, although differences were seen between the stress condition and controls, all animals were subjected to rectal electro-ejaculation, a technique known to have negative effects on

sperm count, motility, and volume (Schneiders, Sonksen, & Hodges, 2004; Sonksen & Ohl, 2002; Yeoman, Sonksen, Gibson, Rizk, & Abee, 1998). Although all the animals were exposed to electro-ejaculation and therefore this technique is not a confounding variable, it is still interfering with sperm quality and a less damaging technique should be utilised. Nevertheless, this study demonstrates that stress has a negative effect on sperm quality in a primate model but one cannot know whether these effects were due to the social isolation or to the repeated physical stress of blood sampling.

Footshock stress is another physical stressor that has been shown to continuously elevate corticosterone levels after each exposure (Retana-Marquez, Bonilla-Jaime, Vazquez-Palacios, Martinez-Garcia, & Velazquez-Moctezuma, 2003a). It involves placing an animal in a box with an electrified grid floor. The animal is then exposed to a shock at an interval, intensity and duration chosen by the researcher. This stressor can be applied for one day or over many days. It was shown that after one exposure to 5 minutes of footshock there was no change in sexual behaviour compared to the control group in rats placed in the same box but not exposed to stress (Retana-Marquez et al., 1996). In contrast, there was a decrease in quality of sexual behaviour after 20 days of five minutes a day of footshock (Retana-Marquez et al., 1996), including a reduction in the number of ejaculations (Retana-Marquez et al., 2003a), as compared to controls. Furthermore, testosterone levels were significantly lower than controls (Retana-Marquez et al., 2003a). In hamsters a reduction in the number of ejaculations was also seen after 12 days of 90 minutes of footshock (Holmer, Rodman, Helmreich, & Parfitt, 2003). These results demonstrate that chronic administration of footshock interferes with the early stages of reproduction (sexual behaviour), as well as reproductive hormonal control (i.e., testosterone), however, sperm quality was not

measured in these studies so it is not known to what extent such effects would impact on such parameters.

The effect of exercise stress on reproduction has also been examined. Three hours a day, five days a week, for four months of swimming exercise was not found to impact on testosterone levels or sperm count (Woody et al., 1998), however sperm count was the only measure of sperm quality taken. Mingoti (2003) however, found that 3 minutes of swimming a day for 15 minutes per day had a significant negative effect on spermatid production but not on testicular weight, seminal vesicle weight, nor the number of foetal pre or post implantations. In both studies stress was not assessed, so the extent to which this level of physical exercise increased HPA activation is not known. The lack of testosterone suppression (Woody et al., 1998) would suggest no HPA activation but other research on exercise stress shows a clear stress response. Running or swimming exercise increased the size of adrenal glands in rats (Droste, Chandramohan, Hill, Linthorst, & Reul, 2007), as well as corticosterone (Contarteze, Manchado, Gobatto, & De Mello, ; Droste et al., 2007), and ACTH levels (Contarteze et al., 2007) compared to stationary control groups of rats. The mechanism of action for exercise is not yet known. Extreme exercise in humans (for example professional cyclists) produces negative effects on sperm quality through increased testicular temperature (Lucia et al., 1996). In animals chronic exercise has also been shown to increase overall body temperature (Gronli et al., 2005). Thus, exercise stress effects on reproductive parameters may be due to a number of secondary effects rather than on HPA activation per se.

Some physical stressors induce pain in the animals (e.g., blood withdrawal, footshock) which itself may elicit reactions from other biological systems, such as an immune, response that could interfere with the HPA/HPG interactions (Kusnecov &

Rossi-George, 2002). Consequently, with physical stressor paradigms reproductive effects could be due to either the psychological stress or physical stress or a combination of both.

3.1.3 Psychological stressor paradigms

Other stressor paradigms capitalise on natural fears among the rodent population, these are known as psychological stressors. Immobilisation stress has previously been shown to elevate corticosterone levels with some mild habituation over time (Retana-Marquez et al., 2003a). It involves placing an animal in a confined space where it is unable to move. Several experiments have been conducted examining the effect of this stressor on different reproductive parameters. Rats exposed to 20 days of 2 hours a day of immobilisation showed a decrease in sexual behaviour, including fewer ejaculations as compared to rats in the control condition (Retana-Marquez, Bonilla-Jaime, Vazquez-Palacios, Martinez-Garcia, & Velazquez-Moctezuma, 2003b). When sperm quality was assessed after 6 hours a day for 60 days of immobilisation stress, a reduction in sperm count was observed (Almeida et al., 1998) and a decreased number of spermatids (Almeida, Petenusci, Franci, Rosa e Silva, & Carvalho, 2000), as well as, an increase in the number of damaged sperm (Almeida, Kempinas et al., 2000). No change in testicular size was seen, however there was a decrease in seminal vesicle weight (Almeida et al., 1998). There was also an increase in pre and post implantation loss in females mating with rats exposed to immobilisation stress as compared to controls (Almeida, Kempinas et al., 2000). A decrease in testosterone levels was also found in rats exposed to just 3 hours of immobilisation stress (Orr & Mann, 1992; Orr, Taylor, Bhattacharyya, Collins, & Mann, 1994).

The immobilisation stressor paradigm has considerable negative impact on several levels of reproduction, such as sexual behaviour, sperm quality and production,

as well as foetal outcome. There may be unintended side effects of this stressor paradigm that may confound results. It is known that increased temperature of the testicles reduces testicular size and sperm production and induces morphological changes in sperm (Bedford, 1991). Immobilisation stress may lead to a rise in the temperature of the testicle by raising the testicles close the body when placing the animal in a tight restraint tube (Almeida et al., 1998; Retana-Marquez et al., 1996).

Other psychological stress models include exposing the animal to predator odour. Blanchard (1998) found that when rats were exposed to cat odour corticosterone levels rose, and unlike many other stressors there was no habituation. After 20 days of exposure to cat odour corticosterone levels peaked at the same high levels as the first exposure. Despite the stress reaction, no differences were found between rats exposed to cat odour and controls in testicular weight or testosterone level. Thus, chronic HPA activation may not impact on these specific reproductive outcomes. However reproductive outcomes were measured at the end of the experiment. Therefore it is possible that there was recovery, thus the state of the reproductive organs at other times during the experiment is unknown.

3.1.4 Multiple stressor paradigms

One problem of previous paradigms using single stressors is that after chronic exposure to a stressor most animals adapt and peaks in corticosterone levels are no longer as high as the first or second exposure to that stressor (with the exception of predator odour). To limit the amount of habituation the chronic mild stress (CMS) paradigm was developed (Gronli et al., 2005; Willner, Towell, Sampson, Sophokleous, & Muscat, 1987). In this paradigm the stressor involves exposing the animals to a different mild stressor everyday for example cage tilt, food deprivation, water deprivation, empty water bottle, wet cage, continuous light, or continuous dark. This

paradigm increases corticosterone levels (Bielajew, Konkle, & Merali, 2002) but also induces anhedonia as demonstrated by reduced sleep and decreased consumption of sucrose (Gronli et al., 2004). After 28 days of exposure to CMS there was a reduction in overall sexual behaviour in rats, including fewer ejaculations as compared to controls, and this effect was seen as early as 2 weeks after the onset of the stressor (Gronli et al., 2005). Although the paradigm produces stress effects, it is impossible to determine the cause of such effects because several different stressors are used. For example, the paradigm includes depriving animals of food and/or water for short periods at a time but over many weeks, and it may be possible that negative effects on reproduction could be influenced by poor nutrition. In rats, food deprivation has been linked to lower sperm motility (Chapin, 1997), as well as, a reduced number of pups per litter (Parshad, 1993). Nevertheless these results indicate a negative stress effect via sexual behaviour and merits further investigation into other reproductive parameters.

3.1.5 Social stress paradigms

Other animal models of stress and infertility attempt to model human social stress. Social stress paradigms induce a stress response through negative social interactions, these include the resident/intruder paradigm, social dominance, the visual burrow system (VBS), aggressive colonies, and/or cage rotations. The resident/intruder paradigm is when a subordinate rat/mouse is placed in the home cage of a more dominant (and usually larger) rat/mouse, this technique is known to increase corticosterone levels in the subordinate animal, but adrenal gland weight does not change (Bhatnagar & Vining, 2003), sexual behaviour also decreases, as well as, testosterone levels (Niikura et al., 2002).

Similar to the resident/intruder paradigm, the social status of the animals is determined through three tests a week for eight to fifteen weeks and then one dominant

and one subordinate animal are housed together. In mice, it was found that the subordinate animal had fewer motile sperm, and sperm count, testicular, and seminal vesicle weights remained unchanged compared to dominant animals (Koyama & Kamimura, 1998).

The VBS is a habitat providing burrows and an open area for mixed-sex rat colonies to interact allowing the experimenters' to observe social interactions (R. J. Blanchard, McKittrick, & Blanchard, 2001). Similar results to the resident/intruder paradigm were found; subordinate rats had increased corticosterone levels, and increased adrenal gland weight compared to controls, providing evidence that the technique did induce a stress response. Although sperm quality was not assessed, testicular atrophy and lowered testosterone levels were found in the subordinate rats compared to control animals (Blanchard et al., 1995).

Instead of examining dominant and subordinate animals directly, one can examine animals with high and low aggression. Taylor (1987) created low and high aggression colonies, the high aggression colony was also labelled as the high stress condition, and to minimize habituation, rats' were daily switched from one cage to another between aggressive colonies. Results demonstrated that although the high aggression (high stress) colonies had the highest corticosterone levels and increased adrenal gland weight they also had higher testosterone levels and higher sperm counts. This could be explained by the fact that aggression and competitiveness in rats is known to increase testosterone levels (Albert, Walsh, Gorzalka, Siemens, & Louie, 1986), and sperm production is highly dependent on testosterone (Hernandez et al., 2007) and therefore possibly blocking or minimizing the effects of stress on reproduction and sexual behaviour. Although stress is generally thought to suppress testosterone (Demura et al., 1989) it appears that this is only the case when testosterone levels are stable.

However, in the presence of challenges to dominance that increase testosterone, stress does not have an effect on testosterone levels (Albert et al., 1986).

However, evidence has been found suggesting that social stress of daily cage change for 28 negatively affects reproduction even when without separation based on aggressive behaviour (Lemaire, Taylor, & Mormede, 1997). Rats were housed in cages of three and on a daily basis the occupiers of each cage were changed (technique by (Mormede et al., 1990)). This procedure was found to increase corticosterone levels, and adrenal gland and seminal vesicle weights compared to controls; other organ weights remained unchanged (epididymal weight, thymus weight) (Lemaire et al., 1997). These interesting and inconsistent results found in reaction to social stress merit further investigation that should include analysis of additional reproductive measures.

3.1.6 Commentary

Table 3.1 summarises effects of the results of studies examining the effect of stress on male reproductive outcomes. Overall there is only partial support that stress does have a negative impact on reproduction as 22 of 39 (56%) of comparisons showed decrease in at least one reproductive outcome. However, there is little consistency between studies in reproductive measures and techniques, making comparisons difficult.

Sperm quality is the most commonly measured outcome, the majority of studies which examined sperm quality found an effect (social isolation/blood withdrawal, footshock, exercise, immobilisation, social dominance), although some contradictory evidence exists in one study where no effect was found (corticosterone injections) and

Table 3.1 Summary of the male reproductive outcome effects according to type of stressor.

Stressor	Sperm Quality				Testosterone	Organ Weights			Sexual Behaviour	Copulatory Plugs	Pre/Post Implantation	Live Births	References
	Vol	Count	Mot	Morph		Testicular	Epididymal	Seminal Vesicle					
Physiological													
Corticosterone Injections		-	-						-	↓	↓	↓	Lerman 1997, Retana-Marquez 1998
Physical													
Social isolation/ Blood withdrawal (Monkey)	↓	↓	↓	↓									Cui 1996
Footshock					↓				↓				Retana-Marquez 1996, Retana-Marquez 2003, Holmer 2003
Exercise		↓			-	-		-			-		Woody 1998, Mingoti 2003
Psychological													
Immobilisation		↓		↓	↓	-		↓	↓		↓		Almeida 1998, Almeida 2000, Retana-Marquez 2003 Orr 1992, Orr 1994
Predator Odour					-	-							Blanchard 1998
Multiple Stressors													
CMS									↓				Gronli 2005
Social													
Resident/intruder					↓				↓				Niikura 2002, Bhatnagar 2003
Social dominance (Mouse)		-	↓			-		-					Koyama 1998
VBS					↓	↓							Blanchard 2001
High aggression		↑			↑								Taylor 1987
Cage change							-	↑					Lermaire 1997

Note. ↓ decrease, ↑ increase, - no change, blank outcome not measured.

another where an increase in sperm count was found (high aggression). Sperm morphology was only affected by the longest of the chronic stressors reviewed (60 days of immobilisation, for example (Almeida, Kempinas et al., 2000; Almeida et al., 1998) and 2 years of social isolation and weekly blood withdrawal (Cui, 1996b)).

Differences in sperm morphology in rodent species are difficult to detect. The curved shape of the sperm head makes subtle differences difficult to detect, such as angle of the curve and requires a very powerful microscope as well as much practice and training. However, in the two studies which examined morphology (monkey, rat) (Almeida, Petenusci et al., 2000; Cui, 1996b) morphological changes were found as would be predicted after exposure to a stressor that lasts longer than one time of ejaculation the sperm is already completely formed, therefore acute stress at spermatogenetic cycle. The 12 days during which sperm is developed in the male reproductive tract is the only time in which sperm morphology could be affected. At the about the time of ejaculation is not likely to affect sperm morphology. Morphological changes can only take place during spermatogenesis and therefore chronic stress that covers this period (12 days) is the most likely to lead to a negative effect on this sperm parameter. Effects on other sperm parameters may indicate that accessory glands and their outputs may be negatively affected by stress which, in turn, could potentially be the cause of deficiencies noted in count or motility. For example, for sperm to be fully motile it requires energy in the form of fructose from the seminal vesicles (Jones, 1997). If the seminal vesicles and its contents have been impaired, for example by stress, then sperm motility may be affected.

Of the studies reviewed here, six examined the effects of stress on testosterone levels; three found a reduction and two found no differences in testosterone after stress, one found an increase in testosterone levels in an aggressive colony. It is clear that the

response of testosterone to stress is not completely understood; there is even contradictory evidence between different types of social stressors (Blanchard et al., 2001; Taylor et al., 1987). The role testosterone plays in aggression, however, has attracted much attention and research. It has been found that testosterone levels rise in males before a physical challenge or social competition (Mazur & Lamb, 1980), and that testosterone levels rise in the winner of an altercation but decrease in the loser. There is an interaction between testosterone and social dominance: position in the social hierarchy influences testosterone levels while high testosterone levels leads to a higher social status (Giammanco, Tabacchi, Giammanco, Di Majo, & La Guardia, 2005). It is, therefore, possible that testosterone plays a different role in the stress response of a social stressor compared to other stressors. The role of testosterone in response to stress still requires further evaluation.

The situation is more consistent with organ weights as most studies did not find an effect on testicular, epididymal or seminal vesicle weight although it was demonstrated in response to immobilisation stress and VBS stress. An increase in seminal vesicle weight was found in response to a cage change stressor. As previously mentioned stress can affect body weight, therefore it is possible that the lack of effect seen on organ weights is due to the fact that the animal's body weight is not being taken into account. All the above mentioned studies report organ weights directly, the contribution of body weight, and/or the ratio of organ weight to body weight has been overlooked. It is possible that the size of the organ relative to the body is an important measure. Again, the effect of stress on organ weights needs further investigation.

The evidence from this literature review suggests that stress does have a negative effect on male reproduction. However, this effect is in no way consistent between stressors including the nature of the stressor and the duration. It would seem

that some of the inconsistency between studies could be due to the confounds between different types of paradigms.

The psychosocial cage change stressor (PCCS)

In light of this review, an adapted paradigm (PCCS paradigm) was developed for the present studies to capitalise on existing methodologies known to induce a stress response, but with modifications aimed at addressing limitations of each. The purpose of the present set of experiments was to model, using rats, human psychosocial stress.

In a recent study conducted by Schmidt (2007) the stress response to exposing mice to a psychosocial stressor was studied. Psychosocial stress was induced by changing the cage mates around twice a week. This stressor was shown to increase corticosterone levels, as well as, adrenal gland weight, compared to the control group (Schmidt et al., 2007). As mice are very aggressive animals, prone to fighting, using rats instead would mean that the cages could be changed everyday instead of twice a week and could therefore be used as a chronic stressor to cover the spermatogenetic cycle if the changes were made over an extended period of time. Mormede (1990) developed a paradigm where cages were changed everyday, each cage included three rats. This technique was successful in inducing a stress response, including increased corticosterone levels. This paradigm however also included a division between levels of aggression, similar to Taylor (1987) mentioned above.

The Mormede (1990) and Schmidt (2007) techniques were adapted in the present study to create the PCCS paradigm used here. Everyday rats were quasi-randomly placed in a new cage either alone, in a pair, or in a cage of three rats, and never placed in the same combination twice, to minimize habituation as has been observed in immobilisation (Retana-Marquez et al., 2003b) and CMS paradigms (Gronli

et al., 2005). This chronic stressor is flexible and can be applied for as few or as many days as required.

In the present study the stressor was administered to be present throughout the spermatogenetic cycle. As noted effects on sperm morphology would not be expected from a stressor applied for less than the 12 days, as it takes that long for spermatids to develop into fully formed sperm. As the PCCS paradigm does not produce secondary physical effects the results would not be confounded by the concomitant effects of physical stress (e.g., testicular heat, physical pain) as observed in swimming exercise (Mingoti et al., 2003; Woody et al., 1998), or footshock studies (Holmer et al., 2003; Retana-Marquez et al., 1996)). As the paradigm does not require special equipment or expertise as in footshock paradigms, and as daily cage changing only takes a few minutes, this technique is not labour intensive or less so than some other paradigms (e.g., resident/intruder). The suitability, simplicity, and low cost, mean this paradigm could be accessible to other research teams interested in psychobiology of stress effects on fertility potential.

3.1.8 The present experiments

The aims of the animal experiments were twofold. First, to develop and test the PCCS paradigm, and second, to test the effects of PCCS on reproductive parameters.

Rats were exposed to the PCCS for the duration of either one (12 days) or two (24 days) spermatogenetic cycles. To assess the stress response to PCCS after completion of the stressors blood from exposed animals was assessed for corticosterone levels. In later experiments the stress response was also measured by adrenal gland weight. Body weight gain was recorded in all experiments as a measure of stress and overall well-being of the animals.

3.1.9 The hypotheses

The hypothesis was that animals exposed to PCCS would have higher circulating plasma corticosterone levels and heavier adrenal gland weights as compared to control animals that were left in pairs in their cages for the duration of the experiment. It was also predicted that the longer animals were exposed to stress (24 versus 12 day conditions), the higher the circulating corticosterone levels and the heavier the adrenal glands were expected to be.

The reproductive outcomes assessed, depending on the experiment, could include circulating plasma testosterone levels, reproductive organ weights (testicular, seminal vesicle, epididymal weights), as well as epididymal sperm quality. The hypothesis was that animals exposed to PCCS would have lower testosterone levels, lower reproductive organ weights, and reduced sperm quality compared to control animals. Further, these effects should be more pronounced in the longer duration stress condition.

3.2 Experiment 1: Test and evaluation of the corticosterone enzymeimmunoassay kit

3.2.1 Introduction

In order to identify HPA activation as the cause of reproductive deterioration an accurate and sensitive measure of corticosterone is required. The principle approach to assessing stress has been to quantify circulating plasma levels of corticosterone, using dangerous radioactive isotopes. In the present experiments the corticosterone enzymeimmunoassay (EIA) kit was used. This technique is a homogenous competitive enzymeimmunoassay. The plasma sample containing an unknown amount of corticosterone binds to the antibody coated 96 well plate. The plate is then incubated with a corticosterone labelled enzyme, the colour is then developed using a

chromogenic substrate and then read using a spectrophotometric micro plate reader. The levels of the unknown samples are then plotted against known levels of hormone which are plotted on a curve (Chan, 1987).

Although other prepared kits that make use of competitive enzymes are in common use, the corticosterone EIA kit, however, has not been frequently cited in peer reviewed published journals. It was therefore necessary to test the limits, range, and advised dilution of the kit to assess its validity and intricacies. To test the range of doses which can be detected by the kit male rats were injected with three different doses of corticosterone, from 0 ng/ml to 25 ng/ml, which is well within the advertised limits of the kit (15 ng/ml to 1800 ng/ml). Blood was collected and the plasma separated. The plasma was then diluted using three different dilutions from a dilution of 0 to 100, including the dilution recommended by the kit (dilution of 10), all samples were tested in duplicate.

Also information included with the kit suggested that handling, sampling techniques, and lab environments can vary greatly and that each laboratory should establish its own normal ranges of circulating plasma corticosterone. The kit indicates that the range of circulating corticosterone levels in rats would fall anywhere from 23 ng/ml to 363 ng/ml.

3.2.1.1 The hypotheses

In the present study it was expected that diluting the plasma samples by a factor of 10, as suggested by the instructions included in the kit, would yield the best results. Samples which were not diluted should yield results which would be highly concentrated and therefore on the high end of the curve and difficult for the kit to detect. Similarly, samples diluted by a factor of 100 should contain too little hormone and fall below the detectable levels of the kit.

A dose of 10 mg/kg of corticosterone represented a dose similar to that of animals exposed to mild stress (Lerman et al., 1997). Therefore it was expected that animals injected with 10 mg/kg of corticosterone would represent circulating corticosterone levels similar to that of animals exposed to stress and levels would be higher than animals injected with vehicle, representing baseline (normal) levels of circulating corticosterone. A dose of 25 mg/kg represented supra-physiologic levels of corticosterone and was expected to be higher than both 0 mg/kg and 10 mg/kg.

3.2.2 Materials and Methods

3.2.2.1 Subjects

Subjects were 12 male hooded Lister rats (supplied by Harlan Olac, UK) weighing an average of 376.3± 3.39 g on the day of the experiment. Animals were house four per cage (50 x 30 x 20 cm).

For all experiments in this chapter animals maintained under climate-controlled vivarium (21°C±2), and 12-h lighting per day (lights on at 08:00). Food and water were available ad libitum. Animals were given one week to acclimatise to the environment before the onset of the experiment, and were randomly assigned to conditions. All experiments were conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" (NIH publication number 86–23, 1985) and the United Kingdom Animals (Scientific Procedures) Act (1986).

3.2.2.2 Corticosterone

Corticosterone and the vehicle propylene glycol were obtained from Sigma Chemical Company (Dorset, UK). Rats (n=4/group) were injected once subcutaneously with 1 ml/kg of either 0 mg/kg which consisted solely of the vehicle propylene glycol (Lerman et al., 1997), 10 mg/kg, or 25 mg/kg of corticosterone. Two hours after the injection animals were anesthetised with isoflurane and decapitated, trunk blood was

then collected. Samples were placed in a centrifuge and spun at 10 000rpms for four minutes at room temperature. The plasma was separated and placed in heparinized epindorphs and frozen at -20℃ until use.

3.2.2.3 Corticosterone enzymeimmunoassay (EIA)

Enzymeimmunoassay for corticosterone content was performed using the IDS OCTEIA Corticosterone Kit (Immunodiagnostic Systems Ltd., Bolden, UK). At the time of assay, the plasma was thawed to room temperature. Controls and samples were diluted by a factor of 0, 10, or 100 with phosphate buffered saline containing horse serum, protein (for a total of 36 samples). Samples and controls were then added in duplicate to the polystyrene 96 well microplate coated with polyclonal rabbit anti-corticosterone antibody. The calibration curve was obtained by loading in duplicate calibrators (0, 1.8, 4.4, 11.6, 28.8, 75.9, 191 ng/ml) in the same plate. The plate was then incubated with corticosterone horseradish peroxidase-labelled overnight at 2-8°C. The wells were then washed three times with phosphate buffered saline. The colour was revealed by adding the enzyme substrate tetramethylbenzidine (TMB) to the plate and incubate at room temperature for 30 minutes before adding hydrochloric acid to stop the reaction. The absorbance was read at 450 nm using a microplate plate reader and Revelation Program (Dynex Technologies), colour intensity developed being inversely proportional to the concentration of corticosterone. A calibration curve was generated, and the percent binding of each calibrator, control and unknown sample was calculated using cubic spline data extrapolation. The values were read from the curve in ng/ml.

3.2.2.4 Statistical analysis

A mixed factorial analysis of covariance (ANCOVA) was conducted, where sample dilution was the within-subjects factor and corticosterone dose was the between subjects factor. Since body weight can affect corticosterone levels body weight from the

day of the experiment was added as a covariate. Significant main effects and interaction were followed-up with simple pairwise comparisons with Bonferroni correction.

3.2.3 Results

A total of five samples were not detected by the kit as the dosage in the sample fell below detectable levels for the kit (all 4 samples at a dose of 0 mg/kg at a dilution of 100 undetected): 1 undetected sample at a dose of 25 mg/ml at a dilution of 0, and 3 undetected sample at a dose of 10 mg/ml at a dilution of 100. Two further samples were labelled as 'not well read' as the dose in those samples fell above the range of the curve produced (samples at a dose of 25 mg/kg at a dilution of 0). Results of the mixed factorial ANCOVA revealed a significant main effect of corticosterone dose ($F(2, 8)=22.125$ $p<.001$), and a significant interaction between corticosterone dose and dilution ($F(4, 16)=11.262$ $p<.001$). No main effect of dilution was found ($F(2,16)=1.023$ $p=.382$ *ns*) (Figure 3.2). Follow-up tests revealed that at a dilution factor of 0 the kit detected significantly less corticosterone in the sample of 0 mg/kg of corticosterone as compared to a dose of 10 mg/kg ($p<.001$) and 25 mg/kg ($p<.01$). At a dilution factor of 10 the kit detected significantly less corticosterone in the sample of 0 mg/kg of corticosterone as compared to a dose of 10mg/kg ($p<.001$) and 25 mg/kg ($p<.001$). At a dilution factor of 100 the kit detected significantly less corticosterone in the sample of 0 mg/kg of corticosterone as compared to a dose of 25 mg/kg ($p<.01$).

At the recommended dilution of 10 the circulating levels of corticosterone fell within the expected range as indicated by the kit (0 mg/kg: M = 22.66, SD = 13.55 ng/ml; 10 mg/kg: M = 78.15, SD = 12.94 ng/ml; 25 mg/kg: M = 90.66, SD = 12.46221 ng/ml).

3.2.4 Discussion

The results of this experiment demonstrate that the enzymeimmunoassay kit is functional and appropriate for use in further stress experiments. The range of doses and the suggested dilution included in the kit instructions are accurate.

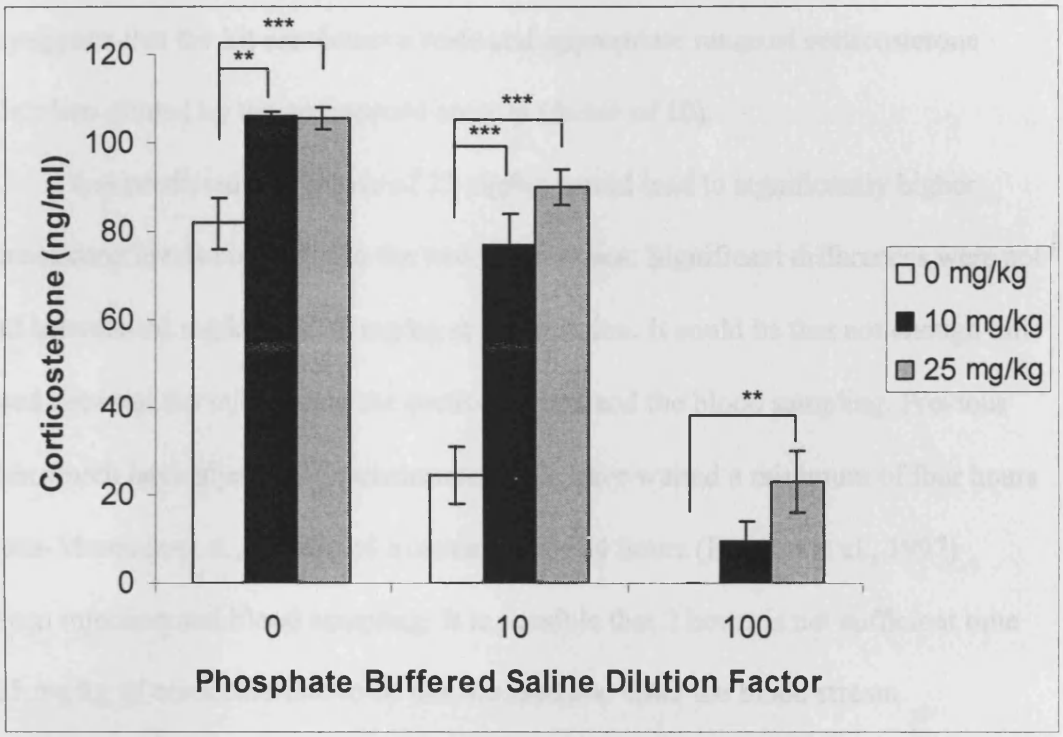

Figure 3.2. Mean circulating corticosterone levels (± SE) after injections of 0mg/kg (n=4), 10mg/kg (n=4), or 25 mg/kg (n=4) of corticosterone diluted by a factor of 0, 10, or 100. 0mg/kg at a dilution of 100 was nor detected by the kit. (**p<.01; ***p<.001).

Samples diluted by a factor of 10 yielded the best results, and were all within range of the kit. Many samples which were not diluted were within the upper limits of the kit but several were labelled as 'not well read' suggesting that although they fell within the range of the kit the sample could not be expected to yield accurate results. It is likely that in these cases there was more circulating corticosterone in the sample then there was polyclonal rabbit anti-corticosterone antibody coated on the plate and

therefore all the available anti-body was bound, therefore the kit could not accurately read how much extra hormone there was in the sample.

Several samples which were diluted by a factor of 100 fell below detectable levels of the kit, especially those samples of rats that were injected with vehicle. It is likely that in these cases there was not enough circulating corticosterone in the sample to bind to the polyclonal rabbit anti-corticosterone antibody coated on the plate. These data suggests that the kit can detect a wide and appropriate range of corticosterone levels when diluted by the appropriate amount (factor of 10).

It was predicted that a dose of 25 mg/kg would lead to significantly higher corticosterone levels compared to the two lower doses. Significant differences were not found between 10 mg/kg and 25 mg/kg at any dilution. It could be that not enough time elapsed between the injection of the corticosterone and the blood sampling. Previous studies which have injected corticosterone levels have waited a minimum of four hours (Retana-Marquez et al., 1998) and a maximum of 24 hours (Lerman et al., 1997) between injection and blood sampling. It is possible that 2 hours is not sufficient time for 25 mg/kg of corticosterone to be metabolised and enter the blood stream.

A dose of 25mg/kg is considered supraphysiological (Lerman et al., 1997), an animal exposed to a exogenous stressor would not have comparable levels of circulating corticosterone. Therefore it is not a great concern that many samples of 25mg/kg were not detected by the kit, nor is it a concern that the injected corticosterone levels were not fully absorbed in the rat's bloodstream as such levels would not be produced in typical experiments.

Although it is recommended that each lab obtain its own range of corticosterone levels, the corticosterone levels found in this experiment (when diluted by 10) were comparable to previous studies using other analysis techniques. Using a

radioimmunoassay kit for corticosterone Lerman (1997) found that when animals were injected with vehicle only, baseline levels of circulating corticosterone mg/ml was M = 31, SD = 5.4, and in the present study comparable baseline levels of M = 22.66, SD = 13.5 were obtained. These results indicate that the kit was accurate in detecting corticosterone levels and as baseline levels between studies was comparable; it should be possible to make direct comparisons between studies.

It was expected that in the following studies examining corticosterone levels after exposure to psychosocial stress, corticosterone levels would be likely to approach levels seen after an injection of 10 mg/kg where samples were diluted by a factor of 10, and therefore the EIA kit was considered to be appropriate for use in these studies.

3.3 Experiment 2a: Testing the effects of acute and chronic unstable social environment stress on reproductive parameters

3.3.1 Introduction

The purpose of this study was to evaluate the PCCS, as well as to investigate its effects on reproductive parameters. The PCCS stressor was developed to model human chronic psychosocial stress, and to test stressor effects on male reproductive parameters. Rats were exposed to the stressor for the duration of one or two spermatogenetic cycles (12 or 24 days, respectively), whereas controls remained caged in the same pair. In order to evaluate acute stress another group of rats were housed alone for 24 hours. It has previously been found that social isolation (even for short periods of time) leads to an increased stress response(Serra, Sanna, Mostallino, & Biggio, 2007). A normal healthy rat requires an environment in which they can explore, find cover, and have social interactions (Balcombe, 2006).

3.3.1.2 The hypotheses

The hypothesis was that rats exposed to the PCCS would have higher circulating plasma corticosterone compared to controls. It was also expected that animals exposed to acute stress would have higher corticosterone levels as compared to controls. Moreover, the longer the animals were exposed to stress, the higher the circulating corticosterone levels were expected to be.

The second hypothesis was that animals exposed to the PCCS would have smaller testicular and epididymal weights and reduced sperm count. The organ weights on rats exposed to acute stress would remain the same as controls as there would be insufficient time for this one-day stress to reduce organ or body weight. All stressor effects on reproductive measurements should be more pronounced as the length of the stressor increased.

Finally, the animals exposed to stress will gain less weight or slightly less than animals in the control or acute stress conditions.

3.3.2 Materials and Methods

3.3.2.1 Subjects

Subjects were 24 male hooded Lister rats (supplied by Harlan Olac, UK) weighing on average 478.8±7.87g at the start of the experiment. Animals were housed in cages sized 42 x 24 x 12 cm, and were weighed on a daily basis

3.3.2.2 Conditions

As indicated by Figure 3.3 the experiment consisted of four experimental conditions. In condition 1, animals were exposed to stress for the duration of two spermatogenetic cycles (24 days) (CS24; n=6), the first day CS24 animals were exposed to stress was considered day 1 of the experiment. CS24 were sacrificed on day 25 of the experiment. In condition 2, animals were exposed to 12 days of chronic social stress

the experiment. Condition 3, animals were in cages in the same pair for the duration of the experiment except for the last 24 hours of the experiment where animals were then exposed to an acute social isolation stress by being housed alone for 24 hours (AS; n=6). Condition 4 was the control condition (CTRL; n=6), all animals were caged in the same pair for the duration of the experiment. To correspond to the final day of the experiment for CS12 and CS24 both the AS and CTRL conditions were split into two sub-conditions; ASa (n=2) and CTRLa (n=4) sacrificed on day 13 of the experiment, as well as, ASb (n=4) and CTRLb (n=2) sacrificed on day 25 of the experiment.

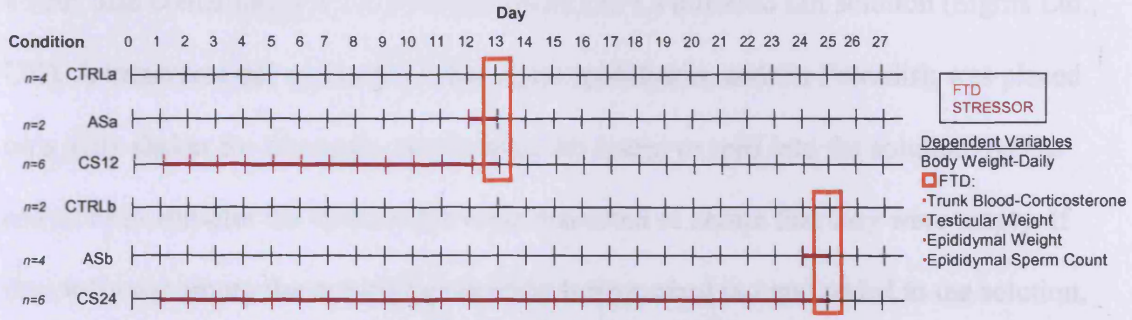

Figure 3.3. Methodology for experiment 2a. CTRLa, control group a; ASa, acute stress group a; CS12, chronic stress for 12 days; CTRLb, control group b; ASb, acute stress group b; CS24, chronic stress for 24 days.

3.3.2.3 PCCS paradigm

Chronic social stress was created by exposing the animals to an unstable social environment. Every 24 hours (between 1200 and 1300) animals were placed in a clean cage alone, or in a cage of two or three rats. All animals received the same amount of exposure to each of the cage arrangements, and were not placed in the same group twice.

3.3.2.4 Corticosterone enzymeimmunoassay (EIA)

Stress levels were measured by assessing circulating plasma corticosterone levels collected from trunk blood on the relevant final test day. The EIA procedure for corticosterone was replicated from Experiment 1 using only a

The EIA procedure for corticosterone was replicated from Experiment 1 using only a dilution of 10.

3.3.2.5 Reproductive parameters

After exposure to the PCCS rats were killed by intraperitoneal injection of Euthatal (1 ml). Trunk blood was then collected in heparinised tubes, plasma was separated using a centrifugation (10 000 rpms for 4 minutes at room temperature) and stored at -20°C until use for hormonal. Left and right testicles, as well as, left and right epididymis were dissected and weighed. The epidymides of each animal were placed in a Petri dish containing 5 ml of warmed Earles (35°C) buffered salt solution (Sigma Ltd., UK). A transverse cut was made in the caput epididymis, and the Petri dish was placed on a plate shaker for three minute allowing the sperm to spill into the solution. At the end of three minutes the epidymides were inspected to assure that they were empty. If they were not empty the remaining contents were pushed out and added to the solution, this was to ensure that the samples were not biased towards sperm that were motile. The solution was gently mixed using a pipette and then 12 µl was placed in a Nauber improved haemocytometer (Sigma Ltd.) and examined using a Leica microscope at magnification of 10X using Analysis D software (technique adapted from (Ernst, 1989)). The outcome measure generated from this procedure was a count of sperm per millilitre X 10^5.

3.3.2.6 Body and organ weight

Body weight was measured using a Compact Scale (OHAUS). Weight gain was quantified by subtracting body weight from the first day of the experiments from body weight on the last day of the experiment. Organ weight was measured using a M-Series Analytical Balance (Denver Instruments). A ratio between body and organ weight was also calculated by dividing organ weight by final day body weight.

3.3.2.7 Statistical analysis

Two t-tests were conducted comparing body weight gain over the first 12 days of the experiment, first between ASa and ASb, second between CRTLa and CTRLb. If not significantly different from each other they were collapsed to create AS and CRTL conditions.

Two one-way analyses of variance (ANOVA) were then conducted comparing body weight gain. First, comparing all conditions on body weight gain over the first 12 days of the experiment. Second comparing body weight gain over 24 days of the experiment for those in the relevant conditions (CS24, ASb, CTRLb).

For all further analyses t-tests were conducted and if ASa and ASb, as well as, CRTLa and CRTLb were not significantly different from each other the AS and CTRL conditions were used.

Corticosterone levels in each condition were compared using a one-way ANCOVA controlling for final day body weight. Since the hypothesis with regards to corticosterone was directional, as supported by previous research a one-tailed test of significance was used (one-tailed).

To account for the effects of body weight on organ weight, two one-way ANOVAs were conducted on testicular and epididymal weights divided by body weight on the final test day (organ to body weight ratio). Epididymal sperm counts for each condition were compared using a one-way ANCOVA controlling for epididymal weight since sperm counts would be affected by the size of the epididymis. Tukey HSD post hoc tests were used.

An alpha level of .05 was used, except when otherwise specified.

3.3.3 Results

3.3.3.1 Body weight

No significant differences were found between ASa and ASb (t(4)=3.43, p=.138 *ns*), as well as, between CTRLa and CTRLb (t(4)=.001, p=.99 *ns*), and these were

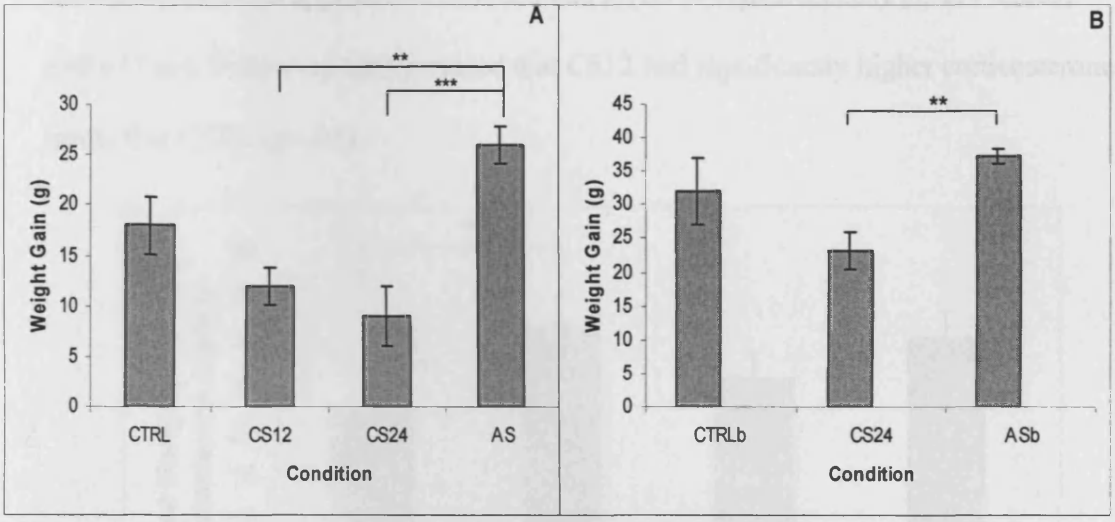

Figure 3.4. (A) Body weight gain (± SE) over the first 12 days of the experiment by condition. (B) Body weight gain (± SE) over 24 days of the experiment by condition (*p<.05, **p<.01, ***p<.001).

therefore combined to test difference in weight gain over the first 12 days of the experiment. Figure 3.4 revealed that there was a significant difference in body weight gain over the first 12 days of the experiment between conditions (F(3,20)=9.67 p=.001). Follow-up test revealed that CS12 and CS24 gained significantly less weight than AS (p<.01, p<.001 respectively).

Similarly, there was a significant difference in body weight gain over 24 days of the experiment between conditions (F(2,9)=7.56 p=.05). Follow- up tests revealed that CS24 gained significantly less weight than ASb (p<.01), whereas ASb and CTRLb were not significantly different from each other (p=.99).

3.3.3.2 Corticosterone

No significant differences were found between the sub-groups ASa and ASb (t(4)=1.46, p=.293 *ns*), and CTRLa and CTRLb (t(4)=.98, p=.378 *ns*) therefore conditions AS and CTRL were used. As shown in Figure 3.5, differences in corticosterone levels between conditions approached conventional levels of significance (F(3, 19)=2.265, p=0.057 *ns*). Follow-up tests revealed that CS12 had significantly higher corticosterone levels that CTRL (p<.05).

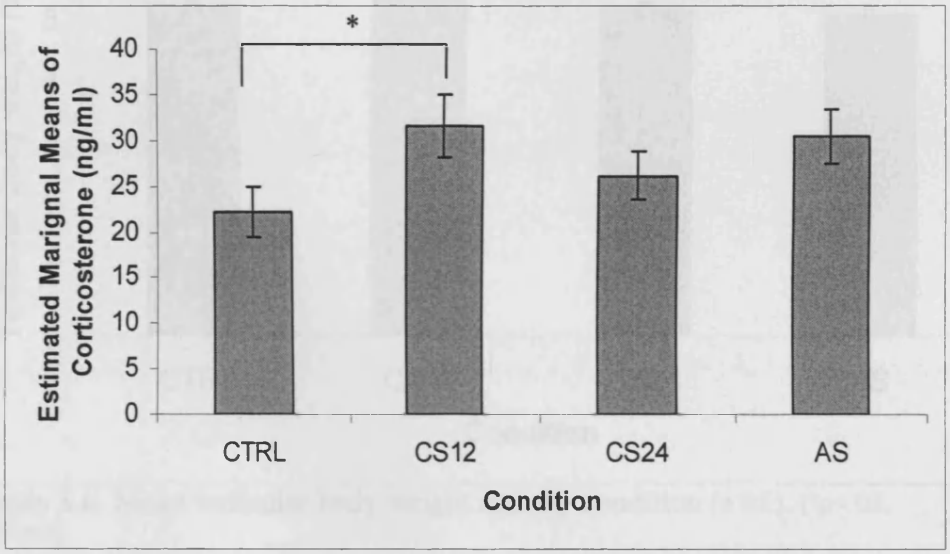

Figure 3.5. Estimated marginal means of corticosterone levels (± SE), after controlling for body weight, by condition. (*p<.05).

3.3.3.3 Reproductive organ weights

No significant differences found between the sub-groups ASa and ASb (t(4)=.204, p=.675 *ns*), and CTRLa and CTRLb (t(4)=.885, p=.40 *ns*) for testicular body weight ration therefore conditions AS and CTRL were used. Results revealed that there was a significant difference in the testicular body weight ratio between conditions (F(3, 20)=5.091, p<.01) (Figure 3.6). Tukey HSD post hoc tests revealed that the testicular to body weight ratio was greater in condition CS12 compared to CS 24 (p<.05), AS (p<.01),

and CTRL ($p<.05$) indicating that animals in CS12 had larger testicles relative to their body size.

No significant differences were found between the sub-groups ASa and ASb ($t(4)=.302$, $p=.612$ *ns*), and CTRLa and CTRLb ($t(4)=.989$, $p=.35$ *ns*) for epididymal body

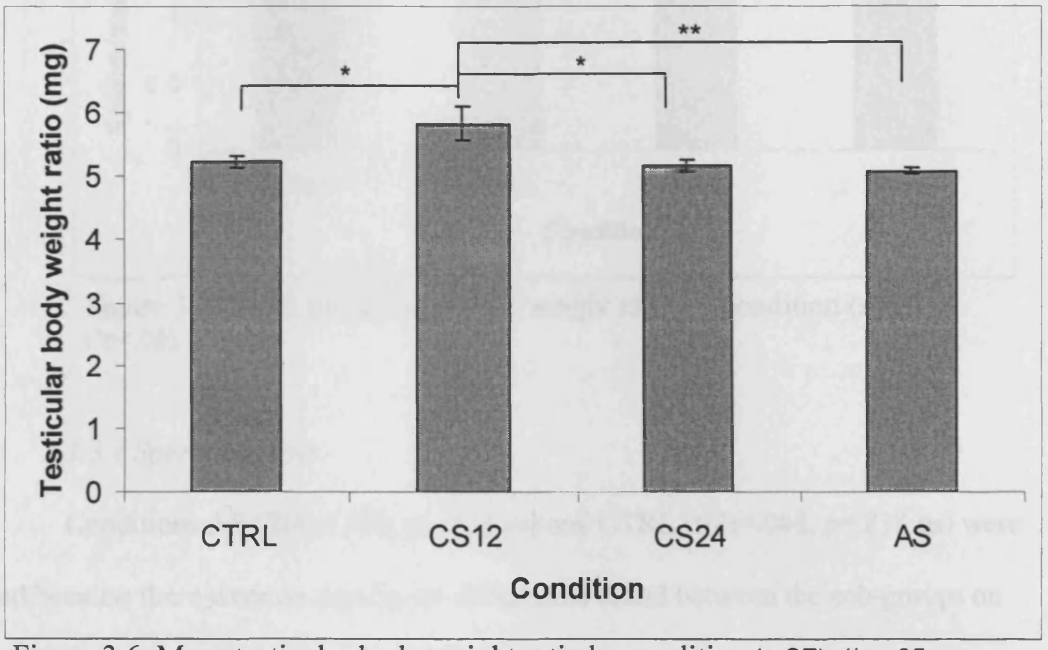

Figure 3.6. Mean testicular body weight ratio by condition (± SE). (*$p<.05$, **$p<.01$).

weight ratio therefore conditions AS and CTRL were used. Results also revealed that there was a significant difference in epididymal body weight ratio between conditions ($F(3, 20)=4.037$, $p<0.05$) (Figure 3.7). Tuckey's HSD post hoc tests revealed the epididymal body weight ratio was greater in CS12 compared to CS24 ($p<.05$), and AS ($p<.05$), but not CTRL indicating a larger epididymis compared to body weight in the CS12 group.

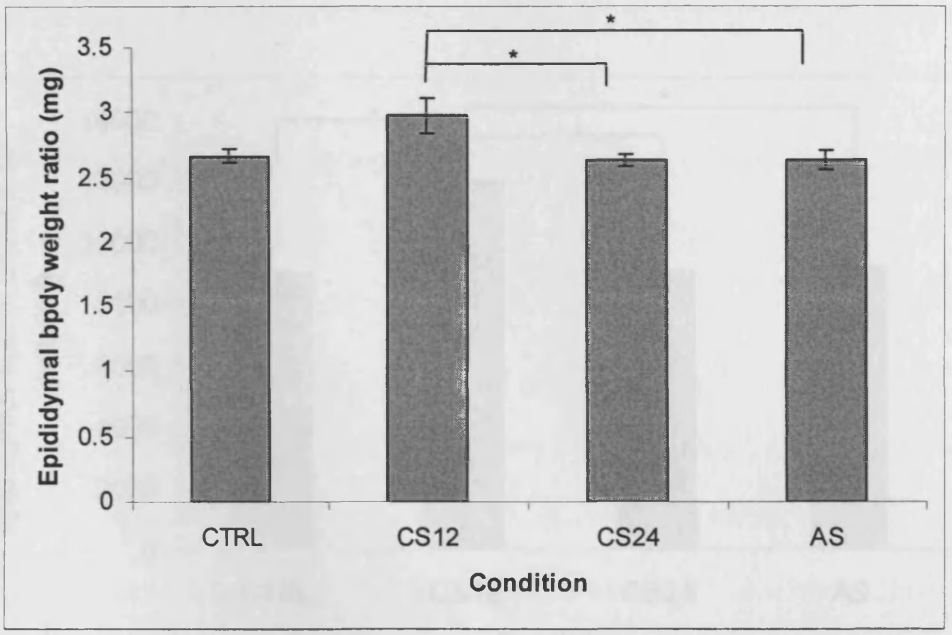

Figure 3.7. Mean epididymal body weight ratio by condition (± SE). (*p<.05).

3.3.3.4 Sperm analysis

Conditions AS (T(4)=.838, p=.412 *ns*) and CTRL (t(4)=.048, p=.838 *ns*) were used because there were no significant differences found between the sub-groups on sperm count. As demonstrated by Figure 3.8 a trend was found (F(3, 19)=2.55, p=0.086 *ns*) in sperm count across conditions. Tukey's HSD post hoc tests revealed that the marginal effect was due to CS12 having a significant higher sperm count than CRTL (p<.05), CS24 (p<.05), and AS (p<.05) groups.

3.3.4 Discussion

This experiment demonstrated that the PCCS induced a stress response in male rats, as demonstrated by elevated corticosterone levels and lower body weight gain. Reproductive parameters were significantly affected by exposure to this psychosocial stress. The effect on sperm quality may operate through the suppression of reproductive hormones by corticosterone.

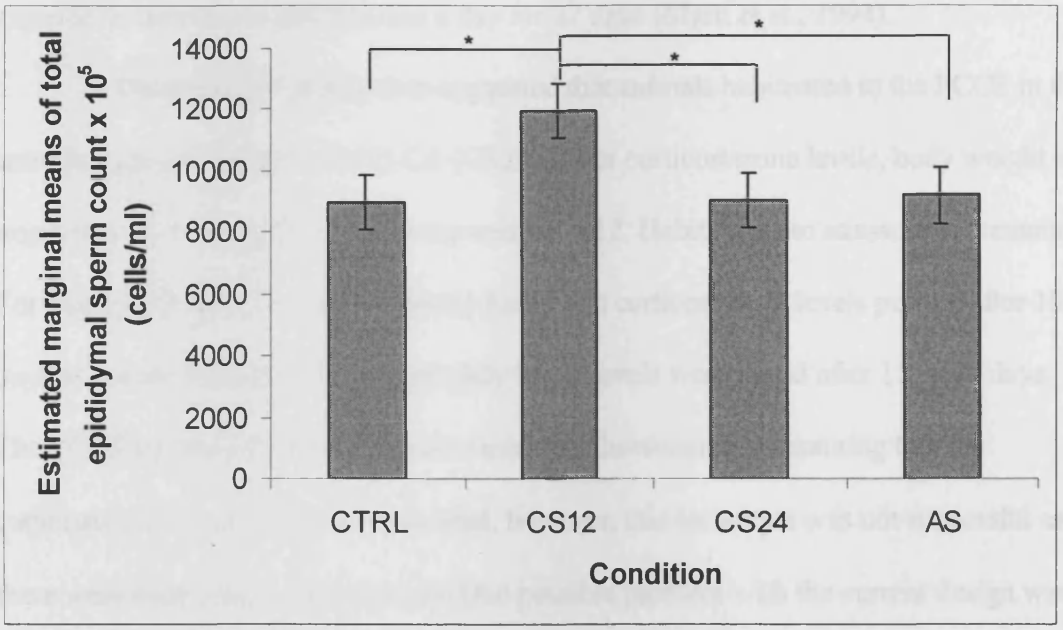

Figure 3.8. Estimated marginal means of total epididymal sperm count (±SE) controlling for epididymal weight, by condition.

The PCCS induced psychosocial stress but this effect was not as pronounced as expected and was moderated by duration of exposure. Similar social stress paradigms have demonstrated increased corticosterone levels (Bhatnagar & Vining, 2003; R. J. Blanchard et al., 2001; Koyama & Kamimura, 1998; Lemaire et al., 1997; Mormede et al., 1990; Niikura et al., 2002; Schmidt et al., 2007; Taylor et al., 1987). In line with this previous work, an increase in corticosterone was seen in animals exposed to PCCS, although only approaching significance. Since this paradigm has been developed based on a combination of other paradigms it is possible the PCCS stressor was not as potent as the original stressors. However, results of body weight gain revealed animals exposed to stress did not gain much weight during the experiment, suggesting that the PCCS is sufficiently stressful to interact with food consumption or metabolic activity. In previous work such interference is only seen in more severe chronic stress, handling does not affect food consumption. Fifteen minutes of handling a day for 6 days was shown to have very

mildly affect food consumption, while food consumption was greatly reduced in animals exposed to restrain for 240 minutes a day for 27 days (Marti et al., 1994).

The pattern of results also suggested that animals habituated to the PCCS in that animals exposed longer to the PCCS (CS24) lower corticosterone levels, body weight and organ (testes, epididymis) ratios compared to CS12. Habituation to stressors is common. For example, Retana-Marquez (2003b) found that corticosterone levels peaked after 10 days of immobilisation stress and slightly lower levels were found after 15 or 20 days. The PCCS paradigm was developed to minimise habituation by assuring that the combinations of rats was never repeated, however, this technique was not successful as there were clear signs of habituation. One possible problem with the current design was that rats changed cages at the same time every day. Predictability is known to reduce the severity of stressors, (Sapolsky, 2005). Therefore, to improve on the paradigm predictability was removed in subsequent experiments so that clear differences between control conditions and stress conditions can be seen.

Contrary to the hypothesis reproductive measures were more favourable in animals with the highest levels of circulating corticosterone; CS12. These effects were particularly striking in the reproductive organ body weight ratios. There was one exception, however, where increased seminal vesicle weight was found after exposure to stress. Lermaire (1997) attributed changes in reproductive organ weights to increase to changes in androgens induced by social interactions, although no androgen was measured. It is possible that social interaction enforced on these animals lead to increased aggression and fighting for dominance. Although, aggression and dominance are not measured in the present study, mild aggression and fighting is normal among rats and would be expected (Taylor et al., 1987). This aggressive behaviour may increase testosterone levels as previously seen in highly aggressive colonies (Taylor et al., 1987). Taylor (1987) found

that highly aggressive colonies have higher testosterone levels and better sperm count despite high levels of circulating corticosterone, which could account for the results found here. It is possible that stress and increased corticosterone levels only have a negative effect in the absence of high testosterone levels. In light of these effects testosterone should be measured as part of the hormonal profile to identify whether aggression may be moderating the effect of stress.

Due to the availability of animals, those used in this experiment weighed, on average, over 500g at the start of the experiment. This is relatively large for rats of this strain. According to the growth chart provided by the animal suppliers Harlan, a rat weighing 500g is approximately 19-20 weeks old (Retrieved September 7). It has previously been shown that older male rats exposed to stress have higher circulating testosterone levels as compared to younger animals exposed to the same stressor (Bowman, Maclusky, Diaz, Zrull, & Luine, 2006). The age could explain the results found in the present study. However, in contrast previous research has also found that there is no difference in social interaction between young and old rats (Boguszewski & Zagrodzka, 2002). Finally, future experiments should use younger lighter animals to be more comparable to animals used in the studies reviewed previously and to reduce age-related complications and confounds.

Another explanation to account for our finding of higher sperm count in the CS12 but not CS24 condition is that the higher sperm count was due to a greater number of non-motile sperm caused by stress-induced changes in spontaneous ejaculations. Previous research has shown that stress decreases the number of ejaculations in the presence of females (Retana-Marquez et al., 2003b) and one can assume that the mechanism for this could also reduce the number of spontaneous ejaculations. Reduced ejaculations, in turn, builds up the reservoir of sperm in the vas deferens and ampulla,

which ultimately could lead to a higher sperm count in the most stressed animals. In humans sexual abstinence has been shown to cause a higher concentration of non-motile sperm, and be a contributing cause to a spoor semen analysis (Jurema et al., 2005). However, in the present study the motility was not evaluated. In order to rule out this possibility it would be imperative to ascertain the sperm motility in subsequent studies. In addition, there is consensus in the human literature that motility is a more important indicator of fertility than count (Jedrzejczak, Taszarek-Hauke, Hauke, Pawelczyk, & Duleba).

In conclusion the PCCS demonstrated validity as a psychosocial stressor and this stressor was shown to impact significantly on reproductive parameters. In order to better understand the mechanism underlying this psychobiological the next experiments used a modified PCCS paradigm and sampled a broader range of reproductive outcomes.

3.4 Experiment 2b

3.4.1 Introduction

Results of Experiment 2a were that exposure to the PCCS marginally increased corticosterone levels, decreased body weight gain, and increased reproductive organ body ratio, especially in the group of rats exposed to the stressor for 12 days. Evidence of habituation to the stressor was found as animals exposed to stress for 24 days showed lower corticosterone levels as compared to animals stressed for 12 days. Similarly, there was no increase in reproductive organ weights as seen in the condition exposed to 12 days of stress.

In the previous PCCS procedure the cage change occurred at the same every day allowing the animal to anticipate the stressor, which would affect how animals responded to the stressor. Indeed Sapolsky (2005) found that decreased predictability and control lead to increased cortisol levels. In Old World monkeys, subordinate

monkeys are subjected to unpredictable aggression from a more senior and dominant monkeys, monkeys that did not have a social outlet (another more submissive monkey to be aggressive towards, or social grooming) had higher cortisol levels then did animals with social outlets for stress (Abbott et al., 2003). Therefore in the current experiment cages were changed at a different time each day instead of within a one hour window as in the previous experiment. Also to minimize effects of handling the rats were only weighed three times during the experiment instead of daily. It was expected that these changes would create a more uniform stressor for animals who would not be able to anticipate the critical cage change event, and lead to a more marked change in corticosterone levels in CS12 and CS24 groups compared to controls.

Aggressive colonies of rats showed increased levels of circulating androgens, which have been hypothesised to be involved in increased sperm quality, and increased reproductive organ weights despite high levels of glucocorticoids (Taylor et al., 1987). It has been suggested that testosterone masks or blocks negative effects of stress (Taylor et al., 1987). It is possible that daily changes in the rats' social environment, as in the PCCS, leads to increased aggressiveness and therefore increased testosterone levels, the interactions between testosterone and corticosterone are not clear and testosterone may play a role in moderating the stress response. The present experiment seeks to investigate the role of testosterone, and therefore circulating testosterone levels are measured.

Male reproduction can be measured in several ways; Experiment 2a included recording reproductive weights as well as sperm count. For a more in depth analysis that could differentiate between motile and immotile sperm video software is needed. Compared to human sperm, rat sperm in very dense, potentially including billions of sperm. A sub-sample of rat sperm can be difficult to count and even more difficult to

assess for motility using the standard techniques applied in human sperm analysis laboratories. To overcome this problem in this experiment a short video clip of rat sperm was taken so that the rat sperm video could be enhanced and slowed down to make for a more accurate reading of motile sperm.

3.4.1.2 The hypotheses

The hypotheses were similar to experiment 2a. It was hypothesised that rats exposed to the PCCS would have higher circulating plasma corticosterone compared to controls. Following results of the previous experiment some habituation was expected after 24 days of stress, therefore it was expected that the CS12 group would have the highest corticosterone levels. All animals exposed to stress were expected to gain less weight than control animals.

If the PCCS produced aggressive behaviour in the stress conditions then testosterone, reproductive organ body weight ratio and sperm quality should be more favourable in these groups compared to controls. In contrast, PCCS was not associated with increased aggression then the reproductive parameters would be expected to be less favourable in the stress conditions, in particular the CS12 conditions, compared to controls. If high aggression exists in the stress conditions then if testosterone did not moderate the impact testosterone level would be expected to be higher in animals exposed to stress as compared to controls. Similarly, if increased testosterone levels were due to increased aggression then reproductive organ which are sensitive to androgens (Nussey, 2001) (as discussed in the general introduction) will be greater in an animals exposed to stress. Spermatogenesis is dependent on testosterone (Nussey, 2001), therefore if increased testosterone levels were due to increased aggression then sperm quality would be greater in an animals exposed to stress.

3.4.2.1 Subjects

Originally subjects were 40 male hooded Lister rats (supplied by Harlan Olac, UK) but due to ill health (undescended left testicle) one animal was removed from the experiment leaving a final sample size of 39 rats. Animals weighed an average of 351.5±2.15 g at the start of the experiment. To minimize stress associated with handling animals were weighed in 6 day intervals throughout the experiment.

3.4.2.2 Conditions

Using G*POWER software (Faul, 1992) a power analysis was conducted using an effect size from the results of corticosterone in the previous experiment (.483), an

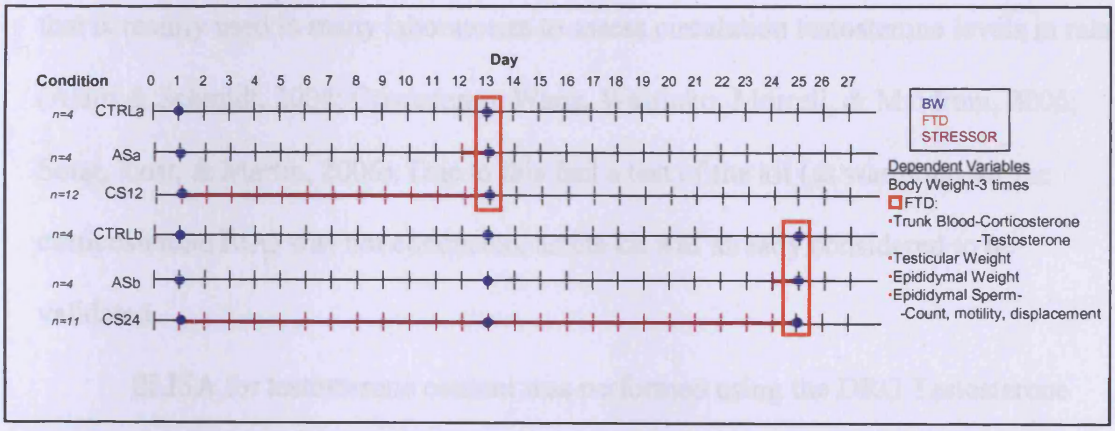

Figure 3.9. Methodology for experiment 2b. CTRLa, control group a; ASa, acute stress group a; CS12, chronic stress for 12 days; CTRLb, control group b; ASb, acute stress group b; CS24, chronic stress for 24 days.

alpha level of .05 (one-tailed test) and a recommended power (i.e., .8) level. The results of the power analysis indicated that 12 animals per condition would be needed; due to animal availability and to minimize the use of animal lives occasionally smaller group sizes were used. Figure 3.9 summarises the methodology used. The experiment consisted of the same four experimental conditions as Experiment 2; CS24: n=11, CS12: n=12 AS: n=8 (ASa; n=4; ASb; n=4), CTRL: n=8 (CTRLa: n=4; CTRLb: n=4).

3.4.2.3 PCCS paradigm

PCCS was used as described in Experiment 2a, however to increase the severity of the stressor the cages were changed at different/random times each day therefore reducing the predictability of the stressor.

3.4.2.4 Corticosterone enzymeimmunoassay (EIA)

The EIA procedure for corticosterone was replicated from and described in Experiment 2a.

3.4.2.5 Testosterone enzyme-linked immunosorbent assay (ELISA)

Testosterone levels were assessed from plasma obtained from trunk blood, and expressed as ng/ml. The Enzyme-Linked Immunosorbent assay (ELISA) is a technique that is readily used in many laboratories to assess circulation testosterone levels in rats (Alam & Schmidt, 2004; Crisostomo, Wang, Wairiuko, Morrell, & Meldrum, 2006; Song, Kost, & Martin, 2006). Due to this fact a test of the kit (as was done for the corticosterone EIA) was not conducted, as the kit was already considered to be validated.

ELISA for testosterone content was performed using the DRG Testosterone ELISA Kit (Immunodiagnostic Systems Ltd., Bolden, UK). At the time of assay, the plasma was thawed to room temperature. Twenty-five microliters of controls and samples were loaded in duplicate into a 96 microtiterwell coated with mouse monoclonal anti-Testosterone anti-body. The calibration curve was obtained by loading in duplicate calibrators (0, 0.2, 0.5, 1.0, 2.0, 6.0,16 ng/ml) in the same plate. The plate was thoroughly shaken for 10 seconds and then incubated with testosterone horseradish peroxidase-labelled at room temperature for 60 minutes.

The wells were then washed three times with phosphate buffered saline. The colour was revealed by adding the enzyme substrate tetramethylbenzidine (TMB) to the plate and

incubate at room temperature for 15 minutes before adding hydrochloric acid to stop the reaction. The absorbance was read at 450 nm using a microplate plate reader and Revelation Program (Dynex Technologies), colour intensity developed being inversely proportional to the concentration of testosterone. A calibration curve was generated, and the percent binding of each calibrator, control and unknown sample was calculated using cubic splice data extrapolation. The values were read from the curve in ng/ml.

3.4.2.7 Reproductive parameters

Additional sperm measures included the analysis of sperm motility. A three second (30 frames per second) video clip at a magnification of 10X, on the Leica microscope using Analysis D software was acquired. The video was played within the grid of the haemocytometer. Sperm which were moving were labelled as motile and those that were not moving were labelled as immotile sperm. Also, motile sperm were measure, in millimetres, for displacement, a straight line from the first position to the final position of the sperm after the 90 frames lapsed.

3.4.2.8 Body and organ weight

Body and organ weight techniques were replicated from experiment 2a.

3.4.2.9 Statistical analysis

Two t-tests were conducted comparing body weight gain over the first 12 days of the experiment, first between ASa and ASb, second between CRTLa and CTRLb. If not significantly different from each other they were collapsed to create AS and CRTL conditions.

Two one-way analyses of variance (ANOVA) were conducted comparing body weight gain (difference between first and last day of the experiment). First, comparing all conditions on body weight gain over the first 12 days of the experiment. Second

comparing body weight gain over 24 days of the experiment for those in the relevant conditions (CS24, ASb, CTRLb).

For all further analyses t-tests were conducted and if ASa and ASb, as well as, CRTLa and CRTLb were not significantly different from each other the AS and CTRL conditions were used to increase statistical power.

Corticosterone levels in each condition were compared using a one-way ANCOVA controlling for final day body weight. Since the hypothesis with regards to corticosterone was directional, as supported by previous research a one-tailed test of significance was used.

A one-way ANOVA was conducted comparing testosterone levels between conditions. Regression was also computed to assess the relationship between corticosterone levels and testosterone levels. A one-way ANOVA was conducted comparing testosterone levels between conditions.

To account for the effects of body weight on organ weight, two one-way ANOVAs were conducted on testicular and epididymal weights divided by body weight on the final test day (organ to body weight ratio).

A one-way ANCOVA was conducted comparing total sperm count across each condition and controlling for epididymal weight. Also, two one-way ANOVAs were conducted, first comparing immotile sperm between conditions, and second comparing sperm displacement between conditions.

An alpha level of .05 was used, except when otherwise specified.

3.4.3 Results

3.4.3.1 Body weight

No differences were found between ASa and ASb ($t(6)=.225$, $p=.652$) nor between CRTLa and CTRLb ($t(6)=2.359$, $p=.175$) on body weight gain over the first

twelve days of the experiment, therefore the collapsed AS and CTRL conditions were used.

No significant difference in body weight gain over the first 12 days of the experiment between conditions ($F(5,33)=1.734$ $p=.154$). Results also revealed that a difference in body weight gain over 24 days of the experiment between conditions CS24, ASb, and CTRLb approached conventional levels of significance ($F(2,16)=3.467$ $p=.056$). Post hoc tests revealed CS24 gained significantly more weight than ASb ($p<.05$).

3.4.3.2 Corticosterone

No differences were found in corticosterone levels between ASa and ASb ($t(6)=1.793$, $p=.229$) nor between CRTLa and CTRLb ($t(6)=2.059$, $p=.201$), therefore the collapsed AS and CTRL conditions were used.

ANCOVA demonstrated that corticosterone levels did not differ significantly between conditions ($F(3, 34)=1.147$ $p= 0.172$ *ns*), however it is worth noting that the CS12 condition presented with the highest corticosterone levels, and the control condition the lowest levels.

3.4.3.3 Testosterone

No differences were found in testosterone levels between ASa and ASb ($t(6)=2.569$, $p=.160$) nor between CRTLa and CTRLb ($t(6)=6.886$, $p=.051$), therefore the collapsed AS and CTRL conditions were used.

A one-way ANOVA demonstrated that there was no significant difference between conditions in levels of testosterone ($F(3, 34)=1.076$ $p=0.372$ *ns*), however as predicted the CS12 condition presented with the lowest testosterone levels.

A significant negative correlation was found between corticosterone and testosterone levels ($r(36)=.509$, $p<0.001$).

3.4.3.4 Reproductive organ weights

No differences were found in testicular body weight ratio between ASa and ASb ($t(6)=1.464$, $p=.272$) nor between CRTLa and CTRLb ($t(6)=5.317$, $p=.061$), therefore the collapsed AS and CTRL conditions were used.

A one-way ANOVA demonstrated a significant difference between conditions in testicular body weight ratio ($F(3, 34)=4.44$, $p<0.01$). Tukey HSD post hoc tests revealed that the CS12 condition had a significantly larger testicular ratio than CS24 ($p<.05$) (Figure 3.10).

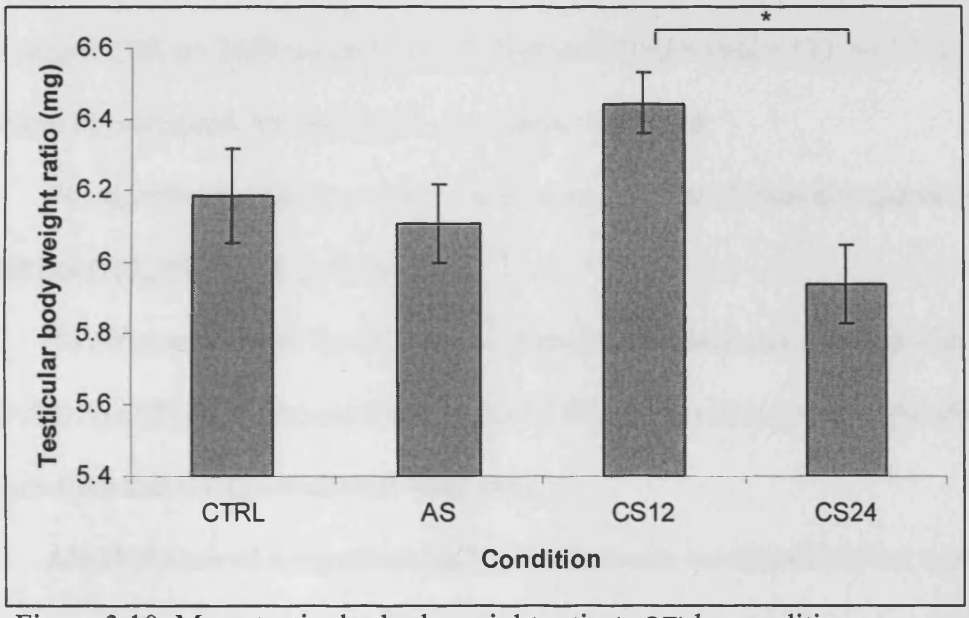

Figure 3.10. Mean testicular body weight ratio (± SE) by condition. (*p<.05).

No differences were found in epididymal body weight ratio between ASa and ASb ($t(6)=4.837$, $p=.070$) nor between CRTLa and CTRLb ($t(6)=2.735$, $p=.149$), therefore the collapsed AS and CTRL conditions were used.

ANOVA demonstrated that difference between conditions in epididymal body weight ratio approached conventional levels of significance ($F(3, 34)=2.656$, $p=0.064$

ns). Tukey HSD post hoc test revealed that CS12 had a marginally larger epididymal ratio than the CS24 condition ($p<.05$).

3.4.3.5 Sperm analysis

No differences were found in epididymal sperm count between ASa and ASb ($t(6)=4.247$, $p=.085$) nor between CRTLa and CTRLb ($t(6)=.532$, $p=.499$), therefore the collapsed AS and CTRL conditions were used.

ANCOVA demonstrated no difference between conditions in total epididymal sperm count ($F(3, 26)=0.977$, $p=0.419$ *ns*) when controlling for epididymal weight.

No differences were found in the number of immotile sperm between ASa and ASb ($t(6)=1.560$, $p=.267$) nor between CRTLa and CTRLb ($t(6)=.133$, $p=.728$), therefore the collapsed AS and CTRL conditions were used.

No significant difference were found in the number of immotile sperm between conditions ($F(2,26)=1.694$, $p=0.193$ *ns*).

No differences were found in sperm displacement between ASa and ASb ($t(6)=.001$, $p=.989$) nor between CRTLa and CTRLb ($t(6)=.031$, $p=.865$), therefore the collapsed AS and CTRL conditions were used.

ANOVA showed a significant difference between conditions in total sperm displacement ($F(3, 26)=3.364$, $p=0.034$). Tukey HSD post hoc tests revealed that CS12 showed sperm with significantly greater displacement than CS24 sperm ($p<.05$) (Figure 3.11).

3.4.4 Discussion

PCCS had an effect on reproductive parameters but not indicators of HPA activation. These results suggest that younger animals may be more resistant to the effects of stress.

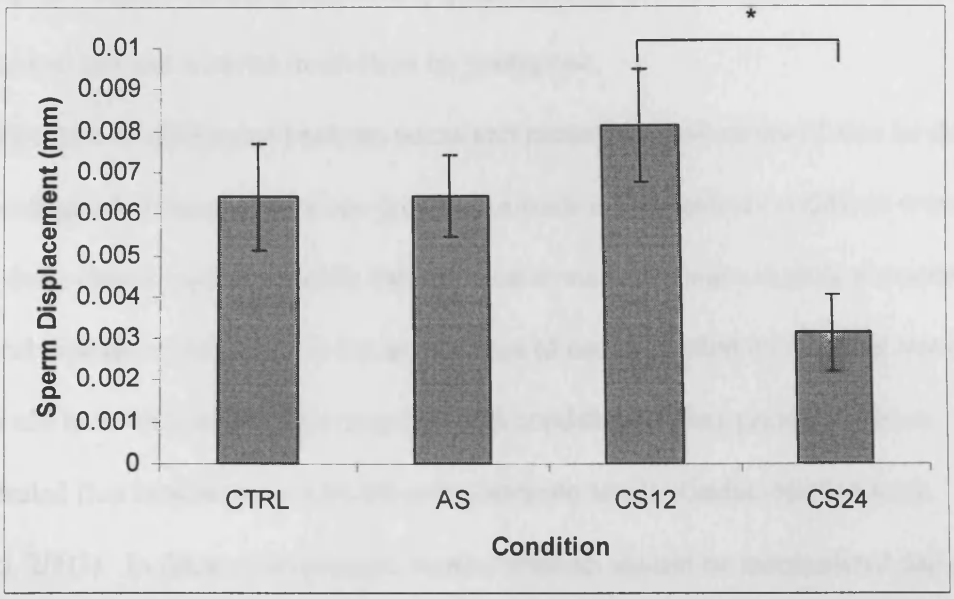

Figure 3.11. Average distance displaced by rat sperm (mm) by condition (± SE). (*p<.05).

Compared to effects observed in older animals in Experiment 2a a significant difference in corticosterone levels between conditions was not found in the present experiment, though the pattern of results was replicated. One could interpret the similarity in the pattern of results across experiments as converging evidence that the PCCS elicits a minor stress response that peaks after 12 days of exposure. It is possible that this minor stressor had a greater impact on older compared to younger animals. For example it was found that older rats had higher levels of corticosterone levels compared to younger male rats after exposure to restraint stress. Older male rats were also impaired in behavioural measures of stress such as remembering where an object used to be (object placement task; Bowman et al., 2006). However, it may also be possible that younger animals are particularly resistant to social stress, and may even thrive on the challenge. Aggressive behaviour and fighting for dominance is normal rat behaviour and it is possible that the PCCS elicits more aggressive behaviour then it does a stress response. If such is the case then the severity of the stressor may need to be increased in

order to detect stress effects on reproductive functioning, so that PCCS is interpreted as a stressor and not just a social interaction by young rats.

The lack of difference between stress and control conditions could also be due to a methodological difference between groups. Animals in the control conditions were not handled daily therefore it is possible that corticosterone levels were slightly elevated in the control animals in response to the acute stress of being handled on the final test day, which could have obscured differences between conditions. It has previously been demonstrated that handling can elevate corticosterone levels (Gadek-Michalska & Bugajski, 2003). In future experiments control animals should be manipulated daily

Effects of the PCCS on reproductive parameters were clear but not in the expected direction. In the present experiment CS12 animals had larger testicles and epididymi (relative to body weight) compared to control animals, and this effect was not due to a reduction in body weight gain. This indicates that the effect of the PCCS was directly on the organ itself. In addition, the CS12 condition showed increased sperm displacement, indicating better sperm motility but not a higher sperm count as was shown in Experiment 2a. Together these findings show significant effects for reproductive parameters, but all effects could be interpreted as being positive not negative effects of stress.

It was hypothesised that the enhancement in reproductive parameters after 12 days of stress was due to increased aggressiveness and therefore increased testosterone levels (Taylor et al., 1987). However, in the present study no differences were found in circulating testosterone levels between conditions. In fact, a negative correlation between corticosterone and testosterone indicating that animals with the highest corticosterone levels, as in CS12, had the lowest testosterone levels. It is possible that testosterone is not the appropriate androgen to measure and the effects are due to other

hormones. As previously discussed in the General Introduction optimal levels of LH, FSH, testosterone, and ABP are needed for spermatogenesis. It is possible that measuring testosterone alone is not sufficient, but a measure of all these hormones and the ratios between them would be needed to understand the effects of stress on reproduction.

Another methodological point is that animals were sacrificed at different time points; animals in condition CS24 were sacrificed 12 days after animals from CS12. This design cannot account for the effects of increased age in CS24 animals (albeit only slight). Nor can the experiment account for environmental effects, events that might have happened in the holding room in those 12 days for example, that could also cause differences in CS12 and CS24 behaviour. Although these effects are accounted for by the fact that animals from control conditions were sacrificed at the same time as each stress condition, all animals should be sacrificed at the same point in the life cycle.

The present study demonstrated that PCCS had a significant effect on reproductive parameters after 12 days of stress, but that young animals were relatively immune to the stressor itself in terms of HPA activation. In order to achieve an effective dose, some minor changes could be made to the stressor so that it would represent a greater challenge to young rats. Also additional stress measures should be taken to establish whether PCCS has an effect on other components of the HPA axis. Similarly, more precise sperm quality measures should be investigated to better understand aspects of the HPG axis affected by the PCCS.

Finally, some methodological issues need to be addressed, first, control animals should be handled daily and animals should be sacrificed at the same age so that inadvertent stress and age effects do not impact on control measures on the day of testing.

3.5 Experiment 2c

3.5.1 Introduction

The purpose of this experiment was to reduce noise produced by potential experiential differences in the paradigm used in Experiment 2B, and to assess a broader range of stress and reproductive parameters.

Several changes were made to the PCCS manipulation and its assessment. Similar patterns of corticosterone levels emerged between study 2a and 2b indicating a consistent stress response. However, results from study 2b were not found to be significantly different between CS12 and CS24 conditions, possibly due to the greater stress resistance of young rats. To enhance the severity of PCCS an extra element was incorporated into the PCCS in the present study. Typically healthy adult male rats will show avoidance behaviour towards odours of conspecifics (Bakker, van Ophemert, & Koos Slob, 1996; Portillo & Paredes, 2003; Xiao, Kondo, & Sakuma, 2004). Therefore the PCCS severity was increased by introducing, into the rats' cages, dirty bedding from rats undergoing an unrelated experiment. Adrenal gland weight has been shown to increase with prolonged secretion of ACTH and corticosterone (Ingle, 1938; Koko, Djordjeviae, Cvijiae, & Davidoviae, 2004; Nussdorfer, 1986). Therefore, to further measure HPA axis activation adrenal gland weight were recorded in the present study. Finally, to increase confidence that the stress response seen in the experiment is due to the chronic stress manipulation and not due to acute stress effects of handling, animals in the control condition received handling compared to that of controls (Gadek-Michalska & Bugajski, 2003).

In terms of reproductive effects, seminal vesicles weight and a stain to more accurately visualise dead sperm cells were used. Briefly, fluorescent stain is only absorbed by dead sperm therefore dead sperm can be visualised under a fluorescent

microscope for an accurate count of dead versus live sperm. Finally, to make sperm quality measurement more compatible and therefore more easily comparable to other studies the WHO, (Jeyendran, 2000) the percent forward progressing sperm was also measured. Forward progression is characterised by sperm swimming in a straight line, not circular for example.

3.5.1.2 The hypotheses

It was hypothesised that rats exposed to the PCCS would have higher circulating plasma corticosterone compared to controls. Following results of the previous experiments, habituation was expected after 24 days of stress, therefore it was expected that the CS12 condition would have the highest corticosterone levels. Animals exposed to stress were also expected to have larger adrenal gland body weight ratios. All animals exposed to stress were expected to gain less weight than control animals.

In terms of reproductive parameters it was expected that younger animals in the stress conditions would have lower testosterone levels, increased reproductive organ body weight ratios and most favourable sperm quality, and that these effects would be particularly pronounced in the CS12 animals

3.5.2 Extenuating circumstances

A number of extenuating circumstances need to be considered in relation to the data collected in Experiment 2c and Experiment 2d. Unfortunately in the following two experiments a high percentage of unexpected physical abnormalities were found on measurements taken on the final test day in the two cohorts of rats. These included atrophied testicles, undescended testicles, and unidentified masses. These abnormalities were not likely caused by any experimental manipulation as abnormalities were found across all experimental conditions including the control conditions as well as in control rats, and rats examined from unrelated experiments. The undescended testicles found in

rats used for the present experiments were atrophied and un-vascularised and sperm production was non-existent. A summary of abnormalities found can be seen in Table 3.12. These defects point to a more serious problem originating with the breeders. The animals weighing 250-300g received from the breeder should have had descended testicles so that the presence of testicular abnormalities points to a developmental problem that existed before the onset of my experiments or the delivery of the animals to our laboratory.

Table 3.12. Summary of developmental abnormalities found in experiment 2c.

	kidney mass	epididymal mass	one undescended testicle	two undescended testicles	atrophied testicles and epidymides	total
CTRL	0	1	1	1	0	3
CS12	0	0	1	1	1	3
CS24	1	1	1	0	0	3
total	1	2	3	2	1	9

This hypothesis was separately confirmed by a consultation with our laboratory affiliated veterinarian Dr T. Rogers (personal communication, March 10, 2006). The supplier took responsibility for these problems and donated rats so that the experiment could be replicated. Assurance was provided that the developmental abnormalities were an isolated incidence.

Unfortunately, many more abnormalities were found after sacrificing animals for experiment 2d. All abnormalities were internal and could only be identified through necropsy on the final test day. A summary of abnormalities found in experiment 2d can be found in Table 3.13. Personal communication with several leading scientists was consistent in that all agreed that these problems were not due to the PCCS experimental manipulation, that the problems were developmental and likely originated with the

supplier (S. Bhatnagar (personal communication, April 22, 2007), D. Funk (personal communication, April 22, 2007), I. Lucki (personal communication, April 22, 2007), D. Choi (personal communication, April 23, 2007), S Morato (personal communication, April 24, 2007), L. Pohrecky (personal communication, April 22, 2007), and L. Fraser (personal communication April 29, 2007)).

Table 3.13. Summary of developmental abnormalities found in experiment 2d.

	kidney mass	epididymal mass	one undescended testicle	two undescended testicle	total
CTRL	4	0	0	0	4
rCTRL	1	0	0	1	2
CS12	2	1	1	0	4
CS24	0	1	5	0	6
total	7	2	6	1	16

The supplier was called for a consultation and was given samples to test. These results have not been communicated to us, despite many attempts to obtain them. It is important to discover the nature and cause of these abnormalities to minimize the future waste of animal lives, by having to discard and replicate experiments. Since these occurrences our laboratory has ceased using this supplier.

To minimize the waste of animal lives I felt it was important to nevertheless analyse the results from the two experiments. It was decided that animals with visible abnormalities would be removed from all analyses. All animals were carefully inspected for problems at the time of sacrifice. The majority of the abnormalities were found on reproductive organs and therefore it was especially important that they not be included in the analyses of reproductive parameters. It was also important to note that removal of the testicles reduces circulating testosterone, which potentiates the effects of stress (Bingaman, Van de Kar, Yracheta, Li, & Gray, 1995). Therefore animals with

abnormalities were also excluded from analyses of stress data. I feel confident that the animals that were retained for analyses were healthy.

And finally, according to Carr (1985) 7.3 pH is optimal to assess rat sperm motility, although the Earles Balanced Salt solution is supposed to be pH balanced at 7.3 it was found to have a pH of 8.7. Unfortunately, this error was not found until after the completion of experiment 2d. The high alkaline solution was sufficient to render all sperm immotile and therefore all sperm related results have been removed from experiment 2d.

3.5.3 Materials and Methods

3.5.3.1 Subjects

Subjects were 27 male hooded Lister rats (supplied by Harlan Olac, UK) with an average weight of 465.7±3.15g at the start of the experiment. Animals were weighed every 6 days throughout the experiment.

3.5.3.2 Conditions

Figure 3.14 summarises the methodology used here. This experiment consisted of 3 experimental conditions. In condition 1, animals were exposed to 24 days of PCCS (CS24; n=9) and were sacrificed on day 25 experiment, the first day of stress exposure for CS24 was considered day1 for the experiment. In condition 2, animals were exposed to 12 days of PCCS (CS12; n=9), animals remained in pairs in the home cage until day 13 of the experiment when exposure to stress began, they were sacrificed on the same day as CS24. Condition 3 was the control condition (CTRL; n=9), all animals were caged in the same pair for the duration of the experiment and sacrificed 25 days after the onset of the experiment at the same time as CS24 and CS12.

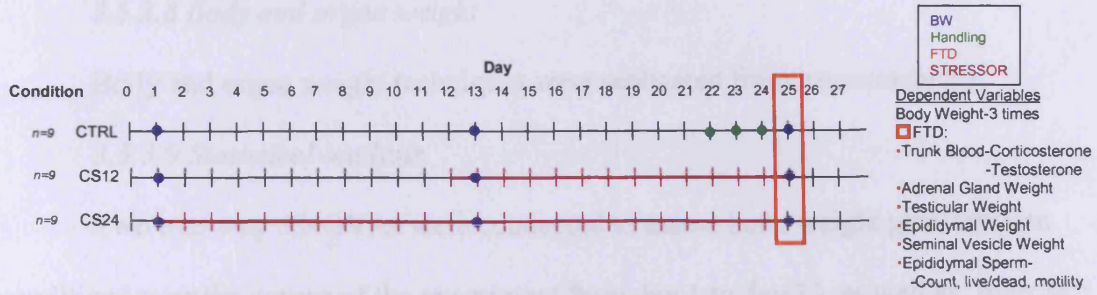

Figure 3.14. Methodology for experiment 2c. CTRLa, control group a; ASa, acute stress group a; CS12, chronic stress for 12 days; CTRLb, control group b; ASb, acute stress group b; CS24, chronic stress for 24 days.

3.5.3.3 *PCCS paradigm*

PCCS was administered as described previously. To further increase the severity of the stressor the bedding in the cages was changed daily, and replaced with dirty bedding from a cage of rats undergoing a separate experiment.

3.5.3.5 Corticosterone enzymeimmunoassay (EIA)

The EIA procedure for corticosterone was replicated from and described Experiment 2a.

3.5.3.6 Testosterone enzyme-linked immunosorbent assay (ELISA)

The testosterone ELSIA procedure was replicated from and described in Experiment 2b.

3.5.3.7 Reproductive parameters

To further examine stress adrenal gland weights were also recorded. To further investigate the HPG seminal vesicle weights were collected along with further sperm measures. A live/dead stain was used to accurately identify dead sperm. Propidium Iodide was introduced into the sample, a dye that is only absorbed by dead cells. The dead cells can then be observed under a fluorescent microscope.

3.5.3.8 Body and organ weight

3.5.3.8 Body and organ weight

Body and organ weight techniques were replicated from experiment 2b.

3.5.3.9 Statistical analysis

Two one-way ANOVAs were conducted to assess body weight gain between conditions over the course of the experiment from day 1 to day 13, as well as, from day 1 to day 24.

Corticosterone levels between conditions were compared using a one-way ANCOVA controlling for final day body weight. An alpha level of $p<.1$ was used.

Testosterone levels were assessed using a one-way ANOVA. A correlation was also computed to assess the relationship between corticosterone levels and testosterone levels.

To account for the effects of body weight on organ weight, four one-way ANOVAs were conducted on adrenal gland, seminal vesicle, testicular, and epididymal body weight ratios. Epididymal sperm counts for each condition were compared using a one-way ANCOVA controlling for epididymal weight. Total sperm count, dead-sperm and the percent of sperm moving with a forward progression were analysed using three separate one-way ANOVAs. Tukey HSD post hoc tests were used.

An alpha level of .05 was used for all statistical tests, unless otherwise stated.

3.5.4 Results

3.5.4.1 Body weight

An ANOVA revealed no significant difference in body weight gain over the first 12 days of the experiment ($F(2,19)=.375$ $p=.694$ *ns*) or over 24 days of the experiment ($F(2,19)=.795$ $p=.463$ *ns*) between conditions.

3.5.4.2 Corticosterone and adrenal gland weight

ANCOVA demonstrated that corticosterone levels between conditions approached conventional levels of significance ($F(2, 23)=2.430$ $p= 0.06$ *ns*). Tukey HSD post hoc test revealed that CS12 had significantly higher circulating levels of corticosterone compared to CTRL ($p<.05$) and CS24 ($p<.05$). (Figure 3.15).

ANOVA demonstrated that differences between conditions in adrenal gland body weight ratio approached conventional levels of significance ($F(2,23)=2.766$ $p=.084$). Tukey HSD post hoc test revealed that CS24 had a marginally larger adrenal gland ratio compared to CTRL ($p=.091$) (Figure 3.15).

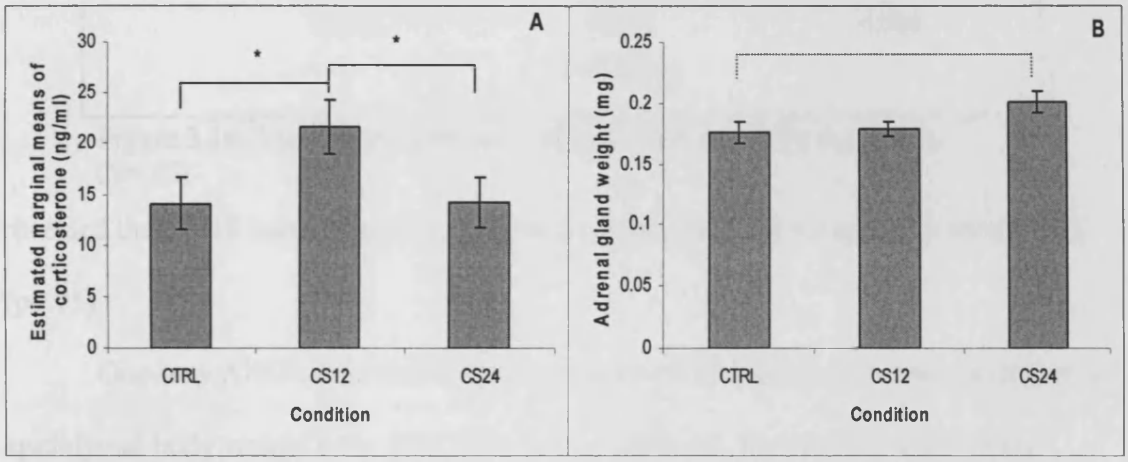

Figure 3.15. (A) Estimated marginal means of corticosterone levels (± SE), after controlling for body weight, by condition. (B) Adrenal gland weight (± SE) by condition. (*p<.05, --- trend p=.091).

3.5.4.3 Testosterone

Results showed no difference between conditions in levels of testosterone ($F(2, 23)=.156$ $p=0.856$ *ns*).

The correlation between corticosterone and testosterone levels was not significant ($r=.304$ *ns*) where only 10 % of the variance in testosterone levels is due to corticosterone.

3.5.4.4 Reproductive organ weights

Figure 3.16 demonstrates a marginally significant difference between conditions in testicular body weight ratio (F(2, 24)=3.18, p=.059). Tukey HSD post hoc test

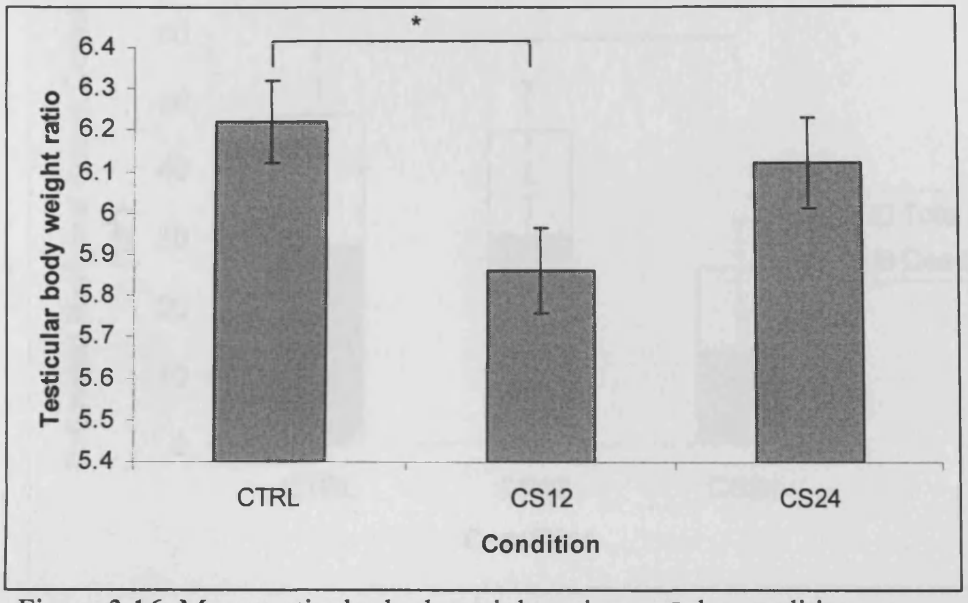

Figure 3.16. Mean testicular body weight ratio (± SE) by condition. (*p<.05).

revealed that CS12 had a significantly smaller testicular body weight ratio than CTRL (p=.05).

One-way ANOVA revealed that there were no difference between conditions in epididymal body weight ratio (F(2, 24)=.063, p=.940 *ns*), or seminal vesicle body weight ratio (F(2, 24)= .528 p=.597 *ns*).

3.5.4.5 Sperm analysis

In the proportion of subjects analysed for sperm quality a one-way ANCOVA demonstrated a significant difference between conditions in total sperm count (F(2, 17)=3.261 , p<0.01) controlling for epididymal weight. Tukey HSD post hoc test revealed that CS24 (p<.05) had significantly less sperm than CTRL (Figure 3.17).

Conditions showed differences in number of dead sperm cells when controlling for epididymal weight approached conventional levels of significance (F(2, 17)=2.766

p=.09). Tukey HSD post hoc test revealed that CS24 (p=.057) had marginally less dead sperm than CTRL. Figure 3.18 illustrates the sperm which have been stained red with PI (dead cells)

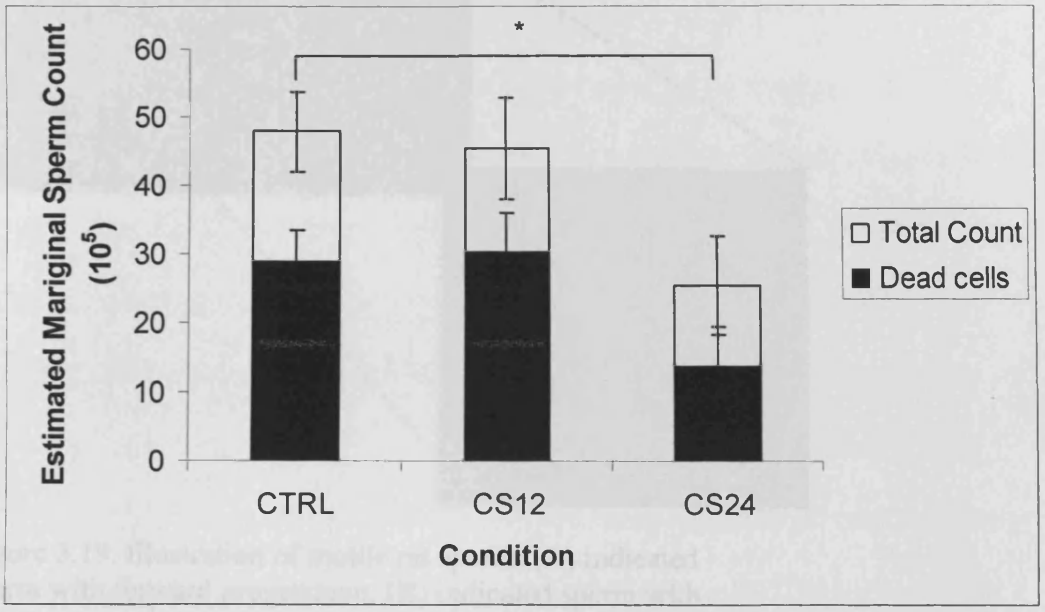

Figure 3.17. Estimated marginal means for total sperm count and total dead sperm cells (±SE) by condition when controlling for epididymal weight.

Finally, differences in percent of forward progressing sperm by condition when covaried for epididymal weight approached conventional levels of significance (F(2, 8)=3.182 p=.096). Tukey HSD post hoc test revealed that CS24 (p=.093) had marginally more percent forward progressing sperm than CTRL. Figure 3.19 illustrates the path of one forward progressing sperm, and one sperm moving in a circular motion.

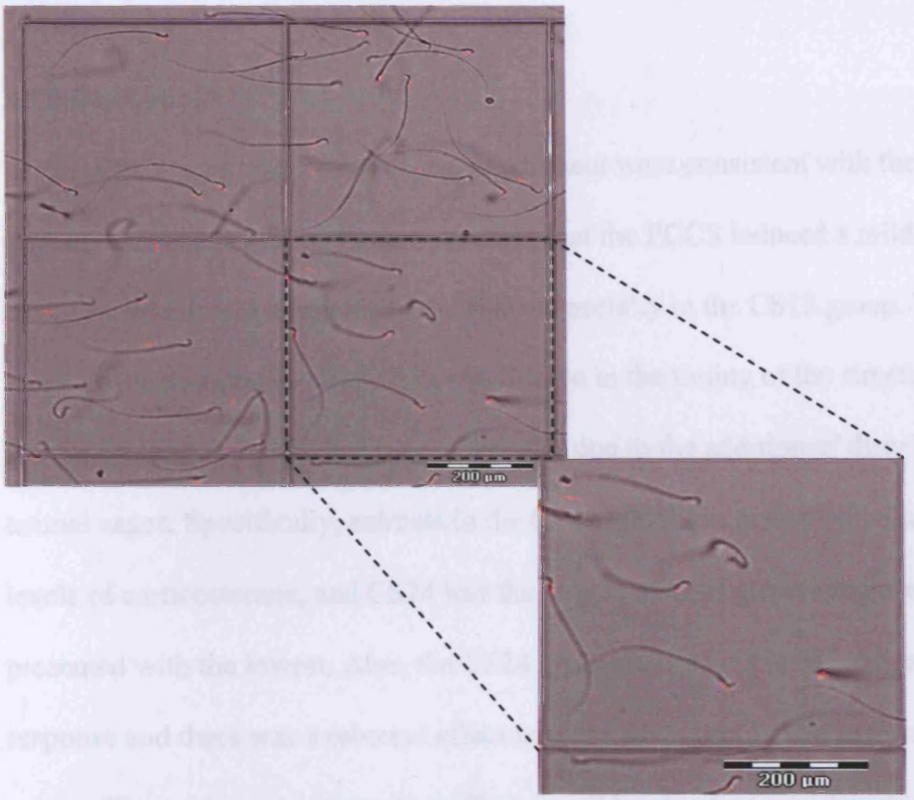

Figure 3.19. Illustration of motile rat sperm. (A) indicated sperm with forward progression, (B) indicated sperm with circular progression.

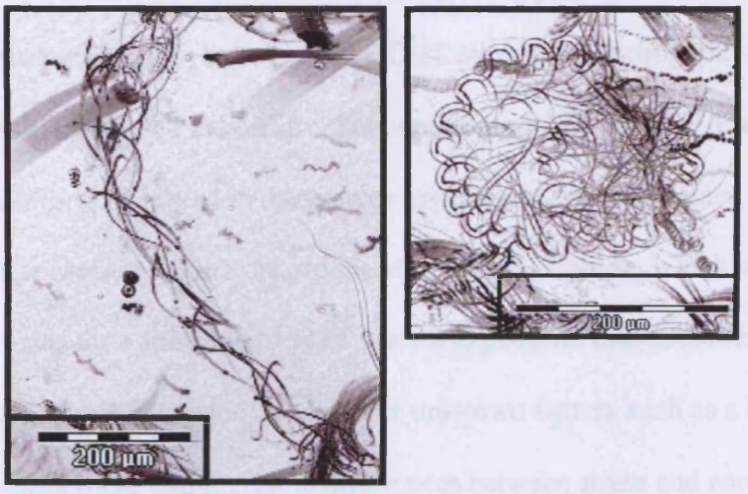

Figure 3.18. Illustration of rat sperm. Sperm dyed red with Propidium Idodide (PI) indicate dead sperm cells.

3.5.5 Discussion

Patterns of responding in this experiment were consistent with the previous experiments and provided further evidence that the PCCS induced a mild stress response, which had a reproductive effect, especially in the CS12 group. The stress results were marginally affected by the change in the timing of the stressor in relation to animal age and the slight increase in severity due to the addition of dirty bedding to the animal cages. Specifically, animals in the CS12 condition presented with the highest levels of corticosterone, and CS24 had the largest adrenal gland weight while controls presented with the lowest. Also, the CS24 group showed signs of habituation in stress response and there was a rebound effect in some aspects of sperm performance.

The evidence to suggest an effect on HPA activation was increase corticosterone levels in CS12 as well as significantly enlarged adrenal glands in the CS24 group. Such distension has been reported for chronic stress in other research (Koko et al., 2004; Nussdorfer, 1986) but repeated CORT assessment throughout the PCCS exposure would be needed to confirm that persistent and chronic HPA activation was the cause of the effects observed in the present experiment. Such repeated assessment might also better capture the effects produced in the CS12 group, which was exposed to the stressor for a shorter period of time. It is possible that stress levels of the control group were slightly elevated due to other unknown factors such as a large, busy, and loud holding room and therefore differences between stress and control conditions were smaller. Comparing untreated rats held in different holding rooms may give an indication as to whether or not stress levels are different in animals between different holding rooms. It may also be that the marginal significance was due to the loss of diseased animals, and a replication with more animals would be required.

Evidence of the reproductive effects produced by the PCCS was mixed. As expected the CS12 and CS24 showed a lower sperm count than did the controls but the CS24 animals had, unexpectedly, more purposeful (i.e., alive and forward moving) sperm than the controls. The lower sperm count results are consistent with previous results where sperm count was the most sensitive sperm parameter and most likely to be affected my stress (Almeida et al., 1998; Orr et al., 1994; Retana-Marquez et al., 2003b; Woody et al., 1998). The effect in CS12 animals could be explained by smaller testes, but this would not explain the effect in CS24 animals. The pattern of results in CS24 animals shows not only recovery but some sort of rebound effect, although this effect has not been previously reported in the literature, and should be investigated further.

As seen in experiment 2b there were no differences in testosterone levels between conditions. Testosterone levels, therefore cannot explain the effects seen on sperm quality. It is possible that assessment of other androgens, or other hormones implicated in the HPG axis (e.g. LH, FSH,) which play important roles in spermatogenesis, may give a better indication of how stress is interacting with sperm quality.

3.6 Experiment 2d

3.6.1 Introduction

Experiment 2c provided evidence that PCCS induced a stress response. PCCS also had an impact on reproductive organs, although it was unclear what that affect was and what was its magnitude. However, due to the health problems seen in the previous experiment the purpose of the present experiment was to replicate Experiment 2c. A blood sample from the tail of each animal was collected every 6 days over the course of the experiment (Vahl et al., 2005) to obtain a more complete profile of corticosterone change throughout the one or two spermatogenetic cycles. Also, to assess whether the

holding room environment impacted on rat stress levels (including the control group) a sub-set of rats were held in a separate, smaller holding room.

3.6.1.2 The hypotheses

It was hypothesised that rats exposed to the PCCS would have higher circulating plasma corticosterone compared to controls. Corticosterone level was expected to increase after the onset of the PCCS but it was expected to decline as the animal habituated to the stressor at some point after the initial 12 days of PCCS exposure. Animals exposed to stress were also expected to have larger adrenal gland body weight ratio then controls, with a more pronounced effect in the CS24 condition.

Weight was assessed as a control variable but weight differences were not expected between conditions. As previously mentioned stress has a greater impact on aged rats (Sapolsky, Krey, & McEwen, 1983), as was seen in experiment 2a where the less severe PCCS lead to marginally significant differences in corticosterone levels between conditions even though a slightly more severe stressor did not elicit a stress response in younger animals in Experiment 2b and 2c. Further evidence of the moderating effect of age was seen on body weight gain, where older but not younger animals exposed to stress gained less weight than controls. The present study uses younger animals more comparable to experiment 2b and 2c.

In terms of reproductive parameters the hypotheses remained unchanged, despite mixed results in previous experiments. Specifically, it was hypothesised that animals exposed to the PCCS would show testosterone suppression and lower testicular body weight. Recall that data from sperm analyses was discarded due to technical problems with pH level in the buffer solution.

3.6.2 Materials and Methods

3.6.2.1 Subjects

Subjects were 26 male hooded Lister rats (supplied by Harlan Olac, UK) with an average weight of 389.1±2.51 g at the start of the experiment. Animals were weighed every 6 days throughout the experiment.

3.6.2.2 Conditions

Figure 3.20 provides a summary of the methodology used. The experiment consisted of four conditions. The same four conditions were used as in experiment 2c. An additional control condition was added where animals house in pairs in a separate room from the other animals in the experiment (rCRTL, n=3). The final sample size per condition was CTRL n=8, rCRTL n=3, CS12 n=8, CS24 n=6.

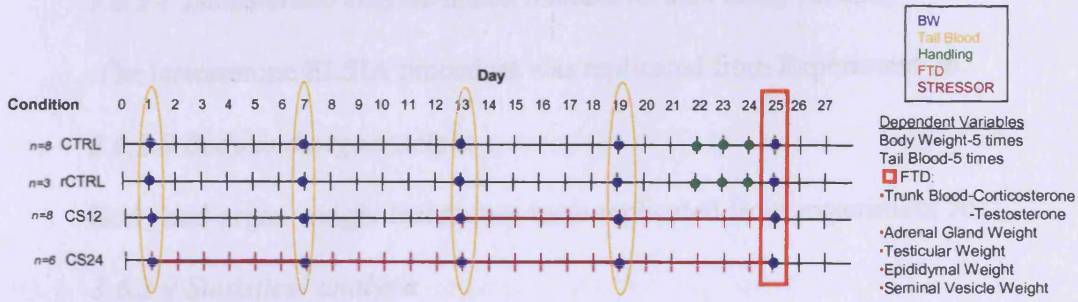

Figure 3.20. Methodology for experiment 2d. CTRLa, control group a; ASa, acute stress group a; CS12, chronic stress for 12 days; CTRLb, control group b; ASb, acute stress group b; CS24, chronic stress for 24 days.

3.6.2.3 PCCS paradigm

PCCS was administered as previously describes in experiment 2c.

3.6.2.4 Tail blood samples

Blood samples were obtained from each animal every six days throughout the experiment namely, on day 1, 7, 13, and 19. On day 25 blood was collected via trunk blood after decapitation as in the previous experiments. On the day of tail blood

sampling (between 1200 and 1400) one cage of rats was transported to a quiet nearby testing room. One rat at a time was removed from the cage and briefly wrapped in a towel by one person and another person (on the first occasion only) then removed less than 1 mm of the distal tip of the tail. On subsequent tail-tipping days, only the scab that had formed from the first tail tip was removed. This technique only induced mild discomfort similar to an injection. The tail was then milked for approximately 200 µl of blood collected in a heparinised epindorph. The procedure was kept under 3 minutes to avoid detection of stress hormones due to the procedure itself (technique adapted from (Vahl et al., 2005)).

3.6.2.6 Corticosterone enzymeimmunoassay (EIA)

The corticosterone EIA procedure was replicated from experiment 2a.

3.6.2.7 Testosterone enzyme-linked immunosorbent assay (ELISA)

The testosterone ELSIA procedure was replicated from Experiment 2b.

3.6.2.8 Body and organ weight

Body and organ weight techniques were replicated from experiment 2c.

3.6.2.9 Statistical analysis

Two one-way ANOVAs were conducted to assess body weight gain between conditions over the course of the experiment from day 1 to day 13, as well as, from day 1 to day 24.

Corticosterone levels between conditions on the final test day were compared using a one-way ANCOVA controlling for final day body weight, one-tailed test were used. Additionally, a factorial trend analysis for corticosterone was conducted. The between subjects factor was condition, and the within subjects factor was time (day 1, 7, 13, 19).

Testosterone levels were assessed using a one-way ANOVA. A correlation was also computed to assess the relationship between corticosterone levels and testosterone levels.

To account for the effects of body weight on organ weight, four one-way ANOVAs were conducted on adrenal gland, seminal vesicle, testicular, and epididymal body weight ratios. Epididymal sperm counts for each condition were compared using a one-way ANCOVA controlling for epididymal weight. Tukey HSD post hoc tests were used.

An alpha level of .05 was used for all statistical, unless otherwise stated.

3.6.3 Results

3.6.3.1 Body weight

No significant differences were found between CTRL and rCTRL on body weight over the first 12 days ($t(9)=1.657$ $p=.230$), or weight gain over 24 days ($t(9)=2.11$ $p=.180$). Therefore the two groups were collapsed into one control condition.

ANOVA results revealed that there was no significant difference in body weight gain over the first 24 days of the experiment between any conditions ($F(2,23)=.324$ $p=.726$ *ns*). Similarly, there was no significant difference in body weight gain over the first 12 days of the experiment between any conditions ($F(2,23)=.530$ $p=.596$ *ns*).

3.6.3.2 Corticosterone and adrenal gland weight

No significant differences were found between CTRL and rCTRL in corticosterone levels on day 1 ($t(8)=.561$ $p=.475$), day 7 ($t(8)=.511$ $p=.495$), day 13 ($t(8)=2.124$ $p=.179$), day 19 ($t(8)=.199$ $p=.667$), day 25 ($t(8)=.053$ $p=.824$). Therefore the two groups were collapsed into one control condition.

Figure 3.21 illustrates the pattern of corticosterone levels hypothesised between conditions and over time. Figure 3.22 shows the actual results. The trend analysis

revealed a significant main effect of condition ($F(2, 14)=12.78$ $p<.001$) no significant effect of within subjects factor time ($F(4, 59)=.9$ $p=.223$ *ns*) and a significant linear trend of time ($p<.05$). There was also a significant interaction ($F(8, 59)=1.98$ $p=.028$) between time and group. Follow-up tests revealed that on day 13 CS24 ($p=.009$) had significantly more circulating corticosterone than the CTRL condition. On day 19 CS12 ($p<.05$) and CS24 ($p<.05$) had significantly more circulating corticosterone than the CTRL condition (Figure 3.22).

When results from the final test day were isolated ANCOVA demonstrated that trunk blood corticosterone levels from day 25 were significantly different between conditions ($F(2, 20)=7.368$ $p< 0.01$). Tukey HSD post hoc test revealed that CS12 ($p<.05$) and CS24 ($p<.05$) had significantly more circulating corticosterone than CTRL (Figure 3.22).

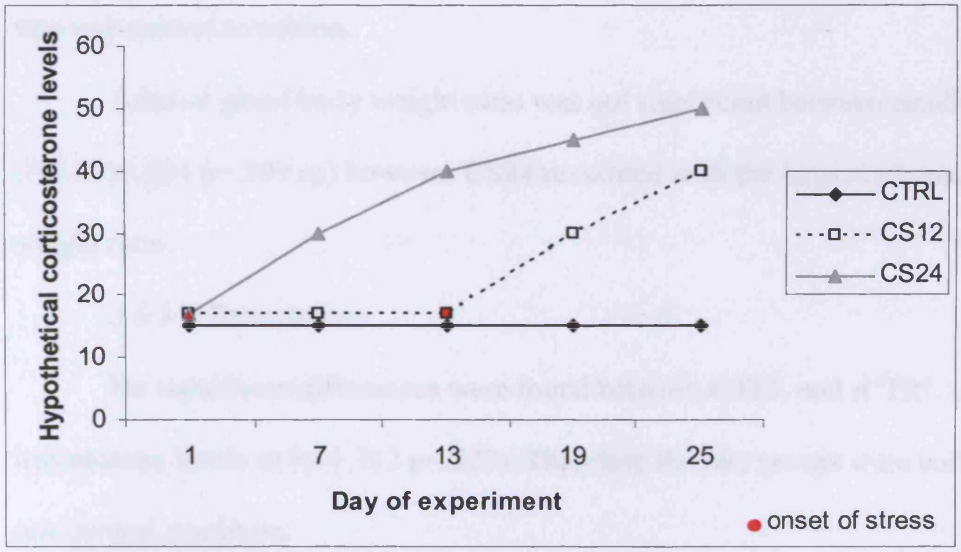

Figure 3.21. Hypothetical patterns of corticosterone levels over time and across conditions. Red indicates onset of PCCS.

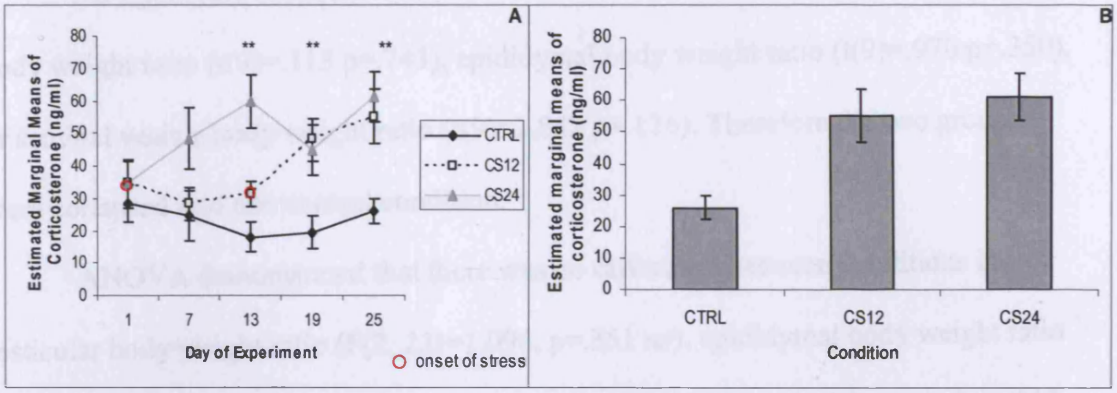

Figure 3.22. (A) estimated marginal means of corticosterone levels (± SE), over time after controlling for body weight, by condition. (B) estimated marginal means of corticosterone levels (± SE), after controlling for body weight, by condition on final day of the experiment.

No significant differences were found between CTRL and rCTRL on adrenal gland body weight ratio (t(9)=1.205 p=.301). Therefore the two groups were collapse into one control condition.

Adrenal gland body weight ratio was not significant between conditions (F(2,23)=.524 p=.599 *ns*) however CS24 presented with the largest adrenal gland body weight ratio.

3.6.3.3 Testosterone

No significant differences were found between CTRL and rCTRL on testosterone levels (t(9)=1.712 p=.227). Therefore the two groups were collapsed into one control condition.

A one-way ANOVA demonstrated that there was no significant difference between conditions in levels of testosterone (F(2, 22)=.157 p=0.855 *ns*). A correlation demonstrated a marginal significant positive relationship between corticosterone and testosterone levels (F(1,35)=3.576, R=.305 p<.067).

3.6.3.4 Reproductive organ weights

No significant differences were found between CTRL and rCTRL on testicular body weight ratio (t(9)=.115 p=.743), epididymal body weight ratio (t(9)=.970 p=.350), or seminal vesicle body weight ratio (t(9)=2.842 p=.126). Therefore the two groups were collapsed into one control condition.

ANOVA demonstrated that there was no difference between conditions in testicular body weight ratio (F(2, 23)=1.096, p=.351 *ns*), epididymal body weight ratio (F(2, 23)=1.637, p=.216 *ns*), seminal vesicle body weight ratio (F(2, 23)= .209 p=.813 *ns*).

3.6.4 Discussion

The PCCS induced a mild stress response but sustained HPA activation was not associated with adrenal organ pathology, and there was no evidence of habituation or recovery.

Also, corticosterone levels did not different between control groups housed in different holding rooms, indicating that stress levels do not differ between holding rooms, and that control groups do not have higher than normal baseline corticosterone levels which would obscure the differences between control and stress conditions.

No evidence of habituation was seen; the CS24 condition had significantly higher levels of corticosterone then controls on the last day of the experiment. The lack of habituation could be explained by the fact that obtaining tail blood samples was in itself a stressor that added a physical stressor to the PCCS. Tail blood samples were collected every 6 days, and even though care was taken so that the blood sample did not reflect the stress of the technique itself, previous research has found that corticosterone levels rise after approximately 3 minutes after the onset of the procedure (Vahl et al., 2005). Therefore PCCS was compounded by the physical stressor of tail blood samples

and overall there was an increase in severity of the stress the animals exposed to the PCCS. It has been found that over time habituation to a repeatedly experienced stressor is common, however, it is less common in more severe stressors (Jean Kant et al., 1985). It is likely that the increase in severity led to decreased levels of habituation in the CS24 condition.

Although adrenal gland weight is a valid measurement of stress (Nussdorfer, 1986), in the present study adrenal gland weight was not found to reflect increases in corticosterone as would have been expected. Adrenal gland weight is a valid measure of prolonged stress, typically longer than 24 days (Koko et al., 2004), since the maximum number of days in the present studies was only 24 days this is the likely cause of the small marginal increase was found in the CS24 condition in Experiment 2c.

Unfortunately, the incidence of animal health problems meant that there was a significant (i.e., 35.7%) reduction from the originally intended sample size and this reduction in power could not be ruled out as an explanation for the lack of significant effect of stress on reproductive parameters (testosterone, testicular weight) that had been observed in previous experiments. However, the pattern of corticosterone results was consistent with Experiment 2a, 2b, and 2c. Furthermore, it was not possible to examine whether sustained HPA activation would have produced negative effects on sperm quality because of the problem with the Earle's Buffered Salt Solution.

A replication of this study, with healthy animals is clearly necessary. As the intention of the PCCS was to model human chronic psychosocial stress, it is important that the PCCS remain a psychosocial stressor therefore employing blood sampling techniques such as a jugular catheter, which does not introduce a physical stressor (as did the tail pinch method) would be useful. Obtaining a hormonal profile of corticosterone over time would be important, especially to determine when

corticosterone levels peak in reaction to PCCS exposure but also when habituation begins.

3.7 General Discussion

Figure 3.23 is a summary of results found in Experiments 2a-2d. Overall the evidence from these experiments indicates that the PCCS is a valid psychosocial stressor that induces a mild stress response that affects reproductive parameters in male rats.

There was converging evidence that the PCCS induced a mild stress response but the magnitude of this effect was not consistent across studies. A similar pattern of differences in corticosterone levels between the stress and control conditions was found in the majority of the studies. Specifically, control rats displayed the lowest levels of corticosterone levels, CS12 with the highest. The CS24 was found to have habituated to the stressor. The only exception to this pattern was in experiment 2d where CS24 animals had the highest levels of corticosterone without habituation but this effect was attributed to the additional stress produced by tail blood samples, a known physical stressor (Vahl et al., 2005). Results were found to be most consistent with the hypothesis when the severity of the stressor was increased slightly (experiment 2c) with the addition of dirty bedding into the cages.

The lack of consistency in the magnitude but not the direction of PCCS induced stress response could be due to several factors. The stressors included in the PCCS were social interactions with new cage mates everyday, cage change at different times every day, and odours from other unknown rats. Experiments which have used a similar stressor paradigm have shown a consistent effect on corticosterone levels (Lemaire et al., 1997; Mormede et al., 1990; Schmidt et al., 2007). However, these studies generally maintain the cage group size at three and change cage occupants each day (Lemaire et

al., 1997; Mormede et al., 1990). It is possible that changing cages is more stressful when there are more animals involved. The magnitude of the stress response found in the present set of studies is less than other studies, which could be explained by the changing number of animals per cage. It is possible that when a rat is moved to a cage of just two animals that this is less stressful than moving into a cage of three. Even though being in a cage alone is generally considered stressful (e.g. social isolation) (Dronjak & Gavrilovic, 2006) it is possible that being housed alone intermittently with either two or three animals is a break from isolation for the animals further reducing the severity of the stressor. Other studies have been conducted on mice where cage occupants are changed once or twice a week only and yet corticosterone levels were still high (Schmidt et al., 2007). It has been found that rats and mice do not have the same social interactions, mice have been found to be more aggressive where fights often end with severe injury (Scott, 1966) which could explain differences in stress responding to differences in social behaviour. Thus the PCCS may have been too mild to induce a potent and reliable HPA response, and this conclusion would be supported by the general lack of effect on other stress indicators (i.e., adrenal gland and body weight, testosterone suppression).

Consistent with findings from corticosterone data, results with other indicators of HPA activation, were also variable in their effect with some results in expected direction and others showing opposite effects. Adrenal gland weight is a valid measurement of stress (Nussdorfer, 1986) especially prolonged stress (Koko et al., 2004). However , a stressor producing a greater stress response (higher corticosterone levels) is more likely to affect adrenal gland weight. Klein (1992) found that cage change between three males and three females for four weeks led to a 43.3 % enlargement in adrenal gland size. It is likely that the variable results found in the

Table 3.23. Summary table for experiments 2a, 2b, 2c, 2d.										
	Stress measures				Reproductive Organ Weights			Sperm Quality		
Exp.	Body Weight	Cort	Adrenal /BW	Testost	Testes /BW	Epi/BW	Sem Ves. Weight/BW	Count	Dead	Motility
2a	*C12<CTRL, CS24*	CS12>CTRL			CS12>CS24, AS, CTRL	CS12>CS24, AS		CS12>CS24, AS, CTRL		
2b	CS24>AS	-		-	CS12>CS24	*CS12>CS24*		-	-	CS12>CS24
2c	-	*C12>CTRL, CS24*	*CS24>CTRL*	-	CS12<CS24	-	-	CS24<CTRL	*CS24<CTRL*	*CS24>CTRL*
2d	-	CS12, CS24> CTRL	-	-	-	-	-			
Note. - no change, blank outcome not measured, *italics* refers to a statistical trend.										

present set of studies where in Experiment 2c CS24 had larger adrenal gland body weight ratio weight than the control condition, but no differences were found between conditions in Experiment 2d, is due to the mild nature of the stressor and/or the lack of statistical power due to the loss in animal.

Another measure of stress is body weight. Reduced body weight gain is considered a sign of anhedonia often induced by severe stress (Gronli et al., 2004). In experiments 2b, 2c, and 2d no differences in weight gain between conditions were found suggesting that the PCCS was a mild stressor not severe enough to reduce consumptive behaviour. Also weight gain is often considered a sign of overall animal health, a dramatic drop in weight is likely to indicate illness. The fact that animals (excludes animals that were not included in the studies due to unknown developmental abnormalities) exposed to stress maintained normal body weight indicates that animals remained in good health during the course of the study. The reproductive changes can be considered a product of stress and not likely mediated or moderated by adverse effects on health. Again, a replication of these studies with healthy animals and an increase in the severity of the stressor would allow insight into the effect of PCCS on adrenal gland weight and body weight gain.

The PCCS consistently produced an effect on reproductive parameters but the parameter affected and the nature of the effect varied from experiment to experiment. In some experiments testicular body weight ratio showed an increase (2a, 2b) in others a decrease (2c) and in still others no effect (2d). Epididymal weight generally did not differ across conditions (2c, 2d) but showed an effect in others (2a, 2b). Seminal body weight was not measured until Experiment 2c and 2d where no effect was seen. Testosterone levels varied across experiments but did not differ between conditions in any experiment. Finally, sperm quality was consistently affected but, again, results were

variable across sperm characteristics. Sperm count was increased in Experiment 2a but decreased in Experiment 2c. Contrary to predictions an increase in sperm motility was seen in conditions exposed to stress (2b, 2c). Thus the PCCS clearly produced reproductive effects but the nature of these effects could not be predicted.

A lack of strong reproductive effects could be due to the mild nature of the PCCS stressor as described previously. The mechanism of action thought to underlie PCCS reproductive effects is interaction between HPA and HPG axis. The current model of HPA-HPG interaction suggests that the way in which stress negatively effects reproduction is an inhibitory pathway from corticosterone through the serial pathway of beta-endorphin to LH and FSH to testosterone (Ferin, 1999). However this model is not supported by the results found here, as no differences between testosterone levels were found. This data may suggest that there is an additional inhibitory pathway by which corticosterone effects sperm quality, or that the inhibitory pathway acts on other hormones from the HPG axis that contribute to spermatogenesis (e.g. LH, FSH, ABP). This hypothesis would have to be further investigated by analysing beta-endorphin, LH, and FSH, and ABP levels after exposure PCCS. Nevertheless the mild nature of HPA activation could explain the mild or non-existent reproductive effects.

But the mild response could not explain why opposite results were sometimes obtained even when there was HPA activation. For example, where CORT differences were observed (2c) a decrease in testicular body weight ratio and sperm count was observed as expected, but improved sperm motility was observed in stressed animals. It is possible that the PCCS contains more than one active component, and that the different components produce different and possibly opposite results. For example, although PCCS is described as a social stressor it is possible that the social interaction is playing an important role in the reproductive effects. The social interaction includes

potentially includes daily fights for dominance which could interfere with reproductive hormones that have direct negative feedback with testosterone (e.g., LH) (Flaherty, 1995). There is a large body of research showing that rat physiology is sensitive to social hierarchy/arrangement. For example Taylor (1987) showed that aggressive colony had better sperm quality compared to a less aggressive colony. Therefore to fully understand the variable (but consistent) PCCS effects, a study that examines the differences between naturally occurring aggression in response to PCCS may be required. It is possible that social stress produces effects that are different from other types of stress (e.g., restraint stress) and that animals, especially young animals, respond to this challenge differently.

Finally, it is possible that effects on sperm quality are mediated or moderated by effects on the accessory glands and the seminal fluid that they produce. Recall that seminal fluid contains contributions from the surrounding reproductive accessory glands. For example, the prostate gland contributes prostaglandins to the seminal fluid, which is thought to be important for sperm transportation through the female reproductive tract (Purvis, Magnus, Morkas, Abyholm, & Rui, 1986). In a study by Purvis (1986) it was found that arousal can affect prostaglandin levels. Seminal fluid was compared between sexual intercourse and masturbatory sample, the sample from intercourse was of a better quality, including higher levels of prostaglandin, an effect thought to be moderated by arousal (Purvis et al., 1986), if seminal fluid can be affected by arousal it is possible that stress can also affect seminal fluid quality, as well as, sperm quality. In light of these findings, it would be important to examine the seminal fluid as well as sperm quality to get a complete account of stress effects. Also to better construct the animal model the sperm analysis ought to examine sperm in seminal fluid instead of an artificial medium, as was done in the present experiments. However,

investigating this possibility would involve collecting a fresh ejaculate sample from rats containing all the constituents of accessory glands to provide a more accurate measure of sperm quality. Unfortunately, to date there is no way of collecting a fresh ejaculated semen sample from the rat. Several techniques were attempted in this doctoral research but none were successful. For example, rats were held lightly in a towel and manually stimulated with warm wet cotton bud but no ejaculate was produced. An artificial rat was developed and placed in a cage with female rats for several days, so it could acquire the smells of female rats. The artificial female was then placed in a cage with a male rat. The male rats demonstrated mild exploratory behaviour but did not engage in sexual activity. And finally, male rats were placed in a cage where females had previously been housed then they were picked up and a vibrating collection tube was placed over the penis of the rat. This technique often led to erections in the rats but never an ejaculation.

The present set of studies contributed important methodological knowledge on the measurement of stress and reproductive parameters. First, it is important to measure more than one component of stress and/or HPA activation. Here, corticosterone the main output of the HPA axis was measured while adopting a new, cheaper, safer, and more accessible method (EIA) of assessing it. Moreover, to obtain a more complete assessment of the stress response to PCCS body weight gain, as well as adrenal gland weight was also taken into account. To establish a timeline for the stress response, a hormonal profile was obtained through the use of repeated tail blood samples.

A second methodological contribution was that reproductive potential was measured in many different ways. As compared to previous research body weight was taken into account when examining the reproductive organ weights. A reproductive organ weight to body weight ratio was used so that effects seen could not be simply due to changes in body weight. Also a detailed sperm analysis was conducted where a dye

was introduced into the sperm sample so that live and dead sperm were easily differentiated from each other and quantified. And finally, a new technique was developed to analyse sperm motility, where a short video clip of the sperm was taken, this led to a more accurate analysis but also to the visualisation of the direction, and displacement of sperm that would not otherwise be seen.

The strengths of the present set of studies were that the PCCS was developed on the basis of a thorough an exhaustive examination of existing paradigms and combined the most active but humane aspect of each design. A series of experiments were carried out to strengthen experimental effects and whilst this goal was not fully achieved the experiments generated much important information about the nature of stress effects in rats and concomitant reproductive effects and how best to measure these. The experimental design included rigorous controls to guard against uncontrolled effects. Finally an extensive array of stress and reproductive factors were assessed.

The main limitations were that the ongoing development of the PCCS meant that each successive experiment used a modified PCCS and this may have introduced noise in the data. This is particularly true for Experiment 2d, where obtaining repeated blood samples from the tail, which lead to a hormonal profile over time, meant that the nature of the stress was changed from psychosocial to psychosocial and physical. In future to obtain an in vivo hormonal profile over time the use of jugular catheter for example may be implemented. Some of the experiments were also under-powered because animals had to be excluded due to health problems associated with breeding protocols. In future, it would be beneficial to replicate experiment 2c with a healthy cohort of animals.

Future studies would also benefit from further increasing the severity of the PCCS, for example, as mentioned earlier it is likely that a larger number of animals per

cage is more stressful, therefore instead of changing cages form 1, 2, or 3 rats, rats could be rotated between 3, 4, or 5 rats per cage. To further investigate the HPA axis and what levels are affected in response to PCCS, circulating ACTH levels or the quantification of CRH and AVP receptor in the PVN may also be quantified. To further investigate the HPG axis circulating LH, FSH, and ABP may be analysed as previously mentioned. These evaluations should show not only absolute measures but also ratios between these hormones as it is possible that optimal ratios between these hormones must exist for optimal sperm quality.

In conclusion this set of experiments provides evidence that PCCS is a valid and reliable stressor that impacts on male reproduction. The severity of the stressor presented in experiment 2c leads to negative effects on reproductive organ body weight ratio as well as sperm count. These results also suggest, as hypothesised, an interaction between the HPA and HPG axes.

Chapter 4

General Discussion

4.1 Main findings

The findings of the set of experiments reported here indicate that the biopsychosocial model is an appropriate, coherent and parsimonious way to explain and describe male infertility. The biopsychosocial model of disease states that biological, psychological and social variables all influence health, and that bi-directional effects occur so that disease affects psychosocial factors and vice versa (Engel, 1977). Overall, the findings of this set of studies provides evidence of inter-relationships between psychological and biological variables that would not easily be explained by the biomedical model of disease.

Infertility was shown to impact on psychological, emotional and physical well being. Men in a couple with persistent infertility and a year of treatment failure experienced psychological suffering and a deterioration of their social environment mental well-being is separate and unrelated to bodily function regardless of the source of their infertility. Moreover, physical stress symptoms, including chest pains, racing heart, shortness of breath, and nausea also increased over time, demonstrating that psychological suffering induced by the persistent strain of infertility resulted in physical stress symptoms. Mental health at the start of treatment predicted social support after a year of unsuccessful treatment.

The animal model of male fertility showed that psychosocial stress combined with social interaction between rats produced consistently variable effects on reproductive parameters across studies. These findings are a demonstration of bi-directional influence of biological, psychological, and social effects that could not be explained by the biomedical model of disease, as the biomedical model of disease specifically states that mental well-being is separate and unrelated to bodily function

4.2 Strengths and limitations of the biopsychosocial model

The strength of the biopsychosocial model is that it coherently and parsimoniously describes the phenomena of male fertility. It is a holistic approach to medicine (Engel, 1977), where the person as a whole is treated and not just the illness (Sadler & Hulgus, 1992). The biopsychosocial model addresses not only the problem of infertility, for example, but also what the person experiences as a consequence of their infertility (Williams, Bischoff, & Ludes, 1992). It acknowledges the multidimensional nature of infertility (Sadler & Hulgus, 1992), where psychological, social, and biological factors all interact to influence reproductive health (Taylor, 1990). The biopsychosocial model accounts for the fact that men who are not able to produce a child suffer psychologically and socially, as well as, the fact that psychosocial stress can interfere with fertility. Support for the biopsychosocial model has also been found in other areas of heath care, for example, in cancer research (Greer & Watson, 1985; Pettingale, 1985), where reducing stress improves physical health (Kop, 1999; Larson et al., 2000; McBride et al., 2000). Similarly, the biopsychosocial model has proven to be particularly useful in the area of cardiovascular disease (Blumenthal, Sherwood, Gullette, Georgiades, & Tweedy, 2002), again, where stress has been found to provoke heart disease (Kop, 1999).

Given converging evidence of the applicability of the biopsychosocial model in different health contexts, it is surprising that it is still not widely applied. The model is not well accepted by medical practitioners (Alonso, 2004), who believe that it is complicated, it does not help with clinical decision making, and it does not define with much specificity the interactions between biological, psychological, and social factors. In contrast, the biopsychosocial model is better accepted in academic communities, such as universities and in research centres concerned with health programmes (Alonso,

2004). Despite the fact that much research has been generated outlining the validity of the biopsychosocial model in disease, the biomedical model continues to be dominant model in medicine (Wade & Halligan, 2004).

One reason for lack of acceptance is that doctors report feeling that the biopsychosocial model is too complicated, it adds another layer of care, for example involving a psychologist (Hertz, 1982). By comparison the biomedical model is preferred because it is simplistic; the underlying biological cause of infertility must be found and treated. Another similar criticism is that it is not pragmatic in the context of today's medical system (Guerrero, Hishinuma, Serrano, & Ahmed, 2003). In today's society medical resources are limited by time and budget; illness and treatment must be prioritised, that is, it is felt by medical staff that it is more effective to treat the underlying biological cause of illness because that is where the best chances of recovery for the patient lies (Guerrero et al., 2003). Addressing psychological and social issues costs too much and takes up too much time and is only likely to increase pregnancy rates for a few select people, or to only slightly increase treatment effectiveness and this type of intervention is not seen as cost or time efficient.

Compounding this problem is the fact that the biopsychosocial model lacks theoretical explanations of the interactions between biological, psychological, and social factors (Molina, 1983). Moreover, the model does not prioritise (Sabelli & Carlson-Sabelli, 1989) and it is therefore not helpful in making clinical decisions. If the model was more precise in explaining the effect size, for example, or the order of importance in which each domain (e.g., biological, social, psychological) should be treated then perhaps it would be more readily accepted by medical professionals.

4.3 Implications for use of the biopsychosocial model

The appropriate use of the biopsychosocial model would lead to better decision making for diagnosis and treatment. Treating the person as a whole rather than a list of physical symptoms as well as recognising what the patient is going through emotionally and not just medically would mean a more patient-centred approach to care (Sadler & Hulgus, 1992). This is important and has serious medical implications, for example increased distress prior to or during in vitro fertilisation has been associated with a poorer biological response and/or a reduced pregnancy rate (Boivin et al., 1995; Smeenk et al., 2001). Due to the interactive nature of biological, psychological, and social factors treatments, interventions that encompass psychological and social factors alongside biological factors are likely to be more successful than treatments that only address one single issue. For example, it has been previously found that counselling and/or detailed education have been shown to decrease negative affect in infertile patients (Domar, Seibel, & Benson, 1990; McQueeney, Stanton, & Sigmon, 1997). Interestingly, men report equal amounts of benefit from counselling as women (Boivin, 2003). Moreover, three studies have also found higher pregnancy rates and shorter time until pregnancy in groups that received psychological interventions (Domar et al., 2000; Sarrel & DeCherney, 1985; Tuschen-Caffier, Florin, Krause, & Pook, 1999).In light of these results it is possible that psychological interventions that addresses the impact of psychosocial stress on male fertility male improve sperm quality and perhaps even increase pregnancy rates. Also, the biopsychosocial model can lead to interventions that not only reduce suffering, and therefore possibly increase the chances of treatment success, but also improve health behaviour by increasing treatment compliance, for example (Boivin, 2003; Taylor, 1990).

In summary, the implications of implementing the biopsychosocial model include generating new research ideas leading to interventions designed to reduce the suffering of men who are in a couple who cannot have a baby, reduce stress and therefore increase the chances of pregnancy (Domar et al., 2000; Sarrel & DeCherney, 1985; Tuschen-Caffier et al., 1999). Hertz (1982) sums it up very eloquently in saying "...the physician who is aware of the psychological and social repercussions to such circumstances [infertility] will be in a better position to modify stress and facilitate adaptation. Further research is needed to develop better methods for predicting reactions to infertility and its treatment" (p. 100).

4.4 Future research

To address some of the limitations of the biopsychosocial model, future research should continue to explore the interactions between biological, psychological, and social factors with regards to male fertility. One way to achieve this goal is to continue research with an animal model to investigate the HPA and HPG axes. More measures of the HPA and HPG should be taken, but also behavioural measures should be included, such as stress behaviours and social interactions. Also research should eventually include the examination of the male/female sexual interaction and outcome measures such as embryo implantation and live births. With a better understanding of how psychosocial factors such as psychosocial stress can affect fertility, and to what extent, then the development of interventions to minimize these affects can be targeted.

Also, work should continue to investigate the links between psychosocial factors and human male fertility. For example, prospective studies with appropriate control groups, examining stress, social environment, sperm quality, and pregnancy outcome would give valuable information about interactions and relative importance of biological, psychological, and social factors with regards to male fertility.

With a better understanding of interactions between biological, psychological, and social factors clear and practically useful information can be built into the model so that it is more straightforward for clinical use. For example, tools can be developed to help medical staff assess which patients would benefit from psychological interventions. These tools could include simple questionnaires filled out by patients which could identify in which patients psychosocial variables could be interfering with their fertility. This type of research may eventually lead not only to the increase of success rates in fertility treatments but also to a decrease in the incidence of infertility and therefore the need for fertility treatment.

4.5 Final word

Most people report having children as a major life goal (Lampic, Svanberg, Karlstrom, & Tyden, 2006) and there is much suffering by both men and women when difficulties obtaining this goal are encountered (Lee et al., 2001; van Balen & Trimbos-Kemper, 1993). It is therefore important that research continues to investigate the reproductive process so as to better understand how it works and what might influence that process. This is particularly important in men as their contribution to conception has been grossly overlooked where the majority of research to date has focused on female reproduction.

For people who are already facing difficulties conceiving it is important that the patient is treated as a whole and not simply as a diagnosis. Further research must be conducted to further understand the male experience of infertility, and to what degree male suffering interferes with treatment either at a biological level by interfering with reproductive hormones for example, at a psychological level, perhaps by reducing treatment compliance, or at a social level where social support deteriorates. Research

must also be conducted on possible interventions to minimize suffering in infertile patients and to possibly increase treatment success.

Finally, a better understanding of the interactions between the HPA and HPG should help identify psychosocial risk factors for infertility and therefore possibly even minimise the incidence of infertility.

References

Abbey, A., Andrews, F. M., & Halman, L. J. (1991). Gender's Role in Responses to Infertility. *Psychology of Women Quarterly, 15*(2), 295-316.

Abbott, D. H., Keverne, E. B., Bercovitch, F. B., Shively, C. A., Mendoza, S. P., Saltzman, W., et al. (2003). Are subordinates always stressed? A comparative analysis of rank differences in cortisol levels among primates. *Hormones and Behavior, 43*(1), 67-82.

Adler, J., D., & Russell, L. B. (1985). The psychological reactions to infertility: Sex roles and coping styles. *Sex Roles: A Journal of Research, V12*(3), 271-279.

Agmo, A. (1997). Male rat sexual behavior. *Brain Research Protocols, 1*(2), 203-209.

Alam, M., & Schmidt, W. J. (2004). Mitochondrial complex I inhibition depletes plasma testosterone in the rotenone model of Parkinson's disease. *Physiology & Behavior, 83*(3), 395-400.

Albert, D. J., Walsh, M. L., Gorzalka, B. B., Siemens, Y., & Louie, H. (1986). Testosterone removal in rats results in a decrease in social aggression and a loss of social dominance. *Physiology & Behavior, 36*(3), 401-407.

Almeida, S. A., Kempinas, W. G., & Lamano Carvalho, T. L. (2000). Sexual behavior and fertility of male rats submitted to prolonged immobilization-induced stress. *Braz J Med Biol Res, 33*(9), 1105-1109.

Almeida, S. A., Petenusci, S. O., Anselmo-Franci, J. A., Rosa-e-Silva, A. A., & Lamano-Carvalho, T. L. (1998). Decreased spermatogenic and androgenic testicular functions in adult rats submitted to immobilization-induced stress from prepuberty. *Braz J Med Biol Res, 31*(11), 1443-1448.

Almeida, S. A., Petenusci, S. O., Franci, J. A., Rosa e Silva, A. A., & Carvalho, T. L. (2000). Chronic immobilization-induced stress increases plasma testosterone and delays testicular maturation in pubertal rats. *Andrologia, 32*(1), 7-11.

Alonso, Y. (2004). The biopsychosocial model in medical research: the evolution of the health concept over the last two decades. *Patient education and counseling, 53*(2), 239-244.

Aslam, H., Rosiepen, G., Krishnamurthy, H., Arslan, M., Clemen, G., Nieschlag, E., et al. (1999). The cycle duration of the seminiferous epithelium remains unaltered during GnRH antagonist-induced testicular involution in rats and monkeys. *J Endocrinol, 161*(2), 281-288.

Bachmann, P. S., & Lock, R. B. (2007). In Vivo Models of Childhood Leukemia for Preclinical Drug Testing. *Current Drug Targets, 8*, 773-783.

Bakker, J., van Ophemert, J., & Koos Slob, A. (1996). Sexual differentiation of odor and partner preference in the rat. *Physiology & Behavior, 60*, 489-494.

Balcombe, J. P. (2006). Laboratory environments and rodents' behavioural needs: a review. *Laboratory Animals, 40*, 217-235.

Baram, D., Tourtelot, E., Muechler, E., & Huang, K. E. (1988). Psychosocial Adjustment Following Unsuccessful Invitro Fertilization. *Journal of Psychosomatic Obstetrics & Gynecology, 9*(3), 181-190.

Bedford, J. M. (1991). Effects of elevated temperature on the epididymis and testis: experimental studies. *Advances in Experimental Medicine and Biology, 286*, 19-32.

Berg, B. J., & Wilson, J. F. (1991). Psychological functioning across stages of treatment for infertility. *J Behav Med, 14*(1), 11-26.

Bhatnagar, S., & Vining, C. (2003). Facilitation of hypothalamic-pituitary-adrenal responses to novel stress following repeated social stress using the resident/intruder paradigm. *Hormones and Behavior, 43*(1), 158-165.

Bielajew, C., Konkle, A. T. M., & Merali, Z. (2002). The effects of chronic mild stress on male Sprague-Dawley and Long Evans rats: I. Biochemical and physiological analyses. *Behavioural Brain Research, 136*(2), 583-592.

Bingaman, E. W., Van de Kar, L. D., Yracheta, J. M., Li, Q., & Gray, T. S. (1995). Castration attenuates prolactin response but potentiates ACTH response to conditioned stress in the rat. *The American Journal of Physiology, 269*(4 Pt 2), R856-863.

Bjørner, J. B., Damsgaard, M. T., Watt, T., Bech, P., Rasmussen, N. K., Kristensen, T. S., Modvig, J. & Thunedborg, K. . (1997). *Dansk Manual for SF-36 (Danish Manual for SF-36).* Copenhagen: Lif Copenhagen.

Blanchard, D. C., Spencer, R. L., Weiss, S. M., Blanchard, R. J., McEwen, B., & Sakai, R. R. (1995). Visible burrow system as a model of chronic social stress: Behavioral and neuroendocrine correlates. *Psychoneuroendocrinology, 20*(2), 117-134.

Blanchard, R. J., McKittrick, C. R., & Blanchard, D. C. (2001). Animal models of social stress: effects on behavior and brain neurochemical systems. *Physiology & Behavior, 73*(3), 261-271.

Blanchard, R. J., Nikulina, J. N., Sakai, R. R., McKittrick, C., McEwen, B., & Blanchard, D. C. (1998). Behavioral and endocrine change following chronic predatory stress. *Physiology & Behavior, 63*(4), 561-569.

Blumenthal, J. A., Sherwood, A., Gullette, E. C., Georgiades, A., & Tweedy, D. (2002). Biobehavioral approaches to the treatment of essential hypertension. *J Consult Clin Psychol, 70*(3), 569-589.

Boccabella, A. V. (1963). Reinitiation and restoration of spermatogenesis with testosterone propionate and otehr hormones after long-term posthypohysectomy regression period. *Endocrinology, 72*, 787-798.

Boguszewski, P., & Zagrodzka, J. (2002). Emotional changes related to age in rats--a behavioral analysis. *Behavioural Brain Research, 133*(2), 323-332.

Boivin, J. (2003). A review of psychosocial interventions in infertility. *Social Science & Medicine, 57*(12), 2325-2341.

Boivin, J., Bunting, L., Collins, J. A., & Nygren, K. G. (2007). International estimates of infertility prevalence and treatment-seeking: potential need and demand for infertility medical care. *Human Reproduction, 22*(10), 2800-.

Boivin, J., Scanlan, L. C., & Walker, S. M. (1999). Why are infertile patients not using psychosocial counselling? *Human Reproduction, 14*(5), 1384-1391.

Boivin, J., & Schmidt, L. (2005). Infertility-related stress in men and women predicts treatment outcome 1 year later. *Fertility and Sterility, 83*(6), 1745-1752.

Boivin, J., Takefman, J. E., Tulandi, T., & Brender, W. (1995). Reactions to infertility based on extent of treatment failure. *Fertility and Sterility, 63*(4), 801-807.

Bowman, R. E., Maclusky, N. J., Diaz, S. E., Zrull, M. C., & Luine, V. N. (2006). Aged rats: Sex differences and responses to chronic stress. *Brain Research, 1126*(1), 156-166.

Broeckel, J. A., Jacobsen, P. B., Balducci, L., Horton, J., & Lyman, G. H. (2000). Quality of life after adjuvant chemotherapy for breast cancer. *Breast Cancer Research and Treatment, 62*(2), 141-150.

Callan, V. J. (1983). Perceptions of parenthood and childlessness:A comparison of mothers and voluntarily childless wives. *Population and Environment, 6*, 179-189.

Campbell, K. M., Meier, A., Carr, C., Enga, Z., James, A. S., Reedy, J., et al. (2001). Health behavior changes after colon cancer: a comparison of findings from face-to-face and on-line focus groups. *Family & community health, 24*(3), 88-103.

Carmeli, Y. S., Birenbaum-Carmeli, D. (1994). The Perdicament of masculinity: toward understanding the male's experience of infertility treatments. *Sex Roles: A Journal of Research, 30*(9-10), 663-679.

Carr, D. W., Usselman, M. C., & Acott, T. S. (1985). Effects of pH, lactate, and viscoelastic drag on sperm motility: a species comparison. *Biology of Reproduction, 33*(3), 588-595.

Chan, D. W., Perlstein, M. T. (Eds). (1987). *Immunoassay: A Practical Guide.* New Jersey: Academic Press Inc.

Chapin, R. E. (1997). Reproductive toxicology. Feed restriction in rats. *Environmental Health Perspectives, 105 Suppl 1*, 381-382.

Clarke, R. N., Klock, S. C., Geoghegan, A., & Travassos, D. E. (1999). Relationship between psychological stress and semen quality among in-vitro fertilization patients. *Human Reproduction, 14*(3), 753-758.

Cohen, S., & Wills, T. A. (1985). Stress, social support, and the buffering hypothesis. *Psychological Bulletin, 98*(2), 310-357.

Connolly, K. J., Edelmann, R. J., Cooke, I. D., & Robson, J. (1992). The impact of infertility on psychological functioning. *J Psychosom Res, 36*(5), 459-468.

Contarteze, R. V. L., Manchado, F. D. B., Gobatto, C. A., & De Mello, M. A. R. (2007). Stress biomarkers in rats submitted to swimming and treadmill running exercises. *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology, In Press, Corrected Proof.*

Cordova, M. J., Andrykowski, M. A., Kenady, D. E., McGrath, P. C., Sloan, D. A., & Redd, W. H. (1995). Frequency and correlates of posttraumatic-stress-disorder-like symptoms after treatment for breast cancer. *Journal of Consulting and Clinical Psychology, 63*(6), 981-986.

Crisostomo, P. R., Wang, M., Wairiuko, G. M., Morrell, E. D., & Meldrum, D. R. (2006). Brief exposure to exogenous testosterone increases death signaling and adversely affects myocardial function after ischemia. *Am J Physiol Regul Integr Comp Physiol, 290*(5), R1168-1174.

Cruess, D. G., Antoni, M. H., McGregor, B. A., Kilbourn, K. M., Boyers, A. E., Alferi, S. M., et al. (2000). Cognitive-behavioral stress management reduces serum cortisol by enhancing benefit finding among women being treated for early stage breast cancer. *Psychosomatic Medicine, 62*(3), 304-308.

Cui, K. H. (1996a). The effect of stress on semen reduction in the marmoset monkey (Callithrix jacchus). *Hum Reprod, 11*(3), 568-573.

Cui, K. H. (1996b). The effect of stress on semen reduction in the marmoset monkey (Callithrix jacchus). *Human Reproduction, 11*(3), 568-573.

Cukierski, M. A., Sina, J. L., Prahalada, S., & Robertson, R. T. (1991). Effects of seminal vesicle and coagulating gland ablation on fertility in rats. *Reprod Toxicol, 5*(4), 347-352.

Dallman, M. F., Akana, S. F., Scribner, K. A., Bradbury, M. J., Walker, C. D., Strack, A. M., et al. (1992). Stress, Feedback and Facilitation in the Hypothalamo-Pituitary-Adrenal Axis. *Journal of Neuroendocrinology, 4*(5), 517-526.

Daniluk, J. C. (1988). Infertility: intrapersonal and interpersonal impact. *Fertility and Sterility, 49*(6), 982-990.

DeLongis, A., Folkman, S., & Lazarus, R. S. (1988). The impact of daily stress on health and mood: psychological and social resources as mediators. *J Pers Soc Psychol, 54*(3), 486-495.

Demura, R., Suzuki, T., Nakamura, S., Komatsu, H., Odagiri, E., & Demura, H. (1989). Effect of immobilization stress on testosterone and inhibin in male rats. *Journal of Andrology, 10*(3), 210-213.

Dhaliwal, L. K., Gupta, K. R., Gopalan, S., & Kulhara, P. (2004). Psychological aspects of infertility due to various causes--prospective study. *Int J Fertil Womens Med, 49*(1), 44-48.

Diamandis, E. P., Arnett, W. P., Foussias, G., Pappas, H., Ghandi, S., Melegos, D. N., et al. (1999). Seminal plasma biochemical markers and their association with semen analysis findings. *Urology, 53*(3), 596-603.

Domar, A. D., Clapp, D., Slawsby, E. A., Dusek, J., Kessel, B., & Freizinger, M. (2000). Impact of group psychological interventions on pregnancy rates in infertile women. *Fertility and Sterility, 73*(4), 805-811.

Domar, A. D., Seibel, M. M., & Benson, H. (1990). The mind/body program for infertility: A new behavioral treatment approach for women with infertility. *Fertility and Sterility, 53*(2), 246-249.

Dronjak, S., & Gavrilovic, L. (2006). Effects of stress on catecholamine stores in central and peripheral tissues of long-term socially isolated rats. *Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas / Sociedade Brasileira de Biofisica ... [et al, 39*(6), 785-790.

Droste, S. K., Chandramohan, Y., Hill, L. E., Linthorst, A. C., & Reul, J. M. (2007). Voluntary exercise impacts on the rat hypothalamic-pituitary-adrenocortical axis mainly at the adrenal level. *Neuroendocrinology, 86*(1), 26-37.

Due, P., Holstein, B., Lund, R., Modvig, J., & Avlund, K. (1999). Social relations: network, support and relational strain. *Soc Sci Med, 48*(5), 661-673.

Dyer, S. J., Abrahams, N., Mokoena, N. E., & van der Spuy, Z. M. (2004). 'You are a man because you have children': experiences, reproductive health knowledge and treatment-seeking behaviour among men suffering from couple infertility in South Africa. *Human Reproduction, 19*(4), 960-967.

Ellison, M. A., & Hall, J. E. (2003). Social stigma and compounded losses: quality-of-life issues for multiple-birth families. *Fertility and Sterility, 80*(2), 405-414.

Engel, G. L. (1977). The need for a new medical model: a challenge for biomedicine. *Science 196*(4286), 129-136.

Ernst, E. (1989). A method for evaluation of epididymal sperm count and motility in the rat. *Scandinavian Journal of Laboratory Animal Science, 16*(2), 67-71.

Faul, F., Erdfelder, E. (1992). GPOWER: A priori, post-hoc, and compromise power analyses for MS-DOS. [Computer Program]. Bonn, FRG: Bonn University; Department of Psychology.

Fenster, L., Katz, D. F., Wyrobek, A. J., Pieper, C., Rempel, D. M., Oman, D., et al. (1997). Effects of psychological stress on human semen quality. *J Androl, 18*(2), 194-202.

Ferin, M. (1999). Clinical review 105: Stress and the reproductive cycle. *J Clin Endocrinol Metab, 84*(6), 1768-1774.

Flaherty, S. P. M., C. D. (Eds). (1995). *The Infertile Male: Advanced Assisted Reproductive Technology.* New York: CSIRO Publishing.

Folkman, S. (1997). Positive psychological states and coping with severe stress. *Soc Sci Med, 45*(8), 1207-1221.

Folkman, S., & Lazarus, R. S. (1988). The relationship between coping and emotion: implications for theory and research. *Soc Sci Med, 26*(3), 309-317.

Gadek-Michalska, A., & Bugajski, J. (2003). Repeated handling, restraint, or chronic crowding impair the hypothalamic-pituitary-adrenocortical response to acute restraint stress. *J Physiol Pharmacol, 54*(3), 449-459.

Gerhard, I., Lenhard, K., Eggert-Kruse, W., & Runnebaum, B. (1992). Clinical data which influence semen parameters in infertile men. *Human Reproduction, 7*(6), 830-837.

Giammanco, M., Tabacchi, G., Giammanco, S., Di Majo, D., & La Guardia, M. (2005). Testosterone and aggressiveness. *Medical science monitor, 11*(4), RA136-145.

Giblin, P. T., Poland, M. L., Moghissi, K. S., Ager, J. W., & Olson, J. M. (1988). Effects of stress and characteristic adaptability on semen quality in healthy men. *Fertility and Sterility, 49*(1), 127-132.

Glass, A. R., Beach, J., & Vigersky, R. A. (1985). Hypogonadotropic hypogonadism in nephrotic rats: Increased sensitivity to negative feedback effects of testosterone. *Metabolism, 34*(6), 574-579.

Goffman, E. (1963). *Stigma: Notes on the Management of Spoiled Identity.* Englewood Cliffs, NJ: Prentice Hall.

Gonzalez, F. J. (2007). Animal Models for Human Risk Assessment: The Peroxisome Proliferator-Activated Receptor Alpha-Humanized Mouse. *Nutrition Reviews, 65*, 2-6.

Gotz, J., Deters, N., Doldissen, A., Bokhari, L., Ke, Y., Wiesner, A., et al. (2007). A Decade of Tau Transgenic Animal Models and Beyond. *Brain Pathology, 17*(1), 91-103.

Greer, S., & Watson, M. (1985). Towards a psychobiological model of cancer: psychological considerations. *Soc Sci Med, 20*(8), 773-777.

Gronli, J., Murison, R., Bjorvatn, B., Sorensen, E., Portas, C. M., & Ursin, R. (2004). Chronic mild stress affects sucrose intake and sleep in rats. *Behavioural Brain Research, 150*(1-2), 139-147.

Gronli, J., Murison, R., Fiske, E., Bjorvatn, B., Sorensen, E., Portas, C. M., et al. (2005). Effects of chronic mild stress on sexual behavior, locomotor activity and consumption of sucrose and saccharine solutions. *Physiology & Behavior, 84*(4), 571-577.

Guerrero, A. P., Hishinuma, E. S., Serrano, A. C., & Ahmed, I. (2003). Use of the mechanistic case diagramming technique to teach the biopsychosocial-cultural formulation to psychiatric clerks. *Academic psychiatry, 27*(2), 88-92.

Haagen, E. C., Tuil, W., Hendriks, J., de Bruijn, R. P., Braat, D. D., & Kremer, J. A. (2003). Current Internet use and preferences of IVF and ICSI patients. *Human Reproduction, 18*(10), 2073-2078.

Hamrick, N., Cohen, S., & Rodriguez, M. S. (2002). Being popular can be healthy or unhealthy: stress, social network diversity, and incidence of upper respiratory infection. *Health Psychology, 21*(3), 294-298.

Handa, R. J., Burgess, L. H., Kerr, J. E., & O'Keefe, J. A. (1994). Gonadal steroid hormone receptors and sex differences in the hypothalamo-pituitary-adrenal axis. *Hormones and Behavior, 28*(4), 464-476.

Harris, R. B. S., Zhou, J., Youngblood, B. D., Rybkin, I. I., Smagin, G. N., & Ryan, D. H. (1998). Effect of repeated stress on body weight and body composition of rats fed low- and high-fat diets. *Am J Physiol Regul Integr Comp Physiol, 275*(6), R1928-1938.

Hernandez, M., Soto-Cid, A., Aranda-Abreu, G., Diaz, R., Rojas, F., Garcia, L., et al. (2007). A study of the prostate, androgens and sexual activity of male rats. *Reproductive Biology and Endocrinology, 5*(1), 11.

Hertz, D. G. (1982). Infertility and the physician--patient relationship: a biopsychosocial challenge. *General hospital psychiatry, 4*(2), 95-101.

Holmer, H. K., Rodman, J. E., Helmreich, D. L., & Parfitt, D. B. (2003). Differential effects of chronic escapable versus inescapable stress on male syrian hamster (Mesocricetus auratus) reproductive behavior. *Hormones and Behavior, 43*(3), 381-387.

Hubert, W., Hellhammer, D. H., & Freischem, C. W. (1985). Psychobiological profiles in infertile men. *J Psychosom Res, 29*(2), 161-165.

Hunt, N., & McHale, S. (2007). Psychosocial Aspects of Andrologic Disease. *Endocrinology & Metabolism Clinics of North America, 36*(2), 521-531.

Ingle, D. J., Kendall, E. C. (1938). Weights of adrenal glands in rats fed different amounts of sodium and potassium. *Am J Physiol Regul Integr Comp Physiol, 122*(2), 585-587.

Jean Kant, G., Eggleston, T., Landman-Roberts, L., Kenion, C. C., Driver, G. C., & Meyerhoff, J. L. (1985). Habituation to repeated stress is stressor specific. *Pharmacology Biochemistry and Behavior, 22*(4), 631-634.

Jedrzejczak, P., Taszarek-Hauke, G., Hauke, J., Pawelczyk, L., & Duleba, A. J. Prediction of spontaneous conception based on semen parameters. *International Journal of Andrology, 0*(0), ???-???

Jeyendran, R. (2000). *Interpretation of Semen Analysis Results: A Practical Guide.* Cambridge: Cambridge University Press.

Johansen, J. A., Jordan, C. L., & Breedlove, S. M. (2004). Steroid hormone masculinization of neural structure in rats: a tale of two nuclei. *Physiology & Behavior, 83*(2), 271-277.

Johnson, E. O., Brady, L., Gold, P. W., & Chrousos, G. P. (1996). Distribution of hippocampal mineralocorticoid and glucocorticoid receptor mRNA in a glucocorticoid resistant nonhuman primate. *Steroids, 61*(2), 69-73.

Johnson, M. H. E., B. J. (Eds). (2000). *Essential Reproduction.* Oxford: Blackwell Science.

Jones, R. E. E. (1997). *Human Reproductive Biology* (2 ed.). London: Academic Press.

Jurema, M. W., Vieira, A. D., Bankowski, B., Petrella, C., Zhao, Y., Wallach, E., et al. (2005). Effect of ejaculatory abstinence period on the pregnancy rate after intrauterine insemination. *Fertility and Sterility, 84*(3), 678-681.

Kedem, P., Mikulincer, M., Nathanson, Y. E., & Bartoov, B. (1990). Psychological aspects of male infertility. *Br J Med Psychol, 63 (Pt 1)*, 73-80.

Kentenich, H., Schmiady, H., Radke, E., Stief, G., & Blankau, A. (1992). The male IVF patient--psychosomatic considerations. *Human Reproduction, 7 Suppl 1*, 13-18.

Klein, F., Lemaire, V., Sandi, C., Vitiello, S., Van der Logt, J., Laurent, P. E., et al. (1992). Prolonged increase of corticosterone secretion by chronic social stress does not necessarily impair immune funnctions. *Life Sciences, 50*(10), 723-731.

Koko, V., Djordjeviae, J., Cvijiae, G., & Davidoviae, V. (2004). Effect of acute heat stress on rat adrenal glands: a morphological and stereological study. *J Exp Biol, 207*(24), 4225-4230.

Koolhaas, J. M., de Boer, S. F., & Buwalda, B. (2006). Stress and Adaptation. Toward Ecologically Relevant Animal Models. *Current Directions in Psychological Science, 15*(3), 109-112.

Kop, W. J. (1999). Chronic and acute psychological risk factors for clinical manifestations of coronary artery disease. *Psychosomatic Medicine, 61*(4), 476-487.

Koyama, S., & Kamimura, S. (1998). Lowered Sperm Motility in Subordinate Social Status of Mice. *Physiology & Behavior, 65*(4-5), 665-669.

Kusnecov, A. W., & Rossi-George, A. (2002). Stressor-induced modulation of immune function: a review of acute, chronic effects in animals. *Acta Neuropsychiatrica, 14*(6), 279-291.

Lampic, C., Svanberg, A. S., Karlstrom, P., & Tyden, T. (2006). Fertility awareness, intentions concerning childbearing, and attitudes towards parenthood among female and male academics. *Human Reproduction, 21*(2), 558-564.

Larson, M. R., Duberstein, P. R., Talbot, N. L., Caldwell, C., & Moynihan, J. A. (2000). A presurgical psychosocial intervention for breast cancer patients. psychological distress and the immune response. *Journal of Psychosomatic Research, 48*(2), 187-194.

Lazarus, R. S., Folkman, S. (1984). *Stress, appraisal, and coping.* New York: Springer Publishing Company.

Lee, T. Y., Sun, G. H., & Chao, S. C. (2001). The effect of an infertility diagnosis on the distress, marital and sexual satisfaction between husbands and wives in Taiwan. *Human Reproduction, 16*(8), 1762-1767.

Leiblum, S. R., Kemmann, E., Colburn, D., Pasquale, S., & DeLisi, A. M. (1987). Unsuccessful in vitro fertilization: a follow-up study. *J In Vitro Fert Embryo Transf, 4*(1), 46-50.

Lemaire, V., Taylor, G. T., & Mormede, P. (1997). Adrenal axis activation by chronic social stress fails to inhibit gonadal function in male rats. *Psychoneuroendocrinology, 22*(8), 563-573.

Lerman, S. A., Miller, G. K., Bohlman, K., Albaladejo, V., Leonard, J. F., Devas, V., et al. (1997). Effects of corticosterone on reproduction in male Sprague-Dawley rats. *Reprod Toxicol, 11*(6), 799-805.

Lewis, M. H., Tanimura, Y., Lee, L. W., & Bodfish, J. W. (2007). Animal models of restricted repetitive behavior in autism. *Behavioural Brain Research, 176*(1), 66-74.

Lin, N. E., W. M. (1989). Life Stress and Health: Stressors and Resources. *American Sociological Review, 54*(3), 382-399.

Lipowski, Z. J. (1977). Psychosomatic medicine in the seventies: an overview. *The American Journal of Psychiatry, 134*(3), 233-244.

Lucia, A., Chicharro, J. L., Perez, M., Serratosa, L., Bandres, F., & Legido, J. C. (1996). Reproductive function in male endurance athletes: sperm analysis and hormonal profile. *J Appl Physiol, 81*(6), 2627-2636.

Ludwig, A. M. (1975). The psychiatrist as physician. *JAMA, 234*(6), 603-604.

Marti, O., Marti, J., & Armario, A. (1994). Effects of chronic stress on food intake in rats: Influence of stressor intensity and duration of daily exposure. *Physiology & Behavior, 55*(4), 747-753.

Matsumoto, A. M., & Bremner, W. J. (1985). Stimulation of sperm production by human chorionic gonadotropin after prolonged gonadotropin suppression in normal men. *Journal of Andrology, 6*(3), 137-143.

Matsumoto, A. M., Karpas, A. E., Paulsen, C. A., & Bremner, W. J. (1983). Reinitiation of sperm production in gonadotropin-suppressed normal men by administration of follicle-stimulating hormone. *The Journal of Clinical Investigation, 72*(3), 1005-1015.

Mazur, A., & Lamb, T. A. (1980). Testosterone, status, and mood in human males. *Hormones and behavior, 14*(3), 236-246.

McBride, C. M., Clipp, E., Peterson, B. L., Lipkus, I. M., & Demark-Wahnefried, W. (2000). Psychological impact of diagnosis and risk reduction among cancer survivors. *Psycho-oncology, 9*(5), 418-427.

McQueeney, D. A., Stanton, A. L., & Sigmon, S. (1997). Efficacy of Emotion-Focused and Problem-Focused Group Therapies for Women with Fertility Problems. *Journal of Behavioral Medicine, 20*(4), 313-331.

Menning, B. E. (1980). The emotional needs of infertile couples. *Fertility and Sterility, 34*(4), 313-319.

Mingoti, G. Z., Pereira, R. N., & Monteiro, C. M. (2003). Fertility of male adult rats submitted to forced swimming stress. *Braz J Med Biol Res, 36*(5), 677-681.

Molina, J. A. (1983). Understanding the biopsychosocial model. *International journal of psychiatry in medicine, 13*(1), 29-36.

Mormede, P., Lemaire, V., Castanon, N., Dulluc, J., Laval, M., & Le Moal, M. (1990). Multiple neuroendocrine responses to chronic social stress: Interaction between individual characteristics and situational factors. *Physiology & Behavior, 47*(6), 1099-1105.

Murphree, L. J., Rundhaugen, L. M., Kelly, K. M., & R. Eugene Ramsay, J. C. C. K. M. K. I. E. L. a. E. P. (2007). Animal Models of Geriatric Epilepsy. In *International Review of Neurobiology* (Vol. Volume 81, pp. 29-40): Academic Press.

Nabeshima, T., Mouri, A., Murai, R., & Noda, Y. (2006). Animal Model of Schizophrenia: Dysfunction of NMDA Receptor-Signaling in Mice following Withdrawal from Repeated Administration of Phencyclidine. *Ann NY Acad Sci, 1086*(1), 160-168.

Nachtigall, R. D., Becker, G., & Wozny, M. (1992). The effects of gender-specific diagnosis on men's and women's response to infertility. *Fertility and Sterility, 57*(1), 113-121.

Newton, C. R., Hearn, M. T., & Yuzpe, A. A. (1990). Psychological assessment and follow-up after in vitro fertilization: assessing the impact of failure. *Fertility and Sterility, 54*(5), 879-886.

Niikura, S., Yokoyama, O., Komatsu, K., Yotsuyanagi, S., Mizuno, T., & Namiki, M. (2002). A Causative Factor of Copulatory Disorder in Rats Following Social Stress. *The Journal of Urology, 168*(2), 843-849.

Nussdorfer, G. G. (1986). Cytophysiology of the adrenal cortex. *International Review of Cytology, 98*, 1-405.

Nussey, S. S., Whitehead, S. A. (Eds.). (2001). *Endocrinology: An Integrated Approach.* Oxford, UK: BIOS Scientific Publishers.

O'Moore, A. M., O'Moore, R. R., Harrison, R. F., Murphy, G., & Carruthers, M. E. (1983). Psychosomatic aspects in idiopathic infertility: effects of treatment with autogenic training. *J Psychosom Res, 27*(2), 145-151.

Orr, T. E., & Mann, D. R. (1992). Role of glucocorticoids in the stress-induced suppression of testicular steroidogenesis in adult male rats. *Hormones and Behavior, 26*(3), 350-363.

Orr, T. E., Taylor, M. F., Bhattacharyya, A. K., Collins, D. C., & Mann, D. R. (1994). Acute immobilization stress disrupts testicular steroidogenesis in adult male rats by inhibiting the activities of 17 alpha-hydroxylase and 17,20-lyase without affecting the binding of LH/hCG receptors. *J Androl, 15*(4), 302-308.

Parshad, R. K. (1993). Effect of restricted feeding of prepubertal and adult male rats on fertility and sex ratio. *Indian Journal of Experimental Biology, 31*(12), 991-992.

Pennebaker, J. W. (1982). *Psychology of Physical Symptoms* New York: Springer-Verlag

Peterson, B. D., Newton, C. R., Rosen, K. H., & Skaggs, G. E. (2006). Gender differences in how men and women who are referred for IVF cope with infertility stress. *Human Reproduction, 21*(9), 2443-2449.

Pettingale, K. W. (1985). Towards a psychobiological model of cancer: biological considerations. *Soc Sci Med, 20*(8), 779-787.

Pook, M., Tuschen-Caffier, B., & Krause, W. (2004). Is infertility a risk factor for impaired male fertility? *Human Reproduction, 19*(4), 954-959.

Porter, D. (1999). *Health, Civilization, and the State: A History of Public Health from Ancient to Modern Times.* Lonodon: NetLibrary.

Portillo, W., & Paredes, R. G. (2003). Sexual and olfactory preference in noncopulating male rats. *Physiology & Behavior, 80*(1), 155-162.

Purvis, K., Magnus, O., Morkas, L., Abyholm, T., & Rui, H. (1986). Ejaculate composition after masturbation and coitus in the human male. *Int J Androl, 9*(6), 401-406.

Quittner, A. L., Glueckauf, R. L., & Jackson, D. N. (1990). Chronic parenting stress: moderating versus mediating effects of social support. *Journal of Personality and Social Psychology, 59*(6), 1266-1278.

Rasmussen, C. A., & Kaufman, P. L. (2005). Primate Glaucoma models. *Journal of glaucoma, 14*(4), 311-314.

Retana-Marquez, S., Bonilla-Jaime, H., Vazquez-Palacios, G., Martinez-Garcia, R., & Velazquez-Moctezuma, J. (2003a). Changes in masculine sexual behavior, corticosterone and testosterone in response to acute and chronic stress in male rats. *Horm Behav, 44*(4), 327-337.

Retana-Marquez, S., Bonilla-Jaime, H., Vazquez-Palacios, G., Martinez-Garcia, R., & Velazquez-Moctezuma, J. (2003b). Changes in masculine sexual behavior, corticosterone and testosterone in response to acute and chronic stress in male rats. *Hormones and Behavior, 44*(4), 327-337.

Retana-Marquez, S., Bonilla-Jaime, H., & Velazquez-Moctezuma, J. (1998). Lack of effect of corticosterone administration on male sexual behavior of rats. *Physiology & Behavior, 63*(3), 367-370.

Retana-Marquez, S., Salazar, E. D., & Velazquez-Moctezuma, J. (1996). Effect of acute and chronic stress on masculine sexual behavior in the rat. *Psychoneuroendocrinology, 21*(1), 39-50.

Retrieved September 7. LH: Lister Hooded (Publication., from Harlan: www.harlaneurope.com

Rowland, R. (1985). The social and psychological consequences of secrecy in artificial insemination by donor (AID) programmes. *Soc Sci Med, 21*(4), 391-396.

Rybkin, I. I., Zhou, Y., Volaufova, J., Smagin, G. N., Ryan, D. H., & Harris, R. B. S. (1997). Effect of restraint stress on food intake and body weight is determined by time of day. *Am J Physiol Regul Integr Comp Physiol, 273*(5), R1612-1622.

Sabelli, H. C., & Carlson-Sabelli, L. (1989). Biological priority and psychological supremacy: a new integrative paradigm derived from process theory. *The American Journal of Psychiatry, 146*(12), 1541-1551.

Sadler, J. Z., & Hulgus, Y. F. (1992). Clinical problem solving and the biopsychosocial model. *The American Journal of Psychiatry, 149*(10), 1315-1323.

Sandlow, J. I. (2000). Shattering the myths about male infertility. Treatment of male factors may be more successful and cost-effective than you think. *Postgrad Med, 107*(2), 235-239, 242, 245.

Sapolsky, R. M. (2005). The Influence of Social Hierarchy on Primate Health. *Science, 308*(5722), 648-652.

Sapolsky, R. M., Krey, L. C., & McEwen, B. S. (1983). The adrenocorticol stress-response in the aged male rat: Impairment of recovery from stress. *Experimental Gerontology, 18*(1), 55-64.

Sarrel, P. M., & DeCherney, A. H. (1985). Psychotherapeutic intervention for treatment of couples with secondary infertility. *Fertil Steril, 43*(6), 897-900.

Schmidt, L. (1996). *Psykosociale konsekvenser af infertilitet og behandling (Psychosocial consequences of infertility and treatment)*. Copenhagen: FADL Book Company.

Schmidt, L., Holstein, B. E., Boivin, J., Tjornhoj-Thomsen, T., Blaabjerg, J., Hald, F., et al. (2003). High ratings of satisfaction with fertility treatment are common: findings from the Copenhagen Multi-centre Psychosocial Infertility (COMPI) Research Programme. *Human Reproduction, 18*(12), 2638-2646.

Schmidt, M. V., Sterlemann, V., Ganea, K., Liebl, C., Alam, S., Harbich, D., et al. (2007). Persistent neuroendocrine and behavioral effects of a novel, etiologically relevant mouse paradigm for chronic social stress during adolescence. *Psychoneuroendocrinology, 32*(5), 417-429.

Schneiders, A., Sonksen, J., & Hodges, J. K. (2004). Penile vibratory stimulation in the marmoset monkey: a practical alternative to electro-ejaculation, yielding ejaculates of enhanced quality. *J Med Primatol, 33*(2), 98-104.

Scott, J. P. (1966). Agonistic behavior of mice and rats: a review. *American Zoologist, 6*(4), 683-701.

Selye, H. (1936). A Syndrome Produced by Diverse Nocuous Agents. *Nature, 138*(4), 32.

Serra, M., Sanna, E., Mostallino, M. C., & Biggio, G. (2007). Social isolation stress and neuroactive steroids. *European Neuropsychopharmacology, 17*(1), 1-11.

Setterlind, S., Larsson, G. (1995). The stress profile: A psychosocial approach to measuring stress. *Stress Medicine, 11*(1), 85-92.

Smals, A. G., Kloppenborg, P. W., van Haelst, U. J., Lequin, R., & Benraad, T. J. (1978). Fertile eunuch syndrome versus classic hypogonadotrophic hypogonadism. *Acta Endocrinologica, 87*(2), 389-399.

Smeenk, J. M., Verhaak, C. M., Eugster, A., van Minnen, A., Zielhuis, G. A., & Braat, D. D. (2001). The effect of anxiety and depression on the outcome of in-vitro fertilization. *Hum Reprod, 16*(7), 1420-1423.

Sofikitis, N., Takahaski, C., Nakamura, I., Kadowaki, H., Okazaki, T., Shimamoto, T., et al. (1990). The role of rat copulatory plug for fertilization. *Acta Europaea fertilitatis, 21*(3), 155-158.

Song, J., Kost, C. K., Jr., & Martin, D. S. (2006). Androgens augment renal vascular responses to ANG II in New Zealand genetically hypertensive rats. *Am J Physiol Regul Integr Comp Physiol, 290*(6), R1608-1615.

Sonksen, J., & Ohl, D. A. (2002). Penile vibratory stimulation and electroejaculation in the treatment of ejaculatory dysfunction. *Int J Androl, 25*(6), 324-332.

Speroff, L. F., M. A. (Eds). (2005). *Clinical Gynecology Endocrinology and Infertility* (7 ed.). Philadelphia: Lippincott Williams and Wilkins.

Stam, R. (2007). PTSD and stress sensitisation: A tale of brain and body Part 2: Animal models. *Neuroscience & Biobehavioral Reviews, 31*(4), 558-584.

Stewart, A. L. W., J. E. (Eds). (1992). *Measuring functioning and well-being: The medical outcomes study approach*. Durham, North Carolina: Duke University Press.

Suls, J., & Rothman, A. (2004). Evolution of the biopsychosocial model: prospects and challenges for health psychology. *Health Psychology, 23*(2), 119-125.

Taylor, G. T., Weiss, J., & Komitowski, D. (1983). Reproductive physiology and penile papillae morphology of rats after sexual experience. *J Endocrinol, 98*(2), 155-163.

Taylor, G. T., Weiss, J., & Rupich, R. (1987). Male rat behavior, endocrinology and reproductive physiology in a mixed-sex, socially stressful colony. *Physiology & Behavior, 39*(4), 429-433.

Taylor, S. (1990). Health psychology. The science and the field. *The American Psychologist, 45*(1), 40-50.

Terje, S., Vivienne, A. R., Heidi, A., Espen Borgå, J., & Mehdi, F. (2005). Rodent Models of Attention-Deficit/Hyperactivity Disorder. *Biological Psychiatry, 57*(11), 1239-1247.

Thoits, P. (1995). Stress, coping, and social support processes: Where are we? What next? *Journal of Health and Social Beavior., 35*, 53-79.

Tilbrook, A. J., Turner, A. I., & Clarke, I. J. (2000). Effects of stress on reproduction in non-rodent mammals: the role of glucocorticoids and sex differences. *Reviews of Reproduction, 5*(2), 105-113.

Toner, J. P., & Adler, N. T. (1986). The pre-ejaculatory behavior of male and female rats affects the number of sperm in the vagina and uterus. *Physiology & Behavior, 36*(2), 363-367.

Toner, J. P., Attas, A. I., & Adler, N. T. (1987). Transcervical sperm transport in the rat: the roles of pre-ejaculatory behavior and copulatory plug fit. *Physiology & Behavior, 39*(3), 371-375.

Tuschen-Caffier, B., Florin, I., Krause, W., & Pook, M. (1999). Cognitive-behavioral therapy for idiopathic infertile couples. *Psychother Psychosom, 68*(1), 15-21.

Vahl, T. P., Ulrich-Lai, Y. M., Ostrander, M. M., Dolgas, C. M., Elfers, E. E., Seeley, R. J., et al. (2005). Comparative analysis of ACTH and corticosterone sampling methods in rats. *American Journal of Physiology, 289*(5), E823-828.

van Balen, F., Trimbos-Kemper, T., & Verdurmen, J. (1996). Perception of diagnosis and openness of patients about infertility. *Patient Education and Counseling, 28*(3), 247-252.

van Balen, F., & Trimbos-Kemper, T. C. (1993). Long-term infertile couples: a study of their well-being. *J Psychosom Obstet Gynaecol, 14 Suppl*, 53-60.

Varady, K. A., & Hellerstein, M. K. (2007). Alternate-day fasting and chronic disease prevention: a review of human and animal trials. *Am J Clin Nutr, 86*(1), 7-13.

Verhaak, C. M., Smeenk, J. M., Eugster, A., van Minnen, A., Kremer, J. A., & Kraaimaat, F. W. (2001). Stress and marital satisfaction among women before and after their first cycle of in vitro fertilization and intracytoplasmic sperm injection. *Fertility and Sterility, 76*(3), 525-531.

Viau, V., Lee, P., Sampson, J., & Wu, J. (2003). A Testicular Influence on Restraint-Induced Activation of Medial Parvocellular Neurons in the Paraventricular Nucleus in the Male Rat. *Endocrinology, 144*(7), 3067-3075.

Voipio, H. N., T. (1998). Improved method for vaginal plug detection in rats. *Scandinavian Journal of Laboratory Animal Science, 25*, 5-9.

Wade, D. T., & Halligan, P. W. (2004). Do biomedical models of illness make for good healthcare systems? *BMJ (Clinical research ed, 329*(7479), 1398-1401.

Walen, H. R., & Lachman, M. E. (2000). Social Support and Strain from Partner, Family, and Friends: Costs and Benefits for Men and Women in Adulthood. *Journal of Social and Personal Relationships, 17*(1), 5-30.

Ware, J., Jr., Snow, K. K., Kosinsky, M., Gandek, B. . (1993). *SF-36 health survey: Manual and interpretation guide.* Boston: The Health Institute, New England Medical Center.

Williams, L., Bischoff, R., & Ludes, J. (1992). A biopsychosocial model for treating infertility. *Contemporary Family Therapy, 14*(4), 309-322.

Willner, P., Moreau, J. L., Nielsen, C. K., Papp, M., & Sluzewska, A. (1996). Decreased Hedonic Responsiveness Following Chronic Mild Stress Is Not Secondary to Loss of Body Weight. *Physiology & Behavior, 60*, 129-134.

Willner, P., Towell, A., Sampson, D., Sophokleous, S., & Muscat, R. (1987). Reduction of sucrose preference by chronic unpredictable mild stress, and its restoration by a tricyclic antidepressant. *Psychopharmacology, 93*(3), 358-364.

Wischmann, T., Stammer, H., Scherg, H., Gerhard, I., & Verres, R. (2001). Psychosocial characteristics of infertile couples: a study by the 'Heidelberg Fertility Consultation Service'. *Human Reproduction, 16*(8), 1753-1761.

Woody, C. J., Weber, S. L., Laubach, H. E., Ingram-Willey, V., Amini-Alashti, P., & Sturbaum, B. A. (1998). The effects of chronic exercise on metabolic and reproductive functions in male rats. *Life Sci, 62*(4), 327-332.

WordNet: An Electronic Lexical Database. http://wordnet.princeton.edu/perl/webwn?s=disease. (Publication. (Retrieved September 7, 2007).

Xiao, K., Kondo, Y., & Sakuma, Y. (2004). Sex-specific effects of gonadal steroids on conspecific odor preference in the rat. *Hormones and Behavior, 46*(3), 356-361.

Yeoman, R. R., Sonksen, J., Gibson, S. V., Rizk, B. M., & Abee, C. R. (1998). Penile vibratory stimulation yields increased spermatozoa and accessory gland production compared with rectal electroejaculation in a neurologically intact primate (Saimiri boliviensis). *Human Reproduction, 13*(9), 2527-2531.