Assessing a chemosterilant for fertility control of rats
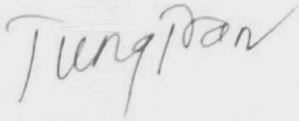
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A thesis submitted for the degree of Doctor of Philosophy
of The Australian National University

Declaration

The research presented in this thesis is my own original and independent work, except where due reference is given in the text. No part of this research has been submitted for any previous degree.

Tran T. Tung

Statement of contribution to international conferences

This thesis is submitted as a series of discrete papers that will be submitted to international peer-reviewed journals. In all papers I was responsible for experimental design, data collection, analysis, interpretation and writing. The main findings in this thesis were successfully presented as oral presentations at three international conferences:

Tung, T. T., Cooper, D. P. and Hinds, L. A. (2010). Effects of different seed extracts and nicotine on the reproductive tracts of laboratory rats (*Rattus norvegicus*). Spoken presentation at the 4th International Conference for Rodent Biology and Management (4th ICRBM), Bloemfontein, South Africa, 12-16 April 2010.

Tung, T. T. and Hinds, L. A. (2010). Laboratory evaluation of alternative baits to impove bait acceptance by ricefield rats (*Rattus argentiventer*). Spoken presentation at the 4th International Conference for Rodent Biology and Management (4th ICRBM), Bloemfontein, South Africa, 12-16 April 2010.

Tung, T. T. and Hinds, L. A. (2011). Effect of multiple periods of treatment with 4-vinylcyclohexene diepoxide (VCD) on the fertility of female rats (*Rattus norvegicus)*. Spoken presentation at the 15th Australasian Vertebrate Pest Conference (15th AVPC), Sydney, 20-23 June 2011.

Tung, T. T. and Hinds, L. A. (2011). Evaluation of bait uptake by ricefield rats (*Rattus argentiventer*) using Rhodamine B as a bait marker under enclosure conditions. Spoken presentation at the 8th European Vertebrate Pest Management Conference (8th EVPMC), Berlin, 26-30 September 2011.

Acknowledgements

Abstract

Fertility control of rodents could be used as an additional approach for the sustainable management of pest populations in agricultural production systems. One potential technique is the use of chemosterilants which induce long-term infertility in female animals. The aim of my research was to assess the effects of an industrial chemical, 4-vinylcyclohexene diepoxide (VCD), on reproduction of female and male laboratory rats (*Rattus norvegicus*). In addition, the uptake of bait types and basic formulations with potential to deliver a fertility control agent to the ricefield rat, *Rattus argentiventer*, a target species for fertility control in Southeast Asia, was undertaken.

In female rats, the effects on ovarian follicle populations were assessed after a period of oral treatment (500 mg VCD/kg/day for 10 days), which was then repeated either once or twice at 14 and 21 day intervals. The effect of three periods of VCD treatment on fertility was examined by mating with fertile, untreated males for 4 breeding cycles. In male rats, the effects of a single 15-day period of oral administration (500 mg VCD/kg/day) on reproductive tissues of juvenile and adult rats were assessed at different times after treatment (days 16 to 90). The effect of VCD treatment on the fertility of adult males was determined by mating with fertile, untreated females for 5 breeding cycles. In the bait-uptake study, laboratory evaluations of bait acceptance by ricefield rats were conducted using different physical forms of bait, vegetable oils, food additives and types of cereal. Based on these laboratory results, the most accepted bait formula plus Rhodamine B (RB) as a bait marker was used under field enclosure conditions to determine the proportion of animals consuming bait and the rate of bait consumption per individual.

In females, oral administration of VCD (500 mg/kg) for all treatment schemes induced significant depletion of primordial and primary follicles in the ovaries. Three periods of VCD treatment significantly enhanced ovarian follicle depletion and induced delayed but irreversible infertility. In male rats, a single oral VCD treatment for 15 days did not induce any effects on either reproductive tissues or fertility suggesting an absence of effect on the reproductive status of male rats.

Under laboratory conditions, bait in kibble form, containing cereal-base (10% broken rice, 30% wheat and 30% rice flours), 5% coconut oil, 20% intralipid (a fatty emulsion), and 5% sugar was shown to be most preferred by ricefield rats. Under field enclosure conditions, this bait formula was highly accepted with approximately 90% of ricefield rats consuming bait, and at a high rate per individual (approximately 9.5% body weight).

This research has demonstrated that VCD has potential as a candidate for fertility control of rats, and that a cereal-based bait formula derived in this study could potentially be used as a carrier for oral delivery of fertility control agents in ricefield rats. In the future, fertility control could be used as an additional integrated tool for improved management of pest rodents and thus could significantly contribute to food security and poverty alleviation programs in developing countries.

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CHAPTER 1

Introduction

1.1 Rodent problems

Rodents have become one of the major constraints to agricultural production in many countries around the world (Myllymäki, 1977; Leirs et al., 1996; Parshad and Ahmad, 1996; Aplin et al., 2003; Singleton, 2003; Singleton et al., 2005a; Singleton et al., 2010a; Sudarmaji et al., 2010) as they cause severe damage both before and after harvest. Rodents can be a major constraint in attempts to alleviate poverty in Africa (Mwanjabe et al., 2002) and Asia (Singleton, 2003).

In Asia, it is estimated that rodents commonly cause annual pre-harvest losses equivalent to 5% to 10% of total rice production. Over the last few decades, the damage caused by rodents has increased significantly, particularly in locations where cropping intensity has increased, from one to two or three crops per year (Singleton, 2003; Huan et al., 2010; Sudarmaji et al., 2010). Chronic yield losses of 20-50% per annum, or even losses up to 100% in certain seasons are not unusual (Aplin et al., 2003; Singleton, 2003; Singleton et al., 2003a; Baco et al., 2010; Singleton et al., 2010a; Sudarmaji et al., 2010). It is estimated that if rice production losses caused by rodents in Asia could be reduced by 5%, or approximately 30 million tons, there would be enough rice to feed 180 million people for one year (Aplin et al., 2003; Singleton, 2003).

Apart from crop damage, pest rodents can also cause damage to native plants, animals (Ohashi and Oldenburg, 1992), household appliances and infrastructure (Caughley et al., 1994). Furthermore, rodents can transmit diseases to humans and livestock - this can occur directly via faeces or urine, or indirectly via vectors such as fleas and ticks (Begon, 2003; Singleton et al., 2003a; Bonnefoy et al., 2008; Meerburg et al., 2009).

A range of factors including climate change and cropping intensity are attributed to outbreaks of rodent populations. The increased frequency of major catastrophic weather events, such as drought and flooding and the increase in cropping intensity, especially additional crops, provide favourable conditions for pests, particularly rodents, and diseases (Baco et al., 2010; Singleton et al., 2010b; Sudarmaji et al., 2010).

In Southeast Asia, widespread rice crop losses caused by rodents can lead to food shortages and threaten national food security. Reduced rice crop yields lead to reduction in the cash income of farmers and alter their

expenditure and investment decisions. Poverty can increase as a result of rodent outbreaks (Singleton, 2003; Singleton et al., 2010a; Sudarmaji et al., 2010).

Given the major impacts of rodents on pre- and post-harvest crop production, it is clear that rodent management must play a significant role in food security and poverty alleviation programs (Singleton, 2003).

1.2 Current methods of rodent management

A range of control methods have been developed in an attempt to minimize the damage caused by pest rodents. Lethal methods (e.g. poisons, shooting, or trapping) are widely used throughout the world, especially in developing countries, as they are considered to be effective, practical and economical (Parshad and Ahmad, 1996; Wood and Fee, 2003). By contrast, non-lethal methods including biological control, habitat manipulation and exclusion, which may provide long-term effects, are rarely adopted (Wilson et al., 1992; Parshad and Ahmad, 1996; Parshad, 1999). One of the most common control measures employed by rice farmers in developing countries is the use of rodenticides (Aplin et al., 2003; Singleton et al., 2003b; Wood and Fee, 2003; Singleton et al., 2007). Unfortunately, indiscriminate use of these poisons has caused serious problems. Residues of rodenticides induce harmful effects on non-target species (Hegdal and Colvin, 1988; Newton et al., 1997; Madden, 2002; Riley et al., 2007), and their continued inappropriate application may lead to increased chemical resistance (Muller and Pelz, 1996; Rost et al., 2004; Lasseur et al., 2005; Pelz et al., 2005; Heiberg et al., 2006), ultimately resulting in major pest outbreaks (Singleton et al., 1999; Pelz et al., 2005; Ngowo et al., 2006). Furthermore, rat meat is a common food for people in some areas of Asia and Africa, and consumers can suffer sub-lethal poisoning from the chemicals used in baits (Khiem et al., 2003; Fisher et al., 2006).

Ecologically-based rodent management (EBRM) has recently been developed and practiced as a sustainable and environmentally-friendly control method (Leung et al., 1999; Singleton et al., 1999; Singleton et al., 2004; Brown et al., 2006; Singleton et al., 2007; Jacob et al., 2010). The aim of EBRM is to control populations of target species based on knowledge of farming systems and ecological factors that contribute to the suppression of populations of pest

rodents (Stenseth, 1977; Stenseth, 1981; Ramsey and Wilson, 2000). EBRM is likely to be successful where a combination of several control techniques is applied (Singleton, 1997). Techniques include the use of trap barrier systems (TBS) to catch the rats (Singleton *et al.*, 2005b; Brown *et al.*, 2006), habitat manipulation (Leung *et al.*, 1999; Brown *et al.*, 2001; Jacob, 2008) and synchronising planting to maximise the fallow period between cropping seasons in order to minimise food sources, shelters and nest sites for target pests (Lam, 1983; Singleton, 1997; Tristiani and Murakami, 1998; Leung *et al.*, 1999; Leung and Sudarmaji, 1999). EBRM not only provides economic benefits to farmers through minimising yield losses but also brings environmental benefits through the use of more environmentally benign techniques replacing inappropriate or excessive use of chemicals (Singleton *et al.*, 2003b).

Public awareness of the environmental, moral and animal welfare issues associated with conventional lethal control methods has increased in recent times. In response to these issues, vertebrate pest control has turned some of its focus to the development of non-lethal alternatives, such as fertility control (Bomford, 1990). Fertility control has been considered a potential additional technique that could contribute significantly to benign strategies for vertebrate pest management (Bomford and O'Brien, 1992; Caughley *et al.*, 1992; Tuyttens and Macdonald, 1998; Hinds *et al.*, 2003; Kirkpatrick *et al.*, 2005; Jacob *et al.*, 2008). Research and development of fertility control for vertebrate pests relies largely on a detailed knowledge of the reproductive biology of the target species.

1.3 Reproductive biology of rodents

1.3.1 Reproductive system of females

The reproductive system of the female rat consists of two ovaries, two oviducts, a uterus, cervix and vagina. The ovaries contain a number of cell types and structures (Fig. 1.1). The ovary is joined to the uterus via the oviduct, which is also known as the Fallopian tube. In rats, the uterus comprises 2 uterine horns (Rowett, 1974).

1.3.2 Ovarian structure and follicular development

In the ovary, the follicle is the basic functional unit, consisting of an oocyte surrounded by granulosa cells and thecal cells. The development and maturation of the oocyte occurs within the ovarian follicle which goes through various distinct stages of development from a primordial to primary, secondary, antral and finally the pre-ovulatory or Graffian follicle (Fig. 1.1). In primordial follicles, the oocytes are arrested in meiosis and do not resume meiotic division until just prior to ovulation (Hirshfield, 1991b). Females are born with a finite number of primordial follicles that cannot regenerate after birth (Hirshfield, 1991b). The total number of ovarian primordial follicles at birth is large, but during the reproductive period, only a small proportion (less than 1%) of the total pool of primordial follicles will successfully develop through each stage of development to ovulation. Instead, most follicles (more than 99%) undergo a natural degenerative process called atresia (Richards, 1980; Hirshfield, 1989, 1991b). The process of atresia is known to occur via physiological cell death, or apoptosis (Hughes and Gorospe, 1991; Tilly et al., 1991).

Continuous recruitment of small follicles into the growing pool is required to ensure that mature follicles, which are capable of releasing ova for fertilization, are produced in every cycle (Hirshfield, 1991b, a). During the female reproductive cycle, the series of changes which take place in the ovaries, oviducts and uteri are regulated by the reproductive hormones produced in the Hypothalamic–Pituitary–Gonadal (HPG) axis (Fig. 1.2). The hypothalamus produces gonadotrophin-releasing hormone (GnRH); the pituitary gland produces the gonadotrophic hormones (luteinizing hormone, LH and follicle-stimulating hormone, FSH); and the ovaries produce 2 major steroid hormones, oestrogen and progesterone (Fig. 1.2). FSH stimulates the development of ovarian follicles which begin to produce oestrogen which in turn stimulates further release of FSH. Oestrogen feedback from the Graafian follicle to the pituitary gland also induces the pre-ovulatory surge of LH which stimulates ovulation. After ovulation the granulosa cells of the follicles cease to produce oestrogen and become luteinised to form corpora lutea (CL) which produce progesterone during the oestrous cycle and throughout gestation. Progesterone has direct stimulatory effects on the endometrium of the uterus, while at the pituitary level it inhibits the production of FSH, thus inhibiting follicular development, but stimulates LH release which in turn stimulates progesterone

production by the CL. If pregnancy does not occur the CL degenerate rapidly, and FSH release resumes, stimulating the recruitment and development of another pool of follicles (Rowett, 1974; Ross *et al.*, 2003).

Primordial follicle

Primary follicle

Secondary follicle

Antral follicle

Graafian follicle

Corpus luteum (CL)/
Corpus albicans (CA)
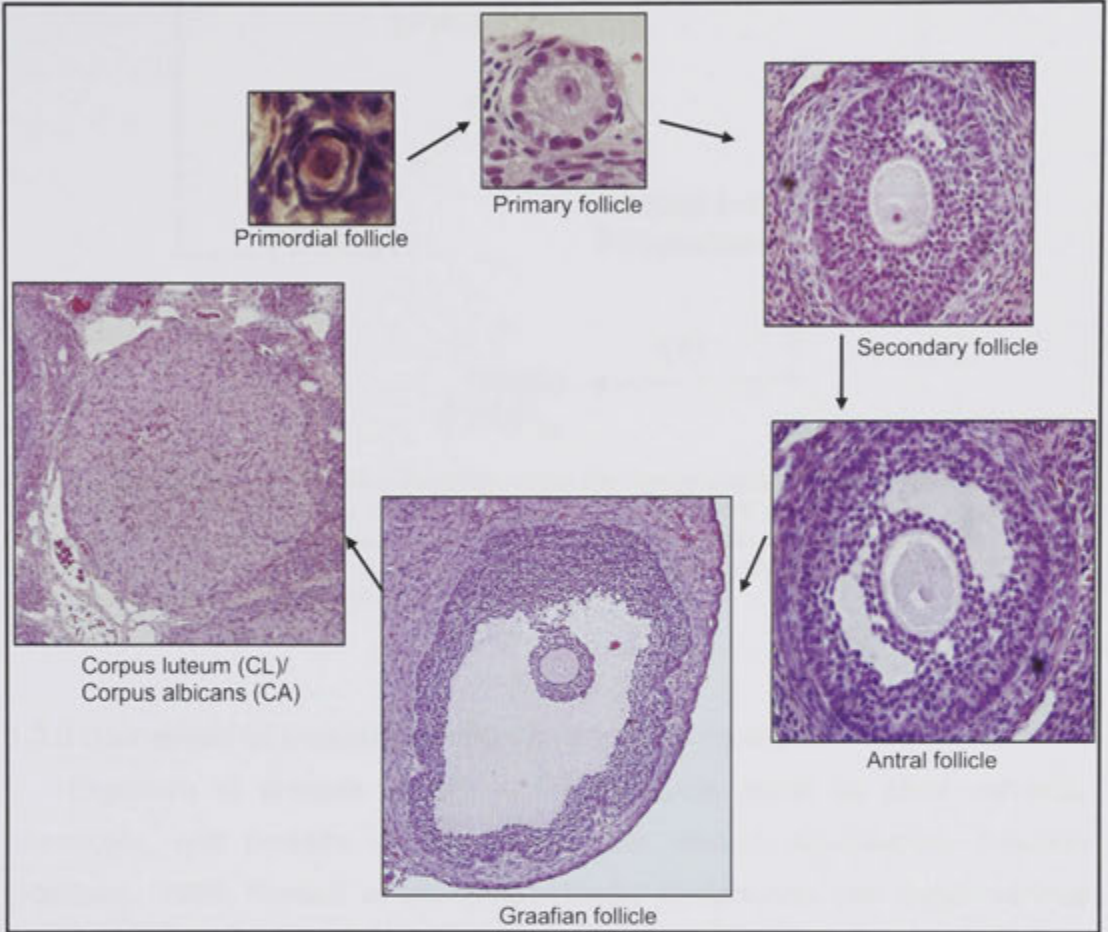
Figure 1.1 Development of ovarian follicles. Primordial follicles are activated to grow and develop through the primary, secondary, antral and Graafian stages of follicular development until ovulation. Most follicles degenerate and become atretic. An ovulated follicle becomes a corpus luteum (CL), which supports pregnancy if fertilization occurs. (Different images are not to the same scale.)

(+) Hypothalamus (-)

| GnRH

(+) Anterior Pituitary (-)

(+)/(-) | FSH/LH

Ovary

Follicle   Corpus Luteum
↑Estrogen   Progesterone ↑

(+)
Uterus
↑ PGF$_{2\alpha}$
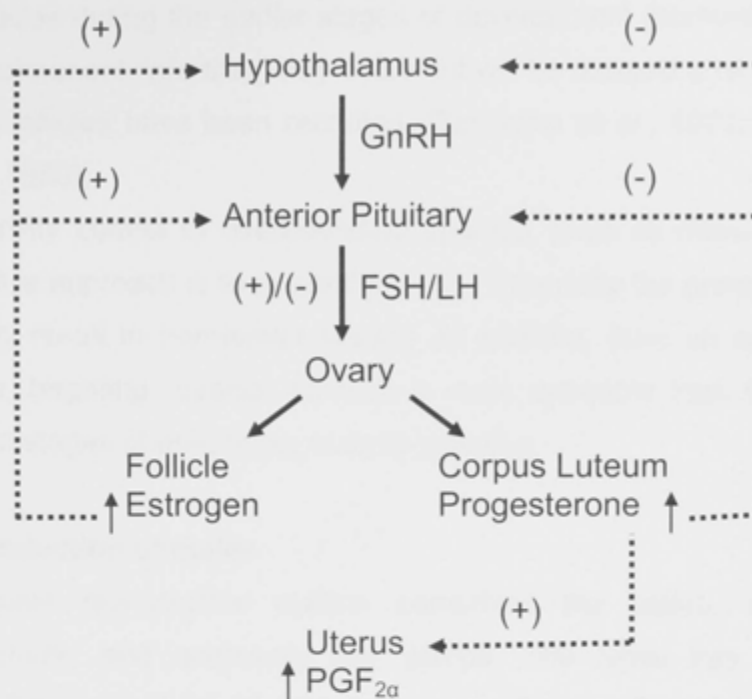
Figure 1.2 Regulation of hormone feedback within the Hypothalamic-Pituitary-Gonadal axis: the oestrous cycle and pregnancy of female mammals is regulated by the various reproductive hormones: Gonadotrophin Releasing Hormone (GnRH), Luteinising Hormone (LH), Follicle Stimulating Hormone (FSH), oestrogen, progesterone and prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$). (+) hormone has positive effect; (-) hormone has negative effect.

1.3.3 Disruption of ovarian function and fertility control

Exposure of animals to different compounds (such as plant extracts, chemicals, self proteins or hormones) can disrupt reproductive function (Mattison, 1985; Burdorf *et al.*, 2006). These compounds can target various sites in the reproductive axis, including the hypothalamus, pituitary and ovary (Mattison and Schulman, 1980; Thomas, 1993) resulting in a disruption of ovarian function that may lead to a reduction in fertility.

The developmental stage at which the follicles are destroyed by exotic compounds will determine the impact of those compounds on reproduction (Hoyer and Sipes, 1996). Compounds that target follicles at later stages of development (secondary or larger follicles) have a temporary effect on reproductive function as these developing follicles can be replaced by recruitment from the pool of primordial follicles in the ovary. Therefore, infertility caused by these compounds may occur soon after the animals are exposed to the compounds but the effect is reversible after treatment is withdrawn (Generoso *et al.*, 1971; Mattison and Schulman, 1980; Jarrell *et al.*, 1991; Davis *et al.*, 1994). In contrast, if the compounds damage the oocytes or granulosa

cells of follicles during the earlier stages of development (primordial or primary follicles), permanent infertility may occur but will be delayed until after all of the later stage follicles have been recruited (Generoso et al., 1971; Mattison and Schulman, 1980).

For fertility control of overabundant species, such as many rodents, the most effective approach is to target the ovary, especially the primordial follicles, as this may result in permanant sterility. In addition, from an animal welfare perspective, targeting ovarian function is more desirable than targeting post implantation stages of pregnancy or early lactation.

1.3.4 Reproduction of males

The male reproductive system comprises the testes, epididymides, excurrent ducts, and accessory sex glands. The testis has two primary functions: the production of sperm, and synthesis of androgens, mainly testosterone, which are essential for spermatogenesis (Ross et al., 2003). Spermatogenesis occurs continuously throughout adult life within the seminiferous tubules of the testis (Figs. 1.3, 1.4) in continuous breeders. During spermatogenesis, in the germinal epithelium of the seminiferous tubules, the stem cells or spermatogonia undergo mitotic division and give rise to spermatocytes which undergo a reduction division to produce secondary spermatocytes. The latter then divide again to form spermatids which eventually develop into mature spermatozoa. Spermatozoa pass into the epididymis and then leave the epididymis through the ductus deferens. Associated with each ductus deferens is a seminal vesicle which produces a viscous secretion which forms much of the volume of the semen (Rowett, 1974).

The spermatogenic cycle can be affected by various factors, such as dietary deficiencies, general or local infections, elevated testicular temperature, steroid and pituitary hormones, radiation and toxic agents such as mutagens, drugs, antimetabolites and pesticides. Exposure to these factors may result in a reduction in fertility (Rowett, 1974; Ross et al., 2003). However, unlike oocytes in females, sperm in male mammals are produced throughout adult life in continuous breeders. Fertility control agents for males are likely to have only temporary effects unless spermatogenesis can be directly and permanently inhibited.
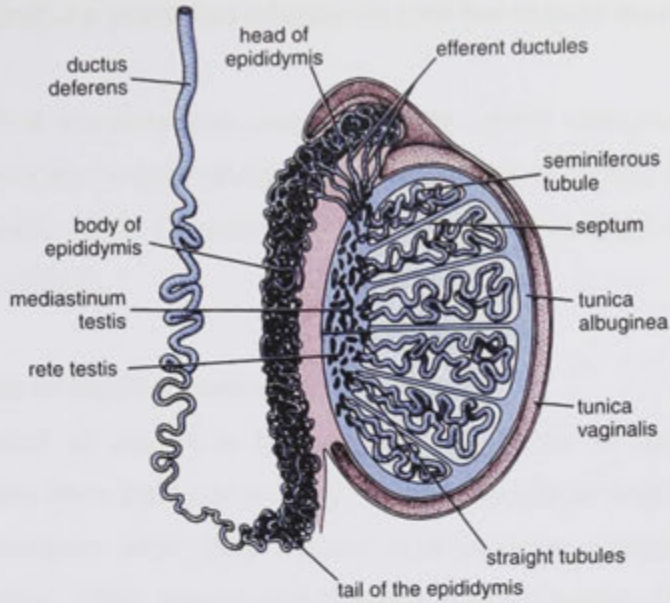
Figure 1.3 Schematic diagram of the testis and epididymis. Septa divide the testis into lobules containing seminiferous tubules in which spermatozoa are produced. Sperm from the seminiferous tubules enter into the rete testis then to the epididymis through the efferent ducts. Sperm mature as they pass through the head (caput) and body (corpus) of the epididymis and are stored in the tail (cauda) of the epididymis before ejaculation through the ductus deferens. Source: Ross *et al.* (2003).
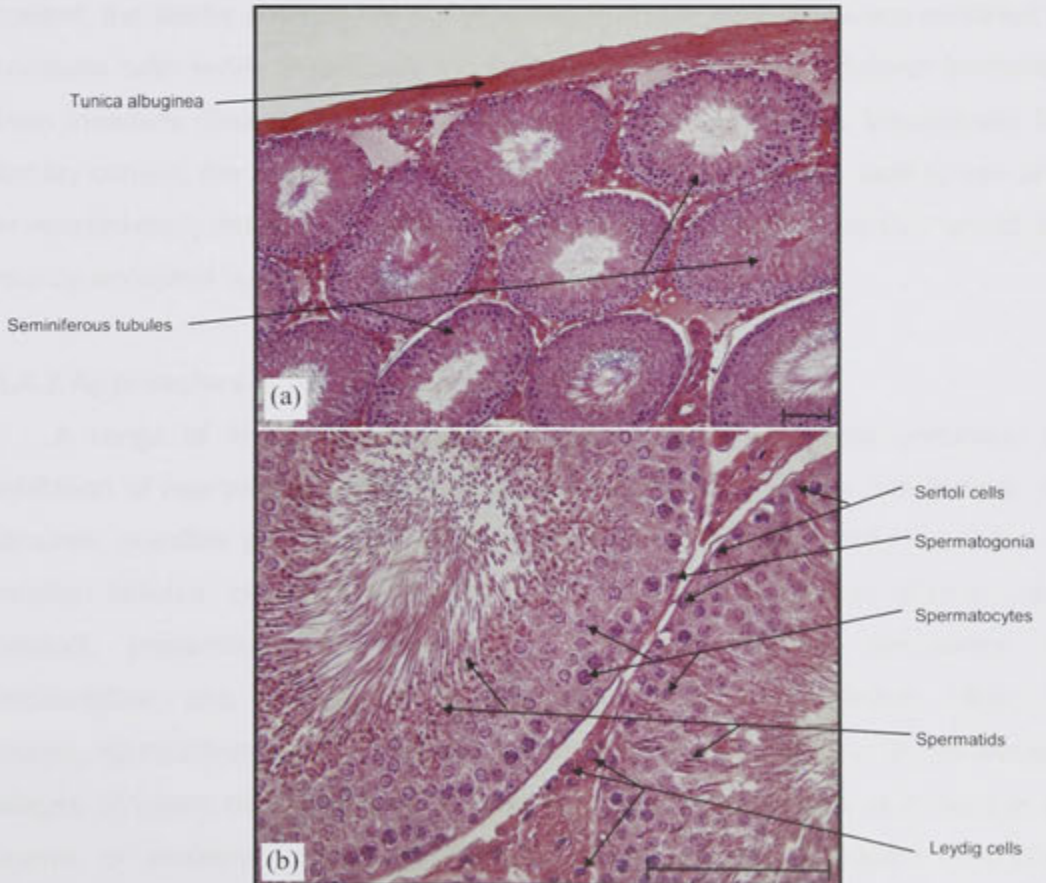
Figure 1.4 Cross-section of testis from a healthy rat showing various seminiferous tubules. Stain: haematoxylin and eosin; scale bar = 100 um; (a) x100 and (b) x400.

1.4 Fertility control – a potential additional tool for rodent management

Fertility control involves the use of agents which disrupt reproduction, leading to a temporary or permanent reduction or loss of fertility (Balser, 1964; Marsh and Howard, 1973; Chambers et al., 1999a; Chambers et al., 1999b; Fagerstone et al., 2002).

1.4.1 Advantages of fertility control

Fertility control of wildlife is favoured for a number of reasons. Firstly, preventing animals from being born may be more practical and humane than reducing their numbers after they mature and become established in their environment (Balser, 1964; Marsh and Howard, 1973; Oogjes, 1997; Jacob et al., 2008). Secondly, controlling populations of rodents by increasing mortality often results in a compensatory response due to an increased rate of reproduction or survival or both (Balser, 1964) as well as increasing immigration into treated areas (Sullivan et al., 2001; Brown and Tuan, 2005). With fertility control, the sterile animals are not removed from the population and continue to compete with fertile individuals for food and mates, and to defend territories from invaders (Balser, 1964; Tuyttens and Macdonald, 1998). Importantly, for fertility control, the agents employed aim to be species specific, safe to use and environmentally-friendly. If these aims are achieved, fertility control would be readily accepted by the public.

1.4.2 Approaches to fertility control

A range of fertility control options are available to enable disruption or inhibition of reproduction in both sexes based on their reproductive biology. In females, possible targets include: prevention of development and maturation of ovarian follicles, inhibition of ovulation, blockage of the passage of ova in the oviduct, prevention of fertilization of ovulated oocytes, prevention of implantation, and interference with gestation or lactation (Balser, 1964). In males, approaches include inhibition of spermatogenesis at one of the various stages of germ cell development, interference with transport and storage of sperm, or inhibition of the function of the male accessory organs. For both sexes, the potential targets can be suppression of or interference with the synthesis and secretion of steroids by targeting the hormones of the

hypothalamus or pituitary (Balser, 1964). For fertility control of pest species in which mating systems are promiscuous, females or both sexes need to be targeted rather than only targeting males in order to obtain the desired effect at the population level (Bomford and O'Brien, 1992; Caughley et al., 1992; Barlow et al., 1997; Fagerstone et al., 2002; Kirkpatrick and Turner, 2008).

Many approaches have been reported to suppress reproduction, for example, the use of steroids (Balser, 1964; Plotka and Seal, 1989; Gao and Short, 1994; White et al., 1994; Croxatto, 2000; Zhang et al., 2004; Coulson et al., 2008); agonists and antagonists of gonadotrophin releasing hormone (GnRH) (Conn and Crowley, 1991; Herbert et al., 2006); immunocontraceptive vaccines (Miller et al., 1998; Chambers et al., 1999a; Redwood et al., 2007; Kirkpatrick and Turner, 2008); natural plant extracts (Farnsworth et al., 1975a, b; Unny et al., 2003; Quresh et al., 2006); and chemicals (Marsh, 1988b; Mayer et al., 2002; Mayer et al., 2004; Nash et al., 2007). These agents may exert their inhibitory effects directly on a specific process within the reproductive system (e.g. development of oocytes and ovarian follicles, implantation), or may indirectly affect a number of sites (e.g. oviduct, uterus, vagina) within the reproductive tract due to changes in hormone synthesis and release by the hypothalamic-pituitary-gonadal (HPG) axis (Fig. 1.2). The effects on reproductive performance may be reversible or permanent over time depending on the mechanism of action.

Because mammalian females are born with a finite number of primordial follicles their reproductive success is dependent on the pool of primordial follicles in the ovary (Hirshfield, 1991b). If the fertility control agents target and destroy the primordial follicle pool, then these follicles will not be able to regenerate and therefore such control agents could be excellent candidates for fertility control as they potentially induce permanent infertility (Hoyer and Sipes, 1996).

1.4.3 Attributes of an ideal fertility control agent

For fertility control of overabundant species, especially pest rodents, an ideal control agent should i) rapidly and irreversibly reduce fertility of a high proportion of females or of both sexes; ii) be deliverable orally to the target species; iii) be safe or neutral to the environment; iv) be stable and effective at low doses for practical field application; and v) be palatable to target species

without side effects on non-reproductive organs. An ideal fertility control agent should be relatively tasteless and odourless, or capable of being masked so as not to cause bait aversion by the target species. The agents should be attractive in the presence of other food sources to ensure a high proportion of the population will consume sufficient quantities of bait to achieve the required effect (Balser, 1964; Marsh and Howard, 1973; Fagerstone *et al.*, 2002).

1.5 Rodents - the ideal species for fertility control

Population dynamics of a species depend on a number of parameters i.e. population numbers, sex ratios, age structure, reproductive and mortality rates, immigration and emigration. These population parameters have major effects on the success of fertility control programs (Curtis *et al.*, 1993; Nielsen *et al.*, 1997). Dolbeer (1998) used population models to compare the relative efficiency of wildlife population management between fertility control and lethal methods. The modelling found fertility control is the most effective method of managing populations of small species with high reproductive rates and females that start reproducing at an early age. Females of these species tend to have a large number of litters, large litter sizes, and low survival rates. Other models by Caughley *et al.* (1992) suggest that the effectiveness of fertility control is critically dependent on the social behaviour of the target species and the mode of diffusion of the control agent. These models demonstrated that in the majority of cases a sterilising agent reduces the productivity of the target populations. Stenseth (1981) and Hone (1992) reported that reducing fertility may be the optimal control method for *r*-selected species, i.e. species with higher fecundity and lower survival rates, but not for *K*-selected species, i.e. species with lower fecundity and higher survival rates.

From the perspective of population dynamics, rodents are likely to be an ideal species for fertility control, as most rodents are *r*-strategist species, with high fecundity, early maturity onset, and short generation time. Most pest rodents have high reproductive potential as they typically have short gestation periods, large litter sizes and females can fall pregnant within a few days of parturition (Aplin *et al.*, 2003). In addition, many rodent species reach sexual maturity at a very early age, which enables populations to increase rapidly when food is available (Aplin *et al.*, 2003). In such a highly fecund group, controlling

the population by poisons or other lethal methods is not effective unless subsequent population recovery and immigration from surrounding areas is prevented or slowed down. Most wild rodents have relatively short life spans (usually <6 months), therefore potentially improved survival of sterilised individuals due to fertility control may not be critical for the management outcome (Jacob et al., 2008).

Because rodents have high reproductive rates, a higher proportion of individuals need to be sterilised when compared to a K- strategist species to achieve the required effect at the population level (Jacob et al., 2008). For example, it is necessary to sterilise 50–80% of females in eruptive house mouse populations (Chambers et al., 1997; Davis et al., 2003) and more than 50% in non-eruptive ricefield rats (Jacob et al., 2004a). However, the proportion of infertile females could be as low as 33% if fertility control can be effectively applied to each new cohort during a breeding season and if the reproductive output of the fertile females in a population is not affected by the presence of infertile females (Davis et al., 2003). Hone (1992) suggested that the best strategic timing to apply fertility control is when populations are already declining in abundance. Therefore, if the population of the target pest is large, the population should initially be reduced by other methods, such as culling, and the fertility control then applied to maintain the reduction of population numbers (Barlow et al., 1997).

Fertility control is a potential method for long-term management of rodent populations and should be considered as an additional tool in an integrated pest management strategy (Parshad, 2006; Jacob et al., 2008). Currently there is no single successful method of fertility control available for the management of rodent populations. However, over the last few decades there have been advances in our understanding of the mechanisms regulating reproductive success and in the development of promising antifertility agents, such as immunocontraceptives and chemosterilants for use in fertility control of pest rodents (Chambers et al., 1999a; Parshad, 2006; Jacob et al., 2008).

Of the pest rodent species, ricefield rats (Rattus argentiventer), one of the most destructive pre- and post harvest pests of rice production systems in Southeast Asia, is considered a potential target for fertility control. Ricefield rats have short breeding seasons (around 8 weeks per cropping season) and their breeding is closely linked to the growing stages of rice crops (from one week

prior to maximum tillering through to harvest) (Lam, 1983; Leung *et al.*, 1999). Survival of ricefield rats during the fallow period between crop seasons is relatively low, leading to high population turnovers (Leung *et al.*, 1999) and therefore, fertility control is likely to be effective in population management (Hone, 1992). The application of fertility control before the onset of breeding would reduce the number of the first or second litters produced in each cropping season, leading to reduction in the overall recruitment within rat populations, and a consequent reduction in the damage to rice crops (Jacob *et al.*, 2008). Female ricefield rats are territorial (Tristiani *et al.*, 2000; Brown *et al.*, 2001; Jacob *et al.*, 2004b), therefore infertile animals may reduce invasion by fertile immigrants. Moreover, as ricefield rats have a short lifespan, carry-over of sterilized individuals in the population due to fertility control may not be critical (Jacob *et al.*, 2008).

1.6 Factors affecting bait uptake by rodents

The major challenge in fertility control is delivery of the control agents in adequate amounts to a high proportion of target populations (Bomford and O'Brien, 1992). In rodent population management, oral delivery of control agents (e.g. poisons, sterilants) using baits is the most practical option under field conditions (Tuyttens and Macdonald, 1998). The success of baiting programs largely depends on bait preference by the target species, particularly when there may be abundant alternative foods in their natural habitats (Majumder *et al.*, 1969; Shafi *et al.*, 1990; Berdoy and Macdonald, 1991; Shafi *et al.*, 1992a; Ahmad *et al.*, 1994; Amjad *et al.*, 2000; Harjit, 2004).

Various studies indicate that bait acceptance by rodents is largely influenced by bait materials, including types of cereal, vegetable oils, bait flavour, sugar and salt, and other food additives, such as chicken eggs and fishmeal (Mason *et al.*, 1991; Flynn *et al.*, 1993; Malhi and Kaur, 1995; Sivaprakasam and Durairaj, 1995; Yamaguchi, 1995; Pervez *et al.*, 1999; Khan *et al.*, 2000; Kaur and Parshad, 2002; Jacob *et al.*, 2003b; Leung *et al.*, 2007). Bait uptake by rodents is also known to be affected by the texture and size of baits and varies between species. Studies of short tailed rats (*Nesokia indica*) found significant variations in food consumption due to the texture and size of the bait used (Shafi *et al.*, 1988). Murids in general prefer powdered and small

soft food over large hard grains (Parshad and Jindal, 1991). Baits with pelleted texture were more acceptable to Sprague Dawley rats compared to bait in a powdered form (Naim *et al.*, 1986). Rats preferred semolina of wheat, rice and millet grains over whole grains (Kaur and Parshad, 2002). In contrast, baits with open texture, such as agglomerated granules may be more attractive to rodents than solid blocks or pellets since it is easier for the rats to detach small portions from the open texture bait than from solid blocks or pellets (Baker, 1998).

In general, because the feeding behaviours of rodents are very complex, no universally accepted bait has been developed. Understanding the factors that influence the bait preference of target species will help improve bait acceptance by rodents and increase the success of baiting programs.

1.7 Aims of present study

For sustainable management of pest rodents, it is essential to develop additional, alternative control techniques that are environmentally-friendly, species specific, cost-effective and more humane. This thesis aims to examine the effects of an industrial chemical (4-vinylcyclohexene diepoxide – VCD) on reproduction of laboratory rats (*Rattus norvegicus*) in an attempt to contribute to the development of fertility control agents as an additional tool for integrated management of pest rodents. VCD was selected for this study as it is known to induce significant depletion of non-regenerating primordial and primary follicles in the ovaries of female rats and mice, thus potentially providing a long-term effect on fertility (Flaws *et al.*, 1994; Springer *et al.*, 1996a; Borman *et al.*, 1999; Devine *et al.*, 2001; Mayer *et al.*, 2002; Devine *et al.*, 2004; Mayer *et al.*, 2004; Lohff *et al.*, 2005; Haas *et al.*, 2007; Sahambi *et al.*, 2008; Kodama *et al.*, 2009). A summary of the effects of VCD on reproduction of female rats and mice for previous studies is presented in Table 1.1.

In addition, an evaluation of bait-uptake by ricefield rats (*Rattus argentiventer*), one of the potential target species for fertility control, was undertaken to determine a suitable bait formulation for delivering fertility control agents for population management of this pest species.

1.8 Thesis outline

This thesis is written as a series of four individual papers (Chapters 2 to 5) that will be submitted for publication. As a result, there is some unavoidable overlap between chapters, particularly in the method sections. For example, there is repetition in the descriptions of the methods of animal care, administration of test compounds, and tissue preparation for histological assessments used in Chapters 2 and 3.

Chapter 2 and 3 present the effects of oral administration of VCD (500 mg/kg body weight) on reproduction of female and male laboratory rats, *Rattus norvegicus*. In Chapter 2, the effects of different periods of treatment with VCD on the ovarian follicle populations and fertility of adult female rats were investigated. Chapter 3 presents the effects of VCD exposure for 15 consecutive days through oral gavage on the reproductive tracts of juvenile and adult male rats as well as the breeding outcomes of adult males when paired with untreated, fertile females.

In Chapters 4 and 5, food uptake by ricefield rats, *Rattus argentiventer*, was evaluated. Chapter 4 evaluates the effects of different forms of bait, vegetable oils, food additives and cereal types on bait acceptance using a series of no choice and choice tests under laboratory conditions. Chapter 5 examines bait uptake by ricefield rats under enclosure conditions using the most accepted bait defined from the laboratory tests, and with Rhodamine B as a bait marker. The final chapter (Chapter 6) presents major findings and conclusions, relevance of thesis results for rodent management and suggestions for future research.

Table 1.1 Effects VCD on reproduction of female rats and mice

Animal	Dose	Route	Duration of treatment	Effects	References
Rats, Mice	62.5, 120, 250, 500, 1000 mg/kg	O	5 days/week for 13 weeks	Numbers of primary and secondary follicles decreased at high doses in mice, but not in rats	(Chhabra *et al.*, 1990a)
Rats	1.125, 2.25, 4.5, 9, and 18 mg/animal	Dermal	5 days/week for 13 weeks	Numbers of primary and secondary follicles decreased in mice at the high dose (1 mg/animal), but not in rats at any doses tested.	(Chhabra *et al.*, 1990a)
Mice	0.0625, 0.125, 0.25, 0.5 and 1mg/animal				
Rats	80 mg/kg	IP	30 days	Oestrous cycles prolonged in adults but not in immature rats; number of primordial and primary follicles reduced to 33 and 38% of control (in adults), and to 19 and 45% of control (in juveniles). Growing follicles were reduced to 54% of control in immature rats, but not in adults.	(Flaws *et al.*, 1994)
Rats	79.8 mg/kg	IP	10 days	Follicular viability reduced via an effect on granulosa cells.	(Springer *et al.*, 1996a)
Rats	80 mg/kg	IP	6, 8, 10, 12, or 15 days.	Numbers of oocytes contained in primordial and primary follicles decreased following 15 days of treatment, numbers of growing follicles not affected.	(Springer *et al.*, 1996b)
Rats, Mice	80 mg/kg	IP	6, 8, 10, or 12 days.	Percent atretic primordial follicles increased 4h after the last dose in mice on Day 8, and in rats on Day 10. Significant loss of primordial and primary follicles first observed on Day 12 in both rats and mice, and follicle damage to a greater extent in mice than in rats.	(Kao *et al.*, 1999)
Rats	80 mg/kg	IP	1(single dose) or 15 days	Significant increase in the number of primary follicles (41%) at 15 days after a single dose with VCD; significant reduction (50%) in the number of primordial and primary follicles after 15 days of treatment.	(Borman *et al.*, 1999)
Rats	79.8 mg/kg	IP	15 days	The numbers of primordial and primary follicles were reduced to approximately 37 and 44% of the control. The numbers of secondary follicles were not affected.	(Devine *et al.*, 2001)
Rats	80 mg/kg	IP	30 days	After 30 days of treatment, animals were killed at days 30, 60, 120, 240 and 360. Treatment with VCD induced significant reduction in the number of primordial follicles (31%) and primary follicles (49%) of the control on day 30. The number of antral follicles in treated animals was reduced relative to control beginning on day 120. Prolonged oestrous cycles observed from day 349-360. Increase in circulating FSH levels (day 120, 240, and 360) compared to control.	(Mayer *et al.*, 2002)

	160 mg/kg	IP	15 days	On Day 15 of dosing, complete loss of primordial follicles; primary follicles reduced to 10% of control. On Day 46, complete loss of primary follicles; secondary and antral follicles reduced to 0.7% and 2.6% of control, respectively.	(Mayer *et al.*, 2004)
Rats	40, 80, 160, 320 mg/kg	IP	Single dose	VCD (320 mg/kg) caused time-dependent decrease in primordial and primary follicles beginning 6 days after the single exposure; larger follicles not affected. Significant reductions in most stages of follicles on Day 36 following a single dose of VCD (320 mg/kg).	(Devine *et al.*, 2004)
Mice	160 mg/kg	IP	15 days	Significant increase in the length of oestrous cycles (10.9 days) from cycle 5 to cycle 12. Mean time to ovarian failure (the time at which oestrous cycle enters the persistent period of dioestrous stage) was Day 85 after the onset of treatment.	(Lohff *et al.*, 2005)
Mice	160 mg/kg	IP	10 or 20 days	Number of primordial and primary follicles reduced by 93.2% and 85.1%, respectively after 10 days of dosing. Nearly complete loss of these follicles after 20 days of dosing. The average time to ovarian failure on Day 135 (for 10-day treatment), and Day 52 (for 20-day treatment).	(Lohff *et al.*, 2006)
Mice	160 mg/kg	IP	15, 17, 20, or 22 days	Significant reduction in primordial follicles after 15 days of dosing. Complete loss of primordial follicles after 17, 20 and 22 days of dosing. Significant reduction in secondary follicles on Day 17.	(Haas *et al.*, 2007)
				Average time to ovarian failure was 45 days (between 36 and 56 days). Significant reduction in fertility in treated animals when mated 20 days after the final dose.	(Haas *et al.*, 2007)
Mice	240 mg/kg	IP	5 days	96% reduction in the numbers of primordial and small primary follicles on Day 16 after starting treatment, and near complete loss of these follicle types on days 37 to 100.	(Sahambi *et al.*, 2008)
Rats	5, 20, 80 mg/kg	IP	14, or 28 days	Significant reduction in the primordial and primary follicle numbers at dose of 80 mg/kg for 2 weeks, or at 20 and 80 mg/kg, for 4 weeks.	(Ito *et al.*, 2009)
Rats	5, 20, 80 mg/kg	IP	14 days before mating to 7 days of gestation	Significant decrease in the number of small follicles, the number of implanted embryos, and rate of implantation at all doses. Increase in pre-implantation loss and decrease in ovarian weight at 80 mg/kg	(Kodama *et al.*, 2009)
Rats	40, 80 mg/kg	IP	30 days	At 40 mg/kg, primordial follicles reduced to 52% (adults) and 60% (juveniles) of the control; primary follicles reduced to 58% (adults) and 80% (juveniles). At 80 mg/kg, primordial follicles reduced to 9% (adults) and 19% (juveniles) of the control; and primary follicles reduced to 13% (adults) and 42% (juveniles) of the control	(Muhammad *et al.*, 2009)
Rats	500 mg/kg	O	15 days	>85% depletion of primordial follicles after 15 days; fertility significantly reduced after 2 rounds of breeding	(L. Hinds *et al.*, unpublished data)

Route of administration: O = oral; IP = Intraperitoneal

CHAPTER 2

Effect of multiple periods of treatment with
4-vinylcyclohexene diepoxide on the fertility of
female rats (*Rattus norvegicus*)

Abstract

Fertility control of rodents has been proposed as an additional management option for pest populations in agricultural production systems. A potential approach is the use of chemosterilants which induce permanent sterility in females. One candidate chemosterilant, 4-vinylcyclohexene diepoxide (VCD), when administered by the parenteral or oral route at doses from 80-500mg/kg for 15 or 30 days to mice and rats induces depletion of primordial ovarian follicles.

In this study, the effects on ovarian follicle populations of female rats (*Rattus norvegicus*) were assessed after repeated 10-day periods of oral treatment with VCD (500mg/kg/day). Six-week old female rats (208 ± 18 g body weight) were divided into four groups and treated as follows: G*roup 1* animals received either corn-oil or VCD for a period of 10 days; Group *2* animals received treatment for two periods of 10 days separated by 21 days; Group *3* animals received treatment for three periods of 10 days separated by 21 and 14 days respectively. At the end of treatment, ovaries were collected for histological assessment. Animals in *Group 4* were treated as in G*roup 3*, but following their last period of oral gavage, they were paired with fertile, untreated males for 4 breeding rounds to assess their fertility. Their ovaries were then collected and follicle numbers determined.

Oral administration of VCD (500 mg/kg) to female rats induced significant depletion in primordial follicles for each treatment protocol (follicle numbers reduced to 34%, 8%, 2.3% and 2% of the controls in Groups 1, 2, 3 and 4, respectively) and in the number of primary follicles for animals treated for two or three periods of 10 days (10%, 6% and 1% of the controls in Groups 2, 3 and 4, respectively). Depletion of primordial and primary follicles resulted in a consequent reduction in the numbers of secondary and larger follicles in treated animals. After three periods of treatment with VCD, the fertility of treated female rats was significantly reduced by the 2nd round of breeding. From the 2nd to the 4th breeding rounds, control animals produced approximately 4 times the number of pups produced by treated animals. Therefore VCD has potential as a chemosterilant for fertility control of pest rodents although the duration of treatment would need to be considerably shorter for practical field delivery.

2.1 Introduction

For fertility control of wildlife species, one of the promising approaches is the use of chemosterilants (Howard, 1967; Kirkpatrick and Turner, 1985) which are chemicals that can induce temporary or permanent sterility in either or both sexes (Marsh and Howard, 1970, 1973). In species where mating systems are promiscuous (e.g. most rodents), chemosterilants that target females or both sexes are preferred (Bomford and O'Brien, 1992; Caughley *et al.*, 1992; Barlow *et al.*, 1997; Fagerstone *et al.*, 2002). An ideal chemosterilant should rapidly and irreversibly reduce fertility. In addition, the control agent should be suitable for oral delivery, non-toxic to non-reproductive organs and environmentally safe.

Female mammals are born with a finite number of primordial follicles (Hirshfield, 1991b); therefore chemosterilants that destroy these follicles can induce premature ovarian failure, leading to a reduction in reproductive success. Chemosterilants that target ovarian follicles, especially primordial follicles, could be considered the most effective fertility control agents for overabundant species as they could achieve permanent sterility. One promising chemosterilant candidate is an industrial chemical, known as 4-vinyl cyclohexene diepoxide (VCD).

VCD is used commercially as a chemical intermediate and reactive diluent for diepoxides and epoxy resins (Huff, 2001). Several studies have demonstrated that VCD induces a significant depletion of primordial and primary follicles in rats and mice, and causes gradual ovarian failure (Chhabra *et al.*, 1990a; Flaws *et al.*, 1994; Springer *et al.*, 1996a; Springer *et al.*, 1996b; Borman *et al.*, 1999; Kao *et al.*, 1999; Devine *et al.*, 2001; Mayer *et al.*, 2002; Devine *et al.*, 2004; Mayer *et al.*, 2004; Lohff *et al.*, 2005; Lohff *et al.*, 2006; Haas *et al.*, 2007; Sahambi *et al.*, 2008; Ito *et al.*, 2009; Kodama *et al.*, 2009; Muhammad *et al.*, 2009). A summary of the doses, routes of administration, duration of treatment and the effects of VCD on reproduction of female rats and mice was presented in Table 1.1, Chapter 1. In these studies, animals were mainly treated using an intraperitoneal (IP) injection and doses ranged from 80 to 320 mg/kg for a single period of 15 or 30 consecutive days. While effects on ovarian follicle numbers were observed and in some cases ovarian failure was reported, few studies included direct assessments of effects on fertility (Haas *et al.*, 2007; Kodama *et al.*, 2009).

A limited number of studies have been conducted on the effects of VCD after oral administration. A thirteen-week study by Chhabra *et al.* (1990a) reported that oral administration of VCD at 500 and 1000 mg/kg induced ovarian atrophy in mice. However, the main aim of this study was to investigate the mechanisms of the toxicity on the ovaries or general toxicity of VCD. VCD was also used as a model chemical for investigation of potential risks of ovotoxic chemicals in humans (Hoyer and Sipes, 1996; Hoyer *et al.*, 2001; Mayer *et al.*, 2004).

In a recent study (L. Hinds *et al.*, unpublished data), oral treatment of laboratory rats with VCD (500mg/kg or 750 mg/kg) for 15 days induced significant depletion (>85%) of primordial follicles and a significant decrease in pups produced after 2 breeding rounds. However, because tolerance of the 750mg VCD/kg dose was poor, subsequent studies have focussed on assessing the effects of oral doses of 500mg VCD/kg on ovarian function and fertility.

The aim of this study was to examine the effects of repeated periods of oral treatment with VCD (500mg/kg body weight) on ovarian follicle populations in female laboratory rats (*Rattus norvegicus*). In addition, the effects of VCD treatment on the oestrous cycle and fertility of female rats were investigated.

With respect to fertility control of pest rodents, the use of multiple 10 day periods of treatment in this study mimics the expected application of VCD in a cropping system where the chemosterilant would be applied at different times during the early development of the crop. Findings from the present study will contribute to the development of a chemosterilant as an additional tool for population management of pest rodents.

2.2 Materials and methods

2.2.1 Chemical

One batch of 4-vinyl-1-cyclohexene diepoxide (97%) was purchased from Sigma Chemical Co., St. Louis, MO, USA. VCD was diluted in corn-oil in order to prepare doses on a per kg body weight basis immediately before each gavaging period.

2.2.2 Animals

Adult laboratory female rats (*Rattus norvegicus*) (208±18 g body weight) were obtained from the Animal Resources Centre, Western Australia. The rats were assigned to weight classes and allocated into experimental groups according to a table of random numbers prior to treatment. The animals were housed in small cages (52cm x 36cm x 21cm) with two animals per cage in an air-conditioned animal room at a temperature of $22^{\circ}C \pm 2^{\circ}C$ and exposed to 12 hours of light (0600-1800h). Shelters in the form of plastic nest boxes and polypipes, sawdust and nesting materials were provided. The rats were fed with standard rat chow (maintenance and breeding pellets, Gordon's Specialty Stockfeeds, NSW, Australia) and water *ad libitum* throughout the course of the experiment. The use of animals was approved by the CSIRO Sustainable Ecosystems Animal Ethics Committee (Number 09-06).

2.2.3 Method of administration

Immediately before oral gavage, animals were lightly anaesthetised by inhalation of 4% Isoflurane in oxygen administered via a VetQuip Compact Anaesthetic machine with a Tec 5 Isoflurane vaporiser. Animals were given either an oral gavage of corn-oil (control) or VCD (500 mg/kg) in corn-oil at a volume of between 200-800ul depending on body weight using a gavage needle (curved stainless steel metal, 2.5mm ball-tipped; 16-18g x 75mm) which was attached to a 1 ml syringe. The oral administration procedure was undertaken between 1300–1600h each day. Animals were observed for their responses to oral gavage as they recovered from the anaesthetic. Some overt responses to dosing were observed and included gastric reflux, salivation and sneezing.

2.2.4 Experimental design

Effects of different periods of VCD treatment on oestrous cycles and reproductive tissues

Adult female rats were divided into three groups and were given either corn-oil (control) or VCD (500mg/kg) orally according to the following treatment protocols: *Group 1* animals (n=4 controls; n=8 VCD treated) were treated for one period of 10

days; *Group 2* animals (n=4 controls; n=8 VCD treated) were treated for two periods of 10 days, separated by 21 days; and *Group 3* animals (n=8 controls; n=8 VCD treated) were treated for three periods of 10 days, separated by 21 and 14 days respectively (Fig. 2.1). Oestrous cycles were monitored during these intervening periods. At the end of the last gavage for each group, all animals were killed and dissected for tissue collection as described below.

Effects of multiple periods of VCD treatment on fertility

Group 4 animals (n=8 controls; n=8 VCD treated) were orally administered with either corn-oil (control) or VCD (500mg/kg) for three periods of 10 days, separated by 21 and 14 days respectively, as in *Group* 3. Following the final gavage, all animals were paired with fertile, untreated males (female:male = 2:1) for four breeding rounds to assess their breeding outcomes (Fig. 2.1). Towards the end of gestation, females were checked daily for the birth of pups. The litter size of the females was determined and pups removed as soon as possible after birth, sexed, and weighed before euthanasia. At the end of the final breeding round, all females were killed for tissue collection as described below.
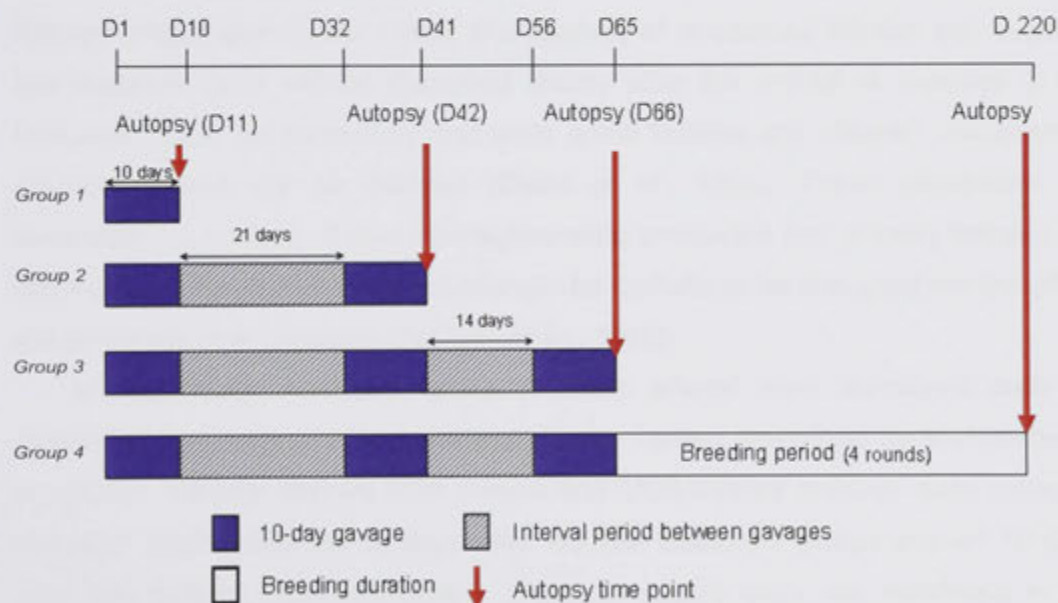
Figure 2.1 Experimental design. Animals were divided into four groups: *Group 1*, 2 and 3 animals received either corn-oil (control) or VCD (500mg/kg) orally for one, two and three periods of 10 days, respectively. At the end of the last gavage, animals were killed and ovaries were collected for histological assessment. *Group 4* animals were treated as in *Group 3*, but following their last period of gavage, they were paired with fertile, untreated males for four breeding rounds. At the end of the last breeding round, animals were killed and the numbers of follicles were examined.

2.2.5 Data collection

Body weights and the weights of reproductive and non-reproductive organs

Over the course of the experiment, the body weights of all animals were recorded daily during treatment periods and three times each week at other times. Prior to autopsy, the animals were euthanased with an overdose of Lethobarb (Virbac Australia Pty. Limited, NSW)(162.5mg/kg), administered intra-peritoneally. Reproductive tissues, including ovaries and uteri were collected, weighed, and fixed in 10% neutral buffered formalin for 24h. Other internal tissues (kidneys, liver, adrenals, heart, lungs, spleen) were also collected, weighed and fixed in 10 % neutral buffered formalin. Observations of the general appearance of these tissues, as well as the oesophagus, stomach, small intestine, buccal cavity, and brain were also made.

Oestrous cycles

Destruction of ovarian follicles leads to changes in the regularity of oestrous cycles and can be used as the first indicator of the toxicity of chemicals on ovarian follicles (Hoyer and Sipes, 1996). If a majority of developed follicles are targeted, the oestrous cycle will be disrupted shortly after the animal is exposed to the toxicants, but if the secondary and early antral follicles are affected, disruption of oestrous cycles will be delayed (Davis *et al.*, 1994). These disruptions are reversible. In contrast, if the non-regenerating primordial and primary follicles are targeted and destroyed, it will take longer for cyclicity to be disrupted but the effect will be permanent (Mattison and Schulman, 1980).

In this study, oestrous cycles of each animal were monitored daily by examination of vaginal cytology following the method described by Marcondes *et al.* (2002). Vaginal smears from control and VCD-treated animals were collected between 1400-1600h for 18 days after the first treatment period and for 10 days after the second treatment period. Oestrous cycles were not monitored in the animals after the 3rd treatment period as they were either killed or mated with untreated males shortly after the last dose. Vaginal secretions were collected with a plastic pipette which was filled with 500 µL of 0.9% phosphate buffered saline (PBS). The contents of the vaginal flush were placed in a flat-bottom well of a 96-

well plate and unstained material was observed under a light microscope. The proportions of epithelial cells, cornified cells and leukocytes were used to determine the phase of the oestrous cycle. The average length of the oestrous cycles of control and treated animals were compared.

Number of ovarian follicles

The formalin-fixed ovaries from each animal were processed, embedded in paraffin and serially sectioned at 5 µm thickness using an automatic microtome (Leica RM 2255). Every 10th section was mounted onto glass slides then stained with Gill's haematoxylin and eosin (H&E) for histological assessment using an automatic staining machine (Shandon Varistain 24-3, ThermoFisher Scientific). The staining procedure is presented in Appendix 1.

The number of ovarian follicles at different stages of development were counted using a light microscope (Diaplan, Leitz Wetzlar Germany). Follicle types were classified according to previous studies (Pedersen and Peters, 1968; Flaws *et al.*, 1994; Borman *et al.*, 1999; Kao *et al.*, 1999). In brief, primordial follicles were classified as those having a single layer of squamous granulosa cells. Primary follicles were identified as those containing at least 50% cuboidal granulosa cells in a single layer. Secondary follicles were identified as having two or more layers of granulosa cells with no antral space in the granulosa layer. Antral follicles were identified as those containing at least two layers of granulosa cells and a fluid-filled antral space (Fig. 1.1, Chapter 1).

The numbers of primordial and primary follicles were counted in every 40th section. The total number of follicles of each type per ovary (N_t) was estimated as follows:

$$N_t = \frac{N_o \times S_t \times t_s}{S_o \times d_o}$$

where N_o = number of follicles observed in the ovary,

S_t = total number of sections in the ovary,

t_s = thickness of the section (µm),

S_o = total number of sections observed, and

d_o = mean diameter of the nucleus of that follicle (~7 µm in rat).

For secondary and larger follicles, initially the number of these follicles (follicles with oocytes present) was counted in every 10th section. However, if every 20th section was counted the same numbers were observed if counts were then multiplied by 2. Subsequently only every 20th section was counted.

Healthy follicles were defined as follicles containing an oocyte surrounded by a well-defined single or multiple layers of granulosa cells and with no leucocytes present among the granulosa cells. Atretic follicles were defined as follicles that had no oocyte or the oocytes had pyknotic nuclei, leucocytes were present among the granulosa cells, and the basement membrane was not intact.

Fertility

The fertility of *Group 4* females was assessed based on the breeding outcomes of pairing with untreated, fertile males. The number of animals giving birth and the time intervals from mating to birth were recorded. Litter sizes for each of the four breeding rounds were determined. Pups from the litters were removed, sexed, and weighed before euthanasia.

2.2.6 Data analysis

Means of body weight (g), the weights of non-reproductive organs (g/kg body weight) and reproductive organs (mg/100 g body weight), lengths of oestrous cycles (days), the estimated numbers of different types of ovarian follicles, and litter sizes from the control and VCD-treated animals were compared using Student's t-tests with a significance level of 5%, using the SPSS software package (Version 16). The absolute weights of reproductive tissues (mg/animal) from control and treated groups were also compared using t-tests. All values are presented as mean $\pm$ standard deviation (SD). Data were transformed to meet requirements for normal distribution prior to analysis as indicated in figures.

Means of litter size, time interval to birth after pairing with a male, the body weights and sex ratio of pups between control and treated groups were also compared.

2.3 Results

2.3.1 Effect of VCD treatment on body weight

During the first several days of each gavaging period, body weights of the treated animals gradually declined, but were at least maintained thereafter. No significant difference ($P>0.05$) in body weights between control and treated animals was found at any time points (Figs. 2.2a-d).

In this study, 32 animals were orally gavaged repeatedly with VCD with a total of 625 individual gavages being performed. However, over the three rounds of treatment, 7 animals were removed due to gavage-related deaths; 3 deaths were recorded on Days 1 and 2 of the first round of gavage, 3 deaths on Days 2, 6 and 7 of the second round of gavage, and 1 death on Day 5 of the third round of gavage. During the gavaging periods, all animals were checked daily and their health closely monitored. Each of the animals which died showed VCD reflux while being gavaged or as they were recovering from the light anaesthetic. These reflux responses can lead to inhalation of VCD, compromise of lung function and lead to death. Autopsies of the dead animals showed damage to the upper lobes of the lungs. Thus, the mortality that occurred in this study was likely due to gavaging errors.

2.3.2 Effects of VCD treatment on non-reproductive organs

The weights of the non-reproductive organs (g/kg body weight), (liver, kidneys, adrenals, spleen, heart and lungs) in the animals treated with VCD in each treatment group were not significantly different ($P>0.05$) compared to control animals (Table 2.1). The appearance of these organs and of the oesophagus, stomach, small intestine, buccal cavity and brain were normal in both the treated and control animals.
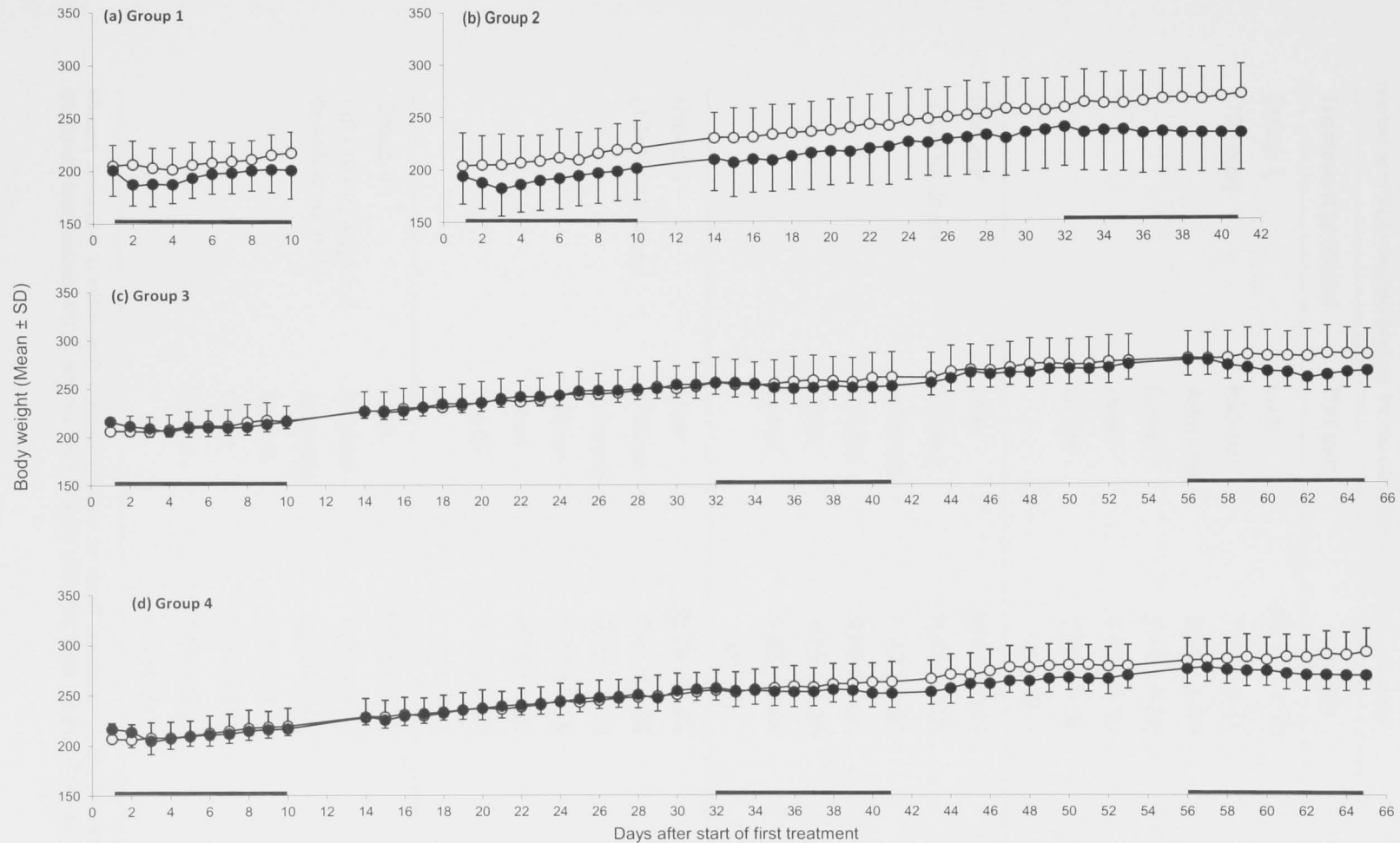
Figure 2.2 Body weights (g, Mean ± SD) of animals receiving either corn-oil (○) or VCD (500 mg/kg)(●) for (a) one period of 10 days, Group 1 (n=4 controls; n=5 treateds), (b) two periods, Group 2 (n=4 controls; n=5 treateds), (c) three periods, Group 3 (n=8 controls; n=7 treateds); and (d) three periods, Group 4 (n=8 controls; n=8 treateds). Bar on x-axis indicates 10 day periods of treatment.

Table 2.1 Weights of non-reproductive organs (g/kg body weight)(Mean ± SD) of female rats from control and VCD-treated groups for the different treatment protocols.

Treatment protocol	Tissues	Control	VCD
Group 1	Liver	39.12 ± 3.29	$43.61 ± 1.54^{ns}$
(10 days)	Kidneys	7.43 ± 0.18	$8.25 ± 0.67^{ns}$
	Adrenals	0.24 ± 0.03	$0.26 ± 0.04^{ns}$
	Spleen	2.12 ± 0.26	$1.77 ± 0.23^{ns}$
	Heart	3.12 ± 0.13	$3.57 ± 0.30^{ns}$
	Lungs	6.09 ± 0.36	$5.33 ± 0.69^{ns}$
		(n = 4)	(n = 5)
Group 2	Liver	38.47 ± 3.87	$39.76 ± 4.81^{ns}$
(10+10 days)	Kidneys	6.69 ± 0.23	$9.12 ± 3.13^{ns}$
	Adrenals	0.22 ± 0.03	$0.30 ± 0.07^{ns}$
	Spleen	2.00 ± 0.29	$1.67 ± 0.19^{ns}$
	Heart	3.15 ± 0.25	$3.45 ± 0.34^{ns}$
	Lungs	5.52 ± 0.44	$5.59 ± 0.71^{ns}$
		(n = 4)	(n = 5)
Group 3	Liver	33.78 ± 1.80	$37.59 ± 6.48^{ns}$
(10+10+10 days)	Kidneys	6.37 ± 0.26	$7.19 ± 0.57^{ns}$
	Adrenals	0.23 ± 0.03	$0.24 ± 0.04^{ns}$
	Spleen	1.71 ± 0.20	$1.53 ± 0.37^{ns}$
	Heart	3.16 ± 0.22	$3.32 ± 0.25^{ns}$
	Lungs	4.71 ± 0.43	$5.50 ± 1.06^{ns}$
		(n = 8)	(n = 7)
Group 4	Liver	33.59 ± 3.49	$31.93 ± 4.58^{ns}$
(10+10+10 days + 4	Kidneys	5.66 ± 0.34	$6.00 ± 0.51^{ns}$
breeding rounds)	Adrenals	0.16 ± 0.02	$0.15 ± 0.02^{ns}$
	Spleen	1.42 ± 0.21	$1.30 ± 0.13^{ns}$
	Heart	2.99 ± 0.17	$3.07 ± 0.15^{ns}$
	Lungs	3.53 ± 0.27	$3.94 ± 0.61^{ns}$
		(n = 8)	(n = 8)

Means between control and treated groups in each treatment protocol were compared using Student *t-test;* values with ^{ns} are not significantly different from control (P>0.05).

2.3.3 Effects of VCD treatment on the weights of ovary and uterus

Significant reductions in ovarian weights (mg/100g body weight) were observed in those animals which underwent three periods of treatment with VCD and 4 breeding rounds compared to their corresponding control animals (Group 4) (t = 4.9, df = 14, P < 0.0001)(Fig. 2.3a). No difference in weights of ovaries was found between the control and treated animals in Groups 1, 2 and 3. However, when expressed as their absolute weights (mg/animal), the total ovarian weight in the treated animals was significantly reduced in Group 2 (animals treated for 2 periods, t = 2.3, df = 8, P = 0.048), Group 3 (animals treated for 3 periods; t = 2.4, df = 13, P = 0.031), and Group 4 (animals treated for 3 periods plus 4 breeding cycles; t = 5.7, df = 14, P < 0.0001)(Fig. 2.3b).

The weights of uteri, either expressed as their absolute weight (mg/animal)(data not shown) or corrected for body weight (mg/100g body weight), were not significantly different (P>0.05) between control and treated animals for any of the groups (Fig. 2.3c).
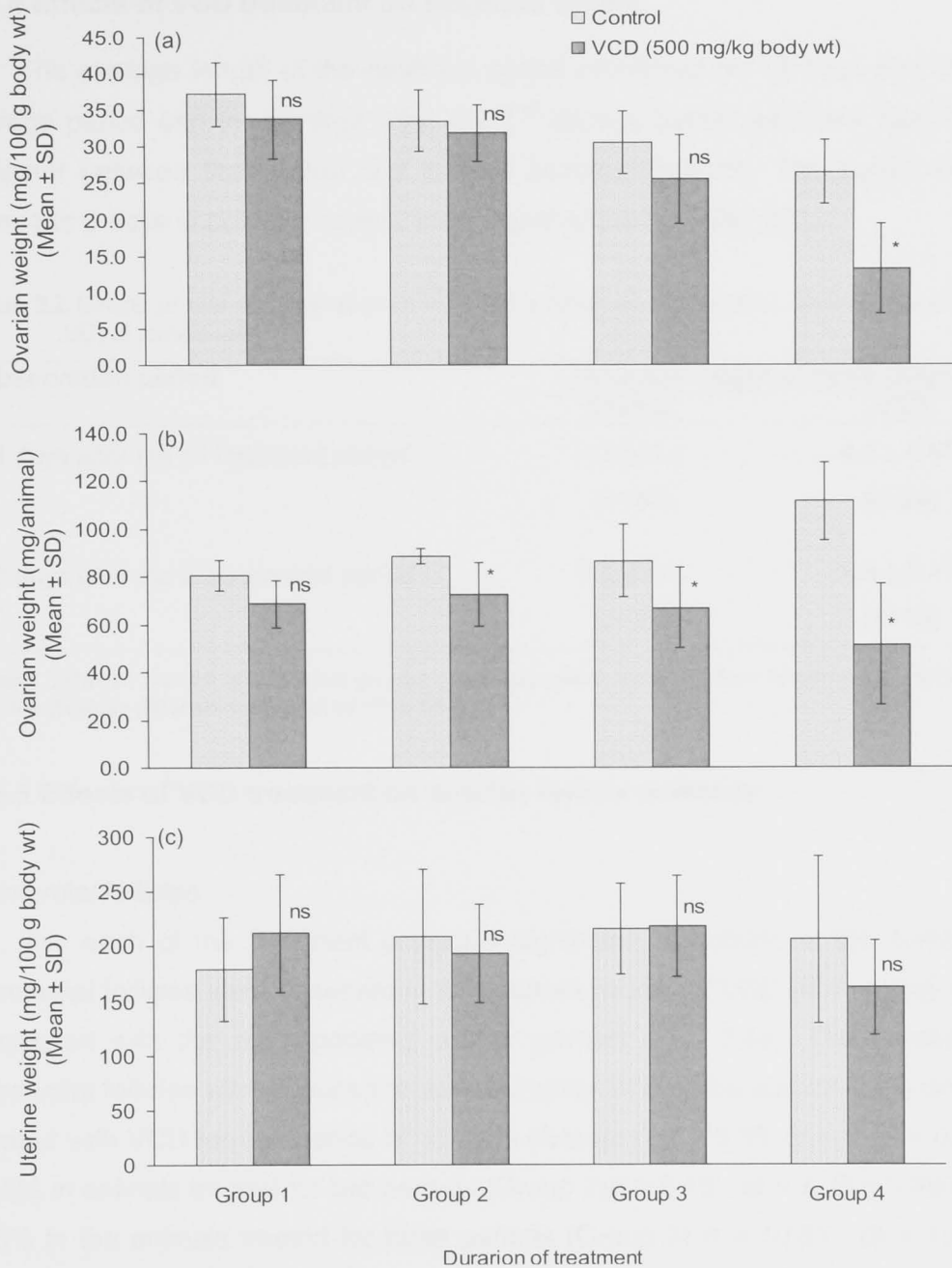
Figure 2.3 Effects of different periods of treatment with VCD (500 mg/kg) on (a) the total ovarian weight corrected for body weight (mg/100g body weight), (b) absolute total ovarian weight (mg/animal), and (c) the total uterine weight corrected for body weight (mg/100g body weight), (Mean ± SD). Group 1 (n= 4 controls, n= 5 treateds), Group 2 (n= 4 controls, n= 5 treateds), and Group 3 (n= 8 controls, n= 7 treateds) animals treated for one, two and three periods of 10 days, respectively and killed shortly after the last dose. Group 4 (n= 8 controls, n= 8 treateds) animals treated as in Group 3, but killed after 4 breeding rounds. The ovarian and uterine weights, corrected for body weights, were transformed to arcsine prior to analyses. Means between control and treated groups were compared using Student *t-test;* columns with * are significantly different (P<0.05), and columns with [ns] are not significantly different from control.

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2.3.4 Effects of VCD treatment on oestrous cycles

The average length of the oestrous cycles monitored for 18 days after the 1st gavage period and for 10 days after the 2nd gavage period were not significantly different between the control and treated animals (P>0.05). The cycles ranged from 4 to 5 days in both the control and treated animals (Table 2.2).

Table 2.2 Effects of oral administration of VCD (500mg/kg) on the oestrous cycle (days) (Mean ± SD) of female rats.

| Observation period	Average length of cycle (days)	
	Control	VCD
18 days after the 1st treatment period	4.5 ± 0.4	4.7 ± 0.6^{ns}
	(n=20)	(n=24)
10 days after the 2nd treatment period	4.6 ± 0.2	4.8 ± 0.4^{ns}
	(n=16)	(n=16)

Means between control and treated groups were compared using Student *t-test;* values with ^{ns} are not significantly different from control (P>0.05).

2.3.5 Effects of VCD treatment on ovarian follicle numbers

Primordial follicles

For each of the treatment protocols significant reductions in the number of primordial follicles were observed in the animals receiving VCD (500 mg/kg) when compared with the corresponding control animals (Fig. 2.4a). The numbers of primordial follicles were reduced to approximately 34% of the control in the animals treated with VCD for one period of 10 days (Group 1) (t = 5.39, df = 7, P = 0.001), to 8% in animals treated for two periods (Group 2)(t = 6.19 , df = 7, P < 0.0001), to 2.3% in the animals treated for three periods (Group 3) (t = 10.81 , df = 13, P < 0.0001), and to 2% in the animals treated the same as Group 3 and killed after 4 breeding rounds (Group 4)(t = 9.46, df = 14, P < 0.0001) (Fig. 2.4a).

Primary follicles

No significant difference (P>0.05) in the number of primary follicles was found between the control animals and those treated with VCD for one 10-day period (Group 1) (Fig. 2.4b).

The number of primary follicles was significantly reduced in the animals treated with VCD for two 10- day periods (Group 2) (t = 9.89, df = 7, P < 0.0001), for three 10-day periods (Group 3) (t = 12.45, df = 13, P < 0.0001), and for three 10-day periods plus 4 breeding rounds (Group 4) (t = 10.77, df = 14, P < 0.0001) when compared with the corresponding control animals. Numbers were reduced to approximately 10% of the control in Group 2, 6% in Group 3, and 1% in Group 4 (Fig. 2.4b).

Secondary and larger follicles

No differences in the number of secondary and larger follicles were observed between control and treated animals in Group 1 (Fig. 2.4c). However, in Groups 2, 3, and 4 the numbers of these follicle types were significantly reduced following oral administration with VCD (500mg/kg). Numbers were reduced to approximately 27.5% of the control in Group 2 (t = 5.48, df = 7, P =0.001), 22% in Group 3 (t = 8.28, df = 13, P < 0.0001), and 3% in Group 4 (t = 11.14, df = 14, P < 0.0001) (Fig. 2.4c).

Total numbers of healthy follicles

Total numbers of healthy follicles (primordial, primary, secondary and larger) in the VCD-treated animals were significantly lower than those of the control animals in each of the treatment protocols (Fig. 2.5a). The total number of healthy follicles was reduced to approximately 46% of the controls in animals treated for one period of 10 days (Group 1) (t = 5.77, df = 7, P = 0.001), to 10% in the animals treated for two periods (Group 2) (t = 9.28, df = 7, P < 0.0001), to 2% in the animals treated for three periods (Group 3) (t = 5.96, df = 13, P < 0.0001), and to 1.7% in animals treated as in Group 3 and killed after 4 breeding rounds (Group 4) (t = 6.42, df = 14, P < 0.0001 (Fig. 2.5a).

Ovaries from VCD-treated animals in Group 4 were almost devoid of all follicle types, whereas the ovaries from control animals contained follicles at all stages of development (Fig. 2.6).

Atresia in secondary and larger follicles

Reduced numbers of atretic follicles (secondary and larger) were found in animals in Group 2 (t = 2.99, df = 7, P = 0.02), Group 3 (t = 4.88, df = 13, P < 0.0001), and Group 4 (t = 11.13, df = 14, P < 0.0001) compared to the control animals (Fig. 2.5b).
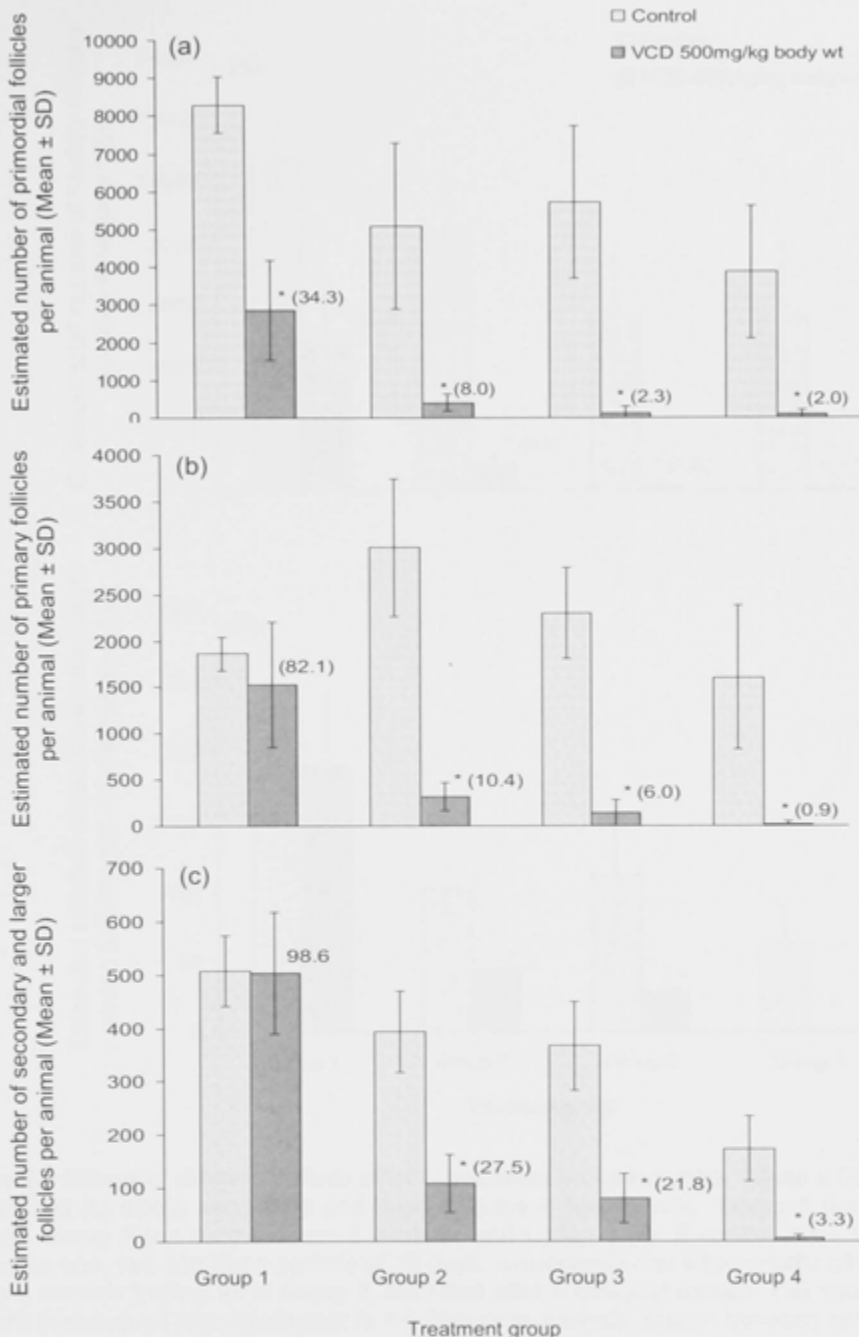
Figure 2.4 Effects of different periods of VCD treatment on the numbers (Mean ± SD) of (a) primordial follicles, (b) primary follicles and (c) secondary and larger follicles in female rats. Group 1 (n= 4 control, n= 5 treated), Group 2 (n= 4 control, n= 5 treated), and Group 3 (n= 8 control, n= 7 treated) animals treated for one, two and three periods of 10 days, respectively and killed shortly after the last dose. Group 4 (n= 8 control, n= 8 treated) animals treated as in Group 3, but killed after 4 breeding rounds. The numbers of follicles (x) were transformed into Squareroot (x + 0.5) prior to analysis; means between control and treated groups were compared using Student *t-test*; columns with * are significantly different from control (P<0.05). Values in brackets represent the percentage of follicle numbers in VCD-treated animals compared to the corresponding control animals.
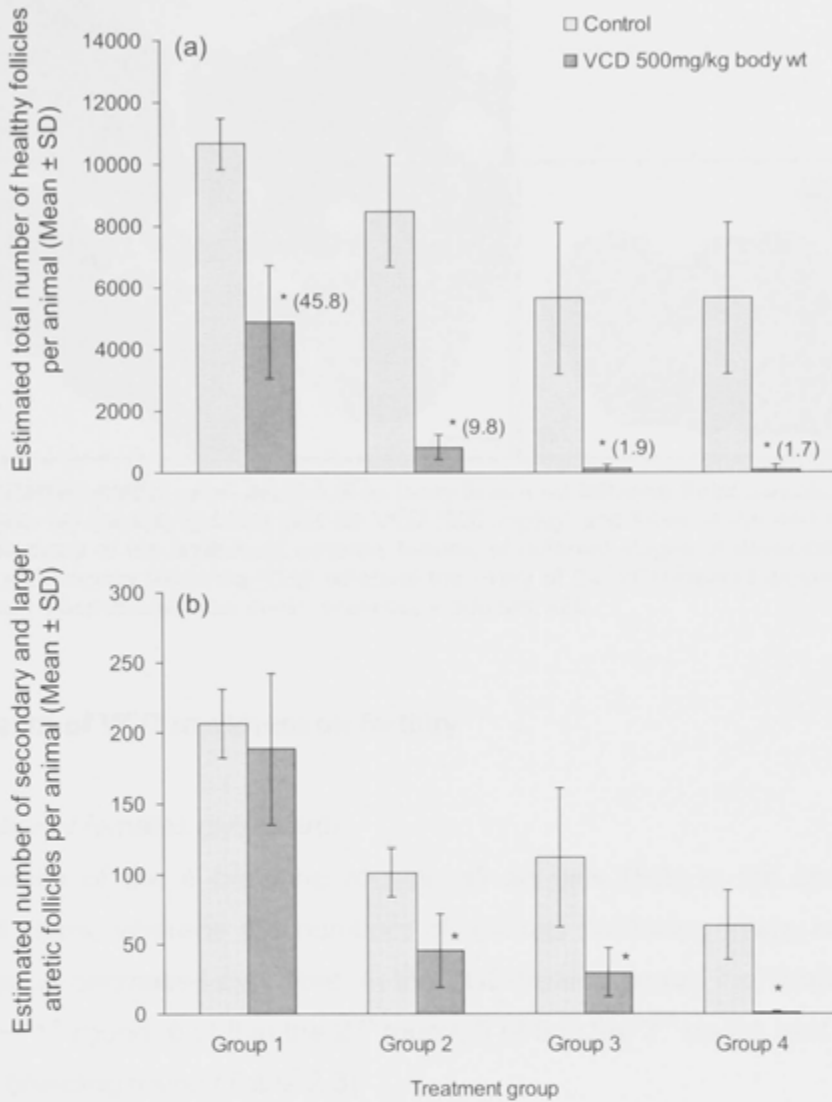
Figure 2.5 Effects of different periods of VCD treatment on total number (Mean ± SD) of (a) healthy follicles and (b) atretic secondary and larger follicles in female rats. Group 1 (n= 4 control, n= 5 treated), Group 2 (n= 4 control, n= 5 treated), and Group 3 (n= 8 control, n= 7 treated) animals treated for one, two and three periods of 10 days, respectively and killed shortly after the last dose. Group 4 animals treated as in Group 3, but killed after 4 breeding rounds. The numbers of follicles (x) were transformed into Squareroot (x + 0.5) prior to analysis; means between control and treated groups were compared using Student *t-test*; columns with * are significantly different from control (P<0.05). Values in brackets represent the percentage of follicle numbers in VCD-treated animals compared to the corresponding control animals.
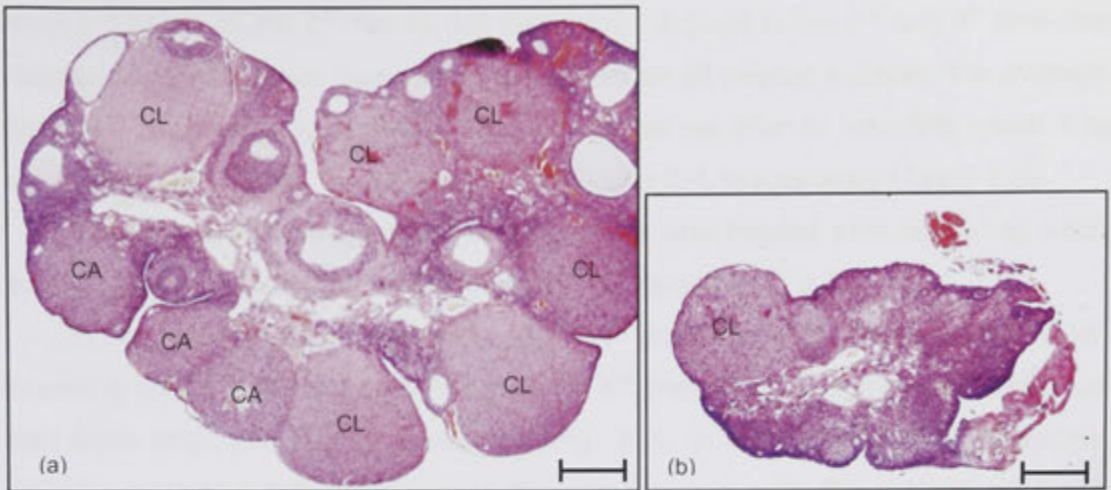
Figure 2.6 Representative cross-section of an ovary from a rat following three periods of 10 days of treatment with (a) corn-oil (control) and (b) VCD (500 mg/kg) and killed at the end of 4 breeding rounds. The ovary of the control rat contains follicles at different stages of development, corpora lutea (CL) and corpora albicantia (CA); whereas the ovary of the VCD-treated animal is devoid of follicles. Stain: haematoxylin and eosin; scale bar = 500 um; x25.

2.3.6 Effects of VCD treatment on fertility

The number of females giving birth

For each of the 4 breeding rounds, all animals (8/8) in the control group produced litters, whereas the numbers of animals producing litters in the VCD-treated group decreased over time. In the VCD-treated group, 7 of 8 animals gave birth in the 1st round, 6 of 8 in the 2nd round, 5 of 8 in the 3rd round, and only 2 of 8 in the 4th breeding round (Table 2.3).

Litter size

The litter sizes for control animals were similar for each breeding round, with an average of 12.4 pups per litter (Table 2.3), ranging from 5 to 18 pups. In contrast, the average litter sizes for VCD treated animals that gave birth decreased in each successive breeding round. Significant reductions in the litter sizes of treated animals compared to control animals were found in the 2nd breeding round (7.7 pups per litter; $t = 2.32$, df = 12, $P = 0.039$), in the 3rd round (3 pups per litter; $t = 6.01$, df = 11, $P < 0.0001$), and in the 4th round (3.5 pups per litter; $t = 3.58$, df = 8, $P = 0.007$) (Table 2.3). Litter sizes in these successive breeding rounds ranged

from 5-13 pups in the 2nd round, but only from 1-5 pups in the 3rd and 4th breeding rounds. When litter size was calculated based on all treated animals, the average litter size progressively declined, from 11.5 pups per litter in breeding round 1 to approximately 6, 2 and 1 pups per litter in rounds 2-4, respectively (Table 2.3).

The changes in body weight of the control and treated animals during each breeding round reflect the sizes of the litters produced (Fig. 2.7).

In the control group approximately 100 pups were produced during each breeding round, such that by the end of the 4th breeding round a total of 397 pups had been produced by the 8 animals (Fig. 2.8). In contrast, the 8 VCD-treated animals produced only 160 pups in the same period, with the majority of these pups (n=92) being delivered by 7 animals in the 1st round of breeding. Thereafter only a further 68 pups were produced from 13 litters delivered in the last 3 breeding rounds.

Table 2.3 Effects of VCD treatment on the number of animals that gave birth and the litter size (Mean ± SD) over 4 breeding rounds.

Treatment		Breeding round			
		1	2	3	4
Control	No. giving birth	8	8	8	8
	Average litter size	13.5 ± 1.5	12.9 ± 4.4	12.0 ± 2.9	11.3 ± 2.8
	Range of litter size	12-16	5-18	7-16	6-15
VCD	No. giving birth	7	6	5	2
(500 mg/kg)	Average litter size[†] (for animals giving birth)	13.1 ± 4.6	7.7 ± 3.8*	3.0 ± 2.0*	3.5 ± 2.1*
	Average litter size [‡] (all animals, n = 8)	11.5 ± 6.3	5.8 ± 4.8*	1.9 ± 2.2*	0.9 ± 1.8*
	Range of litter size	4-19	5-13	1-5	2-5

Following 3 periods of gavage either with corn-oil (n=8) or VCD (500 mg/kg, n=8), animals were paired with fertile, untreated males for 4 rounds. Litter sizes of treated animals were presented either for the animals that gave birth (†) or for all animals of the group (‡); mean between control and treated groups were compared using Student *t-test;* values with * are significantly different from control ($P<0.05$).
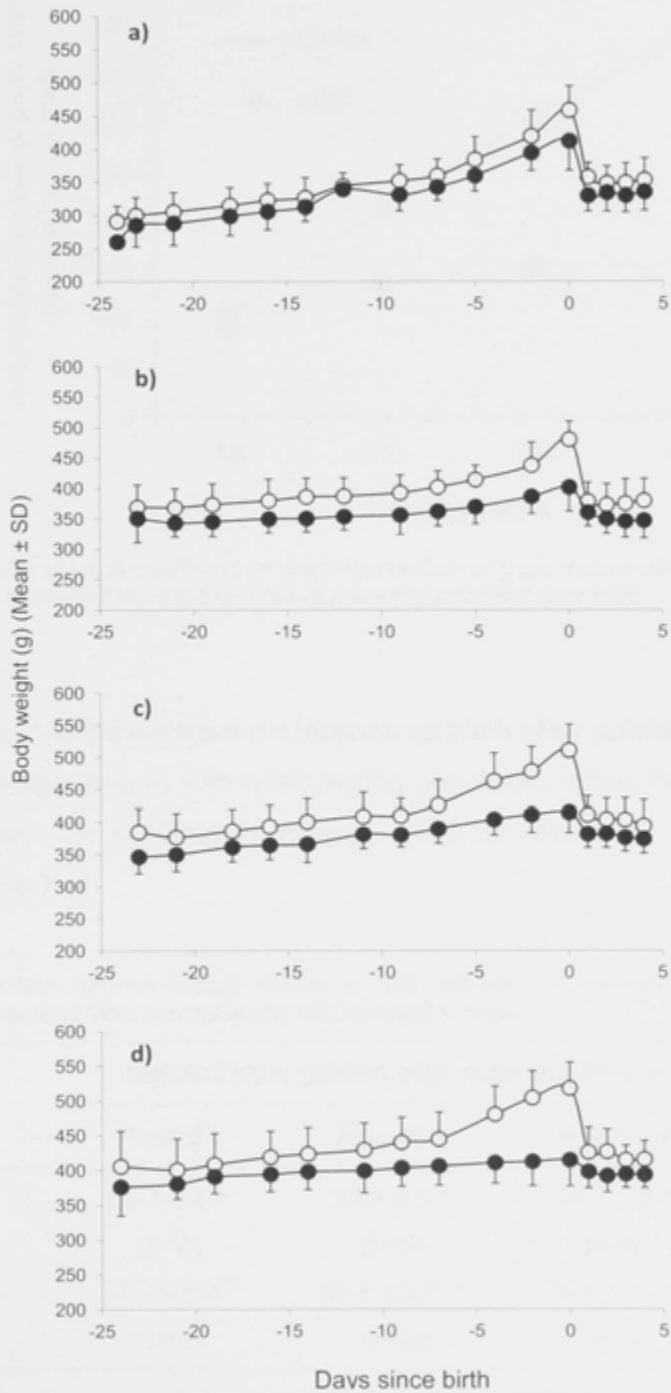
Figure 2.7 Changes in body weights (g) (Mean ± SD) of 8 control animals (o) and 8 animals treated with VCD (500 mg/kg)(●) for three periods of 10 days and then mated with fertile males for 4 successive breeding rounds (a, b, c, d). Weights have been aligned from day of birth (Day 0).
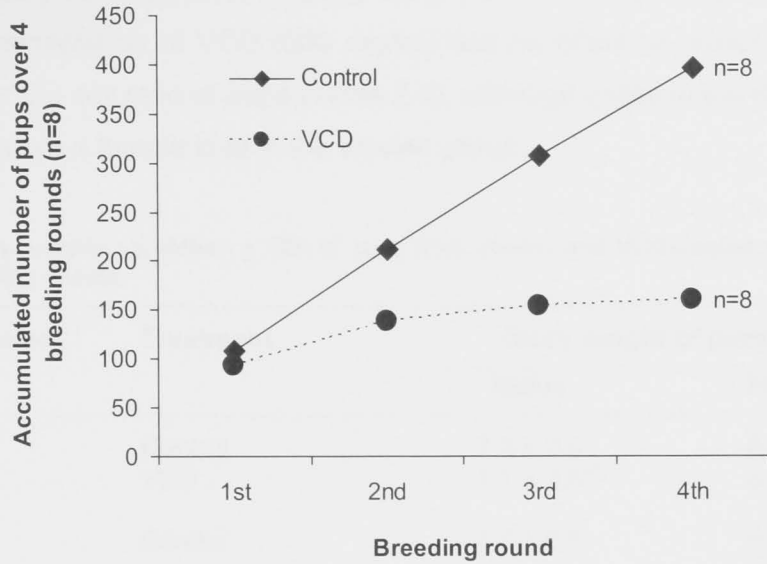
Figure 2.8 Effects of VCD treatment on the total number of pups accumulated over the 4 breeding rounds. Values represent the total number of pups accumulated over time.

2.3.7 Effect of VCD treatment on interval to birth after pairing with males.

The average time to birth after pairing with fertile males ranged from 26 to 34 days and was not significantly different (P>0.05) between control and treated animals (Table 2.4).

Table 2.4 Average interval (days) (Mean ± SD) between introduction of fertile males and subsequent births in control and VCD-treated groups.

Treatment	Interval from pairing with male to births in females (days)			
	Round 1	Round 2	Round 3	Round 4
Control	26.1 ± 4.6	34.4 ± 5.1	26.3 ± 2.7	26.8 ± 5.6
	(n=8)	(n=8)	(n=8)	(n=8)
VCD	27.7 ± 6.8^{ns}	31.7 ± 5.7^{ns}	26.8 ± 4.4^{ns}	27.0 ± 4.2^{ns}
	(n=7)	(n=6)	(n=5)	(n=2)

Mean between control and treated groups were compared using Student *t-test*. Values with ^{ns} are not significantly different from control (P>0.05).

2.3.8 Effect of VCD treatment on body weight and sex ratio of pups.

Oral administration of VCD (500 mg/kg) had no effect on either pup weights (Table 2.5) or the sex ratio of pups (Table 2.6), although unlike in the control group, there was always a female bias in the treated group .

Table 2.5 Body weights (g) (Mean ± SD) of pups from control and VCD-treated animals over 4 breeding rounds.

Breeding round	Treatment	Body weight of pups (g)[†]	
		Males	Females
1	Control	7.3 ± 0.5	6.9 ± 0.4
	VCD	7.1 ± 0.5[ns]	6.9 ± 0.6[ns]
2	Control	7.4 ± 0.6	6.9 ± 0.6
	VCD	8.2 ± 0.8[ns]	7.7 ± 0.7[ns]
3	Control	7.4 ± 1.0	7.0 ± 0.6
	VCD	8.9 ± 1.0[ns]	7.9 ± 0.9[ns]
4	Control	7.8 ± 1.4	7.4 ± 1.4
	VCD	10.1 ± 0.0[ns]	8.5 ± 0.7[ns]

[†] Mean between control and treated groups in each breeding round were compared using Student t-test. Values with [ns] are not significantly different from control ($P>0.05$).

Table 2.6 Sex ratio of pups from control and VCD-treated animals over 4 breeding rounds.

Breeding round	Treatment	Sex ratio (%)[†]	
		Males	Females
1	Control	54.6	45.4
	VCD	45.7[ns]	54.3[ns]
2	Control	51.0	49.0
	VCD	39.1[ns]	60.9[ns]
3	Control	49.0	51.0
	VCD	46.7[ns]	53.3[ns]
4	Control	42.2	57.8
	VCD	42.9[ns]	57.1[ns]

[†] Sex ratio of pups between control and treated groups in each breeding round were compared using Chi-square test; values with [ns] are not significantly different from control ($P>0.05$).

2.4 Discussion

Body weight and non-reproductive organs

In this study, during each 10 day period of treatment with VCD (500 mg/kg) the body weights of treated animals declined over the first several days, but were maintained or increased thereafter. This weight loss was most likely due to poor appetite caused by the toxicity of VCD. However, as the liver enzymes that detoxify VCD became upregulated (Salyers, 1995), this effect dissipated. Body weight losses have been observed in other studies. For example, compared to controls, female rats receiving oral treatment with VCD (500mg/kg and 1000 mg/kg) for 13 weeks lost 7% and 20% of body weight respectively (Chhabra et al., 1990a).

The weights of non-reproductive organs, including liver, kidneys, adrenals, spleen, heart and lungs in VCD treated animals were not significantly different from control animals for any of the treatment protocols in the present study. These results suggest that multiple periods of oral administration with VCD (500mg/kg) did not induce short or long term effects on the non-reproductive organs of female rats. Similarly, no adverse effects on the weights of liver, spleen, kidneys and adrenals were observed in mice following intraperitoneal administration with VCD at 160 mg/kg for 10 or 20 days (Lohff et al., 2006). However, Chhabra et al. (1990a) found significant increases in liver weights in female rats following 13-weeks of oral gavage with VCD at 500 mg/kg, and in liver and kidney weights at a dose of 1000 mg/kg. These differences were likely due to the substantially longer period of treatment and higher VCD doses compared to the present study and most likely reflect the detoxification processes occurring in these organs (Salyers, 1995).

The weights of ovaries and uteri

In the present study, there was no significant difference in normalised ovarian weights (mg/100 g body weight) between the control animals and those treated with VCD for one, two, or three periods and autopsied shortly after the last dose. Although significant reductions in the numbers of primordial and primary follicles were found in the ovaries of treated animals, these reductions were not detected in

a change in overall ovarian weight. Flaws *et al.* (1994) also reported no changes in ovarian weights in rats treated with VCD (80 mg/kg, i.p.) for 30 days.

Significant reductions in normalised ovarian weight were found in treated animals after 4 breeding rounds (Group 4) (ovaries collected on Day 220). Such reductions in ovarian weights have also been observed in mice on Day 135 after treatment with VCD (160 mg/kg, IP) for 10 days and on Day 52 after treatment for 20 days (Lohff *et al.*, 2006). The decrease in ovarian weights in treated animals reflects the absence of all follicle types and that ovarian failure has occurred over time following treatment (Lohff *et al.*, 2005; Lohff *et al.*, 2006).

When expressed as the absolute weight (mg/animal), the weights of ovaries in animals treated with VCD for two or three periods (Groups 2, 3 and 4) were significantly reduced compared to their corresponding controls. The decline in ovarian weights was similar to the sequential depletion in primary and secondary or larger follicles in these treated animals over time and reflects impending ovarian failure.

Ovarian follicles

Oral administration with VCD (500 mg/kg) for one, two and three periods of 10-days reduced the number of primordial follicles to 34%, 8%, and 2.3% respectively in treated compared to control animals. Significant reductions in primary follicle numbers were also found in the animals treated with VCD for two, or three 10-day periods. These results confirm and enhance previously reported observations on follicular depletion (Chhabra *et al.*, 1990a; Flaws *et al.*, 1994; Springer *et al.*, 1996b; Mayer *et al.*, 2002; Mayer *et al.*, 2004; Haas *et al.*, 2007). Indeed, the observed follicle depletion in this study was enhanced compared to the previous reports. For example, thirty consecutive days of treatment with VCD (80 mg/kg, i.p.) reduced the number of primordial follicles in rats to 19-31% compared to the control animals (Flaws *et al.*, 1994; Mayer *et al.*, 2002). A 40% reduction of primordial follicle number was found in rats after treatment with VCD at the same dose (80 mg/kg, i.p.) for 15 days (Springer *et al.*, 1996b). These different responses are most likely due to the differences in doses, durations of treatment and timing of tissue collection. In the previous studies, animals were treated for a single period of 15 or 30 consecutive days and tissues collected shortly after the

last dose, whereas, in the present study, animals were treated for a period of 10 days, and this was repeated one or two times before the ovarian responses were analysed. This repeated administration with VCD resulted in cumulative effects on follicle depletion.

The effects of VCD on follicle populations observed in this study were dependant on the duration of treatment: the greater the number of treatment periods the greater the depletion in all follicle types in the ovary. The results confirm similar observations of dose and time dependant effects of VCD in rats and mice (Springer et al., 1996b; Kao et al., 1999; Devine et al., 2004; Haas et al., 2007). These responses may be explained by the metabolic capacity of the ovary to protect against the ovotoxicity of VCD. If lower doses of VCD are used, longer durations of exposure are required for sufficient accumulation of the bioactive toxicant to induce follicle depletion. Conversely, if higher doses that can overcome the ovarian metabolic capabilities are used, shorter durations of exposure can be sufficient to achieve the same effect (Devine et al., 2004).

VCD has been known to directly target primordial and primary follicles through accelerating the natural process of follicular atresia (Springer et al., 1996b; Borman et al., 1999; Kao et al., 1999). The toxic effects of VCD on the oocytes contained in these ovarian follicles might be through a direct or indirect mechanism (Hoyer and Sipes, 1996). The ovary contains enzymes responsible for biotransformation and detoxification of many xenobiotics. The rat ovary contains epoxide hydrolase, glutathione-S-transferases, and cytochrome P450, which metabolize ovarian toxicants (Mukhtar et al., 1978; Mattison and Thorgeirsson, 1979; Bengtsson and Rydstrom, 1983; Bengtsson et al., 1992). Consequently, biotransformation of a chemical might occur within the ovary and thus oocytes of certain types of follicles could be directly exposed to its toxic effects. Additionally, as oocytes at all stages of follicular development are surrounded by granulosa cells, the toxic effects of a chemical on the oocytes might be indirect if it targets the granulosa cells, resulting in a loss of their ability to maintain the viability of the oocyte (Buccione et al., 1990). The mechanism of the ovarian toxicity of VCD on the primordial and primary follicles has been reported to involve acceleration of the normal rate of atresia of the oocyte, intracellular targeting of mitochondrion-mediated pathways of apoptosis and requires repeated exposure (Hoyer et al., 2001). The present study has not

examined the mechanism of enhanced atresia or apoptosis, but confirms the ovotoxic effects of VCD on primordial and primary follicles in female laboratory rats.

In control animals, the primordial follicle pool declined over time as might be expected with age. There was a similar pattern of follicle reduction in the animals treated with VCD; however, the significant initial reduction in the number of primordial follicles caused by VCD treatment resulted in essentially complete loss of all follicles in the ovaries of animals after 4 breeding rounds (Day 220). This was not the case with control animals at that time, in which cohorts of all follicle types were still available to undergo development and ovulation at that time.

For the growing follicles (secondary and larger follicles), significant follicle reductions were observed in the animals treated with VCD for two or three periods of 10 days. As VCD selectively targets and destroys primordial and primary follicles, reductions in the number of these growing follicles may be due to the initial depletion of the non-regenerating primordial and primary follicles which otherwise are recruited to become growing follicles. If the majority of the primordial and primary follicle pools are depleted, recruitment into larger follicles can no longer occur, and ultimately will lead to ovarian failure and permanent infertility (Hirshfield, 1991b; Hoyer and Sipes, 1996). Significant losses of growing and antral follicles were also reported in female rats following 30 days of intra-peritoneal administration of VCD (80 mg/kg) (Mayer *et al.*, 2002). However, there were no significant effects on growing or preantral follicles in female rats following 6, 8, 10 or 12 days of treatment with VCD (80 mg/kg, i.p.) (Springer *et al.*, 1996b; Kao *et al.*, 1999). It is difficult to compare these results directly with those of the present study as the doses and routes of administration were different. The lack of effect in the previous studies, however, was most likely due to the shorter duration of treatment.

Reduced numbers of atretic follicles (secondary and larger) were found in the animals following two or three periods of treatment with VCD when compared to the control animals. These reductions of atretic follicles in the treated animals may be as a consequence of a reduction in the number of healthy secondary and larger follicles, which otherwise may potentially become naturally atretic during their development.

Oestrous cycles

In the present study, the oestrous cycles of female rats were not affected following oral administration of VCD (500 mg/kg) for one or two periods of 10 days. Histological assessment of ovaries in animals treated for one period (Group 1) and two periods (Group 2) showed large numbers of developing follicles (secondary and larger follicles) and corpora lutea and corpora albicantia. As such, this explains ongoing recruitment of these follicles leading to ovulation and normal oestrous cycle patterns of the treated animals at this time after VCD treatment. Flaws *et al.* (1994) reported no effects on oestrous cycles following intraperitoneal administration with VCD (80 mg/kg) for 30 consecutive days where the rats were immature at the onset of treatment. However, this treatment induced prolonged oestrous cycles in rats that were mature at the onset of dosing. Prolonged oestrous cycles were also found during Days 349-360 in rats treated with VCD (80 mg/kg, i.p.) for 30 days and was associated with increases in FSH concentrations indicating ovarian failure (Mayer *et al.*, 2002). Even though oestrous cycles were not directly monitored during the 4 breeding rounds (Group 4) in this study, the time to conception of animals which conceived were similar between control and treated animals and between breeding rounds indicating normal oestrous cyclicity in these animals. However, those treated animals that did not conceive during the study were presumably not undergoing oestrous cycles as they were experiencing progressive premature ovarian failure.

Fertility

In the first breeding round, there was no significant reduction in the number of animals giving birth and the litter sizes in the treated animals compared with control animals. Because VCD targets only primordial and primary follicles, its effects on breeding outcomes is dependent on the proportion of follicles destroyed. Although a small proportion of primordial and primary follicles remained in the ovaries (2.3 and 6% of control, respectively) of the animals following 3 periods of treatment (Group 3), a higher proportion of larger follicles (22% of control) were still present. These growing follicles could develop, ovulate and release oocytes for fertilization and this would account for the normal fertility in the 1st breeding round in the treated animals (Group 4). From the 2nd to the 4th breeding round, the number of

animals giving birth and their litter sizes in the treated group progressively declined compared to the control group. This reduced fertility over time would appear to be due to the initial depletion of a majority of the non-regenerating primordial and primary follicles, leading to a progressive decline in the number of secondary and larger follicles remaining in the ovaries. Certainly the ovaries from the treated animals killed at the end of the 4th breeding round showed an almost complete loss of primordial, primary and growing follicles (secondary or larger follicles) (2%, 0.9% and 3.3 % of control, respectively), and this would explain the significant reduction in fertility observed in treated animals by this time. Six of the eight animals in the treated group did not have pups in the 4th breeding round indicating premature ovarian failure had occurred in these animals.

Previous studies reported that treatment of female mice with VCD (160 mg/kg, i.p.) for 17 days did not induce a reduction in pregnancy rates or number of live foetuses, when the animals were mated soon after dosing; whereas a significant reduction in fertility was found in treated animals that were mated on day 20 after dosing (Haas *et al.*, 2007). Significant decreases in the number of implanted embryos and rate of implantation, and an increase in pre-implantation losses were also found in female rats following treatment with VCD (160 mg/kg, i.p.) from two weeks before mating to day 7 of gestation (Kodama *et al.*, 2009). In another study in mice given VCD (160 mg/kg i.p.) for 10 or 20 days, the average time to ovarian failure was Day 135 and Day 52 respectively (Lohff *et al.*, 2006). Thus in these studies and in the present study, the duration of treatment and time since treatment are important considerations in ensuring that infertility is achieved.

This study has clearly demonstrated that repeated periods of oral administration with VCD (500 mg/kg) enhanced follicle depletion and induced a delayed but permanent infertility in female rats. With respect to fertility control of pest rodents, such as the ricefield rat, the use of multiple periods of treatment in this study mimics VCD's possible application in the field - the chemosterilant would be applied several times during the late fallow period and soon after planting, and certainly before breeding could be triggered at the maximum tillering stage of the rice crop. Taken together, VCD has potential as a chemosterilant for fertility control of pest rodents although the duration of treatment would need to be considerably shorter for practical field delivery. Findings from this study will contribute to the

development of a chemosterilant as an additional tool for population management of pest rodents.

If we consider the ricefield rat as a target species, then similar experimental studies of the effects of VCD in this species need to be undertaken before further studies in enclosures or at the field scale. In a preliminary study in wild ricefield rats some depletion of the primordial follicle population after oral gavage (500 mg/kg for 15 consecutive days) was observed (N. Herawati and L. Hinds, personal communication). However, no studies of the effects of VCD treatment on fertility have been conducted.

CHAPTER 3

Effects of 4-vinylcyclohexene diepoxide on the reproductive status of male rats (*Rattus norvegicus*)

Abstract

4-vinylcyclohexene diepoxide (VCD) induces depletion of non-regenerating primordial and primary follicles in female rats and mice, but its effects on male reproduction have not been determined. This study investigated the effects of oral administration of VCD on reproductive tissues of juvenile and adult male Sprague Dawley rats (*Rattus norvegicus*) as well as effects on fertility.

Three groups of male rats were used. *Group 1* (juveniles, 6 weeks old, n= 27) and *Group 2* (adults, 8-10 weeks old, n=27) were orally given either corn-oil (control, n=9) or VCD (500 mg/kg body weight, n=18) for 15 consecutive days. After the final dose, a subset of control animals (n=3) was killed at Days 16, 60 and 90, and of the treated animals (n=3) at Days 16, 30, 45, 60, 75 and 90. The body weights and reproductive organ weights were recorded and testes and epididymides fixed for histological assessment. *Group 3* (adults, 8-10 weeks old, n=16) were used to assess the effect of VCD on breeding outcomes. Following 15 days of oral treatment with oil (n=8) or VCD (500mg/kg/day) (n=8) the rats were allowed to mate with different fertile, untreated females over five breeding rounds to assess their fertility. These males were killed at day 95 for collection and assessment of reproductive tissues.

The weights of reproductive organs as well as the histological characteristics of the testes and epididymides of treated juvenile and adult animals were not significantly different to control animals at any time after VCD treatment. All *Group 3* males sired pups and litter sizes of females mated with either treated or control animals were not significantly different within or between breeding rounds. There was no difference in the time intervals from pairing with fertile females to birth of litters between the control and treated groups. Sex ratios of pups and pup weights were not affected by VCD treatment. Normal histological characteristics of the testes and epididymides were observed in the control and treated males at the end of the breeding period.

This study demonstrated no effect of oral treatment with VCD on either reproductive tissues or breeding outcomes of male rats. The results are in contrast to one study in male mice and could indicate that the antifertility effects of VCD show species and/or sex specificity.

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3.1 Introduction

The industrial chemical, 4-vinylcyclohexene (VCH) and its various metabolites, including 4-vinylcyclohexene diepoxide (VCD), have been shown to induce destruction of gonadal germ cells in rodents (National Toxicology Program, 1986, 1989; Smith et al., 1990; Hooser et al., 1995). Mice are more sensitive to VCH and its metabolites than rats (Chhabra et al., 1990; Smith et al., 1990 ; Kao et al., 1999), and there appears to be sex differences in responses (Hooser et al., 1995). Route of delivery (e.g. oral or intraperitoneal injection) also influences the degree of germ cell destruction (National Toxicology Program, 1986, 1989; Chhabra et al., 1990; Hooser et al., 1995).

In female rats and mice, VCD induces a significant depletion of primordial and small primary follicles, leading to premature ovarian failure (Flaws et al., 1994; Springer et al., 1995; Springer et al., 1996a; Borman et al., 1999; Kao et al., 1999; Devine et al., 2001; Hoyer et al., 2001; Mayer et al., 2002; Devine et al., 2004; Mayer et al., 2004; Lohff et al., 2005; Lohff et al., 2006; Haas et al., 2007; Hoyer et al., 2007; Sahambi et al., 2008; Ito et al., 2009; Muhammad et al., 2009). However, relatively few studies of the effects of VCD on reproduction of males have been conducted. In the general toxicity studies of VCD on rats and mice, the National Toxicology Program (1989) and Chhabra et al. (1990a) reported that 13 weeks of exposure to VCD induced germ cell destruction in male mice but not in rats when administered orally (500 and 1000 mg/kg body weight). Histological examination of the testis of VCD-treated mice indicated testicular degeneration, characterized by a decrease in the number of germ cells within the seminiferous tubules. According to Hooser et al. (1995), intraperitoneal administration of VCD in male mice induced a reduction in testis weight and caused significant dose-dependent testicular toxicity.

Although the effects of VCD in terms of general toxicity have been evaluated in male rats (National Toxicology Program, 1989; Chhabra et al., 1990), neither the reproductive effects due to age nor the breeding outcomes were considered. Therefore, the primary objective of this study was to investigate the effects of oral treatment with VCD on the reproductive tracts of juvenile and adult male rats (*Rattus norvegicus*). In addition, breeding outcomes of adult male rats following a period of treatment with VCD were investigated.

3.2 Materials and methods

3.2.1 Chemical

One batch of 4-vinyl-1-cyclohexene diepoxide (97%) was purchased from Sigma Chemical Co., St. Louis, MO, USA. VCD was diluted in corn-oil in order to prepare doses on a per kg body weight basis immediately before each gavaging period. The dose (500 mg/ kg body weight) used in this study was based on previous studies that showed this dose was highly effective in ovarian follicle depletion on female rats (L. Hinds *et al* unpublished results).

3.2.2 Animals

Male laboratory rats of two age groups (juvenile, 6 weeks old, and adult 8-10 weeks old) were obtained from the Animal Resources Centre, Western Australia. The rats were assigned to weight classes and allocated to experimental groups according to a table of random numbers. Two animals were housed in each cage (52cm x 36cm x 21cm) and shelters provided. They were kept in an air-conditioned room at a temperature of $22^{\circ}C \pm 2^{\circ}C$ and exposed to 12 hours of light (0600-1800h). Rats were fed with standard rat chow (Gordon's Specialty Stockfeeds, NSW, Australia) and water *ad libitum* throughout the course of the experiment. The use of the animals was approved by the CSIRO Sustainable Ecosystems Animal Ethics Committee (Number 08-03).

3.2.3 Method of administration

Oral administration of corn oil or VCD was employed under light anaesthesia. Anaesthesia was administered by inhalation using 4% Isoflurane in oxygen, administered via a VetQuip Compact Anaesthetic machine with a Tec 5 Isoflurane vaporiser. All animals were given either an oral gavage of corn-oil (control) or VCD (500 mg/kg) in corn oil using a gavage needle (curved stainless steel metal, 2.5mm ball-tipped; 16-18g x 75mm) which was attached to a 1 ml syringe. The oral administration procedure was undertaken from 13:00h to 16:00h each day, for 15 consecutive days. Animals were observed for their responses to the oral gavage as they recovered from the anaesthetic. Some overt responses to dosing were observed and included gastric reflux, salivation and sneezing.

3.2.4 Experimental design

Effects on reproductive tracts

Male rats of two age groups: *Group 1* (juvenile, 6 weeks old, n = 27) and *Group 2* (adult, 8-10 weeks old, n = 27) were used. In each group, animals received either corn-oil (control, n = 9) or VCD (500mg/kg, n = 18) orally for 15 consecutive days. After the treatment period, animals (n = 3) were euthanized on each of days 16, 60 and 90 for the control group and on days 16, 30, 45, 60, 75 and 90 for the VCD treatment group. At autopsy, reproductive and non-reproductive tissues were collected and weighed. Left testes and epididymides were then prepared for histological assessment (see below). In rats, the length of the spermatogenic cycle is 12.9 days (Clermont and Harvey, 1965); therefore, the autopsy time line used in this study was conducted to assess any short-term or long-term effect of VCD treatment on spermatogenesis of treated animals over approximately 5 cycles.

Effects on fertility

A group of adult male rats (8-10 weeks old, n = 16) (*Group 3)* were used for the breeding trial. Initially, animals received either corn-oil (control, n=8) or VCD (500mg/kg, n=8) orally for 15 consecutive days. To assess their fertility, each control and treated male rat was paired with two fertile, untreated females at 14 day intervals from Day 16 to Day 80. Males were rotated between different pairs of females to avoid any effects of females on breeding outcomes. The females were monitored for pregnancy and weighed twice weekly throughout the experiment. When births were expected, females were checked for the birth of pups daily. The litter size of the females was determined and pups removed as soon as posible after birth, sexed, and weighed before euthanasia. At the end of the last mating, all male rats were euthanased, and their reproductive tissues collected for histological assessment. The experimental design is summarised in Fig. 3.1.
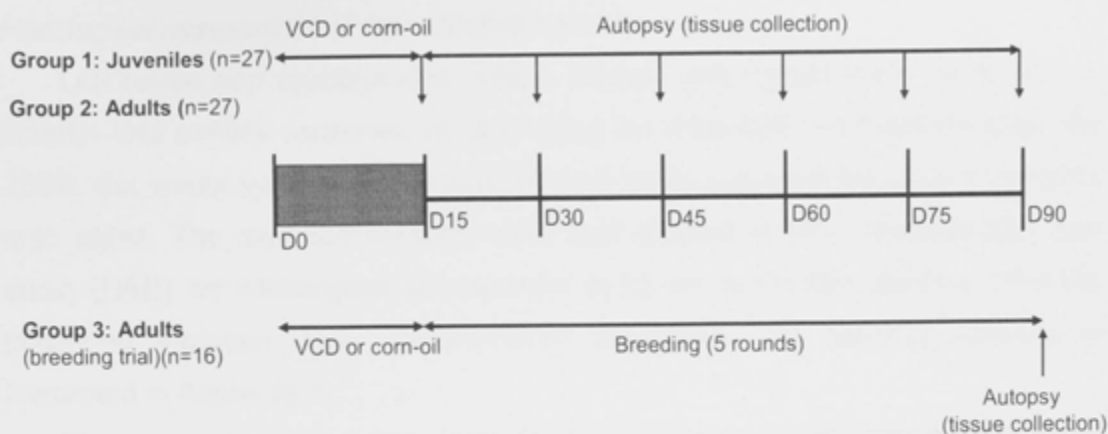
Figure 3.1 Schematic diagram of experimental protocol. Note: control animals (n=3) in Group 1 and Group 2 were autopsied on days 16, 60 and 90 only.

3.2.5 Data collection

Body weight and weights of reproductive and non-reproductive organs

All animals were weighed daily shortly before oral gavage, then twice a week and at autopsy. Prior to autopsy, the animals were euthanased with an overdose of Lethobarb (Virbac Australia Pty. Limited, NSW) (162.5mg/kg), administered intra-peritoneally. Reproductive tissues, including testes, epididymides, seminal vesicles and prostate were collected and weighed.

The length and width of each testis was measured and the testis volume was calculated using the formula for the volume of a prolate spheroid;

$$V = \frac{4}{3} \pi a^2 b$$

where;   V = volume (cm^3)
          a = 0.5 x testis width
          b = 0.5 x testis length

The collected tissues were fixed in 10% neutral buffered formalin or Bouin's for 24-48h. Left testes and epididymides were then prepared for histological assessment. Other internal tissues, including kidneys, liver and adrenals were also collected, weighed and fixed in formalin 10% (v/v) for later use as necessary. In addition, observations of the general appearance of lungs, heart, spleen, oesophagus, stomach, small intestine, buccal cavity, and brain were made at the time of autopsy.

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Histological assessment of reproductive tissues

Left testes and epididymides (caput, corpus, and cauda) were embedded in paraffin and serially sectioned (5 μm) using an automatic microtome (Leica RM 2255). Six randomly-selected sections of each testis and epididymis were mounted onto slides. The mounted sections were then stained in Gill's haematoxylin and eosin (H&E) for histological assessment using an automatic staining machine (Shandon Varistain 24-3, ThermoFisher Scientific). The staining protocol is presented in Appendix 1.

Three stained sections in each testis and of each part of the epididymis (caput, corpus and cauda) were selected for assessment using a light microscope (Diaplan, Leitz Wetzlar Germany). For each selected section of testis, 20 randomly selected seminiferous tubules were examined for the presence of the varying stages of testicular cells (Sertoli cells, spermatogonia, spermatocytes, spermatids) and spermatozoa in the lumen. For each selected sections of the epididymis (caput, corpus and cauda), 20 randomly selected cross sections of the tubule were observed for the presence of the lumen and spermatozoa.

Litter size

The fertility of the control and treated males was assessed based on the breeding outcomes of pairing with untreated, fertile females. The average litter sizes over five breeding rounds were determined, and the times from mating to birth were recorded. Pups from the litters were removed, sexed, and weighed before euthanasia.

3.2.6 Data analysis

Means of body weight (g), reproductive and non-reproductive organ weights, normalised to body weight (g/kg body weight), and litter sizes of the untreated females mated with males in the control and treated groups were compared by Student's t-tests with a significance level of 5%, using the SPSS software package (Version 16). The absolute weights of reproductive tissues (g/animal) between control and treated animals were also compared. All values are presented as mean ± standard deviation (SD).

3.3 Results

3.3.1 Effects of VCD treatment on body weight

Groups 1 and 2: During the first 2-3 days of the treatment period, the body weights of the treated animals in both age groups declined. Thereafter body weights were maintained or increased again but remained at a lower level than the control animals (Fig. 3.2a, b). The body weights in VCD-treated animals were significantly lower ($P < 0.05$) than that of the control animals from Day 13 (for juvenile group, Group 1), and from Day 3 (for adult group, Group 2) to the end of treatment.

At the end of the treatment period, the body weights of the juvenile and adult rats given VCD were, respectively, 10% and 12% lower than those of the matching control animals and these differences were significant ($P < 0.05$). There were no significant differences in body weight between the control and treated animals at Day 30, 45, 60, 75 and 90 days after the onset of treatment period (Table 3.1)

Group 3: Similar trends in the changes of body weight were observed in Group 3 compared to Group 1 and 2; body weights of the treated animals declined over the first 2-3 days of the treatment period, and maintained or gained thereafter. At the end of the treatment period, body weights of treated animals were significantly lower than the control animals, but thereafter body weights of treated animals gradually increased overtime. Although their body weights were consistently lower than control animals between days 20 and 95, the differences were not significant (Fig. 3.3).

3.3.2 Effects of VCD treatment on weights of non-reproductive tissues

There was no significant difference ($P > 0.05$) in the weights of the non-reproductive organs (g/kg body weight) (livers, kidneys, adrenals, spleens, hearts and lungs) between the animals treated with VCD and the control animals in either the juvenile group (Table 3.2) or adult group (Table 3.3) at any time after treatment. These organs and the appearance of the oesophagus, stomach, small intestine, buccal cavity, and brain at autopsy appeared normal in both the treated and control animals. The results suggest that oral administration of VCD (500mg/kg) for 15 days induced no adverse effects on the non-reproductive organs of male rats.
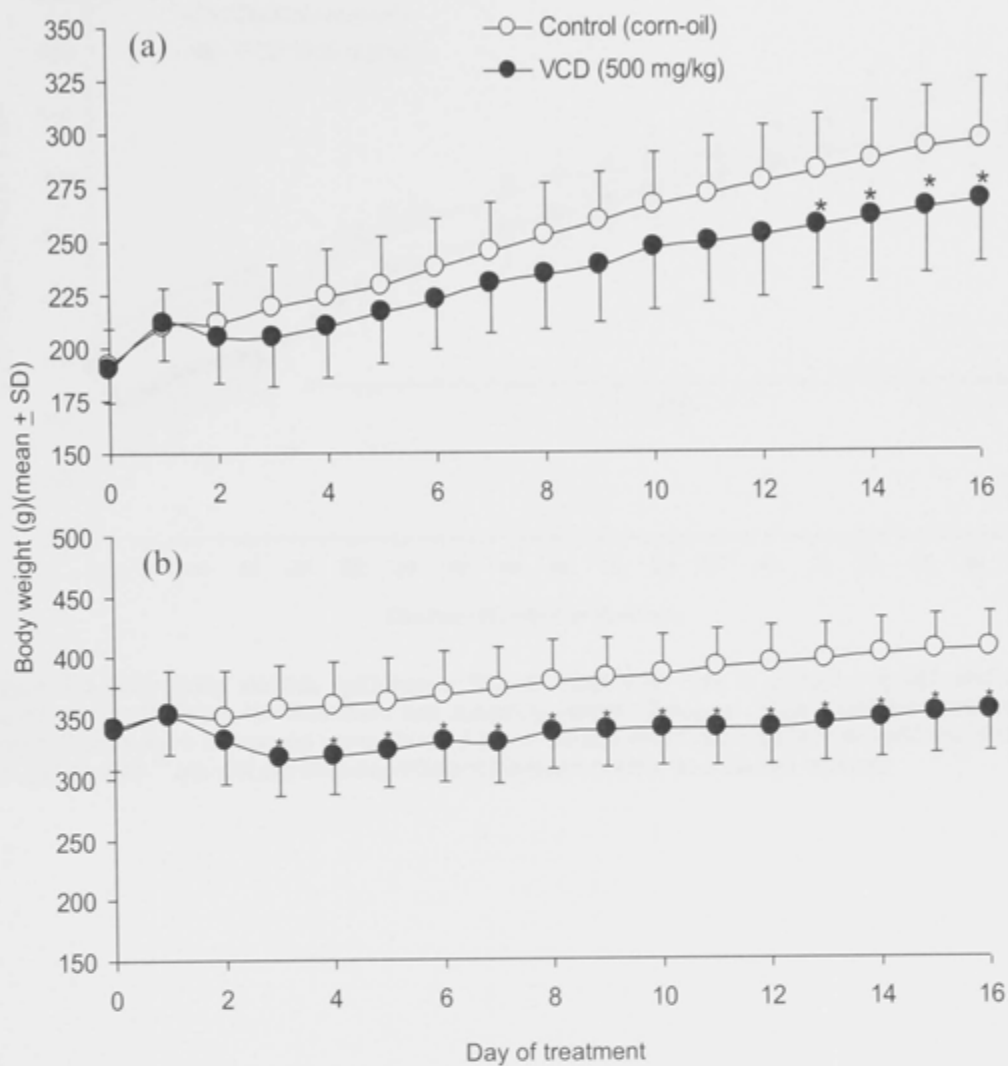
Figure 3.2 Daily body weights (g)(Mean ± SD) of control (○) and VCD-treated (●) male rats over the 15-day period of treatment for (a) juveniles (Group 1) (n=9 controls; n=18 treateds) and (b) adults (Group 2) (n=9 controls; n=18 treateds). Means between control and treated groups were compared using Student *t-test*; values with * are significantly different from control animals (P<0.05).
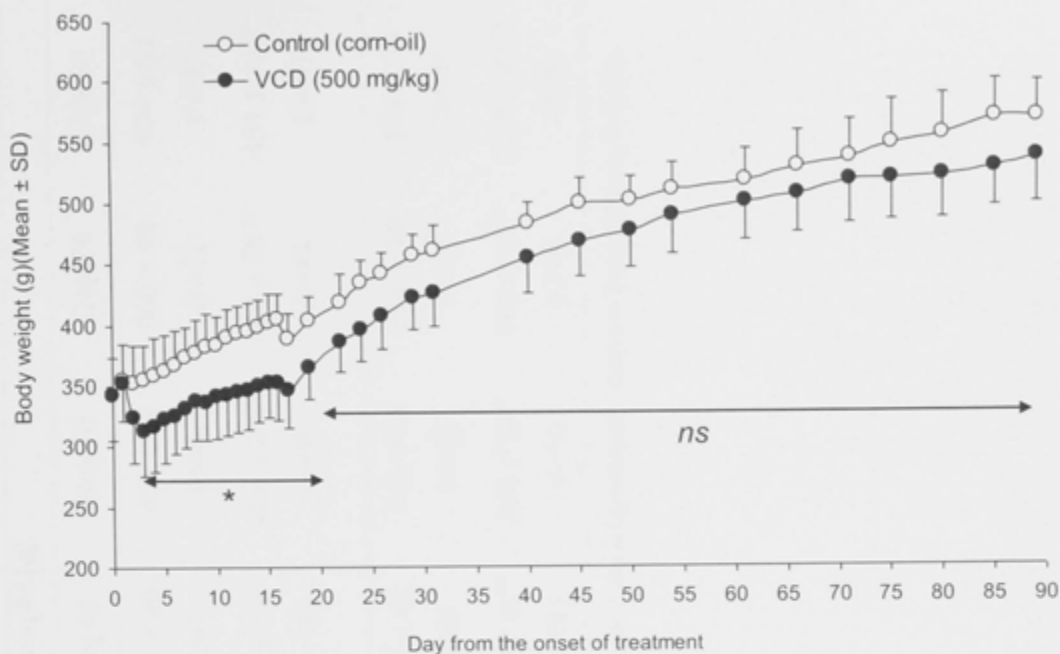
Figure 3.3 Daily body weights (g)(Mean ± SD) of adult male rats in control (○)(n=8) and VCD-treated (●) (n=8) during the treatment and breeding period (Group 3). Means between control and treated groups were compared using Student *t-test*; values with * are significantly different (P<0.05) and values with ^{ns} are not significantly different between control and treated animals.

Table 3.1 Body weight (g)(Mean ± SD) of control and treated male rats at the start of experiment and at the different times of autopsy in juvenile and adult groups

	Treatment	Body weight (g)						
		Day 0	Day 16	Day 30	Day 45	Day 60	Day 75	Day 90
Juvenile	Control	192 ± 17 (n=9)	296 ± 29 (n=9)	365 ± 25 (n=6)	419 ± 29 (n=6)	451 ± 41 (n=6)	502 ± 49 (n=3)	534 ± 53 (n=3)
	VCD	190 ± 16^{ns} (n=18)	268 ± 29* (n=18)	337 ± 37^{ns} (n=15)	405 ± 31^{ns} (n=12)	435 ± 28^{ns} (n=9)	464 ± 39^{ns} (n=6)	481 ± 14^{ns} (n=3)
Adult	Control	338 ± 37 (n=9)	404 ± 32 (n=9)	431 ± 36 (n=6)	474 ± 42 (n=6)	493 ± 53 (n=6)	512 ± 58 (n=3)	538 ± 53 (n=3)
	VCD	340 ± 33^{ns} (n=18)	354 ± 34* (n=18)	418 ± 30^{ns} (n=15)	463 ± 33^{ns} (n=12)	486 ± 35^{ns} (n=9)	492 ± 24^{ns} (n=6)	518 ± 28^{ns} (n=3)

Means between control and treated groups were compared using Student *t-test*. Values with * were significantly different from control (P<0.05), and values with ^{ns} are not significantly different from control.

Table 3.2 Weights (g/kg body weight)(Mean ± SD) of non-reproductive organs of juvenile male rats following 15 days of oral gavage with either corn oil or VCD (500mg/kg)

Organs	Treatment	Weight (g/kg body weight) at autopsy					
		Day 16	Day 30	Day 45	Day 60	Day 75	Day 90
Liver	Control	42.61 ± 0.23			37.69 ± 1.76		40.88 ± 1.63
	VCD	42.15 ± 1.56^{ns}	45.44 ± 4.84	42.46 ± 3.10	39.50 ± 1.77^{ns}	43.61 ± 2.97	38.20 ± 0.87^{ns}
Kidneys	Control	7.86 ± 0.42			6.94 ± 0.80		6.73 ± 0.04
	VCD	7.87 ± 0.37^{ns}	7.58 ± 0.07	6.76 ± 0.28	6.92 ± 0.28^{ns}	6.95 ± 0.26	6.96 ± 0.35^{ns}
Adrenals	Control	0.46 ± 0.04			0.23 ± 0.03		0.17 ± 0.04
	VCD	0.47 ± 0.15^{ns}	0.29 ± 0.07	0.28 ± 0.06	0.29 ± 0.04^{ns}	0.19 ± 0.04	0.18 ± 0.04^{ns}
Spleen	Control	1.98 ± 0.39			1.94 ± 0.14		1.62 ± 0.19
	VCD	2.02 ± 0.21^{ns}	1.99 ± 0.27	1.81 ± 0.17	2.10 ± 0.35^{ns}	1.62 ± 0.18	1.75 ± 0.02^{ns}
Heart	Control	3.42 ± 0.15			2.78 ± 0.11		3.19 ± 0.72
	VCD	3.88 ± 0.30^{ns}	2.98 ± 0.05	3.23 ± 0.19	2.72 ± 0.20^{ns}	3.03 ± 0.39	2.94 ± 0.18^{ns}
Lungs	Control	4.95 ± 0.28			3.70 ± 0.20		3.69 ± 0.14
	VCD	4.52 ± 0.04^{ns}	4.07 ± 0.40	3.92 ± 0.40	3.71 ± 0.36^{ns}	3.44 ± 0.35	3.45 ± 0.10^{ns}

Means between control and treated groups were compared using Student *t-test*. Values with ^{ns} are not significantly different from control (P>0.05).

Table 3.3 Weights (g/kg body weight)(Mean ± SD) of non-reproductive organs of adult male rats following 15 days of oral gavage with either corn oil or VCD (500mg/kg).

Organs	Treatment	Weight (g/kg body weight) at autopsy					
		Day 16	Day 30	Day 45	Day 60	Day 75	Day 90
Liver	Control	39.09 ± 2.08			38.98 ± 2.47		34.58 ± 1.96
	VCD	43.98 ± 4.55^{ns}	45.44 ± 4.84	42.46 ± 3.10	41.69 ± 1.02^{ns}	43.61 ± 2.97	36.80 ± 3.23^{ns}
Kidneys	Control	7.13 ± 0.58			6.24 ± 0.28		6.51 ± 0.62
	VCD	7.94 ± 0.66^{ns}	7.58 ± 0.07	6.76 ± 0.28	7.03 ± 0.18^{ns}	6.95 ± 0.26	6.91 ± 0.32^{ns}
Adrenals	Control	0.35 ± 0.12			0.25 ± 0.09		0.18 ± 0.03
	VCD	0.42 ± 0.07^{ns}	0.29 ± 0.07	0.28 ± 0.06	0.25 ± 0.01^{ns}	0.19 ± 0.04	0.22 ± 0.06^{ns}
Spleen	Control	1.54 ± 0.10			1.56 ± 0.20		1.76 ± 0.47
	VCD	1.68 ± 0.07^{ns}	1.99 ± 0.27	1.81 ± 0.17	1.50 ± 0.05^{ns}	1.62 ± 0.18	1.40 ± 0.31^{ns}
Heart	Control	2.92 ± 0.03			2.88 ± 0.17		2.86 ± 0.25
	VCD	3.28 ± 0.47^{ns}	2.98 ± 0.05	3.23 ± 0.19	2.71 ± 0.25^{ns}	3.03 ± 0.39	2.62 ± 0.03^{ns}
Lungs	Control	4.01 ± 0.31			3.24 ± 0.34		3.37 ± 0.20
	VCD	4.15 ± 0.33^{ns}	4.07 ± 0.40	3.92 ± 0.40	4.01 ± 0.11^{ns}	3.44 ± 0.35	3.46 ± 0.71^{ns}

Means between control and treated groups were compared using Student t-test. Values with ^{ns} are not significantly different from control (P>0.05).

3.3.3 Effects of VCD treatment on testis volume and the weights of reproductive tissues

Testis volume (cm^3/kg body weight) and the weights of reproductive tissues (testis, epididymis, seminal vesicle, and prostate) corrected for body weight (g/kg body weight) were not significantly different ($P>0.05$) between control and treated animals of the juvenile group (Table 3.4) and the adult group (Table 3.5) at any time after the treatment period. Testis volumes and the normalised weights of testes and epididymides declined over time in both the control and VCD-treated animals. These declines are due to the increase in the body weight of the animals during the experiment. The weights of reproductive organs when expressed as their absolute weight (g/animal) (data not shown) also showed no significant difference between control and treated animals.

3.3.4 Effects of VCD treatment on histological characteristics of testis and epididymis

Histological assessment of cross sections of testis from the animals following 15 days of oral gavage with VCD (500mg/kg) showed normal testicular structures. Normal germ cells at all stages of development (spermatogonia, spermatocytes, and spermatids), Leydig cells and Sertoli cells were present in all control as well as treated animals (Fig. 3.4). The epididymis of the treated animals showed normal epithelium and the lumen was filled with normal maturing spermatozoa (Fig. 3.5). These observations suggest that oral administration of VCD (500 mg/kg) for 15 days had no adverse effects on the spermatogenic cycle of male rats.

Table 3.4 Testis volume (cm^3/kg body weight) and the weights of reproductive organs (g/kg body weight)(mean ± SD) of the control and VCD-treated juvenile male rats. Animals were orally administered either corn-oil or VCD (500mg/kg) for 15 days then killed (n=3 per group) on days 16, 60 and 90 in the control group, and on days 16, 30, 45, 60, 75 and 90 in the VCD treated group

Organs	Treatment	Day of autopsy					
		16	**30**	**45**	**60**	**75**	**90**
Testis volume	Control	7.96 ± 0.80			7.08 ± 0.37		6.20 ± 0.62
	VCD	9.98 ± 1.42 ^{ns}	9.64 ± 0.75	7.95 ± 1.07	7.65 ± 0.29 ^{ns}	6.89 ± 0.51	7.77 ± 0.34 ^{ns}
Testis weight	Control	9.69 ± 1.20			7.42 ± 0.44		6.28 ± 0.46
	VCD	11.81 ± 0.89 ^{ns}	9.24 ± 0.59	7.99 ± 0.71	7.75 ± 0.37 ^{ns}	7.10 ± 0.72	7.32 ± 0.29 ^{ns}
Epididymis	Control	7.05 ± 0.34			5.43 ± 0.42		5.14 ± 0.78
	VCD	7.31 ± 1.07 ^{ns}	7.05 ± 0.62	6.38 ± 0.75	5.30 ± 0.17 ^{ns}	5.31 ± 0.82	6.37 ± 0.51 ^{ns}
Seminal vesicle	Control	3.28 ± 0.25			4.92 ± 0.86		2.94 ± 0.42
	VCD	3.29 ± 0.13 ^{ns}	3.97 ± 1.21	4.06 ± 0.32	5.42 ± 0.39 ^{ns}	3.36 ± 0.31	3.02 ± 0.53 ^{ns}
Prostate	Control	1.41 ± 0.27			1.81 ± 0.20		1.70 ± 0.53
	VCD	1.45 ± 0.15 ^{ns}	1.17 ± 0.32	1.56 ± 0.19	1.42 ± 0.18 ^{ns}	1.68 ± 0.19	2.05 ± 0.35 ^{ns}

Means between control and treated animals at each time point were compared using Student *t-test*. Values with ^{ns} are not significantly different from control (P>0.05).

Table 3.5 Testis volume (cm^3/kg body weight) and the weights of reproductive organs (g/kg body weight)(mean ± SD) of the control and VCD-treated adult male rats. Animals were orally administered either corn-oil or VCD (500mg/kg) for 15 days then killed (n=3 per group) on days 16, 60 and 90 in the control group, and on days 16, 30, 45, 60, 75 and 90 in the VCD treated group.

Organs	Treatment	Day of autopsy					
		16	30	45	60	75	90
Testis volume	Control	6.44 ± 0.40			7.16 ± 1.00		6.04 ± 0.30
	VCD	7.98 ± 1.21 ^{ns}	8.63 ± 1.68	7.21 ± 0.74	6.12 ± 1.13 ^{ns}	6.95 ± 1.03	5.21 ± 1.35 ^{ns}
Testis weight	Control	7.55 ± 0.51			7.04 ± 0.52		6.34 ± 0.43
	VCD	8.64 ± 1.09 ^{ns}	9.01 ± 0.67	7.39 ± 0.22	5.87 ± 2.05 ^{ns}	6.72 ± 1.40	6.01 ± 2.06 ^{ns}
Epididymis	Control	6.01 ± 0.48			5.25 ± 0.83		4.97 ± 0.32
	VCD	6.79 ± 0.71 ^{ns}	6.61 ± 1.55	5.38 ± 0.63	4.03 ± 0.82 ^{ns}	5.14 ± 0.59	4.49 ± 0.42 ^{ns}
Seminal vesicle	Control	3.38 ± 0.49			4.74 ± 0.67		3.66 ± 0.29
	VCD	2.86 ± 0.20 ^{ns}	4.91 ± 1.01	4.24 ± 0.79	3.75 ± 0.68 ^{ns}	3.93 ± 0.21	3.78 ± 0.07 ^{ns}
Prostate	Control	1.49 ± 0.23			1.87 ± 0.28		2.42 ± 0.88
	VCD	1.41 ± 0.53 ^{ns}	1.41 ± 0.11	1.78 ± 0.18	1.58 ± 0.40 ^{ns}	1.96 ± 0.20	2.21 ± 0.42 ^{ns}

Means between control and treated animals at each time point were compared using Student *t-test*. Values with ^{ns} are not significantly different from control (P>0.05).
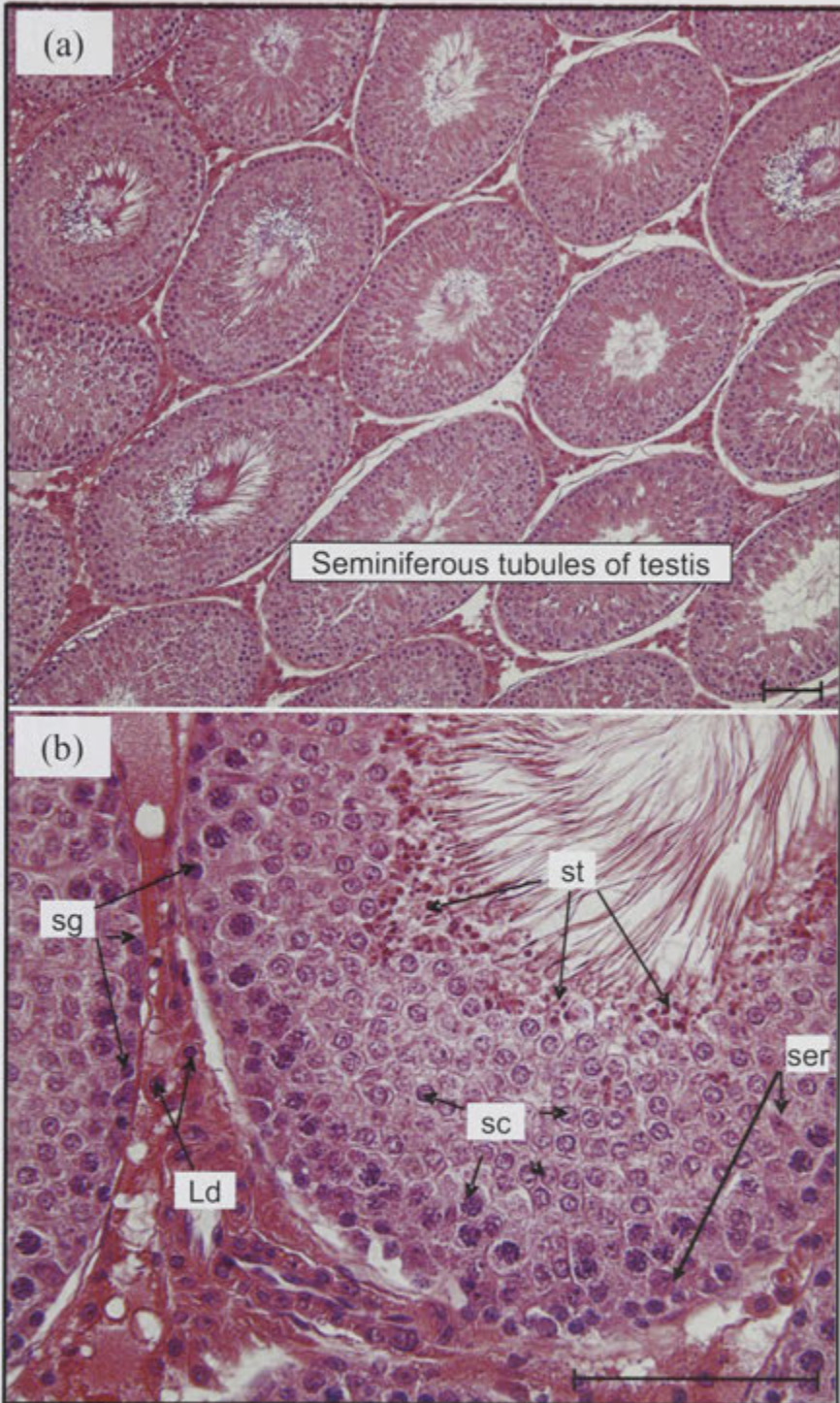
Figure 3.4 Histology of the testis. Representative cross-section of seminiferous tubules from a rat dosed with VCD (500 mg/kg, orally) for 15 days and killed at 24 hours after the last dose showing all normal stages of spermatogenesis (Ld: Leydig cells, ser: Sertoli cells, sg: Spermatogonia, sc: Spermatocytes, and st: Spermatids). Stain: haematoxylin and eosin; scale bar = 100 um; (a) x100 and (b) x400.
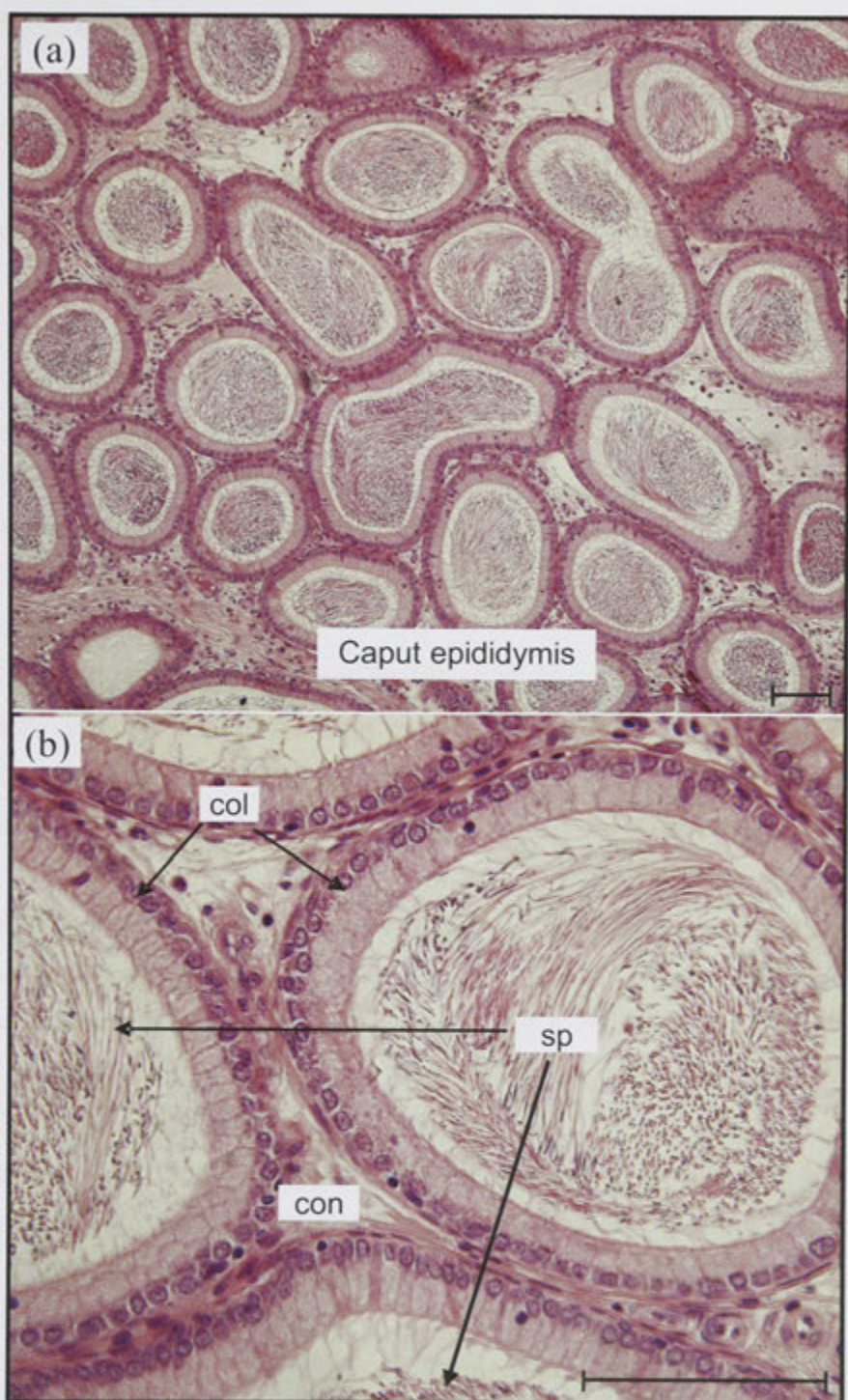
Figure 3.5 Histology of epididymis. Representative cross-section of caput epididymis from a rat dosed with VCD (500 mg/kg, orally) for 15 days and killed 24 hours after the last dose showing normal epithelium with well-defined basal and principal cells and the lumen filled with normal maturing spermatozoa (col: Columnar epithelium, sp: Spermatozoa, and con: Connective tissues). Stain: haematoxylin and eosin; scale bar = 100 um; (a) x100 and (b) x400.

3.3.5 Effects of VCD treatment on fertility

For all males in the control group, after mating with a pair of fertile females, at least one female of each pair produced litters in every breeding round. These findings indicate that all the control males were fertile throughout the trial. In the VCD-treated group, 7 of 8 males were fertile because at least one female in a pair became pregnant and had pups when mated with these males in every breeding round. Only one male in this group did not produce any pups when mated with any pair of fertile females over the breeding period, suggesting this male was infertile. The volume of the testes of this male was only 3.64 cm^3/kg compared to the average of 7.6 cm^3/kg in other animals of the same group. Histological assessment of the testis of this animal showed absence of germ cells at any development stage. This result may have been due to natural infertility of this rat occurring before treatment.

Litter sizes of females mated with either treated or control animals were not significantly different ($P > 0.05$) within or between breeding rounds (Fig. 3.6). The average number of pups per litter was approximately 14.3 ± 2.7 when females mated with control males and 13.2 ± 3.9 when mated with VCD-treated males.

3.3.6 Effects of VCD treatment on time interval from mating to birth, sex ratio of pups and pup weights

There was no significant difference ($P > 0.05$) in the time interval from mating to birth of females mated with either control (24.5 ± 2.7 days) or treated males (26.1 ± 3.4 days) at any breeding round (Table 3.6).

The sex ratio of pups produced from females paired with the control and treated males were not significantly different over 5 breeding rounds (Table 3.7). In addition, there was no significant difference in the birth weights of pups sired by the control and treated males (Table 3.8).
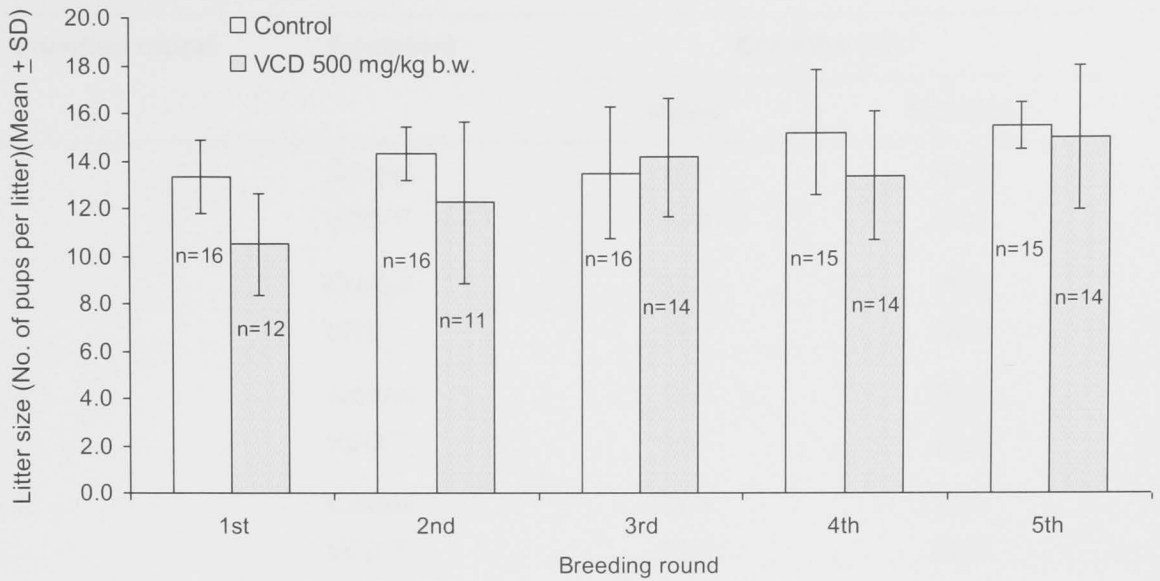
Figure 3.6 Effects of VCD exposure on litter size (Mean ± SD) of fertile female rats paired with control or treated males following 15 days of oral gavage with VCD (500 mg/kg) over 5 successive breeding rounds. Each male was paired with two fertile females and litter size was calculated from females that gave birth in each breeding round (n). Mean litter sizes between control and treated animals in each round were compared using Student *t-test* at the significance level of 5%.

Table 3.6 Average time (days) (Mean ± SD) between introductions of control and VCD-treated males and subsequent births of fertile, untreated females for 5 breeding rounds

Treatment	Time interval from mating to births (days)				
	Round 1	Round 2	Round 3	Round 4	Round 5
Control	23.1 ± 1.2	24.4 ± 3.1	25.8 ± 3.4	24.5 ± 1.3	25.6 ± 4.8
VCD	23.3 ± 1.7^{ns}	26.5 ± 4.4^{ns}	25.9 ± 3.6^{ns}	27.0 ± 4.4^{ns}	27.9 ± 4.0^{ns}

Mean between control and treated groups were compared using Student *t-test*. Values with ^{ns} are not significantly different from control animals (P>0.05).

Table 3.7 Sex ratios of pups from control and VCD-treated animals over 5 breeding rounds.

Breeding round	Treatment	Sex ratio (%) [†]	
		Males	Females
1	Control	45.5	54.5
	VCD [ns]	52.8	47.2
2	Control	53.3	46.7
	VCD [ns]	45.4	54.6
3	Control	44.9	55.1
	VCD [ns]	51.5	48.5
4	Control	48.9	51.1
	VCD [ns]	46.3	53.7
5	Control	47.4	52.6
	VCD [ns]	47.4	52.6

[†] Sex ratio of pups between control and treated groups in each breeding round were compared using Chi-square test; values with [ns] are not significantly different from control group (P>0.05).

Table 3.8 Body weights (g) (Mean ± SD) of pups from control and VCD-treated animals over 5 breeding rounds.

Breeding round	Treatment	Body weight of pups (g) [†]	
		Males	Females
1	Control	7.0 ± 0.6	6.7 ± 0.5
	VCD	7.4 ± 0.5 [ns]	7.0 ± 0.7 [ns]
2	Control	6.7 ± 0.5	6.4 ± 0.5
	VCD	6.5 ± 0.6 [ns]	6.1 ± 0.6 [ns]
3	Control	6.8 ± 0.5	6.2 ± 0.4
	VCD	7.0 ± 0.5 [ns]	6.8 ± 0.6 [ns]
4	Control	6.6 ± 0.2	6.4 ± 0.3
	VCD	7.3 ± 0.9 [ns]	7.2 ± 1.0 [ns]
5	Control	6.8 ± 0.7	6.8 ± 0.5
	VCD	6.4 ± 0.7 [ns]	6.9 ± 0.6 [ns]

[†] Mean between control and treated groups in each breeding round were compared using Student *t-test*. Values with [ns] were not significantly different from control group (P>0.05).

3.4 Discussion

Body weight and the weights of non-reproductive organs

In this study, reduction in body weights of treated animals over the first 2-3 days of the treatment period could have been due to poor appetite caused by VCD. Thereafter, body weights were maintained, possibly due to improved tolerance to the VCD treatment. During the treatment periods, all treated animals remained alert and active. Chhabra et al. (1990a) reported that in male rats, 13-weeks treatment with VCD induced 14% reduction in body weight after dermal administration (60 mg/rat/day), and 6 and 23% after oral treatment (500 and 1000 mg/kg body weight, respectively). In a long-term study, dermal treatment with VCD induced 3-10% reduction in body weight of male and female rats when treated for 15 months (15 and 30 mg VCD/rat/day) or 11-14% when treated for 2 years (30 mg VCD/rat/day). The reduction in body weight of treated animals was possibly as a result of increase in tumours in these animals (Chhabra et al., 1990b).

No significant differences in the weights of non-reproductive organs between control and VCD treated animals in this study suggest that oral administration with VCD (500 mg/kg) for 15 days did not induce adverse effects on these organs. Flaws et al. (1994) also observed no adverse effects on non-reproductive tissues (liver and spleen) of juvenile and adult female rats after intraperitoneal administration with VCD (80 mg/kg, for 30 days). However, significant increases in the weights of liver and kidneys were reported in male rats after 13 weeks of oral treatment with VCD (500 and 1000 mg/kg body weight). The changes in kidney weight were accompanied by renal degeneration of the tubular epithelium, but there were no pathogenic changes in liver (Chhabra et al., 1990a). Dermal application of VCD on male and female rats in a long-term study (5 days per week, for 15 months) did not affect non-reproductive organ weights (Chhabra et al., 1990b).

Reproductive organs and fertility

Results from this study showed that oral administration of VCD (500 mg/kg) for 15 days in male rats of either age group (juvenile or adult) did not induce changes in either the weights of reproductive organs or in the structural characteristics of

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the testis and epididymis at any time after treatment (from day 16 to day 90 after the onset of treatment). Decrease in testis weight is a powerful endpoint for determining spermatotoxicity (Morrissey et al., 1988), and histological examination of testis and epididymis is also one of the most specific and sensitive indicators of spermatotoxicity in rats (Linder et al., 1992). Results from this study suggest that VCD exposure did not affect the spermatogenic cycle of the male rats, at the dosage levels and duration tested.

Significant reductions in the weights of testes were reported in male mice following intraperitoneal administration with VCD at doses from 40 to 320 mg/kg for 30 days, and the effects were dose and time dependant (Hooser et al., 1995). Histological examination of testes showed that VCD at doses of 160 and 320 mg/kg induced destruction and loss of cell types that were undergoing DNA synthesis, such as Type I and B spermatogonia. However, these germ cells repopulated the seminiferous tubules upon cessation of treatment. These results suggested that in male mice VCD causes germ cell destruction through DNA binding and interference with DNA synthesis and cell replication, and VCD may also react with other intracellular macromolecules. Thus it is possible that binding of VCD to other critical intracellular macromolecules could contribute to testicular germ cell destruction (Hooser et al., 1995).

Chhabra et al. (1990a) reported that oral administration of VCD (500 and 1000 mg/kg body weight) for 13 weeks induced reduction in the size of testes and caused significant degeneration of the testis in male mice. These effects were characterised by a decrease in the number of germinal epithelium cells within the seminiferous tubules. However, with the similar doses and treatment period, no effect was reported in male rats. The difference in responses to testicular toxicity caused by VCD between male rats and mice could be due to male rats being less susceptible to VCD, or they may have a greater capacity to detoxify VCD (Hooser et al., 1995). A number of studies have demonstrated that VCD induced germ cell destruction in females of both rats and mice (National Toxicology Program, 1989; Springer et al., 1995; Borman et al., 1999; Mayer et al., 2002; Devine et al., 2004; Mayer et al., 2004; Haas et al., 2007; Ito et al., 2009), whereas destructive effects of VCD were found in male mice, but not male rats (National Toxicology Program, 1989; Chhabra et al., 1990a). It is possible that there may be differences in the

mechanism of VCD-induced germ cell destruction between males and females. Such differences in metabolism (activation and detoxification) and disposition of VCD, as well as differences in the physiology of germ cells between female and male rats could account for their different susceptibilities to VCD (Hooser *et al.*, 1995). The absence of responses to VCD in male rats may also be due to the blood-testis barrier which could limit exposure of the male germ cells to VCD toxicity, whereas, in the female, the vascular supply in the ovaries allows immediate exposure to circulating VCD.

Previous studies demonstrated that age of animals can affect their susceptibility to xenobiotics. For example, immature male rats were more susceptible to toxic effects of ethoxyquin than adults (Manson *et al.*, 1992). In contrast, adult rats have been found to be more sensitive than prepubertal rats, to the toxic effects of lead in terms of the concentration and production of testosterone and sperm (Sokol and Berman, 1991). In the present study, neither age group showed responses to VCD treatment.

There were no significant differences in the breeding outcomes between control and treated animals when mated with fertile females, suggesting that oral treatment with VCD did not induce adverse effects on fertility of male rats. These findings are supported by the histological assessment of the animals in Group 1 and 2, which had the same treatment as Group 3, and showed normal characteristics in their testes and epididymides at each time after treatment.

Results from the present study suggest that 15 days of oral administration with VCD (500 mg/kg) did not induce post-treatment testicular degeneration in male rats of either age group. This study for the first time demonstrates no effect of oral treatment with VCD on either the reproductive tracts of juveniles or adults or the fertility of adult male rats.

CHAPTER 4

Laboratory evaluation of bait uptake in ricefield rats
(*Rattus argentiventer*)

Paper in preparation to be submitted to *Pest Management Science*

Abstract

In population management of pest rodents, the challenge for baiting methods is to achieve adequate bait consumption by the target species, particularly when there are abundant alternative foods in their natural habitats. The aim of this study was to define a suitable cereal-based bait for use in oral delivery of fertility control agents (e.g. chemosterilants) for population management of ricefield rats, *Rattus argentiventer*, one of the major pests of rice production in Asia.

A three phase bait-uptake study was conducted using a series of no-choice and choice tests on wild ricefield rats (body weight = 70-140g, n = 8-10 animals in each test) under laboratory conditions. For Phase 1, a no-choice test on uptake of baits in three different physical forms (kibble, granule and pellet) by rats was determined. Based on the results from this phase, the most accepted bait form was used in Phase 2. No-choice and choice tests were conducted on the uptake of baits containing different vegetable oils (coconut oil, canola oil, sesame oil and neem oil), different food additives (intralipid, anchovies, and sardines), and different ratios of wheat:rice flours as the bait base (1:0, 1:1, and 0:1). In Phase 3, a choice test between the two most accepted bait combinations defined in Phase 2 was performed.

Results from this study indicate that: 1) bait in kibble form was more attractive to ricefield rats than granule and pellet forms; 2) bait containing coconut oil was preferred by the rats over baits containing canola, sesame and neem oils; 3) bait with an addition of intralipid (fat emulsion) was preferred over other baits containing anchovies or sardines; and 4) bait containing rice flour or a combination of wheat and rice flours as the bait base was preferred compared to the bait containing only wheat flour. Taken together, a cereal-based bait, in kibble form, containing broken rice (10%), equal proportions of wheat and rice flours (30% each), coconut oil (5%), intralipid (20%) and sugar (5%) was the most accepted bait by ricefield rats. This bait formula could be used for further study in the development of oral delivery of fertility control agents for population management in ricefield rats.

4.1 Introduction

Ricefield rats (*Rattus argentiventer*) have been identified as one of the major constraints to rice production in Southeast Asia (Aplin *et al.*, 2003; Singleton, 2003; Brown *et al.*, 2006; Singleton *et al.*, 2010a; Sudarmaji *et al.*, 2010). Farmers often use poison baits in their ricefields to control rodents (Aplin *et al.*, 2003; Singleton *et al.*, 2003b; Wood and Fee, 2003; Singleton *et al.*, 2007). However, a major challenge for baiting is to achieve high bait acceptance by the target species. Poor bait acceptance can occur particularly when abundant alternative foods are available in the natural habitat. The success of rodent control programmes therefore largely depends on the type of baiting materials used and when baiting is applied (Majumder *et al.*, 1969; Johnston *et al.*, 2005; Leung *et al.*, 2007).

The ricefield rat is an omnivorous species, primarily inhabiting rice fields and although they feed on a wide range of vegetable food and invertebrates (Jesus, 1960; Lim, 1966; Lavoie *et al.*, 1970; Aplin *et al.*, 2003), when available, their preferred diet is rice (Lam, 1983; Leung *et al.*, 1999). The array of abundant foods in rice fields represents a major challenge for developing an attractive bait as a carrier for control agents. A number of studies have reported that bait acceptance by rodents is generally affected by various factors, including taste, flavour, size, texture, ingredients, and food additives (Shafi *et al.*, 1990; Berdoy and Macdonald, 1991; Shafi *et al.*, 1992a; Shafi *et al.*, 1992b; Ahmad *et al.*, 1994; Amjad *et al.*, 2000; Harjit, 2004). It was believed that increased effectiveness of baiting programmes could be achieved if bait uptake could be increased by improving the attractiveness of the bait to pest species (Johnston *et al.*, 2005). Although many studies have been conducted on bait-uptake by a range of species, including pest rodents (Sridhara and Srihari, 1983; Chakraborty and Chakraborty, 1984; Bean *et al.*, 1988; Marsh, 1988a; Shafi *et al.*, 1991; Asran, 1993b, a; Sahni and Saxena, 1993; Bhadauria and Mathur, 1994; Malhi and Kaur, 1995; Ramesh, 1995; Weihong *et al.*, 1999; Kaur and Parshad, 2002; Parshad, 2002), limited studies on bait uptake by ricefield rats have been published. Otsu (1979) reported that roasted salted fish, roasted crab, roasted shrimp paste and sardine were consumed in small amounts, whereas nine other foods (cassava, sweet potato, sweet corn, polished rice, rice panicle, peanut butter mixed with shrimp liquid, fresh anchovies,

eel, and roasted coconut) were not eaten. Leung *et al.* (2007) found bait uptake by ricefield rats was increased by 65% by replacing maize with rice flour as the bait base. A number of poison baits have been developed and widely used to control a range of rodent species, including ricefield rats (Leung *et al.*, 2007), however, no bait is universally accepted by all species because of the complexity of rodent feeding behaviors.

This study aimed to evaluate the effects of different factors such as the physical form of the bait, type of vegetable oil added, different food additives and different combinations of cereal flours on bait preference by ricefield rats in order to improve bait acceptance of this pest rodent species. Results from this study are essential for defining a suitable bait formula for delivering control agents (e.g. chemosterilants) for population management of ricefield rats.

4.2 Material and methods

4.2.1 Animals

Wild mature ricefield rats (70-140g body weight) were captured from rice fields using trap barrier systems (TBS) around the Indonesian Rice Research Center, Sukamandi, West Java, Indonesia. Captured rats were housed individually in metal cages (20 x 25 x 40cm) and allowed to acclimatise for at least five days before baiting trials commenced. No immature, injured, diseased or pregnant individuals were included. Water was provided *ad libitum* throughout the study, and unhulled rice was provided between test periods. Before the start of each feeding trial, animals were weighed, assigned to weight classes and allocated equally across the experimental groups. The number of animals used in each test was presented in Table 4.1.

Table 4.1 Number of ricefield rats used in the three-phase bait-uptake study

Phase		No. of groups	Females	Males	Total
1	Trial 1 (no-choice, bait forms)	3	8	0	24
2	Trial 2 (no-choice, vegetable oils)	5	8	0	40
	Trial 3 (no-choice, food additives)	5	5	5	50
	Trial 4 (choice, food additives)	6	5	5	60
	Trial 5 (choice, wheat and rice flour)	3	5	5	30
3	Trial 6 (choice, 2 most accepted baits)	2	5	5	20

4.2.2 Bait preparation and experimental design

A three phase bait-uptake study was conducted using a series of no-choice and choice tests on wild ricefield rats under laboratory conditions. For the first phase, uptake of baits of different physical forms (kibble, granule and pellet) was evaluated using a series of no-choice tests (Trial 1). The most accepted bait form defined from this phase was then used to prepare the baits for the next phase. For the second phase, uptake of the most accepted physical bait form containing different vegetable oils (coconut oil, canola oil, sesame oil and neem oil)(Trial 2), different food additives (intralipid, anchovies, and sardines)(Trial 3 and 4), and different ratios of wheat:rice flours as a bait base (1:0, 1:1, and 0:1) (Trial 5) was determined using a series of no-choice and choice tests. For the final phase (Phase 3), a choice test between the two most accepted bait combinations defined in Phase 2 was performed (Trial 6).

In all choice tests, none of the animals used had previously been exposed to either of the baits being tested, except for Trial 6, where an additional group of animals (n=10) had been exposed previously to one of the two test baits for 5 days.

Phase 1 – Uptake of baits of different physical forms

Trial 1. No-choice tests on uptake of baits of different physical forms

Three physical forms of bait were tested: i) Kibble (2-4 mm diameter), ii) Granule (7-8 mm diameter), and iii) Pellet (1 cm x 4-5 cm). Each form of bait contained broken rice (10%) + wheat flour (65%) + coconut oil (5%) + sugar (20%). This high concentration of sugar was used to mimic another proprietary bait which used a sweetening compound (20%) known to induce inhibitory effects on reproduction of female rats (L. Mayer, personal communication). Initially, all the ingredients were weighed then thoroughly mixed using a food blender. The mixture was then moistened before making the appropriate form (Fig. 4.1). The baits were then dried in an oven at 40°C for 24 hours. Dried baits were kept in sealed plastic bags before use.

Figure 4.1 The three forms of bait (kibble, granule and pellet) used in the study.

In this trial, each form of bait was provided in a single feeder placed at the corner of the cage for three days. For each day, a known amount of each form was provided, and the remaining bait, including spillage, was removed on the following day, then dried and weighed. Daily consumption of each bait form was recorded over the period of trial. The calculation method for daily consumption is presented in Section 4.2.3 below. The most accepted bait form determined from this trial was used to prepare baits for the next trials in Phase 2.

Phase 2 – Uptake of baits containing different vegetable oils, food additives and cereal flours

Trial 2. No-choice tests on uptake of baits containing different vegetable oils

This trial was conducted to evaluate the effect on bait-uptake by ricefield rats of three vegetable oils which were available in the local market, including canola oil, coconut oil, and sesame oil. In addition, neem seed oil was also tested as it is known to reduce the fertility of rodents (Dhaliwal *et al.*, 1998; Dhaliwal *et al.*, 1999). Four types of bait containing four vegetable oils were used; i) 5% canola oil (commercial origin), ii) 5% coconut oil (freshly prepared at the local market), iii) 5% sesame oil (commercial origin), and iv) 5% neem seed oil (Neeming Australia Pty Ltd, Queensland, Australia). Each bait type was prepared by mixing the vegetable oil (5%) with the base material which comprised broken rice (10%), wheat flour (65%) and sugar (20%). Similar to Trial 1 this high concentration of sugar was used; however, bait with this sugar concentration was observed to be very attractive to ants, therefore a lower rate of sugar (5%) was used in the rest of the

study. All test baits were prepared in kibble form as defined in Phase 1. The details of bait composition are presented in Appendix 2.1.

In this trial, uptake of the four bait types was evaluated using no-choice tests. Consumption of unhulled rice (a natural preferred food of ricefield rats) was also evaluated and served as a positive control. Bait was provided in a single feeder for three consecutive days, and daily consumption was recorded (see Section 4.2.3).

Trial 3. No-choice tests on uptake of baits containing different food additives

Based on Trial 2, bait containing 5% coconut oil was most accepted by the rats. Therefore, in this trial, coconut oil was used as the base oil to prepare bait with the addition of different additives. Five bait formulas were used in this trial:

      i)   Coconut oil (5%),

      ii) Intralipid (20%) + coconut oil (5%),

      iii) Wax (12%) + coconut oil (5%),

      iv) Anchovies (5%) + coconut oil (5%), and

      v) Sardines (5%) + coconut oil (5%).

Intralipid is a fatty emulsion, containing mainly soya oil (10-30%) + egg lecithin (1.2%) + glycerol (2.25%). All bait types contained the same proportions of broken rice (10%), and sugar (5%). The details of bait formulas are presented in Appendix 2.2. All the baits were made in kibble form (2-4mm) using a food processor. The kibble was then dried at $40^{O}C$ (~ 24 hours) and kept in a sealed plastic bag before use.

In this trial, each bait type was provided in a single feeder placed at the corner of the cage for five days and daily consumption was determined.

Trial 4. Choice tests on uptake of baits containing different food additives

In an attempt to gain insight into the results of the no-choice tests in Trial 3, a series of two-choice tests were conducted. Based on the results from the no-choice tests in Trial 3, four bait formulas were selected for choice tests. These four bait formulas formed six two-choice tests as follows:

1) Intralipid (20%) *vs.* Coconut oil (5%)

2) Intralipid (20%) *vs.* Wax (12%)

3) Intralipid (20%) *vs.* Anchovies (5%)

4) Coconut oil (5%) *vs.* Wax (12%)

5) Coconut oil (5%) *vs.* Anchovies (5%)

6) Wax (12%) *vs.* Anchovies (5%)

Trial 5. Choice tests of kibble bait containing different proportions of wheat and rice flour

Wheat flour has been widely used as a bait base for controlling rodents in ricefields (Jiang *et al.*, 1999; Neena and Parshad, 2002; Kocher and Parshad, 2006; Ocampo and Lontoc, 2006). Although rice-based bait has been used in trials in ricefields (Buckle *et al.*, 1980; Buckle and Kaukeinen, 1988; Akhila and Rani, 2002), no comparisons of the palatability of these two cereals for ricefield rats have been made. The aim of this trial was to compare uptake of baits containing different proportions of wheat and rice flour using two-choice tests.

Three different baits containing different ratios of wheat:rice flours were tested, including: i) Wheat flour only; ii) Wheat:rice flour = 1:1; and iii) Rice flour only. All bait types contained the same proportions of broken rice (10%), coconut oil (5%), and sugar (5%). The details of bait composition are presented in Appendix 2.3, and all baits were prepared in kibble form as described in Phase 1.

Three two-choice tests were performed as follows:

1. Wheat flour only   *vs.* Wheat:rice flour (1:1)

2. Wheat flour only   *vs.* Rice flour only

3. Wheat:rice flour (1:1) *vs.* Rice flour only

Phase 3 - Uptake of the two most accepted baits

Trial 6. Choice tests between the two most accepted baits derived from Phase 2

Based on the choice tests from Phase 2 (Trials 4-5), the two most accepted baits were selected to test against each other. These two baits were intralipid (20%) + coconut oil (5%) (derived from Trial 4), and bait containing coconut oil

(5%) with equal proportions of wheat and rice flours (derived from Trial 5). In this trial, two groups of animals (n=10 per group) were used. In the first group, none of the animals were exposed to either of the test baits before being used. In the second group, all animals had previously been provided with bait containing 5% coconut oil with equal proportions of wheat and rice flours for 5 days before being used for the choice test.

For each choice test (Trials 4-6), baits were provided in two separate feeders placed at the opposite corners of the cage for five consecutive days. The positions of the feeders were interchanged daily to minimise possible positional effects. Daily consumption of each type of bait in each two-choice test was recorded.

4.2.3 Data collection and analysis

Daily bait consumption

In all trials, daily bait consumption (M_C) of each bait was determined as the difference in weight (g) between the amount added (M_A), and the amount remaining (M_R) after drying. A correction was made for moisture changes using a set of control bait samples (n=3). These control baits were exposed to the same conditions as the tested baits but could not be accessed by rats. Correction for moisture changes was calculated as follows:

Moisture correction = $M_A + (M_A \times p)$

where M_A = daily amount (g) added;

p (% change in weight in control samples)

= [(amount of control added – amount of control remaining after drying)/amount of control added] x 100.

Therefore amount consumed, $M_C = [M_A + (M_A \times p)] - M_R$

Number of animals consuming bait

In each test, animals considered to have consumed bait were the animals for which the consumption was equal to or greater than 2% of their body weight for at least two days (of the three-day feeding period) in Trial 1 and 2, or three days (of the five-day feeding period) in Trials 3-6.

Data presented on bait uptake includes: the proportion of animals consuming bait and the average daily consumption calculated for animals that consumed bait as well as for all animals in the group. In Trials 3-6, bait consumption with respect to gender are also presented.

Mean consumption (mean ± standard error) of different baits in each trial were compared using one-way ANOVA for no-choice tests (Trials 1, 2 and 3) and Student's t-test for two-choice tests (Trials 4, 5 and 6) at a significance level of 5%.

In each two-choice test, preference of one bait over the other was also compared using a preference index, which was calculated as follows:

$$\text{Preference index (between bait A and B)} = \frac{\text{Amount of bait A eaten}}{\text{Total amount of bait A and B eaten}} \times \frac{100}{1} - 50$$

Values of zero or close to zero represent no difference in preference between the two baits tested, i.e. similar consumption for both baits; and values close to 50% represent animals consuming one bait but not the other.

4.3 Results

4.3.1 Phase 1 – Uptake of baits of different physical forms

Trial 1. No-choice tests on uptake of baits of different physical forms

Over the 3 day period of feeding, a high proportion of animals (7/8) consumed kibble bait, whereas 4/8 animals consumed the granules or pellets (Fig. 4.2). For those animals that consumed bait (equal to or greater than 2% of body weight), the highest average daily consumption was found for animals given kibble bait (~5.5% body weight), and this was significantly higher ($P<0.05$) than that for the bait in pellet form (~3.8% body weight) (Fig. 4.2).

The average daily consumption for all animals in the group receiving kibble bait was maintained at approximately 5% of their body weight over the feeding

period; whereas the consumption for the granule or pellet bait ranged from 2 to 3 % body weight (Fig. 4.3).

The results of Trial 1 indicated that ricefield rats preferred bait in the kibble form over granules or pellets. Therefore in the following trials, all tested baits were prepared in kibble form.
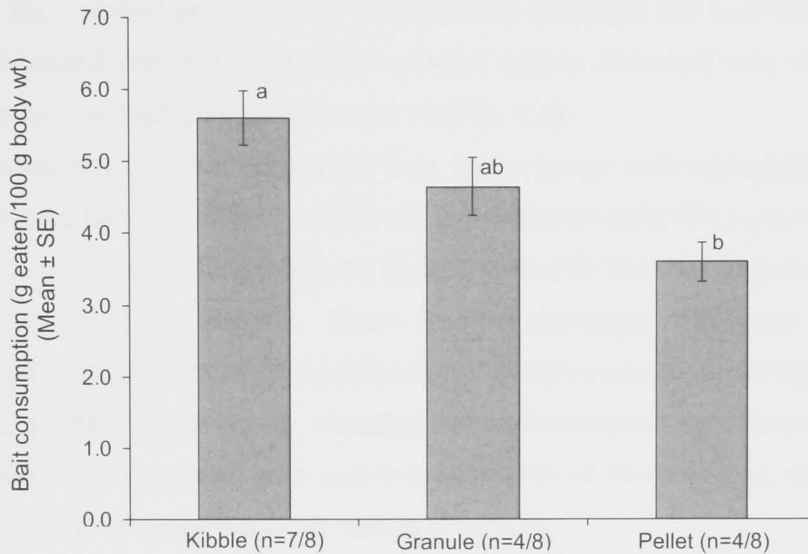
Figure 4.2 Average bait consumption (g eaten/100g body weight) (Mean ± SE) over 3 days in no-choice tests of different physical forms of bait. Each group contained 8 animals, but only consumption for those considered to have consumed bait equal to or greater than 2% body weight (n) is presented. Columns with different letters are significantly different (P<0.05).
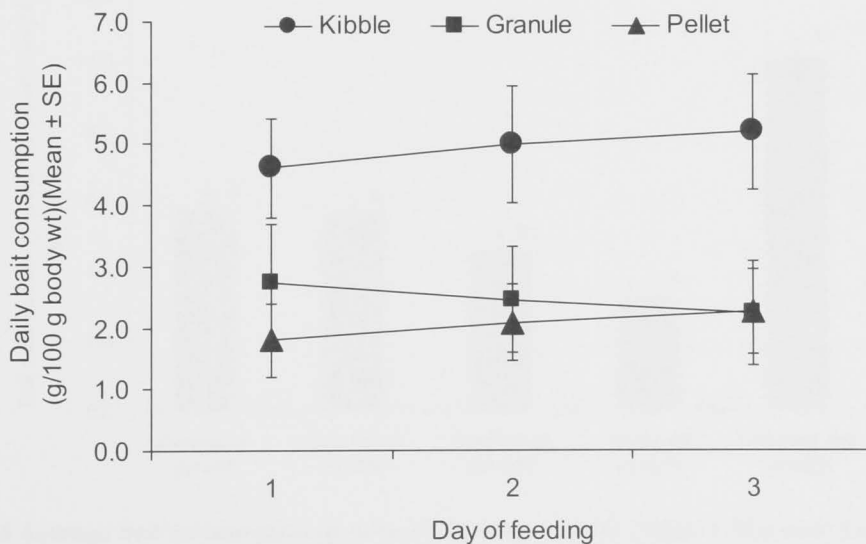
Figure 4.3 Daily bait consumption (g eaten/100g body weight) (Mean ± SE) over the 3-day feeding period in no-choice tests of different forms of bait: kibble (●), granule (■), and pellet (▲). Consumption was calculated for all animals in each group (n=8).

4.3.2 Phase 2 – Uptake of baits containing different vegetable oils, food additives and cereal flours

Trial 2. No-choice tests on uptake of baits containing different vegetable oils

Of the four vegetable oil-based baits tested, bait containing coconut oil was consumed by a higher proportion of animals (7/8 animals); 6/8 animals consumed sesame oil-based bait; 5/8 animals consumed canola oil-based bait; and only 3/8 animals consumed bait containing neem oil (Fig. 4.4).

For those animals that consumed bait, the average daily consumption for the baits containing coconut oil and canola oil was approximately 6% body weight. This level of consumption was significantly higher ($P<0.05$) than for animals fed neem oil-based bait (3% body weight). There was no significant difference ($P>0.05$) in consumption among coconut oil, canola oil and sesame oil-based baits (Fig. 4.4).

For all animals in the group, average daily consumption for coconut oil-based bait over the feeding period was approximately 5% of body weight, and this was higher than the other vegetable oils tested (Fig. 4.5).

In the group fed with unhulled rice (the preferred diet of ricefield rats), 100% of the animals consumed the food at a rate of approximately 10% of body weight (Figs. 4.4, 4.5).
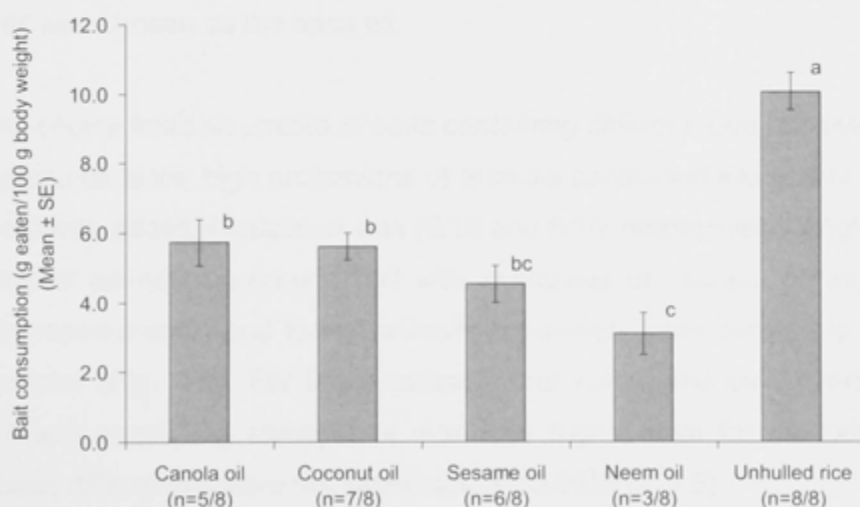
Figure 4.4 Average bait consumption (g eaten/100g body weight) (Mean ± SE) over 3 days in no-choice tests of baits containing different vegetable oils (canola oil, coconut oil, sesame oil and neem oil). Unhulled rice was used as a positive control. Each group contained 8 animals, but only consumption for those consuming equal to or greater than 2% body weight is presented (n); Columns with different letters are significantly different ($P<0.05$).
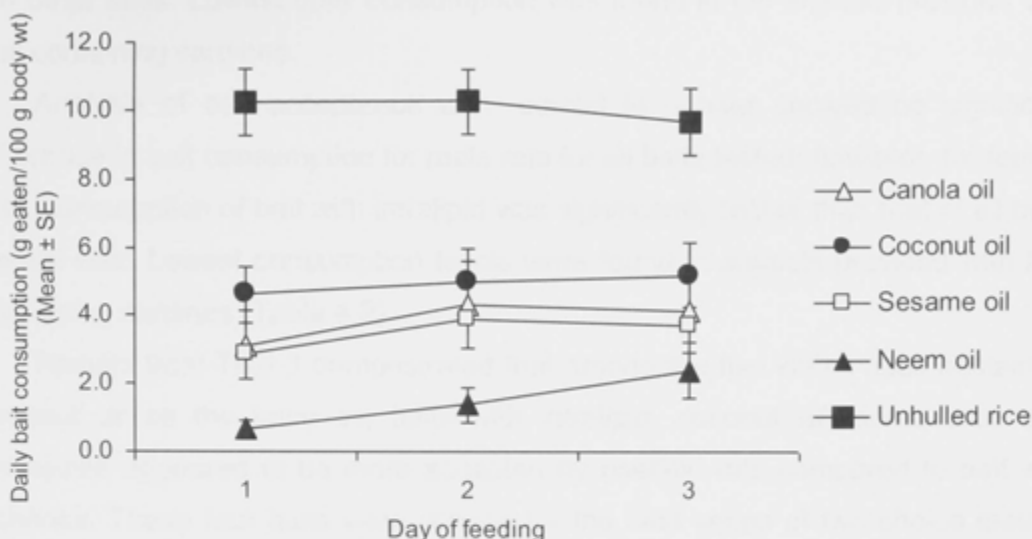
Figure 4.5 Daily bait consumption (g eaten/100g body weight) (Mean ± SE) over the 3-day feeding period in no-choice tests of baits containing different vegetable oils: canola oil (Δ), coconut oil (●), sesame oil (□) and neem oil (▲). Unhulled rice (■) was used as a positive control. Consumption was calculated for all animals in each group (n=8).

Results from Trial 2 suggested that kibble bait containing 5% coconut oil was preferred by ricefield rats over other vegetable oils. However, if increased concentrations of coconut oil (from 5 to 10 and 15%) were offered, this did not alter bait consumption (data not shown). Therefore for further trials of bait-uptake 5% coconut oil was chosen as the base oil.

Trial 3. No-choice tests on uptake of baits containing different food additives

In no-choice tests, high proportions of animals consumed kibble bait (plus 5% coconut oil) with added intralipid or wax (9/10 and 8/10, respectively). Slightly lower proportions of animals consumed bait with anchovies or coconut oil alone (7/10 and 6/10, respectively); and fewer animals consumed baits containing sardines (5/10 animals) (Fig. 4.6). For those animals that consumed bait, average daily uptake of bait containing intralipid or wax was higher than for the other baits, though these differences were not significant (P>0.05)(Fig. 4.6).

Average daily consumption for all the animals in a group gradually increased during the feeding period for each bait (Fig. 4.7). Among the five bait formulas, uptake of baits containing intralipid or wax in coconut oil was higher than that for

the other baits. Lowest daily consumption was found in the animals provided with bait containing sardines.

Analysis of bait acceptance with respect to gender showed no significant difference in bait consumption for male rats for all baits tested; however, for female rats, consumption of bait with intralipid was significantly higher than that of all baits except wax. Lowest consumption levels were found in animals provided with bait containing sardines (Table 4.2).

Results from Trial 3 demonstrated that among the five kibble baits containing coconut oil as the base oil, baits with intralipid, coconut oil alone, wax, and anchovies appeared to be more accepted by ricefield rats compared to bait with sardines. These four baits were chosen for the next series of two-choice tests in Trial 4.
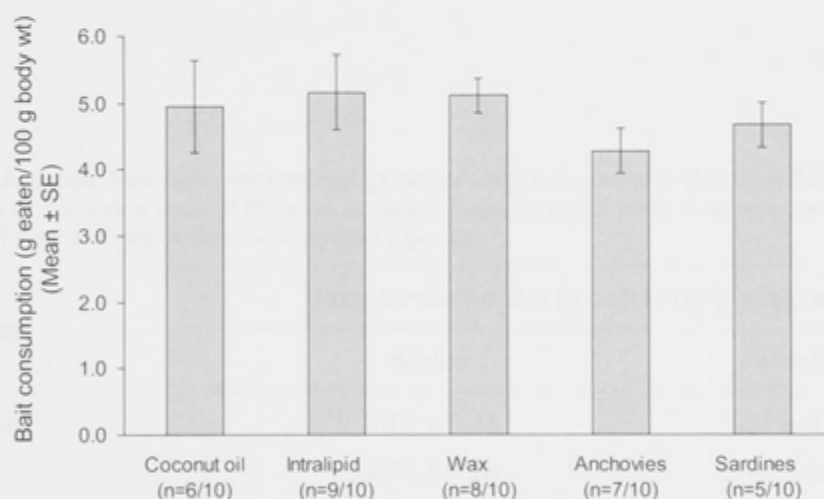
Figure 4.6 Average daily consumption (g eaten/100 g body weight) (Mean ± SE) by ricefield rats over 5 days in no-choice tests of coconut oil-based kibble baits containing different food additives. Average consumption was calculated only for the animals consuming equal to or greater than 2% body weight (n). Means were compared using one-way ANOVA at a significance level of P<0.05
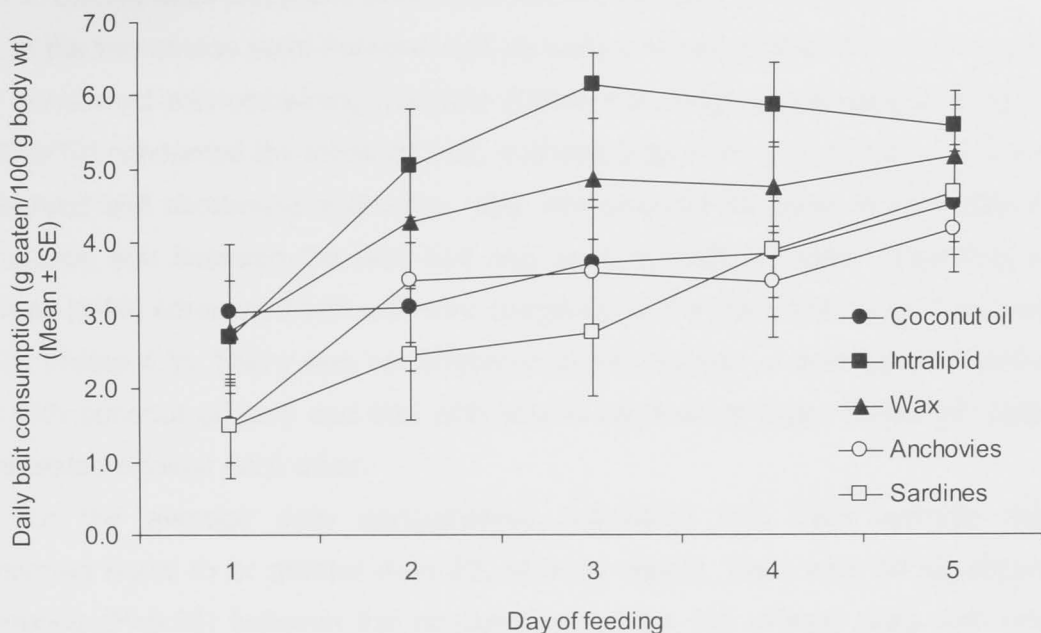
Figure 4.7 Daily bait consumption (g eaten/100g body weight) (Mean ± SE) by ricefield rats over 5-day feeding period in no-choice tests of different coconut oil-based kibble baits: Coconut oil (●), Intralipid (■), Wax (▲), Anchovies (○), and Sardines (□). Average daily consumption was calculated using all animals in the group (n=10).

Table 4.2 Average daily bait consumption (g eaten/100g body weight) (Mean ± SE) of male and female rats in no-choice tests of different coconut oil-based kibble baits. Average consumption was calculated for all animals of each sex (n=5) in a group.

Bait types	Daily consumption (g eaten/100g body weight) [†]	
	Males	**Females**
Coconut oil	3.70 ± 0.43	3.61 ± 0.61 [bc]
Intralipid	4.71 ± 0.28	5.41 ± 0.57 [a]
Wax	4.22 ± 0.47	4.51 ± 0.50 [abc]
Anchovies	3.49 ± 0.49	2.95 ± 0.48 [bc]
Sardines	3.61 ± 0.53	2.50 ± 0.48 [c]

[†] Means from different groups were compared using one-way ANOVA. Values followed by different letters are significantly different (P<0.05).

Trial 4. Choice tests on uptake of baits containing different food additives

In the two-choice tests between bait containing intralipid and other baits, more rats consumed bait containing intralipid (Table 4.3). High proportions of animals (7/10-8/10) consumed the intralipid bait, whereas only 1/10, 4/10 and 5/10 animals consumed bait containing anchovies, wax, and coconut oil baits, respectively. In the choice test between the wax bait and anchovy bait, a higher proportion of animals (6/10) consumed bait with wax compared to the bait containing anchovies (4/10) (Table 4.3). There was no difference in the number of animals consuming bait with coconut oil only and bait with wax in coconut oil when these two baits were tested against each other.

For the average daily consumption, calculated only from animals that consumed equal to or greater than 2% of body weight, there was no significant difference (P>0.05) between the consumption of the two offered baits with one exception; consumption of bait with wax was significantly higher (P<0.05) than that of the bait with anchovies (Table 4.3).

For all animals in group, the total daily bait consumption of the two offered baits was maintained at approximately 5 to 6% of body weight over the 5-day feeding period (Fig. 4.8). Consumption of intralipid bait was significantly higher (P<0.05) than that for bait containing anchovies (Fig. 4.8a) and wax (Fig. 4.8b) and consumption of bait containing wax was significantly higher (P < 0.01) than for the bait with anchovies (Fig. 4.8d).

The analysis of bait preference for each of the choice tests indicated that the rats preferred bait with intralipid over bait with anchovies (Fig. 4.9a), bait with intralipid over bait with wax (Fig. 4.9b), and bait with wax over bait with anchovies (Fig. 4.9d).

Data on bait preference with respect to gender (Table 4.4) shows that male and female rats preferred the bait with intralipid over the baits containing anchovies or wax. Similarly, both sexes preferred the bait with wax over anchovies. In the test between coconut oil-based bait containing wax and bait with coconut oil only, male rats preferred the bait containing wax, while the opposite preference was observed in female rats. Bait containing coconut oil alone was preferred over intralipid by male rats, but not by female rats (Table 4.4).

Apart from the results presented, our study also found that addition of several other food enhancers, including crabs, eggshell and fish oil, into cereal-based bait did not enhance bait acceptance by ricefield rats (data not shown).

Findings from Trial 4 demonstrated that coconut oil-based bait, in kibble form, containing intralipid proved to be most accepted by rice field rats with a rate of daily consumption approximately 5% body weight. Therefore coconut oil-based bait containing intralipid was chosen for further bait-uptake studies in rice field rats.

Table 4.3 Average consumption (g eaten/100g body weight) (Mean ± SE) by ricefield rats given a choice tests of two different kibble baits

Choice test	Number of animals consuming bait	Average consumption (g eaten/100g body weight) [†]
Anchovies	1/10	6.20 ± 0.58
Intralipid	8/10	5.99 ± 0.28
Intralipid	7/10	4.78 ± 0.29
Wax	4/10	4.19 ± 0.62
Coconut oil	5/10	5.02 ± 0.21
Wax	5/10	4.50 ± 0.40
Anchovies	4/10	3.12 ± 0.33*
Wax	6/10	5.95 ± 0.45
Coconut oil	5/10	4.78 ± 0.49
Intralipid	7/10	3.82 ± 0.33
Coconut oil	6/10	3.74 ± 0.34
Anchovies	6/10	3.73 ± 0.39

[†] average bait consumption in each of the two-choice tests were calculated for only animals that consumed bait, and the means were compared using Student's t-test; value with * is significantly different ($P < 0.05$) from the other bait in the choice test.
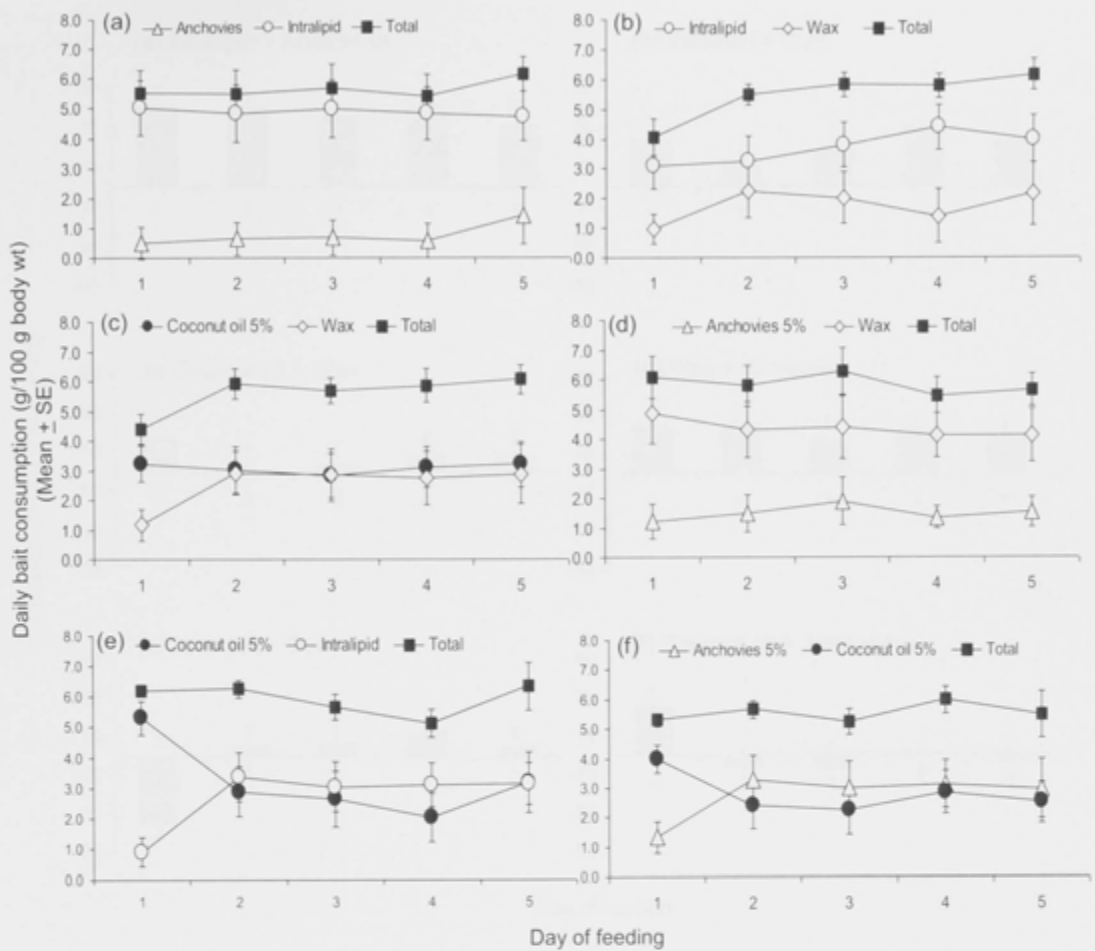
Figure 4.8 Daily bait consumption (g eaten/100g body weight) (Mean ± SE) in choice tests between a) Anchovies (Δ) and Intralipid (○); b) Intralipid (○) and Wax (◊); c) Coconut oil only (●) and Wax (◊); d) Anchovies (Δ) and Wax (◊); e) Coconut oil only (●) and Intralipid (○); and f) Coconut oil only (●) and Anchovies (Δ). n = 10 animals per group.
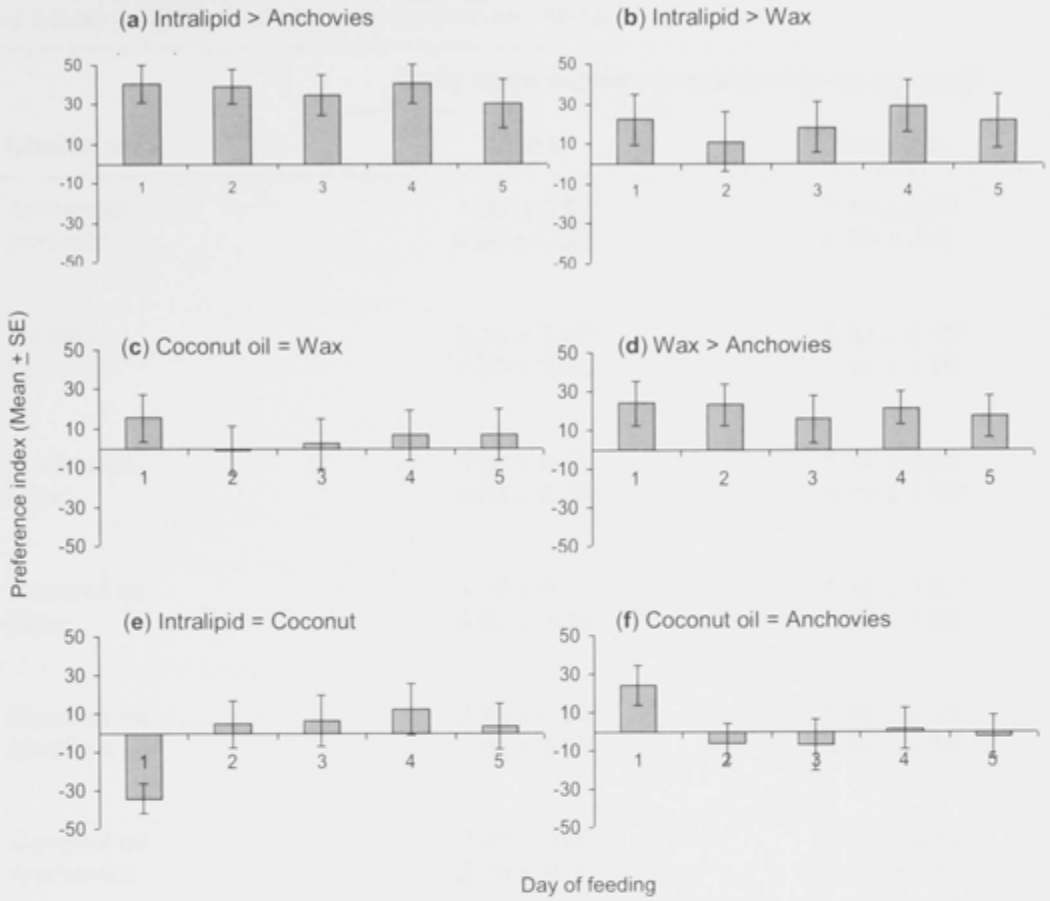
Figure 4.9 Preference index for each of the choice tests (n=10 per group) between (a) Intralipid *vs.* Anchovies, (b) Intralipid *vs.* Wax, (c) Coconut oil *vs.* Wax, (d) Wax *vs.* Anchovies, (e) Intralipid *vs.* Coconut oil, and (f) Coconut oil *vs.* Anchovies over the 5-day feeding period. Values of zero or close to zero represent no difference in preference between two baits tested; the maximum value (50%) represents animals consuming one bait but not the other.

Table 4.4 Average daily bait consumption (g eaten/100g body weight) (Mean ± SE) of male (n=5) and female (n=5) rats in choice tests between two different kibble baits.

Choice test	Daily consumption (g eaten/100g body weight)[†]	
	Males	Females
Anchovies	1.30 ± 0.51	0.26 ± 0.21
Intralipid	4.05 ± 0.39*	5.69 ± 0.65*
Intralipid	3.49 ± 0.42*	3.93 ± 0.55*
Wax	1.63 ± 0.46	1.90 ± 0.60
Anchovies	1.94 ± 0.40	1.19 ± 0.29
Wax	3.34 ± 0.53*	5.38 ± 0.56*
Coconut oil	1.14 ± 0.17	5.02 ± 0.21*
Wax	4.50 ± 0.40*	0.57 ± 0.25
Coconut oil	3.79 ± 0.55*	2.66 ± 0.53
Intralipid	1.96 ± 0.39	3.49 ± 0.49
Coconut oil	3.03 ± 0.40	2.57 ± 0.42
Anchovies	2.19 ± 0.30	3.27 ± 0.62

[†] Average consumption between two baits was compared using Student's t-test. Values with * are significantly different (P<0.05) from the other bait in the choice-test.

Trial 5. Choice tests of kibble baits containing different proportions of wheat and rice flours

Choice tests between baits containing different ratios of wheat and rice flour as the cereal base showed that a higher proportion (8/10) of animals consumed baits containing a combination of wheat and rice flours or rice flour alone than of bait containing wheat flour only (2/10 - 3/10)(Table 4.5). There was no obvious difference in the number of animals consuming baits in choice-tests between bait containing wheat plus rice flour and bait with rice flour only (4/10 and 5/10, respectively).

The average daily consumption (calculated only from the animals consuming bait) for the bait containing either wheat and rice flour or rice flour alone was approximately 5% of body weight, and significantly higher (P<0.05) than that for

the bait with wheat flour only. There was no significant difference in the average consumption between bait with wheat plus rice flour and bait with rice flour only (Table 4.5).

For all animals in the group, the total daily bait consumption of the two baits in each choice test ranged from approximately 4 to 6% of body weight over the 5 days of feeding (Figs. 4.10a-c). Average daily consumption of bait containing wheat and rice flours or rice flour alone was significantly higher than that for the bait containing wheat flour only (Figs. 4.10a,b). Analyses of bait preference between two baits in each choice test using the preference index clearly revealed that rats preferred bait containing wheat and rice flour or rice flour alone over the bait containing only wheat flour (Fig. 4.11).

Bait acceptance data with respect to gender (Table 4.6) revealed that both sexes preferred bait containing wheat plus rice flour or rice flour alone over bait containing only wheat flour. There was no obvious sex preference in consumption of bait containing rice flour alone or wheat plus rice flour as the bait base (Table 4.6).

Results from Trial 5 indicated that baits containing equal proportions of wheat and rice flour, or rice flour alone were preferred over bait containing only wheat flour as the bait base. In the next trial, bait containing equal proportions of wheat and rice flour was used because the kibble prepared with some wheat flour remained intact and did not crumble with handling like the bait containing rice flour only.

Table 4.5 Average consumption (g eaten/100g body weight) (Mean ± SE) over 5 days in choice tests of baits containing different ratios of wheat and rice flours.

Choice test	Number of animals consuming bait	Average consumption (g eaten/100g body weight) [†]
Wheat flour only	3/10	3.41 ± 0.52
Wheat:rice flour (1:1)	8/10	4.79 ± 0.27*
Wheat flour only	2/10	3.27 ± 0.36
Rice flour only	8/10	5.14 ± 0.19*
Wheat:rice flour (1:1)	4/10	3.79 ± 0.32
Rice flour only	5/10	4.67 ± 0.32^{ns}

[†] average bait consumption in each choice test for animals that consumed equal to or greater than 2% of body weight; values with * are significantly different (P<0.05) and value with ^{ns} is not significantly different from other bait in the choice-test.
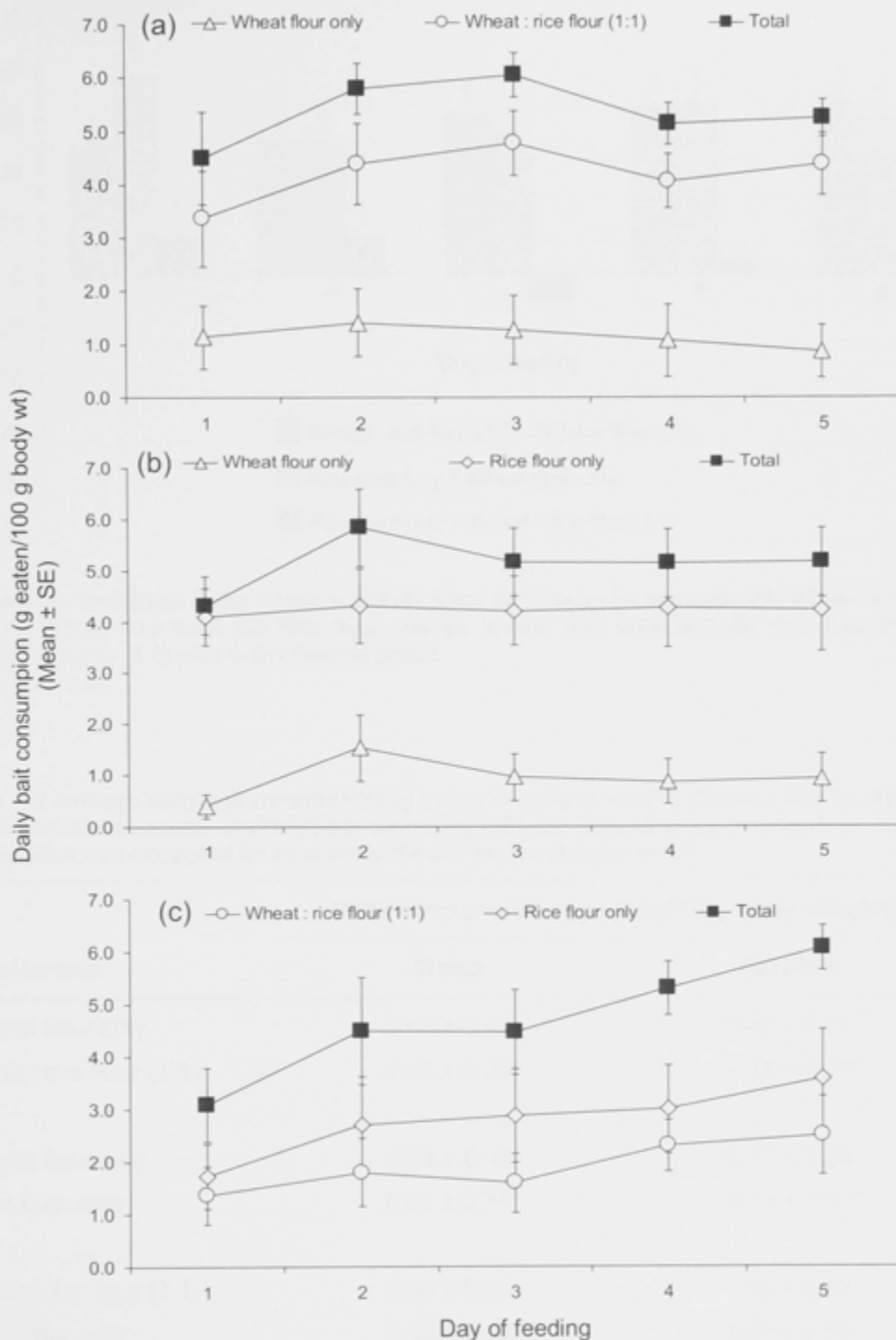
Figure 4.10 Daily bait consumption (g eaten/100g body weight) (Mean ± SE) over 5-day feeding period in choice tests between bait containing a) wheat flour only (Δ) and wheat:rice flour (1:1) (○); b) wheat flour only (Δ) and Rice flour only (◊); and c) wheat:rice flour = 1:1 (○) and Rice flour only (◊). Average consumption was calculated for all animals in the group (n=10).
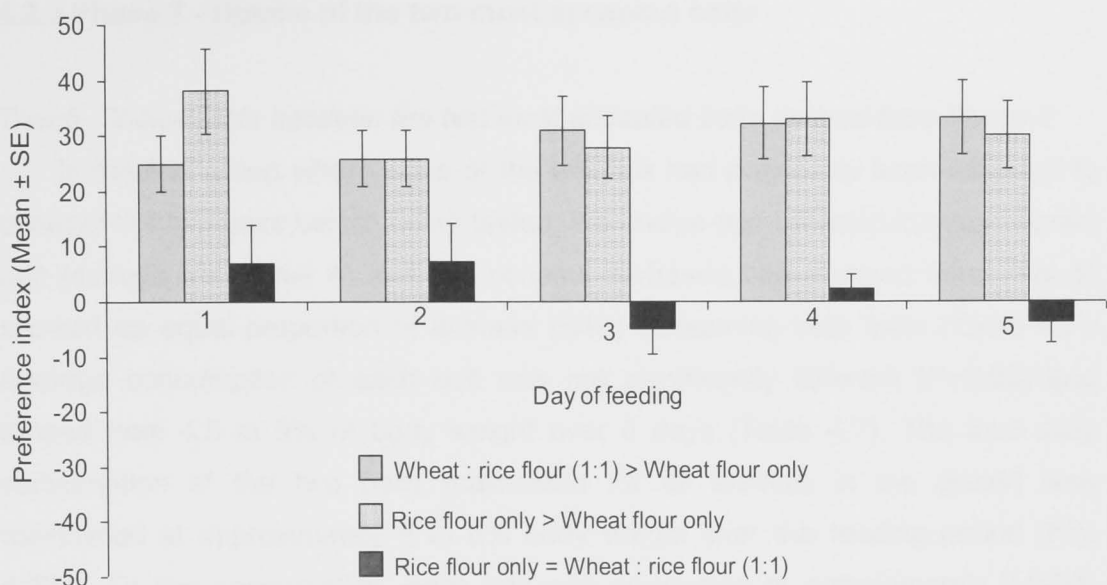
Figure 4.11 Preference index (Mean ± SE) in choice tests between two baits: (□) Wheat:rice flours (1:1) vs. Wheat flour only; (□) Rice flour only vs. Wheat flour only; and (■) Rice flour only vs. Wheat:rice flours (1:1) over 5-day feeding period.

Table 4.6 Average daily bait consumption (g eaten/100g body weight) (Mean ± SE) by male and female rats in choice tests of kibble baits containing different ratios of wheat and rice flour. Average consumption was calculated for all animals of each sex (n=5) in the group.

Choice test	Daily consumption (g eaten/100g body weight) [†]	
	Males	Females
Wheat flour only	1.69 ± 0.47	0.62 ± 0.21
Wheat:rice flour (1:1)	4.23 ± 0.55*	4.16 ± 0.27*
Wheat flour only	1.04 ± 0.32	0.77 ± 0.28
Rice flour only	4.13 ± 0.38*	4.33 ± 0.49*
Wheat:rice flour (1:1)	2.00 ± 0.35	1.82 ± 0.42
Rice flour only	2.56 ± 0.48	2.98 ± 0.58

[†] Mean consumption between baits was compared using Student's t-test; values with * are significantly different (P<0.05) from other bait in the choice-test.

4.3.3 Phase 3 - Uptake of the two most accepted baits

Trial 6. Choice tests between the two most accepted baits derived from Phase 2

In the first group where none of the animals had previously been exposed to either of the two baits before being tested, the choice-test between intralipid-based bait (derived from Trial 4) and 5% coconut oil-based bait (derived from Trial 5) showed an equal proportion of animals (5/10) consuming both baits (Table 4.7). Average consumption of each bait was not significantly different (P>0.05) and ranged from 4.5 to 5% of body weight over 5 days (Table 4.7). The total daily consumption of the two baits (calculated for all animals in the group) was maintained at approximately 5 to 6% body weight over the feeding period (Fig. 4.12a). Of this consumption, each bait was consumed at approximately 2.5-3% body weight. There was no significant difference (P>0.05) in uptake between the two baits. The preference index also showed no obvious preference for either of these two baits (Fig. 4.13).

In contrast, for the group of animals that had previously been exposed to the 5% coconut oil-based bait for 5 days before being used in the choice test, the uptake of this bait was significantly higher than that of the intralipid-based bait. The number of animals consuming 5% coconut oil-based bait was 8/10, whereas only 2/10 animals ate intralipid-based bait (Table 4.7). Average consumption of 5% coconut oil–based bait was significantly higher (P<0.05) than that of the intralipid-based bait (Table 4.7). The total daily consumption of the two baits was maintained at approximately 6% body weight over the feeding period (Fig 4.12b). Of this consumption, rats consumed approximately 5% of coconut oil–based bait and only 1% of intralipid-based bait, and this difference was significant (P<0.05). The preference index also showed a preference for 5% coconut oil-based bait over intralipid-based bait when the animals had previously been provided with 5% coconut oil-based bait before being tested (Fig. 4.13).

Findings from Trial 6 indicated that there was no obvious preference between bait containing intralipid and bait containing coconut oil only with equal proportion of wheat and rice flours when these two baits were tested against each other. However, as intralipid can be used as a carrier for control agents, it is therefore suggested that bait with an addition of intralipid could be used for incorporating

control agents, e.g. chemosterilants, into the bait formula. When the rats had previously been provided with 5% coconut oil-base bait for 5 days prior to being tested, this bait formula was highly preferred over the intralipid-based bait.

The results from this bait-uptake study suggest that bait in kibble form containing 10% broken rice, equal proportions of wheat and rice flours (30%), 5% coconut oil, 20% intralipid, and 5% sugar should be used for further bait-uptake studies on ricefield rats under enclosure and field conditions. This bait formula could potentially be used for oral delivery of control agents for population management of rice field rats.

Table 4.7 Average daily consumption (g eaten/100g body weight) (Mean ± SE) over 5 days in choice test between the intralipid-based bait and 5% coconut oil-based bait.

Choice test	Number of animals consuming bait	Average consumption (g eaten/100g body weight) [†]
Animals that had not previously been exposed to coconut oil-based bait		
Intralipid-based bait	5/10	4.76 ± 0.37
Coconut oil-based bait	5/10	4.67 ± 0.27^{ns}
Animals that had previously been exposed to coconut oil-based bait		
Intralipid-based bait	2/10	2.69 ± 0.71
Coconut oil-based bait	8/10	$5.65 \pm 0.45^{*}$

† average consumption in the choice test was calculated only for animals that consumed equal to or greater than 2% of body weight. Value with [ns] is not significantly different ($P>0.05$) and value with * is significantly different ($P<0.05$) between the two baits.
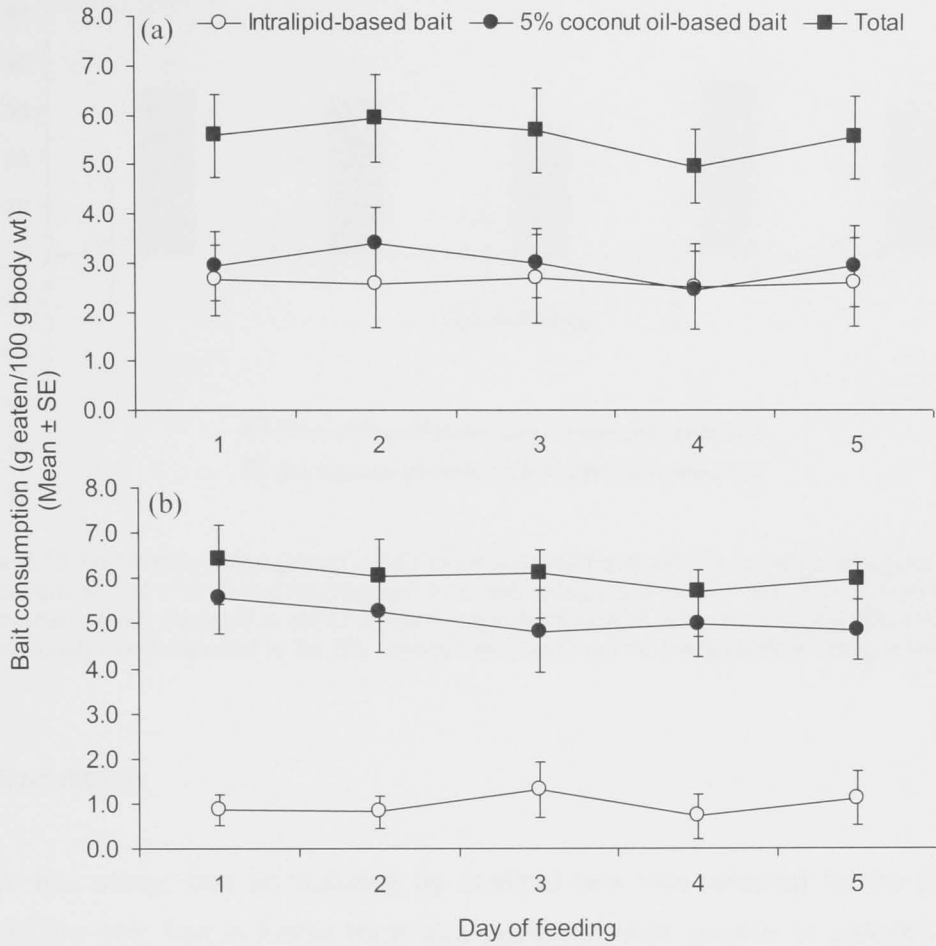
Figure 4.12 Daily bait consumption (g eaten/100g body weight) (Mean ± SE) over 5-day feeding period in choice test between bait containing intralipid (○) and 5% coconut oil (●). Average consumption was calculated from all animals of the group (n=10); (a) animals had not been exposed to either of the two test baits before being tested, and (b) animals had previously been exposed to 5% coconut oil-based bait for 5 days before being tested.
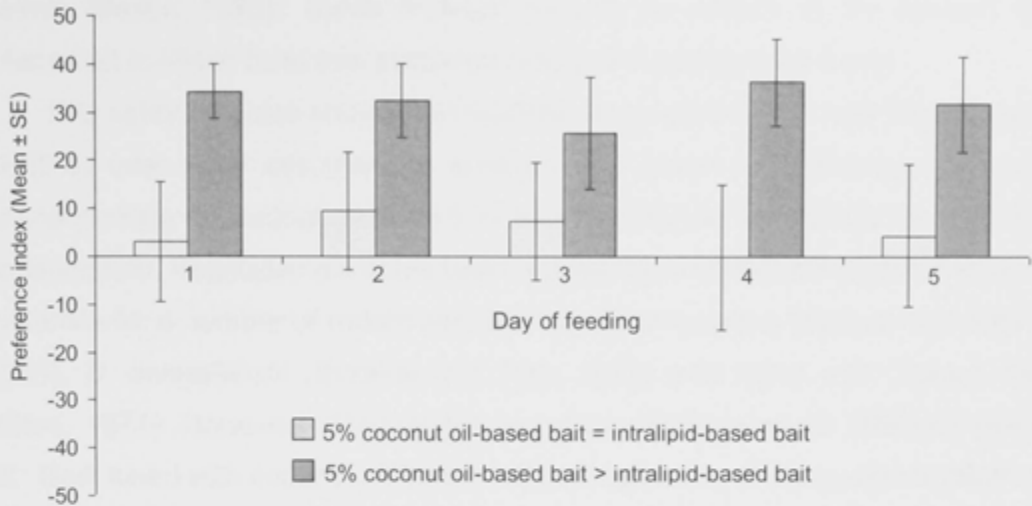
Figure 4.13 Preference index (Mean ± SE) in choice test between 5% coconut oil-based bait and intralipid-based bait over 5-day feeding period in two groups of ricefield rats: (☐) animals that had not previously been exposed to either of the two test baits before being tested, and (■) animals that had previously been exposed to the 5% coconut oil-based bait for 5 days before being tested

4.4 Discussion

In this study, bait acceptance by ricefield rats was affected by the physical form of the bait: bait in kibble form was preferred over granule or pelleted forms. While bait in pelleted form has been widely used for laboratory rats, this form of bait was less accepted by ricefield rats. The effects of texture and size of bait on bait uptake by various rodent species have been reported in a number of studies. Significant variation in food consumption was found in short tailed rats (*Nesokia indica*) due to differences in texture and size of bait (Shafi *et al.*, 1988). Murids in general preferred powdered, small sized and soft food over large and hard grains (Parshad and Jindal, 1991; Sahni, 1992). Pelleted baits were more accepted by Sprague Dawley rats (*Rattus norvegicus*) compared to bait in a powdered form (Naim *et al.*, 1986), and Lesser bandicoot rats (*Bandicota bengalensis*) preferred semolina flour of wheat, rice and millet grains over their whole grains (Kaur and Parshad, 2002). Bait with an open texture, such as agglomerated granules, was shown to be more attractive to rodents than solid blocks or pellets as rats can more easily detach small portions from the open texture bait than from solid blocks or

pellets (Baker, 1998). These findings support the results of the present study where bait in kibble form was preferred over pellet and granule forms.

This study has also shown that ricefield rats preferred bait with added coconut oil (5%) over other oils (canola, sesame, and neem oils). However, increased concentrations of coconut oil (from 5 to 10 and 15%) did not change the rate of bait consumption. Vegetable oils have been reported to improve the preference of food materials for a number of rodent species, e.g. *R. norvegicus* (Barnett and Spencer, 1953), *B. bengalensis* (Durairaj and Rao, 1975) and black rats (*Rattus rattus*) (Khan, 1974). However, different species show preferences for different types of oil. Bait mixed with coconut oil enhanced bait uptake by *B. bengalensis* (Malhi and Sheikher, 1988) and Common Indian field mice (*Mus booduga*) (Sivaprakasam and Durairaj, 1995). Norway rats (*Rattus norvegicus*) preferred coconut, peanut and corn oils, while *R. rattus* preferred corn oil over peanut oil (Meehan, 1984). Addition of 5-15% sesame oil into bait increased bait consumption by house mice (*Mus musculus*) (Asran, 1993a). Strong preference towards mustard oil at 2 and 5% was found in female *R. rattus*, whereas male rats preferred groundnut oil mixed diet over soybean oil, olive oil, sunflower oil and corn oil (Ahmad *et al.*, 1994). Bait mixed with cotton seed oil increased bait acceptance by *B. bengalensis* (Ramana and Sood, 1982). Addition of groundnut oil into bait improved bait acceptance in Indian palm squirrels (*Funambulus pennanti*) (Kaur, 1993), brown spiny mice (*Mus platythrix*) and African grass rats (*Arvicanthus niloticus*) (Suliman *et al.*, 1984; Soni *et al.*, 1985). The low consumption of neem oil-based bait found in this study may be due to its bitter taste (Chinnasamy *et al.*, 1993) and if used could cause bait avoidance by the rats.

In the present study, bait mixed with intralipid was consistently preferred by ricefield rats over baits containing other food additives. This improved acceptance may be due to additional nutritive values of intralipid, which is a fatty emulsion containing mainly soya oil (10-30%), egg lecithin (1.2%) and glycerol (2.25%). However, addition of anchovies or sardines, as an added protein source did not improve bait acceptance by ricefield rats. The addition of other food enhancers (crabs, eggshell and fish oil) did not enhance bait acceptance by ricefield rats, although previous studies reported that addition of various food additives, such as eggshells, egg yolk, yeast, cheese and fish into bait materials enhanced bait

acceptance by a number of rodent species. In *R. rattus*, 2% egg yolk increased bait consumption (Shafi *et al.*, 1990). A strong preference for bait with 2% eggshell or 2% egg yolk was found in Indian gerbils (*Tatera indica*) (Shafi *et al.*, 1991), and in ricefield rats (Khan *et al.*, 2000), while bait containing 2% yeast increased bait acceptance by *R. norvegicus* (Shafi *et al.*, 1992b). Fishmeal was used to improve bait uptake by mice (Jacob *et al.*, 2003b), whereas cheese, chocolate and soap were preferred over wax and oiled wood by *R. norvegicus* (Weihong *et al.*, 1999). Otsu (1979) reported that among 13 foods tested as bait bases in ricefield rats, four kinds (roasted salted fish, roasted crab, roasted shrimp paste and sardine) were consumed in small amounts, whereas nine others (cassava, sweet potato, sweet corn, polished rice, rice panicle, peanut butter mixed with shrimp liquid, fresh anchovies, eel, and roasted coconut) were not eaten. In general, the previous and present studies indicate that increasing the protein content of the bait through addition of food additives did not enhance bait acceptance by ricefield rats.

Cereal flour is the most popular base for baits for rodent control. In the present study, bait containing either a combination of wheat and rice flours (1:1) or rice flour only was preferred over the bait containing wheat flour only. The rice plant is the main dietary item for ricefield rats, and their survival and reproduction are highly reliant on the availability of rice crops in the fields (Lam, 1983; Leung *et al.*, 1999). This may explain the higher acceptance of bait containing rice flour over bait containing only wheat flour. A number of previous studies have reported that different rodent species prefer different kinds of cereal. For example, *N. indica* preferred sorghum and bajra over wheat, maize and cowpea (Ramesh, 1995), and *F. penanti* and *B. bengalensis* preferred cracked millet over cracked wheat, maize and rice (Malhi and Kaur, 1995; Kaur and Parshad, 2002). Wheat was most accepted by *B. bengalensis* (Bhadauria and Mathur, 1994), whereas rice was preferred over other grains by *R. rattus*, *B. indica*, and *T. indica* (Sridhara and Srihari, 1983; Chakraborty and Chakraborty, 1984; Bhadauria and Mathur, 1994). Wheat and sorghum were most preferred by *M. musculus* (Asran, 1993b). According to Leung *et al.* (2007), bait uptake of *R. argentiventer* was increased 65% by replacing maize flour with rice flour as bait base.

The choice test between the two most accepted baits which were determined from the series of no-choice and choice tests in this study, showed that cereal-

based bait containing intralipid (20%) plus 5% coconut oil and bait with 5% coconut oil only were equally accepted by ricefield rats. Intralipid has been proposed as an appropriate carrier for VCD, therefore, a bait formula containing intralipid should be chosen for further bait-uptake studies in ricefield rats.

If animals had previously been provided with 5% coconut oil-based bait for 5 days, this bait was highly preferred over the intralipid-based bait in the choice test. Many rodent species exhibit neophobic responses, that is, an avoidance of novel food or objects (Barnett, 1958; Mitchell, 1976; Lund, 1988), but they become habituated with increasing exposure. Thus the results above are not unexpected as the animals were consuming a familiar food over a novel food. This also suggests that pre-baiting with the plain bait matrix would enhance familiarity and should improve bait uptake by ricefield rats.

Taken together, findings from this study indicate that bait in kibble form containing 10% broken rice, 30% wheat flour, 30% rice flour, 20% intralipid (as a carrier of control agents), 5% coconut oil and 5% sugar was readily accepted by ricefield rats under laboratory conditions. Therefore this bait formula was evaluated for uptake by ricefield rats under enclosure conditions (Chapter 5).

CHAPTER 5

Evaluation of bait uptake by ricefield rats using Rhodamine B as a bait marker under enclosure conditions

Paper in preparation to be submitted to *Wildlife Research*

Abstract

The efficacy of an orally delivered antifertility agent in the field will, at least partly, depend on its rate of uptake by the target species. Field trials of fertility control thus require information on bait consumption by the target species. Although bait uptake studies using bait markers have been undertaken in a range of species, including rodents, there has been a lack of quantitative analysis of bait uptake by ricefield rats (*Rattus argentiventer*), one of the major pests of rice production in Southeast Asia. In this study, consumption of a kibble bait by ricefield rats was assessed under field enclosure conditions, using Rhodamine B (RB) as a bait marker. Wild caught rats were placed into 3 enclosures (each 25 x 50m) (n=45 rats per enclosure) and were acclimatized for 5 days with unhulled rice available in bait stations (n=18 per enclosure). The kibble bait was then offered and uptake monitored until the daily consumption reached an asymptote (~4-5 days). After that, kibble bait coated with RB (0.3% w/w) was offered overnight. To obtain an estimate of the proportion of animals consuming bait, approximately 50% of the animals from each enclosure were killed to collect whiskers for detection of RB. The remaining animals were fed with kibble bait for another 4 days, and daily bait consumption was again recorded. For 2 enclosures, a second round of RB-coated kibble bait was offered 14 days after the first round. Two days later, all animals were caught and killed to collect whiskers for RB detection using a fluorescence microscope.

Total consumption of kibble bait by ricefield rats increased during the first 4 to 5 days of the feeding period. A high proportion of animals (84.6-100%) in each enclosure were shown to have RB bands in their whiskers indicating they had consumed the RB-coated kibble bait. The average daily consumption per individual was approximately 9.1g/100 g body weight. Similarly, after the second round of RB-coated kibble bait, a high proportion (93.8-100%) of animals had fluorescent bands in their whiskers and the average daily consumption per individual was approximately 9.8g/100 g body weight. For these animals, approximately 56.3% from Enclosure 1 and 90% from Enclosure 3 showed 2 RB-bands in their whiskers. There was no significant difference (P>0.05) in bait acceptance between males and females. These results will be used to determine appropriate concentrations of

fertility control agents to be incorporated into formulated baits and to develop mathematical models simulating fertility control for ricefield rats.

5.1 Introduction

For fertility control of overabundant rodents, oral delivery of a control agent via non-toxic bait is considered one of the most practical options (Bomford and O'Brien, 1992; Holland, 1999; Fagerstone *et al.*, 2002; Jacob *et al.*, 2008). However, the efficacy of fertility control agents in the field will largely depend on bait acceptance by target species. One of the major challenges in bait use is achieving consistent bait uptake in sufficient quantities by a high proportion of the target population. For example, to achieve fertility control effects at the population level, 50–80% of female house mice (*Mus domesticus*) (Chambers *et al.*, 1997; Chambers *et al.*, 1999b) and more than 50% of female ricefield rats (*Rattus argentiventer*) (Jacob *et al.*, 2004a) need to be sterilized. Thus, it is likely that an even higher proportion of the target population needs to consume a fertility control bait to ensure success via a baiting program for these species.

The ricefield rat is one of the major pests of rice production in Southeast Asia (Aplin *et al.*, 2003; Singleton, 2003; Singleton *et al.*, 2003; Baco *et al.*, 2010; Singleton *et al.*, 2010; Sudarmaji *et al.*, 2010). This species is highly fecund and has been considered as a potential species for fertility control (Jacob *et al.*, 2008). Information on bait consumption under field conditions is essential for defining the parameters for delivering effective doses of fertility control agents in bait.

A series of trials on bait acceptance by ricefield rats under laboratory conditions determined that wild-caught ricefield rats prefer a kibble bait based on wheat and rice flours and containing coconut oil, intralipid (a fatty emulsion) as a carrier and sugar (see Chapter 4). Field studies on bait uptake using bait markers have been undertaken for a range of species including other rodents (Morgan, 1981; Lindsey, 1983; Cowan *et al.*, 1987; Farry *et al.*, 1998; Fisher, 1999; Spurr, 2002), but there has been no quantitative analysis of bait uptake by ricefield rats. This study therefore was conducted to assess the uptake of the preferred kibble bait using Rhodamine B as a bait marker under field enclosure conditions. The

aims of this study were to determine the proportion of animals consuming the bait and the average quantity of bait consumed per individual ricefield rat. Results from this study will help to define parameters for delivering appropriate concentrations of a fertility control agent in bait and the data is essential for further field studies on fertility control of ricefield rats.

5.2 Materials and methods

5.2.1 Enclosures

The study was conducted in field enclosures located in lowland, irrigated rice fields of the Indonesian Center for Rice Research (ICRR) in Sukamandi, West Java, Indonesia (06°20'S, 107°39'E), during the onset of the 2010 wet season (October–December). The enclosures were set up following the method described by Jacob et al. (2004a). Two enclosures (50m x 50m) built of 3-mm sheet metal (1.5m above ground, 0.2m below ground in a concrete foundation) were used. The metal sheets were stabilised by vertical metal bars (1.5m above ground, 0.2m below ground in a concrete foundation) every 3 m. The metal bars were covered with sheet metal to prevent the rats from climbing up the bars. Each of the 2 enclosures was subdivided into 2 equal enclosures (25m x 50m) with a brown 1-mm thick plastic fence (1.0 m above ground, 0.2 m below ground) that was stabilised with sewn-in bamboo poles (Fig. 5.1).

The outer fence was fitted with plastic netting (mesh size 2mm) on bamboo poles that extended 1.5 m above the metal fence to exclude predators such as cats and mongooses. A moat (50 cm wide) was built along each fence line, inside each of the enclosures, and 2 linear soil banks bisected each enclosure, connecting with the banks along the fence lines. An underground piping system linked to irrigation channels provided water to the moats during the experiment. Mesh wire (1.5 cm mesh size) covered all openings in the irrigation system to prevent rats from escaping but allowed access for insects, snails, small fish and amphibians that were commonly present in the adjacent fields. An earth mound was built (5m x 0.8m x 0.5m) in the centre of each enclosure, and bamboo tubes and rice straw were provided as shelter and nest sites for the rats. All enclosures were under fallow condition at the time of introduction of the rats (Fig. 5.1a). The soil was

maintained moist and weeds were allowed to grow during the experiment (Fig. 5.1b).

Figure 5.1 Appearance of enclosures (25 x 50 m) at (a) start and (b) the end of experiment. Bait stations were placed along the banks at equivalent distances. A moat was built along the fence and plastic netting was fixed on the outer fence to avoid animal losses caused by predation or escape. Water was maintained and grasses were allowed to grow over the period of experiment. Apart from the kibble bait, animals could also access other natural food sources such as grasses, snails, insects, small fish and amphibians available in the enclosures.

5.2.2 Animals

Wild ricefield rats (*Rattus argentiventer*) were caught from the fields around Sukamandi using a linear barrier trap system (LTBS) with multiple-capture traps (20 x 20 x 50 cm), by hunting and by digging burrows along banks. Adult non-

pregnant females and males were checked for general health (alert, active, good coat, no obvious infections or injuries), weighed, tagged with brass ear tags (left ear tagged for females and right ear tagged for males) and then immediately transferred into the enclosures. Forty-five healthy adult rats (29 females, 16 males) (80-150 g) were released into each enclosure.

5.2.3 Baits

Plain kibble bait

Based on the series of laboratory trials described in Chapter 4, a kibble formulation, containing 10% broken rice, 30% wheat flour, 30% rice flour, 5% coconut oil, 20% Intralipid and 5% sugar was the most accepted bait by ricefield rats. In this study, uptake of this bait formulation was examined under field enclosure conditions.

Bait marker

Rhodamine B (RB) (Basonyl Red 540, BASF Australia Ltd, Vic., Australia) was used as a bait marker. RB is a popular non-toxic bait marker which has been used in bait uptake studies for different species (Morgan, 1981; Lindsey, 1983; Cowan *et al.*, 1987; Farry *et al.*, 1998; Fisher, 1998, 1999; Spurr, 2002). It is a xanthene dye which is taken up by cells undergoing mitosis and results in long-lasting markings of keratinous structures such as hair and whiskers (Fisher, 1998). RB generally does not affect palatability at the doses used for bait marking in vertebrates (Fisher, 1999). It has relatively high LD_{50} oral doses for various species of vertebrates, and is not considered a highly toxic substance. After ingestion the systemic bait marker can be detected in several tissues, blood, urine, and faeces under ambient light as well as in hair and whiskers under UV light (Lindsey, 1983; Fisher, 1998).

Bait coated with RB

Kibble bait coated with RB (0.3% w/w) was used to determine the proportion of rats consuming the bait and the average amount of bait consumed per individual

rat. It was prepared by first mixing 0.3 g of RB powder with 20 ml of coconut oil and then mixing this solution thoroughly with 1 kg of kibble bait which was then dried at room temperature before use.

To minimise the potential influence of the colour of RB on bait uptake, the kibble bait was dyed with a pink food dye that was similar to the colour of RB-coated kibble bait (Fig. 5.2).

Figure 5.2 Plain kibble and Rhodamine B–coated kibble bait

5.2.4 Bait stations

Bait was provided in each enclosure using bait stations. The stations were made using a plastic box (30 x 21x 12 cm), with two openings (5 cm x 20 cm) along the sides to allow access to the bait. Kibble bait was placed in a solid glass ashtray (diameter = 10cm) held in position in the centre of the box by three nails. Bait stations were designed to minimise access by birds and had an overhanging plastic cover to protect the bait from rain (Fig. 5.3).

Eighteen individually marked bait stations were used in each enclosure and were placed along the banks equidistant from each other (Fig. 5.4). The position of the stations was fixed throughout the experiment.
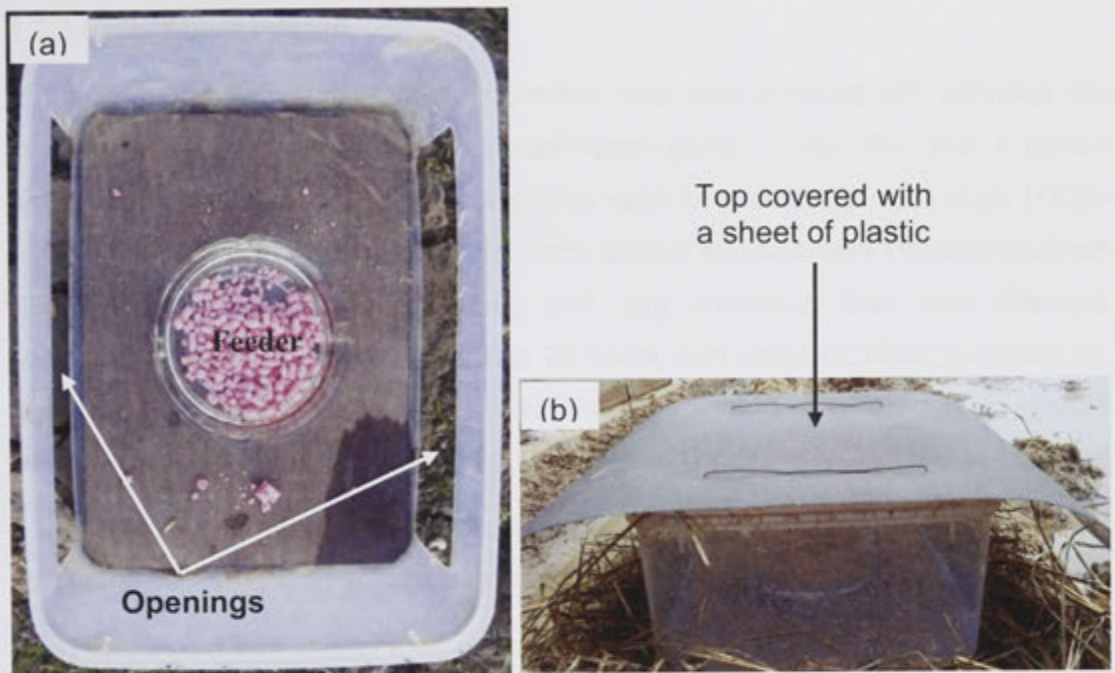
Figure 5.3 Bait station made of (a) plastic box (30 x 21x 12 cm) with two openings (5 cm x 20 cm), feeder (ashtray) and (b) covered by plastic sheet on the top.
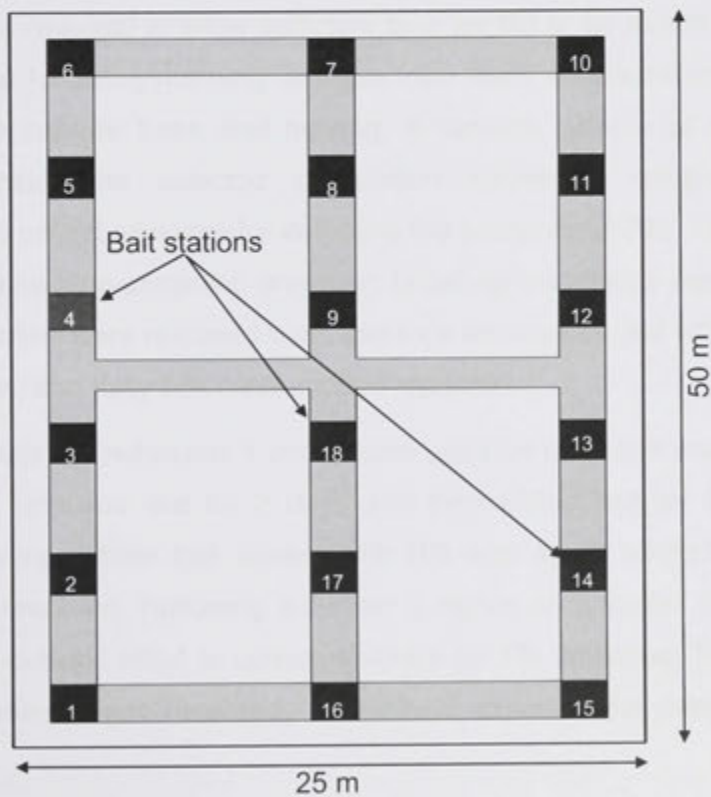
Figure 5.4 Arrangement and identification of bait stations in enclosures

5.2.5 Experiment design

After being released into the enclosures, rats were provided with unhulled rice in each bait station during a 5 day acclimation period. After this time a known amount of kibble bait (~25g) was placed in each bait station around dusk (1730-1800h). To minimise access to bait by birds, all bait stations were checked at dawn (0530-0630h) the following morning and any remaining bait was collected separately, dried in an oven at 40°C for 24 hours, and weighed. After correction for moisture loss, daily consumption was measured as the difference in weight between the amount added and the amount remaining.

If most or all bait was consumed at a bait station during the previous night, an additional 50% of bait by weight was presented thereafter.

Plain kibble bait was offered and uptake monitored until the daily consumption in each enclosure reached an asymptote. Kibble bait coated with RB (0.3% w/w) was then offered overnight and consumption recorded. Unhulled rice was given on the following two nights. This two-night interval after consumption of RB-coated kibble bait was required to allow sufficient time for RB to be detectable in the rats' whiskers. The following morning, animals from each enclosure were trapped out using multiple capture traps and hunting. A random sample of around 50% of trapped animals was selected (population sampling), weighed and killed immediately to collect whiskers for detecting the presence of RB. The reproductive status of females (non-pregnant, pregnant, lactating) and males was recorded. The remaining animals were released back into their enclosures, fed with kibble bait for another 4 days, and daily bait consumption recorded.

The animals in Enclosures 1 and 3 were used for a second round. They were provided with unhulled rice for 2 days and then kibble bait for 3 days. On the following evening, kibble bait coated with RB was again offered overnight and consumption recorded. Following a further 2 nights on unhulled rice the animals were trapped-out and killed to collect whiskers for RB detection. The reproductive status of all animals was recorded. The overall experimental design is presented in Figure 5.5.
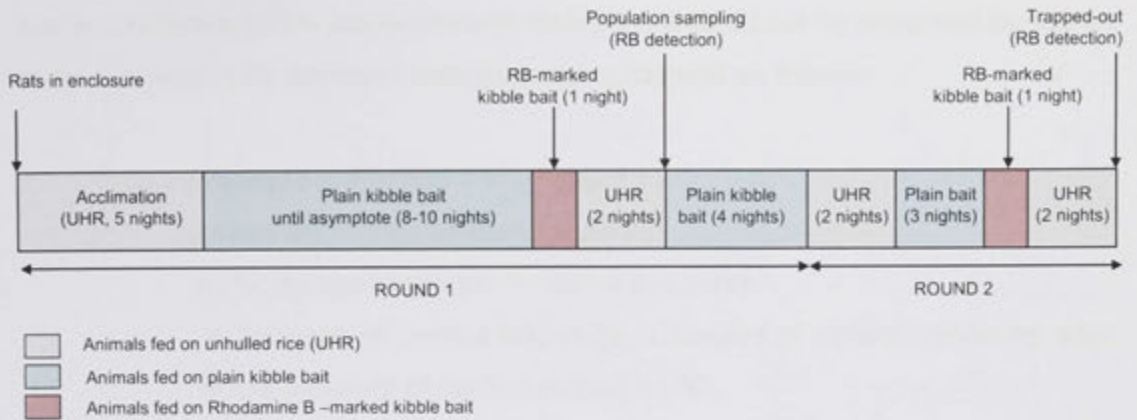
Figure 5.5 Experimental design for bait uptake by ricefield rats using Rhodamine B (RB) as a bait marker under field enclosure conditions.

5.2.6 Detection of Rhodamine B

At autopsy, approximately 8-10 whiskers from each side of the mouth of each animal were collected. Preparation of whiskers for RB examination was undertaken following the method described by Fisher (1998). Briefly, 4-6 whiskers were mounted parallel to each other on a standard laboratory slide using Fluoromount-G (SouthernBiotech) as the mounting medium. The whiskers were then covered with a coverslip and examined under a fluorescence microscope set up with a light source and filter combinations (Leitz Diaplan Fluorescence Microscope, Type 020-437.035512834). A high intensity 200 W mercury lamp light source was used, in conjunction with a permanent BG38 filter and Green filter combination block to select for the emission of RB fluorescence. Under the fluorescence microscope, whiskers marked with RB were identified by the presence of bright orange bands.

5.2.7 Measurement of bait consumption

Daily bait uptake

Daily bait consumption (M_C) was determined as the difference in weight (g) between the amount added (M_A), and the amount remaining (M_R) after drying. A correction was made for moisture changes for the amount added. This was determined using a set of control bait samples (n=3). These were exposed to the

same conditions as the enclosure bait stations but could not be accessed by rats or birds. Correction for moisture changes was calculated as follows:

Moisture correction = $M_A + (M_A \times p)$

where M_A = daily amount (g) added;

p (% change in weight in control samples)

= [(amount of control added (g) − amount of control remaining after drying)/amount of control added] x 100.

Therefore amount consumed, $M_C = [M_A + (M_A \times p)] − M_R$

Proportion of animals consuming bait

The proportion of animals consuming bait (P_{RB}) was estimated as the percentage of animals marked with Rhodamine B,

$P_{RB} = (N_{RB}/N_S) \times 100$;

where N_{RB} = number of individuals marked with RB, and

N_S = total number of animals sampled (killed).

Average bait consumption per individual

Daily average bait consumption per individual (M_I) was estimated as amount of RB-coated kibble bait consumed per individual, $M_I = M_{RB}/N_E$;

where M_{RB} = total amount of RB bait consumed, and

N_E = estimated number of animals consuming RB bait

= [(% animals marked with RB) x (total number of individuals trapped-out)]/100.

The estimates of bait consumption normalised to body weight (g eaten/100 g body weight) were determined from the amounts of RB-coated kibble bait consumed per individual and the average body weights of the RB-marked individuals.

The average bait consumption per individual and proportion of animals consuming bait were also estimated using a removal index (Eberhardt, 1982; Cowan *et al.*, 1987) which has been applied to bait uptake in free-ranging wild populations, and they were calculated as follows:

Average bait consumption per individual

Daily average bait consumption per individual (M_I) was estimated as amount of RB-coated kibble bait consumed per individual, $M_I = M_{RB}/N_E$;

where M_{RB} = total amount of RB bait consumed, and

N_E = estimated number of animals consuming RB bait

= (number of individuals marked with RB)/(estimated proportion of the population removed by sampling, P_b). $P_b = (x_1 - x_2)/x_1$;

where x_1 = consumption of RB-coated kibble bait prior to sampling, and

x_2 = daily consumption of kibble bait after sampling. Consumption after sampling was calculated as the average consumption over 4 days after population sampling. Average consumption was used to minimise potential effects of disturbance (caused by population sampling on consumption) on the first day after sampling.

Proportion of animals consuming bait (P_E) = $(N_E/N_1) \times 100$;

where N_E = estimated number of individuals consuming RB bait (see above), and N_1 = total number of animals trapped-out.

5.3 Results

5.3.1 Loss of rats due to predation and escape.

During the acclimation period it became apparent that rats were escaping from the enclosures. Repairs were made to prevent further losses of animals. The enclosures were trapped out and each was found to have approximately 30 animals before the kibble bait was first provided. During the initial kibble period, animal losses caused by predation were observed in Enclosure 2 (see below).

5.3.2 Daily bait consumption over the course of the experiment

Daily bait consumption by the rats in Enclosures 1 and 3 increased rapidly during the first 4 to 5 days on kibble bait before reaching an asymptote over the next 4 to 5 days (Fig. 5.6). Consumption in Enclosure 2 also increased significantly during the first 4 days, but declined steadily over the next 4 days before reaching

an asymptote. The decline in consumption from day 5 to day 7 in Enclosure 2 was due to some rats escaping and/or due to predation by cats and mongoose. The enclosure was modified at this point to prevent animal losses for the rest of the study period. After population sampling to remove around 50% of the population from the enclosures, total bait consumption by the remaining animals was reduced by about 50%. This consumption was maintained until the end of the experiment (Fig. 5.6).

5.3.3 Proportion of animals consuming bait and the average amount of bait consumed per individual rat

Two days after the first round of RB kibble bait, RB bands were detected in the base of the whisker follicles. Similarly after the second round of RB-kibble bait, bands were again detected in the base of whisker follicles. At this time whiskers were also examined for a second band (resulting from the first feed); if observed these were close to the whisker tip and were "less" fluorescent than the bands in the base of the follicles (Fig. 5.7).

Following the first round of offering RB-coated kibble bait overnight, the whiskers of a high proportion of animals were marked with RB (84.6%, 90.9% and 100% in Enclosures 1, 2 and 3 respectively) (Table 5.1) 2 days later. Average bait consumption per rat for each of the enclosures, ranged from 8.8–9.5 g/100g body weight (Table 5.1). Enclosure 2 was trapped out on Day 17, 6 days after RB kibble bait (Fig. 5.6), and of these animals (n=12), 92% also showed bands in their whiskers.

Similarly, results from the second round of RB-coated kibble bait (Enclosures 1 and 3) indicated a high proportion of animals consumed bait (~ 97%) and at an average rate of ~9.8 g/100 g body weight (Table 5.1).

The bait uptake by ricefield rats after the first round of feeding was also examined using a removal index described by Eberhardt (1982) and Cowan (1987). This analysis gave similar results and demonstrated a high proportion of animals were consuming bait (~80%) at a consumption rate per individual of ~10.5g/100g body weight (Table 5.2).

For the animals that were provided with RB-coated kibble bait twice (14 day interval), the proportion of animals with 2 RB bands (Fig. 5.7) in their whiskers was

56% (9/16 animals) and 90% (9/10 animals) from Enclosures 1 and 3, respectively (Table 5.3).

There was no effect of sex on bait acceptance or bait consumption rates for either round of feeding trial (P>0.5) (Table 5.4)

Assessment of the reproductive status of each female and male rat at the time of autopsy revealed no signs of pregnancy or lactation in females although males all had descended scrotal testes.

5.3.4 Distribution of bait uptake at different stations in the enclosures

The distribution of bait consumption from different bait stations in Enclosures 1 and 3 was analysed using the uptake recorded for the final 4 days of the experiment. More bait was taken from the stations located along the middle banks of each enclosure (Fig. 5.8).

5.3.5 Bait consumption by other species

During the experiment, there was no evidence of access to bait stations by any non-target species other than a very few birds (sparrows) in any enclosures. Access by birds was minimised by placing kibble bait in bait stations at dusk and by collecting any remaining bait at dawn the following morning, before any birds were actively feeding in the fields. It is concluded that birds removed minimal amounts of bait and did not contribute to overall bait consumption.
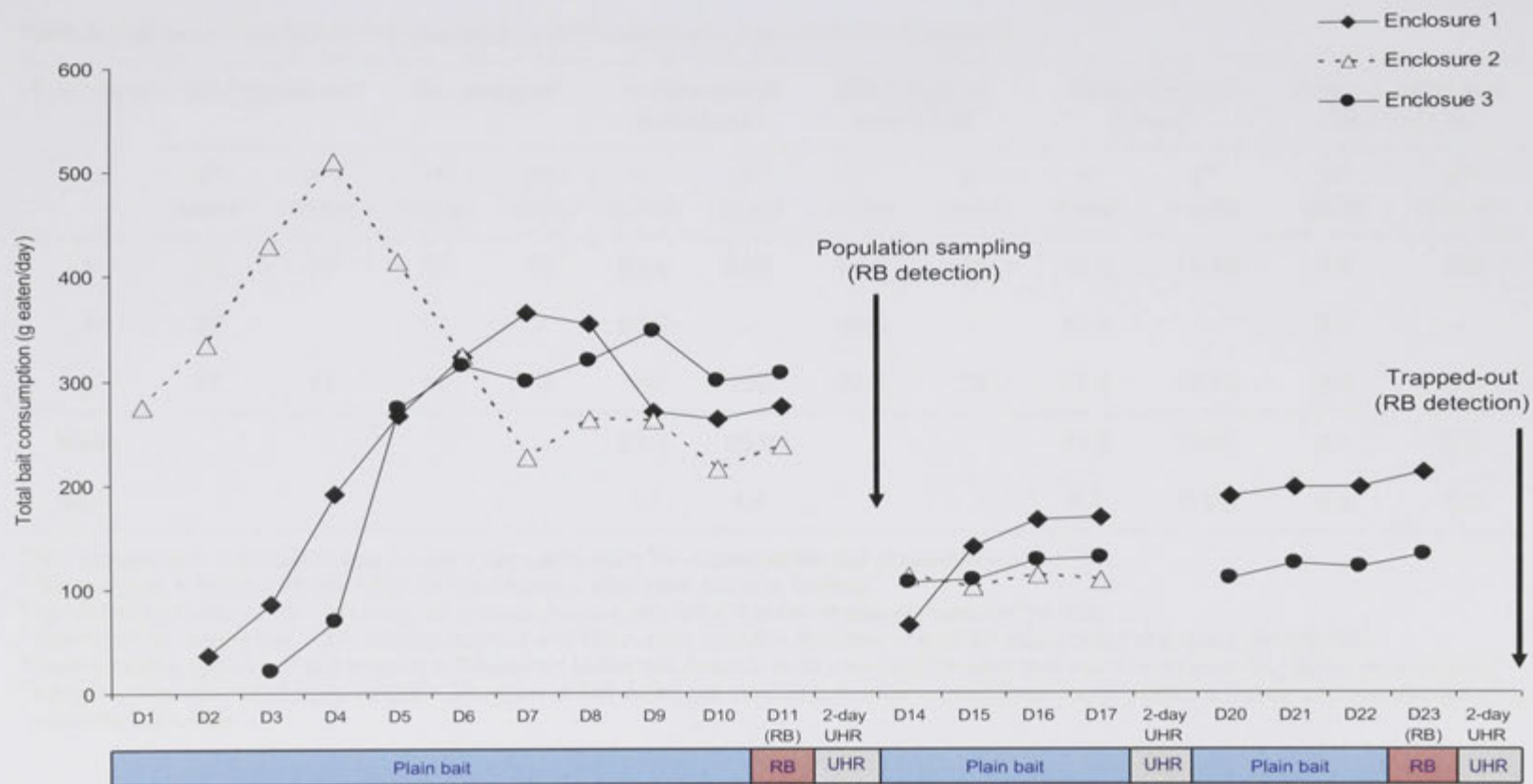
Figure 5.6 Daily bait consumption (g eaten/day) by ricefield rats in three enclosures during the experiment. Bait coated with Rhodamine B (RB) was provided overnight after 8-10 days of pre-feeding with kibble bait. Two days after RB-coated kibble bait was provided, population sampling (kill) was conducted; approximately 50% animals from each enclosure were removed and killed to collect whiskers for RB detection. The remaining animals were provided with kibble baits again for 4 days (days 14-17), and daily consumption recorded. Animals from enclosure 2 were trapped out on Day 17. Animals from Enclosures 1 and 3 were used for a 2nd feeding round; after the completion of the 1st round, rats were fed unhulled rice (UHR) for 2 days then kibble bait for 3 days (days 20-22) before being presented with RB-coated kibble bait overnight. Two days later, all animals were killed to collect whiskers for RB detection.

Table 5.1 Uptake of Rhodamine B-coated kibble bait by ricefield rats under enclosure conditions.

Enclosure	No. trapped out[a]		No. sampled[b]		% RB-marked individuals[c]		Estimated no. eating bait[d]		Amount eaten (g/rat)[e]		Amount eaten per 100g body wt.[f]	
	1st round	2nd round	1st round	2nd round	1st round	2nd round	1st round	2nd round	1st round	2nd round	1st round	2nd round
1	29	16	13	16	84.6	93.8	24.5	15	11.2	13.99	9.5	9.6
2	23		11	-	90.9	-	20.9	-	11.3	-	8.8	-
3	27	13	14	10	100	100	27.0	10	11.4	13.15	9.0	9.9
Mean					91.8	96.9			11.3	13.57	9.1	9.8
SD					7.7	4.4			0.1	0.59	0.4	0.2

[a] No. trapped-out = Number of rats caught 2 days after each RB-coated kibble bait presentation;
[b] No. sampled = Number of rats killed for RB detection after each round of feeding;
[c] % RB-marked individuals = [(Number of animals marked with RB)/(Number of animals sampled)] x 100;
[d] Estimated no. eating bait = [(% animals marked with RB in each round) x (Number of animals trapped-out after each round)]/100;
[e] Amount eaten (g/rat) = (Total amount of RB-coated kibble bait eaten in each round)/(Estimated number of animals eating bait in each round);
[f] Amount eaten per 100 g body weight = [(Amount of bait eaten per individual in each round)/(Mean body weight of individuals marked with RB in each round)] x 100.

Table 5.2 Uptake of Rhodamine B-coated kibble bait by ricefield rats calculated based on a removal index.

Enclosure	No. trapped out after 1st RB	No. sampled[a]	% RB-marked individuals[b]	P_b[c]	Estimated no. eating bait[d]	Amount eaten (g/rat)[e]	Amount eaten per 100g body wt.[f]	Estimated proportion consuming bait[g]
1	29	13	84.6	0.51	21.6	12.8	10.9	74.5
2	23	11	90.9	0.54	18.5	12.8	10.0	80.4
3	27	14	100	0.62	22.7	13.5	10.7	84.1
Mean			91.8			13.0	10.5	79.7
SD			7.7			0.4	0.5	4.8

[a] No. sampled = Number of animals killed after the 1st round of feeding;
[b] % RB-marked individuals = [(Number of animals marked with RB)/(Total number of animals sampled)] x 100;
[c] P_b = Estimated proportions of populations removed by sampling = $(x_1-x_2)/x_1$; where x_1 (x_2) = daily bait consumption before (after) sampling;
[d] Estimated no. eating bait = (Number of animals marked with RB)/P_b;
[e] Amount eaten (g/rat) = (Total amount of RB-coated kibble bait eaten)/(Estimated number of animals eating bait);
[f] Amount eaten per 100 g body weight = [(Amount of bait eaten per individual)/(Mean body weight of individuals marked with RB)] x 100;
[g] Estimated proportion consuming bait = [(Estimated number of animals eating bait)/(Number of animals trapped-out)] x 100.

Table 5.3 Proportion of animals with one and two RB-bands in their whiskers after being provided with RB-coated kibble bait

Enclosure	1st RB bait exposure [†]	2nd RB bait exposure[‡]	
	% animals with 1st round RB-band	% animals with 2nd round RB-band	% animals with 2 RB-bands
1	84.6 (11/13)	93.8 (15/16)	56.3 (9/16)
3	100 (14/14)	100 (10/10)	90.0 (9/10)

[†] Animals were killed two days after the 1st RB kibble bait provided
[‡] Animals were killed two days after the 2nd RB kibble bait provided
Numbers in brackets are the number of animals marked with RB/total number of animals sampled
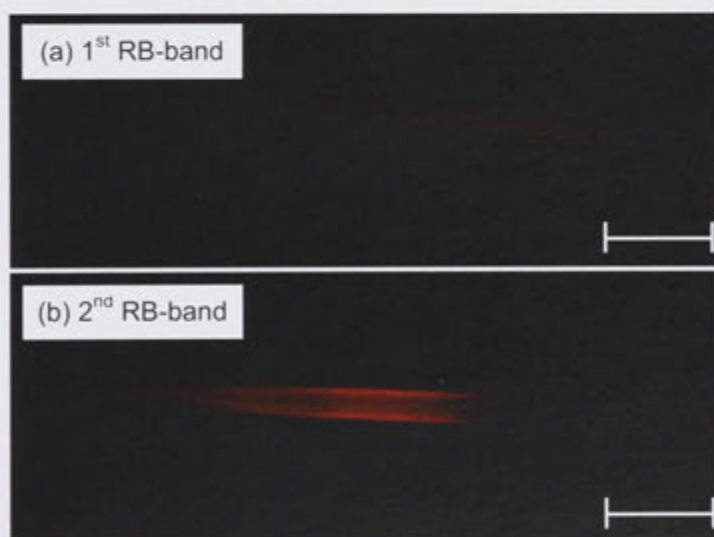
Figure 5.7 Whisker bands observed in ricefield rats after two single intakes (14 days interval) of Rhodamine B-coated kibble bait under a fluorescence microscope. (a) 1st RB band near the tip of the whisker, and (b) 2nd RB band close to the follicle of the whisker; scale bar = 1.0 mm.

Table 5.4 Bait uptake by ricefield rats with respect to sex[c].

	1st RB round		2nd RB round	
	Males	Females	Males	Females
No. sampled	16	22	9	17
No. marked with Rhodamine B[a]	15	20	8	17
% marked with Rhodamine B[b]	93.8^{ns}	90.9	88.9^{ns}	100

[a] Number marked with Rhodamine B = Number of males or females known to have consumed RB-coated kibble bait by detecting the presence of RB in their whiskers;
[b] % marked with Rhodamine B = (Number of males or females marked with RB)/(Total number males or females sampled);
[c] Proportions of males and females consuming bait in each feeding round were compared using Chi-square test at the significance level of 5%. Values with ^{ns} in male group are not significantly different from female group in round 1 (x^2 = 0.81, df = 1, P>0.05) and round 2 (x^2 = 1.96, df = 1, P>0.05).
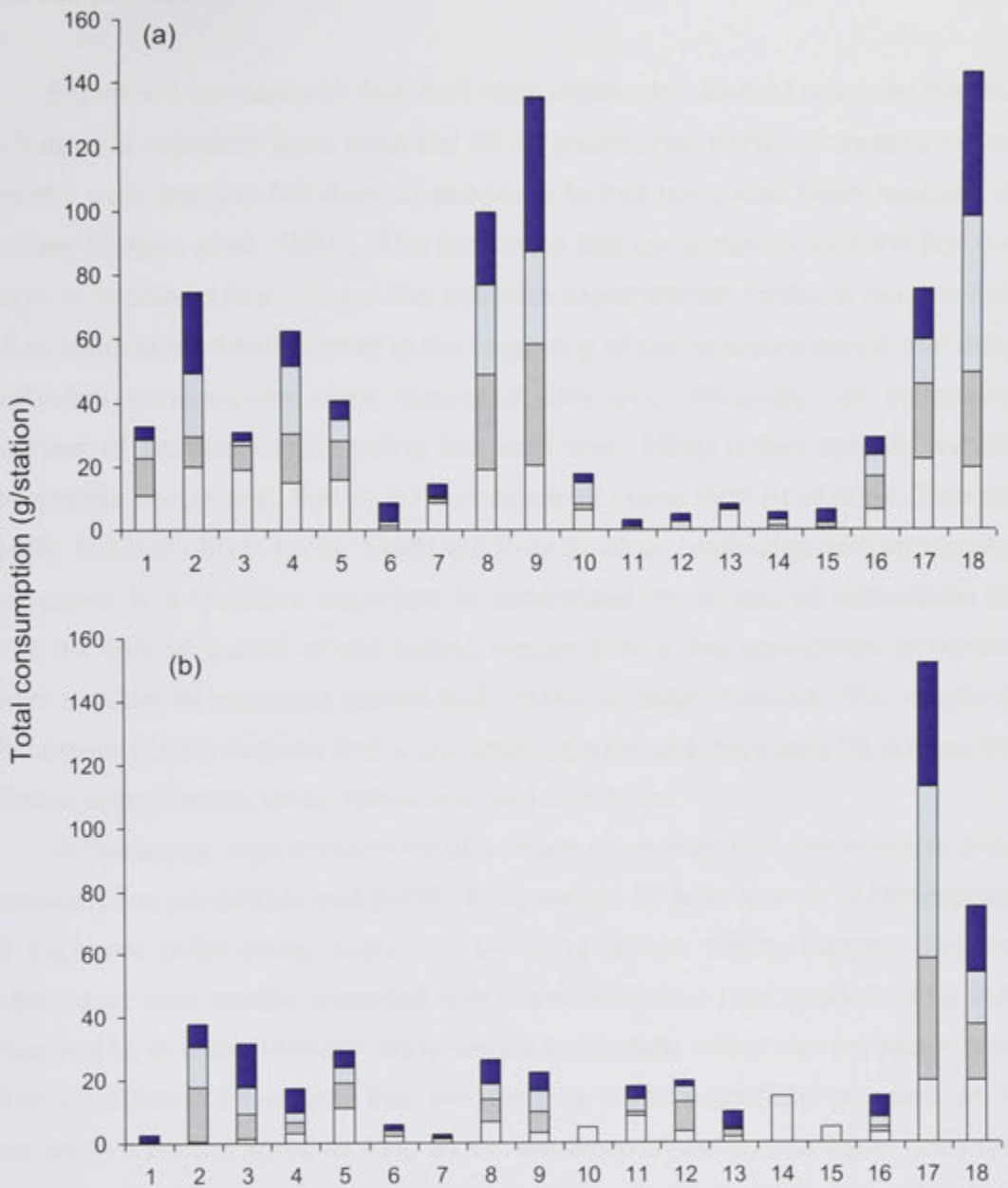
Figure 5.8 Distribution of total bait consumption (g/station) for each of the 18 bait stations in (a) Enclosure 1 and (b) Enclosure 3 during the last 4 days (☐) day 20; (☐) day 21; (☐) day 22; and (■) day 23 of the experiment.

5.4 Discussion

Significant increases in daily bait consumption by ricefield rats over the first 4-5 days of exposure were recorded for all enclosures. Similar increases in bait uptake over the first 5-8 days of exposure to bait have also been reported in rabbits (Cowan *et al.*, 1987). The increasing bait consumption over the first 4-5 days of exposure has at least two potential explanations. Firstly, it may be that all animals started eating baits at the beginning of the exposure period and daily individual consumption rates increased with time. Secondly, an increasing number of animals began eating bait with time. Many rodent species exhibit neophobic responses, that is an avoidance of novel food or objects (Barnett, 1958; Mitchell, 1976; Lund, 1988) but they become habituated with increasing exposure. It is therefore important to understand the drivers of bait-uptake so that an optimal period of pre-baiting occurs before the application of control baits in order to maximise control bait uptake by target species. The results of the present study indicate that a pre-baiting period of 4 days may be optimal for *Rattus argentiventer* under fallow rice-field conditions.

In this study, high proportions of animals consumed bait and average daily consumption per animal was 8-10% body weight for both rounds of Rhodamine B exposure (intervening interval of 14 days). These results suggest that the kibble bait was readily accepted and consumed at a rate equivalent to that required to, at least, maintain body weight by ricefield rats under the fallow rice-field conditions. Therefore, this bait formula could potentially be used as a carrier of a control agent as long as the presence of the control agent does not constrain bait acceptance. For control agents that may influence bait acceptance, such agents should be masked before being incorporated into the bait in order to maintain palatability.

For Enclosures 1 and 3, where the animals were provided twice with RB-coated kibble bait, high proportions of animals showed RB-bands in their whiskers two days after exposure to this bait (84.6% and 93.8% after the 1st and 2nd RB exposures respectively for Enclosure 1, and 100% after both RB exposures for Enclosure 3). However, lower proportions of animals were found with 2 RB-bands (56.3% for Enclosure 1 and 90% for Enclosure 3). For those whiskers with 2 RB-bands, the first round bands were mainly observed at the tips of the whiskers while the second round bands were found close to the base

of the whisker follicles. This difference between enclosures might be partly due to the interval (14 days) between two RB exposures, or lower consumption of RB-coated bait in some animals in the first round leading to the fading or disappearance of the first round RB bands by the time of whisker collection after the second round. The lower proportion of animals consuming RB-coated bait in the first round in Enclosure 1 (84.6%) compared to Enclosure 3 (100%) may reflect the lower proportion of animals with two bands in Enclosure 1 than Enclosure 3. However, the difference in the proportion of animals with 2 RB-bands between the 2 enclosures is not easily explained. The persistence of RB marking depends on the concentration of RB in the bait, the rate of growth of the whisker, which can vary between species, individual animals and even between individual whiskers (Fisher, 1998; Weerakoon and Banks, 2011). For example, persistence of RB-marked bands was 60-100 days in rabbits, and 65 days in lab rats (Fisher, 1995), and 7-50 days in the wood mouse (Apodemus sylvaticus) and bank vole (Clethrionomys glareolus)(Papillon et al., 2002). For ricefield rats, no information about the occurrence and persistence of RB in the whiskers has been published. Results from this study suggest that in ricefield rats persistence may be shorter than in lab rats, and so for future studies on bait-uptake in ricefield rats using RB, shorter intervals between multiple applications of RB-coated baits should be considered.

An analysis of daily bait uptake at different stations in Enclosures 1 and 3 revealed that more bait was taken by the rats from the stations located on the middle bank of the enclosures. This was not unexpected because in each enclosure an earth mound was built on this bank to provide a shelter/nesting place for the rats. However, this result also demonstrates that the rats were less likely to move further from shelter than was "safe" in order to consume bait. This feeding behaviour may be explained in terms of how the animals respond to microhabitats to minimise their predation risk (i.e. giving-up densities, GUDs) (Jacob and Brown, 2000; Stokes et al., 2004). Ricefield rats prefer to use habitats where there is abundant cover (Brown et al., 2001; Brown et al., 2005). Therefore these results suggest that bait stations should be placed close to shelter or other vegetation cover if a control agent was to be applied in the fallow period between rice crops.

In all enclosures, there were no differences in consumption of kibble bait and the bait coated with Rhodamine B (0.3% w/w). These results suggest that

Rhodamine B at the concentration tested did not influence the bait acceptance by ricefield rats, and therefore could be successfully used for bait uptake studies in ricefield rats, as has been reported for a range of species (Morgan, 1981; Lindsey, 1983; Cowan et al., 1987; Farry et al., 1998; Fisher, 1998, 1999; Cagnacci et al., 2007; Wanless et al., 2008; de Tores et al., 2011; Weerakoon and Banks, 2011).

In this study, the kibble bait was provided in the absence of a growing rice crop, but in the presence of other natural food sources, such as grasses, snails, insects, small fish and amphibians. Survival of the rats was high and in contrast to one dietary study in which captive ricefield rats survived for only four to five days when fed exclusively on grasses, rice plants at the tillering stage, crabs, snails, and insects (Goot, 1951, cited by Leung et al. 2007). However, rats fed on starch-based foods such as rice grains, maize, sweet potatoes, peanuts and soy beans survived for several months (Goot, 1951, cited by Leung et al. 2007). Captive ricefield rats were also found to survive for more than two weeks when fed exclusively on rice plants at the ripening stage, whereas survival was poor when they were fed rice plants at an earlier stage of development (tillering and reproductive stages) (Murakami et al., 1990). These findings indicate that rice at the ripening stage is the most important food for the ricefield rats. The high rate of bait consumption by ricefield rats achieved in this study may have been due to the rats consuming the kibble bait as an alternative source of starch in their diet in the absence of their preferred natural food in the field.

The onset of the breeding season of ricefield rats is strongly linked to the growing stage of rice crop, and occurs just before the maximum tillering stage (Lam, 1983; Leung et al., 1999). Therefore for fertility control of this species, a bait containing a fertility control agent must be applied before breeding is initiated (i.e. before maximum tillering stage). During this period, rice grains (the preferred diet) are not available in the fields, so the cereal bait would likely be accepted by the rats. Indeed the high rates of bait consumption in this study suggest that the tested kibble bait could potentially be used as a carrier for control agents, such as chemosterilants. Furthermore, in the present study, adult male and female rats (with the ratio of 1: 2) were used in each enclosure, but during the experiment (30 days) there were no signs of pregnancy or lactation in females, despite an abundance of alternative food (bait). These results support previous findings that breeding of ricefield rats is triggered by

the quality of rice crop rather than the quantity of food (Lam, 1983). Therefore, the use of kibble bait as a carrier for fertility control agents before and during the early development of a rice crop would not be an issue in terms of providing an additional food source that could inadvertently stimulate an earlier onset of breeding by ricefield rats.

Findings from this study will help define parameters for delivering appropriate concentrations of fertility control agents in formulated bait; data is essential for further field studies on fertility control of ricefield rats.

CHAPTER 6

General discussion and future directions

This thesis investigated the effects of a chemosterilant on reproduction of laboratory rats, *Rattus norvegicus*, and evaluated the uptake of a bait type and formulation with potential to be used to incorporate and deliver a fertility control agent to the ricefield rat, *Rattus argentiventer*, a target species for fertility control in Southeast Asia. Each of Chapters 2-5 had its own specific discussion. This final chapter presents a summary of the major findings and their conclusions, and then discusses the relevance of this body of work for rodent management. Directions for future research are proposed.

6.1 Major findings

For fertility control, one potential approach is the use of chemosterilants. In Chapters 2 and 3, the research focused on the effects of an industrial chemical, 4-vinylcyclohexene diepoxide (VCD) on reproductive functions of laboratory rats (*Rattus norvegicus*) of both sexes. Chapter 2 examined the effects of different periods of treatment with VCD (500 mg/kg) on ovarian follicle populations as well as outcomes on the fertility of female rats. Oral treatment with VCD for one, two or three periods of 10 days, separated by 14 and 21 days, induced significant reductions in the number of primordial and primary follicles in the ovaries. These reductions in non-regenerating follicles resulted, over time, in significant reductions in the number of secondary and larger follicles. The effects of VCD on ovarian follicle depletion were time dependent: with increased VCD exposure, there was greater follicle depletion. Following three 10 day treatment periods with VCD, the fertility of the treated animals was significantly reduced from the second breeding round onwards. Because VCD selectively targets non-regenerating primordial and primary follicles, until the remaining larger follicles were recruited or became atretic, the effects on fertility were delayed. However, the eventual outcome was permanent infertility.

Chapter 3 determined the effects of oral administration of VCD (500 mg/kg body weight) for 15 consecutive days on the reproductive status of juvenile and adult male rats. Oral treatment with VCD did not affect either the weights of reproductive organs or the histological characteristics of the testes and epididymides of either juvenile or adult male rats at any time after VCD treatment. In addition, treated males remained fertile: when mated with fertile, untreated females at 14 day intervals over 80 days after the end of VCD

treatment, they all sired pups. Furthermore, the litter sizes of females mated with either treated or control males were not significantly different within or between breeding rounds. These results indicate there were no effects of oral administration of VCD on the reproductive viability of male rats.

In Chapters 4 and 5, the research focus shifted to bait uptake by ricefield rats. Chapter 4 presented the results of a laboratory evaluation of bait uptake by ricefield rats using different physical forms of bait, vegetable oils, food additives and cereal types in a three-phase bait-uptake study using a series of no-choice and choice tests. The results indicated that: 1) bait in kibble form was preferred by ricefield rats compared with granule and pellet forms; 2) bait with coconut oil was preferred over baits with canola, sesame and neem oils; 3) bait with an addition of intralipid (a fatty emulsion) was preferred over baits with added anchovies or sardines; and 4) bait containing rice flour or a combination of wheat and rice flours was preferred compared to bait containing only wheat flour as the bait base. Based on these results, the composition of bait preferred by ricefield rats was defined as follows: cereal-based bait in kibble form, containing broken rice (10%), equal proportions of wheat and rice flours (30%), coconut oil (5%), intralipid (20%) and sugar (5%). This bait, with Rhodamine B as a marker, was then used under field enclosure conditions to assess uptake by ricefield rats. The results of this evaluation were presented in Chapter 5.

Under enclosure conditions, the cereal-based bait formula was highly accepted by the ricefield rats. Consumption by the rats steadily increased over the first 4-5 days of the feeding period. A high proportion of animals consumed bait (80-100%) and a high rate of bait consumption per individual (9-10% of body weight) was observed. These results can be used to determine appropriate concentrations of fertility control agents to be incorporated into formulated baits and to develop mathematical models simulating fertility control for ricefield rats.

6.2 Relevance of thesis results for rodent management

Fertility control is a potential alternative to lethal control methods for the sustainable management of overabundant populations (Oogjes, 1997). This approach has been used successfully for population management of some large animals such as white-tailed deer (*Odocoileus virginianus*), wild horses

(*Equus caballus*) (Kirkpatrick *et al.*, 1997) and African elephants (*Loxodonta Africana*)(Fayrer-Hosken *et al.*, 2000; Delsink *et al.*, 2007). In small mammals, controlling populations by suppressing reproduction of female animals has been tested using surgical sterilisation under controlled enclosure and field conditions; for example, house mice (*Mus domesticus*) (Chambers *et al.*, 1999b), ricefield rats (*Rattus argentiventer*) (Jacob *et al.*, 2004a; Jacob *et al.*, 2006), and European rabbits (*Oryctolagus cuniculus*) (Twigg and Williams, 1999). These studies suggested that a high proportion of female animals need to be sterilised (60-80% for rabbits, 50–80% for house mice, and greater than 50% for ricefield rats) in order to achieve the desired effect at the population level.

However, currently the use of fertility control for the management of wildlife, especially for pest rodents is not practical or cost effective. None of the available fertility control agents can reduce the fertility of rodents immediately and permanently in order to achieve the required long term effect at the population level. Fertility control is generally not considered an appropriate solution on its own for population management, but it could be used to complement other approaches including culling (Barlow *et al.*, 1997; Cowan and Hinds, 2008). Theoretically, for high density pest populations, such as ricefield rats, lethal methods could be used to provide a rapid population knock-down, and then fertility control agents could be applied to maintain the population at below an identified threshold of economic damage. If an appropriately efficient and effective fertility control method was available, its use could be integrated with other ecologically-based rodent management approaches.

Findings in this thesis clearly demonstrate that the chemosterilant, VCD, is a promising candidate as a fertility control agent for population management of rats for a number of reasons. Firstly, VCD reduces fertility in female animals. In this study, treatment with VCD induced a significant depletion of follicle populations in the ovaries of female laboratory rats. As a consequence, the fertility of the rats was significantly reduced over time. Many rodent species have promiscuous mating systems (Wolff and Sherman, 2007) and their increase in population size is largely dependent on the fecundity of females. Therefore, for fertility control of rodents, suppressing reproduction in females is considered to be the most efficient approach to achieve an effect at the population level. Imposing male sterility in species with promiscuous mating

systems is unlikely to be effective in reducing population size (Bomford, 1990; Bomford and O'Brien, 1992; Caughley et al., 1992; Barlow et al., 1997; Fagerstone et al., 2002). This study shows VCD could be a potential candidate for fertility control of female rats, but there will be no additive effect at the population level as male fertility was not affected by this chemosterilant.

Secondly, VCD can be orally delivered. The inhibitory effects of VCD on reproduction of female rats were achieved here through oral administration. For fertility control, one of the major challenges is to develop cost effective methods of delivery of the control agent to the animal. For overabundant free-ranging species, such as many rodents, a most practical and cost-effective means to deliver a control agent is by oral delivery (Miller, 1997; Tuyttens and Macdonald, 1998). Results from this study suggest that VCD could potentially be delivered orally using a formulated bait attractive to the target species (e.g. ricefield rats).

Thirdly, VCD induces permanent infertility. A number of chemicals (e.g. synthetic steroids) have been known to exhibit inhibitory effects on reproduction in animals (Marsh, 1988b; Bomford, 1990). However, these chemicals induced temporary effects and thus have not been considered to be suitable for fertility control of overabundant species, especially pest rodents. In contrast, the effect of VCD on reproduction of female rats is permanent because this chemical targets and destroys non-regenerating primordial and primary follicles in the ovaries (Hirshfield, 1991b). When the majority of these small follicle pools are depleted, ovarian failure occurs and treated animals become irreversibly infertile. Given such permanent effects, VCD may have long-term effects in suppressing growth of the pest population, and therefore lead to reduced damage to agricultural crops.

Further, from an animal welfare perspective, the use of chemosterilants to control fertility is generally more humane and has fewer side-effects compared to lethal methods (Oogjes, 1997). For example, rodenticides can cause suffering before death due to slow-acting mechanisms or sub-lethal doses (Hayes and Gaines, 1959; Rowe et al., 1981; Rost et al., 2004). In addition, a fertility control method which directly induces ovarian failure is more desirable than targetting, for example, post implantation stages of pregnancy or early lactation (Singer, 1974). Oral administration of VCD did not induce adverse effects on non-reproductive organs of treated animals. Furthermore, treated

animals that gave birth had normal, healthy pups and sucessful lactation, before their ovarian function subsequently failed.

Finally, the use of VCD is potentially safe for the environment. Fertility control may provide an alternative to poisons, which can have adverse effects on non-target species and the environment (Saunders and Cooper, 1981; Eadsforth et al., 1996; Murphy et al., 1998). Residues of some chemosterilants (e.g. synthetic steroids) from baits or carcasses could enter the food chain and pose a risk to other wildlife or people (Marsh, 1988b). However, VCD has a very short half-life and is rapidly metabolised to an inactive, non-toxic tetrol (Hoyer and Mayer, 2010); therefore, carry-over effects of this chemosterilant as secondary intake by other species or any residues in the environment will not occur. The effect of VCD requires repeated uptake by the animals, therefore non-target species would be unlikely to be affected by a single direct uptake of a VCD bait.

Although the results from this study demonstrated that VCD is a promising candidate for fertility control of rats, a number of challenges remain. Firstly, the effect of VCD on fertility was delayed until the second breeding cycle. At the population level, compensation responses can have major influences on density. Partial or delayed suppression of breeding may lead to compensation in survival of adults and juveniles produced by the remaining fertile females. As a result, the treatment may not reduce population size sufficiently to reduce damage to crops. For example, compensatory responses in populations have been reported in the simulation of fertility control studies for European rabbits (O. cuniculus) (Twigg and Williams, 1999; Twigg et al., 2000), house mice (M. domesticus) (Chambers et al., 1999b) and ricefield rats (R. argentiventer)(Jacob et al., 2004a). Therefore, for successful fertility control, a high proportion of females need to be sterilised before the start of the breeding season of the target species in order to achieve a decline in population growth rate. For example, for effective control at the population level, 60-80% of female European rabbits (Twigg and Williams, 1999; Twigg et al., 2000), 50–80% of female house mice (Chambers et al., 1997; Chambers et al., 1999b) and more than 50% of female ricefield rats (Jacob et al., 2004a) need to be sterilized. This is also the reason why fertility control approaches should be integrated with other management methods. If after VCD treatments, ricefield rats produced

only one litter soon after the maximum tillering stage, this F_1 litter may have a high rate of survival and may also reproduce before the crop is harvested. However, these F_2 pups would not contribute to damage and yield losses for the current crop season. If the productivity of the founder adult females is reduced to 30-35% for the entire rice crop season, would this rate of population recruitment be low enough to prevent economic damage and increase crop yields? Modeling of the population dynamics of the ricefield rat is required to answer this question.

Secondly, a long treatment period with VCD was required to achieve the observed effects on fertility. Repeated periods of 10 consecutive days of treatment used in the present study are unlikely to be practical for field application. However, if the same effect could be achieved when the treatment period was reduced to 3 to 5 days and repeated at 2 week intervals, the use of this technique would be considered more realistic and practical for field application.

Another major challenge in using VCD for fertility control is its incorporation into a formulated bait for oral delivery in the field. The presence of VCD in the bait must not influence bait acceptance by the target species to ensure that a high proportion of the population consumes the bait in sufficient quantities in order to achieve the requisite effect. VCD itself is not palatable to animals, however, it can be incorporated into a lipid emulsion, such as intralipid to mask the taste as well as to protect it from rapid degradation in the gastrointestinal tract (L. Mayer and C. Dyer, personal communication). This approach could improve its efficacy.

Ricefield rats have a short breeding season (around 8 weeks per cropping season), and their breeding cycle is strongly linked to the different growing stages of the rice crop (Lam, 1983; Leung et al., 1999). Most wild ricefield rats have relatively short life spans, average of 6.2 months (range from 4 months to one year) (Harrison, 1956). In addition, ricefield rats have low survival rates during the fallow period after the wet and dry season crops (Leung et al., 1999). During the fallow periods between crop seasons the rats accumulate in well-defined refuge habitats (Jacob et al., 2003a). These characteristics of breeding biology and ecology of ricefield rats create ideal conditions for the potential application of fertility control as an additional effective management tool for this species. However, the success of a fertility control program will be highly

dependent on the timing of application of the control agents. For ricefield rats, a fertility control program must be implemented before the population increases (i.e. before the start of the breeding season) to ensure that the treatment not only reduces population size but also reduces crop damage.

For population management of pest rodents, such as ricefield rats, delivering control agents through bait is the best option as it is practical under field conditions (Bomford and O'Brien, 1992; Fagerstone et al., 2002; Jacob et al., 2008). However, the success of baiting programs is largely dependent on bait uptake by the target species. To achieve a desired bait consumption, it is important to use baits that are highly palatable and attractive to the target species. In addition, the bait should be applied at the most suitable time, especially when alternative food sources are less abundant in the field and surrounding habitats. Further, costs of materials need to be considered to enable farmers to afford to use baits.

In this study, evaluations of bait uptake by ricefield rats under laboratory and field enclosure conditions revealed that cereal-based bait, containing broken rice, wheat and rice flours, coconut oil, intralipid and sugar was readily accepted by the rats with a high proportion of animals consuming bait (~ 80-100%). With such a high proportion of animals accessing bait, the sterilisation of more than 50% of females in a ricefield rat population using fertility control bait seems feasible. The main materials used in this bait formula were widely available and relatively cheap at the local markets. Intralipid (a fatty emulsion) is the proposed carrier for VCD and this would be formulated separately for subsequent incorporation into the bait. The actual cost of this VCD component is not known.

The enclosure study found bait consumption by ricefield rats steadily increased over the first 4-5 days. This information is important for determining a suitable pre-feeding period of plain bait to improve the subsequent uptake of bait containing control agent. In addition, this study showed a high rate of bait consumption (~9-10% body weight) per individual ricefield rat, therefore it is expected a high amount of control agent could be taken by the target species. This could enhance efficacy, especially if the effect could be achieved with a shorter period of application of bait. Alternatively, given such high rates of bait uptake, a lower concentration of control agents in the bait could be appropriate as it may minimise the development of bait aversion (Parshad and Kochar,

1995; O'Connor and Matthews, 1997; Cook, 1999) by the rats, and also reduce any potential risk to non-target species.

The multiple periods of treatment with VCD used in this study mimicked the expected application routine that farmers would be able to incorporate into their routine activities in and around their ricefields. Thus the fertility control agent could be applied during the fallow period between cropping seasons, and during the time from land preparation to the tillering stage of the rice crops, prior to the onset of breeding season of ricefield rats. During this period, rice grains (the preferred natural food source of ricefield rats) (Goot, 1951, cited by Leung *et al.* 2007; Murakami *et al.*, 1990) are not available in the fields, providing favourable conditions for the rats to consume the bait.

Results from this study indicated that VCD did not affect the fertility of male rats. For fertility control of pest rats using a formulated VCD bait, bait will be consumed by both sexes, thus more bait will be required to ensure access by and efficacy in females. It is therefore important that a final product is highly efficacious and cost-neutral with respect to other management tools (e.g. rodenticides).

In general, the results from this thesis suggest that VCD is a promising fertility control candidate which could be orally delivered to the target species using a formulated bait. Thus VCD could potentially be used as an additional tool for sustainable management of pest rodents, such as ricefield rats. However, at this stage the effect of VCD on ricefield rats is unknown although it would be expected to be similar to that observed in laboratory rats. The effect of VCD on reproduction of female ricefield rats remains to be tested; such an evaluation was outside the scope of this thesis.

6.3 Future directions

The present study clearly demonstrated that multiple periods of oral administration of VCD significantly reduced fertility in female laboratory rats. However, for practical application in the field, it will be essential to reduce the duration of each treatment period without loss of effect and VCD must be incorporated into a formulated bait for oral delivery. It is therefore suggested that further studies should focus on investigations of the effects of shorter periods of treatment with VCD and of varying the number of periods of exposure.

The effects of VCD in formulated bait on follicle depletion and fertility of ricefield rats need to be examined first under laboratory and then under enclosure conditions before further research on development of a VCD bait for broad-scale fertility control of this pest species is undertaken. In addition, to fully understand the effect of VCD on ricefield rats, some studies need to be done on the mechanisms of effect of VCD in this species although again the mechanisms would be expected to be similar to those observed in laboratory rats (Hoyer et al., 2001).

For effective fertility control at the population level, it is essential to reduce the reproductive potential of the target species before the first oestrus of a breeding season in order to minimise population recruitment, and thus reduce the damage caused by rats to maturing stages of the crops. As VCD effects on fertility are delayed due to the presence of the larger unaffected follicular stages, VCD should be applied well in advance of the onset of the breeding season to ensure that nearly complete depletion of the total ovarian follicle pool is achieved before the breeding season starts. An enclosure study on fertility control in ricefield rats using surgical sterilisation of females at different levels (0, 25, 50 and 75%) indicated that sterilisation of more than 50-75% of female rats significantly reduced population numbers and thus reduced accumulative crop damage (Jacob et al., 2004a). Modelling the outcomes of the results from the present study on population dynamics of ricefield rats after one litter is produced should be undertaken. Furthermore determining whether there is a reduction in crop damage and associated increased yields is also essential.

VCD could also be used in conjunction with other agents that target the larger follicles in the ovaries, such as some plant extracts: Melia azedarach (Roop et al., 2005; Mandal and Dhaliwal, 2007), Ferula jaeschkeana (Prakash and Sharma, 1997), and Carica papaya (Joshi and Chinoy, 1996; Chinoy et al., 1997; Raji et al., 2005) and thereby accelerate the time to ovarian failure. Together, these agents could have complementary effects and result in more rapid and permanent changes in ovarian function, leading to irreversible infertility. Furthermore, improved technologies for protecting VCD in the gastrointestinal effects, such as formulating VCD to specifically prevent degradation of the chemical before uptake, could enhance the efficacy and potentially reduce the doses required. Such approaches remain to be tested.

The bait uptake study demonstrated that cereal-based bait in kibble form was highly acceptable to ricefield rats; however, in this study, the bait was tested under the enclosure conditions in the absence of a rice crop (a preferred diet of ricefield rat). Further studies therefore should be conducted to evaluate uptake of this bait formula in the presence of a growing rice crop. However, the best option would still be to present baits prior to the rice crop being available as an abundant, alternative and preferred food source.

A field study on fertility control of ricefield rats using surgical or hormonal based sterilisation at the scale of 10 ha indicated that the treatment was not effective due to immigration of rats from adjacent areas (Jacob et al., 2006). Therefore, future studies on the effect of fertility control for population management of ricefield rats using chemosterilants, such as VCD bait, under field conditions should be conducted at a larger scale to minimise the potential effect of immigration of untreated rats into the treated areas.

Generally, the present study has shown that VCD is a promising candidate for fertility control of female rats. However, further studies remain to be done: 1) reducing the treatment period without loss of efficacy, 2) incorporating VCD into the bait and testing the effect on follicle depletion and fertility of ricefield rats under laboratory and enclosure conditions, and 3) evaluating the effect of VCD bait on reproduction of ricefield rats at the population level under field conditions, including monitoring crop damage. Findings from these studies are essential for further development of VCD bait for fertility control in ricefield rats.

Given the increased cropping intensity and frequency of major catastrophic weather events in recent years in the equatorial region, there has been increasing impacts of rodents on agricultural crop production, especially in Southeast Asia (Baco et al., 2010; Singleton et al., 2010b; Sudarmaji et al., 2010). Therefore, rodent management must play a critical role in food security and poverty alleviation programs (Singleton, 2003). Current control methods rely mainly on the use of poisonous chemicals which can cause various side-effects on non-target species, humans and the environment. It is therefore essential to develop alternative control techniques that are more species specific, safe to use and environmentally-friendly. In this regard, fertility control could potentially be used as an additional tool for sustainable management of pest rodents.

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Appendices

Appendix 1. Haematoxylin and Eosin (H&E) staining procedure for staining tissue sections

Step	Chemical solutions used	Duration (minute'/second")
Deparafinization		
1	Xylene/Histolene (1)	2'
2	Xylene/Histolene (2)	2'
3	Xylene/Histolene (3)	2'
Hydration		
4	Rinse in 100% Ethanol (1)	1'
5	Rinse in 100% Ethanol (2)	1'
6	Rinse in 95% Ethanol	1'
7	Rinse in 70% Ethanol	1'
8	Wash in running H_2O	1'
Staining		
9	Stain in Gill's Haematoxylin	3'45
10	Wash briefly in running H_2O	0'30"
11	Differentiate in 1% Acid alcohol	0'2"
12	Wash briefly in H_2O	0'30"
13	Blue in ammonia solution (1/250)	0'4"
14	Wash in running H_2O	1'
15	Rinse in 80% Ethanol	0'30"
16	Counterstain in Eosin	3'
Dehydration		
17	Rinse in 95% Ethanol	0'10"
18	Rinse in 100% Ethanol (1)	1'
19	Rinse in 100% Ethanol (2)	1'
20	Rinse in 100% Ethanol (3)	1'
Clearing		
21	Xylene/Histolene (1)	2'
22	Xylene/Histolene (2)	2'
Coverslipping	DPX mounting medium	

Appendix 2.1 Composition of baits containing different vegetable oils

Bait material (% in weight)	Bait type				
	Canola oil	Coconut oil	Sesame oil	Neem oil	Unhulled rice
Broken rice	10	10	10	10	0
Wheat flour	65	65	65	65	0
Sugar	20	20	20	20	0
Canola oil	5	0	0	0	0
Coconut oil	0	5	0	0	0
Sesame oil	0	0	5	0	0
Neem oil	0	0	0	5	0
Unhulled rice	0	0	0	0	100

Appendix 2.2 Composition of kibble bait containing different food additives

Bait material (% in weight)	Bait type				
	Coconut oil	Intralipid	Wax	Anchovies	Sardines
Broken rice	10	10	10	10	10
Wheat flour	80	60	68	75	75
Sugar	5	5	5	5	5
Coconut oil	5	5	5	5	5
Intralipid	0	20	0	0	0
Wax	0	0	12	0	0
Anchovies	0	0	0	5	0
Sardines	0	0	0	0	5

Appendix 2.3 Composition of baits containing different ratios of wheat and rice flour

Bait material (% in weight)	Bait type		
	Wheat flour only	Wheat : rice (1:1)	Rice flour only
Broken rice	10	10	10
Wheat flour	80	40	0
Rice flour	0	40	80
Sugar	5	5	5
Coconut oil	5	5	5