In vivo and *in vitro* studies of 4-vinylcyclohexene diepoxide in wild-caught female brushtail possums (*Trichosurus vulpecula*) and Norway rats (*Rattus norvegicus*) and its potential as a fertility control agent

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Anna Mae Burd

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Abstract of a thesis submitted in partial fulfilment of the

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In vivo and *in vitro* studies of 4-vinylcyclohexene diepoxide in wild-caught female brushtail possums (*Trichosurus vulpecula*) and Norway rats (*Rattus norvegicus*) and its potential as a fertility control agent

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In New Zealand (NZ), the brushtail possum (*Trichosurus vulpecula*) poses a major threat to native flora and fauna and it is the main wildlife vector for bovine tuberculosis. Primary control utilizes lethal means but these methods have limited efficacy long-term and are associated with environmental impact concerns. Fertility control has become more popular as it is potentially more effective, sustainable and humane. The industrial chemical, 4-vinylcyclohexene diepoxide (VCD) may serve as an ideal chemosterilant candidate for investigation. Oral gavage studies have shown that VCD reduces the pool of non-regenerating immature ovarian follicles in rodents, resulting in premature ovarian failure and sterility. The first objective of these studies was to examine the effects of orally administered VCD on female possum ovarian follicle populations. Orally delivered VCD had no effect on the primordial follicles of adult female possums and two formulations aimed at improving VCD uptake and efficacy did not change this outcome. The second objective examined the uptake and metabolism of orally administered VCD in female possums and rats *in vivo.* VCD concentration in the blood of rats was significantly greater than in possums while concentrations of VCD in the stomach were comparable between species. VCD dosing did not alter pH of stomach contents of possums while that of rats was increased and sustained for 6 hours. VCD-induced reductions in ovarian and liver glutathione levels were observed in the rat with no effects in the possum. It was determined that the highly acidic environment of the stomach of possums poses an initial barrier for orally delivered VCD. Without sufficient quantities of VCD reaching the liver and ovaries of possums, it was not possible to compare species differences in metabolism *in vivo*. The third objective examined the fate of VCD when exposed to acidic environments and stomach contents and the effects of VCD on liver metabolism *in vitro* in possums and rats. VCD hydrolysis in stomach contents was slower in possums compared with rats suggesting that possum stomach contents are able to retain VCD longer, thus potentially modulating VCD toxicity. GSH levels in possum liver tissue were less affected when incubated with VCD compared with rats, suggesting an increased detoxifying capacity of possums. Preliminary data on the ability of possum liver microsomes to convert VCD's parent compound, 4-vinycyclohexene, to VCD corroborated the GSH

findings. Collectively, these findings suggest that VCD may not be suitable for possum fertility control when delivered orally in a raw unprotected chemical state. Encapsulation of VCD with an additional active compound, triptolide, is being examined as a novel chemosterilant (ContraPest®, SenesTech®, Flagstaff, Arizona). The fourth objective examined ContraPest® efficacy in wild-caught female Norway rats. The ovarian primordial follicle pools of ContraPest®-consuming rats were reduced compared with controls. The proposed protection of the active components within ContraPest® from stomach acid by encapsulation may provide the first step for its potential use in possums. Further work demonstrating the efficacy of ContraPest® in rodents will be required before further inestigation on possums can be proposed.

Keywords: Brushtail possum, *Trichosurus vulpecula*, Norway rat, *Rattus norvegicus*, fertility control, pest control, chemosterilant, bait, 4-vinylcyclohexene diepoxide, ovary, follicle, stomach pH, glutathione, metabolism.

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Thank you also to the furry, charming and eccentric possums used during the course of these studies.

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Chapter 1

General Introduction

The aim of the studies presented here was to examine a potential fertility control agent, 4-vinylcyclohexene diepoxide (VCD), for its potential use for control of New Zealand pest mammals, specifically the brushtail possum. The first research aim was to examine the effects of an oral VCD dose on the primordial follicle populations in adult female possums (Objectives 1a and 1b). Based on results obtained from the first objective, the following 3 objectives were formulated using a flow diagram developed prior to the start of experimentation (Figure 1.1). The uptake and metabolism of orally administered VCD in the possum was next examined (Objective 2a). Comparisons were made to that of wild Norway rats and previous literature in rodents. The effects of acid and stomach contents of each species were then examined *in vitro* for their actions on VCD (Objective 2b). The third research aim compared differences in the metabolism of VCD and its parent compound, 4-vinycyclohexene (VCH), between rats and possums (Objectives 3a and 3b). Finally, a novel chemosterilant (ContraPest®, SenesTech Inc®) was examined for its potential as a rodent fertility control method in wild-caught Norway rats (Objective 4a and 4b). Pending successful application for its use as a rodent chemosterilant, the formulation and application methods for ContraPest® may subsequently provide an opportunity for its use as a control method for possums in New Zealand.

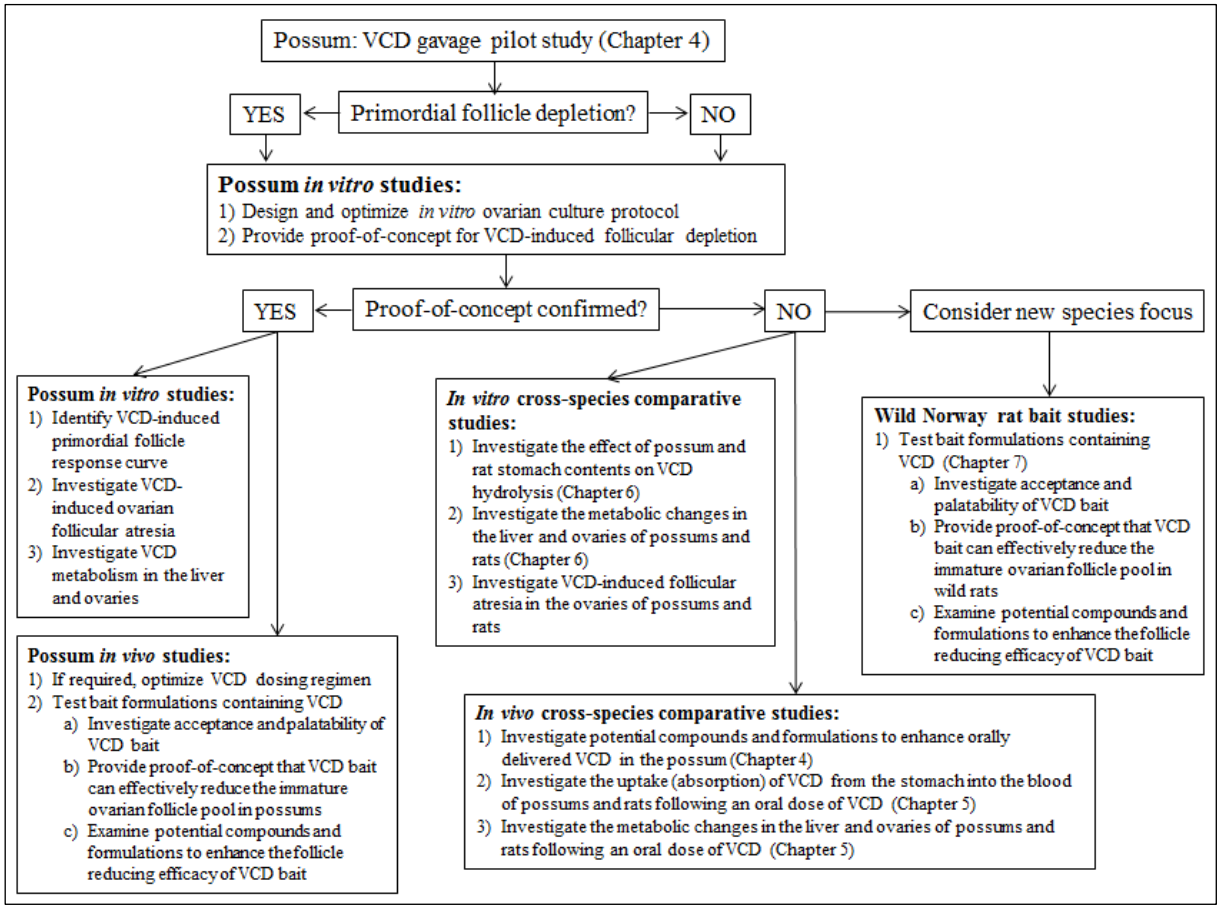

Figure 1.1 Flow diagram of potential research objectives based on the outcome derived from the initial gavage pilot study in possums.

1.1 Research objectives

To achieve the aims outlined above the following questions and research objectives were identified:

1. Will an oral dose of VCD induce primordial follicle reductions in adult female possums?

    a. Identify an effective dosing regimen for orally delivered VCD and examine is effects on female possums (Chapter 4) as they relate to:

        i. The general health of the animal

        ii. The primordial ovarian follicle pool of the animal

    b. Investigate potential formulations to improve the uptake and efficacy of orally delivered VCD in female possums (Chapter 4) as it relates to:

        i. The general health of the animal

        ii. The primordial ovarian follicle pool of the animal

2. Is VCD absorbed from the stomach into the blood of possums in a similar fashion to that of rats? Once absorbed, is VCD metabolised in a similar fashion in possums as documented in rats?

    a. Examine *in vivo* differences in VCD-induced ovarian toxicity between possums and rats (Chapter 5) as it relates to:

        i. VCD uptake from the stomach into the blood of each species

        ii. The effect of a bolus dose of VCD on the stomach acidity of each species

        iii. VCD's effects on metabolism as measured by changes in glutathione levels of the red blood cells, plasma, liver and ovaries of each species.

    b. Verify *in vivo* results of possums and rats through *in vitro* examination of VCD effects (Chapter 5) as they relate to:

        i. The effect of acid on VCD over time and concentrations ranges

        ii. The effect of stomach acidity of each species on VCD

3. Is possum metabolism more similar to that of rats or to that of mice?

    a. Compare and contrast the *in vitro* metabolism of VCD's parent compound, 4-vinylcyclohexene, in possum and rat liver microsomes as measured by cytochrome P450 (Chapter 6)

    b. Compare and contrast the *in vitro* metabolism of VCD in possum and rat liver microsomes as measured by glutathione (Chapter 6)

4. Can ContraPest® serve as a chemosterilant for the control of rodent pests in New Zealand?

    a. Examine palatability and consumption rates of ContraPest® with and without pellet food provided (Chapter 7)

    b. Identify and ideal baiting regimen (i.e. efficacy of a pre-feed period) (Chapter 7)

    c. Examine the effects of ContraPest® on the ovarian follicle populations of rats (Chapter 7)

1.2 General comments

The four data chapters in this thesis (Chapters 4-7) have been written as thesis chapters to avoid redundancy. However, they will be reformatted as manuscripts as per the requirements of each

individual journal. Details on individual publications arising from this thesis are presented in Table 1.1.

Table 1.1 Manuscript details and status of each chapter.

Chapter	Title	Authors	Journal	Status
4	Effects of orally delivered 4-vinylcyclohexene diepoxide on the ovarian follicle populations of female brushtail possums	Burd, AM; Scobie, S; Brown, S; Dyer, CA; Duckworth, JA	Wildlife Research	In preparation
5 and 6	Uptake and metabolism of 4-vinylcyclohexene diepoxide in wild female brushtail possums and Norway rats	Burd, AM; Ketelaars, L; van Grinsven, N; van der Hee, B; Brown, L; Brown, S; Trought, K; Duckworth, JA	PLOS ONE	In preparation
7	Examination of a novel bait containing 4-vinylcyclohexene diepoxide and triptolide in wild female Norway rats	Burd, AM; Mayer, LP; Hinds, LA; Barrell, GK; Duckworth, JA; Dyer, CA	European Journal of Wildlife Research	In preparation

Chapter 2

Literature review

2.1 Pests of New Zealand

Prior to human colonization, the only known mammals present in New Zealand (NZ) were bats and pinnipeds. The lack of land-dwelling mammals favoured the evolution of flightless birds such as the iconic Kiwi. The first records of land-dwelling mammals arrival to NZ was during Polynesian colonization (AD 1250-1300). These included the Kiore (Pacific rat, *Rattus exulans*) and the Kurī (Polynesian domestic dog, *Canis familiaris* subspecies; extinct) which later interbred with the European dog (*Canis familiaris*). European colonization (from circa 1769) resulted in the introduction of 54 mammalian species into New Zealand, some of which have become pests. Of the established NZ pests 57% negatively impact 359 threatened taxa (King, 2005). This review will focus on two of the most common invasive species in NZ, the brushtail possum (*Trichosurus vulpecula*) and the Norway rat (*Rattus norvegicus*). I will discuss the nature of their introduction, current lethal control methods and the development of non-lethal, fertility control management strategies for these NZ pest species. I will present an overview of current fertility control methods being examined with focus on a novel chemosterilant, 4-vinylcyclohexene diepoxide (VCD), which shows promise because of its ability to induce permanent infertility.

2.2 The brushtail possum

In the mid-1850s the Australian brushtail possum was introduced to NZ by European settlers to establish a fur trade (Clout and Ericksen, 2000). Controversy over the damaging effects of possums to the native forests first arose in the 1920s and continued until 1947 when the NZ Government instituted the legalisation of poison baiting and penalized any harbouring or liberation of possums. Between 1950 and 1970, the need for possum control using poisons grew steadily. By the late 1960s it was recognized that possums were likely vectors for bovine tuberculosis (Tb), spurring the Government to continue to increase possum control efforts (Clout and Ericksen, 2000). Brushtail possums are now considered to be the main wildlife vector for Tb in livestock, thus forming a significant threat to NZ dairy, beef and deer product exportation. Presently, possums inhabit 90-95% of mainland NZ (Cowan, 2000) at densities up to ten times higher than those found in Australia (Cowan, 2005; PCE, 2000). Current lethal and non-lethal methods for possum control in NZ are described below.

2.3 The Norway rat

The Norway rat was the first of the four rodents in the Muridae family to be unintentionally introduced to NZ by European settlers. Since their introduction, they have spread rapidly throughout the North

Island and South Island due to their adaptive nature and commensalism with humans. Additionally, it has been documented that they have colonized >60 offshore islands (Innes, 2005). Similar to the brushtail possum, the Norway rat is a predatory pest on native fauna such as ground-dwelling birds like the Kiwi. Furthermore, because they are commensal pests, they cause undesirable effects to society, for example gnawing on items such as electrical wiring in homes, cars, and businesses; spoiling food by defaecation; damaging food containers; and harbouring of zoonoses (infectious diseases that spread from non-human animals to humans). In the hope of negating these effects, control methods have been wide spread throughout NZ and are reviewed in detail below.

2.4 Lethal control methods

Since the mid-1950s, the NZ Government has employed the use of broad spectrum poisons to control pest populations. Sodium monofluoroacetate (also known as 1080), cholecalciferol (Vitamin D3), Feratox® (encapsulated cyanide), and brodifacoum have been widely used throughout NZ forests to control possums, whereas rodent control has mainly focused on eradication from off-shore islands using aerial and ground baiting (primarily with 1080 or brodifacoum) (Cowan, 2005). Recent estimates of 2.8 million hectares (10%) of NZ's land is subject to such control methods (Nugent et al., 2012). Removal of Kiore (*R. exulans*), ship (*R. rattus*) and Norway rats has been highly successful with more than half of the populated islands now rodent-free (Innes, 2005). Of the four rodent species in NZ, only the ship rat has been specifically targeted on the North and South islands. Because of their similarities to possums (impacts, habitat and dispersion) these two species are now routinely co-targeted in mainland poison operations. Control of the house mouse (*Mus musculus*) has been minimal, mainly due to inadequate understanding of their effects in the environment, and has included commercial poisoning, fumigation, trapping and repellent use. Additionally, house mouse populations on off-shore islands have been indirectly targeted following 1080 aerial drops aimed at targeting possums (Innes, 2005).

Poison control efforts often report >80% reduction in pest species, though failures do occasionally occur. Ingestion of a sub-lethal dose can lead to bait shyness (Hardy et al., 1983), hampering control efforts long-term. Reduced interspecific competition can occur due to increased food and resource availability for surviving animals, thereby increasing reproduction potential (Clinchy et al., 2001; Eason et al., 2011; PCE, 2000). Such effects are most prevalent in rodent species due to their high fecundity. Rodents are able to rebound in numbers that can exceed the original population in a short period of time. Immigration and re-establishment of pest species following the cessation of control operations can also diminish effectiveness (Eason et al., 2011; Green and Coleman, 1984; Isaac, 2005; Ji et al., 2004; Nugent et al., 2012). There is also great pressure from the public sector to halt poison control because of its associated costs, need for repeat operations, potential to enter public water-supply catchments, persistence in soil and plants, potential lethal effects to non-target species and its general inhumane nature (Clout, 1999; Eason et al., 2011; PCE, 1994, 2000, 2011; Veitch and Clout, 2001). Death from cholecalciferol and brodifacoum poisoning can take anywhere from 4-36 days

(Eason, 2002) and 15-28 days (Eason et al., 1996) respectively, demonstrating the inhumaneness of their use as a form of possum control. In addition, persistence of brodifacoum in possum muscle and liver tissues poses a risk for transfer of residues through the food chain into wildlife, domesticated animals, and humans (Eason et al., 1996). Studies have shown that ingestion of toxins can occur through direct bait consumption or by indirect methods such as feeding on poisoned carcasses. Non-target species that have been affected include birds, dogs, cats, cattle, sheep, pigs, lizards, frogs and invertebrates (Eason, 2002; Notman, 1989; Spurr and Powlesland, 1997). Collectively, these negative side effects support the need for a more publically acceptable, effective and sustainable control method to reduce and maintain low pest populations, particularly of possums and rodents, throughout mainland NZ. One such option may be to target the reproductive output of the target species.

2.5 Ovarian and follicular development

Development of the undifferentiated gonad through to folliculogenesis during the oestrous cycle is highly conserved in mammalian species. However, differences between eutherians and marsupials do exist, especially regarding the timing of events. Using a rodent model, I will review key events in ovarian development and follicle maturation taking special note of the differences in possum biology where applicable.

In mice, primordial germ cells (PGCs) are first observed in the endoderm (yolk sac) on gestational day (GD) 8 and migrate to the genital ridge by GD 11-12. In possums however, PGCs originate in the nephrostomial canals of the mesonephros and can be first observed migrating to the genital ridge just before birth (GD 15-17.5) (Ullmann, 1996). Following gonadal ridge formation, the PGCs begin to invade and proliferate within the undifferentiated gonad (mouse, GD 14.5; possum, GD 17.5; Figure 2.1). Gonadal sexual differentiation can be determined by GD 13.5 in the rat (GD 12.5 in the mouse) whereas in the possum, this occurs after birth on approximately post natal day (PND) 2 (Eckery et al., 1996; Hirshfield, 1991). In both species, differentiation of the testis precedes that of ovarian differentiation. Initiation of meiosis in the germ cells (GCs) in rodents is initiated around GD 14.5 and by GD 18.5 the maximum amount of GCs is reached (~75,000 in the rat) (Hirshfield, 1991). In the possum, germ cell meiosis is initiated around PND 35-40 with the maximum number reached around PND 50-70 (Eckery et al., 1996; Shackell et al., 1996).

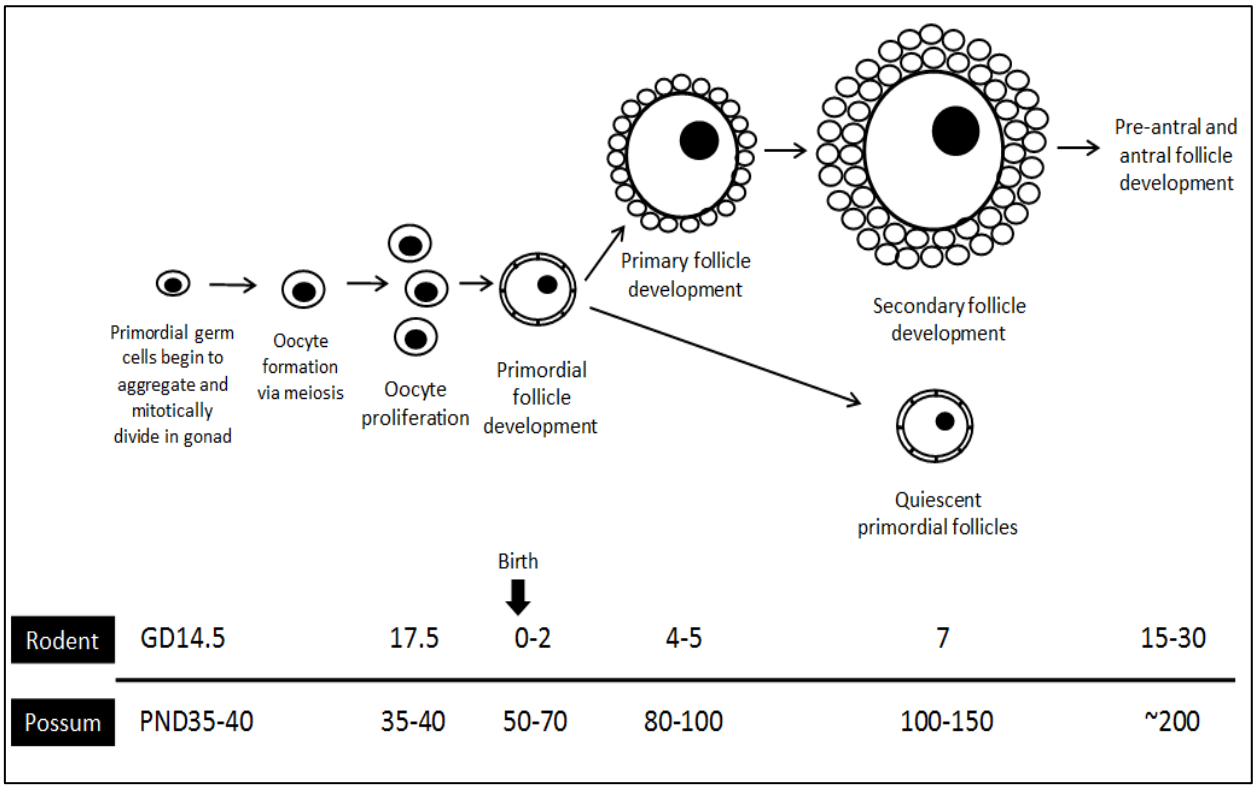

Figure 2.1 Stages of oogenesis (not drawn to scale) during initial follicular recruitment for the rodent (mice and rats) and possum. Adapted from Frankenberg et al. (1996).

Following the cessation of mitotic division, GCs begin to transform into oocytes through meiotic division. In the rat, meiotic GCs are first observed on GD 17.5 (GD 16.5 in the mouse) while in the possum this occurs around PND 35-40 (Eckery et al., 1996; Frankenberg et al., 1996; Hirshfield, 1991). In both species, mass attrition of the GCs populations occurs shortly after with approximately 65% (PND 2) and 80% (PND 80-90) of GCs lost to atresia in the rat and possum, respectively. Rat primordial follicle formation begins around birth and by PND 2 much of the primordial follicle pool has formed (Hirshfield, 1991; McGee and Hsueh, 2000). On the other hand, the primordial follicle pool in possums begins to form around PND 50-70 (Eckery et al., 1996; Eckery et al., 2002b; Shackell et al., 1996; Ullmann, 1996). These species differences in timing of events is continued throughout the transition from primordial to pre-ovulatory follicles with numerous preantral and antral follicles present by PND 30 in the rat and ~200 days of possum pouch life (Eckery et al., 1996; Hirshfield, 1991; Shackell et al., 1996) (Figure 2.1). For clarification, pre-pubertal development of follicles will be referred to as initial recruitment whereas post-pubertal follicle development occurring during each oestrous cycle will be referred to as cyclic recruitment (Figure 2.2) (McGee and Hsueh, 2000).

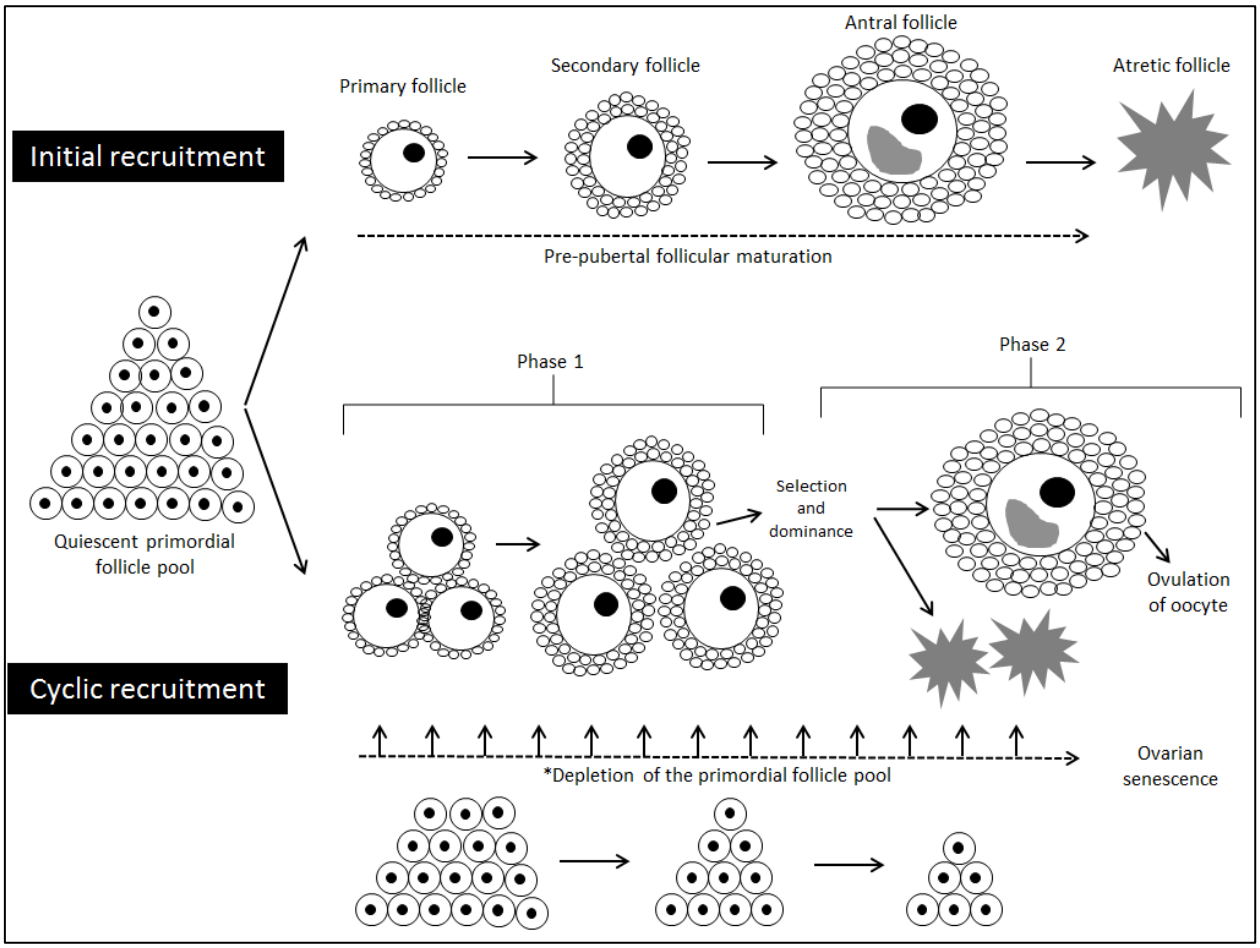

Figure 2.2 The folliculogenesis life cycle depicting initial and cyclic recruitment of follicles. Adapted from McGee and Hsueh (2000).

Rats become sexually mature around 32-36 days of age (Goldman et al., 2007) while possums reach sexual maturity at 1 year (Tyndale-Biscoe, 1955). The age at which each species reaches sexual maturity reflects the differences in their life cycles (rodents, 1-2 year life span (Berg and Simms, 1960; Solleveld et al., 1984); possums, 8-9 years (Clinchy et al., 2004)). The oestrous cycle, which is regulated by the hypothalamic pituitary gonadal (HPG) axis feedback loop (Figure 2.3), lasts for 4 to 5 days in the rat (Goldman et al., 2007) and 26.5 ± 1.3 days in the possum (Duckworth et al., 1998).

The biphasic pattern of cyclic recruitment of follicles during the oestrous cycle is similar between the species with the exception that rodents are polyovulatory while possums are monovulatory (McGee and Hsueh, 2000; Rodger et al., 1992). Phase 1 of follicular cyclic recruitment involves maturation of a small cohort of primordial follicles and their oocytes (Figure 2.2) (Rodger et al., 1992). Once follicular growth is initiated, primordial follicles are committed to either growing to ovulation (i.e. dominant follicle) or they will become atretic and die (Figure 2.2) (Crawford et al., 2011). Primordial follicles are both nonsteroidogenic and gonadotrophin and steroid hormone independent (Skinner, 2005). The factors that regulate the initiation and growth of primordial follicles is not well understood, although it is widely accepted that local growth factors are involved (Crawford et al., 2011). Details on

these local growth factors as they related to eutherians and marsupials are reviewed in Skinner (2005) and Crawford et al. (2011), respectively.

The transition from a primordial follicle to a primary follicle involves several changes. The GCs change from squamous to cuboidal (Hirshfield, 1991) and begin expressing follicle stimulating hormone (FSH) receptors (Eckery et al., 2002c; McGee and Hsueh, 2000). Theca interstitial cells (differentiated stromal cells) are recruited and begin to surround the primary follicles. In eutherians, expression of luteinizing hormone (LH) receptors in theca cells is first observed at this stage (McGee and Hsueh, 2000). However, in possums, LH receptor expression in theca cells does not occur until the time of antrum formation (phase 2, discussed below) (Crawford et al., 2011). In addition, the zona pellucida (ZP) begins to form around the oocyte within the primary follicle (Hirshfield, 1991; Mate, 1998). The ZP is a glycoproteinaceous matrix which functions as a species-selective substrate for sperm binding, an agonist for spermatozoon acrosome reaction, defence against polyspermy, and as protection for the embryo during the early stages of development until implantation of the blastocyst occurs (Mate, 1998; Wassarman and Litscher, 2008). The ZP matrix of mice and possums contains three glycoproteins, designated ZP1, ZP2 and ZP3 while a fourth glycoprotein (ZP4) has recently been reported in species such as rats, hamsters, humans and monkeys (Ganguly et al., 2008; Hoodbhoy et al., 2005; Hughes and Barratt, 1999; Izquierdo-Rico et al., 2009; McCartney and Mate, 1999). The follicles and their oocytes continue to grow, increasing both in size and number of GCs present. During this time FSH does not seem to be essential, rather it appears to be involved in a facilitating manner (Crawford et al., 2011; McGee and Hsueh, 2000). For a full cellular description of each follicle stage and the associated follicle diameters for rats and possums refer to the histology section of Chapter 3.

Phase 2 commences when the oocytes have reached their maximum diameters. At this stage only growth of the follicles occurs and formation of a fluid-filled antrum begins (Figure 2.2) (McGee and Hsueh, 2000; Rodger et al., 1992). Within the cohort of growing follicles, the selection for the most dominant follicle is believed to be achieved through the expression of LH receptors in their associated GCs. In possums, LH receptor expression can be found in the GCs of all healthy antral follicles (Crawford et al., 2011). Thus, selection for the dominant follicle is less clear but it is thought that down-regulation of anti-Müllerian hormone (AMH) GC expression may help GC differentiation and maturation of the follicle in possum (Crawford et al., 2011). Follicles not selected for ovulation undergo atresia (Figure 2.2).

Gonadotrophin releasing hormone (GnRH), which is derived from the hypothalamus, controls the secretion of the gonadotrophins (LH and FSH) from the anterior pituitary gland (Figure 2.3) (Crawford et al., 2011; Skinner, 2005). The secretion profiles of LH and FSH in possums is, in many ways, very similar to that of eutherians (Crawford et al., 2011). As a follicle develops in preparation for ovulation (follicular phase of oestrous cycle), LH induces theca cells to increase the conversion of cholesterol to androgens (androstenedione and testosterone) while the GCs begin to increase their

capacity to biosynthesize and secrete oestradiol (E_2) and progesterone (P_4) under the influence of FSH (Craig et al., 2011; Skinner, 2005). Ovarian-derived E_2 and P_4 secretion provides positive feedback on the hypothalamus, allowing for increased GnRH secretion and subsequently, LH and FSH secretion (Figure 2.3). This positive feedback HPG axis loop increases the levels of circulating LH, FSH, E_2 and P_4. Ovulation of the oocyte is preceded by an LH surge (10-12 hours in rats) which is concomitant with an E_2 and FSH surge. For successful rupture of the follicle wall and subsequent oocyte release an increase of intrafollicular P_4 is required (Robker et al., 2009).

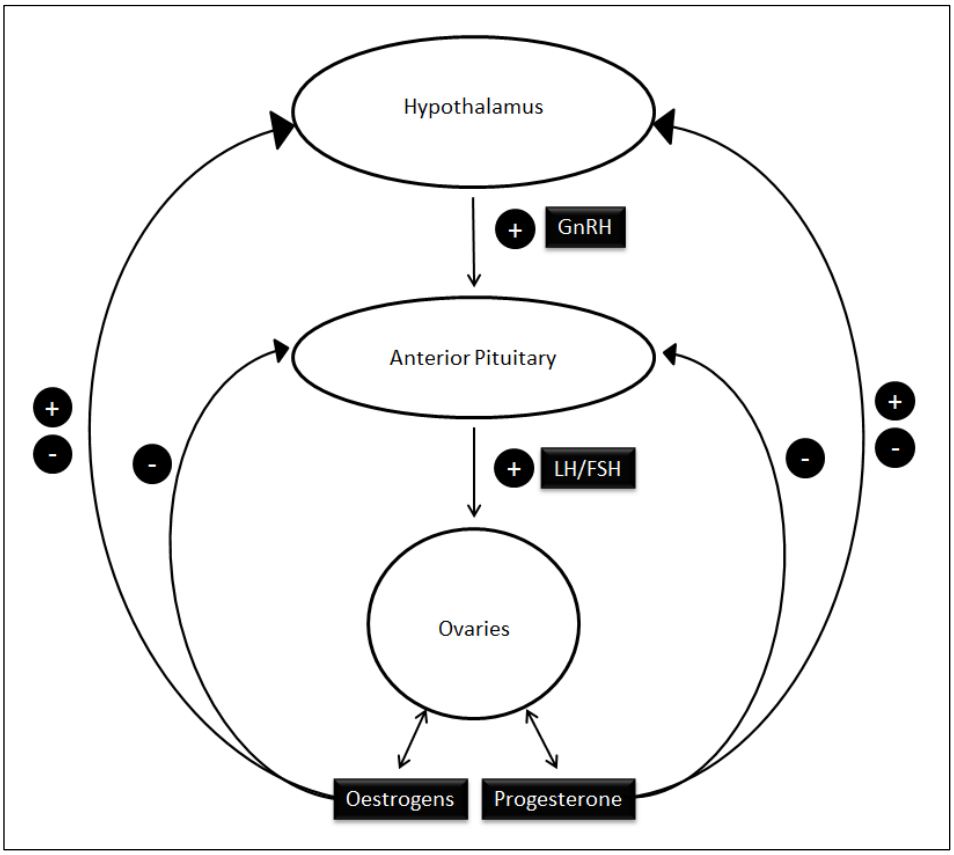

Figure 2.3 The hypothalamic-pituitary-gonadal (HPG) feed-back loop. Hormonal feedback may be positive (+) or negative (-) depending on the stage of the reproductive cycle. GnRH: gonadotrophin releasing hormone; FSH: follicle stimulating hormone; LH: luteinizing hormone.

After ovulation of the oocyte, the luteal phase of the oestrus cycle begins. It is characterized by the luteinisation of the surrounding GCs under the influence of LH forming a progesterone-producing organ, the corpus luteum (CL). At the end of an oestrous cycle or pregnancy, prolactin release then induces luteolysis (luteal cell death) forming a non-steroidogenic corpus albicans (CA) (Hirshfield, 1991).

A clear difference in gestation and lactation lengths exists between rodents and marsupials. In rodents, if insemination occurs, then the CL is maintained and continues to secrete P_4 to support the pregnancy

which lasts for 21 ± 1 days (Whittingham and Wood, 1983). In contrast, pregnancy (16.5 ± 1 day) in possums occurs within the luteal phase of the oestrous cycle (26.5 ± 1.3 days) (Duckworth et al., 1998; Eckery et al., 2002a; Hinds, 1990). Thus, pregnancy occurs during the period when P_4 is being secreted by the CL and comprises approximately 60% of the oestrous cycle. There is no evidence that pregnancy affects the function of the possum CL although it is required at parturition (Eckery et al., 2002a; Hinds, 1990). In both rats and possums, the oestrous cycle is suppressed during lactation which lasts for 21 ± 1 days for rodents (Whittingham and Wood, 1983) and approximately 6 months for possums (Cowan, 1989).

Clearly, there are considerable differences between rats and possums in the timing of reproductive events. However, there are similarities in the stages of development and growth within the ovary. Development of a successful fertility control method will require a detailed understanding of the reproductive biology of a target species. If the aim is to utilize the same (or a similar) fertility control method for several species, understanding of the similarities and differences in their reproductive biology becomes even more important. Below I discuss current methods being examined for mammalian fertility control with special focus on their relevance for rats and possums.

2.6 Non-lethal (fertility) control methods

Research investigating fertility control as an alternative, humane, and non-lethal approach to controlling pest populations has increased in recent years. Ideally, an useful contraceptive agent should have the following properties: 1) long-term, reversible or permanent sterility; 2) low production and application cost; 3) easy delivery to the pest population of interest; 4) minimal or no harmful effects on target species other than reduced fertility; 5) no negative impacts on non-target species; and 6) environmentally neutral (DeNicola et al., 1997; Humphrys and Lapidge, 2008). Fertility control may provide the advantage of reducing toxin application rate, thereby reducing risk of environmental contamination, associated application costs, and primary and secondary poison risks to non-target species (Duckworth et al., 2006).

The effectiveness of fertility control in rodent populations has been demonstrated using surgical sterilization and sex steroid inhibition. It has been estimated that 70-80% female sterility will be required to significantly reduce the population size of a specific species (Chambers et al., 1999b; Jacob et al., 2004; Jacob and Matulessy, 2004). Furthermore, fertility control effects may be more pronounced in an open field population where rats may be exposed to predation and other management strategies, such as the use of rodenticides and traps. Models for brushtail possum populations have shown that fertility control can provide a long-term, sustainable approach to maintaining pest populations at a low level. Spurr (1981) predicted that a population will return to its former level within 10 or 14 years following a 70% or 90% poison kill, respectively. In contrast, if 70% of a population is permanently sterilized, it will take 23 years for the population to return to its former level (Spurr, 1981). Similarly, Barlow (1991) demonstrated that a 70% kill would reduce and

sustain low population levels of possums for up to 8 years. In contrast, 50-60% sterilization, followed by an annual sterilization rate of 18%, would be sufficient to continually maintain a population at 35-40% below its natural equilibrium long-term (Barlow, 1991). A five year field study examining the effect of surgical sterilization on possum population dynamics demonstrated that a 50% sterility rate would reduce *per capita* recruitment by 50% when a population was close to carrying capacity (Ramsey, 2005). Furthermore, studies have shown that fertility control may reduce breeding related contact between possums, thereby reducing horizontal disease transmission between animals (Caley and Ramsey, 2001; Ramsey, 2007).

Collectively, these modelling and experimental field studies illustrate that fertility control could offer an effective and sustainable alternative method for the long-term control of pest populations in addition to the potential to reduce disease transmission between individuals. Current approaches being investigated include hormonal manipulation and immunological and chemical sterilization. For the purposes of this review only significant findings in rodents and marsupials will be discussed in detail.

2.6.1 Immunological sterilization

An immunological fertility control agent works by stimulating an immune response against reproductive proteins (or antigens), thereby interfering with reproduction (Arthur et al., 2007; Barlow, 2000; Miller et al., 1998). Immunological fertility control has been investigated extensively in eutherian mammals (Barfield et al., 2006; Barlow, 2000; Chambers et al., 1999a; Gupta and Bansal, 2010; Hardy et al., 2006) and marsupials such as the tammar wallaby, koala, eastern grey kangaroo and the brushtail possum (Bradley et al., 1999; Cowan, 1996; Cowan, 2000; Duckworth et al., 2006; Mate and Hinds, 2003; Mate et al., 1998).

One area of focus for fertility control has been on targeting the ZP proteins surrounding the oocyte. Wildlife populations such as feral horses (*Equus calballus*) (Kirkpatrick et al., 1990), white-tailed deer (*Odocoileus virginianus*) (Kirkpatrick et al., 1995), and captive zoo animals (Frank et al., 2005; McShea et al., 1997) are currently being controlled using porcine ZP (pZP) immunocontraceptive vaccines (Kirkpatrick et al., 2011). pZP contraceptive control of these species is highly effective (75 – 100%), although annual boosters are required during the first 2-3 years. pZP is also beneficial because it causes minimal behavioural effects, no debilitating side effects and, although infertility is temporary, this allows for better control and management of these specific populations over time (Kirkpatrick et al., 2011). New vaccine formulations offer greater efficacy and reduced treatment costs (Miller et al., 1998). The current route of administration is via hand injection after capture or using remote dart delivery. Although remote delivery will not be feasible for control of most species (e.g. rodents), in the case of large bodied mammals which can be easily accessed routinely in large groups (e.g. captive animals; seasonal breeders), such methods are suitable.

Successful reductions in fertility following immunization with pZP or bacterially produced recombinant ZP3 (rZP3) have also been demonstrated in several other species including bonnet

monkeys (Bagavant et al., 1994; Govind et al., 2002; Kaul et al., 2001), baboons (Govind and Gupta, 2000), dogs (Gupta et al., 2011; Mahi-Brown et al., 1985; Srivastava et al., 2002), rabbits (Bhatnagar et al., 1992; Dietl et al., 1982; Kerr et al., 1999; Wood et al., 1981), mice (Clydesdale et al., 2004; Hardy et al., 2003; Jackson et al., 1998; Li et al., 2007; Lloyd et al., 2003; Millar et al., 1989), hamsters (Hasegawa et al., 1992), wallabies (Kitchener et al., 2002), and brushtail possums (Duckworth et al., 1999; Duckworth et al., 2007). Again, the current route of administration is via injection and therefore alternative routes (i.e. oral/nasal) for ZP delivery have been examined in mice (Zhang et al., 2011) and possums (Cui et al., 2010; Duckworth and Cui, 2004; Walcher et al., 2008).

Bacterial ghost vectors (BGVs) and virus-like particles (VLPs) have been examined as potential delivery systems for ZP antigens. BGVs consist of the cell membranes of recombinant bacteria which have been genetically engineered to express target reproductive molecules (Mayr et al., 2005) while VLPs are lipoprotein nanoparticles artificially created to resemble virus capsids that incorporate target reproductive antigens (Pattenden et al., 2005). Both BGVs and VLPs lack genetic material and therefore cannot replicate (Cui et al., 2010; Duckworth and Cui, 2004; Walcher et al., 2008). VLPs expressing ZP3 have been shown to reduce litter sizes and suppress fertility in mice when subcutaneously injected (Choudhury et al., 2009). To date, there have been no reports on orally or nasally delivered VLPs expressing ZP antigens. Intranasal co-administration with mouse ZP (mZP) DNA and protein vaccines encapsulated in chitosan reduced fertility and mean litter size of female C57BL/6 mice while not affecting normal follicular development (Zhang et al., 2011). In female possums, oral and intranasal delivery of a BGV expressing possum ZP2 or ZP3 antigens produces successful humoral and mucosal immune responses as well as reduced fertilization rates (36% relative to controls) (Cui et al., 2010; Duckworth and Cui, 2004; Walcher et al., 2008). It seems that an oral/nasal route utilizing BGVs may be feasible for inducing successful ZP-induced immunocontraception although further development will be required for its use on large scale pest control operations.

Viral vectored immunocontraception (VVIC) has been examined as a potential immunological control agent because of its potential for a low cost-benefit ratio, its natural disseminating properties and it can be species-specific (Chambers et al., 1999a; Hardy et al., 2006; McLeod et al., 2008; Redwood et al., 2008). Murine cytomegalovirus (MCMV) has been examined as a potential vector for the expression of a murine ZP3 (mZP3) gene (Farroway et al., 2002; Hardy et al., 2006; Lloyd et al., 2010; Lloyd et al., 2003; Redwood et al., 2007; Singleton et al., 2001; Smith et al., 2005). MCMV is a mouse-specific betaherpesvirus which is readily carried in wild house mouse populations and is naturally occurring in Australia. Anti-mZP3 antibodies block the ability of sperm to penetrate the egg for fertilization, thereby inducing infertility (Wassarman, 1999). Laboratory trials have demonstrated infertility in nearly all mice inoculated with recombinant MCMV expressing mZP3 (Lloyd et al., 2007; Lloyd et al., 2003; O'Leary et al., 2008; Redwood et al., 2005) but infectivity of the modified viruses was compromised (Hardy et al., 2006). These findings, however noteworthy, need to be repeated in wild, outbred rodent populations. Release of any self-disseminating fertility control agents would require a

thorough understanding of their potential impacts and would need to meet local and international regulatory requirements to safe guard against unintended consequences (Williams et al., 2001).

Various VVIC methods have also been examined in possums with varying results. Zheng (2007) identified two possum intestinal enteroviruses, W1 and W6, as potential VVICs (Zheng, 2007). Infection with the W1 or W6 strain did elicit antibody responses in both captured and wild-dwelling possums. However, antibody response was variable, short-lived and distribution of the enterovirus was limited (Zheng and Chiang, 2007; Zheng et al., 2010). In Australia, macropod herpesvirus (MaHV) infection is common amongst macropod marsupials and at least one strain has been identified and tested as a potential vector in NZ possums. Possums infected with MaHV-1 showed transient infection at inoculation sites but a systemic and latent infection was not generated (Zheng et al., 2001; Zheng et al., 2004). Although there is no evidence that eutherians can be infected with MaHV, it is highly pathogenic and can cause negative side effects including transient infertility, lingual ulcers, discharge from the eyes and nose and, in extreme cases, death (Speare et al., 1989). Because of these undesirable effects and the lack of infectivity, no further research has been reported on the use of MaHV for control of NZ possums.

Other possum-specific viruses that have been identified as potential disseminating delivery systems include adenovirus and coronavirus (Rice and Wilks, 1996; Thomson et al., 2002), papillomavirus (Perrott et al., 2000), and a type D retrovirus identified as TvERV(D) (Baillie and Wilkins, 2001). Much work will be required to determine the individual viral infectivity of each potential disseminating delivery system and ultimately its ability to reduce possum fertility. In addition, there is concern for accidental or intentional (illegal) transfer of a disseminating delivery system to Australia where possums are endemic and protected (Gilna et al., 2005). Therefore, if successful, use of a VVIC for possum control will need to be carried out with caution.

Immunization utilizing endogenous hormones has also been widely examined. Gonadotrophin releasing hormone (GnRH) is a decapeptide present in both males and females. Multiple reports have demonstrated effective blocking of fertility in a range of species with the use of GnRH made immunogenic via coupling with various carrier proteins (Gupta and Bansal, 2010; Kirkpatrick et al., 2011; Miller et al., 1998). Miller et al. (1997) injected Norway rats with GnRH vaccines made immunogenic via coupling to the carrier protein keyhole limpet haemocyanin (KLH). Through the action of antibodies binding to GnRH, reductions in FSH and LH from the anterior pituitary gland and eventual atrophy of the gonads is induced, leading to temporary infertility (Figure 2.3). The authors demonstrated 100% infertility in both male and female rats for the duration of the study (12 months) and predicted infertility to last through the rodent's lifetime (1-3 years). However, several booster injections over a three month period were required to establish infertility (Miller et al., 1997).

The GnRH vaccine, GonaCon™, is a single shot, injectable vaccine developed by the USDA National Wildlife Research Center (Miller et al., 2004) and has demonstrated long-term infertility (2-4 years) in a range of species, both male and female (Kirkpatrick et al., 2011; Mauldin and Miller, 2007).

GonaCon™ halts reproduction by eliciting antibodies to native GnRH (Figure 2.3). GonaCon™ was examined as a potential management strategy for black-tailed prairie dogs (*C. ludovicianus*) and the high antibody titres indicate that it will likely control prairie dog fertility for ≥1 year (Yoder and Miller, 2010). Further work will be required to determine if GonaCon™ will be effective as a contraceptive agent for prairie dog populations.

A long-term study examined the effects of GonaCon™ on the fertility and behaviour of male and female tammar wallabies, *Macropus eugenii* (Snape et al., 2011). GonaCon™ vaccination resulted in 100% infertility in adult males (>2 years) and females (>4 years) for the duration of the study. Behavioural observations during the study revealed that males, when vaccinated as juveniles, showed reduced rates of sexual behaviour compared with controls. Interestingly, when control males were housed with vaccinated males, they showed decreased rates of agonistic behaviour compared with when they were housed with untreated males. Although behavioural changes were observed during the study, it was determined that animal welfare was not negatively impacted.

GonaCon™ use in brushtail possums has demonstrated efficacy and examination into an oral or nasal route is underway (Cross et al., 2011). A single injection of GonaCon™ produced anti-GnRH antibodies and rendered >70% females infertile for 2 years (Cross et al., 2011). A proof of concept study examined the effects of poly(ethylcyanoacrylate) (PECA) nanoparticles containing D-Lys^6-GnRH when administered into the caecum of brushtail possums. Their results demonstrated that sufficient bioactive peptide was able to reach the pituitary gland to evoke a response as demonstrated by increased blood LH levels (Kafka et al., 2011). Further investigation into potential oral or nasal delivery will be necessary for this method to be used in a large-scale operation for control of possum populations in NZ.

At the moment, there remain several limitations to immunological fertility control. These include the generally temporary nature of the infertility, the need for repeat exposure to maintain infertility, inconsistent responses, and a reliance on an immune response for effectiveness. In some cases animals do not generate an immune response to the vaccine (non-responders) (Mann et al., 2009). There is concern that if there is a genetic basis to this non-responsiveness then natural selection for non-responsive progeny may occur over time resulting in populations that develop resistance to the reproductive agent (Cowan, 2001; Magiafoglou et al., 2003). Furthermore, modelling studies have demonstrated that permanent sterilization will be more effective in reducing and then sustaining lower pest populations compared with temporary sterilization (Spurr, 1981), supporting the case for an irreversible fertility control management system. In order for such technology to be feasible for wild pest populations, an oral route of delivery will be necessary.

2.6.2 Chemosterilization

Chemical sterilization (chemosterilization) offers another option for fertility control. A chemosterilant can be defined as a chemical, whether endogenous (hormonal manipulation) or exogenous (xenobiotic

manipulation), that can induce temporary or permanent sterility (Marsh and Howard, 1970). Chemosterilants can inhibit fertility by altering the hormones which control ovarian or testicular function (Gao and Short, 1993, 1994a, b) or by directly affecting the function and physiology of the ovary or testis.

Hormonal chemosterilization

Endocrine signalling by hormones plays a vital role in reproduction (Figure 2.3), thus it was one of the first targets for early investigations of fertility control. Hormonal chemosterilization is accomplished by suppressing reproductive activity with the use of hormone agonists, antagonists, or toxins.

Proof of concept studies have been carried out investigating the use of a GnRH toxin conjugate, GnRH-PAP (pokeweed antiviral protein) in possums (Eckery et al., 2001). Possum pituitary cells cultured with GnRH-PAP showed decreased LH secretion and LH-containing cell numbers suggesting that the toxin was able to induce cellular death. Possums treated with GnRH-PAP by injection showed depressed circulating levels of LH and FSH although the effect was temporary and hormone levels returned to normal within 3 weeks (Eckery et al., 2001). Delivery of GnRH through an oral (or oronasal) route of administration in the possum examined the use of the previously discussed VLPs (Cross et al., 2011). Orally delivered VLPs chemically conjugated to GnRH (GnRH-VLP) resulted in an immune response in possums, although the response was lower than when injected (Cross et al., 2011).Although these studies demonstrate proof of concept, hormonal sterilization was temporary, reversible, not species-specific, and the current routes of administration are not practical for wildlife populations.

A hormonal implant containing a GnRH agonist, deslorelin, has been examined as a potential method for fertility control delivery. The action of deslorelin on the anterior pituitary results in suppression of steroidal hormone production, follicular development and ovarian cycles (Figure 2.3). Its use as a potential fertility control method has been investigated in several marsupials including the brushtail possum (Eymann et al., 2013; Eymann et al., 2007; Lohr et al., 2009), the tammar wallaby (*M. eugenii*) (Herbert et al., 2007, 2012; Herbert et al., 2004a; Herbert et al., 2004b, 2005) and the eastern grey kangaroo (*M. giganteus*) (Herbert et al., 2006; Wilson et al., 2013; Woodward et al., 2006). Eymann et al. (2007) examined the effects on fertility of subcutaneous deslorelin implants in male and female NZ possums. Following deslorelin implantation, female reproduction was inhibited for at least one breeding season, but such effects were temporary and reversible while males showed no reduction in fertility despite reduced circulating testosterone and FSH levels (Eymann et al., 2007). Similar results were reported in brushtail possums in Australia where 80% of treated females showed no evidence of breeding at study completion (18 months) (Lohr et al., 2009). Recent examination of the effects of deslorelin implants in rodents demonstrated reductions in ovarian weight, pre-antral follicle numbers, circulating gonadotrophin levels, and overall disruption of the oestrous cycle (Alkis et al., 2011; Cetin et al., 2012; Grosset et al., 2012; Smith et al., 2012). The current route of administration (surgical implantation or dart gun) (Herbert et al., 2010) and the temporary (6 to 18 months) and

reversible nature of this method make it suitable for veterinary or captive (e.g. zoo) animal use. However, these same characteristics are not appropriate for wild pest populations, especially considering reports on decreased sexual behaviour in many species (Bertschinger et al., 2001; Patton et al., 2006; Woodward et al., 2006).

Hormonal manipulation has also be achieved through the use of the cholesterol mimic, 20,25-diazacholesterol (DiazaCon™) which inhibits cholesterol production. Cholesterol is required for the synthesis of steroid reproductive hormones, such as testosterone and progesterone. Therefore, inhibition of cholesterol production indirectly inhibits reproduction. Oral treatment with DiazaCon™ has caused reduced reproductive output in black-tailed prairie dogs (*Cynomys ludovicianus*) (Nash et al., 2007) and evidence for its effectiveness has been demonstrated in female grey squirrels (*Siurus carolinensis*) (reduced cholesterol levels (Mayle et al., 2012) and antibody titre response (Yoder et al., 2011)). Intraperitoneal injection of 20,25-diazacholesterol dihydrochloride (SC 12937) caused antispermatogenic and antifertility effects in male Parkes strain mice (Singh and Chakravarty, 2003). Although DiazaCon™ administration demonstrates effectiveness via the oral route, the effects have been shown to last only up to 6 months and it may not be suitable for wild animal populations if alteration of the reproductive hormones affects sexual behaviour. Furthermore, DiazaCon™ is cleared slowly and bioaccumulation does occur with repeated treatment (Nash et al., 2007; Yoder et al., 2011).

Natural compounds occurring in various parts of plants have been found to disrupt or inhibit reproduction and therefore have been examined for their contraceptive potential (Tran and Hinds, 2013). Plant-derived compounds which specifically target the ovary have been identified and a full review by Tran and Hinds (2013) discusses their effects when orally administered. Cessation of treatment with any of the currently examined plant-derived compounds does result in the return of normal fertility (Tran and Hinds, 2013). However, 6 plant species (*Azadirachta indica, Hibiscus rosasinensis, Melia azedarach, Momordica charantia, Trichosanthes cucumerinas* and *Tripterygium wilfordii*) have been identified as potential candidates for contraceptive use as they specifically target ovarian follicle development and function (Tran and Hinds, 2013). For the purposes of this review, focus will be on the active ingredient, triptolide (Figure 2.4) derived from *T. wilfordii* (thunder god vine).

Triptolide

Triptolide (TR) was first discovered to have anti-fertility effects in males (e.g. oligozoospermia and asthenozoospermia) (Huynh et al., 2000; Lue et al., 1998; Qian et al., 1986; Qian et al., 1988; Zhen et al., 1995) and these effects have been confirmed in wild-caught male laboratory black rats (*R. rattus*) treated with a bait containing TR (Singla et al., 2012). More recently, the effects of orally administered TR in females rats have been examined (Liu et al., 2011; Xu and Zhao, 2010). Oral administration of 60 or 120 µg TR/kg/day for 35 days to female Sprague Dawley (SD) rats caused a significant increase in both healthy and apoptotic secondary ovarian follicles compared with controls (Xu and Zhao, 2010). No observed changes in the primordial or primary follicle pools were noted. Additionally, the

oestrous cycles were lengthened in animals receiving the higher TR dose (120 µg TR/kg/day) relative to controls (Xu and Zhao, 2010). A similar study examining the effects of orally delivered TR (100, 200 or 400 µg/kg/day, 90 days) on the fertility of female SD rats found that the two highest TR doses caused decreases in ovarian and uterine weight, reduced serum levels of E_2 and P_4, increased serum levels of FSH and LH and overall reduced staining for the presence of the E_2 receptor alpha (ERα) in the uterus and ovaries relative to controls (Liu et al., 2011). The only observed effect following treatment with a low TR dose (100 µg/kg/day; 90 days) was reduced P_4 levels and ERα staining relative to controls.

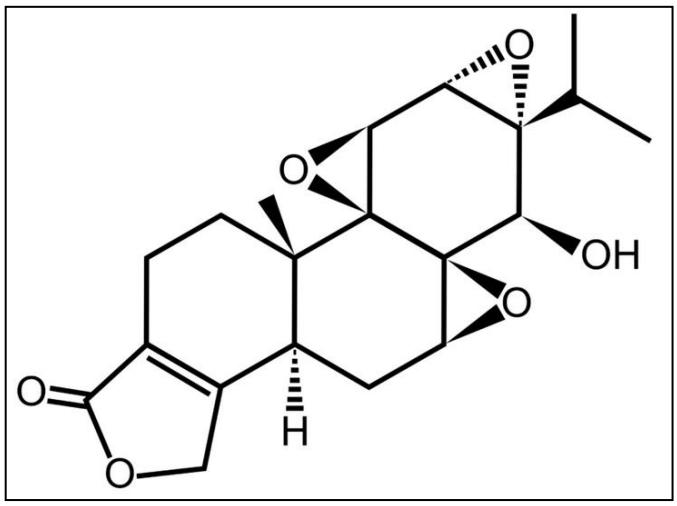

Figure 2.4 The chemical structure of triptolide (360.4 g/mol).

Lue et al. (1998) first suggested that TR's method of action was involved in alterations of the intracellular calcium (Ca^{2+}) influx pattern (Lue et al., 1998). Following specific [^{3}H]triptolide-binding activity, Leuenroth et al. (2007) identified the Ca^{2+} channel, polycycstin-2 (PC2), as a putative target protein for TR binding (Leuenroth et al., 2007). PC2 is a product of a gene mutation found in type 2 autosomal dominant polycystic kidney disease (ADPKD) and is a member of a subfamily of the transient receptor potential (TRP) superfamily (Koulen et al., 2002). It was determined that TR causes an increase in intracellular Ca^{2+} release through a PC2-dependent pathway (Leuenroth et al., 2007). Signal transduction pathways that control the decision for a cell to divide, differentiate or die are activated by increases in intracellular Ca^{2+} (Ermak and Davies, 2002; McKinsey et al., 2002; Orrenius et al., 2003). Additionally, intracellular Ca^{2+} influx following cellular stress can be sufficient to trigger or modulate apoptosis (Orrenius et al., 2003). Therefore, their findings suggest that TR is acting directly on the ovarian follicle populations rather than the steroids involved in the HPG axis (Figure 2.3). Further evidence suggesting TR's involvement in calcium-dependant cellular regulation is TR's ability to induce apoptosis in tumor cells as well as sensitizing tumor cells to apoptosis via α-mediated tumor necrosis factor (TNF) (Lee et al., 1999) without being mutagenic (Kupchan, 1977; Shamon et al., 1997).

Current investigation utilizing TR in combination with another chemosterilant, 4-vinylcyclohexene diepoxide (VCD), is being undertaken by researchers at SenesTech Inc® (Flagstaff, AZ, USA). Preliminary findings from their laboratory have demonstrated that female SD rats consuming a bait containing VCD and TR have reduced numbers of primordial, primary and secondary follicles (Dyer et al., 2013) as well as reduced litter sizes (Drs L. P. Mayer and C. A. Dyer, unpublished). A full review of the ovarian follicle depleting effects of VCD is discussed below.

Non-hormonal chemosterilization

Non-hormonal chemosterilants that induce premature ovarian failure could potentially serve as fertility control agents due to their permanent fertility-inhibiting characteristics. In a young, cycling ovary, recruitment of primordial follicles for development occurs continuously, yet little is known about the factors that control this process. After initial recruitment of any given follicle, it can progress through several stages of growth (primary, preantral, antral, and pre-ovulatory) to reach ovulation. During the process of follicular maturation many follicles and their oocytes undergo atresia (apoptosis or programmed cell death) with only ~400 of the ~2 million oocytes contained within primordial follicles progressing to ovulation (Hirshfield, 1991; Hirshfield and Midgley Jr, 1978; Palumbo and Yeh, 1994). Some recent investigations have suggested that stem-cell populations may exist that could give rise to follicular germ cells following ovarian failure, but this concept has yet to be confirmed (Johnson et al., 2004; White et al., 2012; Zou et al., 2009).

The immature ovarian follicle pool can be affected by a variety of xenobiotic compounds (see review and references cited in Table 2.1). Chemical groups which have been shown specifically to target ovarian function in laboratory animals include: chemotherapeutic agents such as cyclophosphamide (CPA) and cisplatin; ionizing radiation; polycyclic aromatic hydrocarbons (PAHs; commonly found in cigarette smoke) such as 7,12-dimethylbenz(a)anthracene (DMBA), 3-methylcholanthrene (3-MC), and benzo[a]pyrene (BaP); endocrine distruptors such as bisphenol-A (BPA), medroxyprogesterone acetate (MAP) and diethylstilboestrol (DES); and occupational chemicals containing an epoxide moiety (which are able to bioactivate by epoxidation) such as 1,3-butadiene (BD) and vinylcyclohexene (VCH) and its epoxide metabolite 4-vinylcyclohexene diepoxide (VCD). Although the xenobiotic mechanisms for ovarian toxicity are not fully understood, in general they target and destroy the oocytes located within the follicles which ultimately lead to loss of these follicle structures and onset of premature ovarian failure (POF; menopause in humans). Depending on the dosing regimen and route of exposure, development of ovarian tumours (granulosa cell tumours, mesothelial adenomas, and mixed benign tumours) are often associated with the loss of ovarian follicles. A general review of the positive and negative side effects of commonly studied ovarian toxicants is provided in Table 2.1.

Table 2.1 A summary of commonly studied ovarian toxicants and the positive effects (*as they relate to pest fertility control), and negative effects (as they relate to animal humaneness). Due to the exhaustive bodies of work on these topics, reviews which cover each topic in depth have been provided. [Δ]Effects evident >90 days of daily gavage; [‡]Effects also observed in humans.**

Chemical	Primary animal model	*Positive effects	**Negative effects
Benzo(a)pyrene (BaP) (Kappeler and Hoyer, 2012; Mark-Kappeler et al., 2011a)	Mouse	Depletion of primordial and primary ovarian follicles via necrosis	[‡]Carcinogenic; ovarian tumours
Bisphenol-A (Cabaton et al., 2013; Kappeler and Hoyer, 2012; Mark-Kappeler et al., 2011a; Rogers et al., 2013)	Mouse; rat	Decreased primordial follicles; increase in growing follicles; endocrine disruptor	Meiotic disturbances; disruption of energy metabolism and brain function; alteration of immune response
2-Bromopropane (2-BP) (Ichihara, 2005)	Rat	Depletion of primordial, primary and antral follicles; increased rates of follicular atresia; disruption of estrous cycle; reduced ovarian and uterine weights and ovulated ova	[‡]Neurotoxic; [‡]hematopoietic depletion; reduction of bone marrow (males)
1,3-butadiene (BD) (Kappeler and Hoyer, 2012; Mark-Kappeler et al., 2011a; Maronpot, 1987)	Mouse	Depletion of preantral and antral follicles	[‡]Carcinogenic
Cisplatin (Kappeler and Hoyer, 2012; Mark-Kappeler et al., 2011a)	Rat	Depletion of all ovarian follicle types; increased atretic secondary and antral follicle numbers	[‡]Carcinogenic
Cyclophosphamide (CPA) (Kappeler and Hoyer, 2012; Mark-Kappeler et al., 2011a)	Mouse; rat	Reduced health and depletion of all ovarian follicle types; reduced implantation sites; malformed offspring	[‡]Mutagenic; [‡]carcinogenic
Diethylstilboestrol (DES) (Kappeler and Hoyer, 2012; Mark-Kappeler et al., 2011a; Marselos and Tomatis, 1993)	Mouse; rat	Reduced oocyte quality; endocrine disruptor; reduced litter size	[‡]Vaginal carcinomas; [‡]Uterine, oviduct and ovarian abnormalities; chromosomal aberrations and DNA disruption; genotoxic effects
7,12-Dimethylbenz(a)anthracene (DMBA) (Bhattacharya and Keating, 2011; Kappeler and Hoyer, 2012; Mark-Kappeler et al., 2011a)	Mouse; rat	Depletion of all ovarian follicle types via apoptosis	[‡]Carcinogenic; ovarian tumours

Table 2.1 continued:

Chemical	Primary animal model	*Positive effects	**Negative effects
Isopropyl methanesulphonate (IMS) (Kappeler and Hoyer, 2012; Mark-Kappeler et al., 2011a)	Mouse	Destruction of oocytes within small follicles	‡Mutagenic; ‡carcinogenic; ovarian tumours
Medroxyprogesterone acetate (MAP) (Barnes and Meyer, 1964; Hayden et al., 1989; Keskin et al., 2009; Loretti et al., 2004; Oguge and Barrell, 1996)	Rat; rabbit; cat; dog	Reduced ovulation rate; ‡cessation of ovulation; impairment of gestation or parturition; foetal death ‡endocrine disruptor	Abnormal mammary gland growth; ovarian cysts; pyometra; cystic endometrial hyperplasia
3-Methylcholanthrene (3-MC) (Kappeler and Hoyer, 2012; Mark-Kappeler et al., 2011a)	Mouse	Depletion of primordial and primary ovarian follicles via necrosis	‡Carcinogenic; ovarian tumours
1,4-Di(methanesulfonoxy)-butane (busulfan; Myleran) (Bishop and Wassom, 1986; Kappeler and Hoyer, 2012; Mark-Kappeler et al., 2011a)	Mouse; rat	Destruction of oocytes within small follicles; depletion of all ovarian follicle types	Cytotoxicity; teratogenic; mutagenic; hematopoietic depletion;
Trimethylenemelamin (TEM) (Kappeler and Hoyer, 2012; Mark-Kappeler et al., 2011a)	Mouse	Destruction of oocytes within small follicles	‡Mutagenic; ‡carcinogenic; ovarian tumours
4-Vinylcyclohexene diepoxide (VCD) (Kappeler and Hoyer, 2012; Mark-Kappeler et al., 2011a; NTP, 1986)	Mouse; rat	Depletion of primordial and primary ovarian follicles; ovarian and uterine atrophy	ΔHyperplasia of the forestomach; Δtoxic nephrosis

Of all the known ovotoxic chemicals one in particular, VCD, has been singled out as a model chemical for studying ovarian toxicity (Hoyer and Sipes, 1996; Kappeler and Hoyer, 2012; Van Kempen et al., 2011). VCD, a cyclohexene formed during synthesis of industrial chemicals, is an epoxide metabolite of its parent compound, VCH (Figure 2.5). Toxicology studies have shown that a short duration of treatment with VCD (3-30 days) specifically targets and depletes the pool of primordial and small primary ovarian follicles in rode ts (Figures 2.2 and 2.6) (Devine et al., 2004; Kao et al., 1999; Mayer et al., 2001; Mayer et al., 2002; Sahambi et al., 2008; Smith et al., 1990b). In addition, short duration treatment with VCD in rodents results in minimal to nil effects on other organs such as the liver, spleen, kidneys, and adrenal glands (Burd, 2009; Haas et al., 2007; Mayer et al., 2004; Mayer et al., 2010; Muhammad et al., 2009; Sahambi et al., 2008). Reduced ovarian and uterine weights in rodents following VCD treatment have been reported (Lohff et al., 2005; Lohff et al., 2006; Mayer et al., 2004; Mayer et al., 2002; Muhammad et al., 2009; Sahambi et al., 2008) although these are likely downstream effects resulting from loss of the ovarian follicle pools and disruption of the HPG axis (Figure 2.3).

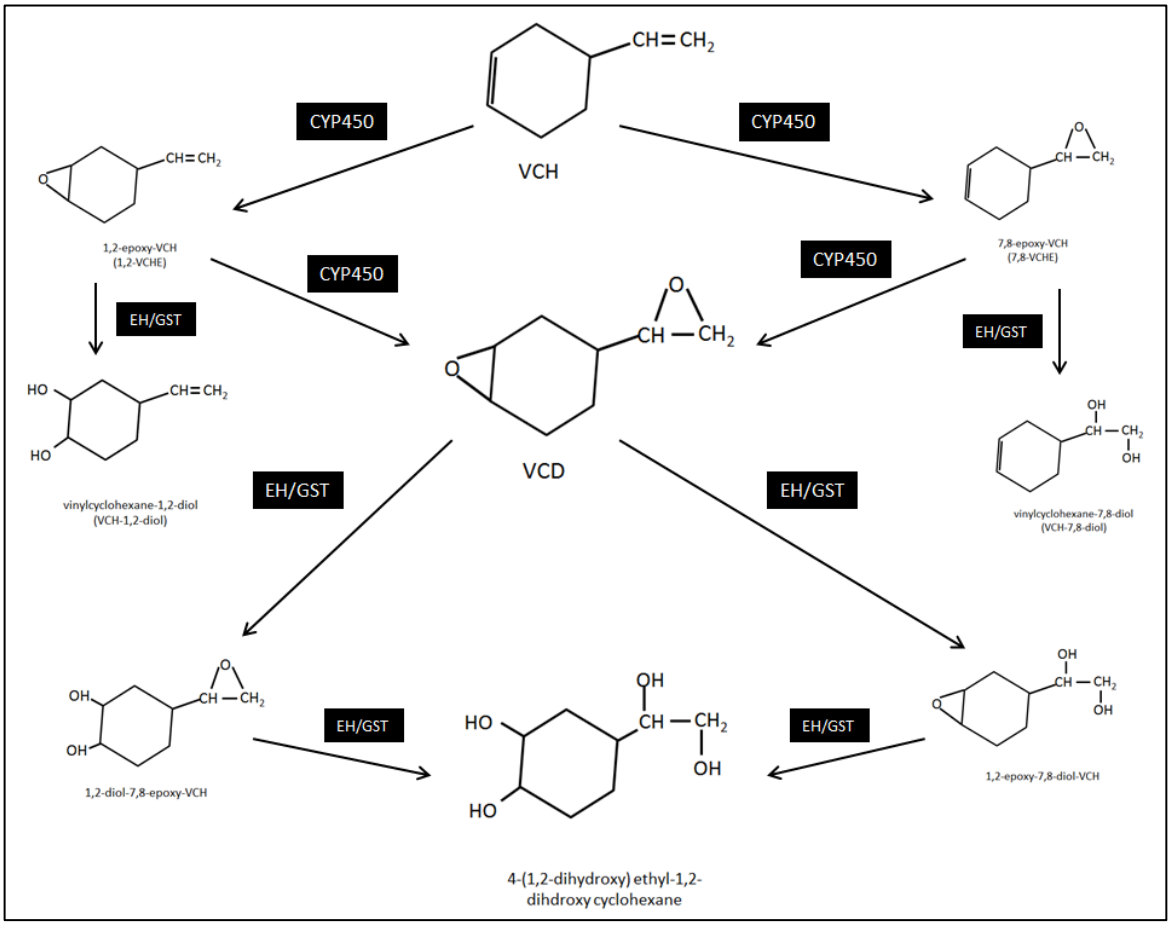

Figure 2.5 Metabolic pathway of VCH and VCD. VCH: 4-vinylcyclohexene; VCD: 4-vinylcyclohexene diepoxide; CYP450: cytochrome P450; EH: epoxide hydrolase; GST: glutathione S-transferase. Adapted from Salyers (1995).

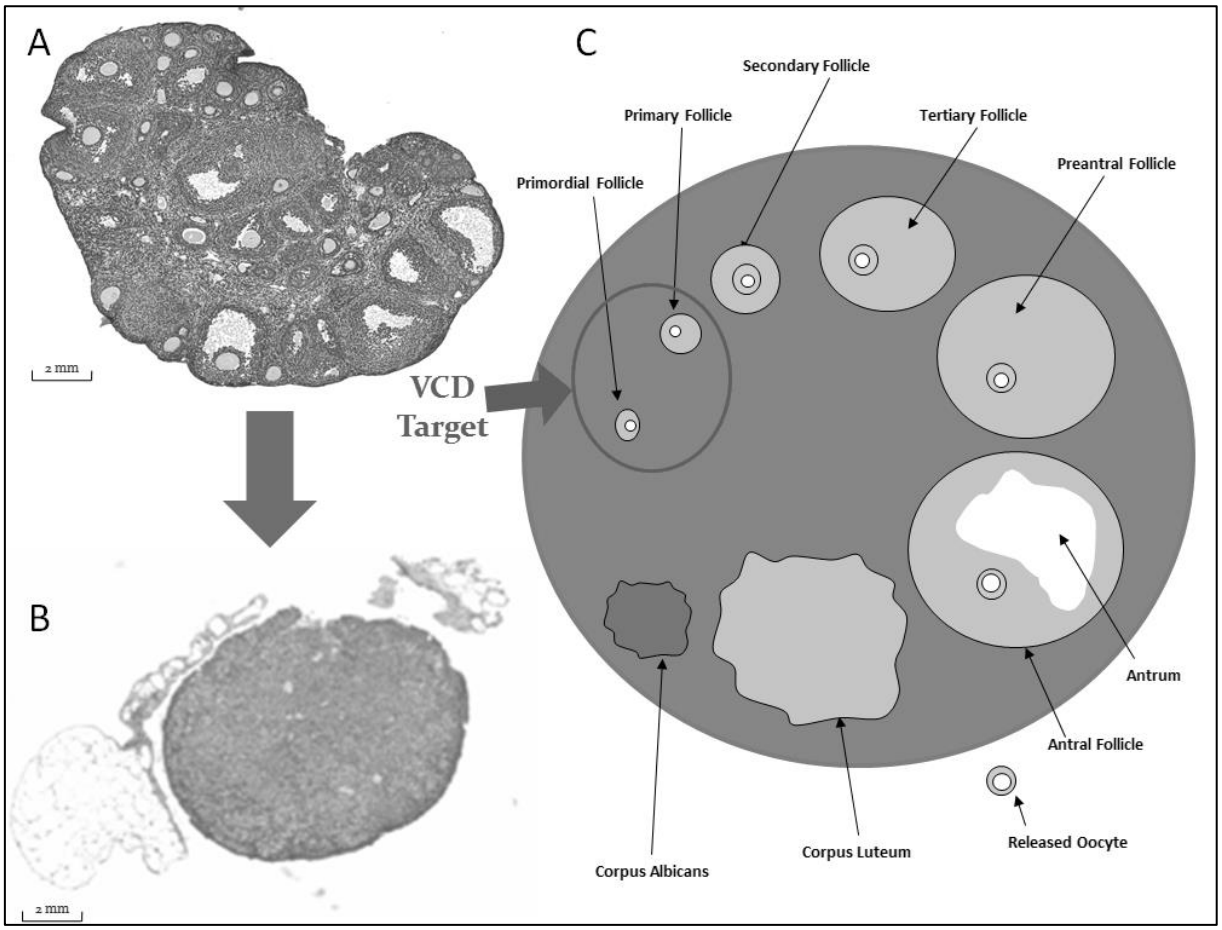

Figure 2.6 Ovary schematic depicting the follicular stages and the downstream effects of VCD. Histological images, adapted from Mayer et al. (2004), are representative images for a (A) healthy and (B) VCD-treated ovary of an adult Sprague Dawley rat. The schematic of follicle types within the ovary (C; images not drawn to scale) depicts the order in which follicles mature from the most immature (primordial) to pre-ovulatory (antral) as well as the post-ovulatory structures (corpus luteum, corpus albicans).

In order to simulate human worker exposure the National Toxicology Program (NTP) investigated the toxicological effects and carcinogenic activity of VCD over a 14-day, 13-week, and 2-year study in male and female rats and mice (NTP, 1986). The most prevalent effects were following 2 years of daily gavage with VCD (200 or 400 mg/kg/day) which included forestomach hyperplasia, adenomas and carcinomas, ovarian neoplasms and death. The NTP concluded that there was clear evidence for VCD-induced carcinogenicity following chronic exposure to the chemical (NTP, 1986). However, such extreme dosing regimens are not required to induce ovarian senescence; rather acute, short duration treatment is sufficient with no reported mutagenic or carcinogenic side-effects (Burd, 2009; Haas et al., 2007; Mayer et al., 2004; Mayer et al., 2010; Muhammad et al., 2009; Sahambi et al., 2008). Therefore, concern for its potential carcinogenic properties is not warranted for use of VCD as an ovotoxic model or chemosterilant. A large body of work has been dedicated to understanding VCD's follicle-depleting mode of action and its metabolic pathways. Here I will review the current body of knowledge on VCD with focus on its use as a potential chemosterilant for pest mammals.

VCD: method of action

Studies in rodents to elucidate the mode of action of VCD-induced follicular depletion initially determined that VCD treatment increases the natural process of atresia (Fernandez et al., 2008; Hu et al., 2001a; Hu et al., 2001b; Hu et al., 2006; Kao et al., 1999; Mayer et al., 2002; Springer et al., 1996a; Takai et al., 2003). The selective nature of VCD ovarian toxicity is believed to be due to the poor ability of primordial and primary follicles to convert VCD to its inactive tetrol metabolite (Flaws et al., 1994). VCD treatment increases the expression and activation of *bax* and Bad (proteins that promote apoptosis), caspase-3 (a proteolytic enzyme present during induction of apoptosis), and mitogen activated protein kinases (MAPKs which are associated with intracellular signalling pathways for apoptosis) (Hoyer et al., 2001; Hoyer and Sipes, 2007). However, more recent findings suggest that these effects may reflect downstream responses to VCD induced ovarian toxicity rather than initiating effects (Figure 2.7) (Fernandez et al., 2008; Keating et al., 2009; Keating et al., 2010; Mark-Kappeler et al., 2010; Mark-Kappeler et al., 2011b).

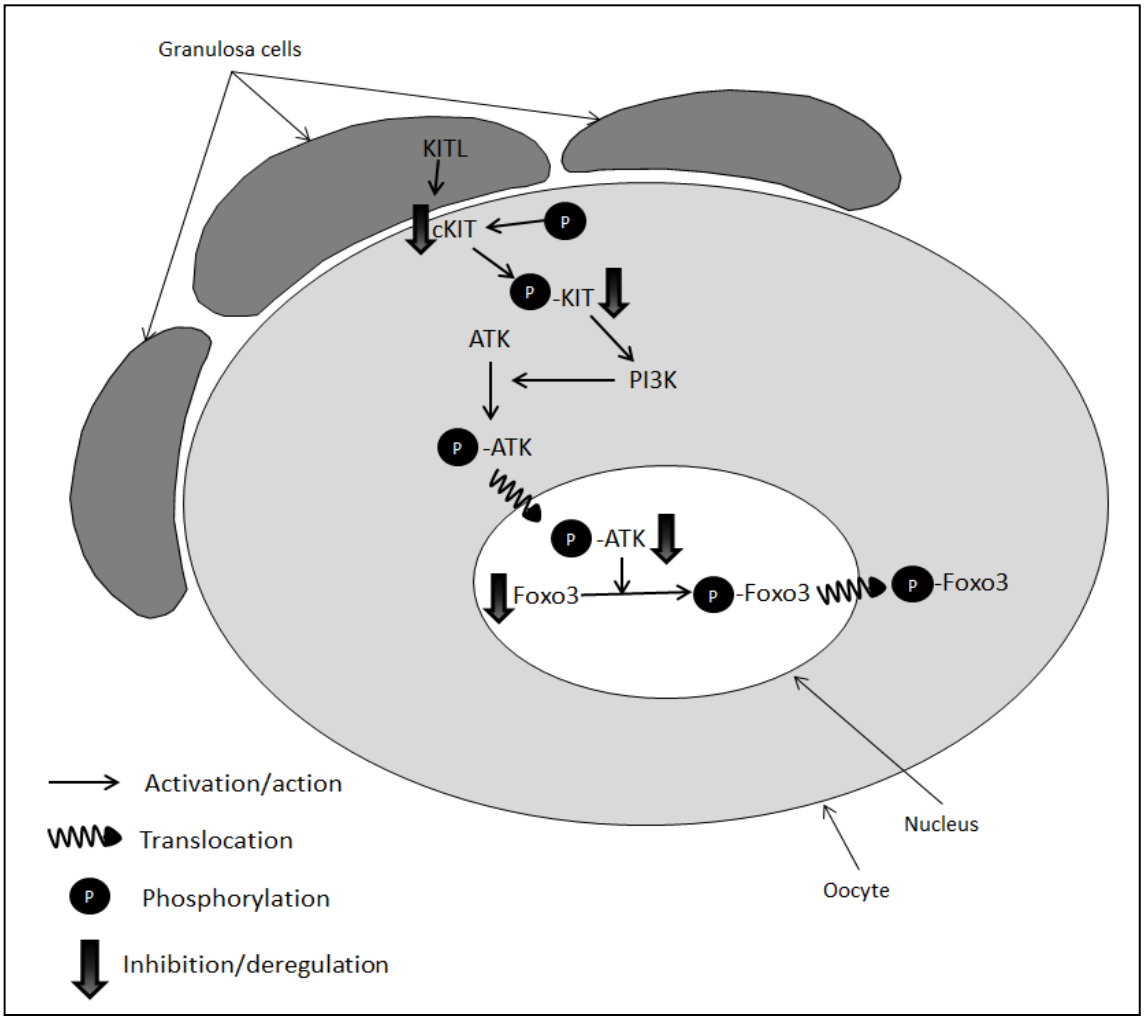

Figure 2.7 Currently proposed VCD method of action in primordial and primary follicles. Adapted from P. J. Devine (unpublished).

Fernandez et al. (2008) examined cell survival signal pathways in immature follicle populations of post natal day (PND) 4 Fischer 344 rat ovaries following *in vitro* exposure to VCD. Findings revealed that VCD treatment reduced oocyte-derived *Kit* mRNA, a pro-survival gene, which preceded an increase in granulosa cell-derived *Kitl* mRNA (Fernandez et al., 2008). The ligand for KIT, KITL is produced in granulosa cells and is able to initiate folliculogenesis, stimulate oocyte and primordial follicle development, and act as an antiapoptotic factor on primordial follicles. These findings suggest that the oocyte may be initially affected by VCD and as a feedback response to decreased oocyte survival signals the granulosa cells produce more KITL. Furthermore, addition of exogenous KITL resulted in a dose-dependent attenuation of VCD-induced primordial follicle loss, supporting the suggestion that VCD compromises the KIT/KITL survival signalling pathway of primordial and primary follicles (Figure 2.7) (Fernandez et al., 2008).

A study was conducted to determine if KIT was directly targeted by VCD as the ovarian toxic mechanism in immature follicles (Mark-Kappeler et al., 2011b). Results demonstrated that a change in KIT receptor phosphorylation is an initial and critical component of VCD-induced ovarian toxicity, supporting previously reported findings (Figure 2.7). Furthermore, the high expression of oocyte derived KIT and granulosa cell derived KITL during immature follicle growth helps to explain and support evidence for the ability of VCD to target primordial and primary follicle populations while not affecting other tissues in the body. Although other tissues expressing KIT may be a target of VCD, such tissues are able to repair themselves whereas primordial follicles are incapable of being replaced following cellular destruction. This may explain why there are no observed long-term effects in other tissues following VCD exposure and, hence, the physiological specificity of VCD on immature follicle populations.

To examine further the role of VCD in the KIT/KITL signalling pathway, Keating et al. (2009) studied the role of phosphatidylinositol-3 kinase (PIK3) inhibition on VCD induced ovarian toxicity (Figure 2.7). KIT activates the PIK3 kinase signalling pathway which has been shown to activate and recruit immature follicles (Reddy et al., 2005). Using LY294002 to inhibit the PIK3 signal pathway, the primordial follicle pool was protected from VCD-induced follicle loss while primary follicle numbers were depleted. The authors concluded that primordial follicles are not the direct target of VCD; rather VCD targets and destroys small primary follicles, resulting in increased recruitment of primordial follicles into the primary follicular pool (Keating et al., 2009). In fact, Mark-Kappeler et al. (2010) demonstrated supporting results when examining anti-Müllerian hormone (AMH) protein expression during VCD treatment. AMH protein is expressed in granulosa cells of maturing primary follicles and inhibits primordial follicle recruitment into the growing follicle pool. Results showed that AMH protein expression was reduced following VCD treatment. The authors concluded that the reduced expression of AMH protein following VCD treatment may be contributing to the acceleration of primordial follicle recruitment thought to be caused by VCD (Mark-Kappeler et al., 2010).

To elucidate further the role of PIK3 and its downstream signal pathway members, Keating et al. (2010) examined time points prior to VCD-induced ovarian toxicity *in vitro* using post natal day (PND) 4 Fischer 344 rat ovaries. Their results demonstrated that the initial effects of VCD are a result of altered sub-cellular oocyte-derived distributions of KIT protein and two downstream signals of PIK3, phosphorylated AKT (pAKT) and forkhead transcription factor family (FOXO3) proteins (Figure 2.7). AKT has been shown to play an important role in the PIK3 signalling pathway and FOXO3 is involved in regulation of primordial follicle recruitment (Castrillon et al., 2003; Reddy et al., 2005). VCD-induced ovarian toxicity was not associated with changes in mRNA encoding the *Kit, Akt1,* and *Foxo3* genes. Therefore, it seems that VCD is targeting the post-translational protein signalling pathway and that any decrease in gene transcription is a subsequent response. Overall, these findings seem to indicate that VCD-induced ovarian toxicity is targeting the oocyte rather than granulosa cells in primordial and primary follicles (Keating et al., 2010).

VCD: metabolism and detoxification

4-Vinylcyclohexene (VCH), the parent compound of VCD, is metabolized to VCD in a Phase I reaction (also referred to as biotransformation) primarily by the hepatic-derived enzyme superfamily cytochrome P450 (CYP450) (Doerr-Stevens et al., 1999; Doerr et al., 1996; Springer et al., 1996c) (Figure 2.5). Phase I metabolism is usually a prerequisite for Phase II conjugative metabolism (also known as detoxification) wherein lipophilic compounds are converted to more water-soluble metabolites for excretion (Rushmore, 2002; Xu et al., 2005). Through Phase II reaction, VCD is further metabolized in the liver and, to a lesser extent, in the ovary to a non-active tetrol metabolite ([1,2-dihydroxy] ethyl-1,2-dihydroxycyclohexane) (Figure 2.5) though the action of microsomal epoxide hydrolase (mEH) and glutathione-S-transferase (GST) (Cannady et al., 2002; Devine et al., 2001; Flaws et al., 1994; Keating et al., 2008a; Keating et al., 2008b; Salyers, 1995). mEH catalyses the hydration of alkene epoxides and arene oxides while GST catalyses glutathione (GSH) conjugation with compounds to increase their elimination from the body.

It has been hypothesized that VCD ovarian toxicity is largely due to repeat exposure, thereby overwhelming and ultimately reducing the capacity of the detoxifying enzymes. Keating et al. (2008a) demonstrated *in vitro* that continual exposure to VCD in $B_6C_3F_1$ mouse ovaries initially increases expression of GST enzymes (day 4). However, by day 6 to 8 there was no change in GST expression compared to controls, suggesting the overwhelming nature of repeated VCD treatment on the ovarian detoxifying pathways (Keating et al., 2008a). In a follow-up study, the authors demonstrated GST's ability to down regulate pro-apoptotic activity, thereby increasing its protective role in the ovary (Keating et al., 2008a). It was revealed that GSTp is able to form a protein complex with the pro-apoptotic c-jun N-terminal kinase (JNK) and its downstream molecule c-jun, thereby down-regulating their activity (Hu et al., 2002; Keating et al., 2010). Collectively these studies suggest that the VCD can be readily detoxified. However, with repeated or continual exposure, the detoxifying enzymes can become overwhelmed, allowing VCD-induced ovarian toxicity to surpass enzymatic activity.

Species differences in VCD uptake and metabolism have been demonstrated in laboratory-bred mice and rats (Kao et al., 1999; Keller et al., 1997; Salyers, 1995; Smith et al., 1990a; Smith et al., 1990b). It has been demonstrated that VCD distributes faster from the blood into the tissues of mice compared with rats while excretion of VCD metabolites was primarily through the urine in both species (Salyers, 1995; Smith et al., 1990a). In addition, VCD metabolites are eliminated faster in rats and the urinary metabolite profile of each species suggests different pathways for VCD metabolism (Salyers, 1995; Smith et al., 1990a). Comparison of enzymatic metabolism of VCH and VCD revealed that mice have higher Phase I metabolic activity but slower Phase II activity rates compared with rats, resulting in the capability of mice to metabolise VCH faster than rats. As VCH is detoxified to VCD via CYP450, VCD accumulates faster in mice compared with rats due to their slower Phase II activity and are mice are therefore more susceptible to VCD-induce ovarian toxicity (Doerr-Stevens et al., 1999; Doerr et al., 1996; Smith et al., 1990a; Smith et al., 1990b).

VCD: effective oral fertility control method?

In order for VCD to be an effective fertility control agent for pest species it must be deliverable by an oral route. In an assessment of VCD as a potential fertility control management strategy, several studies have examined the effects of oral VCD administration on the fertility of female rats (Burd, 2009; Herawati et al., 2010). Two paired long-term breeding studies examined oral VCD administration effects on the fertility of female SD rats and their *in utero* exposed female offspring (Burd, 2009). Primordial follicle counts from pregnant females orally dosed with 500 mg VCD/kg for 10 days (gestational days 8-17) were reduced by 82% compared with controls. When exposed *in utero* to VCD, female offspring primordial follicle counts were reduced by 28% and 31-35% of controls (500 mg VCD/kg, gestational days 6-20 and 8-17, respectively) (Burd, 2009). It is well understood that the biological properties of the placenta allow small (<1000 g/mol), lipid-soluble molecules, such as VCD (140.14 g/mol), to cross rapidly in a flow-dependent manner (Morgan, 1997). Thus, the properties of VCD would suggest that placental transfer of the chemical would occur readily. The follicle reducing effects of VCD have also been examined on outbred wild population of female ricefield rats (*R. argentiventer*). Results demonstrated that 15 days of oral gavage with VCD (0, 500 or 750 mg/kg/day) caused a dose dependent decline in primordial follicle numbers (Herawati et al., 2010).

Effectiveness of orally delivered VCD on the fertility of male rats and mice has also been examined (Hooser et al., 1995; Schmuki, 2009). Male mice intraperitoneally injected with VCD (40 – 320 mg/kg/day; 5 – 30 days) had reduced testicular weights and testicular damage. Cessation of treatment resulted in recovery of the testicular tissue (Hooser et al., 1995) suggesting that if fertility effects had occurred, such effects were likely reversible. Fifteen days of oral VCD gavage (500 mg/kg/day) caused SD rat testicular and epididymal weights to increase (day 47 post-treatment). No effects on reproductive function of treated males were reported (Schmuki, 2009). These findings in male rodents are not surprising considering the continual nature of the spermatogenesis cycle (Clermont, 1972;

Clermont and Harvey, 1967) and demonstrate VCDs specificity in its permanent sterilizing effects on female reproduction.

As previously mentioned, researchers at SenesTech Inc® (Flagstaff, AZ, USA) have established efficacy of the chemosterilant bait, ContraPest®, at reducing the immature and pre-antral follicle pools in adult female SD rats. The current formulation of ContraPest® contains both VCD and TR. Therefore, because TR is known to reduce male and female fertility (e.g. oligozoospermia and asthenozoospermia) (Huynh et al., 2000; Lue et al., 1998; Qian et al., 1986; Qian et al., 1988; Zhen et al., 1995), this may provide an additional avenue for the control of pest mammals.

Recently, VCD's use as a chemosterilant for Australian and NZ marsupials has been proposed. Although the timing of follicle development and maturation in marsupials and rats is different (as previously discussed in section 2.5), the underlying events are similar. Therefore, it can be postulated that VCD-induced primordial follicle depletion observed in rodents may have an equivalent effect in a marsupial species. To date, there is only one published report on the effects of VCD in marsupials (Koehn, 2008). Adult female and male tammar wallabies (*Macropus eugenii)* were treated with subcutaneous (s.c.) injections of 0, 46 or 183 mg VCD/kg/day for 15 days (n = 5-6/treatment) or with intramuscular (i.m.) injections of 0 or 100 mg/kg/day VCD for 9 and 15 days (n = 2-3/treatment). There were no changes in the primordial follicle pool of treated animals which may, in part, be due to the low robustness of the study (i.e. low sample size) or the route of administration. In addition, injection site reactions occurred in all VCD treated groups (males and females), requiring cessation of treatment on day 5 in one group (183 mg VCD/kg/day) (Koehn, 2008). Considering the severity of injection site reactions in captive wallabies, investigation of the possibility for an oral route of VCD administration is warranted for wild-dwelling marsupials such as the NZ brushtail possum.

2.7 Summary

There is an on-going need for sustainable, effective, humane and affordable methods of pest control in NZ. The chemosterilant VCD could potentially satisfy such needs as it is an orally active compound that causes permanent infertility in female mammals. The purpose of the studies reported here was to examine the effects of orally delivered VCD on wild-caught adult female possums' health, internal organs and immature ovarian follicle populations. Potential species differences in the fate and metabolism of VCD were also examined *in vivo* and *in vitro* in wild female rats and possums. In addition, the palatability and follicle depleting effectiveness of ContraPest® (SenesTech Inc®) was tested in wild-caught female Norway rats.

Chapter 3

General Methodology

3.1 Reagents

4-Vinylcyclohexene diepoxide (batch # BCBB9360 and BCBC1500) was purchased from Sigma-Aldrich (Auckland, NZ; cat. 94956). Sunfield sunflower oil, used as a carrier liquid for VCD and for vehicle control, was purchased from Tasti Products Ltd (Auckland, NZ). Cyclohexanone, purchased from May and Baker LTD, Dagenham, England (cat. BC00595) and 4-vinyl-1-cylohexene 1,2-epoxide (VCH 1,2-isomer; lot #MKBL783V; cat. 106-86-5) were used as internal standards for gas chromatography. Ethyl acetate, used to extract VCD and its metabolites, was obtained from Fisher Scientific (cat. 141-78-6). Attane™ isoflurane, purchased from Bomac Laboratories Ltd (Auckland, NZ; cat. 26675-46-7), was used as a general inhalation anaesthetic during gavage procedures. Sodium pentobarbital, used as the euthanizing agent, was purchased from ProVet (Auckland, NZ).

3.2 Animals

Mature female brushtail possums (live weight 2.5 - 4.0 kg) and Norway rats (live weight 100 - 300 g) were captured in North Canterbury, NZ using cage traps set along farmland-bush margins. Animals were housed and acclimatized indoors for 4 - 6 weeks in individual cages with constant temperature (20 ± 5°C) and a light:dark cycle that followed the external environmental photoperiod. On a daily basis, animals were provided with cereal-based pellets formulated for possums or rats (CRT Reliance Feeds, Rolleston, NZ), fresh fruit and vegetables and water ad libitum. All experimental procedures were carried out with the approval of the Animals Ethics Committee of Landcare Research, Lincoln and in accordance with Part 6 of the New Zealand Animal Welfare Act 1999.

3.3 Glutathione analysis

Glutathione (GSH) activity levels in red blood cells (RBCs), plasma and hepatic (liver) and ovarian tissues were analysed using methods described in the GSH assay kit (Sigma, cat# CS0260) with the following changes: a stock solution of 10 mM GSH was stored in aliquots for up to 3 months at -20°C and then diluted down to create the GSH standards as needed; 96-well plates were kept on ice until commencement of the 5 min incubation; the plate was shaken for 5 seconds prior to absorbency reading to ensure even distribution of sample and reagents; the plate reader was kept at 37°C during readings. To reduce enzymatic degradation, all samples were slowly defrosted on ice prior to analysis and all samples were run in triplicate. Total GSH content (nmol/µL or mg tissue) in each sample was calculated using the following formula: $[\Delta A_{412}/\text{min(sample)} * \text{dil}]/[\Delta A_{412}/\text{min(1 nmole)} * \text{vol}]$ where $\Delta A_{412}/\text{min(sample)}$ is the slope generated by the sample with background absorbency removed;

ΔA_{412}/min(1 nmole) is the slope generated from the standard curve for 1 nmole of GSH; dil is the dilution factor of the original sample; and vol is the volume (mL) of sample in the reaction. Final GSH concentrations were then converted to µmol and multiplied by the 5-sulfosalicylic acid (SSA) dilution factor (RBCs and plasma, 1x; liver and ovary, 28x) to achieve µmol GSH per µL or mg of tissue. For details on the GSH standard curve please refer to section 3.6.1.

3.4 Gas chromatography assay sample preparation

VCD and its metabolites were extracted and quantified on gas chromatography (GC) by placing samples into glass scintillation vials and then VCD extracted by the addition of two times the sample volume of ethyl acetate (EA) containing an internal standard. In three successive rounds, samples were vortex mixed for 30 seconds and then shaken for 10 minutes at 200 rpm. Phases were separated by centrifugation at 3,000 x g for 10 minutes and the organic layer analysed for VCD quantification as outlined in section 3.5.

3.5 Gas chromatography analysis

VCD quantification in biological samples was achieved utilizing a 6890N Network GC (Agilent Technologies) equipped with a DB5-ms column (60 m x 0.25mm; J&W Scientific, Folsom, CA) and a flame ionization detector (FID). The nitrogen carrier gas flow rate was 0.5ml/min. The FID gas flow rates for hydrogen and air were 50 and 400 ml/min respectively, with a combined flow of 60ml/min. Split injection was used with a split ratio of 20:1, a spit flow of 10.5 ml/min and a total flow of 13.6 ml/min at 15 psi. The injection volume was 1.0 µL. The initial oven temperature was held at 100°C and then ramped up to 130°C at a rate of 2°C/min and held at the final temperature of 200°C at a rate of 13°C/min for 15 minutes. The injection and detector temperatures were held at 250°C and 280°C, respectively. VCD chromatography revealed two peaks. Thus, VCD was quantified by first adding together the area under the curve (AUC) for each VCD peak. Total VCD AUC and the internal standard AUC were then compared against the standard curve of each corresponding analyte for total VCD recovery and correction factors for loss due to methods. The retention times for the analytes were as follows: VCD peak 1, 10.8 min; VCD peak 2, 11.0 min (Figure 3.1); VCH, 2.7 min (Figure 3.2); VCH 1,2-isomer, 4.7 min (Figure 3.3); cyclohexanone, 3.4 min (Figure 3.4); ethyl acetate, 2.0 min (Figures 3.1 – 3.4). All samples were run in triplicate. The practical quantitation limit (PQL) for VCD was 0.371 mM. For details on the VCD and internal standard curve and assay validation test refer to section 3.6.2.

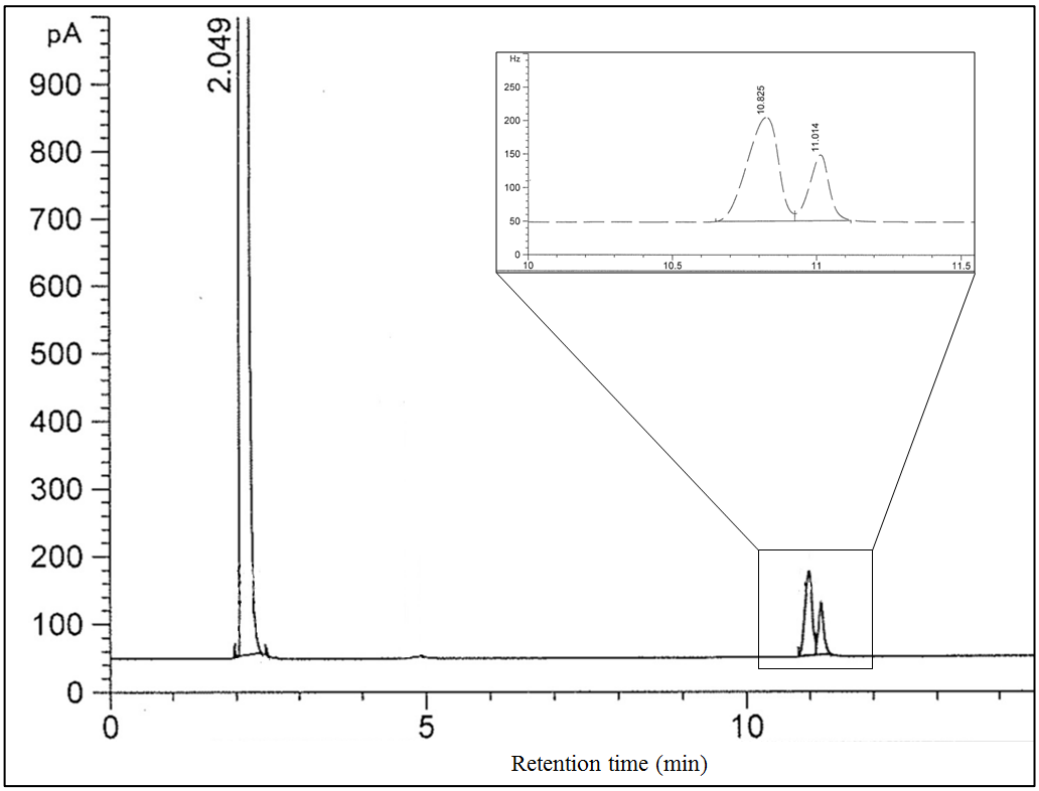

Figure 3.1 Representative image of a gas chromatograph of 4-vinylcyclohexene diepoxide (VCD; 0.913 mM). VCD peak 1, 10.825 minutes; VCD peak 2, 11.014 minutes; ethyl acetate, 2.049 minutes.

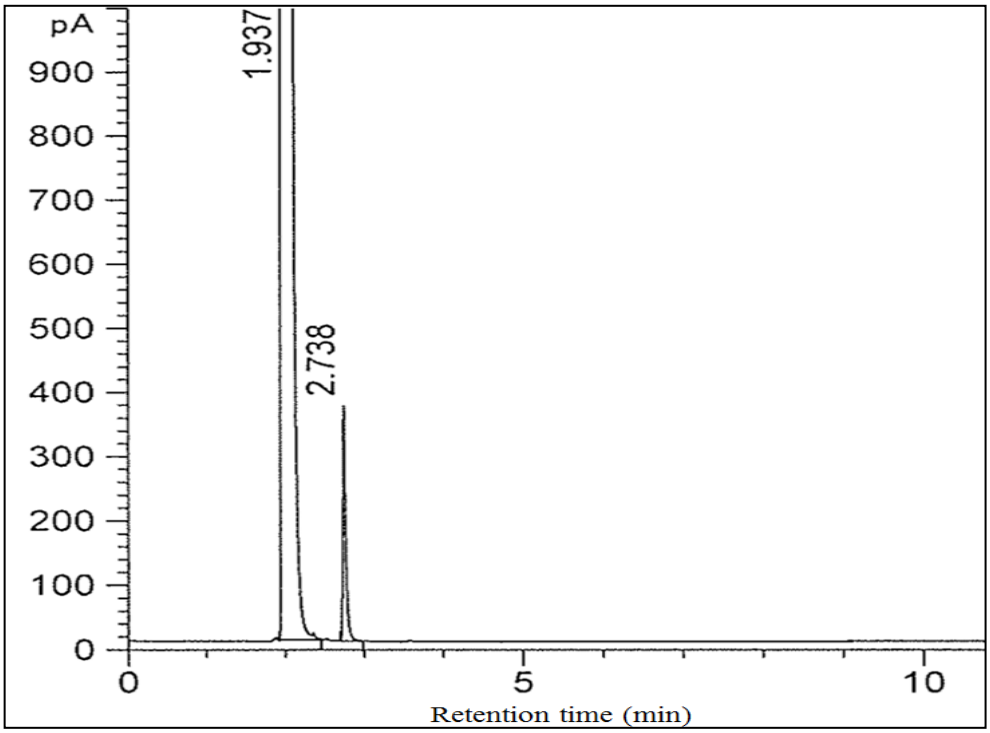

Figure 3.2 Representative image of a gas chromatograph of 4-vinylcylohexene (VCH; 0.87 mM). VCH, 2.738 minutes; ethyl acetate, 1.937 minutes.

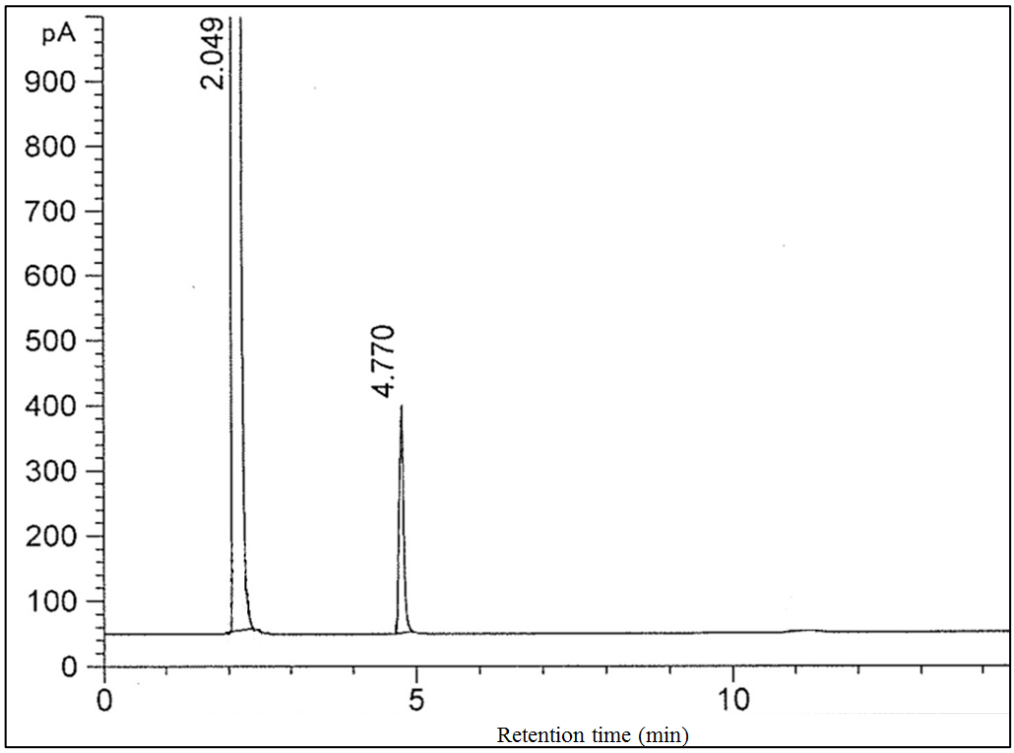

Figure 3.3 Representative image of a gas chromatograph of 4-vinyl-1-cylohexene 1,2-epoxide (VCH-isomer; 1.05 mM). VCH-isomer, 4.77 minutes; ethyl acetate, 2.049 minutes.

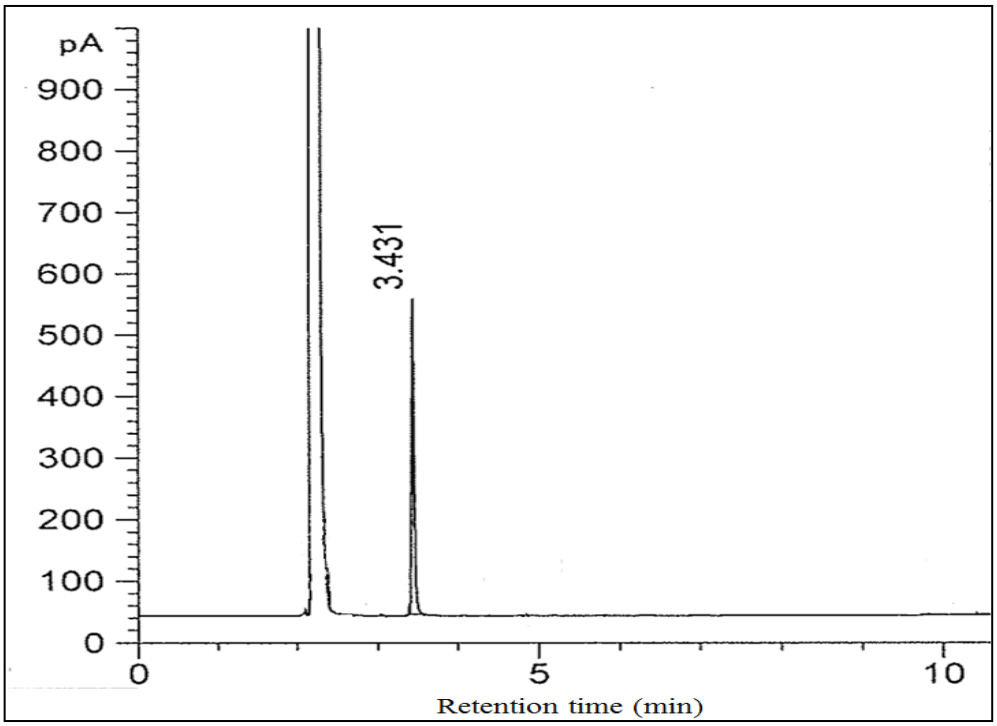

Figure 3.4 Representative image of a gas chromatograph of cyclohexanone (1.18 mM). Cyclohexanone, 3.4 minutes; ethyl acetate, 2.05 minutes.

3.6 **Preparation of standard curves**

3.6.1 GSH

Standard GSH samples were created by diluting a stock solution of GSH (10 mM in ultrapure water; w/v) with a 5% solution of 5-sulfosalicylic acid, creating a standard GSH range from 0.78 to 50 µM. All standards were run in duplicate and were made fresh for each run. The standard curve was generated by subtracting the background absorbency from each standard absorbency reading and then the average absorbency of each standard pair was calculated and graphed against the standard concentration range (Figure 3.5).

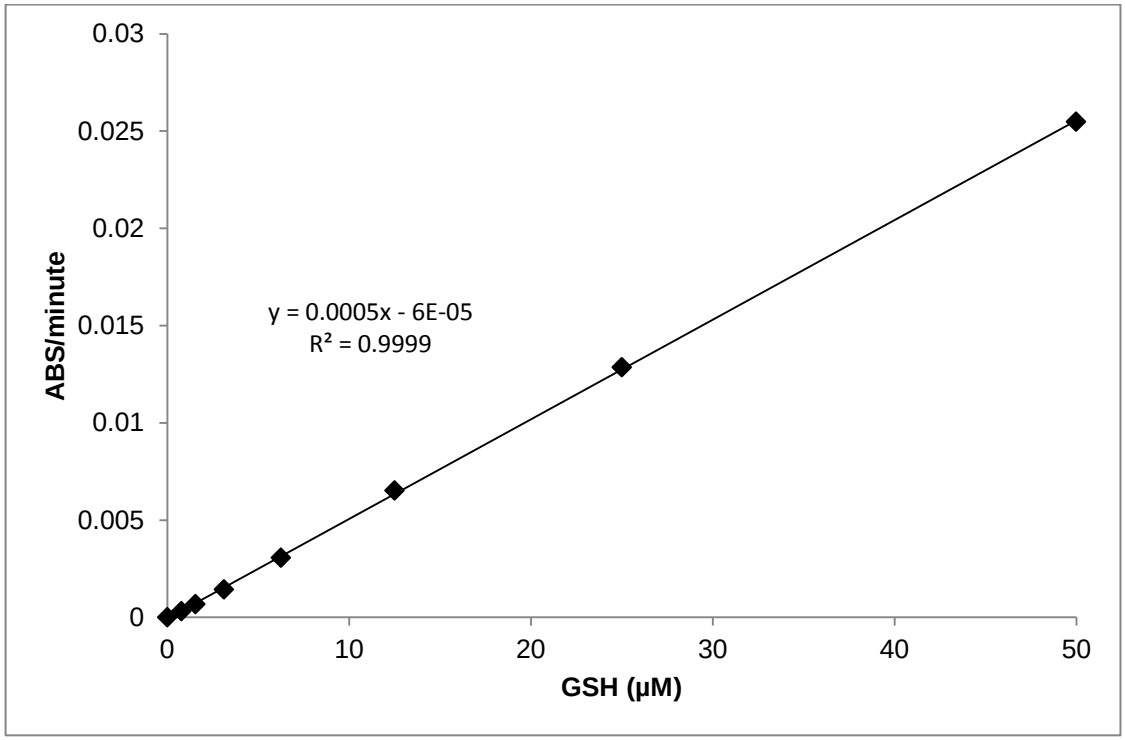

Figure 3.5 Representative image of a standard curve for glutathione (GSH).

3.6.2 VCD, VCH and VCH 1,2-isomer

For each chemical, standards were made up fresh for each run in EA and then diluted down to the desired concentration. All standards were run in triplicate. For the standard curve for each chemical, refer to Figures 3.6 to 3.9.

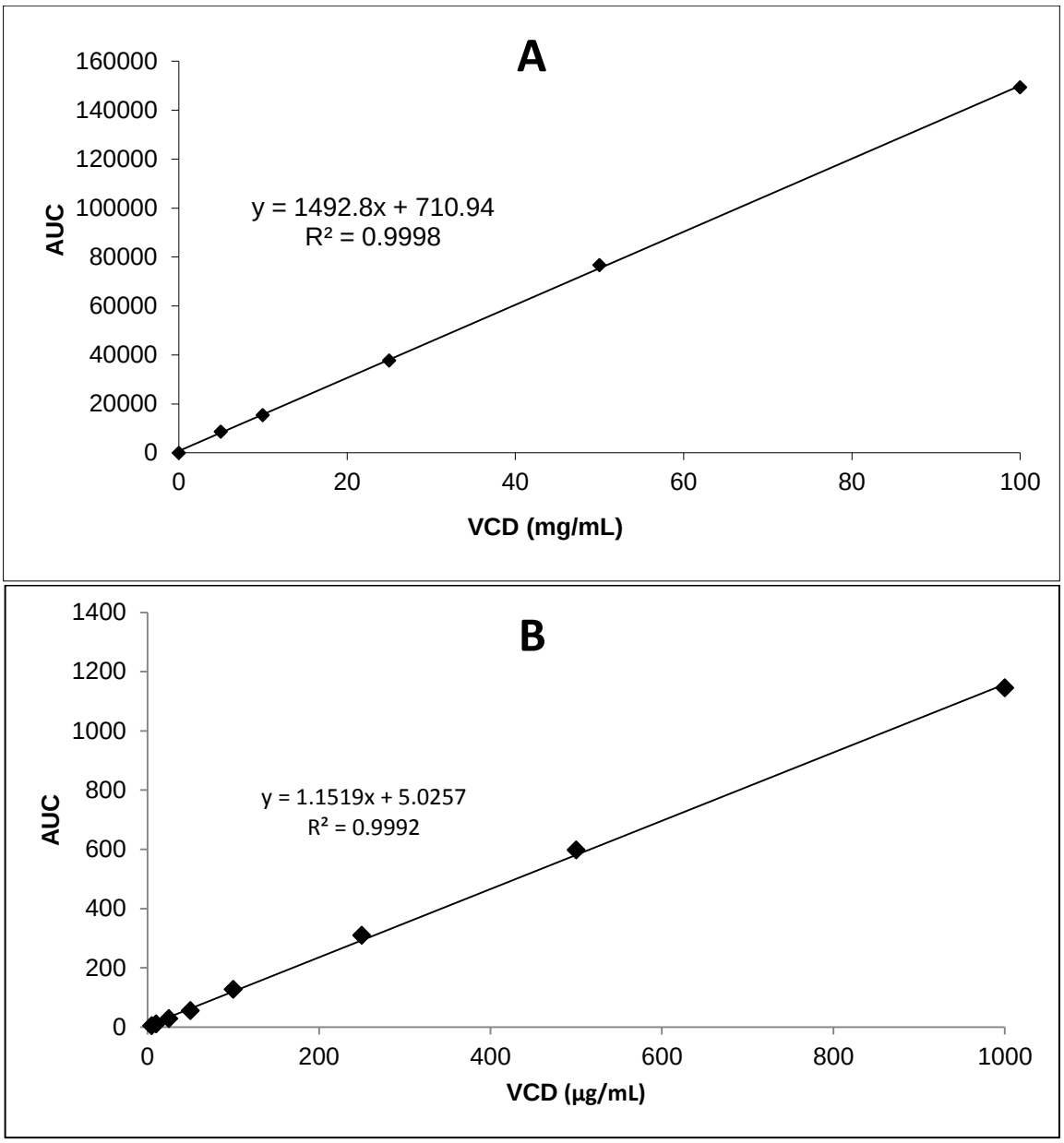

Figure 3.6 Representative image of standard curves for upper 4-vinylcyclohexene diepoxide (VCD) concentration range (A; 5.0 – 100 mg/mL) and lower VCD concentration range (B; 5.0 – 1000 µg/mL).

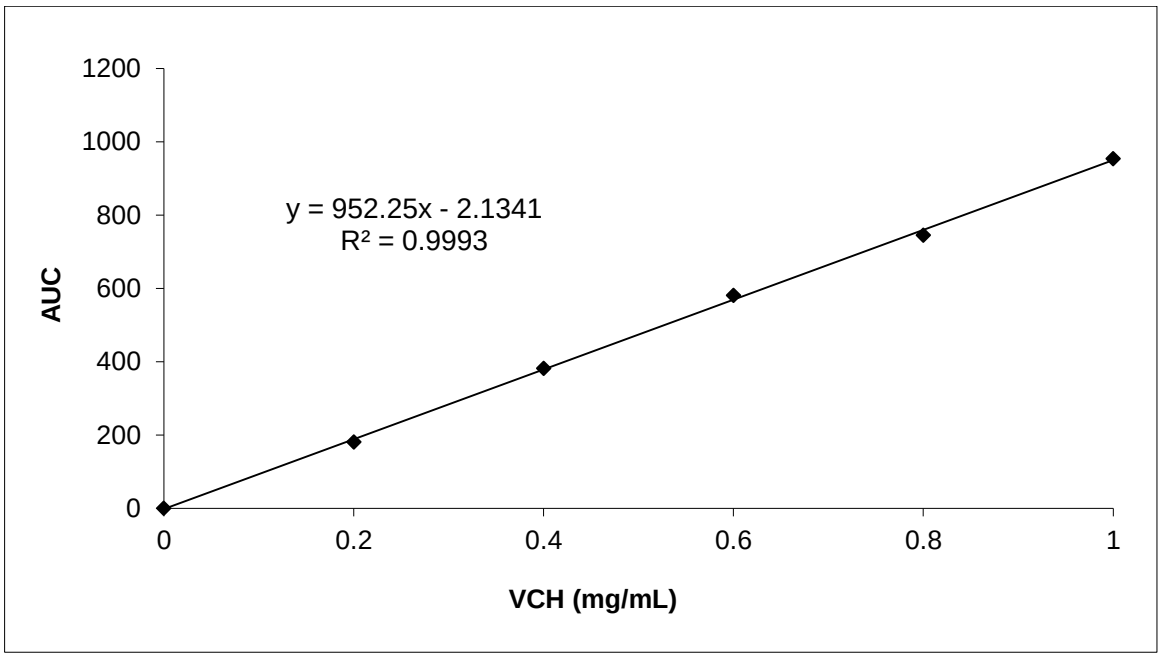

Figure 3.7 Representative image of a standard curve for 4-vinylcyclohexene (VCH).

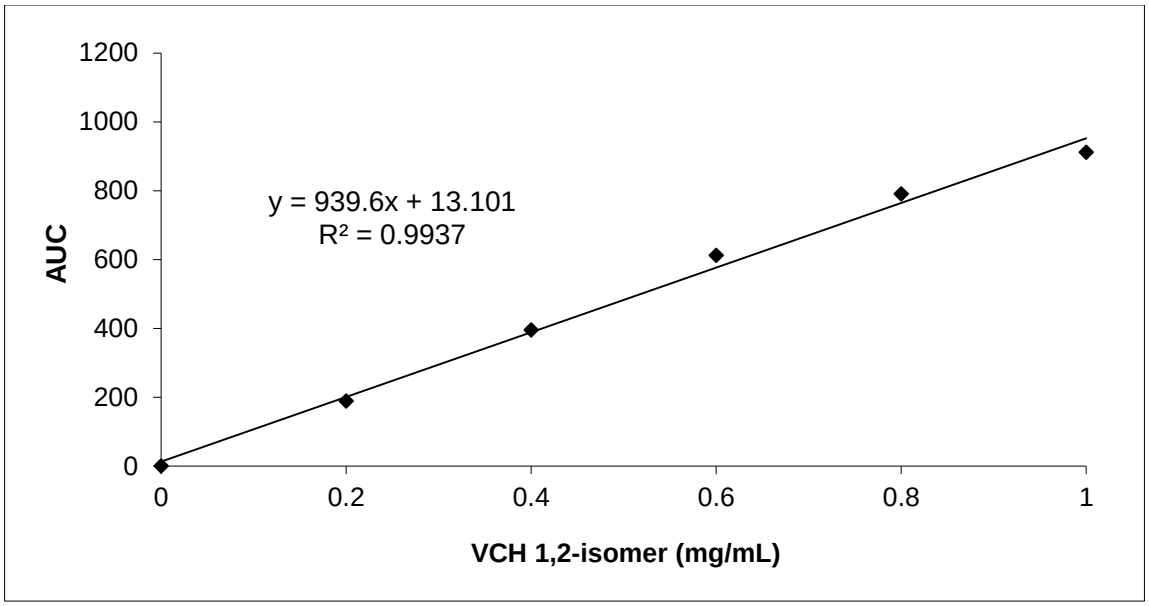

Figure 3.8 Representative image of a standard curve for 4-vinyl-1-cylohexene 1,2-epoxide (VCH 1,2-isomer).

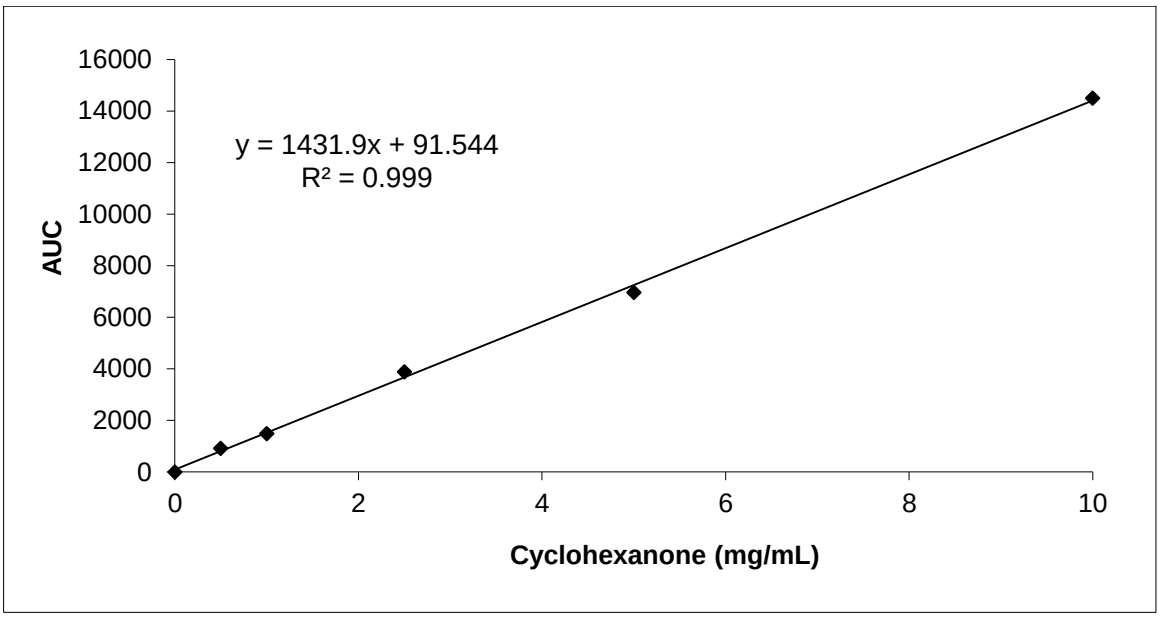

Figure 3.9 Representative image of a standard curve for cyclohexanone.

3.7 **Histology and Follicle Analysis**

For each species, ovaries were trimmed of fat, weighed and placed into 10% neutral-buffered formalin solution for 48 hours and then transferred to 70% ethanol until processing. Ovaries were paraffin-embedded, serially sectioned (5 µm), mounted, and stained with Harris hematoxylin and Eosin Y (1%) prior to cover-slip application. Ovarian follicles were counted in every 20th section to avoid double counting of larger follicles. To ensure consistency, follicles were assessed by the same examiner who was blinded to experimental conditions. Follicles were identified based on the following criteria: primordial (oocyte surrounded by a single layer of ≥ 50% elongated flattened granulosa cells; rat, ≤ 20µm diameter; possum, ≤ 60 µm diameter; Figure 3.10); primary (oocyte surrounded by a single layer of ≥ 50% cuboidal granulosa cells; rat, 20-70 µm diameter; possum, 60-200 µm diameter; Figure 3.11); secondary (oocyte surrounded by two or more layers of cuboidal granulosa cells as well as a somatic layer that may or may not contain a fluid filled antrum with a diameter less than the oocyte; rat, 70-390 µm diameter; possum, 200-450 µm diameter; Figure 3.12); and antral (oocyte surrounded by multiple layers of granulosa cells containing a fluid filled antrum with a diameter larger than the oocyte; rat, 80-320 µm diameter; possum, ≥ 450 µm diameter; Figure 3.13) (Hirshfield and Midgley Jr, 1978; Pedersen and Peters, 1968). The corpus luteum (CL) was comprised of healthy, uniformly-sized luteal cells (rat, >320 µm diameter; possum, >4 mm diameter; Figure 3.14). Total primordial and primary follicle counts were estimated using a correction factor formula (Gougeon and Chainy, 1987) as follows: $N_t = (N_0 \times S_t \times t_s)/(S_0 \times d_0)$ where N_t : total number of follicles, N_0 : number of follicles observed in the ovary, S_t : total number of sections in the ovary, t_s : thickness of the section (µm), S_0 : total number of sections observed, and d_0 : mean diameter of the nucleus of that follicle type. Counts for secondary and antral follicles and copora lutea were directly collated.

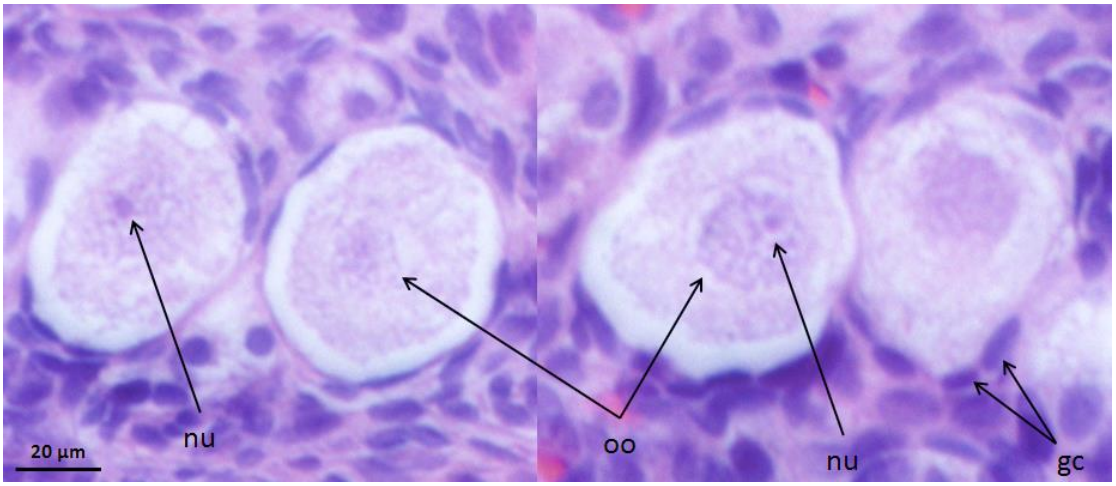

Figure 3.10 Representative image of primordial ovarian follicular structures (*T. vulpecula,* x40). Primordial follicles were classified as an oocyte surrounded by a single layer of elongated flattened granulosa cells; rat, ≤ 20µm diameter; possum, ≤ 60 µm diameter. Oocyte (oo); nucleus (nu); granulosa cells (gc).

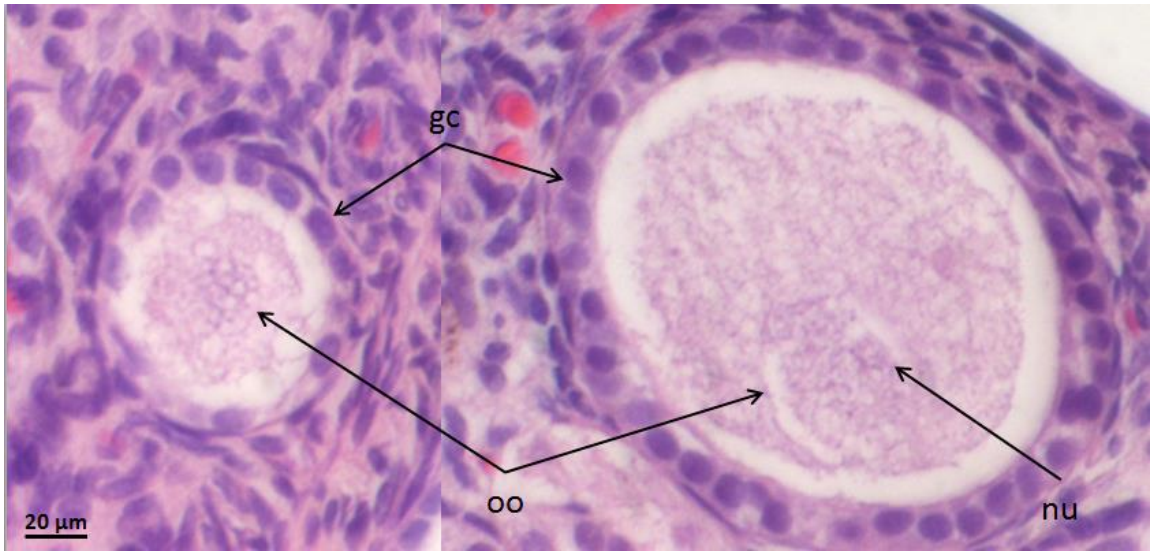

Figure 3.11 Representative image of primary ovarian follicular structures (*T. vulpecula,* x40). Primary follicles were classified as an oocyte surrounded by a single layer of ≥ 50% cuboidal granulosa cells; rat, 20 - 70 µm diameter; possum, 60 - 200 µm diameter. Oocyte (oo); nucleus (nu); granulosa cells (gc).

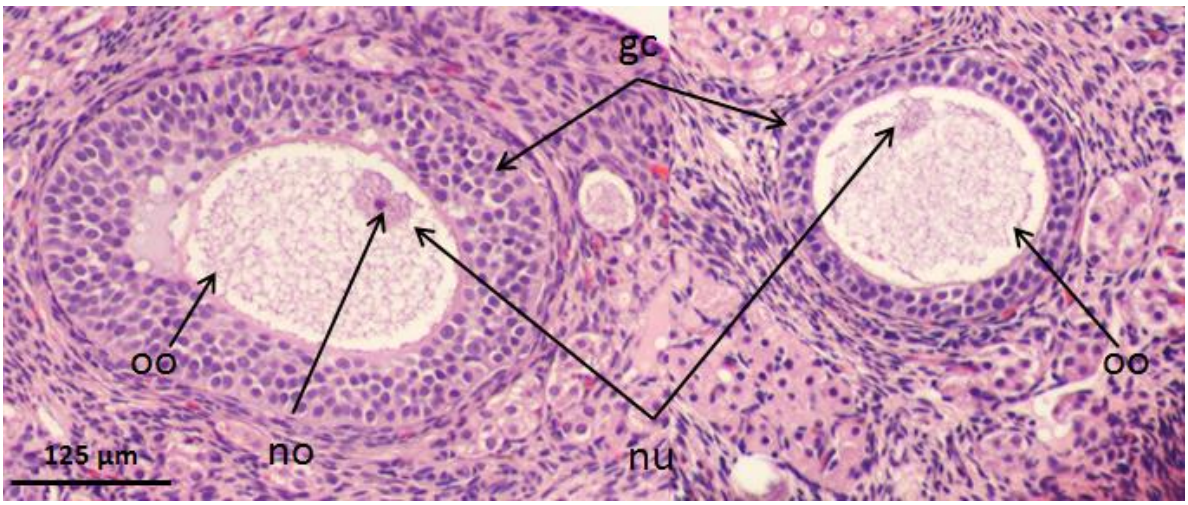

Figure 3.12 Representative image of secondary ovarian follicular structures (*T. vulpecula,* x40). Secondary follicles were classified as an oocyte surrounded by two or more layers of cuboidal granulosa cells as well as an additional somatic layer that may or may not contain a fluid filled antrum with a diameter less than the oocyte; rat, 70 - 390 µm diameter; possum, 200 - 450 µm diameter. Oocyte (oo); nucleus (nu); nucleolus (no); granulosa cells (gc).

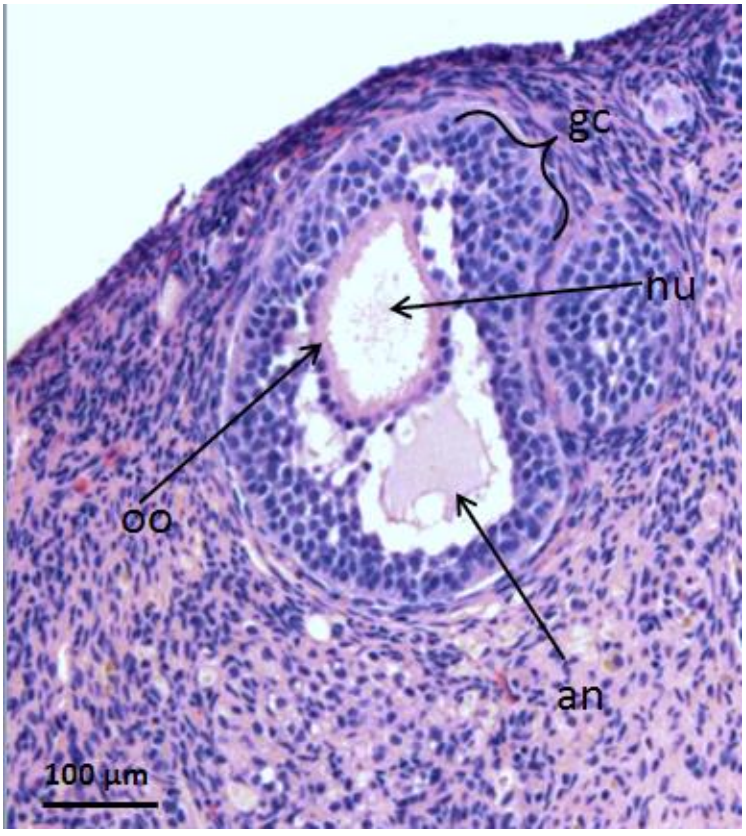

Figure 3.13 Representative image of antral follicular structures (*T. vulpecula,* x25). Antral follicle were classified as an oocyte surrounded by multiple layers of granulosa cells containing a fluid filled antrum with a diameter larger than the oocyte; rat, 80 - 320 µm diameter; possum, ≥ 450 µm diameter. Oocyte (oo); nucleus (nu); antrum (an); granulosa cells (gc).

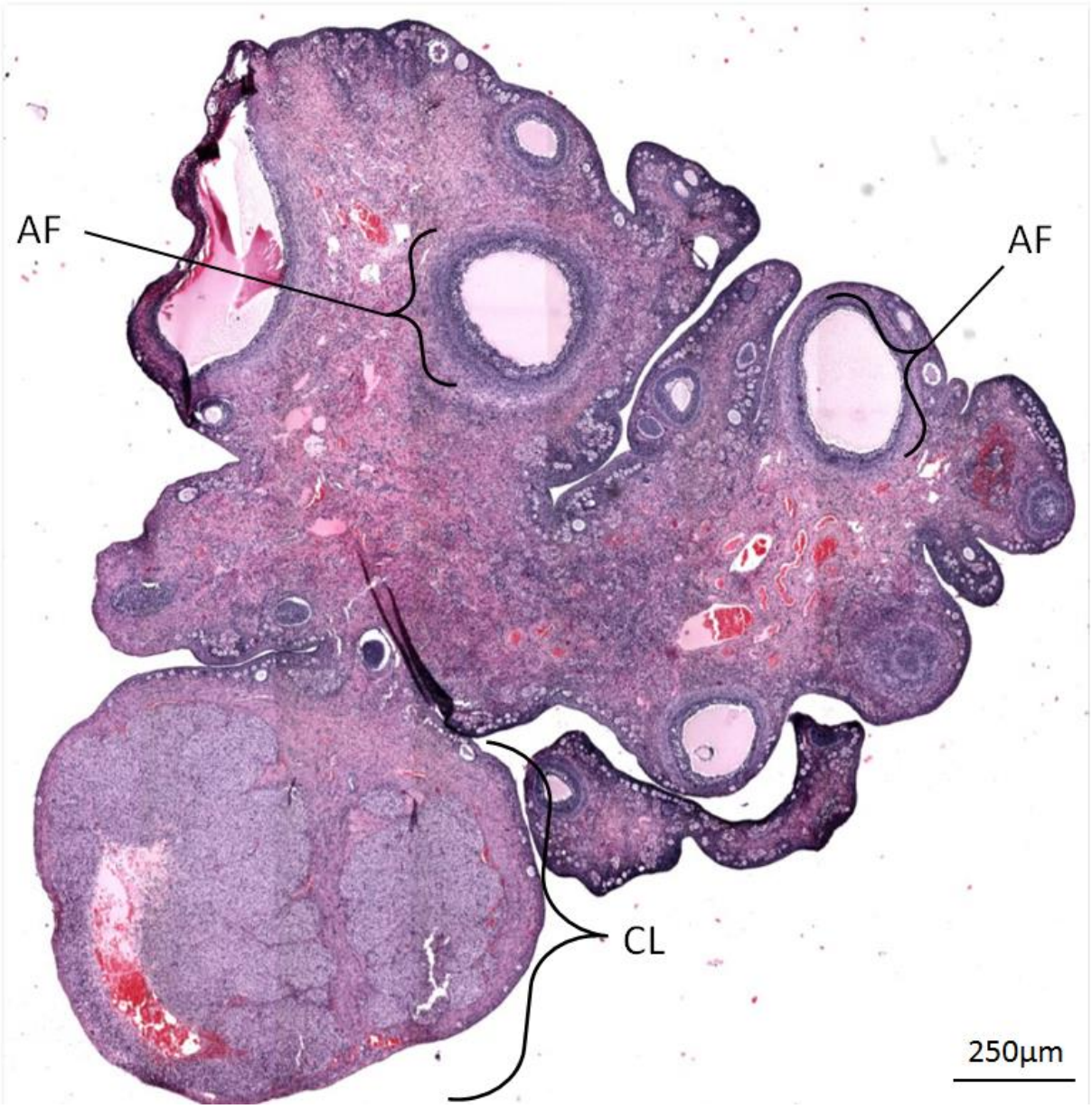

Figure 3.14 Representative image of corpus luteum (CL) within possum (*T. vulpecula)* ovarian tissue (x10). A CL was classified as a postovulatory follicle containing luteal cells; AF: antral follicle; rat, >320 µm diameter; possum, >4 mm diameter.

3.8 Statistical analysis

Statistical significance was established at α=0.05. Normally distributed data were analysed by Student's *t*-test or one-way ANOVA (analysis of variance). Tests for normality (Shapiro-Wilk) and variance (Levene) were performed to ensure that ANOVA assumptions were not violated. Non-normally distributed data were analysed using a Mann-Whitney or Kruskall-Wallis test. Where appropriate, Box-Cox transformations were performed for non-normally distributed data. All data were analysed in using JMP® 10.2 (SAS, Cary, NC) software. Data are presented as means ± SEM (standard error of the mean).

Chapter 4

Effects of orally delivered 4-vinylcyclohexene diepoxide on the health and ovarian follicle populations of female brushtail possums

4.1 Introduction

Throughout New Zealand the brushtail possum (*Trichosurus vulpecula*) poses a serious threat to native fauna and flora and, through its role as a primary vector of the disease, bovine tuberculosis, to the health of domestic livestock and feral game species (Cowan, 2005). Current methods for control include poisons and trapping. Although effective in the short-term, there are public concerns about the animal welfare and environmental impacts of conventional lethal control methods (PCE, 2011). New cost-effective and more humane approaches to possum control are needed.

Fertility control has received much interest as an alternative and more publicly acceptable method of pest control. One approach is to utilize chemicals that specifically target the reproductive function of pest species and can induce permanent sterility (Marsh and Howard, 1970; Mauldin and Miller, 2007). 4-Vinylcyclohexene diepoxide (VCD), an organic compound formed during industrial processes and used as a reactive diluent for diepoxides and epoxy resins, shows potential as a chemosterilant in mammals. Toxicology studies in rodents have shown that VCD reduces the pool of primordial ovarian follicles, resulting in a rapid onset of ovarian senescence and permanent sterility (Kao et al., 1999; Springer et al., 1996b; Springer et al., 1996c).

VCD-induced reductions in immature follicle populations have primarily been achieved via injection (intramuscular or intraperitoneal) of the chemical (Hoyer and Sipes, 2007). However, to utilize VCD as a fertility control agent for pest species, an oral route for exposure will be essential. A paired reproductive study demonstrated that 10 days of VCD treatment (500 mg/kg/d) during gestation caused an 82% depletion of parental dam primordial follicles compared with controls. Additionally, offspring exposed *in utero* to 10 or 15 days of VCD (500 mg/kg/d) had a 28% and 31-35% reduction in primordial follicles compared with controls, respectively (Burd, 2009). Oral dosing of female Sprague Dawley laboratory rats with 500 or 750 mg/kg VCD for 15 consecutive days caused 58% and 88% depletion of primordial follicles, respectively (Dr. L.A. Hinds, CSIRO, Australia, unpublished data). Orally administered VCD has also been demonstrated to reduce the immature follicle pool in wild ricefield rats (*R. argentiventer*) in a dose dependant manner (Herawati et al., 2010). Collectively, these studies demonstrate that VCD can reduce the immature follicle populations in rodents through the oral route of delivery.

To date, no studies on the efficacy of orally-delivered VCD have been undertaken in marsupial species such as the brushtail possum. The purpose of the studies reported here was to: 1) investigate the effects

of orally administered VCD on the health and ovarian follicle populations of adult female possums; 2) identify the optimal dosage and duration of treatment to induce primordial follicle depletion; and 3) identify formulations to improve the efficacy of orally delivered VCD in adult female possums.

4.2 Materials and Methods

4.2.1 Reagents

VCD, sunflower oil, Attane™ isofluorane, and sodium pentobarbital were obtained from vendors and used for experimental procedures as previously outlined in Chapter 3. Quick-EZE® tablets (780 mg $CaCO_3$, 130 mg Mg CO_3 and 130 mg $Mg_2O_8Si_3$ per tablet), used as an antacid pre-treatment, were obtained from Nestle (Auckland, NZ). Intralipid® (20% fat emulsion containing glycerol, 22.3 g/L; triglycerides, 202 g/L; and phospholipids, <0.5 g/L), a sterile lipid emulsion, was used as a gavage carrier liquid and was purchased from Pharmatel (Auckland, NZ; cat. 835041-54). Liquid carbon dioxide, purchased from BOC (Christchurch, NZ), was used to freeze teeth to the microtome base for aging analysis.

4.2.2 Animal procedures

For each study, possums were randomly allocated into treatment groups using a randomized block design stratified by live weight prior to trial start. Under anaesthesia (5% isoflurane in O_2 at 2 L/min) blood samples (2-3 mL) were collected from the ventral tail vein prior to trial start and on the final treatment day and plasma was stored at -20°C for analysis (see section 4.2.4). Possums were anesthetized daily and, once lightly sedated, gavaged with control or treatment solution (3.5 – 8.5 mL). Gavage tubes consisted of commercially purchased vinyl tubing (ID 3 mm, OD 5 mm, 30 cm long) attached to a 3-way stop cock and a 10 mL syringe. For each treatment group one tube was used and tubes were discarded daily. Live weight was measured daily prior to treatment to adjust VCD dose and monitor general animal health during the study. In an attempt to minimize handling and anaesthetic stress, possums were prepared for anaesthesia by transferring each possum in their nest box to the treatment area. Isofluorane was administered through a tube directly into the nest box. Following treatment, possums were placed back into their nest box and transferred into their cage before regaining consciousness, thus allowing the animal to wake naturally in a safe and familiar environment. Animals were visually monitored for up to 4 hours post-treatment.

Treatment protocol for Study 1

Animals were gavaged daily for 13 days with sunflower oil (vehicle control, n = 8), or a low dose of VCD (500 mg/kg mixed 1:3 w/v) in oil (n = 8) or a high dose of VCD (750 mg/kg mixed 1:3 w/v) in oil (n = 8). The treatment duration and selected doses were based on previous rodent studies demonstrating significant immature follicle depletion following oral treatment (Burd, 2009; Herawati et al., 2010).

Treatment protocol for Study 2

To improve the effectiveness of oral VCD two new formulations were tested. These included a lipoprotein formulation (Intralipid® 20%) shown to improve uptake and palatability of VCD in rodents (Dr. C. A. Dyer, SenesTech Inc., USA, pers. comm.) and the use of antacids (Quick-EZE® tablets) to reduce the breakdown of VCD to its inactive tetrol metabolite by acid conditions in the stomach. Both formulations were predicted to increase the uptake and effectiveness of VCD. Animals were gavaged daily for 10 days in 2 blocks of 5 days with 2 rest days between treatment phases. Control groups included (1) 2.05 mL/kg lipoprotein solution (Intralipid 20%; n = 6), (2) 3.0 mL antacid pre-treatment solution (containing 1.275 g of powdered Quick-EZE® tablets; 956.3 mg $CaCO_3$, 156.8 mg Mg CO_3, 156.8 mg $Mg_2O_8Si_3$) suspended in distilled water followed by 2.05 ml/kg sunflower oil (n = 6), or (3) 2.05 ml/kg sunflower oil only (n = 6). Hereafter control treatment groups will be reported as C-IT (group 1), C-QE (group 2), and C-O (group 3). VCD treatment groups included (1) 750 mg VCD/kg mixed 1:3 (w/v) in lipoprotein solution (n = 10) or (2) 3.0 mL antacid pre-treatment solution (containing 1.275 g powdered Quick-EZE® tablets suspended in distilled water) followed by 750 mg VCD/kg mixed 1:3 (w/v) in sunflower oil (n = 10). Hereafter VCD treatment groups will be reported as VCD-IT (group 1) and VCD-QE (group 2).

4.2.3 Tissue collection

Prior to euthanization, intracardiac blood was collected into lithium heparinised tubes and then animals were euthanized with an intracardiac injection of pentobarbitone (125 mg/kg) and the following tissues excised, trimmed of fat, weighed and examined for gross pathology: complete reproductive tract, ovaries, liver, kidneys and adrenal glands. In addition, the length and width of each uterus, vaginal culs-de-sac and ovarian follicles (>1 mm) were recorded. Unovulated follicles and ovulation sites were enumerated. Ovaries, adrenal glands, kidneys and a portion of the liver were fixed in 10% buffered-neutral formalin solution for histological analysis.

4.2.4 Inductively coupled plasma optical emission spectrophotometer analysis

To determine if calcium toxicity was occurring following the pre-treatment with antacid, total calcium (Ca) and magnesium (Mg) were measured in pre- and post-treatment plasma collected from Study 2 animals. Plasma samples were diluted 1:5 (w/v) with ultrapure water to for analysis preparation. Samples were analysed with an axially aligned inductively coupled plasma optical emission spectrophotometer (ICP-OES) (Varian 720, Mulgrave, Australia). The ICP ultrasonic seaspray nebulizer and cyclonic spray chamber operating conditions and flow injection system used are listed in Table 4.1. Calibration standards and internal standards were serially diluted from Merck ICP standard solutions.

Table 4.1 Inductively coupled plasma optical emission spectrophotometer (ICP-OES) instrumental parameters employed for the determination of total plasma calcium and magnesium concentration.

Forward power	1.2 kW
Nebulizer flow rate	0.9 L/min
Plasma gas flow rate	15.0 L/min
Auxiliary gas flow rate	1.5 L/min
Solution uptake rate	1.0 mL/min
Analytical wavelengths	Ca: 370.6, Mg: 279.6

4.2.5 Tooth cementum analysis

To determine the age of individual possums the cementum deposition layers were counted using an adaptation of published methods (Clout, 1982; Pekelharing, 1970). The right mandible of each animal was boiled in water for 1-2 hours and the second and third molars removed. Teeth were decalcified for 4 hours in the commercial decalcifying agent RDO (Clout, 1982) and then gently rinsed with water. Using a freezing microtome (Reichert Jung 1205, Vienna, Austria) 10 - 20 sections (20 µm) were cut serially through the cementum peninsula (central portion of tooth between the roots). Sections were mounted onto glass slides and air dried for 24 hours. Slides were stained with Erlich's haematoxylin and water (50:50 v/v) for one hour and then gently rinsed with water and a coverslip applied. Cementum layers (narrow dark-staining bands) were counted at x40 or x100 magnification on 3 - 5 sections to determine the age of each animal. To ensure consistency, cementum layers were assessed by a single examiner who was blinded to experimental conditions.

4.2.6 Histology and follicle analysis

Ovaries, liver and kidneys were trimmed of fat, weighed and then prepared for histological examination as previously outlined in Chapter 3. Complete blood counts were measured at Gribbles Veterinary (Christchurch, NZ) and analysis performed as outlined in section 4.2.8. Liver and kidney sections were assessed by an experienced veterinary pathologist (Gribbles Veterinary) for signs of abnormal pathology. The qualitative remarks were divided into 5 major categories of background changes for each tissue. The liver background categories included: 1) lymphocyte aggregates within lobules or cells (range: few; moderate; many); 2) macrophage clusters; 3) periacinar hepatocytes containing brown granular material (range: few; moderate; many); 4) Kupffer cells containing brown granular material (range: few, moderate; many); 5) hepatocytes containing brown granular material (range: few, moderate; many). Kidney background changes included: 1) collecting tubules containing dark blue granular material in lumen; 2) outer cortical, focal infiltrate of lymphocytes (range: single; few; many); 3) scattered granules containing black material; 4) tubules with syncytial-like cells; 5) tubules that lack the eosinophilia and have large nuclei. For each sample, a score ranking from 0-3 was applied as follows: 0 = 0 to 1 categories present; 1 = 1 to 2 categories present; 2 = 2 to 3 categories present; 3 = 4 to 5 categories present.

4.2.7 *In vitro* stomach pH analysis

The effects of Quick-EZE® (QE) and Intralipid® (IT) on stomach pH of possums were analysed *in vitro.* Stomach contents from four female possums were pooled and the initial pH measured. Stomach contents were then divided into 5 gram aliquots and treated in triplicate as follows: 1) control (no additives); 2) low QE dose (214.0 µL); 3) high QE dose (500.0 µL); 4) low IT dose (83.0 µL); 5) high IT dose (123.0 µL). The low QE dose was calculated by dividing the sample size (5.0 g) by a high average weight of possum stomach contents (70.0 g), resulting in 7.14 % w/w. This percentage was then multiplied by the volume of QE dose provided in the study (3.0 mL), resulting in a 214.0 µL of QE spike treatment. The high QE dose was calculated by dividing the sample size (5.0 g) by a low average weight of possum stomach contents (30.0 g), resulting in 16.7 % w/w. This percentage was then multiplied by the volume of QE dose provided in the study (3.0 mL), resulting in a 500.0 µL of QE spike treatment.

Intralipid treatment was calculated based on the treatment regimen outlined in study two (controls, 2.05 mL Intralipid/kg; VCD, 2.05 mL VCD mixed 1:3 v/v with Intralipid/kg). The low dose, representing VCD treatment, was calculated by dividing an average VCD Intralipid dose (2.05 mL/kg * 3.0 kg live weight = 6.15 mL dose * 67.0 % (1:3 v/v) = 4.12 mL Intralipid) by an average weight of possum stomach contents (50.0 g), resulting in an 83.0 µL Intralipid spike treatment. The high dose, representing control treatment, was calculated by dividing an average Intralipid dose (2.05 mL/kg * 3.0 kg live weight = 6.15 mL Intralipid) by an average weight of possum stomach contents (50.0 g), resulting in a 123.0 µL Intralipid spike treatment. Treatment vials were vortex mixed for 15 seconds and the pH measured at 5, 45 and120 minutes post-treatment. Stomach vials were kept in a 37ºC water bath during the length of the experiment to replicate *in vivo* conditions.

4.2.8 Statistical analysis

All general statistical procedures were run as previously mentioned in Chapter 3. In addition, live weights during treatment were analysed using a repeated measure test with animal identification as a random variable and treatment group defined by time. Ovarian follicle estimates were tested for covariance to ovarian weight and animal age at death and normalized where appropriate. Live weight covariance was determined for all post mortem organ weights. Data were normalized to live weight where appropriate by dividing the organ weight by live weight. Individual pre- and post-treatment CBC data from Study 1 were analysed against reference ranges using a Z-test. Additionally, mean CBC pre-treatment levels were tested against post-treatment levels to determine if any time-dependent effects had occurred. Liver and kidney histological reports were analysed using a Wald test with an ordinal logistics fit.

4.3 Results

4.3.1 Study 1

Treatment and necropsy parameters

Animal live weight, food consumption and body condition were monitored daily during treatment (13 days). Animals lost an average of 19.3 grams of live weight per day, regardless of treatment ($p < 0.0001$; Figure 4.1). Following treatment on day one, 5 of 8 animals treated with a low VCD dose (500mg/kg) and 2 of 8 animals treated with a high VCD dose (750 mg/kg) vomited. On treatment day 2 only one animal treated with a high dose of VCD vomited. No other adverse signs were reported during treatment across all groups. Animal age was similar across all treatment groups ($p > 0.713$) with an average age of 3.47 ± 0.31 years (Table 4.2). There were no significant differences between control and VCD treatment groups in final live weight ($p > 0.494$) or the weights of the liver ($p > 0.980$), paired kidneys normalized to live weight ($p > 0.171$), paired adrenal glands ($p > 0.927$), paired uterine tracts ($p > 0.351$), or paired ovaries ($p > 0.483$) (Table 4.2). The data from three animals were removed from the study due to: 1) on treatment day 9, one control animal was mistakenly given a VCD dose (500 mg/kg); 2) one control animal was found to be 10 years old and, thus, considered to be an outlier; and 3) one VCD treated animal (500 mg/kg) was found dead in her cage on the final day of treatment. A necropsy revealed normal appearance and parameters for the oesophagus, trachea, lungs and vital and reproductive organs. There were no signs of aspiration; however, the gastrointestinal tract appeared pale and contained loose, watery stool. The cause of death was undetermined.

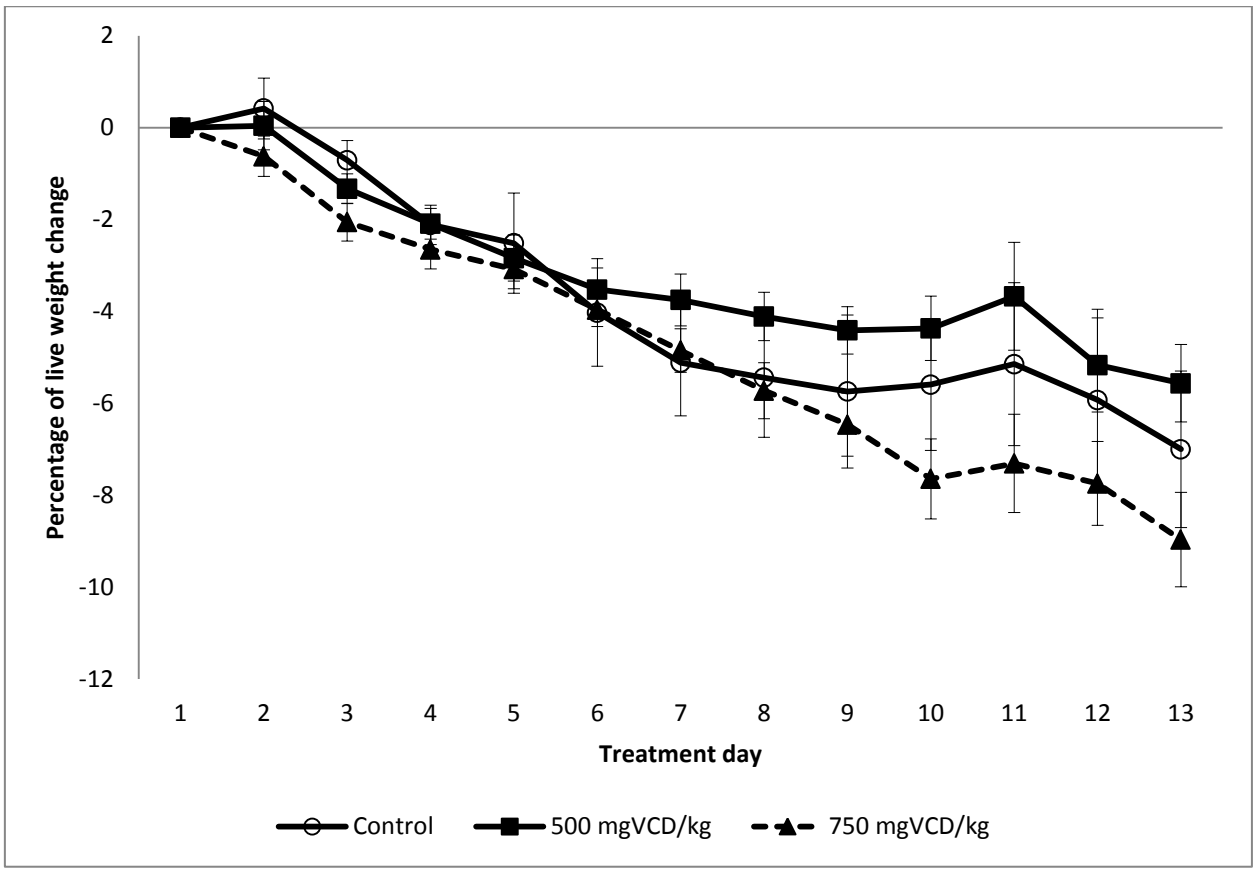

Figure 4.1 Change in live weight (as % starting weight) of wild-caught female brushtail possums during 13 days of daily oral VCD administration (0, 500 or 750 mg/kg; Study 1). Control (oil only), n = 6; 500 mg VCD/kg/d, n = 7; 750 mg VCD/kg/d, n = 8. Vertical bars represent ± SEM.

Table 4.2 The effect of VCD administration on live weight (LW) and somatic and reproductive tissues of wild-caught female brushtail possums during Study 1. Data are means ± SEM.

Measured parameter	Treatment group		
	Control	VCD low dose (500 mg/kg)	VCD high dose (750 mg/kg)
N	6	7	8
Age at death (year)	3.8 ± 0.7	3.6 ± 0.5	3.1 ± 0.5
Final LW (kg)	2.83 ± 0.09	3.01 ± 0.09	2.86 ± 0.14
Liver weight (g/kg LW)	21.1 ± 0.57	21.4 ± 1.40	21.2 ± 1.0
Paired kidney weight (g)	12.4 ± 0.41	12.5 ± 0.53	11.3 ± 0.46
Paired adrenal weight (g)	0.42 ± 0.03	0.42 ± 0.03	0.43 ± 0.02
Paired uteri weight (g)	13.1 ± 1.84	13.4 ± 3.35	8.98 ± 1.69
Paired ovary weight (g)	0.29 ± 0.03	0.28 ± 0.04	0.23 ± 0.04

Toxicology results

Complete blood counts (CBC) were measured pre- and post-treatment to check for general toxicity. CBC results revealed differences between pre- and post-treatment levels across all treatment groups in

haematocrit (HCT; $p < 0.014$), mean cell volume (MCV; $p < 0.008$), mean cell haemoglobin concentration (MCHC; $p < 0.0001$), neutrophils ($p < 0.008$), lymphocytes ($p < 0.017$), and eosinophils ($p < 0.027$). However, these effects were not treatment related ($p > 0.057$; Table 4.3). Pre- and post-treatment CBC levels were also tested against the mean reference range for each parameter. The data revealed that post-treatment MCHC levels of controls and low dose VCD treatment groups were significantly higher than the reference range ($p < 0.002$ and $p < 0.001$, respectively; Table 4.3). Animals treated with a high dose of VCD had elevated MCHC levels compared with the reference range both pre- and post-treatment ($p < 0.032$ and $p < 0.0001$, respectively; Table 4.3). There were no other differences between pre- or post-treatment CBC parameters when compared with their corresponding reference ranges ($p > 0.087$; Table 4.3). The liver enzymes gamma-glutamyl transferase (GGT) and glutamate dehydrogenase (GLDH) were measured to determine liver toxicity. There were no treatment differences in GGT or GLDH levels across all treatment groups ($p > 0.109$ and $p > 0.407$, respectively; Table 4.3).

Table 4.3 Haematological and liver enzyme parameters of wild-caught female brushtail possums recorded pre- and post-treatment during Study 1. Reference ranges were obtained from Gribbles Veterinary (Christchurch, NZ). ₸ indicates post-treatment levels are different ($p < 0.05$) from pre-treatment levels; * indicates levels are different ($p < 0.05$) from the reference range. Data are means ± SEM.

Measured parameter	Reference range	Pre-treatment			Post-treatment		
		Control	VCD low dose (500 mg/kg)	VCD high dose (750 mg/kg)	Control	VCD low dose (500 mg/kg)	VCD high dose (750 mg/kg)
Sample size	N/A	6	7	8	6	7	8
Red blood cells (10^{12}/L)	5.1 – 7.2	5.73 ± 0.30	6.04 ± 0.22	5.91 ± 0.20	5.50 0.24	5.73 0.33	6.16 0.31
Haemoglobin (g/L)	114 - 169	130.2 ± 5.80	132.4 ± 5.27	136.6 ± 3.83	124.8 4.94	126.3 8.41	141.4 5.75
Haematocrit (%)	35 – 51	0.39 ± 0.02	0.40 ± 0.01	0.41 ± 0.01	₸0.37 ± 0.01	₸0.36 ± 0.02	₸0.40 ± 0.02
Mean cell volume (fL)	64 – 77	67.0 ± 1.44	64.6 ± 0.69	67.4 ± 1.24	₸66.5 ± 1.06	₸64.0 ± 0.65	₸65.5 ± 1.0
Mean cell haemoglobin (pg)	20-30	22.8 ± 0.31	21.9 ± 0.40	23.0 ± 0.27	22.8 ± 0.31	22.0 ± 0.44	23.1 ± 0.30
Mean cell haemoglobin concentration (g/L)	317 – 329	332.8 ± 4.06	333.3 ± 3.17	*335.9 ± 4.45	* ₸341.5 ± 1.78	* ₸343.0 ± 5.78	* ₸ 351.9 ± 1.99
White blood cells (10^9/L)	2.3 – 14.3	7.97 ± 1.16	9.31 ± 1.40	9.48 ± 1.25	7.80 ± 0.94	8.97 ± 1.32	9.35 ± 1.0
Neutrophils (10^9/L)	1.4 – 5.1	2.98 ± 0.59	3.44 ± 0.70	2.61 ± 0.49	₸ 3.50 ± 0.55	₸ 4.17 ± 1.26	₸ 4.43 ± 0.74
Lymphocytes (10^9/L)	0.8 – 13.2	4.48 ± 1.15	5.19 ± 0.87	5.99 ± 1.05	₸ 3.87 ± 0.74	₸ 4.17 ± 0.77	₸ 4.33 ± 0.85
Monocytes (10^9/L)	0.1 – 0.5	0.37 ± 0.15	0.37 ± 0.11	0.50 ± 0.08	0.28 ± 0.06	0.51 ± 0.15	0.44 ± 0.07
Eosinophils (10^9/L)	< 1.2	0.12 ± 0.03	0.33 ± 0.10	0.33 ± 0.11	₸ 0.10 ± 0.03	₸ 0.16 ± 0.04	₸ 0.18 ± 0.10
Basophils (10^9/L)	< 0.4	0.0 ± 0.0	0.01 ± 0.01	0.08 ± 0.04	0.03 ± 0.02	0.0 ± 0.0	0.01 ± 0.01
Gamma-glutamyl transferase (IU/L)	0.0 – 10.0	N/A	N/A	N/A	4.17 ± 0.48	6.0 ± 0.58	7.38 ± 1.68
Glutamate dehydrogenase (IU/L)	0.0 – 10.0	N/A	N/A	N/A	17.8 ± 3.26	18.3 ± 5.07	22.5 ± 3.77

Follicle counts

To determine if primordial follicle counts decrease with age or are correlated with ovarian weight, covariance was first determined for each variable. The data demonstrate no correlations between primordial follicle counts and age or ovarian weight ($p > 0.943$ and $p > 0.095$, respectively). There were no statistical differences in primordial follicle counts of animals dosed with a low (500 mg/kg) or a high concentration of VCD (750 mg/kg) compared to controls ($p > 0.687$, Figure 4.2).

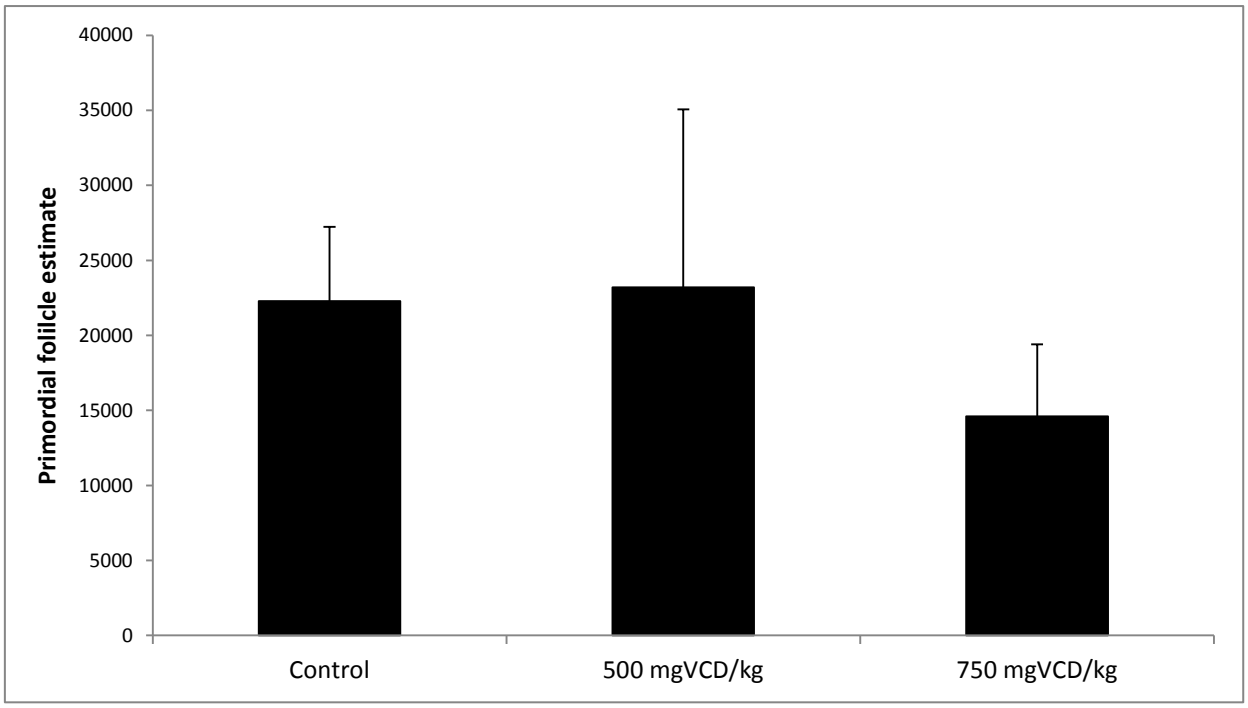

Figure 4.2 Effect of daily oral administration (13 days; Study 1) of VCD on mean total primordial ovarian follicle estimates in wild-caught female brushtail possums. Counts were recorded from the left ovary of each animal and total counts estimated using a correction factor formula (Gougeon and Chainy, 1987). Control (oil only), n = 6; 500 mg VCD/kg, n = 6; 750 mg VCD/kg, n = 7. Vertical bars represent + SEM.

4.3.2 Study 2

Treatment and necropsy parameters

Animals were monitored daily for food consumption, body condition and live weight during treatment (10 days). Food consumption and body condition of animals appeared normal yet animals lost weight across all treatment groups during the 10 day study ($p < 0.0001$; Figure 4.3). Furthermore, VCD-IT and VCD-QE treatment resulted in a greater loss of live weight relative to control groups ($p < 0.0002$). Two VCD-IT animals vomited following treatment on day 2 while only one VCD-QE animal vomited following treatment on day 4. No other adverse signs were observed during treatment. Animal age was not significantly different across all treatment groups ($p > 0.262$) with an average age of 2.69 ± 0.31 years (Table 4.4). There were no significant differences between control and VCD treatment groups in

final live weight ($p > 0.641$) or the weights of the liver ($p > 0.249$), paired kidneys ($p > 0.181$), paired adrenal glands ($p > 0.689$), paired uteri tract ($p > 0.768$), or paired ovaries ($p > 0.514$) (Table 4.4). Five animals were removed from the study due to various reasons: 1) one VCD-IT animal had lost >10% live weight by day 6 and was removed from further treatment, 2) one C-O animal was found to be 15 years old, thus, considered to be an outlier, and 3) three VCD-QE animals died during treatment. A full necropsy was performed for each animal. The oesophagus, trachea, lungs and somatic and reproductive organs of all animals appeared normal. Although there were no signs of aspiration in any of the animals, the gastrointestinal tract of two animals appeared pale and contained loose, watery stool. The cause of death was undetermined.

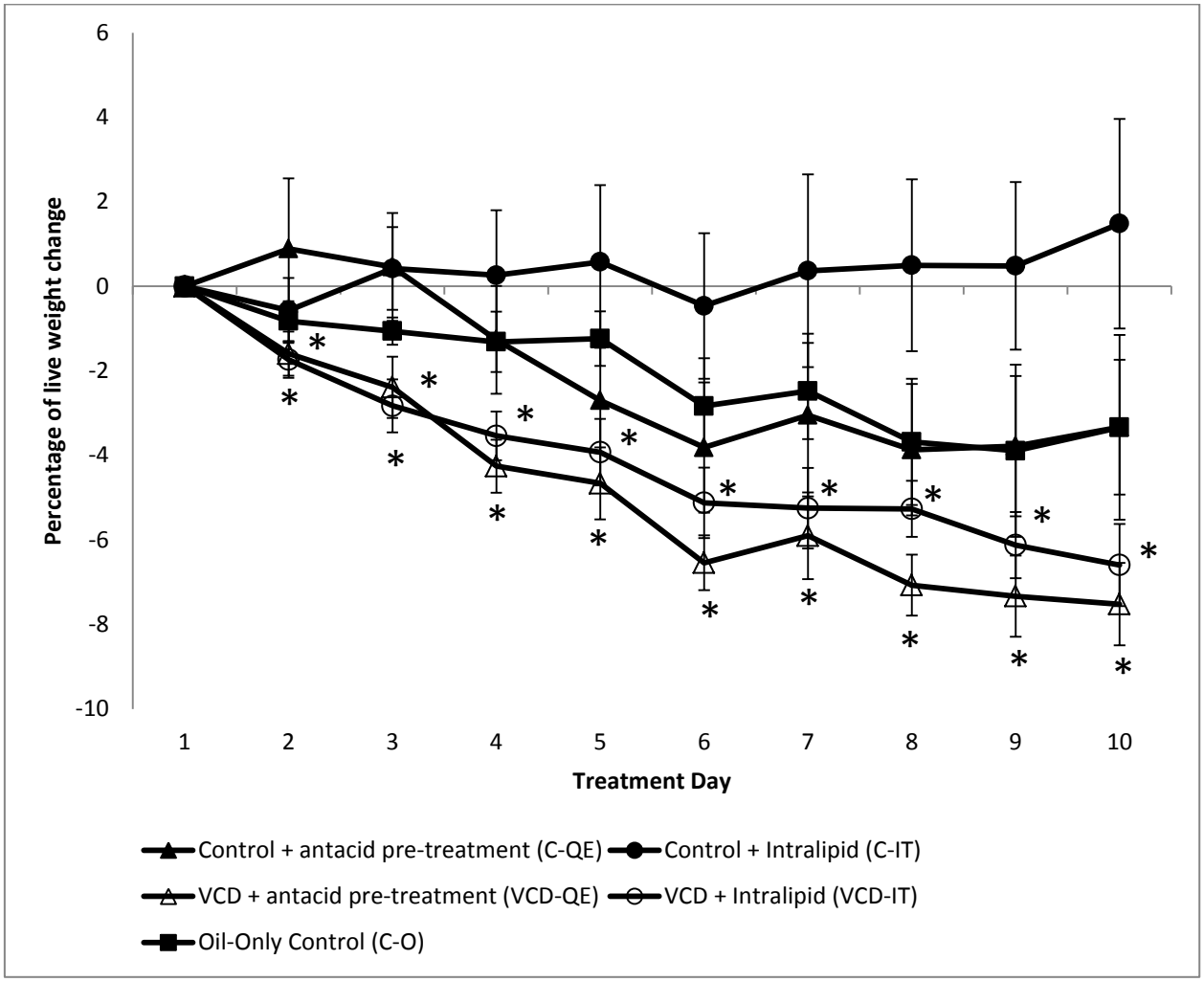

Figure 4.3 Change in live weight (as % starting weight) of wild-caught female brushtail possums during 10 days of daily oral VCD administration (0 or 500 mg/kg; Study 2). Oil-only control (C-O), n = 6; Control + antacid pre-treatment (C-QE), n = 6; Intralipid control (C-IT), n = 6; 750 mg VCD/kg carried in oil + antacid pre-treatment (VCD-QE), n = 7; 750 mg VCD/kg carried in Intralipid (VCD-IT), n = 9. * indicates different ($p < 0.05$) from control. Vertical bars represent ± SEM.

Table 4.4 The effect of VCD administration on live weight (LW) and weight of somatic and reproductive organs of wild-caught female brushtail possums during Study 2. Data are means ± SEM.

Measured parameter	Treatment group				
	Oil control (C-O)	Antacid + oil control (C-QE)	Intralipid control (C-IT)	Antacid + VCD (750 mg/kg) in oil (VCD-QE)	VCD (750 mg/kg) in Intralipid (VCD-IT)
Sample size	5	6	6	7	9
Age at death (year)	2.2 ± 0.7	3.0 ± 0.8	2.8 ± 0.9	1.9 ± 0.6	3.3 ± 0.6
Final LW (kg)	2.85 ± 0.09	2.99 ± 0.08	3.12 ± 0.12	3.04 ± 0.12	3.00 ± 0.11
Liver weight (g)	63.3 ± 5.49	65.9 ± 4.09	68.0 ± 7.23	79.8 ± 5.91	69.7 ± 4.10
Paired kidney weight (g/kg LW)	4.81 ± 0.18	4.99 ± 0.28	4.42 ± 0.15	4.96 ± 0.17	4.62 ± 0.13
Paired adrenal gland weight (g)	0.30 ± 0.03	0.31 ± 0.02	0.33 ± 0.04	0.30 ± 0.04	0.29 ± 0.02
Paired uteri weight (g)	7.79 ± 1.18	8.38 ± 1.57	13.9 ± 3.95	9.82 ± 1.73	12.2 ± 4.35
Paired ovary weight (g)	0.42 ± 0.05	0.37 ± 0.04	0.40 ± 0.07	0.31 ± 0.03	0.33 ± 0.04

Toxicology results

To determine if animals pre-treated with an antacid were affected by the high calcium levels in the Quick-EZE® tablets, total calcium (Ca) and magnesium (Mg) levels were measured in pre- and post-treatment plasma on ICP-OES. When Ca and Mg levels were compared within treatment groups, there were no significant differences between pre- and post-treatment levels among all groups ($p > 0.091$; Figure 4.4). Comparison of pre- and post-treatment Ca and Mg levels between oil-only controls and control and VCD animals pre-treated with antacids revealed no significant differences ($p > 0.171$; Figure 4.4).

When control groups for liver scores were compared with each other, there were no differences in scores ($p > 0.588$) so control data were pooled. Findings revealed no treatment effects in the liver of VCD-QE or VCD-IT treated animals when compared against controls ($p > 0.339$). For the kidney, the C-QE group had significantly higher scores than C-IT and C-O groups ($p > 0.024$; Table 4.5) and therefore control groups were not pooled. The data revealed no significant differences in kidney scores following treatment when control groups were compared with their corresponding VCD groups ($p > 0.401$; Table 4.5).

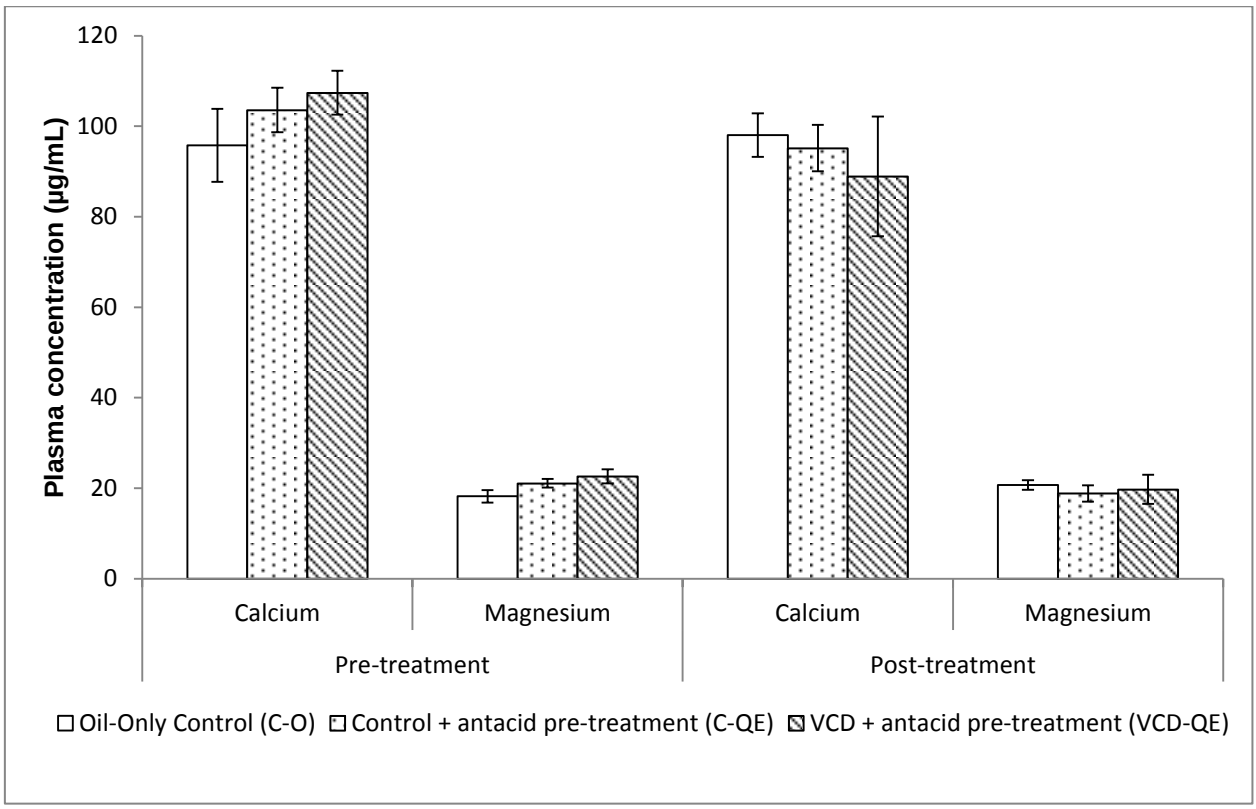

Figure 4.4 Effect of daily oral administration (10 days; Study 2) of VCD on total plasma calcium and magnesium concentrations measured in female brushtail possums before and after treatment. Oil-only control, n = 6; Control + antacid pre-treatment, n = 6; 750 mg VCD/kg carried in oil + antacid pre-treatment, n = 7. Vertical bars represent ± SEM.

Table 4.5 Histopathological scores (see text) of liver and kidneys of wild-caught female brushtail possums following oral treatment with two formulations aimed at improving the follicle depleting effects of VCD (Study 2). Values with different superscript letters are significantly different ($p < 0.05$). Data are means ± SEM.

Measured Parameter	Treatment group				
	Oil control (C-O)	Antacid + oil control (C-QE)	Intralipid control (C-IT)	Antacid + VCD (750 mg/kg) in oil (VCD-QE)	VCD (750 mg/kg) in Intralipid (VCD-IT)
N	5	6	6	7	9
Liver	1.8 ± 0.2	2.0 ± 0.26	2.17 ± 0.31	2.29 ± 0.36	2.11 ± 0.35
Kidneys	0.8 ± 0.37^{AC}	2.0 ± 0.26^{B}	0.83 ± 0.31^{ABC}	1.85 ± 0.4^{ABC}	1.11 ± 0.35^{ABC}

Follicle counts

Age and ovarian weight covariance with primordial, primary and secondary follicle estimates were examined. It was determined that age was not correlated with follicle counts ($p > 0.054$). However, ovarian weight was correlated with primary ($p < 0.002$) and secondary ($p < 0.015$) follicle numbers but

not with primordial follicle counts ($p > 0.095$). Therefore, primary and secondary follicle counts were normalized to ovarian weight and then checked for treatment differences. There were no differences in primordial, primary or secondary follicle counts of C-QE compared with VCD-QE ($p > 0.336$) and C-IT compared to VCD-IT ($p > 0.615$; Figures 4.5 and 4.6). There were no significant differences in the control group's primordial, primary or secondary follicle counts ($p > 0.254$). Therefore, all control group data were pooled for comparison against VCD treatment groups. Compared with controls, there were no significant differences in VCD-IT and VCD-QE primordial, primary and secondary follicle counts ($p > 0.367$; Figures 4.5 and 4.6).

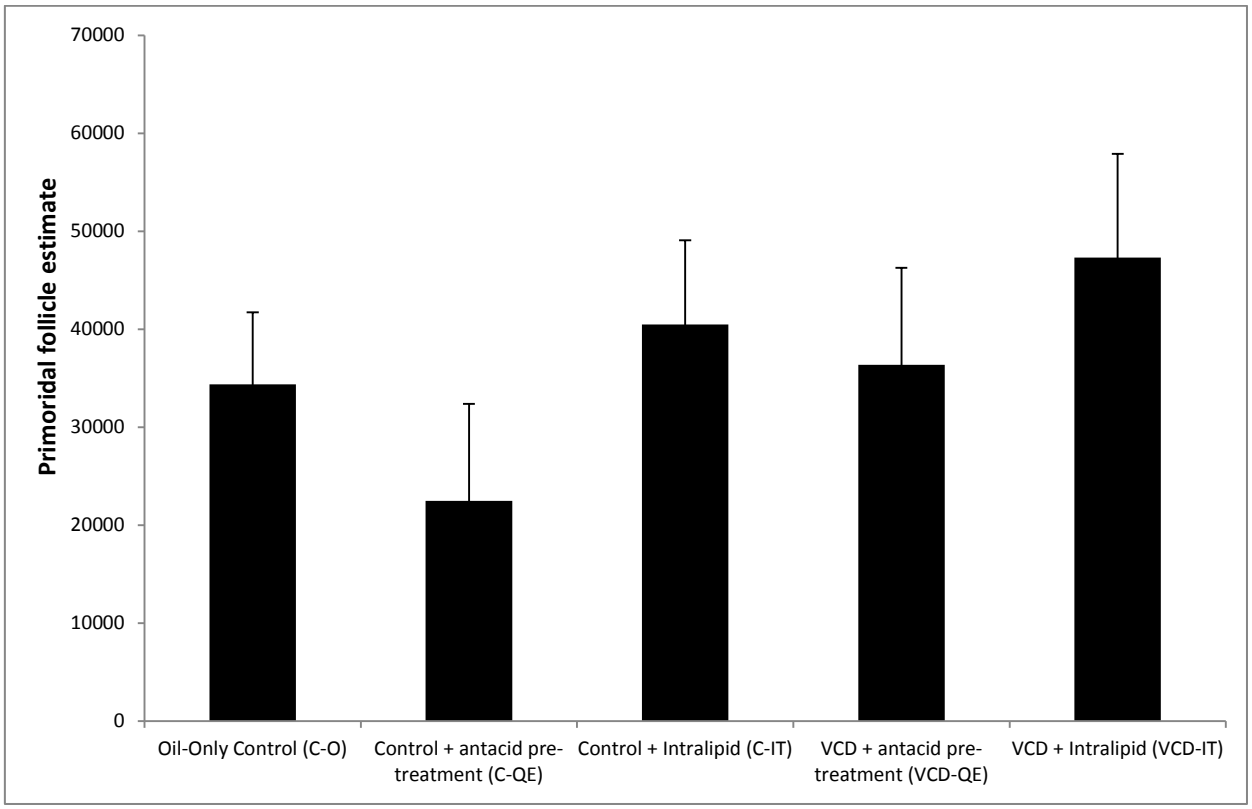

Figure 4.5 Effect of daily oral administration (10 days; Study 2) of VCD on mean total primordial ovarian follicle counts in wild-caught female brushtail possums. Counts were recorded from the left ovary of each animals and total counts estimated using a correction factor formula (Gougeon and Chainy, 1987). Oil-only control (C-O), n = 5; Control + antacid pre-treatment (C-QE), n = 6; Intralipid control (C-IT), n = 6; 750 mg VCD/kg carried in oil + antacid pre-treatment (VCD-QE), n = 8; 750 mg VCD/kg carried in Intralipid (VCD-IT), n = 9. Vertical bars represent + SEM.

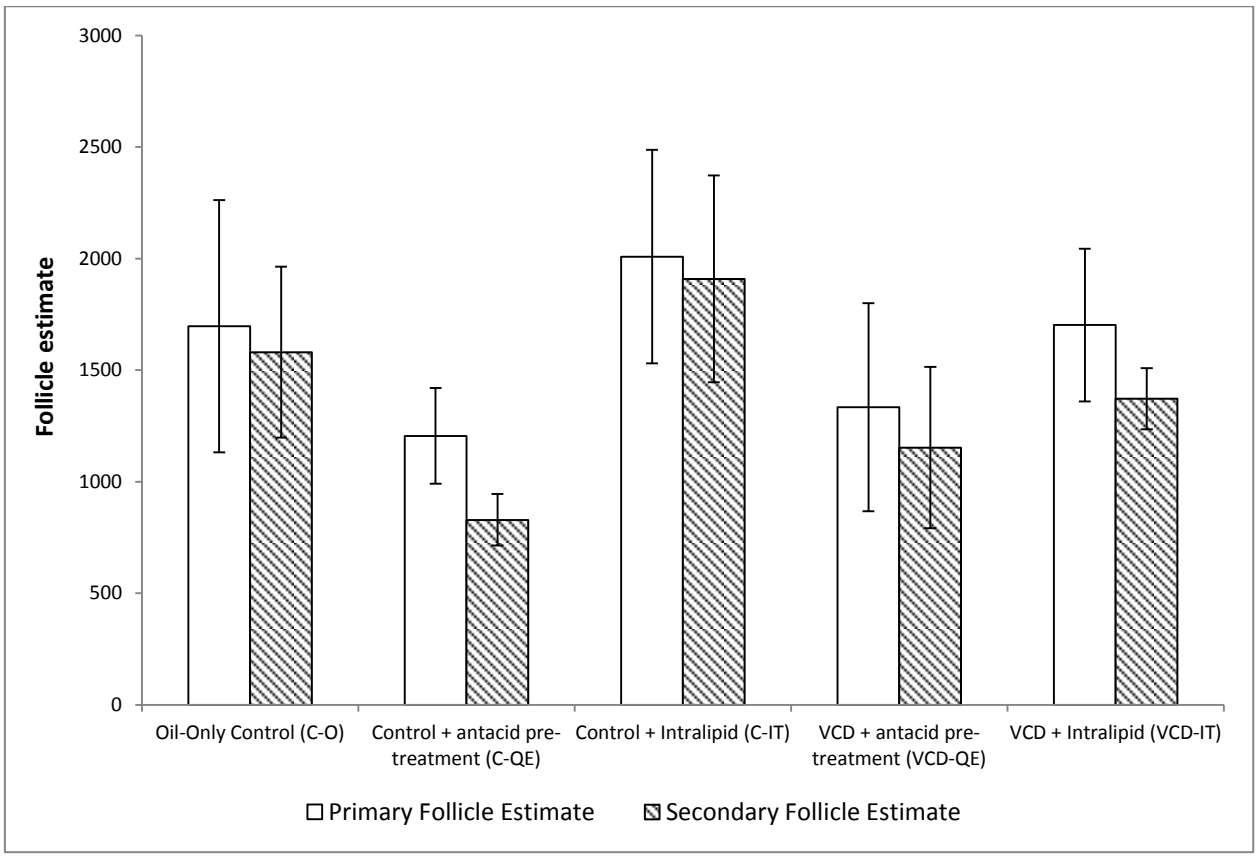

Figure 4.6 Effect of daily oral administration (10 days; Study 2) of VCD on mean total primary and secondary ovarian follicle counts in wild-caught female brushtail possums. Counts were recorded from the left ovary of each animals and total counts estimated using a correction factor formula (Gougeon and Chainy, 1987). Oil-only control (C-O), n = 5; Control + antacid pre-treatment (C-QE), n = 6; Intralipid control (C-IT), n = 6; 750 mg VCD/kg carried in oil + antacid pre-treatment (VCD-QE), n = 8; 750 mg VCD/kg carried in Intralipid (VCD-IT), n = 9. Vertical bars represent ± SEM.

Formulation effects on stomach contents pH

The effects of Quick-EZE® (QE) and Intralipid® (IT) on stomach contents pH of possums were analysed *in vitro*. Addition of a low dose of the antacid Quick-EZE to possum stomach caused the pH to rise from 1.37 (starting pH) to 4.33 ± 0.08 within 5 minutes. The pH then stabilized at 5.19 pH ± 0.03 by 45 minutes and remained at that pH up to 120 minutes. Similarly, a high dose of Quick-EZE caused possum stomach pH to quickly rise from 1.37 to 5.25 ± 0.06 within 5 minutes. At 45 minutes, the pH reached 6.17 ± 0.01 and stabilized at 6.68 ± 0.07 by 120 minutes. Treatment with a low and high dose of Intralipid resulted in minimal pH change with time (120 min) from 1.37 (starting pH) to 1.69 ± 0.06 and 1.38 ± 0.07, respectively. Control stomach contents did not alter during the 120 minute trial period (pH 1.37-1.38 ± 0.04).

4.4 Discussion

The chemosterilant VCD was examined as a potential humane and sustainable pest control method for NZ brushtail possums. The primordial ovarian follicle pools of possums orally treated with either 500 or 750 mg VCD/kg for 13 consecutive days were unaffected compared with controls. Previous studies examining the effects of oral administration of VCD (500 mg/kg) in Sprague Dawley rats have demonstrated significant primordial follicle depletion after 10 (Burd, 2009), 15 or 30 (Schmuki et al., 2011) consecutive days. One possible reason for the lack of effects observed here is the highly acidic stomach environment of brushtail possums. Possum stomach contents pH ranges between 1.0 and 2.0, regardless of food consumption or digestion (Drs. J. A. Duckworth & F. Molinia, pers. comm.). Conversely, rodent stomach contents pH ranges between 3.2 (fed) and 3.9 (fasted) (McConnell et al., 2008). When VCD is exposed to a pH of 1.0 *in vitro*, >99.9% of VCD is hydrolysed within 3 minutes (Chapter 6). It was hypothesized that the highly acidic stomach environment of the possum hydrolysed the VCD dose before the compound could be absorbed and then reach the target sites in the ovary. Therefore, a second study was designed to reduce the potential acidic breakdown of VCD in the possum stomach.

Two formulations which were believed to protect and improve the oral efficacy of VCD were examined. The first formulation utilized a pre-treatment with the antacid Quick-EZE® prior to dosing possums with VCD carried in oil. Previous work had shown that the gastric contents pH of possums orally gavaged with a suspension containing two Quick- EZE® tablets was increased and remained elevated (pH 4.5-6.5) for at least seven hours (Drs. J. A. Duckworth & F. Molinia, pers. comm.). It was predicted that an oral pre-treatment of antacids would reduce stomach acidity prior to VCD administration, thereby decreasing VCD hydrolysis. Results show that ten days of oral VCD treatment (750 mg/kg) administered with an antacid pre-treatment had no effect on immature follicle populations compared with controls. When possum stomach contents were incubated *in vitro* with a comparable Quick-EZE® dose, the pH was increased from 1.37 to 5.19 – 5.25 within 5 minutes of exposure. These *in vitro* results support previous reports (Drs. J. A. Duckworth & F. Molinia, pers. comm.) and suggest that Quick-EZE® may have been able to reduce the acidic breakdown of VCD to some extent. Nevertheless, the treatment did not appear to have any observable effects on the ability of orally delivered VCD to deplete immature follicle populations in the ovaries of VCD treated possums.

The second approach to improve the oral efficacy of VCD was the use of a lipoprotein emulsion, Intralipid®, as an alternative carrier liquid for VCD. Intralipid® is a sterile, homogenous fat emulsion containing soybean oil (20%), egg yolk phospholipids (1.2%) and glycerine (2.25%) and is readily absorbed across the gastric lining. VCD is lipophilic in nature and should therefore readily incorporate into lipid components of a carrier such as Intralipid®. In addition, previous studies have demonstrated that Intralipid® improves the palatability of VCD in rodents (Dr. C. A. Dyer, pers. comm.). It was predicted that replacing the normal carrier liquid (oil) with Intralipid would improve VCD absorption from the gastric lining into the blood and may provide some protection from acidic degradation. VCD

(750 mg/kg) carried in Intralipid had no depleting effect on the immature follicle populations of possums. *In vitro* examination of possum stomach contents pH following Intralipid addition only slightly altered the pH from 1.37 to 1.71 after two hours. Therefore, despite the potential ability of Intralipid to increase VCD absorbency, it is likely that it was unable to protect VCD from the highly acidic environment of the possum stomach.

The effect of orally delivered VCD on the health and body condition of female possums was also examined. Thirteen consecutive treatment days resulted in weight loss, irrespective of treatment. The possible cause of weight loss across all treatment groups may be a result of anaesthetic and handling stress. Wild brushtail possums living in Australia showed stress signs (wobbliness, low body mass) and decreased numbers of pouch young and reduced adult survivability following routine monitoring and handling procedures (Clinchy et al., 2001). During the trials reported here all attempts were made to keep animal stress to a minimum. However, it is likely that the possums experienced some stress as a result of repeated handling and anaesthesia as evidenced by the reduced weight gain observed across all treatment groups.

To determine if any VCD-induced toxicity effects were occurring, internal organs were examined following each study and complete blood counts (CBC) and liver enzyme levels were analysed following Study 1. Oral VCD administration (10-13 days, 500-750 mg/kg) did not have any obvious effects on somatic or reproductive organs. These results could be expected based on similar VCD dosing regimens in rodents (Ito et al., 2009; Kodama et al., 2009; Muhammad et al., 2009). Comparison of pre- versus post-treatment CBC levels revealed that all animals, regardless of treatment, demonstrated altered CBC levels that were indicative of stress. However, only one parameter (MCHC) was outside of the reference range suggesting any stress-induced effects were minimal. In addition, liver enzyme levels and liver and kidney histological samples indicated normal organ function. Taken together, these data suggest that there were no VCD-induced toxicity effects, rather the altered CBC results were likely due to stress caused by repeated daily handling and anaesthesia. Similar haematological reports have been observed in wild possums following capture (Buddle et al., 1992). The blood profiles of those animals returned to levels similar to that of frequently captured possums following caged housing for 1-3 weeks (Buddle et al., 1992) suggesting habituation to handling and the housing environment. Thus it can be postulated that the altered CBC parameters reported here would return to similar levels measured during pre-treatment upon elimination of daily handling and anaesthesia.

Possums treated for 10 days with VCD carried in Intralipid or an antacid pre-treatment followed by VCD carried in oil lost more live weight than controls. The weight loss cannot be attributed simply to the high dose of VCD delivered (750 mg/kg) as possums treated with the same VCD dose for 13 days did not experience significant weight loss associated with treatment. Rather it is likely that the formulations (Intralipid and antacids) used to increase VCD efficacy may have indirectly augmented weight loss. VCD-related effects on weight gain and food intake have been observed in rodents (Burd,

2009; Haas et al., 2007; Muhammad et al., 2009). It is possible that the formulations did in fact protect VCD to some extent, causing prolonged gastrointestinal exposure to VCD and the resulting effects on food intake and weight gain. It must be noted that food consumption during the studies was not measured directly, but rather observed. VCD treatment may have caused internal irritation resulting in VCD treated animals consuming less food than controls and thus affecting weight gain. VCD has been shown to cause irritation to the oesophagus, skin and stomach in rodents following long-term VCD treatment (NTP, 1986). Although the formulations to increase VCD efficacy may have worked, there may still have been insufficient amounts of the chemical reaching the target organs, ovaries, to induce follicle depleting effects.

Potential VCD related deaths occurred during each study. One VCD treated animal (500 mg/kg) was found dead on the final day in Study 1 and three VCD animals receiving an antacid pre-treatment died during Study 2. Although most of the internal organs appeared normal, three of the four animals showed signs of irritated gastrointestinal tracts and disruption of water balance (pale tissue containing watery stool). It was initially speculated that the animals receiving VCD with an antacid pre-treatment died as a result of calcium toxicity from the Quick-EZE® tablets. However, the post-treatment calcium and magnesium levels were similar to levels measured during pre-treatment. Therefore, it is more likely that these animals died as a result of VCD treatment rather than calcium toxicity following antacid administration. What may have occurred is that the antacid associated protection of VCD caused increased irritation to the gastrointestinal tract from prolonged VCD exposure (NTP, 1986). Thus is seems that, to some extent, VCD may have been protected from hydrolysis in the stomach, thereby enabling increased treatment related effects.

VCD dosing regimens and formulations examined here had no obvious effect on the pool of immature follicles in healthy adult female possums. The use of Intralipid or antacid pre-treatment to increase VCD absorbency did seem to work as evidenced by treatment related weight loss, although it appears that insufficient levels of the chemical reached the ovaries to induce follicle depletion. Further understanding of the uptake and metabolism of VCD in the possum will be necessary if the chemical is to be considered for use as a chemosterilant. Therefore, in the following chapter the differences in enzymatic metabolism and uptake and distribution of orally delivered VCD in possums and rats will be investigated.

Chapter 5

Fate and metabolism of 4-vinylcyclohexene diepoxide in wild-caught female brushtail possums and Norway rats: a comparative study

5.1 Introduction

In the previous chapter the effects of orally administered 4-vinylcyclohexene diepoxide (VCD) on the health and ovarian follicle populations of adult female possums was examined. Two formulations aimed at improving the absorbency and efficacy of orally delivered VCD (Study 2) in possums was also investigated. There were no decreases in the immature ovarian follicular pools in healthy adult female possums when they were orally dosed with VCD. However, the observed treatment-related live weight loss during Study 2 suggested that there were some VCD treatment-related effects and that formulations may have increased VCD absorbency to some degree. The dosing regimens tested in those trials were similar to ones used in previous studies that demonstrated significant primordial follicle depletion following oral VCD treatment in the rat (Burd, 2009; Herawati et al., 2010; Mayer et al., 2010). The lack of observed effects on possum ovarian primordial or primary follicle populations suggests differences in the uptake, metabolism, and fate of VCD in possums compared with rats and thus merits examination.

4-Vinylcyclohexene (VCH), the parent compound of VCD, is metabolized to VCD in a Phase I reaction primarily by the hepatic-derived enzyme superfamily cytochrome P450 (CYP450) (Doerr-Stevens et al., 1999; Doerr et al., 1996; Springer et al., 1996c). Phase I metabolism is usually a prerequisite for Phase II conjugative metabolism wherein lipophilic compounds are converted to more water-soluble metabolites for excretion (Rushmore, 2002; Xu et al., 2005). Through Phase II reaction, VCD is further metabolized in the liver and, to a lesser extent, in the ovary to a non-active tetrol metabolite ([1,2-dihydroxy] ethyl-1,2-dihydroxycyclohexane) through the action of microsomal epoxide hydrolase (mEH) and glutathione-S-transferase (GST) (Cannady et al., 2002; Devine et al., 2001; Flaws et al., 1994; Keating et al., 2008a; Keating et al., 2008b; Salyers, 1995). mEH catalyses the hydration of alkene epoxides and arene oxides while GST catalyses conjugation of reduced glutathione (GSH) with compounds to increase their elimination from the body.

Species differences in VCD uptake and metabolism have been demonstrated in laboratory-bred mice (*Mus musculus*) and laboratory-bred Norway rats (Kao et al., 1999; Keller et al., 1997; Salyers, 1995; Smith et al., 1990b). A disposition study revealed that VCD distributes faster from the blood into the tissues of mice compared with rats while excretion of VCD metabolites was primarily through the urine in both species (Salyers, 1995). It was also demonstrated that VCD metabolites are eliminated

faster in rats and the urinary metabolite profile of each species suggests different pathways for VCD metabolism (Salyers, 1995). Comparison of enzymatic metabolism of VCH and VCD revealed that mice have higher Phase I metabolic activity but slower Phase II activity rates compared with rats, resulting in the capability of mice to metabolise VCH faster than rats. As VCH is detoxified to VCD via CYP450, VCD accumulates faster in mice compared with rats due to their slower Phase II activity and mice are therefore more susceptible to VCD-induced ovarian toxicity (Doerr-Stevens et al., 1999; Doerr et al., 1996; Springer et al., 1996c). It is possible to speculate that a similar species difference in VCD metabolism may exist between possums and rats. Therefore, the studies reported here examined the rate of VCD clearance from the stomach and uptake into the blood in wild-caught adult female brushtail possums and wild-caught Norway rats. In addition, hepatic and ovarian levels of GSH were measured to detect any metabolic changes in both species. It was predicted that possums would metabolize VCD to its non-active tetrol metabolite more rapidly than rats.

5.2 Materials and Methods

5.2.1 Reagents

VCD, sunflower oil, cyclohexanone, ethyl acetate, Attane™ isoflurane, and sodium pentobarbital were obtained from vendors and used for experimental procedures as previously described in Chapter 3.

5.2.2 Possum experimental procedures

Possums (n = 24) were randomly allocated into treatment groups using a randomized block design stratified on live weight prior to trial start. Under anaesthesia (5% isoflurane in O_2 at 2 L/min) blood was collected from the tail vein prior to gavage treatment (control time point 0). Possums were then gavaged with a single bolus dose of sunflower oil (vehicle control, n = 8) or VCD (750 mg/kg mixed 1:3 w/v) in oil (n = 8). Gavage tubes consisted of commercially purchased vinyl tubing (3 mm diameter, 30 cm length) attached to a 3-way stop cock and a 10 mL syringe. A different tube was used for each treatment group and tubes were discarded daily. Possums were divided into three sub-groups which were killed at different time points (either at 0 or 15 minutes or 24 hours post-treatment). For group 1 possums (n = 4 per treatment) animals were killed immediately after treatment with an intracardiac pentobarbitone overdose (125 mg/kg). For group 2 possums (n = 4 per treatment), while under anaesthesia, blood was collected at 1, 3, 5, 10 and 15 minutes post-treatment from the tail vein. Group 2 possums were then killed at 15 minutes post-treatment with an intracardiac pentobarbitone overdose (125 mg/kg). For group 3 possums (n = 4 per treatment), blood was collected from the tail vein at 5, 15, 30, 60, 120 and 360 minutes post-treatment. Animals remained under anaesthesia during the 5 and 15 minute time points. For all other time points possums were re-anaesthetised to obtain each blood sample. At 24 hours post-treatment, group 3 possums were anaesthetised and a final blood sample collected via intracardiac injection and then animals were then euthanized with an intracardiac pentobarbitone overdose (125 mg/kg). For each animal, no more than 5% of circulating blood volume

(2.0-4.0 mL/time point) was collected to ensure normal circulatory function (Diehl et al., 2001). All blood samples were collected into lithium heparin vacutainer tubes (BD Vacutainer®), inverted several times and then prepared for GSH or gas chromatography (GC) measurements as outlined below. The liver and ovaries were immediately removed and prepared for GSH measurements as stated below. The stomach was excised, contents removed and weighed, the pH measured and contents prepared for GC analysis as outlined below. The reproductive tract, adrenal glands and kidneys were each weighed separately and then placed into 10% neutral-buffered formalin.

5.2.3 Rat experimental procedures

Rats (n = 56) were randomly allocated into treatment groups using a randomized block design stratified on live weight prior to trial start. Under anaesthesia (5% isoflurane in O_2 at 2 L/min) rats were gavaged with a single bolus dose of sunflower oil (vehicle control, n = 2-4 per time point) or VCD (750 mg/kg mixed 1:3 w/v) in oil (n = 2-4 per time point) using a steel gavage feeding tube (1.2 mm diameter, 76.2 mm length). Blood (3.0 ± 1.0 mL) was collected at 0, 5, 15, 30, 60, 120, 180 or 360 minutes post-treatment by intracardiac puncture into lithium heparin vacutainer tubes (BD Vacutainer®), inverted several times and then prepared for GSH or GC measurements as outlined below. Animals were then euthanized at each of the above time points with an intracardiac pentobarbitone overdose (125 mg/kg). The liver and ovaries were immediately removed and prepared for GSH measurements as outlined below. The stomach was excised, contents removed and weighed, the pH measured and then prepared for GC analysis as outlined below. The reproductive tract, adrenal glands and kidneys were each weighed separately and then placed into 10% neutral-buffered formalin.

5.2.4 Glutathione assay sample preparation

Blood treatment

Blood (0.5 mL) was placed into an Eppendorf tube and centrifuged for 5 minutes at 1,500 x *g*. Plasma was removed and placed into an Eppendorf tube wherein 1 times the sample volume of ice-cold 5% w/v 5-sulfosalicylic acid (SSA) solution was added to deproteinize the sample. The plasma sample was vortex mixed for 10 seconds, incubated at 4°C and then centrifuged for 10 minutes at 10,000 x *g*. The supernatant (~400 µL) was transferred to a cryostat vial, snap frozen with liquid nitrogen and stored at -80°C until analysis. In two successive rounds, the red blood cells (RBCs) were suspended and washed in 3 times the sample volume with 1x neutral-buffered saline solution (PBS), centrifuged for 5 minutes at 1,500 x *g* and then the supernatant discarded. PBS contained 1368.9 mM NaCl, 26.8 mM KCl, 101.4 mM Na_2HPO_4 and 17.6 mM KH_2PO_4. An equal volume of ice-cold 5% SSA solution was added to the volume of RBCs and then vortex mixed for 10 seconds, incubated at 4°C and then centrifuged for 10 minutes at 10,000 x *g*. The supernatant was transferred to a cryostat vial, snap frozen with liquid nitrogen and stored at -80°C until analysis.

Liver and ovary treatment

Whole liver and individual ovary weights were recorded. Ovaries were individually snap frozen whole with liquid nitrogen and the right ovary was stored at -80°C while the left ovary was placed into aluminium foil in preparation for deproteinization treatment with SSA. The liver was perfused with ice- cold 1x PBS and then the left lateral lobe (LLL) was removed and placed into aluminium foil, sealed and snap frozen with liquid nitrogen. The remaining liver was cut into 5-10 gram pieces, snap frozen and stored at -80°C. Individually, the left ovary and the LLL were broken into small pieces using a mallet and then 0.1 - 0.2 g measured into an ice-cold glass homogenizer and 7 times the sample weight of ice-cold 5% SSA was added and tissue homogenized with 10 passes of the pestle while on ice. The solution was incubated at 4°C for 10 minutes and the homogenate transferred to a conical tube and centrifuged at 10,000 x *g* for 10 minutes. Supernatant aliquots (1.0 mL) were placed into cryostat vials and snap frozen with liquid nitrogen and stored at -80°C until analysis.

5.2.5 Gas chromatography analysis

For each species, VCD was extracted from the stomach contents with the addition of 2 times the stomach weight of extraction solution (ES; ethyl acetate containing 1.18 mM cyclohexanone as the internal standard). VCD was extracted from the blood of each animal by collecting 2 aliquots of 0.5 mL whole blood and then adding 2 times the volume of ES. Extraction methods and VCD quantification on GC were performed as outlined in Chapter 3. Final VCD concentrations in blood and stomach contents are represented as µmoles VCD/L ES, unless otherwise stated.

To determine the percentage of VCD recovered in the stomach of each animal the total estimated VCD concentration in the stomach immediately following gavage was first calculated as follows. The amount of VCD stock administered to the animal (mL) was multiplied by the VCD concentration in the gavage liquid (365.33 mg/mL oil) and then divided by the amount of ES (mL) added to the sample tissue. Because ES was always added at the same rate (2 times the stomach contents weight), VCD concentration were standardized to stomach volume for each animal. The amount of recovered VCD (mg/mL ES; determined by GC) was then divided by the estimated VCD concentration (mg/mL ES) in the stomach and the resultant multiplied by 100. These calculations assume equal rate of acid-induced VCD hydrolysis and absorption (uptake) into the gastrointestinal epithelium between species and therefore must be accepted with caution.

5.2.6 Glutathione analysis

GSH concentration (nmol/µL or mg tissue) in RBCs, plasma and hepatic (liver) and ovarian tissues were analysed using methods described in the GSH assay kit (Sigma, cat# CS0260) with changes as outlined in Chapter 3.

5.2.7 Statistical analysis

All general statistical procedures were run as described in Chapter 3. In addition, the concentrations of VCD and GSH were analysed using a repeated measure test with animal identification as a random variable and treatment group defined by time.

5.3 Results

5.3.1 VCD concentration in blood and stomach tissue of possums and rats

The amount of VCD recovered in the blood and stomach of rats and possums following a bolus dose (750 mg/kg) was quantified by GC. At 15 minutes post-gavage the stomach contents of possums contained 57.4 ± 20.2 % (90.74 ± 34.3 mM VCD) of the calculated initial dose (158.68 ± 19.77 mM VCD) and by 24 hours no VCD was detected (Table 5.1). Following gavage, VCD was detected in possum blood up to 15 minutes post-gavage, (Table 5.2).

VCD was detected in rat stomach contents up to 6 hours post-gavage (Table 5.1). Of the calculated initial dose (156.64 ± 15.99 mM VCD) the amount of VCD recovered from the stomach contents of rats was substantial from 3 to 120 minutes post-gavage (27.5 – 50.1 % of given dose) but was reduced after 180 minutes post-gavage (18.1 – 21.4 % of given dose) (Table 5.1). VCD levels in rat blood were elevated initially but declined rapidly and were very low at 31 minutes post gavage (Table 5.2).

At 15 minutes post-treatment there was no significant difference between species in the proportion of VCD dose recovered from the stomach ($p > 0.944$; possum, 57.4 ± 20.2 % of dose; rat, 43.2 ± 2.42 % of dose; Table 5.1). However, there were significantly higher levels of VCD present in the blood of rats compared with that of possums from 3 to 30 minutes post-treatment ($p < 0.037$; Table 5.2). For further between-species comparisons the aim was to collect data from rats at 24 hours. However, VCD-treated rats did not survive past 7 hours. This was presumably a direct result of the VCD-related treatment because the control rats survived perfectly well. It is not understood why the wild-strain of Norway rat, when dosed with VCD, is unable to survive to 24 hours as survivability in laboratory Norway rats dosed daily for 10-30 days with similar oral VCD regimens is well documented (Burd, 2009; Schmuki et al., 2011). Thus, this discrepancy may be due to the wild strain being unable to cope with VCD treatment. A necropsy revealed normal appearance and parameters for the oesophagus, trachea, lungs and vital and reproductive organs and there were no signs of aspiration in each rat. The cause of death was undetermined.

Table 5.1 Change in VCD concentration in the stomach (mM) of female brushtail possums and Norway rats following a single oral dose of oil or VCD (750 mg/kg) suspended in oil (1:3 v/v). Statistical significance was set at p < 0.05. Data are means ± SEM; individual data are listed for sample sizes ≤ 2.

Time (minutes)	Species			
	Possum		Rat	
	Control treatment	VCD treatment	Control treatment	VCD treatment
0	0.0 ± 0.0 (n = 4)	0.0 ± 0.0 (n = 4)	0.0 ± 0.0 (n = 4)	0.0 ± 0.0 (n = 4)
1	-	-	-	-
3	-	-	0.0 ± 0.0 (n = 4)	82.7 ± 15.3 (n = 4)
5	-	-	0.0/0.0 (n = 2)	40.4/91.7 (n = 2)
15	0.0 ± 0.0 (n = 4)	90.7 ± 34.3 (n = 4)	0.0 ± 0.0 (n = 4)	58.4 ± 35.4 (n = 4)
30	-	-	0.0/0.0 (n = 2)	49.2/81.1 (n = 2)
60	-	-	0.0/0.0 (n = 2)	58.2/82.6 (n = 2)
120	-	-	0.0/0.0 (n = 2)	27.6/36.4 (n = 2)
180	-	-	0.0 ± 0.0 (n = 4)	29.2 ± 4.7 (n = 4)
360	-	-	0.0 ± 0.0 (n = 4)	23.4 ± 5.9 (n = 4)
1440 (24 hours)	0.0 ± 0.0 (n = 4)	0.0 ± 0.0 (n = 4)	-	-

Table 5.2 Change in VCD concentration in the blood (µM) of female brushtail possums and Norway rats following a single oral dose of oil or VCD (750 mg/kg) suspended in oil (1:3 v/v). * indicates difference (p < 0.05) between VCD-treated possums and VCD-treated rats. Data are means ± SEM; individual data are listed for sample sizes ≤ 2.

Time (minutes)	Species			
	Possum		Rat	
	Control treatment	VCD treatment	Control treatment	VCD treatment
0	0.0 ± 0.0 (n = 4)	0.0 ± 0.0 (n = 4)	0.0 ± 0.0 (n = 4)	0.0 ± 0.0 (n = 4)
1	0.0 ± 0.0 (n = 8)	221.7 ± 0.07 (n = 8)	-	-
3	0.0 ± 0.0 (n = 4)	192.0 ± 0.09 (n = 4)	0.0 ± 0.0 (n = 4)	*1048.7 ± 137.5 (n = 4)
5	0.0 ± 0.0 (n = 8)	178.7 ± 79.5 (n = 8)	0.0/0.0 (n = 2)	*480.5/*1412.6 (n = 2)
10	0.0 ± 0.0 (n = 8)	31.9 ± 1.02 (n = 8)	-	-
15	0.0 ± 0.0 (n = 8)	2.45 ± 1.38 (n = 8)	0.0 ± 0.0 (n = 4)	*385.1 ± 211.9 (n = 4)
30	0.0 ± 0.0 (n = 4)	0.0 ± 0.0 (n = 4)	0.0/0.0 (n = 2)	*94.5/*115.5 (n = 2)
60	0.0 ± 0.0 (n = 4)	0.0 ± 0.0 (n = 4)	0.0/0.0 (n = 2)	0.0/0.0 (n = 2)
120	0.0 ± 0.0 (n = 4)	0.0 ± 0.0 (n = 4)	0.0/0.0 (n = 2)	0.0/0.0 (n = 2)
180	-	-	0.0 ± 0.0 (n = 4)	0.0 ± 0.0 (n = 4)
360	0.0 ± 0.0 (n = 4)	0.0 ± 0.0 (n = 4)	0.0 ± 0.0 (n = 4)	0.0 ± 0.0 (n = 4)
1440 (24 hours)	0.0 ± 0.0 (n = 4)	0.0 ± 0.0 (n = 4)	-	-

5.3.2 Gastric pH levels following treatment

The pH of stomach contents following VCD treatment for each species is reviewed in Table 5.3. Compared with controls, the pH of possum stomach contents treated with VCD was elevated by

212.5% and 150.0% of controls at 15 minutes and 24 hours, respectively; however this trend was not significant ($p > 0.100$ and $p > 0.238$, respectively). Rat stomach pH did increase by 30.4% of controls by 15 minutes post-treatment although this was not significant ($p > 0.065$). The mean pH of rat stomach contents treated with VCD did increased from 3 to 360 minutes post-treatment compared with controls ($p < 0.031$; control mean, 3.17 ± 0.28 pH; VCD mean, 4.29 ± 0.18 pH). There were no between-species differences in stomach pH at 15 minutes following treatment (control, $p > 0.221$; VCD, $p > 0.637$; Table 5.3).

Table 5.3 Gastric pH levels in the stomach contents of female brushtail possums and Norway rats following a single oral dose of oil or VCD (750 mg/kg) suspended in oil (1:3 v/v). Statistical significance was set at $p < 0.05$. Data are means ± SEM; actual values are listed for sample sizes ≤ 2.

Time (minutes)	Species			
	Possum		Rat	
	Control treatment	VCD treatment (n = 8)	Control treatment	VCD treatment
0	1.00 ± 0.2 (n = 4)	1.13 ± 0.13 (n = 4)	2.88 ± 0.13 (n = 4)	2.88 ± 0.32 (n = 4)
1	-	-	-	-
3	-	-	3.50 ± 0.50 (n = 4)	3.75 ± 0.25 (n = 4)
5	-	-	0.5/3.5 (n = 2)	4.0/5.0 (n = 2)
15	1.00 ± 0.0 (n = 4)	3.13 ± 1.05 (n = 4)	3.25 ± 0.25 (n = 4)	3.75 ± 0.25 (n = 4)
30	-	-	2.0/3.5 (n = 2)	4.0/5.0 (n = 2)
60	-	-	3.0/4.0 (n = 2)	4.0/5.0 (n = 2)
120	-	-	0.5/4.5 (n = 2)	4.0/6.0 (n = 2)
180	-	-	3.67 ± 0.33 (n = 4)	3.50 ± 0.50 (n = 4)
360	-	-	3.67 ± 0.60 (n = 4)	4.83 ± 0.17 (n = 4)
1440 (24 hours)	0.50 ± 0.29 (n = 4)	2.50 ± 1.66 (n = 4)	-	-

5.3.3 GSH concentration in the blood and liver and ovarian tissue of possums and rats

To determine if any differences in VCD metabolism exist between possums and rats, GSH concentration was measured in hepatic and ovarian tissues of each species. Because possums served as their own controls their pre-treatment (baseline control) GSH concentration in plasma and RBCs were compared against post-treatment levels within each group to determine if there were any effects on GSH concentration as a result of anaesthesia or handling stress. Within each group, there were no significant differences between pre- and post-treatment GSH concentration in RBCs ($p > 0.331$; Figure 5.1) and plasma ($p > 0.237$; Figure 5.2). Therefore, any differences between groups can be attributed to effects of the VCD-related treatment.

Possum plasma and RBC GSH concentrations were not significantly different between treatment groups ($p > 0.059$; Figures 5.1 and 5.2). Both control and VCD treated possum hepatic GSH levels

were significantly decreased 15 minutes after treatment ($p < 0.011$ and $p < 0.008$, respectively). Twenty four hours after treatment, VCD treated possum hepatic GSH levels were increased compared to their pre-treatment and 15 minute post-treatment levels, although this was not significant ($p > 0.202$ and $p > 0.061$, respectively; Figures 5.3). Compared to their pre-treatment levels, ovarian GSH levels of control treated possums were elevated at 15 minutes ($p < 0.022$) and 24 hours post-treatment ($p < 0.019$) (Figure 5.4). Similarly, elevated ovarian GSH levels of VCD treated possum were observed at 15 minutes ($p < 0.019$) and 24 hours post-treatment ($p < 0.019$) compared with their pre-treatment levels (Figure 5.4).

The effect of VCD treatment on GSH concentration in rat RBCs, plasma, liver and ovaries was examined. VCD treatment of rats caused significant decreases in mean GSH concentration in the RBCs ($p < 0.029$; -15.0% of control mean) and in liver ($p < 0.0001$; -77.5% of control mean) and ovarian tissues ($p < 0.019$; -83.0% of control mean) during the 6 hours following treatment (Figures 5.1, 5.5 and 5.6 and Table 5.4). Mean plasma GSH concentration in VCD-treated rats was decreased by 25.9% of control mean, although this was not significant ($p > 0.206$; Figure 5.2 and Table 5.4).

Species comparisons were made between GSH concentration of possums and rats 15 minutes following treatment. Rat RBC and plasma GSH levels at pre-treatment and 15 minutes post-treatment were lower than that of possums; however this was not significant ($p > 0.06$; Figure 5.7 and 5.8). Pre-treatment hepatic GSH levels were similar between rats and possum, regardless of treatment ($p > 0.087$; Figure 5.9). However, 15 minutes post-treatment, VCD-treated rat hepatic GSH levels were significantly lower than that of control and VCD treated possums ($p < 0.002$). Pre-treatment ovarian GSH concentrations of control and VCD treated rats were significantly higher than that of possum pre-treatment levels ($p < 0.001$; Figure 5.10). However, regardless of the elevated ovarian GSH levels of rats at pre-treatment, VCD-treated rats were significantly decreased compared to both control and VCD-treated possums 15 minutes post-treatment ($p < 0.022$).

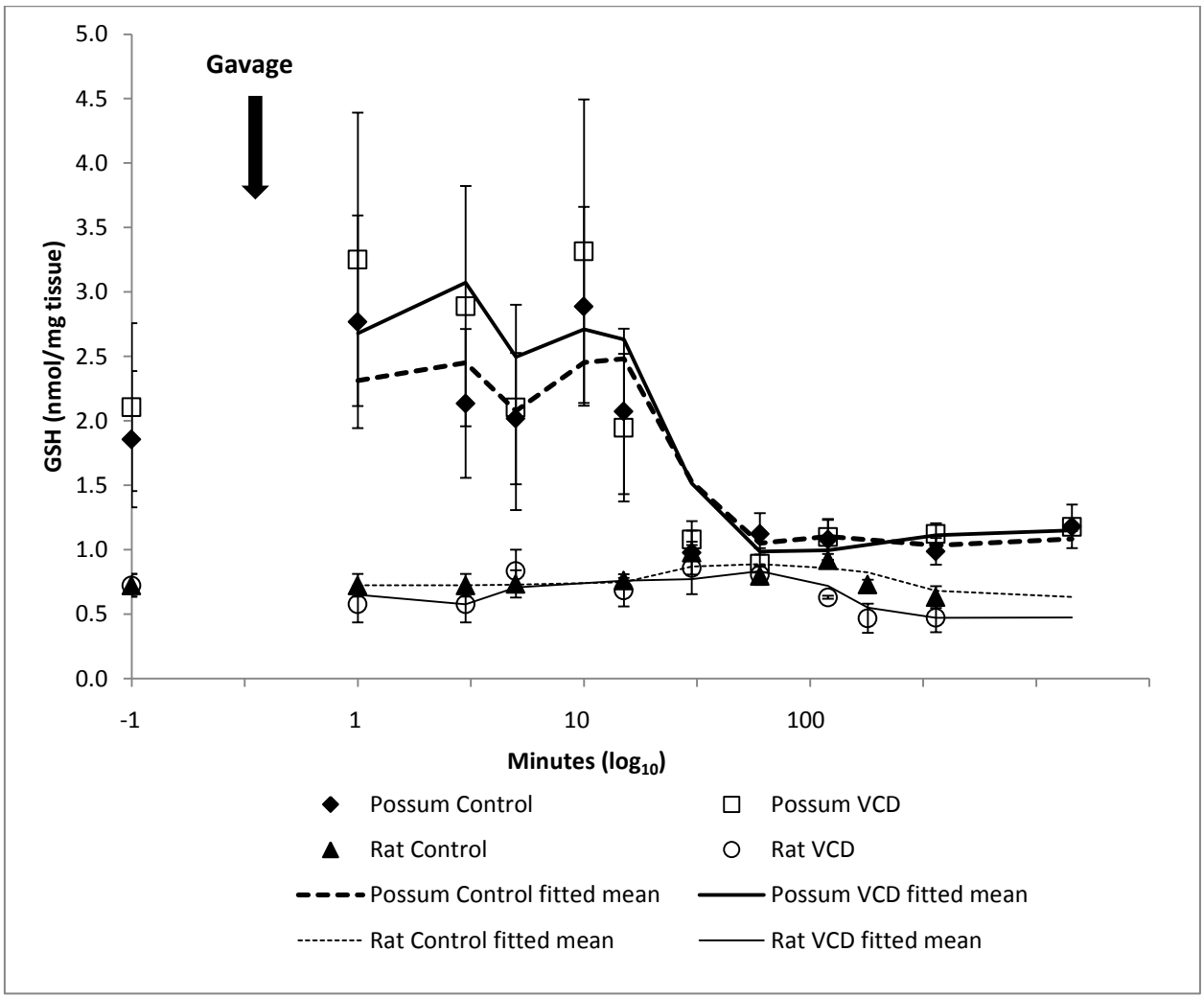

Figure 5.1 Red blood cell GSH levels of female brushtail possums and Norway rats following a single oral dose of oil or VCD (750 mg/kg) suspended in oil (1:3 v/v). Rats, n = 2/treatment at 1, 3, 5, 10, 30, 60 and 120 minutes post-treatment; n =4/treatment at -1 (pre-treatment), 15, 180 and 360 minutes post-treatment. Possums, n = 4/treatment at -1 (pre-treatment), 1, 3, 5, 10, and 15; n = 4/treatment at 30, 60, 120, 360 minutes and 24 hours post-treatment. Time axis is plotted on a logarithmic scale where -1.0 represents the pre-treatment sample. Statistical significance was set at $p < 0.05$. Vertical bars represent ± SEM.

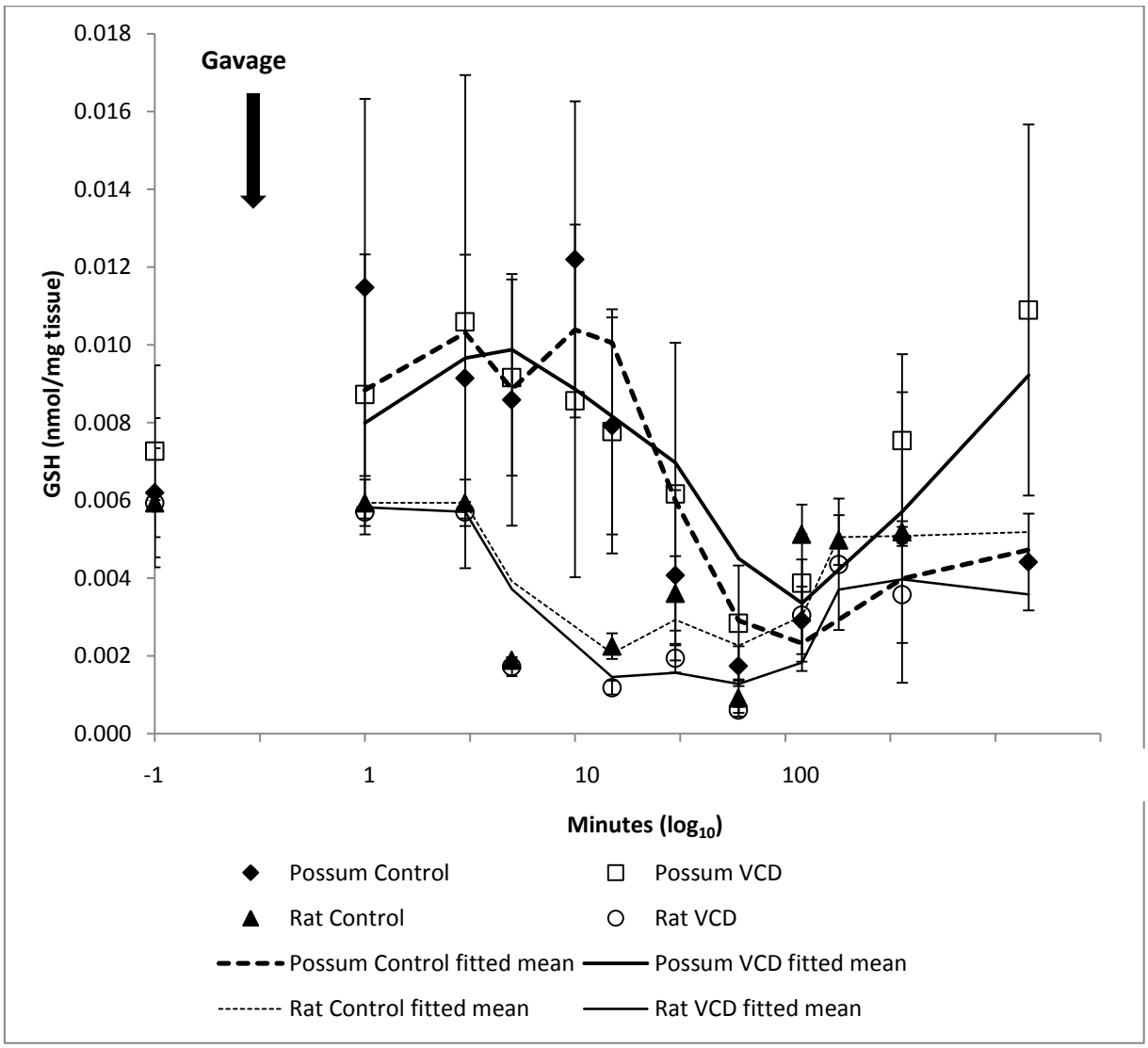

Figure 5.2 Plasma GSH levels of female brushtail possums and Norway rats following a single oral dose of oil or VCD (750 mg/kg) suspended in oil (1:3 v/v). Rats, n = 2/treatment at 1, 3, 5, 10, 30, 60 and 120 minutes post-treatment; n =4/treatment at -1 (pre-treatment), 15, 180 and 360 minutes post-treatment. Possums, n = 4/treatment at -1 (pre-treatment), 1, 3, 5, 10, and 15; n = 4/treatment at 30, 60, 120, 360 minutes and 24 hours post-treatment. Time axis is plotted on a logarithmic scale where -1.0 represents the pre-treatment sample. Statistical significance was set at $p < 0.05$. Vertical bars represent ± SEM.

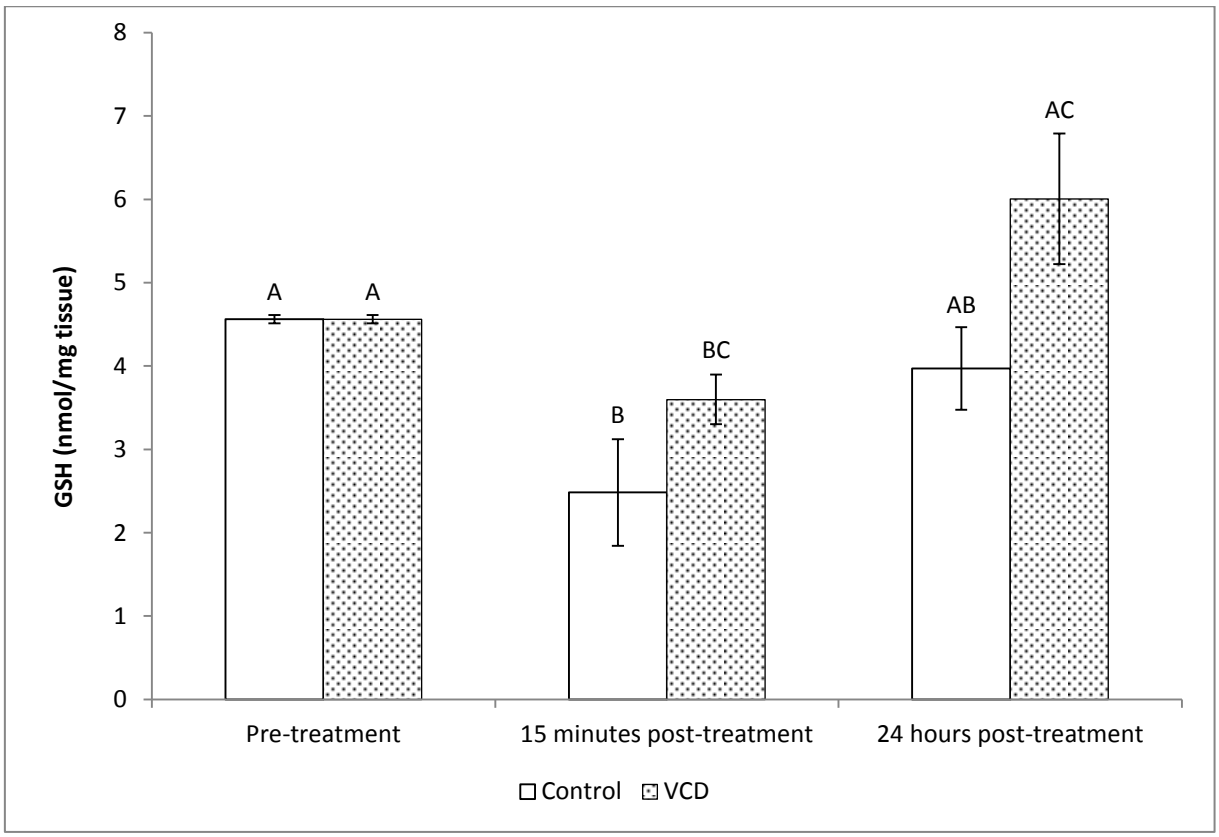

Figure 5.3 Average hepatic GSH levels of female brushtail possums following a single oral dose of oil or VCD (750 mg/kg) suspended in oil (1:3 v/v). Control treatment, n = 4/time point; VCD treatment, n = 4/time point. Values with difference letters are significantly different ($p < 0.05$). Vertical bars represent ± SEM.

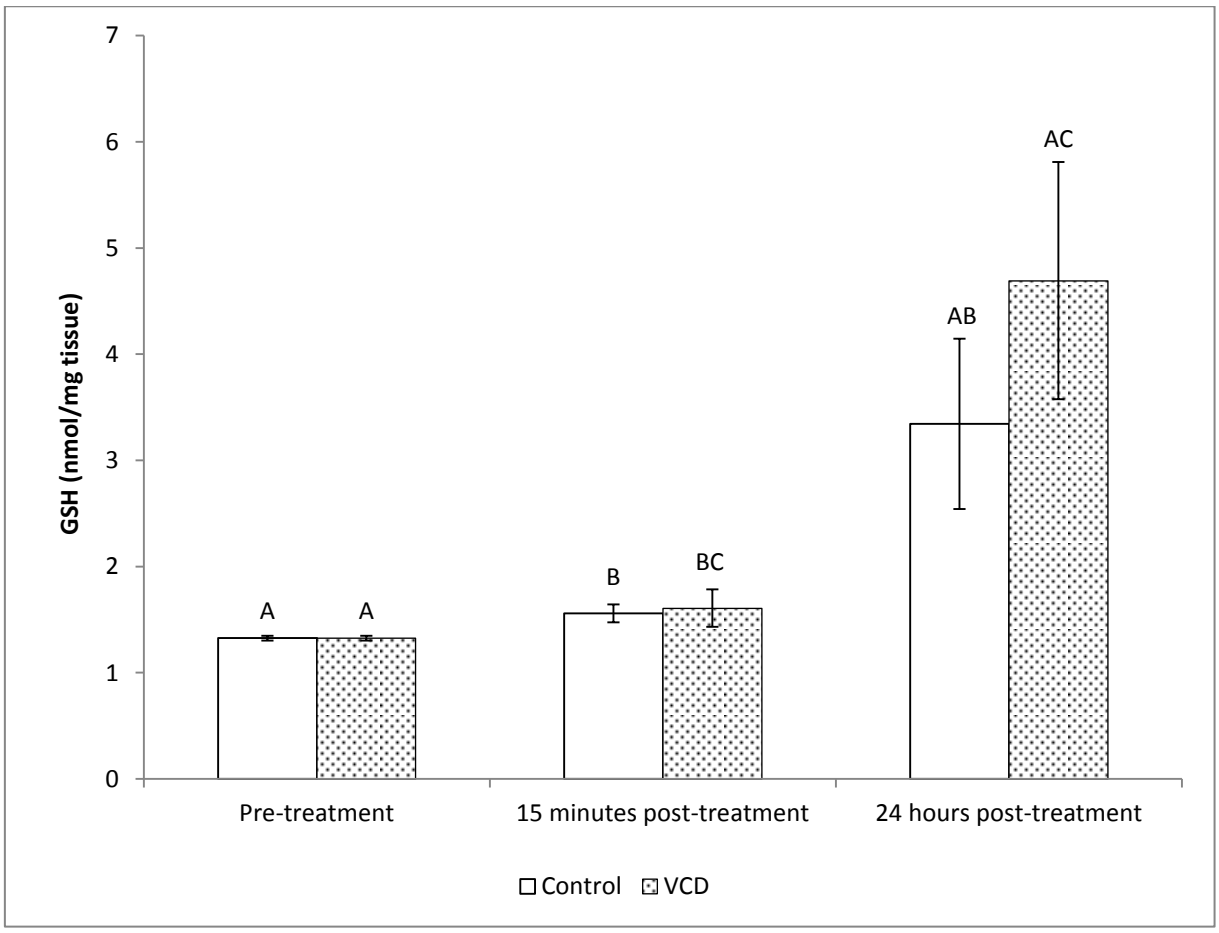

Figure 5.4 Average ovarian GSH levels of female brushtail possums following a single oral dose of oil or VCD (750 mg/kg) suspended in oil (1:3 v/v). Control treatment, n = 4/time point; VCD treatment, n = 4/time point. Values with difference letters are significantly different ($p < 0.05$). Vertical bars represent ± SEM.

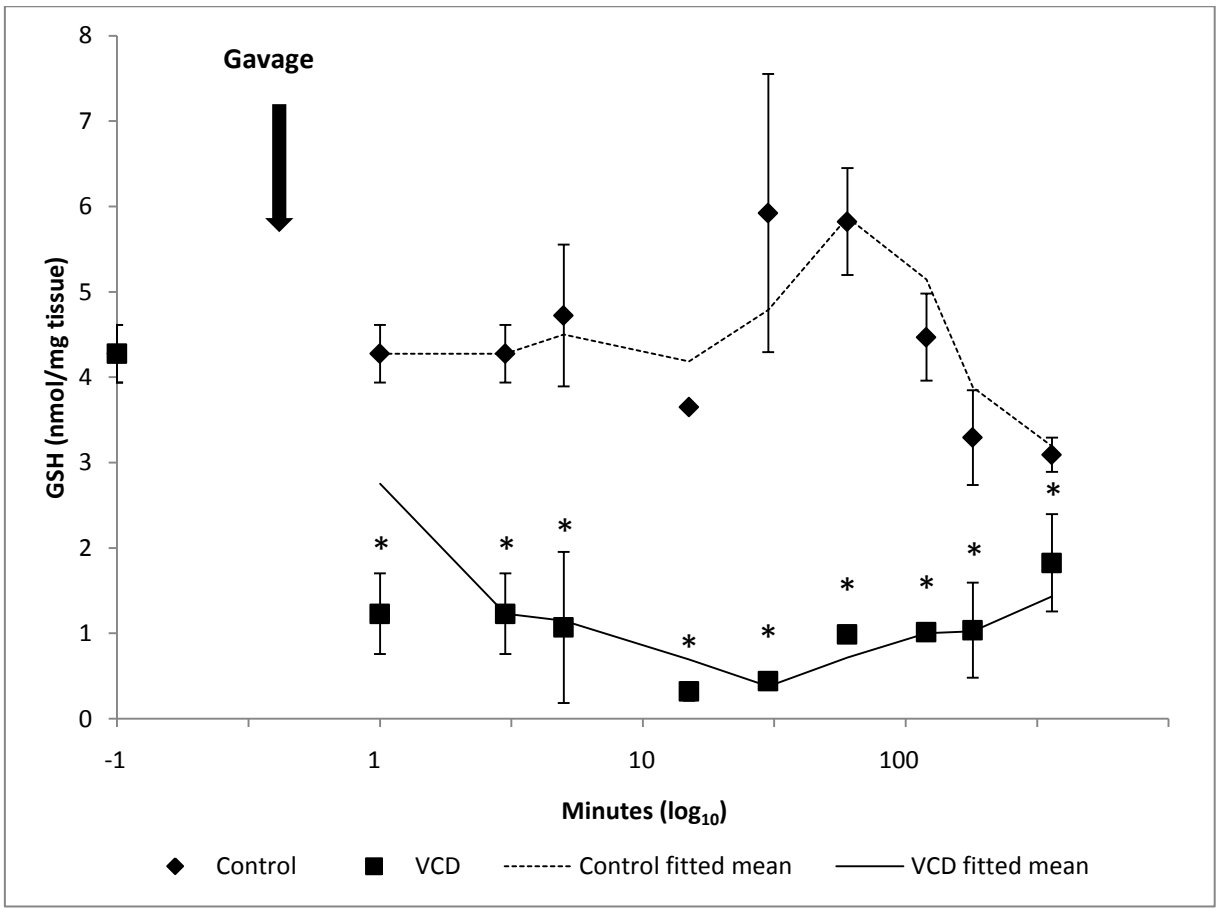

Figure 5.5 Hepatic GSH levels of female Norway rats following a single oral dose of oil or VCD (750 mg/kg) suspended in oil (1:3 v/v). N = 2/treatment at 1, 3, 5, 10, 30, 60 and 120 minutes post-treatment; n =4/treatment at -1 (pre-treatment), 15, 180 and 360 minutes post-treatment. Time axis is plotted on a logarithmic scale where -1.0 represents the pre-treatment sample. * indicates different ($p < 0.05$) from control. Vertical bars represent ± SEM.

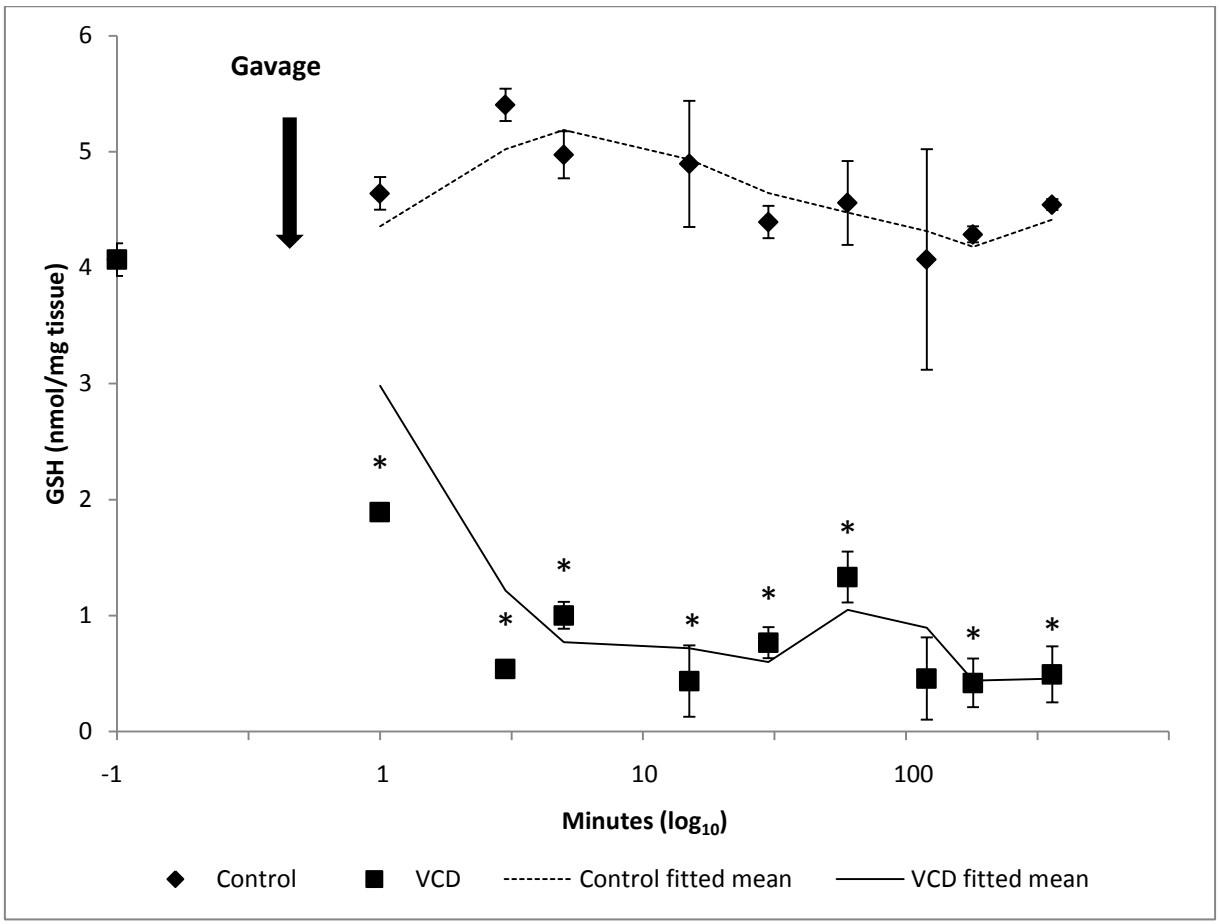

Figure 5.6 Ovarian GSH levels of female Norway rats following a single oral dose of oil or VCD (750 mg/kg) suspended in oil (1:3 v/v). N = 2/treatment at 1, 3, 5, 10, 30, 60 and 120 minutes post-treatment; n =4/treatment at -1 (pre-treatment), 15, 180 and 360 minutes post-treatment. Time axis is plotted on a logarithmic scale where -1.0 represents the pre-treatment sample. * indicates different ($p < 0.05$) from control. Vertical bars represent ± SEM.

Table 5.4 Average GSH concentrations in red blood cells, plasma, liver and ovaries of wild-caught female Norway rats during the 6 hours following a single oral dose of oil or VCD (750 mg/kg) suspended in oil (1:3 v/v). * indicates different from control ($p < 0.05$). Vertical bars represent ± SEM.

Tissue	GSH (nmol/µL or mg tissue)	
	Control treatment (n = 28)	VCD treatment (n = 28)
Red blood cells	0.79 ± 0.07	*0.67 ± 0.12
Plasma	0.004 ± 0.001	0.003 ± 0.001
Liver	4.41 ± 0.59	*0.99 ± 0.35
Ovaries	4.64 ± 0.31	*0.68 ± 0.20

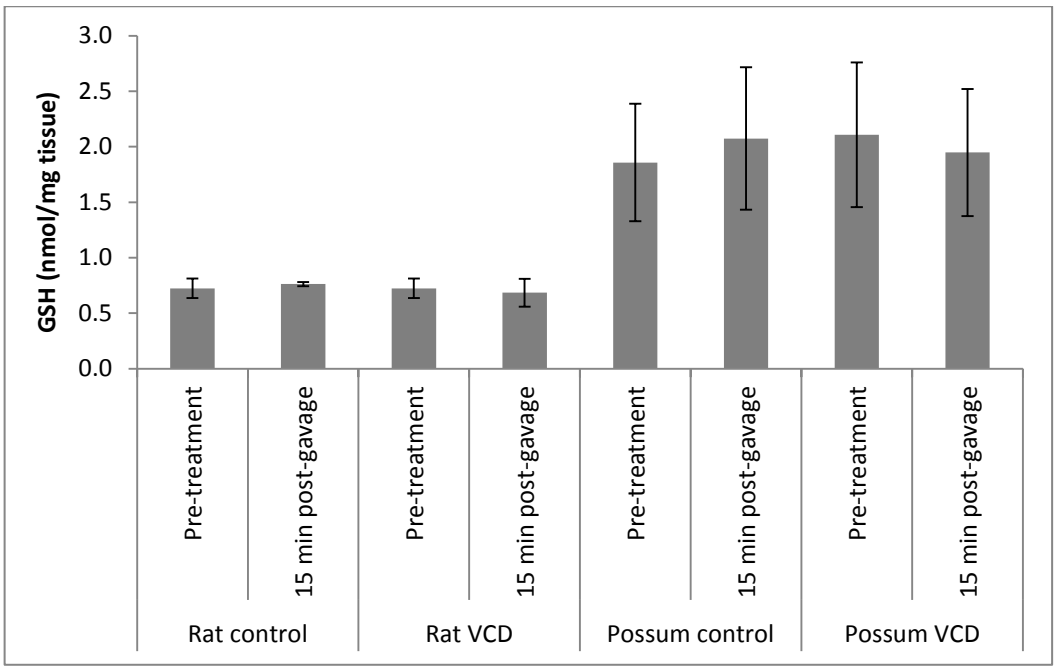

Figure 5.7 RBC GSH levels of wild-caught female brushtail possums and Norway rats during pre-treatment and 15 minutes following a single oral dose of oil or VCD (750 mg/kg) suspended in oil (1:3 v/v). Rats, n = 4/treatment/time point; possums, n = 4/treatment/time point. Statistical significance was set at $p < 0.05$. Vertical bars represent ± SEM.

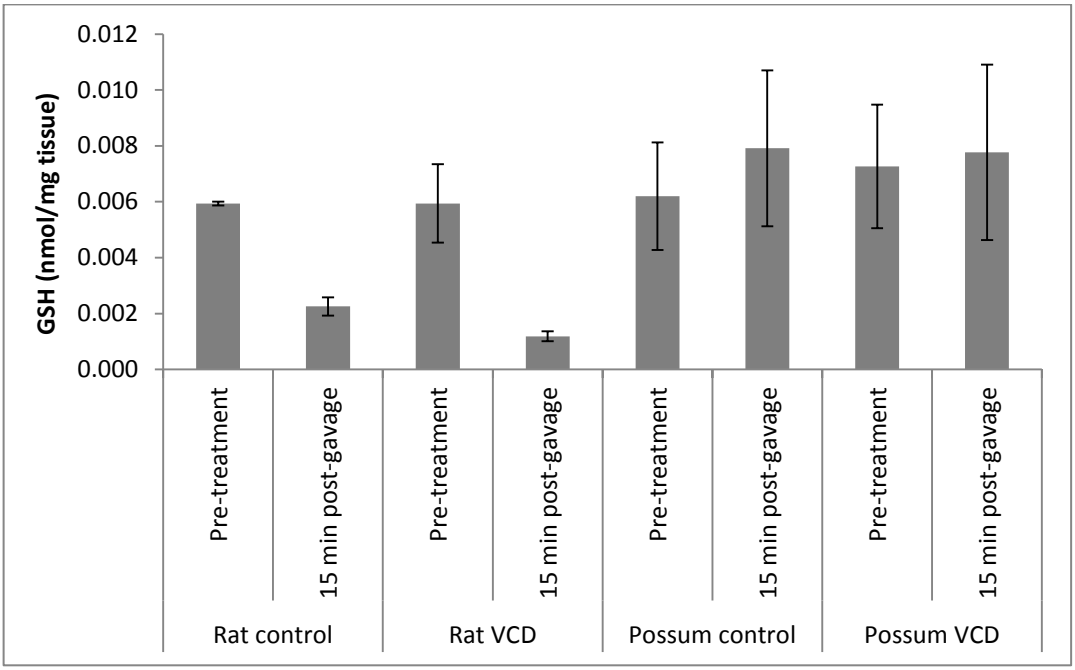

Figure 5.8 Plasma GSH levels of wild-caught female brushtail possums and Norway rats during pre-treatment and 15 minutes following a single oral dose of oil or VCD (750 mg/kg) suspended in oil (1:3 v/v). Rats, n = 4/treatment/time point; possums, n = 4/treatment/time point. Statistical significance was set at $p < 0.05$. Vertical bars represent ± SEM.

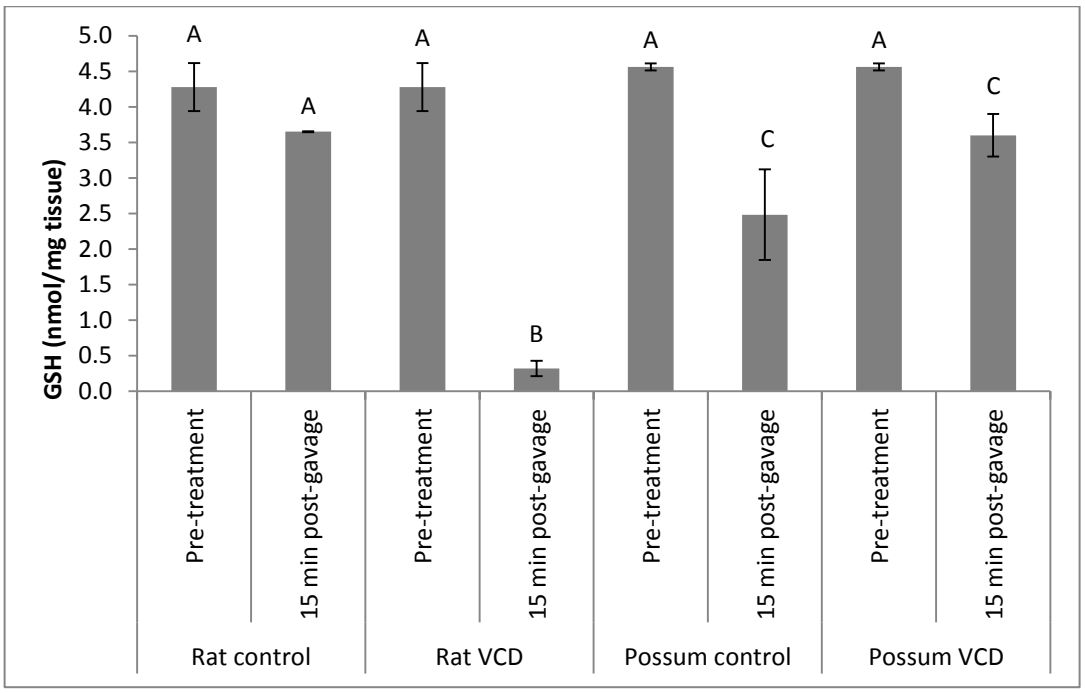

Figure 5.9 Hepatic GSH levels of wild-caught female brushtail possums and Norway rats during pre-treatment and 15 minutes following a single oral dose of oil or VCD (750 mg/kg) suspended in oil (1:3 v/v). Rats, n = 4/treatment/time point; possums, n = 4/treatment/time point. Values with different letters are significantly different ($p < 0.05$). Vertical bars represent ± SEM.

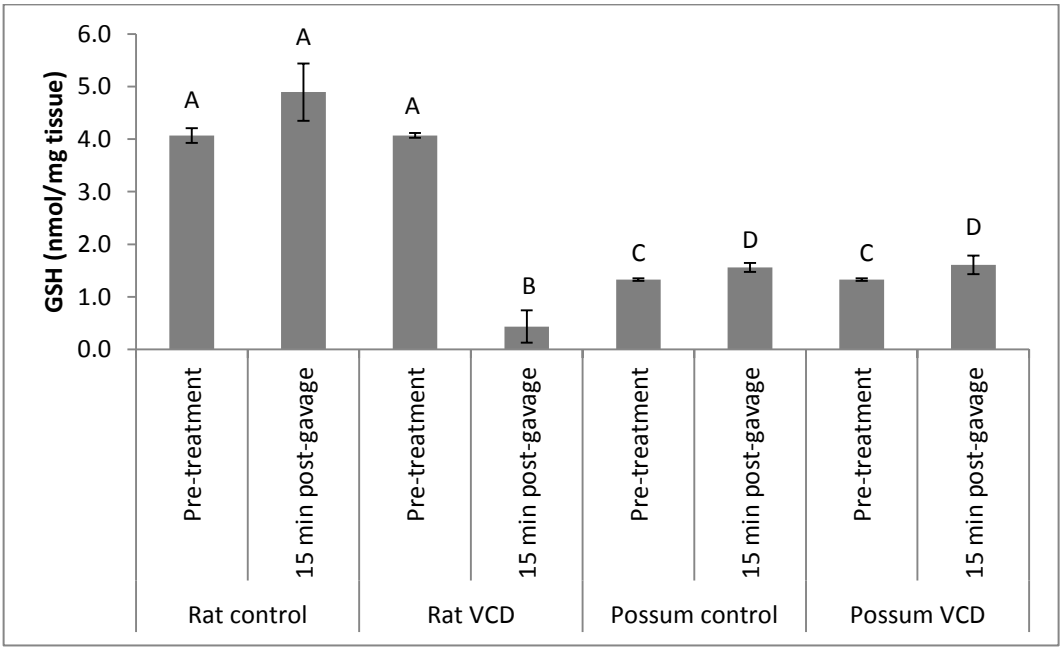

Figure 5.10 Ovarian GSH levels of wild-caught female brushtail possums and Norway rats during pre-treatment and 15 minutes following a single oral dose of oil or VCD (750 mg/kg) suspended in oil (1:3 v/v). Rats, n = 4/treatment/time point; possums, n = 4/treatment/time point. Values with different letters are significantly different ($p < 0.05$). Vertical bars represent ± SEM.

5.4 Discussion

In an attempt to ascertain species differences in VCD-induced ovarian toxicity of wild Norway rats and brushtail possums, the fate and metabolism of orally administered VCD was examined. VCD was detected in the blood of possums and rats as early as 1 to 2.5 minutes following treatment, demonstrating that VCD, once present in the stomach, is rapidly absorbed. The concentration of VCD in the blood was significantly greater in rats compared with possums. One reason for this could be that the amount of VCD available for absorption from the stomach into the blood was higher in the rats. However, 15 minutes after treatment there were no differences in the amount of VCD recovered in the stomach contents of each species. It is reasonable to assume that any VCD not present in the stomach had either been absorbed into the blood stream or was rapidly hydrolysed by stomach acid. Therefore, these differences in blood VCD concentration during the first 15 minutes following treatment may suggest differences in the stomach environment or the VCD uptake rate for each species.

VCD, when exposed to acidic environments, is hydrolyzed to an inactive tetrol metabolite (Figure 2.5). The pH of possum stomach contents ranges between 1.0 and 2.0 and does not appear to be affected by time since feeding (Drs J. Duckworth & F. Molinia, pers. comm.). Conversely, pH of rodent stomach contents ranges between 3.2 (fed) and 3.9 (fasted) (McConnell et al., 2008). Considering these species differences in stomach pH it is plausible to assume that VCD administered orally to possums would be hydrolysed at a faster rate than in rats. Fifteen minutes after VCD treatment, the stomach pH of both possums and rats was elevated but this effect was more pronounced in the possum stomach (possum: pH increase of 212% of control; rat: pH increase of 30% of control). These species differences in stomach pH following oral administration of VCD may explain why VCD was still detected in the blood of rats for at least 30 minutes post-treatment and why VCD had disappeared by 10 to 15 minutes post-treatment in the possums. However, as there were only three measurements of stomach pH recorded from the possums, these conclusions should be treated with some caution.

VCD treatment caused GSH depletion in the liver and ovary of the rat up to 6 hours following treatment. Previous studies in rats and mice treated with VCD show similar patterns wherein GSH is reduced from 1 to 6 hours following treatment (Giannarini et al., 1981; Salyers, 1995). Rodent studies have also revealed that the two expoxides of VCD are substrates for GSH conjugation which form VCD-monoGSH and VCD-diGSH adducts (Rajapaksa, 2007). In mice, VCD-monoGSH and VCD-diGSH adducts do form in both the liver and ovaries and by 24 hours post-treatment it is presumed that the adducts are excreted via the urine or bile (Salyers, 1995) as levels are undetectable (Rajapaksa, 2007).

Conversely, VCD treatment of possums did not cause hepatic or ovarian GSH levels to differ from controls. However, it must be noted that there are only two post-treatment time points at which samples were collected. One possibility is that VCD did induce reductions in possum GSH levels that were missed by this sampling regimen. Interestingly, hepatic and ovarian GSH levels of the VCD

treated possums were higher at 24 hours than at pre-treatment and 15 minutes. Previous research has demonstrated that following initial VCD-induced GSH reduction in rodents hepatic and ovarian GSH levels rebound and surpass both their initial levels and those of controls by 25 hours (Salyers, 1995). This rebound effect of liver GSH following initial depletion in rodents is common and elevated GSH can be sustained for several days (Viña, 1990). It is possible that the elevated GSH levels of VCD-treated possums measured at 24 hours were responding in a similar fashion. If so, this would give support to the possibility that VCD-induced effects were occurring to some degree in the liver and ovaries of possums. VCD-treated rats did not survive to 24 hours so it is not possible to speculate whether a similar pattern in overcompensation of GSH would have occurred during this study.

An alternative and more likely explanation for the lack of effects to possum GSH levels is that there were insufficient amounts of VCD reaching the liver and ovaries in the possums to impact on GSH metabolism in those tissues. This is supported by the low levels of VCD detected in possum blood and similar patterns observed in GSH levels of control possums at 24 hours. It is more likely that the observed increase in GSH levels measured at 24 hours represents a recovery (rebound) period following anaesthetic-induced GSH reduction. Possums were continuously kept under anesthesia for approximately 15 to 17 minutes during pre- and post-treatment blood sampling. Hepatic GSH levels in both control and VCD-treated possums at 15 minutes post-treatment are slightly reduced compared to pre-treatment levels which may be indicating effects from anesthesia. Although published studies have demonstrated that isoflurane/O_2 anaesthesia does not affect plasma GST levels (Allan et al., 1987; Hussey et al., 1988) or induce hepatotoxicity in rodents and humans (Raper et al., 1987), the present observation could be interpreted as evidence for isoflurane having hepatotoxic effects in the possum.

There were no differences in hepatic GSH pre-treatment levels of untreated (control) rats and possums. Following treatment with VCD GSH levels in the liver and ovary of rats were much lower at 15 minutes than in the possums. At first glance, this may seem to suggest that the rat is more sensitive to VCD-induced GSH detoxification but again the very low amount of VCD measured in the blood of the possums indicates that there may have been insufficient levels of VCD reaching the liver and ovaries of possums to induce any reductions in GSH. The limited number of data points available from this study makes it difficult to compare VCD detoxification rates between possums and rats. It is well established that there are species differences in metabolism between laboratory-bred mice and rats (Kao et al., 1999; Keller et al., 1997; Salyers, 1995; Smith et al., 1990b) and so it is possible that there are differences in VCD metabolism between possums and rats. In rodents, metabolism of VCD is also achieved through the action of EH (Hoyer and Sipes, 2007; Van Kempen et al., 2011). Measurement of EH content following VCD treatment in possums may bring to light potential species differences in VCD metabolism. It was my aim to examine VCD-induced effects on hepatic and ovarian EH levels in each species. However, the methods were beyond the scope of this project.

Potential similarities or differences between possum and rat metabolism may further be explained by examining other metabolic pathways. 4-Vinylcyclohexene (VCH), the parent compound of VCD, is

metabolized to VCD in a Phase I reaction primarily by CYP450 (Doerr-Stevens et al., 1999; Doerr et al., 1996; Springer et al., 1996c). Rats and marsupials, including possums, the tammar wallaby (*Macropus eugenii*) and the koala (*Phascolarctos cinereus*), have demonstrated similarities in the content, activity and inhibition of CYP450 (Stupans et al., 2001). Although the effect of VCH treatment has not been compared between rats and possums, it can be hypothesised that because of these CYP450 similarities, possums may metabolize VCH via a Phase 1 reaction in a similar fashion to that of rats. It may be further hypothesized that similarities in Phase II reactions (i.e. GSH and EH) exist between these two species. In this report, untreated rats and possums showed similarities in hepatic GSH content, supporting the potential for species similarities in Phase II metabolism. However, without a broader understanding of the effects of VCD on the content and activity of possum Phase II metabolism little can be concluded on the differences or similarities between rats and possum metabolism.

The dietary evolution of possums may suggest a potential enhanced ability for metabolic clearance of xenobiotics such as VCD. Brushtail possums evolving in Australia relied greatly on specific *Eucalyptus* species, which are high in plant secondary metabolites (PSMs), as their major food source (Stupans et al., 2001). As a result of this dietary specialization, it has been postulated that they evolved highly efficient Phase I and II enzyme systems for metabolism of PSMs. Therefore, although there may be species similarities in the GSH content, it is possible that possums are simply more efficient at metabolizing xenobiotics such as VCD. A study examining the toxicity of a commonly used poison, brodifacoum, demonstrated that the length of sickness and time to death were shorter in Norway rats compared with possums at the same dose per kg live weight (Littin et al., 2000). There were no species differences in absorption and distribution of the poison (Littin et al., 2000), therefore suggesting that rats have reduced detoxification capacity compared to possums. In addition, it has been demonstrated that carnivorous marsupials are able to metabolize the commonly used poison, sodium monofluoroacetate (1080), more readily than carnivorous eutherians of comparable size and diet (feral cat, *Felis catus*; dingo, *Canis familiaris dingo*) (McIlroy, 1981). Collectively, these studies suggest that marsupials possess an enhanced metabolic ability for detoxifying xenobiotics such as VCD than that of rodents.

In order to utilize VCD as a chemosterilant for wild pest populations such as possums and rats, it should be deliverable through an oral route. The data presented here support the conclusion that the high acidic environment of the possum stomach poses an initial barrier for orally delivered VCD. If sufficient quantities of VCD were able to overcome the high stomach acidity of possums, the questions would be whether ovarian toxicity could occur or would the presumed high efficiency of the possums phase II hepatic reaction negate any possible effects on the ovarian follicle populations? It was our aim to provide proof-of-principle for VCD-induced primordial follicle depletion in possums through *in vitro* ovarian follicle culture. However, due to the difficulties discussed in Chapter 6, these trials were not conducted. Therefore, examination of VCD and VCH *in vitro* hepatic metabolism in

possums and rats is described in the following chapter. In addition, there is an exploration of the *in vitro* effects of stomach acidity on VCD hydrolysis for each species.

Chapter 6

The fate of 4-vinylcyclohexene diepoxide in blood and stomach tissues and its effects on liver metabolism of female brushtail possums and Norway rats

6.1 Introduction

In the previous chapter the effects of orally delivered VCD on stomach acidity and on VCD's chemical clearance rates, its uptake into the blood, and its effects on liver and ovarian glutathione (GSH) levels in possums and rats were examined. VCD treatment resulted in rat stomach pH increasing within 5 minutes of treatment and it remained elevated for up to 6 hours whereas the pH of possums' stomachs did not change. The highly acidic nature of possums' stomach contents (pH between 1.0 and 2.0; Drs J. Duckworth & F. Molinia, pers. comm.) and their ability to maintain low pH levels following VCD treatment would infer that VCD hydrolysis should occur rapidly; yet some of the VCD dose was still detected at similar levels in both rats and possums at 15 minutes post treatment. This, in combination with the lower VCD levels detected in possum blood compared with that of rats, may indicate that the stomach contents of possums are able to absorb and protect the chemical from acid hydrolysis to some degree.

Protection of VCD may prolong absorption of the chemical from the stomach and intestines into the blood. It is believed that VCD's ovarian toxicity is largely due to repeated exposure, thereby overwhelming and ultimately reducing the capacity of the detoxifying enzymes (i.e. liver and ovarian derived GSH and epoxide hydrolase) (Hu et al., 2002; Keating et al., 2008a; Keating et al., 2010). Therefore, the presumed slower rate of VCD absorption in the possum gastrointestinal tract may in fact prove serendipitous as it could offer a prolonged period of time for the detoxifying enzymes to become overwhelmed by repeated, slow exposure to VCD.

It was further speculated in Chapter 5 that the dietary evolution of possums may explain, in part, why VCD is not effective at reducing their immature ovarian follicle populations. Brushtail possums evolving in Australia consumed specific *Eucalyptus* leaves, which are high in plant secondary metabolites (PSMs) (Stupans et al., 2001). As a result of this dietary specialization, it has been postulated that possums have evolved highly efficient Phase I and II enzyme systems for metabolism of PSMs. Therefore, it was proposed that possums would likely metabolise VCD more rapidly and efficiently compared with rats; presuming sufficient quantities of VCD were able to reach the liver and ovaries.

The studies presented here first examine the *in vitro* effects of acid on VCD hydrolysis and whether the presence of stomach contents of each species alters the rate of hydrolysis. VCD's effects on GSH

are further examined through *in vitro* incubation of liver tissue from possums and rats with VCD. It was predicted that the stomach contents of possums would absorb, retain, and protect VCD more effectively than those of rats, thereby providing a potential protection mechanism of possums against xenobiotic toxicity. In addition, it was predicted that possum liver tissue would metabolize VCD more readily than the liver tissue of rats.

A pilot study examining potential differences in phase I metabolism of each species was also investigated. 4-Vinylcyclohexene (VCH), the parent compound of VCD, is metabolized to VCD in a Phase I reaction primarily by the hepatic-derived enzyme superfamily cytochrome P450 (CYP450) (Doerr-Stevens et al., 1999; Doerr et al., 1996; Springer et al., 1996c). Therefore, liver microsomes of possums and rats were incubated *in vitro* with VCH and changes to CYP450 concentrations compared over time. It was predicted that possums would be more efficient than rats at metabolizing VCH.

6.2 Materials and Methods

6.2.1 Reagents

VCD, VCH 1,2-isomer, cyclohexanone, ethyl acetate, sunflower oil, Attane™ isofluorane, and sodium pentobarbital were obtained from vendors and used for experimental procedures as previously mentioned in Chapter 3. M199 medium (M4530) was purchased from Sigma-Aldrich (Auckland, NZ). 4-vinyl-1-cyclohexene (VCH; lot #A0332276; cas. 100-40-3) was purchased from Acros Organics (New Jersey, USA). Sodium hydrosulphite, glycerol and sucrose were purchased from Sigma-Aldrich (Auckland, New Zealand). EDTA and TrisCl , used to create buffers, were purchased from BDH (Radnor, USA). Carbon monoxide was purchased from BOC gases (Wellington, New Zealand).

6.2.2 Animal procedures

Animals (rats, n = 6; possums, n = 11) were anaesthetized (5% isoflurane in O_2 at 2 L/min) and once sedated blood samples were collected via intracardiac puncture into lithium heparinised tubes (BD Vacutainer®) and then treated as outlined below. Animals were euthanized with an intracardiac injection of pentobarbitone (125 mg/kg). The stomach was excised and the contents removed, weighed and pH measured using pH indicator strips (Merck) and then treated as outlined below. The liver was immediately removed, weighed and then prepared for GSH or CYP450 analysis as outlined below.

6.2.3 VCD hydrolysis in a range of acidic environments

In order to better understand the effects of acid on VCD hydrolysis, VCD was exposed to a range of acid environments for a set period of time. The physiological buffer solution, M199, was used as the standard diluent for all experimental procedures. In a pilot study, the effect of diluting with ultrapure water and M199 were compared to ensure that the additives in the M199 medium were not influencing VCD hydrolysis or protecting VCD from acid degradation. The pH of the ultrapure water was adjusted to 1.0 or 2.5 pH (± 0.2) while M199 was adjusted to 1.0, 2.5, or 5.0 (± 0.2). Both diluents were

warmed to 21 ± 1 °C and then aliquoted into glass scintillation vials (2.0 mL/vial). VCD (0.0, 1.84, 3.69, 5.53, or 7.40 mM; mixed 1:3 v/v in oil) was added to each of the solution combinations, mixtures were vortex mixed for 15 seconds and then placed in a shaking incubator (300 rpm, 21°C) for 15 minutes. Reactions were then terminated by the addition of 2 times the sample volume of ethyl acetate (EA) containing the internal standard 4-vinyl-1-cyclohexene-1,2-isomer (VCH-isomer; 1.05 mM). VCD and its tetrol metabolites were extracted from each solution in three successive rounds wherein vials were vortex mixed for 30 seconds and shaken for 10 minutes at 500 rpm. Phases were separated by centrifugation (3000 x g for 10 min, 21°C) and three aliquots (500 µL) of the organic layer (top layer) were placed into 2.0 mL amber Crimp-Top vials. The samples were run on gas chromatography (GC) in triplicate and VCD quantified as outlined in Chapter 3. Following this pilot study there was some evidence that a portion the VCH-isomer (internal standard) was degraded during the EA extraction step. This was indicated by the presence of VCD in the control vials (no VCD added) following acid treatment. An additional trial utilizing the internal standard cyclohexanone (1.18 mM) was run as verification for the presence of VCD in the control vials. Following positive confirmation, the effect of VCH-isomer conversion to VCD was thereafter corrected by subtracting the concentration of VCD in control samples from the VCD concentrations in each corresponding treatment vial. From these studies it was determined that there were no differences in the hydrolysis rate of VCD diluted in either water or M199. Therefore, all subsequent experiments used M199 as the diluent.

6.2.4 VCD hydrolysis in stomach contents and blood

Stomach treatment

The effect of stomach acid and stomach contents on VCD hydrolysis was examined in possum and rats. To mimic temperatures found *in vivo*, *in vitro* conditions were matched by incubating the stomach contents of each species at 37°C throughout the experiment. The pH of possum stomach contents ranges between 0.5 and 2.0 (Chapter 5; Drs J. Duckworth & F. Molinia, pers. comm.) while that of a rodent stomach ranges between 0.5 and 3.7 (Chapter 5). For between-species comparisons and to standardise pH levels across replicates, the pH of possum stomach contents was adjusted to 1.0 ± 0.1 or 2.5 ± 0.1 (i.e. typical possum *in vivo* pH range) and rodent stomach contents were adjusted to a pH of 2.5 ± 0.1 (i.e. typical rat *in vivo* pH range). Stomach contents were collected fresh from animals just prior to the trial starting. For each species, equal aliquots of stomach contents (0.1 – 0.2 g) were placed into glass scintillation vials and 4 times the sample volume of M199 (heated to 37°C) was added and the pH of each sample was then altered to within ±0.1 of desired range. For each unique treatment a corresponding blank (M199 only, equal pH and volume as stomach contents) and a corresponding neutral blank (pH 7.0) were examined at each time point. To avoid stomach contents adhering to the sides of the container, samples were centrifuged for 2 minutes (3000 x g at 37°C) to ensure all stomach contents were retained within the solution. Reactions began by the addition of VCD (0.0, 1.22 or 2.43 mM mixed 1:3 v/v in oil) and gently vortex mixed (500 rpm) for 15 seconds to

ensure even distribution throughout the stomach contents matrix. Samples were placed into a shaking incubator at 37°C and reactions terminated at 3, 5, 10, 15, 30, 60, 120 and 360 minutes with the addition of EA containing the internal standard, VCH-isomer. VCD was extracted and samples prepared for GC analysis as stated in section 6.2.3.

Whole blood treatment

The aim of this experiment was to examine if VCD is affected by the blood matrix as it is transported to the target organ, the ovary. Whole blood (3.0 - 4.0 mL) was collected into lithium heparin vacutainer tubes (BD Vacutainer®) which were inverted several times, and then kept at 37°C during the experimental period. For each species' blood sample, two treatments were prepared in glass scintillation vials: A) whole blood (0.167 – 0.375 mL) diluted with 4 times the sample volume of M199 (heated to 37°C) or B) whole blood only (0.67 – 1.5 mL; volumes were matched to treatment A). A corresponding blank (M199 only; final volume matched to treatments A and B) was also examined at each time point. Reactions began by the addition of VCD (0.0, 0.185 or 0.364 mM mixed 1:3 v/v in acetone) and gently vortex mixed (500 rpm) for 15 seconds to ensure even distribution throughout the blood matrix. Samples were placed into a shaking incubator at 37°C and reactions terminated at 3, 10, 15, 20, and 30 minutes with the addition of EA containing the internal standard, VCH-isomer. VCD was extracted and samples prepared for GC analysis as stated in section 6.2.3.

6.2.5 Glutathione assay sample preparation and analysis

The effect of VCD on possum and rat Phase II metabolism was examined by measuring *in vitro* changes in hepatic GSH concentrations. Originally the aim was also to examine *in vitro* the effect of follicle stage (primordial, primary, and secondary follicles) on the GSH response to VCD; hence, the selection of M199 as the sample diluent. However, follicle isolation techniques (Flaws et al., 1994) proved difficult and we could not procure sufficient amounts of ovarian tissue from appropriately aged animals (pouch young aged 80-100 days: age at which primordial follicles are first formed) (Eckery et al., 1996) during the course of the studies. Attempts at *in vitro* culturing of 1 x 1 mm explants (Wandji et al., 1996) from the cortex of adult possum ovaries were also made (D. Eckery, personal comm.). Although harvesting of the explants was successful, the resulting tissue destruction resulted in low follicle survival rates. In addition, primordial follicles were not evenly distributed throughout the cortex of each ovary and therefore very large replicates would be required to obtain statistically significant results. It was evident that variation would likely affect the power of any statistical analyses and solving such difficulties was beyond the scope of this project.

For each species, livers were perfused with ice-cold PBS and then placed into aluminium foil, sealed and snap frozen with liquid nitrogen. Individually, frozen livers, wrapped in aluminium foil, were broken into small particles using a mallet and then combined into one liver stock preparation. Initially, microsomes, prepared as outlined for the CYP450 experiments (section 6.2.6), were utilized for these GSH experiments. However, interference with the microsomal buffers occurred when running the

GSH assay. In addition, purified microsomal GSH concentrations were below the detection limit. Therefore, supernatant 9000 (S9) liver fractions were prepared by adding 2.5 g of liver stock into an ice-cold glass homogenizer and then 4 times the volume of ice-cold PBS was added and tissue homogenized with 10 passes of the pestle while on ice. The homogenate was transferred to conical tubes and centrifuged at 10,000 x g for 30 minutes at 4°C. The concentrated supernatant was then transferred to cryostat vials and immediately snap frozen. The protein content of the S9 fraction was measured using the Bradford protein assay (Bio-Rad Laboratories) using bovine serum albumin (BSA) as a standard.

For each species, the S9 fraction was slowly defrosted on ice to prevent activation or denaturing of enzymes. The solution was diluted to a protein content of 15 mg/mL using ice-cold PBS and then divided into two glass scintillation vials. One vial was kept on ice while the other vial was placed in a boiling water bath (100 ± 5°C) for 10 minutes to ensure all protein was denatured and then the sample was divided evenly into two glass scintillation vials, creating two treatments: 1) negative control: heat-shocked S9 fraction (no additives); 2) negative treatment: heat-shocked VCD-treated S9 fraction (7.13 mM). Samples were allowed to cool to room temperature before trial start. The live S9 fraction sample kept on ice was divided evenly into two glass scintillation vials, creating two treatments: A) positive control: live S9 fraction (no additives); and B) positive treatment: live VCD-treated S9 fraction (7.13 mM). Live sample vials were then placed into a shaking incubator (37^0C) for 10 minutes at 200 rpm to allow enzymes to activate. The trial began with the addition of VCD into the live and heat-shocked treated vials. At 0, 5, 10, 15, 20 30, 45 and 60 minutes 100 µL of each solution was removed, placed into a cryostat tube and snap frozen with liquid nitrogen. All vials were kept in a heated shaker (37°C) during the incubation period and all experimental procedures were run in duplicate.

In preparation for GSH concentration quantification, all samples were slowly defrosted on ice. Samples were then deproteinized by removing 20 µL of sample and adding it to 560 µL (28 times the sample volume) of ice-cold 5% SSA. Samples were incubated at 4°C for 10 minutes and then centrifuged at 10,000 x g for 10 minutes at 4°C. GSH concentration (nmol/mg protein solution) was measured in triplicate for each sample using methods described in the GSH assay kit (Sigma, cat# CS0260) with changes as outlined in Chapter 3.

6.2.6 CYP450 pilot study

Hepatic CYP450 sample preparation

For each species, livers were perfused with ice-cold PBS and then snap frozen with liquid nitrogen. For each species, (possums, n = 8; rats, n = 8) the livers of individual animals were combined to make one stock, wrapped in aluminium foil and then broken into small particles using a mallet. Approximately 5 g of liver stock was measured into an ice-cold glass homogenizer and 4 times the sample volume of GET buffer (20% glycerol, 0.5M EDTA, 1.0M TrisCl, pH 7.4) was added and liver homogenized with 15 passes of the pestle. The homogenate was then aliquoted into ultracentrifuge

tubes and centrifuged at 9000 x g for 30 minutes at 4^0C using a Beckman JL-90 ultracentrifuge fitted with a JA-20 rotor. The supernatant was pooled and then transferred into ultracentrifuge tubes and centrifuged at 105,000 x g for 60 minutes at 4^0C. The supernatant was discarded and the pellet suspended with SET buffer (1.0 mL/g organ weight; 25% sucrose, 0.5M EDTA, 1M TrisCl, pH 7.4). To ensure the pellet was well redistributed, the mixture was transferred to an ice-cold homogenizer and homogenized with 5 passes of the pestle while on ice. The homogenate was transferred back into ultracentrifuge tubes and centrifuged at 105,000 x g for 60 minutes at 4^0C. The supernatant was discarded and the pellet suspended in GET buffer (1.0 mL/g organ weight). The suspension was snap frozen (1.0 mL aliquots) in liquid nitrogen and stored at -80^0C until analysis. Protein content was analysed using the Bradford protein assay (Bio-Rad Laboratories) using BSA as a standard.

CYP450 assay method development and validation

Optimization of carbon monoxide exposure method and incubation time

To determine the optimal method for measuring CYP450 content in possum and rat hepatic microsomes, two carbon monoxide (CO) exposure methods were tested and compared (Choi et al., 2003; Omura and Sato, 1964). For each species, hepatic microsomes were transferred from a -80^0C freezer into a -20^0C freezer for 30 minutes and then placed on ice to allow samples to defrost slowly. Microsomes were diluted to 1.0 mg protein/mL with GET buffer and then bubbled with CO for 30 seconds (0.5 mL aliquots in glass scintillation vials) (Omura and Sato, 1964) or placed into a 96-well flat bottom plate (200 µL aliquots). The plate was then placed into a CO chamber (Figure 6.1), the chamber was sealed and then CO was allowed to flow freely into the chamber for 2 minutes (0.5 L/min) while on a shaker (300 rpm) (Choi et al., 2003). The exit hose was placed into a water-filled beaker to ensure gas flow was continuous. The CO chamber was located within a fume hood to avoid any potential harm from gas vapours. For each method, following CO treatment samples were transferred into disposable cuvettes (10mm light path; LPI, Italy) and the baseline absorbency recorded. The haemoproteins in the sample were then reduced (solubilized) by the addition of 5.0 mg of sodium hydrosulphite (SHS) powder, left to stabilise for 2 minutes, and then the resulting spectra between 400 and 500 nm were measured (Omura and Sato, 1964). It was determined that the CO chamber (Choi et al., 2003) would be the most appropriate method to use as both methods offered similar repeatability but absorbency readings were higher for each species (Figure 6.2) and sample analysis would be more efficient and precise when using the CO chamber.

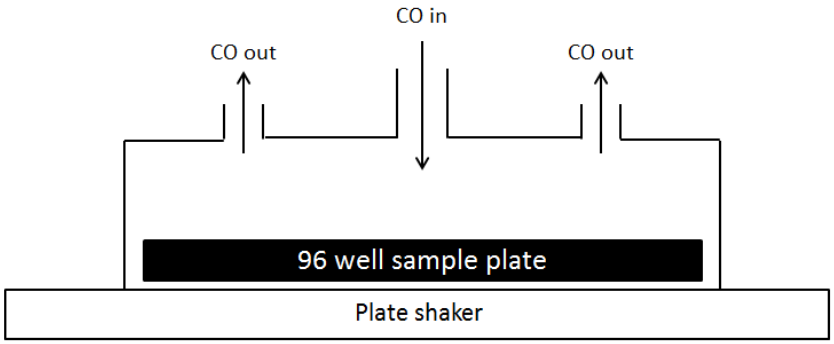

Figure 6.1 The carbon monoxide (CO) chamber (15 x 10 x 4 cm). A 96-well flat bottom plate containing samples is placed inside the chamber, the chamber is sealed and then CO is allowed to flow freely into the chamber. The exit hose was placed into a water-filled beaker to ensure gas flow was continuous. All procedures were carried out within a fume hood. Adapted from Choi et al. (2003).

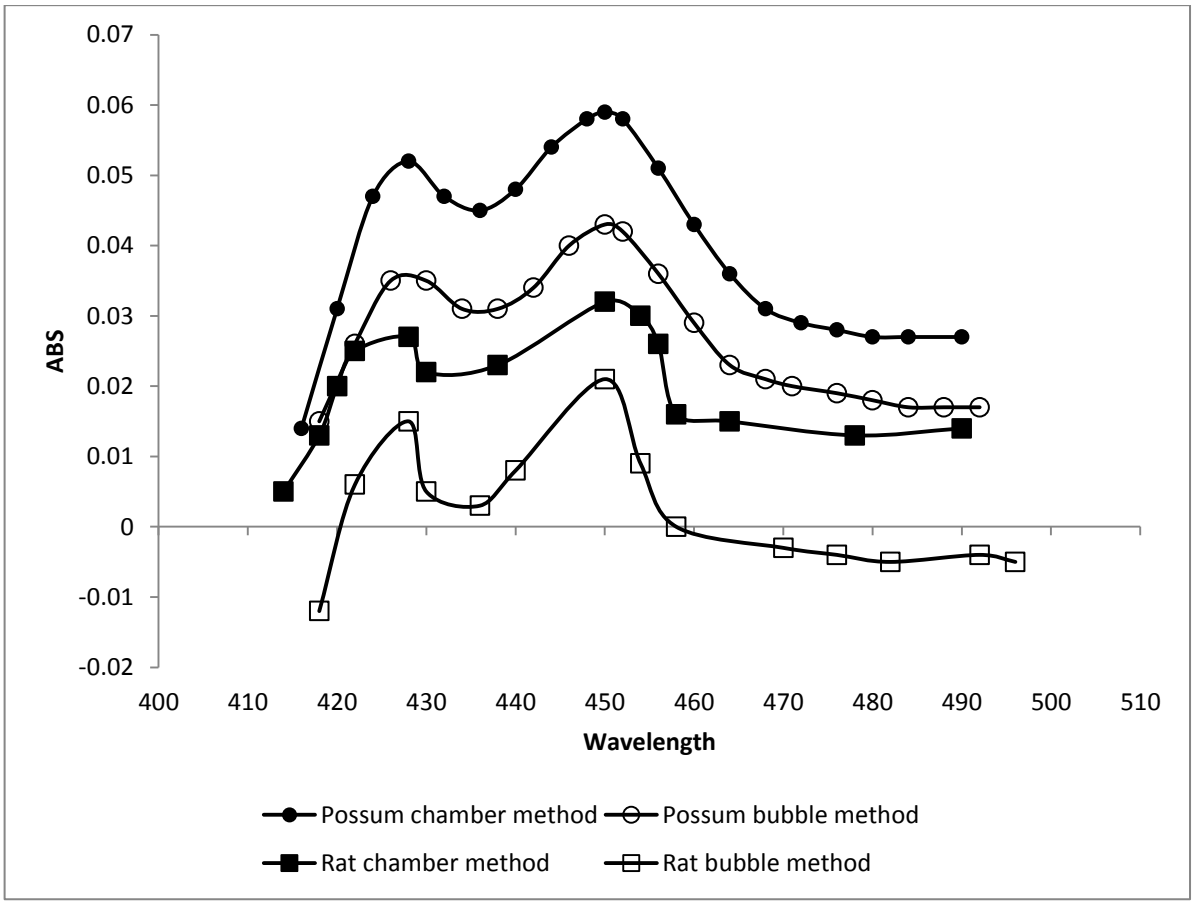

Figure 6.2 Comparison of absorbency potential of female possum and rat hepatic microsomes using the CO chamber or the CO bubbling method. Absorbance (ABS) was measured between 400 and 500 nm.

Optimization of CO chamber incubation time

To determine the optimal CO chamber incubation time, hepatic microsomes were diluted to 1.0 mg protein/mL GET buffer and 6 samples (200µL) were prepared as follows. Three replicates were designated as reduced P450 (PR) samples (negative control). To prevent the P450-CO complex forming in these PR samples, the wells were sealed with tape to ensure no CO access. The other 3 samples were designated as reduced P450-CO complex (PC) samples (positive control) and the wells were allowed full exposure to the CO chamber environment. In addition, the PR and PC samples were kept at a maximum distance from each other within each plate to further ensure separate treatment parameters were met. The plate was then placed into the CO chamber, the chamber was sealed and CO administered for 0 to 12 minutes as previously described. The plate was removed from the CO chamber, the PR sample tape was removed and PR and PC samples reduced with 10.0 µL of a 0.5 M SHS solution (in ultrapure water) (Choi et al., 2003) using a multichannel pipette. SHS, when in a liquid state, oxidizes on contact with air. Therefore, SHS powder was pre-measured before each experiment and ultrapure water was added just prior to its use (< 2 minutes). Absorbance was measured at 450 nm (Soret peak) and 490 nm (Isobestic point) on a microplate reader (VarioSkan Flash, Thermo Scientific, USA) at 37^0C. The change in absorbency was calculated using the following formulas: $\Delta A_{PC} = A_{PC}450 - A_{PC}490$ and $\Delta A_{PR} = A_{PR}450 - A_{PR}490$, where ΔA_{PC} is the absorbance of the P450-CO complex (PC) sample and ΔA_{PR} is the absorbance of the P450-reduced (PR) sample (Choi et al., 2003). The absorbency difference was calculated by subtracting ΔA_{PC} from ΔA_{PR}. The concentration of CYP450 was calculated using the following formula: [CYP450](mM/mg microsomal protein) = $\Delta A_{PC} - \Delta A_{PR})/91$ where 91 is the millimolar difference extinction coefficient (Omura and Sato, 1964). An optimal incubation time of 2 minutes within the CO chamber was selected (Figure 6.3).

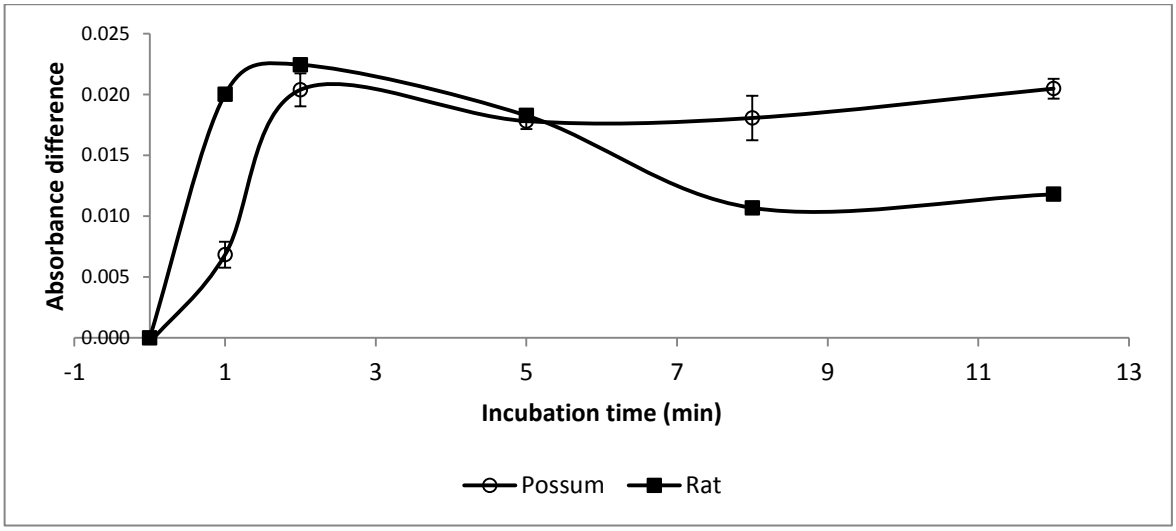

Figure 6.3 Optimization of CO chamber incubation time for female possum and rat hepatic microsomes.

Optimization of SHS reduction volume

To determine the optimal SHS reduction volume hepatic microsomes (possum or rat) were diluted to 1.0 mg/mL and then 3 replicates (200 µL) for each PR or PC treatment were placed into the wells of a 96-well flat bottom plate. The PR samples were sealed with tape, the plate placed into the CO chamber and samples exposed to CO as previously described. The plate was then removed from the CO chamber, the PR sample tape was removed and samples were reduced with 0.0, 5.0, 10.0 or 15.0 µL of a 0.5 M SHS solution and then the difference in absorbency measured as previously described. It was determined that a volume of 10.0 µL of SHS was most stable and efficient at reducing the haemoproteins in each species (Figure 6.4).

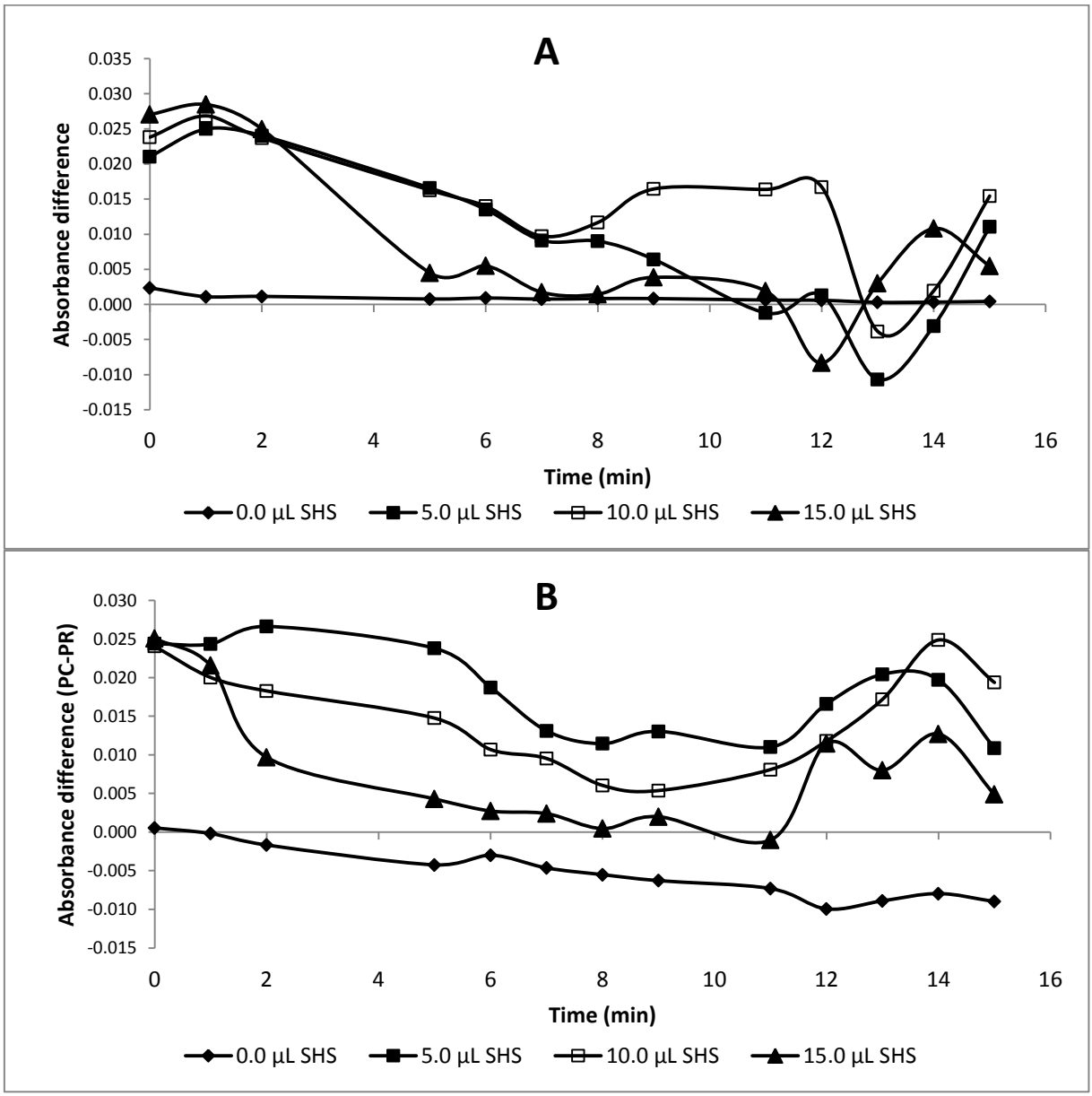

Figure 6.4 Optimization of SHS volume addition for reduction of female possum (A) or rat (B) microsomes.

Optimization of protein content

To determine the optimal protein concentration for each species, hepatic microsomes were diluted to 0.0, 0.05, 0.09, 0.19, 0.38, 0.75, 1.5, 3.0 or 6.0 mg protein/mL in GET buffer. Three aliquots of 200 µL per concentration were added to a 96-well flat bottom plate and placed into the CO chamber for 2 minutes, reduced with 10.0 µL of SHS and the difference in absorbency measured as previously described. Changes in absorbency showed a strong linear relationship over time and a 1.0 mg/mL protein concentration was selected for subsequent assays (Figure 6.5).

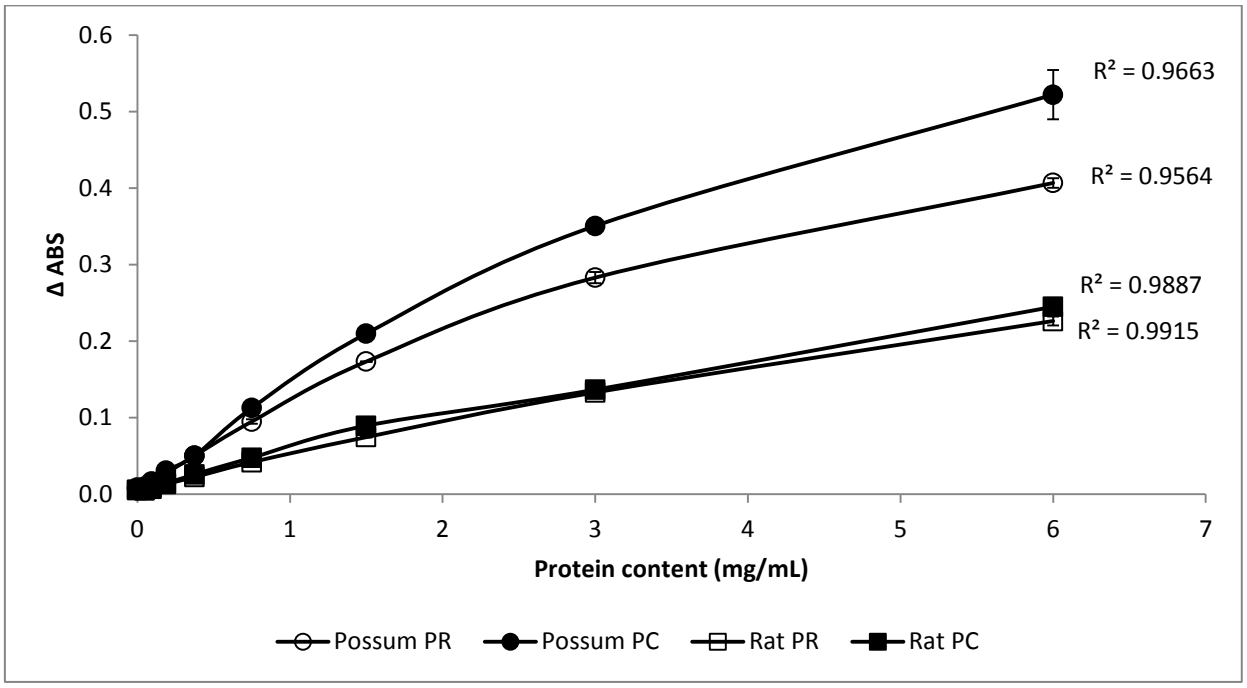

Figure 6.5 Optimization of microsomal protein content for female possum and rat hepatic microsomes.

CYP450 analysis

For each species, hepatic microsomes were transferred from a -80^{0}C freezer into a -20^{0}C freezer for 30 minutes and then placed on ice to allow samples to defrost slowly. Samples were diluted to 1.0 mg protein/mL using GET buffer and then divided evenly into two glass scintillation vials. One vial was kept on ice while the other vial was placed in a boiling water bath (100 ± 5°C) for 10 minutes to ensure all protein was denatured. To avoid any disruption from the protein residues created following boiling, the sample was filtered through a tissue sieve (Cellectortm, 75µm, Belco Glass Inc., New Jersey, USA) and then the sample was divided into glass scintillation vials for the following treatments: 1) negative control: dead microsomes (no additives); 2) negative treatment: dead VCH-treated microsomes (0.0, 4.0 or 8.1 mM). Samples were allowed to cool to room temperature before treatment started. The live microsome sample was kept on ice and aliquoted into two glass scintillation vials for the following treatments: A) positive control: live microsomes (no additives); B) positive

treatment: live VCH-treated microsomes (0.0, 4.0 or 8.1 mM). Live sample vials were then placed into a shaking incubator (37^0C) for 10 minutes at 200rpm to allow microsomes to activate. The trial began with the addition of VCH to the live and dead treatment vials. Vials were placed into a shaking incubator (37°C, 200 rpm) and at each time point (possums: 3, 10, 15, 30, 60 and 120 minutes; rats: 3, 15, 30, 60 and 120 minutes) 6 aliquots of 200 µL were removed from each treatment and transferred to a 96-well flat bottom plate. Three aliquots of each treatment were designated as PR samples and tape was placed on these wells to ensure no CO access. In addition, the PR and PC samples were kept at a maximum distance from each other within each plate to ensure treatment parameters were met. The plate was then placed into the CO chamber, exposed to CO, samples reduced with 10.0 µL SHS and the difference in absorbency was recorded as previously described. Three readings (0, 1, and 2 minutes) were collected and the average absorbency was used to calculate cytochrome P450 (CYP450) content as outlined above.

6.2.7 Statistical Analysis

All general statistical procedures were run as previously outlined in Chapter 3. The concentrations of VCD and GSH were analysed using a repeated measure test with treatment group defined by time.

6.3 Results

6.3.1 VCD hydrolysis in a range of acidic environments

To determine the effects of acid on VCD hydrolysis, VCD was incubated at room temperature (21 ± 1 °C) in a range of acid levels (1.0, 2.5 or 5.0) in either ultrapure water or M199 medium and then the percentage of the total VCD dose remaining after 15 minutes was compared with a VCD dose incubated in a neutral environment (pH 7.0). There were no differences in VCD hydrolysis when incubated in water or M199, regardless of pH ($p > 0.117$). Therefore, only the effect of the medium M199 on VCD will be reported in detail. Incubating two concentration levels of VCD (1.84 or 3.69 mM) in an M199 pH-neutral solution for 15 minutes resulted in 44.4 ± 0.13 and 70.5 ± 0.22 %, respectively, of the total chemical concentration being hydrolysed (Figure 6.6). At an acidity level of pH 1.0 and 2.5, 99.9 ± 0.01 and 99.0 ± 0.02 %, respectively, of VCD was hydrolysed, regardless of the initial concentration (Figure 6.6 and Table 6.1). A pH of 5.0 caused 88.0 ± 2.0 % of VCD to hydrolyse, regardless of the initial concentration (Figure 6.6). Acid significantly reduced VCD concentrations (1.84, 3.69, 5.53, or 7.38 mM) at all pH exposure levels (1.0, 2.5, or 5.0 pH) compared with a pH of 7.0 ($p < 0.015$; Figure 6.6).

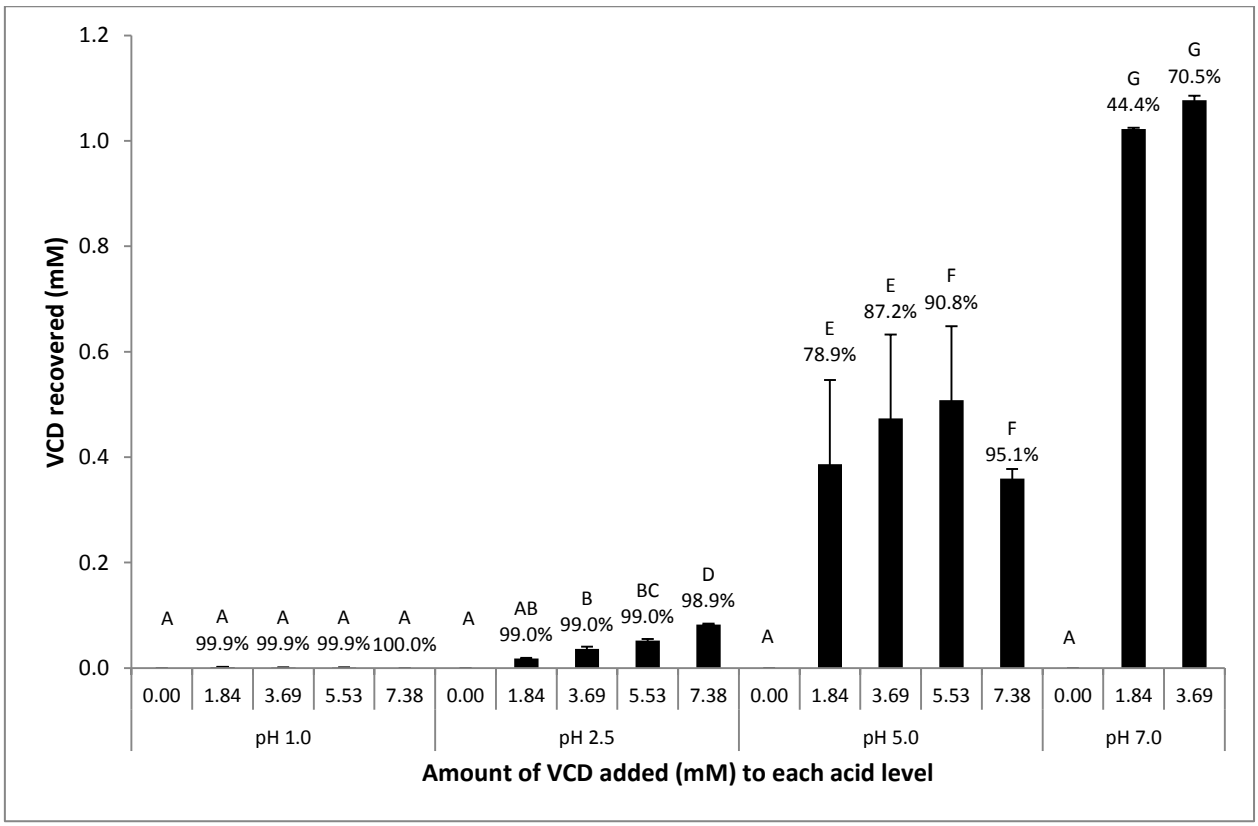

Figure 6.6 The effect of acid on VCD hydrolysis when incubated in pH-altered M199 at 21 ± 1 °C for 15 minutes. Following incubation, remaining VCD was extracted using ethyl acetate and quantified on GC. Percentages above each data set represent the amount of VCD that was hydrolysed during incubation. Levels not connected by the same letter are significantly different ($p < 0.05$). Vertical bars represent + SEM.

Table 6.1 Percentage of VCD hydrolysed when incubated for 15 minutes at a pH of 2.5 in M199 at room temperature (21 ± 1 °C), M199 medium heated to biological temperature (37 ± 1 °C) or the stomach contents of rats or possums mixed 1:4 (v/v) in M199 medium heated to 37 ± 1 °C. Data are means ± SEM.

Matrix	Amount of VCD added (mM)	Amount of VCD remaining (mM)	% VCD hydrolyzed
M199 @ 21°C	0.00	0.00 ± 0.00	N/A
	1.84	0.02 ± 0.001	99.0 ± 0.06
	3.69	0.04 ± 0.001	99.0 ± 0.11
	5.53	0.05 ± 0.003	99.1 ± 0.06
	7.38	0.08 ± 0.002	98.9 ± 0.2
M199 @ 37°C	0.00	0.00 ± 0.00	N/A
	1.22	0.24 ± 0.12	79.3 ± 6.59
	2.43	0.54 ± 0.27	77.9 ± 11.1
Rat stomach contents + M199 @ 37°C	0.00	0.00 ± 0.00	N/A
	1.22	0.0 ± 0.0	100.0 ± 0.00
	2.43	0.16 ± 0.02	93.5 ± 0.70
Possum stomach contents + M199 @ 37°C	0.00	0.00 ± 0.00	N/A
	1.22	0.41 ± 0.002	66.3 ± 0.19
	2.43	0.90 ± 0.01	63.2 ± 0.26

6.3.2 VCD hydrolysis in stomach contents

VCD was incubated in pH-controlled M199-diluted stomach contents (1:4, v/v; 37 ± 1 ºC) of rats or possums or in pH-controlled M199 only (37 ± 1 ºC) and the rate of chemical hydrolysis was compared between species and treatments. When 1.22 mM VCD was incubated in the stomach contents of rats (pH 2.5), VCD was only detected at minute 3 of incubation (Figure 6.7). A higher dose of VCD (2.43 mM) incubated in rat stomach contents (pH 2.5) resulted in the chemical being detected up to 15 minutes but by 30 minutes it was undetectable (Figure 6.8). When rat stomach contents were neutralized (pH 7.0), incubation with 1.22 or 2.43 mM VCD resulted in chemical concentrations peaking at 5 or 10 minutes then dropping off and stabilizing from 15 to 120 minutes ($p > 0.169$) and by 360 minutes VCD concentrations had fallen significantly ($p < 0.005$; Figures 6.7 and 6.8). Incubation of 2.43 mM VCD at pH 2.5 resulted in a lower mean VCD concentration in rat stomach contents compared with M199 only ($p < 0.049$; Figures 6.8 and 6.9).

Possum stomach contents at a pH of 1.0 caused 100 % hydrolysis of VCD by 3 minutes, regardless of the initial chemical concentration ($p < 0.001$; Figures 6.7 and 6.8). Similarly, VCD incubated at 1.0 pH in M199 only was 100 % hydrolysed within 3 minutes ($p < 0.001$; Figure 6.9). When the pH in possum stomach was raised to 2.5, VCD concentrations (1.22 or 2.43 mM) were highest at 3 to 5 minutes and then consistently dropped after 10 minutes until no VCD was detected at 120 minutes (Figures 6.7 and 6.8). There were no differences in VCD concentration incubated in possum stomach contents (pH 2.5) or M199 only (pH 2.5) from 3 to 60 minutes ($p > 0.077$; Figures 6.8 and 6.9). However, at 120 and 360 minutes VCD concentrations levels were significantly lower in possum stomach contents than in M199 ($p < 0.023$ and $p < 0.009$, respectively). VCD (1.22 or 2.43 mM) incubated in neutralized possum stomach contents had a peak concentration at 5 minutes and then remained somewhat stable for up to 360 minutes of incubation (Figures 6.7 and 6.8). There were no differences in VCD concentrations in neutralized possum stomach contents compared with neutralized M199 ($p > 0.081$) with the exception of lower VCD levels in possum stomach contents at 30 and 60 minutes ($p < 0.0004$ and $p < 0.022$, respectively; Figures 6.8 and 6.9).

Incubation in stomach contents at pH 2.5 resulted in higher concentrations of VCD, regardless of initial dose, in possums compared with rats ($p < 0.001$; Figure 6.7 and 6.8 and Table 6.1). Additionally, VCD was detected for a longer period of time in possum stomach contents (1.22 mM: up to 60 min; 2.43 mM: up to 120 min) compared with rat stomach contents (1.22 mM: up to 3 min; 2.43 mM: up to 15 min). When a low dose of VCD (1.22 mM) was incubated in a neutralized stomach environment, more VCD was recovered in possum stomach contents then that of rats ($p < 0.023$; Figure 6.7 and 6.8). However, there were no between-species differences in VCD concentration following incubation with a high dose in a neutral environment (2.43 mM; $p > 0.605$).

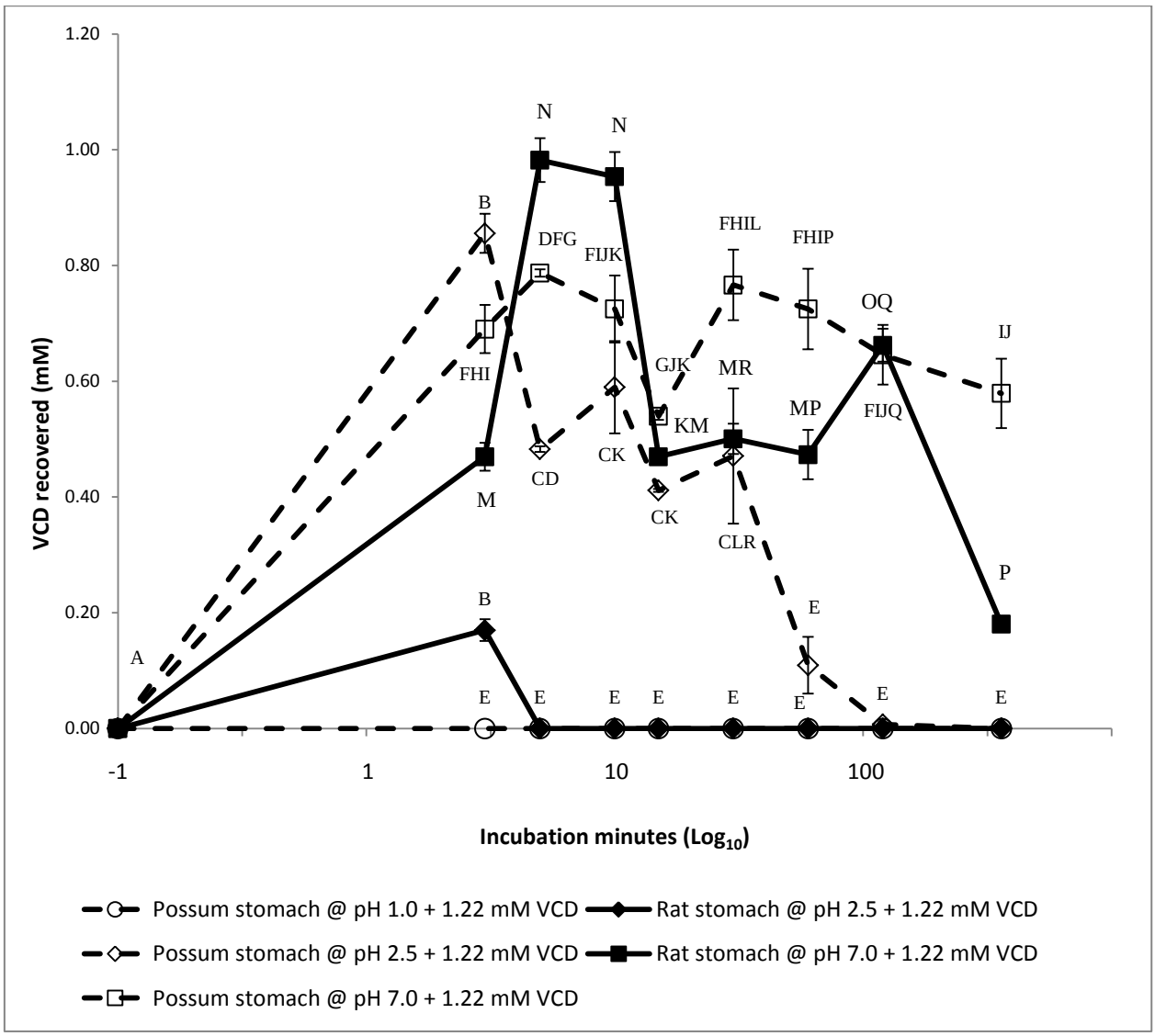

Figure 6.7 The effects on 1.22 mM VCD when incubated in pH-controlled rat or possum stomach contents (37 ± 1 °C). At each time point, VCD was extracted from a subsample using ethyl acetate and quantified on GC (n = 3 – 6 replicates/time point). Values not connected by the same letter are different ($p < 0.05$). Vertical bars represent ± SEM.

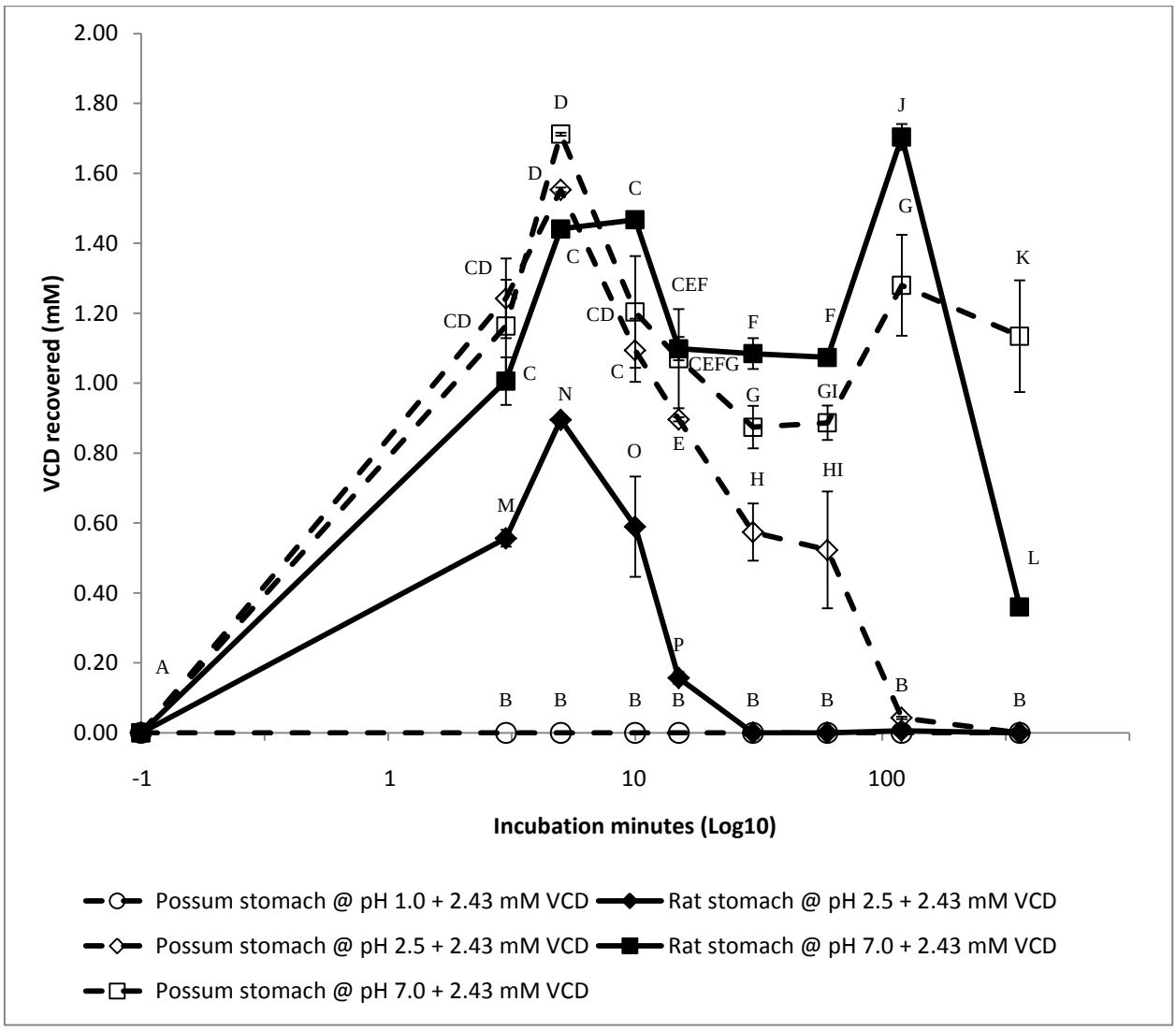

Figure 6.8 The effects on 2.43 mM VCD when incubated in pH-controlled rat or possum stomach contents (37 ± 1 °C). At each time point, VCD was extracted from a subsample using ethyl acetate and quantified on GC (n = 3 – 6 replicates/time point). Values not connected by the same letter are different ($p < 0.05$). Vertical bars represent ± SEM.

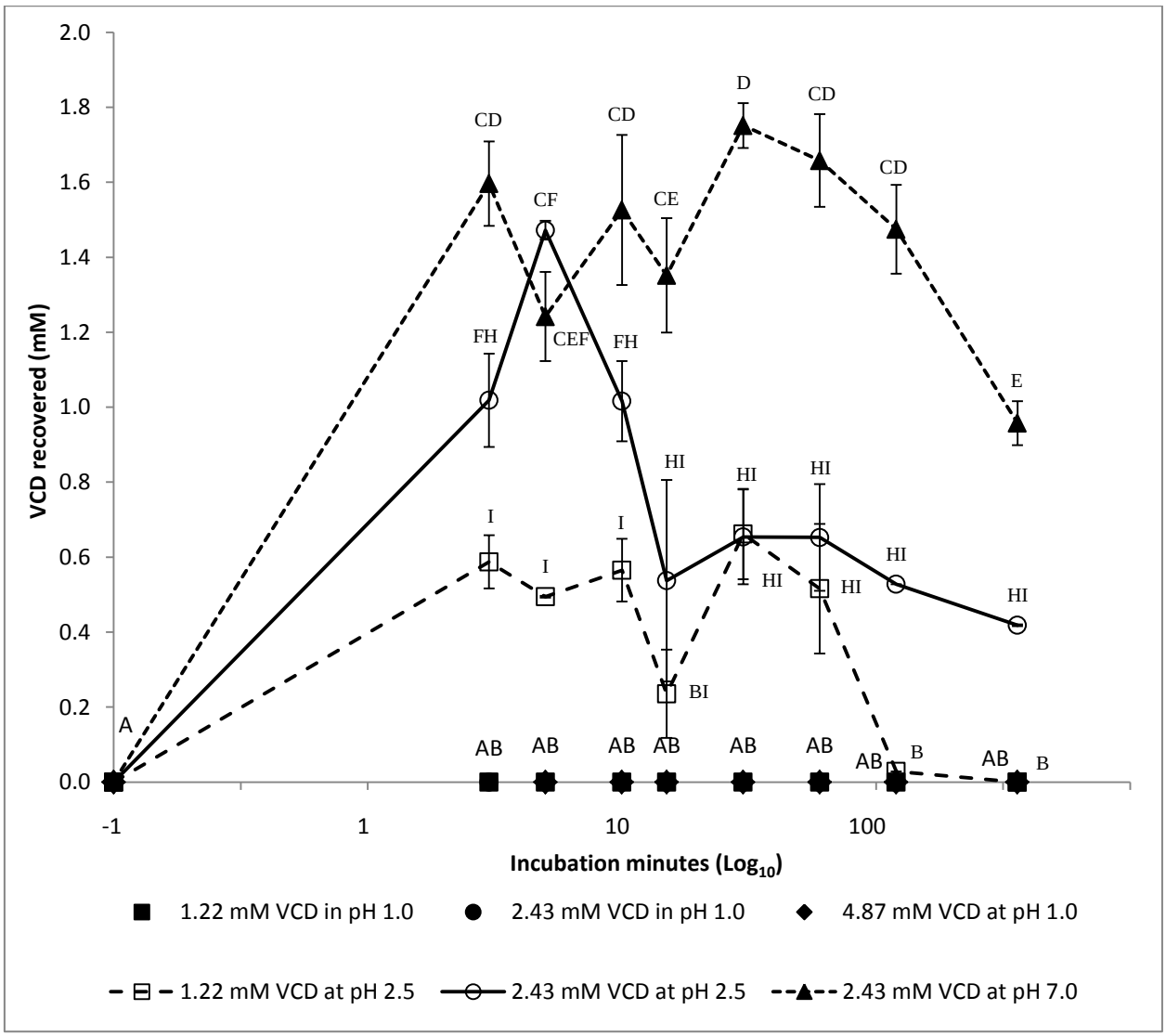

Figure 6.9 The effect of acid and heat (37 ± 1 °C) on VCD hydrolysis when incubated in pH-altered M199. At each time point, VCD was extracted from a subsample using ethyl acetate and quantified on GC (n = 3 – 6 replicates/time point). Values not connected by the same letter are different (p < 0.05). Vertical bars represent ± SEM.

6.3.3 VCD hydrolysis in blood

VCD was incubated in undiluted blood or M199-diluted blood (37 ± 1 ºC) of rats and possums or M199 only (37 ± 1 ºC) and the rate of chemical hydrolysis was compared between species and treatments. VCD, when incubated in M199-diluted or undiluted rat blood, was only marginally affected by time and temperature (Figure 6.10 and Table 6.2). There were no changes in VCD concentration (0.185 or 0.364 mM) in M199-diluted or undiluted rat blood from 3 to 10 minutes ($p > 0.117$) but from 15 to 30 minutes, VCD's concentration consistently dropped ($p < 0.049$; Figure 6.10 and Table 6.2). Conversely, there were no effects on VCD concentrations when it was incubated in M199-diluted or undiluted possum blood at all time points examined ($p > 0.096$; Figure 6.11 and Table 6.2).

The addition of M199 to the blood of each species did result in lower VCD concentrations throughout the incubation period ($p < 0.026$; Figure 6.11 and Table 6.2). However, there were no differences in the rate of VCD hydrolysis in M199-diluted blood of each species compared with M199 only ($p > 0.201$; Table 6.2). There were no between-species differences in VCD concentrations over time when incubated in M199-diluted blood (0.185 mM VCD, $p > 0.061$; 0.364 mM VCD, $p > 0.429$) or in undiluted blood (0.185 mM VCD; $p > 0.071$) of each species (Figures 6.10 and 6.11). However, when 0.364 mM VCD was incubated in undiluted blood of each species, more VCD was recovered from rat blood than that of possums ($p < 0.001$; Figures 6.10 and 6.11).

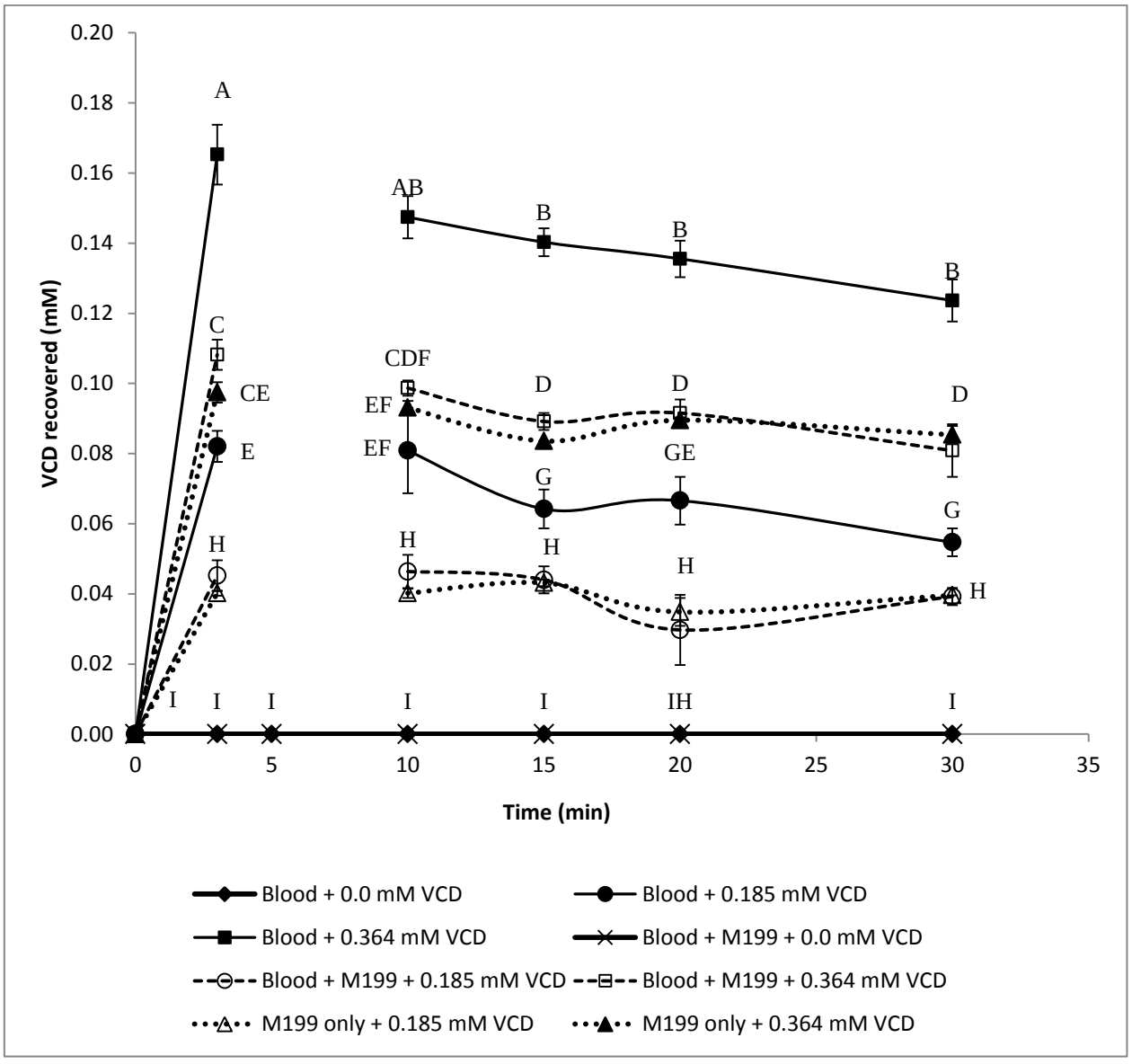

Figure 6.10 VCD concentration over time when incubated in undiluted blood or diluted blood (M199, 1:4, v/v) of female rats (n = 6) or M199 only. Values not connected by the same letter are different ($p < 0.05$). Vertical bars represent ± SEM.

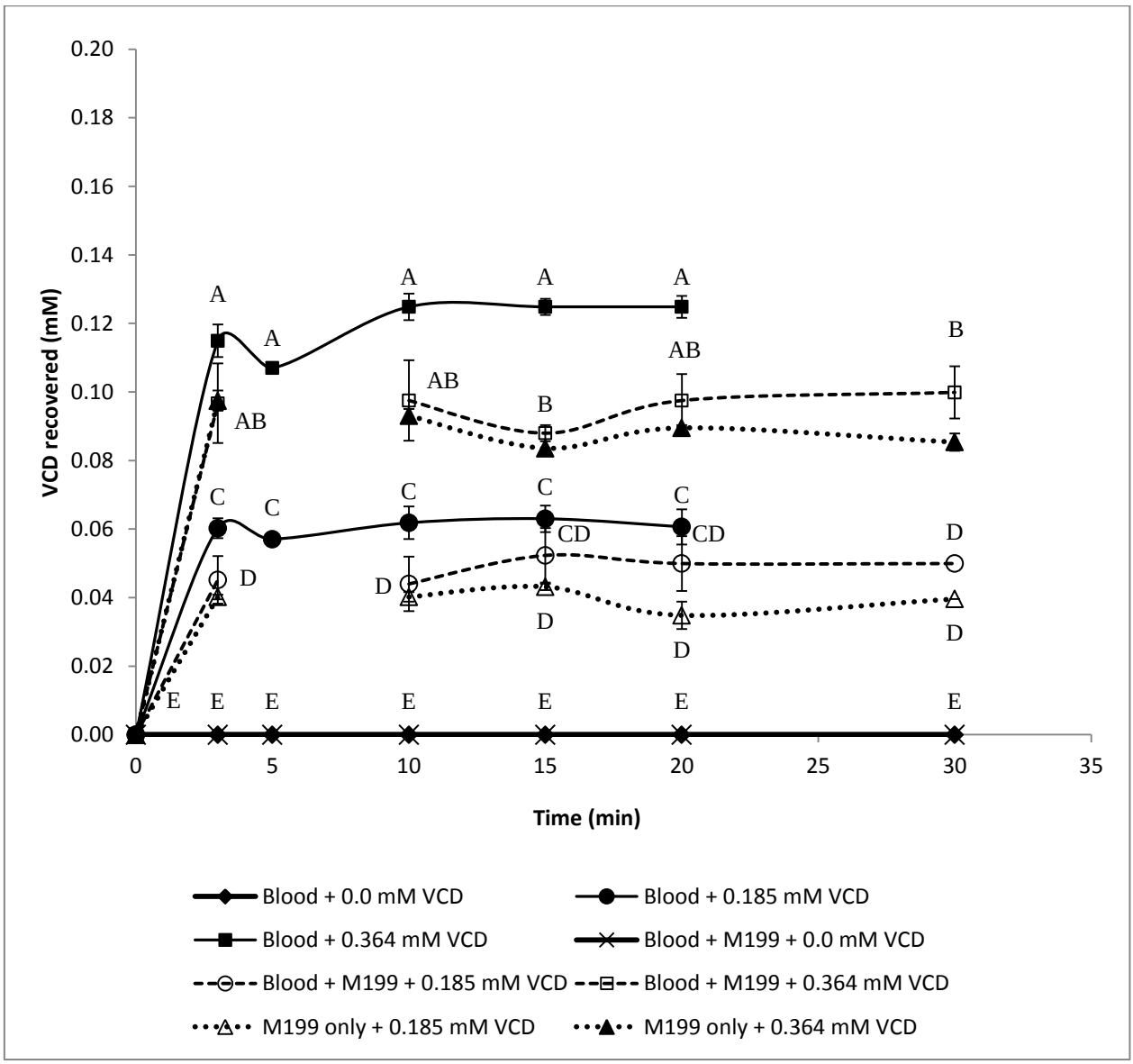

Figure 6.11 VCD concentration over time when incubated in undiluted blood or diluted blood (M199, 1:4, v/v) of female possums (n = 11) or M199 only. Values not connected by the same letter are different ($p < 0.05$). Vertical bars represent ± SEM.

Table 6.2 VCD in whole diluted or undiluted blood of possums or rats or in M199 only. VCD was incubated for 0, 3, 5, 10, 15, 20 or 30 minutes whole blood or whole blood diluted (1:4, v/v) in M199 (37°C). Data are means ± SEM.

Matrix	Amount of VCD added (mM)	Amount of VCD (mM) remaining at 20 minutes	Rate of VCD change over time
Rat whole blood	0.00	0.00 ± 0.00	-
	0.185	0.07 ± 0.007	-1.1 µM/min
	0.364	0.14 ± 0.005	-1.5 µM/min
Rat whole blood diluted in M199	0.00	0.00 ± 0.00	-
	0.185	0.03 ± 0.010	-0.4 µM/min
	0.364	0.09 ± 0.004	-1.0 µM/min
Possum whole blood	0.00	0.00 ± 0.00	-
	0.185	0.06 ± 0.005	+0.2 µM/min
	0.364	0.13 ± 0.002	+0.9 µM/min
Possum whole blood diluted in M199	0.00	0.00 ± 0.00	-
	0.185	0.05 ± 0.008	+0.2 µM/min
	0.364	0.10 ± 0.008	+0.1 µM/min
M199 only @ 37°C	0.00	0.00 ± 0.00	-
	0.185	0.04 ± 0.004	-0.4 µM/min
	0.364	0.09 ± 0.008	-0.1 µM/min

6.3.4 Hepatic GSH concentrations following in vitro incubation with VCD

The effect of VCD on liver metabolism was examined in rat and possum hepatic GSH concentrations following *in vitro* incubation with VCD. Incubation with VCD (7.13 mM) caused GSH concentrations in live S9 hepatic cells of rats to decrease significantly from 1 to 60 minutes compared with live controls ($p < 0.001$; Figure 6.12). There were no differences in GSH concentration in heat-shocked control and heat-shocked VCD-treated rat S9 hepatic cells ($p > 0.211$; Figure 6.12). GSH levels overall were lower in heat-shocked VCD-treated rat S9 hepatic cells compared with live VCD-treated cells, although this was not significant ($p > 0.054$; Figure 6.12). GSH concentrations in rat S9 hepatic cells were lower in heat-shocked controls than that of live controls ($p < 0.04$).

Live possum S9 hepatic cells incubated in VCD had no differences in GSH concentration from that of live controls from 0 to 10 minutes ($p > 0.081$; Figure 6.13). However, from 15 to 60 minutes VCD treatment caused GSH levels to decrease significantly compared with controls ($p < 0.049$; Figure 6.13). There were no differences in GSH concentration in heat-shocked controls compared with live controls ($p > 0.081$) and heat-shocked VCD-treated possum S9 hepatic cells ($p > 0.392$; Figure 6.13). GSH levels overall were lower in heat-shocked VCD-treated possum S9 hepatic cells compared with live VCD-treated cells, although this was not significant ($p > 0.192$; Figure 6.13).

Between-species comparisons revealed that mean GSH concentrations in live, untreated possum S9 hepatic cells (0.95 ± 0.03 nmol/mg protein content) were 10.3% lower than those of untreated rats (1.06 ± 0.01 nmol/mg protein content; $p <0.0001$). However, treatment with VCD resulted in the GSH

concentrations in rat S9 cells being reduced by 76.8% (0.09 ± 0.05 nmol/mg protein content) compared with VCD-treated possums (0.38 ± 0.11 nmol/mg protein content; $p < 0.001$). Within 1 minute of VCD addition, rat GSH levels were reduced by 72.6% of controls and by 30 minutes 100% of the GSH stock was depleted. On the other hand, possum GSH levels at 1 minute post-VCD addition were reduced by only 34.3% of controls but by 30 minutes 98.1% of their GSH stock was depleted. There were no between-species differences in mean GSH concentrations in heat-shocked controls or heat-shocked VCD-treated S9 hepatic cells ($p > 0.166$).

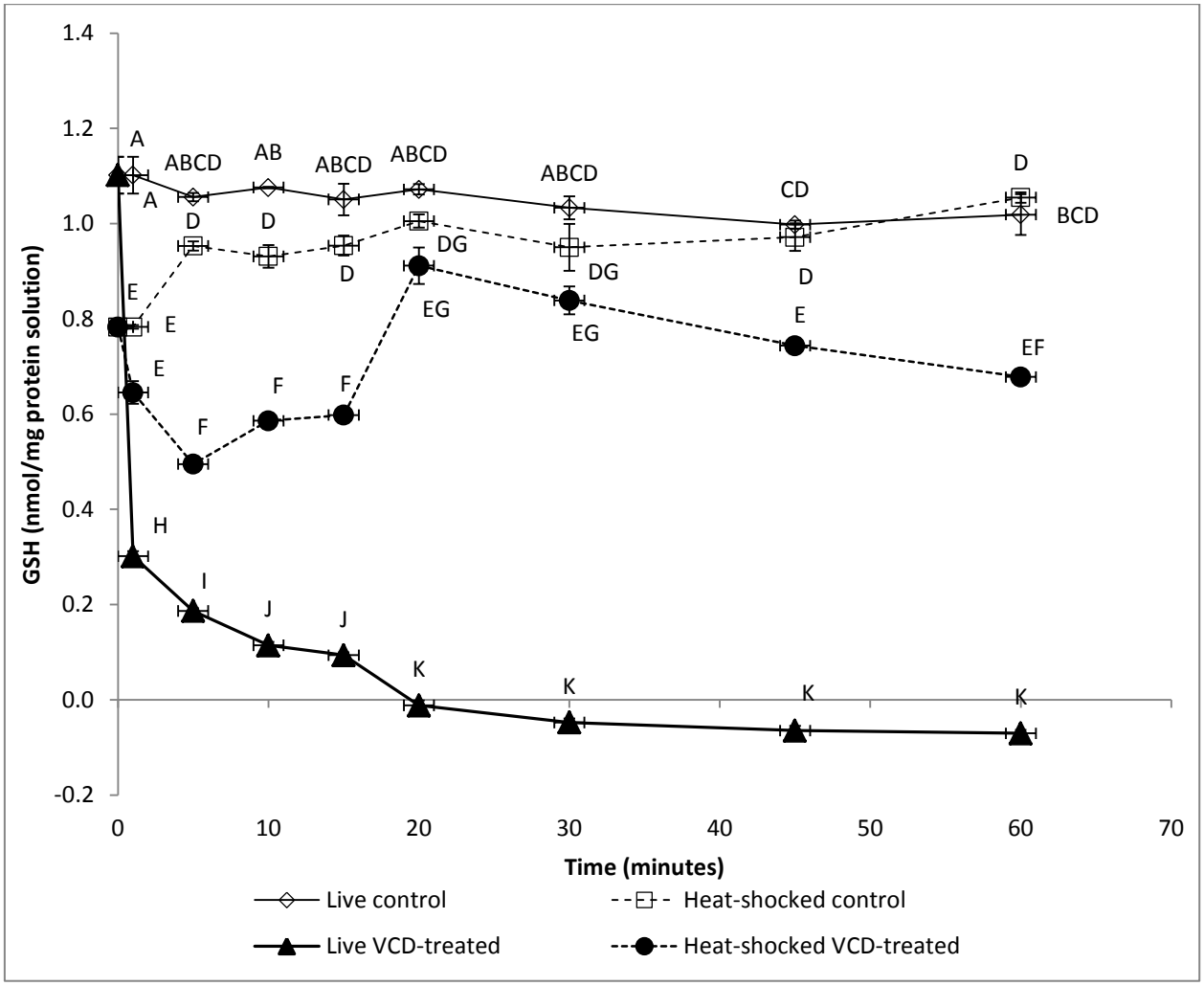

Figure 6.12 Change in rat hepatic GSH levels following *in vitro* incubation with 7.13 mM VCD. N = 3 replicates/time point; levels not connected by the same letter are different ($p < 0.05$). Vertical bars represent ± SEM.

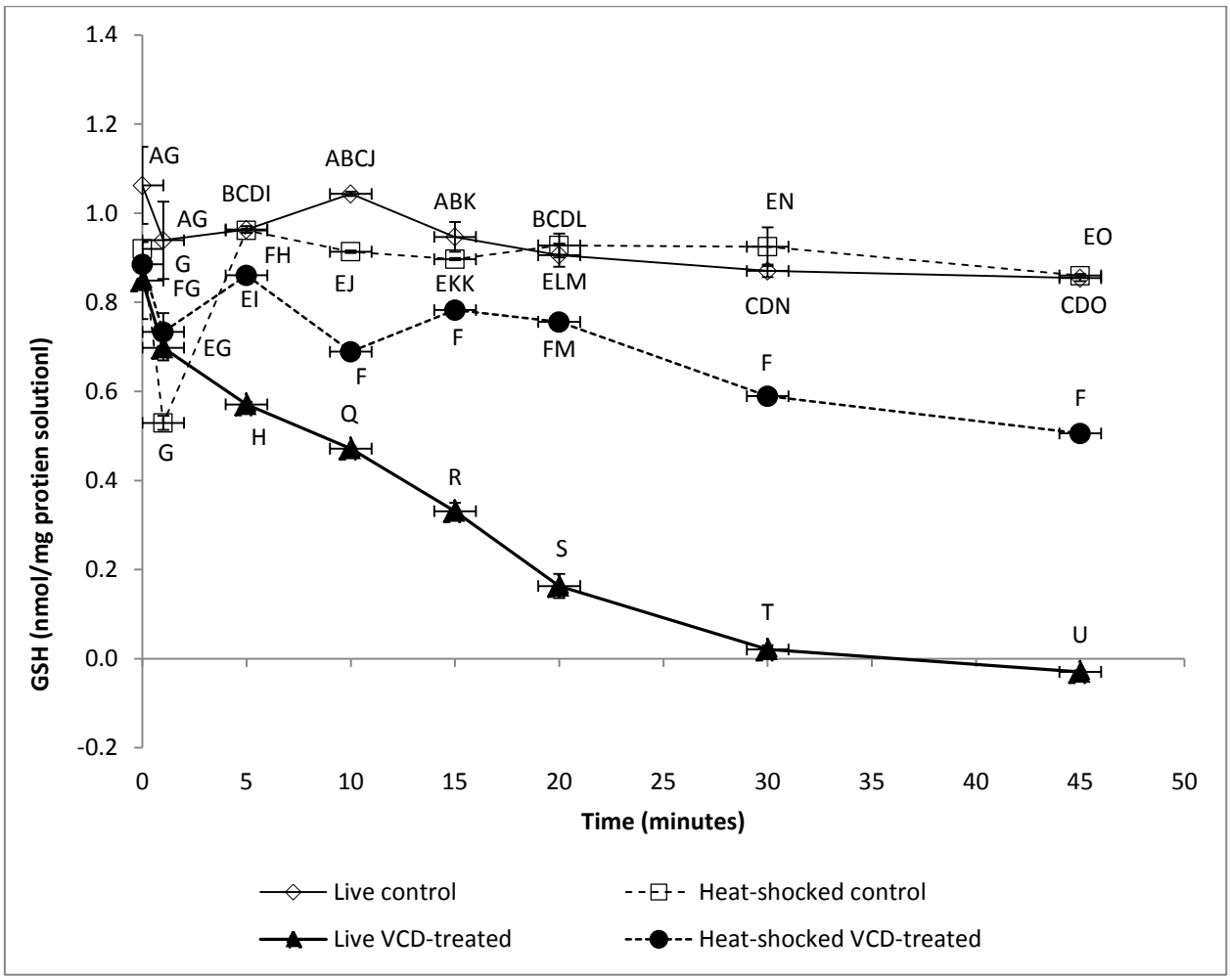

Figure 6.13 Change in possum hepatic GSH levels following *in vitro* incubation with 7.13 mM VCD. N = 3 replicates/time point; levels not connected by the same letter are different ($p < 0.05$). Vertical bars represent ± SEM.

6.3.5 CYP450 concentration following in vitro incubation with VCH

VCH-induced Phase I metabolism was examined in rats and possums by measuring the liver concentration of CYP450 following *in vitro* incubation with VCH. Compared with controls, mean CYP450 concentration in rat microsomes was significantly reduced following incubation with VCH (4.0 mM, $p < 0.0001$; 8.1 mM, $p < 0.0004$; Figure 6.14). There were no differences in mean CYP450 concentrations between heat-shocked rat microsomes treated with a high dose of VCH (8.1 mM) and the heat-shocked controls ($p > 0.081$). However, heat-shocked rat microsomes treated with a low dose of VCH (4.0 mM) had reduced mean concentrations of CYP450 compared with heat-shocked controls ($p < 0.002$; Figure 6.14).

Mean CYP450 concentrations in possum microsomes were significantly reduced following incubation with VCH (4.0 or 8.1 mM) compared with controls ($p < 0.0001$; Figure 6.15). There were no differences in mean CYP450 concentrations in heat-shocked possum microsomes treated with VCH compared with heat-shocked controls (4.0 mM, $p > 0.417$; 8.1 mM, $p > 0.120$; Figure 6.15).

There were significant differences in the CYP450 concentrations of control-treated ($p < 0.0001$) and heat-shocked ($p < 0.008$) rat and possum hepatic microsomes. Mean rat CYP450 levels in live and heat-shocked microsomes were 71.8 ± 2.42 % and 80.3 ± 74.5 % lower, respectively, than levels measured in possums. In addition, mean CYP450 levels measured in rat VCH-treated microsomes (4.0 or 8.1mM) were 77.1 ± 11.4 and 83.3 ± 8.4 % lower, respectively, compared with possums. Three minutes after addition of VCH, rat CYP450 levels in live microsomes were reduced by 97.7% (4.0 mM) and 100% (8.1 mM) to that of live controls. On the other hand, possum CYP450 levels in live VCH-treated microsomes were not reduced below those of controls until 10 (8.1 mM; 6.0%) and 30 minutes (4.0 mM; 79.6%).

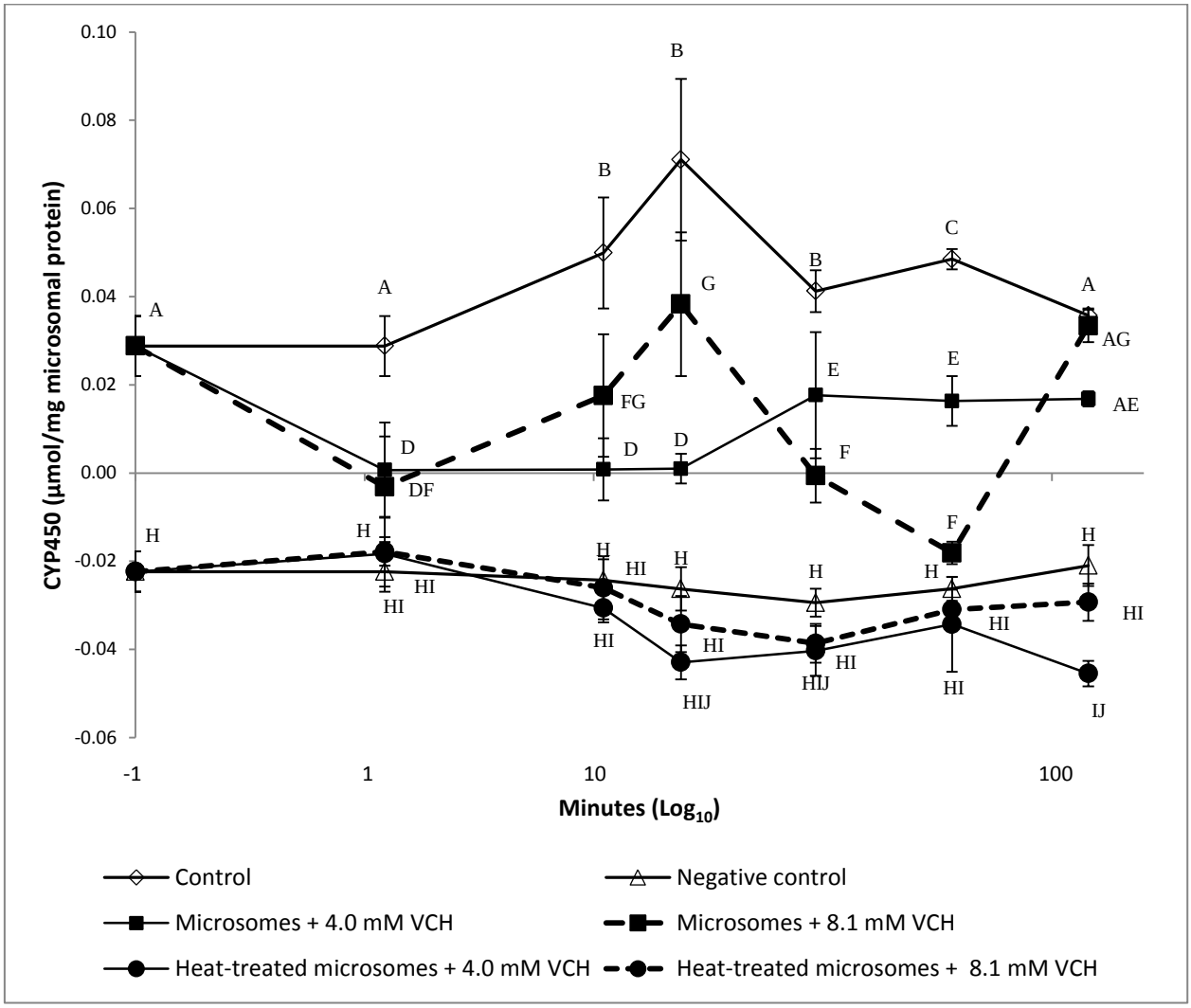

Figure 6.14 Changes in female rat hepatic CYP450 concentration levels following *in vitro* incubation with VCH (4.0 or 8.1 mM). Time scale is represented by Log_{10} (minute) where -1 represents pre-treatment CYP450 levels. Levels not connected by the same letter are different ($p < 0.05$). N = 6 replicates/time point. Vertical bars represent ± SEM.

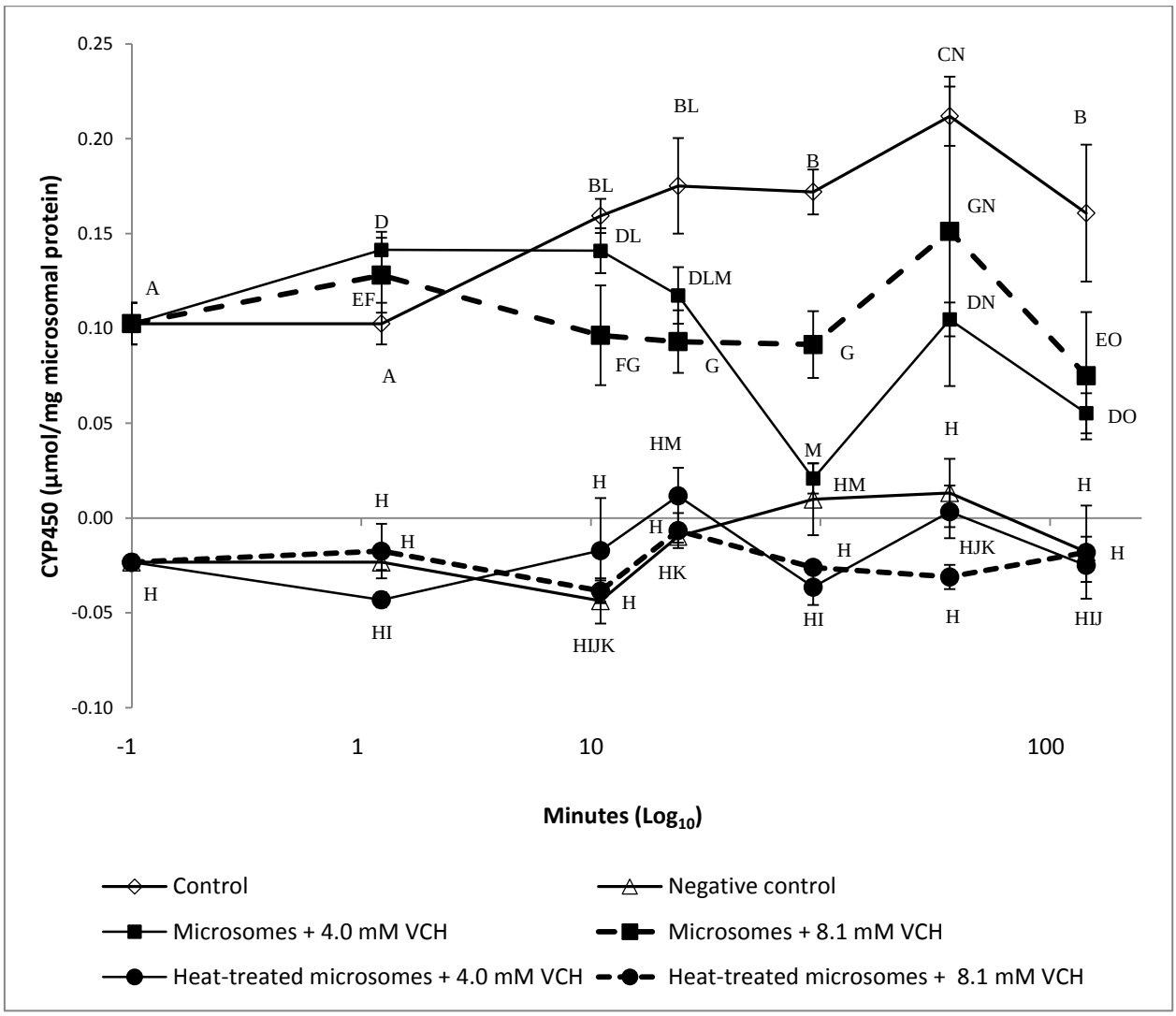

Figure 6.15 Changes in female possum hepatic CYP450 concentration levels following *in vitro* incubation with VCH (4.0 or 8.1 mM). Time scale is represented by Log_{10} (minute) where -1 represents pre-treatment CYP450 levels. Levels not connected by the same letter are different ($p < 0.05$). N = 6 replicates/time point. Vertical bars represent ± SEM.

6.4 **Discussion**

These results confirm the implications from the previous findings (Chapters 4 and 5) that orally administered VCD is handled differently in possums than in rats. The utilization of VCD as a potential oral chemosterilant for the control of vertebrate pest populations has been proposed with evidence supporting its efficacy in rodents (Burd, 2009; Herawati et al., 2010). However, the results presented in Chapter 4 demonstrated that the VCD dosing regimens and formulations examined had no effect on the pool of immature ovarian follicles of healthy adult female possums. The present study has attempted to investigate the species difference in VCD ovarian toxicity between possums and rats by determining the fate of VCD in the blood and stomach and its effects on metabolism through the *in vitro* measurement of hepatic-derived GSH.

At a pH of 1.0, 100% of VCD was hydrolysed within minutes of incubation in possum stomach contents. These data support the hypothesis that a large portion of an oral VCD dose is quickly reduced once it arrives in the stomach of possums. Furthermore, as with the *in vivo* studies, the proposed protective effects of possum stomach contents were demonstrated when the pH was increased to 2.5. Higher concentrations of VCD were recovered in the stomach contents of possums (up to 60 or 120 minutes) compared with rats (up to 3 or 15 minutes). Both rats and possums were fed the same pelleted diet. Thus, the contents of their stomachs would have been very similar suggesting a species effect in their ability to retain VCD in the stomach. VCD is a lipophilic chemical and, although lipid content was not measured, reasons for these between-species differences at pH 2.5 may be that the stomach contents of possums contain a higher proportion of lipids than those of rats. Alternatively, the species difference may be due to differences in particle size of the digesta or rates of stomach emptying which would influence the rate of VCD degradation. It is difficult to determine how relevant this between-species difference is *in vivo* as the average stomach contents pH range for possums is usually pH 1.0 to 2.0, significantly lower than in the rat.

VCD, when incubated in whole blood of possums and rats, was not affected and its chemical concentration remained stable. This finding seems to suggest that the blood is not involved in a detoxifying capacity; rather it primarily serves as a transportation medium for VCD. However, in these *in vitro* studies the only observation recorded was the presence (or disappearance) of VCD over time with no measurements of the detoxifying agents (i.e. GSH or epoxide hydrolase). Following an oral dose of VCD, mean intracellular GSH content in red blood cells of rats was decreased (Chapter 5). This suggests that, given sufficient levels of VCD present in the blood, GSH may serve a detoxification role to some degree. It should be noted that, in the plasma, much of the GSH originates from the liver and thus serves as an indicator of liver GSH content (Viña, 1990). In addition, extracellular GSH has a short half-life (1.9 min in the mouse) (Viña, 1990) suggesting that any GSH detoxification occurring in the blood is likely to be a result of red blood cell-derived GSH action rather than plasma-derived GSH. In the *in vitro* studies presented here, an NADPH regeneration system was not employed. Glutathione reductase, the enzyme responsible for converting glutathione disulphide (GSSG) to GSH, is NADPH-dependant and without a suitable *in vitro* culture set-up, the reformation of GSH from GSSG was not possible (Akerboom and Sies, 1980). Thus, while VCD concentrations appeared to be stable over time, the contribution of GSH cycling to the detoxification of VCD may have been underestimated in the present study.

VCD incubation with possum and rat hepatic S9 cell fractions caused GSH levels to become depressed and species differences in the timing and degree of GSH depletion were evident. VCD-induced reduction of rat liver GSH content occurred within the first minute (69.8% reduction of controls) and this was comparable to the reduction in GSH levels measured in the rat at 1 minute following an oral VCD dose (Chapter 5; 70.5% reduction of controls). Although GSH content in possum liver cells was also reduced following incubation with VCD, this occurred at a slower rate compared with rats. These results support the hypothesis that possums have a higher capacity for detoxifying xenobiotics, such as

VCD, compared with rats. From this it can be presumed that, even if sufficient quantities of VCD were able to avoid degradation by the high stomach acidity of possums, the increased hepatic metabolism of VCD would likely negate any potential effects on fertility.

CYP450 levels were reduced in both possum and rat liver microsomes following 2 hours of incubation with VCD's parent compound, VCH. Treatment with VCH has been shown to induce hepatic CYP450 levels in mice (Doerr-Stevens et al., 1999; Fontaine et al., 2001a; Fontaine et al., 2001b). However, these studies measured CYP450 induction following 10 days of daily intraperitoneal (i.p.) injections with VCH (7.5 mmol/kg/day) and therefore the increase in CYP450 levels over a long period of time is not surprising. Smith et al. (1990b) reported reduced hepatic CYP450 content levels 1 hour following an i.p. dose of VCH. However, the CYP450 inhibitor, chloramphenicol, was utilized as a pre-treatment and therefore CYP450 reductions were likely a direct result of chloramphenicol. The effect of VCH on CYP450 content without the use of a chloramphenicol pre-treatment was not examined (Smith et al., 1990b).

To my knowledge, examination of the short-term effect (<1 day) of VCH on hepatic CYP450 levels has not been examined *in vivo* nor *in vitro*. It is difficult to determine if the results presented here would be a true reflection of the short-term effects of VCH on CYP450 levels experiences *in vivo*. However, studies examining the short-term effects of various other xenobiotics have reported reduced or inhibited activity levels of CYP450 from 1 to 24 hours following treatment (Gueguen et al., 2006; Vuppugalla and Mehvar, 2004). It should be noted that the pilot *in vitro* study presented here did not utilize an NADPH regenerating system. Therefore, similar to the GSH studies, it is possible that the reduction in CYP450 reflected loss of NADPH over time and thus a suspension of CYP450 activity (Vernieulcn, 1996). However, the methods utilized to measure CYP450 did not require an NADPH regeneration system on a short-term time scale (Choi et al., 2003). Therefore, the change in CYP450 level from 0 to approximately 15 minutes is likely to be a true reflection of VCH's effects, whereas any interpretation of data past 15 minutes should be made with caution.

Indications for species differences in CYP450 content and response to VCH treatment were noted. The reduced levels of CYP450 content in untreated (control) microsomes of rats compared with possums suggest that possums may have a higher capacity for Phase I metabolism. This is further supported by species differences in the pattern of CYP450 reduction following VCH treatment. CYP450 levels in rat hepatic microsomes were rapidly decreased within 3 minutes following the addition of VCH and remained depressed until 120 minutes. On the other hand, possum CYP450 levels did not begin to drop until approximately 15 to 30 minutes after the addition of VCH. The delay in possum CYP450 reduction may have been partly due to the higher CYP450 content of their microsome levels prior to trial start compared with rats. Nevertheless, the delay in VCH-induced CYP450 reduction observed in possums suggests that rat's capacity for metabolizing VCH may not be as robust as possums. These data reflect and are supported by the between-species differences observed in the GSH data previously discussed here.

The potential differences between possums and rats in VCH and VCD metabolism may reflect similar differences to those previously reported between mice and rats (Keller et al., 1997; Smith et al., 1990a; Smith et al., 1990b). Mice have an increased capacity for VCH metabolism yet they are more susceptible to VCD treatment due to reduced hepatic and ovarian GSH and epoxide hydrolase activity compared with rats (Salyers, 1995; Smith et al., 1990a). These species similarities between possums and mice suggest that possums may respond to *in vivo* VCH treatment in a similar fashion to that of mice. Further speculation on these species similarities would indicate that possums should be susceptible to VCD-induced effects. Yet, results presented in Chapters 4 and 5 showed that a VCD gavage dose which induces follicle loss in rodents (500 – 750 mg/kg/day) (Burd, 2009; Herawati et al., 2010), when administered to possums, had no effect on the immature follicle populations of possums nor did it induce hepatic or ovarian GSH changes. The GSH data presented here clarify these results and support the hypothesis that possums have a higher capacity for Phase I and II metabolism than rats.

In preparation for the CYP450 and GSH experiments, the aim was to create negative control and negative VCD-treated samples by submerging the S9 liver fractions in boiling water for 10 minutes. Although this was successful for the CYP450 enzyme experiment, the GSH data suggest otherwise. GSH levels in heat-shocked samples were similar to live, nonheat-shocked samples indicating that this method was not successful and may have been inappropriate for the inactivation of GSH. GSH, a tripeptide, plays a vital role in cellular protection from environmental insults including toxic chemicals, ionizing radiation, and heat (Arrick and Nathan, 1984). Cellular thermotolerance can be achieved short-term (<1 hour) through the action of heat-shock proteins and their facilitation of the GSH-redox cycle (Baek et al., 2000; Kregel, 2002). Intracellular GSH levels will increase in response to temperatures of 43 to 45.5°C for up to 1 hour with reversal effects occurring past 1 hour (Mitchell et al., 1983; Russo et al., 1984). In the study reported here, the boiling temperature reached 100 ± 5 °C and coagulation of denatured proteins was visualized. It is difficult to imagine that intracellular GSH would survive for 10 minutes at such an extreme temperature yet GSH levels in the heat-shocked samples indicate activity was still present. Regardless, VCD treatment seems to have amplified the reduction of GSH levels in heat-shocked possum and rat samples to some degree. Future work should utilize a GSH inhibitor for a true reflection of negative control and treated samples.

The findings presented here support the previous results and hypotheses presented in Chapter 5. The rapid hydrolysis of VCD in the presences of varying acidity levels was described. The greater ability of possum stomach contents to retain and thus inadvertently protect VCD from acid degradation compared with rats provides some explanation for why possums may be less susceptible to VCD ovarian toxicity. Evidence for the proposed increased capacity of possum liver tissue to detoxify VCD compared with rats' livers presented here is corroborated by the similar effect of VCH on CYP450 concentrations in each species. Initial considerations of these data suggest that VCD may not be a suitable agent for fertility control in possums. However, a full summation of these findings and those

presented in Chapters 4 and 5 plus their implications for VCD's use in possums and rats will be provided in the General Discussion (Chapter 8).

Chapter 7

Examination of a novel rodent fertility control bait, ContraPest®: palatability, acceptance and health and reproduction effects in wild-caught female Norway rats

7.1 Introduction

The studies reported here examined the intake and palatability of ContraPest® in wild-caught female Norway rats (*Rattus norvegicus*) and its effects on the health and ovarian follicle populations of treated animals. ContraPest® bait has been developed by researchers at SenesTech, Inc® (Flagstaff, Arizona, USA) for control of rodent pest populations. The current formulation contains the orally active ingredients, 4-vinylcyclohexene diepoxide (VCD), an industrial chemical, and triptolide (TR), a plant extract from the thunder god vine (*Tripterygium wilfordii*). Extensive toxicological studies have demonstrated the efficacy of VCD at reducing the pool of primordial and small primary ovarian follicles in mice and rats while not directly affecting numbers of preantral and antral follicles (Danilovich and Sairam, 2006; Hoyer et al., 2001; Mayer et al., 2004; Mayer et al., 2002). In addition, it has been demonstrated that orally-delivered VCD is effective at reducing the immature ovarian follicle pool of laboratory bred Norway rats (Burd, 2009; Mayer et al., 2010; Schmuki et al., 2011) and their *in utero* exposed offspring (Burd, 2009) as well as immature follicle numbers in wild-caught ricefield rats (*R. argentiventer*) (Herawati et al., 2010). TR, on the other hand, targets the pool of larger developing follicles, specifically the secondary follicle population, by reducing the number of developing follicles and increasing the number of atretic follicles (Liu et al., 2011; Xu and Zhao, 2010). In addition, treatment with TR has been shown to reduce circulating oestrogen and progesterone (P_4) levels, increase circulating levels of follicle stimulating hormone (FSH) and luteinizing hormone (LH), and to cause prolonged oestrous cycles in laboratory-bred rodents (Liu et al., 2011; Xu and Zhao, 2010).

Preliminary results have demonstrated that ContraPest® is effective in reducing the primordial, primary and secondary ovarian follicle populations of adult female Sprague Dawley (SD) rats following 15 days of bait consumption (Dyer et al., 2013). Investigation of the long-term fertility effects in female SD rats following ContraPest® consumption is underway (Drs. L.P. Mayer and C. A. Dyer, personal comm.). In an effort to determine if similar effects can be achieved in wild rodents, researchers at the Commonwealth Scientific and Industrial Research Organisation (CSIRO) in Australia have examined the effects of ContraPest® consumption in wild house mice (*M. domesticus*). Initial findings revealed high palatability and acceptance rates and up to 50% depletion of primordial

follicles in mice presented with control emulsion or ContraPest® emulsion (Dr. L.A. Hinds, unpublished).

To date, no work has been undertaken examining the effectiveness of ContraPest® as a chemosterilant in wild Norway rats. The studies described in this chapter were designed to examine if consumption of ContraPest® by wild-caught female Norway rats would achieve similar findings to those reported for the laboratory strain of Norway rats and for wild mice. In the first study a liquid ContraPest® emulsion was tested in wild type Norway rats to determine: 1) effects on general health and food and water consumption, 2) palatability, and 3) the effectiveness of pre-baiting with control (non-active) emulsion on acceptance of the active formulation. The second study tested the addition of a sweetener, sodium saccharine, to the liquid formulation of ContraPest® and examined the effects on: 1) emulsion acceptance rates, 2) effects on general health and food and water consumption, and 3) effects on ovarian follicular populations.

7.2 Materials and Methods

7.2.1 Reagents

VCD, cyclohexanone, Attane™ isoflurane, and sodium pentobarbital were obtained from vendors and used for experimental procedures as previously described in Chapter 3. Control emulsion and ContraPest® emulsion was developed and provided by SenesTech Inc® (Flagstaff, AZ, USA). The proprietary formulation was a mixture of food grade, generally regarded as safe (GRAS) emulsifier, soy bean oil (80%), laboratory grade water (20%) with different concentrations of microencapsulated VCD and TR. Saccharin was used as an emulsion sweetener for Study 2 (Necta Sweet, NSI Sweeteners Inc, Lincolnshire, IL, USA).

7.2.2 Gas chromatography analysis of VCD concentration contained within ContraPest® emulsion

Two batches of emulsion were prepared by SenesTech Inc® in Flagstaff, Arizona for use during the studies and air transported to New Zealand by courier. Study 1 used emulsion from batch 1 while Study 2 used emulsion from batch 2. The effect of international transportation time and temperature on VCD stability within the ContraPest® emulsion was examined within 4 ± 2 days of emulsion arrival using gas chromatography (GC). It must be noted that TR concentration analysis was not performed after arrival in NZ as the addition of this active compound was decided shortly before the emulsion was prepared and no suitable method for quantifying TR was available.

For each batch, two 1.0 mL aliquots were collected into glass scintillation vials upon arrival and following each trial completion (± 3 days). VCD was extracted from the emulsion by the addition of three times the sample volume of extraction solution (ES; ethyl acetate containing 1.18 mM cyclohexanone as the internal standard). Extraction methods and VCD quantification on GC were performed as outlined in Chapter 3. To determine VCD extraction efficiency from the emulsion

matrix, three equal aliquots of control emulsion were spiked with 1.0 mg VCD/mL (7.134 mM). The samples were vortex mixed and then VCD was extracted as previously described. VCD extraction efficiency from VCD-spiked control emulsion was 36.9 %. Therefore, VCD concentrations in ContraPest® emulsions were estimated by dividing the measured concentration of VCD by the emulsion extraction efficiency, 0.369 ± 0.03.

7.2.3 Temperature stability analysis of VCD contained within the ContraPest® emulsion

The stability of VCD contained within the ContraPest® emulsion at different temperatures was examined to determine if temperature and time affect VCD concentration. VCD concentrations in batches were measured by colourimetric analysis in Arizona prior to being transported. Upon arrival, control and ContraPest® emulsion was stored at 4°C until use. Emulsion was prepared for the temperature stability experiment by collecting three 5.0 mL aliquots of control emulsion or ContraPest® emulsion and treated as follows: 1) control emulsion (no VCD added); 2) control emulsion spiked with 0.913 mM VCD; or 3) ContraPest® emulsion. Samples were stored at 4, 21 or 37°C and, on days 0, 1, 3, 6, and 20, two 0.5 mL samples were collected from each treatment vial and VCD extracted and quantified on GC as previously described in Chapter 3. VCD extraction efficiency from VCD-spiked control emulsion was 39.8 %. Therefore, VCD concentrations in ContraPest® emulsions were estimated by dividing the measured concentration of VCD by the emulsion extraction efficiency, 0.398.

7.2.4 Study 1

At the beginning of the study water and pellet consumption and live weight (LW) were monitored daily for 6 days to determine normal reference ranges for each animal. Rats often display neophobic behaviour (avoidance of novel stimuli) (Barnett, 1958; Mitchell, 1976). Therefore, to reduce any potential influence of neophobia, a 50 mL glass beaker, intended to hold the emulsion during the trial, was placed into a wire rack in the corner of each cage (Figure 7.1A). On a daily basis, fresh water was added to the glass beaker to allow animals to acclimate to consuming from the beaker. Animals were then randomly allocated into two groups (G) using a randomized block design stratified by LW during the monitoring phase. In the first treatment phase (P1; choice trial) animals were offered either control (group 1, phase 1; G1P1; n = 4) or ContraPest® emulsion (group 2, phase 1; G2P1; n = 6) at a rate of 5-10% LW for 6 nights (Figure 7.2). The concentration of the active ingredients within the ContraPest® emulsion was 2.96 mM (8.3 mg/kg LW) for VCD and 5.55 µM for TR (100 µg/kg). LW and consumption of pellets, water and control and ContraPest® emulsion were measured and recorded each day. Palatability of control and ContraPest® emulsion was determined with the following formula: (emulsion consumption weight) / [(pellet consumption weight) + (emulsion consumption weight)]. Beakers were cleaned and refilled with fresh emulsion each day. To determine evaporation rates, 3 water containers and 3 beakers containing control or ContraPest® emulsion were placed evenly

throughout the room and replaced as necessary. Evaporation rates for water and control and ContraPest® emulsion were recorded and subtracted from their corresponding daily consumption rates each day. The presence of faeces within the emulsion or signs of emulsion being spilt were recorded. These aberrations were often accompanied by consumption measurements registering above 0.5 g when it was believed that the animal did not consume any emulsion. Based on these observations, it was decided that if an animal's consumption rate was below <0.6% mg emulsion/g LW/day, then consumption would be registered as zero. Percentage LW rates of water and pellet consumption were determined by dividing the consumed weight (g) by the animal's LW (g) and multiplying by 100.

In the second phase (P2) to determine if pre-feeding with control emulsion would increase subsequent acceptance of the active ContraPest® emulsion formulation, the animals which were previously fed with control emulsion (G1P1) were switched to pellets plus ContraPest® emulsion for 6 nights (G1P2) (Figure 7.2). Emulsion storage conditions, the amount of emulsion provided, evaporation rate measurements and the consumption of pellets, water, and emulsion were collected and consumption rates calculated as stated for P1.

To examine the acceptance rate of ContraPest® emulsion when offered as the only source of food animals which were provided with ContraPest® emulsion in the first phase (G2P1) were placed into a no choice trial (phase 2, G2P2; Figure 7.2). During the no choice trial, pellets were removed and only ContraPest® emulsion and water were provided. Before treatment started an end point for the no choice trial was put in place wherein the trial would be stopped, emulsion removed and pellets replaced if animals experienced significant LW loss (>10 %) since trial start. Following the no choice trial (P2) animals were rested for 24 hours prior to the commencement of phase 3.

In the third phase (P3) to examine whether exposure to ContraPest® would influence palatability of the non-active control emulsion (i.e. cause aversion), G2 animals were offered control emulsion and pellets for 3 nights (G2P3) (Figure 7.2). Emulsion storage conditions, the amount of emulsion provided, evaporation rate measurements and the consumption of pellets, water, and emulsion were collected and consumption rates calculated for P2 and P3 as stated for P1.

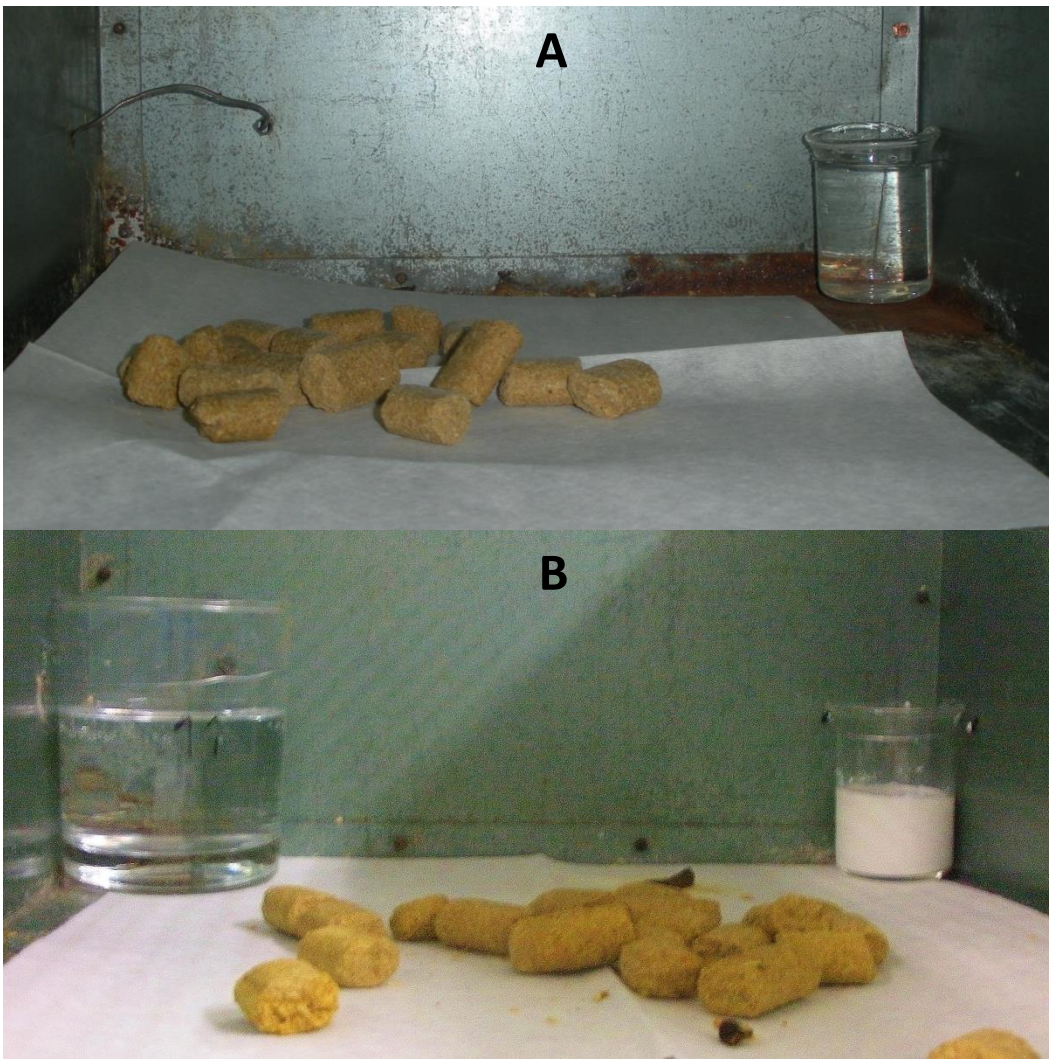

Figure 7.1 Representative images of cage set up during the monitoring phase (A) and treatment phases (B). During the monitoring phase, water was provided in the glass beaker which was intended to contain the emulsions during treatment. When treatment began, the water glass was replaced so that control and active emulsions could be provided in the glass beaker.

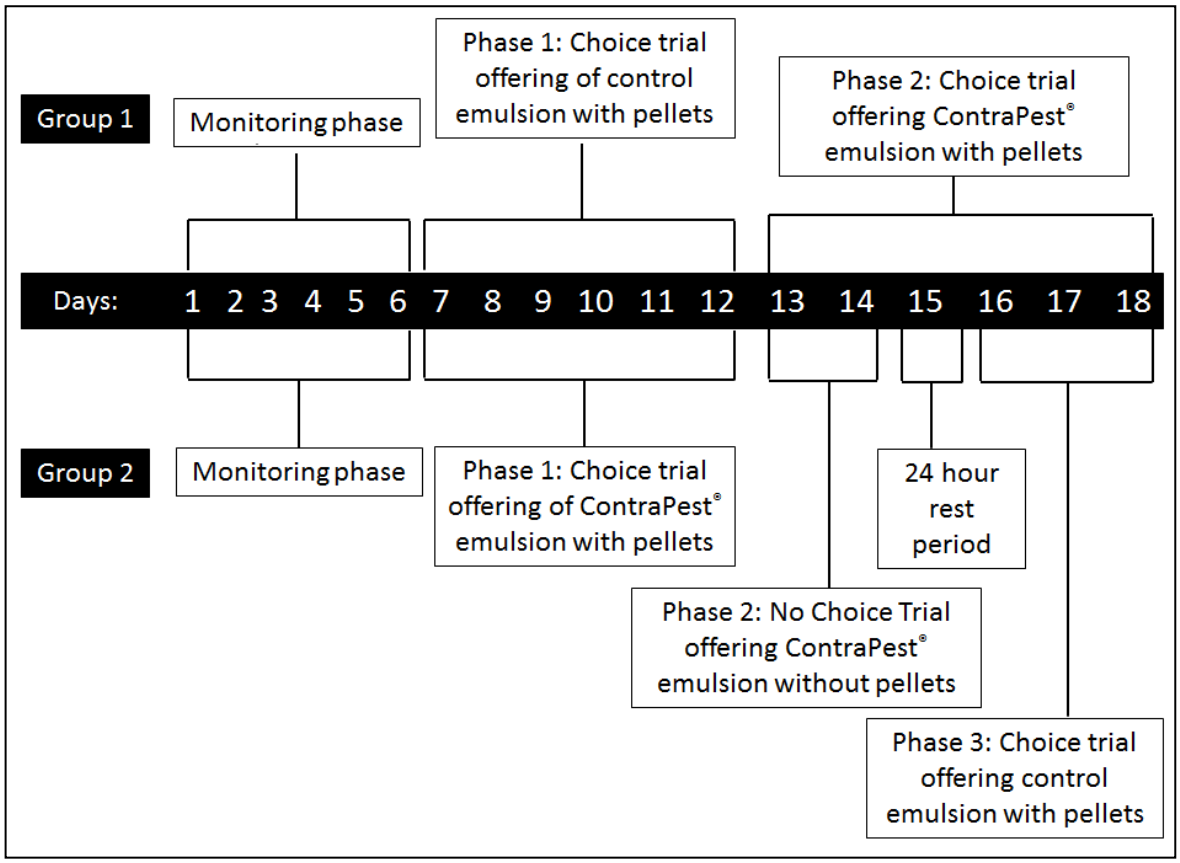

Figure 7.2 Study 1 trial design for Group 1 and 2.

7.2.5 Study 2

A new formulation of ContraPest® emulsion containing an artificial sweetener (ContraPest®-S), saccharin (1.94 mg/mL), was examined for its effects on palatability, animal health and immature ovarian follicle populations. One week prior to trial start a 50 mL glass beaker was placed into each animal's cage and fresh water added daily as outlined in Study 1 (Figure 7.1A). At trial start water and pellet consumption and LW were monitored daily for 4 days to determine normal reference ranges for each animal (Figure 7.3). In addition, all animals were pre-fed with control emulsion at a rate of 5-10% LW for 4 days. Animals were then randomly allocated into treatment groups using a randomized block design stratified by LW during the pre-feeding phase. Animals were provided with pellets plus control or ContraPest®-S emulsion at a rate of 5-10% LW for 15 days in 2 blocks with 7 rest days between treatment phases (7-8 days per phase; Figure 7.3). The concentration of the active ingredients within the ContraPest®-S emulsion was 4.68 mM (13.1 mg/kg LW) for VCD and 11.0 µM for TR (200 µg/kg). Emulsion storage conditions, the amount of emulsion provided, evaporation rate measurements and the consumption of pellets, water, and emulsion were collected and consumption rates calculated as stated for Study 1. Animals were euthanized 3 days post-treatment with an intracardiac injection of pentobarbitone (125 mg/kg). The liver, kidneys, adrenal glands, uterus, and ovaries were excised, trimmed of fat, weighed, examined for gross pathology and then fixed in 10%

neutral-buffered formalin for histological purposes. The eyes were removed and treated as outlined below for age analysis.

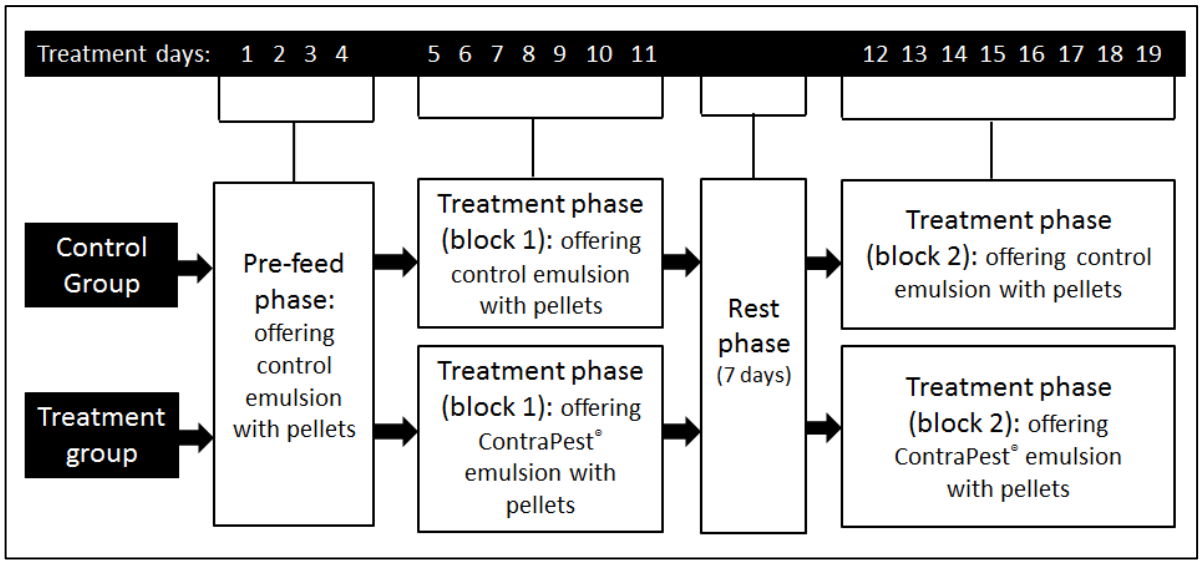

Figure 7.3 Study 2 trial design.

7.2.6 Rodent aging

Animal age was determined using methods described by Hardy, Quy and Huson (1983) with some modifications. Eye balls were fixed in 10% neutral-buffered formalin for 4 weeks. Eye balls were then slit open and the lenses removed by applying light pressure. Lenses were individually stored in open-topped Eppendorf tubes and air dried for 67 hours at 80 ± 1 °C. Lenses were weighed and then reweighed at 115 hours to verify stability of lens weights. To minimize atmospheric moisture absorbance, lenses were removed from the oven individually immediately prior to being weighed. Animal age was determined using the following female-specific, species-specific correction formula: $Y = (10^{xm + b}) - 22$ where Y: age in days, x: paired lens weight (mg), m: 0.021 (slope); b: 1.297 (intercept); and 22 represents the average gestation length in days for *R. norvegicus* (Hardy et al., 1983).

7.2.7 Histology and follicle analysis

Ovaries were trimmed of fat, weighed, prepared for histological examination, analysed and quantified as previously outlined in Chapter 3.

7.2.8 Statistical analysis

All general statistical procedures were run as previously outlined in Chapter 3. In addition, LW and water, pellet and emulsion consumption rates during treatment were analysed using a repeated measure test with animal identification as a random variable and treatment group defined by time as a

covariant. Ovarian follicle estimates were tested for covariance with ovarian weight and animal age and normalized where appropriate. LW covariance was determined for all organ weights and data were normalized to LW where appropriate.

7.3 Results

7.3.1 Concentration analysis of ContraPest® emulsion

Initial concentrations of VCD in each ContraPest® emulsion were 57.6 mM (batch 1) and 50.3 mM (batch 2) (Table 7.1). On average, international transportation resulted in hydrolysis of 1.96 ± 0.3 mM VCD per day. At the end of each study, the concentration of VCD in batch 1 and 2 was estimated to be 2.96 ± 0.06 and 4.68 ± 0.35 mM, respectively (Table 7.1). It should be noted that the methods used to quantify VCD pre-transportation (colorimetric analysis) differ from that of post-transportation (gas chromatography). Therefore, the differences in VCD concentrations between pre- and post-transportation should be accepted with caution. To determine if VCD loss was a result of potential high temperatures during shipment or natural hydrolysis within the emulsion matrix, the VCD concentration in unopened bottles (batch 2; stored at 4°C) was measured at 11 and 47 days post-emulsion creation. VCD hydrolysis in VCD-spiked control emulsion and ContraPest® emulsion during storage at the research facility (4ºC) occurred at a rate of 0.13 ± 0.07 and 0.05 ± 0.03mM/day, respectively.

Table 7.1 The concentration of VCD contained within ContraPest® emulsion batches 1 and 2 prior to international transport and following trial end. Initial VCD concentrations were measured by colorimetric analysis. Final VCD concentrations were measured by gas chromatography. Data are means ± SEM.

Batch number	Initial concentration of VCD (mM)	Final concentration of VCD (mM)	Percentage of VCD loss due to international transport, storage and time	Time from emulsion production (AZ) to arrival (NZ) (days)	Time from trial start to final VCD measurement (days)
Batch 1	57.6	2.96 ± 0.06	94.9 %	15	14
Batch 2	50.25	4.68 ± 0.35	90.7 %	7	28

7.3.2 Temperature stability analysis of ContraPest® emulsion

To further understand the rate of natural hydrolysis and the effect of temperature on VCD contained within ContraPest® emulsion, a temperature stability study was performed. When stored at 4°C, the VCD concentration in VCD-spike control emulsion and ContraPest® emulsion was not affected by time or temperature. In fact, the VCD concentration in both emulsions increased slightly from day 1 to day 20 which was likely due to evaporation ($p < 0.001$; Figures 7.4 and 7.5). Storage of emulsions at 21°C caused VCD concentrations to decline steadily from 3 – 20 days and this effect was emphasised

when emulsions were stored at 37°C ($p < 0.0001$; Figures 7.4 and 7.5). VCD loss per day in VCD-spike control emulsion and ContraPest® emulsion was 0.04 and 0.17 mM/day, respectively, when stored at 21°C and 0.05 and 0.22 mM/day, respectively, when stored at 37°C.

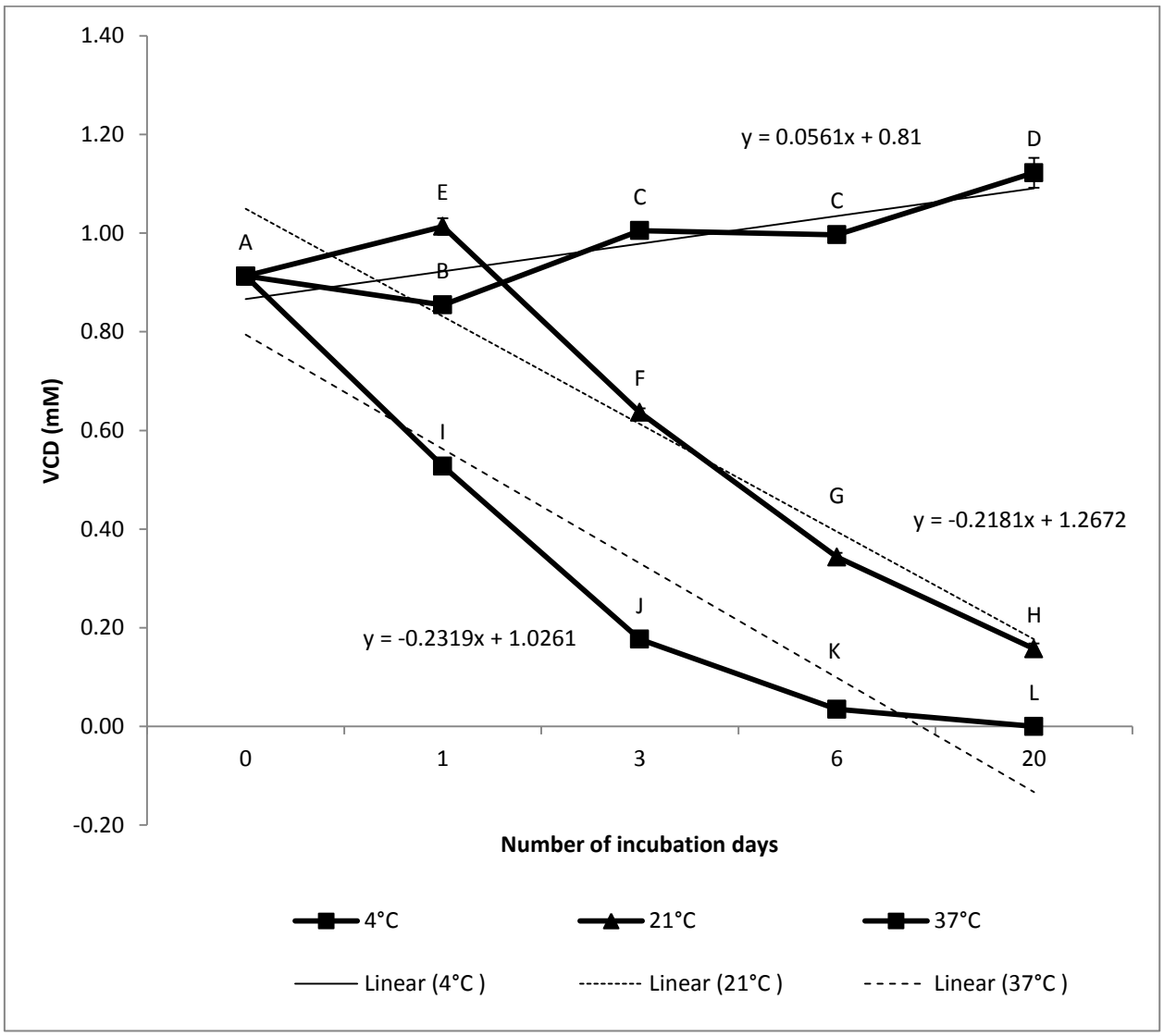

Figure 7.4 The effect of time and temperature on VCD hydrolysis within control emulsion spiked with 0.913 mM VCD. Replicates at each time point were analysed in triplicate on gas chromatography. Values with different letters are significantly different ($p < 0.05$). Vertical bars represent ± SEM.

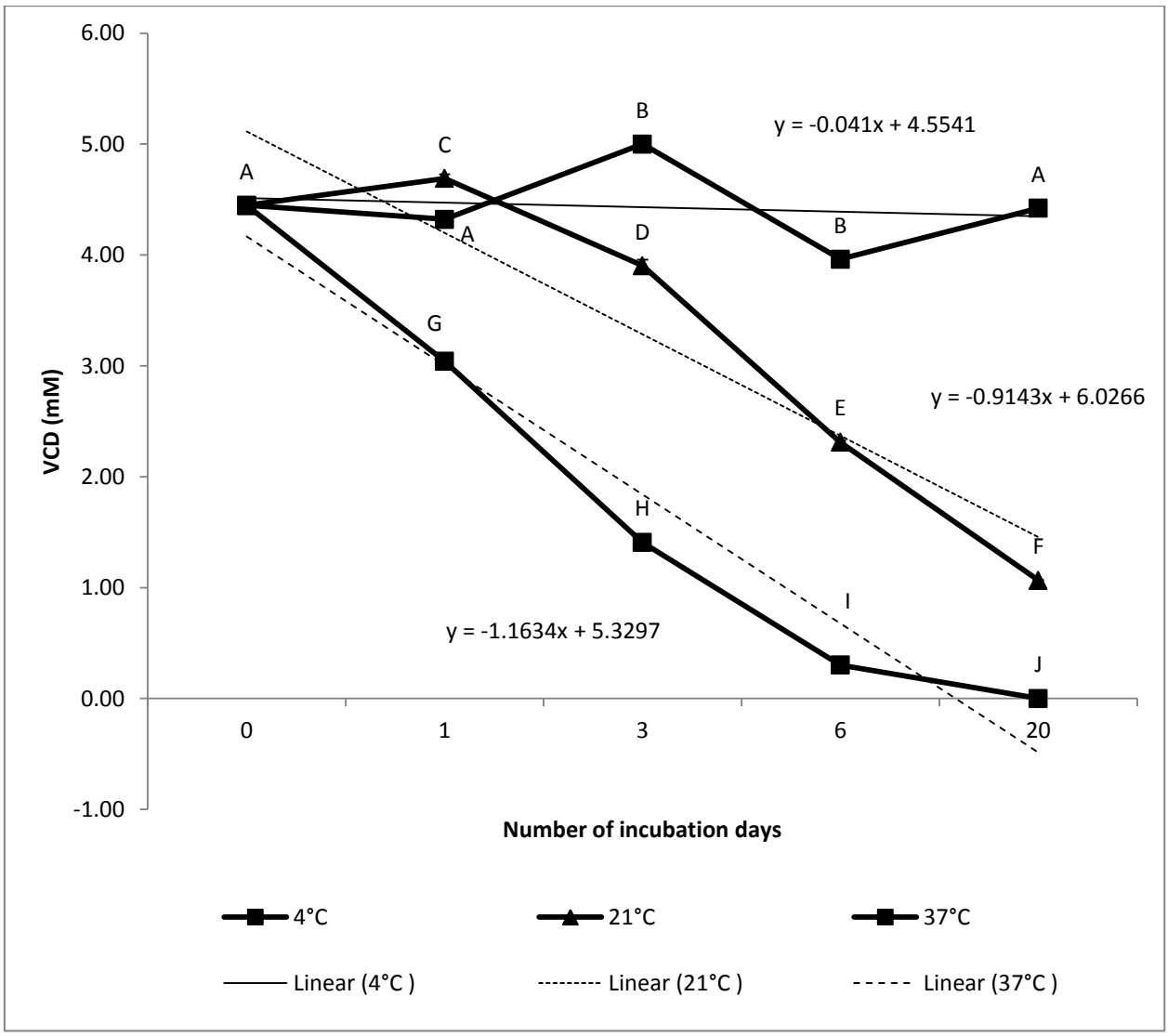

Figure 7.5 The effect of time and temperature on VCD hydrolysis within ContraPest® emulsion (batch 2). Replicates at each time point were analysed in triplicate on gas chromatography. Values with different letters are significantly different (p <0.05). Vertical bars represent ± SEM.

7.3.3 Study 1

Choice study (Group 1 and 2, phase 1)

During the monitoring phase, there were no significant differences between groups in percentage weight gain or water and pellet consumption ($p > 0.364$; Figures 7.6 - 7.8). When these parameters were compared between the monitoring phase and phase 1 (P1), there was no change in the mean LW of each group ($p > 0.295$; Figure 7.8). Animals offered control emulsion (G1) consumed less water ($p < 0.012$; Figure 7.6A) and pellets ($p < 0.037$; Figure 7.7A) on average compared with their consumption levels measured during the monitoring phase. Animals offered ContraPest® emulsion (G2) consumed the same amount of pellets ($p > 0.667$; Figure 7.6B) but significantly more water ($p < 0.014$; Figure 7.7B) during P1 than during the monitor phase. During treatment, G2 animals consumed

more pellets ($p < 0.017$; Figure 7.7) and gained more weight ($p < 0.014$; Figure 7.8) than G1 control animals.

The ContraPest® emulsion consumption rate of G2 animals was significantly reduced compared with G1 consumption of control emulsion throughout P1 ($p < 0.0001$, Figure 7.9 and Table 7.2). All G1 animals consumed control emulsion on a regular basis (Table 7.3). However, of the G2 animals, 3 rats did not consume ContraPest® emulsion while the other 3 consistently consumed throughout P1 (Table 7.3).

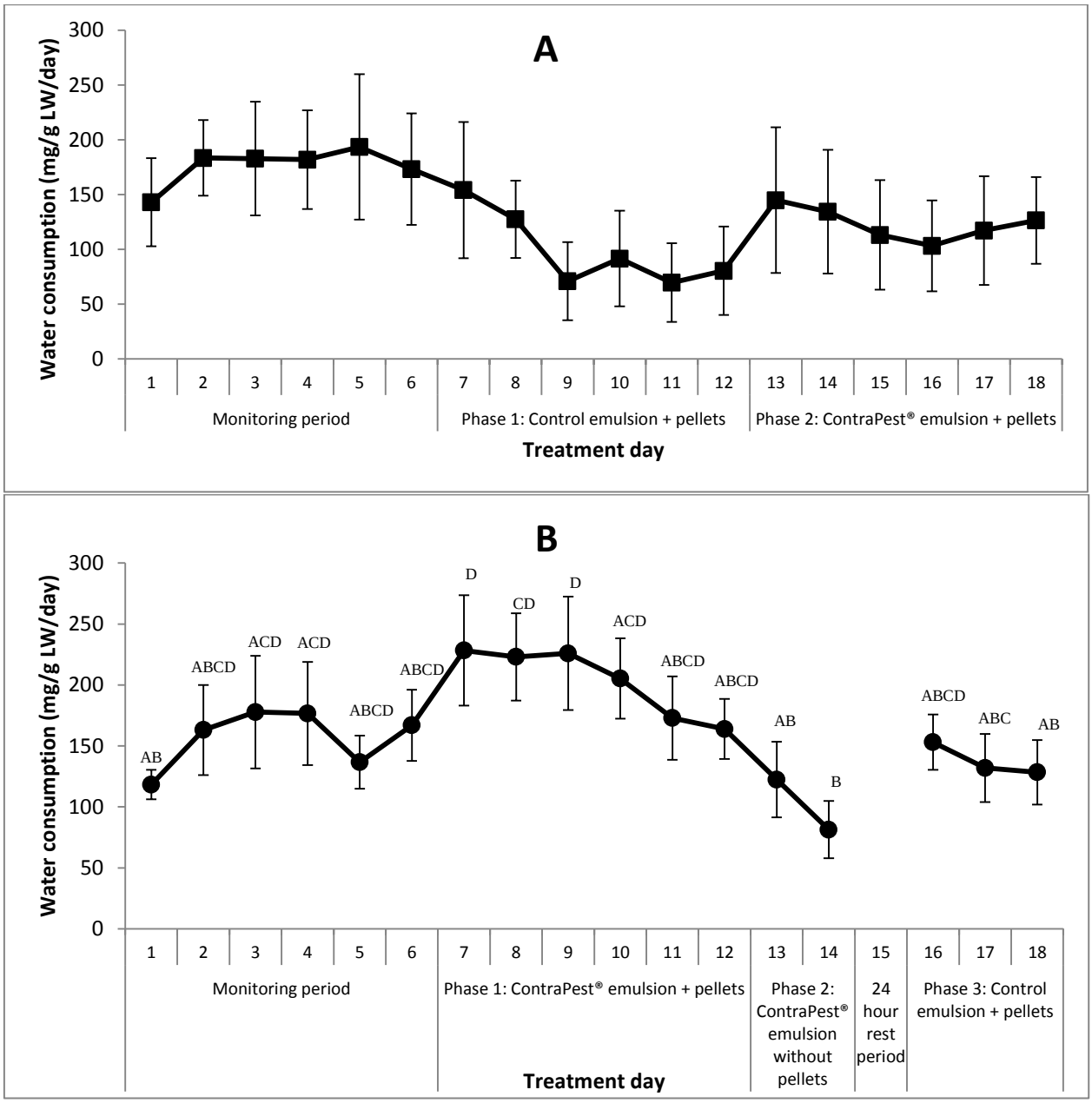

Figure 7.6 Water consumption in wild-caught female rats during Study 1 for group 1 (A; n = 4) and group 2 (B; n = 6). Values with different letters are significantly different ($p < 0.05$). Vertical bars represent ± SEM.

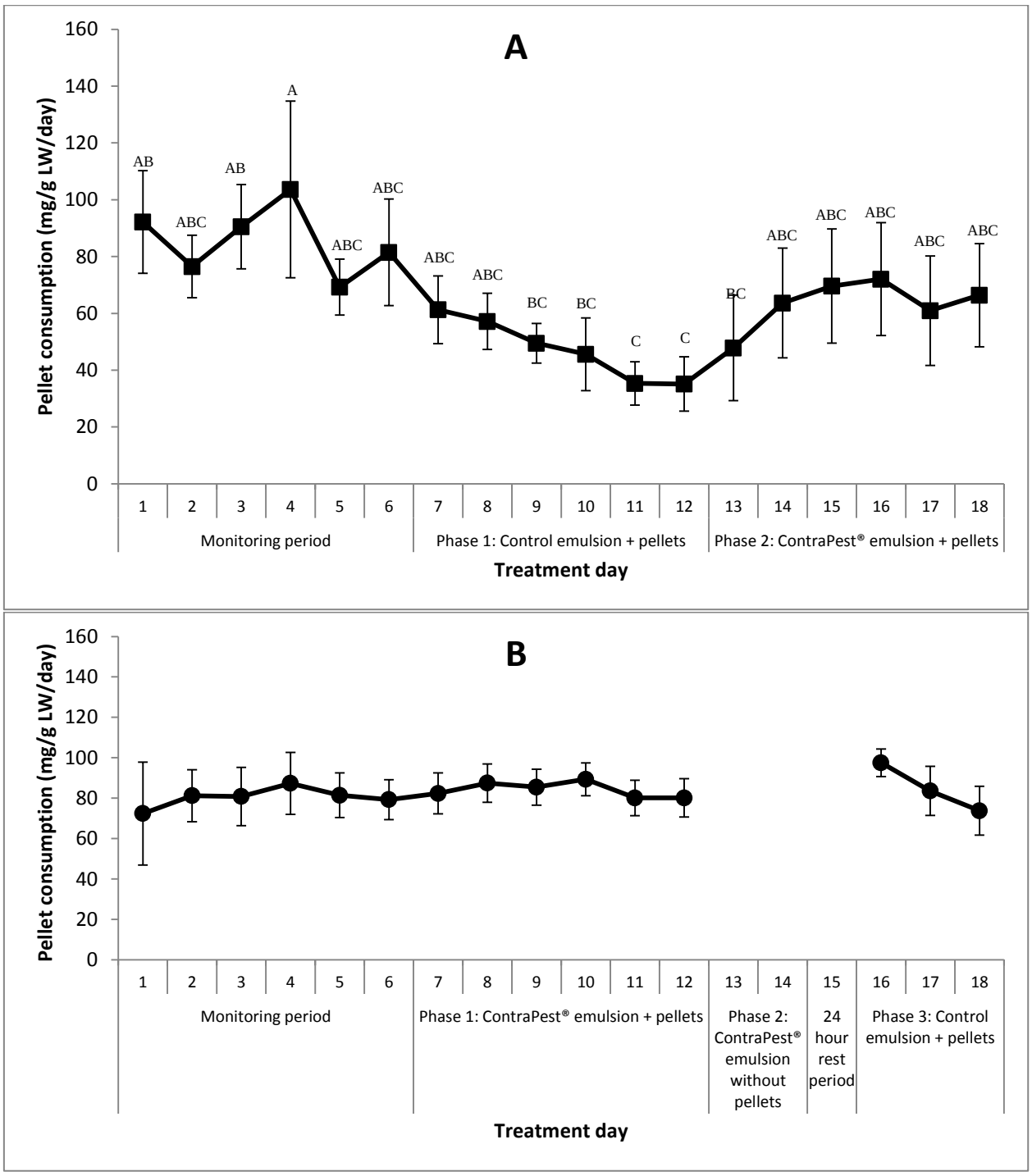

Figure 7.7 Pellet consumption in wild-caught female rats during Study 1 for group 1 (A; n = 4) and group 2 (B; n = 6). Values with different letters are significantly different ($p < 0.05$). Vertical bars represent ± SEM.

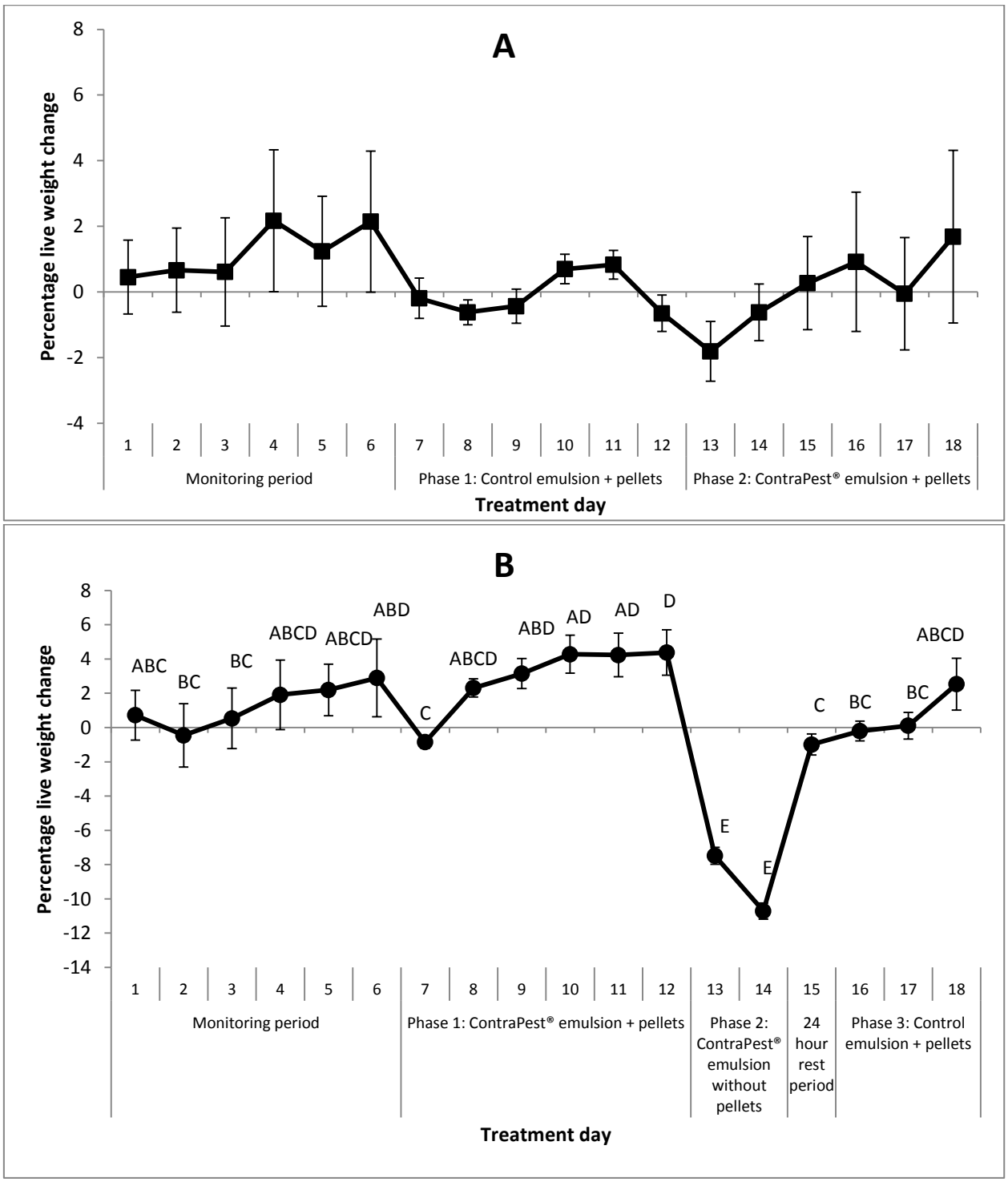

Figure 7.8 Percentage live weight change in wild-caught female rats during Study 1 for group 1 (A; n = 4) and group 2 (B; n = 6). Values with different letters are significantly different ($p < 0.05$). Vertical bars represent ± SEM.

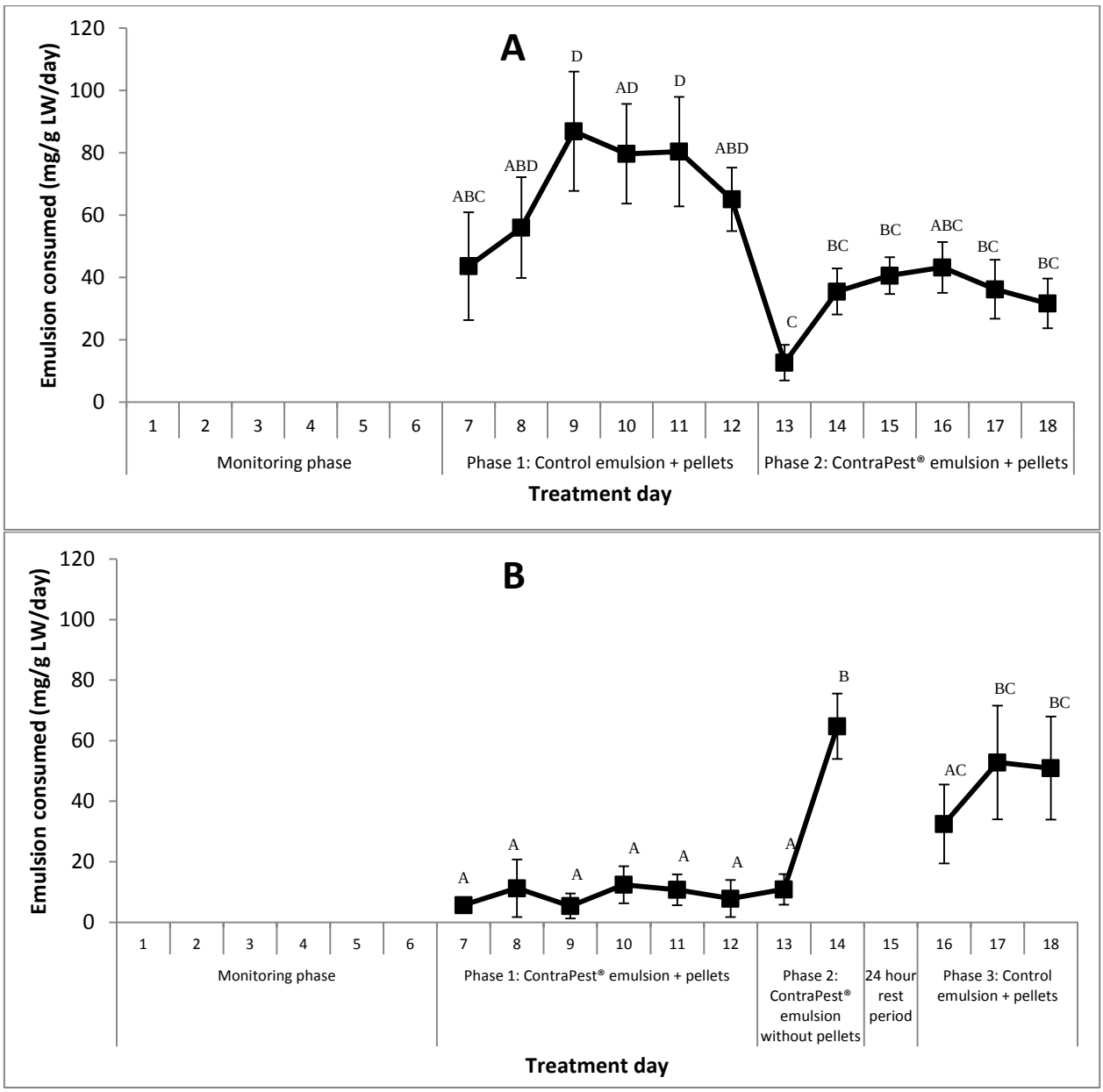

Figure 7.9 Emulsion consumption in wild-caught female rats during Study 1 for group 1 (A; n = 4) and group 2 (B; n = 6). Values with different letters are significantly different ($p < 0.05$). Vertical bars represent ± SEM.

Table 7.2 Consumption parameters for control and ContraPest® emulsion for group 1 (G1, n = 4) and group 2 (G2; n = 6) rats during Study 1 and control-treated (C; n = 8) and ContraPest®-treated (CP; n = 9) rats during Study 2. Statistical significance was set at $p < 0.05$. LW: live weight. For each measured parameter, values with different letters are different ($p < 0.05$). Data are means ± SEM.

Measurement Parameter	Study 1					Study 2			
	Phase 1		Phase 2		Phase 3	Monitoring Phase		Treatment Phase	
	G1	G2	G1	G2	G2	C	CP	C	CP
Emulsion provided	Control + pellets	ContraPest® (batch 1) + pellets	ContraPest® (batch 1) + pellets	ContraPest® only (batch 1)	Control + pellets	Control + pellets	Control + pellets	Control + pellets	ContraPest® (batch 2) + pellets
VCD concentration (mM) in ContraPest®	N/A	2.96 ± 0.1	2.96 ± 0.1	2.96 ± 0.1	N/A	N/A	N/A	N/A	4.68 ± 0.7
VCD consumption (mg/kg LW/day)	N/A	^{A}3.68 ± 0.95	^{B}20.0 ± 5.0	^{B}15.7 ± 4.1	N/A	N/A	N/A	N/A	^{B}14.7 ± 1.2
TR consumption (µg/kg LW/day)	N/A	^{A}17.8 ± 4.6	^{B}96.3 ± 24.1	^{B}75.6 ± 19.8	N/A	N/A	N/A	N/A	^{B}88.6 ± 7.0
Emulsion intake (mg/g LW/day)	^{AB}68.6 ± 6.7	^{E}8.9 ± 2.3	^{C}33.3 ± 3.4	^{C}37.8 ± 9.9	^{C}45.4 ± 9.2	^{A}73.3 ± 6.4	^{A}72.4 ± 5.3	^{B}58.5 ± 1.9	^{D}22.4 ± 1.8
Emulsion palatability	^{AEF}0.58 ± 0.03	^{B}0.08 ± 0.02	^{CD}0.36 ± 0.03	N/A	^{D}0.32 ± 0.06	^{EF}0.59 ± 0.03	^{E}0.64 ± 0.03	^{F}0.58 ± 0.01	^{G}0.25 ± 0.02

Effect of pre-feeding with control emulsion (Group 1, phase 2)

During P2, G1 animals previously offered control emulsion were offered ContraPest® emulsion to determine the effects of pre-feeding on palatability. Animals consumed significantly less ContraPest® emulsion during P2 compared with control emulsion consumed during P1 ($p < 0.0001$; Figure 7.9A and Table 7.2). Of the four rats, 2 rats consumed ContraPest® emulsion at a similar rate as measured during P1 (Table 7.3). However, the other 2 rats' consumption of ContraPest® emulsion was decreased compared with P1. G1 rats consumed significantly more ContraPest® emulsion ($p < 0.0001$) during P2 compared with G2 rats consumption during P1 (Figure 7.9 and Table 7.2).

The amount of VCD consumed by G1 animals during P2 was compared with previous reports demonstrating VCD-induced ovarian primordial follicle reduction with oral gavage. G1's mean consumption of VCD during P2 (20.0 ± 5.0 mg VCD/kg LW/day) was 96.0 % lower than the amount of VCD that would be delivered through via oral gavage (500 mg VCD/kg LW/day) (Table 7.2). Similarly, TR consumption by G1 rats during P2 was compared with previous reports demonstrating TR-induced ovarian follicle effects through oral gavage. G1 rats' mean consumption of TR during P2 (96.3 ± 24.1 µg TR/kg LW/day) was only 3.8 % lower than the amount of TR that would be delivered through an oral gavage route (100 µg TR/kg LW/day) (Table 7.2).

There were no changes in G1 LW ($p > 0.955$) or pellet ($p > 0.273$) and water consumption ($p > 0.113$) between P1 and P2 (Figures 7.3A – 7.5A). When P2 parameters were compared against the monitoring phase, there were no changes in G1 LW ($p > 0.435$, Figure 7.5A). However, G1 water ($p < 0.006$; Figure 7.6A) and pellet ($p < 0.043$; Figure 7.7A) consumption were significantly lower during P2.

No choice study (Group 2, phase 2)

G2 animals previously offered ContraPest® emulsion for 6 days (P1) were placed into a no choice trial for 48 hours (P2) to measure the acceptance rate of the ContraPest® emulsion. When given no other food option, G2 animals consumed more water ($p < 0.008$; Figure 7.6B) and ContraPest® emulsion ($p < 0.006$; Figure 7.9B and Table 7.2) during P2 compared with P1. However, G2 animals lost a significant amount of LW (-9.1 ± 0.58 %; $p < 0.008$; Figure 7.8B) and therefore the no choice treatment was stopped after 48 hours due to animal welfare concerns.

Choice trial (Group 2, phase 3)

Following a 24 hour rest period, G2 animals were moved into the third trial phase (P3) wherein they were offered control emulsion (3 days) for an intake comparison with ContraPest® emulsion. G2 animals consumed significantly more control emulsion during P3 than ContraPest® emulsion during P1 ($p < 0.05$; Figure 7.9B and Table 7.2). In contrast, there were no differences between control emulsion consumed during P3 and ContraPest® emulsion consumed during P2 ($p > 0.073$).

To determine if control emulsion consumption rates during P3 were affected by the previous exposure to ContraPest® emulsion during P1 and P2, a comparison was made between G2P3 and G1P1 control emulsion consumption rates. G2 animals consumed less control emulsion during P3 compared with

that of G1 animals during P1, although this was not significant ($p > 0.073$; Figure 7.9 and Table 7.2). Of the G2 rats, the 2 which did not consume ContraPest® emulsion during P1 again did not consume control emulsion during P3 (Table 7.4).

There were no changes in G2 water ($p > 0.088$; Figure 7.6B) or pellet ($p > 0.975$; Figure 7.7B) consumption between P1 and P3. However, G2 water consumption dropped significantly during P3 compared with P2 ($p < 0.023$). G2 mean LW dropped significantly during P2 ($p < 0.002$) and, although it recovered slowly during P3, it was still significantly lower ($p < 0.042$) compared with P1 (Figure 7.8B).

Table 7.3 Consumption parameters of control or ContraPest® for individual animals in group 1 during phase 1 and 2 (Study 1). Data are means ± SEM.

	Treatment phase	Rat A	Rat B	Rat C	Rat D
Phase 1 (6 days)	Emulsion consumption (mg emulsion/g LW/day)	39.1 ± 6.8	72.8 ± 10.9	108.4 ± 9.5	54.0 ± 5.6
	Percentage emulsion intake relative to LW	3.9 ± 0.7 %	7.3 ± 1.1 %	10.8 ± 1.0 %	5.4 ± 0.6 %
Phase 2 (6 days)	Emulsion consumption (mg emulsion/g LW/day)	38.0 ± 4.7	45.0 ± 5.3	30.7 ± 8.6	19.4 ± 4.4
	Percentage emulsion intake relative to LW	3.8 ± 0.5 %	4.5 ± 0.5 %	3.1 ± 0.9 %	1.9 ± 0.4 %

Table 7.4 Consumption parameters of control or ContraPest® for individual animals in group 2 during phase 1, 2 and 3 (Study 1). Data are means ± SEM.

	Treatment phase	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6
Phase 1 (6 days)	Emulsion consumption (mg emulsion/g LW/day)	22.1 ± 8.3	0.5 ± 1.2	0.1 ± 0.6	0.0 ± 0.7	9.4 ± 2.6	22.1 ± 4.8
	Percentage emulsion intake relative to LW	2.2 ± 0.8%	0.0 ± 0.1 %	0.0 ± 0.1 %	-0.1 ± 0.1 %	0.9 ± 0.3 %	2.22 ± 0.5 %
Phase 2 (2 days)	Emulsion consumption (mg emulsion/g LW/day)	23.9 ± 1.4	42.0 ± 41.0	40.0 ± 38.6	43.6 ± 43.6	43.4 ± 32.7	33.9 ± 7.0
	Percentage emulsion intake relative to LW	2.4 ± 0.1 %	4.2 ± 4.1 %	4.0 ± 3.9 %	4.4 ± 4.4 %	4.3 ± 3.3 %	3.4 ± 0.7 %
Phase 3 (3 days)	Emulsion consumption (mg emulsion/g LW/day)	84.0 ± 15.9	2.2 ± 0.6	30.6 ± 9.9	0.2 ± 0.4	78.0 ± 10.2	77.5 ± 4.2
	Percentage emulsion intake relative to LW	8.4 ± 1.6 %	0.2 ± 0.1 %	3.1 ± 1.0 %	0.0 ± 0.0 %	7.8 ± 1.0 %	7.8 ± 0.4 %

7.3.4 Study 2

Treatment parameters

ContraPest® emulsion containing the artificial sweetener (ContraPest®-S), saccharin, was examined for its effects on palatability, animal health and immature ovarian follicle populations. To determine if there were any changes between the monitoring/pre-feed phase and treatment phase, LW and consumption of pellets and water were compared within each group. LW change and consumption of water, pellets and control emulsion of the controls were unaltered between the phases ($p > 0.104$; Figures 7.10 - 7.13). Similarly, the percentage LW gain of the ContraPest®-S group during treatment did not differ between phases ($p > 0.436$; Figure 7.13). However, the ContraPest®-S group consumed more water and pellets during treatment compared with the pre-feeding phase ($p < 0.001$) and when compared with controls during treatment ($p < 0.001$; Figures 7.11 and 7.12). Throughout the trial all animals gained weight, regardless of treatment ($p < 0.001$; Figure 7.13).

Animals were pre-fed with control emulsion for 4 days to optimize the acceptance of ContraPest®-S emulsion. During the 4 day pre-feed phase, both groups consumed an average of 72.9 ± 4.1 % of the total amount of control emulsion offered (Figure 7.10 and Table 7.2). When switched to ContraPest®-S emulsion, consumption rates fell by 69.1% during the 15 day treatment period ($p < 0.0001$; Figure 7.10 and Table 7.2). Interestingly, consumption of control emulsion during treatment also fell by 20.2% ($p < 0.033$; Table 7.2). The minimum and maximum consumption of control emulsion during treatment was 50.3 ± 3.96 and 73.5 ± 6.81 mg/g LW/day, respectively. The minimum and maximum consumption of ContraPest®-S emulsion was 1.75 ± 0.61 and 57.3 ± 4.36 mg/g LW/day, respectively. Of the 9 rats offered ContraPest®-S emulsion, 2 consumed <0.6% g emulsion/g LW, 3 consumed 0.6 – 2.0% g emulsion/g LW, and the remaining 4 consumed 3.2 – 3.5% g emulsion/g LW.

The amount of VCD consumed during Study 2 was compared with previous reports demonstrating VCD-induced ovarian primordial follicle reduction through oral gavage. The amount of VCD consumed during the trial (14.7 ± 1.2 mg VCD/kg LW/day) was 97.1% lower than the amount of VCD that would be delivered through an oral gavage route (500 mg VCD/kg LW/day). This figure was minimally affected when the animals which did not consume ContraPest®-S emulsion on a regular basis (<0.6% g emulsion/g LW) were removed from the data set (23.3 ± 4.1 mg VCD/kg LW/day; 95.3% decrease).

Similarly, the amount of TR consumed during Study 2 was compared with previous reports demonstrating TR-induced ovarian follicle effects through oral gavage. The mean consumption of TR during the trial (88.6 ± 7.0 µg TR/kg LW/day) was 11% lower than the amount of TR that would be delivered through an oral gavage route (100 µg TR/kg LW/day). When the animals which did not consume ContraPest®-S emulsion on a regular basis were removed from the data set, TR consumption (140.8.0 ± 24.5 µg TR/kg LW/day) was 40.9% higher than that of an oral TR dose.

Dietary choices in rats are often influenced by social interactions and with age and experience, animals learn avoidance of and preferences for certain food items (Galef and Clark, 1971). Therefore, animal age and consumption rates of control or ContraPest®-S emulsion were examined for correlations. There were no age correlations indicated in animals consuming control emulsion ($p > 0.790$; Figure 7.14). Conversely, there was a positive correlation for age and ContraPest®-S emulsion consumption ($p < 0.032$; Figure 7.14).

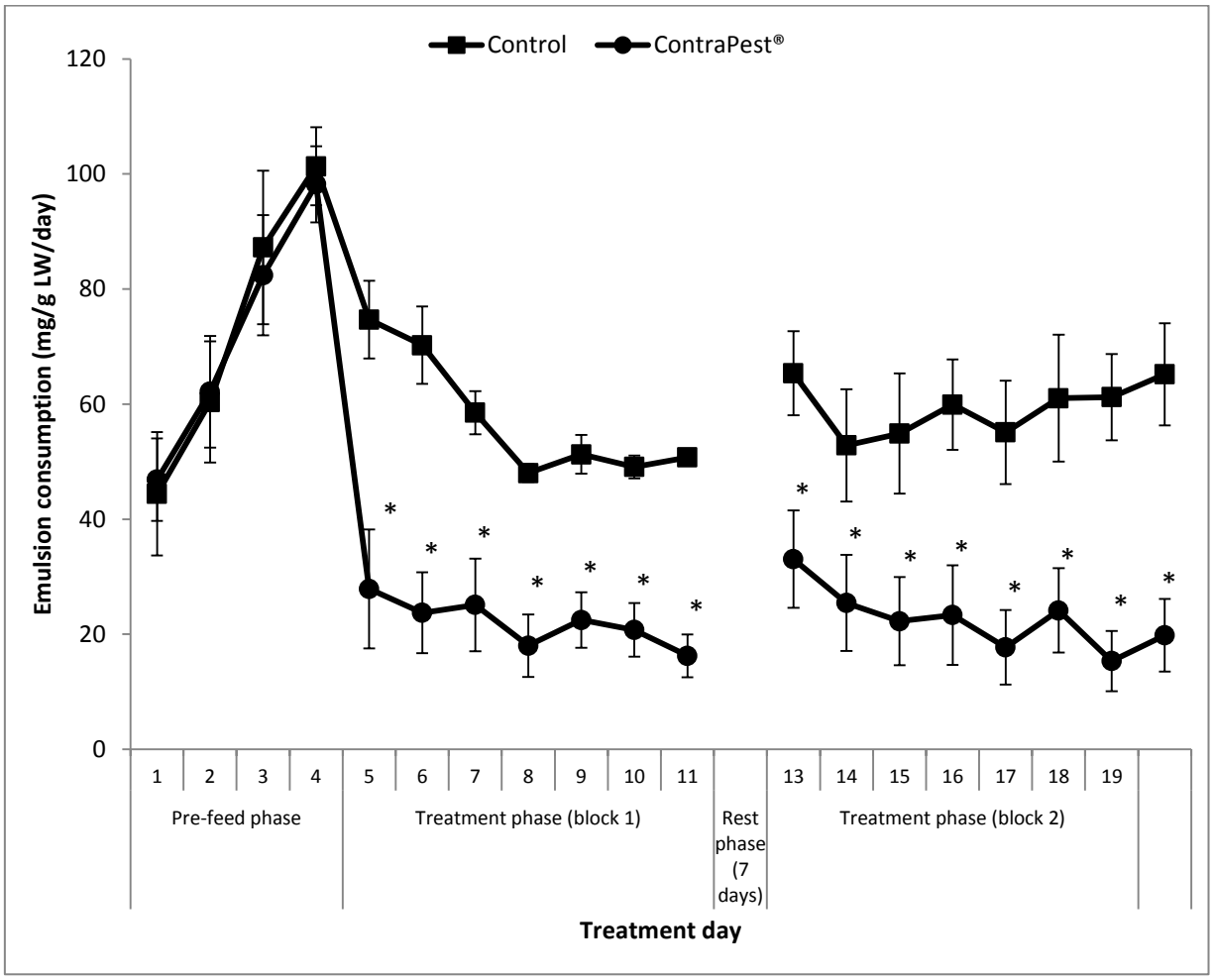

Figure 7.10 Emulsion consumption rates in wild-caught female rats during Study 2 (control, n = 8; ContraPest®-S, n = 9). * indicates different ($p < 0.05$) from control. Vertical bars represent ± SEM.

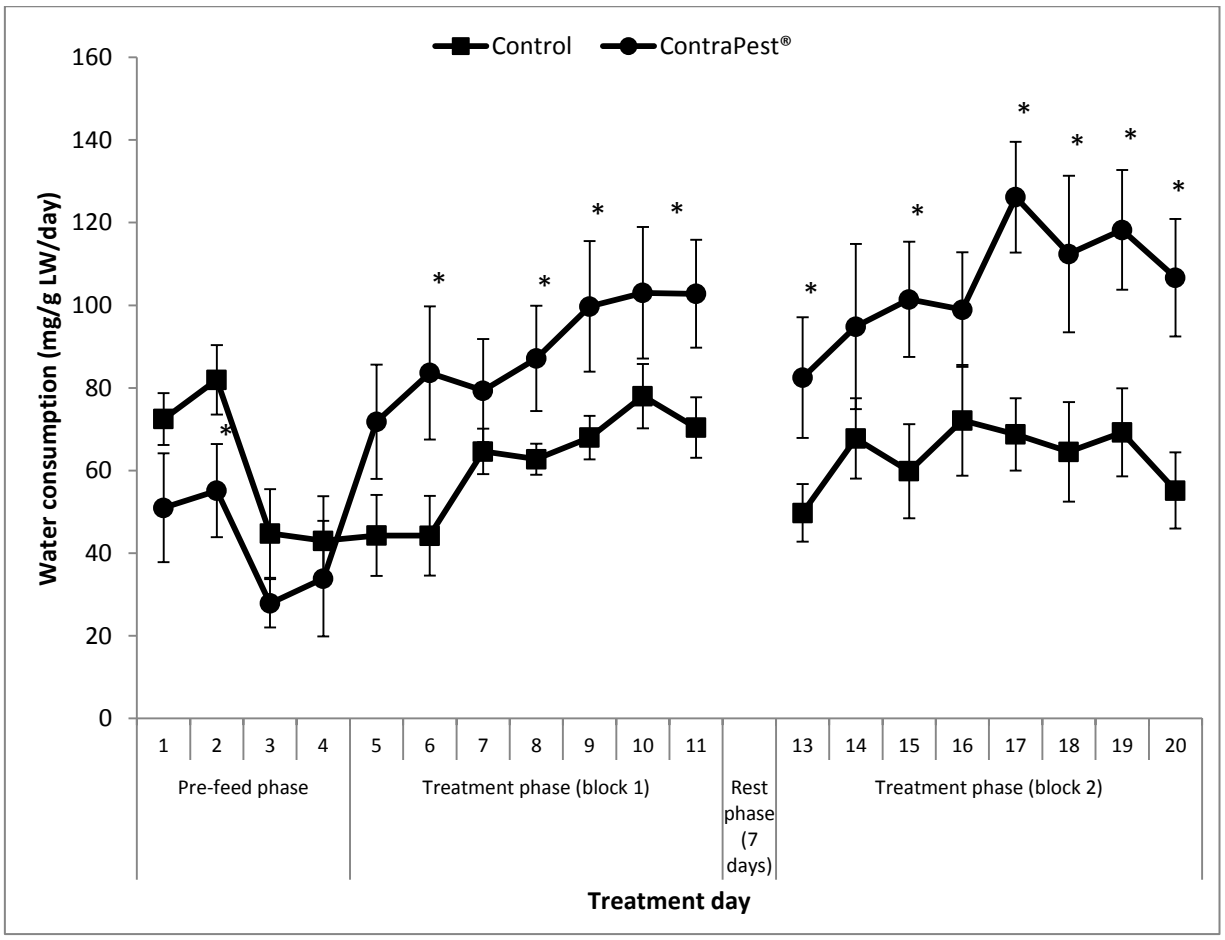

Figure 7.11 Water consumption rates in wild-caught female rats during Study 2 (control, n = 8; ContraPest®-S, n = 9). * indicates different (p <0.05) from control. Vertical bars represent ± SEM.

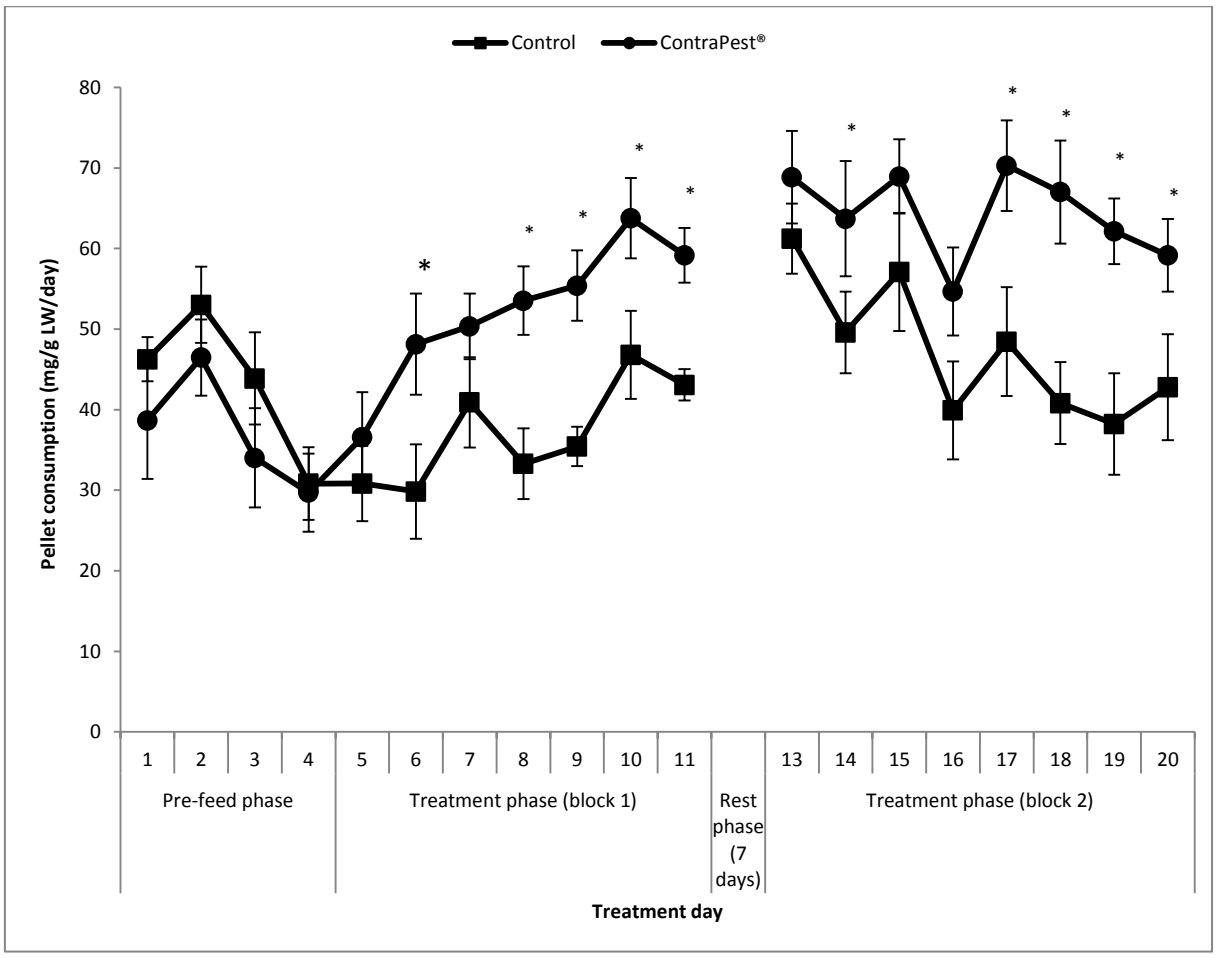

Figure 7.12 Pellet consumption rates in wild-caught female rats during Study 2 (control, n = 8; ContraPest®-S, n = 9). * indicates different (p < 0.05) from control. Vertical bars represent ± SEM.

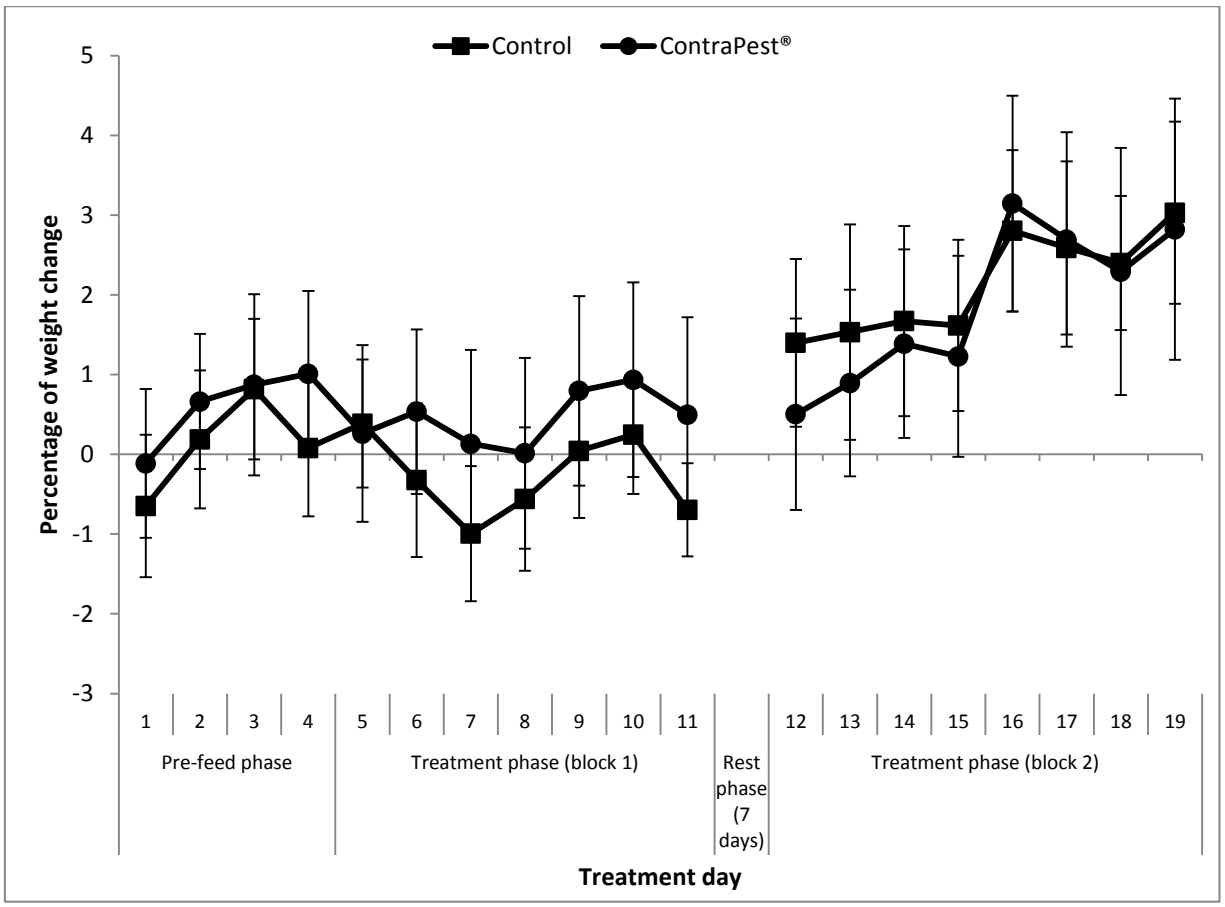

Figure 7.13 Percentage live weight change in wild-caught female rats during Study 2 (control, n = 8; ContraPest®-S, n = 9). Vertical bars represent ± SEM.

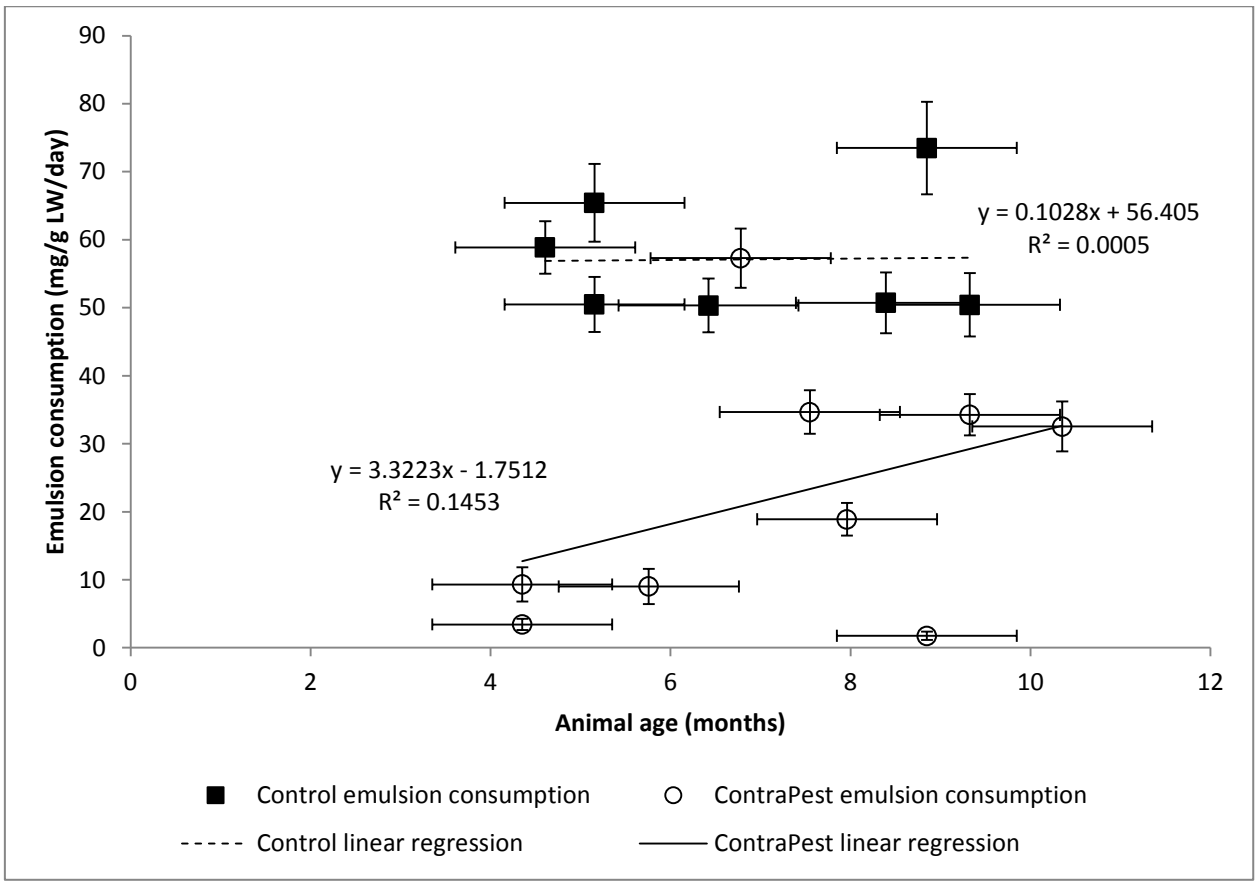

Figure 7.14 Emulsion consumption levels of wild-caught female rats during Study 2 graphed against animal age (months). Vertical and horizontal bars represent ± SEM.

Comparisons of emulsion consumption between Study 1 and 2

To determine if the addition of the sweetener saccharin would increase palatability, the consumption rates of sweetened control and ContraPest®-S emulsion during Study 2 was compared with consumption rates during Study 1. There were no differences in consumption of sweetened or unsweetened control emulsion ($p > 0.601$; Table 7.2). Pre-feeding with control emulsions resulted in greater intake of unsweetened ContraPest® (Study 1, G1P2: 33.3 ± 4.1 mg/g LW/day) than that of ContraPest®-S emulsion (Study 2: 22.3 ± 1.72 mg/g LW/day) ($p < 0.015$; Table 7.2).

Necropsy parameters

There were no significant differences in post mortem LW or the weights of liver, paired kidneys, paired adrenal glands, uterine or paired ovaries between treatment groups ($p > 0.2275$; Table 7.5). In addition, there were no differences in age between treatment groups ($p > 0.891$; Table 7.5).

Table 7.5 Animal age, live weight (LW) and somatic and reproductive tissue weights in wild-caught female rats when offered control or ContraPest®-S emulsions daily for 15 days during Study 2. Somatic and reproductive tissues were normalized to final LW. Statistical significance was set at p < 0.05. Data are means ± SEM.

Measured parameter	Treatment group	
	Control	ContraPest®
N	9	9
Age at death (month)	7.11 ± 0.77	7.25 ± 0.71
Final LW (g)	234.8 ± 17.6	257.6 ± 17.5
Liver (mg/mg LW)	42.2 ± 1.4	43.4 ± 1.4
Paired kidney (mg/mg LW)	6.94 ± 0.29	7.44 ± 0.29
Paired adrenal gland (mg/mg LW)	0.40 ± 0.03	0.39 ± 0.02
Uterus (mg/mg LW)	1.97 ± 0.17	1.99 ± 0.24
Paired ovary (mg/mg LW)	0.29 ± 0.02	0.30 ± 0.02

Ovarian follicle estimates

There was no correlation between age or ovarian weight with primordial, secondary and antral follicles ($p > 0.132$). However, age and CL counts were positively correlated ($p < 0.001$). Therefore, CL counts were normalized to age prior to examining for treatment effects by dividing the CL counts by animal age. Animals receiving ContraPest®-S emulsion had fewer primordial follicles (827.7 ± 198.87) than controls (1312.0 ± 160.30); however this was not significant ($p < 0.076$; Figure 7.15). Because not all animals consumed ContraPest®-S emulsion (n = 2), primordial follicle counts were re-examined in the rats which consumed emulsion on a regular basis (>0.6% g emulsion/g LW; n = 7). In this group, primordial follicle counts were significantly reduced in rats which consumed ContraPest®-S emulsion (592.59 ± 131.76) when compared with controls (1307.53 ± 181.69; 54.7% reduction; $p < 0.009$; Figure 7.15). Rats which did not consume ContraPest®-S emulsion were within the range of control animals (1650.48 ± 434.44). There were no significant differences in numbers of secondary ($p > 0.683$) and antral ($p > 0.909$) follicles or CL ($p > 0.757$) between treatment groups (Figures 7.16 and 7.17). There was a trend for reduced secondary follicles in rats which consumed ContraPest®-S emulsion (10.29 ± 1.43) compared with controls (13.25 ± 1.30); however this was not significant ($p > 0.132$, 22.4% reduction). Rats which did not consume ContraPest®-S emulsion were within the range of control animals (13.50 ± 0.5).

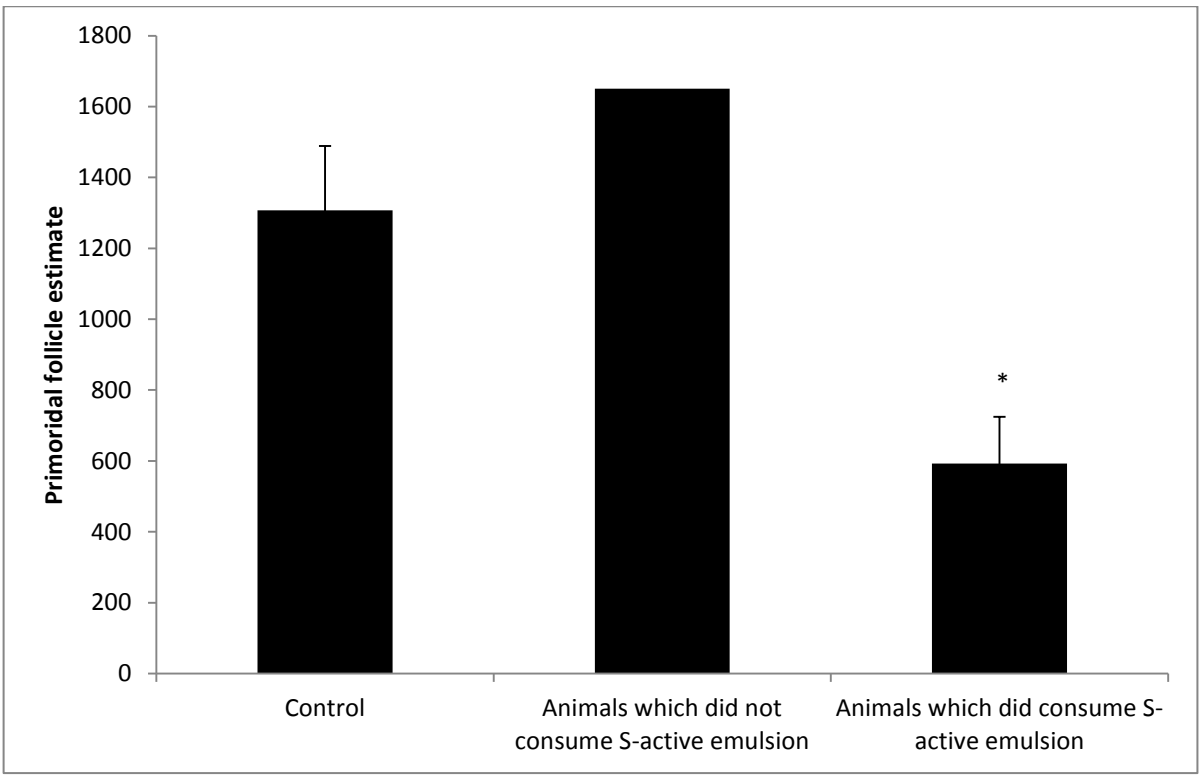

Figure 7.15 Mean total primordial ovarian follicle estimates in wild-caught female rats when offered control or ContraPest®-S emulsions daily for 15 days during Study 2. Counts were recorded from the left ovary of each animal and total counts estimated using a correction factor formula (Gougeon and Chainy, 1987). Control, n = 8; animals which did not consume ContraPest®-S, n = 2; animals which did consume ContraPest®-S, n = 7. * indicates different ($p < 0.05$) from control. Vertical bars represent + SEM.

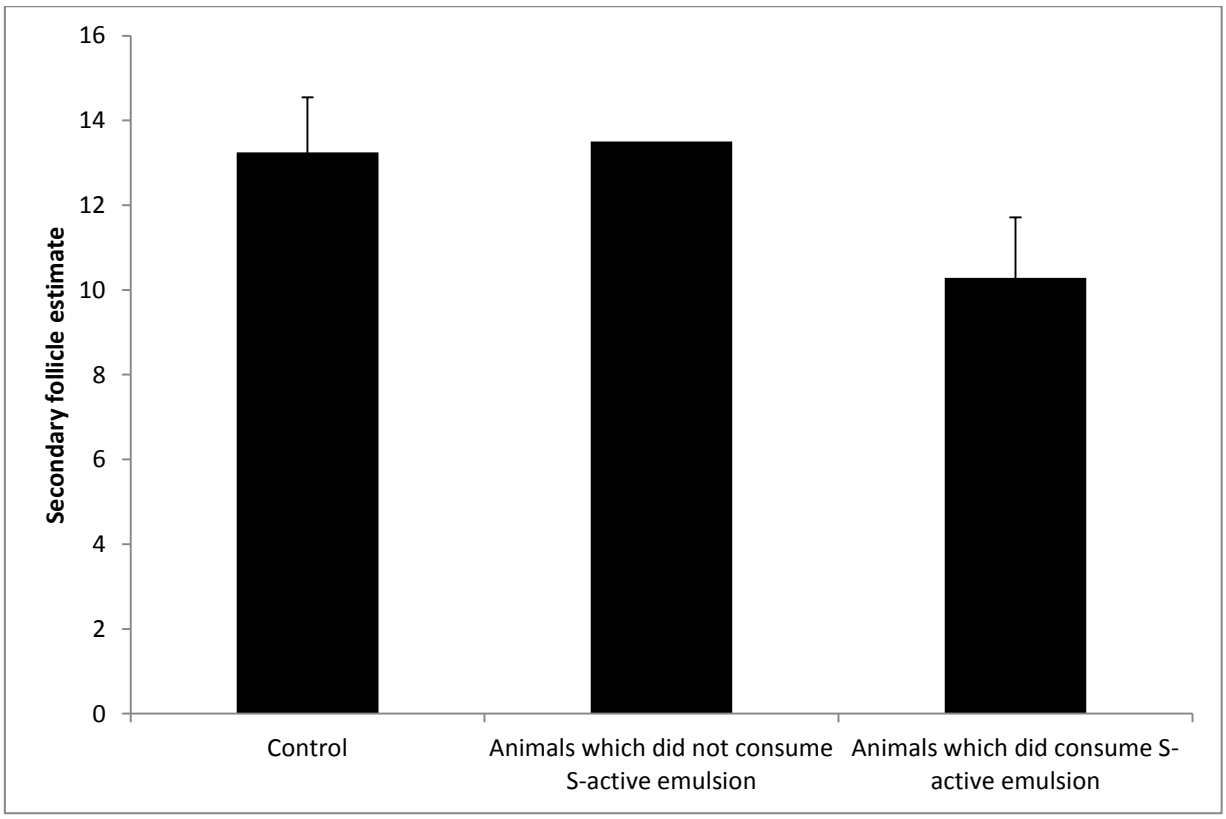

Figure 7.16 Mean total secondary ovarian follicle estimates in wild-caught female rats when offered control or ContraPest®-S emulsions daily for 15 days during Study 2. Counts were recorded from the left ovary of each animal and total counts estimated using a correction factor formula (Gougeon and Chainy, 1987). Control, n = 8; animals which did not consume ContraPest®-S, n = 2; animals which did consume ContraPest®-S, n = 7. Vertical bars represent ± SEM.

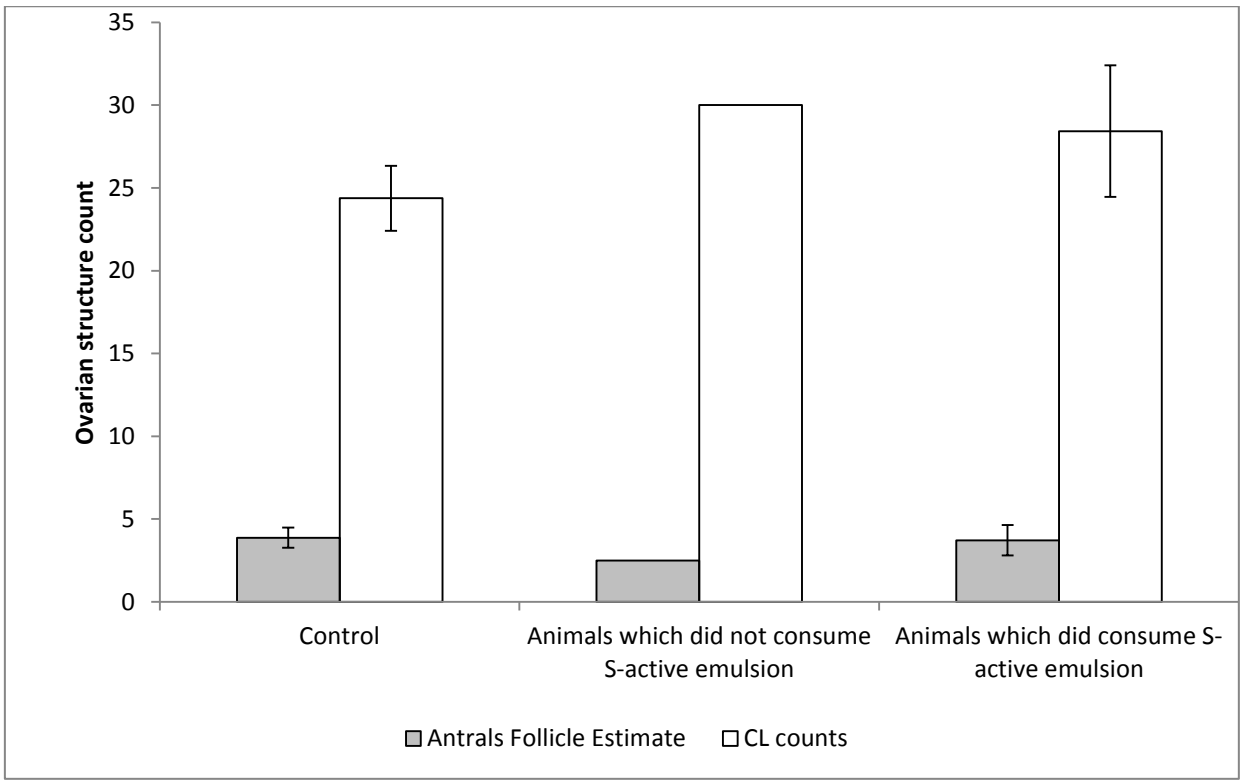

Figure 7.17 Mean total antral ovarian follicle counts and corpus luteum counts in wild-caught female rats when offered control or ContraPest® -S emulsions daily for 15 days during Study 2. Counts were recorded from the left ovary of each animal. Control, n = 8; animals which did not consume ContraPest® -S, n = 2; animals which did consume ContraPest® -S, n = 7. Vertical bars represent ± SEM.

7.4 Discussion

To find a more humane and sustainable alternative solution for pest control in NZ, the chemosterilant emulsion, ContraPest®, was examined for its palatability and effects on animal health and the ovarian follicle populations of wild-caught Norway rats. The ultimate success of ContraPest® as a pest control method will lie in its ability to be delivered as bait that is palatable, effective and able to induce permanent fertility cessation in females.

Consumption rate of ContraPest® emulsion during each study was less than that of the control emulsion, indicating that rats were able to detect the active components (whether the aversion is to VCD or TR, or both is yet to be determined). Within rodent genomes, rats have ~1400 functional olfactory receptors (or ~6% of its genes) whereas humans have only ~400, thus highlighting the importance of olfaction in this species (Ache and Young, 2005). To help overcome the highly developed sense of smell in rodents, the active ingredients were microencapsulated within the emulsion. It is believed that microencapsulation of VCD and TR helps to reduce their olfactory and palatability deterring effects (Dyer et al., 2013). In addition, microencapsulation of these active components may help improve their uptake into the gastrointestinal tract as well as provide the chemicals with a protective barrier from stomach acid (Carr et al., 1996; Florence, 2005; Jani et al.,

1990). VCD, when in direct contact with the oral or nasal cavity, can cause irritation and lesions (personal observation) as well as epithelial hyperplasia of the fore stomach (NTP, 1986). It must be noted that these pathological findings were a result of oral gavage doses (200 – 5000 mg/kg/day, 14 days to 2 years) far above what would be achieved through consumption of VCD contained within ContraPest® emulsion. Nevertheless, microencapsulation of VCD and TR should help to reduce potential irritation during consumption of the emulsion bait.

The reduced intake of ContraPest® emulsion compared with controls would suggest that rats were able to detect some chemical traces within the emulsion. This may have been due to incomplete removal of any unincorporated active ingredients during washing or due to the microcapsules breaking down during international shipping and storage. The temperature stability study demonstrated that VCD, when stored at 4°C, is relatively stable. ContraPest® emulsion was stored at 4°C following arrival at the research facility and throughout the course of each study. Therefore it can be assumed that microcapsule degradation was minimal throughout each study period. However, during international transport (batch 1: 15 days; batch 2: 7 days), it is reasonable to assume that the ContraPest® emulsions would have been exposed to warmer temperatures (>21°C; ambient) which have been shown to affect VCD, regardless of whether the chemical is microencapsulated or not. In fact, VCD concentrations in the ContraPest® emulsions were substantially reduced following international transport (95%) and this was likely a result of microcapsule degradation due to warm transportation temperatures. Thus, reduced ContraPest® emulsion consumption may have, in part, been due to release and hydrolysis of the active ingredients from the microcapsules resulting in olfactory and palatability chemical detection. Despite the deterring effects from the active ingredients, rats did consume ContraPest® to some degree, demonstrating that the emulsion formulation used during these studies was somewhat successful.

Pre-feeding with control emulsion increased the acceptance of ContraPest® emulsion but did not completely overcome the aversion. It is well understood that rodents are neophobic (avoidance of novel stimuli) (Barnett, 1958; Mitchell, 1976) and often require time to adjust to changes in their environment and food sources. The practice of pre-baiting with a non-toxic bait has been utilized in an attempt to increase poison control operation success and to help overcome ‘conditioned bait aversion’ (Buckle and Smith, 1994) . In an open field environment, pre-baiting draws in animals from surrounding areas and allows the animals to habituate to the bait, the bait boxes and any potential environmental changes that may have occurred as a result of human interference. In these studies, animals were exposed to a water-filled glass beaker for 6-7 days prior to the start of the trial in order to reduce any potential rejection of emulsion as a result of neophobic behaviour. No avoidance behaviour was noted. In the first study, consumption rates of ContraPest® emulsion with and without pre-feeding with control emulsion confirmed the effectiveness of the pre-feeding period. Thus, in the second study, a pre-feeding strategy was adopted to optimize intake of the ContraPest® emulsion.

Interestingly, during each study ~50% of rats consumed ContraPest® emulsion at comparable levels to those observed during the control emulsion pre-feed period while the other rats consumed ContraPest® emulsion either at very low levels or not at all. It is not clear why this 50/50 split was observed across the studies. However, in any population, animals that do not respond to treatment (i.e. non-consumers, non-responders) will inevitably exist. A study examining neophobic tendencies of wild Norway rats in geographically separated areas revealed that, on average, 8-10% of individuals show no neophobic behaviours while 4-10% show extreme neophobia (Macdonald et al., 1999). It is possible that we were observing similar results on a smaller scale.

There was a positive correlation between age and ContraPest®-S emulsion consumption during Study 2. It was observed that older animals consumed more ContraPest®-S emulsion per gram live weight compared with that of younger animals. Although this finding was unexpected, it may be that the older animals were more willing to sample the emulsion in small quantities while the younger animals avoided the emulsion all together. Dietary choices in rats are often influenced by social interactions and with age and experience, animals learn avoidance of and preferences for certain food items (Galef and Clark, 1971; Galef et al., 1992). Rats typically consume their meals in small bouts and, when offered new food items, they will often take a small sample and only return to that food item pending no ill effects (Jensen et al., 1983; Lee and Clifton, 2002; Macdonald et al., 1999). Such behaviours are believed to result in bait shyness following a sub-chronic dose of poisoned bait (Barnett and Spencer, 1949; Bhardwaj and Khan, 1979). Full avoidance of the emulsion would indicate that the olfactory cues alone were able to deter some of the rats. Consumption of ContraPest®-S emulsion by the older animals could indicate that they were either not detecting the active ingredients or that they found no ill effects from consuming the emulsion and therefore no avoidance behaviour developed. In addition, the rats which did consume ContraPest®-S emulsion regularly may have been showing preference for the non-active ingredients (e.g. saccharine, high lipid content), whether for their masking effects of the active ingredients or for their palatability preferences, or both.

Saccharin was employed during Study 2 to help disguise any potential deterring factors from the active components in the ContraPest® emulsion. Research has demonstrated that female rats prefer sweetened water to unsweetened water and, when given a choice between glucose or saccharine, they prefer the latter (Mook, 1974; Valenstein et al., 1967). Surprisingly, there was no effect from the addition of a sweetener on consumption rates of ContraPest®-S emulsion. ContraPest® emulsion is a mixture of oil and water (20:80%, v/v). Because consumption rates of ContraPest® emulsion following a control pre-feed period were higher during Study 1 compared with Study 2, this may indicate that the rats were in fact showing preference for the lipids in the emulsion rather than the saccharine. It has been suggested that a rats preference for sweetened solutions is in fact an indicator for a fluid food source (thus informational) rather than for pleasure (Mook, 1974). When offered a choice between saccharin-sweetened or bland liquid diets, rats rarely showed preference between the two. Thus, if there are food-associated cues present, sweetness becomes redundant (Mook, 1974). In addition, rats offered minced liver, flour, wheat grains and sugar did sample from all food types but sugar was

consumed the least (Barnett, 1956). Therefore, it is possible that the rats were consuming ContraPest®-S emulsion for its fat source rather than for its sweetened properties.

Alternatively, the lack of success with the use of saccharine may be that the active ingredients in the ContraPest®-S emulsion overpowered the smell and taste of the sweetener, negating any potential benefits. The VCD content was higher in the batch used during Study 2 and this may have caused an increased deterrent effect. Saccharine has been used in the past to mask rat olfactory cues of toxins (e.g. lithium chloride) presented in a liquid state (Rusiniak et al., 1976). When given a choice between saccharine-sweetened liquid with or without toxins, it was more difficult for rats to distinguish between the liquids but still possible (Rusiniak et al., 1976). Nevertheless, the proposed benefits of the sweetener did not increase the palatability of the ContraPest®-S emulsion in the present case.

For successful application of ContraPest® as a rodent control method, it will be imperative to reduce, remove or disguise any potential olfactory or palatability deterrents. Currently, researchers at SenesTech® (Flagstaff, AZ, USA) are working on reducing the olfactory and palatability cues in ContraPest® emulsion. Their preliminary work in adult female laboratory-bred Sprague Dawley rats has demonstrated high consumption rates of 6-10% of their live weight in new formulations of ContraPest® emulsion (Drs. C.A. Dyer and L.P. Mayer, unpublished data).

When rats were offered ContraPest®-S, primordial follicle populations were significantly reduced in those animals that consumed the emulsion. This result is promising considering that the VCD dose in ContraPest®-S was 95% below that of a VCD dose (500 mg/kg/day) which has shown efficacy in depleting the immature ovarian follicle pool in rats by oral gavage (Burd, 2009; Herawati et al., 2010; Mayer et al., 2010). In addition, the level of primordial follicle reduction in this study (54 - 64.1%; 15 days; 14.7 – 20.0 mg/kg/day) is comparable to oral gavage studies in rats (58 - 88%, 15 days, 500 – 750 mg/kg/day; Dr. L. A. Hinds, CSIRO, Australia, unpublished data). One reason for the primordial follicle reducing success of ContraPest®-S may be due to the incorporation of VCD into the microcapsules as previously discussed. Encapsulation of VCD likely protected the chemical from acidic breakdown or digestion in the gastrointestinal tract or alternatively by promoting uptake across the mucosal surfaces into the blood (Carr et al., 1996; Florence, 2005; Jani et al., 1990).

The success of ContraPest®-S in reducing the primordial follicle pool of consuming rats may also, in part, be due the feeding behaviour of rats. Rat feeding behaviours have been extensively studied in both laboratory- and field-based settings (Berdoy and MacDonald, 1991; Bernstein, 1975; Galef and Clark, 1971; Galef et al., 1992; Galef Jr and Giraldeau, 2001). Rats typically consume their meals at night in small bouts (1-3 g food/meal; 10-15 meals/day) (Jensen et al., 1983; Lee and Clifton, 2002) with females foraging in multiple small trips while male forage trips are fewer and longer (Macdonald et al., 1999). It has been hypothesized that VCD ovarian toxicity is largely due to repeat exposure, thereby overwhelming and ultimately reducing the capacity of the detoxifying enzymes glutathione (GSH) and epoxide hydrolase (EH) (Keating et al., 2008a). Thus, because of the nature of female rat feeding habits, it is likely that the rats in Study 2 were continually ingesting VCD on a nightly basis. If

indeed this was the case, the detoxifying enzymes could become overwhelmed, allowing for the follicle-reducing effects of VCD to occur over the span of the nocturnal feeding window. It seems that the rodent feeding patterns may prove serendipitous for the use of ContraPest® as a rodent fertility control agent.

Interestingly, there was no observed effect of TR on the secondary follicle populations in ContraPest®-consuming rats. A recent study by Dyer et al. (2013) examined the effects of a bait containing 1% VCD plus three different concentrations of TR on the ovarian follicle populations of laboratory bred Sprague Dawley female rats. Ovarian follicle counts (primordial, secondary, antral) were reduced and no corpora lutea (CL) were present in animals consuming a bait containing VCD (1%) + a medium dose of TR (50 µg/kg) for 15 days compared with controls. However, 15 days of bait consumption with VCD (1%) plus a low (25 µg/kg) or high (100 µg/kg) dose of TR did not affect the ovarian follicular pools compared with controls. In fact, the number of CL's was increased in the low and high TR dose groups compared with controls (Dyer et al., 2013).

The reason for TR's ability to cause effects to the follicle pool based on dosage level is not clear. It is thought that TRs method of action is through alteration of the intracellular calcium influx pattern (Leuenroth et al., 2007; Lue et al., 1998). Signal transduction pathways that control the decision for a cell to divide, differentiate or die are activated by increases in intracellular calcium concentrations (Ermak and Davies, 2002; McKinsey et al., 2002; Orrenius et al., 2003). Thus, TRs ability to increase intracellular calcium levels can lead to cessation of follicular development and the onset of follicular apoptosis. Assuming sufficient quantities of TR were reaching the ovaries of rats which consumed ContraPest®-S, it is likely that apoptosis of the secondary follicle pool would have occurred. The numbers of healthy secondary follicles were reduced in rats which consumed ContraPest®-S when compared with rats which did not consume ContraPest®-S. Although this was not significant, the lower secondary follicle numbers may indicate TR-induced apoptosis of the secondary follicle pool was occurring. Without quantitative measurement of atretic secondary follicles in rats which consumed ContraPest®-S, we can only speculate on the potential effects from TR contained within the ContraPest®-S emulsion.

Results presented here and by Dyer et al. (2013) suggest a possible interaction between VCD and TR which may be enhancing their effects on the primordial follicle pool. As previously discussed, the concentration of VCD that was being consumed during this study was well below that of an effective oral dose yet primordial follicles were significantly reduced. Dyer et al. (2013) observed this same effect in the medium TR dose group. A report on the effects of TR alone (60 or 120 µg/kg/day, 35 days) showed that the number of healthy and atretic secondary follicle numbers were increased while the primordial follicle pool was unaffected (Xu and Zhao, 2010). As expected, TR-induced suppression of follicle development resulted in an accumulation of secondary follicles with no impact on the primordial follicle pool. It is possible that the reduction in primordial follicles observed here is strictly a result of VCD's effects, although evidence for potentiation of VCD plus TR is evident.

The studies presented here have demonstrated the effectiveness of a pre-baiting system and its ability to increase the palatability of ContraPest® emulsion. Microencapsulation of the active components seems to have protected the chemicals from warm temperatures encountered during international transportation and from stomach acidity and digestion sufficiently to induce effects on the immature follicle pool of ovaries in consuming rats. Although the VCD concentration in the ContraPest®-S emulsion was well below an average gavage dose, consumption rates were sufficient to reduce the primordial follicles pools of ContraPest®-consuming rats to levels comparable with those observed in oral gavage studies. Collectively, these studies have provided evidence that ContraPest® shows potential as a chemosterilant bait for control of rodent populations. Successful application of ContraPest® will required an understanding of foraging behaviours of the target species. Reduction or elimination of potential deterring effects from the environment (i.e. bait, bait boxes) and within the emulsion itself (i.e. active components) will be essential for success. Increased protection of the active components within ContraPest® from warmer temperatures during international transport and laboratory and field use will also prove efficacious.

Chapter 8

Synthesis and Conclusions

8.1 Chapter summary

The aim of the studies presented in this thesis was to examine the potential of the chemosterilant, 4-vinylcyclohexene diepoxide (VCD), for fertility control of New Zealand (NZ) pest mammals, specifically the brushtail possum. To achieve this aim four main objectives were identified: 1) determine if an oral VCD dose delivered to female possums would result in the depletion of their ovarian primordial follicle pools (Chapter 4); 2) examine the uptake and metabolism of orally administered VCD in rats and possums (Chapter 5); 3) investigate the *in vitro* metabolic differences and effects of acid and stomach contents on VCD in rats and possums (Chapter 6); and 4) Investigate the potential use of ContraPest® as a chemosterilant for the control of rodent pests in NZ (Chapter 7). This chapter synthesises the results obtained from this research and presents recommendations for improvement and future study. In addition, the implications of these findings for science and vertebrate pest management are discussed.

8.2 Summary of thesis findings

The main findings of this research were:

1. VCD, when orally delivered does not induce primordial follicle depletion in adult female possums.

    a. The health of animals was not affected by VCD dosing. However, there was evidence that anaesthetic-induced stress and handling stress may negatively impact on the animal's well-being.

    b. There was no evidence that Intralipid and QuickEZE®, two formulations believed to improve VCD uptake and efficacy, were effective in the possum.

2. VCD-induced toxicity was less evident in possums compared with rats. It was hypothesized that this was due to a combination of the possum's stomach contents being naturally more acidic resulting in greater protection (i.e. containment) of VCD in their stomach compared with rats. The ability of possum stomachs to contain and inadvertently protect VCD was evidenced by:

    a. *In vitro* VCD hydrolysis in the stomach contents (pH 2.5) of possums was less compared with rats

b. Reduced *in vivo* levels of VCD measured in the blood of possums compared with the blood of rats

c. Ovarian and liver glutathione (GSH) levels of possums were unaffected whereas rat GSH levels in each tissue were depleted following *in vivo* treatment

3. The capacity to metabolize VCD and its parent compound, 4-vinylcyclohexene (VCH), appear to be greater in possums compared with rats as evidenced by:

    a. Higher *in vitro* concentrations of hepatic CYP450 in untreated possum microsomes compared with rat

    b. Reduced *in vitro* effects of VCH on hepatic CYP450 levels in the possum compared with that of the rat

    c. Reduced *in vitro* effects of VCD on hepatic GSH levels in the possum compared with that of the rat

4. Rats consuming an emulsion of ContraPest® had reduced ovarian primordial follicle populations. In addition, it was determined that:

    a. Pre-feeding with control emulsion proved efficacious for increasing the acceptance of ContraPest® emulsion

    b. Consumption rates of ContraPest® emulsion were unaffected by the addition of a sweetener (saccharine)

8.3 Synthesis of findings

8.3.1 Possums

The findings presented here have demonstrated that an oral dose of VCD does not induce primordial follicular depletion in the ovaries of possums. The lack of VCD-induced ovarian toxicity is likely due to a combination of factors. First, the typical *in vivo* pH of possum stomach contents ranged between 0.5 and 1.0 while that of the rat ranged between 0.5 and 3.6 (Chapter 5). Because VCD hydrolysis is pH dependant (Chapter 6), these natural pH differences between species would suggest that VCD hydrolysis in the stomach of possums would occur more rapidly compared with the rat. In the possum, this initial effect would likely hydrolyse a large portion of the VCD dose to its inactive tetrol form, ultimately reducing the amount of VCD available for absorption into the blood. The use of an antacid, QuickEZE®, was employed to help reduce the hydrolysis of VCD following oral gavage (Chapter 4). Although dosing possums with bicarbonate or QuickEZE® formulations increases and maintains a higher pH in stomach contents (pH 5.0 – 6.5 for 3 – 7 hours), this formulation did not affect VCD ovarian toxicity *in vivo*.

Secondly, differences in digestion of possums and rats may also be contributing to differences in VCD efficacy. Species differences in food retention times, food passage, and partitioning of fluid components from solid food would all contribute to VCD containment time within the stomach and ultimately VCD efficacy. VCD was more readily contained in the stomach contents of possums compared with rats (Chapter 6). Foods in the stomach of possums are not well mixed; so much so that discrete, homogenous food layers can often be identified in the order they were eaten (Nugent et al., 2000). Such an effect may inadvertently provide protection of VCD from acid hydrolysis, resulting in reduced rates of VCD uptake from the stomach and intestines into the blood stream of possums. If sufficient amounts of VCD were retained in the stomach, uptake of VCD may in fact be occurring over a longer period of time in the possum. This, in combination with the feeding behaviour of possum (1-3 bouts per night with 2-3 hours between each bout) (Nugent et al., 2000) could seem serendipitous considering that VCD ovarian toxicity is believed to occur due to repeat exposure to by overwhelming, and ultimately reducing, the capacity of the detoxifying enzymes (Hu et al., 2002; Keating et al., 2008a; Keating et al., 2010). However, daily oral dosing with VCD for 10 or 13 days (Chapter 4) did not affect the immature ovarian follicle pools of possums. Thus, it can be predicted that either the concentration of VCD was insufficient to induce ovarian toxicity or hepatic and ovarian detoxifying enzymes remained functional and at high enough levels to metabolise VCD efficiently. It is likely a combination of these two factors that explains why, in part, VCD is ineffective when administered to possums.

Third, there was no effect of VCD on stomach acidity of possums following oral gavage (Chapter 5). On the other hand, the stomach contents pH of rats was increased and remained elevated for up to 6 hours. This would imply that, in the possum, any VCD not contained by the stomach contents or taken up into the blood stream would be exposed to a highly acidic degrading environment, ultimately reducing VCD concentrations in the stomach even further. In the rat, however, acid hydrolysis of VCD in the stomach would not occur as quickly and the amount of VCD available for uptake into the blood would be greater than that of possums. In fact, this was demonstrated by higher levels of VCD measured in the blood of rats over a longer period of time compared with possums (Chapter 5).

Fourth, hepatic GSH concentration levels of possums were not as severely depleted *in vivo* (Chapter 5) and *in vitro* (Chapter 6). These findings together suggest that possums have a higher capacity to detoxify VCD compared with rats. The GSH results were further corroborated by the *in vitro* CYP450 results. Possum hepatic CYP450 levels were both higher and more robust than that of rats when exposed to VCD's parent compound, VCH, *in vitro*. Considering these issues, greater effort to protect VCD from acid degradation in the stomach and improve its uptake into the blood will be needed if a VCD-induced follicle-depleting effect is ever to be achieved in the possum. Further discussion on the requirements needed for consideration of VCD's use in possums is discussed below.

8.3.2 Rats

The findings presented here have confirmed previous reports examining the effects of VCD on hepatic and ovarian metabolism as measured by GSH. The fate and uptake of VCD, once present in the rat stomach, was also revealed (Chapter 5 and 6). In addition, the first study on the use of the potential chemosterilant, ContraPest®, for its effects on the ovarian follicle populations of wild Norway rats was conducted here (Chapter 7). As previously discussed for the possum, several factors affect the degree to which VCD-induced ovarian toxicity will occur; and, in the case of the rat, these factors appear to be advantageous.

First, in contrast with the possum, the in vivo stomach contents pH range of rats was higher and overall less acidic (control mean, 3.17 ± 0.28 pH; Chapter 5) implying that the rate of acid hydrolysis of VCD was likely to be slower in the rat stomach. Usually following consumption of food, the stomach pH of a rat decreases in order to aid digestion (McConnell et al., 2008) and therefore might increase the rate of acid hydrolysis in the stomach. Interestingly, however, the effect of a bolus dose of VCD/oil caused the stomach pH of rats to increase (Chapter 5) which would increase both the availability of VCD for absorption into the blood as well as prolong the period over which VCD would be available for absorption. This effect was corroborated by higher levels of VCD in the blood of rats over a longer period of time compared with possums following an oral bolus dose of VCD (Chapter 5). The pH of rat stomach contents following consumption of the ContraPest® emulsion was not measured but it is possible that a similar effect on pH may have occurred. Regardless, the encapsulation of VCD and triptolide contained within the ContraPest® emulsion is likely to have protected them to some degree, from acid degradation in the stomach and concomitantly aided their absorption into the blood stream.

Second, the feeding behaviour of rats (i.e. multiple small feeding bouts) may actually increase the efficacy of VCD due to repeated and continual exposure to the chemical (Chapter 7). Repeated exposure to VCD is thought to cause reduced capacity of the detoxifying enzymes glutathione (GSH) and epoxide hydrolase (EH) (Keating et al., 2008a). Third, the capacity of rats to metabolize VCD *in vivo*, as measured by their liver and ovarian GSH concentrations, was lower than that of possums (Chapter 6). This finding was corroborated by the reduced capacity of rat liver S9 cell fractions to metabolize VCD and VCH *in vitro* compared with that of possum liver S9 cell fractions (Chapter 6). The combination of rat feeding behaviour and their reduced capacity to metabolize VCD demonstrate, in part, why VCD reduces their primordial and primary ovarian follicle numbers.

8.4 Recommendations for future study

If future consideration for VCD's use in the possum is undertaken, it will be imperative to demonstrate that VCD is ovotoxic in the possum. It was my aim to develop an ovarian culture system for the possum (Figure 1.1) in order to provide proof-of-concept for VCD ovarian toxicity although unforeseen issues (as discussed in Chapter 6) prevented this objective being achieved. Species

similarities in ovarian folliculogenesis (reviewed in Chapter 2) allow for speculation that VCD's effects on the ovaries of possums may be similar to the effects observed in rats. However, the findings presented in Chapters 4, 5 and 6 suggest that, even if VCD ovarian toxicity was demonstrated with an *in vitro* culture system, the issues associated with stomach acidity and metabolism would likely negate any potential effects of orally delivered VCD on the ovaries of possums.

Although these issues may be difficult to overcome, there are methods which may protect VCD from breakdown in the gastrointestinal tract and increase its efficacy once absorbed into the blood stream and which should be considered for future research. Formulations such as those found in the ContraPest® emulsion (i.e. encapsulation of active ingredients, lipid emulsion matrix for increased absorption) may prove efficacious for future use in the possum. Evidence was presented in Chapter 4 which suggested that an antacid pre-treatment may have protected VCD from acid degradation in the stomach of possums to some degree. Thus, antacid inclusion as an additional emulsion ingredient or outer coating around bait may be considered for future formulations.

Second, additional *in vivo* or *in vitro* examination of the capacity of possums to metabolize VCD may aid in developing different formulations for VCD and their potential application for possum control. A reassessment of the ovarian, gastric and metabolic effects following an oral VCD dose in combination with protective formulations (i.e. encapsulation) should be considered.

Third, if additional *in vitro* studies are to be carried out for the possum, several areas for improvement should be noted. First, the effects on both ovarian GSH and EH should be measured *in vitro* to determine the full effects of VCD and the capacity of the possum ovary to metabolise it. Assuming an *in vitro* ovarian culture system is not developed for the possum, ovarian S9 cell fractions (as outlined in Chapter 6) could be utilized as an alternative for such experiments. Secondly, a re-evaluation of liver metabolism, as measured by GSH and EH and their associated inhibitors, should be performed in conjunction with an *in vitro* culture protocol (i.e. inclusion of an NADPH regeneration system) to mimic *in vivo* conditions as closely as possible. Third, it may be useful to measure the rate of disappearance of VCD (Chapter 5) from hepatic and ovarian tissues under *in vitro* conditions. Such results would help to confirm the rate of conversion of VCD to its inactive tetrol metabolites by these tissues. This, in turn, could also be useful if future examination of the effects of VCD's parent compound, VCH, on possum metabolism were to be undertaken. Collectively, *in vitro* examination of VCD (or similar compounds) may help in the formulation of methods to improve VCD efficacy for fertility control in the possum.

8.5 Implications for vertebrate pest management

To my knowledge, the findings presented on the effects of orally applied VCD in possums were the first of their kind. The summation of these findings indicates that the use of VCD for the control of fertility in the possum is not plausible at this time. Furthermore, the implication of these findings in the possum is that VCD may not be an effective management tool for other marsupials or eutherians

species with similar physiology or dietary habitats. It has been hypothesized that possums are effective metabolizers of VCD due to their dietary evolution (i.e. consumption of eucalypt foliage which is high in plant secondary metabolites (PSMs); Chapter 4). Thus, it may be hypothesized that species which regularly consume foliage high in PSMs would likely respond to VCD treatment in a similar manner to that of possums.

To date, the only other examination of VCD's effects on a marsupial were carried out on the tammar wallaby (*Macropus eugenii*) (Koehn, 2008). Fifteen days of subcutaneous or intramuscular injections of VCD did not reduce immature ovarian follicle pools of treated females. Although no direct correlations can be made, it is possible that, in part, the lack of VCD effects were due to the tammar wallaby having evolved an efficient metabolic capacity for detoxifying xenobiotics such as discussed and demonstrated here for the possum. In fact, tammar wallabies and koalas have shown similarities in their metabolism of xenobiotics to that of possums (Stupans et al., 2001) further supporting the hypothesis that VCD's effects on the ovarian follicular pools of species which regularly consume foliage high in PSMs may be similar.

The success of ContraPest® emulsion at reducing the primordial follicle pools of wild Norway rats (Chapter 7) is encouraging for its potential use as a fertility control management strategy for rodents. For successful application of a chemosterilant, such as ContraPest®, future work will require several aspects for development and investigation. First, reduction or elimination of deterring odour and taste cues from the active ingredients within the bait may be necessary, especially for species, such as rodents, which rely on their highly sensitive olfactory and gustatory cues to identify acceptable food sources (Jensen et al., 1983; Lee and Clifton, 2002; Macdonald et al., 1999). If an ideal chemosterilant emulsion or bait can be produced which demonstrates high acceptability by a species then consideration for its use in the field could begin. It will be important to understand the effects of the fertility control bait on all components of the population of interest such as males, pregnant females, lactating females and juveniles. In addition, the effects of the bait on non-target species and the environment will need to be to be undertaken.

Other factors that may improve bait acceptance for the species of interest would be identification of an optimal pre-baiting protocol and how the bait should be applied within the environment. Pre-baiting with non-active emulsion increases the acceptance and intake of the active emulsion formulation (Chapter 7). In addition, pre-baiting may reduce or prevent neophobic-induced behaviours (Barnett, 1958; Buckle and Smith, 1994; Mitchell, 1976), thus further increasing acceptance of the active formulation. It would be advantageous to utilize local knowledge and vertebrate pest control experts, where possible, to improve the success of each baiting scheme (Buckle and Smith, 1994). For international applications, it will be important to further understand the effects of transportation time and temperature on all active ingredients within the fertility control bait. The effect of time and temperature on the active ingredients would also apply for developing bait strategies in the field.

Development and integration of a new pest control product, such as a chemosterilant, will require an in depth understanding of its impacts on the species of interest. The impact of a chemosterilant will vary depending on the species' ability to absorb, metabolize, and detoxify the active components as well as the efficacy of the active components on the target organ and its downstream effects on fertility. For the successful application of a chemosterilant bait, its acceptance and palatability needs to be understood in the target species. Understanding the reproductive biology (i.e. age at first litter, size of litters) of the target species may also aid in development of treatment protocols and strategies for dealing with acute (i.e. mouse plagues) and chronic pest populations.

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