Gossypol's effects on ingestive behaviour in mice: The first step in a systematic process to define gossypol's suitability for use in murine pest management

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Abstract

Gossypol, synthesised by the cotton plant, *Gossypium,* causes physiological and behavioural changes in mammals, suggesting it may be suitable for murine pest management. One of the most under-studied responses to gossypol, especially in the house mouse, *Mus musculus*, is its effect on ingestive behaviour, with some authors reporting anorexia and others observing no effect on energy metabolism. Importantly, there has been no systematic analysis of gossypol's effect on food intake in mice. Therefore, the goal of this thesis was to provide the initial step in defining gossypol's effect on feeding behaviour in mice by observing their responses after exposure to precise doses of gossypol delivered via injection. Mice underwent two injection intraperitoneal (IP) paradigms; acute and chronic (11 daily injections) exposures to 0 (vehicle), 10, 30, 100 and 300 mg/kg b.wt., and 0 (vehicle) and 100 mg/kg b.wt. respectively. The intakes of bland (chow) and palatable tastants were measured during the acute exposure. An increase in chow intake was observed at 1 and 3 hr post-exposure with 300 mg/kg b.wt. dose. An increase in glucose intake was also observed in mice injected with 100 mg/kg b.wt. at 1 and 12 hr post-exposure. Neither of these hyperphagia responses showed a complementary increase in body weight. During the chronic exposure, body weight and chow intake were measured throughout the injection period and on select days of the 40 day post-exposure period. While there was no difference in food intake and body weight during the gossypol exposure, on day 10 post-exposure, food intake had increased by 50% and was still elevated on day 40, but no differences in body weight were noted. To examine whether this increase in food intake was an effect of a long-term anxiogenic response associated with a likely post-exposure malaise, several anxiety assessment tests were performed, showing no change. This thesis shows that gossypol affects feeding behaviour in mice. Interestingly, no anorexigenic effect was observed, but in fact moderate hyperphagia without changes in body weight was shown in both acute and chronic paradigms. The data from this thesis can be built upon with future studies using oral administration to develop the understanding of this

important aspect of gossypol and to more precisely determine gossypol's suitability for enhancing murine pest management.

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1 <u>Literature Review</u>

1.1 History of Gossypol Research

Gossypol is a naturally occurring compound, synthesised by one of the world's most commonly commercially grown plants, *Gossypium*, the cotton plant. It has potential as a male contraceptive (Coutinho, 2002), cancer chemotherapeutic (Lian *et al.*, 2012)and a Human Immuno-deficiency Virus (HIV) replication inhibitor (An *et al.*, 2012). It is a cheaply produced molecule containing only carbon, oxygen and hydrogen, arranged in two biphenolic chains, each with an aldehyde group, connected by a single C-C bond (Lin *et al.*, 1989). This gives the structure two optical isomers. Many studies have shown the (-) gossypol enantiomer to cause more severe physiological and behavioural responses than the (+) enantiomer (An*, et al.*, 2012; Lian*, et al.*, 2012; Lin*, et al.*, 1989; Matlin *et al.*, 1985; Zhong *et al.*, 2013). The vast majority of studies on gossypol available in the literature and referenced in the current thesis used the (-) enantiomer. Synthesis of this compound, by the cotton plant, is thought to have evolved as a means of defence against insect grazers (Coutinho, 2002; Stipanovic *et al.*, 1999). The anthropogenic uses of the compound are much more varied.

Research into applications of gossypol was initially sparked in 1957, when it was reported that Wang village in Jiangsu, China had not had a single new born in a decade. This drop in fecundity was correlated with a change in cooking oils, from soybean oil to cottonseed oil. This was of interest to the Chinese government, that at the time were trying to slow the growth of a population that consisted of one quarter of the world's total (Wilson, 2001).

From 1972 evidence began to be published that gossypol negatively affected male fertility. These studies culminated in over 8000 men in China being administered the drug in clinical trials sponsored by the Chinese

government. The conclusions of these studies showed that gossypol had a very reliable contraceptive effect (Qian & Wang, 1984).

As the studies continued, the negative effects of gossypol became apparent. Human trials of gossypol were published only two years after the first animal studies were published, and this lapse in scientific ethics caused numerous side effects of the drug to be first seen in human participants (Yu & Chan, 1998). One of the most interesting long term effects of chronic gossypol exposure was permanent sterility in a minority of participants (Meng *et al.*, 1988a). The potential to develop permanent sterility was dependent on many factors; period of exposure, dose and individual susceptibility, amongst others (Yu & Chan, 1998). Coutinho *et al.*, 2000, observed that men who ingested 7mg/day and 10mg/day of gossypol had a 21% and a 16% chance respectively of remaining azoospermic 12 months after the last exposure to gossypol.

1.2 Gossypol's Side Effects

Permanent infertility was seen in conjunction with other effects. These are the reason why the World Health Organisation made the decision in 1986 to no longer support any research studying gossypol's contraceptive potential (Waites *et al.*, 1998). Gastrotoxicity (Kitada *et al.*, 2008), hypokalemia (Wang & Yeung, 1985), and sterilization (Meng*, et al.*, 1988a; Meng *et al.*, 1988b) were amongst the effects that lead to this decision.

1.2.1 Gastrotoxicity

Gastrotoxicity has been observed only in animal studies. This was associated with chronic exposures to gossypol at high concentrations. Many studies have reported this phenomenon. A dose of 10mg/kg b.wt. was injected in rats for 5 weeks. This exposure caused severe gastrotoxicity along with reduced body growth (Gåfvels *et al.*, 1984). At a

pig farm in Illinois, USA, where a herd was mistakenly fed a high concentration of gossypol over a 4 week period, a high mortality rate with gastrotoxicity symptoms was recorded (Haschek *et al.*, 1989).

These cases of gossypol induced gastrotoxicity were not the first. In 1915, Withers and Caruth published findings that gossypol extracted from cottonseed was toxic to rabbits. This work was later expanded on by Harms and Holley, 1951, who observed intestinal haemorrhages in rabbits fed cottonseed meal . In a more recent study the physiology of the gastrotoxicity of gossypol was described. The mice, which were gavage-fed gossypol, displayed behaviour suggesting they were suffering from gastrotoxicity. This was confirmed when an ultrasound showed a distended gastrointestinal system, while histology and pathology showed damage to the mucosal lining and intestinal wall including loss of villi, ulceration and necrosis (Kitada*, et al.*, 2008).

Although gastrotoxicity is a side effect of many drugs which are either prescribed freely or can be bought over the counter, the gastrotoxicity seen in these gossypol studies was severe. It should be emphasised that this reaction was observed only at high doses. The effect on food ingestion has not been fully elucidated. Romualdo *et al.*, 2002, noted that exposure to gossypol caused a loss of body weight in rats compared with controls, but observed no difference in food intake. This finding suggests that the gossypol induced weight loss is caused by an increased metabolic rate. This in turn suggests that gossypol has little noticeable toxicity upon exposure, with few visceral effects.

1.2.2 Hypokalemia

Gossypol causes an inhibition of potassium absorption, thus many cases of hypokalemia were reported during the first decades of gossypol research (Qian, 1985). In China, of the almost 9000 men trialling gossypol, the vast majority had reduced potassium levels. In most cases these levels were still within the healthy range, but 1% had very severe hypokalemia resulting in temporary paralysis. Geographically, the

incidence of a severe reaction to gossypol was much higher in regions with low dietary intakes of potassium (Qian, 1985). However, Yu and Chan, 1998, state that the conclusion that gossypol-induced hypokalemia was correlated with areas of potassium poor diets is unfounded. They argue that the hypokalemia seen in some patients was not due to gossypol and that the geographical correlation published was misinterpreted.

The mechanism of gossypol induced hypokalemia was detailed by Michael, 1998. He stated that gossypol inhibited the enzyme 11β hydroxysteroid dehydrogenase (11βHSD). This enzyme reserves mineralocorticoid receptors (MR) for mineralocorticoids; without its activity, glucocorticoids outcompete the target molecules. Regulated renal potassium excretion requires specific mineralocorticoid bound MR. When 11βHSD is inhibited by gossypol, MR activation is unregulated due to incessant binding of glucocorticoids. This causes excessive absorption of potassium by the kidney, resulting in high concentrations of potassium excreted via the urine (Chen *et al.*, 2009).

This effect of gossypol induced hypokalemia can be overcome with a potassium supplement. Langur monkeys exposed to gossypol were also exposed to the potassium supplement, potassium chloride. After 180 days the animals still had normal serum potassium levels. The control group of monkeys, exposed to gossypol alone, showed fatigue, diarrhoea, vomiting and very low serum potassium levels. Both of these groups were still azoospermic (Kumar *et al.*, 1997).

The WHO statement that gossypol-induced hypokalemia was an issue severe enough to halt contraception studies was controversial. In one study, a sample of men from China, Brazil, Nigeria and Kenya were given gossypol for 40 weeks. None of them showed symptoms of hypokalemia, although there was a minor trend of slightly lowered serum potassium levels (Coutinho *et al.*, 2000). This study also called in to question the legitimacy of the WHO's use of hypokalemia as a reason to stop research in to the efficacy of gossypol as a male contraceptive (Coutinho, 2002). Gossypol does cause a decrease in serum potassium (Coutinho, 2002;

Qian, 1985; Sharma *et al.*, 1999), but this effect is dose dependent; the low doses used for human trials may have caused hypokalemia only in those particularly susceptible (Reidenberg *et al.*, 1993), while the higher doses used in animal trials were more likely to cause severe potassium deficiency (Wu & Reidenberg, 1993).

1.2.3 Permanent Infertility

Gossypol has caused permanent infertility in a minority of human participants in trials (Coutinho, 2002; Meng*, et al.*, 1988a, 1988b). In the most recent human fertility study, 19% of patients were still azoospermic one year after stopping gossypol treatment (Coutinho, 2002; Coutinho*, et al.*, 2000). This study used participants from various ethnic groups, including Brazil, China, Kenya and Nigeria. When compared with the original Chinese studies, researching the response that a chronic exposure to gossypol has on the reproductive system, similar results were reported. Meng *et al.*, 1988, found 22% of all participants were azoospermic after, on average, 1.9 years of exposure. In the Chinese meta-study, exposing ~8800 men to gossypol, for between 6 months and 4.5 years, 10% of the participants were still azoospermic (Qian & Wang, 1984). These studies have suggested that gossypol has a significant, long-term effect on the male reproductive system.

Gossypol's effect on the mammalian reproductive system has been thoroughly studied. This evidence shows inter-species differences, and even inter-study differences for the same species. The mouse, along with other members of the Rodentia order, shows many of these same discrepancies in responsiveness to gossypol. Amini and Kamkar, 2005, gavage fed mice gossypol, resulting in a decrease in epididymal and testicular weight. This result has been repeated in rats and mice many times with differing methods and modes of exposure (Gåfvels*, et al.*, 1984; Romualdo*, et al.*, 2002; Wazir *et al.*, 2006), although some studies have shown otherwise. Coulson, *et al.*, 1980, reported that a subcutaneous injection of gossypol resulted in testes weighing equal to or greater than the controls.

In rats, sperm production decreased by as much as 100% compared to the controls in one study, although this was with a high dose (Sotelo *et al.*, 1982). Other studies have found decreases in sperm production of 67% (Amini & Kamkar, 2005) and 54% (Romualdo*, et al.,* 2002). Studies into the contraceptive effect of gossypol, as reviewed by Qian and Wang, 1984, found infertility in 99.07% of men exposed to gossypol. This discrepancy between the human and animal studies is probably due to the extended treatment in the human studies, in some cases over two years.

The infertility in these human studies would not only consist of a decrease in sperm production, but the viability of the sperm would also have an impact. Sperm viability was also a factor in mice and rats exposed to gossypol. Immobile, tailless and deformed sperm have all been reported (Gåfvels*, et al.*, 1984; Romualdo*, et al.*, 2002). What is gossypol actually doing to cause these morphological changes and defunct sperm?

Gametogenesis is regulated by different cell types within the testes, which each control a certain aspect of the development of the sperm. The Sertoli cells form a tissue that secretes growth factors, hormones and lactate allowing the post-meiotic germ cells to develop (Riera *et al.*, 2001). Gossypol has been observed to affect these functions. An exposure of gossypol to cultured rat Sertoli cells caused a dose-dependent decrease in viability. Lactate, the preferred substrate for developing sperm cells, initially increased in concentration with increasing gossypol , then dropped with higher gossypol concentrations (Monsees *et al.*, 1998).

This initial increase in lactate would have been due to gossypol's ability to de-couple oxidative phosphorylation (Reyes *et al.*, 1986). This would have increased the concentration of lactate via accelerating the glycolysis pathway. The cause of the decrease in lactate concentration at higher doses of gossypol would be due to the decreased viability of the Sertoli cells. Leydig cells line the seminiferous tubule of the testes, and are involved in secreting androgen hormones, including testosterone. These cells are also effected by gossypol, but not to the same extent as Sertoli cells (Zhuang *et al.*, 1983). Their viability is decreased in the presence of

high concentrations of gossypol. Androgen secretion was not effected at any gossypol exposure. The combination of these factors would cause defunct sperm and morphological changes in the testes.

Biochemically, gossypol's contraceptive effect is owed to its two aldehyde groups. When these groups were substituted with less toxic chains the contraceptive property of these gossypol derivatives was decreased. An *et al.*, 2012, tested the spermicidal activity of amino acid side chain gossypol derivatives on mouse sperm. They observed that all of the derivatives had a lower spermicidal activity than gossypol. After 20 min of exposure to gossypol the viable sperm count was below 5%, whereas after 40 min of exposure to any of the amino acid derivatives the viable sperm count was >15%.

The endocrine system of the hypothalamic – pituitary – gonadal axis (HPGA) affects many different aspects of the vertebrate life cycle. Of these, reproduction, in both males and females, is perhaps the most important (Sower *et al.*, 2009). The HPGA controls the production of sperm by controlling the production of, and binding potential of, testicle produced testosterone, with luteinizing hormone (LH) and follicle stimulating hormone (FSH) respectively, both secreted by the pituitary gland. Gossypol's effect on the HPGA has been studied thoroughly. Upon exposure to gossypol some of the pituitary's functions are compromised. Gossypol exposure to rats has shown that the secretory abilities of the pituitary FSH cells were affected. Reports of dilated endoplasmic reticulum and an increase in granulation of the cytoplasm have been published (Nair & Bhiwgade, 1990; Udoh *et al.*, 1992). The effect of this cellular function on serum FSH levels has been tested.

These tests have shown that gossypol alone does not cause a change in serum FSH (Yang *et al.*, 2011), while an exposure to gossypol and a mix of steroids (desogestrel/mini-dose ethinylestradiol/testosterone undecanoate) causes a decease (Yang *et al.*, 2004). There have also been reports of changes to LH cells in rats after exposure to gossypol. LH cells were seen to be degranulated (Nair & Bhiwgade, 1990). This process

was suggested to cause a decrease in serum LH (El-Sharaky *et al.*, 2010). A decrease in LH will be reflected in a decrease in testosterone released from the testes. Testosterone is necessary for spermatogenesis, by activating the Sertoli cells (Griswold, 1998).

Gossypol was thoroughly studied for decades as a male contraceptive, but its effect on females has not been as carefully studied. Although few in number, these studies have suggested that gossypol has a dose-dependent effect on the female reproductive system. Treatment with medium and large doses of gossypol in female rats has resulted in irregular and infrequent oestrous cycles (Bender *et al.*, 1988; Lin *et al.*, 1985). This infrequency in female rats has been observed as extended oestrous periods in many studies (Bender*, et al.*, 1988; Gu & Anderson, 1985; Lagerlöf & Tone, 1985; Lin*, et al.*, 1985), due to an increase in the time spent in diestrous (Gu & Anderson, 1985; Lin*, et al.*, 1985).

This oestrous cycle disrupting effect of gossypol in rodents has not been observed consistently throughout the literature. A recent study, using female Wistar rats, observed that upon exposure to cottonseed oil the animals kept regular oestrous cycles (Akinola *et al.*, 2006). The gossypol content of the cottonseed oil used in this study was not confirmed. Gossypol does not cause any deleterious effects to the oestrous cycle when in low concentrations (Bender*, et al.*, 1988; Gu & Anderson, 1985). The gossypol content of the cottonseed oil may have been below this effective threshold.

Similar results were seen in female lesser bandicoot rats, *Bandicota bengalensis,* which ingested cottonseed oil mixed into their food source at a ratio of 5% and 10%. The gossypol content of the cottonseed oil was established to be 0.01%. This made the amount of gossypol ingested miniscule. However the 10% cottonseed oil diet significantly reduced the sperm viability and motility of the male rats (Singla & Garg, 2013). In another study, gossypol was gavage-fed to female hamsters at concentrations high enough to cause toxicity, but no change to the oestrous cycle (Wu *et al.*, 1981). However the majority of the evidence

indicates that gossypol does affect the oestrous cycle of rodents by causing them to be irregular and to have an extended diestrous period.

Gossypol has also been shown to effect pregnancies in rodents. A daily oral dose of gossypol acetic acid decreases the success rate of pregnancies of rats. The treatment rats were observed to have fewer pregnancies, a reduced maintenance of pregnancies with loss of many embryos that were viable at day 13 of gestation, and a decrease in the weight of offspring born from those embryos which were viable (Lagerlöf & Tone, 1985). Another study showed that an intramuscular injection of gossypol during the first 8 days of pregnancy prevented all rats tested from maintaining their pregnancies (Lin*, et al.*, 1985). This was observed in conjunction with a decrease in progesterone and estradiol 17 β, suggesting that gossypol causes an endocrine dysfunction which affects pregnancy maintenance.

The female reproductive system can be affected by gossypol, but how this happens has not been as thoroughly defined as the effect of gossypol on the male reproductive system. As previously stated, Lin *et al.*, 1985, observed a decrease in the serum levels of progesterone and estradiol 17 β in pregnant rats treated with gossypol. This observation has been partially repeated in hamsters, Wu *et al.*, 1981, reported that an individualised response of decreased progesterone levels was observed in some of the hamsters treated with gossypol. This study also measured levels of pituitary hormones, and found that gossypol exposure was correlated with a decrease in FSH, but not LH. Thus gossypol potentially has an effect on some aspects of the endocrine system, so what does it target to have this effect? This question is largely unanswered, but a decrease in ovary weight has been observed when rats were exposed to gossypol (Gu & Anderson, 1985). This observation was not repeated by Bender *et al.*, 1988, who reported finding no changes in the ovaries, uterus, vagina or adrenal glands.

1.2.4 Toxicity of Gossypol

Gossypol has recently been studied as a potential HIV replication inhibitor (An*, et al.*, 2012; Keller *et al.*, 2003; Lin*, et al.*, 1989). These studies have found that gossypol inhibits the action of HIV-T1 by inhibiting the fusing of the virus to the target cell, mimicking the action of the clinical drug T20 (Yang *et al.*, 2012). These studies into the HIV replication inhibitory action of gossypol have also highlighted another of its effects; immunosuppression (Xu *et al.*, 2009). BALB/c Mice were injected intraperitoneal (IP) with gossypol, alongside cultured lymphocyte cells exposed to gossypol. These experiments resulted in treatment mice with lighter weight spleens and thymus glands than those of controls. These mice also showed a decreased ability to proliferate lymphocytes compared with controls. This decrease in lymphocyte proliferation was mirrored in the cultured lymphocyte cells. This also suggested that exposure to gossypol could induce apoptosis in a dose- and time-dependent manner (Xu*, et al.*, 2009).

Gossypol causes responses in different organs, tissue types and cell types. These effects all derive from gossypol's toxicity, including antagonising 11βHSD (Michael, 1998), decreasing serum FSH levels (Wu*, et al.*, 1981), and decoupling oxidative phosphorylation (Reyes*, et al.*, 1986). The majority of the symptoms of gossypol toxicity have been well documented. The symptoms that detract from the applied use of the chemical could potentially be overcome, either with a reduced dose (Randel *et al.*, 1992) or with mineral supplements to combat hypokalemia and other deficiencies (Haschek*, et al.*, 1989; Kumar*, et al.*, 1997).

Overall, it should be emphasized that previous studies into gossypol's effect on mice have produced inconsistent outcomes in terms of the spectrum and intensity of responses observed in this species. An expanded knowledge of these effects in mice is necessary to understand more precisely its potential as a pest management chemical for this invasive species.

1.3 House mouse, *Mus musculus*, population management

The house mouse, *Mus musculus,* is one of the world's most widely distributed mammals, due to a long-standing commensal relationship developed with humans (King, 2005). The *Mus* genus evolved in Africa and Asia approximately 12-14 Mya (Suzuki *et al.*, 2000), and since the mouse has dispersed to North America, South America, Europe, Australia, New Zealand and Polynesia, currently inhabiting every continent except Antarctica (Auffray *et al.*, 1990).

This dispersal has taken the species to areas with naïve, native prey, and into habitats with abiotic features allowing them to breed at high rates. House mice are a major agricultural, ecological and disease carrying pest especially in these areas (Fitzgerald *et al.*, 2004; Jacob *et al.*, 2003). Human-derived population management often attempts to reduce the impact of this pest.

1.3.1 Mouse Foraging Behaviour

The natural behaviour of the mouse offers one of the major challenges for a pest management technique to overcome. Mice are neophobic and prone to developing conditioned taste aversions (Andrews & Horn, 2006). These behaviours have been selected over time because mice and other rodents have no physiological or neurological ability to vomit risky foods once eaten (Horn *et al.*, 2013). Successful mouse populations are those that have evolved a set of behaviours that cause them to be cautious in what they ingest.

On discovery of a new food source, a mouse eats a small amount, and after a few hours, if the mouse has not felt any negative effects and if it is palatable, it will return to the food source to continue ingestion of what it has discerned to be safe. If the mouse has experienced any negative effects from the food source, it will associate the taste with the negative

feeling and will not ingest any more of the food source (Clapperton, 2006). If this is repeated a few times in succession, a conditioned taste aversion (CTA) is developed (Watkins *et al.*, 1998). The development of a CTA will ensure that when the animal tastes the risky food source again it will stop instantly (O'Connor & Matthews, 1999).

This behaviour is one of the severest challenges for mouse population control using food baits. If the bait is unpalatable it will not be ingested, if it causes a negative effect, a CTA will be developed.

1.3.2 Lethal Population Management

There are two broad forms of animal pest management; lethal and non-lethal, both of which are applicable to mouse populations. These two very different forms of pest management have advantages and disadvantages relative to each other. Lethal pest management for mice includes physical, biological and chemical techniques.

1.3.2.1 Physical Methods of Population Management

Physical, lethal methods for population control are effective in small commensal populations of mice, but are too intensive and inefficient for the control of mice in a large area. Mice, as with other pest rodents, breed rapidly and are neophobic towards new items in their territory, such as traps (Clapperton, 2006). Hence, physical methods, such as trapping, are generally not used to control mice in any large scale area.

1.3.2.2 Biological Methods of Population Management

Biological techniques include the release of a disease or predator into the affected ecosystem. These are arguably the most cost effective and most immediate of all population management techniques, if they work. They are, however, also very unpredictable and irreversible. Releases of natural predators of pests have occurred all around the world with the aim of controlling populations. These have often resulted in negative consequences due to a lack of prey specificity and the addition of new pathogens to the ecosystem (Atkinson, 2001; Courchamp *et al.*, 2003).

Deliberate release of a mammalian predator to a new habitat for the sole purpose of preying on a given pest has not been done for many decades, but some areas are still dealing with the consequences of past introductions (Atkinson, 2001).

The release of a disease into a population is a cheap and non-intensive option as a means of control, but it carries major disadvantages. Myxomatosis, a virus specific to European rabbits, (*Oryctolagus cuniculus*), has been deliberately released in Australia, the United Kingdom and France. This virus, native to South America, was released initially in Australia as a means to control the population of introduced rabbits, which it did with great success, decreasing the population to a sixth its size within two years. Since, the rabbit population has slowly recovered even though the virus persists. This is due to genetic resistance in the population with those with the greatest resistance breeding more successfully and the virus becoming less virulent and pathogenic (Angulo & Cooke, 2002; Kerr, 2012).

The private release of myxomatosis, subsequently performed in France, was a different case, because the target population of rabbits was native. This release was followed by the decimation of the native rabbits across Europe and the collapse of an ecosystem suddenly lacking a significant herbivore and prey (Fenner & Fantini, 1999). This example highlights the short term outcome of using a disease as a lethal method of population reduction, and the lack of control of the consequences. To the authors' knowledge, these weaknesses of the technique have prevented any release of disease for the control of pest mouse populations anywhere.

1.3.2.3 Chemical Methods of Population Management

Poisoning is the most suitable lethal chemical method used to control mouse populations. It is a very powerful technique, and is used as the primary technique for total population eradications from offshore islands (McClelland, 2011). On the other hand, this method, although powerful, is often expensive, non-species specific, controversial, potentially environment damaging, and intensive (Courchamp*, et al.,* 2003).

Poisons used to control mice include; first generation anticoagulants such as diphacinone and coumatetralyl; metal phosphides such as zinc phosphide; and calciferols such as cholecalciferol. Each of these different types of poison has various advantages and disadvantages.

First generation anticoagulants are the most widely used rodenticide throughout the world (Bankes & Garthwaite, 1998; Timm, 1994). These poisons effect the cardiovascular system and the ability of the blood to clot, leading to death by internal haemorrhaging (Thijssen, 1995). First generation anticoagulants are not potent toxins, so a large dose must be ingested to be lethal, and thus they rely on palatability to be effective. Ingestion of a lethal dose results in a slow death, the animal's health slowly deteriorates, usually over several days post-ingestion (Albert *et al.*, 2010; Fisher, 2005; O'Connor & Booth, 2001). This slow action usually prevents target animals from learning conditioned taste aversions against first generation anticoagulants (Wright, 2011).

First generation anticoagulants are also not rodent-specific, they affect other mammals and birds (Albert*, et al.*, 2010; Wright, 2011). This inspecificity is especially apparent in the common secondary poisoning of predators. A lethal dose of a first generation anticoagulant will eventually make the animal very easy prey for a predator (Cox & Smith, 1992). This along with the poison's ability to bio-accumulate, especially in the liver, and its slow half-life, makes secondary poisoning problematic for native non-target predators, but beneficial for introduced, pest predators (Albert*, et al.*, 2010; Chapuis *et al.*, 2001).

Metal phosphides are another commonly used rodenticide. These poisons were developed in the early 1900's, with zinc phosphide being first used as a rodenticide in 1911 (Marsh, 1987). They have recently become superseded by the development of second generation anticoagulants (Eason *et al.*, 2011). Once the metal phosphide has been ingested, the poison reacts with the acidic stomach contents, which causes a release of phosphide gas. This gas is very toxic, reducing the membrane potential of mitochondria, interrupting cellular respiration, also forming the hydroxyl

free radical (Proudfoot, 2009). This action causes a single dose to be lethal (Hood, 1972).

Zinc phosphide is very potent against rodents, but is not particularly toxic to many other mammalian families. Importantly, it is also very toxic to some bird species (Caughley, 1998; Marsh, 1987). When these poisons are used in an agricultural setting for rodent control, the bird populations likely to be affected will be abundant and potentially pests in their own right, whereas non-rodent mammals will not be affected (Marsh, 1987; Sugihara *et al.*, 1995). These poisons have been used to manage mouse plagues in rural Australia with great success (Caughley, 1998). On the other hand, because zinc phosphide is a single dose lethal poison, conditioned taste aversions can easily develop if a sub-lethal dose is ingested (Mushtaq *et al.*, 2010; Parshad & Kochar, 1995; Sridhara & Srihari, 1980). For this reason it is a poison best used in conjunction with another.

The calciferol rodenticides are vitamin D derivatives. The poison's mode of action is to cause death by hypercalcemia, which is observed as a release of Ca^{2+} from the bone, and this increase in Ca^{2+} in the blood causes mineralisation of the blood vessels resulting in cardiac failure in rodents. This lethal hypercalcemia causes death in 4 – 7 days after the ingestion of a single lethal dose. This long time lapse was initially hypothesised to reduce the development of conditioned taste aversions (Greaves *et al.*, 1974; Marshall, 1984), but more recent reports state that bait shyness occurs after a sub-lethal dose, suggesting that negative effects can be felt shortly after ingestion (Prescott *et al.*, 1992; Zeinelabdin & Marsh, 1991).

Cholecalciferol is the most commonly used calciferol poison for rodents. Non-target species are not particularly affected by cholecalciferol. When various bird species were fed a very high acute dose, mallard ducks were not affected, canaries were minimally affected and chickens were negatively affected (Eason *et al.*, 2000). Mammalian predators likely to be at risk of secondary poisoning (dogs and cats) have been shown in the lab to be unaffected by cholecalciferol (Eason*, et al.*, 2000), whereas non-lab

based reports suggest that cats and dogs are affected (Fooshee & Forrester, 1990; Moore *et al.*, 1988). This lethal poison, as with the other poisons listed above, is not ideal for rodent population management. The mouse's neophobic, non-emetic and extreme dietary selectivity behaviour ensures that no single lethal poisoning technique will ensure permanent population management.

1.3.3 Non-lethal Population Management

Mice, as with the majority of rodents, have an extremely high reproductive output and short life expectancy (Tyndale-Biscoe, 1994). This combined with their selective foraging behaviour, ease of forming a conditioned taste aversion and exploratory behaviour constrains the suitability of lethal poisons (Chambers *et al.*, 1999a; Shilova & Tchabovsky, 2009).

Non-lethal fertility control has been suggested to be a better alternative (Chambers*, et al.*, 1999a). For example, mice have a short life expectancy of 6 months in Australian grain farms, yet in this time they have the potential to produce 600 offspring (Singleton *et al.*, 2001). Sterilization of 67% of the females in a population caused a significant decrease in population growth compared with the control (Chambers *et al.*, 1999b). This suggests a potential alternative to lethal population control methods. Fertility control would be humane, with less chance of conditioned taste aversions reducing population growth (Hardy *et al.*, 2006; Littin, 2010; McLeod *et al.*, 2007; Tuyttens & Macdonald, 1998).

Studies into fertility-control tools have explored two different techniques for delivering the sterilant; immunocontraception and chemosterilisation. Immunocontraception uses the individuals' own immune system to inhibit fertility (Hardy*, et al.*, 2006). Chemosterilisation uses a chemical or hormonal compound (Alemany *et al.*, 2008).

1.3.3.1 Immunocontraception for Population Management

This ingenious idea was first suggested in the 1980s, when an alternative human contraception method was being studied using primate models

(Talwar & Gaur, 1987). An antigen of a hormone or protein necessary for reproduction is introduced into the target animal. This induces an immune response to the target protein or hormone, removing it from the reproduction system, the loss of which should induce infertility (van Leeuwen & Kerr, 2007).

The target of choice is the glycoprotein matrix of the zona pellucida in females. There are three to four proteins in this matrix dependent on the species, but each of these has a different potential for causing infertility; for instance, in rabbits, using zona pellucida protein C as an anitgen caused a high proportion of sterility, whereas zona pellucida protein A as an antigen had little effect (van Leeuwen & Kerr, 2007). These immunocontraceptives have been used to control the fecundity of wild populations of some species for over a decade now as vaccines, in intensive, capture, dose, release programs (Kirkpatrick *et al.*, 2011; Miller *et al.*, 1998).

Virally Vectored Immunocontraceptives (VVIC) is the term enveloping all immunocontraceptives transmitted by a recombinant organism. This tool has the potential to control pest populations inexpensively, humanely and species-specifically (McLeod*, et al.*, 2007). At the time of writing there have been no releases of VVIC into wild populations, because the technology is still in its infancy. Currently there are four pest species being targeted by VVIC research; the house mouse (Redwood *et al.*, 2005), rabbit (Mackenzie *et al.*, 2006), red fox (*Vulpes vulpes*) (Strive *et al.*, 2006) and the Australian brushtail possum (*Trichosurus vulpecula*) (Cowan *et al.*, 2008). The ideal immunocontraceptive would be disseminating, long lasting, immune to host resistance, and without serious side effects (McLeod*, et al.*, 2007).

VVIC have the potential to be a cheap and effective tool for pest management. A single release into a population has the potential to infect all individuals in the population, and if there is contact between populations the single release could reduce fertility in many populations connected geographically. The success of the VVIC is dependent on the

vector. Shellam, 1994, put forward criteria for a successful biological vector for an immunocontraceptive, as outlined in Table 1.

Introduced mice in Australia exemplify the damage caused by this invasive species, especially to agriculture, therefore significant effort in that country has been invested into research on controlling populations of this pest. Certain environmental conditions arise sporadically which favour a large increase in mouse reproduction and survival rates, which lead to mouse plagues (Saunders & Giles, 1977). This results in massive damage to agricultural crops and huge economic loss (Brown & Singleton, 1999).

The use of VVIC could potentially reduce the substantial cost and workload required by traditional trapping and poisoning methods while still controlling the population of the mouse (Chambers, *et al.*, 1999a). The murine cytomegalovirus (MCMV) has been researched as a potential vector for VVIC of the mouse (Cunningham *et al.*, 2010; Shellam, 1994). Table 1 compares MCMV against the criteria put forward by Shellam, 1994.

Although immunocontraceptives have the potential to be very effective, they also have severe drawbacks. For example, brushtail possums targeted with a species-specific vector are pests where they are introduced in New Zealand, but in their natural home range in Australia they are important members of ecosystems (Angulo & Cooke, 2002; Gilna *et al.*, 2005), and even protected in some areas (Cowan, *et al.*, 2008). The release of the myxomatosis virus, as explained above, emphasizes the difficulty in controlling and regulating VVIC. The anthropogenic aspect is impossible to predict. Currently the major technological drawbacks of this technology are the attenuation of virulency, innate and acquired resistance, and transmission rates, amongst others (Arthur *et al.*, 2009; McLeod, *et al.*, 2007; Redwood *et al.*, 2007). These important aspects are the reason why no VVIC technology has been applied in the field.

Table 1: Table illustrating the suitability of Murine Cytomegalovirus (MCMV) as a biological vector of an immunocontraceptive for the control of pest mouse populations. Shellam, 1994, put forward seven criteria that a biological vector must comply with to successfully transmit an immunocontraceptive.

Criteria for a successful biological vector of an immunocontraceptive	Suitability of MCMV as a vector for an immunocontraceptive
Vector naturally infects the target species	Lab studies on inbred and wild outbred mice have shown that MCMV naturally infects mice (Lloyd *et al.*, 2003; Scalzo *et al.*, 2005b).
Vector is species-specific	MCMV is species specific, mice will be infected while Norway rats, *Rattus norvegicus,* will not (Smith *et al.*, 2005).
Vector reaches high prevalence in the target species	Inbred lab mice have shown strong innate resistance to the MCMV virus, while free living mice in Australia only very rarely have this resistance. This enables the virus to reach high levels within the population (Scalzo *et al.*, 2005a).
Vector is readily transmitted in the target species	MCMV is transmitted between individual mice via saliva. Exposure to saliva of other mice is common, via sharing of food or water sources, aggressive behaviour and grooming (Shellam *et al.*, 2006). Although some models show that the transmission can be very slow (Arthur*, et al.*, 2009).
Vector persists at low host densities	The virus is capable of staying in a latent state in the lungs of infected mice. This enables it to persist in a small population in low densities (Kurz *et al.*, 1997).
Vector does not normally cause lethal infection	MCMV is a herpes virus, and is non-lethal to adult mice. New born mice are more susceptible to associated thymus damage for the first 6 days after birth, causing death in the vast majority of cases (Shellam*, et al.*, 2006).
Recombinant vector can be introduced and maintained in the presence of existing infection	Multiple MCMV strains have been shown to be capable of infecting a single host mouse (Booth *et al.*, 1993).

1.3.3.2 Chemosterilization for Population Management

Chemosterilization is the process of directly exposing the target species to a pharmaceutical inducing a contraceptive reaction. This method has the advantage over immunocontraception of being fully controllable, and less likely to produce any form of acquired resistance. The disadvantages of chemosterilization are a much higher expense and a heavier work load, as the pharmaceutical must be continually applied, especially if it is reversible. Another disadvantage is discerning the exposure route of the pharmaceutical. If it is to be ingested, it must be highly palatable. There are two strategies when using chemosterilization; either hormonal or non-hormonal (Jewgenow *et al.*, 2006).

The hormonal strategy has been studied thoroughly, and many hormones, agonists and antagonists have been researched. Females are more often targeted for hormonal manipulation of reproduction than males, because the oestrous cycle is easily disrupted during specific periods. Male spermatogenesis is much more difficult to suppress, especially if it is important to avoid changing testosterone levels, which could cause potentially unwanted changes in behaviour (Jewgenow *, et al.*, 2006).

Hormonal chemosterilants act by disrupting the reproductive cycle of the individual exposed to the pharmaceutical (Gao & Short, 1993). Hormonal chemosterilants tend to have different effects on males and females, due to the differences in the hormonal cocktail of the reproductive cycles. The three hormonal pathways that have shown the most promise as pest management tools are outlined below; quinestrol (Lv & Shi, 2011), progestin (Jewgenow *, et al.*, 2006), and GnRH agonists (Jewgenow *, et al.*, 2006). GnRH agonists have been the most successful in many animals of both sexes. Exposure to GnRH agonists, such as deslorelin, causes an increase in pituitary release of LH and FSH causing feedback inhibition of these hormones, disrupting the oestrous cycle or spermatogenesis (Asa *et al.*, 2010). GnRH antagonists have also been synthesised, but their expense makes them uneconomical as a pest management tool (Gobello, 2007).

All GnRH analogs are susceptible to proteases, which means they cannot be delivered by ingestion as the stomachs proteases would degrade them before they could have any effect. So GnRH analogs have to be applied subcutaneously, requiring all animals to be caught before being treated (Gobello, 2007). This is feasible with large animals in urban settings, such as white tailed deer in urban centres of the USA, and in large feral ungulates (Turner & Kirkpatrick, 2001; Wellman et al., 2009). Hormonal chemosterilants are reversible, which again makes them suitable for some roles and less for others.

Non-hormonal chemosterilants have varied sources and many targets, while all have one of two goals – reversible or non-reversible infertility. These chemosterilants are either chemically synthesised or natural plant extracts, and both have shown potential as pest management tools. Chemically synthesised chemosterilants have the advantage of being cheaper to manufacture compared with plant extracts. Targets for reversible infertility are the reproduction stimulating hormones. This was the case in a recent field trial of the chemosterilant, 20,25-Diazacholesterol, willingly ingested as a bait on a natural population of the protected black-tailed prairie dog (Nash et al., 2007).

Although the indigenous black-tailed prairie dog is threatened, it is also seen as an agricultural pest in the USA. The often overcrowded populations must be controlled to protect them from disease and illegal eradication by property owners. A reversible chemosterilant is the most humane and legal form of population control for this species and others with similar formal protection. 20,25-Diazacholesterol inhibits cholesterol production, which in turn inhibits a precursor for sex hormones to be produced in the pituitary gland. This approach has been shown to inhibit fertility in the bandicoot rat (Hikim, 1987), the house mouse (Singh & Chakravarty, 2003) and many bird species (Cyr & Lacombe, 1992; Johnston et al., 2001). After 10 treatments the prairie dog populations had a significant decrease in recruitment of young (approx. 50%), and this infertility was still observed 3 months post-treatment. This is one of the few

non-hormonal chemosterilants to have been successful in the field (Singh & Chakravarty, 2003).

Non-reversible chemosterilants will often be female-specific, and target the finite supply of ovarian follicles. When all ovarian follicles are removed, total sterilization is achieved because ovarian follicles are the precursor cells to oocytes. Female mice with all follicles removed will react as does a woman in menopause; the loss of the hormones associated with ovulation may change some behaviours (Mayer *et al.*, 2004).

Many plants have adapted to grazing by producing chemical defences, some of which are non-hormonal chemosterilants. Of the plants tested so far as synthesising potential non-hormonal chemosterilants, *Azadirachta indica, Hibiscus rosasinensis, Melia azedarach, Momordica charantia, Trichosanthes cucumerinas and Tripterygium wilfordii* have shown the greatest potential. Unfortunately these extracts all targeted the semi-mature ovarian follicles, while the immature supply of follicles remained. This implies that the outcome would be reversible infertility (Tran & Hinds, 2013). If a chemosterilant could be found which targets the immature supply of ovarian follicles, this would have a greater chance of causing permanent sterility. A female chemosterilant used in conjunction with a male chemosterilant could have very significant effects on a population.

Mouse population management is a dynamic field. New technologies are being researched with ever increasing effectiveness, humaneness, and practicality. The effect of mice as commensal, agricultural and ecological pests is not yet fully understood. Future research into new technologies and tools will be able to better target mice, increasing efficiency. With the move away from lethal management and towards fertility control, mouse population management over large areas will become more socially acceptable, thus removing another hurdle to ecosystem and agricultural protection.

1.3.4 Importance of Food Intake and Body Weight in Pest Management

Food preference and intake are fundamental aspects for the control of a pest species. The level of food intake, impacts on many aspects of life, such as social hierarchies, fecundity, and life-span (Koyama & Kamimura, 1998; Lathe, 2004). Food preference is the individual or species choice in what food sources are ingested, this can be related to nutritional and caloric needs, competition, defence, and abundance (Emlen, 1966; Nicotri, 1980; Provenza, 1995). Food preference is regulated by the reward system and the stress system. A food source which has previously resulted in a hedonistic, satiating feeling will be ingested readily, while a food source which has previously resulted in visceral distress will induce an aversion response (Provenza, 1995).

A smaller individual often has a lower rank in a social hierarchy (Van de Weerd *et al.*, 1997), and will thus have less chance of producing offspring (Osadchuk *et al.*, 2007). The caloric intake, modulated by food preference and the rate of ingestion, controls the body weight of an animal. In social and non-social animals, a lower body weight can indicate less fat reserves, which decreases fecundity by reducing the energy stores required to search for a mate. These two aspects can be altered to directly lead to an advantage for attempts at population management. Food preferences can also be used to define the best bait to use as a vector for a pest management chemical or to bait a trap. This preference depends on the pest species.

Palatable tastants are deemed palatable if the animal perceives a pleasant initial taste and perceives reward after ingestion. The initial taste is defined by the chemical composition of the tastant. Taste receptors in the oral cavity ligate to specific tastant molecules. For instance a sugar will ligate to the T1R2+T1R3 taste receptor, this will result in a sweet taste being registered by the cerebral cortex, defining it as a safe and palatable food source (Sclafani & Ackroff, 2012). A separate response will also be recorded if the reward system is activated by a food source of high caloric value, because ingestion of it will result in a stimulation to eat, even if it is

metabolically unnecessary. The detection of a sweet taste causes a release of opioids in the brain. This pathway makes the act of eating palatable food a hedonistic and rewarding experience. Overstimulation of this pathway can result in addiction to palatable tastants (Olszewski & Levine, 2007) . A bait or pest management chemical capable of stimulating or overstimulating this pathway will be an effective population control agent.

1.4 Gossypol's Effects on Food Intake and Body Weight in Murine Pest Management

There is convincing evidence that supports gossypol's ability to decrease fecundity in mice. In this context, gossypol has been trialled as a pest management tool once, in the bandicoot rat but that study focused exclusively on gossypol's well demonstrated, contraceptive abilities, and neglected to assess food intake and body weight changes (Singla & Garg, 2013).

While data on the effect of gossypol on fecundity are abundant for mice, one of the most strikingly understudied topics is how gossypol influences food intake and the resulting body weight. The very few papers published thus far indicate that some authors have observed an anorexigenic influence of the compound (Amini & Kamkar, 2005), whereas others state that gossypol does not decrease appetite or body weight in mice (Xu*, et al.*, 2009). Interestingly it has also been suggested that gossypol does cause a decrease in food intake in rats (Romualdo*, et al.*, 2002). From the standpoint of pest management, it is crucial to understand changes in feeding patterns, food preferences, energy metabolism and, finally, resulting body weight in mice exposed to gossypol either acutely or chronically. This highlights the importance of generating systematic analyses of ingestive behaviour in gossypol-treated mice.

The gustatory system refers to the neurological and physiological aspects of the sense of taste and the resulting responses (Smith & Margolskee, 2001). The success of an ingested pest management chemical is utterly reliant on this system. The cautious foraging behaviour of mice prevents them from ingesting unpalatable food or food that causes gastric discomfort. Gossypol is known to cause gastrotoxicity at high doses and over long chronic exposures (Kitada, *et al.*, 2008; Sharma, *et al.*, 1999). However lower doses for shorter periods have not all observed gastrotoxicity, while still effecting fertility (Saksena & Salmonsen, 1982). This effect of gossypol still requires more study to understand these inconsistencies.

The palatability of gossypol to mice has not been reported, although in other mammalian studies, it was deemed as unpalatable, but easily masked (Semon, 2012). These gaps in the literature limit the range of possible experiments on toxicity, as these initial reactions must be identified first.

Upon exposure to certain chemicals the regulation of the gustatory system can be changed, which can be a cause of dysgeusia (a change in the perception of a tastant) (Carr *et al.*, 2012). For a pest management chemical, dysgeusia could be a positive or negative attribute. A desensitization of the bitter or sour taste receptors by an antagonist, could result in increased ingestion of an unpalatable pest management chemical. An agonist or an over-excitation of the sweet taste receptors could also result in higher rates of ingestion. However, the inverse response could be observed instead, in that ingestion could be reduced by a dysgeusic response, negatively affecting the gustatory system. An increase in the palatability of a tastant could lead to an increased reward response. This would be an advantageous effect for use in pest management, as it could lead to addictive behaviour toward the palatable bait. There have again been no studies identifying gossypol's effect on this aspect of the gustatory system.

High doses of gossypol have been shown to cause anorexia (East *et al.*, 1994; Zelski *et al.*, 1995). This symptom of gossypol toxicity may partially be caused by a change in metabolic homeostasis. Kitada *et al.*, 2008, observed that long term exposure to gossypol results in the de-villification of the small intestine. This retards the absorption of nutrients, hindering body weight stasis. Romualdo *et al.*, 2002, observed that, along with a decrease in weight, the mice which were being exposed to gossypol also ate less, during the chronic exposure. A decrease in food intake suggests that the de-villification is not the entire cause for the gossypol-induced anorexia, due to the increased caloric requirements after the loss of nutrient absorption capabilities, a malaise may be experienced as well.

The decrease in food intake and anorexia may be partially metabolically driven. A metabolically driven, decrease in food intake and body weight could be an advantage for pest management, as the mice would have less impact on other species. However if this decrease in food intake and body weight was toxically driven, it would suggest that gossypol is not suitable for pest management. This important aspect of gossypol remains to be clarified.

1.4.1 A Systematic Way to Understand Gossypol's Effect on Food Intake and Body Weight

The current conflict in the data surrounding food intake in gossypol-exposed mice, demands a systematic approach to define the true response. Certain responses to gossypol have been shown to be dose dependent (Coulson*, et al.*, 1980; Lin *et al.*, 1990). Importantly it has also been shown that ingested gossypol is quickly excreted via the faeces (Abou-Donia *et al.*, 1970). This finding emphasizes our inability to control the effective dose of gossypol when delivered via the gastrointestinal tract.

In oral administration of gossypol, one of the greatest uncontrollable variables is the definition of the exact amount of the drug that enters circulation. This is due to several aspects of this route of administration

that can hugely affect absorption. These include; the amount of food already present in the gastrointestinal tract, the rate of ingestion of the chemical and other foods, and the individual health of the subjects (DeSesso & Jacobson, 2001). Gossypol's physiological and histological effects could affect its ability to be absorbed into the circulatory system (Chen*, et al.*, 2009; Kitada*, et al.*, 2008; Lienhard *et al.*, 2012). Therefore, in initial studies on the effects of pharmaceuticals on food intake, the preferred route of delivery to expose animals to a precise dose is via injection into the body cavity. This is the best technique to confidently identify any dose-dependent or minor responses in food intake behaviour.

In a pest management scenario using bait as the vector, there are two possibilities for the animal to encounter a pest management chemical; either through a single, acute ingestion or via a chronic ingestion, consisting of the animal regularly exposing itself by ingesting multiple baits over time. To imitate this variable, injections need to be performed both acutely and chronically. These two different forms of exposure have the potential to cause different ingestive behaviour responses, this will increase the depth of the study.

Importantly, in environmental/field research, it is impossible to determine the form and frequency of exposure of wild animals, which further decreases the ability to accurately assess the data when the length of exposure to gossypol cannot be controlled. Controlled laboratory studies are required, initially, to allow interpretation of data later collected in the field.

Voluntary food intake is controlled by and affects numerous, somatic and neural pathways. To test if gossypol affects the energy requirements of the mice, the intake of normal lab chow can be measured. To test if the reward system is altered, intake of sugars and fats can be measured. These palatable tastants contain calories. To verify if the intake of these tastants has been changed due to an effect on the energy requirements, the intake of non-caloric sweeteners can be measured, such as saccharin.

These injection studies will unveil the physiological responses of food intake. Exposures to gossypol via the gastrointestinal tract will deepen this knowledge. To define whether the dose dependent responses still occur when gossypol is ingested, a precise dose can be delivered via gavage-feeding. The next step in this systematic approach will require exposing the mice to gossypol via food. The dose cannot be precisely controlled in this experiment, adding a substantial variable. Still, the responses observed from this experiment will suggest how palatable gossypol is to mice. Depending on the observations recorded from these experiments, field studies can be considered from this point on. Data from field studies can be accurately interpreted only after we have a clear understanding of what the effects of gossypol are.

1.5 Aims, Approaches and Outline of Thesis

The research undertaken in this study used a systematic approach aimed to define gossypol's effect on ingestive behaviour in mice, to further determine its suitability in pest management by documenting gossypol's effects beyond the regulation of reproductive functions. This aim was met by performing a series of injection experiments using tastants differing in palatability and caloric content/density. Studies on acute exposure to gossypol were carried out to determine whether this compound induces any changes in bland food intake (solid diet: standard laboratory chow) and palatable diet intake (liquid diets: glucose, sucrose and saccharin solutions). Possible effects on water intake were also assessed.

This acute exposure approach is a crucial step in the process of determining the feasibility of using gossypol in pest management, due to the wide ranging effects that even short-lived changes in food intake have on mice, as explained in section 1.3.4. Subsequently, a chronic injection experiment was used to determine gossypol's effects on food intake and resulting body weight.

As changes in appetite and body weight, especially in the long-term context, can stem from the animal's anxiety profile, the effects of gossypol on anxiety-related behavioural parameters were also established. This was accomplished using the open-field activity anxiety test, light/dark chamber exploration anxiety test and the novel item burying anxiety test. Importantly, stress is a critical factor in pest management as it changes behavioural responses to: food intake (Bale *et al.*, 2000), reproduction (Jeong *et al.*, 1999), predation (Adamec *et al.*, 2006), exploration (Strekalova *et al.*, 2004) and life span (Holzenberger *et al.*, 2002).

To better understand gossypol's effect on food intake in mice, a systematic approach needs to be undertaken, using the steps listed in section 1.4.1.

The lack of evidence on the effect of gossypol on the regulation of ingestive behaviour and body weight calls for systematic observation of behaviours related to energy homeostasis. The overarching goal of the current thesis was to provide the first step in the evaluation of ingestive behavioural responses to gossypol administered via injection, and ultimately to define gossypol's suitability as a pest management chemical. To achieve this goal, two specific programmes were undertaken:

1. Define the responses of ingestive behaviour in mice upon exposure to an acute dose of gossypol (Chapter 2).

2. Define the responses of ingestive and stress related behaviour in mice to a chronic exposure to gossypol (Chapter 3).

Chapter 4 includes the conclusions drawn from the results obtained, and potential future experiments that are needed to further evaluate gossypol as a potential mouse population management tool.

2 Effects of an Acute Exposure of Gossypol on the Ingestive Behaviour of Mice

Abstract

There is a lack of data in the literature regarding the effects of acute exposures to gossypol on the ingestive behaviour and the resulting body weight of mice. This chapter takes the first step in systematically defining this response. A series of acute injections IP, of 0 (vehicle), 10, 30, 100 and 300 mg/kg b.wt. (300 mg/kg b.wt. used in the bland diet experiment only) gossypol, were carried out to elucidate any changes in the ingestive behaviour of mice. The intakes of a bland diet (standard laboratory chow), palatable diets (glucose, sucrose and saccharin solutions) and of water were measured, along with corresponding body weights. The intake of the bland diet changed only with exposure to 300mg/kg b.wt. gossypol in which food intake increased at 1 and 3 hr post-exposure. The intake of the sucrose or saccharin solutions both produced no change, whereas, the intake of glucose was increased at 1 and 12 hr post-exposure to 100 mg/kg b.wt. gossypol. There was no significant change in water intake with exposure to gossypol, but a potential trend to decrease water intake with an increased dose of gossypol was suggested. These results suggest that exposure to gossypol does have an effect on the ingestive behaviour in mice, causing an increase in bland food intake and glucose intake with no change in body weight.

2.1 Introduction

Few studies on the response to acute exposures of gossypol in mice have been reported in the literature, with none reporting responses of ingestive behaviours. One of these few acute studies was reported in Tilyabaev *et al.*,2010. In this study the LD_{50} of gossypol for mice was suggested. No observations of changes in ingestive behaviour were reported. A high dose of gossypol, such as delivered in Tilyabaev *et al.*, 2010, may have had any effect on ingestive behaviour; changes in food intake, rate of intake, caloric intake, food preference or rewards, amongst others.

The sensing of flavours is performed by taste receptors, each taste group has its corresponding taste receptor. Exposure to certain exogenous chemicals can cause changes to the flavours sensed and the responses caused (Monleon *et al.*, 1995; Moran, 2010). The taste receptors can have their standard activity disrupted through ligation by agonists (Nakagawa *et al.*, 2013), antagonists or enhancers (Servant *et al.*, 2010). For example the action of the sweet taste receptor, T1R2+T1R3, has been shown to be altered through the binding of an allosteric enhancer, this increased the sweetness perceived of sucralose (Servant*, et al.*, 2010). A change in the gustatory action of a taste receptor induced by a chemical could have positive or negative effects for use in pest management.

In this experiment acute exposures to gossypol were injected IP in mice to observe responses in ingestive behaviour. The responses to these injections to the intake of food, water and three palatable tastants; glucose, sucrose and saccharin, were measured.

2.2 Methods

2.2.1 Animals

This study was performed on twenty C57BL/J mice, supplied by AgResearch Ltd (Hamilton, NZ). Standard laboratory chow (chow) and tap water were provided *ad libitum* unless stated otherwise. LD 12:12, lights on at 0600h, constant temperature at 23-25^{0}C. The mice were acclimatised to single housing 3 days prior to the experiment.

2.2.2 Pharmaceuticals

Gossypol was supplied by Tocris, Minneapolis, USA. The gossypol powder was dissolved in 3% DMSO and then diluted in saline.

2.2.3 Effect of an Acute Exposure to Gossypol on Food Intake

2.2.3.1 Treatment

Mice were deprived of chow overnight. The following morning (7:00am), measured quantities of chow were returned to hoppers. Just prior to regaining access to food, the animals were injected IP with 0 (vehicle), 10, 30, and 100 of gossypol in 0.1 ml volume (n=5/group).

The hoppers were weighed 1, 3 and 24 hr post-injection to determine food consumption. Body weights were recorded just prior to the overnight food deprivation and immediately after the 24 hr food intake measurement.

This regime was repeated twice. The third series of injections consisted of 0, 100 mg/kg b.wt. (n=5/group) and 300 mg/kg b.wt. (n=10/group) with the same measurements recorded.

2.2.4 Effect of an Acute Exposure to Gossypol on Water Intake

2.2.4.1 Treatment

Mice were deprived of water and chow 1 hr prior to injections. Three hours before lights out the mice were injected IP with 0 (vehicle), 10, 30, and 100 of gossypol in 0.1 ml volume (n=5/group).

Known volumes of water were returned to the mice directly after injections and water intake was measured 1 and 12 hr post injection. Known weights of food were returned to the hoppers one hour post injection and food intake was weighed 12 hr post-injection. Body weights were measured 1 hr prior to injection and 12 hr post-injection. This experiment was repeated twice.

2.2.5 Effect of an Acute Exposure to Gossypol on Palatable Tastant Intake

2.2.5.1 Treatment

Mice were deprived of water and chow 1 hr prior to injections. Three hours before lights out the mice were injected IP with 0 (vehicle), 10, 30, and 100 of gossypol in 0.1 ml volume (n=5/group).

In place of water, a palatable solution was given to the mice directly after the injections and intake was measured 1 and 12 hr post-injection. Food was returned to the hoppers 1 hr post-injection and intake was weighed 12 hr post-injection. Body weights were measured 1 hr prior to injection and 12 hr post-injection.

The palatable solutions were; 10% glucose, 2% sucrose and 0.1% saccharin. Prior to the injection the mice were given an acclimatisation period of 2 days to each solution. This experiment was repeated twice for each palatable tastant.

2.2.6 Statistical Analysis

The mean of each category of the data was calculated. An analysis of variance (ANOVA) was used to test for any statistical significance between means of categories. If significance was calculated two post hoc tests were performed to define whether the significance was calculated between the control and a gossypol treatment. The post hoc tests used were the Newman-Keuls test and the Duncan test. Histograms display the upper standard deviation from the mean with error bars displaying 2 standard deviations.

2.3 Result

2.3.1 Effect of an Acute Exposure to Gossypol on Food Intake

Exposure to gossypol caused no significant change in food intake except at the highest dose. No change in food intake was observed after IP injection with 10, 30 and 100 mg/kg b.wt. gossypol. This lack of change was observed at 1, 3 and 24 hr post-injection (Figure 1).The 300 mg/kg b.wt. dose was followed by a significant increase in food intake at 1 and 3 hr post-injection, but not at 24 h (Figure 1).

There was no change in body weight in any of the gossypol-treated groups, including the 300 g/kg group that showed an increase in consumption (Figure 2).
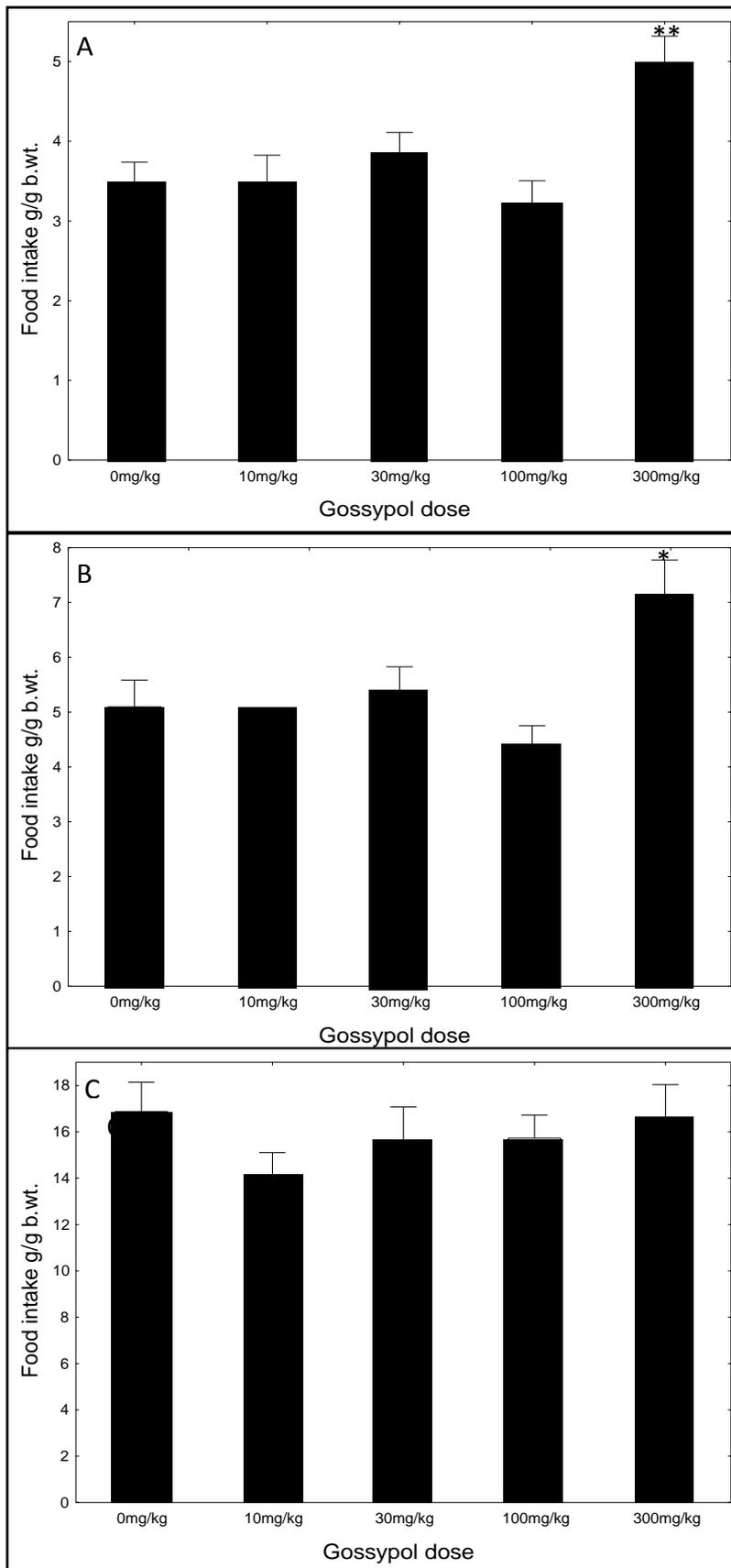
Figure 1: Food intake in mice acutely injected IP with 0 (vehicle), 10, 30, 100 and 300 mg/kg b wt. of gossypol at 1 (A), 3 (B) and 24 hours (C) post-injection. * p ≥ 0.05, ** p ≥ 0.01.
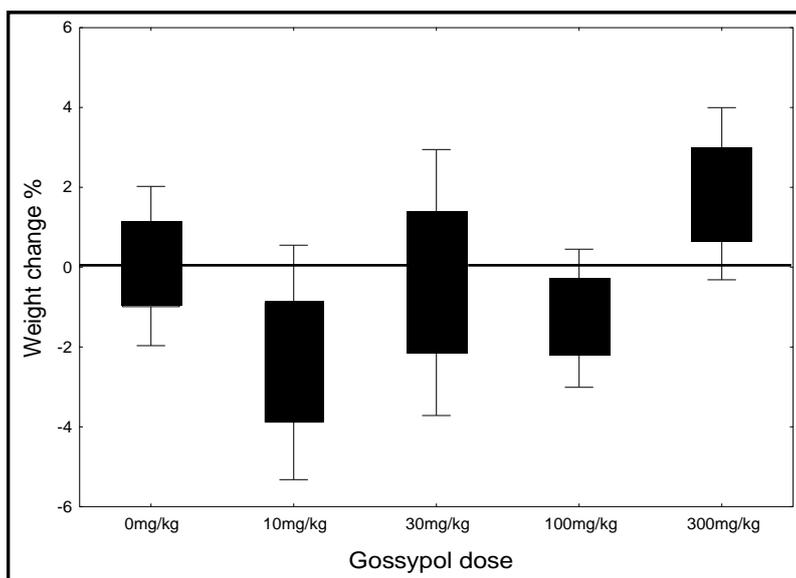
Figure 2: Mice were acutely injected IP with 0 (vehicle), 10, 30, 100 and 300 mg/kg b wt. of gossypol. Body weight change was measured 24 hr post-injection.

2.3.2 Effect of an Acute Exposure to Gossypol on Water Intake

An IP injection of gossypol had no significant effect on the intake of water in the mice. No effect of the compound was seen 1 hr post-injection (Figure 3). Although there was no significant difference in water consumption at 12 h post-injection, animals injected with the highest, 100 mg/kg b.wt., dose showed a trend towards a decrease in water intake (p=0.07 (Duncan post hoc) p=0.14 (Newman-Keuls post hoc)) (Figure 3).

Figure 3: Water intake in mice acutely injected IP with 0 (vehicle), 10, 30, 100 mg/kg b.wt. gossypol at 1 (A) and 12 (B) hours post-injection.

2.3.3 Effect of an Acute Exposure to Gossypol on Palatable Tastant Intake

2.3.3.1 Glucose

An IP injection of gossypol caused a significant response in glucose intake. At 1 and 12 hr post-injection the 100 mg/kg b.wt. dose caused a significant increase in glucose intake (p=0.044 (Newman-Keuls) p=0.022 (Duncan) and p=0.021(Newman-Keuls) p=0.021 (Duncan), respectively). The other doses had no response (Figure 4).
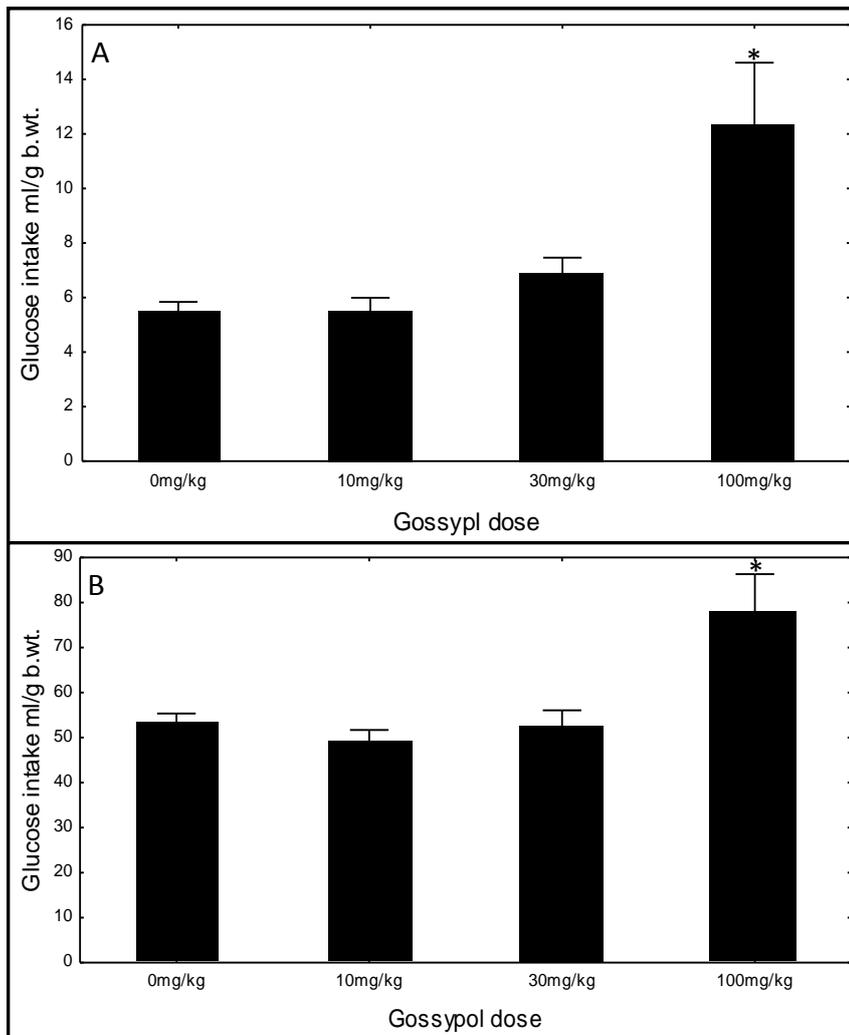
Figure 4: Glucose intake in mice acutely injected IP with 0 (vehicle), 10, 30, 100 mg/kg b.wt. gossypol at 1 (A) and12 (B) hours post-injection. * p ≥ 0.05.

2.3.3.2 Sucrose

Exposure to gossypol caused no change in sucrose intake in mice. Measurements at 1 and 12 hr post-injection resulted in no response in sucrose intake in any of the doses of gossypol that the mice were treated with (Figure 5).
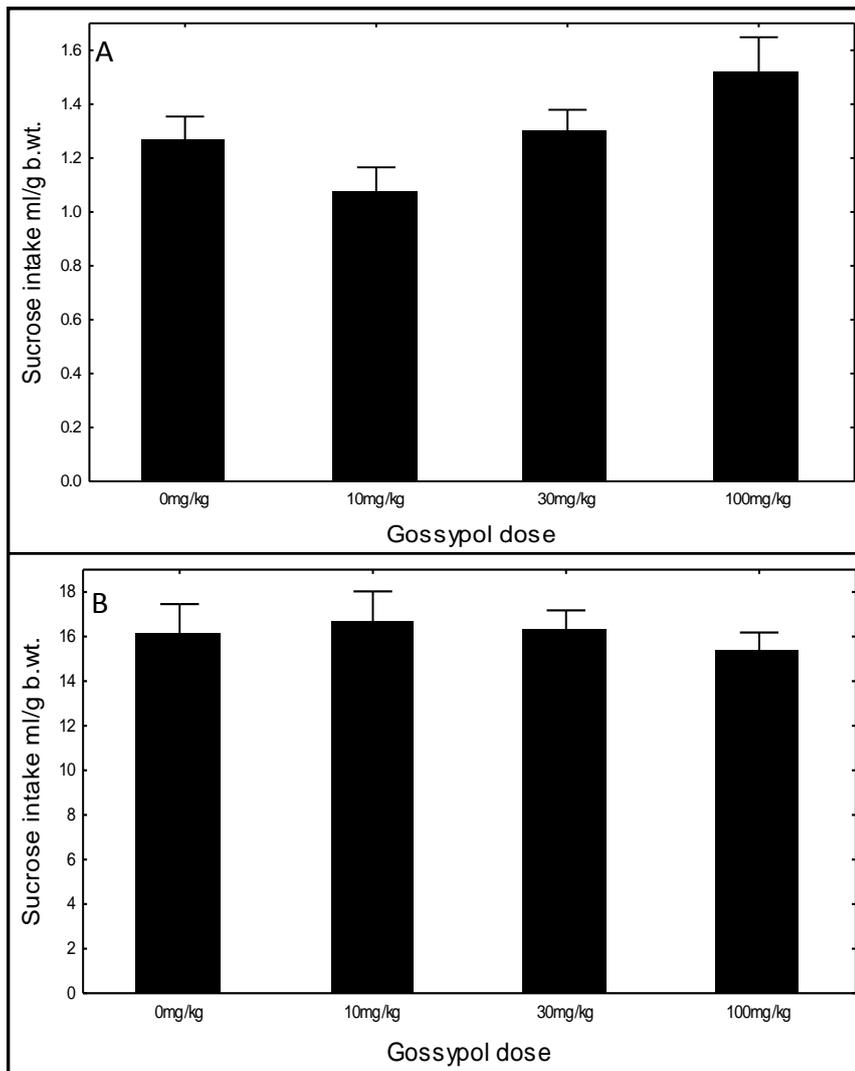
Figure 5: Sucrose intake in mice acutely injected IP with 0 (vehicle), 10, 30, 100 mg/kg b.wt. gossypol at 1 (A) and 12 (B) hours post-injection.

2.3.3.3 Saccharin

Similar results were observed in saccharin intake compared to sucrose intake after the gossypol treatments. No changes in saccharin intake were seen with increasing concentrations of gossypol exposure (Figure 6).

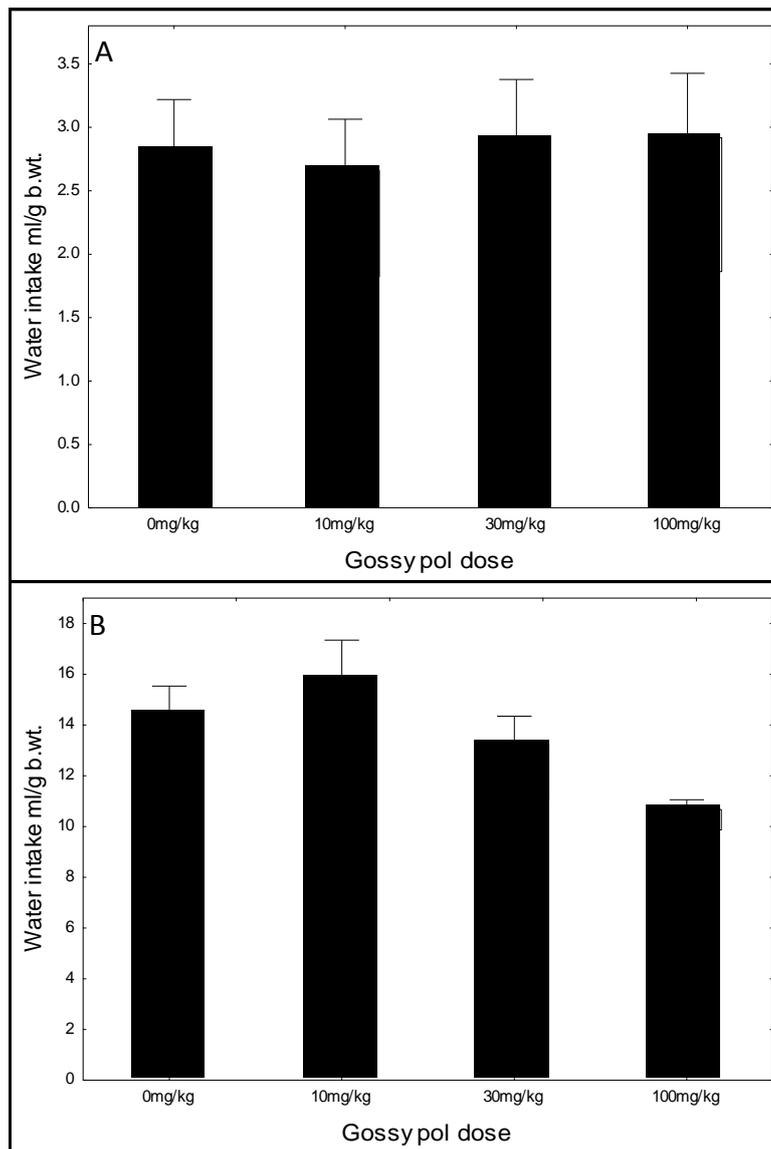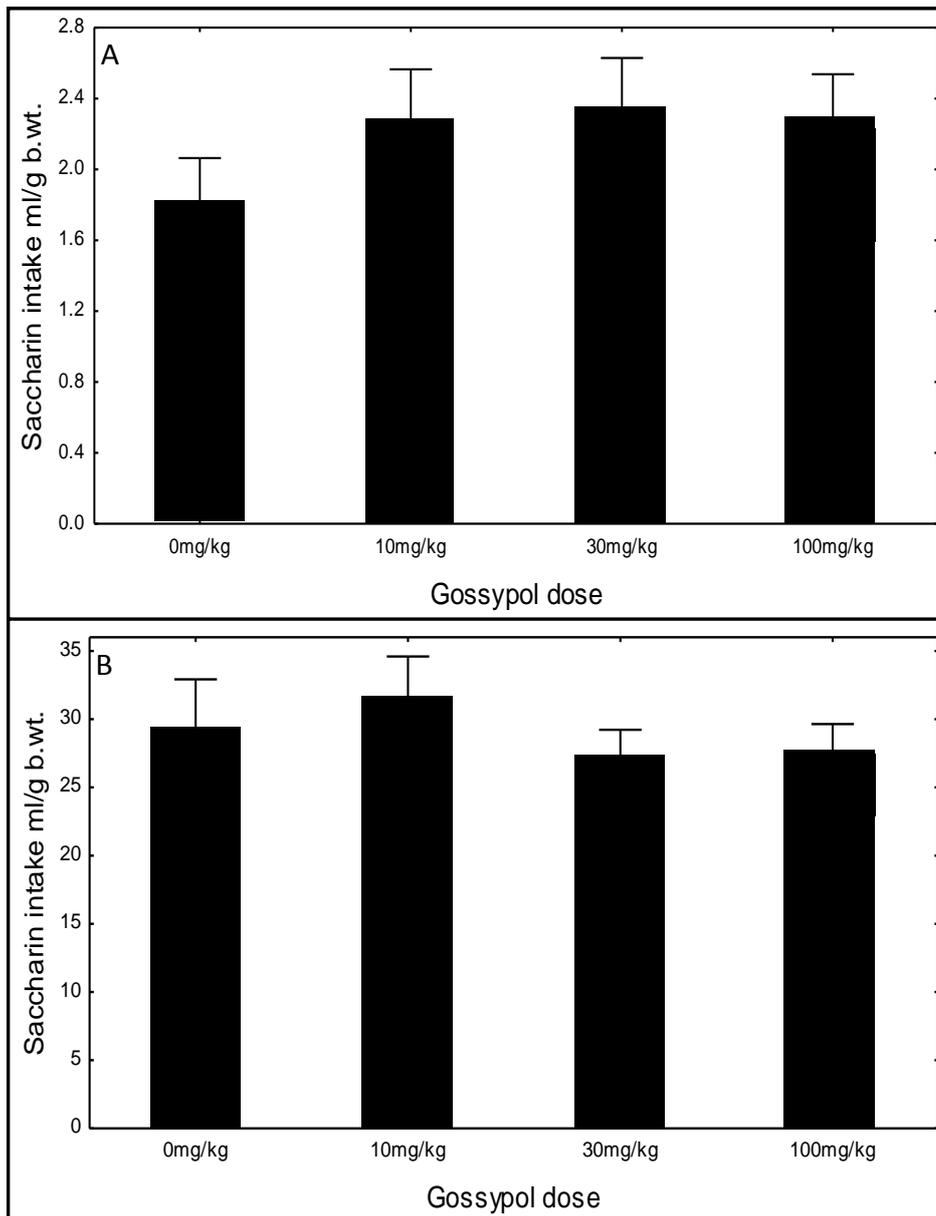Figure 6: Saccharin intake in mice acutely injected IP with 0 (vehicle), 10, 30, 100 mg/kg b.wt. gossypol at 1 (A) and 12 (B) hours post-injection.

2.4 Discussion

This experiment was the first step in a systematic process designed to define the behavioural responses of mice to gossypol. For this reason extrapolations from this data to conclusions on the potential of gossypol to improve real pest management operations may be imprecise.

Understanding gossypol's effect on the intake of water, bland, and palatable tastants is critical to understanding its suitability as a murine pest management tool. The results, described above, direct us to some of these responses.

 The response of the food intake of mice to an acute exposure of gossypol was measured. The only significant change was an increase in food intake observed in the mice treated with 300 mg/kg b.wt. gossypol during the first three hours after exposure, but this effect had dissipated after 12 hr. The mice also had their water intake measured during an acute exposure to gossypol. After the initial hour post-injection there was no response, but later, at 12 hr post-injection, there was an insignificant trend suggesting that higher doses cause a decrease in water intake. Gossypol's effect on the intake of palatable tastants was also tested. Measurements of glucose intake revealed a dose dependent response. A significant increase in glucose intake was observed in the mice treated with 100 mg/kg b.wt. gossypol. Saccharin and sucrose intakes showed no change in response to the acute exposure to gossypol.

2.4.1 Effect of an Acute Exposure to Gossypol on Food Intake

The effect on food intake caused by the acute exposure to gossypol was significant only at the highest dose, 300 mg/kg b.wt. This very large dose may have been toxic. After the injection the 300 mg/kg b.wt. treatment mice had a significantly higher intake of food, an increase in ingestion is a common response to toxicity in rodents. Rodents are non-emetic, they do

not have the anatomy, physiology or neurology capable to regurgitate food (Horn*, et al.*, 2013). A behavioural attribute that has been selected to overcome this phenomenon, is toxin induced pica-like behaviour. When toxicity is detected in the gut, via a visceral sensation, mice will respond by eating clay, dirt and other indigestible items (Andrews & Horn, 2006). It has been suggested that this behaviour was selected to dilute and bind the toxin still in the gastrointestinal system (De Jonghe & Horn, 2008). In the current study there were no suitable indigestible items for the mice to ingest, so the increase in chow may have been a substitute for the mice. But how toxic was the 300 mg/kg b.wt. dose?

An experiment defining the acute LD_{50} of gossypol in an unidentified strain of white mice has recently been completed (Tilyabaev*, et al.*, 2010). This study observed that an acute dose of 154 mg/kg b.wt. was concentrated enough to be lethal to half of the population. As with my study, the doses were dissolved in DMSO and were delivered via injection IP. The highest dose to which I exposed the mice, with no fatalities, was almost double the LD_{50} that was reported in Tilyabaev *et al.*, 2010. This discrepancy between the two similar studies is difficult to resolve, partially due to the lack of experimental detail reported in Tilyabaev *et al.*, 2010. Full details for my study are given above: by contrast, Tilyabaev *et al.*, 2010, reports only that white mice were injected with 154 mg/kg b.wt. gossypol dissolved in DMSO, sourced from the Institute of Bioorganic Chemistry, Uzbek Academy of Sciences. This is all the experimental detail that is available in the publication. Factors that could have caused the disparity include differences in gossypol purity, mouse strain and lab conditions.

Other publications studying gossypol's toxicology may help us to understand the reason why a high dose of gossypol caused an increase in food intake. It is important to emphasize that there have been few acute gossypol studies, so these results must be interpreted using chronic gossypol studies. A chronic exposure in mice has suggested that, a dose of 62 mg/kg b.wt. gossypol, gavage fed for 5 days on, 2 days off, will be lethal to 50% of a population after 16 days (Kitada*, et al.*, 2008). This dose totalled 744 mg/kg b.wt. over the 16 days, almost 2.5x the highest single

dose injected into the mice in my acute study. The different reactions observed between the two studies, mine and Kitada *et al.,* 2008, are due to the excretion (Abou-Donia & Dieckert, 1974; Abou-Donia*, et al.*, 1970) and storage of gossypol in certain areas of the body, especially the spleen, liver and testis (Gamboa *et al.*, 2001; Kalla & Sud, 1990).

For example, Abou-Donia and Dieckert, 1974, used C^{14} marked gossypol to study its excretion in pigs. They observed that the majority of the radioactivity was being detected in the faeces of the pigs. After 20 days, 94% of the original radioactivity injected was detected in the faeces. Similar results were also seen in rats (Abou-Donia*, et al.*, 1970). These studies suggest that a single dose of gossypol, although very concentrated, may be slowly excreted out of the body via the faeces, whereas a smaller, chronic dose cannot be removed from the body before another dose is given.

The gossypol Materials Safety Data Sheet (MSDS) states that an acute dose 2315 mg/kg b.wt. is the LD_{50} for rats (Register of Toxic Effects of Chemical Substances, 2010). This is substantially higher than any of the doses in my experiment. Although the average weight of the C57BL/J mice in my study was 29.05 g, the average weight of the control group of 90 day old, male Wistar rats in a diabetes study was ~300g (Nakhooda *et al.*, 1977). Assuming that the two species have similar reactions to gossypol, weight for weight, the acute LD50 of 2315 mg/kg b.wt. for a 300g rat should be 10x the amount for a 30g mouse. If this assumption was correct, then the C57BL/J mice should have an acute LD50 of 231.5 mg/kg b.wt. The acute exposure to gossypol in my study, of 300 mg/kg b.wt., had no fatalities, compared to the 50% fatalities recorded in Tilyabaev *et al.,* 2010, at a dose of 154 mg/kg b.wt. This suggests that mice are relatively more tolerant per body weight to gossypol than rats. Rats, themselves, have been suggested to be particularly tolerant to gossypol toxicity (Waites*, et al.*, 1998).

Food intake was not affected in the mice exposed to an acute dose of gossypol of 10, 30 and 100 mg/kg b.wt. This suggests that an acute dose

injected IP does not cause any visceral response. It is of interest that the 100 mg/kg b.wt. dose did not cause a change in food intake, as it was a highly concentrated dose. As stated previously a dose of 154 mg/kg b.wt. was shown to be the LD_{50} of a population of mice (Tilyabaev*, et al.*, 2010). The highest dose with no response, in my study, was merely two thirds of the dose that was lethal to 50% of a population of the same species. This discrepancy between my study and this LD_{50} study highlights the requirement for more acute studies into gossypol's effect on ingestive behaviour and the need for a systematic approach to be taken to fully understand any responses observed.

The gossypol content of cottonseed is minute, but in early studies cottonseed was shown to be unwillingly ingested by rabbits (as reviewed in(Semon, 2012). This is potentially due to toxicity or a bitter taste. There is a small amount of evidence to suggest that gossypol is the cause of the bitter taste in cottonseed. Weiss *et al.*, 2011, has suggested that *Drosophila* senses gossypol as bitter. This was concluded by testing neuron activation when the labellum (taste organ) was exposed to gossypol . These results cannot be directly extrapolated to mammalian taste sensing. Basic palatability tests in a variety of mammals were performed in the first half of the 20th century (Semon, 2012). Rabbits and guinea pigs that both refused to eat cottonseed would willingly ingest cottonseed mixed with sweet molasses (Withers & Carruth, 1915). In my study the acute exposure to the lower concentrations of gossypol did not affect food intake in the mice. This exposure was via injection IP, which does cause it to bypass the majority of the taste receptors in the oral cavity. If, at a later stage, gossypol is shown to be unpalatable to mice, this could be overcome by masking the flavour with a more palatable tastant.

2.4.2 Effect of an Acute Exposure to Gossypol on Water Intake

Acute exposure to gossypol did not show any effect on the intake of water initially. After the first hour post injection, all gossypol treated mice did not show any change in water intake compared with the control group. However, at 12 hours post injection, an insignificant trend was observed. This suggested that a larger dose of gossypol might cause a decrease in water intake. This trend may have been more pronounced earlier in the post injection period, as there was a large gap between observations. What did gossypol react with to cause this potential reaction?

Thirst is triggered either by cellular dehydration or by a decrease in the volume of blood (McKinley *et al.*, 2003). The blood volume will decrease with dehydration. This decrease in blood volume triggers the renin-angiotensin system (Lehr *et al.*, 1973). Angiotensin II is synthesised in the lungs and regulates vasoconstriction and the feeling of thirst, and this action has been shown to be inhibited by gossypol (Hasegawa *et al.*, 1993).

The inhibition of this role of angiotensin II was caused by gossypol's inhibitory effect on the angiotensin II-regulated-release of endothelium-derived relaxing factor (EDRF) (Hasegawa*, et al.*, 1993). Without the release of EDRF, caused by gossypol, vasoconstriction is unregulated. A disruption to the regulation of vasodilation and vasoconstriction (vasospastic) causes decreased thirst and fluid intake in humans (Teuchner *et al.*, 2004). Gossypol inhibits angiotensin's action as a vasoconstrictor, thus the vascular system is unregulated, which may in turn reduce the feeling of thirst in the mice.

2.4.3 Effect of an Acute Exposure to Gossypol on Palatable Tastant Intake

Of the three palatable tastants measured in my study, glucose, sucrose and saccharin, only the intake of glucose was significantly altered by

exposure to gossypol. How was the intake of this sweet tastant altered when gossypol was injected IP, bypassing the oral cavity?

Sweet flavours can be detected right throughout the digestive system, from the mouth to the small intestine. The G protein coupled T1R family of receptors are involved with detecting sweet tastes along with other taste types, and these have been found in the mouth and in the gut (Nelson *et al.*, 2001; Young, 2011; Zhao *et al.*, 2003). The sweet taste receptor is the heterodimeric T1R2+T1R3. This is a broad spectrum receptor, binding sugars, artificial sweeteners and some proteins as ligands found in both the oral cavity and the gut (Sclafani & Ackroff, 2012). The gut also contains the sugar transporter sodium glucose co-transporter 1 (SGLT1). This regulates the movement of glucose from the gut to the enterocytes. The expression of SGLT1 is up-regulated by the detection of sugars by T1R2+T1R3 (Dyer *et al.*, 2007; Margolskee *et al.*, 2007).

Unlike the taste receptors in the oral cavity, the gut taste receptors are not involved in detecting the flavour of the ingestants. The detection of sweet molecules in the gut is involved with many processes including; nutritional detection and conditioning, generating the feeling of satiety, and glucose homeostasis (Sclafani & Ackroff, 2012). The injection of gossypol into the peritoneal cavity could have had an effect on any of these pathways. What process did gossypol change to cause the increase in glucose intake?

The sweet receptor through the entire digestive tract is T1R2+T1R3. This receptor has been found to have a broad spectrum of ligands, including glucose, sucrose and saccharin (Lewis *et al.*, 2005; Shirazi-Beechey *et al.*, 2011). This indicates that this receptor is not affected by gossypol, if it were, a change in all three of the tastants intake would have been observed.

Another possibility to explain the gossypol-induced increase in glucose intake is a change in SGLT-1's expression or function. SGLT-1 regulates the movement of ingested glucose out of the lumen into the enterocytes. If expression or activity of SGLT-1 was interrupted, this would decrease the blood glucose level requiring an increase in intake of glucose. This would

result in a craving for a sweet tastant, which in turn would be observed as an increase in intake of any sweet tastant. My results rule out this explanation.

I have focused only on gossypol's effect on the digestive system. This is because, of the three palatable tastants tested, two of them contain glucose. Sucrose is a disaccharide comprised of fructose and glucose. This molecule is lysed upon digestion by the enzyme sucrase, releasing fructose and glucose separately. This enzyme is found primarily in the duodenum of the small intestine (Gray, 1971). This suggests that any activity of gossypol downstream of the duodenum and intestinal transport of glucose would have an effect on the intake of sucrose as well. Unfortunately in my experiment the concentration of sucrose used was based upon the ideal concentrations used in various other studies (Bachmanov *et al.*, 2001; El Yacoubi *et al.*, 2003; Monleon*, et al.*, 1995). The 2% sucrose solution used, once digested, consisted of 1% glucose. Whereas in the glucose test, I used a 10% glucose solution. This tenfold dilution in glucose, in the sucrose intake study, may have masked a similar effect that was observed in the glucose test. This unfortunate mistake may have masked a non-gustatory effect of gossypol on glucose and sucrose.

This study has revealed some of gossypol's effects on the sweet gustatory system. Certain potential areas that it could have affected have been discussed above and the data in the literature suggests that the sweet taste receptor, T1R2+T1R3, and the glucose transporter, SGLT1, are not targets. This process of elimination could continue on for an extended period. Unfortunately a potential effect on sucrose intake may have been masked due to an experimental mistake in the concentration of sucrose presented to the mice. This will make any precision in the interpretation of these results difficult to come by.

2.4.4 The Pest Management Context
The experiments described and discussed above are merely the first steps in a thorough, systematic process to understand an aspect of gossypol's

activity that could determine its suitability in pest management. Gossypol's negative effect on the fecundity of mice is well documented (Randel, *et al.*, 1992). This aspect of its activity has already been recognised for its potential in pest management (Singla & Garg, 2013), but our understanding of this compound requires broadening before it is used in this area.

The reproductive inhibition of this compound has already been thoroughly established (Randel, *et al.*, 1992). This important aspect of gossypol is required for it to have an effect on pest mouse populations. Importantly its effect on feeding behaviour, and the consequent implications for bait additives, has never been thoroughly explored in mice or other closely related rodents (Kitada, *et al.*, 2008; Romualdo, *et al.*, 2002; Xu, *et al.*, 2009).

Hence, the most interesting result from the acute exposures documented here is the significant increase in glucose intake. This response to an acute exposure to gossypol has not been reported before in the literature. Glucose could potentially be used as the vector bait in which gossypol is ingested in the field. The increase in glucose intake observed in this study suggests that ingestion of a glucose bait containing gossypol may increase the intake of the bait, potentially increasing the dose of gossypol and the subsequent response.

The present study used injection IP to expose the mice to gossypol. This method of exposure allowed a reliable and repeatable amount of gossypol to be used. By contrast, in a pest management scheme, the application of a pest management tool is only very rarely administered via injection. It can be done on large animals which can be darted or rounded up for treatment, to reduce the recruitment rate to populations via contraception (Miller, *et al.*, 1998; Wellman, *et al.*, 2009). Rodent pests are typically neophobic, sexually mature at a young age and have a high reproductive output (King, 2005). These traits make the pest management strategy of contraceptive via injection unsuitable.

Are the results obtained through the present study comparable to a pest management tool that would be applied orally? The plasma concentration of gossypol has been shown to be much higher after a single injection compared to a single oral exposure (Jia *et al.*, 2008). This suggests that the reactions observed in the current study may be exaggerated compared to an oral exposure. The injection IP was chosen because it can deliver a precise dose, thus allowing comparison between different doses. The next step from here is to expose the mice to gossypol orally. To ensure the correct dose is ingested, the mice will have to be exposed via gavage feeding. The observations reported in this current thesis will need to be repeated by gavage feeding in order to confirm the results.

3 Effects of a Chronic Exposure to Gossypol on the Food Intake, Body Weight and Anxiety of Mice

Abstract

The ingestive behavioural responses in mice chronically exposed to gossypol have had contradictory reports in the literature. This chapter explains in depth, a systematic experiment measuring this response in mice. Mice were injected IP with 0 (vehicle) and 100 mg/kg b.wt. daily for 11 days. Food intake and body weight were measured at certain points throughout this period and the following 40 day "post-exposure" period. No responses to this injection were observed by the end of the injection period, but measurements taken during the 40 day post-exposure period showed that the mice increased their food intake with no corresponding increase in body weight. The anxiety levels of the mice were tested to detect any possible long-term gossypol-induced anxiogenic effect affecting food intake. This showed no changes, therefore the food intake response was probably due to a gastro-intestinal or metabolic effect of gossypol. This study suggests that a chronic exposure to gossypol IP does have an effect on ingestive behaviour, causing an increase in food intake post-exposure.

3.1 Introduction

The majority of the studies available throughout the literature have been of chronic exposures to gossypol, showing a variety of effects observed in numerous species. These have included; weight loss, damage to tissue of the testis, hypokalemia, pulmonary oedema, gastrotoxicity and a decrease in lymphocytes, amongst others (Braga *et al.*, 2012; Coutinho, 2002; East*, et al.*, 1994; Lohiya *et al.*, 1990; Monsees*, et al.*, 1998; Xu*, et al.*, 2009). The responses of mice to chronic exposures have resulted in contradictory reports. For example a report of gossypol having no effect on the body weight or food intake of mice (Xu*, et al.*, 2009) is confounded by a report of gossypol causing a decrease in food intake and a corresponding decrease in body weight (Amini & Kamkar, 2005).

Some chronic exposures to gossypol have resulted in a dose dependent decrease in weight (Kitada*, et al.*, 2008), with higher doses recording larger reductions (Lin*, et al.*, 1990) in rodents. The cause of this decrease in body weight is unknown. Gossypol has been suggested to cause gastrotoxicity (Kitada*, et al.*, 2008), which in itself suggests a reduction in food intake that would aid the drop in body weight.

Mice are naturally highly-stressed, anxious animals, and their cautious behaviour is one of the main barriers to population management (King, 2005). Certain pharmaceuticals have been shown to increase anxiety levels in laboratory animals (Simon *et al.*, 1994; Simon *et al.*, 1993). Gossypol's effect on anxiety has never been determined. The current study is aimed to define the outcome of a chronic exposure of gossypol on long-term anxiety levels in mice. This may have important implications for the suitability of gossypol in pest management, because higher anxiety may be correlated with lower willingness to sample novel baits.

This study was performed to define the responses of food intake and body weights to a chronic exposure of gossypol in mice by IP injection. The anxiogenic response was studied during a post-exposure period of 40

days after the injection period, when any long term chronic responses might be expected.

3.2 Methods

3.2.1 Animals

This study was performed on twenty C57BL/J mice, supplied by AgResearch Ltd (Hamilton, NZ). Standard laboratory chow and tap water were provided *ad libitum* unless stated otherwise. LD 12:12, lights on at 0600h, constant temperature at 23-25^{0}C. The mice were acclimatised to single housing 3 days prior to the experiment.

3.2.2 Pharmaceuticals

Gossypol was supplied by Tocris, Minneapolis, USA. The gossypol powder was dissolved in 3% DMSO and then diluted in saline.

3.2.3 Chronic Exposure to Gossypol

3.2.3.1 Treatment

Daily injections were given to mice for 11 days of either 0 (vehicle) or 100 mg/kg b.wt. (n=10/group) just prior to lights out (4:00pm). Immediately post-injection, the chow, water and the weight of the mice were measured to discern food and water intake and body weight.

After the 11-day injection period was complete, food and body weight of mice were recorded daily for the initial 10 days of the post-exposure period, then twice weekly for another 30 days.

3.2.4 Measuring Anxiety Levels after Chronic Exposure to Gossypol

The mice were individually tested in 3 different protocols to define their levels of anxiety. The three tests used were: the openfield activity anxiety test, the light/dark chamber exploration anxiety test, and the novel item

burying anxiety test. These tests took place during the post-exposure phase of the chronic exposure to gossypol experiment.

3.2.5 Openfield Activity Anxiety Test

The openfield activity anxiety test, performed on Day 29, assessed whether treatment with gossypol would have an effect on the animal's willingness to expose itself away from cover. A decrease in the amount of time spent in the open is correlated to an increase in anxiety (Heisler *et al.*, 1998).

3.2.5.1 Apparatus

A 44cm (L) x 44cm (W) x 49cm (H) square enclosure was used. The walls and floor of this box were whited out. In the centre of this floor, was a 14.7cm x 14.7cm central square, demarcated with a thin black line.

3.2.5.2 Treatment

Before being placed within the enclosure, each mouse had a white mark (correcting fluid) placed on its neck to show the observer which field the mouse was in. A mouse was placed in a corner of the enclosure. The total time spent in the central square (open field) was recorded for 10 min, after which it was replaced in its cage. The enclosure was cleaned with 95% ethanol to remove any animal scent and left for 3 min to dry before the next mouse was tested.

3.2.6 Light/dark Chamber Exploration Anxiety Test

The light/dark chamber exploration anxiety test, performed on Day 30, tested the animals' choice between exploring a novel environment (a light chamber), and staying in the relative safety of a dark chamber (Martin *et al.*, 2002). An anxious mouse will prefer to stay in the safety of the dark chamber, whereas a less anxious mouse will be more likely to expose itself in the light chamber to explore whether there is a reward.

3.2.6.1 <u>Apparatus</u>

Two chambers of 30cm (L) x 24.5cm (W) x 50cm (H) connected by a 6cm (W) x 10cm (H) doorway were used. One of the chambers had blacked out walls and floor and a translucent black lid (dark chamber), the other chamber had whited out walls and floors and no lid (light chamber).

3.2.6.2 <u>Treatment</u>

Before being placed within the chamber, each mouse had a white mark (correcting fluid) placed on its neck to show which chamber the mouse was in. Individually a mouse was placed in the corner furthest from the doorway in the dark chamber. The total time spent in the light chamber was recorded for 10 min, after which it was replaced in its cage. The chambers were cleaned with 95% ethanol and left for 3 min to dry before the next mouse was tested.

3.2.7 Novel Item Burying Anxiety Test

The novel item burying anxiety test, performed on Day 34, measured the change in anxiety levels towards novel objects in an acclimatised environment, and hence the adaptation of mice towards neophobia. Aversion to new objects is heightened with anxiety, causing the mice to bury more of the objects (Homma & Yamada, 2009).

3.2.7.1 <u>Apparatus</u>

This experiment used each mouse's acclimatised single house cage with a layer of bedding approximately 2 cm deep. Fifteen marbles were placed equidistantly in a 5 x 3 pattern on top of the bedding.

3.2.7.2 <u>Treatment</u>

The mouse was removed from its cage to a holding enclosure. In the mouse's acclimatised cage the bedding was flattened and the marbles were placed on the bedding. The mouse was replaced in its own acclimatised cage for 20 min. After this 20 min the mouse was removed

from the cage and the number of marbles buried was recorded. The marbles were then collected and cleaned in a 50% ethanol bath. The mouse was replaced after removal of the marbles.

3.2.8 Statistical Analysis

The Student's T-test defined the significance between the two treatment groups in food intake, body weight and anxiety. Histograms display the upper standard deviation from the mean with error bars displaying 2 standard deviations.

3.3 Results

3.3.1 Effect of a Chronic Injection of Gossypol on Bodyweight of Mice

There was no significant difference between the body weights of the control and treatment group at the end of the injection period, at day 10 of the post-exposure period or at day 40 of the post-exposure period. However at the end of the injection period there was an insignificant trend of decreased body weight compared to the pre-injection measurement in both the control and gossypol treated groups, of 3% and 12.7% respectively. Also over the post-exposure period another insignificant trend appeared in which the control group regained body weight to within 0.4% of their pre-injection weight within 10 days, while by day 40 of the post-exposure period the gossypol treatment group were still 5.1% lighter than the pre-injection weight (Figure 7).

Figure 7: Body weights in mice chronically exposed to gossypol. Mice injected IP with 0 (vehicle) and 100 mg/kg b.wt. of gossypol for 11 days with a post-exposure period had body weight measured at Day 1 of the injection period (A), at Day 11 at the end of the injection period (B), at Day 21, 10 days into the post-exposure period (C), and at Day 51, 40 days into the post-exposure period (D).

3.3.2 Effect of a Chronic Injection of Gossypol on Food Intake in Mice

Food intake was not significantly changed by the end of the injection period in either the control or gossypol treatment groups. However there was a significant increase in food intake in the gossypol treatment group compared with the control group at day 10 of the post-exposure period, and at day 40 of the post-exposure period (p=0.005 and p=0.003 respectively). Both the control and gossypol treated groups were potentially showing an insignificant trend to decrease food intake at the end of the food intake period compared with their corresponding pre-injection measurements (Figure 8).

Figure 8: Food intake of mice chronically exposed to gossypol. Mice injected IP with 0 (vehicle) and 100 mg/kg b.wt. of gossypol for 11 days with a post-exposure period had food intake measured at Day 1 of the injection period (A), at Day 11 at the end of the injection period (B), at Day 21, 10 days into the post-exposure period (C), and at Day 51, 40 days into the post-exposure period (D).

3.3.3 Effect of a Chronic Exposure to Gossypol on the Anxiety Levels of Mice

The three different anxiety tests each studied a different aspect of anxiety. Neither the openfield activity anxiety test, light/dark chamber exploration anxiety test or the novel item burying anxiety test showed any increase in anxiety in the gossypol treated group.
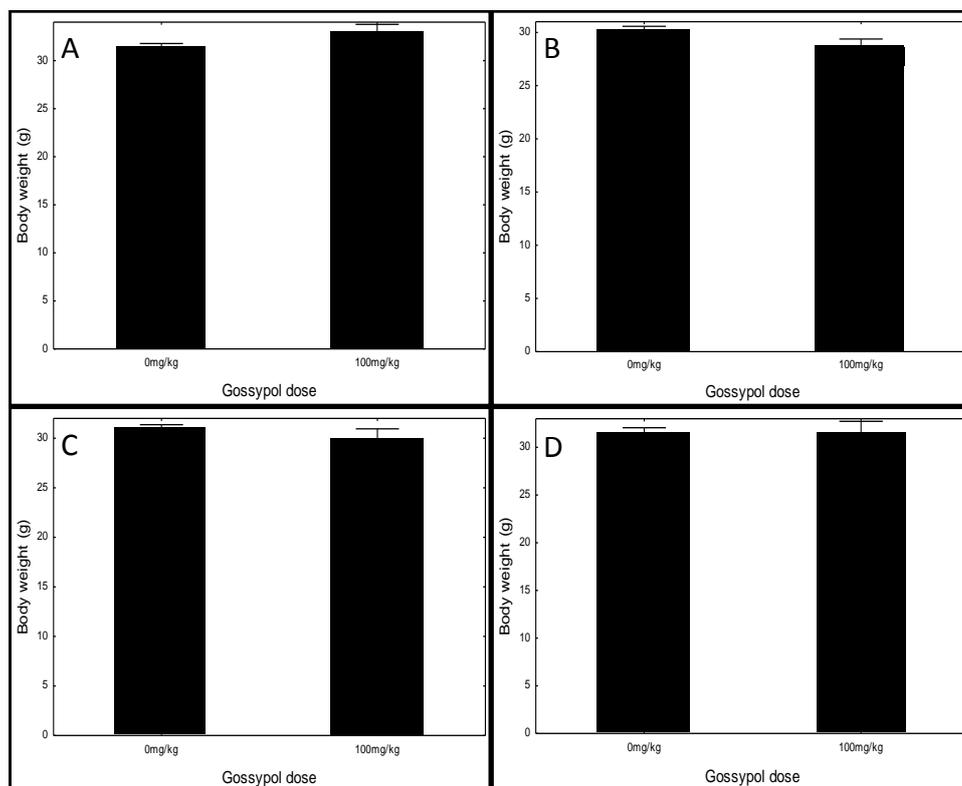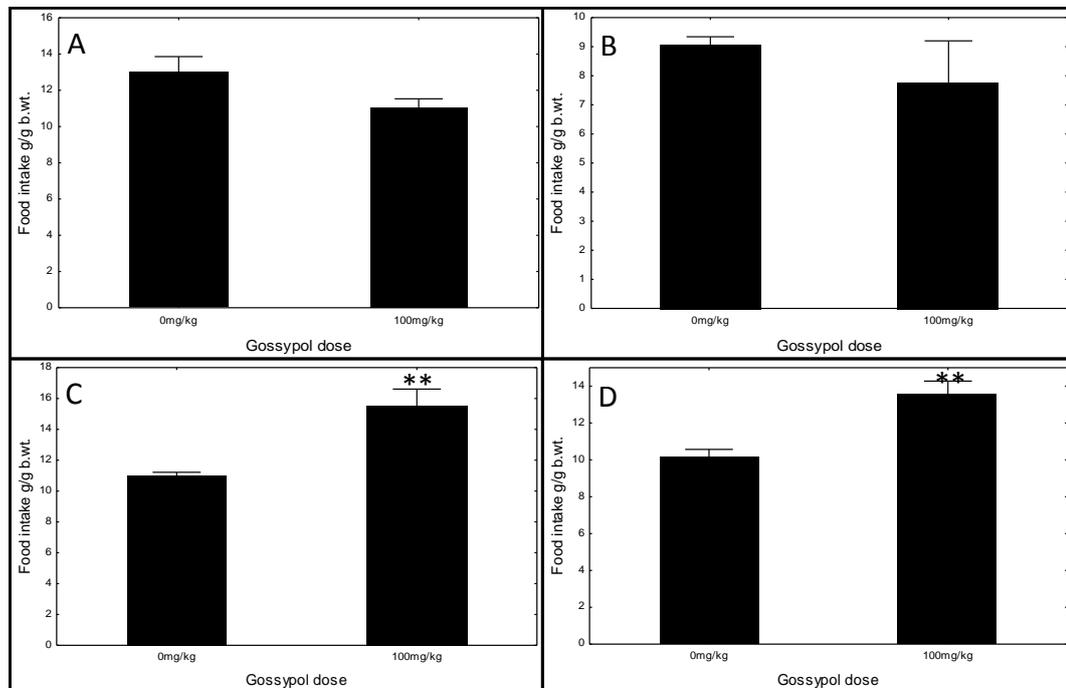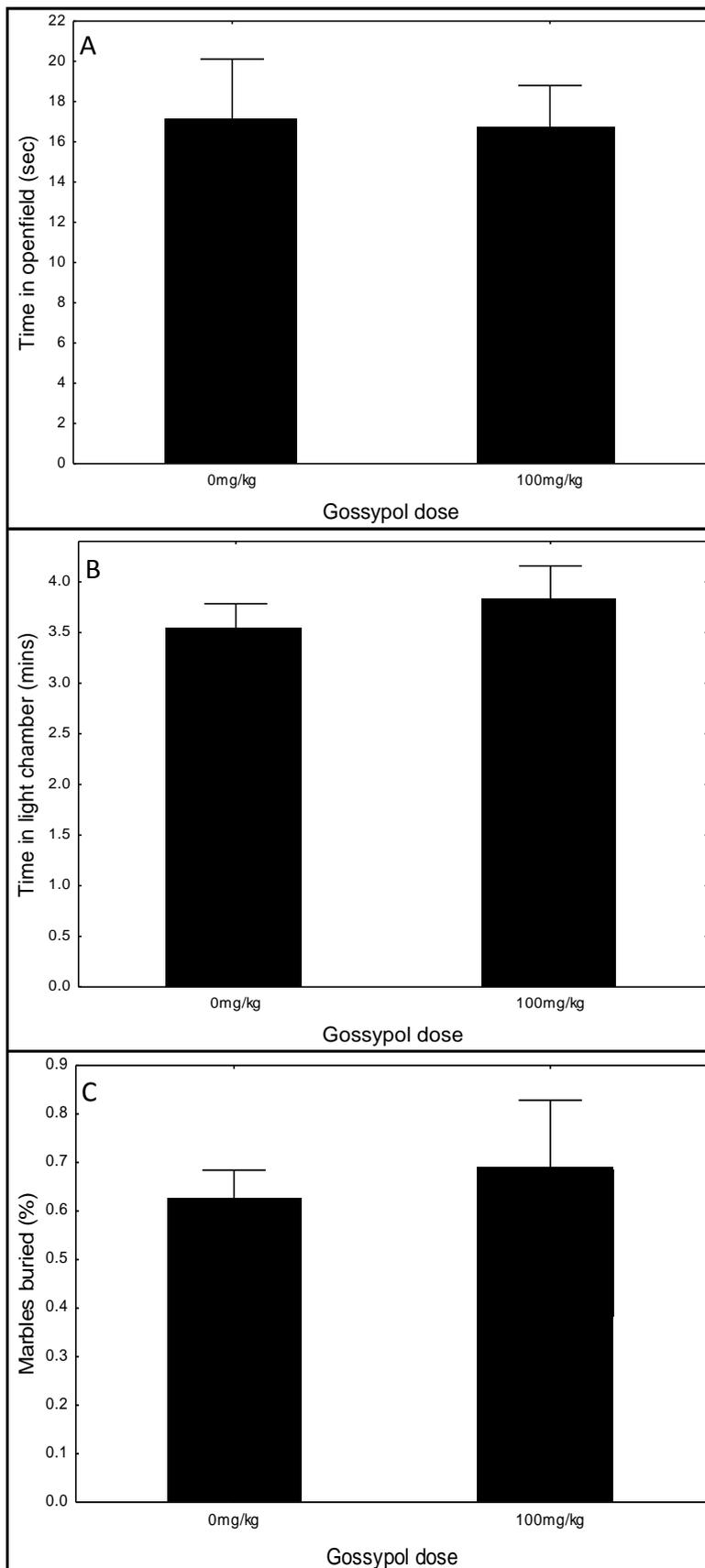
Figure 9: Anxiety levels of mice chronically exposed to gossypol. Mice injected IP with 0 (vehicle) and 100 mg/kg b.wt. of gossypol for 11 days with a post-exposure period were submitted to the openfield activity anxiety test, on day 29 (A), light/dark chamber exploration anxiety test, on day 30 (B) and the novel item burying anxiety test, on day 34 (C).

3.4 Discussion

In practice, the usage of gossypol has been hindered by certain responses to chronic exposures. These have prevented its use as a male contraceptive (Lohiya*, et al.*, 1990; Waites*, et al.*, 1998), and slowed its clinical usage as an HIV replication inhibitor (Royer *et al.*, 1995) and a cancer chemotherapeutic (Baggstrom *et al.*, 2011). Gossypol toxicity is a well characterized set of these effects that has been reported in response to chronic exposures to high doses or long periods of gossypol in various species (East*, et al.*, 1994; Haschek*, et al.*, 1989; Hudson *et al.*, 1988; Kitada*, et al.*, 2008; Zelski*, et al.*, 1995). These reactions are many and varied.

This experiment showed that after 11 days of exposure to gossypol the mice had a near-significant drop in food intake and weight. The mice were then allowed to recover for 40 days. During this period food intake increased significantly while the body weight was retarded in the gossypol treatment mice. This effect was seen right throughout the post-exposure period, as the body weight of the gossypol treatment mice slowly increased to that of the controls.

3.4.1 Effect of a Chronic Injection of Gossypol on the Bodyweight of Mice during the Injection Period

The drop in body weight in the current thesis was insignificant, but a significant response due to a chronic exposure of gossypol has been reported before in mice (Gåfvels*, et al.*, 1984; Kitada*, et al.*, 2008). Mice are not the only species to have this reaction, it has also been observed in rats (Bender*, et al.*, 1988; Gåfvels*, et al.*, 1984; Lin*, et al.*, 1990), cynomolgus monkeys (Heywood, 1988) cows (Zelski*, et al.*, 1995) and goats (East*, et al.*, 1994). This decrease in body weight is not universal throughout the animal kingdom. Sheep fed cottonseed meal kept a constant weight (Braga*, et al.*, 2012), as did langur monkeys

subcutaneously injected with gossypol (Sharma, *et al.*, 1999). Also, of the ~8800 men who trialled gossypol as a contraceptive during the 1970's and 1980's, there were no reports or complaints of weight loss (Qian & Wang, 1984).

Although weight loss has been reported in mice chronically exposed to gossypol (Gåfvels, *et al.*, 1984; Kitada, *et al.*, 2008), other studies have shown consistent body weight with chronic exposures. Xu, *et al.,* 2009, found that after 7 daily exposures the body weight and energy intake of the mice had not been altered, while its effects on the immune system were apparent.

The trend of weight loss observed in this current thesis was exacerbated by the decrease in food intake. There was a decrease in food intake in both the treatment animals and the control animals. This suggests that the vehicle of 3% DMSO may have produced a toxic response. DMSO has many physiological effects, as documented in its Material Safety Data Sheet, which states that DMSO causes nausea, headache and fatigue (Sigma-Aldrich, 2013). Gossypol solutions commonly use DMSO as a solvent, and these solutions have been injected into animals either subcutaneously or intraperitoneally (Ko *et al.*, 2007; Rao *et al.*, 1985; Xu*, et al.*, 2009). In my study the 3% DMSO may have been the cause behind the control animal's decrease in food intake, but the stress involved in daily injections would have exacerbated this effect.

3.4.2 Effect of a Chronic Injection of Gossypol on the Food Intake of Mice during the Injection Period

There were no significant changes in food intake during the injection period, but the food intake of the gossypol treatment mice suggested a declining trend more obvious than that of the control mice. This suggests that a chronic exposure of gossypol may cause anorexia or gastrotoxicity. Romualdo *et al.*, 2002, reported a similar observation in rats. In this study it was observed that during a 70 day chronic exposure to 15 mg/kg b.wt.

gossypol the body weight decrease was correlated to a decrease in food intake. This report adds evidence to the suggested anorexigenic effect of gossypol.

Kitada *et al.*, 2008, recently reported that gossypol does cause gastrotoxicity in mice. This was observed when orally treating the mice with a chronic exposure of 63 mg/kg b.wt. five times weekly for three weeks. Inspection of the intestinal tract showed damage to the mucosal epithelium and loss of villi . A response like this would probably have a corresponding visceral effect, which would cause a decrease in food intake.

Fatalities in the gossypol treatment group reached 50% during my experiment, but there were no fatalities in the control mice. This difference highlights the toxic effect of a high dose of gossypol. The current study used 100 mg/kg b.wt. for 11 daily injections, which was a very high dose compared with other studies observing a chronic exposure on mice. Kitada *et al.,* 2008, gavage-fed 62 mg/kg b.wt. gossypol to mice for 15 days over 3 weeks, which resulted in 100% fatality within 19 days. The chronic exposure to gossypol reported by Kitada *et al.,* 2008, was also observed to cause necrosis of the liver, and spleen toxicity. B cell counts in the spleen showed gossypol treated mice had a significantly lower level than other groups of mice (who were treated with another gossypol derivative).

Another chronic exposure to gossypol in mice was reported by Amini and Kamkar, 2005. The dose used was much lower, 13 mg/kg b.wt. gossypol orally for 15 days, and resulted in no deaths in the treated mice. Although this was not a lethal dose, numerous toxic responses were observed. These included difficulty breathing, irregular heartbeats, damage to the gastrointestinal tract, and enlarged livers, amongst other responses.

3.4.3 Effect of a Chronic Injection of Gossypol on the Body Weight of Mice during the Post-exposure Period

During the post-exposure period the body weight of the gossypol treatment mice showed a very slow increasing trend compared with the controls. This was particularly interesting considering that these mice were ingesting up to 37% more chow than was measured at the beginning of the study. I have not been able to find any similar observations in any other published reports. All other chronic exposures to gossypol that have observed a recovery period have reported that either the treatment animals showed no change in bodyweight (Kumar*, et al.*, 1997), or they regained weight at a rate similar to that of the controls (Sharma*, et al.*, 1999; Yang*, et al.*, 2004). The lack of reports of this phenomenon, long-term body weight decrease with increased food intake, suggests that this response has been observed for the first time in this study.

Gossypol is a toxic substance (Haschek*, et al.*, 1989), and the symptoms and effects of its toxicity are well documented (Haschek*, et al.*, 1989; Heywood, 1988; Hudson*, et al.*, 1988; Kitada*, et al.*, 2008; Zelski*, et al.*, 1995). The increase in food intake and the long-term body weight decrease are potentially related, because chronic oral exposure to gossypol has caused severe gastrotoxicity, resulting in de-villification of the intestine (Kitada*, et al.*, 2008). With the de-villification of the small intestine significantly reducing the surface area, fewer nutrients will be absorbed, causing an increase in food intake to meet energy demands, while decreasing growth rates. This explanation could be confirmed with an autopsy of the mice after the post-exposure period, unfortunately this was beyond the scope of this thesis.

There is a crucial difference between this study and that of Kitada *et al.,* 2008. They exposed the mice to gossypol via ingestion, which would have brought the compound into direct contact with the intestinal lining, whereas in my study, gossypol was injected into the peritoneal cavity, allowing it to reach the circulatory system more efficiently while bypassing the gastrointestinal tract. Other responses may have affected bodyweight too.

Chronic exposure to gossypol causes hypothyroidism in rats (Lin, *et al.*, 1990). In this study, all thyroid hormones were significantly decreased when exposed to 5 mg/kg b.wt. and 10 mg/kg b.wt. gossypol daily for 15 days. Hypothyroidism causes exaggerated body weight gain, (Roberts & Ladenson, 2004), in contrast to my results, although in Lin *et al.*, 1990, no recovery period was observed. The decrease in thyroid hormones may be a short term effect, observed only during gossypol treatment, because gossypol is not stored in the thyroid or brain (Kalla & Sud, 1990). During the post-exposure period the thyroid may have attempted to recuperate the reduced thyroid hormone levels and vastly overshot it, causing hyperthyroidism. Hyperthyroidism causes increased appetite and body weight decrease (Cooper, 2003). A mild case of hyperthyroidism could be the cause of the increased food intake with reduced body weight gain, unfortunately to conclude this confidently is beyond the scope of this thesis.

3.4.4 Effect of a Chronic Injection of Gossypol on the Food Intake of Mice during the Post-exposure Period

The injection period was followed by a 40 day post-exposure period. Initially this recovery period was planned to be observed for 10 days. The results recorded from this 10 day period suggested an interesting, ongoing response to the chronic exposure to gossypol, so the post-exposure period was extended to 40 days.

An increase in food intake with no corresponding increase in bodyweight was recorded. Some measurement points of food intake during this post-exposure period indicated that the average gossypol-treated mouse was consuming 37% more food than the control mice. This increased food intake was observed until the end of the experiment, 40 days after the end of the injection period. The difference declined through the post-exposure period, but was still significant at the final measurement, on day 51 of the experiment.

This response to a chronic exposure to gossypol has never been observed before. It suggests that gossypol has a long term effect on mice. The increase in food intake by the treatment mice suggests that exposure to gossypol could affect the satiation and/or hunger responses controlled by certain areas of the brain. Gossypol has many times been theorized to cross the Blood Brain Barrier (BBB) (Semon, 2012; Wu *et al.*, 1986), however, there is only one study which has published evidence to suggest that it does. When adult patients with gliomas, cerebrospinal tumours, were treated with gossypol to inhibit the growth of the tumour, some patients showed a positive response, but, certain other medications were also taken during the treatment (Bushunow *et al.*, 1999). This is fairly strong evidence as to gossypol's ability to pass the BBB, but there is also evidence suggesting that gossypol either doesn't pass the BBB or that it isn't stored in the brain. In rats and rabbits chronically exposed to gossypol, in order to define its distribution throughout the body, very low levels were found in the rabbit brain, but none in the rat brain (Kalla & Sud, 1990). More evidence is needed before either action of gossypol can be confirmed.

While only vague speculations can be made as to whether gossypol has affected a neural path, giving rise to the increased food intake, there are many somatic effects of gossypol which could be the cause of this increased food intake. As discussed previously, chronic exposures to gossypol have resulted in a loss of surface area of the intestine and thus nutrient absorption (Kitada*, et al.*, 2008). This lack of addition to the energy balance would cause an increase in food intake.

The increase in food intake due to the chronic exposure to gossypol could also have been hormonally driven. Leptin has an important role in the regulation of food intake. Leptin is synthesised in adipocytes and released into the circulatory system, so the concentration of leptin depends on the amount of adipose tissue in the body. Increased adipose tissue is followed by increasing leptin production and secretion, which interacts with the hypothalamus to produce a feeling of satiety (Casanueva & Dieguez, 1999). It has been reported that a small proportion of gossypol is stored in

adipose tissue in Qiao-Qin *et al.*, 1987. If so, the increased food intake may have been due to a change in leptin release. However, Qiao-Qin *et al.*, 1987, also noted that a small proportion of a dose of gossypol was stored in the testes. The testes are not a significant site of storage for gossypol, yet the compound heavily affects their function. This suggests that the minor amount of gossypol stored in the adipose tissue may contribute a larger response than initially expected. Further evidence has shown that gossypol-enriched cottonseed oil causes a decrease in leptin *in vitro* (Zhong*, et al.*, 2013). This decrease may hinder the satiety feeling in mice resulting in hyperphagia ultimately attributable to a change in leptin activity.

3.4.5 Effect of a Chronic Injection of Gossypol on Anxiety Levels of Mice

An increase in anxiety often results in an increase in food intake (Crawley, 1985), but the potential anxiogenic effect of gossypol on food intake in mice had not been studied. The current study found no detectable long term effect on the anxiety levels of the mice after chronic exposure to gossypol, when tested by three different methods. At the time of study the gossypol-induced hyperphagia and retarded body-weight gain were still being observed.

Chronic exposures to gossypol have produced an accumulation of gossypol in various tissues (Gamboa*, et al.*, 2001; Kalla & Sud, 1990), which will have increased the duration of the toxic presence of gossypol in the treatment mice. This suggests that if an anxiogenic or anxiolytic effect was observed in the treatment animals, it would be due not to the initial toxicity of gossypol, but to the long term presence of it in the tissues or a semi/permanent effect caused by the exposure. The presence of accumulated gossypol in the tissues of the mice, and the other effects caused by gossypol on food intake and body weight, had no effect on the anxiety levels of the mice.

3.4.6 The Pest Management Context

Gossypol's effect on the reproductive output of rodents has been observed only after chronic exposures (Randel*, et al.*, 1992). This decrease in fecundity is the major effect which has brought gossypol to the attention of pest management science (Singla & Garg, 2013). It is necessary to understand the food intake and body weight responses to a chronic exposure of gossypol to define its potential in this field. The chronic exposure tests performed in the current thesis demonstrates the initial step in this process of defining the compounds suitability. However, it is not valid to extrapolate from the laboratory data recorded by an injection study to predict gossypol's effects in the field.

The results described above indicate that the effects on food intake after a chronic exposure can differ markedly from effects caused by an acute exposure. To understand both of these forms of exposure is important, because the initial exposures to the compound will determine its future ingestion. A conditioned taste aversion is learnt a lot more readily to a new food source (Riley & Tuck, 1985). To ensure that wild mice will take an effective dose, they must maintain a chronic exposure in the field. This current experiment has begun the process of understanding how that could be achieved and what effects this may result in.

During the post-exposure period of the chronic exposure experiment, the gossypol treated mice increased their food intake. This had never been reported before throughout the gossypol literature. This finding suggests that a chronic exposure to a high dose of gossypol has an effect on some aspect of the gustatory system for a period after exposure. This effect may have a substantial influence on gossypol's potential as a pest management chemical. Before any conclusions can be made, a chronic exposure experiment via ingestion will need to repeat this result.

Anxiety levels are an important variable in pest management. All rodents, including mice, cannot vomit (Horn*, et al.*, 2013), hence are very neophobic to new food (Andrews & Horn, 2006). Neophobia can be exacerbated with increased anxiety (Ennaceur *et al.*, 2006). Exposure to

some pharmaceuticals have been shown to increase anxiety (Simon, *et al.*, 1994). The chronic exposure to gossypol did not cause a prolonged anxiogenic effect on the mice. This and the other results from the chronic exposure suggest that gossypol's potential in murine pest management is still worthy of investigation.

4 <u>Conclusions and perspectives</u>

My research aimed to define gossypol's effect on ingestive behaviour in mice, because the literature on this aspect of gossypol's effect is inconsistent and often contradictory. The experiments performed in this thesis were planned as a systematic approach towards documenting this aspect of gossypol on mice. Injections IP were used to expose the mice to a precise dose of gossypol, allowing accurate measurements of dose-dependent responses. However, the behavioural results from this study cannot be extrapolated confidently to post-exposure behaviour in the field or even to oral doses.

This study is the first to consider exploiting gossypol's physiological effects as a pest management tool for mice, and only the second for any rodent. Singla & Garg ,2013, considered the effect of gossypol-containing cotton seed oil on the sexual organs and reproductive success of both sexes of the lesser bandicoot rat, *Bandicota bengalensis*. The cottonseed oil was exposed to the rats via their diet. This study reported that the gossypol content damaged the testes of the male rats, while not affecting their ability to reproduce. Similar results of low dose gossypol exposure for a short period have been observed many times before (Randel*, et al.*, 1992). The readiness of animals to ingest gossypol-containing substances willingly has been thoroughly studied in early experiments (Semon, 2012; Withers & Carruth, 1915). However its effects on ingestive behaviour in response to an acute and chronic exposure had not been studied.

Some of the results from the acute and chronic exposures to gossypol published in this thesis have never before been reported in the literature. This highlights the importance of this initial step in this systematic process to understand gossypol's effect on ingestive behaviour for the benefit of its potential use as a pest management chemical. If, for example, this initial step had been skipped, a change in food intake was observed after ingestion of a bait containing gossypol, there would be no clue as to what caused it. With the data reported in this thesis, a reason for this response

could be put forward. It could be an increased ingestion of glucose, or the dose may be too high, causing a pica-like response, or it could be an increased food intake after a chronic exposure. These possibilities can now be distinguished in future experiments.

It is important to emphasize the point that the results published in this thesis cannot be used to directly infer gossypol's suitability in pest management, although one result suggests that it may have potential as an additive to lethal baits to increase ingestion. An acute exposure to 100 mg/kg b.wt. caused a substantial increase in glucose ingestion in mice. This suggests that gossypol + glucose + lethal poison added to a bait may increase intake of the poison, thus decreasing sub-lethal doses and reducing conditioned taste aversions. Prior to this being studied, the systematic approach to understanding gossypol's effect on ingestive behaviour must be completed. This affect has not been observed after an oral exposure to gossypol.

As gossypol is an expensive compound to extract and purify, this form of the compound will be unsuitable for the meagre budgets often available for pest management schemes. A much cheaper alternative is available, cottonseed flour and oil both contain a low concentration of gossypol. The gossypol content of these are high enough to effect the reproductive parameters of rodents (Akinola*, et al.*, 2006; Singla & Garg, 2013; Sotelo*, et al.*, 1982; Wazir*, et al.*, 2006). The effects on ingestive behaviour in mice of cottonseed flour and oil are not known, so will need to be measured systematically as well.

The results discussed in this thesis may, eventually, facilitate gossypol's use in pest management, they could also be used to assist its use in medicine. Gossypol and its derivatives are being studied as cancer chemotherapeutics (Baggstrom*, et al.*, 2011; Lian*, et al.*, 2012; Liu, 2009) and HIV replication inhibitors (Royer*, et al.*, 1995; Royer, 1995). Studies of ingestive behaviour will be required to understand how the patients will react to treatment with gossypol. Also studies such as these may identify foods that will be ingested readily during treatment, as during treatments

of certain drugs, especially cancer chemotherapeutics, ingestive behaviour changes (Ovesen *et al.*, 1991).

The work done during this thesis was the very first step towards understanding gossypol's effects on ingestive behaviour in mice. If a systematic process of studies is followed from this point, the level of understanding of this area of this compound will increase dramatically, especially compared with the current data available in the literature. Depending on the results obtained from this systematic process, gossypol's suitability as a pest management chemical may be determined.

5 <u>References</u>

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