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Over the past decades, the number of managed honeybee colonies in the United States has been declining. One hypothesis proposed to explain the decline is that exposure to xenobiotics might affect bee health without killing the bees directly. The gut is important to bee health because it functions as the first physical barrier after honeybees ingest xenobiotics. My research investigated the proliferation rate of intestinal stem cells (ISCs) as a biomarker of honeybee health after bees were exposed to 12 relevant xenobiotics. The study sought to determine whether the xenobiotics showed sub-lethal effects on ISC proliferation. I hypothesized that xenobiotics affect ISC proliferation at concentrations that do not significantly affect mortality. I studied acute and latent effects of selected xenobiotics and one combination of xenobiotics on honeybee lifespan and ISC proliferation. Except for a few xenobiotics, I found that most xenobiotics did not show ISC proliferation effects. Specifically, a high but sub-lethal concentration of hydroxytetracycline decreases proliferation, while tau-fluvalinate only decreases ISC proliferation at lethal doses. On the other hand, low concentrations of methoxyfenozide increased proliferation without any mortality effects. Thus, I identified several xenobiotic that have an effect on ISC proliferation. My results demonstrate sub-lethal effects of xenobiotics in a novel, health-relevant context but suggest that ISC proliferation is not a general honeybee health indicator. My study does not support a central role of the gut physiology in the recent health declines but cannot rule out specific effects that contribute to the general decline in honeybee health.

INVESTIGATING THE EFFECT OF XENOBIOTICS ON HONEYBEE INTESTINAL STEM CELL PROLIFERATION

by

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Approved by

_____
Committee Chair

To my family and friends. For the words of encouragement and your endless love.

APPROVAL PAGE

This thesis has been approved by the following committee of the Faculty of The Graduate School at the University of North Carolina at Greensboro.

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CHAPTER I

INTRODUCTION

Importance of Honeybees

The European honeybees, *Apis mellifera* (L.), are very important to the agricultural economy. Honeybees are important because they produce honey but more significantly, they serve as major insect pollinators. Worldwide, the value of honey in 2006 was estimated to be about 1.25 billion U.S. dollars and the value of insect pollination about 198 billion U.S. dollars, representing approximately 9.5% of the total value of agricultural production (Gallai, Salles *et al.* 2009). Particularly in North America, the economic value of honeybees to agriculture is an estimated 18.6 billion U.S. dollars, representing about 11% of the total North American agricultural economy (Gallai, Salles *et al.* 2009). Specifically in North Carolina, honeybee pollinators are essential for crops such as blueberries, apples, cucumbers, alfalfa, cotton, and peanuts. Averaged between the years of 2000-2004, honeybee pollination resulted in approximately $88 million in annual fruit and vegetable production and approximately $154 million in total annual crop productivity (N.C. Cooperative Extention 2005). Although honeybees are not the only animal pollinators, they are the most important species. As generalized feeders, they are useful in a variety of farms. For some crops such as almond, apples and blueberries they make up more than 90% of the animal pollinators aiding in pollination (Morse and Calderone 2000)

Honeybee Decline

Despite their importance, over the past decades, the number of managed honeybee colonies in the United States (U.S.) has been declining, regardless of efforts to replenish lost colonies using measures such as colony splitting and imports (Ellis, Evans *et al.* 2010). From 1947-2008 the number of managed honey producing colonies has dropped from 5.9 million to 2.3 million (vanEngelsdorp and Meixner 2010). In 2006, increased colony losses were reported and associated with new symptoms: bee colonies left with few or no adult bees and no dead bodies. In addition, capped brood and food stores usually remained undisturbed by other bees or bee pests. This phenomenon was termed colony collapse disorder (CCD) (Ellis, Evans *et al.* 2010). Between 2008 and 2009, CCD attributed honeybee losses decreased, although overall bee population decline increased during the same time (vanEngelsdrop, Hayes *et al.* 2010). Regardless of whether CCD is a real phenomenon or not, European and U.S. honeybee populations are rapidly declining and more research is needed to study potential causes.

Potential Causes of Honeybee Decline

A specific cause for the increased honeybee losses has not yet been identified but several hypotheses have been suggested. The proposed causes include known pathogens, such as Israeli acute paralytic virus and *Nosema ceranae*, new pathogens or more virulent forms of known pathogens that may not yet have been identified, poor nutrition, poor commercial bee management practices, agrochemicals and api-chemicals, or an interaction between two or more of the above proposed causes (Ellis, Evans *et al.* 2010; vanEngelsdrop, Hayes *et al.* 2010). Other threats linked to honeybee decline include the

introduction of invasive species such as the Africanized honeybees and intensive land use causing loss of habitats. (Berenbaum, Bernhardt *et al.* 2007).

Some important honeybee pests are hive mites such as tracheal mites (*Acarapis woodi* Rennie), which infect the trachea (Bradbear 2009), and *Varroa* mites. These latter mites are native to the eastern honeybee, *Apis cerana*, which are naturally resistant to them (Bradbear 2009). However, since the introduction of *Varroa* mites to the European honeybee, in the U.S., the rate of decline per year of honeybee colonies has risen from 0.06% ± 0.5 to 1.5% ± 0.7 (Ellis, Evans *et al.* 2010). A wide range of additional pathogens such as fungus, bacteria, and viruses can infect honeybees. Fungal pathogens include the recently reclassified *Nosema apis*, a microsporidian that infects epithelial cells of the midgut of adult honeybees, and *Ascosphaera apis, which* competes with the bee larvae for food and then ultimately consumes the larvae in a disease called Chalk Brood. Bacterial pathogens include *Paenibacillus larvae ssp. larvae* and *Melissococcus plutonius* which cause American Foulbrood and European Foulbrood, respectively (Sanford 1987). Lastly, honeybees are infected by numerous viruses, most of which are from the family Dicistroviridae and include the deformed wing virus, black queen virus (Knowles 2007) and Israeli acute paralytic virus, which has been linked to CCD through a correlation study (Cox-Foster, Conlan *et al.* 2007).

Like pests and pathogens, poor nutrition has been linked to reduced honeybee fitness. Honeybees are generalist feeders and thus feed on many different pollen types. Monoculture farm pollination forces them sometimes to feed on only one kind of pollen (Alaux, Ducloz *et al.* 2010). However, feeding on polyfloral pollen increases the baseline

immuno-competence of bees compared with monofloral diets (Alaux, Ducloz *et al.* 2010). In addition, honeybees fed different quality pollen, high quality (royal jelly), mid quality (pollen), and low quality (pollen mixed with cellulose) showed an increase in intestinal stem cell (ISC) proliferation as food quality decreased (Willard, Hayes *et al.* 2011). The proliferation effect of nutrition is presumably due to increased digestive stress caused by poor quality diets (Ward, Coleman *et al.* 2008; Willard, Hayes *et al.* 2011).

Xenobiotics

Focusing on one group of the above-mentioned potential causes, my research will examine the possible effect of xenobiotics on honeybee health. Xenobiotics are chemicals such as drugs, pesticides and carcinogens not normally found in organisms. Their presence in honeybees might therefore lead to physiological effects, which in turn might cause a decline in honeybee health. For honeybees, substances that could act as health-deteriorating xenobiotics include insecticides used to control arthropod pest and antimicrobials used to alleviate honeybee diseases. While most of these xenobiotics might not cause lethal harm to honeybees, there might be sub-lethal effects not yet known, which might reduce honeybee health and thus life expectancy.

Some insecticides are highly lethal and /or cause behavioral, cognitive, fecundity and, developmental sub-lethal effects to honeybees (Decourtye, Lacassie *et al.* 2003; Desneux, Decourtye *et al.* 2007; Johnson, Ellis *et al.* 2010; Gregorc and Ellis 2011). For example, some insecticides, such as imidacloprid, tau-fluvalinate, coumaphos, and chlorothalonil, cause increased midgut epithelial cell death during larvae development (Gregorc and Ellis 2011). However, the highly relevant midgut of adult honeybees has

not yet been studied. Specifically, nothing is known about the effect of xenobiotics on the epithelial and stem cells of the intestine discussed below.

Ingested antimicrobials might also cause a disruption of the natural microbial flora of the bee guts. Because some microorganisms are beneficial to honeybee food digestion, disturbing the microbiota might cause a reduced ability of honeybees to properly digest food and thus increase stress to the midgut epithelial cells (Gilliam 1997; Rada, Máchová *et al.* 1997; Kacaniova, Chlebo *et al.* 2004). Not only might such a disturbance decrease epithelial cell viability by increasing digestive demand, but disturbing the balanced microflora might also increase pathogen virulence (Gilliam 1997). Thus, the effect of antimicrobials on honeybee midgut cells needs to be studied.

There are many different classes of insecticides and this study focuses on insecticides from five classes to account for this variety. The insecticides included in the study, the classes they are associated with, and their relevance to honeybee health is explained next.

Coumaphos is an organophosphate used in bee hives to control *Varroa* mites and hive beetles, *Aethina tumida*. The commercial product Checkmite+™ contains 10% coumaphos embedded in a plastic strip (U.S. Enviromental Protection Agency 2003). Coumaphos kills mites by inactivating acetylcholinesterase and thus interfering with nerve signaling by causing hyperexcitation (Johnson, Ellis *et al.* 2010). Coumaphos has an ld_{50} of 20.3µg per bee (Johnson, Pollock *et al.* 2009). Additionally, it has the potential to accumulate in the hive at median concentration levels of 1240ppb and high levels of

91900ppb in hive wax and 13.1ppb median levels and 5828ppb high levels in pollen stores (Mullin, Frazier *et al.* 2010).

Tau-fluvalinate (Apistan™) is a pyrethroid used as a miticide by beekeepers to combat *Varroa* mites. Tau-fluvalinate is highly toxic to honeybees with an acute contact LD_{50} (the dose at which 50 percent of the bees die during a given period) of 0.2 µg/bee (U.S. Enviromental Protection Agency 2006). For this reason, the EPA recommends that this insecticide not be used in the hives while bees are present. However, in honeybee hives, pollen samples contained 40.2ppb median and 2670ppb high concentration levels. In wax samples, a median of 3595ppb and a high of 204000ppb were found (Mullin, Frazier *et al.* 2010).

The accumulation of these two insecticides in beeswax makes their co-occurrence in hives likely (Johnson, Ellis *et al.* 2010; Mullin, Frazier *et al.* 2010). The co-occurrence of these two pesticides in the hives might have synergistic effects on bee health, even at sub-lethal concentrations. Such synergistic effects have been found when coumaphos-treated larval bees were first pretreated with tau-fluvalinate or vice versa, increasing honeybee mortality (Johnson, Pollock *et al.* 2009). Coumaphos might not be toxic alone but competition with tau-fluvalinate for the detoxification enzyme cytochrome P450 might be the cause of the synergistic interaction (Johnson, Ellis *et al.* 2010). Coincidently, honeybees compared with other insects have fewer genes encoding for detoxification enzymes in general (Claudianos, Ranson *et al.* 2006).

Imidacloprid is a neonicotinoid, a class of insecticides that act as acetylcholine receptor agonists. They cause hyperexcitation and eventual death of affected organisms

by consistently activating nicotinic cholinergic receptors (Johnson, Ellis *et al.* 2010). The acute oral LD_{50} for honeybees has been recorded at 30ng/bee (Decourtye, Lacassie *et al.* 2003). In honeybee hives, median concentration levels of 20.5ppb and high levels of 206ppb of Imidacloprid in honeybee pollen and a median concentration of 8ppb and a high of 13.6ppb in wax were detected (Mullin, Frazier *et al.* 2010). Neonicotinoids have been associated with adverse neurological effects and cause abnormal foraging behavior even at sub-lethal levels (Decourtye, Lacassie *et al.* 2003; Yang, Chuang *et al.* 2008). Although the three above-mentioned pesticides are classified as neurotoxins and are therefore unlikely to target the gut, they are clearly relevant to honeybee health and my tests in this study might expose unspecific or systemic effects.

Methoxyfenozide is an insect growth regulator and an agonist of the molting hormone 20- hydroxyecdysone (20-HE). Although structurally different from 20-HE, methoxyfenozide has the same mode of action and thus can cause precocious molting and death of susceptible insect larvae (Thacker 2002). Intrepid 2F® and 80 WSP® are brand name products containing methoxyfenozide. They are registered for use to control insect pests of pome fruit and cotton. The contact LD_{50} for honeybees is 100µg/bee, which is considered relatively non-toxic (California Department Of Pesticide Regulation 2003). In honeybee hives, a median concentration of 42.3ppb and a high of 495ppb were found in wax samples and a median concentration of 22.3ppb and a high of 128.0ppb were found in pollen samples (Mullin, Frazier *et al.* 2010). 20-HE was tested as a xenobiotic and can serve as a positive control for methoxyfenozide. In larvae of many insects, ecdysone, specifically its active form, 20-HE, regulates cell proliferation and differentiation

(Smagghe, Vanhassel *et al.* 2005). Ecdysone is present in low amounts in some foraging honeybees, and can activate a cascade of gene expression in the brain (Velarde, Robinson *et al.* 2009). If ecdysone receptors exist in the adult honeybee ISCs, exogenic ecdysone or ecdysone agonists like methoxyfenozide could have a proliferative effect on these cells.

Cry1ac, cry1acmod, and cry22 are all toxins produced by the gram-positive soil-dwelling bacterium *Bacillus thuringiensis* (*Bt*). To make transgenic crops, the genes coding for these toxins are incorporated into the genome of the respective crops through genetic engineering. If successful, the crops express these genes and exhibit the insecticidal activity of the toxins. The difference between cry toxin subtypes is their species-specific insecticidal activity. The insecticidal specificity of cry toxins is due to the need for an interaction with specific receptors located on the host midgut epithelial apical microvilli cell surface (Saberon and Bravo 2008).

Cry toxins are ingested as protoxins by arthropods and are activated by the organism's midgut proteases. Activated toxins then bind to the cadherin receptor on the microvilli membrane causing a conformational change. Additionally, cleaving a fragment, helix α-1, from the amino-terminal region exposes hydrophobic regions needed to form an oligomer. This oligomer allows the toxin to bind to aminopeptidase, which subsequently facilitates the insertion of the toxin into the epithelial cell membrane. This forms pores in the membrane causing the cells to leak out their contents (Saberon and Bravo 2008).

The insecticidal action of cry1ac is targeted at lepidopteran insects. The difference between cry1ac and cry1acmod is a modification that allows cry1acmod to

form the oligermeric structures necessary for pore formation without a need for cadherin receptor binding (Saberon and Bravo 2008). Presently, studies have produced no evidence for harm to honeybees caused by the use of *Bt* crops in the United States (Johnson, Ellis *et al.* 2010) but sub-lethal effects cannot be ruled out and cry1ac is commonly used. On the other hand, cry22 was isolated because of its action on Hymenoptera (Crickmore 2007), especially ants. Although cry1ac is more prevalent than cry22 (Saberon and Bravo 2008), cry22 is more likely to affect honeybees, specifically the intestinal epithelium.

The other xenobiotics tested are antimicrobials, specifically fungicides and antibiotics. The purpose for testing this group of xenobiotics is to assess non-specific, side effects on the intestinal epithelium, which may be due to changes of the honeybee gut microflora with effects on the gut physiology. The few microorganisms that make up the gut microflora of honeybees are important, as they seem to have co-evolved with pollinating bees in general (Martinson, Danforth *et al.* 2011).

Two fungicides, chlorothalonil, and fumagillin will be studied. Chlorothalonil is used in agriculture mainly as a fungicide. However, it also has bactericidal, algaecidal, microbiocidal and insecticidal activity (Extension Toxicology Network 1994). Its mechanism of action is not known, but it is considered relatively non-toxic to honeybees (vanEngelsdorp, Evans *et al.* 2009). The LD_{50} is >40µg per bee (Food and agriculture organization of the United Nations 2006). Additionally, vanEngelsdorp *et al.* (2009) described a new phenomenon of chlorothalonil-contaminated pollen entombed by honeybees. In other words, pollen is encapsulated with wax by the bees. While feeding of

entombed pollen did not lead to increased mortality, this fungicide might have unknown physiological effects on the bees causing the entombing behavior.

Fumagillin, the second fungicide, is used to combat the previously described honeybee pathogen *Nosema*. It changes the honeybee gut microflora by increasing yeast populations (Rada, Máchová *et al.* 1997), potentially affecting honeybee digestion, and health. Fumagillin is not very stable in heat and light and thus does not persist in hives (Assil and Sporns 1991).

Lastly, the broad-spectrum antibiotic hydroxytetracycline (U.S. Enviromental Protection Agency 1988) is used to cure honeybee colonies afflicted with American foulbrood and European foulbrood. This antibiotic could also potentially harm the normal flora of microorganisms in the honeybee gut (Kacaniova, Chlebo *et al.* 2004) and thus affect the honeybee's ability to digest food. Stressed bees harbor more yeast and yeast-like organisms in the midgut (Rada, Máchová *et al.* 1997). Disturbing the natural microbial flora of the honeybee midgut might decrease epithelial cell viability by increasing digestive demand (Ward, Coleman *et al.* 2008), and perhaps pathogenic stress (Gilliam 1997) and thus increase the rate of epithelial cell death and ISC proliferation (Amcheslavsky, Jiang *et al.* 2009; Buchon, Broderick *et al.* 2009).

The Insect Alimentary Canal

The alimentary canal, or digestive tract, is the first point of contact between the xenobiotics and the individual bee after ingestion. The alimentary canal of insects is divided into the foregut, midgut, and hindgut. The foregut includes the pharynx and esophagus, which assist with food ingestion and transportation, and the crop, which can

store food before it is transported to the midgut (Snodgrass 1956). The midgut starts directly after the proventriculus and ends directly before the start of the Malpighian tubules and the ileum. Food digestion and absorption in the honeybee takes place in the midgut. Due to this role, the midgut epithelium also acts as the first barrier to ingested xenobiotics.

The midgut epithelium is primarily made up of absorptive columnar cells with apical microvilli and secretory goblet cells (Smagghe and Tirry 2001). These epithelial cells undergo constant turnover as new cells replace damaged or infected cells (Ohlstein and Spradling 2006). Lining the lumen of the midgut is a chitin-protein matrix, the peritrophic membrane. This membrane prevents direct contact between ingested food and the midgut cells minimizing mechanical but not chemical damage. The waste products after digestion move on to the hindgut were water, salts, amino acids, and sugars are reabsorbed before excretion of the remainder through the rectum (Smagghe and Tirry 2001; Hakim, Baldwin *et al.* 2010).

Intestinal Stem Cell Proliferation

Ward *et al* (2008) confirmed the presence of a population of replicative cells in the adult honeybee midgut near the basal lamina, originally suggested by Snodgrass (1956). These replicative cells have also been identified in adult *Tenebrio molitor (*Nardi, Bee *et al.* 2010*)* and adult *Drosophila melanogaster (*Micchelli and Perrimon 2006*)*. These ISCs are round in shape with very little cytoplasm but a large spherical nucleus (Raes, Verbeke *et al.* 1994). The mitotically active ISCs are found in regenerative crypts

with only one actively proliferating cell. This cell is usually located closest to the basement membrane (Smagghe and Tirry 2001; Ohlstein and Spradling 2006).

In order to maintain homeostasis, ISC proliferation and differentiation are regulated by the ISC niche through many interacting signaling pathways. Some identified signaling pathways include JNK, Jak-Stat, p38, EGFR, Hippo, and Notch (Liu, Singh *et al.* 2010; Ren, Wang *et al.* 2010; Biteau and Jasper 2011). The proliferative activity of ISCs is also influenced by environmental factors. Midgut epithelial stem cell nuclei of *Spodoptera littoralis* have ecdysteroid receptors and thus proliferation and differentiation is increased when ecdysteroid titers are increased (Smagghe, Vanhassel *et al.* 2005). In addition, feeding of cry toxins from Bt strains AA 1–9 correlated with an increase in stem cell differentiation of *Heliothis virescens* larvae midgut cells (Loeb, Martin *et al.* 2001). When epithelial cells are damaged or stressed in adult *Drosophila*, EGRF ligands are released which in turn leads to increased ISC proliferation and differentiation (Jiang, Grenley *et al.* 2011). Furthermore, feeding-tissue damaging chemicals, such as dextran sulfate sodium and bleomycin, increased ISC proliferation and differentiation in *Drosophila* (Amcheslavsky, Jiang *et al.* 2009). In adult honeybees, ISC proliferation increases as digestive demand is increased (Ward, Coleman *et al.* 2008; Willard, Hayes *et al.* 2011). This increase in proliferation is presumably due to an increase in epithelial cell death. ISC proliferation activity is responsive to environmental conditions and thus it may be useful as a sub-lethal indicator of honeybee health when bees are exposed to xenobiotics.

CHAPTER II

HYPOTHESIS AND SPECIFIC AIMS OF STUDY

Hypothesis

I hypothesized that xenobiotics damage epithelial cells and thus cause an increase in ISC proliferation at lethal and sub-lethal concentrations based on previous results in larvae (Jiang, Grenley *et al.* 2011). Alternatively, a decrease in proliferation might have been predicted. Increased requirements for replication could lead to an exhaustion of ISC replicative capacity. Thus, while there might be an immediate increase in ISC proliferation, there could be an eventual decrease in the number of replicating cells. In addition, ISC poisoning could occur, potentially leading to ISC death or cell cycle arrest. This would result in a more immediate decrease in the number of ISCs proliferating (Yan and Wajapeyee 2010). To summarize, there are six possible effects of xenobiotics on ISC proliferation activity. Proliferation rate could increase, decrease, or be unaffected by the treatment at two different time points: directly after treatment or in the long-term (Figure 1)

Specific Aims

Aim 1. My first aim was to test 12 relevant xenobiotics at relatively high concentrations for mortality and ISC proliferation effects in honeybee workers.

Aim 2. Based on the initial screening experiments, my second aim was to test xenobiotics that affected ISC proliferation at high concentrations for their effects on honeybee lifespan and ISC proliferation at sub-lethal concentrations.

Aim 3. My third aim was to test the combination of tau-fluvalinate and coumaphos for their possible synergistic effects on honeybee lifespan and ISC proliferation at sub-lethal concentration.

CHAPTER III

MATERIALS AND METHODS

In order to meet the aims of the study, three sets of experiments were performed. First, a short- term experiment screened 12 relevant xenobiotics, which were fed or exposed to the bees for 7 days, for their effects on honeybee mortality and ISC proliferation rate at high concentrations comparable to concentrations bees would encounter in hives or during foraging (Table 1). In addition, I used colchicine as a positive control for a reduction of cellular proliferation. Colchicine acts to arrest cell division by inhibiting spindle fiber formation (Borisy and Taylor 1967). Thus, treatment with colchicine was predicted to decrease ISC proliferation significantly. In addition, negative vehicle controls were conducted. The results from the first set of experiments was used to develop the list of chemicals tested in the second set of longer-term experiments. For the long-term study, xenobiotics showing an effect on ISC proliferation rate at high concentration were fed to the bees at three sub-lethal concentrations for 7 days and bees were monitored for up to 22 days. In addition, coumaphos and tau-fluvalinate (Apistan™) were administered in combination and the bees were again monitored for up to 22 days. These two experiments examined in detail the effect of the tested xenobiotics on lifespan and ISC proliferation rate. The methodology of my study was based on previous studies (Ward, Coleman *et al.* 2008; Willard, Hayes *et al.* 2011) and my own preliminary research.

Honeybees

All honeybees used in the experiments were newly emerged (< 24 hours old adults) worker bees (*Apis mellifera L.*). Before emergence, brood combs were collected at random from *A. mellifera* colonies of mixed descent. Brood combs were kept for no more than two days in an incubator. The incubator was set for 24 hours of darkness, 35^{o}C, and a relative humidity at 60-70%. Newly emerged bees were counted and randomly assigned to an experimental treatment. Treatment groups were kept in separate Plexiglas® feeding cages (10cm x 7.5cm x 10cm) in the incubator. During the feeding experiments, the cohorts were fed queen candy (9:3:1, powdered sugar: water: honey). Throughout the experimental period, dead bees were removed from the cage, counted, and recorded. Mortality between treatment groups was compared with Kaplan-Meier log-rank tests, accounting for any censored data.

Aim 1- Effect of High Concentration of Xenobiotics on Honeybee Lifespan and ISC Proliferation Rate

Preliminary experiments were performed during the summer of 2010 to establish protocols for sub-lethal treatments and to screen for mortality and ISC proliferation effects of high concentrations of the 12 relevant xenobiotics (Table 1). The relative humidity and temperature of the incubator during the feeding period were set for this experiment at 35% RH and 25^{o}C. Each experimental group consisted of four replicates each with 25 individual bees per cage.

The tested concentrations of the xenobiotics were determined by the highest concentration found in hives for hydroxytetracycline, imidacloprid, coumaphos, chlorothalonil, and methoxyfenozide (Thompson, Waite *et al.* 2005; Mullin, Frazier *et al.*

2010). In the case of the Cry protoxins, the highest concentration reportedly found in genetically engineered cotton was used (Han, Niu *et al.* 2010). 20-HE concentration was taken from highest concentration found to have an effect on *Plodia interpunctella* larval midgut cells (Rharrabe, Bouayad *et al.* 2009). For the antimicrobial fumagillin, no hive accumulation data were available and consequently the highest recommended use dosage as printed on the product label was fed to the bees. These xenobiotics and their vehicles controls were mixed with queen candy and provided ad-libitum to the caged bees (concentrations specified in Table 1). Tau-fluvalinate was purchased as pre-soaked strips (Apistan™) and half strips were placed in the bee cage. All xenobiotics were purchased from commercial suppliers (Table 1), except for the Cry protoxins, which were a gift from our collaborators (Table 1).

Aim 2 - Effect of Sub-Lethal Concentrations of Xenobiotics on Honeybee Lifespan and ISC Proliferation

The xenobiotics for the second set of experiments were chosen for their significant effects on ISC proliferation in the first set of experiments. Methoxyfenozide, hydroxytetracycline, and tau-fluvalinate were tested using sub-lethal concentrations (Table 2). Because bees are not typically exposed to tau-fluvalinate orally, I exposed the bees to Apistan™ strips as in aim 1, but drastically shortened the exposure time (Table 2).

For each of these treatment groups, I adhered to the feeding regime used in the first experiment. However, to allow easy asses to the food for changing daily, cut centrifuge tubes were used instead of feeding plates. In addition, a larger number of bees were used to determine long-term honeybee life expectancy. Since sample bees were

needed immediately after xenobiotic feeding was stopped to assess acute proliferation effects and then again at an older age to assess long-term proliferation effects, the number of bees per treatment was increased to two replicate cages of 120-155 bees per treatment. For a few treatments (fluvalinate, fluvalinate with coumaphos and hydroxytetracycline), insufficient brood was available from UNCG colonies and therefore brood from a local beekeeper, and the Wake Forest apiary was used. To facilitate the practical implementation of this large-scale experiment, no more than 6 cages were initiated per day in a pseudo-randomized design over a period of 20-30 days for a given treatment. The cohorts were monitored twice per day for mortality and any dead bees were immediately removed. A subset of ten bees from each cage were sampled at random after day seven for quantification of the replication rate of the intestinal stem cells (see below). After the seventh day of feeding, surviving bees were fed untreated queen candy for up to 22 days. A second sample was taken when the bees were between ages 19-22 days old or when mortality of the experimental cohort had reached ≥90% before that.

Aim 3- Effect of Combined Coumaphos and Tau-Fluvalinate

In addition to using pesticides singularly to assess treatment effects, one combination was tested for synergism. Pesticides described in the literature to have synergistic effects on midgut cells are coumaphos and tau-fluvalinate (Johnson, Pollock *et al.* 2009; Johnson, Ellis *et al.* 2010). This pair was used in combination to assess possible synergistic effects on ISC proliferation rate. The same experimental protocol described in Aim 2 was used. In the first set of experiments, coumaphos did not show any significant effect on ISC proliferation, although mortality effects were observed. Thus, to

minimize mortality, coumaphos concentration was reduced 10-fold for all treatment groups. The only variation between treatment groups were the tau-fluvalinate exposure times (Table 2). As described above, samples of ten workers per cage to measure ISC proliferation were taken at 7 days and 21 days of age.

Proliferation Assays

Following established methods (Ward, Coleman *et al.* 2008; Willard, Hayes *et al.* 2011), stem cell replication was measured by labeling and quantifying replicating cells using an immuno-histochemical staining of 5-bromo-2-deoxyuridine (BrdU) (Invitrogen, B23151) in intestinal cross-sections. BrdU is a thymidine analogue that does not occur naturally, but it is readily incorporated into newly synthesized DNA. Consequently, it is a reliable indicator for DNA synthesis, usually indicating cell replication (Ward, Coleman *et al.* 2008). At the time of ISC proliferation assessment, focal bees were fed a 5 mg/ml BrdU in 25% queen candy *ad libitum* for a 24-hour period. Subsequently, bees were anaesthetized by chilling and their midguts were dissected. The midguts were fixed in Carnoy's fixative (Table 3) for 24 hours and embedded in Paraplast™ (Fisher, 23-021-399) wax for sectioning (10µm) using a microtome. These sections of tissue were placed in warm water baths on Superfrost Fisher plus™ microscope slides (Fisher, 22-034-979). To allow the tissue to adhere to the slides, the water was allowed to evaporate on slide warmers (40^{o}C) for 24 hours.

The prepared slides were de-paraffinized in xylene (Fisher, X3P-1GAL), rehydrated in graded alcohols (2x 100%, 90%, then 70%), and permeabilized in phosphate-buffered saline with triton X-100 (PBST, Table 3). The DNA was then

denatured using 2N hydrochloric acid (Fisher, SA56-1). After three washes in PBST, unspecific cell antigens were blocked with normal goat serum (Thermo scientific, PI-31873) and the sections were subsequently incubated with a primary anti-BrdU antibody (Phoenix Flow Systems, PRB1U) for 24 hours at 4^{o}C. After the 24 hour incubation and several washings (3x PBST and 2x PBS, Table 3), the tissue was incubated at roo temperature with a peroxidase-conjugated secondary antibody (Jackson Immuno Research, 115-035-003) that binds to the BrdU-bound primary antibody. The peroxidase cleaved added diaminobenzidine (Sigma, D4418) in a colorimetric reaction. Thus, any cell that contained DNA synthesized after ingestion of BrdU, was stained brown. The slides were then counterstained blue for approximately five minutes using gill hematoxylin (Fisherbrand, CS400-1D) followed by bluing solution (Table 3) for approximately one minute. The tissue cross-sections were then dehydrated for five minutes each in graded alcohols (50%, 70%, 95%, and 2x 100%) and three times in Citrisolv (Fisher, 22-143-975).

Labeled cell counts represent a direct measure of the replication rate of the intestinal stem cells (Ward, Coleman *et al.* 2008). Labeled cells were blindly counted in one cross-section of the intestine of each individual to estimate overall replication rates (Figure 2). Because the number of cells per tissue could differ amongst tissue sections, I also standardized ISC proliferation counts relative to the number of labeled cells per crypt.

Analysis

Survival analyses were performed with Kaplan-Meier tests, comparing each treatment group to its respective control group. In one instance, (15min tau-fluvalinate exposure combined with 500ppb coumaphos feeding), no appropriate acetone control group was available and a water-fed control group was used instead. If survival was comparable or higher than the control, the treatment was considered sub-lethal. For experiment two, three survival analyses were performed. Acute mortality was assessed during the first 7 days. Latent mortality effects were assessed by comparing survival of treatment and control groups between 7 days and the end of the experiment (19-22 days). Overall mortality was also assessed for the entire experimental period.

ISC proliferation was analyzed using a ANOVA. Individuals from treatment groups were compared with individuals from control groups matched by emerging date. When no exactly matched control group existed, a control group of the closest emerging date was chosen. Replicate cage differences were assessed and if no difference was found, cages were combined. In addition, analyses were done to evaluate age effects. For each treatment group and various water control groups ISC proliferation was compared between the 7-day-old workers and workers collected at the end of the experiment (19-22 days old).

Lastly, a correlational analysis was performed between mean mortality age and the mean number of ISC proliferation cells per crypt for the screening experiments. For the sub-lethal experiments, correlational analysis was performed for the mean survival age and the mean number of proliferating cells per crypt.

CHAPTER IV

RESULTS

Aim 1: Screening Xenobiotics - Mortality

All vehicle control groups exhibited mortality rates that were not significantly different from the water controls (Table 4). Isopropanol had the highest bee mortality amongst the vehicle controls (5%). During the 7-day feeding period, more than 50% of the bees fed colchicine, coumaphos, hydroxytetracycline, imidacloprid, and fumagillin died. Tau-fluvalinate-treated bees showed more than 50% mortality by day 3 (Figure 3). Compared with their controls, all xenobiotics except for 20-HE and methoxyfenozide showed a significant increase in mortality (Table 4).

Aim 1: Screening Xenobiotics - ISC Proliferation Rate

There were a total of 12 xenobiotics and 5 controls tested. No vehicle control showed a significant difference in mean cell counts compared with water controls (Table 5). Labeled cell counts per cross-section ranged from 49 to 461cells. The group means ranged from 62 to 323 cells (Table 6). Compared with their controls, colchicine, hydroxytetracycline, and tau-fluvalinate showed a significant decrease in ISC proliferation based on the preliminary count of the total number of cells per cross section. Only methoxyfenozide caused a significant increase in ISC proliferation (Table 6). However, when the number of cells per crypt was used as the basis for comparison, the effect of methoxyfenozide on proliferation rate was no longer significant, ($p = 0.114$).

Aim 2: Sub-lethal Xenobiotic Concentrations – Mortality

Hydroxytetracycline

In the short-term, hydroxytetracycline did not cause any significant differences in mortality at any concentration (Table 7). Therefore, all concentrations tested were considered sub-lethal (Figure 4). Honeybees fed the low and the high concentration of hydroxytetracycline showed a latent survival mean higher than the control bees (Table 8). In addition, there were no significant difference between controls and the experimental group for the medium concentration fed. Therefore, latent mortality was sub-lethal for all concentrations fed (Figure 5). Cumulative mean survival over the entire observation period was significantly higher than the respective control for honeybees emerging on 7/11/11 and fed the low concentration of hydroxytetracycline. All other feeding groups did not show a significant difference between treatment and controls. Thus, overall all hydroxytetracycline treatments were sub-lethal (Figure 6).

Methoxyfenozide

In the short-term, there was no significant difference in mean age of survival when bees fed 40ppb methoxyfenozide were compared to controls. However, bees fed 400 or 2000ppb methoxyfenozide responded with a higher mean survival age (Table 7). All three concentrations of methoxyfenozide were sub-lethal during the treatment period (Figure 7). There was an increase in latent mean survival age for bees emerging on July 11 that were given 40ppb methoxyfenozide (Table 8). On the other hand, there was no significant difference in latent mortality for all other feeding groups (Table 8). Consequently, latent mortality for all the experimental groups was considered sub-lethal

(Figure 8). With an increase in mean cumulative survival for bees fed 400ppb, 2000 ppb and 40ppb emerging on 7/11/11 but no significant difference in mean survival for bees emerging on 7/12/11 who were also fed 40ppb of methoxyfenozide, all methoxyfenozide treatments were considered to be sub-lethal overall (Figure 9).

Tau-fluvalinate

Honeybees randomly exposed to three times one minutes of tau-fluvalinate per day did not show a significant difference in acute mean survival times when compared to controls (Table 7). Thus, this exposure was considered sub-lethal (Figure 10). However, bees emerging on 7/11/11 and exposed to three sequential minutes of tau-fluvalinate per day responded with a significantly decreased mean survival time when compared to controls (Table 7). With exposure times of 15 sequential minutes, bees emerging from brood combs acquired from the Wake Forest honeybee lab apiary showed a significantly decreased acute mean survival time (Table 7). On the other hand, honeybees emerging from brood combs acquired from a nearby North Carolina beekeeper showed an increase in acute mean survival time when exposed to 15 sequential minutes of tau-fluvalinate per day (Table 7). Tau-fluvalinate exposure did not lead to increased latent mortality except for bees emerging on 6/20 that were exposed to tau-fluvalinate for 15 minutes (Figure 10). These bees had a significantly decreased mean survival age (Table 8). Cumulative mortality over the entire observation period was sub-lethal for all concentrations examined (Figure 11), except for bees emerging on 6/20/11 which originated from Wake Forest and were exposed to 15 minutes of tau-fluvalinate (Table 8).

Tau-fluvalinate with Coumaphos

In the short term, when tau-fluvalinate exposure was combined with 500ppb coumaphos, only the lowest exposure time was sub-lethal (Figure 13).The combination of tau-fluvalinate with coumaphos significantly increased mortality at all concentrations compared to tau-fluvalinate alone (Figure 14). Latent mortality was comparable to control bees except for bees from 6/20/11, which responded with a decreased mean survival (Table 8). Thus, all group except the feeding group from 6/20/11 showed sub-lethal effects after treatment (Figure 15). Lastly, cumulative mortality over the whole observation period was not significantly increased for any group, except for bees emerging on 6/20/11 exposed to 15 minutes of fluvalinate and bees emerging on 7/11/11 exposed for 3 minutes sequentially (Figure 16).

Sub-lethal Xenobiotic Concentrations: ISC Proliferation Rate

Hydroxytetracycline

In the short-term, hydroxytetracycline ingestion did not cause proliferative effects. However, hydroxytetracycline showed a negative latent effect on ISC proliferation, dependent on concentration. Only the highest concentration caused a significant decrease in ISC proliferation at older ages (Table 10). This effect was not observed in bees obtained from Wake forest, which emerged on 6/20/11 (Table 10). Comparison of acute and latent proliferation effects showed that later proliferation was lower for the two highest concentrations despite water controls not showing this effect (Figure 17).

Methoxyfenozide

The lowest concentration of methoxyfenozide fed caused a significant increase in proliferation in the short term, although this effect was only significant when the two performed experiments were pooled (Table 11). There was no significant effect on proliferation in the long term. The highest and the lowest treatment groups showed a significant decrease in proliferation from the younger to the older ages (Figure 18). The 400ppb treatment group showed the same trend but not significantly so. There were no concentration effects in the short-term or the long-term.

Tau-fluvalinate

There was no significant difference in proliferation when the three exposure times of tau-fluvalinate were compared to no exposure bees (Table 11). There were no significant differences between younger and older test bees for any exposure time (Table 11,). No bees were left at the end of day 19 for bees exposed to tau-fluvalinate for 3 minutes randomly, preventing a study of ISC proliferation at older ages in that group. Analyzing for concentration effect of tau-fluvalinate showed that there was no effect of increasing exposure on short term or long- term proliferation (Figure 19).

Tau-fluvalinate with Coumaphos

There were no acute or latent effects on ISC proliferation of tau-fluvalinate combined with coumaphos (Table 13). There were no significant differences between the two time points for any exposure time (Table 13). Because there were no bees left at the end of day 19 for bees exposed to tau-fluvalinate and coumaphos for 3 minutes randomly, latent verses acute comparisons could not be done. With the combination of tau-

fluvalinate and coumaphos, analyzing for concentration effects showed no effect of increasing exposure on short term or long- term proliferation (Figure 20). Analyzing the effect of adding coumaphos to tau-fluvalinate showed no significant effect of the addition, except in bees that were exposed for 15 minutes to tau-fluvalinate showed a significant increase in proliferation (Figure 21). Between the two treatments, older bees did not show a significant difference in proliferation between the two treatment groups (Figure 21).

Correlation analysis of mortality means with proliferation mean differences showed that there is a negative correlation of mortality and proliferation (Figure 22). However, in the second year, this negative relationship was not significant in the short-term or long-term dataset (Figure 23).

CHAPTER V

DISCUSSION

Honeybee health is compromised and toxins are likely to play some role in the ongoing decline of honeybees. My study exposed adult worker bees to 12 xenobiotics and one xenobiotic combination and determined bee survival and ISC proliferation. The initial screening suggested a negative impact on honeybee survival for almost all xenobiotics but ISC proliferation effects for only three. The follow-up studies of these three xenobiotics used a slightly modified experimental paradigm, which affected honeybee survival in control and treatment groups. Thus, the two sets of experiments are not directly comparable. However, treatment effects in each year could be assessed relative to the relevant control groups. In sum, my results suggest sub-lethal effects of hydroxytetracycline and methoxyfenozide on ISCs, a positive correlation of survival and ISC proliferation, and a decline of ISC proliferation with age.

The results of aim one of this study showed the effects of high concentrations of several xenobiotics on survival and ISC proliferation. Among the 12 xenobiotics screened only 20-HE did not have an effect on mortality or proliferation. The absence of a proliferation effect was unexpected because 20-HE is the main proliferative hormone found in honeybees and it stimulates cell proliferation during larval stages (Smagghe, Vanhassel *et al.* 2005). The results of my study could be due to the receptors being

different in the adult honeybee or the wrong concentration been feed to the honeybees (Siaussat, Porcheron *et al.* 2009). I was expecting to see no mortality effect because adult honeybees do not need to molt and thus won't be affected by a premature molt as larval insects are (Thacker 2002).

Several of the xenobiotics decreased survival without causing a change in ISC proliferation. These included the two fungicides chlorothalonil and fumagillin. These results were not expected because chlorothalonil fed to larval honeybees increased cellular apoptosis in the midgut (Gregorc and Ellis 2011). Thus, I expected that midgut cells of the adult honeybee would also respond to chlorothalonil with increased cell death, leading to an increase in ISC proliferation to replace the dead cells. The difference of my results from Gregorc and Ellis result (2011) could stem from the differences in concentration used. While I used 1000ppb, which was slightly higher than the highest concentration found in honey (Mullin and Frazier et al. 2010), Gregorc and Ellis (2011) used 400ppm. Another potential explanation is a difference in larval and adult gut physiology (Mansour 1927). In any case, my results suggest that chlorothalonil does not affect adult ISC proliferation, even at concentrations that decrease survival.

The other fungicide used was fumagillin. Fumagillin has been found to change the natural microflora of the midgut by causing an increase in yeast cell populations (Rada, Máchová *et al.* 1997). Given the specificity of the possibly co-evolved microbiota of corbiculate bees, I expected that changes in the microflora would have caused an increase in digestive demand, causing a change in cellular proliferation (Ward, Coleman *et al.* 2008; Martinson, Danforth *et al.* 2011; Willard, Hayes *et al.* 2011). Fumagillin reduced

bee survival significantly in my experiment and detrimental changes in the gut are likely, although I did not confirm effects on the gut microflora. Regardless, ISC proliferation was not significantly affected in the surviving bees. The same mortality and ISC proliferation results were obtained for the miticide coumaphos, which was also observed by Gregorc and Ellis (2011) to cause an increase in honeybee larval midgut cell death. The difference between the studies may be explained by a difference between juvenile and adult gut physiology, similarly to the difference for chlorothalonil. However, in both cases the missing effect of the xenobiotic could also be due to demographic selection, meaning that the bees that were least affected were the bees that survived and were studied for ISC proliferation.

Another xenobiotic that did not affect ISC proliferation but decreased survival was imidacloprid, the only neonicotinoid tested. While this class of pesticide has been shown to cause adverse neurological effects, it is considered a systemic insecticide (Decourtye, Lacassie *et al.* 2003; Yang, Chuang *et al.* 2008; Johnson, Ellis *et al.* 2010) and unspecific effects on the ISCs might have been possible. However, no effects were identified.

Likewise, none of the three cry toxins caused an effect on proliferation, although all lowered bee survival. The absence of proliferation effects was expected for cry1ac and cry1ac-mod because these cry toxins affect specifically Lepidopteran hosts. Their specificity is due to their dependence on an appropriate host pH, needed for toxin activation and host-specific receptors needed for pore formation and thus ion leakage and subsequent cell lysis (Bravo, Gill *et al.* 2007). On the other hand, cry22 has been found to

be active in ants, which are also found in the order Hymenoptera, like honeybees. Thus, I expected that cry22 would cause increased midgut cell death and result in an increase in cell proliferation (de Maagd, Bravo *et al.* 2003). However, this was not the case. My results complement Porcar and Gomez *et al* (2008) who found that the Bt strain PS86Q3 which was also active in ants and sawflies did not affect honeybees. Thus, honeybees seem to be different from other Hymenoptera and relatively robust against cry toxins. However, all cry toxins affect mortality, which must be due to other effects that need to be further studied.

Three xenobiotics decreased both survival and proliferation. These xenobiotics were colchicine, the negative control, along with tau-fluvalinate and hydroxytetracycline. However, because of the decrease in survival the fed or exposed dose was not considered sub-lethal. Colchicine was expected to completely halt cell proliferation (Borisy and Taylor 1967). However, as in the case of hydroxyurea proliferation was not completely stopped (Ward, Coleman *et al.* 2008). Thus, it seems colchicine is not able to complete stop spindle fiber formation in all the ISCs. Perhaps, it can be detoxified, it might not reach the ISCs, or the concentration used was too low. However, the severe reduction of survival argues that it did physiologically affect the bees, just like hydroxyurea (Ward, Coleman *et al* 2008). This finding strengthens the case for the existence of important proliferative cells in other tissues of the adult honeybees. The decrease of ISC proliferation by hydroxytetracycline and fluvalinate was accompanied by a significant increase in mortality. Therefore, these two substances were subject to more detailed studies at lower concentrations, which are discussed below.

Methoxyfenozide did not affect survival and was the only xenobiotic to increase the rate of ISC proliferation compared with its acetone control. However, this effect was only significant when the absolute number of labeled cells was counted, not relative to crypt number. A positive effect was expected, based on the similarity of methoxyfenozide to 20-HE, which has a general proliferative effect (Thacker 2002). In contrast to 20-HE, methoxyfenozide probably did not degrade in my experiment or was dosed correctly to elicit a proliferation increase. Thus, methoxyfenozide was also subsequently tested at different concentrations in order to determine sub-lethal concentration effects. The results from this study are also discussed below.

Overall, there was an increase in mortality from the first set of experiments to the second set. The increase in mortality could be due to the changes made in the experimental protocol from the first summer to the second summer. These changes included an increase in honeybee density per cage, changes made in the feeding methods, relative humidity, and temperature of the incubator. These differences make it difficult to compare the results from the first set of experiments to those from the second set. However, similar results were obtained by comparisons within these experiments, which were valid because treatments were always compared to controls. Overall, the detailed studies of tau-fluvalinate, methoxyfenozide, and hydroxytetracycline in the second year confirmed the previous results but some differences were observed and more detailed observations could be made.

In the short-term (7 day feeding period), long-term (>12 days after feeding period) and, cumulatively (combined observations time), all tested doses of

hydroxytetracycline were sub-lethal when compared to their vehicle controls. The highest sub-lethal concentration (0.006%) showed a negative proliferative effect compared with the control group. This effect was only seen in the older bees and thus this effect was not an acute effect as was observed in the first experiment. There was also a negative but latent proliferative effect when 0.006% hydroxytetracycline was compared to 0.00012% hydroxytetracycline the lowest concentration of hydroxytetracycline fed.

Hydroxytetracycline tests were performed with bees of different origin. Post-hoc analysis of bees fed the highest concentration of hydroxytetracycline revealed that the proliferative effect observed was not observed in honeybees originating from Wake Forest. The control bees from this apiary however, had a lower ISC proliferation rate compared to all other control bees. Thus, the threshold for a decrease in proliferation caused by further external stressors might not have been observable. The 0.006% hydroxytetracycline effect on proliferation seems to be a delayed effect. The negative proliferative effect could be due to a loss of the natural microflora of the midgut causing an increase in digestive demand and thus an increase in ISC proliferation rate, exhausting replicative capacity (Kacaniova, Chlebo *et al.* 2004; Ward, Coleman *et al.* 2008; Willard, Hayes *et al.* 2011). Alternatively, the changes in the microflora may have allowed for secondary health infections later in life that compromised the ISC proliferation.

In combination, my results from both years suggest that the effect of hydroxytetracycline on proliferation is dose-dependent. The highest but lethal concentration fed showed acute proliferation effects, while the highest sub-lethal concentration only showed delayed effects, and lower concentrations did not have any

effects. The persistent hydroxytetracycline effects may be due to indirect effects on the microflora. Alternatively, the decrease in latent proliferation may have been caused by direct damage to the ISC, which could not be alleviated in the long term (Yan and Wajapeyee 2010).

Methoxyfenozide was sub-lethal at all concentrations and time points. Comparing each concentration of the xenobiotic with its control showed that methoxyfenozide at the lowest concentration increased acute proliferation. This result corroborated my finding in the first year. Furthermore, the highest and the lowest concentration fed showed a significant decrease in latent mean proliferation when compared to acute mean proliferation. The effect of methoxyfenozide could be due to its hormonal activity (Thacker 2002), upregulating ISC proliferation in young bees. The decrease in proliferative activity from bees assayed directly after the feeding to bees assayed later could be due to the exhaustion of replicative capacity (Ward, Coleman *et al.* 2008). This is in contrast to my results from the hydroxytetracycline treated bees, where the latent decrease in proliferation was presumably due to long lasting damage to the ISCs.

Acute mortality for bees exposed to tau-fluvalinate was increased, except for bees exposed to three randomized minutes of fluvalinate and bees obtained from a nearby beekeeper emerging on 6/22/11 and exposed to 15 minutes for fluvalinate. Latent mortality was also higher for this group of bees when it could be measured. Bees exposed for 3 days during the first set of experiments, were not kept for more than the feeding period and thus old age data was not available. The initial finding of decreased ISC

proliferation in fluvalinate-exposed bees could not be confirmed for lower exposures, regardless of mortality effects. Because fluvalinate exposure to individual bees could not be controlled it is possible that bees which were exposed to fluvalinate enough to cause proliferation changes also experienced death before assays were done (demographic selection), preventing the detection of a potential effect. In sum, I can conclude that fluvalinate, which caused the lowest midgut epithelium cell death in larval bees (Gregorc and Ellis 2011), only showed proliferation effects at a lethal concentration in adult bees.

While coumaphos did not show any proliferation effects by its self in the first set of experiments, the combination of fluvalinate with coumaphos showed an additive effect on ISC proliferation and survival when compared to fluvalinate alone. The combination treatment showed significant effects on acute survival, except for the randomized three-minute treatment. However, only the honeybees originating from Wake Forest exposed to fluvalinate for 15 minutes daily responded with an increase in latent mortality. As was the case for fluvalinate alone, the combination of fluvalinate with coumaphos showed no proliferative effects relative to its controls. However, comparison of honeybees exposed to either fluvalinate alone or fluvalinate combined with coumaphos showed that there was a two-fold increase in proliferation when coumaphos feeding was added to fluvalinate exposure. The additive effect was probably due to the added concentration of coumaphos competing for the same xenobiotic detoxification enzymes needed to detoxify fluvalinate (Johnson, Pollock *et al.* 2009). The mortality results from the combined xenobiotics were as expected as the combination of fluvalinate with coumaphos significantly decreased survival. Despite the mortality effect, I had to reject my hypothesis that there will be a

multiplicative change in cell proliferation with the combination of sub-lethal concentrations of fluvalinate and coumaphos. Even if the worker bees used in my experiment had higher levels of cell death as suggested by the results of Gregorc and Ellis (2011), ISC proliferation did not increase acutely to replace the dead cells. Thus, coumaphos and fluvalinate at sub-lethal concentrations even when exposed together do not affect adult ISC proliferation, although their combination puts a particular burden on the honeybees' detoxification systems (Johnson, Pollock *et al.* 2009).

The positive relationship between ISC proliferation and survival suggested that decreased ISC proliferation is associated with increased mortality. However, because treatment with fluvalinate was stopped after three days and hydroxytetracycline had a low population survival but a high mean survival age due to increased mortality after BrdU feeding the correlation is unclear. In addition, the combination of the two miticides, tau-fluvalinate, and coumaphos were studied for synergistic effects. After studying these four treatment groups, a post hoc correlation analysis was done. The analysis showed that there was no correlation between survival and proliferation in the short-term or the long-term. Thus, it is unclear whether ISC proliferative activity is an indicator of life expectancy in honeybees.

CHAPTER VI

CONCLUSIONS

This study examined the impact of xenobiotics on the ISC proliferation of the honeybee and possibly the health of honeybees. Different xenobiotics have specific effects on ISC proliferation and many have no effects at all. The study supports previous findings (Ward, Coleman *et al*. 2008; Willard, Hayes *et al.* 2011) that ISC proliferation is flexible and varies with environmental conditions. My results could be explained by direct effects on the ISCs, rather than affecting the need for proliferation (Willard, Hayes *et al.* 2011). However, at least the effect of hydroxytetracycline may also be caused by interactions with the gut microfauna and more research is needed. I confirmed the effect of aging on proliferation (Ward, Coleman *et al.* 2008), although it seems to depend on the availability of an additional stressor. This stressor could be the daily tasks of food processing and brood care in an observational hive but in cage studies, it could be the ingestion of xenobiotics. In addition, the positive stimulation of ISC proliferation by methoxyfenozide could mean that there are ecdysteroid receptors located in the midgut, which a future study could investigate.

Considering the availability of xenobiotics in the environment and accumulation in the hive (Mullin, Frazier *et al.* 2010) and the many detrimental effects these xenobiotics could have, decoupling mortality effects from behavioral, physiological, and anatomical effects at sub-lethal concentrations seems necessary. Simple toxicological

tests of pesticides need to be replaced with a battery of tests that take possible interactions, latent, and sub-lethal effects into account if we want to ensure the health of honey bees. The midgut is an important target organ and my results do not suggest that it can be neglected. Even though few compounds affected the ISC proliferation rate, the employed assay is easy, relevant, and quantitative and may allow us to predict the health consequences of novel pesticides for honeybees.

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APPENDIX A

TABLES AND FIGURES

Table 1. Xenobiotic concentrations used in aim one.

Xenobiotic	Supplier	Concentration	Citation for concentrations (Author, year)
Colchicine	Sigma-Aldrich	0.5%	(Sullivan and Castro 2005)
20-hydroxyecdysone	MP Chemicals	200ppb	(Rharrabe, Bouayad et al. 2009)
DMSO [a]	Acros Chemical	0.001%	
Acetone [b]	Mallinckrodt chemicals	0.01%	
Isopropanol [c]	Fisher	200ppb	
Sodium Bicarbonate (SB) [d]	Fisher	300ppb	
Cry22 Bt protoxin	Nicolas Desneux	300ppb	(Han, Niu *et al.* 2010)
Cry 1ac Bt protoxin	Mario Soberon Bruce Tabashnik	300ppb	(Han, Niu et al. 2010)
Cry1acmod Bt protoxin	Mario Soberon Bruce Tabashnik	300ppb	(Han, Niu et al. 2010)
Fumagillin	Mann Lake Ltd	0.2%	Highest dose allowed on Label
Tau-fluvalinate (Apistan™ strips)	Mann Lake Ltd	half a strip	
Hydroxytetracycline	Sigma- Aldrich	0.3%	(Thompson, Waite *et al.* 2005)
Imidacloprid	Sigma-Aldrich	500ppb	(Johnson, Ellis et al. 2010)
Coumaphos	Sigma-Aldrich	5000ppb	(Johnson, Ellis et al. 2010)
Chlorothalonil	Fluka	1000ppb	(Johnson, Ellis et al. 2010)
Methoxyfenozide	ChemSevice Inc.	400ppb	(Johnson, Ellis et al. 2010)

a, b, c, d are vehicle controls. They were used as solvents for a = Imidacloprid, b= Methoxyfenozide, Coumaphos and Chlorothalonil c = 20-HE and d= Cry1ac, Cry1acmod and Cry22

Table 2. Xenobiotic concentration used in aims two and three.

Xenobiotic	**Supplier**	**Concentration or Exposure Time**		
		High	**Mid**	**Low**
Methoxyfenozide	ChemSevice Inc.	2000ppb	400ppb	40ppb
Tau-fluvalinate *	Mann Lake Ltd	15 minutes	3 minutes sequential	3 minutes randomized
Hydroxytetracycline	Sigma-Aldrich	0.006%	0.003%	0.00012%
Tau-fluvalinate * + 500ppb Coumaphos	Mann Lake Ltd Sigma-Aldrich	15 minutes	3 minutes sequential	3 minutes randomized

* Tau-fluvalinate was exposed to bees using the commercial Apistan™ strip. A full strip was placed in each cage for the indicated exposure time per day for 7 days.

Table 3. Buffers and solutions used in proliferation assay.

Solutions and Buffers	**Recipe**
PBS	Add 8g of NaCl, 0.2g of KCl, 1.44g of Na2HPO4, and 0.24g of KH2PO4 to 1 L of water and adjust to PH 7.4.
PBST	Add 500µl of triton X-100 to 1000ml of 1 X PBS
Carnoy's Fixative	60% ethanol + 30% chloroform + 10% glacial acetic acid
Hematoxylin Bluing Solution	Add 1 gram sodium bicarbonate to 1000ml distilled water.

Table 4. Worker bee mortality during xenobiotic screening.

Treatment	Total N	N surviving after day 7	Percent Survived
20-HE	100	96	96.00%
Chlorothalonil[b]	100	95	95.00%
Coumaphos[a b]	100	23	23.00%
Cry1AC[a b]	100	87	87.00%
Cry1ACmod[a b]	100	83	83.00%
Cry22[a b]	100	92	92.0%
Tau-fluvalinate[a]	101	23	22.77%
Fumagillin[a]	101	42	41.58%
Imidacloprid[a b]	100	47	47.00%
Hydroxytetracycline[a]	103	7	6.79%
Methoxyfenozide	100	99	99.00%
Controls			
Water	100	99	99.00%
Colchicine[a]	78	5	6.41%
Acetone	100	100	100.00%
DMSO (Imidacloprid)	25	25	100.00%
Isopropanol (20-HE)	100	95	95.00%
Sodium bicarbonate	100	100	100.00%

a. feeding these xenobiotics results in a significant decrease in mortality as compared to the water control. b. feeding these xenobiotics results in a significant decrease in mortality as compared to the respective vehicle controls.

Table 5. Mean difference in absolute number of proliferating cells between xenobiotics and respective controls.

Control	Mean	Treatment	Mean	Mean Difference (Control-Treatment) ± SE	ANOVA
Water	261.7.	Colchicine*	169.3	92.4 ± 30.7	$F_{(1,11)} = 9.1$, $p = 0.012$
		Acetone	245.9	15.8 ± 27.7	$F_{(1,21)} = 0.3$, $p = 0.575$
		DMSO	252.9	8.8 ± 22.6	$F_{(1,17)} =$, 0.2 $p = 0.701$
		Isopropanol	280.6	-18.9 ± 27.5	$F_{(1,18)} = 0.5$, $p = 0.501$
		Sodium bicarbonate	238.3	-23.5 ± 27.8	$F_{(1,20)} = 0.7$, $p = 0.408$
		Fumagillin	250.1	11.6 ± 23.6	$F_{(1,19)} = 0.2$, $p = 0.629$
		Hydroxytetracycline*	62.3	199.4 ± 29.2	$F_{(1,11)} = 46.6$, $p < 0.001$
		Tau-fluvalinate*	164.3	97.5 ± 29.4	$F_{(1,16)} =$, $p = 0.004$
		Cry1ac	269.8	-8.1 ± 33.7	$F_{(1,18)} = 0.1$, $p = 0.813$
		Cry1acmod	248.5	13.3 ± 25.9	$F_{(1,19)} = 0.3$, $p = 0.615$
Acetone	245.9	Coumaphos	275.9	-30.0 ± 32.7	$F_{(1,20)} = 0.8$, $p = 0.371$
		Chlorothalonil	275.5	-29.6 ± 35.4	$F_{(1,22)} = 0.7$, $p = 0.411$
		Methoxyfenozide*	323.1	-77.2 ± 30.3	$F_{(1,21)} = 6.5$, $p = 0.019$
DMSO	252.9	Imidacloprid	263.0	-10.1 ± 39.9	$F_{(1,18)} = 0.8$, $p = 0.386$
Isopropanol	280.6	20-HE	254.1	26.5 ± 28.2	$F_{(1,20)} = 0.9$, $p = 0.359$
Sodium bicarbonate	238.3	Cry22	290.7	-52.5 ± 31.3	$F_{(1,21)} = 2.8$, $p = 0.108$

*Xenobiotics showing a significant mean difference from control.

Table 6. Mean difference of the relative number of proliferating cells per crypt between xenobiotics and respective controls.

Control	Mean	Treatment	Mean	Mean Difference (Control-Treatment) ± SE	ANOVA
Water	4.9.	Colchicine*	3.1	1.8±0.7	$F_{(1,9)}$ =7.4 , p = 0.024
		Acetone	4.7	0.2±0.5	$F_{(1,18)}$ = 0.2 , p = 0.662
		DMSO	5.4	-0.5±0.5	$F_{(1,16)}$ =0.8 , p = 0.399
		Isopropanol	4.8	0.1±0.6	$F_{(1,15)}$ =0.0 , p = 0.849
		Sodium bicarbonate	5.5	-0.6±0.4	$F_{(1,17)}$ =2.0 , p = 0.177
		Fumagillin	4.6	0.3±0.5	$F_{(1,17)}$ =0.5 , p = 0.509
		Hydroxytetracycline*	1.8	3.1±0.4	$F_{(1,13)}$ = , p < 0.001
		Tau-fluvalinate*	3.3	1.6±0.7	$F_{(1,10)}$ =6.1 , p = 0.033
		Cry1ac	4.5	0.4±0.4	$F_{(1,18)}$ =1.1 , p = 0.316
		Cry1acmod	4.8	0.02±0.4	$F_{(1,18)}$ =0.0 , p = 0.958
Acetone	4.7	Coumaphos	5.3	-0.6±0.5	$F_{(1,17)}$ =1.4 , p = 0.249
		Chlorothalonil	4.8	-0.1±0.4	$F_{(1,20)}$ =0.02 , p = 0.887
		Methoxyfenozide	5.4	-0.7±0.5	$F_{(1,20)}$ =2.7 , p = 0.114
DMSO	5.4	Imidacloprid	5.3	0.1±0.6	$F_{(1,16)}$ =0.04 , p = 0.851
Isopropanol	4.8	20-HE	4.8	-0.3±0.7	$F_{(1,18)}$ =0.2 , p = 0.630
Sodium bicarbonate	5.5	Cry22	5.3	0.2±0.6	$F_{(1,19)}$ =0.1 , p = 0.733

*Xenobiotics showing a significant mean difference from control.

Table 7. Data on mortality during the xenobiotic feeding in the second set of experiments (Acute)

Bee Emerging Date	Conc.	Total N	N of Deaths	Estimated Mean Survival Age	Kaplan Meier test
Hydroxytetracycline					
7/11/11	0.00012%	151	54	6.6 ± 0.2	$\chi2 = 0.0$, p = 0.970
	Control	155	60	7.0 ± 0.1	
7/12/11	0.00012%	152	79	6.7 ± 0.1	$\chi2 = 0.0$, p = 0.992
	Control	148	70	6.2 ± 0.2	
8/2/11 & 8/10/11	0.003%	601	313	6.3 ± 0.1	$\chi2 = 0.7$, p= 0.409
	Control	446	248	6.4 ± 0.1	
8/10/11	0.006%	314	185	6.0 ± 0.1	$\chi2 = 0.0$, p = 0.962
	Control	149	85	5.9 ± 0.2	
Methoxyfenozide					
7/11/11	40ppb	153	35	7.3 ± 0.1	$\chi2 =1.2$, p = 0.265
	Control	151	41	6.8 ± 0.2	
7/15/11	40ppb	179	116	5.6 ± 0.2	$\chi2 =0.2$, p = 0.618
	Control	160	100	5.8 ± 0.2	
7/15/11	400ppb	316	171	6.4 ± 0.1*	$\chi2 =7.2$, p= 0.007
	Control	160	100	5.8 ± 0.2	
7/15/11	2000ppb	309	155	6.8 ± 0.1*	$\chi2 =16.1$, p < 0.001
	Control	160	100	5.8 ± 0.2	
Tau-fluvalinate					
8/2/11	3 min. rand.	295	165	6.5 ± 0.1	$\chi2 =0.2$, p= 0.631
	Control	297	163	6.7 ± 0.1	
7/11/11	3 min. seq.	147	73	6.4 ± 0.1[a]	$\chi2 =6.9$, p= 0.009
	Control	155	60	7.0 ± 0.1	
7/12/11	3 min seq.	156	94	5.9 ± 0.1[a]	$\chi2 =5.2$, p= 0.023
	Control	148	70	6.2 ± 0.2	
6/20/11	15 min seq.	150	91	6.0 ± 0.2[a]	$\chi2 =20.4$, p < 0.001
	Control	150	64	7.1 ± 0.1	
6/22/11	15 min seq.	154	40	7.0 ± 0.2*	$\chi2 =11.6$, p= 0.001
	Control	150	67	6.3 ± 0.2	
Tau-fluvalinate with Coumaphos					
8/3/11	3 min. rand.	296	174	6.2 ± 0.1	$\chi2 = 0.8$, p= 0.371
	Control	309	184	6.0 ± 0.1	
7/11/11	3 min. seq.	149	75	6.7 ± 0.1[a]	$\chi2 =11.1$, p= 0.001
	Control	151	41	6.8 ± 0.2	
7/15/11	3 min seq.	149	127	4.4 ± 0.1 [a]	$\chi2 = 45.3$, p < 0.001
	Control	160	100	5.8 ± 0.2	
6/21/11	15 min seq.	300	154	6.1 ± 0.1 [a]	$\chi2 =10.9$, p= 0.001
	Control	150	64	7.1 ± 0.1	

*Estimated mean survival is significantly higher than control. [a]Estimated mean survival is significantly lower than control (treatment not considered sub-lethal).

Table 8 : Mortality data for the period after xenobiotics were fed (Latent) in the second experimental set

Date Bees Emerged	Conc.	Total N	N of Deaths	Estimated Mean Survival Age	Kaplan Meier test
Hydroxytetracycline					
7/11/11	0.00012%	87	58	15.4 ± 0.6 *	$\chi2 = 5.5$, p= 0.019
	Control	85	75	14.3 ± 0.7	
7/12/11	0.00012%	63	33	19.9 ± 0.4*	$\chi2 = 23.0$, p < 0.001
	Control	68	54	16.1 ± 0.7	
8/2/11	0.003%	141	111	14.3 ± 0.3	$\chi2 = 0.8$, p= 0.386
	Control	114	93	14.7 ± 0.3	
8/10/11	0.003%	107	85	11.6 ± 0.3	$\chi2 = 3.4$, p= 0.065
	Control	54	41	12.4 ± 0.5	
8/10/11	0.006%	109	69	13.8 ± 0.3*	$\chi2 = 8.5$, p= 0.004
	Control	54	41	12.4 ± 0.5	
Methoxyfenozide					
7/11/11	40ppb	108	77	13.7 ± 0.6 *	$\chi2 = 6.1$, p= 0.013
	Control	100	86	12.8 ± 0.5	
7/15/11	40ppb	53	38	13.8 ± 0.8	$\chi2 = 0.4$, p= 0.538
	Control	50	37	15.9 ± 0.6	
7/15/11	400ppb	125	89	14.3 ± 0.5	$\chi2 = 0.0$, p= 0.936
	Control	50	37	15.9 ± 0.6	
7/15/11	2000ppb	55	35	15.4 ± 0.7	$\chi2 = 2.5$, p= 0.117
	Control	50	37	15.9 ± 0.6	
Tau-fluvalinate					
8/3/11	3 min. rand.	110	90	14.3 ± 0.3	$\chi2 = 2.5$, p= 0.117
	Control	114	93	14.7 ± 0.3	
7/11/11	3 min seq.	63	39	16.4 ± 0.8*	$\chi2 = 7.5$, p= 0.006
	Control	85	75	14.3 ± 0.7	
7/15/11	3 min seq.	52	40	15.6 ± 0.9	$\chi2 = 0.0$, p= 0.902
	Control	68	54	16.1 ± 0.7	
6/20/11	15 min seq.	43	39	15.7 ± 0.8*[a]	$\chi2 = 48.3$, p < 0.001
	Control	85	22	18.9 ± 0.4	
6/22/11	15 min seq.	104	53	15.1 ± 0.5*	$\chi2 = 6.2$, p= 0.013
	Control	54	35	12.9 ± 0.6	
Tau-fluvalinate with Coumaphos					
8/3/11	3 min. rand.	102	80	13.9 ± 0.4*	$\chi2 = 11.1$, p= 0.001
	Control	105	62	15.2 ± 0.5	
7/11/11	3 min seq.	64	34	17.9 ± 0.6*	$\chi2 = 36.0$, p < 0.001
	Control	100	86	12.8 ± 0.5	
7/15/11	3 min seq.	12	2	15.5 ± 5.5	$\chi2 = 1.3$, p= 0.251
	Control	50	37	15.9 ± 0.6	
6/20/11	15 min. seq.	40	16	17.5 ± 1*[a]	$\chi2 = 4.9$, p= 0.026
	Control	85	22	18.9 ± 0.4	
6/22/11	15 min seq.	66	15	19.6 ± 0.6*	$\chi2 = 32.8$, p < 0.001
	Control	54	35	12.9 ± 0.6	

*Estimated mean survival is significantly different from control. [a]Estimated mean survival is not sub-lethal when compared to control.

Table 9: Overall mortality of workers in the second experimental set

Date Bees Emerged	Conc.	Total N	N of Deaths	Estimated Mean Survival	Kaplan Meier test
Hydroxytetracycline					
7/11/11	0.00012%	151	112	11.2 ± 0.6	$\chi2 = 2.5$, p= 0.114
	Control	155	135	10.7 ± 0.6	
7/12/11	0.00012%	152	112	12.0 ± 0.6*	$\chi2 = 8.1$, p= 0.004
	Control	148	124	10.3 ± 0.6	
8/2/11	0.003%	326	275	9.5 ± 0.3	$\chi2 = 0.1$, p= 0.755
	Control	297	256	9.4 ± 0.3	
8/10/11	0.003%	275	233	7.6 ± 0.3	$\chi2 = 0.2$, p= 0.685
	Control	149	126	7.6 ± 0.4	
8/10/11	0.006%	314	254	8.1 ± 0.3	$\chi2 = 2.3$, p= 0.113
	Control	149	126	7.6 ± 0.4	
Methoxyfenozide					
7/11/11	40ppb	153	112	11.6 ± 0.6*	$\chi2 = 6.9$, p= 0.009
	Control	151	127	10.3 ± 0.5	
7/15/11	40ppb	179	154	7.4 ± 0.4	$\chi2 = 0.5$, p= 0.460
	Control	160	137	8.4 ± 0.5	
7/15/11	400ppb	316	260	9.0 ± 0.3*	$\chi2 = 4.8$, p= 0.029
	Control	160	137	8.4 ± 0.5	
7/15/11	2000ppb	309	247	9.6 ± 0.3*	$\chi2 = 13.0$, p < 0.001
	Control	160	137	8.4 ± 0.5	
Tau-fluvalinate					
8/2/11	3 min. rand.	295	255	8.998	$\chi2 = 1.7$, p= 0.191
	Control	297	256	9.4 ± 0.3	
7/11/11	3 min. seq.	147	112	10.3 ± 0.6	$\chi2 = 0.1$, p= 0.747
	Control	155	135	10.7 ± 0.6	
7/12/11	3 min seq.	156	134	8.6 ± 0.5	$\chi2 = 3.2$, p= 0.072
	Control	148	124	10.3 ± 0.6	
6/20/11	15 min. seq.	144	130	8.4 ± 0.5*	$\chi2 = 70.0$, p < 0.001
	Control	159	86	13.4 ± 0.6	
6/22/11	15 min. seq.	154	93	12.2 ± 0.6*	$\chi2 = 25.4$, p < 0.001
	Control	131	102	8.3 ± 0.5	
Tau-fluvalinate with Coumaphos					
8/2/11	3 min. rand.	296	254	8.4 ± 0.3	$\chi2 = 1.5$, p= 0.223
	Control	309	246	8.6 ± 0.4	
7/11/11	3 min. seq.	149	109	11.2 ± 0.6	$\chi2 = 3.0$, p= 0.085
	Control	151	127	10.3 ± 0.5	
7/12/11	3 min seq.	149	129	5.1 ± 0.4*[a]	$\chi2 = 40.3$, p < 0.001
	Control	160	137	8.4 ± 0.5	
6/20/11	15 min. seq.	148	114	8.2 ± 0.5*[a]	$\chi2 = 47.1$, p < 0.001
	Control	159	86	13.4 ± 0.6	
6/22/11	15 min. seq.	132	71	12.9 ± 0.7	$\chi2 = 19.8$, p < 0.001
	Control	131	102	8.3 ± 0.5	

*Estimated mean survival is significantly different from control. [a]Estimated mean survival is not sub-lethal when compared to control.

Table 10: Effect of hydroxytetracycline feeding on intestinal stem cell proliferation rate (cells/crypt)

Sampled Time	Concentration (N)	Mean	Control Mean	Water Control (N)	Mean Difference ± SE	ANOVA
Acute	0.00012% (11)	4.138	4.1	(10)	0.08 ± 0.5	F = 0.0, p = 0.984
	0.003% (8)	4.35	3.52	(9)	-0.84 ± 0.4	F = 4.4, p = 0.540
	0.006% (7)	3.620	3.142	(15)	-0.48 ± 0.4	F = 1.2, p = 0.286
Latent	0.00012% (10)	3.43	3.318	(5)	-0.11 ± 0.6	F = 0.0, p = 0.843
	0.003% (8)	3.715	3.028	(5)	0.69 ± 0.5	F = 2.3, p = 0.161
	0.006% (7)	2.169	2.802	(15)	0.63 ± 0.6	F = 1.3, p = 0.273
	0.006% [a] (4)	2.636	2.345	(10)	-0.2 ± 0.8	F = 0.1, p =0.710
	0.006% [b] (3)	1.545	3.715	(5)	2.17 ± 0.4*	F = 28.97, p = 0.002

Comparison of mean acute proliferation and mean latent proliferation

Concentration	Acute proliferation Sample (N)	Mean	Latent proliferation Sample (N)	Mean	Mean Difference ± SE	ANOVA
0.00012%	11	4.138	10	3.432	0.71 ± 0.4	F = 2.7, p = 0.117
0.003%	8	4.355	8	3.028	1.33 ± 0.4 *	F = 11.3, p = 0.005
0.006%	7	3.620	7	2.169	1.5 ± 0.4 *	F = 12.6, p = 0.004
0.006% [a]	4	3.738	4	2.636	1.10 ± 0.6	F = 3.4, p = 0.115
0.006 [b]	3	3.462	3	1.545	1.92 ± 0.3 *	F = 41.3, p = 0.003

*Significant difference in mean cells per crypt per tissue. Since bees for 0.006% were acquired from different sources, they were also analyzed separately. [a] Bees originating from Wake Forest apiary. [b] Bees originating from UNCG apiary.

Table 11: Effect of methoxyfenozide feeding on intestinal stem cell proliferation rate (cells/crypt)

Sampled Time	Concentration (ppb) (N)	Mean	Control Mean	Acetone control (N)	Mean Difference ± SE	ANOVA
Acute	40 7/11/11 (5)	3.843	2.728	(4)	-1.12 ± 0.5	F = 5.3, p = 0.054
	40 7/15/11 (5)	5.289	4.093	(5)	-1.20 ± 0.6	F = 4.1, p = 0.078
	Combined 40 (10)	4.566	3.487	(9)	-1.08 ± 0.5 *	F = 4.7, p = 0.045
	400 (9)	3.491	4.093	(5)	0.60 ± 0.4	F = 1.8, p = 0.200
	2000 (10)	3.887	4.093	(5)	0.21 ± 0.4	F = 0.3, p = 0.609
Latent	40 7/11/11 (12)	2.640	2.768	(10)	0.13 ± 0.2	F = 0.7, p = 0.419
	400 (11)	3.186	2.768	(10)	-0.42 ± 0.3	F = 1.6, p = 0.220
	2000 (8)	2.727	2.768	(10)	0.04 ± 0.3	F = 0.0, p = 0.894

Comparison of mean acute proliferation and mean latent proliferation

Concentration	Acute proliferation Sample (N)	Mean	Latent proliferation Sample (N)	Mean	Mean Difference ± SE	ANOVA
40 7/11/11	5	3.843	12	2.640	1.20 ± 0.3*	F = 18.9, p = 0.001
40 7/15/11	5	4.093	N/A	N/A	N/A	N/A
Combined 40	10	4.566	12	2.640	1.93 ± 0.4*	F = 25.4, p < 0.001
400	9	3.491	11	3.186	0.31 ± 0.4	F = 0.5, p = 0.487
2000	10	3.887	8	2.727	1.16 ± 0.4*	F = 8.5, p = 0.010
Acetone	5	4.093	10	2.768	1.33 ± 0.2*	F = 39.4, p < 0.001

*Significant difference in mean cells per crypt per tissue.

Table 12: Effect of tau-fluvalinate exposure on intestinal stem cell proliferation rate (cells/crypt)

Sampled Time	Concentration (N)	Mean	Control Mean	Water Control (N)	Mean Difference	ANOVA
Acute	3 Randomized[a] (11)	3.516	3.753	9	-0.24 ± 0.4	F = 0.3, p = 0.587
	3 minutes sequential (7)	4.280	3.886	(15)	-0.40 ± 0.5	F = 0.6, p = 0.454
	15 min (10)	3.005	3.093	Combined (11)	0.09 ± 0.3	F = 0.1, p = 0.798
Latent	3 minutes sequential (10)	3.077	3.318	(5)	0.24 ± 0.7	F = 0.2, p = 0.669
	15 minutes (13)	3.314	3.385	Combined (15)	0.07 ± 0.3	F = 0.5, p = 0.820

Comparison of mean acute proliferation and mean latent proliferation

Concentration	Acute proliferation Sample (N)	Mean	Latent proliferation Sample (N)	Mean	Mean Difference ± SE	ANOVA
3 minutes seq.	7	4.28	10	3.077	1.20 ± 0.6	F = 4.4, p = 0.052
15 minutes	10	3.005	13	3.314	-0.31 ± 0.2	F = 1.7, p = 0.200

[a]Latent proliferation effects could not be assessed because no samples were available.

Table 13: Effect of tau-fluvalinate exposure with coumaphos feeding on intestinal stem cell proliferation rate (cells/crypt)

Sampled Time	Exposure Time (N)	Mean	Control Mean	Control (N)	Mean Difference	ANOVA
Acute	3 randomized[b] (8)	4.391	4.782	0.02% Acetone (8)	0.39 ± 0.6	F = 0.4, p = 0.517
	3 sequential (8)	3.845	4.782	0.02% Acetone (8)	0.93 ± 0.6	F = 2.4, p = 0.147
	15 minutes (10)	2.622	2.594	Water[a] (6)	-0.03 ± 0.4	F = 0.0, p = 0.941
Latent	3 sequential (11)	3.535	3.366	0.02% Acetone (10)	-0.17 ± 0.4	F = 0.2, p = 0.701
	15 minutes (10)	2.986	2.345	Water[a] (10)	-0.64 ± 0.5	F = 1.6, p = 0.228

Comparison of mean acute proliferation and mean latent proliferation

Concentration	Acute proliferation Sample (N)	Mean	Latent proliferation Sample (N)	Mean	Mean Difference ± SE	ANOVA
Acetone	8	4.782	10	3.366	1.42 ± 0.6*	F = 5.3, p = 0.036
3 minutes seq.	8	3.845	11	3.535	0.31 ± 0.4	F = 0.6, p = 0.445
15 minutes	10	2.622	10	2.986	-0.36 ± 0.3	F = 1.6, p = 0.227

*Significant difference in mean cells per crypt per tissue. [a] Water was used as the control because of the bees originating from Wake Forest and which had no acetone control. [b]Latent proliferation effects could not be assessed because no samples were available.

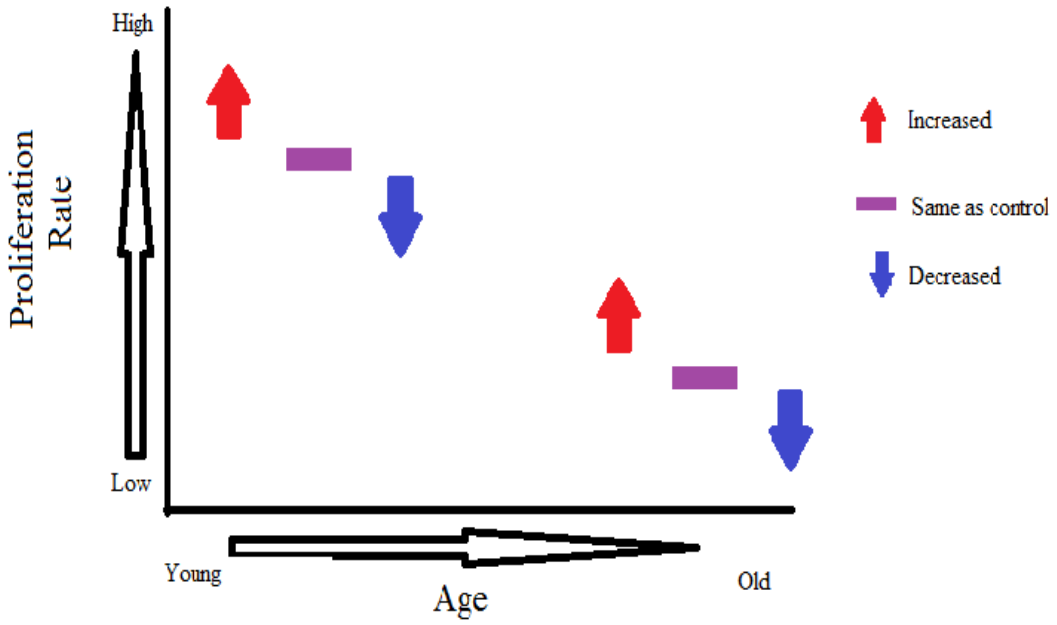

Figure 1. Possible acute and latent effects of xenobiotics on ISC proliferation. At each age at which proliferation is assessed, proliferation could either increase stay the same or decrease relative to control.

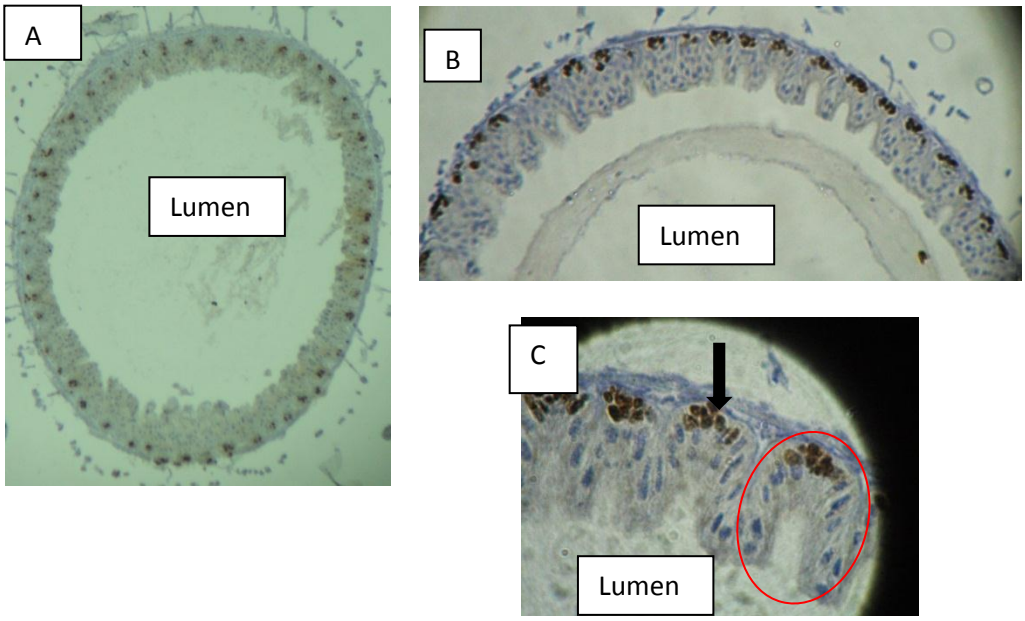

Figure 2: Cross-section of honeybee midgut. A is 40x magnified, b is 100x magnified and c is 400x magnified. Black arrow points to brown stained newly synthesized cell. Red circle surrounds a stem cell crypt. For data analysis, only cells found in well-defined cone shaped crypts were counted.

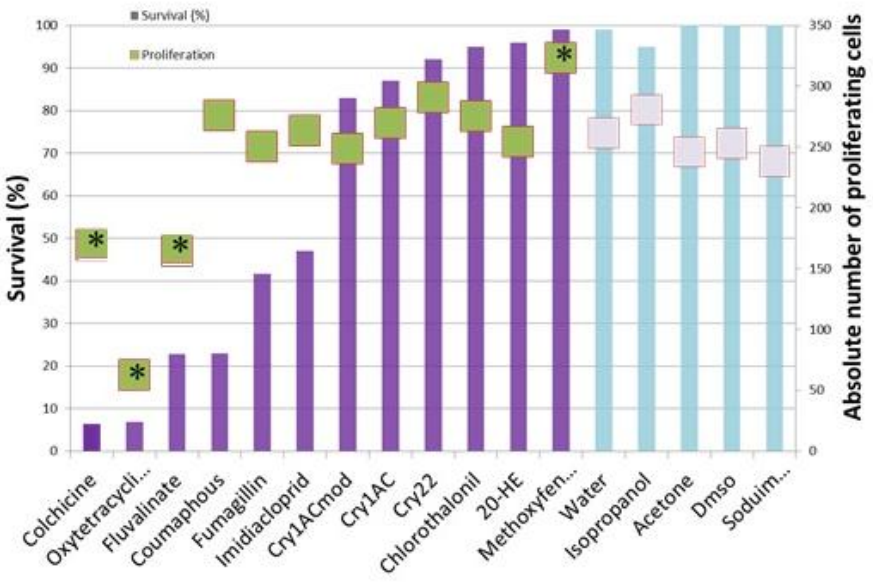

Figure 3. Percent of sampled population surviving past treatment period (7 days) (left y-axis indicated as bars) and absolute number of proliferating cells per tissue section (right y-axis indicated by squares). Due to high mortality, the feeding period for tau-fluvalinate was 3 days. Vehicle controls shown with light blue bars and grey squares. All xenobiotics except for 20-HE and methoxyfenozide did not have an effect on survival

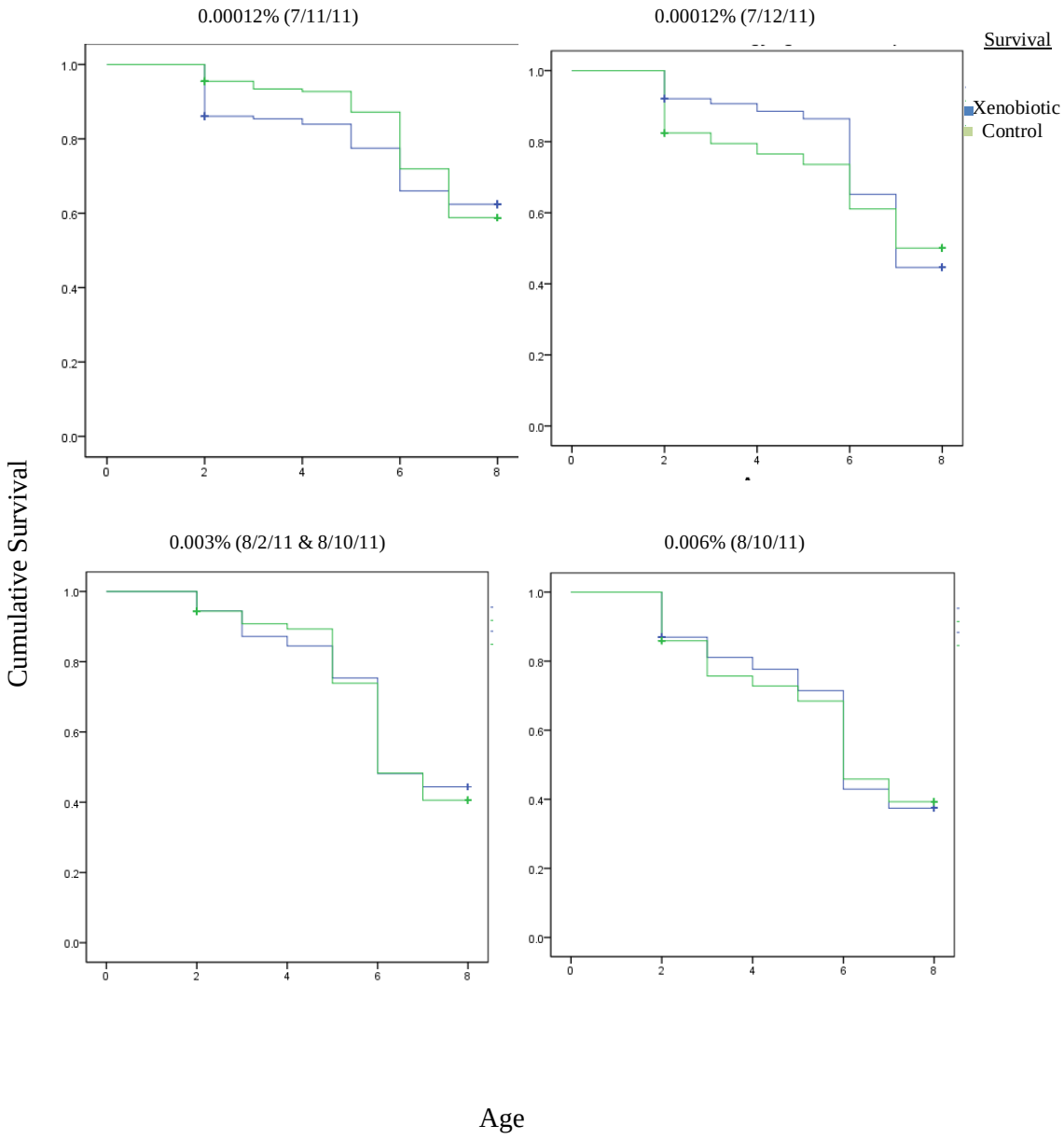

Figure 4: Acute survival curves of honeybees feed hydroxytetracycline. A) Honeybees emerging on 7/11/11/11 fed 0.00012% hydroxytetracycline. B) Honeybees emerging on 7/12/11 fed 0.00012% hydroxytetracycline. C) Honeybees emerging on 8/2/11 and 8/10/11 fed 0.003% hydroxytetracycline. D) Honeybees emerging on 8/10/11 fed 0.006% hydroxytetracycline. None of the concentrations fed caused a reduction in mortality compared to the control bees.

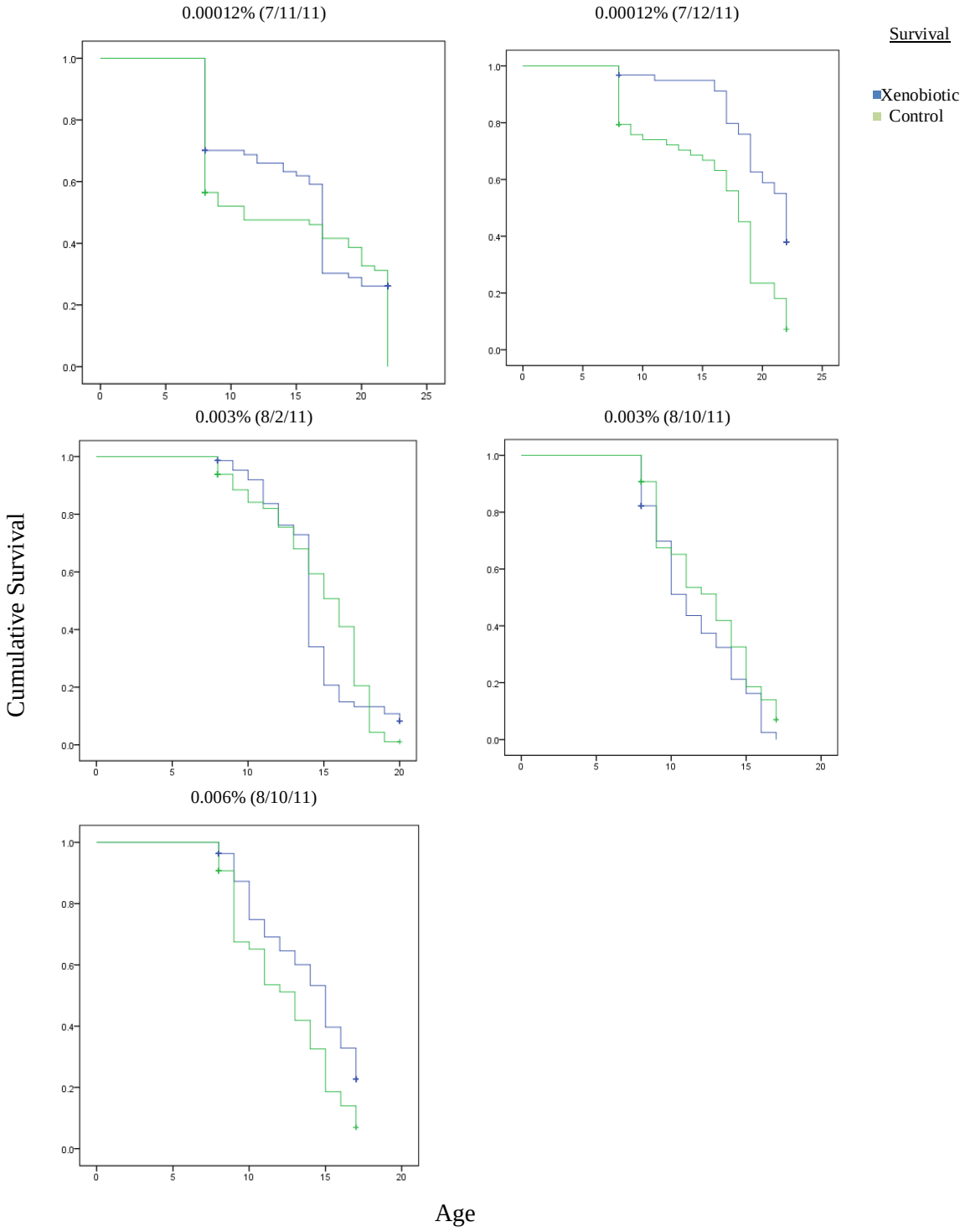

Figure 5: Latent survival curves of honeybees after hydroxytetracycline feeding. All concentrations tested were sub-lethal.

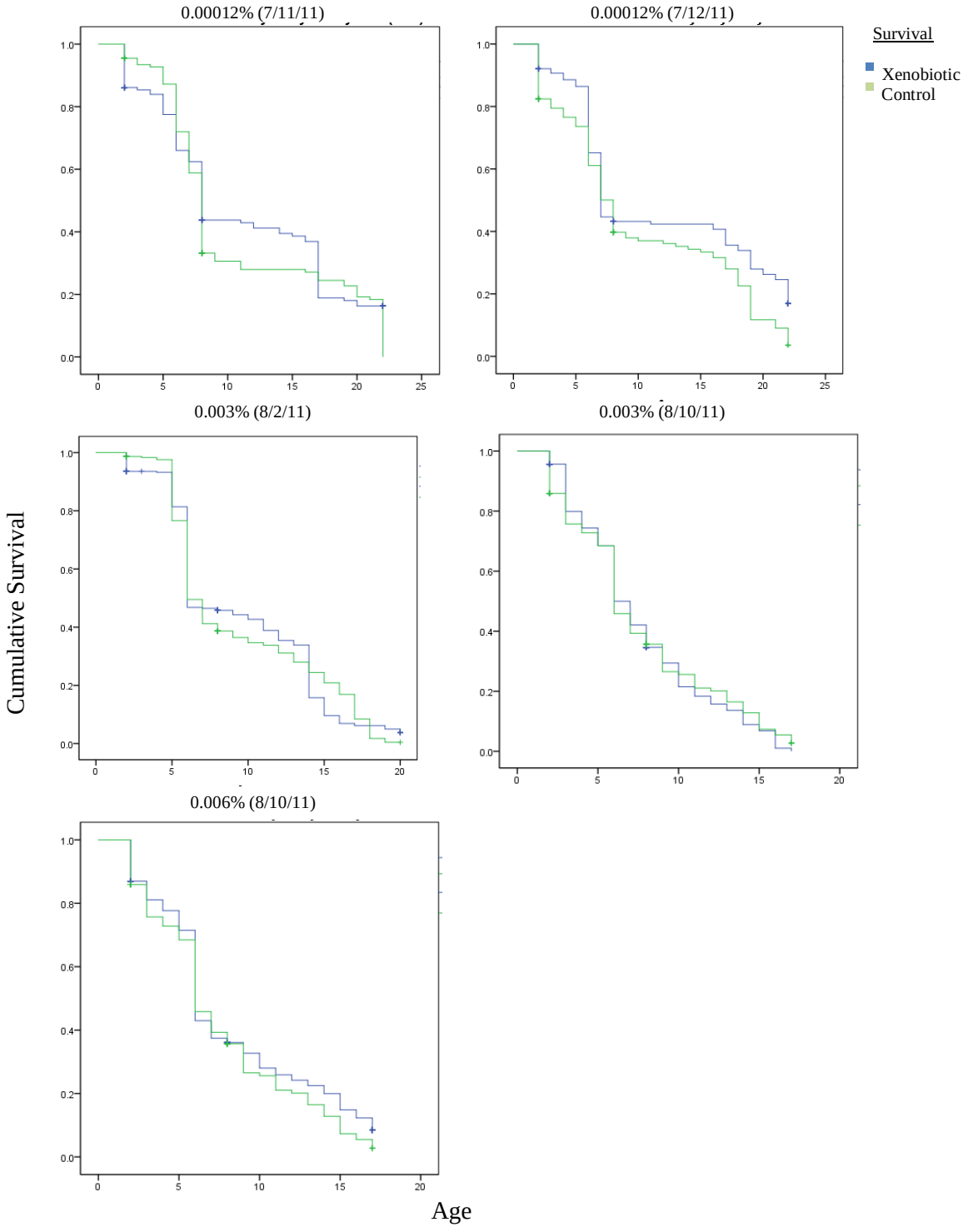

Figure 6: Cumulative survival curves of honeybees over 19-22 days of bees fed hydroxytetracycline. All concentrations were sub-lethal when compared to control.

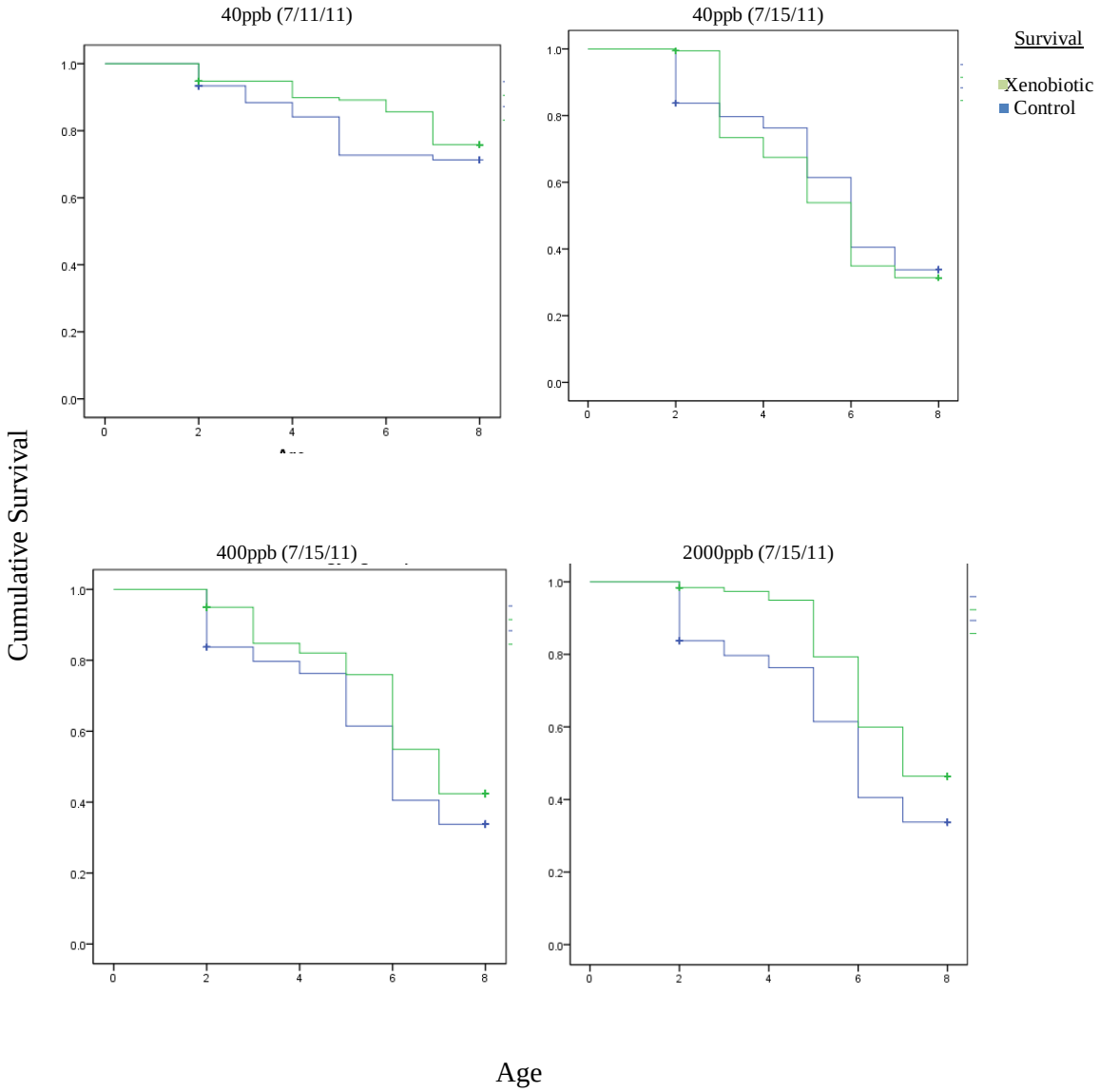

Figure 7: Acute survival curves of honeybees fed methoxyfenozide. All three concentrations were sub-lethal when compared to the acetone control.

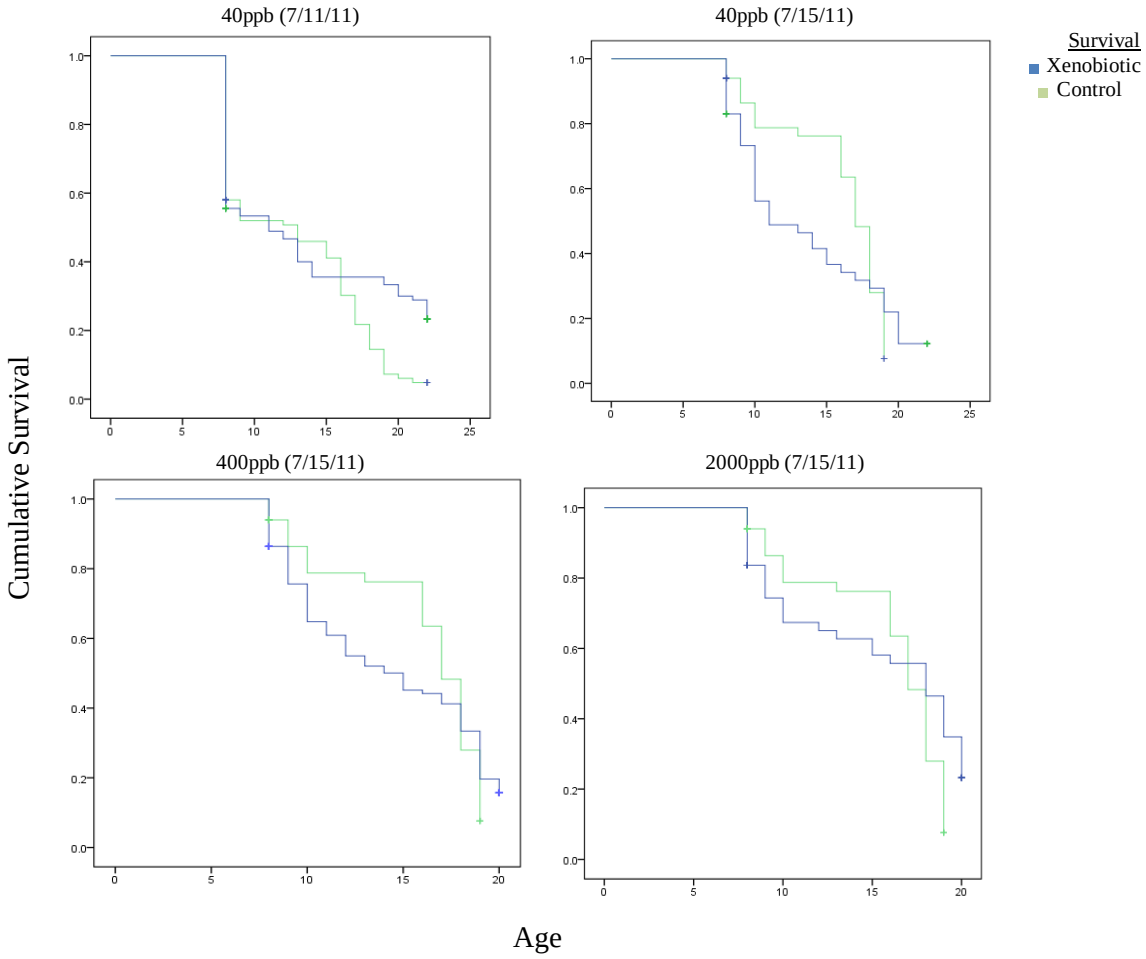

Figure 8: Latent survival curves of honeybees after methoxyfenozide feeding. All concentrations tested were sub-lethal.

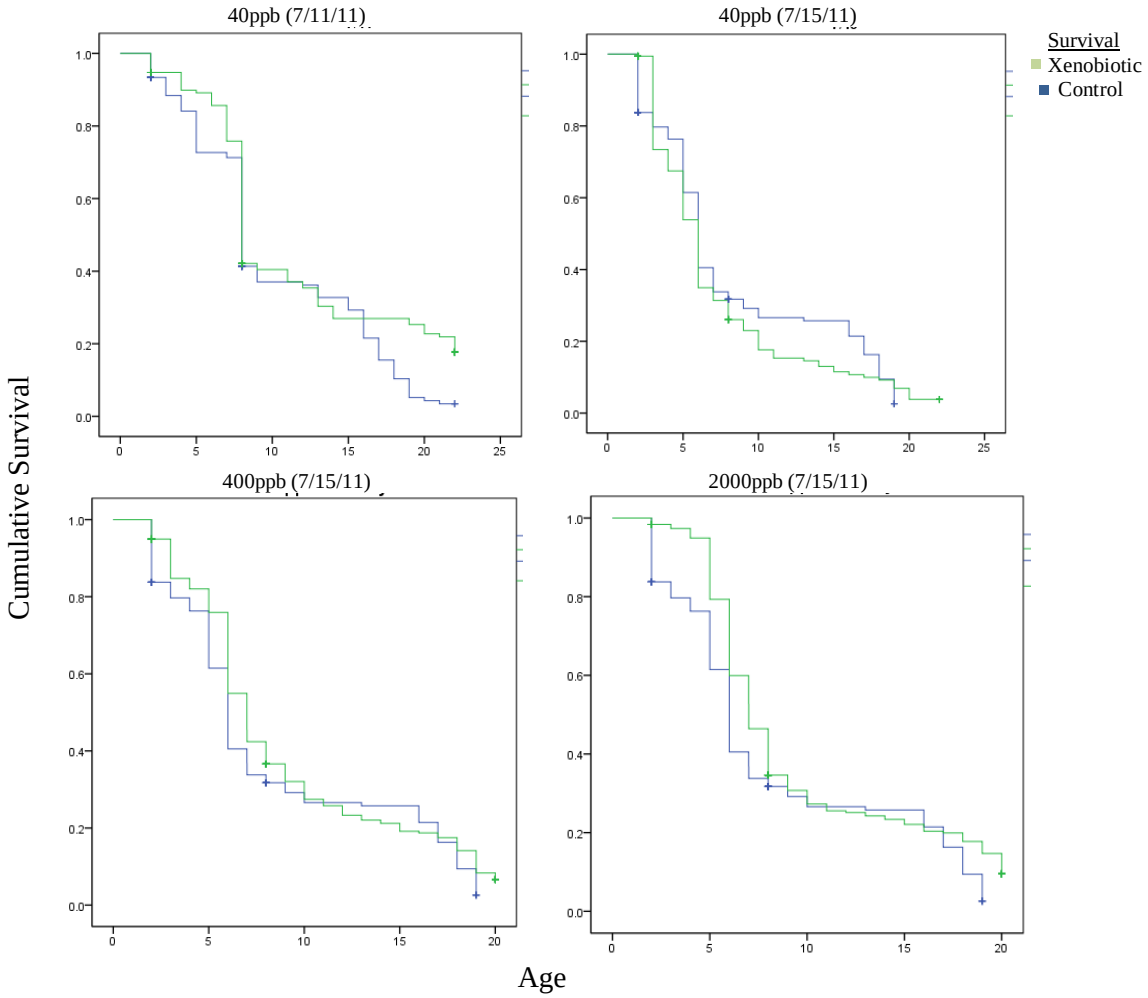

Figure 9: Cumulative survival curves of honeybees over 19-22 days of bees fed methoxyfenozide. All concentrations were sub-lethal when compared to control.

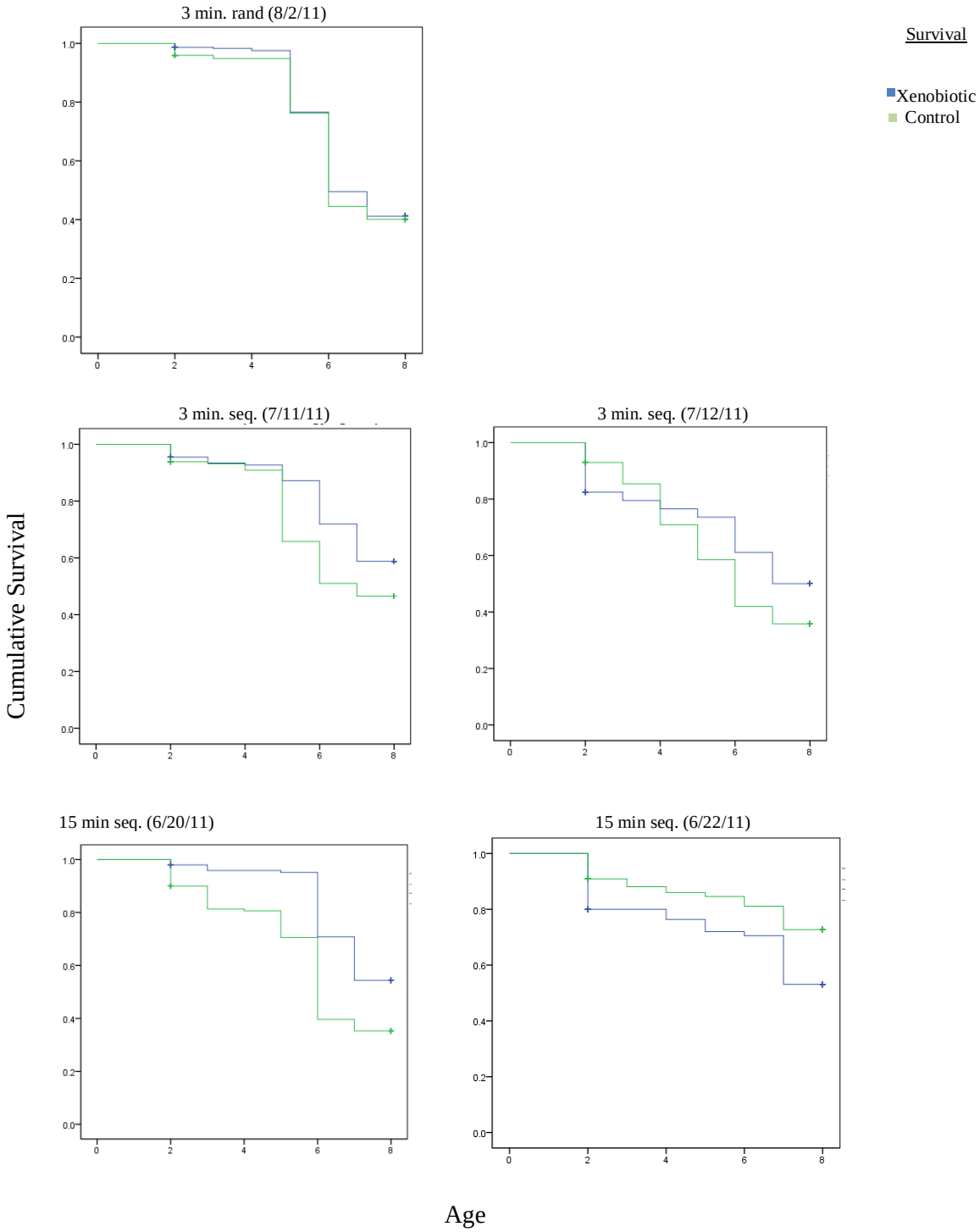

Figure 10: Acute survival curves of honeybees exposed to tau-fluvalinate. Bees exposed to 3 sequential minutes of tau-fluvalinate responded with a decrease in survival compared to water controls (p= 0.009 for bees emerging on 7/11/11 and p = 0.023 for bees emerging on 7/12/11). Honeybees exposed to 15 minutes of tau-fluvalinate originated from either Wake Forest's apiary or from a local beekeeper differed: Only the bees from Wake Forest responded to the treatment with an increase in mortality ($p < 0.001$).

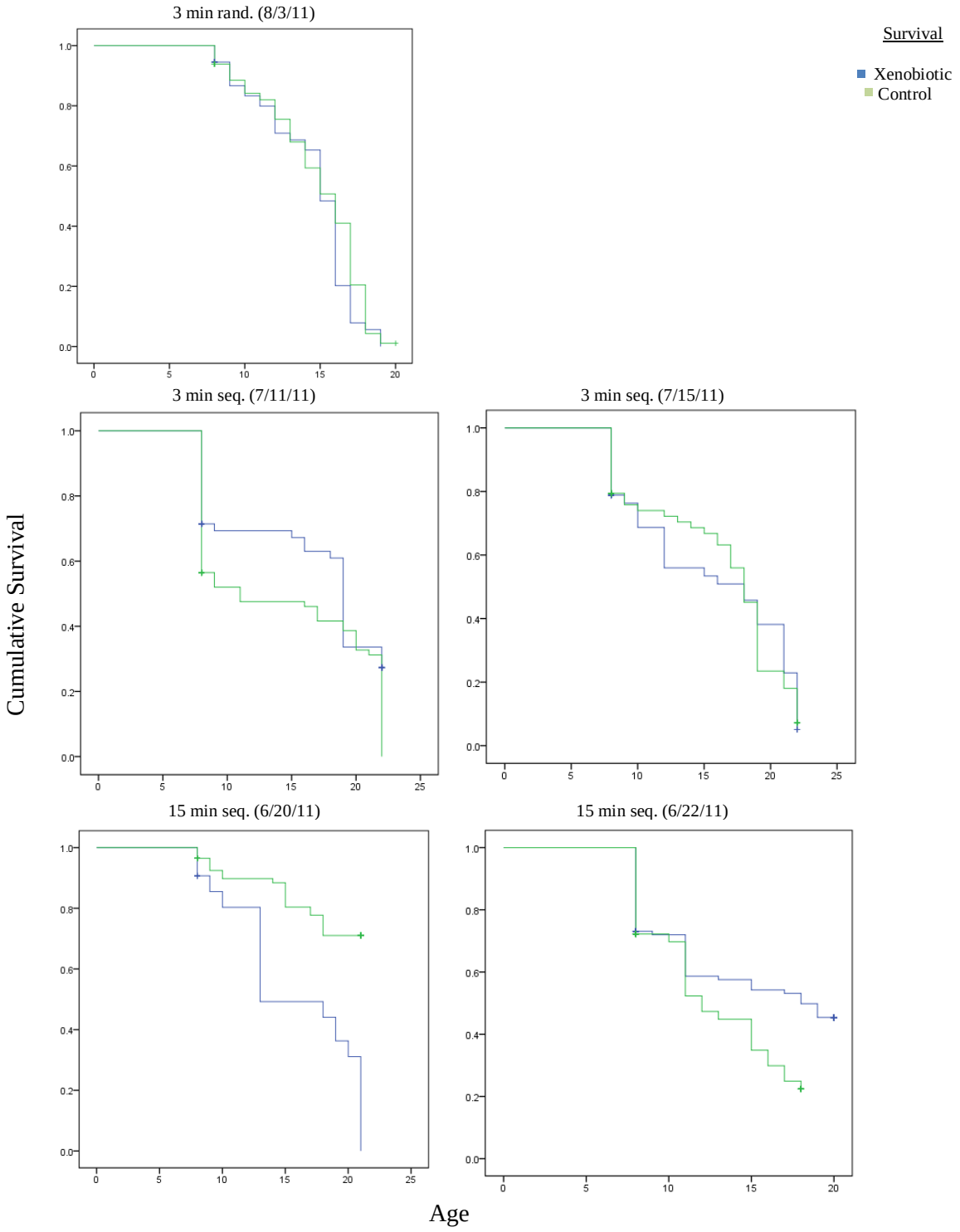

Figure 11: Latent survival curves of honeybees after tau-fluvalinate exposure. All concentrations were sub-lethal except for bees from 6/20/11 or bees acquired from Wake Forest University. These bees have a significantly decreased mean survival age ($p < 0.0001$) when treated.

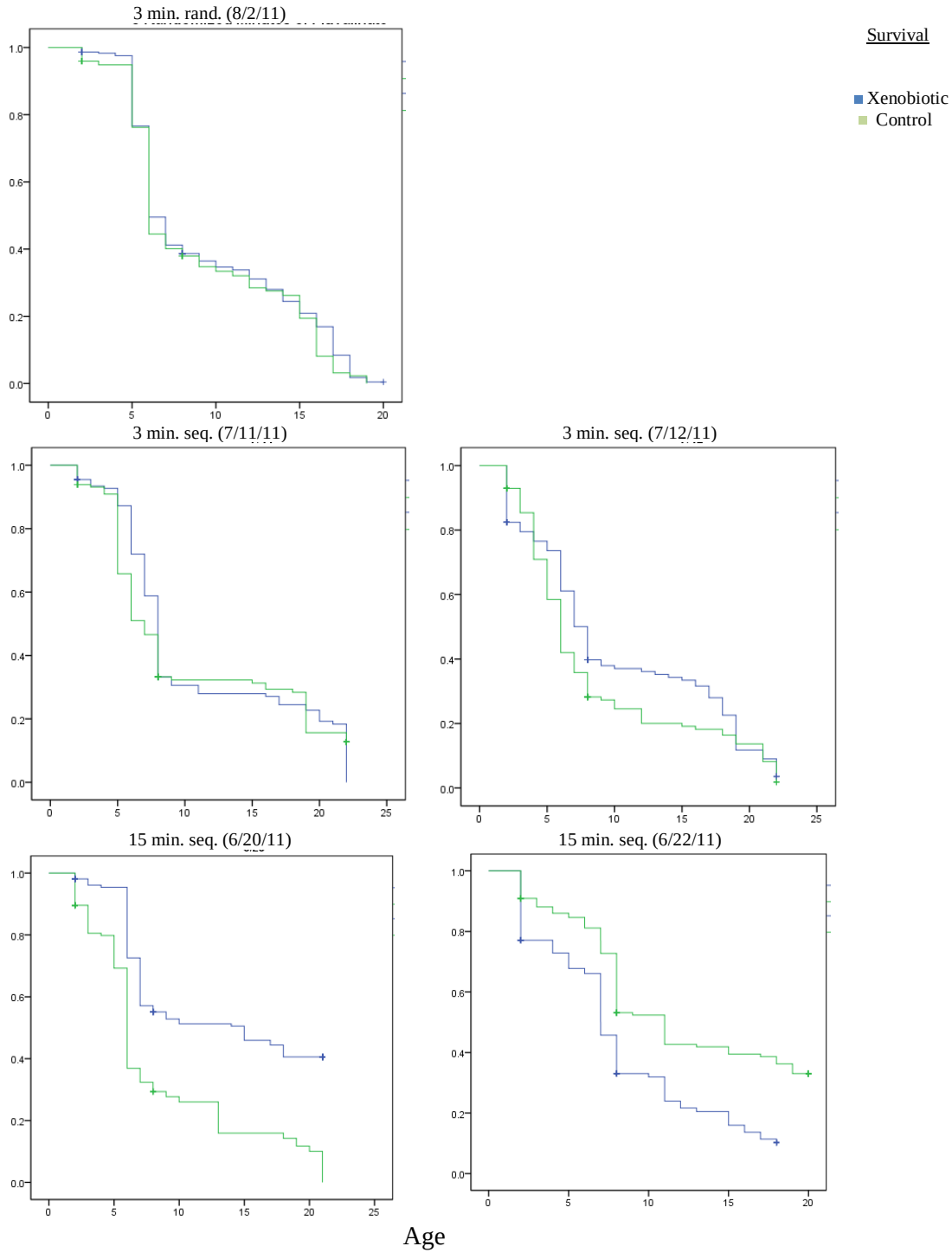

Figure 12: Cumulative survival curves of honeybees over 19-22 days of bees exposed to tau-fluvalinate. All concentrations were sub-lethal except for bees from 6/20/11 or bees acquired from Wake Forest. These bees had a significantly decreased mean survival age when treated ($p < 0.0001$).

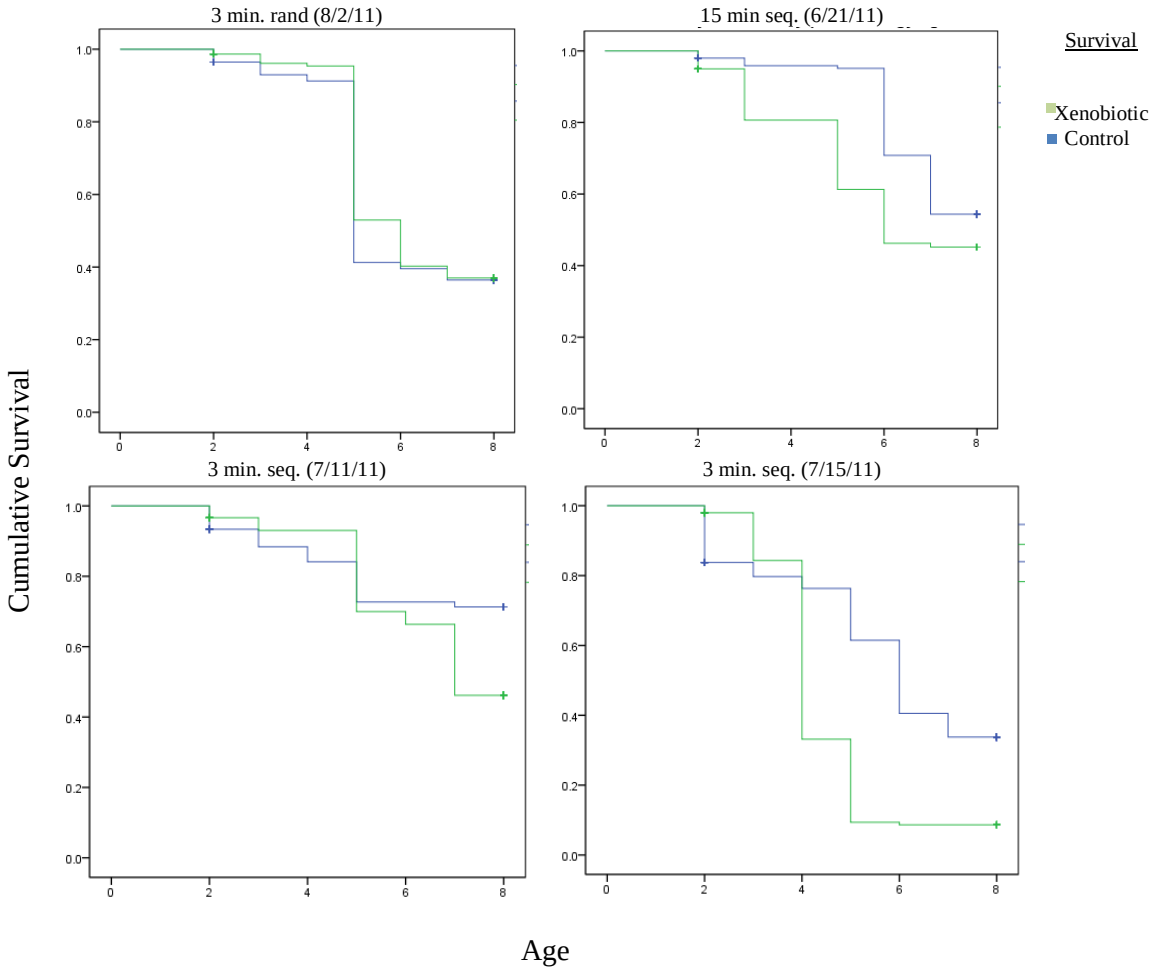

Figure 13: Acute survival curves of honeybees exposed to tau-fluvalinate with coumaphos feeding. Only the lowest exposure time (3 minutes randomized exposure) did not show significant departures from the survival of the respective controls.

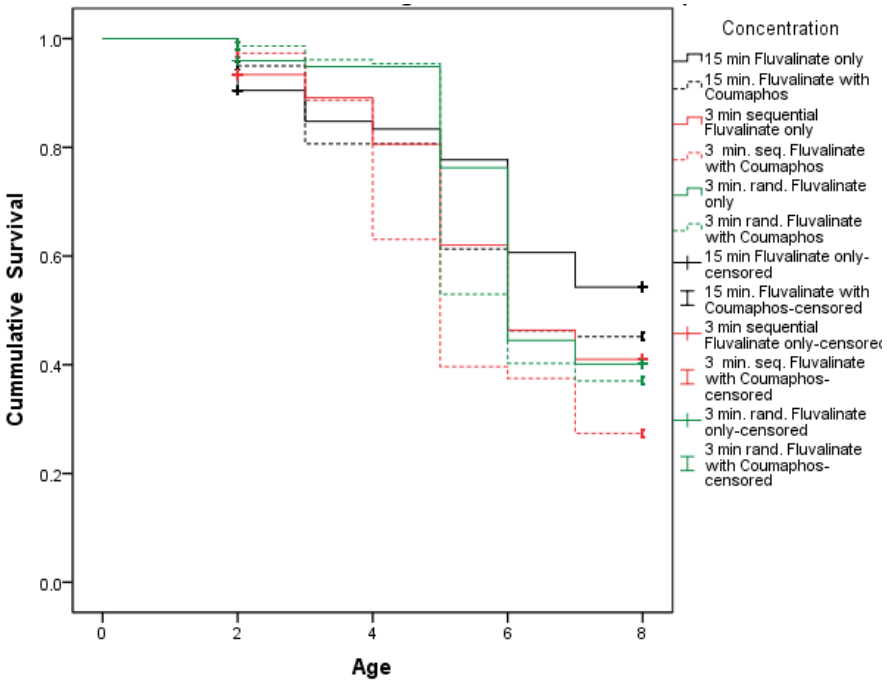

Figure 14: Comparing tau-fluvalinate only exposure and exposure with fluvalinate and coumaphos. The comparison showed that there was a decrease in estimated mean survival for all concentrations tested when tau-fluvalinate was combined with coumaphos. P= 0.015, 0.000, 0.049 for 15 minutes, 3 minutes sequential and 3 minutes randomized exposure, respectively.

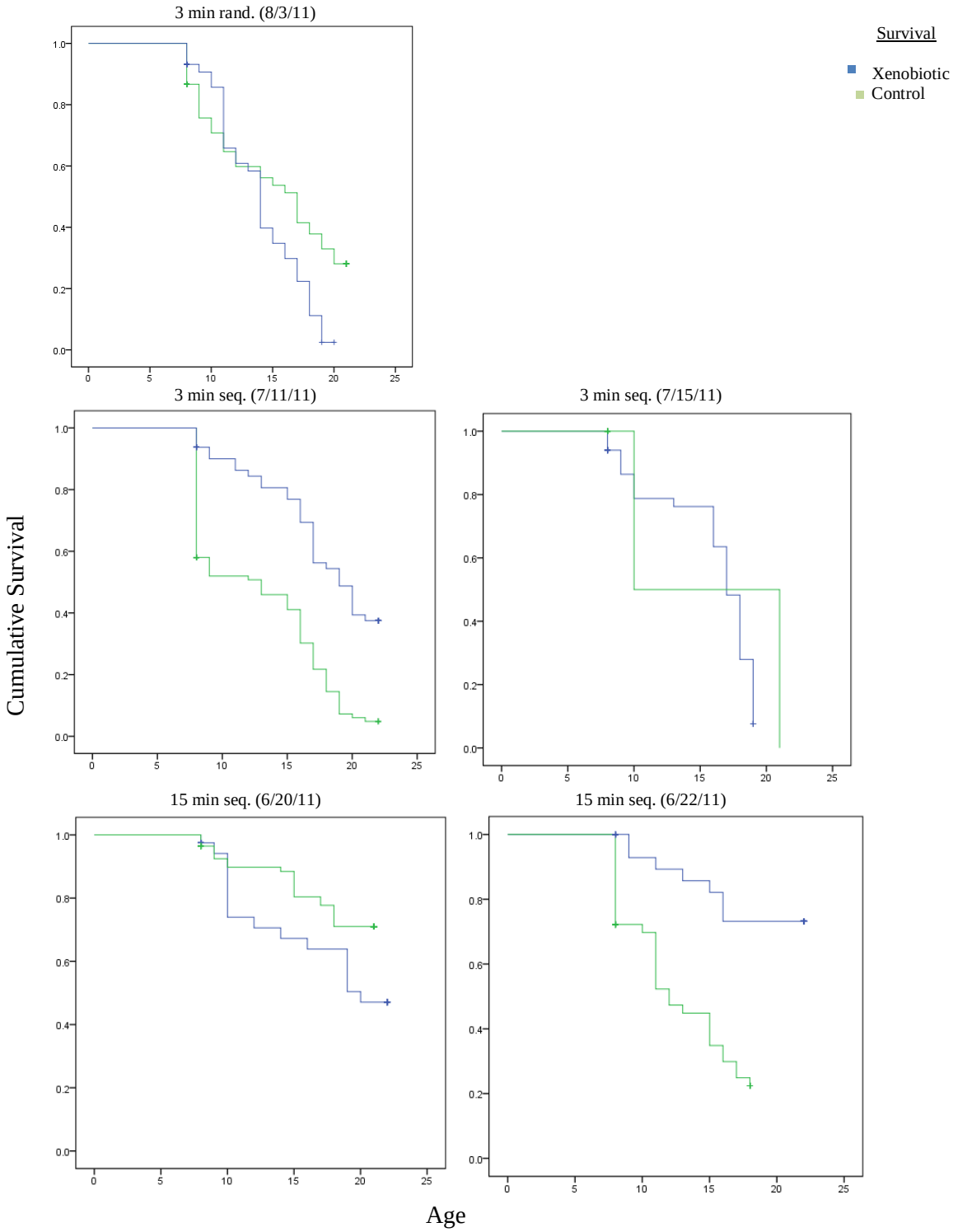

Figure 15: Latent survival curves of honeybees after tau-fluvalinate exposure with coumaphos feeding. All concentrations were sub-lethal except for bees from 6/20/11 or bees acquired from Wake Forest University. These bees had a significantly decreased mean survival age when treated (p=0.026).

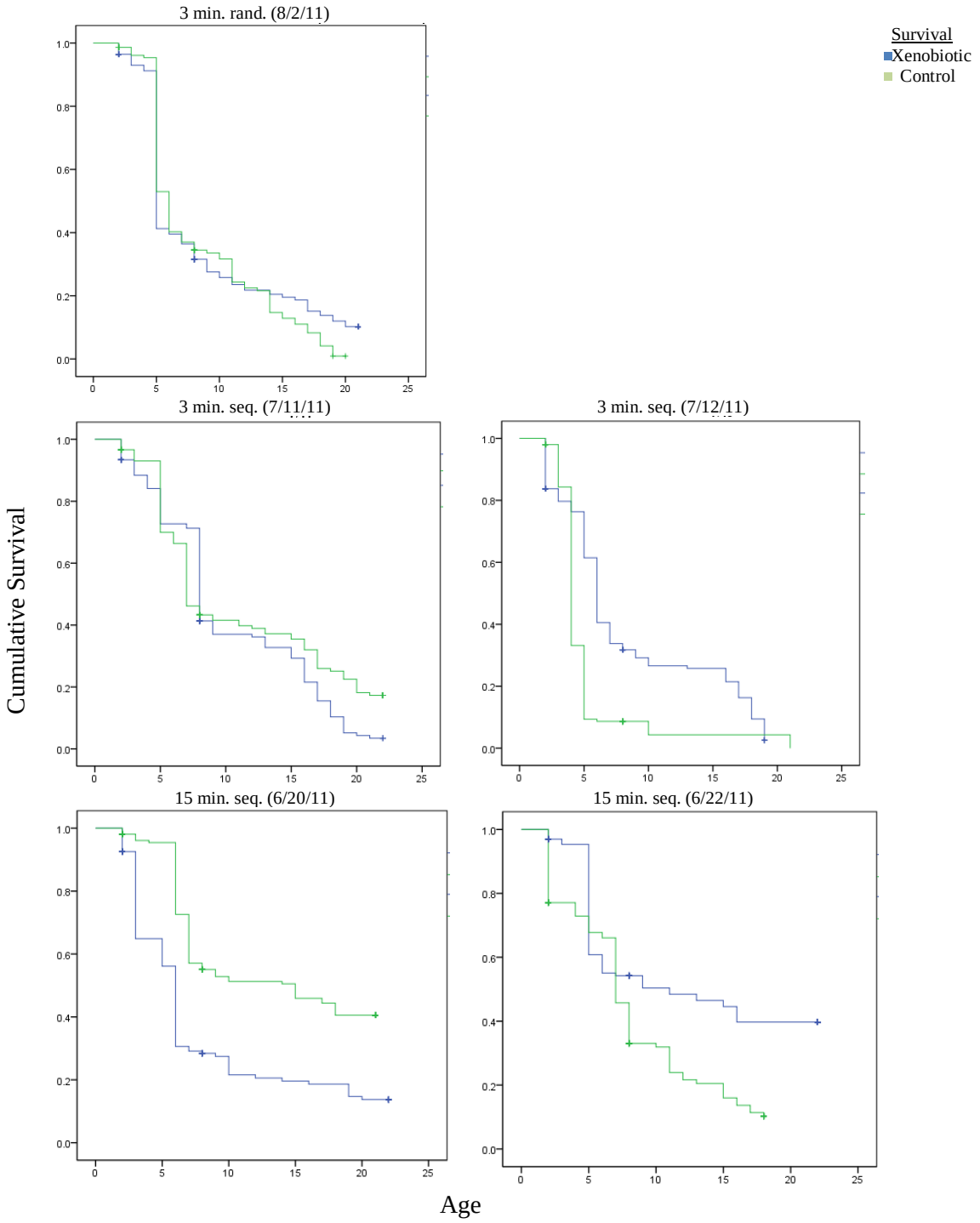

Figure 16: Cumulative survival curves of honeybees over 19-22 days of bees exposed to tau-fluvalinate with coumaphos feeding. All concentrations were sub-lethal except for bees from 6/20/11 or bees acquired from Wake Forest University ($p < 0.0001$) and bees emerging on 7/12/11 exposed 3 sequential minutes of tau-fluvalinate ($p < 0.0001$).

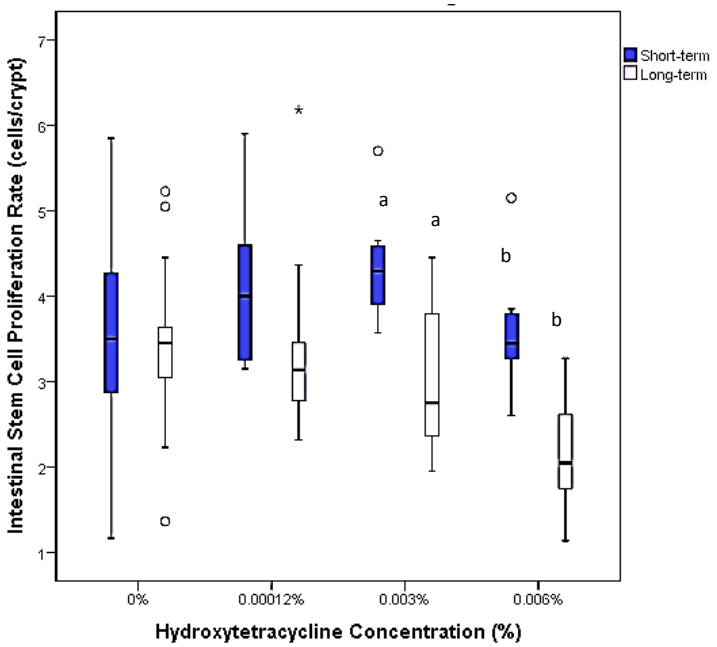

Figure 17: Effect of hydroxytetracycline ingestion on intestinal stem cell proliferation rate immediately after proliferation (Acute) and 19-22 days after proliferation (Latent). There was a significant difference between bees as they age for the two highest concentrations fed. $p = 0.005$ for 0.003% and $p = 0.004$ for 0.006% hydroxytetracycline. The concentration effect on proliferation between 0.006% and 0.003% [b] $p = 0.024$.

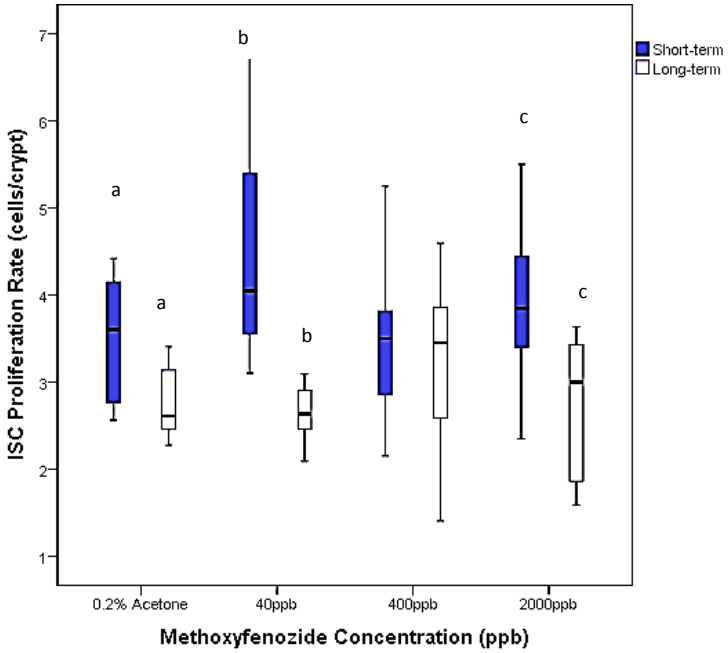

Figure 18: Effect of methoxyfenozide ingestion on intestinal stem cell proliferation immediately after proliferation (Acute) and 19-22 days after proliferation (Latent). There were no dosage effects. The 40ppb[b] and the 200ppb[c] caused aging effects ($p = 0.001$ and $p = 0.01$ respectively). There was also an age effect between short-term acetone samples and long-term acetone samples[a] ($p= 0.036$).

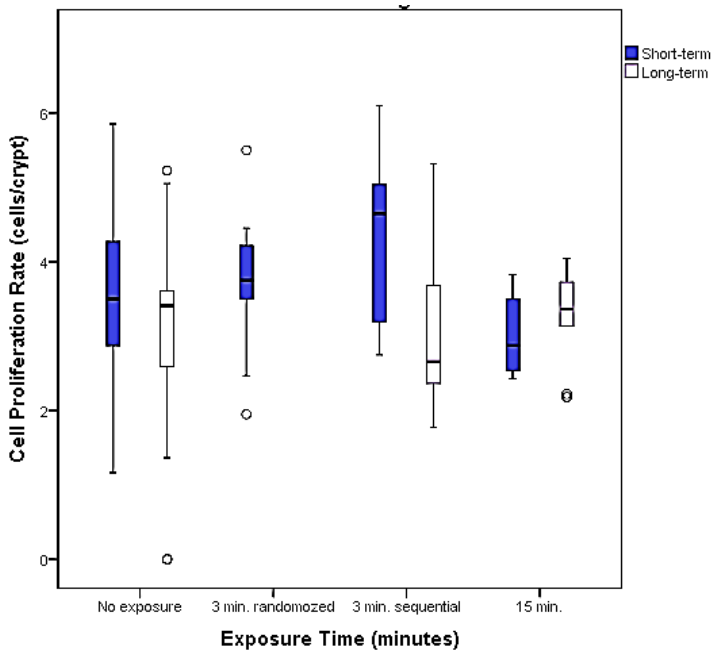

Figure 19: Effect of tau-fluvalinate exposure on intestinal stem cell proliferation immediately after proliferation (Acute) and 19-22 days after proliferation (Latent). There were no dose effects of tau-fluvalinate exposure. However there was a trend towards an age effect when bees were exposed to 3 minutes of tau-fluvalinate sequentially ($p = 0.052$).

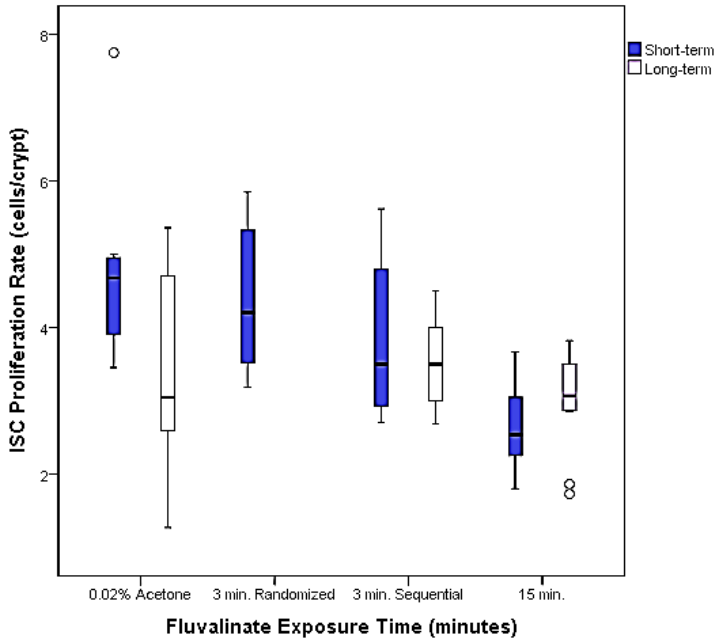

Figure 20: Effect of tau-fluvalinate exposure with coumaphos ingestion on intestinal stem cell proliferation immediately after proliferation (Acute) and 19-22 days after proliferation (Latent). For bees sampled immediately after exposure there was a significant decrease in proliferation between the highest exposure time and the control acetone ($p = 0.001$) or the lowest exposure time ($p= 0.006$). However, it should be noted that the bees from the highest exposure experiment originated from Wake Forest and a local beekeeper, not from the UNCG apiary. The only age effect seen was within the acetone control bees ($p = 0.036$)

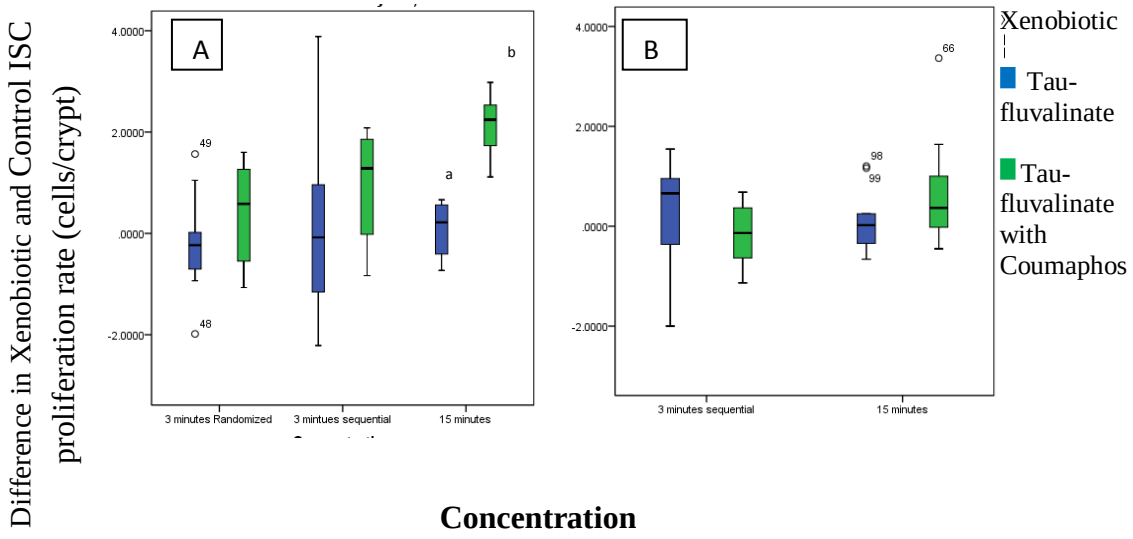

Figure 21: Comparison of proliferative effects of adding coumaphos to tau-fluvalinate. A) Bees sampled after day 7 directly after the xenobiotic exposure, acute proliferation assay. B) Bees sampled at older ages, latent assay. There were no bees from 3 minutes of randomized exposure after 19 observation days left. Thus, BrdU assays could not be done. Only bees exposed to 15 minutes of tau-fluvalinate showed a significant increase in proliferation when coumaphos feeding was combined with exposure ($p < 0.001$), with a mean difference to the control of 2.07 labeld cells /crypt. Bees assayed after 19 days of obsevation did not show a significant differnce in proliferation between the two treatment groups.

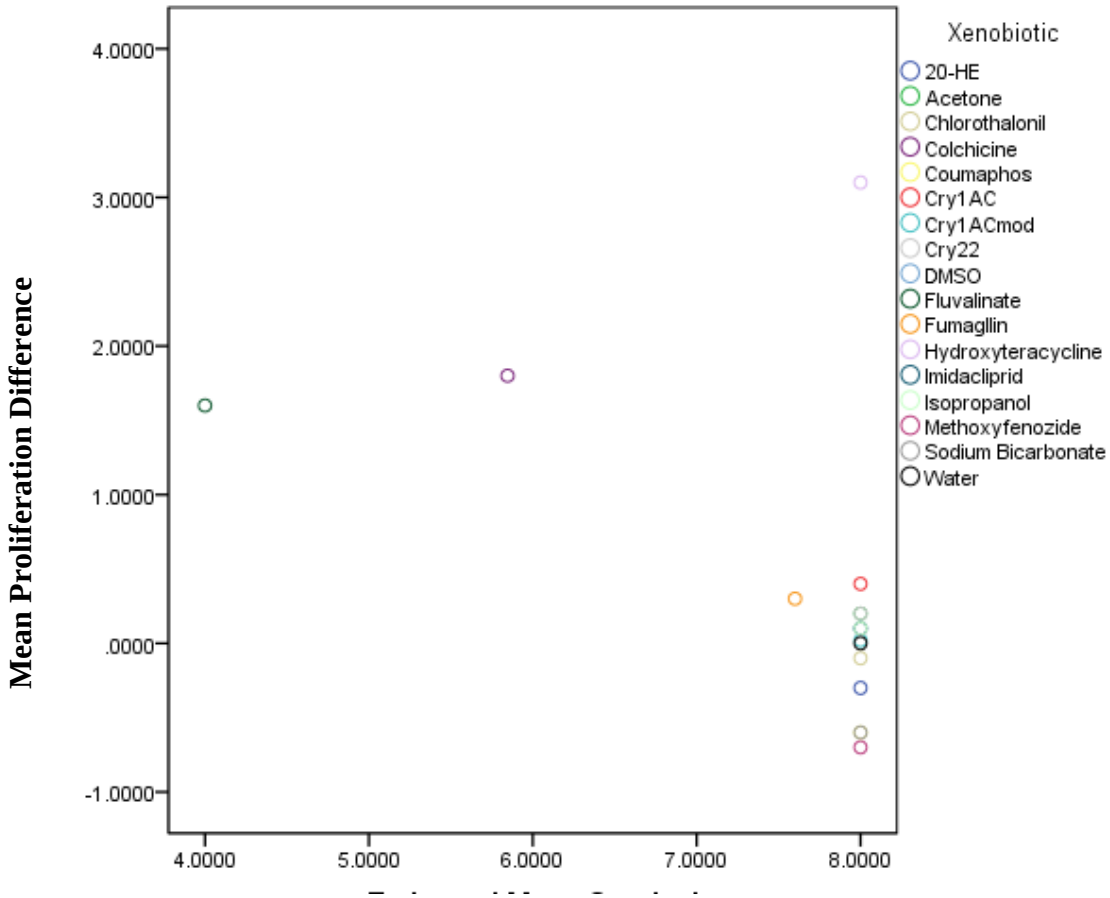

Figure 22: Mean survival difference of xenobiotic treatments from respective control treatments compared to the mean proliferation difference of xenobiotic treatments from respective control. Treatments show a negative correlation between relative ISC proliferation and mortality (r= -0.493, p= 0.04).

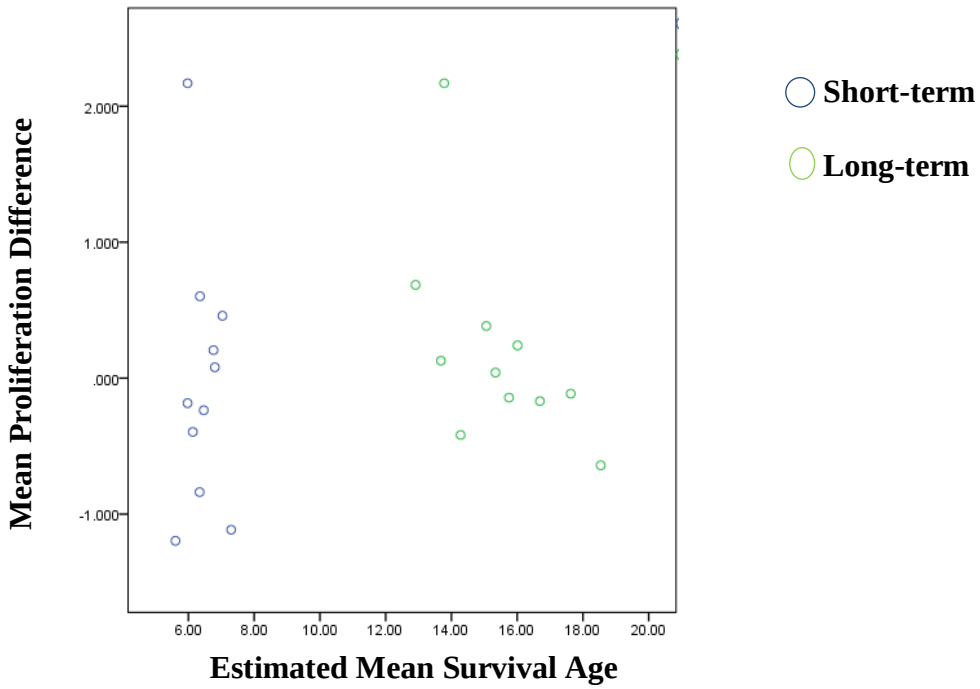

Figure 23: Acute and latent survival correlated to acute and latent intestinal stem cell (ISC) proliferation rate mean differences from controls. Survival is not correlated to ISC proliferation rate short-term (r= -0.207, p= 0.477) or long-term (r=-0.580, p= 0.061).