

Bt maize and frogs: An investigation into possible  
adverse effects of Bt toxin exposure to  
amphibian larvae

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## Abstract

Genetically modified maize expressing the Bt-protein Cry1Ab (Bt maize) is planted widely in South Africa. Crop residues of Bt maize often end up in aquatic ecosystems where aquatic organisms are exposed to Cry1Ab protein. The effect of this protein on non-target aquatic organisms has not yet been studied in South Africa. The aim of this study was to evaluate the possible effect of exposure to Bt maize on morphological development of *Xenopus laevis* and *Amietophrynus gutturalis* tadpoles. Three experiments were conducted with each of *X. laevis* and *A. gutturalis*. Five of these were conducted in the bio-secure Amphibian Biology laboratory and one with *A. gutturalis* in a shade-house facility where microcosms were exposed to natural conditions. In the first experiment of *X. laevis* and *A. gutturalis*, which was replicated three times, large portions of maize leaves were placed in the bottoms of microcosms. *X. laevis* received supplementary pulverised leaves in suspension while *A. gutturalis* tadpoles fed on provided leaves. For both control and experimental groups microcosms were divided in three groups receiving respectively 15, 30 and 45 g of maize leaves. In the second and third experiment tadpoles only received pulverised Bt maize leaves in suspension. Each replicate (microcosm) contained 50 one-day old tadpoles. Experiment two was conducted to determine whether the Bt-protein has adverse effects on *A. gutturalis* tadpoles when tadpoles are exposed to the protein in the water but not feeding on the plant material. A total of 100 tadpoles were used during the experiment and tadpoles were placed individually in 250 ml plastic cups that were filled with 100 ml water with contained an extract of either Bt and non-Bt maize leaves. Tadpoles were fed twice a week with TetraTabimin bottom-feeding fish pellets in suspension. Experiment three was conducted to determine whether the Bt-protein will have adverse effects on *A. gutturalis* tadpoles when tadpoles feed on Bt maize leaves. Tadpoles were divided into a treatment in which 50 tadpoles were fed Bt maize leaves and a control treatment in which 50 tadpoles were fed non-Bt maize leaves. Tadpoles were placed individually in 250 ml plastic cups that were each filled with 100 ml borehole water. On a weekly basis 10 randomly selected tadpoles were collected, measured and staged for morphological development, using the Nieuwkoop and Faber Normal Table for *X. laevis* and Gosner stages for *A. gutturalis* tadpoles. The significant effects observed in some life history parameters of tadpoles exposed to Cry1Ab protein cannot be ascribed to the effect of the protein. Poor husbandry turned out to be the single most important confounding factor. Before follow-up studies are conducted husbandry practices should be optimized.

Keywords: Bt maize, Bt-protein, Cry1Ab, *Xenopus laevis*, *Amietophrynus gutturalis*, Nieuwkoop and Faber Normal Table, Gosner staging

## Uittreksel

Geneties-gemodifiseerde mielies (Bt mielies) wat die Bt-protiën Cry1Ab vervaardig, word algemeen in Suid-Afrika aangeplant. Oesreste van Bt-mielies beland dikwels in akwatiese ekosisteme waar akwatiese organismes aan die Cry1Ab toksien blootgestel word. Die effek van hierdie proteïen op nie-teiken akwatiese organismes is nog nie in Suid-Afrika bestudeer nie. Die doel van die studie was om die moontlike effek van blootstelling aan Cry1Ab op die morfologiese ontwikkeling van *Xenopus laevis* en *Amietophrynus gutturalis* paddavissies te evalueer. Ses eksperimente is uitgevoer. Vyf eksperimente is onder gekontroleerde toestande in die biosekuur amfiërlaboratorium van die NWU uitgevoer en een blootstellingseksperiment is in 'n buitenshuise groeikamer met skadunet uitgevoer. In die eerste eksperiment met *X. laevis* en *A. gutturalis* is paddavisse blootgestel aan heel mielieblare wat op die bodem van die glasbakke gehou is deur 'n glas raam. Die kontrole en eksperimentele groep is onderverdeel in drie groepe wat onderskeidelik aan 15, 30 en 45g mielieblare blootgestel is. In hierdie eksperiment is paddavissies aan die Bt-proteïen wat uit die blare geloog het blootgestel, terwyl die filtervoedende *X. laevis* paddavissies aanvullende voedsel in die vorm van verpoëerde mielieblare in suspensie ontvang het. *Amietophrynus gutturalis* paddavissies het geen bykomstige voeding ontvang nie aangesien hulle bodemvoeders is. In die tweede en derde *X. laevis* eksperimente het die paddavissies net verpoëerde Bt en nie-Bt mielieblare onderskeidelik in suspensie ontvang. In die tweede en derde *A. gutturalis* eksperimente is 100 paddavissies individueel in 250 ml glasies, elk met 100 ml water daarin geplaas. Eksperiment twee is in twee groepe verdeel. Vyftig paddavissies het Bt-mielieblare as voeding ontvang en 50 as kontrole nie-Bt mielieblare. Die oogmerk met hierdie eksperiment was om vas te stel of enige nadelige effekte waargeneem kan word wanneer paddavisse blootgestel word aan die Bt proteïen in die water sonder dat hulle toegelaat is om aan die blare te vreet. In eksperiment 3 wat soortgelyk as eksperiment 2 opgestel is, is die 50 paddavissies individueel blootgestel aan stukkies Bt en nie-Bt blare en toegelaat om daaraan te vreet. Albei groepe het TetraTabimin as voeding ontvang. Op 'n weeklikse basis is 10 paddavissies lukraak gekies, gemeet en die vlak van morfologiese ontwikkeling met behulp van die Nieuwkoop en Faber Normale Tabel vir *X. laevis* en die Gosner staduims vir *A. gutturalis* paddavissies bepaal. Probleme is met die studie ondervind en die betekenisvolle verskille wat wel gevind is kan nie aan die teenwoordigheid van die Cry1Ab proteïen toegeskryf word nie aangesien praktiese probleme met die aanhou en versorging van die proefdiere ondervind is. Hierdie studie het wel bygedra om te dien as protokol ontwikkeling. Alvorens enige verder evaluasies van die effek van Bt mielies op paddavisse gedoen word, is dit nodig dat die eksperimentele tegnieke verfyn word.

Sleutelwoorde: Bt-mielies, Bt-proteïen, Cry1Ab, *Xenopus laevis*, *Amietophrynus gutturalis*, Nieuwkoop en Faber Normale Tabel, Gosner staduims

## **Declaration**

I declare that the work presented in this Masters dissertation is my own work, that it has not been submitted for any degree or examination at any other university, and that all the sources I have used or quoted have been acknowledged by mean of complete references.

Signature of Student:.....

Signature of Supervisor:.....

# CHAPTER 1

## INTRODUCTION AND LITERATURE REVIEW

### 1.1. History of genetically modified crops

Through the use of scientific developments such as the genetic manipulation of plants to produce certain traits, food production may be able to keep pace with the increasing human population over the next few decades. The most important breakthrough in the development of genetically modified (GM) crops occurred when Murai *et al.*, (1983) produced sunflower (*Helianthus annuus*) tissues carrying a seed-protein gene from French bean (*Phaseolus vulgaris*), and through this demonstrated that a plant gene can express effectively after transfer to a taxonomically distinct botanical family. Scientists use various methods to produce elite cultivars, for example artificial crossing or hybridization, in the development of desirable traits. When desired characteristics, such as insecticidal properties are unavailable in cultivated plants, genes from other organisms such as *Bacillus thuringiensis* (Bt) are used and incorporated into the genome of the crop plant (Conway, 1999). After this discovery it took more than a decade for the release of the first commercial GM crops. The first commercial release of a GM crop took place in 1994 in the USA the FLAVR SAVR™ tomato (*Solanum lycopersicum*) (Nap *et al.*, 2003). The first herbicide tolerant and insect-resistant traits in soybean (*Glycine max*), cotton (*Gossypium* spp.), maize (*Zea mays*) and canola (*Brassica napus*) were launched into the global market in 1996 (Nap *et al.*, 2003).

The main goal of these traits was to benefit farmers directly, to reduce labour inputs, increase productivity, reduce chemical usage and production costs, and improve grower health (Gianessi & Carpenter, 1999; Fernandez-Cornejo *et al.*, 1999; Perlak *et al.*, 2001; Pray *et al.*, 2001; Ismael *et al.*, 2001; Traxler *et al.*, 2001; Huang *et al.*, 2002; Bennett *et al.*, 2004; Shankar & Thirtle, 2005). By 1997 the Department of Agriculture in South Africa issued the first conditional commercial release permits for GM crops in South Africa. These were genetically modified cotton and maize (insect-resistant maize) (Biowatch South Africa, 2011).

Bt maize with insecticidal properties is one of the most widely planted GM crops in the world. The genes from different strains of *Bacillus thuringiensis* produce toxins

effective against different groups of insects. By using genetic engineering, modified novel genes from *B. thuringiensis* (Bt) were introduced into maize, to control lepidopteran stem borers. The product is Bt maize which has inherent resistance to stem borers due to the presence of proteins produced by these genes. These proteins (Cry1Ab and Cry2Ab) were found effective against lepidopteran stem borers (*Chilo partellus* and *Busseola fusca*), (Tende *et al.*, 2005; Van Rensburg, 2007; Kruger *et al.*, 2009).

*Bacillus thuringiensis* is a ubiquitous gram-positive soil bacterium that forms crystalline protein inclusions during sporulation. Plants which are modified to produce an insecticidal protein from Bt are known as Bt-protected plants. The inclusion bodies consist of proteins (referred to as cry-proteins) which are selectively active against a specific range of insects and, as a class of proteins, are effective against a wide variety of insect pests. Cry-proteins are produced as protoxins that are proteolytically activated upon ingestion. Cry-proteins bind to specific sites (i.e., receptors) in the midgut cells of susceptible insects and form ion-selective channels within the cell membrane. The cells swell due to influx water which leads to cell lysis and ultimately the death of the insect (Van Rie *et al.*, 1989; English & Slatin, 1992; Gill *et al.*, 1992; Ferré & Van Rie, 2002; Betz *et al.*, 2000). The three major GM crops that are planted in South Africa are maize, cotton and soybeans. Maize occupies the largest area, accounting for 89% of the total GM crops planted in South Africa and consists of either herbicide tolerant or insect resistant crops as well as cultivars with stacked traits (contains both herbicide tolerance and insecticidal properties) (James, 2010).

## **1.2. Adoption rates of GM crops in South Africa**

South Africa is a developing country and for the first 12 years of commercialization of GM crops, 1997 to 2008, South Africa was the only country on the African continent to plant GM crops. In Africa there is a lead country commercializing biotech crops in each of the principal regions: South Africa in southern and eastern African region, Burkina Faso in West Africa and Egypt in North Africa (James, 2010). Africa is the continent that represents the biggest challenge in terms of adoption and acceptance of GM crops (James, 2011). South Africa is ranked ninth in the world for planting GM

crops with a total of 2.2 million hectares and accounts for one percent of the global production of GM crops (James, 2011). The adoption rate of GM crops in South Africa was slow until 2005 when the adoption rate increased rapidly until 2008 (James, 2010). The rate of increase between 2009 and 2011 was more gradual (James, 2011).

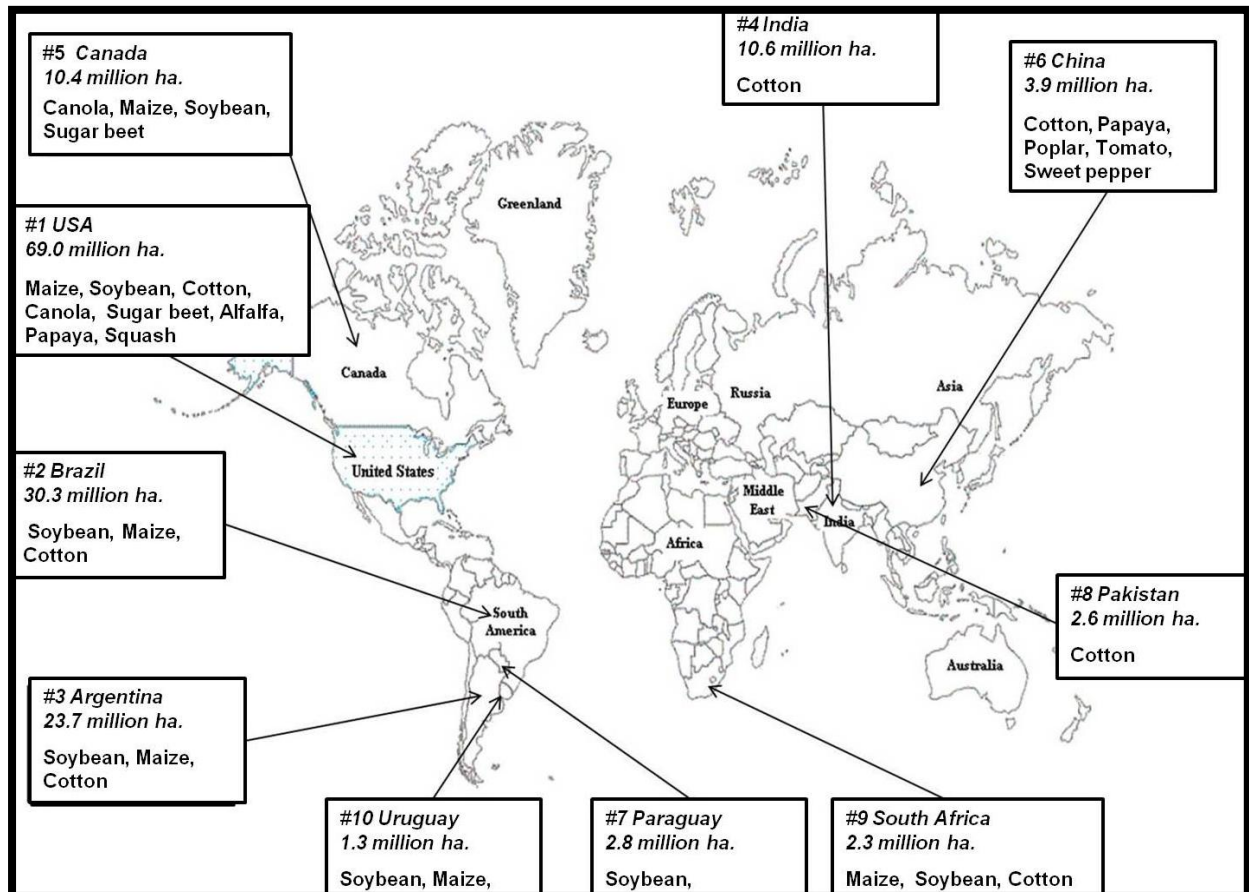


Figure 1.1. Ten mega-countries growing 50,000 hectares, or more, of biotech crops in the world (Modified from James, 2011).

### 1.3. Bt-proteins in aquatic ecosystems

Crop residues of Bt maize often end up in aquatic ecosystems where inhabiting organisms are exposed to Cry1Ab protein (Fig 1.2) (Rosi-Marshall *et al.*, 2007; Tank *et al.*, 2010). The frequency of dissolved Cry1Ab in stream water suggests that streams are integrating the patchy distribution of Cry1Ab containing debris. It is however unknown if there are ecological consequences for stream-dwelling organisms that are exposed to the dissolved Cry1Ab protein (Tank *et al.*, 2010). In the Mid-Western United States Tank *et al.*, (2010) observed that although Cry1Ab

positive maize debris co-occurred with Cry1Ab protein in stream water 75% of the time, there were multiple pathways through which Cry1Ab protein could enter streams (Fig 1.2). Cry1Ab proteins can be introduced into agricultural soils through root exudates and from maize biomass, with the exuded and leached protein persisting for up to 180 days and 3 years, respectively. Cry1Ab protein binds strongly to surface soils containing clay minerals, humic acids, organo-mineral complexes, and it has the potential to enter adjacent streams through surface runoff and erosion (Tank *et al.*, 2010). Each entry route is largely influenced by human activity, wind, rain and soil runoff events and includes erosion of soil and adsorbed protein, surface runoff of freely soluble protein, aerial deposition of pollen and crop dust, and movement of plant tissue and senescent crop residue (Carstens *et al.*, 2011).

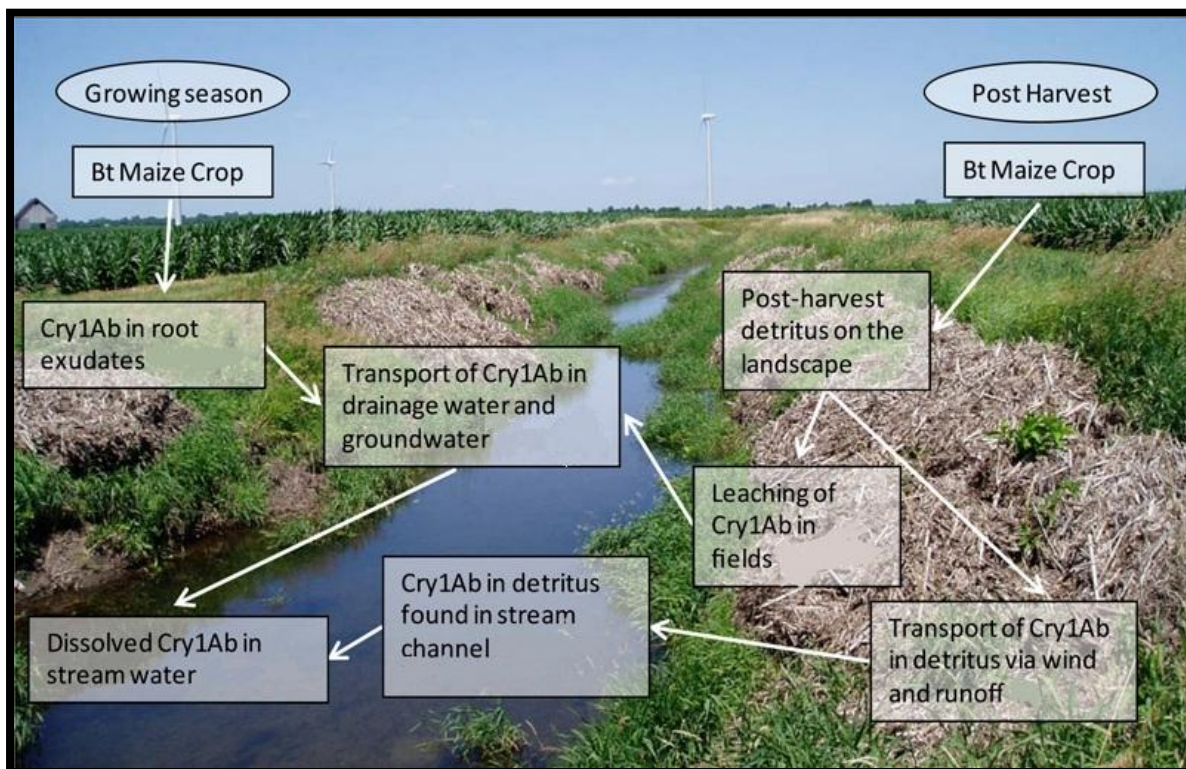


Figure 1.2. Diagram illustrating pathways of maize and Cry1Ab proteins entering stream ecosystems. The photograph depicts maize accumulation in the riparian zone and active stream channels (Tank *et al.*, 2010).

Griffiths *et al.* (2009) studies the decomposition of allochthonous organic matter in agricultural streams and explored how technological advances in agriculture (genetic engineering) might affect the rate at which crop debris is incorporated into stream

food webs. These above mentioned aspects were addressed by comparing decomposition and microbial respiration rates of Bt and non-Bt maize in agricultural streams, because microbial activity was likely an important driver of organic-matter decomposition in high-nutrient, low gradient agricultural streams (Griffiths *et al.*, 2009). Results showed Bt maize had a faster decomposition rate than non-Bt maize, while microbial respiration rates did not differ between Bt and non-Bt maize (Griffiths *et al.*, 2009). Decomposition rates were not negatively affected in GM cultivars, probably because the Bt-protein does not adversely affect the aquatic microbial assemblage involved in maize decomposition (Griffiths *et al.*, 2009).

Substrate quality and in-stream nutrient concentrations may also influence microbial respiration on maize. Comparison of the respiration rates of maize and red maple (*Acer rubrum*) leaves showed significantly higher rates of microbial respiration on maize (Griffiths *et al.*, 2009) and higher rates in agricultural compared to forest streams. The elevated nutrient status of agricultural streams and the problem of maize debris result in a rapid incorporation of maize leaves into the aquatic microbial food web (Griffiths *et al.*, 2009). Overall, the conversion of native vegetation to row-crop agriculture appears to have altered the quantity, quality, and predictability of allochthonous carbon inputs into headwater streams, with unexplored effects on stream ecosystem structure and function (Griffiths *et al.*, 2009).

#### **1.4. Non-target organisms and possible risk**

Wolt and Peterson (2010) presented a scenario where Cry1Ab protein accumulation and loss were estimated in water. When maize expressing this protein was grown in an agro-ecosystem, data indicated that a wetland may be the most affected by the Cry1Ab protein when it accumulates in the water. Conservative environmental fate models are used to synthesise the quantities and partitioning of Cry1Ab proteins generated throughout the crop growing cycle into estimated environmental concentrations for aquatic species. This was done for species of concern in or near maize fields, because these estimates are a current unanswered consideration for aquatic non-target organism risk assessments (Wolt & Peterson, 2010). Species sensitivity distributions are used to estimate the threshold concentrations of concern for presumed sensitive aquatic organisms. The outcomes of screening assessments

which use data reported in literature and conservative modelling assumptions, would point to clarification of the exposure assumptions rather than ecotoxicity testing as the appropriate first step for a more robust environmental risk assessment (Wolt & Peterson, 2010). The modelling was clearly conservative when considering that all Cry1Ab present in the field in plant debris at harvest will be available to the presumed susceptible species. If refined exposure analysis were employed to determine more realistic exposure due to protein degradation as well as the feeding habits of the non-target aquatic species, one would anticipate substantially lower levels of exposure and, therefore, even lower probable risk (Wolt & Peterson, 2010).

### **1.5. Effects of genetically modified organisms on vertebrate organisms**

Not many studies on effects of GM crops on vertebrates have been conducted. Although much criticized, studies by Séralini *et al.* (2012a) indicated that studies on the effects of GM crops on vertebrate organisms should consist of long term studies and not just 90 day tests. Studies such as Séralini *et al.* (2012a) is a good starting point for long term studies. Séralini *et al.* (2012a) demonstrated that lower levels of agricultural glyphosate herbicide formulations, at concentrations well below officially set safety limits, adversely affected rats exposed to these compounds

A study on pigs conducted by Walsh *et al.* (2012) showed short term feeding of Bt MON810 maize to weaned pigs resulted in increased feed consumption, less efficient conversion of feed to gain and a decrease in goblet cells/mm of duodenal villus. There was also a tendency for an increase in kidney weight, but this was not associated with changes in histopathology or blood biochemistry. Showing further need for long term studies. Effects of Cry1Ab protein on freshwater organisms

### **1.6. The effects of GM crops on non-target aquatic organisms ought to be further investigated due to the little research done on the topic.**

Rosi-Marshall *et al.* (2007) provided evidence that crop debris and pollen enter agricultural streams. Her studies showed that *Lepidostoma libia* (Trichoptera: Lepidostomatidae) that were fed Bt maize leaves in a laboratory experiment exhibited suppressed growth rates and increased mortality compared to their

counterparts fed conventional maize. However, the study was limited to one laboratory feeding experiment using one taxon and one variety of Bt maize and did not include isolines or a field examination of macro-invertebrate responses.

Criticism against the study by Rosi-Marshall *et al.* (2007) was that there were errors in the experimental design and that no appropriate control treatments were used in the experiments. It was therefore impossible to draw the conclusion that Bt-crops have impacts on aquatic insects (Beachy *et al.*, 2008). Because maize hybrids differ in many traits, any trait that differs between the hybrids, e.g., the level of trypsin inhibitors present, could explain the results observed by Rosi-Marshall *et al.* (2007) (Parrott, 2008). Since isogenic lines were not used, it is impossible to attribute the observed effect to Bt as opposed to any other factor that differed. It is possible that the claimed negative impacts on larval growth were attributable to chemical components in the tissue and not to the Bt-protein (Beachy *et al.*, 2008; Parrott, 2008). Rosi-Marshall *et al.* (2007) failed to identify and to quantify the Bt-protein, other leaf chemicals, and agricultural chemicals in stream waters, making it impossible to repeat the study or to draw conclusions from the data (Beachy *et al.*, 2008; Parrott, 2008).

Chambers *et al.* (2010) build on the work of Rosi-Marshall *et al.* (2007) by investigating the effects of Bt maize leaf debris on aquatic invertebrates in headwater streams using combined field and laboratory approaches. In the laboratory, multiple feeding trials were conducted, using debris of two varieties of Bt maize, with a leaf-shredding trichopteran (*Lepidostoma liba*) (Trichoptera: Lepidostomatidae), a shredding amphipod (*Hyalella azteca*) (Amphipoda: Hyalellidae), and a snail (*Gyraulus* sp.) (Pulmonata: Planorbidae). *Lepidostoma liba* individuals grew significantly slower when fed Bt maize compared to non-Bt maize. There was no mortality in *Hyalella* or *Gyraulus* growth experiments (Chambers *et al.*, 2010). *Hyalella* growth did not differ when fed Bt or non-Bt maize. *Gyraulus* growth also did not differ between Bt and non-Bt maize treatments. Invertebrate colonization of maize debris using litterbags containing either Bt or non-Bt leaves were measured and no significant differences were observed in litterbag colonization (Chambers *et al.*, 2010).

While field studies were designed to provide real-world relevancy, the laboratory components of the above mentioned study allowed for a controlled assessment of Bt-effects without confounding environmental factors (Chambers *et al.*, 2010). Highly tolerant taxa, such as oligochaetes and chironomids, were dominant in both Bt and non-Bt streams, and macro-invertebrate community composition was relatively constant across seasons (Chambers *et al.*, 2010). The field observations did not support laboratory results, most likely because the streams are highly degraded and subject to various, persistent anthropogenic stressors (channelization, altered flow, nutrient and pesticide inputs). Invertebrate communities in streams are a product of the degraded conditions, and the impact of a single stressor, such as Bt-proteins, may therefore not be readily discernible (Chambers *et al.*, 2010). These results add to growing evidence that Bt-proteins may have sub-lethal effects on non-target aquatic taxa, but this evidence should be considered in the context of other anthropogenic impacts and alternative methods of pest control influencing streams that drain agricultural regions (Chambers *et al.*, 2010).

In Jensen *et al.* (2010) study showed that the input of maize debris into a stream after harvest was extended over a period of several months. Using laboratory bioassays based on European corn borer (*Ostrinia nubilalis*) (Lepidoptera: Crambidae), Jensen *et al.* (2010) found no bioactivity of Cry1Ab protein in senesced maize tissue after two weeks of exposure to terrestrial or aquatic environments. *Ostrinia nubilalis* has been used as a sensitive indicator of the toxin since Bt maize was first developed. Bt near-isolines impact growth and survivorship of some species of invertebrates. Of the four non-target invertebrate species fed Bt near-isolines, growth of two closely related trichopterans (*Lepidostoma* spp. and *Pycnopsyche* cf. *scabripennis*) was not negatively affected, whereas a tipulid crane fly (*Tipula* (*Nipopotipula*) *abdominalis*) (Diptera: Tipulidae), exhibited reduced growth rates. The isopod, *Caecidotea communis* (Isopoda: Asellidae), exhibited reduced growth and survival on the Cry1Ab near-isoline but not on the stacked Cry1Ab and Cry3Bb1 near-isoline. Due to a lack of evidence of bioactivity of Cry1Ab protein after two weeks and because of lack of non-target effects of the stacked near-isoline, Jensen *et al.* (2010) suggested that tissue-mediated differences, and not the presence of the Cry1Ab protein, caused the different responses among the species. Overall, the results provided evidence that adverse effects on aquatic non-target shredders

involved complex interactions arising from plant genetics and the environment that cannot be ascribed to only to the presence of Cry1Ab proteins (Jensen *et al.*, 2010).

Studies on a non-target model organism (*Daphnia magna*) (Diplostraca: Daphniidae), in Norway, investigated whether Bt maize have negative impacts either through direct toxic effects or through reduced energy availability. This was done by comparing the fitness performance of *D. magna* fed Bt maize and its isogenic control. The measured response variables were survival, growth, individual fecundity, population fecundity, frequency of maturation, and age at maturation. Results demonstrated increased mortality, reduced growth and a lower number of eggs produced in *D. magna* feeding on Bt maize, likely due to a toxic response to the Bt maize (Bøhn *et al.*, 2008).

In another experiment by Bøhn *et al.* (2009) food quality of different GM maize varieties expressing Cry1Ab protein was evaluated over the life-cycle of *D. magna*. Demographic responses were compared between individuals fed Bt maize or isogenic non-Bt maize, with and without the addition of an additional stressor in the form of predator smell. Data on survival, fecundity and population growth rate generally disfavoured Bt maize as food for *D. magna* compared to individuals fed on non-Bt maize. Decomposition of age-specific effects revealed that the most important contributions to a reduced population growth rate in the Bt-fed group came from both fecundity and survival differences early in life. It was concluded that juvenile and young adult stages were the most sensitive experimental units. These stages are often absent in toxicological/ecotoxicological studies and in feeding trials (Bøhn *et al.*, 2009; Viktorov, 2011).

Studies in Brazil showed genotoxicity as well as embryo toxic effects of Bt proteins on Zebrafish, (*Danio rerio*) (Cypriniformes: Cyprinidae). Ecotoxicological evaluations of four Bt-proteins: Cry1Aa, Cry1Ab, Cry1Ac, and Cry2A from *B. thuringiensis* were carried out on Zebrafish, to explore the possible negative effects on their genome and embryos. The presence of Cry1Aa increased the micronucleus frequency in peripheral erythrocytes of adult *D. rerio*, while Cry1Ab, Cry1Ac and Cry2A did not show genotoxicity after 96 hours of exposure at a concentration of 100 mg/L. Exposures to binary mixtures (Cry1Aa + Cry 1Ac, 50:50 mg/L) and (Cry1Aa + Cry2A,

50:50 mg/L) for 96 hours also resulted in significant increases in micronucleus frequency. Other evaluated binary mixtures did not show genotoxicity. In the embryonal study, all tested proteins showed embryo toxicity and developmental delay after exposure to the concentrations of 25, 50, 100 and 150 mg/L for 96 hours. However, each protein presented a different pattern of toxic response suggesting that different approaches should be used for its toxicological evaluations (Grisolia *et al.*, 2008).

A study conducted by Douville *et al.* (2008) revealed that freshwater mussels, (*Elliptio complanata*) (Unionoida: Unionidae), was indirectly exposed to Cry1Ab genes by feeding on bacteria or particles, that are maintaining or stabilizing the genes in the aquatic environment. These results further suggest that horizontal gene transfer from GM plants to bacteria took place. The adverse effects of these biotechnology products in mussels are not clear in such altered, agriculture-dominated environments, but a trend analysis did reveal that condition factor and oxidative status were significantly related to their presence. However, simultaneous contamination by chemical pollutants and their potential adverse effects in these agricultural watersheds cannot be excluded (Douville *et al.*, 2008).

### **1.7. Interactions between herbicides, pesticides and amphibians**

While no studies have been conducted on the effects of Cry proteins on amphibians several studies have been conducted on the effects of herbicides and pesticides on aquatic fauna.

Pesticides have the potential to affect many aquatic taxa. The impacts on amphibians have been of particular concern during the past decade because of the apparent global decline of many species (Kiesecker *et al.*, 2001). Amphibians in nature frequently experience multiple applications of pesticides over time (Relyea & Diecks, 2008). It is therefore important to evaluate the effect of pesticides on these organisms as well as the synergistic effects that could result from additional stressors such as the presence of predators.

In laboratory tests four North American tadpole species (*Anaxyrus americanus*, *Lithobates sylvatica*, *Lithobates pipiens*, and *Lithobates clamitans*) were shown to have lower lethality values for glyphosate than glyphosate formulations containing poly-ethoxylated tallowamine (POEA) (Edginton *et al.*, 2004, Howe *et al.*, 2004). Under more natural conditions of aquatic mesocosms, and with only a single application, glyphosate can still be highly toxic to a variety of amphibian larvae (Relyea, 2005a). Glyphosate had substantial direct negative effects on the tadpoles, reducing total tadpole survival and biomass by 40%. However, glyphosate had no indirect effects on the amphibian community via predator survival or algal abundance (Relyea *et al.*, 2005a).

Relyea (2005b) conducted static renewal studies of glyphosate toxicity in the laboratory on six species of amphibians from the Midwestern United States (*L. sylvatica*, *L. pipiens*, *L. clamitans*, *Lithobates catesbeiana*, *A. americanus*, and *Hyla versicolor*). Consistent with studies of tadpoles in Canada (Edginton *et al.*, 2004), it was observed that LC50 values for these six species were relatively low, ranging from 0.6 to 2.5 mg active ingredient per litre (Relyea, 2005b). The addition of predatory stress to *L. sylvatica* resulted in glyphosate being twice as lethal (Relyea, 2005b). This discovery suggested that synergistic interactions between predatory stress and pesticides may indeed be a common phenomenon in amphibians.

According to Relyea (2006) pH, predatory stress, and a single application of an insecticide (carbaryl) could affect the survival and growth of larval bullfrogs (*L. catesbeiana*) and green frogs (*L. clamitans*) in outdoor mesocosms. A decreased pH had no effect on survival of the tadpoles, but resulted in greater tadpole growth. Low concentrations of carbaryl had no effect on either species, but high concentrations caused lower survival and larger growth in *L. catesbeiana*. Predatory stress and reduced pH did not make carbaryl more lethal, probably because of the rapid breakdown rate of carbaryl in outdoor mesocosms. This is contrary to what is found under laboratory conditions where pH and predator-associated stress results in carbaryl having a more lethal effect than using repeated applications of carbaryl. These stressors did not interact under mesocosm conditions when a single application of carbaryl was used (Relyea, 2006).

*Lithobates pipiens* tadpoles showed lower survival with high glyphosate concentrations compared with low or no herbicide treatment in water at a pH of 5.5. A trend of earlier mortality for *L. pipiens* was observed when it was exposed to high herbicide concentrations and high food availability. There were no significant effects of pH alone but there were significant effects of pH in both low concentration and high concentration herbicide treatments, further demonstrating a significant pH by herbicide interaction (Chen *et al.*, 2004).

The formulated mixture of glyphosate as well as its components, isopropylamine (IPA) salt of glyphosate and the surfactant MON 0818 (containing POEA)) were separately tested in 96h acute toxicity tests against tadpoles (Moore *et al.*, 2011). These tests were done with larval anurans at Gosner stage 25 (*L. pipiens*, *L. clamitans*, *L. catesbeiana*, *Anaxyrus fowleri* and *Hyla chrysoscelis*) that were reared from egg masses and exposed to a series of 11 concentrations of the original formulation of glyphosate herbicide, nine concentrations of MON0818 and three concentrations of IPA salt of glyphosate in static (non-renewal) aqueous laboratory tests. In these studies, *L. pipiens* was shown to be the most sensitive of five species. No significant mortality was observed during exposures of 96h for any of the five species exposed to glyphosate IPA salt at concentrations up to 100 times the predicted environmental concentration (PEC) (Moore *et al.*, 2011).

Studies suggest that under laboratory conditions, ecologically relevant concentrations of glyphosate can cause substantial mortality in some species of amphibian larvae and that this death is primarily due to the POEA surfactant (Mann & Bidwell, 1999, Perkins *et al.*, 2000, Lajmanovich *et al.*, 2003, Relyea, 2005c, Moore *et al.*, 2011).

The insecticide malathion was reported to have a number of direct effects on tadpoles. While high concentrations of malathion can directly kill larval anurans, more ecologically relevant concentrations can have positive effects on larval anurans in mesocosms by removing predatory insects (Relyea *et al.*, 2005c). The abovementioned results indicate that pesticides can have both direct and indirect effects in natural communities and that these effects critically depend upon the composition of the aquatic community.

Relyea (2009) examined how a single application of five insecticides (malathion, carbaryl, chlorpyrifos, diazinon, and endosulfan) and five herbicides (glyphosate, atrazine, acetochlor, metolachlor, and 2,4-D) at low concentrations (2–16 p.p.b.) effected aquatic communities. The larval amphibians used in the study were gray tree frogs (*H. versicolor*) and leopard frogs (*L. pipiens*). *Lithobates pipiens* tadpoles suffered an apparent direct toxic effect and endosulfan resulted in 84% mortality. An indirect effect induced by diazinon resulted in 24% mortality and very high mortality (99%) with a mix of insecticides or all ten pesticides while metamorphs were smaller with diazinon but larger with endosulfan (Relyea, 2009). These mixtures did not influence mortality or time to metamorphosis in *H. versicolor* tadpoles and, as a result, this species grew nearly twice as large due to reduced competition with *L. pipiens* tadpoles (Relyea, 2009). Gray tree frog metamorphs emerged larger with atrazine, the mix of insecticides, and the mix of all ten pesticides (Relyea, 2009).

Organisms in nature frequently experience multiple applications of pesticides over time rather than a single constant concentration. Using outdoor mesocosms, Relyea and Diecks (2008) examined how low concentrations of malathion (a common insecticide) applied at various amounts, times, and frequencies affected aquatic communities containing zooplankton, phytoplankton, periphyton and larval amphibians for 79 days. The reduced periphyton had little effect on wood frogs (*Lithobates sylvatica*), however, leopard frogs (*L. pipiens*) had a longer time to metamorphosis, and experienced reductions in growth and development, which led to fatality as the environment dried out. Malathion which rapidly breaks down did therefore not directly kill amphibians, but indirectly resulted in amphibian mortality (Relyea & Diecks, 2008). Importantly, repeated applications of the lowest concentration of malathion caused larger impacts on many of the response variables than single “pulse” applications of which concentrations were 25 times higher. These results are not only important because malathion is a commonly applied insecticide often present in wetlands, but it provides the possibility of general predictions for the way in which many insecticides impact aquatic communities and populations of larval amphibians (Relyea & Diecks, 2008).

### **1.8. Legislation of South Africa**

The Genetically Modified Organisms act (Act No. 15 of 1997) requires regular monitoring and reporting on the effect of GM maize on target and non-target organisms. This act supports safe use of GM crops that are introduced into South Africa, and was developed to promote the responsible development, production, use and application of genetically modified organisms and to ensure that activities are carried out in such a way as to limit possible harmful consequences to the environment and human health (South Africa, 1997). Although the *Cry* proteins produced in transgenic maize are considered to be target specific, some side effects on non-target species have been reported (Rosi-Marshall *et al.*, 2007, Tank *et al.*, 2010, Chambers *et al.*, 2010). Depending on future results it may be necessary to monitor certain amphibian species in aquatic ecosystems.

### **1.9. Background of frog species used in this study**

Frogs form an important component of aquatic ecosystems in and also occur in agro-ecosystems where GM maize is cultivated. In this study two frog species were used, i.e. the Guttural Toad *Amietophrynus gutturalis* (Anura: Bufonidae) and the Clawed frog *Xenopus laevis* (Anura: Pipidae). These species have a high probability to come in contact with Bt-proteins because they are wide spread and common in farmland areas. These two species were used to investigate if shredder tadpoles (*A. gutturalis*) and filter feeding tadpoles (*X. laevis*), having different feeding methods, showed the same trend in survival, growth and development if they ingested Bt maize leaves or were exposed to Bt protein in suspension. Overlapping of the major maize production area in South Africa and *A. gutturalis* (Fig 1.4) and *X. laevis* (Fig 1.6) distribution areas, increases the potential of tadpoles to be exposed to Bt protein in aquatic ecosystems.

### 1.9.1. *Amietophrynus gutturalis*



Figure 1.3. *Amietophrynus gutturalis*.

*Amietophrynus gutturalis* has a wide distribution in South Africa (Fig. 1.4). Its distribution is centred in the northeast, particularly in KwaZulu-Natal, Mpumalanga, Gauteng, central Limpopo, eastern North-West, and eastern Free State provinces as well as Swaziland. In the Northern Cape Province its distribution extends westward along the Orange River as far as Goodhouse (Du Preez *et al.*, 2004). Breeding takes place in open pools, dams, vleis and other semi-permanent or permanent bodies of water, such as garden ponds. In areas where permanent water bodies do not exist breeding is initiated by the first heavy spring rains. As many as 25,000 eggs, 1.4-1.5 mm in diameter, are laid in two gelatinous strings (Du Preez & Carruthers, 2009; Measey *et al.*, 2009). Strings of eggs are often twined around aquatic vegetation. The tadpoles hatch after a week and mass together as small, very black, forms. These bottom feeding tadpoles have toxins which make them unpleasant to birds and mammals but are eaten by clawed frogs and aquatic insects. After 5-6 weeks, small metamorphs begin to leave the water and move into the surrounding habitat (Measey *et al.*, 2009). Adults are mostly terrestrial, attracted to light at night and feed on insects.

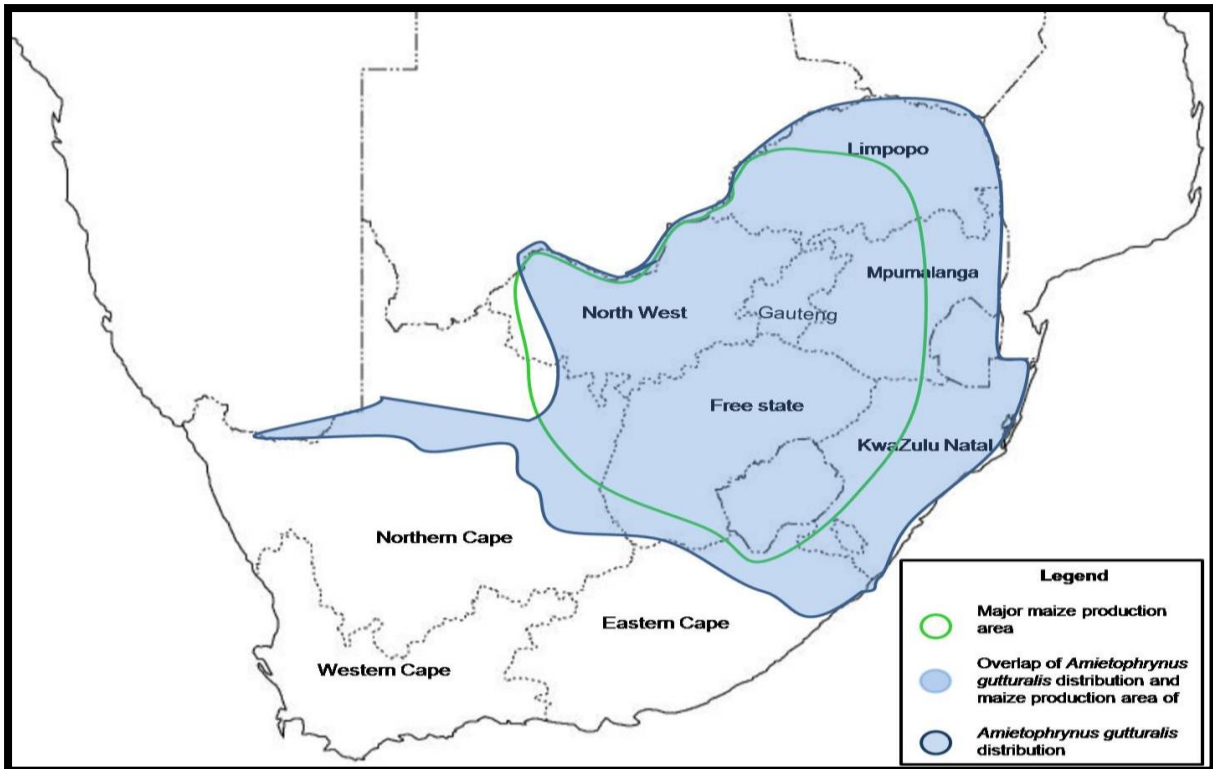


Figure 1.4. Map showing the distribution of *Amietophrynus gutturalis* and the major maize production areas in South-Africa.

### 1.9.2. *Xenopus laevis*



Figure 1.5. *Xenopus laevis*.

*Xenopus laevis* (African Clawed Frog / Platanna) is the most widespread amphibian on the African continent. Within South Africa *X. laevis* is a common species which occurs from the Western Cape Province northwards, excluding the extreme northern parts of Kwazulu-Natal, eastern parts of Mpumalanga and Limpopo (Fig.1.6).

The African Clawed Frog has shown plasticity in habitat characteristics such as food availability, vegetation, substrate, turbidity, salinity, water temperature, and hydrology. This makes a precise characterization of habitat characteristics difficult. Highest densities of frogs are reached in permanent, eutrophic, fish-free waters that have soft substrates and submerged vegetation, and do not freeze over but remain above 20°C for most of the year (Crayon, 2011). *Xenopus laevis* occupies permanent bodies of water such as ponds, dams, streams, rivers and waterholes (Weldon, 1999). Populations occur in disturbed or human-made bodies of water, such as drainage ditches, flood control channels, golf course ponds, manmade lakes, irrigation canals, cattle tanks, and sewage plant effluent ponds. This affinity for opportunistic colonizing of disturbed habitats is also seen in the parts of Africa where the species' range is expanding. Human-made irrigation canals, lakes and ponds are especially favoured habitats for the expanding range of this species (Curtis *et al.*, 1998).

Adults are primarily aquatic consumers of slow-moving invertebrates, they are often characterized as rather unskilled at capturing actively swimming prey. They rely upon olfaction and the lateral line system retained after metamorphosis to detect waterborne scents and the movements of aquatic prey, they can even find food and feed when blinded (Crayon, 2011). Tadpoles filter feed while suspended in open water. Food items include phytoplankton, especially unicellular algae and diatoms, protozoans, and bacteria. Larvae are free-swimming within 1–2 days after hatching but they are weak swimmers. Larvae are especially vulnerable to fish predation, and they school in the middle of deeper water to feed, rather than hiding in shallows (Crayon, 2011).

In bodies of water where there are limited prey, adults will cannibalize young. Tadpoles may act as collectors of nutrients such as seasonal single-celled algal

blooms, which are unavailable to adults. Adults that cannibalize these larvae can thus rely indirectly on this phytoplankton food base. Cannibalism allows clawed frogs to colonize a body of water that does not offer a large prey base for the adults or to stay in a body of water that has been depleted of prey (Schramm, 1987).

*Xenopus laevis* has become a common laboratory animal since the detection of its suitability for hormonal reactions and it is commonly used in toxicity studies (Slooff & Baerselman, 1980).

*Xenopus laevis* are filter-feeding tadpoles, meaning that they receive their food in suspension, whereas *Amietophrynus gutturalis* tadpoles are bottom feeding, meaning they are able to feed on leaves and debris.

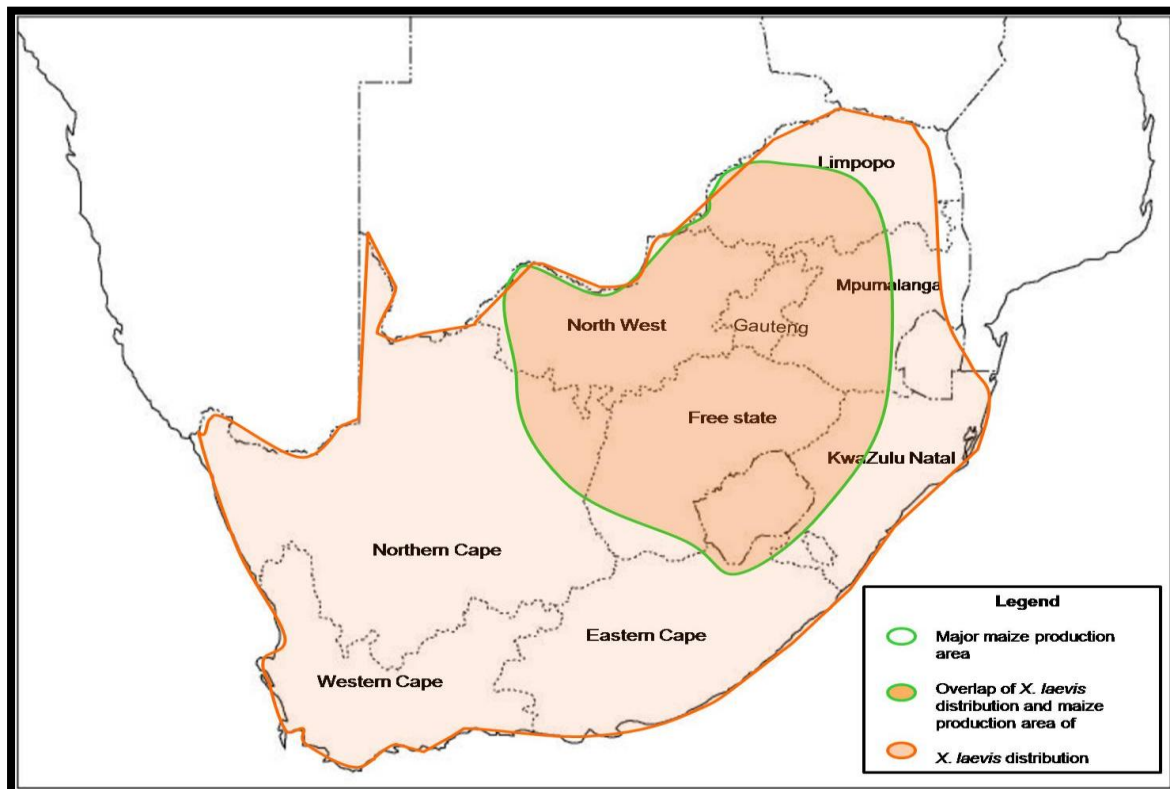


Figure 1.6. Map showing the distribution of *Xenopus laevis* and the major maize production areas in South-Africa.

## **1.10. Objectives**

### **1.10.1. General objectives**

The general objectives of this study were to evaluate if Cry1Ab protein expressed in leaves of Bt maize plants affect the development of *Xenopus laevis* and *Amietophrynus gutturalis* tadpoles.

### **1.10.2. Specific objectives**

- to evaluate the effect of Bt maize leaves as food source on the morphological development of filter-feeding tadpoles of *Xenopus laevis*.
- to evaluate the effect of Bt maize leaves as food source on the morphological development of bottom-feeding tadpoles of *Amietophrynus gutturalis*.

## CHAPTER 2

### MATERIAL AND METHODS

#### 2.1. Frog species used in experimental studies

##### 2.1.1. *Xenopus laevis*

In order to obtain hatchling *X. laevis* tadpoles, spawning of females was induced using commercially available chronic gonadotrophin (Pregnyl). Through the use of injections, three field-collected males each received 250 IU (international units) Pregnyl on three successive days. Three field collected females received 50 IU on the second day and 500 IU on the third day. The injections were made into the dorsal lymph sac, piercing the skin of the thigh and passing through the septum between the lymph sacs of the thigh and the back. After receiving the final Pregnyl treatment males and females were placed together as pairs into breeding tanks. Each tank was fitted with a raised mesh floor to protect the eggs against potential damage cause by adults. The temperature inside the temperature-controlled room was set at  $24\pm 2^{\circ}\text{C}$ . Spawning took place during the night following the last dosage. On the day after spawning the adult frogs (Fig 2.1) were removed from the tanks. The water with eggs was aerated for the rest of the experiment. Tadpoles that hatched from eggs were, pooled and used in the experiments.



Figure 2.1. *Xenopus laevis* adults.

### **2.1.2. *Amietophrynus gutturalis***

Eggs and adult frogs (Fig. 2.2) in amplexus were collected at various ponds in Potchefstroom e.g. Botanical Gardens throughout the study. The egg strings were brought to the laboratory, pooled and placed in a microcosm with water and aerated. Eggs hatched approximately three days later with the tadpoles clinging to the sides of the container. After three days the tadpoles became free swimming. The tadpoles were removed from the microcosm when they reached a Gosner development stage of 23-25, after which they were used in the experiments. The Gosner development stages are described below, under Fig.2.9.



Fig 2.2. *Amietophrynus gutturalis* adult.

## **2.2. Housing and experimental containers for test animals**

Experiments were conducted in glass microcosms (29cm x 40cm x 25cm) (Fig 2.3). Each glass microcosm contained 20 litres of water and the water level was maintained throughout the study. Experiment 1 and 3 contained tap water. The reason for this was that experiment 1 was a pilot experiment and experiment 3 was conducted in a shade-house facility (Fig 2.4) on where was no borehole water available. For the other experiments borehole water was used. Borehole water is used because the water is less likely to contain chlorine.



Figure 2.3. Microcosms used in all the experiments. Each microcosm contained 20 l water that was aerated throughout each experiment.

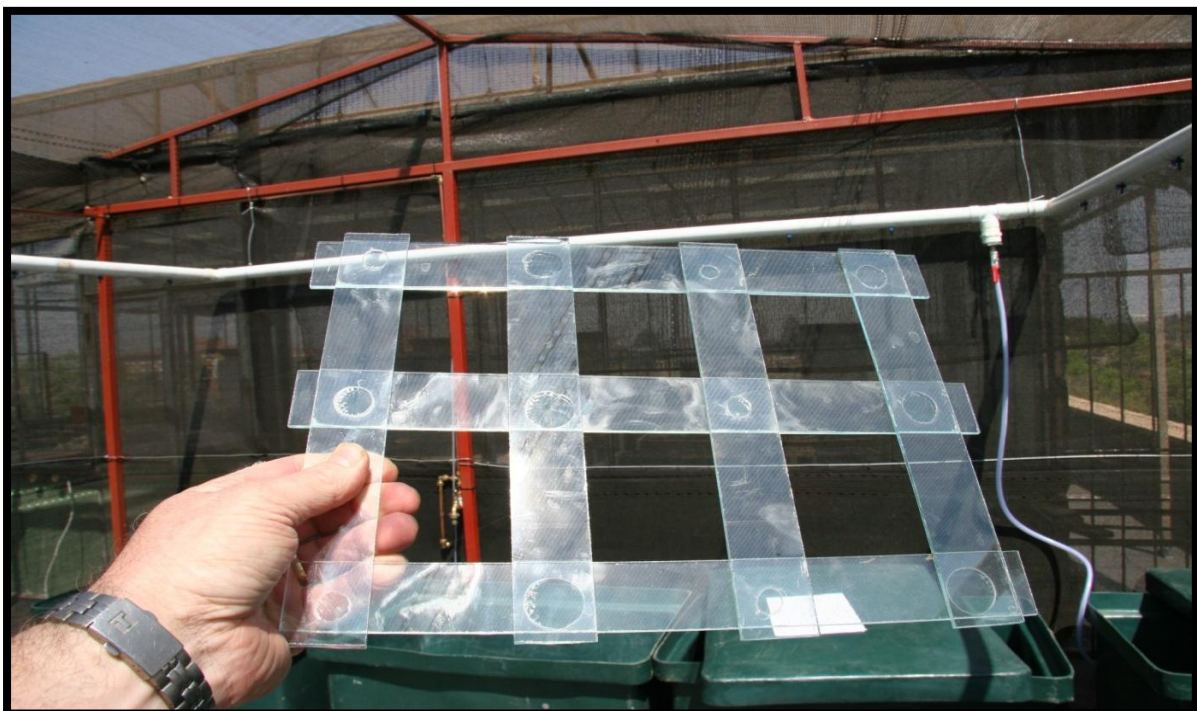


Figure 2.4. Glass grids was used to keep maize leaves submerged in the shade-house facility were Experiment 1 of *Amietophrynus gutturalis* was done.

### **2.3. Environmental parameters**

Three experiments were conducted with *X. laevis* and three with *A. gutturalis*. Five of these were conducted in the bio-secure Amphibian Biology laboratory and one with *A. gutturalis* was conducted in a shade-house facility (Fig 2.4) where microcosms were exposed to natural conditions (ambient temperatures, rainfall and partial sunlight).

### **2.4. Experiments**

All maize leaves used in this study were collected from maize grown in a plant growth tunnel at North-West University. Leaves were air dried for two months and then used in the experiments since literature indicated that maize leaves enter aquatic ecosystems over a period of approximately six months after harvest (Jensen *et al.*, 2010; Tank *et al.*, 2010; Carstens *et al.*, 2011). MON810, expressing Cry1Ab protein and its isoline were used in all experiments.

Air dried maize leaves collected from the plant growth tunnel were pulverised into a very fine powder at the Agricultural research council (ARC) food processing department in Potchefstroom.. To ensure that the nutritional value of the Bt and Non-Bt leaves are comparable split samples of both were blind tested by Inspectorate M&L (Pty) Ltd. The calorific value for the Bt leaves were found to be 16.47 and for the Non-Bt 15.82. Thus the nutritional value for both is comparable.

#### **2.4.1. *Xenopus laevis***

##### **2.4.1.1. Experiment 1**

The aim of this experiment was to simulate conditions in a roadside pond where maize leaves accommodate and where the protein leach out in the water in order to investigate the effect of the Bt-protein in the water on free swimming *X. laevis* tadpoles. The reason for using different amounts of leaf material was to create a “dose-effect” since it was unclear what the level of maize leaf material is inside aquatic systems in South Africa. Both control and experimental groups contained large pieces of leaves at the bottom of the microcosms which were kept submerged

under the water with the use of glass grids that were placed on top of the leaves (Fig. 2.4).

The experiment was conducted from 12 March 2010 to 29 April 2010. Tadpoles were maintained in microcosms with maize leaves submerged in water. Since *X. laevis* tadpoles are filter feeders tadpoles were fed every second day on pulverized maize leaves that were homogenized in water. Tadpoles were divided into two groups: control group (non-Bt maize leaves) and experimental group (Bt maize leaves). Both the experimental and control groups were further divided into three sub-treatments with a different amount of submerged leaves (Fig. 2.6). These groups consisted of 15 g, 30 g and 45 g leaves per microcosm respectively. A volume of 15 ml of maize leaves were placed in 100 ml tap water and mixed with a magnetic stirrer. Tadpoles were fed every second day with 9 ml of this suspension of pulverised Bt maize leaves, or non-Bt maize leaves for each microcosm. Pulverised leaves were kept at room temperature in air tight Schott glass bottles. The concentration of Cry1Ab protein inside maize leaves or inside the water was not determined. The experiment was replicated three times and consisted of 18 microcosms (Fig. 2.5).



Figure 2.5. Experimental design and layout of different treatments in microcosms.

Fifty 4-day old tadpoles were introduced into each microcosm. The experiment was conducted for a period of eight weeks and was terminated when the tadpoles showed deformities which were ascribed to incorrect husbandry practices.

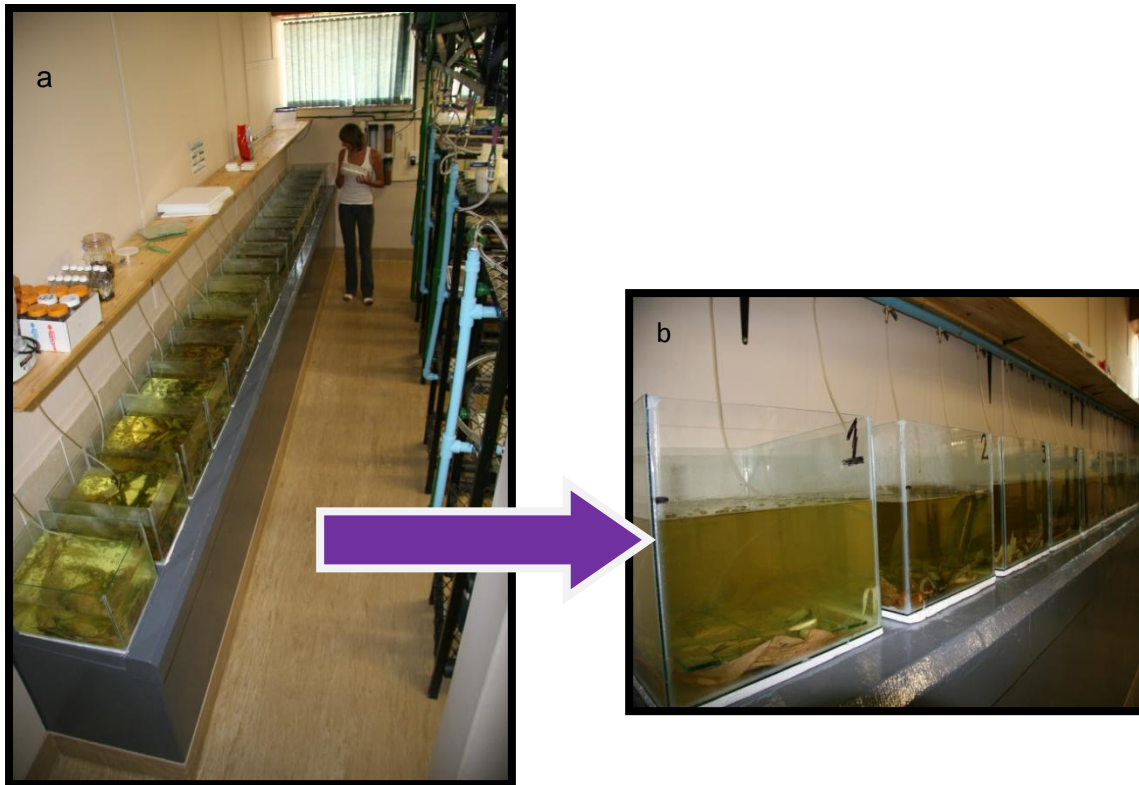


Figure 2.6. Experimental layout of Experiment 1, a) showing 18 microcosms with maize leaves, b) close-up of microcosms.

#### 2.4.1.2. Experiment 2

The aim with this experiment was to determine whether Bt-proteins will have adverse effects on *X. laevis* tadpoles when tadpoles feed on pulverised Bt leaves. The experiment was conducted between 9 June 2010 and 10 November 2010. Treatments were divided into two groups which was replicated six times (12 glass microcosms per treatment). The control group received pulverised non-Bt maize leaves and the experimental group received pulverised Bt maize leaves. In this experiment the filter-feeding tadpoles were only feeding on the suspension of either 9 ml pulverised Bt or non-Bt maize leaves per microcosm (that were prepared in the same manner as in Experiment 1) that they received twice a week. Each microcosm contained fifty 4-day old tadpoles. There were no maize leaves at the bottom of the microcosms. The experiment was conducted for a period of 19 weeks and was terminated when the tadpoles showed a very slow development rate and growth over this period.

### **2.4.1.3. Experiment 3**

The aim of this experiment was to determine whether Bt-proteins will have adverse effects on *X. laevis* tadpoles when tadpoles feed on pulverised Bt leaves with better husbandry. This experiment was conducted between 14 July 2011 and 13 October 2011. Two treatments were evaluated in this experiment, a) the experimental group (pulverised Bt maize leaves) and, b) the control group (pulverised non-Bt maize leaves). Each treatment was replicated six times. In this experiment the microcosms was cleaned and water replaced every week, in contrast to experiment 1 and 2 where water was topped up at regular intervals. Each microcosm contained thirty 4-day old tadpoles.

Feeding was done every second day, each microcosm received 15ml of pulverized maize leaves (either Bt or non-Bt) in suspension. The experiment was conducted for a period of 14 weeks. It was then decided to terminate the experiment since tadpole growth was unsatisfactory and very slow.

### **2.4.2. *Amietophrynus gutturalis***

#### **2.4.2.1. Experiment 1**

The aim of this experiment was to simulate conditions in a roadside pond where maize leaves accumulate and where the protein leach out in the water in order to investigate the effect of Bt-protein in the water on free swimming *Amietophrynus gutturalis* tadpoles. The experiment was conducted from 9 September 2010 to 3 February 2011. Tadpoles were divided into two groups: control group (non-Bt maize leaves) and experimental group (Bt maize leaves). Both the experimental and control groups were further divided into three sub-treatments with a different amount of submerged leaves. These groups consisted of 15 g, 30 g and 45 g leaves per microcosm respectively. The concentration of Cry1Ab protein inside maize leaves or inside the water was not determined. The experiment was replicated three times and consisted of 18 microcosms.

For both groups large pieces of maize leaves were placed at the bottom of the microcosms using glass grids (Fig 2.7) since this species is a known bottom-feeder. The experiment was further divided into three sub-treatments (different

concentrations), consisting of 15, 30 and 45 g leaves per microcosm respectively. The experiment was replicated three times and consisted of 18 microcosms. Fifty tadpoles were placed in each microcosm. In this experiment tadpoles were exposed to the Bt-protein in two ways. Firstly, to Bt-protein that leached out from the leaves, and secondly, from the emerged maize leaves that they fed on. The experiment was conducted for a period of 21 weeks and was terminated when the percentage survival was low and most of the tadpoles reached metamorphosis.



Figure 2.7. Microcosms in the shade-house facility were Experiment 1 of *Amietophrynus gutturalis* was done.

#### **2.4.2.2. Experiment 2**

The aim with this experiment was to determine whether the Bt-protein will have adverse effects on *Amietophrynus gutturalis* tadpoles when tadpoles feed on Bt maize leaves. A total of 100 tadpoles were used for this experiment from 24 October to 8 December 2011. Tadpoles were placed individually in 250 ml plastic cups that were each filled with 100 ml borehole water.

The experiment was divided into a treatment in which 50 tadpoles were fed Bt maize leaves and a control treatment in which 50 tadpoles were fed non-Bt maize leaves. Small circular (5 mm diameter) pieces of maize leaf were provided as food. A single-hole paper punch was used to cut Bt and non-Bt maize leaves into circular shapes. At the beginning of the experiment tadpoles received three circular maize leaf-cuts per week for the first three weeks and as the tadpoles developed ten pieces were provided per week from week four until the end of the experiment. The water inside the containers was replaced each week when new food was also provided. The experiment was conducted for a period of six weeks. The experiment was then terminated since, although survival was high, the tadpoles showed almost no growth during this period.

### 2.4.2.3. Experiment 3

The aim with this experiment was to determine whether the Bt-protein will have adverse effects on *Amietophrynus gutturalis* tadpoles if tadpoles are exposed to the protein in the water but not feeding on the plant material. A total of 100 tadpoles were used during the experiment which was conducted between 24 October and 8 December 2011. Tadpoles were placed individually in 250 ml plastic cups that were filled with 100 ml water containing an extract of either Bt- and non-Bt maize leaves. The maize leaf water extract was prepared by placing 11.25 g of Bt or non-Bt maize leaves in 5 l borehole water for three days.

$\frac{45 \text{ g maize leaves}}{20 \text{ litres of water}}$	$= 2.25 \text{ g maize leaves per litre water} \times 5 \text{ litres} = 11.25 \text{ g of maize leaves needed}$
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The experiment was divided into a treatment where 50 tadpoles were exposed to Bt maize leaf water and a control treatment where 50 tadpoles were exposed to non-Bt maize leaf water. Tadpoles were fed twice a week with TetraTabimin bottom-feeding fish pellets in suspension. This food-suspension was made up by putting one and a quarter fish tablets (0.375 g) were pulverised and suspended in 25 ml borehole water. A micropipette was used to add 250 µl suspended food into the glass container of each tadpole. The food-solution for the experiment was prepared three days prior to measurement of the tadpoles. The reason for this was to ensure that

Cry1Ab leached out of the Bt maize leaves prior to the extract being used. The water was replaced once a week. The experiment was conducted for a period of six weeks and was terminated when the percentage survival was low with Bt-water survival at 26% and non-Bt water at 20%.

## 2.5. Data collection

Data were collected on a weekly basis until metamorphosis occurred or until termination of the experiment. On a weekly basis ten randomly selected tadpoles were collected from each microcosm by means of an aquarium fish net. Tadpoles were then placed in a large glass petridish where they were temporally anaesthetized with MS 222 (3-amino benzoic acid ethyl ester) diluted in water.

The length (in mm) of each tadpole was measured and the developmental stage was established according to the Normal Table of development for *X. laevis* (Nieuwkoop & Faber, 1994) and Gosner stages (Gosner, 1960) for *A. gutturalis* tadpoles.

In two of the three experiments (experiments 2 & 3) with *A. gutturalis* tadpoles were measured each week with the use of a Nikon SMZ1500 stereo microscope fitted with a dedicated Nikon DXM1200 digital camera and connected to a computer with Nikon NIS Elements software that took a reading of the length of the tadpoles. Their morphological development was determined according to the Gosner stages.

Data were analysed using STATISTICA version 10 (StatSoft Inc., 2011). Non-parametric Mann-Whitney U tests were done using developmental stages for all the *X. laevis* experiments and *A. gutturalis* experiment 1. T-tests were done at the end of the all experiments *X. laevis* experiments and *A. gutturalis* experiment 1. Repeated measures analysis of variance (ANOVA) was used to compare to compare average length in all the experiments tadpoles over time except *A. gutturalis* experiment 2 and 3. These ANOVAs were done using data on tadpole growth collected weekly. *Amietophrynus gutturalis* experiment 2 and 3 data were analysed using SAS software (SAS Institute Inc, 2011) Mixed procedure. The advantages of this hierarchical linear modelling method are multilevel analyses exploit the information contained in cluster samples to explain both the between and within

cluster variability of an outcome variable interest (Hancock & Mueller, 2010). The model also allows predictors to be used on an individual as well as a group level to explain the variance in the dependent variable (Hancock & Mueller, 2010).

*Xenopus laevis* survival was not determined due to the adverse effect that handling has on small tadpoles. Adverse effects of handling of this commonly used laboratory animal have previously been reported (National Research Council, 1974). Collecting of tadpoles from microcosms in which leaf material was put (*Xenopus laevis* experiments 1) was very difficult due to the dark brown colour of water and decaying leaves in the water. Due to the difficult task collecting tadpoles, only ten individuals were collected at each sampling interval. It would have been almost impossible to collect all of the tadpoles in the microcosms with the maize leaves and the 20l of dark-coloured water. The survival of the *X. laevis* tadpoles in experiment 2 was not determined as it was mortality was not part of the aim of the experiment.

The description and assessment of the development of anuran embryos and larvae is facilitated by the use of staging tables such as the Nieuwkoop and Faber normal table of development for *Xenopus laevis* (1994) shown in Fig 2.8, was used for all the *X. laevis* experiments, and Gosner staging table in Fig 2.9. These staging tables are indispensable to many studies involving frog life-history materials (Gosner, 1960). The Gosner (1960) staging table is a simplification of other more complex staging tables and was developed to facilitate staging of most tadpoles. The Gosner staging table was used in all the *A. gutturalis* experiments.

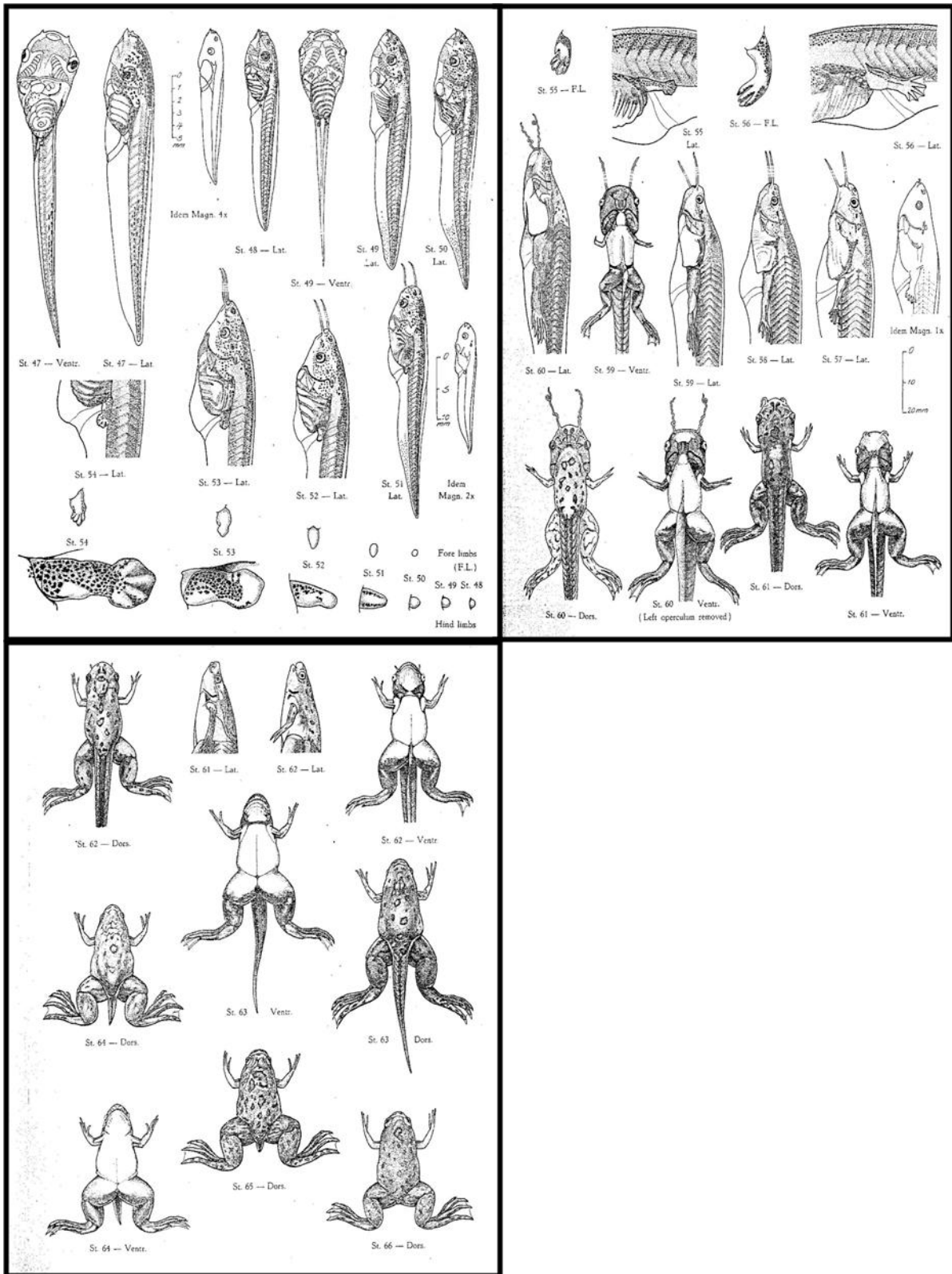


Figure 2.8. The Nieuwkoop and Faber (1994) staging table used for determining developmental stages of *Xenopus laevis* tadpoles.

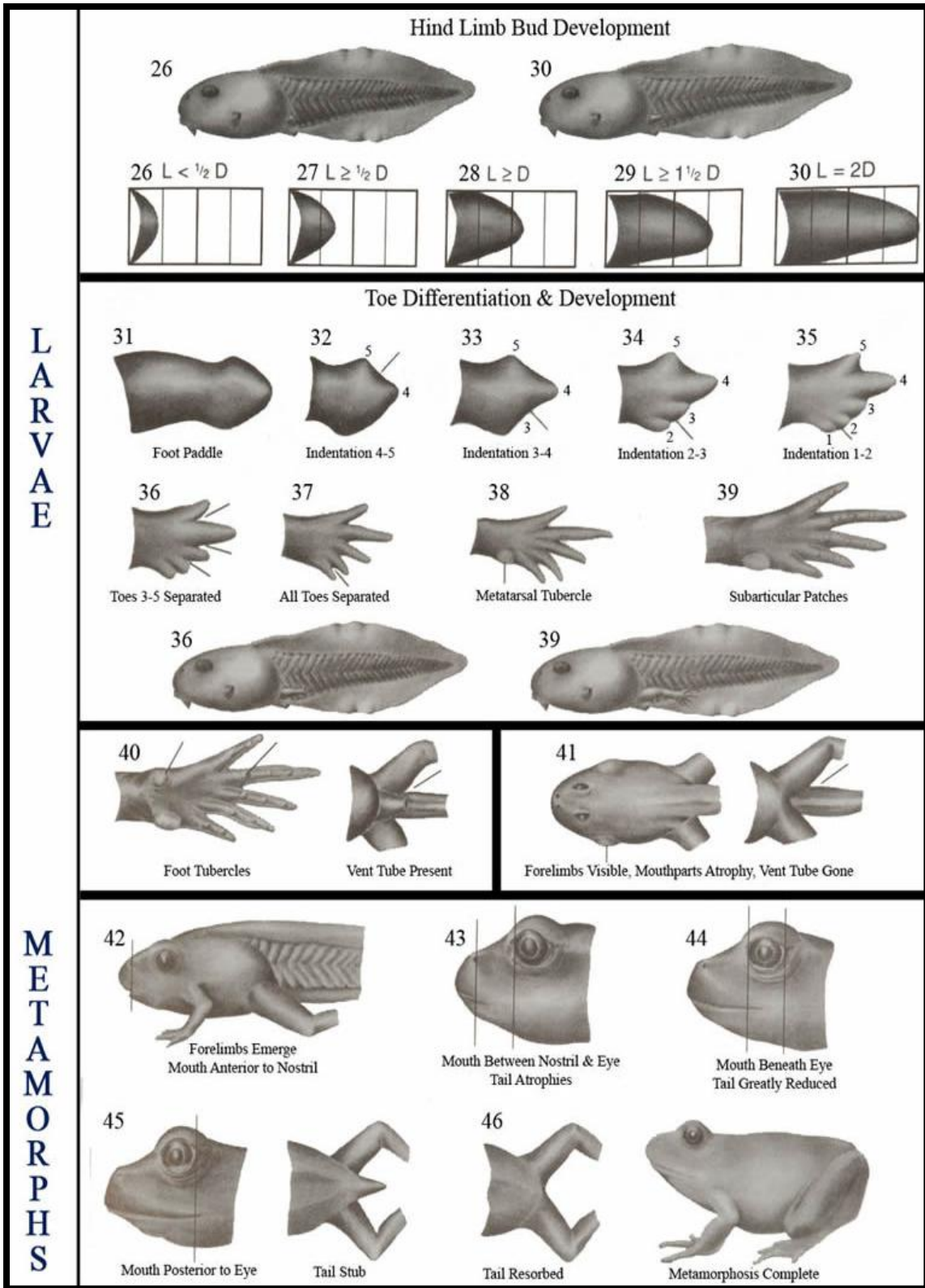


Figure 2.9. The simplified Gosner (1960) staging table used to describe *Amietophrynus gutturalis* tadpoles developmental stages.

## CHAPTER 3

### RESULTS AND DISCUSSION

#### 3.1 *Xenopus laevis*

##### 3.1.1. Experiment 1

This experiment was terminated at week six since a significant proportion of the tadpoles showed developmental deformities. The presence of deformities was ascribed to poor husbandry. The development rate was similar for the first two weeks for all treatments (Fig. 3.1). In spite of early termination, interesting tendencies were observed in tadpole growth and development and are reported herein. Differences in the mean lengths of Bt and non-Bt feeding tadpoles became evident from week 2 and increased over time. In all three treatments the length of tadpoles feeding on Bt leaves were shorter than those that fed on non-Bt maize (Fig. 3.1). There was a significant difference ( $F = 4.8747$ ,  $p = 0.028905$ ) between the Bt and non-Bt groups. There was a significant interaction ( $F = 9.3285$ ,  $p = 0.000159$ ) between Bt and non-Bt tadpoles with regard to length. In the 45 g non-Bt treatment, the mean length of tadpoles was significantly shorter than all the other treatments, which did not differ from each other (Fig 3.1).

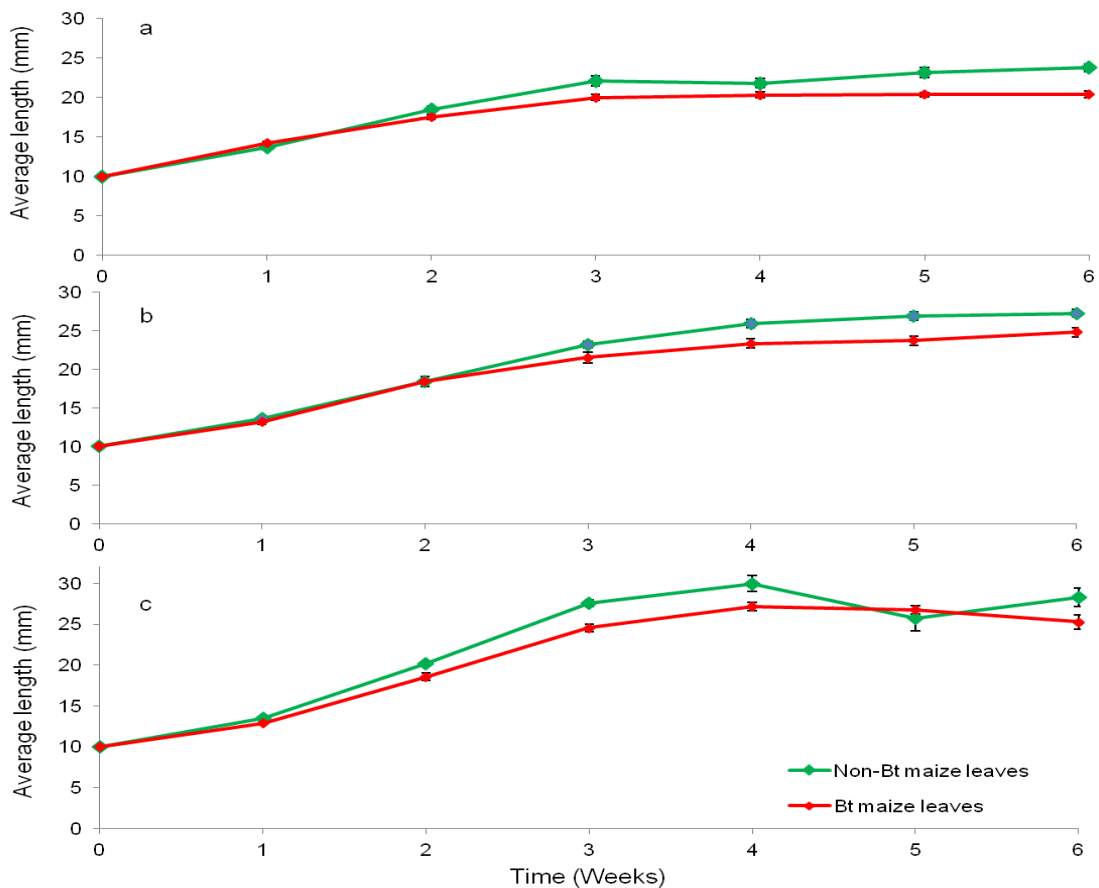


Figure 3.1. The average length of *Xenopus laevis* tadpoles over time. Tadpoles were maintained in 20 l microcosms containing (a) 15, (b) 30 and (c) 45 grams of maize leaves. Bars indicate standard error.

The comparatively advanced developmental stages of tadpoles that fed on non-Bt maize indicated that they developed at a faster rate than their Bt-feeding counterparts (Fig. 3.2), in spite of their lengths being similar. Tadpoles that were fed on non-Bt maize leaves were further developed according to the Nieuwkoop and Faber (NF) table of normal development than tadpoles fed Bt maize leaves. This trend continued until the end of the experiment. The significant differences ( $F = 8.956$ ,  $p = 0.003277$ ) that were observed showed that the Bt and non-Bt feeding tadpoles did not develop at the same rate. At the end of the experiment tadpoles in the 45 g treatment of non-Bt feeding maize, were significantly ( $Z = -3.41255$ ,  $p = 0.0006$ ) less developed than the Bt-feeding tadpoles. However, no significant differences in tadpole development were observed in the 15g and 30 g treatments.

In the light that a large proportion of the tadpoles developed deformities no definitive conclusions could be made from this experiment. Although significant differences were found in this experiment, it is not conclusive that Bt have an effect on tadpole growth if exposed to 45 g of maize leaves, because the husbandry in this experiment was not up to standard.

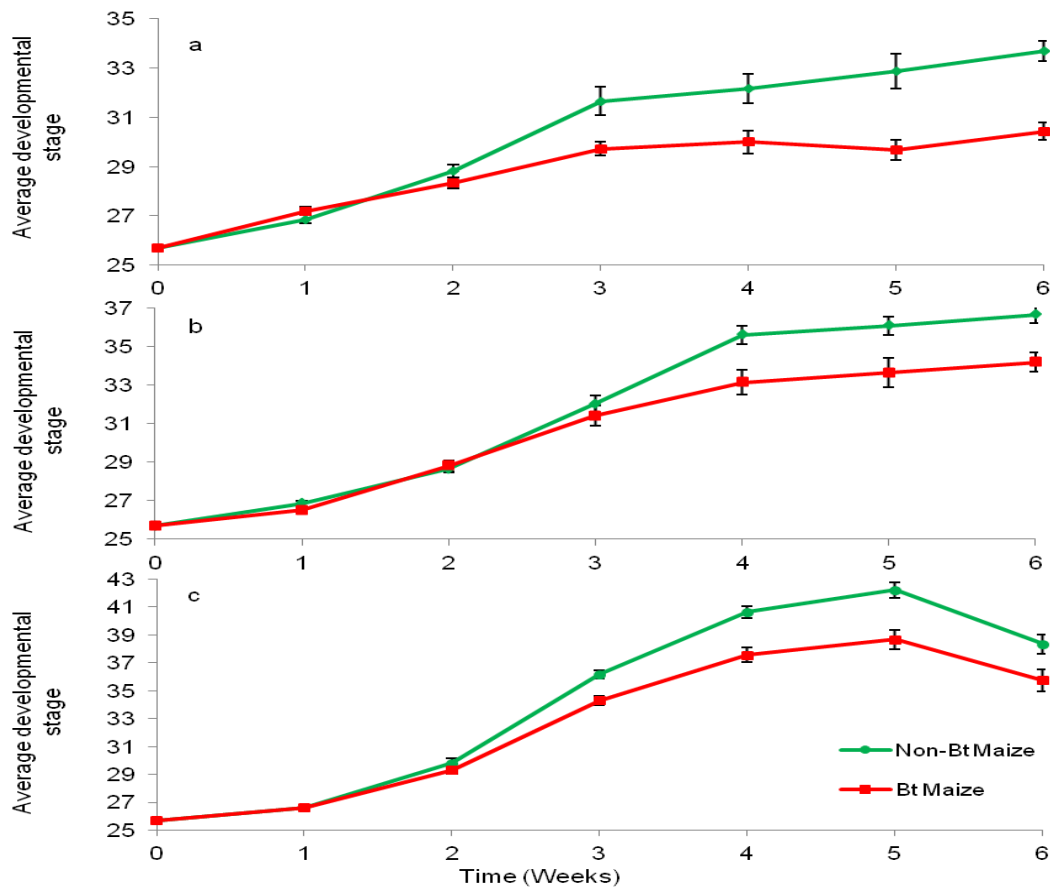


Figure 3.2. The average Nieuwkoop and Faber (1994) developmental stage of *Xenopus laevis* tadpoles over time. Tadpoles were maintained in 20 l microcosms containing (a) 15 (b) 30 and (c) 45 grams of maize leaves. Bars indicate standard error.

### 3.1.2. Experiment 2

Tadpoles that were fed on non-Bt maize leaves were slightly longer than those fed on Bt maize leaves for the first nine weeks of the experiment (Fig. 3.3a). From ten weeks onwards, Bt-fed tadpoles were of similar length than the non-Bt feeding tadpoles and no significant differences were observed ( $t = 0.386138$ ,  $p = 0.699740$ ).

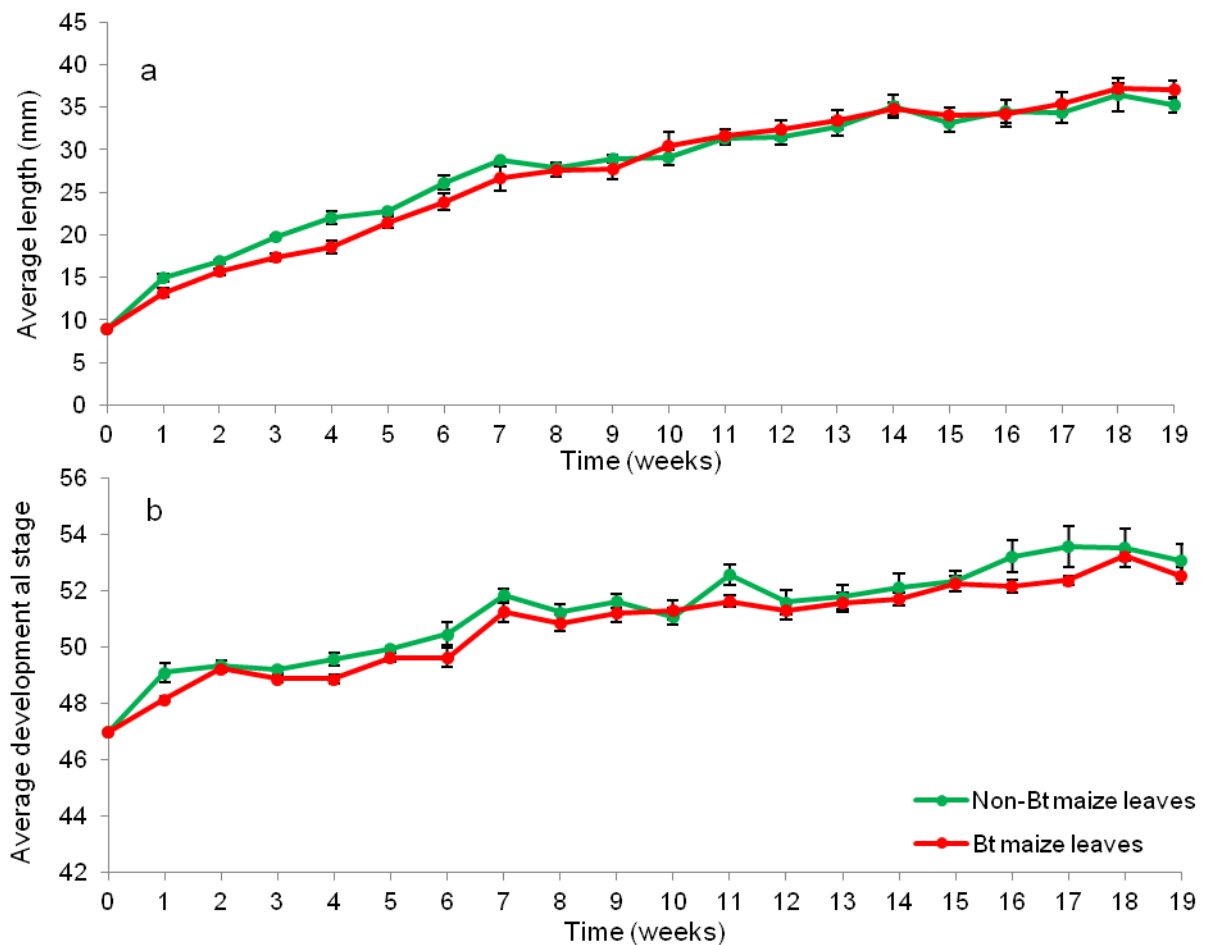


Figure 3.3. The a) average length and b) morphological development of *Xenopus laevis* tadpoles feeding on Bt maize leaves compared to non-Bt maize leaves over time. Bars indicate standard error.

At week 19, when the experiment was terminated the Bt-fed tadpoles had an average length of 37.1 mm where the non-Bt fed tadpoles had an average length of 35.2 mm (Fig. 3.3 (a)). The development of the tadpoles was gradual throughout the course of the experiment. The average NF stage (Fig.3.3 b) of the tadpoles that fed on pulverised non-Bt maize leaves was slightly more advanced than that of tadpoles feeding on Bt maize leaves. However, no significant differences were observed in the NF development stages between treatments at the end of the experiment ( $Z = 1.723775$ ,  $p = 0.084749$ ) (Fig 3.3).

At the end of the experiment Bt-feeding tadpoles and non-Bt feeding tadpoles had developed at the same rate, but non-Bt feeding tadpoles had an average NF stage of 53.1 whereas Bt-feeding tadpoles had an average of 52.5. T-tests done on data

collected at the final assessment of tadpole growth and development showed that the mean NF stage differed significantly ( $t = 1.983592$ ,  $p = 0.048451$ ) between Bt and non-Bt feeding tadpoles.

Comparing Fig. 3.3 (b) with Fig. 3.2 the *X. laevis* Bt and non-Bt feeding tadpoles in Experiment 2 developed further than in Experiment 1. A possible reason for this may be the duration of experiments. Experiment 1 was maintained for a period of six weeks while Experiment 2 lasted for 19 weeks so the tadpoles had more time to develop as well as to make sure that they had no deformities. Another reason may be the tadpoles (Experiment 2) had no maize leaves present in the water that could have made it unfavourable for the tadpoles to develop.

### **3.1.3. Experiment 3**

There were no noticeable differences in terms of length and developmental stage between non-Bt and Bt-feeding tadpoles for the first three weeks of the experiment. The same trend was observed in the growth and development (Fig. 3.4). Average length of the Bt-feeding tadpoles was notably reduced compared to the non-Bt feeding tadpoles from week seven onwards until termination of the experiment. The Bt-feeding tadpoles were longer with an average length of 36.50 mm while the non-Bt feeding tadpoles had an average length of 35.75 mm (Fig 3.4 a). However, this difference was not significant from that of non-Bt feeding tadpoles at the end of the experiment ( $Z = 1.351042$ ,  $p = 0.176683$ ).

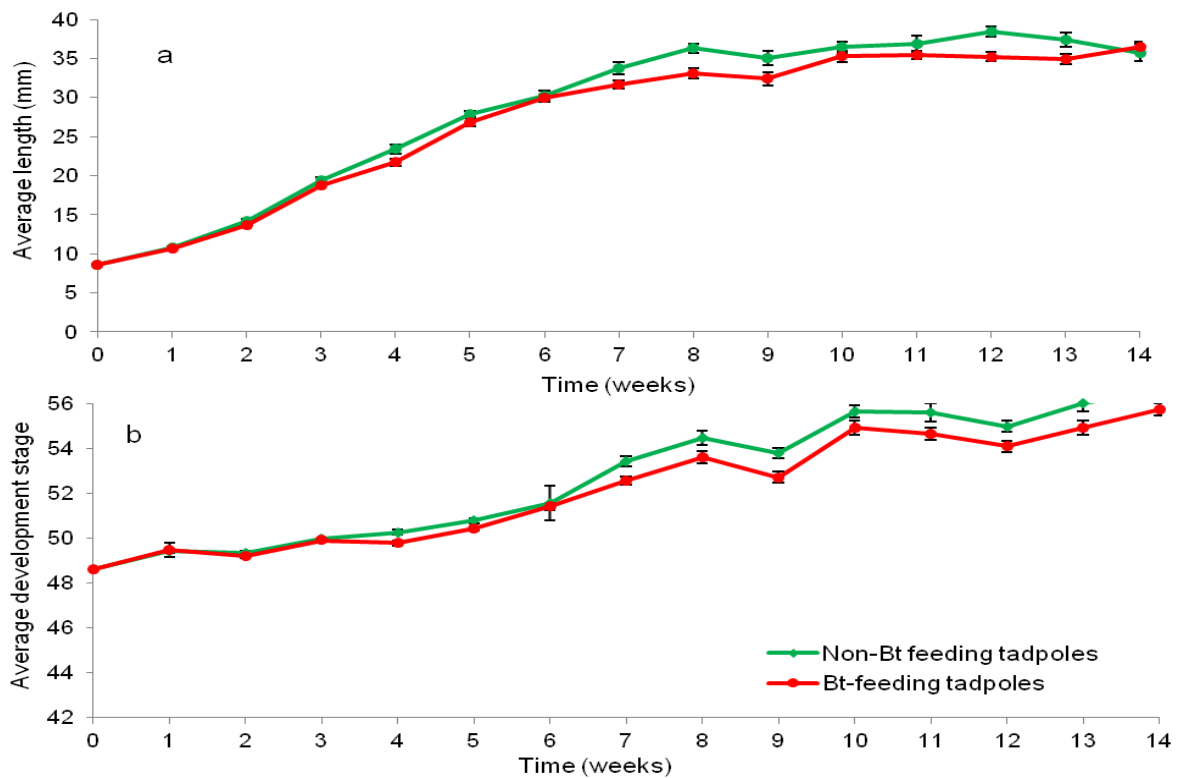


Figure 3.4. The a) average length and b) morphological development of *Xenopus laevis* tadpoles feeding on Bt maize leaves compared to non-Bt maize leaves over time. Bars indicate standard error.

Comparing NF stages of non-Bt feeding *X. laevis* tadpoles to the Bt-feeding tadpoles showed that the average NF stages were slightly more advanced in the former. The average NF stage for non-Bt feeding tadpoles was 56.6 compared to the Bt-feeding tadpoles (average NF stage 55.7) from week four to the end of the experiment (Fig. 3.4 b). These differences were however not significant ( $Z = 1.391549$ ,  $p = 0.164060$ ).

It is clear from Fig. 3.5. that the Bt-feeding tadpoles had a higher survival than the non-Bt feeding tadpoles during week two to six. This trend continued until the end of the experiment. The reason for the lower survival of the non-Bt feeding tadpoles is unclear because in Fig. 3.4 it seems non-Bt feeding tadpoles were doing better than Bt-feeding tadpoles in terms of length and development. Survival data for these experiments are clearly essential for a better understanding of the data.

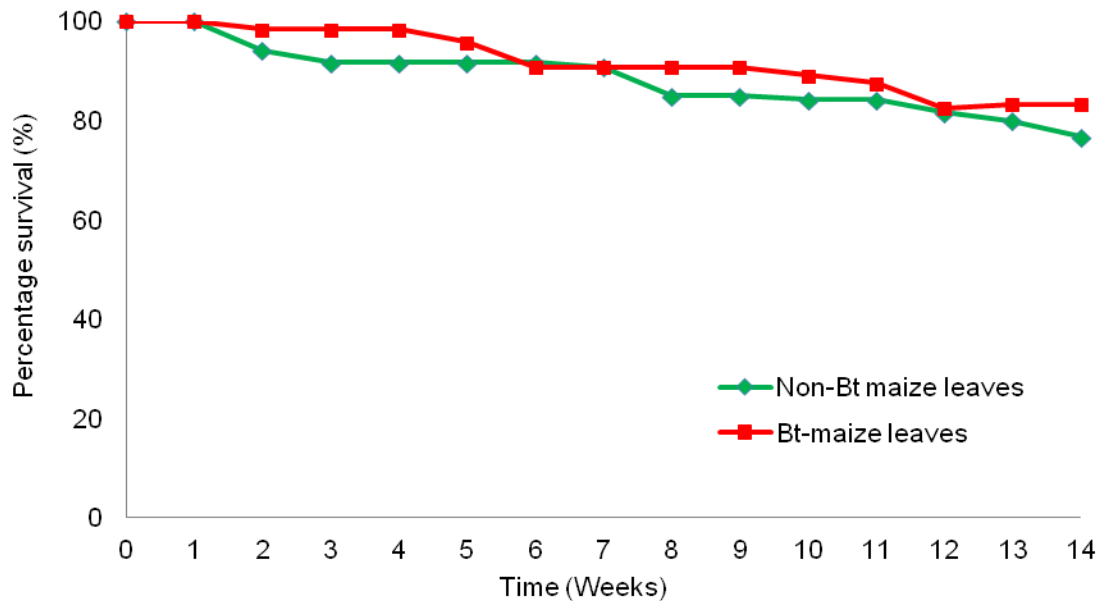


Figure 3.5. Percentage survival *Xenopus laevis* tadpoles over time in microcosms feeding on Bt or non-Bt maize leaves.

The disappointing results in terms of tadpole development and length in the above mentioned three experiments is ascribed to poor husbandry practices which resulted from attempts to establish large enough samples (50 individuals per microcosm) and not to disturb the tadpoles too often. According to guidelines developed by the National Research Council (1974) tadpoles should be fed daily and water in experiments should be replaced every three days. Experiment 3 showed the best results of the three experiments but still no metamorphs emerged after 14 weeks, where it usually takes five to six weeks for tadpoles to reach metamorphosis (National Research Council, 1974). These long development periods indicate that experiment conditions were not optimal. Although tadpoles received sufficient amounts of food, these results could indicate that the feed value maize leaves might not be a good food source for these animals.

## **3.2. *Amietophrynus gutturalis***

### **3.2.1. Experiment 1**

The tadpoles exposed to the different treatments, developed at the same rate in the beginning of the experiment during which there was a gradual increase in length (Fig 3.6) and Gosner development stage (Fig 3.7). The length and development of the Bt-feeding tadpoles followed the same tendency for the different treatments showing a delayed effect (lag effect) in growth for the middle part of the experiment. In all three treatments the lag effect was present in the following weeks the 15 g treatment week four to 12, the 30 g treatment week four to nine and the 45 g treatment from week four to nine showed. The average length of the Bt-feeding tadpoles for the different treatments were 25.8mm for the 15g, 27.0mm for 30g and 24.3mm for the 45g (Fig. 3.6), whereas the non-Bt feeding tadpoles average length were 25.8 for the 15g, 28mm for the 30g and 32.5mm for the 45g treatment (Fig. 3.6). No significant interaction was observed regarding tadpole length between groups (Bt and non-Bt) and treatments (15g, 30g & 45g) ( $F = 0.99469$ ,  $p = 0.370793$ ). T-tests were done on the mean length over the experiment showed significant differences ( $t -2.31309$ ,  $p = 0.021232$ ) with the Bt feeding tadpoles (14.06 mm) being shorter than the non-Bt feeding tadpoles (16.71 mm).

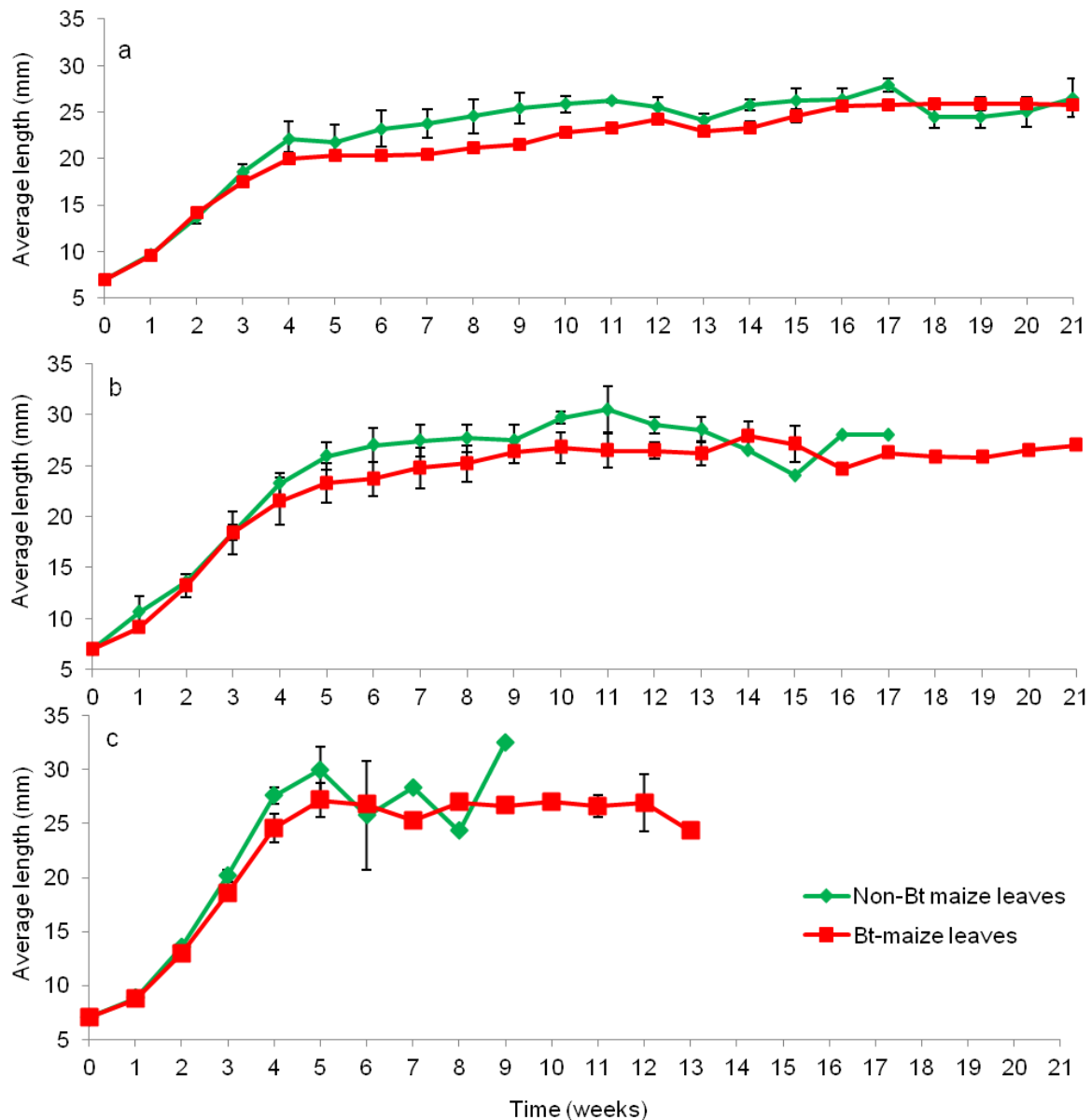


Figure 3.6. The average length of *Amietophrynus gutturalis* tadpoles over time. Tadpoles were maintained in 20 l microcosms containing (a) 15, (b) 30 and (c) 45 grams of maize leaves. Bars indicate standard error.

Bt-feeding *A. gutturalis* tadpoles had a similar developmental stage as non-Bt feeding tadpoles and that the three different maize treatments (15, 30 and 45g) did not have a significant ( $p > 0.05$ ) effect on development at the end of the experiment (Fig. 3.7). The morphological development of Bt feeding tadpoles for the different treatments were more gradual compared to non-Bt feeding tadpoles. At 15g maize leaves there were still tadpoles of both the Bt and non-Bt treatment that did not complete metamorphosis after 21 weeks (Fig 3.6 a) with 20% survival (Fig 3.8). The average Gosner stage was 36 for Bt-feeding tadpoles and 38 for non-Bt feeding

tadpoles at 15g maize leaves (Fig 3.7 (a)). The non-Bt feeding *A. gutturalis* tadpoles of the 30g treatment (Fig 3.7 b) almost completed metamorphosis at week 17 (average Gosner stage 41) while the Bt-feeding tadpoles were only at Gosner stage 38 at week 17.

Comparison of the Bt group with the non-Bt group at the end of the experiment showed a significant difference between the Gosner stages of Bt and non-Bt feeding tadpoles (t-value = -2.45; p = 0.01 Bt-feeding tadpoles that did not complete metamorphosis at the end of week 21 when they reached an average Gosner stage of 36 (Fig 3.7 b). The non-Bt feeding *A. gutturalis* tadpoles (Fig 3.10) of the 45g treatment took only ten weeks (average Gosner stage 40) to metamorphosis where the Bt-feeding tadpoles completed metamorphosis at week 14 with an average Gosner stage 43 at week 13.

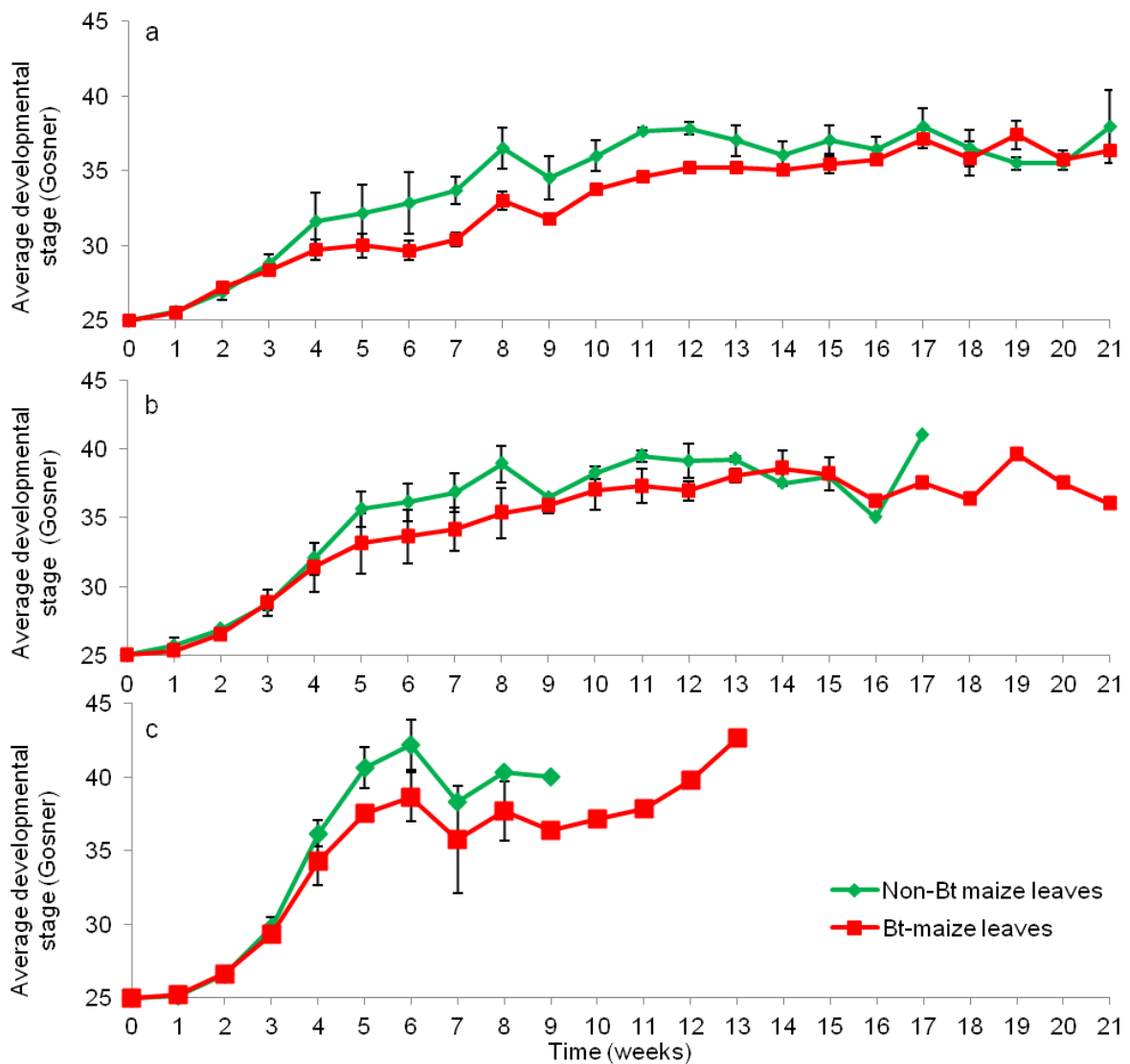


Figure 3.7. The average Gosner developmental stage of *Amietophrynus gutturalis* tadpoles over time. Tadpoles were maintained in 20 l micocosms containing (a) 15, (b) 30 and (c) 45 grams of maize leaves. Bars indicate standard error.

This data show a possible lag effect that *A. gutturalis* Bt-feeding tadpoles could have experienced during the initial stages of the experiment which did not affect mortality (Fig 3.8), but only “delayed” development of the tadpoles. According to Berill *et al.* (1998) *Anaxyrus americanus* pre-metomorphs with well-developing hind legs but un erupted front legs, (possibly Gosner developmental stage 41), exposed to endosulfan for 96 hours revealed swelling and discoloration after 24 hours, at the end of the 96 hour exposure eight were dead, none metamorphosed of the remaining seven tadpoles exhibited further swelling and discoloration and the animals had rigid hind legs. The control treatment tadpoles all behaved normally after 24 hours and after 96 hours eight tadpoles metamorphosed with the remaining

seven tadpoles had normal avoidance responses, had erupting forelimbs, and subsequently completed metamorphosis (Berill *et al.*, 1998). Jones *et al.*, (2009) showed that *A. americanus* individuals also showed a significant time-lag effect in development when exposed to the insecticide endosulfan for 4 days (10% mortality) and a mortality that ranged between 60-90% five days later in clean water. Weir *et al.* (2012) conducted acute and chronic toxicity tests with the herbicide trifluralin on *Lithobates clamitans* tadpoles. Chronic trifluralin exposure resulted in significantly smaller tadpoles at low concentrations (20 mg/L) compared with controls and 200-mg/L treatments (Weir *et al.*, 2012) showing low concentrations of the herbicide could affect tadpole fitness and growth.

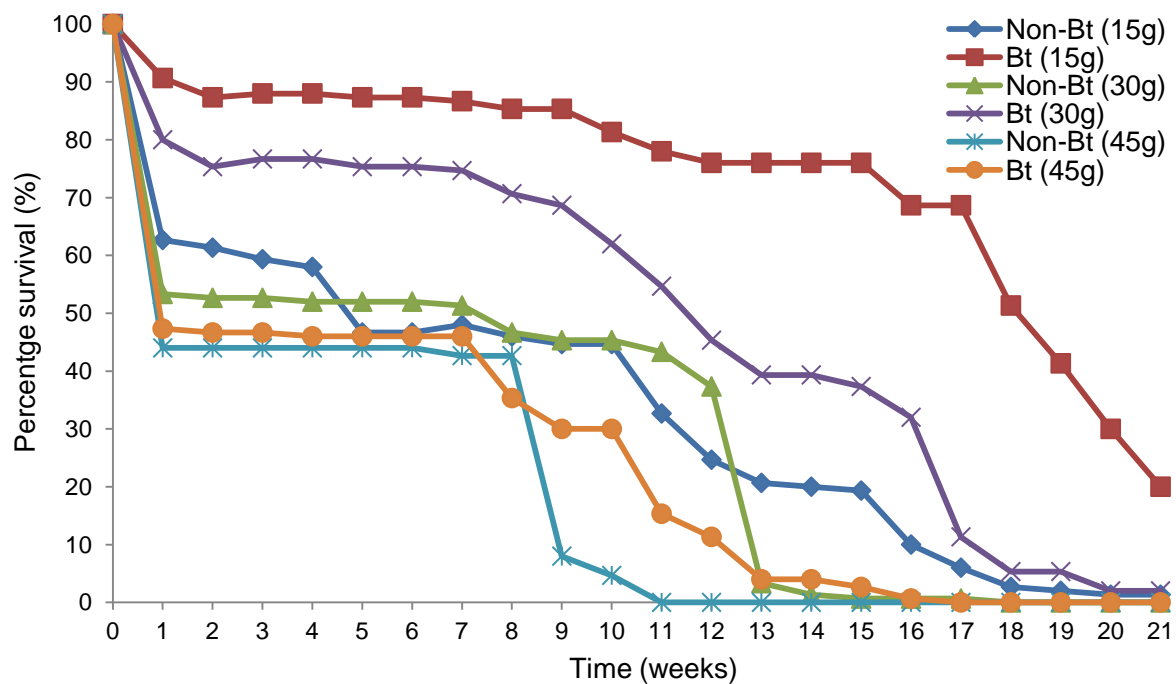


Figure 3.8. Percentage survival *Amietophrynus gutturalis* in microcosms containing different amounts of Bt and non-Bt maize leaves.

Table 1. LT50-values of *Amietophrynus gutturalis* tadpoles subjected to different treatments of feeding on Bt and non-Bt maize leaves

Treatments	LT50 (weeks)	95% Fiducial limits
Bt treatment 15 g	18.28	17.63-19.01
Bt treatment 30 g	9.54	9.20-9.88
Bt treatment 45 g	4.23	3.83-4.61
Non-Bt treatment 15 g	6.28	5.85-6.7
Non-Bt treatment 30 g	4.89	4.46-5.29
Non-Bt treatment 45 g	3.54	3.20-3.85

The period until 50 % tadpole mortality (LT50) of the Bt-feeding tadpoles in the 15 g treatment was 18.36 weeks (95%fiducial limits in Table 1). This was significantly longer compared to non-Bt feeding tadpoles subjected to the 15 g treatment (6.28 weeks). For the 30 g treatment LT50 values were significantly different between treatments with the Bt-exposed tadpoles having a LT50 value of 9.54 weeks and the non-Bt feeding tadpoles having a value of 4.89 weeks. This could be explained by Fig 3.8 were there was a high rate of mortality in the first week of the experiment and the 15 g and 30 g Bt treatments had the highest survival during this time and for the remainder of the experiment. The 45 g treatment LT50 values did not differed significantly, the Bt-feeding tadpoles LT50 value was 4.23 weeks and the non-Bt feeding tadpoles LT50 value was 3.54 weeks.

As the weight of the maize leaves increased the average developmental stage also seemed to increase (Fig 3.7) as well as the average weight of the metamorphs (Fig 3.9) and time to metamorphosis was faster (Fig 3.10). The reason for this could be that the 15g feeding tadpoles did not receive sufficient food to complete metamorphosis as well as this could indicate that maize leaves alone are inadequate diet for these tadpoles. The higher rate of metamorphosis in the 45g treatment of maize leaves is also an indication that the tadpoles could have had more food available than the 15g and 30g maize leaves (Fig 3.9). Another explanation for this may be that according to Griffiths *et al.* (2009) Bt maize has a faster decomposition

rate than non-Bt maize leaves. Thus Bt maize leaves loses its nutrients and feeding value at a higher rate than non-Bt maize leaves and these tadpoles then have then less food readily available to them causing them to starve resulting in a slower development. The mass of the 15 g metamorphs was significantly lower ( $F = 6.3156$ ,  $p = 0.002023$ ) than the other treatments, but the 30 g and 45 g treatments did not differ from each other. No significant interaction between groups (Bt and non-Bt) and treatments (15g, 30g & 45g)  $F = 1.3747$ ,  $p = 0.254890$  were observed. *Rana dalmatina* tadpoles showed no significant reduction in survival, larval growth (mass), snout–vent length, stage development or number metamorphosed with the acute exposure experiment to chlorpyrifos (Bernabo *et al.*, 2011). In contrast, chlorpyrifos exhibited significant chronic toxic effects on larval development, manifested as the appearance of abnormalities, including tail flexure, skeletal and muscle defects in later stages of development in tadpoles exposed to all ecologically relevant concentrations tested (Bernabo *et al.*, 2011). Bt-proteins may not affect tadpole length and development stage but may negatively impact tadpoles over time by delaying their development and increasing the risk of predation, desiccation and starvation.

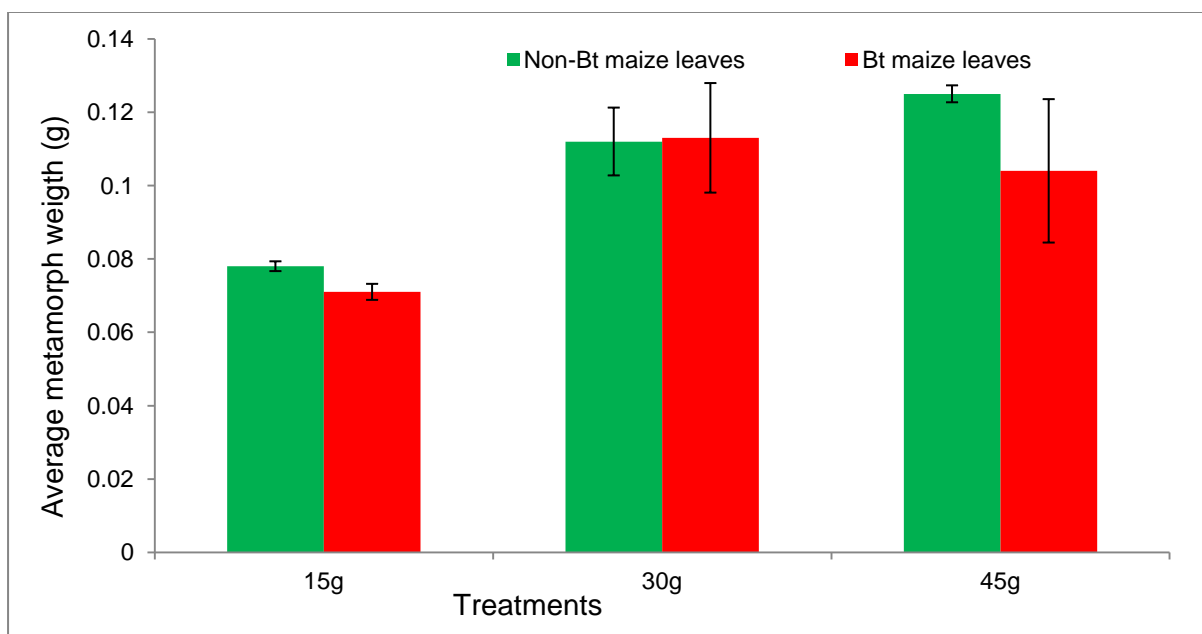


Figure 3.9. Average weight of *Amietophrynus gutturalis* metamorphs reared in microcosms on different amounts of Bt and non-Bt maize leaves for a period of 21 weeks. Bars indicate standard error.

*Amietophrynus gutturalis* are active between August to April and sometimes warmer periods in the winter months in South Africa. According to Du Preez *et al.* (2004) breeding usually starts as soon as the temperatures rise in spring (September) often before the first rains arrive in places where permanent small water bodies are present. In places where there are no permanent water bodies breeding is initiated by the first heavy rains. Metamorphosis is usually complete after 5 to 6 weeks (Du Preez *et al.*, 2004). In this experiment however, some tadpoles did not complete metamorphosis after 21 weeks (146 days).

The “lag” effect that tadpoles experienced in development (Fig. 3.7) and metamorphosis (Fig. 3.11) during this study could have a negative effect on *A. gutturalis* populations. Tadpoles in water bodies on maize farms could feed on maize debris that are left on fields after harvest and which may end up in the water bodies. This may result in longer development time to metamorphosis, this could lead to population declines if environmental conditions become unfavourable (drought). However, the results of this study cannot at all be extrapolated to field conditions since the animal husbandry as well as food supply to tadpoles and several other factors differed between the laboratory microcosms that were used and that experienced by tadpoles under natural conditions. In certain areas in South Africa there is a temporal overlap between breeding of *A. gutturalis* populations and harvesting of maize, making it possible for these tadpoles to come in contact with GM maize. An example of where *A. gutturalis* individuals may be exposed to Bt maize due to this temporal overlap is illustrated in Fig. 3.10, which is a typical environment where *A. gutturalis* occur inside maize cropping systems, in this case Bothaville in the Free State Province.



Figure 3.10. A typical environment where *A. gutturalis* occur inside maize cropping systems, in this case Bothaville in the Free State Province.

Under field conditions large masses of maize debris can enter streams during floods and with high winds and can accumulate in substantial amounts of debris inside dams and streams (Griffiths *et al.*, 2009). Maize inputs into streams tend to be temporally variable, yet they potentially occur throughout the year, particularly when no-till agricultural practices are used (Chamber *et al.*, 2010). Crop debris can therefore constitute a temporally variable but important substrate for microbial decomposers and invertebrate consumers. However, the potential effect is not yet understood and large gaps exist in our knowledge of frog biology and interaction with crop residues in agro-ecosystems. Similar shortcomings in understanding of the systems were highlighted by Rosi-Marshall *et al.*, (2007). If the results observed in this laboratory study can be extrapolated to field conditions it could be speculated that tadpoles may experience a delay in morphological development when Bt maize debris is present in high enough volumes farm water bodies. There might be a potential “lag” effect in development as illustrated in Fig 3.4 where Bt-feeding

tadpoles experienced slower development and metamorphosed later than non-Bt feeding tadpoles (Fig 3.11).

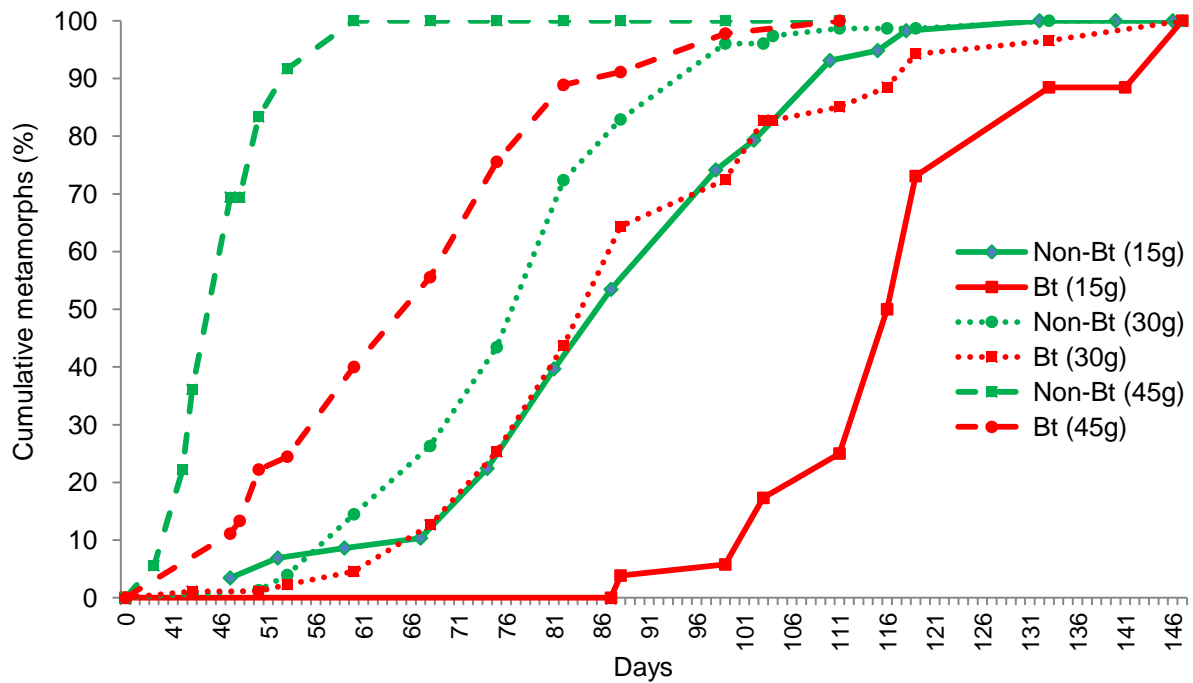


Figure 3.11. Cumulative percentage *Amietophrynus gutturalis* metamorphs developing over time in microcosms containing different amounts Bt and non-Bt maize leaves.

These results are however in no way conclusive since several known and unknown factors contributed to poor husbandry which may have influenced results. For example, maize leaves may not be a good food source. Further protocol development and research is necessary in order to extrapolate laboratory findings to field conditions. Realya (2007) indicated that there are interactions between stressors and tadpole development and argued that there are multiple ways that predators can influence larval growth and development directly and indirectly (via induction, thinning, and selection). Studies are therefore needed to evaluate if the presence of additional stressors such as predators (e.g. predatory beetles or dragonfly nymphs) together with Bt maize as food source in a more natural environment would impact on development rate of this frog species.

### 3.2.2. Experiment 2

This experiment differed from the one above in that tadpole individuals were kept individually in small containers and not in large groups. *Amietophrynus gutturalis* tadpoles were kept in 250ml plastic cups in 100ml borehole water where they were fed Bt and non-Bt maize leaves in sample quantities. There was a slight increase in the average length (Fig 3.12 a) in both Bt and non-Bt feeding tadpoles during the first two weeks of the experiment, but no significant differences in the average length between the two groups were observed at the end of the experiment ( $F = 2.50$ ,  $p = 0.1144$ ). At week six the average length of Bt-feeding tadpoles was 14.09mm and that of non-Bt feeding tadpoles was 14.31mm (Fig 3.12 a).

The average Gosner developmental stage of non-Bt feeding tadpoles increased slowly during the 6-week period until it reached an average of 25 at end of experiment (Fig 3.12 b). The Bt-feeding tadpoles showed little development through the experiment and at week six had an average Gosner stage of 25 the same as the non-Bt feeding tadpoles, but with no decrease in average development at week six. The Gosner stages differed significantly ( $F = 105.31$ ,  $p < 0.0001$ ) with non-Bt feeding tadpoles having a higher Gosner stage than the Bt feeding tadpoles. Although significant, in reality, this difference in Gosner stage was extremely small, and is not expected to have any effect on fitness of individuals in natural environments.

Survival of both groups was 100% for the first three weeks of the experiment (Fig 3.12 (c)). At week 4 the non-Bt feeding tadpoles remained without any mortality while in the Bt-feeding tadpoles 98% survival was recorded. At Week 5 the survival was 98% for both groups and remained 98% for the non-Bt tadpoles, but the Bt-feeding tadpoles declined with a further two percent to 96% in week six.

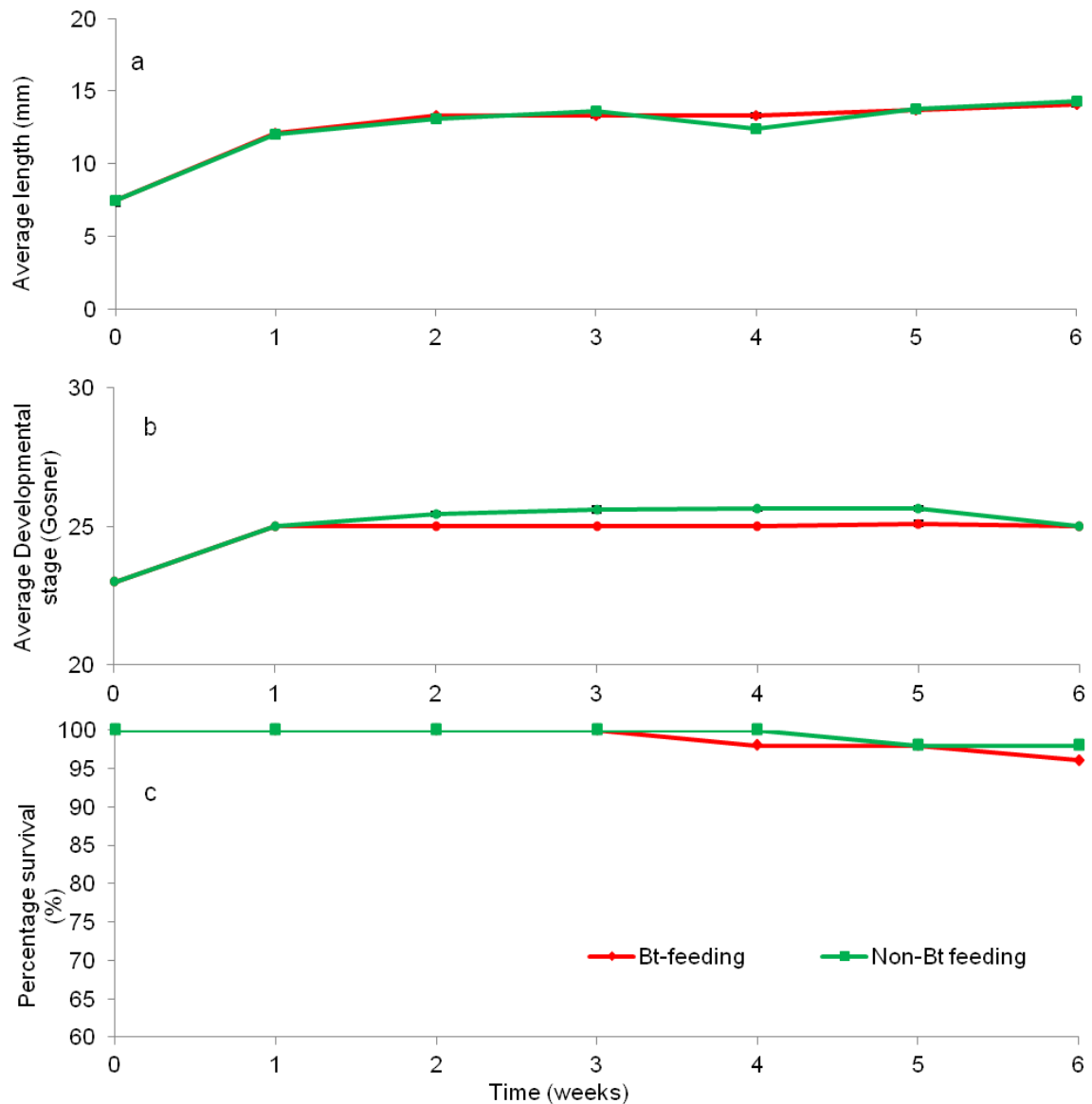


Figure 3.12. The a) average length, b) average Gosner developmental stage and c) percentage survival of tadpoles in 100ml water feeding on Bt or non-Bt maize leaves over time. Bars indicate standard error.

Experiment 2 (Fig. 3.12) showed low development of Bt and non-Bt feeding tadpoles over the course of 6 weeks and although survival was high, this experiment was terminated due to unsatisfactory levels of growth.

### 3.2.2. Experiment 3

Tadpoles in non-Bt treatment had an average length of 23.1mm (Fig 3.13 a) and an average Gosner stage of 40.9 (Fig 3.13 b) at the end of week 6 while tadpoles in Bt-water had an average length of 21.91mm (Fig 3.13 a) and average Gosner stage of 26.7 (Fig 3.13 b). The tadpoles in the non-Bt treatment therefore showed faster morphological development than tadpoles in the Bt-treatment. Although significant differences (F-value 155.44;  $p < 0.0001$ ) in length as well as in Gosner developmental stage (F-value = 1485;  $p < 0.0001$ ) were observed no conclusions should be made from this due to the high rate of mortality in this experiment. Tadpoles in the non-Bt treatment showed a high mortality rate at the beginning of the experiment and continued with this trend until the experiment was terminated. The mortality of tadpoles in Bt- treatment was lower than those in the non-Bt treatment. The survival of the tadpoles in the Bt and non-Bt treatments were 26% and 20% respectively at the end of the experiment (Fig 3.13 c).

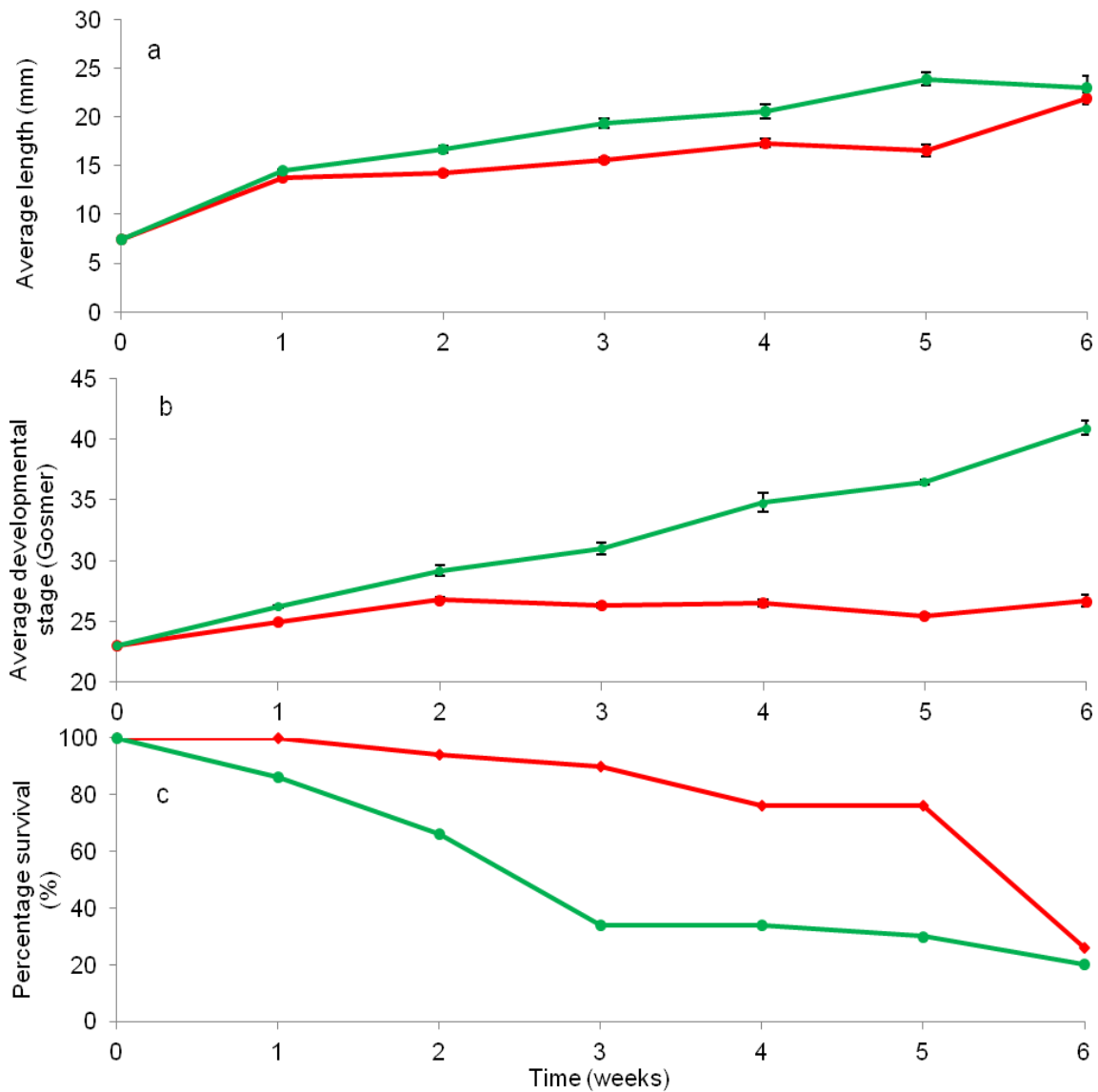


Figure 3.13. The a) average length, b) average Gosner developmental stage and c) percentage survival of tadpoles in 100ml maize leaves water (Bt and non-Bt) over time. Bars indicate standard error.

Although larval development in Experiment 3 was satisfactory (Fig. 3.13), the experiment was terminated due to low survival rates.

## CHAPTER 4

### CONCLUSIONS AND RECOMMENDATIONS

The following important findings were made during these studies:

1. Length may be an inappropriate measurement of the effect that a potential stressor such as a Cry1Ab protein produced by Bt maize could have on tadpole growth. The reason for this is that the growth stage as scored by the Normal Table of *Xenopus laevis* development (Nieuwkoop & Faber, 1994) and Gosner (1960) stages for *A. gutturalis* did not always correlate with length measurements. Combining tadpole length with tadpole mass as well as developmental stages could provide us with a clearer insight of what a potential stressor like Bt maize have on tadpole growth (AMA, 2008). The problem of this method is all the tadpoles must be dried off to the same degree, this could cause additional stress for them and could lead to death. Behavior studies with video-tracking could indicate early effects of toxicity (Denoël *et al.*, 2012).
2. The optimum treatment for evaluating the potential effects of maize cultivars on tadpoles seemed to be 30g/20l of water. *Amietophrynus gutturalis* tadpoles in Experiment 1 displayed slower development and higher survival than the 45g maize leaves in 20l of water. Based on metamorph weight, 30g/20l water were the closest to each other when comparing Bt to non-Bt maize leaves, and the highest number of metamorphs emerged from this treatment.
3. Results from this study should be interpreted carefully since it seems that the maize leaf diet on its own is not optimum. Under field conditions, *X. laevis* take approximately two months to reach metamorphosis while in this study it took approximately five months. *Amietophrynus gutturalis* takes approximately 6 weeks under field conditions, while it took 21 weeks in this study for them to reach metamorphosis. It is necessary for future studies to make use of mesocosm experiments to evaluate the effect of Cry1Ab as a potential stressor on tadpoles where tadpoles may be more exposed to field conditions and not only feed on maize leaves.

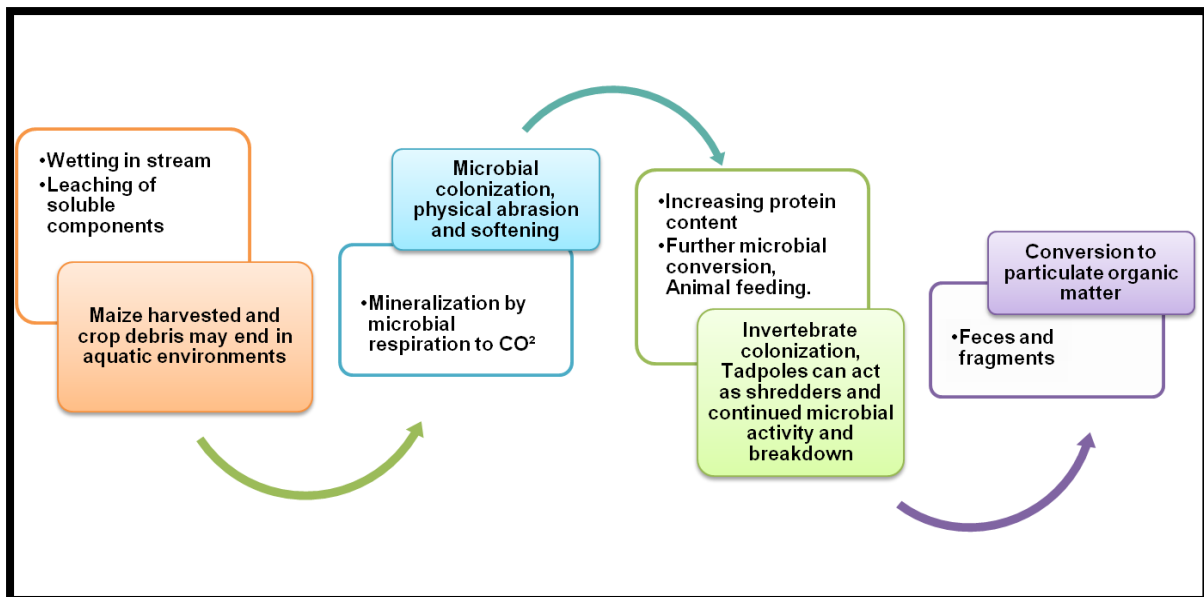


Figure 4.1. Leaf breakdown process in natural streams (adapted from Carstens *et al.*, 2011).

4. According to Fig. 4.1 tadpoles may be present in a water body that may contain Bt maize leaves. The maize leaves are not the only food source that will be available to the tadpoles and some of them might not even feed on the maize leaves. Although tadpoles may act as shredders in the decomposition of maize leaves they might not be exposed in sufficient amounts to have an effect on the tadpoles. It is therefore necessary that the tadpoles must be exposed to more environmental factors and other food sources at different maize leaf concentrations to determine if the tadpoles are in any sense negatively affected by Bt maize leaves. A food preference test must also be done to determine if the tadpoles will actually feed on maize leaves when other food sources are available.
5. Future studies should address concentrations of Cry1Ab proteins in aquatic systems exposed to Bt maize leaves and should take into account temporal differences that may occur in the exposure levels.
6. In this study, protocol development was key and reasonably successful. Towards the end of this study we got hold of a document titled “The proposed husbandry for *X. laevis* experiments”. This document by the National

Research Council of the United States provides guidelines on *the breeding, care and management of laboratory animals* (National Research Council, 1974).

The essential information relevant to this study includes that:

- (a) the development of *X. laevis* tadpoles are usually rapid at 20-22°C.
- (b) the amount of finely ground (pulverized) food administered in measured amounts should permit clearing of the water after four to five hours (National Research Council, 1974).
- (c) tadpoles should be fed daily with fresh food.
- (d) tadpoles should not be netted until they had fed for a week and larval density should not exceed six to eight tadpoles per litre water and the water should be changed and containers cleaned every three days (National Research Council, 1974).

Under the conditions described above, *X. laevis* metamorphosis should begin at five to six weeks after fertilization. Metamorphosis takes 15-20 days. After forelimb eruption tadpoles stop feeding and the tail is absorbed (National Research Council, 1974). Results from this study were not close to the ranges described in the guidelines to development of the different life history parameters (National Research Council, 1974).

The significant effects observed in some life history parameters of tadpoles exposed to Cry1Ab protein cannot be ascribed to the effect of the protein. Poor husbandry turned out to be the single most important confounding factor and before a follow-up study is undertaken further development studies, aimed at optimising husbandry practices should be undertaken.

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