

The effect of glyphosate and Cry1Ab proteins on the growth and survival of tadpoles of two amphibian species

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List of acronyms and definitions

Acronyms

AE:	Acid equivalence
AI:	Active ingredient
AMPA:	Aminomethyle phosphonic acid
Bt:	<i>Bacillus thuringiensis</i>
ELISA:	Enzyme linked immunosorbent assay
EPSP:	-enolpyruvylshikimate-3-phosphate
GH:	Growth hormone
GM:	Genetically modified
ICGEB:	International centre for genetic engineering and biosafety
IPA:	Isopropylamine salt
LC50:	Lethal concentration of 50%
NRC:	National Research Council
OECD:	Organisation for Economic Co-operation and Development
PCR:	Polymerase chain reaction
POEA:	Polyoxyethylene amine
RIA:	Radioimmunoanalysis
TH:	Thyroid hormone
TSH:	Thyroid stimulating hormone
T3:	Triiodothyronin
T4:	Thyroxine

Definitions

- Acid equivalence: "Theoretical yield of parent acid from a pesticide active ingredient which has been formulated as a derivative" (Nordby & Hager 2004);
- Acclimatize: Process in which an organism gradually adjusts to change in its environment (Chinathamby *et al.* 2006);
- Active ingredient: "Component of a pesticide formulation responsible for its toxicity or ability to control the target pests" (Nordby & Hager 2004);
- Adjuvant: Any substance present in a herbicide formulation or is added to the herbicide to improve the herbicidal activity (Curran *et al.* 2014);
- Control: Test organisms are exposed to water which contains no toxicants (LeMay 2014);
- Endocrine gland: Secretes hormone directly into the blood (Upshall 1992);
- Exocrine: Secretes hormones onto a surface of a target organ (Upshall 1992);
- Half-life: The amount of time needed to allow the radioactivity of the isotope to decrease to half its value (Upshall 1992);
- Hard water: High mineral content containing calcium, magnesium, limestone, chalk and dolomite (Jayasumana *et al.* 2014);
- Heterocrine gland: A gland that secretes a hormone into both the bloodstream and surface of target organs;
- LC50: The concentration that kills 50% of a population over a certain time period (Relyea 2004);
- Nephrotoxic: Metals damaging to the kidneys (Jayasumana *et al.* 2014);
- Non-target: Not being the main goal of the pesticide;
- Parotid glands: Gland situated in front of the ear (Upshall 1992);
- Sensitivity: The degree of being sensitive (Upshall 1992);
- Septicaemia: Blood poisoning (Upshall 1992);
- Sexual dimorphism: The difference between the male and females' phenotype (physical appearance);

- Tillage:** The soil preparation by means of mechanical disturbances example digging, stirring and overturning (Upshall 1992);
- Watershed:** The ridge that separates water flow to different aquatic areas (Upshall 1992; Farlex 2000).
- Xenobiotic:** Unfamiliar substance or toxin found in and unrecognized by the body (Lincoln *et al.* 1998).

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Abstract

Studies have shown that pesticides have negative effects on non-target organisms. Lately more studies are focusing on amphibians due to their rapid world-wide decline. This study addressed the potential effects of glyphosate (herbicide) and Cry proteins produced by genetically modified Bt maize (insecticide), and focuses on the development of tadpoles. Crops can be genetically modified by inserting a specific gene, in this case that of the soil bacterium *Bacillus thuringiensis* (Bt), into their genomes. This gene encodes for proteins which have insecticidal characteristics. This protein can then protect the crops from certain Lepidopteran pests. Glyphosate, the herbicide used in this study, is a non-selective systemic herbicide chosen due to its popularity as an active ingredient in many herbicides. Genetically modified Bt maize was selected because of its wide-scale cultivation and possible adverse effects that the insecticidal protein (Cry1Ab) found in aquatic systems may have on amphibians. *Xenopus laevis* and *Amietophrynus gutturalis* tadpoles were chosen based on their wide distribution in South Africa and because they are found in areas where herbicide tolerant and Bt maize are planted. It is believed that each amphibian species thrive in different conditions. For this reason a husbandry study was developed for *X. laevis*, where it was found that *X. laevis* thrives while in groups and *A. gutturalis* thrives individually. The aims of our study were to expose these tadpoles to different concentrations of glyphosate (laboratory based study) and genetically modified Bt maize leaves (laboratory and mesocosm based study) and monitor their development. We hypothesised that glyphosate will affect the development of the tadpoles exposed to the higher dosages and that the tadpoles exposed to Bt maize leaf infusions will develop slower than in the control treatment. After exposing tadpoles to Bt and non-Bt infusions, it was found that both treatments have adverse effects on the development of *X. laevis* tadpoles. However, the severity of the toxicity could depend on the hybrid or its genetic background. Exposure to the three highest glyphosate concentrations resulted in 100% mortality after three weeks. However, tadpoles exposed to the lowest concentration of glyphosate had a similar growth and developmental pattern as those in the

control group, although only 50% reached metamorphosis when compared to that of the control treatment. The results for the degradation of the Cry1Ab proteins were inconclusive. However, an increase in degradation was followed by a sudden decrease before reverting to a steady increase at the end of the test. The Cry1Ab protein concentration flattened off between 10 and 21°C temperatures, and no decrease in concentration was seen during the exposure periods between one hour and sixteen days. During a mesocosm experiment it was also found that the development and growth of tadpoles was less affected in the Bt and non-Bt infusions than in the control. Considering these results, we conclude that the presence of the Bt toxin at environmentally relevant concentrations, does not effect on the development or survival of *Amietophrynus gutturalis* tadpoles.

Keywords: *Bacillus thuringiensis*, genetically modified maize, *Xenopus*, *Amietophrynus*, Cry1Ab degradation.

Uittreksel

Vorige navorsing het bewys dat plaagdoders 'n negatiewe effek op nie-teikenorganismes kan hê. As gevolg van die wêreldwye afname in getalle van amfibië het navorsing op hierdie organismes oor die afgelope aantal jare toegeneem. Hierdie studie het gefokus op die effek van verskillende konsentrasies van 'n onkruidodder (glifosaat) en insekdodende Cry proteïen op paddavisse se ontwikkeling. Gewasse kan geneties gemodifiseer word deur 'n spesifieke geen in hul DNA in te bou. Die geen in hierdie geval is die van 'n grondbakterie genaamd *Bacillus thuringiensis* (Bt). Hierdie geen veroorsaak dat plante 'n insekdodende proteïen produseer wat dit beskerm teen sekere Lepidoptera plae. In hierdie studie is glifosaat gebruik omdat dit 'n algemene aktiewe bestanddeel van onkruiddoders is, terwyl Bt mielies gebruik is omdat dit op groot skaal aangeplant word en moontlike negatiewe effek op nie-teikenorganismes mag hê. *Xenopus laevis* en *Amietophrynus gutturalis* paddavisse is vir hierdie studie gekies omdat hul verspreiding met die verbouingsarea van mielies ooreenstem. Die oogmerk was om paddavisse van hierdie spesies aan verskillende konsentrasies glifosaat (laboratorium studie) en Bt mielieblare (laboratorium en mesokosmos-studie) bloot te stel en die ontwikkeling en groei van die paddavisse te monitor. Ons hipotese is dat glifosaat 'n effek op paddavisse sal hê en dat die effek verband sal hou met die konsentrasie daarvan. 'n Verdere hipotese is dat paddavisse wat in water aan Bt blootgestel is 'n soortgelyke algemene ontwikkeling sal hê as die wat blootgestel is aan water wat nie Bt bevat nie. Die volgende gevolgtrekkings is gemaak: elke amfibiese spesie het hul eie voorkeure (geoordeel aan die hand van groei, ontwikkeling en oorlewing). *Xenopus laevis* paddavisse het beter ontwikkel en meer het oorleef indien hulle in groepe geplaas is, terwyl *A. gutturalis* beter oorleef het wanneer hulle individueel aangehou word. Na blootstelling van paddavisse aan Bt en nie-Bt behandelings, is gevind dat beide 'n effek het op die groei en ontwikkeling van *Xenopus* paddavisse, maar dat die graad van die effek bepaal word deur die mieliebaster wat gebruik word of die genetiese agtergrond van die mielies. Die blootstelling aan die drie hoogste glifosaat-konsentrasies het gelei tot 100% mortaliteit na drie weke, maar by die laagste

konsentrasie is soortgelyke groei en ontwikkelingspatrone waargeneem as by paddavisse in die kontrolebehandeling. Blootstelling het egter gelei tot 'n 50% afname in die aantal individue wat metamorfose bereik het. Geen gevolgtrekking kon gemaak word rakende die degradasie van die Cry1Ab proteïen in water nie. Die proteïen-konsentrasie het aanvanklik toegeneem, toe weer gedaal waarna dit weer toegeneem het. Beide Bt en nie-Bt behandelings is by drie verkillende temperature vir 16 dae gehou. Die Bt konsentrasie het by 10 en 21°C begin afplat, maar geen totale degradasie van die proteïen is gesien nie. Dus was die blootstelling tydperk nie lank genoeg nie. Die mesokosmos het getoon dat paddavisse se groei en ontwikkeling die vinnigste was wanneer hulle blootgestel was aan die Bt en nie-Bt behandelings as in die kontrole. Hierdie studie het getoon dat Bt teen omgewingsrelevante konsentrasies, geen nadelige effek het op die ontwikkeling en oorlewing van *Amietophrynus gutturalis* paddavisse nie.

Sleutelwoorde: *Bacillus thuringiensis*, geneties-gemodifiseerde mielies, *Xenopus*, *Amietophrynus*, Cry1Ab degradasie.

Preface

This dissertation is in fulfilment of the requirements for the degree *Magister Scientiae* in Environmental Sciences at the North-West University (Potchefstroom Campus). The following authors have ownership of this data:

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Submission of this dissertation has been approved by Prof L.H. du Preez and Prof J. van den Berg. The format of the dissertation is based on the instruction for authors of the *African Zoology Journal* (Appendices)

CHAPTER 1

LITERATURE REVIEW

1.1. Legislation and regulations

Healthy environments as well as a healthy agricultural environment are both of the utmost importance for humanity. Agriculture is instrumental in providing food, but this can inevitably have an impact on the environment. This is mainly due to the conversion of natural habitats to agricultural fields. Agricultural fields need to use natural resources like water, soil and synthetic inputs like pesticides and fertilizers in order to sustain life. When carefully managed, the environment is quite resilient, however overgrazing, soil erosion and chemical pollution can have a negative impact on the environment. The increased global human population places an unprecedented demand on agricultural resources. Because of huge demands and capitalist societies, there are constant risks that the carefully balanced scale of nature would tip and cause damage to the environment. This would exceed acceptable boundaries and calls for regulations through legislation.

Legislations are an important governmental instrument that aids in organizing society, protecting individuals and also determines the rights and responsibilities of citizens as well as authorities (depending on who the legislation applies to). Regardless of the importance of legislation, they are of no value if not enforced or enforced without discipline (De Jager 2000).

In 1989, a framework of the NRC (National Research Council) in USA was written in order to regulate the use of genetic modification technology (Nap *et al.* 2003). The guidelines of the OECD 1993 (Organisation of Economic Co-operation and Development) for the applications of genetically modified (GM) organisms on an industrial level lead to extending the evaluation of GM organisms as well as the safety assessment thereof on the environment (Nap *et al.* 2003). The Cartagena protocol on biosafety is a more recent framework for the implementation by individual countries (Nap *et al.* 2003). There are currently different databases that provide

information on competent authorities and relevant legislations and regulations of individual countries which include *UNIDO BINAS Moodle*, *ICGEB* (International Centre for Genetic Engineering and Biosafety) and *AGBIOS* (Nap *et al.* 2003).

South African legislations and regulations

Regulatory systems are in place to ensure effective evaluation of biosafety of GM crops. There are different organisations that help generate such systems in each country. The Constitution of the Republic of South Africa as well as several acts refers to protection of people and the environment.

Constitution of the Republic of South Africa (No. 108 of 1996)

It is important to remember that, though ecologists and environmentalists strive to protect the world and all creation in it, the rights of every person must be considered. This is where the Constitution of the Republic of South Africa (No. 108 of 1996) comes into play. It states that all people of South Africa have a right to dignity, freedom and equity (108/1996: S7 (1)) as well as a right to give his or her opinion in matters that can affect them. The public has a right to an environment that isn't harmful to their health and that is protected for future generations by preventing pollution and promoting conservation (108/1996: S24). The public also has a right to water, food (108/1996: S27), housing (108/1996: S28) and the right to work or create work (108/1996: S23). Whether or not the human population continues to grow, the country still has a right to use as much water and soil resources as it requires. Even though these rights allow the exploitation of the environment and its resources, the environment and its resources have their own set of *rules* which will now be discussed.

Genetically Modified Organisms Act-GMO Act (No. 15 of 1997)

According to this Act, no activity may be undertaken where genetic modification is involved unless a suitable risk assessment is made as to its effect on the environment and human health (15/1997: S3, 1). It also states that a lack of scientific knowledge on the use of a GMO

will not be interpreted as a specific risk level (15/1997: S3, 2). This means that a GMO will not be seen as a threat to the ecosystem, unless there is research that supports this claim.

The public also has a right to receive a notice when new GMO are released as well as a right to give their opinion on the matter (15/1997: S6, 2 & 6). The GMO Act states that the effects of GM plants (in this study, maize) on target and non-target organisms should be monitored and reported regularly. It supports the safe use of GM crops in South Africa and promotes the responsible development, production as well as application of GMOs in the agricultural sector. It also ensures that any such activities are done in a way that will reduce any negative consequences to the environment and on human health (15/1997).

National Environmental Management Act-NEMA (No. 107 of 1998)

This Act aims to achieve environmental governance co-operation by providing decision making principles on environment related matters. It stipulates that people have a right to a healthy environment, but if the health and wellbeing of people are not threatened, the environment should be managed as best as possible during developmental activities. The principles of NEMA give a general framework in which environmental management and implementation programs should be formulated (107/1998: S1, 1b). It also insists that the people and their needs are of the utmost concern (107/1998: S1, 2). However, when developing, it must be socially, environmentally and economically sustainable (107/1998: S1, 3).

Sustainable development as described in this Act requires that if disturbance of an ecosystem is inevitable, as well as loss of biodiversity, pollution and degradation of environments, it must be minimized (107/1998: S2, 4ai, ii). Section 2 (4) aviii of the Act therefore states that any negative impacts on the environment must be prevented or minimized. The purpose of environmental implementation and management plans is to create a balance between conserving the environment and the needs of the human population (107/1998: S12).

National departments of each province responsible for environmental affairs must prepare an environmental implementation plan every four years. This includes the department of Environmental Affairs and Tourism, Land Affairs, Agriculture and Water Affairs and Forestry.

National Water Act-NWA (No. 36 of 1998)

This Act provides laws related to water resources as well as to revoke other specific laws that pose a threat to the resources. This Act recognises the importance of water to people, its scarcity and the need for effective management thereof. It ensures that the nation's water resources are protected, conserved, managed and controlled (36/1998: S2) while taking into account the rights of people (Constitution of the Republic of South Africa) (107/1998: S2a). Section 2(g) ensures the protection of aquatic and associated ecosystems and their biological diversity, while being solely responsible for the development of monitoring plans for aquatic resources.

Part one, two and three of Chapter 3 give different measures that should be followed to ensure the protection of all water resources, while part four and five is associated with prevention measures for pollution of water resources. Chapter 4 S21 (i) states that the Act is also implemented to prevent any sort of pollution that can alter the bed, banks, course or characteristics of a water source.

Water Service Act (No. 108 of 1997)

The main objective of this Act is to allow access to water and sanitation by setting national standards (Department of Water affairs 2009). This Act also strives to provide developmental plans, monitoring and frameworks for water services, water boards and services establishments as well as the powers and duties of the committee. This Act also provides finances to assist the institution of water services (108/1997).

Water Research Act (No. 34 of 1971)

Here research related to Water Affairs is promoted and has led to the establishment of the Water research Commission and Water Research fund (Department of Water Affairs 2009).

The Biodiversity Act strives to manage and conserve the biodiversity of South Africa while staying within the framework of the National Environmental Management Act (NEMA) (1998). NEMA includes the protection of ecosystems and species, the sustainable use of indigenous resources and equal benefits of biological resources (10/2004).

PESTICIDES

1.2. Pesticides in South Africa

Agriculture plays a vital role in the South African economy as it provides employment, food and foreign exchange income (Greyling & Vink 2012). In order to ensure high yield, certain technologies have been developed to limit yield losses that weeds, diseases and pests have been developed. An example of this is the development of pesticides to protect crops from harmful plants and insect pests. Pesticides are the collective term for both herbicides and insecticides and are beneficial to crop production in that it targets destructive plant or insect species without affecting the crops. This is because most crops are herbicide and insecticide resistant (Wagner *et al.* 2013). Despite possible benefits (including that of yield increase), there remains a need to assess the possible impacts of these pesticides on non-target species (Relyea & Jones 2009a; Jones *et al.* 2010).

Many studies have been done on the effects that pesticides could have on certain non-target organisms. Pesticides are most-likely to end up in aquatic systems as a result of runoff, spray drift or inadvertent overspray from both ground and aerial application (Relyea 2005c; Jones *et al.* 2010; Edge *et al.* 2011; Jones *et al.* 2011; Perez *et al.* 2011; Relyea 2012; Güngördü 2013; Hanlon *et al.* 2013; Hanlon & Parris 2014). Amphibians for example, have been decreasing in number and it is thought to be partly due to the presence of pesticides in water (Relyea 2005a; Relyea 2005b; Relyea *et al.* 2005; Edge *et al.* 2011; Hanlon & Parris 2014).

Today more attention is given to identify the effects of certain pesticides on amphibians, but few amphibians have shown a decline in survival at pesticide concentrations found in nature

(Relyea 2005a). According to Relyea (2005b) the lack of data on the effects of pesticides on amphibians is due to the “*federal regulations for registering pesticides require testing birds, mammals, fish, and aquatic invertebrates, but not amphibians*”. When studying the effects of any pesticide on amphibians (or any organism for that matter) in the laboratory, it is important for the setup to be as environmentally relevant as possible (Relyea 2005a).

Herbicides

Herbicides are not only used in agriculture, but also in silviculture to limit competition with other plants (Thompson *et al.* 2004; Edge *et al.* 2011; Edge *et al.* 2013). Herbicides are widely used because they kill target plants and micro-organisms, but not animals or herbicide tolerant GM crops (Jaan 2012; Relyea 2012; Lanctôt *et al.* 2013; Yadav *et al.* 2013). This selectivity of certain herbicides, in most cases glyphosate (found as an active ingredient in Roundup®), on the target plants that are not genetically modified to be tolerant to these herbicides is due to the active ingredient of the herbicide compound targeting the synthesis of aromatic amino acids via the shikimate pathway (discussed below; Edge *et al.* 2011; Wagner *et al.* 2013; Yadav *et al.* 2013), a process that does not occur in animals. Animals obtain proteins through their diet and therefore do not need this pathway. In other words, herbicides will *directly* impact the producers (plants), but not the herbivores or predators (Relyea 2005c). However, herbicides will affect the herbivores and predators *indirectly* by creating a trophic cascade if host plants are removed from the ecosystem (Relyea 2005c).

Insecticides

Insecticides on the other hand, may directly affect invertebrate predators and not the producers (Relyea 2005c). Insecticides can either be chemical or biological in origin (Ware *et al.* 2004). This means that it can either be found as a chemical (normally applied externally) or as a protein expressed by a GM crop (modified to express insecticidal traits) (Ware *et al.* 2004). Insecticides control insect pests differently by disrupting the nervous system (Ware *et al.* 2004; Relyea 2012), damaging their exoskeleton or repelling the insects (Ware *et al.* 2004). Insecticides are available in different packages which include sprays, dust, gels and baits

(Ware *et al.* 2004), and for these reasons, insecticides may have different risk levels to the environment, non-target organisms and even humans (Ware *et al.* 2004).

1.3. Roundup®, a glyphosate-based herbicide

Glyphosate-based products are well-known and amongst the most widely used herbicides in the world. These products are sold under different commercial names (Roundup®, Roundup® Original®, Roundup® WeatherMax®, Touchdown®, Glyphos®, Rodeo®, Aqua Master®, Vision®, and VisionMax®) by several manufacturers such as the Monsanto Company (Relyea 2005a; Relyea 2005b; Jones *et al.* 2010; Jones 2011; Relyea 2012; Lanctôt *et al.* 2013). Although the popularity of glyphosate indicates its worldwide usage, very little is known on how this formulation interacts with natural stressors in the aquatic system (Jones 2011).

Roundup® is used in both agriculture and forest management and can be used on land and in aquatic environments to control unwanted plants (Costa *et al.* 2008; Lanctôt *et al.* 2013). This product consists of water, isopropyl-amine salts (IPA-glyphosate salts) and polyoxyethylene amine, also known as polyoxyethylene amine (POEA), as the surfactant (Relyea 2005a; Costa *et al.* 2008; Jones *et al.* 2010; Edge *et al.* 2011; Jones *et al.* 2011; Relyea 2012; Güngördü 2013). "Surfactant" refers to a substance that reduces the surface tension of a liquid that it's dissolved in (Brannon 2007; Jones *et al.* 2011). Although manufacturing companies of Roundup® have stated that POEA has no effect on animals (due to lack of the shikimate pathway found only in plants, Archaea and Bacteria), there have been studies that contradict this statement, indicating that the surfactant is harmful to animals and humans. Tsui and Chu (2003) did a study on the toxicity of the ingredient of Roundup® and found that the POEA was the most toxic ingredient, followed by Roundup®, glyphosate acid and only then the IPA salt of glyphosate. They did report that the glyphosate acid had a higher toxicity than the IPA salt due to its acidity and that each organism was effected differently, but overall the POEA proved to be most toxic (Tsui & Chu 2003).

The surfactant or POEA aids the penetration of the herbicide through the cuticle and into the plant epidermis, leaving the plant defenceless against the glyphosate (Relyea 2005b; Relyea *et al.* 2005; Costa *et al.* 2008; Jones *et al.* 2010). Glyphosate then acts by disrupting the synthesis of essential amino acids required for growth and development of the plant (see below). A study done by Perkins *et al.* (2000) indicated that the Roundup® formulation of glyphosate is more toxic than that of the glyphosate formulation in Rodeo®. Since the latter product lacks the POEA surfactant, the greater toxicity of Roundup® was concluded to be due to the presence of POEA (Perkins *et al.* 2000; Güngördü 2013). Indeed, POEA alone is more toxic to fish, amphibians and other aquatic invertebrates than the active ingredient, glyphosate (Lajmanovich *et al.* 2003; Güngördü, 2013). Comparative studies have found that, after four days exposure, POEA had a LC50 of 6.8 mg ae/l. A Rodeo® formulation was reported to have a LC50 of 7.3 mg ae/l while Roundup® formulation had a LC50 of 9.3 mg ae/l (Perkins *et al.* 2000). In contrast, Yadav *et al.* (2013) found that after four days the LC50 for Roundup® was 3.39 mg ae/l. These differences could possibly be ascribed to different environmental conditions in the presence of the two formulations.

Previous studies reported glyphosate to have a water solubility of 15,7 mg/l and a half-life of up to 200 days (in pond water) depending on environmental conditions (Relyea 2005b; Relyea 2005c; Relyea *et al.* 2005; Costa *et al.* 2008; Jones *et al.* 2010; Jones *et al.* 2011; Relyea 2012; Wagner *et al.* 2013). Under laboratory conditions glyphosate, however, had a half-life of seven to eight days (Jones *et al.* 2010). The surfactant (POEA) on the other hand, had a half-life of twenty-one to twenty-eight days, depending on conditions (Relyea 2005b). According to Lanctôt *et al.* (2013) glyphosate dissipated at a faster rate in natural aquatic environments than under laboratory conditions. Under the former conditions glyphosate was more prone to faster degradation under variable environmental conditions, including water pH, temperature, sorption, microbial degradation and biota uptake (Lanctôt *et al.* 2013).

1.3.1. History of Roundup®

Roundup® was developed in 1974 for the “sustainability of agricultural crops and environmental protection”, although the main purpose of Roundup® was to destroy weeds (Jones *et al.* 2011; Perez *et al.* 2011). The benefits associated with the use of herbicides included less tillage and the amount of herbicides used, and saving costs on fuel for mechanical tilling of the soil (Perez *et al.* 2011; Wagner *et al.* 2013; Monsanto 2014). Soybean, the first genetically modified glyphosate resistant crop, was commercially approved for cultivation in the United States of America in 1996. This improved crop production and followed shortly thereafter by the production of glyphosate resistant cotton, maize, canola, alfalfa and sugar beet (Perez *et al.* 2011).

Roundup® is a non-selective herbicide consisting of a mixture of glyphosate, surfactants and water (Relyea 2005c; Lajmanovich *et al.* 2003; Perez *et al.* 2011; Lanctôt *et al.* 2013; Wagner *et al.* 2013; Hanlon *et al.* 2014; Monsanto 2014). This herbicide is effective the moment it comes into contact with the green and growing areas of the plant. Once Roundup® is sprayed on the plant (not herbicide resistant) the POEA provides a pathway, allowing the glyphosate to be absorbed and translocated through the plant tissue (Monsanto 2014) where it disrupts the respiratory membrane of cells (Costa *et al.* 2008; Edge *et al.* 2011; Jones *et al.* 2011; Jones *et al.* 2010; Perez *et al.* 2011; Relyea 2005b; Relyea 2005c; Relyea 2012). Once glyphosate is inside the plant tissues it is transported through the phloem to the meristems (Perez *et al.* 2011) where it blocks the enzyme EPSP synthase (5-enolpyruvylshikimate-3-phosphate synthase) (Fig. 1.1).

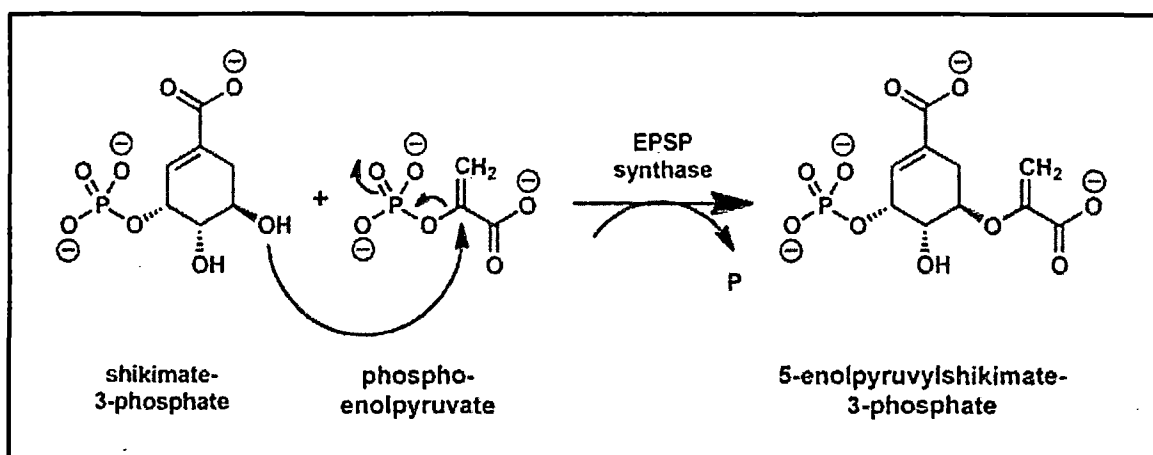


Figure 1.1: EPSP synthase (Moran 2007)

Blocking of EPSP prevents the plant from synthesizing three amino acids (phenylalanine, tyrosine and tryptophan), but all 20 amino acids (including phenylalanine, tyrosine and tryptophan) are essential for plant growth and development (Relyea *et al.* 2005; Moran 2007; Perez *et al.* 2011; Relyea 2012; Güngördü 2013; Wagner *et al.* 2013; Yadav *et al.* 2013; Hanlon *et al.* 2014; Monsanto 2014). After glyphosate application, plants start to wilt and turn yellow and finally brown after five to ten days as plant tissue deteriorates (Monsanto 2014). As the plant tissues decompose, so do the roots and rhizomes and in the end the whole plant dies (Monsanto 2014).

1.3.2. Chemistry and biochemistry of glyphosate

The chemistry of glyphosate is only briefly discussed in this review. The chemical name for glyphosate is N-(phosphonomethyl) glycine (Fig. 1.2), and it belongs to the chemical group phosphoglycine (Perez *et al.* 2011). The main degradation product of glyphosate is known as aminomethyl phosphonic acid (AMPA) (Perez *et al.* 2011). The name glyphosate is a contraction from the name *glycine* and *phosphate*. It has a water solubility of 1.2% at 25°C (i.e. a low solubility in water) and is insoluble in other solvents (Perez *et al.* 2011). The most common formulation of glyphosate is isopropylamine salt (IPA) (Fig. 1.2), but other chemical forms are also available (Perez *et al.* 2011).

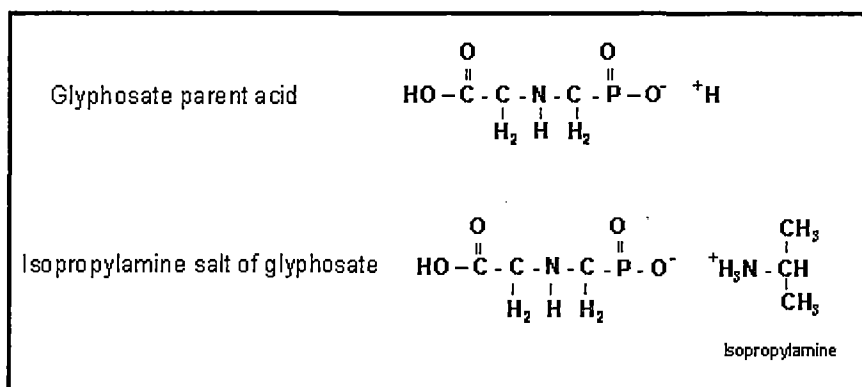


Figure 1.2: Glyphosate molecule (acid and salt molecule) (Hartzler 2001)

The effect of glyphosate is localized to the pathway that synthesises aromatic amino acids (shikimate pathway), but it may also have effects on photosynthesis, respiration as well as the synthesis of proteins and nucleic acids (Perez *et al.* 2011).

1.3.3. Effects of glyphosate on aquatic organisms

According to Perez *et al.* (2011), the most sensitive non-target species is that of aquatic plants. Aquatic plants play an important role in the functioning of aquatic ecosystems (Perez *et al.* 2011). Their functions include:

- the stabilizing of sediments,
- the change on sedimentation, flow velocity, recirculation and the uptake of nutrients,
- shelter for aquatic vertebrates,
- substrate for surface-living organisms,
- and food for other organisms (aquatic plants and algae).

Perez *et al.* (2011) stated that glyphosate alone is not as toxic as commercial formulations with the surfactant. It was observed that green algae (*Selenastrum capricornutum*) (Sphaeropleales: Selenastraceae) were affected more severely when exposed to the commercial formulation, Roundup®, than the IPA salt of glyphosate (Tsui & Chu 2003). Similar results were found when the macrophyte *Lemna minor* (Alismatales: Araceae) was exposed to the same treatment (Cedergreen & Streibig 2005). Other authors found a lower toxicity than the previous two examples, but still higher than the IPA salts (Saenz *et al.* 1997). Tsui and

Chu (2003) also found that *Vibrio fischeri* (a common marine bacterium) (Vibrionales: Vibrionaceae) was more sensitive to Roundup® than the glyphosate acid. They also reported a higher sensitivity with *Euplotes vannus* (Hypotrichida: Euplotidae) and *Tetrahymena pyriformis* (Hymenostomatida: Tetrahymenidae) (ciliates).

Perez *et al.* (2011) stated that fish and amphibians had a low sensitivity to glyphosate itself and that fish exposed to glyphosate had a LC50 ranging between 130 mg ai/l (active ingredient per litre) to 620 mg ai/l for the *Ictalurus punctatus* (channel catfish; Folmar *et al.* 1979) (Siluriformes: Ictaluridae) and *Cyprinus carpio* (carp; Neskovic *et al.* 1996) (Cypriniformes: Cyprinidae) respectively. Mann and Bidwell (1999) exposed four species of South-western Australian frog tadpoles (*Crinia insignifera*, *Heleioporus eyerei*, *Limnodynastes dorsalis*, and *Litoria moorei*) to two treatments (glyphosate IPA salt and glyphosate acid) and found that the tadpoles exposed to IPA salt had a LC50 of between 340 and 460 mg ae/l (acid equivalence per litre) while those exposed to the acid had a LC50 from 82 to 121 mg ae/l. This indicates that amphibians are less resistant to the surfactant than the glyphosate itself. It was also reported that glyphosate affects energy metabolism by causing a higher demand for energy in the teleost fish, *Leporinus obtusidens* (Characiformes: Anostomidae) (Güngördü 2013).

Previous studies have shown that Roundup® reduced tadpole growth-and development-rate (Howe *et al.* 2004). Hanlon *et al.* (2013) investigated the possible reasons for these findings. The latter study hypothesised that Roundup® affected tadpole foraging by damaging the mouthparts of larvae. This proposal was made based on previously documented work showing that the mouthparts of tadpoles were altered by exposure to coal ash deposition or pesticides (Rowe *et al.* 1996; Lajmanovich *et al.* 2003). Unfortunately, Hanlon *et al.* (2013) did not determine the cause of the reduced growth rates in the exposed tadpoles (no correlation between Roundup® and the damaged mouthparts), but suggested that further histological investigations should be undertaken to determine the levels of different hormones important for tadpole growth and development (Lanctôt *et al.* 2013).

Lajmanovich *et al.* (2003) and Mann and Bidwell (1999) described comparable results when amphibians were exposed to Roundup® and Glyphos®. Both formulations contained the surfactant, polyoxyethylene amine (POEA) and had varying LC50 values ranging from 2.6 mg/l Glyphos® (tadpoles of *Scinax nasicus*) (Anura: Hylidae) to 11.6 mg ae/l Roundup® (tadpoles of *L. moorei*) (Anura: Hylidae) respectively. Another study by Cauble and Wagner (2005) showed a mortality of 50% for the *Rana cascadae* (Anura: Ranidae) tadpoles when exposed to 1.94 mg ai/l of Roundup®. The study also showed an increased metamorphosis rate when exposed to 1 mg ai/l Roundup®. In contrast, Relyea (2004) showed a reduced rate of metamorphosis when exposed to 2 mg ai/l Roundup®. Perez *et al.* (2011), concluding that the surfactant (POEA) present in the herbicide was the most toxic component, and that this surfactant was the most harmful component found in different formulations.

Güngördü (2013) did a similar study by comparing the effect of glyphosate and methidathion on three different amphibian species, namely *Pelophylax ridibundus* (Anura: Ranidae), *Pseudepidalea viridis* (Anura: Bufonidae) and *Xenopus laevis* (Anura: Pipidae). This study reported that four days of exposure to glyphosate had a LC50 of 5.05 mg ai/l in *X. laevis*, 19.6 mg ai/l in *P. ridibundus* and 22.7 mg ai/l in *P. viridis*.

Wagner *et al.* (2013) reported that the effects of sub-lethal concentrations glyphosate-based herbicide on amphibians included unnatural deformities of larvae, endocrine disruption, altered rates of developmental, inhibition of certain enzymes, effects on the embryos and lastly behavioural changes.

1.3.4. The presence of glyphosate in aquatic ecosystems

Glyphosate could find its way into the aquatic ecosystems by means of runoff and spray drift. Previous studies were done on the adsorption of glyphosate by soil particles. It was assumed that glyphosate would bind to soil particles quickly and tightly and therefore would not find its way to any water body through runoff; however the results were contradictory (Perez *et al.* 2011; Wagner *et al.* 2013). Laboratory and field studies of Rueppel *et al.* (1977), Crisanto *et*

al. (1994) and Roy *et al.* (1989) demonstrated the immobility of glyphosate in soil. In contrast, Perez *et al.* (2007) showed traces of glyphosate in water channels and streams one day after application, though this was restricted to a short window period (Perez *et al.* 2011). Perez *et al.* (2011) also reported that in natural waters, glyphosate dissipated quickly due to its adsorption to suspended particles and sediments followed by degradation (Wagner *et al.* 2013).

Edwards *et al.* (1980) collected valuable data on glyphosate concentrations in runoff caused by rainfall after early spring application of this herbicide to no-till agricultural soils. The highest glyphosate concentration (5.2 mg/l) was found in runoff one day after a treatment with Roundup® at 8.6 kg/ha. This 5.2 mg/l was only 1.85% of the glyphosate applied (Edwards *et al.* 1980). Feng *et al.* (1990) reported increased glyphosate concentrations (between 1.1 and 1.5 mg/l) one day after application (after rainfall) in a treated watershed (Feng *et al.* 1990). Some formulations (Rodeo® and AquaMaster®) were specifically developed to kill aquatic weeds. These glyphosate-based herbicides must therefore be directly applied to the aquatic ecosystem. The glyphosate concentration in that area was therefore expected to be higher than that of non-aquatic herbicides (Perez *et al.* 2011).

According to Perez *et al.* (2011), glyphosate transported long distances in canals and streams could affect unwanted crop free or aquatic areas. It was reported that up to 58% of the applied glyphosate (metered in canal water) was found between eight and 14.4 km downstream from the application area (Comes *et al.* 1976). In nature, the highest concentrations found in water were 1.24 mg ae/l, 1.54 mg ae/l, 2.8 mg ae/l, and 5.2 mg ae/l (Perez *et al.* 2011). The maximum glyphosate concentrations recorded in the study of Wagner *et al.* (2013) and Relyea (2005b) was 0.17 mg ae/l to 0.70 mg ae/l (measured in environment without intervention), 0.27 mg ae/l to 3.10 mg ae/l (measured after application or in runoff during the first heavy rain) and 1.43 mg ae/l to 7.60 mg ae/l (estimated worst-case scenario).

1.4 Genetically modified Bt maize

1.4.1. History of genetically modified crops

Maize is the most important field crop produced globally (Tank *et al.* 2010). In South Africa it is most commonly grown in the North-West province, Free State, Highveld of Mpumalanga and the Midlands of KwaZulu-Natal (Fig. 1.3) and is important in providing the human diet with carbohydrates (Greyling & Vink 2012). Due to its importance, the production of maize is obligated to keep up with the country's growing population, and in order to meet this demand various biotechnologies have been developed (Marvier *et al.* 2008). Assistance in this regard comes from GM crops, although this form of biotechnology (field trials of cotton) has only been permitted in South Africa since 1992 (Mayet 2001).

In 2004 South Africa had planted 0.5 million hectares of GM crops, and of this 155 000 hectares was GM Bt maize (James 2010). By 2009, GM crops increased to 2.1 million hectares (maize, soybean and cotton) (James 2009). From 1996 to 2013 the ISAAA reported that on a global scale herbicide resistant crops occupied 99.4 million hectares with the most common trait being glyphosate tolerance (ISAAA 2014).

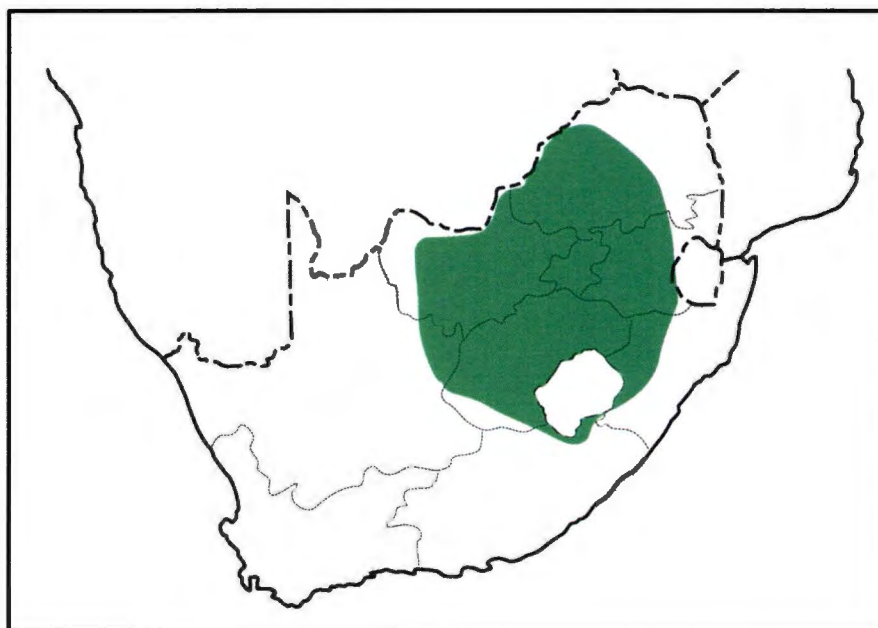


Figure 1.3: The maize production region of South Africa where genetically modified maize is produced

Genetic modification refers to the process of adding certain small sections of genes of an organism's DNA (deoxyribonucleic acid) to introduce a desired trait (Whitman 2000; Nap *et al.* 2003). In the case of GM maize, the crop is rendered insect resistant or has tolerance to herbicides, or both (Nap *et al.* 2003). Insect resistant maize has a specific gene inserted into its DNA that allows the plant to encode a protein with inherent pesticidal activity (Betz *et al.* 2000; Whitman 2000). This is known as Bt-maize.

The crop gets its name from the organism from where the gene originated *viz.* *Bacillus thuringiensis* which is a microbial soil bacterium that has a natural ability to produce a crystalline toxin (referred to as Cry proteins or toxin) during sporulation that has insecticidal traits (Betz *et al.* 2000; Whitman 2000; Baumgarte & Tebbe 2005; Bravo *et al.* 2007; Rosi-Marshall *et al.* 2007; Chambers *et al.* 2010; Tank *et al.* 2010).

GM crops provide distinct economic benefits compared to non-GM crops in that a higher crop yield may be achieved (insecticide savings and yield advantages although variable among geographical regions; Letourneau & Burrows 2001). Bt protected crops were developed because it: i) reduces the overall usage of insecticide treatments, ii) improves efficiency in insect pest control, iii) reduces yield losses due to pests and v) supposedly provides protection of non-target insects (Betz *et al.* 2000).

1.4.2. Bt-proteins and the environment

Cry proteins are reported to be toxic to only certain insect species. This selectivity is because specific receptor sites, pH levels and enzymes that are needed to activate and bind the toxin to the mid-gut cells of the target insect (Betz *et al.* 2000; Chambers *et al.* 2010). This toxin causes the intestinal tissue to disintegrate (and loss of mid-gut bacteria) and supposedly causes starvation or septicaemia which lead to the death of the insect (Fig. 1.4; Betz *et al.* 2000; Broderick *et al.* 2006; Chambers *et al.* 2010). Another mechanism in the case of Bt sprays is that the crystalline spores ingested by the pest are provided access to the hemocoel due to cell lysis, leading to septicaemia and death. According to Broderick *et al.* (2006), this is

not the case in larvae gypsy moth (*Lymantria dispar*) (Lepidoptera: Erebidiae). Their study concluded that the toxicity of the Bt toxin depends on “*the interaction with microorganisms of the normal gut community*” (Broderick *et al.* 2006).

The effect of Bt proteins, either applied by means of spray applications or produced by GM plants with this trait, is discussed below (Fig. 1.4). If an insect feeds on maize sprayed with a Bt insecticide, it ingests the bacteria or its spores. The alkaline environment of the insects' gut causes sporulation, releasing crystalline spores. These crystals puncture the intestinal wall allowing the gut bacteria and spores into the hemocoel leading to septicaemia and later death (Broderick *et al.* 2006; Deacon 2011). Insects that feed on GM crops producing Bt proteins (signalled by inserted gene), ingests protoxins which are produced by plants. The protoxin must first be activated before it can have any effect on the insect (Broderick *et al.* 2006; Deacon 2011). In certain Lepidopteran species, the pH (9.5) of the gut is conducive to solubilize the protoxin, activating it. The gut protease cleaves the protoxin, producing an active toxin known as *delta-endotoxin*. This endotoxin binds to the mid-gut epithelial cells creating pores in the cell membrane. In the end, it causes the immobilization of the gut, lyses of epithelial cells and ultimately death due to both septicaemia and starvation (Deacon 2011).

In South Africa, plants with Bt-traits protect themselves against stem borer damage, notably *Busseola fusca* (Lepidoptera: Noctuidae), *Chilo partellus* (Lepidoptera: Crambidae) and *Sesamia calamistis* (Lepidoptera: Noctuidae) (Midega *et al.* 2005; Van den Berg & Van Wyk 2007; Van Rensburg 2007; Kruger *et al.* 2009). Pesticides containing Bt DNA have been used since 1961 and are favoured by organic farmers for pest control because of their specificity and lack of effect on non-target species (Betz *et al.* 2000; Bøhn *et al.* 2008). The first time that a Cry protein was cloned was in 1981 (Betz *et al.* 2000) and since then the process of genetic modification of crops has grown rapidly. Different hybrids are produced that express different Cry endotoxins (Cry1Ab, Cry1Ac, Cry1F etc.) that are species specific, such as Cry1 and Cry2 that are toxic to Lepidoptera and Cry3 that is toxic to Coleoptera (Baumgarte & Tebbe 2005;

Bravo *et al.* 2007; Swan *et al.* 2009; Hellmich & Hellmich 2012). In this study, Cry1Ab Bt maize was used.

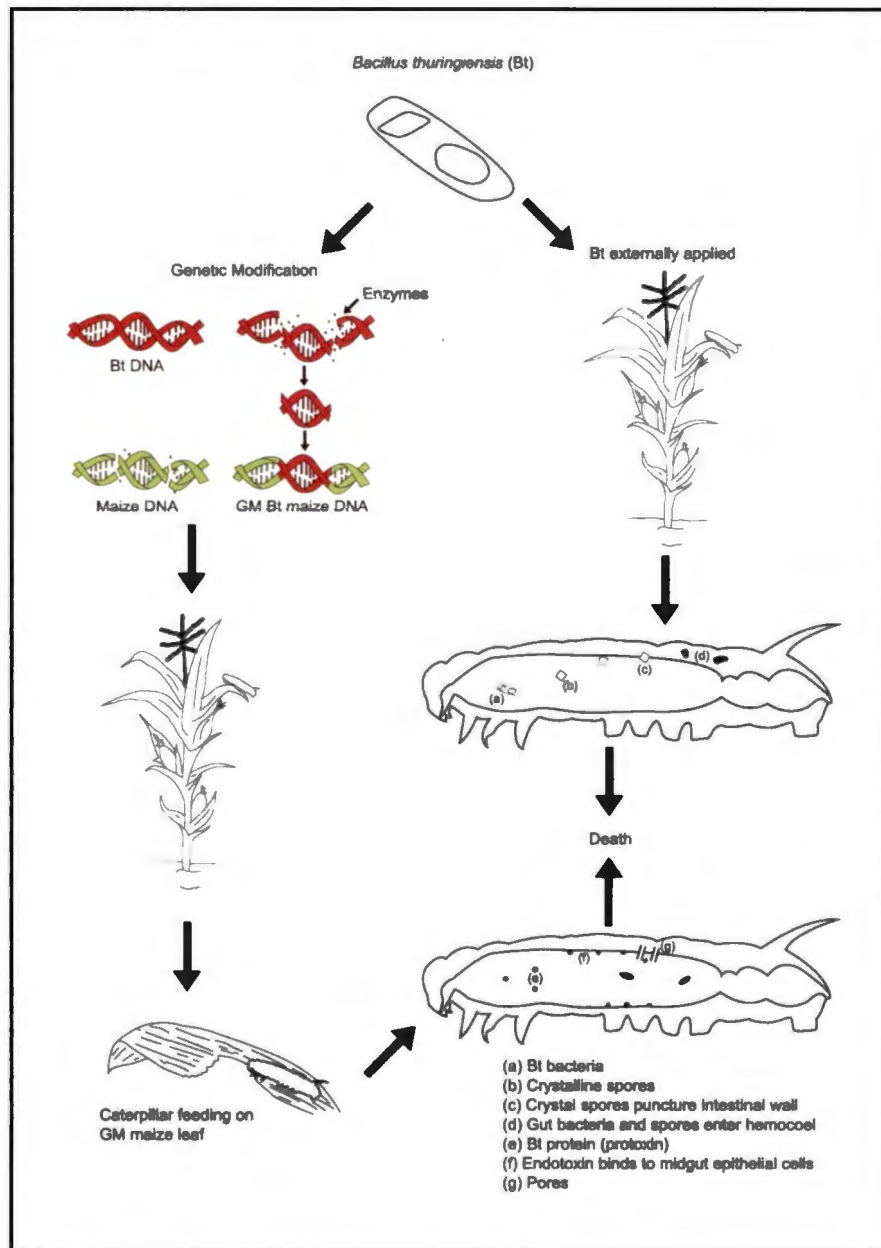


Figure 1.4: Diagrammatic presentation of different mechanisms that could function in a larva if it was to feed on Bt sprayed or GM Bt maize leaves

Bt crops have become extremely popular because of its convenience. Once Bt crops are planted, pesticides are no longer needed against the target species (Hellmich & Hellmich 2012). This means that farmers spend less time applying chemicals leading to improved

health, not only of farmers and/or their workers, but of the environment as well (Hellmich & Hellmich 2012). Other benefits of Bt maize includes improved grain quality due to less pest damage (Hellmich & Hellmich 2012), while unlike the externally applied Bt-containing insecticides, the plants that produce Cry proteins are not affected by rain or sun and is therefore cost effective (Betz *et al.* 2000).

Although the benefits of Bt maize is well recognised, there are potential risks, especially the potential unknown effects on non-target species (Baumgarte & Tebbe 2005; Hellmich & Hellmich 2012). So far there has been little evidence of effects on non-target species. However, very little research has been done in this regard (Hellmich & Hellmich 2012).

1.4.3. Concentrations of Bt in the environment

Bøhn *et al.* (2008) showed that Cry1Ab levels in maize had an average of 67 ng per 1 g dried grain tissue. A study done by Tank *et al.* (2010) indicated that the presence of Cry1Ab in streams containing maize detritus was above the minimum detection limit in 19% of the sites sampled and that 25% of the samples had concentrations higher than 14 ± 5 ng/l with the two maximum concentrations being 21 ng/l and 32 ng/l (Tank *et al.* 2010). Palm *et al.* (1994) found that the concentration of Bt toxins extracted from soil depended on the soil type. For example 27 to 60% (high clay and organic matter content to low clay and organic matter content, respectively) of *B. thuringiensis* var. *kurstaki* (Btk) toxins was recovered from soil. The higher the clay and organic matter, the stronger the toxin binds to it, making extraction difficult (Palm *et al.* 1994).

Zwahlen *et al.* (2003) did a study on how long the toxin remains in the plant tissues when left on the field after harvesting by using an enzyme-linked immunosorbent assay (ELISA) to determine the Cry1Ab concentration during autumn, winter and spring. They found that the degradation rate of the Cry protein is temperature dependant. This means that as the soil temperature increases, so does the concentration of the Cry proteins. The initial Cry concentration in Bt leaves was 15.4 ± 4.3 mg/g dry weight at a soil temperature of 14 and 8°C.

The temperatures decreased from 8 to 3°C during mid-November to mid-December, leading to a significant change in concentration (from 5.4 to 3.0 mg/g dry weight). Five months later, 1.5% of the initial Cry concentration was still present in the remaining plant residues. They also found that even up to eight months after the introduction of the plant material, the Cry protein was still detected.

1.4.4. Aquatic ecosystems

According to Relyea (2005c), pesticides could impact the diversity and productivity of the aquatic community even over a short period (two weeks), although such impact could be pesticide specific. Cry proteins can enter aquatic systems by leaching into the soil through the fusion of plant material (Fig. 1.5). According to Saxena *et al.* (1999) Cry toxins remain active in the soil where it binds quickly and tightly to humic acid and clays (Saxena *et al.* 1999; Carstens *et al.* 2012). Though bound, the Cry toxin maintains its insecticidal characteristic and, since it is bound to soil particles, it is protected from degradation. Depending on the soil type, the toxin can persist for at least 234 days (Saxena *et al.* 1999).

Different organisms are exposed to the Cry1Ab protein once crop residues enter aquatic systems (Rosi-Marshall *et al.* 2007; Böll *et al.* 2013). During both the growing season and after harvest, toxins can still enter streams (Fig. 1.5). Tank *et al.* (2010) suggested that the dissolved Cry protein in stream water mixes with patches of Cry1Ab containing detritus (Tank *et al.* 2010). Leaf detritus can be left on the field to provide soil with nutrition, but as a result, Bt proteins can leach into the ground and make its way to a stream or water body (Swan *et al.* 2009; Chambers *et al.* 2010; Carstens *et al.* 2012). Indeed, maize debris containing Cry proteins is consistent with the amount of Cry protein found inside stream water (Tank *et al.* 2010). There is however, more than one pathway whereby Cry proteins can eventually find their way into an aquatic system (Fig. 1.5).

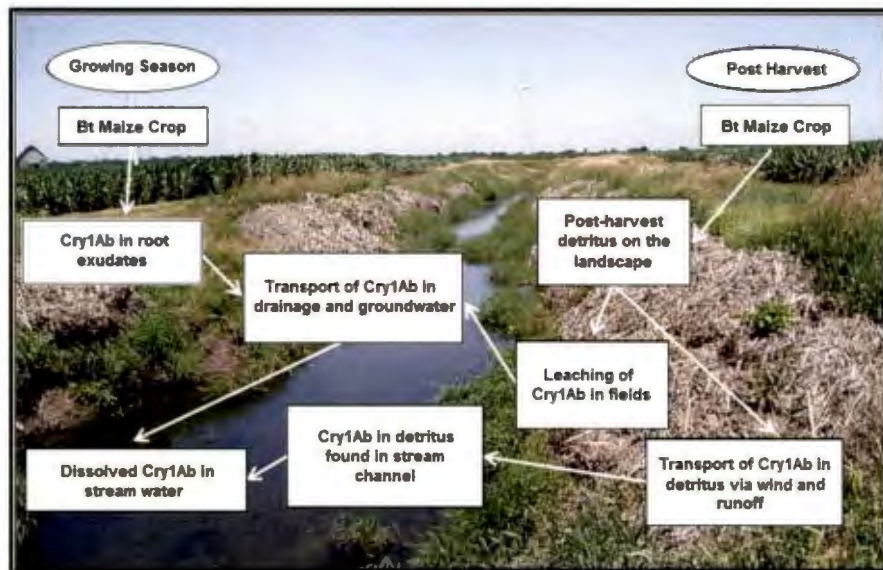


Figure 1.5: Pathways through which Cry1Ab protein may enter aquatic systems (Tank *et al.* 2010)

As depicted in Figure 1.5, Cry proteins may be introduced into soils by slowly being exuded from roots and maize biomass into the soil. These proteins may also persist for up to 180 days or for as long as three years in the case of maize biomass. Moreover, the Cry proteins have the potential to transfer to other nearby streams through erosion and surface runoff (Rosi-Marshall *et al.* 2007; Tank *et al.* 2010; Böll *et al.* 2013).

1.4.5. Effects of Cry1Ab protein on aquatic organisms

Rosi-Marshall *et al.* (2007) studied the effect of Bt on aquatic organisms (*Lepidostoma liba*) (Trichoptera: Lepidostomatidae) and found that those that were exposed to Bt demonstrated reduced growth rates as well as higher mortality than those that were exposed to non-Bt maize. The latter study, however, was criticized for its design flaws and the lack of a control treatment which lead to inconclusive findings (Parrott 2008). Parrott (2008) goes on to suggest that the variety of maize hybrids used in the study of Rosi-Marshall *et al.* (2007) could explain the results since every hybrid has a different trait (e.g. different levels of trypsin inhibitors). Moreover, another factor is the failure of the study to quantify and identify the amount and type of Bt that was present in the pollen (Parrott 2008).

Chambers *et al.* (2010) also investigated the effect of Bt protein on aquatic invertebrates in headstreams. Their study extended the work of Rosi-Marshall *et al.* (2007). Chambers and colleagues (2010) included multiple feeding trials using two varieties of Bt debris, but different taxa. This study showed that *L. liba* had a slower growth rate when fed Bt maize compared to those that were fed non-Bt maize, while other taxa were not differentially influenced by Bt and non-Bt maize (Chambers *et al.* 2010).

Jensen *et al.* (2010) undertook a study on the European corn borer (*Ostrinia nubilalis*; Lepidoptera: Crambidae) and found no bioactivity of the Cry protein in older maize tissue after it was exposed to terrestrial and aquatic environments for two weeks. Due to the lack of bioactivity of the Cry protein after two weeks and a lack of non-target effects, the authors suggested that the different responses in the taxa were probably caused by differences in exposed tissue and not the toxin (Jensen *et al.* 2010). Jensen *et al.* (2010) indicated that the negative effects caused by Cry proteins on aquatic organisms cannot only be ascribed to the presence of the Cry protein since complex interactions between plant genetics and the environment also play important roles (Jensen *et al.* 2010).

AMPHIBIANS

1.5. Importance of amphibians

Amphibians are good bio-indicators of healthy ecosystems due to their sensitive larval stages. Biotic and abiotic stressors (Howe *et al.* 2002; Du Preez & Carruthers 2009) may affect development of larvae or tadpoles. Frogs absorb water through their skin and in the process absorb substances present in water, including nutrients and toxins (Du Preez & Carruthers 2009; Böll *et al.* 2013). Amphibians can be used as indicator groups to monitor any direct or indirect adverse effects of genetically modified plants or crops.

They are also suitable for use in monitoring programs due to the following characteristics summarized by Böll *et al.* (2013); Du Preez & Carruthers (2009); Güngördü (2013); Thompson *et al.* (2004) and Wagner *et al.* (2013):

- abundance of knowledge on amphibians,
- role in different trophic levels,
- breeding sites are predictable,
- and easy accessibility.

Furthermore, larval development and migration period occurs during the same time of the year as the application of pesticides (Böll *et al.* 2013). Tadpoles are mostly herbivores and therefore should be exposed to Cry proteins in detritus of genetically modified plants (Böll *et al.* 2013).

Amphibians have experienced a great decline and more than 30% of the known species are listed as threatened (Relyea 2005a; Relyea 2005b; Relyea *et al.* 2005; Du Preez & Carruthers 2009; Edge *et al.* 2011; Denoël *et al.* 2013; Hanlon & Parris 2014). Due to the occurrence of amphibians in rivers, streams or ponds where pesticides may be present, it is believed that pesticides might be a contributing factor to the reduction in amphibian fitness along with habitat degradation or environmental changes, including ozone depletion, predation, competition, pH, temperature, ultraviolet radiation, habitat fragmentation and parasitism (Lajmanovich *et al.* 2003; Relyea 2004; Relyea 2005a; Relyea 2005b; Costa *et al.* 2008; Du Preez & Carruthers 2009; Edge *et al.* 2011; Jones *et al.* 2011; Relyea 2012; Güngördü 2013; Lanctôt *et al.* 2013; Yadav *et al.* 2013; Hanlon & Parris 2014).

The effects of glyphosate on amphibians depend on the proximity of amphibians to these pesticides (Relyea 2005a; Costa *et al.* 2008; Böll *et al.* 2013). Most amphibians breed during spring, which is normally when application of pesticides is done on crops (Thompson *et al.* 2004; Böll *et al.* 2013; Denoël *et al.* 2013; Güngördü 2013; Wagner *et al.* 2013).

1.5.1. Amphibian development

Various hormones, each with their own set of functions, play an important role in the physiology and behaviour of amphibians (Taylor 2012). Hormones are secreted from specialised endocrine glands situated in different areas of the body and all vertebrates (fish, amphibians, reptiles, birds and mammals including humans) share the same endocrine glands (Hiller-Sturmhöfel & Bartke; 1998; Taylor 2012) with little or no difference in function (Bergman, *et al.* 2015). While exocrine glands (such as gastric, tear, and sweat glands) secrete their active substances directly into an organ or into a selected region (stomach, eye, skin), endocrine glands secrete their hormones directly into the blood (Hiller-Sturmhöfel & Bartke 1998), for example, thyroxine from the thyroid gland, cortisol from the adrenal gland and insulin from the pancreas (Hiller-Sturmhöfel & Bartke 1998; Taylor 2012). Endocrine hormones target peripheral organs and the brain that are a far distance from their point of release and are responsible for regulation of metabolic, mineral and water balance, as well as thermostatic, sexual, stress and developmental responses that are critical for survival (Table 1.1) (Hiller-Sturmhöfel & Bartke 1998). A number of endocrine hormones including growth hormone (GH) and thyroid hormone (TH) play central roles in tadpole development.

Miyata and Ose (2012) defined the metamorphosis of tadpoles when they said: "*tadpoles become frogs through new formation, retraction and reconstruction of structures and functions from larval tissues to adult tissues*". Growth hormone is the most important hormone affecting tadpole development (responsible for growth and development) (Hiller-Sturmhöfel & Bartke 1998; Du Preez & Carruthers 2009; Lanctôt *et al.* 2013), and is secreted by the pituitary glands (Taylor 2012). The anterior pituitary gland produces hormones (such as the TSH) that stimulate target glands such as the thyroid gland to secrete its hormones (T3 or T4) or affects the target organs directly (Hiller-Sturmhöfel & Bartke 1998). The main function of the thyroid gland is similar to that of GH, and in tadpoles it controls metamorphosis via the release of thyroxine (T4) and is not species specific (Buchholz *et al.* 2006; Du Preez & Carruthers 2009; Lanctôt *et al.* 2013). According to Du Preez and Carruthers (2009) water temperature

significantly influences the production of T4, so that for example cold water inhibits thyroid activity whereas warm water enhances it (Du Preez & Carruthers 2009). This plays a role in the survival of tadpoles, especially if frogs breed in water bodies that dry out.

Table 1.1: Summary of endocrine glands, their hormones and functions thereof in amphibians

(Norris & Lopez 2005; Hiller-Sturmhöfel & Bartke 1998; Taylor 2012)

Types of endocrine glands	Respective hormones	Function
Pancreas (heterocrine gland)	<i>alpha cells</i>	
	Glucagon	Responsible for raising blood glucose levels
	<i>beta cells</i>	
	Insulin	Stimulates growth
Hypothalamus	Thyrotropin releasing hormone (TRH)	TRH stimulates pituitary gland to release TSH
	Growth hormone-releasing hormone (GHRH)	GHRH stimulates the release of the growth hormone (GH)
	Growth hormone-inhibiting hormone (GHIH)	GHIH inhibits the release of the GH
	Gonadotropin-releasing hormone (GnRH)	GnRH stimulates the release of the FSH and Luteinizing hormone
	Corticotropin-releasing hormone (CRH)	CRH stimulates the adrenocorticotrophic hormone release
	Antidiuretic hormone (ADH) (secreted by pituitary gland)	ADH prevents water loss
	Somatostatin	Inhibits the pituitary hormone secretion and pituitary cell growth
	Dopamine	Regulator of prolactin secretion
	Mesotocin	Physical role is unclear
Parathyroid gland	Parathyroid hormone (PTH)	Control levels of calcium in the blood
Pineal gland	Melatonin	Regulates sleep-wake cycle (circadian rhythm)

Table 1.1: Summary of endocrine glands, their hormones and functions thereof in amphibians continues

Types of endocrine glands	Respective hormones	Function
Pituitary gland	<i>Anterior pituitary</i>	
	Growth hormone (HGH)	HGH stimulates growth, repair and reproduction
	Thyroid stimulation hormone (TSH)	TSH stimulates the thyroid gland
	Follicle stimulation hormone (FSH)	FSH stimulates follicle cells of gonads to produce gametes (ova and sperms)
	Luteinizing hormone (LH)	LH stimulates gonads to produce sex hormones (estrogen and testosterone)
	Prolactin (PRL)	Stimulates growth in tadpoles
	Adrenocorticotrophic hormone (ACTH)	ACTH stimulates adrenal cortex to produce adrenal hormones
	<i>Posterior pituitary</i>	See hypothalamus
	Oxytocin	See hypothalamus
	Antidiuretic hormone (ADH)	
Thyroid gland	Calcitonin	Calcitonin reduces calcium ion concentration in the blood by aiding the adsorption of calcium into the bone matrix
	Triiodothyronine (T3)	Initiates metamorphosis
	Thyroxine (T4)	Initiates metamorphosis

Table 1.1: Summary of endocrine glands, their hormones and functions thereof in amphibians continues

Types of endocrine glands	Respective hormones	Function
Adrenal gland	<i>Adrenal cortex</i>	
	Glucocorticoids	Breaks lipids and proteins down to produce glucose, reduces inflammation and immune response
	Mineralocorticoids	Regulates the mineral concentration in the body
	Androgens	Regulates the growth and activity of cells that are receptive to male hormones
	<i>Adrenal medulla</i>	
	Epinephrine	Both help increase the flow of blood to the brain and muscles to improve the <i>fight or flight</i> response
Ovary	Norepinephrine	
	Estrogen	Stimulates development
	Progesterone	Causes maturation of oocyte
Testis	Androgens	Stimulates development

The thyroid gland secretes T3 and T4 hormones into the blood, and together they are known as the TH (Buchholz *et al.* 2006; Heimeier & Shi 2010; Miyata & Ose 2012). Upon release, T3 and T4 influence development of the limbs, tail and organs (collectively known as the periphery). Low circulating levels of T3 and T4 in the blood stimulate the release of a thyroid stimulating hormone (TSH) in the pituitary gland (Buchholz *et al.* 2006) that stimulates the thyroid gland to release more T3/T4 (Fig. 1.6).

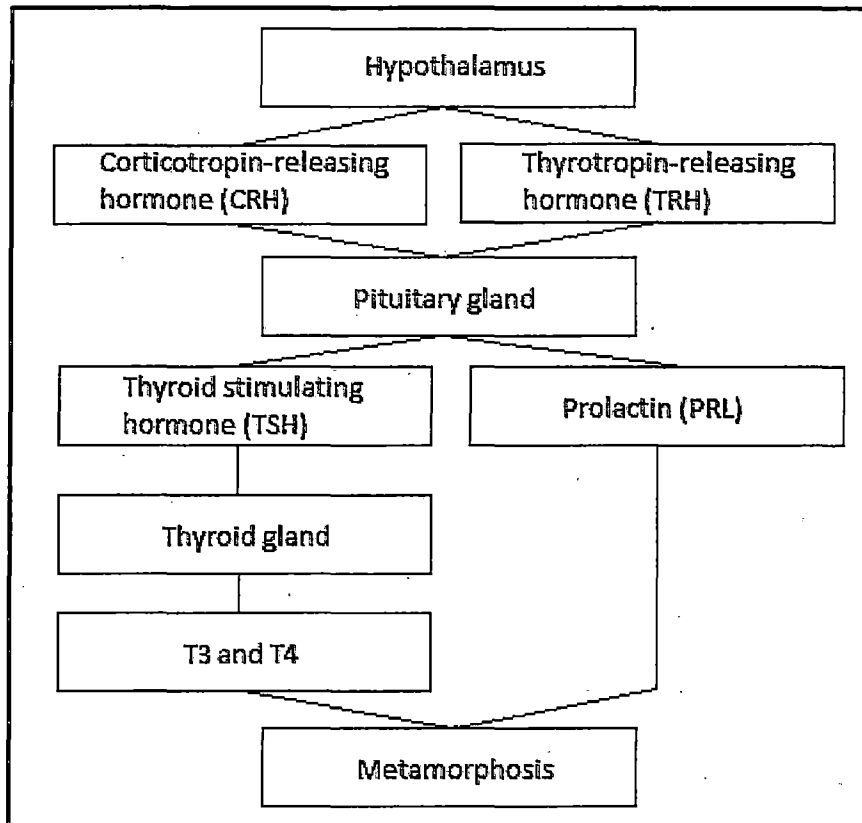


Figure 1.6: Summary of the hormonal pathways responsible for amphibian metamorphosis

In *X. laevis* tadpoles, there are three different metamorphic stages, namely (i) premetamorphosis (N/F stage 45-53) (Nieuwkoop and Faber 1956) where the thyroid hormone is absent (period until hind limbs appear), (ii) prometamorphosis (N/F stage 54-58), which refers to the period where the forelimbs appear, and (iii) metamorphic climax (N/F stage 59-66) referring to the development of lungs and resorption of the tail and gills (Buchholz *et al.* 2006; Miyata & Ose 2012). The last two stages are characterized by an increase in TH in order to allow the correct timing of changes between developmental stages (Buchholz *et al.*

2006). Only during the prometamorphosis stage does the thyroid gland mature allowing the levels of T3/T4 to peak at the metamorphic climax where after it decreases to a lower level and remains low until adulthood (Helbing *et al.* 2006; Zhang *et al.* 2006). In *X. laevis* tadpoles, the thyroid gland is situated in the head (although not part of the brain) between the eyes at stage 51 (Fig. 1.7). However, due to the changes that occurs during metamorphosis, the location of the gland shifts with each stage (Miyata & Ose 2012).



Figure 1.7: Cross section of *Xenopus laevis* tadpole head (Stage 51). Arrows indicates the thyroid gland (Miyata & Ose 2012)

In 1955, Jurands (cited by Rot-Nikcevic & Wassersug 2004) reported that it was possible for amphibians to not reach metamorphosis and remain in the tadpole phase. He discovered that tadpoles that stopped developing had no thyroid gland, thus emphasizing its importance in tadpole development. According to Rot-Nikcevic and Wassersug (2004) the thyroid gland influences metamorphosis, but not the development of the gonadal structures (Rot-Nikcevic & Wassersug 2004). This suggests that although tadpoles may not reach metamorphosis, their reproductive organs may still develop.

According to Böll *et al.* (2013) certain pesticides are endocrine disruptors that target the thyroid gland. Howe *et al.* (2004) showed that glyphosate-based herbicides affected growth and development of tadpoles by disrupting the thyroid axis. In order to determine whether there is an effect of pesticides on the T3 and T4 of amphibians, Radioimmunoanalysis (RIA), enzyme linked immunosorbent assay (ELISA), polymerase chain reaction (PCR) and staining of the thyroid gland have been used in previous studies to investigate presence and extent of these adverse effects (Zhang *et al.* 2006; Helbing *et al.* 2007; Li *et al.* 2009; Korte *et al.* 2011; Zhao *et al.* 2013). With amphibian metamorphosis recognised as being important in the eventual fitness and survival of tadpole, the thyroid gland is therefore an important focus of study. This is one of many reasons why it is believed that amphibian larvae are sensitive to environmental contaminants (pesticides in this case) that can interfere with thyroid hormone function (Helbing *et al.* 2006; Heimeier & Shi 2010).

1.5.2. *Amietophrynus gutturalis* (Anura: Bufonidae): An overview

This species was first described by Power in 1927 and is commonly known as the Guttural toad (Du Preez & Carruthers 2009; Harper *et al.* 2010). In Taita Hills, it is known as Kiwandu, which means “*peoples frog*” because it is found among the Kitaiti people (Measey *et al.* 2009).

Locality

Guttural toads have a wide distribution and are found in countries such as Angola, Botswana, Congo, Kenya, Lesotho, Malawi, Mozambique, Namibia, Somalia, Swaziland, Tanzania, Uganda, Zambia, Zimbabwe and South Africa (Compilation 2014). In South Africa, this species occurs in KwaZulu-Natal, Mpumalanga, Gauteng, central Limpopo, eastern North-West, eastern Free State provinces, Swaziland and as a local invasive in Cape Town (Fig. 1.8) (Du Preez *et al.* 2004). According to De Villiers (2006), it is possible that eggs or tadpoles of this species arrived at a new location with the consignment of aquatic plants (Measey & Davies 2011). By this time the Cape Invasive Alien Animal Working Group became worried about the protection of the endangered Western Leopard toad (*Amietophrynus pantherinus*) and decided to take action by capturing and euthanizing all stages of Guttural toads (eggs,

tadpoles and adults) (Measey & Davies 2011). Due to its wide spread distribution and tolerance to a broad range of habitats, its conservation is of least concern (Poynton *et al*, 2013).

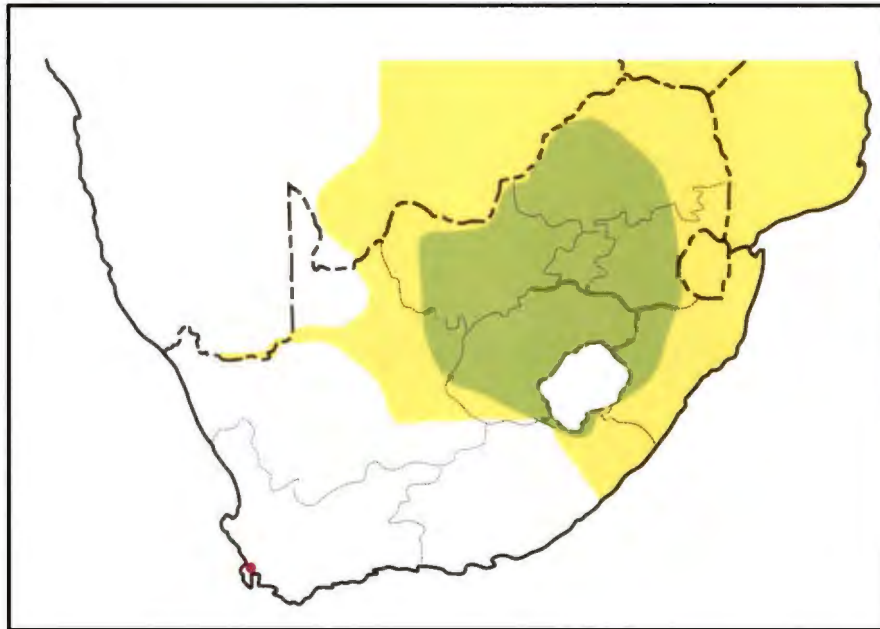


Figure 1.8: Map showing the distribution of *Amietophrynus gutturalis* (indicated in yellow), their invasive area (indicated in red) and the major maize production area (indicated in green) in South-Africa

Description

Amietophrynus gutturalis is a large toad characterised by a pair of dark blotches on the snout and a pair behind the eyes leaving a pale cross on the head and red infusion in the groin and behind the hind legs (Du Preez *et al.* 2009; Harper *et al.* 2010). A pale mid-dorsal stripe extends from the snout down the back, while the toes are slightly webbed at the base (Harper *et al.* 2010) (Fig. 1.9). In *A. gutturalis* the patches on the dorsal side are brown in colour while the parotid glands are very distinct behind the eyes (Fig. 1.9) (Du Preez *et al.* 2009; Harper *et al.* 2010). There is sexual dimorphism in *A. gutturalis*, which includes the size of the sexes (female larger than male), while the male (when sexually mature) has a dark coloured throat as well as dark nuptial pads on the outer fingers (Du Preez *et al.* 2009; Harper *et al.* 2010).



Figure 1.9: *Amietophrynus gutturalis* (photo by L.H. du Preez)

Reproduction

For this species, breeding takes place in dams, rivers and other permanent or semi-permanent water bodies during spring (Du Preez & Carruthers 2009; Measey *et al.* 2009; Harper *et al.* 2010; Böll *et al.* 2013). Under natural conditions, 10, 000 to 25,000 eggs can be laid by a single adult female (this includes both fertilized and non-fertilized eggs). However, the overall survival (from egg to adult) is a lot less due to predation and other environmental conditions that are not suitable for tadpoles (Du Preez & Carruthers, 2009; Harper *et al.* 2010; Compilation 2014). Each egg is between 1.4 to 1.5 mm in diameter and is laid in two jelly-like strings. These strings are usually found between aquatic vegetation (Fig. 1.10) (Du Preez & Carruthers 2009; Measey *et al.* 2009; Compilation 2014). One week after spawning, bottom feeding tadpoles hatch and group together. Five to six weeks later metamorphosis is reached and the metamorphs start to leave the water bodies and enter the terrestrial habitat (Measey *et al.* 2009; Harper *et al.* 2010; Compilation 2014). According to Measey *et al.* (2009) the adults are terrestrial (except when breeding) and feed on insects, which is why this species is usually found close to light (Measey *et al.* 2009; Harper *et al.* 2010).



Figure 1.10: Strings of *Amietophrynus gutturalis* eggs (Schwartz 2014)

These small vertebrates have a defence mechanism (in a form of a toxin) against predators such as birds, making it distasteful when stressed or eaten. This toxin (containing epinephrine that may be fatal to small mammals), forms part of a family of toxins known as bufadienolides (Garg *et al.* 2008), and is secreted in the form of a milky substance from the parotoid gland situated behind the eyes (Du Preez *et al.* 2009; Measey *et al.* 2009; Harper *et al.* 2010).

1.5.3. *Xenopus laevis* (Anura: Pipidae): An Overview

Xenopus laevis is an economically important species for humans, not only because they are used as laboratory research animals, but because their skin contains magainin (peptide) which is an antibiotic/anti-parasitic/antifungal and an antiviral compound with wound-healing properties (Garvey 2000). Moreover, they are an important contributor to ecosystems as well as a measure of ecosystem health (Garvey 2000; Böll *et al.* 2013).

This species adapts quickly to a new habitat, which makes the characterization of preferred habitats difficult (Garvey 2000). *Xenopus laevis* prefers warm and still grassland ponds and streams in arid or semi-arid areas where it thrives in water with temperatures ranging between 15 to 27°C and has the ability to survive in different ranges of water pH (Garvey 2000; Measey

& Davies 2011). However, the presence of metal ions was reported to be lethal or toxic to this species. Although they are aquatic amphibians, they leave the water if forced to migrate (Garvey 2000; Du Preez *et al.* 2009).

Locality

Xenopus laevis (common name, African Clawed Frog) was first described by Daudin in 1802, but no information on the location was given with the description, making it difficult to say where this species originated. However other specimens collected during this time was located in the Cape Province (Picker 1985; Measey & Davies 2011). These collections were made after the Cape was colonized, meaning that farming, along with its associated agricultural infrastructure, was already underway (Measey & Davies 2011). It is therefore likely that this species already invaded the farming areas in the early 19th century (Measey & Davies 2011). *Xenopus gilli* is endemic to the south Western Cape and its habitat was then disturbed by the invasion of *X. laevis*. This invasion was well documented and soon conservational actions were taken to prevent interbreeding between these two species (Measey & Davies 2011; Picker 1985).

In 2011 South African National Parks (SANParks) and the South African National Biodiversity Institute (SANBI) removed over 800 *X. laevis* individuals of which 93% were juveniles and gravid females from the waters of Cape Point (Measey & Davies 2011). Today the African Clawed Frog, more commonly known as the Platanna, is well known across the African continent and is found from the Western Cape extending northwards to the central, northern and north-west regions of the country excluding the northern areas of KwaZulu-Natal and the eastern areas of Mpumalanga and Limpopo (Fig. 1.11).

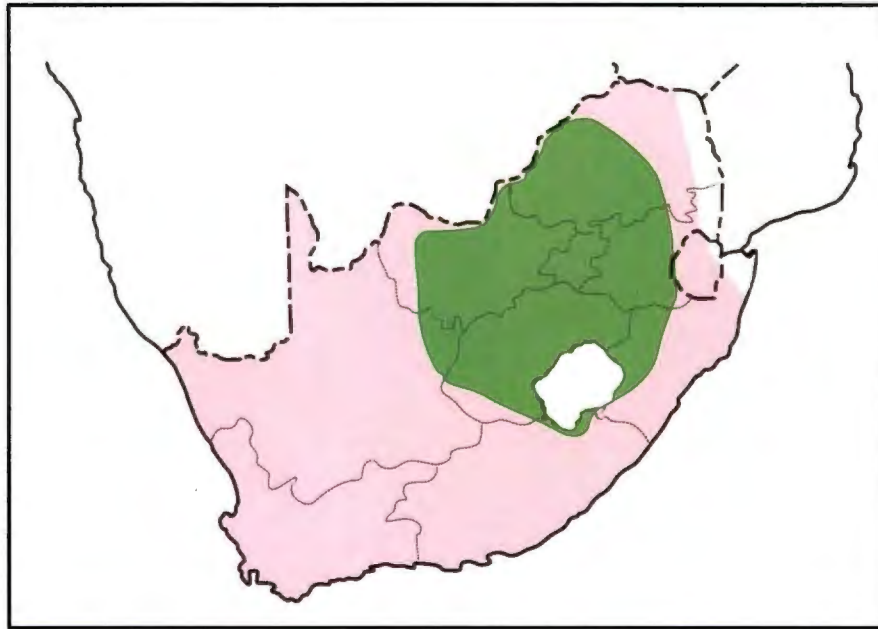


Figure 1.11: Map showing distribution of *Xenopus laevis* (indicated in purple) and the major maize production area (indicated in green) in South-Africa

Description

Descriptively, *X. laevis* are different than the terrestrial amphibians. They lack a tongue as well as visible ears. They have a flattened body with two eyes (immobile eyelids) situated on the top of the head and have small forelimbs and large webbed hind legs with three claws on either side (Fig. 1.12) (Garvey 2000; Du Preez *et al.* 2009). Their skin is slippery and smooth with small darker patches of grey or brown on their dorsal side while the belly is a creamy white colour. Sexual dimorphisms is present in this species and is seen in the size of the sexes as well as the dark colour of the hand palms of the male during breeding season and the presence of a swollen cloacal fold in females (Du Preez *et al.* 2009). Females are bigger than the males and can be as much as 140 g heavier (Garvey 2000; Du Preez *et al.* 2009). A well-developed lateral stream line system is present that detects vibrations in the water (Du Preez *et al.* 2009).



Figure 1.12 Adult female *Xenopus laevis* (photo by L.H du Preez)

Reproduction

Xenopus laevis is a popular experimental species because mating takes place throughout the year (although mostly in spring) and up to four times annually (Garvey 2000). Once males are ready to mate, their palms turn black in colour (Garvey 2000; Du Preez *et al.* 2009). Mating occurs mostly at night. When females are ready to mate, their cloacal folds turn reddish and become swollen (Du Preez *et al.* 2009). The female releases thousands of eggs into the water within three to four hours after mating (Garvey 2000). The change from egg to small frog can take up to six to eight weeks (Garvey 2000).

This species can live up to sixteen years in the wild and up to twenty years in captivity, their longevity making them susceptible to environmental change. Despite this apparent shortcoming, their adaptability to change is a distinct advantage. *Xenopus laevis* are inactive and are scavengers, using a hyobranchial pump to suck food particles into its mouth (Garvey 2000; Du Preez *et al.* 2009). If starved, it will feed on its own tadpoles. The tadpoles on the other hand are filter feeders making them sensitive to certain substances or chemicals in the water (Garvey 2000; Du Preez *et al.* 2009).

These species were selected for the study because of their likelihood of coming in contact with water and/or crop-borne Bt protein and glyphosate. This can be seen in Figure 1.8, 1.11 and 1.13 where there is a distinct overlap between the distribution areas of the two amphibian species and the areas of maize production in South Africa.

A laboratory assessment was done to ensure a controlled environment. However, a controlled environment is the least favoured in science because of the large difference between that and natural environments. These differences include a lack of natural conditions such as fires, floods, temperature, predators and prey, nutrients and minerals, lack of flow (found in rivers and streams) as well as a lack of water plants.

1.6. Interactions between herbicides, insecticides and amphibians

Relyea (2009) studied the effect of low concentrations of five insecticides and herbicides in combination as well as separately on various aquatic communities. The study provided evidence that tadpoles of one particular taxon, viz. the leopard frog, *Lithobates pipiens*, suffered a direct adverse effect following exposure to the insecticide, endosulfan. Exposure resulted in 84% mortality, while a 24% mortality rate was reported following exposure to diazinon (another insecticide), and 99% mortality following exposure to a mixture of these pesticides (i.e. herbicides and insecticides) (Relyea 2009). The opposite effect was noted on tadpoles of the grey tree frog (*Hyla versicolor*). In this case, the mixture did not affect the mortality or time of metamorphosis, but due to the lack of competition between the two species, the grey tree frog grew almost twice as large as the leopard frog. Metamorphs of the grey tree frog were also found to be larger after exposure to atrazine (herbicide), following exposure to a mixture of insecticides and the mix of all ten pesticides (Relyea 2009).

The study of Relyea (2009) provided evidence that separate and combined use of pesticides, even at low concentrations, can have serious direct or indirect effects on the aquatic environment. The author found that the extent of the indirect effect was dependent on the particular pesticide, and that individual herbicides occasionally impact the individual taxa,

although no clear indication of indirect effects following the addition of herbicides was evident (Relyea 2009).

Currently there is a poor understanding of how GM crops can impact human and environmental health. According to Betz *et al.* (2000) Bt (Cry protein) is nontoxic to mammals and the impact on the environment “*will be minimal to non-existent*”. However, very little research has been undertaken regarding the impact on non-target organisms, except those few studies that have focused on mortality and behaviour (Betz *et al.* 2000).

1.7. Aims and objectives

Aims:

- determine the appropriate husbandry for *X. laevis* tadpoles,
- determine whether Cry1Ab protein present in Bt maize, affects the growth and development of *X. laevis* tadpoles,
- determine how glyphosate, the active ingredient of a herbicide, affects development of *A. gutturalis* tadpoles,
- determine the release and degradation rate of the Cry1Ab protein released from Bt maize leaves into water over a period of time,
- determine the effects of the Cry 1Ab protein released from Bt maize leaves on the development of *A. gutturalis* tadpoles,
- adapt the OECD guideline for Amphibian Metamorphosis Assay (AMA).

Objectives:

- to expose *X. laevis* tadpoles to different treatments, in different containers and with different food types,
- to expose *X. laevis* tadpoles to Bt maize leaf infusions over a period of time,
- to analyse Cry protein levels in Bt maize leaf-infused water using ELISA,
- to analyse the growth and development of *A. gutturalis* tadpoles after them to Bt and non-Bt infusion in mesocosms,

- to adapt the OECD guidelines for South African amphibian species (*Amietophrynus* sp.) and specific pesticides (Bt) used in South Africa.

1.8. Hypotheses

We hypothesize that:

- Incorrect husbandry practices will play a major role in the survival of tadpoles,
- Cry1Ab proteins will have little or no effect on the growth and development *X. laevis* tadpoles,
- Glyphosate will affect growth and development of *A. gutturalis* tadpoles,
- Cry1Ab protein levels released from Bt maize leaves will increase over time and then start to decline,
- The *A. gutturalis* tadpoles will develop at a slower rate when exposed to Bt leaves submerged in water than the tadpoles exposed to the control or non-Bt infusion.

CHAPTER 2

MATERIAL AND METHODS

In this chapter, the experiments performed will be fully explained as well as the materials used during experiments. A summary of the experiments and their respective objectives and treatments is provided in Table 2.1.

Table 2.1: Summary of all experiments

Experiment number	Focus of experiment	Species used	Treatments used	Main objectives
1	Husbandry	<i>Xenopus laevis</i>	Borehole water, non-Bt infusion.	Best food type, Container type, Treatment type.
2	Effect of Bt leaf infusion Laboratory study	<i>Xenopus laevis</i>	Borehole water, Bt infusion, non-Bt infusion.	Does Bt effect growth and development?
3	Effect of glyphosate	<i>Amietophrynus gutturalis</i>	Borehole water, Roundup® (ai glyphosate)	Does glyphosate effect growth and development?
4	Cry1Ab concentration	-	Bt infusion	Determine release and degradation rate of Bt maize leaves
5	Effect of Bt leaf infusion Mesocosm study	<i>Amietophrynus gutturalis</i>	Borehole water Bt infusion, non-Bt infusion.	Does Bt effect growth and development?

LABORATORY EXPERIMENTS

2.1. Experiment 1 – Which husbandry practices lead to higher levels of survival?

Husbandry refers to how animals, in this case tadpoles, are cared for to obtain maximum survival. The aims of this experiment were to determine the appropriate husbandry practices for *X. laevis* tadpoles. The following factors' effects on tadpole development and growth was determined:

- the effect of food type (Tetra-TabiMin™ or specially made *Xenopus* pellets),
- container type (tank or cups),
- and treatment type (borehole water or non-Bt maize leaf infusion).

Obtaining *Xenopus laevis* tadpoles

Collection of Xenopus laevis

Traps were set in the Botanical Garden (North-West University) for three days, after which the traps were inspected for adult individuals. Two of the largest females and males were selected and males and females were placed in separate glass tanks filled with 15 l of borehole water (less likely to contain chlorine) and oxygen. The frogs were left for three days to acclimatize to their new environment, before inducing spawning (Fig. 2.1 b). Spawning was induced by injecting a gonadotrophic hormone into females. This is known as a brood stock, which refers to cultured animals used to produce test organisms via reproduction (Environmental protection agency 1996).

Dilution of hormone

The gonadotrophic hormone Ovitrelle™ (active ingredient: choriogonadotropin alfa) (250 µg/0.5 ml) was used. The concentrated hormone was diluted into two 5 ml glass bottles with screw on caps. The bottles were marked either A or B. In A, 0.05 ml Ovitrelle™ and 5 ml water was added and in B 0.2 ml Ovitrelle™ and 2 ml water, respectively. The males were injected once daily for three days with the same concentration Ovitrelle™. The females were only injected on the second and third day with a higher dosage of the hormone before placing the males and females together (Table 2.2 for concentrations). The correct concentration of the

hormone must be injected as to make sure that the ovaries are not over stimulated (too much hormone) as well as to have successful spawning (spawning fails if too little hormone is injected).

Table 2.2: Concentration of hormone injected every day for three days

Day	Male		Female	
	Concentration Ovitrelle™	Volume of dilution	Concentration Ovitrelle™	Volume of dilution
1	250 IU*	0.2 ml of B	No injection on first day	-
2	250 IU	0.2 ml of B	50 IU	0.4 ml of A
3	250 IU	0.2 ml of B	500 IU	0.4 ml of B

*IU- International Units

Injection of hormones

Each adult frog was held with a firm, but gentle grip in such way that their heads faced the wrist of the researcher and the hind legs faced forward on either side of the researcher's finger. The frogs were placed on a paper towel and the head and forelimbs covered. A one ml syringe (needle bent slightly) was used. The needle was inserted from just below the lateral line between the skin and body muscles, slipping it in towards the head into the dorsal lymph sac (Vize 2013).

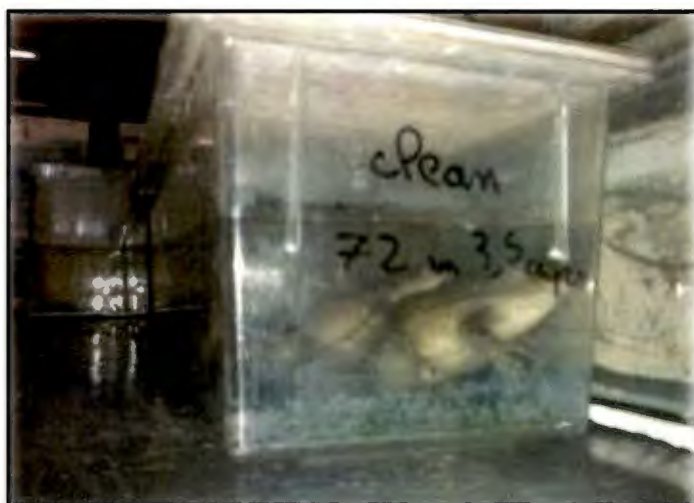
As indicated in Table 2.2, on all three days all males were injected with 0.2 ml dilution B, while the females received no injection. After the second day the hand palms of the male turned black in colour. On the second day the females received 0.4 ml of dilution A and on the third day, the females received the same volume, but of dilution B.

Husbandry of eggs and larvae

Once the final hormonal injection was administered, males and females were placed in pairs into two separate breeding tanks (Fig. 2.1 a) of which the water was maintained at room temperature. To acquire water at room temperature, the water was placed in buckets for 24 hours in a laboratory of which the ambient temperature was maintained at 21°C, before adding the adult frogs, leaves or tadpoles. Each tank was fitted with a raised mesh floor in order to protect the eggs from damage that the adult frogs may cause. Since frogs prefer to spawn at night, a black cloth was placed over the breeding tanks. Spawning took place the first night following the last hormone administration, where after the adults were removed from the breeding tanks and the water aerated. After a few hours of aeration, clean water maintained at room temperature was added and the mesh floor gently removed, once all the developing eggs were no longer attached to it.



a



b

Figure 2.1: a, Breeding tanks in which the two *Xenopus* adults are placed after the three-day hormone treatment. b, two frogs in amplexus. A mesh prevents the frogs from damaging the eggs

The eggs were allowed to develop and were monitored for three days followed by removal of the dead and unfertilized eggs. During the first few days of development, no independent feeding takes place and for this reason, it was not necessary to feed the larvae. Once the

larvae reached N/F stage 46, the tadpoles were fed for the first time by administering 500 µl of finely ground Tetra-TabiMin™ (fish pellets) and lucerne pellets into the water. After a week the larvae were placed into a new tray with clean aerated water (Van Wyk & Du Preez 1984). The trays were cleaned every few days and filled with clean water maintained at room temperature.

Obtaining non-Bt and Bt maize leaves

Maize leaves were collected at the Eco-Rehab Facility at NWU. The maize leaves were air-dried for three weeks. In this experiment the hybrid Phb30Y79B (GM Bt maize) and its isoline Phb30Y83 (non-GM maize) were used.

Experimental setup

This experiment was conducted over a period of five weeks and made use of ten 25 l glass tanks and ten trays which were set up in a laboratory at the North-West University. These ten trays held fifteen individual cups that contained either borehole water or a non-Bt maize leaf infusion. The groups were marked from A to J and the cups in each tray were marked one to fifteen. A tray (Fig. 2.2) and tank was grouped as one. The first six groups contained 15 l of borehole water and the last four contained 15 l of a non-Bt infusion of leaves and water made with submerged leaves in borehole water for 24 hours (Fig. 2.3). The first and last four groups were measured weekly. The cups and tanks were cleaned once a week and replaced with new non-Bt infusion.



Figure 2.2: Tray which can hold up to fifteen plastic cups

Each cup contained one tadpole and was filled with 250 ml borehole water or non-Bt infusion. In the case of the tanks which housed more than one tadpole, the amount of borehole water or infusion added was based on a ratio of one tadpole per 250 ml of borehole water/infusion. Each tank of 15 l could accommodate sixty tadpoles. Every cup and tank was provided with oxygen. The non-Bt infusion was prepared by submerging the non-Bt leaves in borehole water for two hours before removing the leaves. For every 5 l, 12 g of leaves are added so that the tanks all received 36 g of non-Bt leaves. A separate bucket containing 15 l of borehole water plus 36 g of leaves was used to prepare the infusion for the cups.



Figure 2.3: Experimental setup for the husbandry experiment with tanks at the back and trays with fifteen cups in each in front

Husbandry for tadpoles

The cups were cleaned and filled with newly prepared infusion on a weekly basis. The tadpoles were temporarily relocated to another cup filled with borehole water at room temperature. No soap was used during the cleaning of the cups to prevent foaming as well as contamination with other chemicals present in the soap. All the tadpoles in the cups were measured to determine their length and approximately three tadpoles were randomly selected from the tank to determine their size and developmental stage using the Nieuwkoop and Faber (N/F) Normal Table (Nieuwkoop & Faber 1956) (Fig. 2.4). Mortalities were noted daily as well as odd behaviour displayed by the tadpoles.

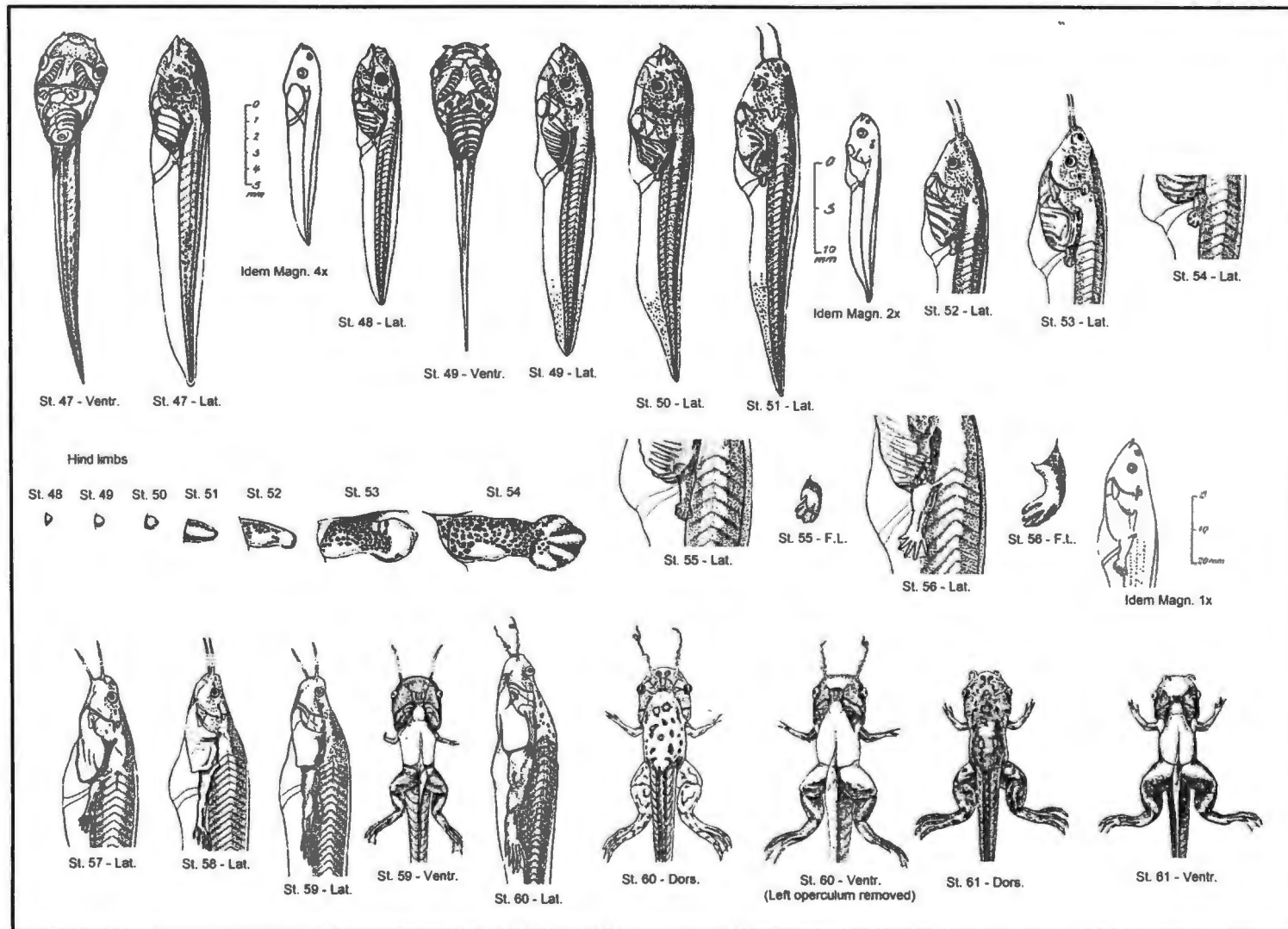


Figure 2.4: The Nieuwkoop and Faber staging Table used for determining developmental stages of *Xenopus laevis* tadpoles

Feeding

In this experiment two food types were used, viz. Tetra-TabiMin™ tablets and custom made *Xenopus* pellets (has the same contents as lucerne pellets). The first two groups maintained in borehole water and non-Bt infusion as well as the first control group received Tetra-TabiMin™ and the rest received the *Xenopus* pellets. Both the pellets were ground into a fine powder using a mortar and pestle, added to 50 ml borehole water and left for five minutes and then liquidized. Five-hundred µl was added to each of the cups and 30 ml was added to each tank. The food differed in nutrients as described in Table 2.3.

Table 2.3: Percentage nutrients found in the two different food types (Tetra-TabiMin™ and *Xenopus* pellets) as well as Dry weight of nutrients found in lucerne pellets

Nutrients	Percentage nutrients found in the different food types (%)		Mass dry weight (g/kg)
	Tetra-TabiMin™ (Ramsay 2010)	<i>Xenopus</i> or Rabbit (lucerne) pellets	<i>Xenopus</i> or Rabbit (lucerne) pellets
Protein	43	32	160
Fibre	1.5	34	170
Moisture	8	24	120
Oil	5	-	
Ash	9.5	-	
Fat	-	5	25
Calcium	-	3.6	18
Phosphorus	-	1.4	7

2.2. Experiment 2 - Effects of Cry1Ab in Bt maize leaves on *Xenopus laevis* tadpole development

This experiment was done to determine whether the Cry proteins in GM Bt maize have adverse effects on the morphological growth, size and survival of *Xenopus laevis* tadpoles.

Obtaining *Xenopus laevis* tadpoles

*Collection of *Xenopus laevis**

Traps were set out in the Botanical Garden, NWU, and then collected two to three days later. Three adult females and three adult males were selected and the males and females placed in separate glass tanks filled with 15 l of borehole water and supplemented with oxygen. The frogs were again left for three days to become accustomed to their new environment before spawning was induced.

Dilution of hormone

The hormone Ovitrelle™ (250 µg/0.5 ml) was used to induce spawning, using the same concentration and volume as that in the first experiment (2.1; Table 2.2).

Injection of hormones

The method of injecting a *Xenopus* was the same as described in 2.1. All three males were injected with 0.2 ml dilution B on the first day (as well as day two and three), while the females received no injection on day one, received 0.4 ml of dilution A on day two and 0.4 ml of dilution B on day three.

Husbandry of eggs and larvae

The husbandry of the eggs and larvae were the same as that described in 2.1.

Obtaining Bt and non-Bt maize leaves

Dried maize leaves collected from the Eco-Rehab Facility were used in this experiment. Hybrid Phb30Y79B (GM Bt maize) and Phb30Y83 (non-GM maize) were used. Dried maize leaves were also collected from the Agricultural Research Council (ARC) facility in Potchefstroom which was used as another non-Bt control group (DKC-80-10).

Experimental setup

Sixteen 25 ℓ tanks were divided into four groups (Fig. 2.5). Group one contained 10 ℓ borehole water per tank (without any leaves). Group two was a second control filled with DKC 80-10 infusion made by 36 g of DKC 80-10 leaves submerged in 10 ℓ borehole water. Group three contained Phb30Y79B Bt infusion made by 36 g of Phb30Y79B leaves submerged in 10 ℓ borehole water. The last group contained 10 ℓ of the Bt isoline, non-Bt infusion (36 g of Phb30Y83 leaves left in 10 ℓ borehole). The leaves of each infusion remained submerged for 24 hours before removing the leaves. Sixty tadpoles were added to every tank and every tank was aerated.



Figure 2.5: Experimental setup for second experiment (four groups of four tanks)

Husbandry for tadpoles

Tanks were cleaned and filled with new infusion on a weekly basis. The tadpoles were relocated until new infusion was placed in the tank and then returned. No less than twenty tadpoles from every tank were measured once a week to determine the length of individual tadpoles as well as their N/F stages (Fig. 2.4) using a Combination microscope (AZ100). The tadpoles were fed three times a week with finely ground lucerne pellets.

After feeding, the water was filled again so as to prevent an increase in toxin concentration. Mortalities as well as odd behaviour were noted daily during cleaning of the tank. Tadpole mortality and length were monitored for eight weeks.

Feeding

Rabbit food pellets (lucerne pellets) were ground into a fine powder (with a hammer mill) at the ARC in Potchefstroom. Two teaspoons of the powder was liquidized in 400 ml borehole water and 25 ml of this suspension was added to every tank every second day. The rabbit pellets had exactly the same nutrients as the *Xenopus* pellets (Table 2.3).

2.3. Experiment 3 - Effects of glyphosate on *Amietophrynus gutturalis* tadpole development

This experiment was done to identify the possible adverse effects of the glyphosate-containing herbicide, Roundup®, on *Amietophrynus gutturalis* tadpole development. Tadpoles were exposed to typical exposure concentrations of Roundup® as well as a worst case scenario (i.e. 6 ng/l; Tank *et al.* 2010) and a control.

Obtaining *Amietophrynus gutturalis* tadpoles

During the months of September to November in 2013, trips in and around Potchefstroom were taken to areas with streams, dams and ponds. Notices were placed in newspapers asking people to contact one of the authors if frogs were calling in their gardens. At the beginning of November 2013, two *Amietophrynus gutturalis* were found in amplexus and taken to the laboratory where they were placed in a glass 25 l tank filled with 3 l of borehole water and a brick set in the middle. The brick was used as a step for the adults because they are not aquatic amphibians and keeping them in water may cause them to stress. Once the *Amietophrynus* spawned, they were removed from the tank and taken back to their original location.

Husbandry of eggs and larvae

The eggs were left in an aerated tank for five days. During this period, the unfertilized eggs were removed to prevent the likelihood of any adverse effects of chemical compounds on the larvae and clean water was added. During the first few days of development, no independent feeding takes place and for this reason, it was not necessary to food the larvae (Gosner 1960). Once the larvae reach N/F stage 26, the tadpoles were fed for the first time by administrating 500 µl of finely ground lucerne pellets into the water.

Glyphosate concentration

Hundred ml Roundup® was purchased with the lot registration number L0407. This product contains 441 g of potassium salt and has 360 g/l of the active ingredient, glyphosate. This product is used in gardens for the control of weeds. According to the literature, the amount of glyphosate found in natural streams, ponds or dams varies between 1.24 mg ae/l and 5.2 mg ae/l (Perez *et al.* 2011). To accommodate these values, the following volumes were selected (Table 2.4):

Table 2.4: The volume of Roundup® used to acquire different ae and ai values

Roundup® (µl)	Acid equivalence (mg ae/l)	Active ingredient (mg ai/l)
0	0	0
3.2	0.2	0.52
32	2	5.22
63	4	10.44
127	8	20.88

In total, five treatments were used each with three replicates (Fig. 2.6). The control treatment consisted of unadulterated borehole water containing no Roundup®. The other treatments were various doses of Roundup®, viz. 3.2 µl, 32 µl, 63 µl and a worst case scenario, 127 µl, in a total volume of 5 l.

The amount of Roundup® was determined by using the four selected acid equivalence values (0.2 ae/l, 2 ae/l, 4 ae/l and 8 ae/l). The dosage of active ingredient was calculated for later comparison.

Experimental setup

Four containers were filled with 5 l borehole water and each one was treated with one of the Roundup® volumes described in Table 2.4. Fifteen trays (each of which can take fifteen plastic cups) was set on a long concrete table and labelled clearly (Fig. 2.6). The cups in each tray were filled with 125 ml of the corresponding concentration of Roundup® and a single tadpole was placed into each cup. Every cup was supplied with oxygen. The tadpoles were fed three times a week and measured once a week to determine their developmental stage.

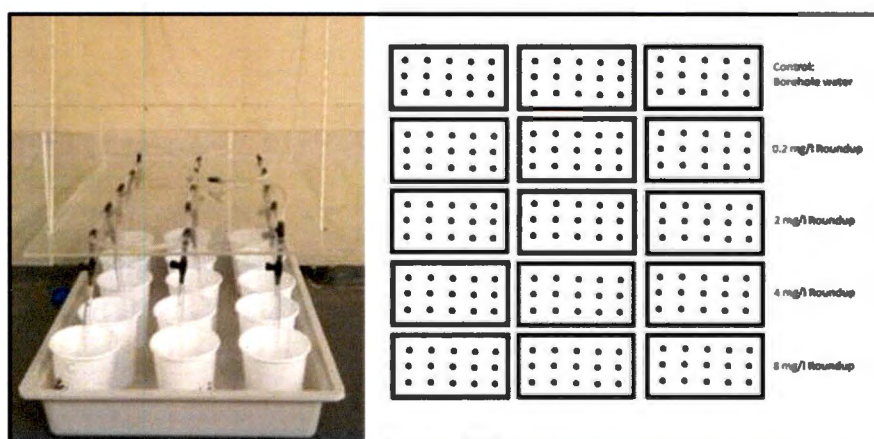


Figure 2.6: Tray with fifteen plastic cups each with an oxygen supply and the experimental design

Husbandry for tadpoles

The cups as well as the glass pipettes supplying each cup with oxygen were cleaned twice a week using a sponge and hot water (containing no soap; OECD 2008). When measuring was to take place, a glass Pasteur pipette (not a Transfer pipette) was used to limit any stress the tadpole might experience during measurements using the Gosner staging Table (Fig. 2.7). The tadpoles were fed three times a week (see feeding).

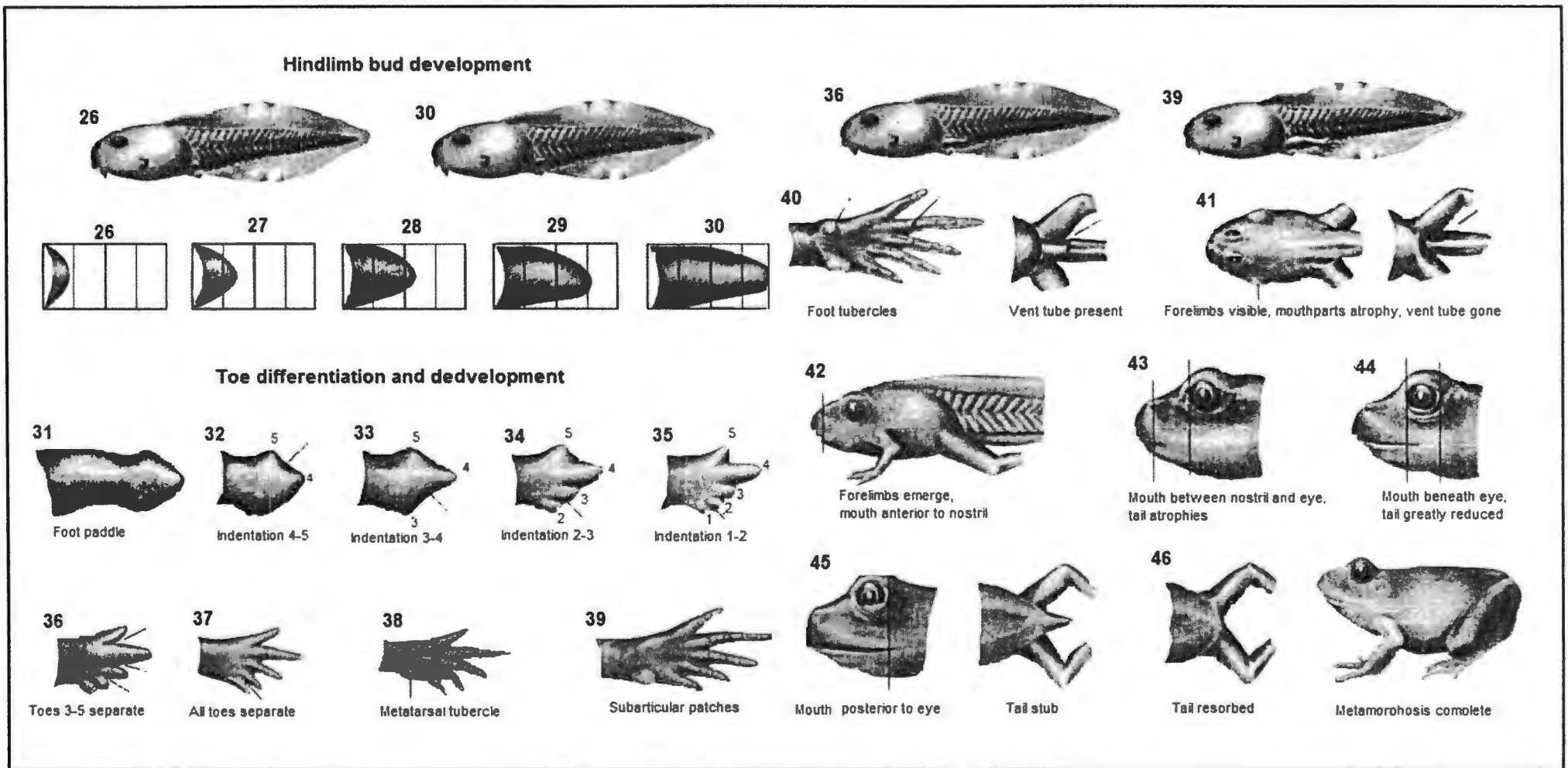


Figure 2.7: Gosner stages 26 to 46

Feeding

Lucerne pellets usually used as rabbit feed were finely ground and a teaspoon size of ground pellets were liquidized in 50 ml borehole water. Once the solution was made, 500 µl was pipetted into each cup. The content of the lucerne pellets is described in Table 2.3.

2.4. Experiment 4 - Bt protein concentration in water over time

This experiment was performed to determine the release-and degradation rate of the Cry1Ab protein from GM maize leaves after being submerged in water. An enzyme-linked immunosorbent assay (ELISA) was performed on water samples collected after various exposure times to determine the Cry1Ab concentration (ng/ml) in each sample.

In this study, the leaves were left submerged throughout the trial period. These results could provide useful information regarding when the infusion should be replaced and how long the leaves should be left in the water before removal during laboratory studies.

Experimental setup

This experiment was conducted at three different temperatures (10°C, 21°C and 30°C) to account for a night, room and day temperature. At each of these temperatures, there were two treatments consisting of borehole water and purified water. Each treatment was replicated three times. The infusions were made by submerging 24 g of Bt leaves into 1 l of either purified or borehole water. This was repeated for the non-Bt treatment. Maize hybrids DKC 7815B and CRN 3505 were used as the Bt and non-Bt hybrids respectively (Fig. 2.8)



Figure 2.8: Experimental setup: Bt maize leaves submerged in borehole water

Collection of samples

Samples were taken at specific times after exposure, viz. one, two, four, eight and sixteen hours, one day, two, four, eight and sixteen days, and then frozen at -80°C . Each sample consisted of 9 ml infusion. The samples were pooled by collecting 3 ml of each replicate and placing it in the corresponding 15 ml centrifuge tube. This was repeated three times. Once all the samples were acquired, they were analysed using ELISA to determine the concentration of Cry1Ab protein in each sample.

2.4.1. ELISA analysis

ELISA uses antibodies and colour changes (spectrophotometry) to identify and quantify the amount of a particular substance in a solution. Briefly, the induced colour change is proportional to the quantity of substance being analysed, viz. the darker the colour, the higher the concentration of toxin and vice versa. The assay uses a solid phase enzyme immunoassay (EIA) to detect a specific substance in an aqueous sample (BioRad Laboratories 2014). EnviroLogix QualiPlate assay Kit for Cry1Ab/Cry1Ac was used to detect the Cry1Ab protein in the maize leaves.

This kit contained the following (Fig. 2.9):

- 96 well plate,
- sachet buffer salts,
- pure Cry1Ab protein,
- enzyme conjugate,
- substrate,
- stop solution.



Figure 2.9: Contents of ELISA kits

All the aqueous samples were allowed to reach room temperature. Before the samples were prepared, 1.5 ml eppendorf cuvettes were labelled. After each analysis, the sample numbering may change depending on whether the optical density (OD) is above the accurate reading. The samples were numbered as follows: (a) undiluted or (b) diluted samples. If the wash buffer was not yet prepared, the sachet containing buffer salts (phosphate buffered saline, pH 7.4 – Tween 20) from the ELISA kits were dissolved in 1 l double distilled water.

Making the stock solution

Once the sample and reagents were at room temperature, an analytical scale was prepared by placing a dry beaker on the scale with a 50 ml centrifuge tube. The scale was tared, whereupon 5.3 µl of the Cry1Ab reference (95 µl/mg Cry1Ab) was added to the centrifuge tube using a 10 µl pipette. With an accurate pipette, the droplet weighed 0.0053 g. The wash buffer was slowly and carefully pipetted into the tube until the solution weighed 50 g (± 0.02 g). This was then known as the stock solution (10 ng/ml of Cry1Ab). This procedure was repeated.

Preparing the Standard series or Calibration curve

Each stock solution, consisting of twelve eppendorf cuvettes labelled S1 to S12, were placed on eppendorf cuvettes stands. The data provided in Table 2.5 was used as a guideline for the preparation of the standard series. The amount of stock solution and wash buffer, as indicated in Table 2.5, was pipetted into the corresponding eppendorf cuvettes.

Table 2.5: Guidelines for the preparation of the standard series (calibration curve)

Standard series	Volume 10 ng/mL diluted stock (µL)	Volume wash buffer (µL)
1	-	1000
2	3	997
3	6	994
4	12	988
5	24	976
6	50	950
7	100	900
8	150	850
9	200	800
10	250	750
11	300	700
12	350	650

Preparation of samples

Once all the samples were at room temperature, each eppendorf tube was mixed using a vortex mixer before 500 µL of the sample was pipetted into each cuvette labelled (a). For each sample, a new tip was used. Hundred and fifty µL was removed from eppendorf cuvettes labelled (a) and pipetted into the corresponding eppendorf cuvettes labelled (b) along with 1350 µL of wash buffer, making a 1:10 dilution.

In some cases, the 1:10 dilution was either not enough or too much; therefore other samples were diluted 2 or 20 fold (this was only done after the first analysis).

Preparation of analysis (Fig. 2.10)

Two plates were removed from the ELISA kits. Each plate contained twelve strips, although some strips were removed from one plate because the wells were not used. The next step was performed one strip at a time because the samples must be added as soon as the conjugate is placed in the well. An eppendorf multichannel pipette was used to add 50 µl **Cry1Ab enzyme conjugate** to each well followed by the 50 µl standard series, **positive control** and the samples in their corresponding well. Each sample was mixed before pipetting into the wells to ensure suspension of the Cry1Ab proteins. After completing each strip, the wells were mixed in circular motions for thirty seconds, whereupon parafilm was used to cover the entire plate to prevent evaporation. The covered plate was incubated on an orbital shaker at 200 rounds per minute (rpm) for two hours with a polystyrene covering to reduce the intake of light.

The following steps were followed in a dark room due to light sensitivity. After the incubation period, the parafilm was removed and the contents of the wells lightly patted out on a paper towel. Using a multi-channel eppendorf pipette, the wells were flooded with 200 µl wash buffer, mixed in a circular motion and patted out again. This was repeated four times to ensure rinsing out of any other substances in the sample (except the Cry1Ab). After the fourth wash, the plates were patted continuously on a paper towel to ensure dryness. An eppendorf multi-channel pipette was used to add 100 µl of the **substrate** into each well. The plates were mixed, the parafilm replaced and the plate incubated on an orbital shaker for a half an hour. After this step, the wells turned blue in colour indicating the presence of the Cry protein (Fig. 2.11). Lastly, 100 µl of the **stop solution** was added to each well, causing the contents to turn yellow, and then mixed in circular motions (Fig. 2.11).

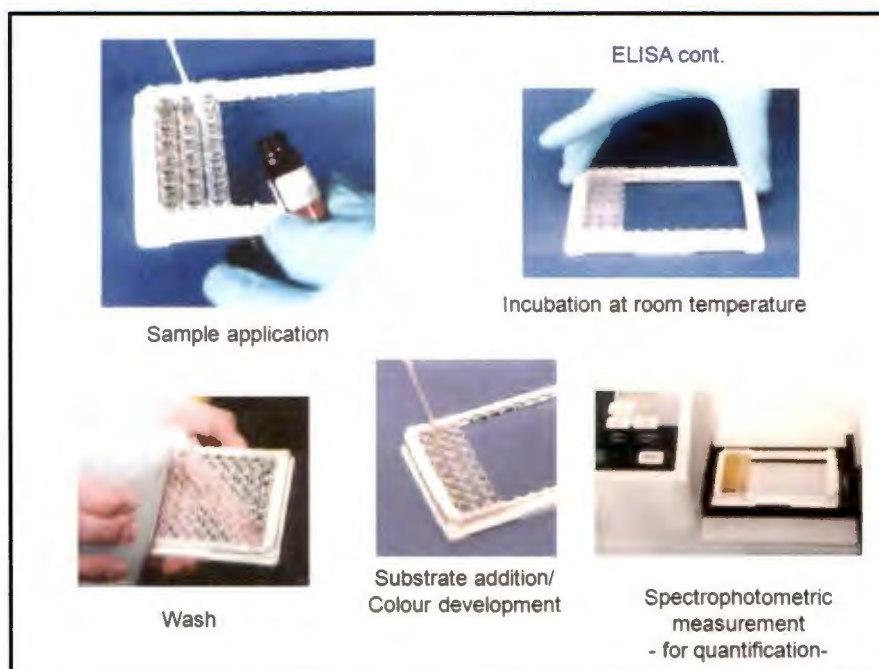


Figure 2.10: Steps for the analysis of water samples (Connolly 2010)

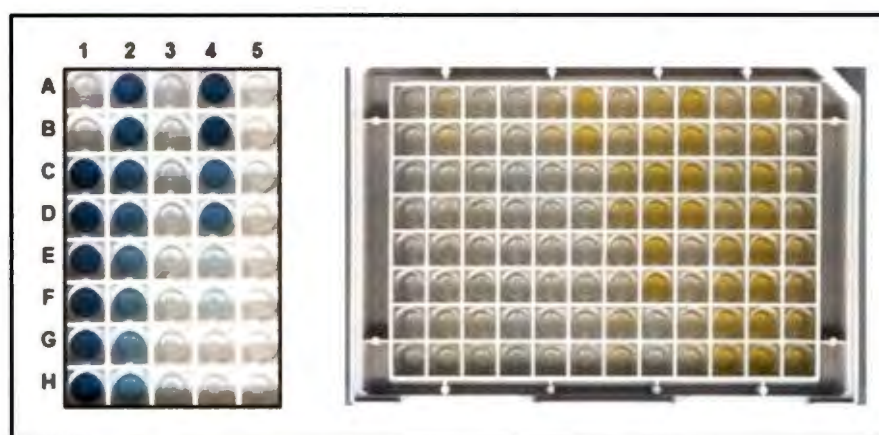


Figure 2.11: Substrate added and wells with Cry protein present turns blue; stop solution added changing the colour to yellow (Connolly 2010)

ELISA apparatus

The plates were placed into the Microplate Reader (wavelength set at 450 nm) and the optical density (OD) of the Cry1Ab protein analysed using the program *MicroWin* (Fig. 2.12). The entire protocol was repeated three times in order to improve the accuracy of the results.



Figure 2.12: Microplate Reader

MESOCOSM STUDY

2.5. Experiment 5 - Effect of Bt maize leaf infusion on *Amietophrynus gutturalis* tadpoles

Bt concentration

Maize leaves were collected at the Eco-Rehab Facility at NWU and were air-dried for three weeks. The hybrid DKC 7815B (GM Bt maize) and its isoline CRN 3505 (non-GM maize) were used. The concentration of the Cry1Ab protein in the infusion was determined by means of ELISA testing, and to ensure that the mass of leaves that is to be added into the water was environmentally relevant.

Preparation of Bt maize leaf infusions

Two containers were set up in the laboratory, each filled with 1 ℓ borehole water. Twenty-four g of dried Bt and non-Bt maize leaves were submerged in borehole water. The leaves remained submerged for four days after which the leaves were sieved out. The remaining water was poured into two separate 1 ℓ glass bottles with screw on caps. An ELISA was performed in order to determine the Cry1Ab concentration after four days. The ELISA

methodology described in experiment 4 was used. This concentration was then used to determine the required volume of the infusion when added to 380 ℓ aged tap water. The following equation was used:

$$V1 \times C1 = V2 \times C2$$

Equation 1: Volumes and concentrations

C1 is the concentration Cry1Ab proteins after four days (ng/ml), while C2 is the concentration required, in this case that of 0.014 ng/ml (Tank *et al.* 2010). V1 is the volume of the infusion which is unknown and V2 is the volume water in which the infusion is mixed (380 ℓ). It was determined that 155 ml of the infusion was required to get a concentration of 0.014 ng/ml. The pH of the water samples was also taken.

Determining the pH of maize leaf infusions

The pH reader (Fig. 2.13) was calibrated to a pH of 4 and 7 by holding the probe in a calibration solution that has a pH of either 4 or 7. The probe was rinsed off with distilled water after placing it into a liquid. The Bt infused water sample was vigorously mixed followed by placing the probe in the sample. The button *ENT* was used (indicated in Fig. 2.13 as number 5) was pressed when the pH had to be read. In other words, once the probe was placed in the Bt infusion, *ENT* was pressed. The probe was kept still while the pH value flashed (1). Once the flashing stopped, the value was noted. The probe was rinsed off before continuing to a new sample. This was repeated with the non-Bt and borehole water samples.



Figure 2.13: pH Reader (1-pH reading; 2-temperature reading; 3-on/off; 4-probe; 5-enter; 6-calibrate; 7-cap with salt water to protect probe)

Obtaining *Amietophrynus gutturalis* tadpoles

At the beginning of September in 2014, trips to areas with streams, dams and ponds were taken in and around Potchefstroom. Strings of eggs were collected and taken to the NWU laboratory and placed in a 5 ℓ tray filled with 3 ℓ borehole water.

Husbandry of eggs and larvae

The eggs were left in an aerated tank for seven to ten days (may vary) during which time the unfertilized eggs were removed and clean water added to prevent the possible adverse effects that unfertilized eggs could have on developing larvae. During the first few days of development, no independent feeding takes place and for this reason, it was not necessary to

feed the larvae (Gosner 1960). Once the larvae reach Gosner stage 26, the tadpoles were fed for the first time by administering 500 µl of finely ground lucerne pellets into the water.

Experimental setup

Twenty four g Bt and non-Bt leaves were submerged in 1 l borehole water and left for four days. After the four days have passed and before the tadpoles were added to the ponds, an ELISA analysis was done to determine the concentration Cry proteins were present in the 1 l infused water. This concentration was then used to determine the volume of the infusion that should be added to the 120 l water in the ponds to make up 0.014 ng/ml Cry1Ab proteins. The same volume non-Bt was added to its corresponding ponds. The temperature readings were taken every hour for fifteen days. Three ponds were located in full sun throughout the day, three more were located in a shaded area where the ponds were shaded from 11:00 onwards. The last three ponds were located in a semi-shaded area where the ponds become shaded at 15:00.

Nine small ponds with a diameter of 125 cm were used in this study (Fig 2.14). All nine ponds were filled with 120 l tap water. Two large 400 l drums were also filled with tap water (used to fill up the ponds and to make new diluted infusion the next week). These were left for five days to allow the chlorine present in the water to dissolve. Once the eggs hatched and the larvae reached stage 24 of the Gosner staging table (seven to 10 days) they were placed into different treatments set up on the roof. In total, three treatments with three replicates were used in this experiment (viz. tap water, non-Bt and Bt). The tap water was used as control treatments. Hundred and fifty-five ml of the Bt and non-Bt infusions were added into the corresponding ponds. A brick and 35 x 16 cm tile (leaning on brick) was placed in the middle of each pond. One-hundred tadpoles were then put into each pond. The lengths of the tadpoles were measured weekly for three weeks, including the day the tadpoles were first exposed to the treatments. The ponds were cleaned once a week by pumping all but three cm of the water out and replaced by aged tap water.

Forty-five ml samples were taken twice a week which was then analysed using the ELISA method explained in experiment 4. An extraction was done before the analysis because the Cry proteins were below the detection limit. The samples were taken directly after the first Bt and non-Bt infusions were added and then a week later just before replacing the water.



Figure 2.14: Experimental setup on roof: three treatments with three replicates each

Extraction of Cry1Ab proteins

The samples that were frozen was first left to reach room temperature. Centripreps filters were used for the extraction of the proteins (Fig. 2.15). These filters were labelled as well as 15 ml centrifuge tubes. The sample container was weighed as was then filled with 15 ml of the corresponding sample and then weighed again. These samples were then centrifuged at 1500 GS for 15 minutes. The lid of the centriprep was removed and the water in the filtrate collector was poured into the corresponding 15 ml centrifuge tube (Fig. 2.16). The centriprep was then centrifuged again for 10 minutes followed by five minutes after the water was poured out. The sample container was weighed again before another 15 ml of the same sample was added. The same process was repeated three times. It is important to take the last mass of the sample container after the last 15 ml of the sample was centrifuged three times. The remaining sample in the sample container was then used for the ELISA analysis. From here the ELISA process of experiment 4 was followed.

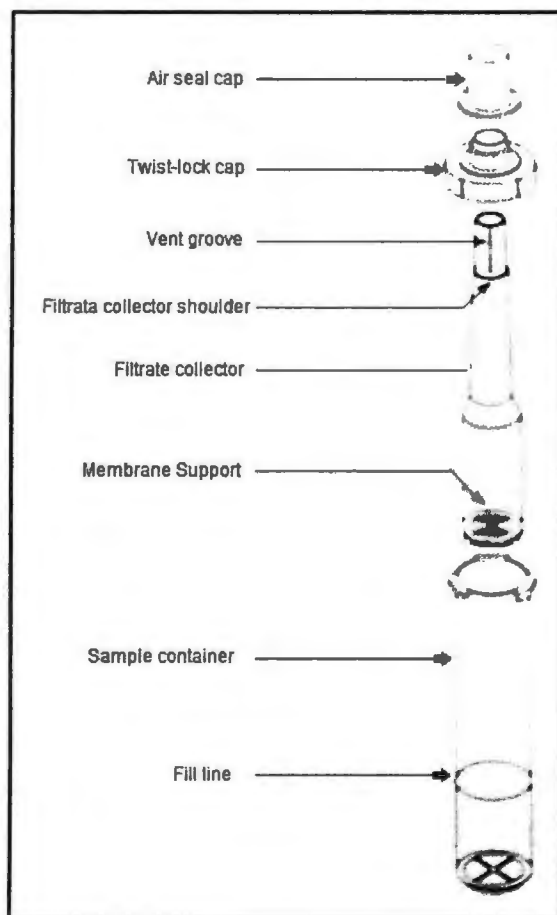
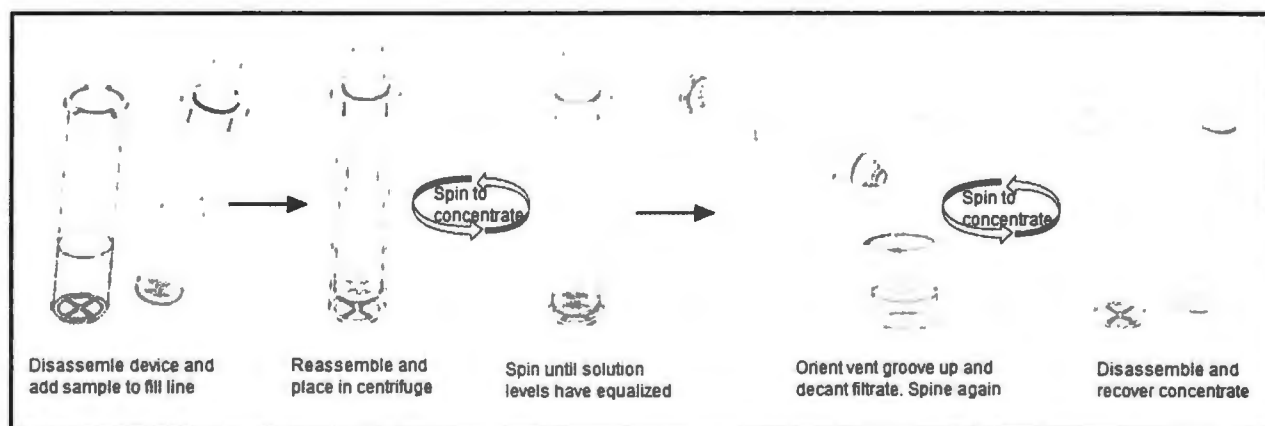


Figure 2.15: a, Labelled centriprep. b, method for extracting Cry proteins (edited from ELISA kit manual)

a



b

Husbandry for tadpoles

Very little husbandry was done in this experiment as a more natural environment was sought. When measuring tadpole length, a glass pipette was used to move them from one container to another. Two-hundred and fifty ml of the prepared ground and liquidized lucerne pellets was added to each mesocosm once a week.

2.6. Data analysis

Statistical analysis was performed using *IBM SPSS Statistics*. The normality of the data was pre-determined as well as kurtosis and skewness. Skewness describes whether the data is evenly distributed. The skewness is hand divided by the *Standard Error of Skewness* to get the Z-value (Kim 2013). According to Kim (2013) if the Z-values for samples below a number of 50 are larger than 1.96, then the null hypothesis is rejected, indicating a non-normal distribution. If the Z-value for a number of samples between 50 and 300 is over 3.29, the distribution is non-normal. Kurtosis describes whether the distribution of the data is peaked or flat (Wittig, 2014). The kurtosis is hand divided by the *Standard Error for Kurtosis* to get the Z-value. If greater than 1.96, then data has kurtosis (Kim 2013). Levene's test can also be done to determine whether equality of variance is significant or not. A t-test was undertaken with maximum two factors and an ANOVA for those with three or more factors. The Cohen's D value was also determined which allows the evaluation of the effect size in a study and is independent of scale (Cohen 1992). A small effect size is denoted by a Cohen's D value of between 0 and 0.2, a medium effect size for values between 0.2 and 0.5, and a large effect size denoted by values larger than 0.5. The most important component of the statistical analysis is to determine whether the factors are significantly different from one another or not. To acquire this information, the t-test or ANOVA was first performed and the significance was read off the information table provided. All statistical analysis was performed in consultation with the Statistical Consultation Service at NWU.

CHAPTER 3

RESULTS AND DISCUSSION

The results of the five experiments will be discussed followed by a detailed description and discussion of each experiment. Table 3.1 provides a summary of all the experiments undertaken in this study.

Table 3.1: Summary of experiments undertaken during this study

Experiment number	Experimental type	Main objectives
1	Husbandry	Best food type, container type, treatment type.
2	Effect of Bt infusion Laboratory study	Does Bt affect tadpole growth and development?
3	Effect of glyphosate	Does glyphosate affect tadpole growth and development?
4	Cry1Ab concentration	Determine degradation rate of Bt maize leaves and release of Cry1Ab toxin
5	Effect of Bt infusion Mesocosm study	Does Bt maize leaves affect tadpole growth and development?

3.1. Experiment 1 – Which husbandry practices lead to higher levels of survival?

This experiment was done to determine the appropriate husbandry practices for *Xenopus laevis* tadpoles. There were three factors that played a role in this experiment, namely the food type, container type and treatment type. After five weeks, the acquired data was read into the program *IBM SPSS* and the results presented graphically followed by an interpretation thereof. The standard deviation bars were set at a confidence interval of 95%. When the normality of

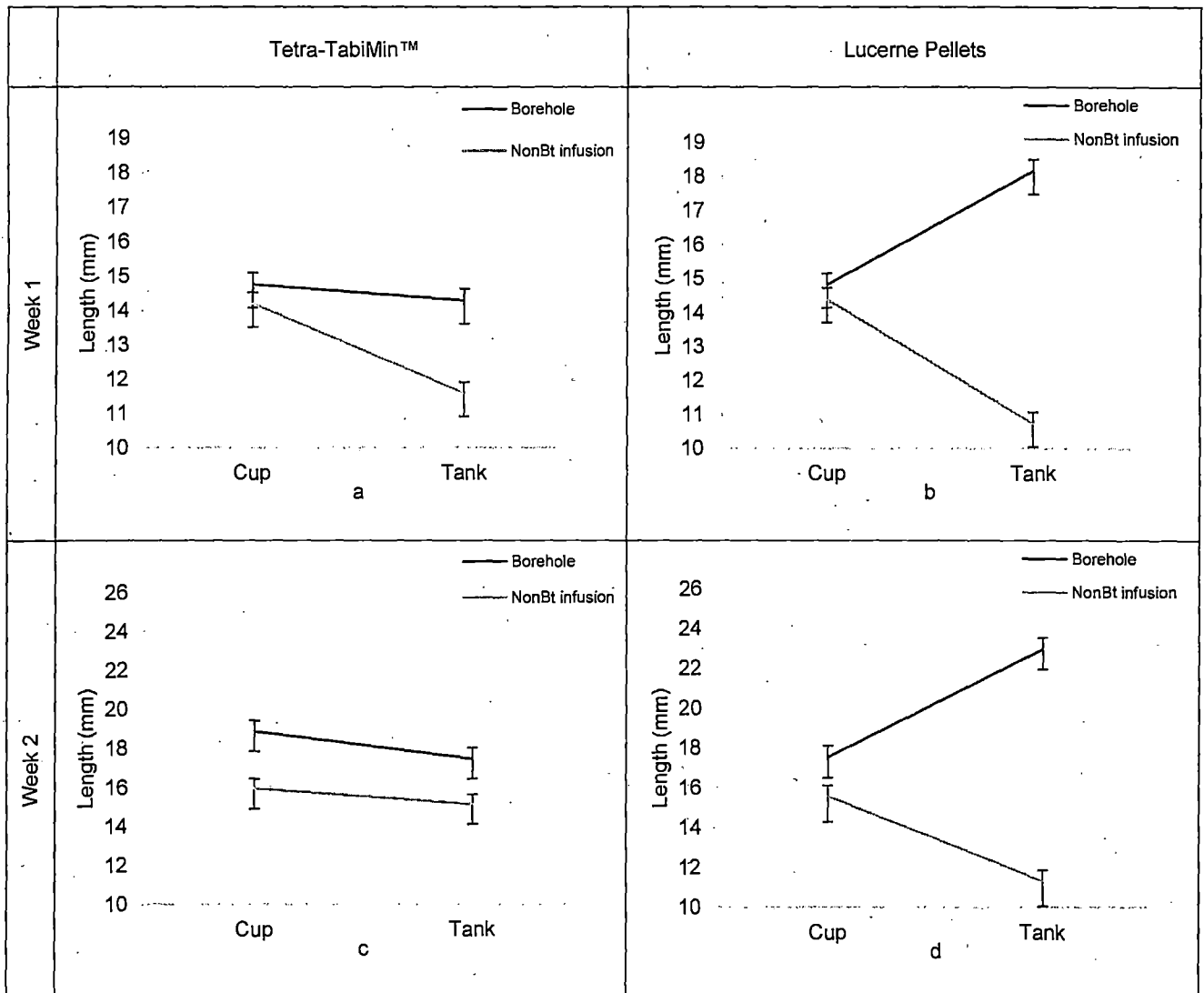
this data was determined, no skewness (0.879) or kurtosis ($Z = 0.908$) was found. This experiment had three factors and for this reason an ANOVA was performed. A repeated measures ANOVA would have been an appropriate analysis to perform. However, the tadpoles measured in the tanks were not the same tadpoles each week and thus a univariate analysis of variance (factorial ANOVA) was performed. The F- and p-values for this experiment are noted in Table 3.2 and will often be referred to during the description of the data.

Table 3.2: F- and p-values of the different factors compared during the five weeks

Dependent Variable:	Week 1		Week 2		Week 3		Week 4		Week 5	
Factors	F	P	F	P	F	p	F	p	F	p
Food	4.649	.033	.000	.983	.018	.894	2.057	.156	.133	.717
Treatment	54.026	.000	59.620	.000	37.958	.000	19.953	.000	24.810	.000
Container	4.972	.028	.171	.680	11.172	.001	36.916	.000	93.665	.000
Food*Treatment	9.307	.003	11.675	.001	13.526	.000	8.394	.005	13.036	.001
Food*Container	3.329	.071	1.934	.167	3.886	.052	.392	.533	.608	.439
Treatment*Container	36.522	.000	13.809	.000	5.265	.024	.095	.759	.620	.434
Food*Treatment*Container	10.350	.002	17.689	.000	22.827	.000	18.952	.000	25.078	.000

During the course of the five weeks, it was observed that the interaction between all three factors was the largest at the end of the fifth week, the interaction between the treatment and container type and the food and container type, however, was not significant. A large interaction ($F = 93.665$) was shown with respect to container type, followed by an interaction between the treatment types ($F = 24.810$), both of which were significant ($p < 0.001$). The food types, however, showed no interaction or statistical significance ($p = 0.717$; $F = 0.133$), although it did appear to have an effect on the development of the tadpoles (number of tadpoles with scoliosis found).

Within each factor, the length of the tadpoles in relation to the different types of food, container and treatment were compared on a weekly basis (Table 3.1 a-j). However, the developmental stages were not determined due to the low number of tadpoles harvested from the tanks. In future studies, it is recommended that no less than twenty tadpoles per tank be used to determine developmental stages.



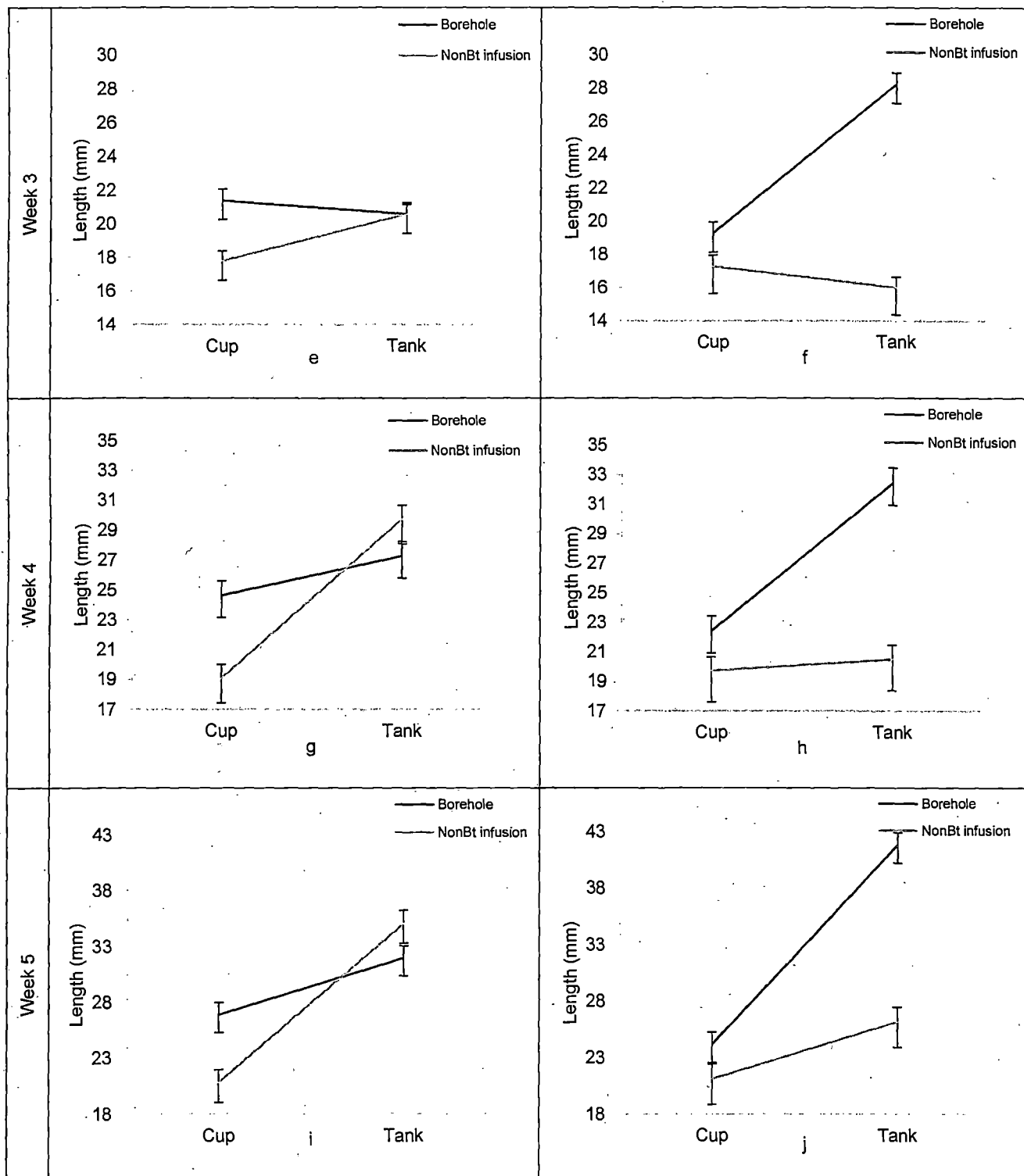


Figure 3.1: Graphs illustrating the length of the tadpoles when fed with Tetra-TabiMin™ or lucerne pellets, housed in cups or tanks and exposed to either borehole water or non-Bt infusion over a period of five weeks. Bars indicate standard error

Figure 3.1 graphs illustrating the overall difference between tadpole lengths after feeding on the two food types while being housed and exposed to two different containers and treatments, respectively. Malformations were observed during the course of the five weeks (Fig. 3.2 & 3.3), as will be discussed later.

When comparing the length of the tadpoles in regard to the treatment types, tadpoles exposed to the borehole water and residing in cups were consistently longer than tadpoles in the tanks, regardless of the food type. However, during the last three weeks the tadpoles fed with Tetra-TabiMin™, and housed in tanks and exposed to non-Bt infusion, had a faster growth rate than those exposed to the borehole water (non-Bt infusion: week 4: 29.74 mm; week 5: 35.08 mm; borehole water: week 4: 27.28 mm; week 5: 31.98 mm; Fig 3.1 g, i). The tadpoles exposed to non-Bt infusion had a growth rate consistently slower than those kept in borehole water, except for the last two weeks. During the last two weeks, this was exchanged leading to the tadpoles exposed to non-Bt having a faster growth rate than in the borehole water. When the complete five weeks of Tetra-TabiMin™ treatment is considered, it was seen that tadpoles exposed to the non-Bt infusion slowly started to increase in length over time. This could be explained by the tadpoles needing to acclimatize to their new environment (Chinathamby *et al.* 2006). If the exposure period was lengthened to eight or nine weeks, the stabilization of the non-Bt pattern might have been seen. However, it remains unclear why tadpoles fed with Tetra-TabiMin™ and housed in the tanks showed a greater growth rate compared to tadpoles exposed to borehole water.

After the first week, tadpoles fed with Tetra-TabiMin™ and exposed to borehole water, showed a difference in length of 0.47 mm between the two container types, whereas tadpoles exposed to the non-Bt infusion had a difference of 2.61 mm between the two container types. The tadpoles exposed to borehole water (fed with lucerne pellets) had a mean difference of 3.35 mm and 3.67 mm (non-Bt infusion) between the container types (Fig. 3.1 a).

Comparing the length of the tadpoles during the five week exposure period while being fed with lucerne pellets and exposed to borehole water, it was found that the tadpoles thrived while being housed in the tanks, always being longer length (week one: 18.18 mm; week five: 41.80 mm; Fig. 3.1 b, j) than tadpoles housed in the cups (week one: 14.83 mm; week five: 24.14 mm; Fig. 3.1 b, j). The length of the tadpoles housed in tanks and exposed to non-Bt infusion, however, were at first shorter than tadpoles in the cups (tanks: 10.73 mm; cups: 14.00 mm; Fig. 3.1 b), although tadpole length slowly increased over time. At the end of the fifth week, tadpoles were longer when housed in tanks as opposed to cups (tanks: 26.17 mm; cups: 21.10 mm; Fig. 3.1 j). This can also be explained by the tadpoles exposed to the non-Bt infusion requiring an acclimatization period of two to three weeks (regardless of the food type) before the development rate could start to stabilize. However, to confirm this assumption, a longer exposure time should be considered.

When comparing the treatment types, specifically tadpoles fed with Tetra-TabiMin™ and lucerne pellets, the difference between the two was significant. During week one (Fig. 3.1 a, b) tadpole length differed with 2.88 mm (borehole water) and 1.06 mm (non-Bt infusion) between the two food types. At the end of the fifth week, these differences were 12.58 mm and 9.18 mm, respectively. The non-Bt does not contain the Bt toxins and therefore should not be significantly different from borehole water, however, this was not the case. A significant difference was seen in the tadpole length between the non-Bt infused and borehole water ($p < 0.001$; $F = 24.810$). The non-Bt infusion was murky green which made the collection of tadpoles for measurement very difficult. This change in water clarity could have possibly affected growth rate and survival. Chlorophyll, located in the chloroplast of plants and that give leaves their green colour, may have leaked into the water from damaged chloroplasts, giving the water a green appearance. Chlorophyll itself could adversely affect the tadpoles, but this remains speculation. Further research in this regard is needed.

Regarding the size of tadpoles housed in the tanks, tadpoles exposed to borehole water and fed with lucerne pellets for five weeks showed the fastest growth (41.80 mm) than when exposed to non-Bt infusion (26.17mm; Fig. 3.1 j) or fed with Tetra TabiMin™ (31.98 mm and 35.08 mm respectively; Fig. 3.1 i). *Xenopus* are known to be social and cluster in a group when still in their larval stage (Baird 1983), which explains the faster growth rate of tadpoles in the tanks. However the individual cups were much smaller in size, thus restricting social behaviour and thereby adversely affecting tadpole growth. The tadpoles that were kept individually throughout the five week period may have been under stress due to isolation or due to the small housing space, while those in groups or in larger housing conditions appeared to thrive. The average length of the tadpoles in tank vs. cup containers did not differ significantly from one another during the second week ($p = 0.680$; $F = 0.171$). This indicated the possibility of a two-week habituation period. After the tadpoles adjusted to their new environment and at the end of the five week period, their average length differed significantly between the two types of containers ($p < 0.001$; $F = 93.665$).

When looking at all the graphs, it is seen that a large interaction is present between all three factors ($F = 25.078$). Throughout the five weeks, tadpoles fed with the lucerne pellets thrived. Borehole water also proved to be a preferred treatment, especially when fed with lucerne pellets. The tanks also proved to be favoured by the tadpoles as the best container. At the end of five weeks, the best husbandry combination proved to be grouped housing (tanks), fed with finely ground lucerne pellets and exposed to borehole water. These factor interactions were significantly different, with a p-value of < 0.001 .

The malformation observed can be described as scoliosis due to the bending of the spine (Fig. 3.2). This developmental abnormality suggests malnutrition and/or the presence of an environmental toxin.

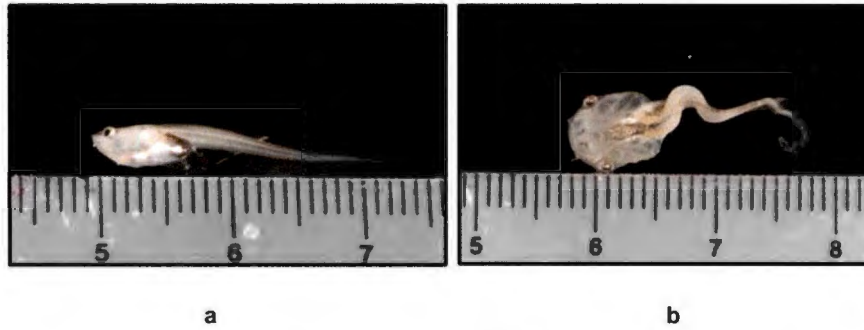


**Figure 3.2: Image of tadpoles, both the same age. (a) Without scoliosis and (b) with severe scoliosis
(photos by L.H. du Preez)**

Figure 3.2 shows the difference between a tadpole without (a) and with scoliosis (b), as observed in this study. Both tadpoles were of the same age (three weeks). The tadpole in Figure 3.2 (a) was housed in borehole water and fed with the lucerne pellets, whereas the tadpole in Figure 3.2 (b) was housed in the non-Bt infusion and fed with Tetra-TabiMin™. During the study, odd swimming behaviour was noted in tadpoles with scoliosis. The bent spine may lead to vibrating circular swimming (the severity depends on the level of scoliosis) and may account for the abnormal swimming behaviour observed. It would be interesting to do a study on locomotor activity (using video-tracking) of the tadpoles with scoliosis. The treatment that resulted in the highest percentage malformations of tadpoles were found to be those housed in the non-Bt infusion and fed with Tetra TabiMin™ (Fig. 3.3).

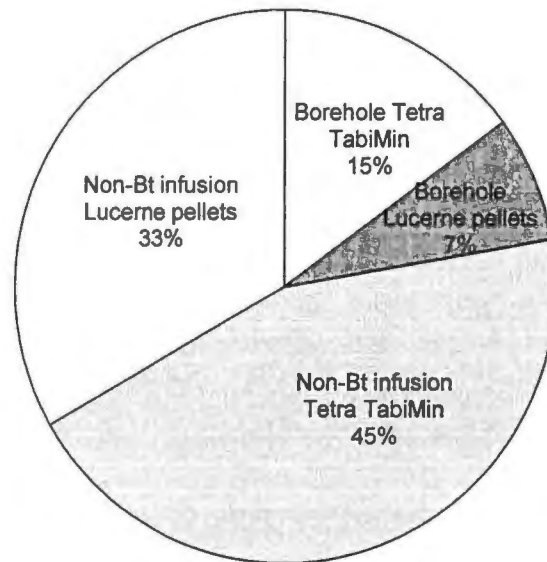


Figure 3.3: Pie chart of the maximum malformed tadpoles found in borehole water and non-Bt infusion as well as the percentage thereof when fed Tetra TabiMin™ or lucerne pellets

Tadpoles exposed to the non-Bt infusion had an overall higher percentage scoliosis (78%) than those in borehole water (22%) regardless of the food type, but the tadpoles fed with Tetra-TabiMin™ had an overall higher percentage scoliosis (60%) than those fed with lucerne pellets (40%). This may indicate that the Tetra TabiMin™ may not have the appropriate nutrients needed for the development of *X. laevis* tadpoles.

Moreover, it can be concluded that the non-Bt infusion also had an effect on the development of tadpoles, albeit less than that noted in the non-Bt group, with a 50% incidence of scoliosis noted in tadpoles exposed to non-Bt-infusion vs. 22% for those housed in borehole water. This data was derived from the tadpoles housed in the cups and not the tanks as it was not possible to keep track of individual growth patterns in the latter. The fact that a variable level of malformations was evident in tadpoles fed both food types (Tetra-TabiMin™ and lucerne pellets), in the presence and absence of Bt, suggests that the content of the leaves may have had adverse effects on tadpole development or at least on their intake of food. However, this is only speculation and further investigations should be undertaken.

Mortality is an important endpoint that was studied on a daily basis throughout the study. This study has shown that healthy tadpole growth and development is indicative of preferred conditions for *Xenopus*. However, by the same token so can mortality. When looking at mortality as a factor (Fig. 3.4), it is important to consider that fatalities during the first week may not necessarily be caused by factors such as food, treatment or container type, but by natural selection (McDiarmid & Altig 1999). This means that some tadpoles or larvae are stronger than others allowing them to collect more food than the weaker tadpoles. This could lead to an increase in strength of the healthy tadpoles and a decrease in strength of the weaker tadpoles (McDiarmid & Altig 1999). The weaker tadpoles become less efficient (diminished fitness) in competing for food and eventually die.

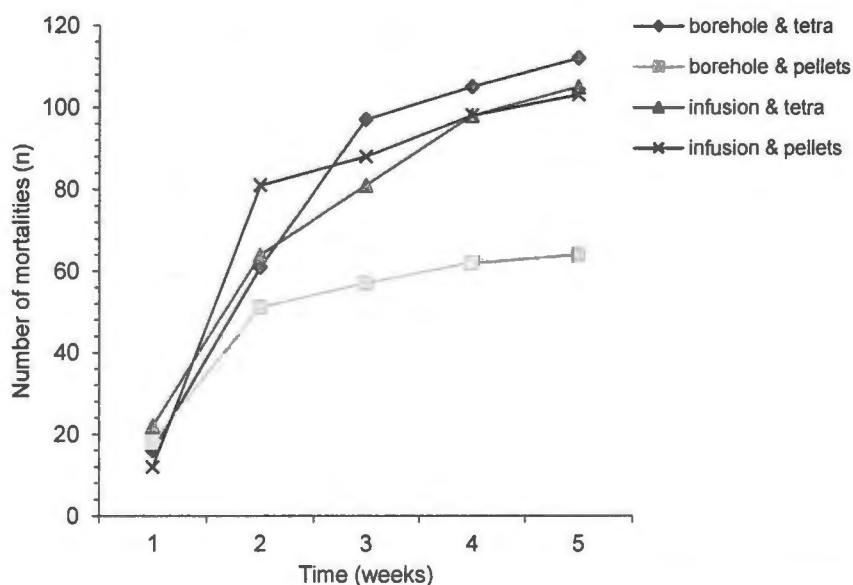


Figure 3.4: A pooled number of tadpole mortalities in cups and tanks over a period of five weeks

Figure 3.4 shows the number mortalities during the five week study. In this graph, the mortalities of tadpoles in both the cups and tanks were pooled together allowing a better understanding of the more appropriate husbandry practices. The two factors that resulted in the lowest mortality ($n = 64$) were that of tadpoles housed in borehole water and fed with lucerne pellets. The tadpoles housed in borehole water and fed with Tetra TabiMin™ had the highest number of deaths ($n = 112$), closely followed by those housed in the non-Bt infusion

(non-Bt infusion and Tetra TabiMin™ = 105; non-Bt infusion and lucerne pellets = 103). The mortality of tadpoles in the two infusions differed with the death of two individuals, indicating that the infusion of maize leaves and borehole water might have an unknown deleterious effect on the survival of the tadpoles.

It is not known why the Tetra TabiMin™ played such a significant role in the survival of the tadpoles housed in borehole water. Possibly a lack of appropriate nutrients could have given rise to developmental malformations leading to the higher mortality described. However, this does not explain why tadpoles fed with Tetra TabiMin™ have a lower mortality when housed in non-Bt infusion vs. those housed in borehole water.

It is concluded that the optimum husbandry practices for *X. laevis* are the following:

- This species prefers to be in large groups while developing and therefore the use of 25 l tanks is recommended instead of individual cups.
- In order for this species to acquire all the necessary nutrients, and hence to show normal development, the most appropriate food type to use is finely ground lucerne pellets liquidized in borehole water.
- Borehole water treatment is the most adequate for this species.

3.2. Experiment 2 - Effects of Cry1Ab in Bt maize leaves on *Xenopus laevis* tadpole development

This experiment was undertaken to determine whether Bt infusions could have an adverse effect on the developmental and/or growth of *X. laevis* tadpoles. This experiment involved four different treatments: borehole water (control 1), non-Bt infusion (control 2), Bt infusion, and a non-Bt infusion (Bt isolate). The experiment was conducted over a period of eight weeks. The length (Fig. 3.5), developmental stage (Fig. 3.6) and mortality (Fig. 3.7) of tadpoles undergoing the different treatments are graphically presented followed by an interpretation thereof.

Each of the treatments had four replicates which were pooled to obtain the average length (mm), developmental stage (N/F stage) and mortality (%).

Data analyses were done using *IBM SPSS* and a confidence interval of 95% was set. When the normality of this data was determined, no skewness was found at the end of the eight week treatment period (skewness = 0.549), although a Z-value exceeding 1.96 was obtained indicating kurtosis ($Z = 2.149$). Only two factors were compared at a time, and for this reason a t-test was performed. In order to correct the *Family Wise Error*, a Bonferroni correction was performed by multiplying the acquired p-value of the t-tests with the number of interactions which, in this experiment, amounted to six.

The development of the exposed tadpoles is important in this experiment, and for this reason no less than twenty tadpoles were used to determine the mean length (Fig. 3.5) and N/F stage (Fig. 3.6).

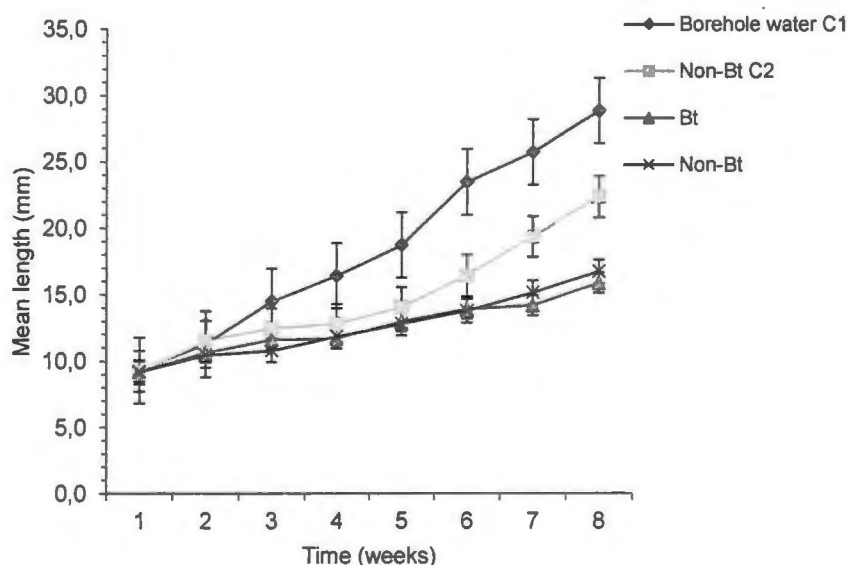


Figure 3.5: Mean length of tadpoles exposed to four different treatments (borehole water C1, non-Bt C2, Bt, non-Bt) over a period of eight weeks. Bars represent standard error

Figure 3.5 provides data on the length of tadpoles exposed to four different treatments. The length of tadpoles in the two control groups (borehole water and non-Bt control) were significantly different with a large effects size observed ($p < 0.001$; $t = 9.602$; Cohen's $D =$

1.173). In experiment 1 it was found that tadpoles exposed to borehole and non-Bt infused water had a visible effect on tadpole length. The data presented in Figure 3.5 supports this conclusion.

When comparing the two treatments (Bt and its isoline), tadpoles exposed to Bt treatment were shorter in length (15.9 mm) than those exposed to the non-Bt treatment (16.8 mm; Fig. 3.5), however this difference was not significant at the end of the experiment ($p > 1$; $t = 0.360$; Cohen's $D = 0.050$). This could indicate that each Bt and its isoline hybrid might follow the same pattern. In other words, if the non-Bt control and its Bt hybrid was included as a third control, it would have the same pattern as the two test treatments. However, further study is required to confirm this.

Of particular interest was that a significant difference with a large effect was observed between the two non-Bt infusions ($p = 0.031$; $t = 4.733$; Cohen's $D = 1.99$; Fig. 3.5). The treatments in this study were a Bt and non-Bt maize hybrid, viz. Phb30Y79B (Bt) and its isoline Phb30Y83 (non-Bt), as well as another non-Bt treatment (DKC-80-10). Each maize hybrid was grown in different fields and at different locations. These factors can possibly play a role in the effects of the infusion. If a third control is brought in as a Bt control (the same hybrid as the non-Bt control), it can be expected that the non-Bt control treatment would show a similar pattern as that of the isoline, indicating no significance, but with a faster growth rate. This would then suggest that each maize hybrid would affect tadpoles differently.

Comparing the length of the tadpoles exposed to the Bt and non-Bt control infusions, it was found that the tadpole growth was significantly reduced with a medium effect in the Bt group (non-Bt control: 22.3 mm; Bt: 15.9 mm) ($p = 0.012$; $t = 3.190$; Cohen's $D = 0.288$).

The Bt infusion was also compared to the borehole water, indicating a significantly greater degree of growth in the borehole water at the conclusion of the treatment period ($p = 0.009$; $t = 3.268$; Cohen's $D = 2.331$; Fig. 3.5). There is a possibility that tadpoles exposed to the infusions used more energy to survive in a treatment, which is not ideal for them.

For this reason there is less energy available for growth and development and therefore were smaller than tadpoles in borehole water. Indeed, tadpoles housed in borehole water had the fastest development (average 28.4 mm, stage 50; Fig. 3.6) after eight weeks.

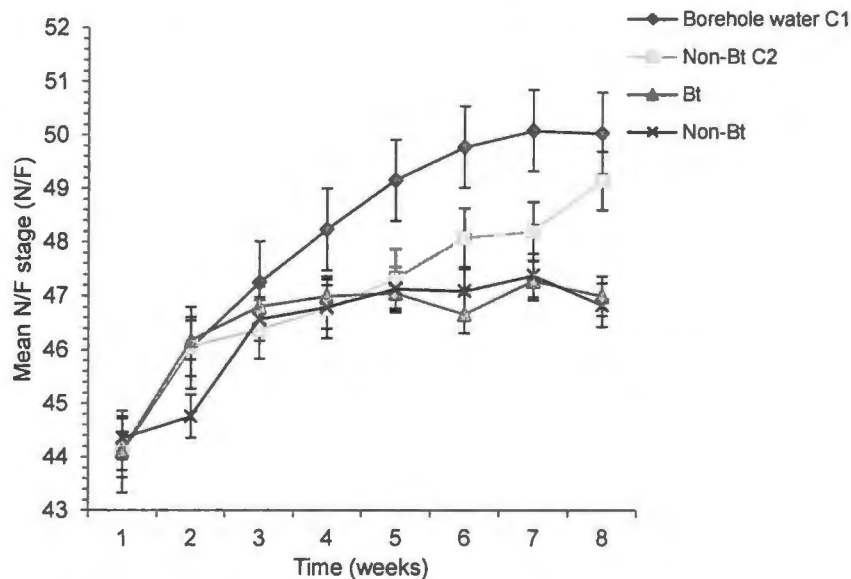


Figure 3.6: Mean developmental stage (N/F) of tadpoles exposed to four different treatments (borehole water C1, non-Bt C2, Bt, non-Bt) over a period of eight weeks. Bars represent standard error

Figure 3.6 presents the mean development of the tadpoles exposed to the four different treatments. The tadpoles exposed to the borehole water (control 1) developed at a faster rate than tadpoles exposed to the other treatments including that of the second control however only slightly ($p = 0.125$; $t = 2.333$; Cohen's $D = 0.333$). The length of the tadpoles exposed to borehole water did not differ significantly when compared to tadpoles housed in the Bt infusion ($p = 0.390$; $t = 3.268$, Cohen's $D = 1.073$). After comparing the lengths of the tadpoles housed in the two non-Bt infusions, it was deduced that they were significantly different with a large effect ($p = 0.014$; $t = 2.088$; Cohen's $D = 0.861$). When comparing the two treatments (Bt and non-Bt), no significance differences regarding the developmental stage of the tadpoles were noted ($p > 1$; $t = 0.130$; Cohen's $D = 0.058$). In order to understand the reason why the two treatments (Bt and its isolate) have similar effects on growth and development requires further studies. However, it could be that the different hybrids have other effects on tadpole growth and development and this should be looked into.

In a recent paper, Parrot (2008) explained that the use of different hybrids used in a study could explain the different results. Studies should therefore strive to use the same hybrid to avoid these complications.

Relyea (2009) found similar results in a study with tadpoles, predators and vegetation in a controlled ecosystem. Different species of tadpoles were exposed to different pesticides as well as a mixture of pesticides. This study showed that the tadpoles reacted differently to every pesticide. Reactions included reduced growth rates and higher mortality rates.

Xenopus laevis can easily adapt to their environment and our study suggests that its development is affected by any type of maize leaf infusion. Moreover, different hybrid infusions affect them in different ways. Rot-Nikcevic and Wassersug (2004) found that the metamorphosis of *Xenopus* tadpoles can be restrained under laboratory conditions, and that tadpoles would continue to grow in size, but no longer *developed* once their limbs started to develop.

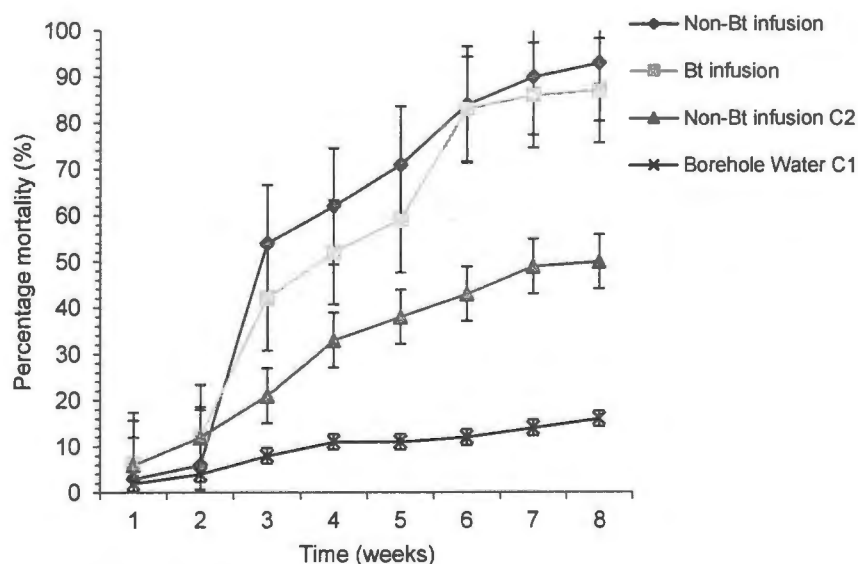


Figure 3.7: Mortality rate of tadpoles housed in the different treatments (borehole water C1, non-Bt C2, Bt, non-Bt) over a period of eight weeks. Bars represent standard error

Figure 3.7 presents the mortality of the tadpoles over a period of eight weeks. Tadpoles housed in borehole water had the lowest mortality (16%) compared to the other three

treatments (non-Bt control: 50%; Bt: 87% and non-Bt: 93%). This data was similar to that of the growth (Fig. 3.5) and development (Fig. 3.6) of the tadpoles, depicting them as thriving with respect to growth, development and survival when housed in borehole water, but the opposite when exposed to the infusions. The Bt and its isoline infusion has similar mortalities regarding tadpole survival (Fig. 3.7), although tadpoles exposed to the two different isoline hybrids were different in mortality. This could also be due to the use of different maize hybrids, as explained above for Figure 3.5 and 3.6. The difference in genetic structure and leaf contents could depend on the company that produced the seeds as well as where the crops were planted and in what type of soil.

3.3. Experiment 3 - Effects of glyphosate on *Amietophrynus gutturalis* tadpole development

This experiment was done to determine whether Roundup® could have an effect on the development and/or growth of *A. gutturalis* tadpoles. This experiment involved four different concentrations of glyphosate (0.52 mg ai/l, 5.22 mg ai/l, 10.44 mg ai/l and 20.88 mg ai/l) as well as a control (0 mg ai/l) (see Table 3.3 for the different volumes Roundup® to acquire the above mentioned glyphosate concentrations). Each concentration, including the control (0 mg ai/l), had four replicates.

Throughout this experiment, the Roundup® concentration will be referred to as treatments. The data of the replicates were pooled in order to determine the average length (Fig. 3.8), developmental stage (Fig. 3.9) and mortality (Fig. 3.10) of the tadpoles exposed to the different concentrations of Roundup®. This experiment continued for 21 weeks due to the time it took for most individuals to reach metamorphosis. The results are graphically presented followed by an interpretation thereof.

Table 3.3: Volume of Roundup® used to provide certain glyphosate concentrations

Roundup® (µℓ)	Active ingredient (mg ai/ℓ)
0	0
3.2	0.52
32	5.22
63	10.44
127	20.88

The statistics of this experiment was done using *IBM SPSS* with the confidence intervals set at 95%. When normality of the data was determined, the data was found to be skew (2.256) and presenting with kurtosis ($Z = 4.114$). Results for the control and lowest Roundup® concentration (3.2 µℓ) were compared at the end of 21 weeks. In this instance the data were analysed using a t-test and not a repeated measures ANOVA. If all five treatments were compared, the latter test would have been appropriate. However, due to the 100% mortality of the tadpoles exposed to the three highest concentrations at week three, this was not necessary. However, in order to correct the *Family Wise Error*, a Bonferroni correction was performed by multiplying the p-value with five (number of interactions considered in this experiment).

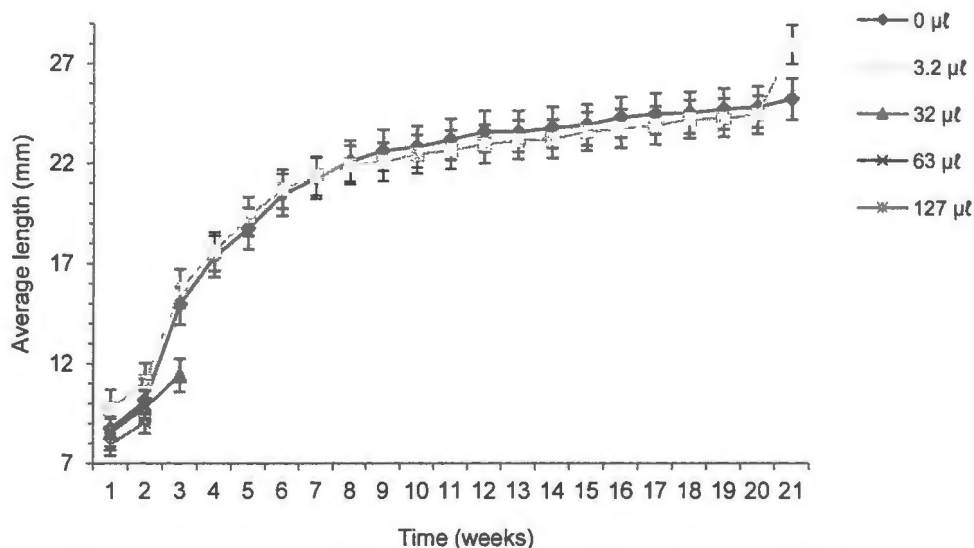


Figure 3.8: Mean length (mm) of tadpoles when exposed to different Roundup® treatments over a period of 21 weeks. Bars represent standard error

Figure 3.8 presents the overall length of the tadpoles exposed to the five different treatments. The only significant difference between the length of tadpoles exposed to the control and 3.2 µl Roundup®, was observed during week two, with p-value of < 0.001 ($t = 4.302$; Cohen's $D = 0.877$). The following weeks, however, failed to show significant differences between the two treatments (week 21: $p > 1$; $t = 0.723$; Cohen's $D = 0.307$). Tadpoles exposed to the 32 µl treatment only reached a mean length of 11.41 mm before that treatment was terminated (due to mortality), but still attaining significant differences compared to control ($p < 0.001$; $t = 4.449$; Cohen's $D = 1.873$). From the first week and up to the 20th week, the tadpoles showed similar growth rates. However, at week 21 the tadpoles exposed to 3.2 µl (27.9 mm) were on average 2.7 mm longer than those in the control group (25.2 mm). This is because most of the tadpoles in the control group had already reached metamorphosis (Fig. 3.10), which is Gosner stage 46 (Fig. 3.9). The highest Roundup® volume (127 µl) as well as the second highest (63 µl) only reached a mean length of 9.06 mm and 9.95 mm, respectively, after two weeks of exposure before the experiment was terminated due to high mortalities. In this short period, the two highest doses of Roundup® lead to a significant difference in tadpole length, with a p-value of < 0.001 ($t = 4.774$; Cohen's $D = 0.965$). When comparing the length of tadpoles in the control treatments to the treatment with the 2nd highest concentration of Round, no significant

difference was found ($p > 1$; $t = 0.951$; Cohen's $D = 0.196$), although a significant difference was found between control and the treatment with the highest Roundup® concentration ($p < 0.001$; $t = 5.235$; Cohen's $D = 1.038$). After three weeks, tadpoles exposed to the treatments with the three highest doses of Roundup® had reached a mortality rate of 100% (Fig. 3.10).

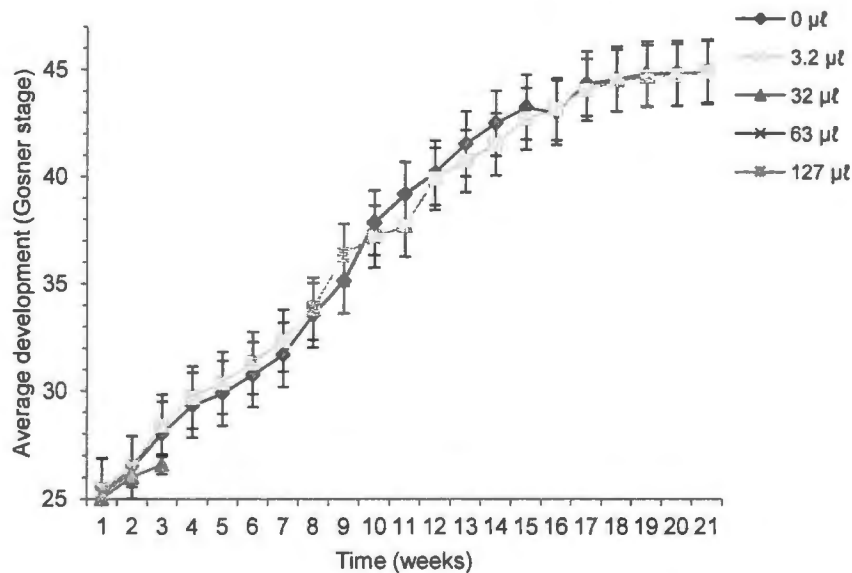


Figure 3.9: Mean developmental stage (Gosner) of tadpoles exposed to different Roundup® treatments over a period of 21 weeks. Bars represent standard error

Figure 3.9 illustrates the developmental stages of the tadpoles when exposed to the different treatments. The development of tadpoles had a similar pattern as tadpole growth (Fig. 3.8). There was no significant difference found between the length of tadpoles in the control treatment and the highest Roundup® volume (127 µl) ($p = 0.929$; $t = 1.336$; Cohen's $D = 0.290$). When comparing the control with the lowest Roundup® volume (3.2 µl), significance occurred only during week seven ($p = 0.021$; $t = 2.637$; Cohen's $D = 0.655$). However, no significance between these two treatments were found at the end of the exposure period (21 weeks), ($p > 1$; $t = 0.007$; Cohen's $D = 0.002$). Considering tadpole development during the first two weeks (all tadpoles exposed to the 127 µl died after two weeks), no significance was found in tadpoles exposed to the highest and lowest Roundup® treatments (3.2 µl and 127 µl; $p = 0.703$; $t = 1.489$; Cohen's $D = 0.338$). The Roundup® treatment (63 µl) showed did also not result in a significant difference in length of tadpoles compared to the control treatment with a

p-value of > 1 ($t = 1.077$; Cohen's $D = 0.212$), reaching only stage 26. The two highest treatments ($63 \mu\text{l}$ and $127 \mu\text{l}$) also showed no significance ($p > 1$; $t = 0.227$; Cohen's $D = 0.051$), also reaching stage 26. The only two treatments that did not result in complete mortality were that of the control and $3.2 \mu\text{l}$ Roundup® treatment. This allowed most of the tadpoles to reach metamorphosis (Gosner stage 46) during the last three weeks. Although both treatments resulted in metamorphosis, the number of metamorphosed individuals differed (Fig. 3.10).

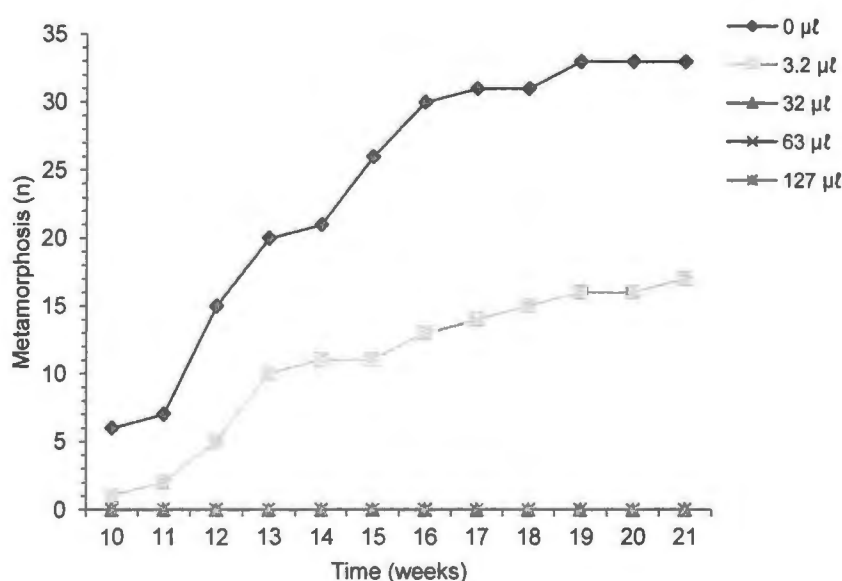


Figure 3.10: Number of individuals that reached metamorphosis (n) when exposed to different Roundup® treatments over a period of 21 weeks

Figure 3.10 presents the number of individuals that reached metamorphosis when exposed to the different Roundup® treatments over a period of 21 weeks. No metamorphosis took place before week 10 and for this reason the graph only starts at week ten. More individuals reached metamorphosis when exposed to the control (33 individuals) than those exposed to the $3.2 \mu\text{l}$ treatment (17 individuals). The tadpoles exposed to the three highest Roundup® concentrations died (Fig. 3.11) within the first three weeks. Throughout the last eleven weeks, a greater number of individuals reached metamorphosis in the control than in the $3.2 \mu\text{l}$ Roundup® treatment. However, it has been noted earlier that tadpole growth and development can be inhibited when housed under laboratory conditions (Rot-Nikcevic & Wassersug 2004).

It has been suggested that amphibians may have a low sensitivity to glyphosate (Perez *et al.* 2011), although they are very sensitive to most toxins, hence the rationale for focusing on amphibians in this study. The data in Figure 3.10 is in agreement with that of Howe *et al.* (2004), who noted that Roundup® can lead to reduced growth and development in tadpoles.

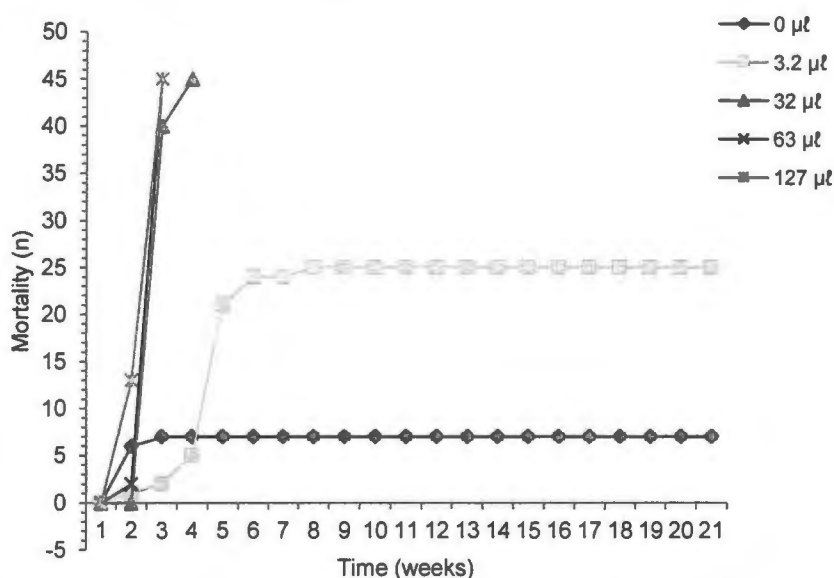


Figure 3.11: Numbers mortality (n) of tadpoles when exposed to different Roundup® treatments over a period of 21 weeks

Mortality is an important endpoint which should be noted on a daily basis (OECD 2008). Figure 3.11 presents the number of mortalities during the course of 21 weeks. The number of mortalities increased during the first five weeks, after which no further deaths were recorded. A possible reason for this could be explained by natural selection or acclimation of tadpoles as discussed in experiment 1. The highest doses Roundup® treatments (32 µl, 63 µl 127 µl) had the highest mortality early in this study due to the toxicity of Roundup®. Conversely tadpoles exposed to the control had the lowest mortality with a total number of seven deaths. More than half of the tadpoles exposed to the 3.2 µl had died, with a total of 25 deaths. This is congruent with that of the previous data (Fig. 3.10).

3.4. Experiment 4 - Bt protein concentration in water over time

This experiment was performed to obtain information on the release and degradation rate of the Cry1Ab proteins present in Bt (Fig. 3.12 & 3.13) and non-Bt (Fig. 3.14) maize leaves when submerged in purified and borehole water over a period of sixteen days. There is currently no data on the release and degradation rate of Cry1Ab proteins, and therefore this study was necessary. The concentrations (ng/ml) of the water samples containing the various maize leaf extracts were determined at three different temperatures (10°C, 21°C, 30°C) by means of ELISA tests. The reason for considering three different temperatures is to account for both a night, room and day temperature. No statistical analysis was done for this experiment and the results are graphically presented followed by a description thereof.

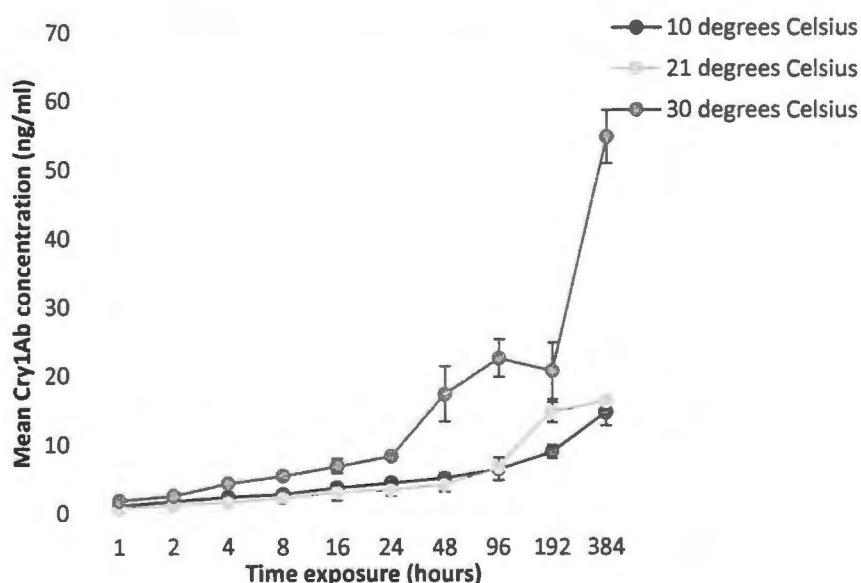


Figure 3.12: Mean Cry1Ab concentration as determined in purified water and Bt maize leaf infusion at temperatures of 10, 21 and 30°C over a period of sixteen days. Bars indicate standard error

Figure 3.12 presents the Cry1Ab concentrations in purified water over a period of sixteen days at three different temperatures. The concentration of the Cry1Ab protein at 10°C was the lowest, with its concentration increasing during the experiment eventually reaching its highest concentration of 14.76 ng/ml at the end of the 16 days.

According to Husby (2000), an increase in temperature leads to an increase in nutrient release in plants due to membrane denaturation. If the time of exposure was to be lengthened, the concentration would possibly continue to gradually increase.

The concentration of Cry1Ab in the infusion maintained at 21°C was less constant, slowly increasing during the first eight days and then appearing to stabilize at 192 hours (14.76 ng/ml). If the time exposure was to be lengthened, the concentration of the Cry proteins could either remain at a constant level or slowly start to decrease. However to confirm this, a second study should be done at a longer time interval.

The concentration Cry proteins at 30°C increased dramatically during the first four days followed by a sudden decline during days four and eight and a large increase between days eight and sixteen. The treatment in this temperature had the highest Cry concentration throughout the experiment and can be explained by Husby (2000), who noted an increase in nutrient release at increasing temperatures. In order to identify when the protein will start to degenerate, this study should be repeated at a longer time interval. Based on Husby's data, and that an increase in temperature leads to an increase in nutrient release in plants, we expected to see an increasing release of Cry1Ab protein as temperatures escalated. This was indeed noted, although nutrient release was not measured, but rather that of Cry1Ab proteins.

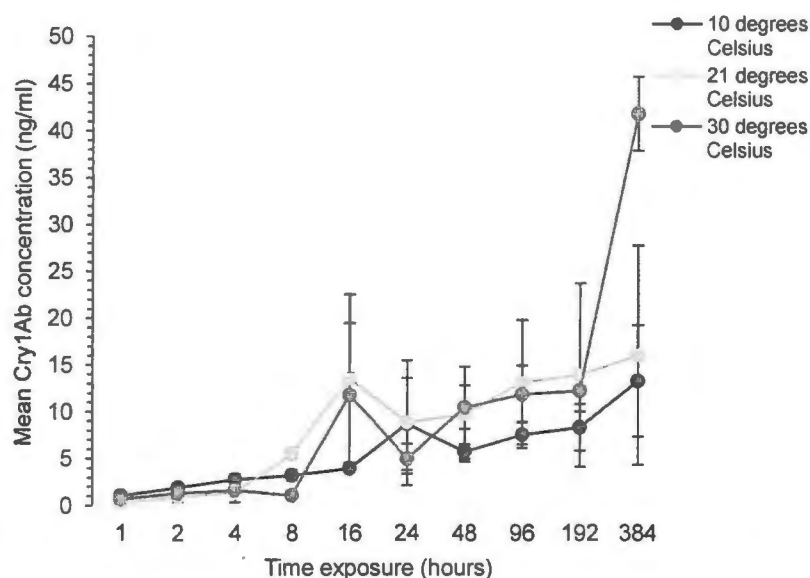


Figure 3.13: Mean Cry1Ab concentration as determined in borehole water and Bt maize leaf infusion exposed to 10, 21 and 30°C over a period of sixteen days. Bars indicate standard error

Figure 3.13 presents the concentration of Cry1Ab proteins present in borehole water released from submerged Bt maize leaves over a period of sixteen days. The Cry1Ab concentration at 10°C was lower than that observed at the other temperatures (21°C and 30°C). The concentration at 21°C was similar to that of the 21°C concentration in purified water described in Figure 3.12. A sudden spike was seen in all three temperatures at day one, which was not present in the purified water data (Fig. 3.12).

The Cry1Ab concentration at 21°C had a similar pattern as that of the 21°C in purified water after the spike in concentration (Fig. 3.12). The Cry1Ab concentration at this temperature was not constant and increased during the first day (13.60 ng/ml) then decreased to 8.87ng/ml, where after it started to stabilize at 192 hours (13.96 ng/ml). If the time exposure was to be lengthened, the concentration of the Cry1Ab proteins could either remain at a constant level or slowly start to decrease. However to confirm this, a second study should be done at a longer time interval.

The Cry1Ab concentration exposed to a temperature of 30°C showed what appears to be a constant increase after 196 hours of exposure with the last reading being 41.87 ng/ml.

The treatment at this temperature had the highest Cry1Ab concentration throughout the experiment and is congruent with the previous graph (Fig. 3.12) showing that an increase in temperature could lead to an increase in plant membrane denaturation. In order to identify when the protein will start to degenerate, this study should be repeated at longer time intervals as well as more sampling.

When the two Bt infusions (Fig. 3.12 & 3.13) were visually compared, all three temperatures showed the same pattern. Both concentrations at 10°C had the lowest amount of Cry1Ab proteins varying between 13 and 15 ng/mL. The infusions exposed to 30°C had the highest Cry1Ab concentrations varying between 41 and 55 ng/mL and those exposed to room temperature between 13 and 17 ng/mL.

A possible reason for the purified water having a higher Cry1Ab concentration is that it was assumed that the purified water has been regularly cleaned and checked, however no test was done to confirm this. For this reason different bacteria could be present which could lead to a higher bacterial interaction. Borehole water should only contain minerals and therefore should not present a possible interaction by contaminating bacteria. This however, is only speculation and a study on the contents of the borehole and purified water should be examined to establish the reliability of this statement.

The non-Bt infusion was tested in order to make sure that the Bt isolate has no Cry1Ab proteins present. The non-Bt leaves were also submerged in both purified (Fig. 3.14 a) and borehole water (Fig. 3.14 b) as well as exposed to the same temperatures (10, 21, and 30°C) as the Bt infusions.

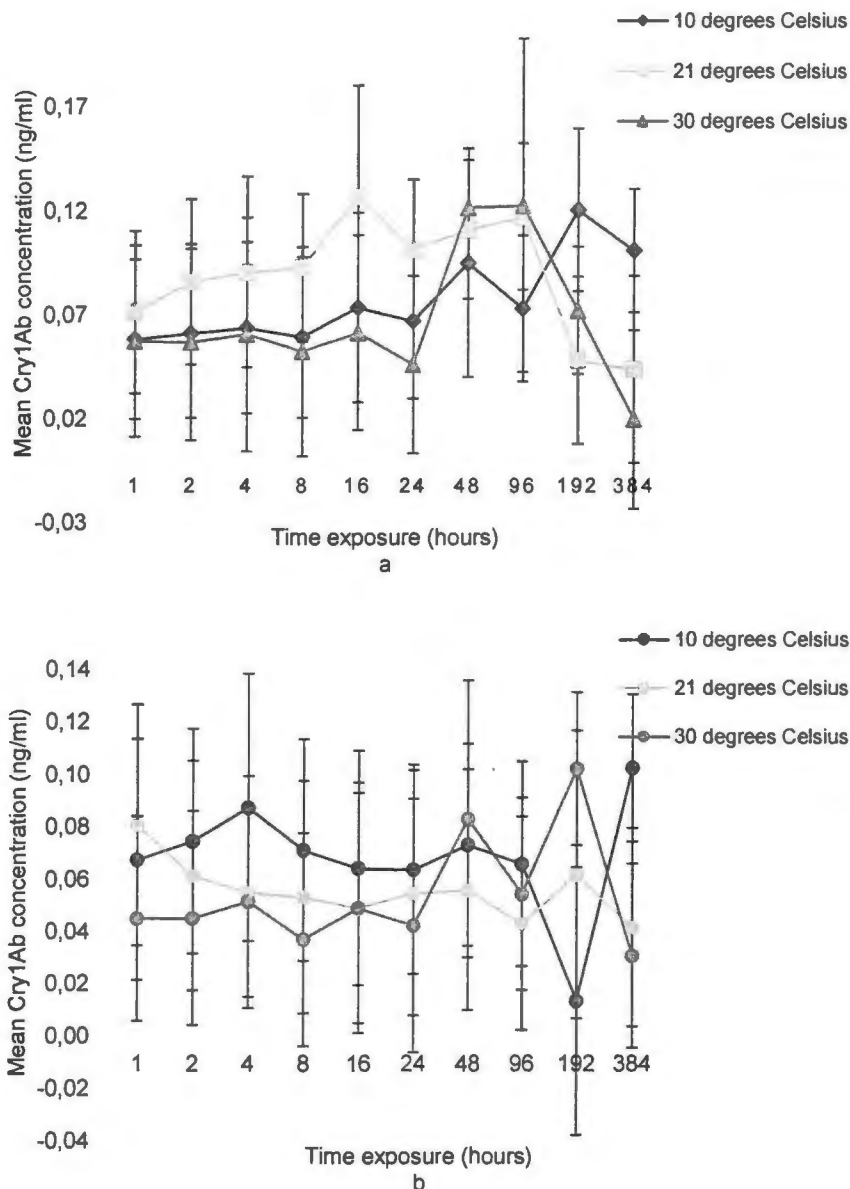


Figure 3.14: Mean Cry1Ab concentration in purified (a) and borehole (b) water when non-Bt maize leaf infusions are exposed to 10, 21 and 30°C over a period of sixteen days. Bars indicate standard error

Figure 3.14 presents the readings of the concentration Cry1Ab proteins recorded during the ELISA process with both purified (a) and borehole water (b). The non-Bt maize leaves were submerged for sixteen days at three different temperatures (10, 21 and 30°C). This test was repeated on the isoline to ensure that no Cry1Ab proteins were present in the infusions. Figure 3.14 indicated that the non-Bt contained less than 0.2 ng/ml Cry1Ab proteins in the two types of water samples. These values ranged between 0.013 ng/ml and 0.127 ng/ml, with the highest concentration 0.127 ng/ml in purified water and 0.102 ng/ml in borehole water. The

lowest concentration found in both Bt treatments was 0.65 ng/ml and 0.32 ng/ml in the purified and borehole water respectively. It is important to stress that these acquired positive values are ascribed to background noise during the ELISA process and is not Cry1Ab proteins. All isolines do not contain any DNA sections from the *Bacillus thuringiensis* organism.

Feng *et al.* (2011) found that soil temperature has an effect on the degradation of Cry1Ab proteins and that high temperatures cause a faster degradation rate of Cry1Ab proteins. This agrees with our results, concluding that water temperature also has a significant effect on the degradation of the Cry protein.

3.5. Experiment 5 - Effect of Bt on *Amietophrynus gutturalis* tadpoles (mesocosm study)

During this experiment the length (Fig. 3.15) and stage (Fig. 3.16) of tadpoles were determined. The pH (Table 3.5) of the Bt and non-Bt infusions were also measured during the incubation period of the ELISA. Both infusions (Bt and its isoline) were prepared twice because a single preparation would not be enough to prepare the next mixture of tap water and infusion. The pH of these infusions was determined although, due to the small data set, no statistical analysis was performed on these values. At the end of this experiment, the following aspects were considered, namely the tadpoles, the temperature and the Cry protein levels in each pond.

Table 3.4: pH values of the Bt and non-Bt infusions

Maize hybrid	Rep* 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6	Average
Bt							
DKC 7815B	6.70	6.72	6.71	6.82	6.82	6.83	6.77
Non-Bt							
CRN 3505	6.25	6.24	6.25	6.71	6.71	6.71	6.48

*Rep = Replicate

pH values determined for the Bt and non-Bt infusions are presented in Table 3.4. Replicates 1 to 3 were determined the day before the experiment commenced while replicates 4 to 6 were determined two weeks into the experiment. When determining the pH of the first two infusions it was important to consider that these bottles had been frozen for a week at -81°C , while the second two infusions were frozen at -81°C for one day. The length of time that the samples spend in a frozen state could in itself decrease the pH value. This is possible due to the CO_2 build-up in the enclosed bottles. When thawing the frozen water more CO_2 is mixed with the infusions and the higher the CO_2 , the lower the pH (Lenntech 2009). The same hybrids (DKC 7815B and CRN 3505) were used for the preparation of the infusions. Thus, the type of maize hybrid played no role in determining the different pH values.

Feng *et al.* (2011) undertook a study to determine whether temperature would affect the degradation rate of Cry1Ab. These authors found that pH values were neutral (pH 7.0) at a temperature of 25°C . In this study the pH values ranged between 6.25 and 6.83 when determined at a room temperature of 21°C . Feng *et al.* (2011) concluded that soil temperature had a significant effect on the degradation of Cry1Ab protein, although pH had no obvious effect. Although pH was considered not to affect degradation rate, the question could be asked whether the Cry1Ab protein may affect the pH.

Tadpoles

Data analyses were performed using *IBM SPSS* at a confidence interval of 95%. When the normality of this data was determined, no skewness was found with respect to length and developmental stage at the end of the four week treatment period (skewness = 0.549, 0.543 respectively), although a Z-value exceeding 3.29 was obtained for the developmental stage indicating kurtosis ($Z = -0.501, 5.743$ respectively). Two treatments were compared to at a time, and for this reason a t-test was performed. A repeated measures ANOVA could not be done because it was impossible to keep track of a single individual. In order to correct the *Family Wise Error*, the Bonferroni correction was done by multiplying the p-value with three.

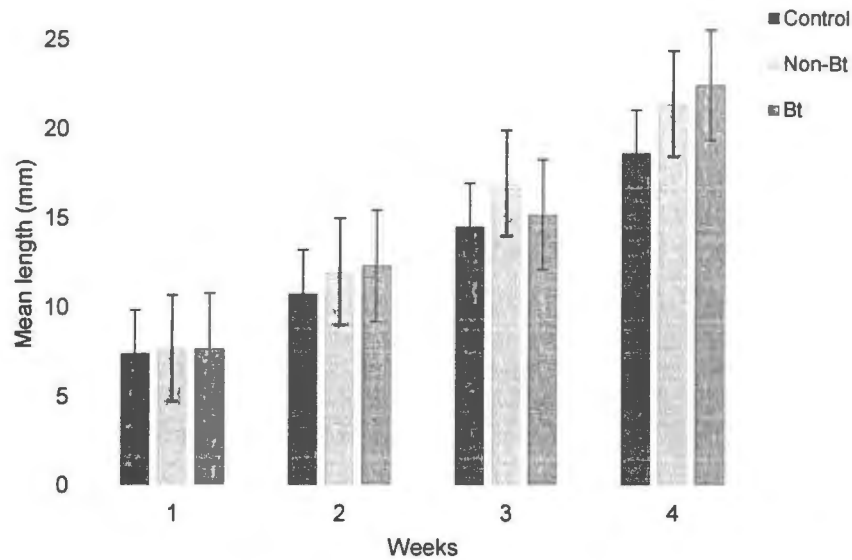


Figure 3.15: Mean length of tadpoles exposed to the different treatments over a period of four weeks.

Bars represent standard error

Figure 3.15 presents the length of the tadpoles as measured during the four-week exposure period and for the three different treatments. By the end of the fourth week, tadpoles in the control treatment were significantly shorter in length compared to those exposed to Bt treatment ($p < 0.001$; $t = -8.924$; Cohen's $D = 1.614$). This result differs from that of the laboratory experiment in which borehole water was used (experiment 2). In this experiment tadpoles were housed in tap water, not borehole water. Before assuming that differences between treatments were caused by the fact that the two studies were performed under different conditions (i.e, laboratory and outside on the roof), it should be kept in mind that different water types and husbandry was also used. When comparing the tadpole length between the control and non-Bt treatment, it was seen that tadpoles in the non-Bt infusion was also significantly larger than those in the control ($p < 0.001$; $t = -7.348$; Cohen's $D = 1.23$). Tadpoles subjected to the Bt treatments were significantly longer than those in the non-Bt treatment ($p = 0.024$; $t = -2.697$; Cohen's $D = 0.448$), but were also significantly larger than tadpoles in the additional control group ($p < 0.001$; $t = -8.924$). During this experiment, it was observed that the Bt and non-Bt mesocosms had more algal growth than that of the control mesocosms.

More algae implies more food for the tadpoles. Indeed, algae are a natural food source for tadpoles and are better suited to promoting tadpole growth (Anon 2014). Tadpoles subjected to the control treatment only received 200 ml liquidized lucerne water once a week whereas the other tadpoles received their weekly amount of food together with a constant supply of algae (present in the water). This explains the faster growth rate of tadpoles in the test treatments (Bt and non-Bt). At the end of this experiment, the length of the tadpoles in each treatment greatly differed from the data presented in experiment 2. Here, tadpoles exposed to the Bt infusion were the longest (22.44 mm), followed by those in the non-Bt infusion (21.89 mm) and lastly those in the control group (18.65 mm).

This data also confirm our suspicions that water clarity may play a role in determining the growth and development of tadpoles. In the second experiment (laboratory experiment), water clarity was poor and tadpoles were hardly visible in the tanks, as a result of infusions not being diluted as in experiment 5. The water in the roof ponds were almost clear, considering that only 155 ml of the infusion was added to these 120 l ponds. Furthermore, two different amphibian species were used (*Xenopus laevis* and *Amietophrynus gutturalis*) in experiment 2 and 5 respectively. It is recommended that a study on tadpole growth is done in different levels of water clarity using different species. A reason for this is because *X. laevis* is a very tolerant amphibian species, and can be found in extremely poor conditions such as polluted water.

When comparing the developmental stages (Fig. 3.16), similar results were found to that of the growth. Tadpoles in the test treatments had more advanced stages of development than those in the control. Considering the statistical analysis, no skewness was found (0.543), although kurtosis was present ($Z = -4.187$) after the four week period. As with the length of the tadpoles, a t-test was subsequently performed on this data. The p-value is again multiplied by three to correct the *Family Wise Error*.

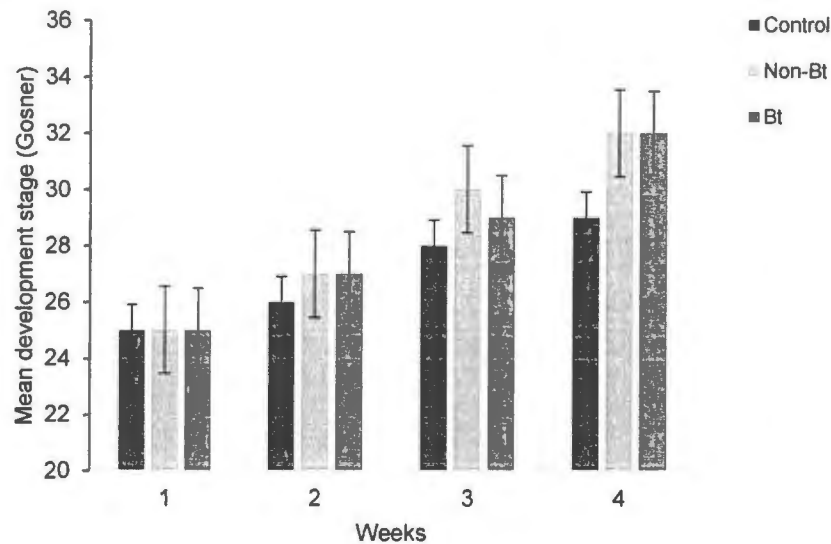


Figure 3.16: Mean developmental stages of tadpoles exposed to the different treatments over a period of four weeks. Bars represent standard error

Figure 3.16 presents the development of tadpoles exposed to the different treatments over four weeks. These results agrees with previous data which showed that tadpoles developed faster in the Bt and non-Bt treatments than in the control treatment. Tadpoles exposed to the controls were significantly shorter (Gosner stage 29) than those exposed to the infusions (Gosner stage 32) (non-Bt: $p < 0.001$; $t = 7.194$; Cohen's $D = 1.311$; Bt: $p < 0.001$; $t = 6.625$; Cohen's $D = 1.068$) at the end of four weeks. The availability of food (algae present in water) could again explain these results.

When focussing on the difference in stage development between the two infusion, no significance was found ($p > 1$; $t = 0.740$; Cohen's $D = 0.119$). This is similar to the results of experiment 2 where the two infusion isolines had similar effects on the growth and development of tadpoles. The reason for this could be that the same hybrids have similar effects on tadpole growth and development. However, a further study is required to confirm this.

Unfortunately the study did not continue until the tadpoles reached metamorphosis due to lack of time. Consequently, the number of metamorphs could not be compared to the mean stage

of tadpole development. This study therefore needs to be repeated in order to allow the experiment to continue through to when tadpoles reach metamorphosis (Gosner stage 46).

Cry protein levels

Before accusing Bt of being toxic to the tadpoles, an ELISA was done after an extraction of Cry proteins were completed. To make sure that the extraction of the protein was done correctly, an extract efficiency test was also done to see how much of the protein was lost during the extraction.

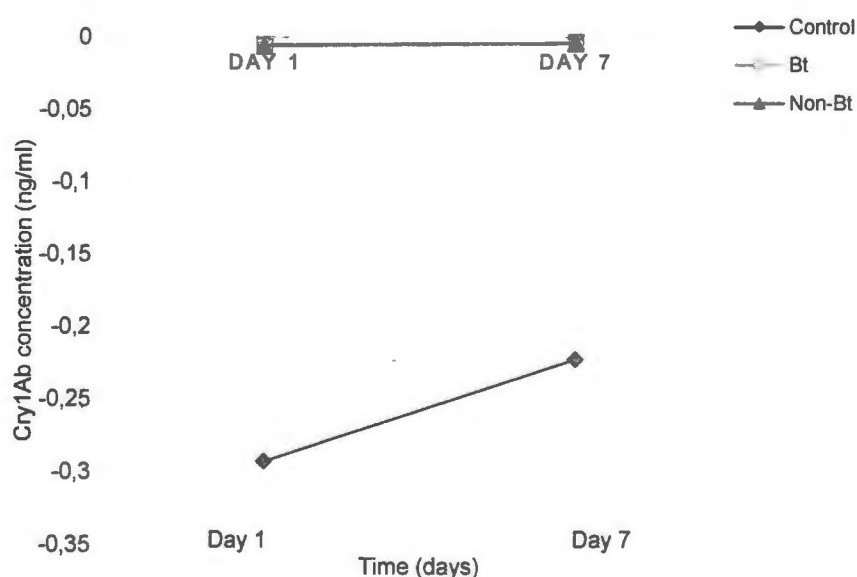


Figure 3.17: Mean Cry1Ab concentration at the beginning and end of the weekly exposure

Figure 3.17 presents the mean Cry1Ab concentration found in the different treatments one and seven days after maize leaves were put into water to prepare infusions. During this experiment, samples were taken twice a week, once after the new infusions were added to the 120 l water (day one) and once before the water was removed from the pond (day seven). These samples were analysed by first extracting the Cry1Ab proteins present in the sample followed by ELISA. The analysis showed that the Cry1Ab concentration present in the Bt and non-Bt infusions were the same, with the Bt infusion having -0.00497 and -0.00353 ng/ml and the non-Bt infusion -0.00577 and -0.0039 ng/ml on day one and seven, respectively. Tap water had the lowest concentrations of Cry1Ab. The negative values indicate the concentrations

below the detection limit. This means that the Microplate Reader could not pick up an accurate concentration of the Cry1Ab proteins. These findings suggest that the extraction procedure has not been sufficiently optimised and further development is required. In order to confirm this, we conducted an extract efficiency test that demonstrated an average recovery of the Cry proteins of 22.5% during the course of the extraction and sample preparation. The initial concentration of 0.014 ng/ml Cry1Ab decreased to -0.00315 ng/ml after the analysis was done. Further protocols that produce a higher recovery rate of proteins should be investigated.

Temperature

Earlier work has found that soil temperatures affect the degradation rate of Cry protein (Feng *et al.* 2011), so it is not unlikely that water temperature could have a similar effect. This will then account for the low concentration of protein found in the Bt treatment.

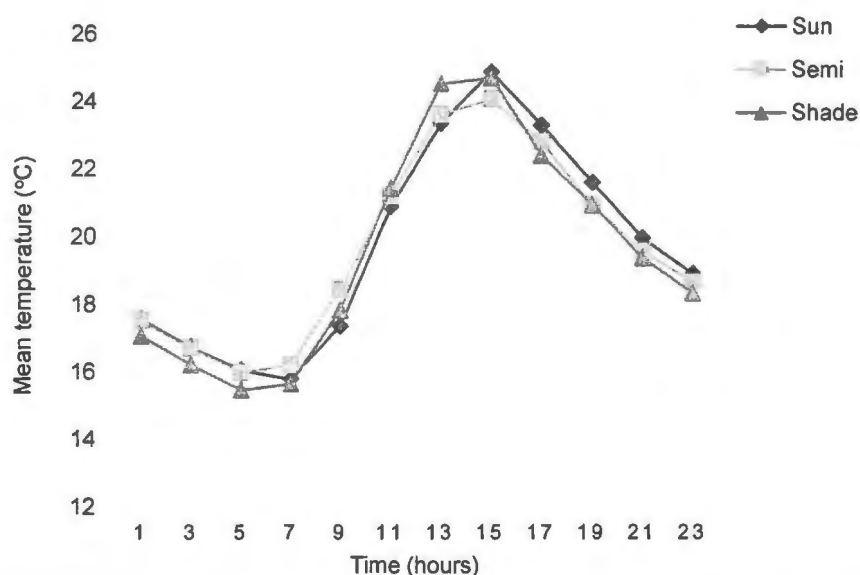


Figure 3.18: Variation in temperatures between different hours of the day while ponds were in the sun, shade and semi-shade

Figure 3.18 presents the water temperature during the course of the experiment. The temperature readings were measured every hour for fifteen days and the mean temperature measured over this period then calculated.

The results of the mean temperatures of the pond water were so similar that it was concluded that the times that the ponds were shaded or not, played no obvious part in the temperature change.

CHAPTER 4

CONCLUSIONS AND RECOMMENDATIONS

4.1. Recommendations for future studies

During the course of these studies, obstacles were encountered which influenced the gathering and accuracy of data. Further studies on the effect of GM maize and Roundup® on tadpoles are needed, but to do this certain changes and modifications are recommended. These are listed below with a detailed description and reason for the change.

Experiment 1 - Which husbandry practices lead to higher levels of survival?

- For this experiment, a study on the contents of maize leaves is recommended to identify possible reasons for the delayed growth reaction in *X. laevis* when exposed to non-Bt maize leaves, and the possible effect that a characteristics of the particular hybrid itself may have on results.
- Our data suggest that the decreased clarity of the water has more pronounced effect on growth and development than food type. To understand the possible role of chlorophylls leaching into the water, a study could be done in which *X. laevis* tadpoles are exposed to different levels of water clarity. The lack of clear vision could be a stressor, limiting tadpole growth and development.
- Another recommendation would be to house the tadpoles in groups and those individually in the same size container, eliminating housing space as a possible effect.

Experiment 2 - Effects of Cry1Ab in Bt maize leaves on *Xenopus laevis* tadpole development

- In this experiment, only two control treatments were used, namely borehole water and a non-Bt control. It was observed that the Bt and non-Bt resulted in similar growth and development patterns of tadpoles and the question can be asked that if the non-Bt controls Bt isolate was used as a third control, a similar pattern will be seen.

However, to confirm this, another study should be done in which several hybrids are used. This will also show if and how the different hybrids influence tadpole development.

- At the end of this experiment, it was found that the length of the tadpoles may not always be the best indicator of the effect of stressors. This is because the growth stages described by the Nieuwkoop and Faber Table do not always correlate with the length of tadpoles. For example, under field conditions *X. laevis* tadpoles takes approximately two months to reach metamorphosis, while in this study, they took four months. However Rot-Nikcevic and Wassersug (2004) note that it is common for the metamorphosis of *Xenopus* tadpoles to be restrained while under laboratory conditions. For this reason, the length of the tadpole should be taken into account together with its weight, which might give a more accurate picture on how stressors may affect tadpole development (OECD 2008). By doing this, these stages can be standardized for our specific laboratory conditions and experiments so that suitable adjustments can be made when predicting an expected time of metamorphosis in the study and when comparing our values to that described in the above table.

Unfortunately even this method has its disadvantages. One concern is that of the time factor. All the tadpoles should be measured and weighed on the same day and time of day in order to maintain consistency with respect to age. A second concern is that in order to weigh the tadpoles, they need to be lightly dabbed dry so that the extra drops of water do not introduce a weight discrepancy. This will cause the tadpole stress, unless they are temporarily euthanized.

- The only stressors in this experiment were that of the Cry1Ab protein and the translocation of the tadpoles from their tanks to the microscope for measurement. However, natural habitats present with a diverse range of stressors such as predators, stream flow, weather changes and other food types. In future studies, more naturalistic stressors should be introduced by creating a mesocosms to make it more environmentally realistic.

- When the husbandry of *X. laevis* tadpoles in the current study was compared to that of a publication of the National Research Council, entitled "*The proposed husbandry for Xenopus laevis experiments*", a number of differences are worth noting. The techniques and recommendations described therein in relation to our experimental conditions are as follows:
 - (1) Optimum temperature for rapid growth is between 20-22°C, which is in agreement with that applied in this experiment (21°C).
 - (2) Once the tadpoles are a week old, they should be fed for a week and left undisturbed before being handled. In this study the tadpoles were transferred into treatment tanks a week after feeding had begun.
 - (3) Tadpole density should not be more than six to eight per litre of water. In this study, tadpole density was six per litre.
 - (4) Food must be finely ground where after the water must be allowed to clear for five hours. In this experiment the *Xenopus* and lucerne pellets were finely ground and allowed to stand for 5 minutes before being liquidized and immediately added to the water.
 - (5) Tadpoles should be fed fresh food every day, whereas tadpoles in the current study were fed every second day.
 - (6) Water and containers must be cleaned every three days with warm water and no soap, whereas in this study the tanks were cleaned once a week without soap.
- Lastly, it is recommended that the concentration of Cry1Ab proteins in the infusion are determined before adding the tadpoles.

Experiment 3 - Effects of glyphosate on *Amietophrynus gutturalis* tadpole development

- During this experiment, it was difficult to establish the relevance and impact of the data without a second non-glyphosate containing herbicide for comparison. For future studies it is recommended that tadpoles in addition be exposed to one such herbicide, such as Acetochlor®.

- Since Roundup® contains many co-ingredients and excipients, further studies should expose tadpoles to herbicides with and without surfactant (POEA), or expose them to pure glyphosate and not Roundup®.

Experiment 4 - Bt protein concentration in water over time

- At the end of this experiment, the degradation rate of Cry1Ab could not be determined accurately due to incorrect exposure timing. Zwahlen *et al.* (2003) found traces of Cry1Ab even after 200 days, although by then it had decreased to approximately 0.3% of the original Cry1Ab concentration. For this reason, it is recommended that continued exposure for at least 40 days be carried out. However, decomposition of leaves over this extended period will lead to problems such as interference by microbial growth.
- In order to keep experiments as environmentally relevant as possible it is important to have knowledge of the pathways of maize leaves in the environment after the harvest, most of which is worked into the top layer of soil. Soil has different types of microbial bacteria that feed on the buried leaves and assists with the decomposition of the leaves, creating compost for the soil. Other leaves enter water bodies where it too is broken down by microbial bacteria. Both of these processes take place either beneath the soil or in water and not above the soil where the higher ambient temperature may have a significant role. For this reason soil and water temperatures should be determined over a period of thirty days at a depth of between 5 and 10 cm (over spring/autumn, winter and summer) and the average temperature for each season determined. These averages should then be used as the exposure temperatures instead of the 10°C, 21°C and 30°C used in this experiment.

Experiment 5 - Effect of Bt on *Amietophrynus gutturalis* tadpoles (mesocosm study)

- It is recommended that this study be undertaken using tap and borehole water in order to determine the effects of these different media on tadpole survival and development. Such a study may assist in explaining the results obtained in this experiment.

- Another study could focus on comparing naturally occurring food (algae) and processed food (lucerne pellets) and whether there is a large difference between the two food types with respect to tadpole growth and development.
- It would be informative to see whether the different hybrids differently affect pH values of the water. Importantly, the samples should not be frozen and the pH should be taken immediately after the samples have been collected.
- *Xenopus laevis* and *Amietophrynus gutturalis* was used in experiment 2 and 5, respectively. A recommendation is to undertake a study on tadpole growth at different levels of water clarity using either one or both species.
- This experiment did not continue until the tadpoles reached metamorphosis due to a lack of time. The number of individuals reaching metamorphosis could not be compared to the mean stage of tadpole development. It is recommended that the experiment be allowed to continue until the tadpoles reach metamorphosis in order to allow the development of tadpoles to be compared to the actual number of metamorphs present and whether the two go hand in hand.
- It is recommended that a procedure be developed that will indicate the volume water that should be used for the extraction of Cry protein that will allow the Microplate Reader to accurately quantify the protein concentration.
- Satiety test:

Another study that can be added to this experiment is that of fitness tests. In order to determine the effect of an environmental toxin or xenobiotic on normal amphibian development, it would be beneficial to identify a behavioural trait that could be studied to provide an accurate measurement of fitness. Hatching fitness can be studied by exposing a certain number of eggs to environmentally relevant Cry1Ab concentrations and to count the number of larvae that hatch compared to the number hatching in a control treatment. In the case of the tadpole and/or metamorphs, these two behaviours could for example be centred on the need to eat (goal-driven) or to avoid being eaten (predation, escape-driven).

The drive to eat can be used as the primary measurable endpoint that in turn would be dependent on the fitness to feed (or locomotion activity). Thus, the ability of the metamorph to feed and survive depends on their feeding fitness. The metamorphs at the end of the experiment should be removed from the mesocosm and placed individually into small (500 mL) transparent containers with lids. The metamorphs used should all be of the same age (Gosner stage 46) in order to limit factors such as age differences, that might play a role in the fitness test. It is important to not overwhelm the metamorph with too many crickets and should therefore be well-controlled. In order to determine feeding fitness, the amount of crickets eaten per unit of time can be counted. This type of test can be described as a satiety test, referring to a process that stops the animal from feeding once satiety is reached (Livingstone 2010).

- Video tracking:

This study has focussed on the possible adverse effects of two types of pesticides on the growth and development of tadpoles. Apart from evidence regarding the growth and development of tadpoles being dependant on the treatment exposed to, behavioural and pathological (e.g. changes in thyroid hormones) evidence is lacking. Addressing this should be viewed as an important extension to this work. Neurodevelopmental toxicity, if severe enough, may be expressed in the animal as deficits in locomotor activity. Recent advances in digital video tracking and analysis has provided an opportunity to monitor not only the behaviour of larger animals, but also fish and amphibians. Denoël *et al.* (2013) recently studied the effect of insecticides on tadpoles using digital video tracking. In line with the long-term goals for our study, these authors determined whether endosulfan impaired locomotion and, secondly, if any observable behavioral alterations could be related to mortality. Reduced locomotor performance would have significant effects on the long-term viability of tadpoles. If we consider that impaired movement will adversely affect the ability of the tadpoles to explore and navigate their habitat, such deficits will give them less chance of gathering enough food as well as make them less efficient at escaping from predators. Indeed, if the levels of exposure to the environmental toxin are long and severe

enough, these deficits may threaten the survival of the species. The paper by Denoël *et al.* (2013) provides evidence of the usefulness of video tracking in ecotoxicology, and would be a valuable extension to the current work.

- Since a number of developmental stages of tadpoles are dependent on thyroid hormones, a valuable extension to this study would be to determine the levels of TSH and TH (T3 and T4) in the blood and in the brain. Any such changes could be correlated to deficits in growth and development observed in our study. More over this study can complement that of the fitness and locomotor activity test

Overall recommendations

- It is recommended that the maize leaves be analysed to determine Cry1Ab concentration in the leaves and not just its concentration in the surrounding water. According to Andow (2002), MON810 Bt maize expresses 0.19-0.39 mg/g Cry1Ab toxin in the grain, 10.34 mg/g in the leaves and less than 0.09 mg/g in the pollen. The entire plant has on average 4.65 mg/g Cry1Ab toxin. This analysis can be performed by an ELISA technique similar to that used in experiments 4 and 5 of this study.
- In order to ensure reliable results, it is recommended that no less than 20 tadpoles from each treatment are analysed.
- It is recommended that experiments be continued unabated until the tadpoles reach metamorphosis.

4.2. Conclusion

Each amphibian species has its own preference regarding husbandry. The *Xenopus laevis* tadpoles were healthier when maintained in groups than in individual cups, whereas the *Amietophrynus* species developed better when housed individually. In identifying species preferences, the effects of poor husbandry practices may have on results is reduced, thereby yielding more accurate data. In this study the husbandry practices played a role in the survival of tadpoles and for this reason, the first hypothesis is accepted, namely that incorrect husbandry practices will play a major role in the survival of tadpoles.

After studying the effect of Cry1Ab proteins on tadpole growth and development under laboratory conditions, it was found that both Bt and non-Bt infusion treatments adversely affected *Xenopus* tadpole development and growth. The severity of the effect seems to depend on the specific hybrid used in the study. For this experiment, the hypothesis has not been rejected or accepted, namely that Cry1Ab proteins will have little or no effect on the growth and development of *X. laevis* tadpoles. However, a new question was developed: whether the severity of the effect of maize leaves per se is dependent on the hybrid used. It is essential in future studies that the concentration of Cry proteins is determined before adding tadpoles to water of the different treatments, since this information will facilitate more accurate interpretation of results and observations.

On the other hand, exposing *Amietophrynus* tadpoles to different concentrations of Roundup® had a significant effect on the tadpole survival. The three highest Roundup® concentrations tested resulted in 100% mortality after three weeks. Tadpoles exposed to the lowest concentration, however, had almost similar growth and developmental patterns as those in the control. However, only half of the tadpoles reached metamorphosis when compared to that of the control. According to prior research (Perkins *et al.* 2000; Güngördü 2013), the glyphosate alone is not toxic to plants without the surfactant (POEA) present in the Roundup® formulation. This poses the question of whether it is the POEA of glyphosate that exerts the strongest influence on the development of the tadpoles.

If the hypothesis stated that Roundup® affects tadpole development, the hypothesis could be accepted. However the hypothesis stated that glyphosate affects tadpole development and for this hypothesis the data was inconclusive. It is not known whether the surfactant (POEA), the active ingredient (glyphosate) or the combination thereof is ultimately responsible for the developmental deficits and/or mortalities noted in *Amietophrynus* tadpoles.

Regarding the degradation rate of the Cry1Ab protein, the data was inconclusive and therefore the hypothesis that Bt protein first increases then decreases as a function of time can neither be accepted nor rejected. The protein did increase and at certain time periods decreased, only to continue to increase again. The exposure period for this type of study needs further optimisation.

During the mesocosm experiment the opposite was found when comparing results from the laboratory and mesocosm studies (experiment 2 and 5). The tadpoles thrived in the maize leaf-infused treatments with the opposite taking place in the controls, leading to the rejection of the hypothesis, that tadpoles exposed to Bt will have a slower growth rate than those exposed to the other treatments.

In conclusion, pesticides can be lethal at extremely high concentrations. However in these studies Bt was not shown to be a contributor to mortality or any other adverse effect found in the previous experiments. Most of the protein concentrations used in these experiments were environmentally relevant, meaning that it can be assumed that Bt proteins at environmentally relevant levels will not adversely affect the tadpoles species evaluated in this study.

CHAPTER 5

GUIDELINES FOR TESTING THE EFFECTS OF MAIZE PLANT PRODUCED CRY PROTEINS ON *AMPHIBIOPHYRUS* SPECIES

(Format based on the OECD guideline for the testing of chemicals: The Amphibian Metamorphosis Assay-AMA)

Introduction

The need to identify the effects the Cry protein produced by Bt maize plants might have on invertebrates have increased due to the increased production of genetically modified Bt crops. There are concerns that Bt crops might have adverse effects of the environment. Declining numbers of Amphibians have in the past been of great concern, and it is believed that the two go hand in hand and that the toxicity of pesticides may play a role in the amphibian decline. The aim of this document was therefore to develop a guideline for the use in studies on effects of Bt crops on amphibians. This guideline is the outcome of the experience and knowledge gained during the study.

Principle of the test

- The amphibian metamorphosis assay (AMA) is an assay which is used to identify toxins that might disrupt the function of the hypothalamic-pituitary thyroid axis. However, in this guideline, it is used to discern between visual effects of possible toxins such as Cry proteins on growth and physical development of tadpoles. For more in depth information on the AMA, the OECD Guideline for the testing of chemicals: The Amphibian Metamorphosis Assay (OECD 2008) should be consulted.
- The overall experimental design entails exposing *Amietophrynus gutturalis* tadpoles to different treatments, maintaining tadpoles in mesocosms until metamorphosis and regular collection of data on growth and development.

Methodology

Test species

Amietophrynus gutturalis is used due to its wide distribution and occurrence in the maize production regions of SA. Reproduction is harder to induce in *Amietophrynus* than in *Xenopus laevis* because it only breeds during spring. Out of their breeding season, even human chorionic gonadotropin (hCG) won't induce spawning. The larvae collected during breeding season can be used in stage-specific tests reared in large numbers. The optimum condition would be to find eggs that are derived from in house adults. If this cannot be done due to insufficient knowledge or incorrect breeding season, eggs or embryos can alternatively be the shipped to the test performing laboratory, but this is not favoured.

Equipment and supplies

The following equipment and supplies are needed in order to conduct this study:

- 9 mesocosms,
- Breeding tanks,
- Crickets (all the same size),
- Maize leaves of genetically modified Bt maize leaves and its isoline,
- Ground lucerne pellets,
- Ovitrelle (hCG)- if induced spawning is required,
- Small transparent containers with lids,
- Vaseline,
- Combination microscope (AZ100),
- Ethyl-m-amino benzoate methanesulfonate salt (CAT number: 103106).

Chemical testability

According to the AMA draft Test Guideline (OECD 2008), the AMA is based on an aqueous exposure protocol where the product to be tested is administered into the chambers by means of a "flow through system". In this study, the chemical used is a Cry protein that is produced

by a gene derived from *Bacillus thuringiensis* and is toxic to certain insect species. This gene is genetically inserted into the DNA of maize, providing the plant with insecticidal traits. Leaves of GM maize plants are used to test the possible effect of Cry protein produced in these leaves, effect on exposed tadpoles. The leaves are submerged in borehole water and added at smaller amounts weekly. When testing other chemicals, consult the *OECD Guidance Document on Aquatic Testing of Difficult Substances and Mixtures* (OECD 2000) to accommodate the type of chemicals used.

Exposure system

The preferred system for use with aqua organisms is that of the flow through system which can be explained by using the salmonid aquaculture sector as an example. This system makes use of clean cool water moved by means of a pump and or gravity from a water stream that passes through the aquaculture and then is discharged into the water stream again (Fig. 5.1) (State Government of Victoria 2010).

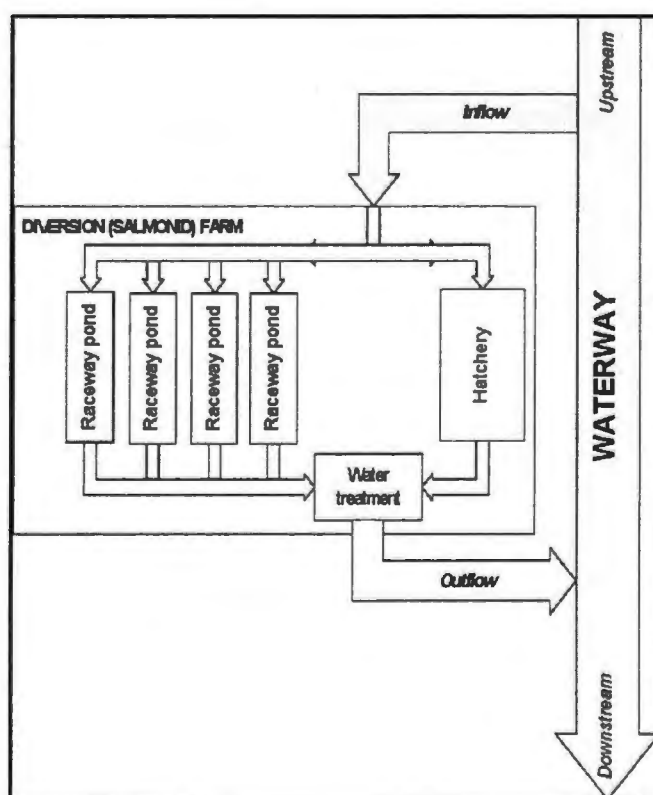


Figure 5.1: Flow-through aquaculture system (State Government of Victoria 2010)

At times the properties of the substance (physical or chemical) will not be suitable for a flow through system. There are alternatives such as static and static renewal systems (Fig. 5.2). A static system is usually used to breed endemic species for food, stocking and on-growing. This system receives its water from streams, irrigation channels, rainwater run-of, dams and bores and is added to replace any water lost through evaporation and or seepage during the managing of water quality (State Government of Victoria 2010). It is a test system where “the test solutions and test organisms are placed in test chambers and kept there for the duration of the test without renewal of the test solution” (Environmental Protection Agency 1996).

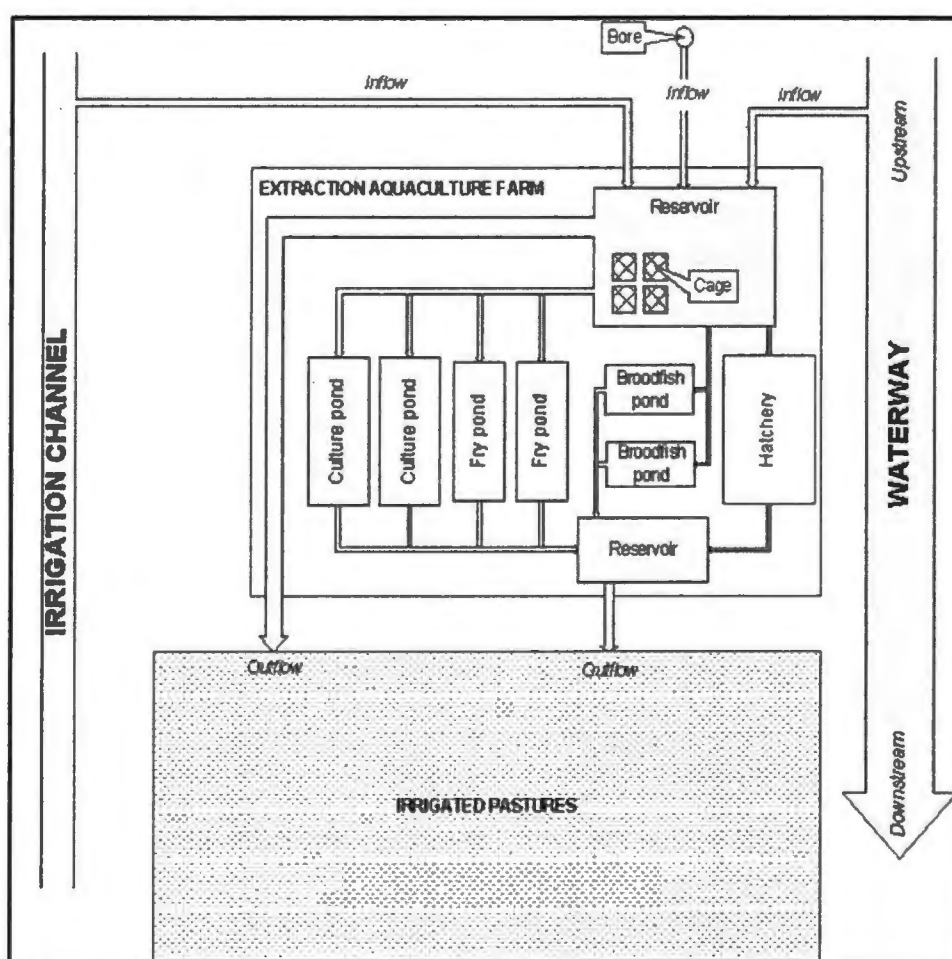


Figure 5.2: Static aquaculture system (State Government of Victoria 2010)

The last system type is known as the static renewal system, which is similar to the static system, except for the test solution which is replaced at certain intervals. In most laboratory studies, this system test is used.

When setting up one of the systems, it is important to make use of glass, stainless steel and/or Teflon® or any other type of plastic which will have no effect on the study (OECD 2008). When making use of tanks, stainless steel or glass (as in our case). The depth of the tanks should be minimum 10 to 15 cm and maximum of 25 cm (maximum volume of 25 l). The system should be able to support four or more replicates, including a control (OECD 2008). Positioning of these treatments should be random to reduce positional or block effects.

The following conditions should be accounted for:

- light intensity,
- temperature,
- pH,
- dissolved oxygen,
- and evaporation.

In this guideline the static renewal system is used. The stability of the experiments should be documented as well as renewal periods which should not exceed 72 hours with a minimum of 24 hours. This is however for laboratory experiments. When trying to imitate or simulate a natural environment, it is best to try and keep all aspects of the study untouched. It is recommended to do ELISA tests every four days along with the water quality parameters such as pH, total dissolved solids and dissolved oxygen. If renewal test is done, then these parameters should be determined at the end of the renewal period.

Water quality

According to the AMA draft test guidelines (OECD 2008), a fluorescent light should be used to give a photoperiod of twelve hours light and twelve hours dark. It is recommended that water temperature of all treatments be maintained at $21^{\circ} \pm 1^{\circ}\text{C}$ with a pH between 6.5 and 8.5. The dissolved oxygen concentration should be maintained below 3.5 mg/l (> 40% of air saturation) (OECD 2008). If it is not possible to maintain these conditions, it should be measured and recorded weekly. In doing this, possible effects can be explained by the recorded data.

When selecting water in which *Amietophrynus* tadpoles will be housed, it is important to use water that will not influence the normal growth and development of tadpoles. Water quality differs between sources and areas and because of this, it is important to analyse water quality prior to experiments. When doing water quality analyses, it is important to make sure that the water is copper, chloramines and chlorine free. These contaminants are toxic to all stages of frog species. Anions such as chlorate, perchlorate and fluoride should also be taken into account seeing that they are “the substrates of the iodine transported of the thyroid gland” (OECD 2008). Elevated levels of these anions may influence the outcome of experiments.

Iodide concentration in test water

For the thyroid gland to produce thyroid hormones an adequate amount of iodide is required. Under natural conditions these compounds are supplied both from food and water sources for the larvae. There are currently no guidelines for the correct iodide concentrations, but the presence of iodide could have an effect on the responsiveness of the thyroid system. Iodide is also known to modulate thyroid gland basal activity that should receive attention when interpreting histopathology results. For this reason, the iodide concentration in water should be recorded. According to available information on validation studies, the appropriate iodide concentrations ranged between 0.5 and 10 µg/l (OECD 2008).

Animal husbandry

Adult care and breeding

To acquire eggs for use in experiments, a male and female adult should be found in amplexus and placed in a large tank (approximately 25 l) with water to a depth of 3 cm. This water should not contain any contaminants such as those mentioned above. A hard substrate (e.g. a brick) should be placed inside the tank as a step for the adults as they are not aquatic amphibians. The eggs that are fertilized in this environment will be free from diseases and contaminants that may be found in uncontrolled environments. Tadpoles for use in studies should be disturbed as little as possible, and should not be sourced from the wild.

If induced spawning is required, Ovitrele™, a hormone that is injected into both males and females, would be easiest to use (Van Wyk & Du Preez 1984). While there are guidelines for inducing spawning in *Xenopus* species (AMA draft test guidelines; OECD 2008), no guidelines exist for *Amietophrynus* species.

Egg and larval care and selection thereof

Adults should be removed from the breeding tanks and the water aerated directly after spawning. After a few hours of aeration, clean water maintained at room temperature should be added. The eggs should be allowed to develop and it is recommended to monitor the eggs for three days followed by removal of dead and unfertilized eggs. During the first few days of development, no independent feeding takes place and for this reason, it was not necessary to feed the larvae (Gosner 1960). Once the larvae reach N/F stage 26, the tadpoles were fed for the first time by administering 500 µl of finely ground lucerne pellets into the water.

After a week the larvae should be placed into a new tank with clean aerated water (Van Wyk & Du Preez 1984). The tanks must be cleaned at weekly intervals and filled with clean water at room temperature. To prevent contamination of tanks with chemicals, no soaps should be used when cleaning the tanks. It is recommended to scrub the tank with hot water. Make sure cloth or sponge is clean before using it

Larval food preparation

Five lucerne pellets should be ground into a fine powder using a mortar and pestle, then added to 50 ml borehole water and liquidized. Five-hundred µl should be added for each tadpole. Table 5.1 provides information on nutritional values of commercially available lucerne pellets.

Table 5.1: Nutrient contents quantified in lucerne pellets

Nutrient	Mass dry weight (g/kg)	Percentage nutrients (%)
Protein	160	32
Fibre	170	34
Moisture	120	24
Fat	25	5
Calcium	18	3.6
Phosphorus	7	1.4

When selecting larvae for any experiments, it is important to do a random selection. Larvae which are attached to the sides of the tank are always the healthiest and should be selected for experiments.

If the experiment requires other food sources, then it is recommended to feed the tadpoles liquidized lucerne pellets during the pre-exposed period. The feeding course should be carefully planned to provide the tadpoles with the appropriate nutrients (regardless of the food type). This means that when tadpoles reach premetamorphosis (Gosner stage 26-40), prometamorphosis (Gosner stage 41-42) or metamorphic climax (Gosner stage 43-46), the amount of food given should increase to compensate for the amount of energy each developmental stage requires (Buchholz *et al.* 2006; Miyata & Ose 2012).

The duration of the experiment is determined by the initial aims as well as development rate of the test species. It is important to plan the experiment before starting. Knowledge of the species used as well as the toxin is vital. When the experiment takes place depends on when eggs can be found. The length of the experiment depends on the time it takes the tadpole to reach metamorphosis. Information on environmental concentrations of the toxin studied should be identified as well as possible affects it might have on tadpoles.

Without careful planning, the study might be regarded as untrustworthy or with flaws.

Preparation of water medium

Before conducting any study with any potential toxin, it is important to determine at which concentrations of the compound the study should be conducted at. Bt maize leave infusions should be prepared by submerging the dried Bt maize leaves in borehole water. The mass of the dried maize leaves and the volume water depends on the concentration Cry proteins the experiment requires. Is it recommended to determine a worst case scenario concentration by submerging 24 g Bt maize leaves in 1 ℓ borehole water for four days and analysed using an enzyme-linked immunosorbent assay (ELISA). Determine the volume of the infusion required to create a solution with the needed concentration. This solution should also be tested to ensure the correct concentration has been created before the study commences. This provides data on the concentration of Cry proteins present in infusions and provides data on environmental relevance. It will also facilitate extrapolation of findings to field conditions or indicate worst case scenarios.

ELISA uses antibodies and colour changes to identify and quantify the amount of a particular substance in a solution. Briefly, the induced colour change is proportional to the quantity of substance being analysed, viz. the darker the colour, the higher the concentration of toxin and vice versa. The assay uses a solid phase enzyme immunoassay (EIA) to detect a specific substance in an aqueous sample (BioRad Laboratories 2014). EnviroLogix QualiPlate assay Kit for Cry1Ab/Cry1Ac was used to detect the Cry1Ab protein in the maize leaves which.

This kit contains the following:

- 96 well plate,
- sachet buffer salts,
- pure Cry1Ab protein,
- enzyme conjugate,
- substrate,
- stop solution.

A stock solution should be made in order to prepare the standard series for the determining of the calibration curve. this can be done by pipetting 5.3 μl of the Cry1Ab reference (95 $\mu\text{l}/\text{mg}$ Cry1Ab) into a 50 mL centrifuge tube followed by wash buffer until the solution measured 50 g (± 0.02 g). Once the stock solution is complete, the standard series can be prepared. The data provided in Table 5.2 was used as a guideline for the preparation of the standard series.

Table 5.2: Guidelines for the preparation of the standard series for Cry1Ab (calibration curve)

Standard series	Volume 10 ng/mL diluted stock (μl)	Volume wash buffer (μl)
1	-	1000
2	3	997
3	6	994
4	12	988
5	24	976
6	50	950
7	100	900
8	150	850
9	200	800
10	250	750
11	300	700
12	350	650

The samples as well as the standard series and positive Cry protein control should mixed well before 500 μl of each is pipetted into marked cuvettes.

The 96 well plate is prepared by adding 50 μl Cry1Ab enzyme conjugate to each well followed by the 50 μl standard series, positive control and the samples in their corresponding well. The

wells are mixed in circular motions and incubated on an orbital shaker at 200 rounds per minute (rpm) for two hours with a polystyrene covering to reduce the intake of light.

After the incubation period, the wells are flooded with 200 μl wash buffer, mixed in a circular motion and patted out. This was repeated four times to ensure rinsing out of any other substances in the sample (except the Cry1Ab). After the fourth wash, the plates are patted dry. Hundred μl of the substrate is then added into each well. The wells are mixed again and incubated on an orbital shaker for half an hour.

The wells turn blue in colour to indicate the presence of the Cry protein (Fig. 5.3). Lastly, 100 μl of the stop solution is added to each well, causing the contents to turn yellow, and then mixed in circular motions (Fig. 5.3).

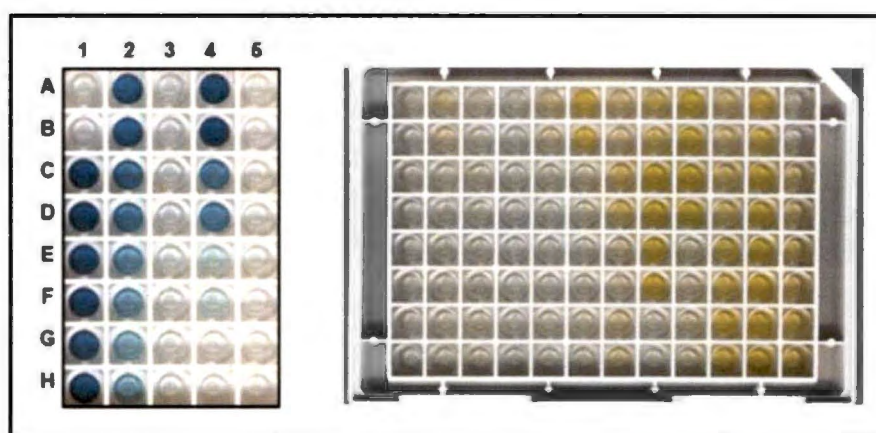


Figure 5.3: Substrate added and wells with Cry protein present turns blue; stop solution added changing the colour to yellow (Connolly 2010)

The plates are then placed into the Microplate Reader (wavelength set at 450 nm) and the optical density (OD) of the Cry1Ab protein analysed using the program *MicroWin*. The entire protocol should be repeated three times in order to improve the accuracy of the results.

Chemical delivery

Chemicals or in this case pesticides can be administered in two different ways. Either by means of the test animals' diet, by feeding Bt maize leaves to tadpoles, or through the aquatic medium itself by means of infusion made by submerging leaves in water.

Selection of test concentrations

Establishing the high test concentration

To determine which concentrations of Cry proteins should be used in this study, data previously reported on the concentrations of Cry1Ab proteins found in natural dam or river systems were used. It was previously reported that Cry1Ab concentration in water varies depending on the flow rate, depth, temperature and distance from where genetically modified Bt crops are planted.

It was reported that Cry1Ab proteins were present in streams that contain Bt maize detritus and in some streams a concentration of 14 ± 5 ng/l was found (Tank *et al.* 2010).

Test concentration range

It is recommended that a study of this nature should have at least three different concentrations of the test compound as well as a control treatment and that the experiment be replicated at least four times. The control should exist of non-treated water. It is recommended that environmentally relevant concentrations be used, including a concentration somewhere in between the maximum and minimum.

Experiment procedure

The evaluation of *Amietophrynus* development is conducted over a period of six weeks since this is the approximate period taken from egg hatch to metamorphosis into adults. Measurements are taken at weekly intervals, with first evaluation being done at day zero. The process of evaluation is described below.

Test initiation and conduct

Day 0 of exposure

When using *Amietophrynus* species, it is recommended that exposure to the different treatments commence once the most tadpoles reached stage 26 (Gosner) in the tank where hatching took place (Fig. 5.4). Since all tadpoles should be at same developmental stage at commencement of experiment healthy tadpoles at the sides of the tank should be selected

and placed in a Petri dish with sufficient water. A microscope can be used to identify the stage of each tadpole. Since tadpoles are extremely sensitive to handling, especially at the beginning of the larval stage, it is important to be gentle in order to minimize handling stress and avoid injury (OECD 2008).

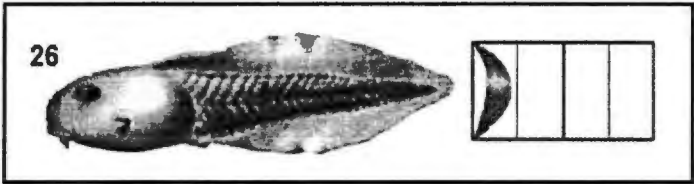


Figure 5.4: Tadpole at stage 26 on the Gosner staging table

Evaluation of the development stage of a tadpole should be done as accurately as possibly, preferably though the use of a microscope. According to Gösner (Gosner 1960), the primary developmental landmark for stage 26 is the hind limb development. It is recommended to consult the entire Gosner stage guide when studying the development of a tadpole, but stage determination can be done using the prominent morphological landmarks (Table 5.3).

Table 5.3: Prominent morphological staging landmarks based on Gosner instructions (Gosner 1960)

Prominent morphological landmarks	Developmental stage								
	26 – 38	39	40	41	42	43	44	45	46
Hind limb	X	X	X						
Forelimb			X	X	X				
Craniofacial structure					X	X	X		
Olfactory nerve morphology						X	X	X	
Tail length							X	X	X

Tadpole size can be used in addition to the developmental stage to determine possible chemical affect. To do this the total body length (TBL) should be measured at day 0 of exposure and not the snout vent length (SVL), tail length or hind limb length (HLL). The reason for this is that the SVL, tail length and HLL is used to determine developmental affects. It is however, the developmental stage which is the main parameter when determining when each tadpole is ready to be exposed to different treatments. Any malformed or injured tadpoles should be removed from the study and excluded from the assay. The tadpoles are divided into two different tanks. All the tadpoles that meet the description above of Gosner stage 26 are placed in on tank and the tadpoles that do not, are placed in the other.

Once this process is complete, the tadpoles are evenly, but randomly divided into the number of treatments. No less than twenty 26 Gosner staged tadpoles per replicate should be measured and weighed weekly.

Once exposure starts, any mortalities and odd deformities noticed during the regular inspections are noted down. If irregularities are observed during the first week of exposure, these can be ascribed to factors other than the treatments and these individuals should be replaced with newly selected healthy individuals. After the first week of exposure, no tadpoles should be replaced. Dead ones should be removed to prevent other deaths caused by toxins released from dead tadpoles.

Day 7 measurements

After a week of exposure, five randomly selected tadpoles should be taken from each replicate and carefully measured and developmental stage determined. When a tadpole's weight and mouth regions are studied, it is recommended that they be temporarily euthanized. The main reason for this is that, in order to inspect its mouth region and weight, the tadpole should be gently placed on its back to avoid sudden movement and the mouth region inspected. Thereafter it should be lightly patted dry to rid it of excess water and then weighed. This approach prevents unnecessary stress to the tadpole. A Combination microscope is

recommended for collecting data on tadpole development since it facilitates freezing of images, making the measuring of the total body length, snout-vent length, tail length and limb length easier.

Tadpoles should be replaced in the correct treatment and replicate after data collection.

Day14 and 21 measurements

The duration of the experiment is determined by the initial aims as well as development rate of the test species. Once an experiment is completed all remaining tadpoles' should be euthanized, rinsed with clean water and dried in order to facilitate final data collection and determination of tadpole mass which cannot be done with live individuals. If needed, all larvae can be stored for future research by placing them in a fixative, either as whole body samples or trimmed head samples which contain the lower jaw for histological studies.

For histological studies, five tadpoles should be collected from each replicate per treatment. These individuals should have the same stage due to follicular cell height being stage dependant (OECD 2008). This means that the tadpoles must be staged prior to preservation.

When selecting individuals for the final data collection process, five larvae of the appropriate stage should be randomly selected. If there are fewer than five appropriate stage larvae, five tadpoles from a lower or upper stage should be selected, depending on the available numbers.

Determination of biological endpoints

Prior to conducting experiments and exposure of tadpoles to pesticides, the biological endpoints should be decided upon. Table 5.4 indicates which endpoints should be measured at which stages of the experimental period.

Table 5.4: Biological endpoints and the intervals to record these during the experiment

Biological endpoints	Daily	Day 7	Day 14	Day 21	Test termination
Mortalities	X				
Gosner stage		X	X	X	X
Hind limb length		X	X	X	X
Snout-vent length		X	X	X	X
Total body weight		X	X	X	X
Malformations		X	X	X	X
Histology					X

Apical endpoints

The above mentioned endpoints are from the Amphibian Metamorphosis Assay (AMA) and accounts for all amphibian species. Each of the endpoints will briefly be discussed below. The difference between biological and apical endpoints is the following: Biological endpoints refers to markers that are used to help affect progression after the exposure to foreign materials, in this case, that of the Cry1Ab toxin (Pharmacelsus 2014). Apical endpoints on the other hand refer to the outcome of the exposure such as developmental abnormalities, changes in size and mortality (Ball 2007).

Developmental stage

The developmental stage of *Amietophrynus* species is determined by the staging criteria described in the Gosner scale. These development stages are used to determine whether the development of larvae is accelerated or delayed when exposed to certain stressors. An asynchronous development is reported when timing of the morphogenesis is different between treatments, but no visible malformations or abnormalities are present (OECD 2008).

Hind limb length

The growth and change of the hind limbs are major developmental landmarks when determining the developmental stage. For consistency's sake, the left hind limb of the tadpole is measured on day 7, 14 and 21, depending on the experimental period. When measuring the hind limb, it should originate from the body wall following the midline though any angular deviations (OECD 2008).

Body length and weight

The determination of the body length and weight is standard protocol when assessing possible effects of substances on the growth rate of tadpoles. It is also useful for the detection of toxicity of compounds. These measurements are taken once a week or on day 7, 14 and 21 and/or on the day of test termination. The measurement of the body length includes the total body length, snout-vent length and hind limb length (Fig. 5.5).

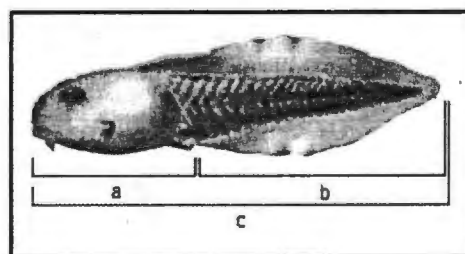


Figure 5.5: Different apical endpoints that can be measured: (a) snout to vent length, (b) tail length and (c) total body length

Thyroid gland histology

It is important to remember that developmental delay or acceleration cannot be a diagnostic indicator of change in thyroid activity on its own. Diagnostic criteria should also include:

- thyroid gland hypertrophy or atrophy,
- follicular cell hypertrophy,
- follicular cell hyperplasia,
- follicular lumen area,
- colloid quality and follicular cell shape or height.

The severity of the mentioned criteria should be reported grading it to 4. When looking into the thyroid gland histology, it is recommended to consult the *Amphibian Metamorphosis Assay: Part 1 & 2* (Braunbeck *et al.* 2007a, b) for information on collecting and processing histological samples.

Mortality and other additional observations

This endpoint should be observed daily to record numbers of dead tadpoles. The number of the tadpoles that died should be recorded as well as the date, concentration of the treatment in which it was housed and replicate number. If the mortality rate exceeds 10%, it could indicate inadequate husbandry conditions or toxic effects of the pesticide. If any other observations such as abnormal behaviour, malformations and lesions are noticed, they too should be recorded including the date, concentration, and tank number. To distinguish between normal and abnormal behaviour see the following (OECD 2008):

Normal behaviour

- tadpoles are suspended in water,
- tail elevated above the head,
- regular tail fin beating,
- surfacing at fixed intervals,
- operculating,
- responsive to stimuli.

Abnormal behaviour

- floating on water surface,
- laying at bottom of tank,
- irregular swimming,
- lack of surfacing activity,
- non-responsive to stimuli.

If any malformations are seen, they should be noted and compared to tadpoles in the control treatment. If the occurrence of the malformation is more in the exposure tanks than the control, then these can be regarded as possible evidence of toxicity, but statistical tests should be done before such conclusion can be made. A few malformations and lesions may include (OECD 2008):

- morphological abnormalities (i.e. scoliosis),
- hemorrhagic lesions,
- bacterial infections,
- or fungus infections.

Data and reporting

Data collection

According to the *AMA Draft Test Guideline*, study data should include the following:

- properties of the test substance (physical, chemical),
- stability of the test substance,
- information of biodegradability of substance,
- method of preparing dilutions,
- frequency of preparing dilutions,
- actual and nominal concentrations of test substance,
- stock and test solutions may require test chemical measurements,
- as well as solvent used and reason therefore.

It is also important to document all test conditions which include the following (OECD 2008):

- ambient temperature,
- test temperature,
- photoperiod,
- flow rates,
- feeding regime,

- water levels,
- pH,
- dissolved oxygen,
- alkalinity,
- hardness,
- conductivity,
- and total iodine.

When collecting data for the results, it is important to keep the following observations in mind (OECD 2008):

- Daily observations:
 - mortality,
 - food consumption,
 - abnormal swimming behaviour,
 - lethargy (lack of energy),
 - malformations,
 - and lesions.
- Collected data at predetermined intervals:
 - developmental stage,
 - hind limb length,
 - snout-vent length,
 - weight.

Histological data should also be presented and includes the severity of the toxins effect from one to four as well as descriptive observations. Any other observations and descriptions which do not fit in any of the previously described categories are added to ad hoc observations (OECD 2008).

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