

Effects of Bt crop residues on the development, growth, and reproduction of the freshwater snail, *Bulinus tropicus*

K Minnaar
20570899

Dissertation submitted in fulfillment of the requirements for the degree *Magister Scientiae* in Environmental Sciences at the Potchefstroom Campus of the North-West University

Supervisor: Prof H Bouwman

September 2014

Contents

.....	1
Contents	ii
Acknowledgments	v
List of abbreviations	vi
Abstract.....	ix
Chapter 1: Introduction.....	10
Chapter 2: Literature review	13
2.1 Genetically modified crops.....	13
2.1.1 <i>Bacillus thuringiensis</i>	15
2.1.2 The pathway of endotoxins	16
2.1.3 Bt crops in South Africa.....	18
2.1.4 GM crops in the environment	19
2.1.5 Non-target effects.....	20
2.2 Molluscs as bio indicators.....	22
2.2.1 Why use molluscs as bio indicators.....	22
2.2.2 <i>Bulinus</i> genus	23
2.2.2.1 The distribution of <i>B. tropicus</i>	23
2.2.2.2 Feeding habits of <i>Bulinus</i>	24
2.2.2.3 Eggs, egg packets, and the development of embryo's	27
2.2.2.4 Reproduction by hermaphrodites	27
2.3 Endocrine Disruptive Chemicals	29
2.3.1 The endocrine system.....	29
2.3.2 What are EDCs	30
2.4 Conclusion.....	31
Chapter 3: Materials and methods	32
3.1 Test conditions	33
3.2 Exposures	34
3.2.1 Development and growth	35

3.2.2 Reproduction.....	37
3.2.3 Dissections.....	37
3.3 Concentrations of Cry1Ab.....	39
3.4 Statistics.....	40
Chapter 4: Results	41
4.1 Concentration of Bt toxins.....	41
4.2 Cotton.....	42
4.2.1 Embryo growth.....	42
4.2.2 Hatching success.....	45
4.2.3 Growth after hatching.....	47
4.2.4 Fecundity	51
4.2.5 Survival after hatching.....	53
4.2.6 Male reproductive organs.....	54
4.3 Maize.....	57
4.3.1 Embryo growth.....	57
4.3.2 Hatching success.....	60
4.3.3 Growth after hatching.....	61
4.3.4 Fecundity	65
4.3.5 Survival after hatching.....	67
4.3.6 Male reproductive organs.....	68
4.4 Supplemental fecundity metrics for both experiments.....	70
4.5 Flow diagram summaries.....	71
Chapter 5: Discussion	73
5.1 Concentration of Bt toxins.....	73
5.2 Cotton.....	74
5.2.1 Embryo growth and hatching success.....	74
5.2.2 Growth after hatching.....	76
5.2.3 Fecundity	78
5.2.4 Survival after hatching.....	79

5.2.5 Male reproductive organs.....	79
5.3 Maize.....	80
5.3.1 Embryo growth and hatching success	80
5.3.2 Growth after hatching	82
5.3.3 Fecundity	84
5.3.4 Survival	86
5.3.5 Male reproductive organs.....	87
Chapter 6: Synthesis, conclusions, and recommendations	88
6.1 Nutritional differences.....	88
6.1.1 Energy allocation.....	89
6.2 Developmental instability	89
6.3 Final remarks.....	90
6.4 Recommendations.....	91
Appendix A	92
References	93

Acknowledgments

The following people and institutions contributed greatly to this project, ensuring its success, and I would like to thank each one for the role they have played:

- Prof. Henk Bouwman for all the late nights and early mornings, the weekends, the unknowing words of encouragement, and your seemingly never-ending patients with me.
- Prof. Johnnie van den Berg for guiding me through the world of GM crops, and always having maize leaves available no matter the season.
- Emeritus Prof. Kenny de Kock and Ignatius Viljoen for teaching me all they know about *Bulinus tropicus*.
- The BSA (Biosafety South Africa) - who funded this project.
- My friends and family – without your support I would have never finished this project.
- Finally, I would like to thank God for blessing me with intelligence, strength, and persistence to learn more of His wonderful creation each day.

List of abbreviations

Abbreviation	Meaning
A	
ADAM	artificial <i>Daphnia</i> medium
ANOVA	analysis of variance
B	
Bt	insecticidal crop trait derived from <i>Bacillus thuringiensis</i>
C	
Ca ⁺²	calcium cation
CaCl ₂	calcium chloride
CaCl ₂ ·2H ₂ O	calcium chloride dehydrate
Cry	crystal
D	
DI	developmental instability
DNA	deoxyribonucleic acid
E	
EDC	endocrine disruptive chemical
ELH	Egg-laying hormone
ELISA	enzyme-linked immunosorbent assay
F	
FA	fluctuating asymmetry
G	
GAS	general adaptation syndrome
GM	genetically modified
GMO	genetically modified organism
H	
ha	hectare
HDPE	high density polyethylene

I

ISO International Organization of Standards

K

KCl potassium chloride

L

LOD limit of detection

N

N nitrogen

NaHCO₃ sodium bicarbonate

NH₃ ammonia

M

MgSO₄·7H₂O magnesium sulphate heptahydrate

MIP's molluscan insulin-like peptides

mRNA messenger RNA

O

OL opening length of the shell

OW opening width of the shell

P

PCA principle component analysis

PCB polychlorinated biphenyl

PS penis length

PPS preputium length

PSPLR penis sheath-preputium length ratio

R

R²

RNA ribonucleic acid

S

SD standard deviation

SEM	Scanning Electron Microscope
SL	shell length
<i>spp.</i>	several species belonging to the same genus or family
T	
Ti	tumour-inducing
U	
USA	United States of America
USEPA	United States Environmental Protection Agency

Abstract

Genetically modified (GM) crops were introduced in South Africa in 1989 and commercially available by 1998. Legislation to control the use of GM crops was only implemented in 1999, with the genetically modified organisms (GMO) act (15 of 1999). In 2012 2.9 million ha of GM crops were planted in South Africa alone. GM Crops, such as Bt maize, are promoted as safer for the environment since no chemical pesticides are needed. However, recently GM crops have been making headlines as more and more studies find adverse effects of these crops on non-target organisms. The effects on aquatic environments have not yet been fully determined, even though traces of Bt residue have been found in water systems surrounding agricultural lands. The aim of this study was to establish the effects of the Bt toxin on fecundity, development and growth of *Bulinus tropicus*, a freshwater snail.

The experiment made use of a static renewal tests to expose *B. tropicus* to 50 cm² Bt maize and cotton leaves in 900 ml of synthetic freshwater. The snails were exposed for the duration of one full life cycle (embryo to adult). Endpoints measured included the development, growth, fecundity, and deformities of the reproductive organs.

The results obtained showed retarded development and low embryo survival when the snails were exposed to cotton leaves, irrespective of the presence or absence of Bt, indicating to the possibility of trace residues of chemical pesticides may have been present on the leaves. Initial stimulated growth of hatchlings was observed for both Bt cotton and maize exposures, but after sexual maturity has been reached, 'surplus' energy was probably shared between growth and fecundity, resulting in a reduction of growth rate. Energy is gained from their diet, thus a sub-optimal diet would result in less energy available to functions such as growth and fecundity. Signs of developmental instability were found in the formation of the shell opening of the snails exposed to Bt. Fecundity decreased significantly after snails had been exposed to Bt maize / cotton leaves. No differences were found in the penis sheath-preputium length ratio, indicating that Bt had no deleterious effects on the reproductive organs.

Key words: Genetically modified crops, Growth, Fecundity, Bt maize, Bt cotton, Molluscs, Freshwater systems, Agriculture

Chapter 1: Introduction

Insect pests have devastating implications to crop yield. Even with the use of pesticides the estimated average yield lost each year is 14% (Kumar, 2003). Without any pesticides the loss of yield in the United States of America alone would be approximately 30% (Yu, 2008). The significantly increased use of chemical pesticides in the 1950s (Castillo *et al.*, 2010) tipped the scale in the battle between pests and human food security. In 2004, roughly \$33.59 billion (US) was spent on chemical pesticides (Yu, 2008), increasing 1 – 2% per annum. Unfortunately the widespread use of pesticides lead to adverse effects on the environment and human health (Stenersen, 2004; Kumar, 2003).

Chemical pesticides are not restricted to the areas they are applied to. Long-range atmospheric transported chemical residues have been reported in some of the most remote locations, including Antarctica (Iwata *et al.*, 1993). Pesticides have been found in cloud water, rain, fog, and even snow. The concentrations found are dependent on the volatility of the chemical, as well as the method and extent of application (Unsworth *et al.*, 1999). Pesticides are not only transported via the atmosphere but also via surface water and suspended sediment from catchments surrounding or near agricultural lands (Castillo *et al.*, 2010).

Unfortunately, the wide over-use of pesticides has resulted in many adverse effects in the environment. Mortalities on all trophic levels due to pesticide exposure have been reported from as early as the 1960s (Castillo *et al.*, 2010). Humanity is exposed to these toxins on a daily basis. It is present on the fruit and vegetables we eat (Curl *et al.*, 2003); the toys children play with; and the carpets in our homes (Shalat *et al.*, 2003). Chronic exposure to pesticides have effects on human health including, but is not limited to; i) the inhibition of normal hormonal pathways concerning the growth and development of a foetus up until adulthood, and sex determination; ii) an increased probability to develop cancer; and iii) neurological effects including reduced intelligence (Alavanja, 2009; Stenersen, 2004; Pastor *et al.*, 2003).

The global human population is constantly increasing, with roughly seven billion people to feed, and an estimated nine billion in 30 years (Lutz & Samir, 2010). The fast growing population has placed a high demand on food producers for larger yields, making pesticides a necessity for the foreseeable future.

The scientific and technological advances made in the agricultural sectors not only have the potential to lessen world hunger and malnutrition and increase food security (Quist *et al.*, 2013); but have the potential to drastically reduce the amount of chemical pesticides currently used. This is especially true for insecticidal Bt crops. A study conducted on small-scale farmers in China showed that the adoption of Bt crops not only brought about a reduction in the amount of pesticides sprayed, from 60.7 kg ha⁻¹ for non-Bt users to only 11.8 kg ha⁻¹ for Bt crops; and the frequency of applying pesticides has more than halved (Huang *et al.*, 2003). A study determining the impact of Bt adoption on income for both small-scale and large-scale farmers showed that the saving on pesticides was ranked the most important benefit by both groups (Ghouse *et al.*, 2003). Bennet and his colleagues (2006) reported a significant ($p < 0.001$) decrease of pesticide use over the course of a three year study, when small-scale farmers in South Africa adopted Bt cotton.

However, questions remain: do the benefits outweigh the potential risks associated with the wide-spread adoption of GM crops over the last two decades? Or is this a case of replacing one potentially dangerous agent (chemical pesticides) with another (GM crops)? The risks accompanying the rapid implementation of GM crops have not yet been fully verified. The literature currently contradicts itself, and no concrete conclusion can be drawn.

The debate reached a pinnacle last year with the European public calling for a ban on all GM crops (van Noorden, 2013), forcing seed companies such as Monsanto to abandon new product applications for approval by the European Union. The public outrage followed the appearance of a peer reviewed article by Séralini and his colleagues (2012) in September of the previous year, concluding an increased risk for cancer in rats reared on Roundup-tolerant maize. According to Arjo *et al.* (2013) the publication sparked public reaction within hours of its release, resulting in both the Russian Federation and Kazakhstan to immediately ban the importation of that specific maize variety, and Kenya placing a ban on the import of all GM food (Owino, 2012). Due to the public concern over the true safety of GM food products, Kyrgyzstan's parliament has moved to pass a bill forbidding the cultivation, production, import or sale of any GM foods within the country (Ibraimov, 2014). This debate is far from over, as more and more risk assessment studies on GM crops gets published.

This study aimed to use the freshwater pulmonate snail, *Bulinus tropicus*, as a biological indicator to determine the effects of the dissolved crystalline Bt proteins, exuded from genetically modified crops, on the development, growth, and fecundity within a controlled static-renewal experiment. This was achieved by:

- Determining the effects of Bt crop residues in water on the development and hatching of embryos, the growth of the hatched snails until sexual maturity, and their fecundity.
- Investigating potential endocrine disruption due to exposure to Bt crop residues, by measuring the male reproductive organs.

Chapter 2: Literature review

A good understanding of the literature is necessary to fully contextualise the results obtained during the course of this study.

2.1 Genetically modified crops

Genetically modified (GM) crops are engineered to obtain a trait that will enhance their survival. These crops may be resistant to herbicides, or act as their own insect control. This means that less conventional insecticides need to be used (Willey *et al.*, 2008).

The engineering of GM crops involves the insertion of a novel gene into the plant's DNA. This is achieved through the process of transgenesis. The novel gene may originate from almost any biological source (animals, bacteria or plants), adding a novel trait or ability to the crop (Rissler & Mellon, 2000; Nabors, 2004).

During the process of transgenesis, a primary gene functioning unit, the segmented DNA from a foreign organism designed to modify the receiving organism, is transported to a specific region on the DNA of the receiving organism (Nabors, 2004). This is achieved using a tumour-inducing (Ti) plasmid as a DNA vector (Willey *et al.*, 2008). Plasmids are circular extrachromosomal DNA molecules that occur naturally in prokaryote organisms. They can be used as a carrier of foreign DNA to eukaryotic cells (Garret & Grisham, 2013; Garrett & Grisham, 1997). A Ti plasmid is usually derived from the bacteria *Agrobacterium tumefaciens*. This soil bacterium is a plant pathogen that causes crown gall tumours by genetically transforming the plant cells. The tumours form as a consequence of a segment of the bacterial DNA, the T-DNA (transferred DNA), being inserted and expressed in the plant genome (Klee *et al.*, 1983). Naturally occurring Ti plasmids cannot be used for transgenesis because i) a transformed crop plant is not able to reach maturity; ii) and the plasmids, ranging between 200 and 800 kb, are too large. During transgenesis the Ti plasmid based vectors are therefore created using the bacterium (Hoekema *et al.*, 1983). A basic Ti plasmid vector requires (Traavik *et al.*, 2007):

- a) A eukaryotic promoter, that will control the timing and level of expression of the transgene (Garret & Grisham, 2013);
- b) A multicloning site, a section of DNA that restricts the insertion of genes to a specific site of complementary DNA sequencing to bind with the plasmid;
- c) Eukaryotic stop signals for both the transcription of the foreign DNA sequence and the translation of the mRNA (Schnell *et al.*, 1996);

- d) The DNA sequence encoding for the polyadenylation of the mRNA 3' end, ensuring the stability and translation of the mRNA (Colgan & Manley, 1997);
- e) And a selection of marker genes to optimize the expression of the foreign gene in the host, by deleting or changing certain introns on the DNA of the organism.

Trangensis into plants can be achieved through a number of processes. Earlier methods of transgenesis required that the characteristically robust plant cell walls first be removed from the protoplasts. Unlike animal cells, plant cells have a primary and secondary cellulose structure (wall) surrounding the plasma membrane reducing water loss (Nabors, 2004). Only after the removal of the cell wall can DNA insertion take place. The protoplasts can be maintained as individual cells in a cultured medium where new cell walls can later be regenerated, and a whole plant is formed. Later methods were developed to introduce cloned genes into only a few cells of a plant tissue, bypassing the need to isolate protoplasts, and regenerating a new plant from that segment of plant tissue. The method most commonly used is microprojectile bombardment (Traavik *et al.*, 2007; Stanford *et al.*, 1987). During this process, a spherical particle of gold, with a diameter of approximately 0.4 - 1.2 μm , is coated with foreign DNA. The DNA is first precipitated in either polyethylene glycol or calcium chloride (CaCl_2) (Klein *et al.*, 1988). These coated particles are then inserted into the plant cell through the intact wall and membranes at a speed of between 300 and 600 m/s, using a particle gun. Due to the low particle density, insertion does not damage the cell wall significantly and the cells are able to restore the damage. Once the particle is inside the cell, the DNA detaches and fuses into the plant DNA (Traavik *et al.*, 2007).

The herbicide resistance trait found in RoundupReady crops is the most popular transgenic trait on a commercial basis. Bt crops, with DNA derived from the bacterium *Bacillus thuringiensis*, is an insecticidal crop. This insecticidal trait is the second-most used trait. Bt genes most often used are Cry1Ab, Cry1Ac, Cry2Ab, and Cry9C and are currently used commercially in maize and cotton (Shelton *et al.*, 2002).

2.1.1 *Bacillus thuringiensis*

Bacillus thuringiensis is a common gram positive soil bacterium and an opportunistic pathogen (Stenersen, 2004). It has the ability to produce crystal proteins (Cry proteins) in the sporangium during the sporulation process (Thomas & Ellar, 1983), consequently killing a target insect. This ensures a nutrient rich environment for dormant spores ready to germinate (de Maagd *et al.*, 2001).

Different strains of *B. thuringiensis* have been identified from which over 300 different Cry proteins has been characterized (Stenersen, 2004; Crickmore *et al.*, 1998). The subspecies are classified according to their flagellar H-antigens (Schneph *et al.*, 1998; Höfte & Whiteley, 1989). Some of the most common variations are listed in Table 2.1.

Table 2.1: The different sub-species of *Bacillus thuringiensis* produces different forms of the crystal proteins, and each of these proteins are target specific.

Variations	δ-endotoxin	Target taxon	Source
<i>kurstaki</i>	Cry1A	Lepidoptera	(Yu, 2008)
	Cry2	Diptera	(Höfte & Whiteley, 1989)
<i>aizawai</i>	Cry1Ab	Lepidoptera	(Stenersen, 2004)
<i>san diego</i>	Cry3A	Coleoptera	(Kumar, 2003)
<i>tenebrionis</i>	Cry3A	Coleoptera	(Stenersen, 2004)
<i>israelensis</i>	Cry4	Diptera	(Höfte & Whiteley, 1989)
<i>thuringiensis</i>	Cry1B	Lepidoptera	(Höfte & Whiteley, 1989)
<i>berliner</i>	Cry1Ab	Lepidoptera	(Kumar, 2003)

Each of these toxins is specific (Knowles *et al.*, 1986) to insect larvae of one of the orders Lepidoptera, the moths and butterflies (Fiuza *et al.*, 1996); Coleoptera, the beetles (Donavan *et al.*, 1992); and Diptera, the flies and mosquitos (Knecht & Nentwig, 2010). Some of these Cry proteins have been found to also be toxic to certain nematodes (Marroquin *et al.*, 2000) and hymenopterans (Baur & Boethel, 2003).

The toxins are classified into 54 groups according to the similarities in their amino-acid sequence. The name is composed of five parts; the mnemonic Cry or Cryt, and four hierarchical classes (Stenersen, 2004). These classes are constructed using numbers, capital letters, lower case letters and again numbers; and are based on the percentile similarity in the sequencing identity of the proteins. The primary number (e.g. Cry1, Cry2, Cry3) indicates a less than 45% sequencing identity between classes, while a 78% and a 95% similarity constitutes the secondary and tertiary ranks (de Maagd *et al.*, 2001). These proteins vary greatly in size due to the presence of absence of the cysteine-rich C-terminal (Schneph *et al.*, 1998). They may either range between 130 - 140 kDa, or be only

approximately 70 kDa in length (Pigott *et al.*, 2008). The elongated C-terminal is believed not to form part of the active toxin, and is lost in the midgut of the insects when conformation of the protein takes place. It is however believed that the C-terminal plays a role in the formation of the crystals (de Maagd *et al.*, 2001).

2.1.2 The pathway of endotoxins

The Cry protein crystal, also referred to as δ -endotoxin (Schneph *et al.*, 1998), consists of three domains (Figure 2.1). Domain I is composed of an α -helix, with a hydrophobic inner helix surrounded by six amphipathic helices. It is located at the N-terminal of the protein and is responsible for inserting a hairpin and pore formation in the membrane of the gut epithelial cells. Domain II consists of three antiparallel β -sheets forming a β -prism with three loops at its apex. These loops are important for receptor recognition and binding. Two antiparallel β -sheets at the C-terminal forms a β -sandwich structure known as Domain III. Domain III, together with Domain II, is involved in recognizing the receptor and binding to it (Grochulski *et al.*, 1995; de Maagd *et al.*, 2001).

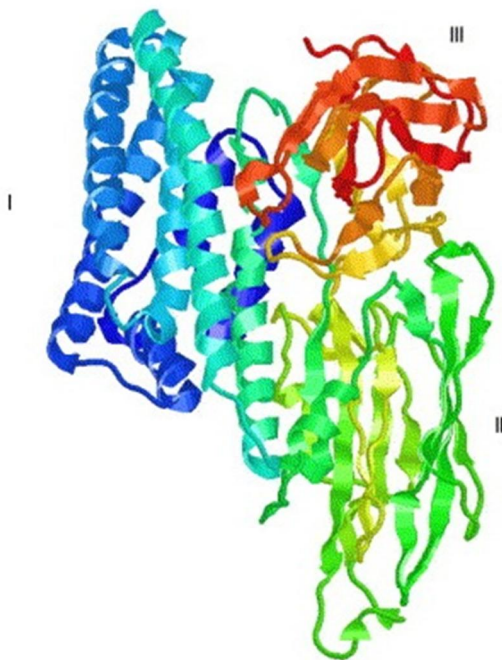


Figure 2.1: The 3-dimensional structure of the activated Cry1Aa toxin. Domain II (green) and Domain III (yellow-red) recognizes the receptors on the midgut epithelial cells' membranes and bind to it. The α -helix of Domain I (blue) is inserted into the cell membrane forming pores. Image acquired from de Maagd *et al.* (2001).

Following the ingestion of the crystals by the insect, the crystals are dissolved in the alkaline environment of the gut. The gut proteases activate a hydrolytical breakdown of both the N-

and C-terminals (Tojo & Aizawa, 1983). Domains II and III then bind to receptors in the apical microvilli of epithelial cells in the midgut. Changes to the tertiary structure in domain I forms a α -helix hairpin which triggers channel forming into the membrane of the epithelial cell, creating a nonspecific pore that allows water and ions to move through (Figure 2.2). This flux results in massive colloid-osmotic swelling and finally lysis. The intake of these δ -endotoxins by an insect causes its death within one to two days (Knowles & Ellar, 1987).

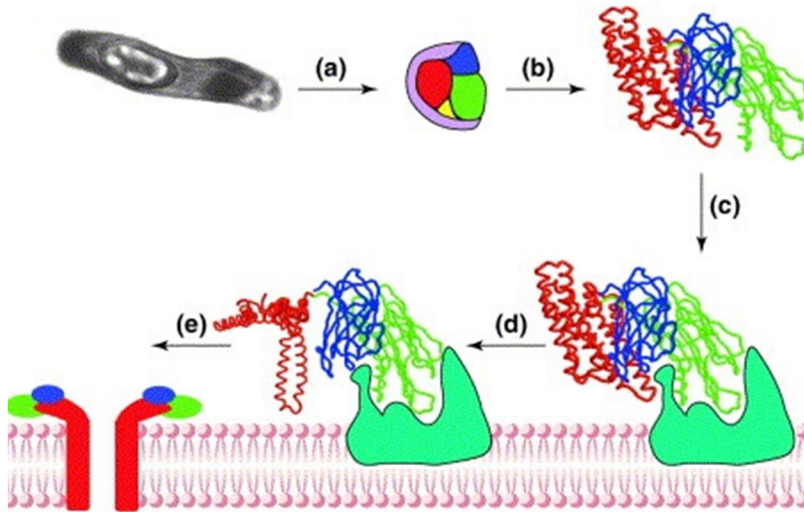


Figure 2.2: The mode of action the Cry proteins take after ingestion. (a)The crystals are dissolved in the gut. (b)The proteases in the insect gut cuts off the short N-terminal (yellow) and the longer C-terminal (purple). (c)This activates the protein (see Figure 1.1) allowing Domain II (blue) and III (green) to be able to detect and bind to the specific binding site on the gut epithelial cells. (d)Domain I (red) forms the “hairpin” to be implanted into the cell membrane. (e) The pore is formed later leading to lysis. Image taken from de Maagd *et al.* (2001).

Resistance to the Cry proteins in insects may take place in a number of ways. Oppert and his colleagues (1994) reported a decreased rate of activation of the protoxin in the gut of resistant insects, leading to a decrease in the quantity of active toxins in the insect midgut and a chance the insect could recover. They later found the absence of the trypsin-like enzyme in the gut, which is responsible for the activation of the protoxins (Oppert *et al.*, 1997). There is evidence suggesting that some insect species have evolved the ability to break down the toxins in the midgut (Foracada *et al.*, 1996). The most frequent mode of resistance reported is a change in the binding site of the midgut epithelial cells (Tang *et al.*, 1996). This modification of the binding sites leads to a lower affinity of the receptors to bind with the toxin, and therefore a reduction of binding receptors in the midgut (Sun *et al.*, 2003; Frutos *et al.*, 1999).

2.1.3 Bt crops in South Africa

South Africa was the first country on the African continent to adopt GM crops (Wolson, 2007; Gouse *et al.*, 2003), with only Egypt following (James, 2008). Some of the first field trials with Bt cotton were conducted in South Africa in 1989. The first commercially grown GM crops were insect-resistant (Bt) cotton planted during the 1997/1998 season, followed by insect-resistant (Bt) yellow maize for cattle feed the next season (1998/1999) (Wolson, 2007; Gouse *et al.*, 2003). The regulation of genetically modified organisms (GMO) Act (Act 15 of 1999) was only implemented in 1999, 10 years after the first Bt cotton was planted. The purpose of the act was to decide which GM applications should be approved. Moreover, the Act plays an active part in questioning facets of each application.

Table 2.2: GM crops were rapidly adopted in South Africa. A rapid increase of agricultural land used for GM crops each year occurred.

Year	Hectares	Source
2001	6 000	(Wolson, 2007)
2003	84 000	(James, 2003)
2004	155 000	(James, 2004)
2006	609 000	(James, 2006)
2008	1 800 000	(James, 2008)
2009	2 100 000	(James, 2009)
2012	2 900 000	(James, 2012)

South Africa was also the first country to allow the introduction of a GM food staple in 2001 when Bt white maize was commercialised. GM cotton was rapidly planted since there were no consumer-acceptance concerns (Wolson, 2007).

The adoption of GM crops over the last decade in South Africa has expanded 500-fold as shown in Table 2.2. In 2001 there was only 6 000 ha of GM crops cultivated in South Africa. In 2012, South Africa was ranked 8th in GM crop production, behind giants such as the United States of America, Canada, China, and Brazil (James, 2012). GM crops are grown across South Africa, but in large parts crop production is primarily found in the wetter Eastern regions of the country (Figure 2.3).



Figure 2.3: The major crop growing areas for maize and cotton in South Africa.

2.1.4 GM crops in the environment

Horizontal gene transfer, or pollination, raises the concern of releasing the novel genes into the environment. This could lead to uncontrollable weeds in the agricultural environment and the distribution of undesired traits in wild plant species (Quist, 2007; Rissler & Mellon, 2000). Hybridization of crop relatives could lead to ecological changes in unmanaged natural environments (Dale *et al.*, 2002). If, for instance, the gene encoding for draught resistance is transferred to a wild relative, the species would be able to inhabit drier regions. This plant species might not be suitable as a food source for many of the herbivore species in that region, probably leading to unintended changes in the ecology (Rissler & Mellon, 2000).

Bt crops were developed as part of an integrated pest management plan, aimed at preserving the natural enemies of pests and managing insect resistance (Schnepp *et al.*, 1998). As the use of Bt crops increased, researchers have been investigating the potential effects of Bt crops and their δ -endotoxins on the environment, but conflicting results were reported (Bøhn *et al.*, 2008; Rosi-Marshall *et al.*, 2007; Clark *et al.*, 2005). Saxena and Stotzky (2000) reported that δ -endotoxins from Bt plants are released into the environment by 1) transgenic plant root exudates; and 2) decomposing plant material. These Cry proteins can bind to elements in the soil, stabilizing them, and can remain active for several months (Clark *et al.*, 2005). A study done by Sims and Ream (1997) found that a mature transgenic cotton plant adds an average of 1.6 μg Cry proteins to 1 g soil. The half-life of these proteins in the soil from plant material was calculated to be 1.6 days, and from the pure protein was 8.3 days (Sims & Holden, 1969). However, the active toxin has been reported in soil samples six – seven months after harvest (Baumgarte & Tebbe, 2005; Tapp & Stotzky, 1998). Flores and colleagues (2005) found Bt maize, cotton and potato plant

material decomposed slower per biomass in soil than their non-transgenic isolines, but no difference in breakdown could be found in a stream ecosystem (Swan *et al.*, 2009; Rosi-Marshall *et al.*, 2007). After the harvest, variable amounts of crop byproducts scatter around agricultural lands, and some ends up in water systems. Rosi-Marshall *et al.* (2007) found that the average byproduct to end up in these water systems is between 0.1 g and 7.9 g/m² of ash-free dry mass. Pollen also end up in the water systems with 0.1 – 1.0 g.m⁻² deposited annually. The amount of Cry proteins in the water systems were below the limit of detection (ELISA, 1 µg/L). After concentrating the stream water samples, Tank *et al.* (2010) were able to determine an average of 0.014 µg/L Cry1Ab protein in streams within 500 m from agricultural activities. The half-life of these proteins, in spiked water samples, proved to be 4.4 days (Douville *et al.*, 2005).

2.1.5 Non-target effects

The fate of Cry proteins in soil is an important parameter influencing exposure of non-target organisms. Dale *et al.* (2002) defined non-target effects as undesirable effects of the novel genes on beneficial organisms in the environment. This is especially a risk to species closely related to the target species, or those that have a similar physiology. The first reports of deleterious effects on non-target organisms appeared in 1999. Larval survival of the non-target monarch butterfly (order Lepidoptera) decreased significantly when they ingested Bt maize pollen (Losey *et al.*, 1999). In 2001, Hellmich and his colleagues also found a significant reduction in survival, during a laboratory study, when monarch butterfly larvae ingested Bt maize pollen, or the purified Cry1Ab protein (Hellmich *et al.*, 2001). However, in the same year Sears *et al.* (2001) reported that the impact of Bt maize on monarch butterfly populations in the field is negligible. A field study comparing the differences in the canopy arthropod communities in Bt and non-Bt cotton fields, found the differences only to be significant when a sufficient number of lepidopteron larvae were present as food source (Whitehouse *et al.*, 2014). Another close relative to the lepidopterans, which is negatively impacted by the Cry proteins, is the trichopteran *Lepidostoma liba*, an important detritivore in the Midwestern United States. A significantly lower growth rate after ingesting Bt maize leaves was reported during two laboratory studies, but no mortality was reported (Chambers *et al.*, 2010; Rosi-Marshall *et al.*, 2007).

The exudates of Cry proteins in soil were tested on two common species of earthworms. A significant loss of body mass (18%), compared to the 4% mass gain in the non-Bt treatments, was found after exposing *Lumbricus terrestris* to Cry1Ab for 200 days in both the field and laboratory (Zwahlen *et al.*, 2003). Likewise, a significant reduction ($p < 0.001$) of

body mass for the earthworm *Eisenia andrei* was found after two weeks of exposure to 37.5 g Bt maize leaves mixed with 338 g of soil (van der Merwe *et al.*, 2012). However, *Eisenia fetida* showed no effect from exposure to the Cry proteins after 28 days. Though the springtail, *Folsomia candida*, showed a reduction in the number of offspring they produced (Clark & Coats, 2006). The reduction in both body mass and reproduction may be due to a possible difference in lignin content of the maize leaves. Saxena and Stotzky (2001) reported higher (33 – 97 %) lignin content in Bt crop plants than their non-Bt isolines, depending on the cultivar. The higher lignin content between Bt, and non-Bt, as well as between different cultivars, results in prolonged breakdown of leaf litter and ultimately a nutritional variation.

A field study assessing the long-term effects of Cry1Ac cotton on 22 arthropod species in Arizona (USA) showed a decrease in density of the butterfly *Drasteria divergens*, but an increase in density of the spider *Dictyna reticulata*. The same study found a significant decrease in five predator species with a multi-year analyses (1999 – 2003), but the authors concluded that this reduction was not ecologically important because the use of conventional chemical insecticides showed a greater reduction in arthropod species (Naranjo, 2005). Another two-year field study on three predator species (*Coleomegilla maculata* (Coleoptera), *Chrysoperla carnea* (Neuroptera), and *Orius insidiosus* (Heteroptera)) found no adverse effects when feeding on *Ostrinia nubilalis* (Lepidoptera) in maize fields (Pilcher *et al.*, 1997). Tian and his colleagues (2014) used Bt-resistant prey as feed for the predators *O. insidiosus* and *Geocoris punctipes*. The prey was given Bt cotton and maize to feed on before they were given to the predators as food. No adverse effects were reported for either of the predators over two consecutive generations. Meta-analyses on the potential effects of Bt maize and cotton on non-target invertebrates confirmed that Bt crops had no effect on the abundance of predator species (Mavier *et al.*, 2007), and therefore no effects on ecological functioning (Wolfenbarger *et al.*, 2008). Feeding Bt maize leaves, expressing an average of 0.203 µg of Cry1Ab per gram fresh leaf tissue, to the aphid *Sitobion avenae* (Homoptera) resulted in no adverse effects on the survival, development, or demographic parameters (Ramirez-Romero *et al.*, 2008).

In contrast to the above-mentioned studies, there have been significant deleterious effects reported on non-target organisms when exposed directly or indirectly to the Cry proteins. Increased mortality and prolonged development was reported in the predatorial green lacewing, *C. carnea*, after ingesting both *O. nubilalis* and *Spodoptera littoralis* raised on Bt maize (Hilbeck *et al.*, 1998). Using the shell diameter and body mass as indications of development during a chronic exposure of the land snail *Cantareus aspersus* to Bt maize as feed resulted in a lower growth rate, fecundity (number of eggs/snail) and fertility (number of

hatchlings/snail) at Cry1Ab protein concentrations of 10.3 – 16.8 mg/kg (Kramarz *et al.*, 2009). The effect of the Cry proteins in water systems on non-target organisms showed deleterious effects on some species. A significantly lower mass gain was reported in the detritivorous Caddisfly larvae, during a laboratory exposure to Bt maize. A low survival rate (43%) of the crustacean *Caecidotia communis* exposed to Bt maize detritus in water was also noted (Jensen *et al.*, 2010; Swan *et al.*, 2009). Bøhn and his colleagues (2008) used the crustacean *Daphnia magna* in a chronic exposure. Bt maize and its isoline were used as feed. A significant decrease in survival was reported. The non-Bt treatment was larger, and had higher fecundity. A very low percentage reached sexual maturity (36.7%) but they did mature faster when exposed to Bt.

2.2 Molluscs as bio indicators

The phylum Mollusca is the second largest phylum of invertebrates, consisting of more than 130 000 species (Oehlmann *et al.*, 2007). The phylum is divided into eight classes of which only two, Gastropoda and Bivalvia, are found in freshwater systems; the remainder are marine. The name Mollusca is derived from Latin meaning soft, referring to their most distinctive characteristic; the soft body (Dillon, 2000; Hickman *et al.*, 2006).

2.2.1 Why use molluscs as bio indicators

Molluscs have been used in an array of toxicological studies, including heavy metals (Zhang *et al.*, 2013; Conti *et al.*, 2012; Zaldibar *et al.*, 2006), endocrine disruptive chemicals (Greco *et al.*, 2011; Duft *et al.*, 2003; Schulte-Oehlmann *et al.*, 1995), bioaccumulation (Campanella *et al.*, 2005; van der Oost *et al.*, 1988), and other anthropogenic contaminants (Byrne & O'Halloran, 2001).

They inhabit a wide range of environments, from the cold polar seas to the warmer tropics. They can be found at altitudes exceeding 7000 m. Molluscs are found in streams, ponds and lakes, in sediment, on vegetation, and on mudflats. They are found in the breaking surf and in the open ocean, from the surface to the abyssal depths (Hickman *et al.*, 2006; Appleton, 2002). This array of habitats makes molluscs a useful tool to generate data for environmental risk assessments of various compounds (Matthiessen, 2008), in water and sediment (Chang *et al.*, 2007).

Different body shapes and sizes are found, ranging from microscopic to giant squids. Some are burrowers, some borers, bottom feeders, or pelagic forms, and some are sessile (Appleton, 2002). They play a key part in the healthy functioning of ecosystems at different trophic levels (Oehlmann *et al.*, 2007). A great variety in reproductive modes is also present

in the phylum; hermaphroditic (both male and female sex organs present in one organism); gonochoric (only one of the sex organs present in each individual); and parthenogenetic (a form of reproduction where the unfertilized egg can develop into an embryo) (Ketata *et al.*, 2008; Oehlmann *et al.*, 2007; Hickman *et al.*, 2006).

Molluscs show higher levels of bioaccumulation factors for persistent pollutants (e.g. Tributyltin) than any other taxa due to the lack of adaptation to metabolise and eliminate pollutants (Oehlmann *et al.*, 2007; Legierse *et al.*, 1998; Lee, 1985). The result is that molluscs may show deleterious effects at lower environmental concentrations than other bio-indicators.

2.2.2 *Bulinus* genus

The *Bulinus* genus is part of the subclass Pulmonata (for full classification, see Appendix A). It consists of 37 species (Stothard *et al.*, 1996), and they can often be challenging to identify. They are classified into four groups based on taxonomic characteristics comprising of shell morphometry, chromosomal numbers, soft part anatomy (including morphometrics of the male reproductive system), DNA analysis, protein electrophoresis and immune-diffusion studies (Stothard *et al.*, 1996). The *B. africanus*-, *B. tropicus*/ *truncatus*- and *B. forskalii*-groups are found in Southern Africa with the *B. reticulatus*-group occurring in the Northern regions of Africa up into the Middle East (Appleton, 2002; Stothard *et al.*, 1996; Brown, 1981).

The genus *Bulinus* has hemolymph using haemoglobin as oxygen carrier. This adaptation has enabled *Bulinus* spp. to be able to adapt to environments with very low oxygen tension and large temperature fluctuations (van Aard & van Eeden, 1976).

2.2.2.1 The distribution of *B. tropicus*

Bulinus tropicus is the most widespread freshwater snail in South Africa and especially abundant in the Eastern and central regions of South Africa (Figure 2.4) (Joubert *et al.*, 1983; van Aard & van Eeden, 1976). They prefer slow-flowing waters (Combrinck & van Eeden, 1970), both temporal and permanent shallow clear pools, with plenty of sunlight and vegetation (Stiglingh & van Eeden, 1977).

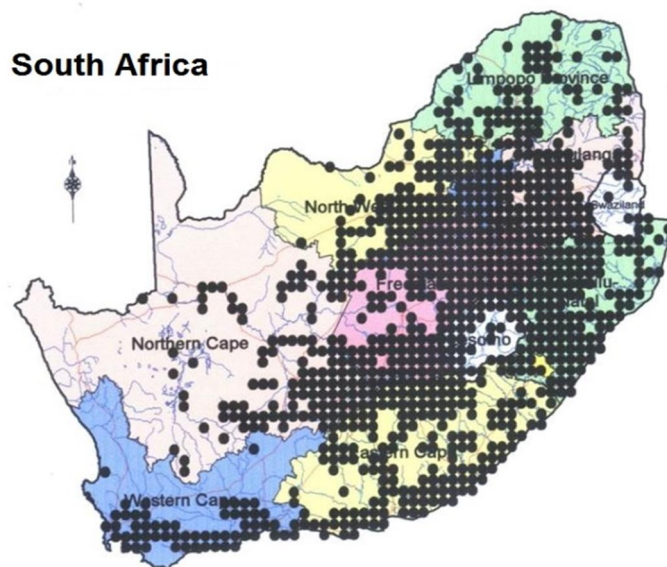


Figure 2.4: The distribution of *B. tropicus* in South Africa. Black dots indicate quarter-degree squares where *B. tropicus* has been found.

2.2.2.2 Feeding habits of *Bulinus*

The buccal mass of *Bulinus* (Figure 2.5) consists of the jaw, radula, odontophora cartilage, salivary glands, and the associated muscles (Dillon, 2000; Runham, 1975). The radula is a unique structure only found in the phylum Mollusca, with bivalves the only exception (Appleton, 2002). The radula is a ribbon-like structure, with numerous transverse and longitudinal rows of small backwards curving teeth (Hickman *et al.*, 2006). Each tooth has a number of raised points, depending on the position and function of the tooth, called cusps (Figure 2.6) (Appleton, 2002). Each row consists of central, lateral, and marginal teeth. The central and lateral teeth are used to gouge, stroke and rasp food particles of the substrate, while the marginal teeth rakes the loosened particles together and upwards into the oesophagus (Appleton, 2002; Stiglingh & van Eeden, 1970). Both the arrangement and number of teeth varies immensely between different species (Owen, 1966), and is used as a tool during classification (Hickman *et al.*, 2006; Hubendick, 1978). Pulmonates typically have between 50 and 150 teeth in each transverse row, and well over 100 longitudinal rows (Dillon, 2000). A large number of the longitudinal rows are incompletely developed and held in reserve to replace teeth as they wear (Appleton, 2002; Dillon, 2000). This “ribbon” is stretched over the odontophora, giving it support and allowing movement of the radula back and forth through the buccal cavity (Hickman *et al.*, 2006; Purchon, 1968). The odontophora has a centre of cartilage, though not true cartilage as found in vertebrates (Owen, 1966). It consists of a homologous structure of muscle fibre supported by connective tissue

(Hubendick, 1978) forming a hydrostatic skeleton of glycogen containing vesicular cells. The glycogen not only gives structure to the cartilage but doubles as energy store (Runham, 1975).

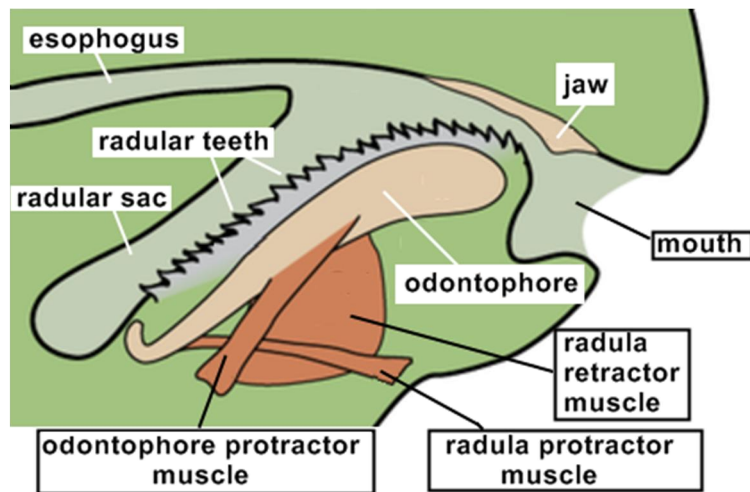


Figure 2.5: The buccal cavity of pulmonates, showing the buccal mass consisting of the jaw, radula, odontophora cartilage, and the associated muscles. Taken from http://barneгатshellfish.org/radula_02.htm on 02/04/2014.



Figure 2.6: Scanning Electron Microscope (SEM) image of the central and lateral cusps found on the radula of pulmonate snails. Taken from <http://scienceblogs.com/deepseanews/2008/03/21/on-how-mollusks-are-cooler-tha-1/> on 02/04/2014.

The thickening of the cuticle inside the buccal cavity forms the jaw. The shape of the jaw is determined by the abrasions the radula makes. The jaws of some pulmonates are robust and can aid in biting of pieces of food, if the need for this action arises due to the food item. Whether *B. tropicus* uses their jaws for biting is still unknown.

B. tropicus mainly feed on leaves (Stiglingh & van Eeden, 1970), diatoms, cyanobacteria, and other algae (Appleton, 2002; Dillon, 2000; Combrinck & van Eeden, 1970). They are not known to feed on living vegetation but will feed on decaying vegetation (Stiglingh & van Eeden, 1977). The tough cell wall of plant cells, as mentioned earlier, can be digested by *B. tropicus* due to the presence of amylase and trypsin in their saliva (Runham, 1975; Purchon, 1968). The salivary glands are located on either side of the oesophagus (Hubendick, 1978). The saliva ducts are situated postero-dorsally in the buccal cavity (Purchon, 1968). The main function of saliva is to lubricate the oesophagus during feeding; and it assists in removing food particles from the radula (Runham, 1975). Stiglingh and van Eeden (1970) also noted that *B. tropicus* will feed on moths that fall into the water, and they will feed on the remains of dead snails. We have also noticed *B. tropicus* feeding on a maize cob that fell in a stream (Figure 2.7). In the laboratory, snails consume, bread, corn, dried spinach, chocolate, cous cous, cheese, and many other small pieces of foodstuffs.



Figure 2.7: Photograph taken in Potchefstroom, courtesy of Ig Viljoen.

2.2.2.3 Eggs, egg packets, and the development of embryo's

The eggs of freshwater pulmonate snails are generally small, ranging between 70 - 200 μm . They are laid in clusters of about 20 eggs, encompassed by a gelatinous capsule filled with albumen, onto submerged surfaces (Appleton, 2002). *B. tropicus* is strictly aquatic and therefore the eggs are deposited onto submerged rocks or vegetation (Stiglingh & van Eeden, 1977).

The eggs are rather poor in intercellular yolk; the surrounding albumin filled capsule functions as the main nutrition source for the embryos. The function of the intercellular yolk is predominantly to supply the hydrolytic enzymes necessary for digestion. Albumen filled vesicles are taken up through narrow channels in the embryo cell membrane. Once inside the cell, the vesicles form an albumin filled vacuole which then ruptures and releases the albumin into the cytoplasm.

The osmotic pressures on the inside and outside of the eggs differ greatly. The outer capsule membrane is freely permeable to water and any inorganic ions. It is therefore necessary to develop a special adaptation in an environment with a greater water potential and low osmotic pressure. Pulmonates develop a wide cleavage cavity from the two cell stage onwards. This cavity opens periodically to the exterior and discharges any excess water and ions.

In most pulmonate species, including *B. tropicus*, embryonic development takes place solely in the eggs (Appleton, 2002) until hatching of the adolescent snails. Basommatophora species have a reduction of the larval, trochophora and veliger, stages (Raven, 1964). When the snails hatch, they already resemble the adults externally. However, the digestive system may still be only partially differentiated and the gonads are undeveloped (Raven, 1975).

2.2.2.4 Reproduction by hermaphrodites

Snails belonging to the family Pulmonata are oviparous hermaphrodites (Appleton, 2002; Runham, 1988). The eggs and sperm are formed in a single but regionally differentiated ovitestis. The ovitestis is located next to the digestive gland in the dorsal region of the visceral hump (Fretter & Graham, 1964). It has been reported that sperm production precedes the formation of oocytes. Snails therefore mature as males before becoming female (Duncan, 1975). From the ovitestis, both the egg and sperm cells mature in a seminal vesicle region after passing through the narrow hermaphrodite duct. Here, the reproduction cells may be stored until use or later discarded if not used. When the snail is ready to copulate, the egg and sperm cells enter the carrefour region. Connecting to the carrefour is the albumin gland, and male and female ducts (Dillon, 2000).

The sperm received in copulation will reach the carrefour to fertilize the eggs. The albumin gland surrounds the fertilized egg with a nutritious secretion before it moves into the female duct (Stiglingh & van Eeden, 1976). The eggs are provided with a gelatinous capsule inside the duct before deposition onto a submerged surface (Duncan, 1975; Fretter & Graham, 1964).

Sperm is transferred into the male duct through the penial complex to a copulatory partner. The basic penial complex includes the penis and its sheath, the preputium, and the attached muscles (Runham, 1988). The morphology of the penial complex is a helpful tool in taxonomy since the structure is different in different species (Fretter & Graham, 1964).

In unstable habitats, where drought or floods are common, chance meetings with a female and a male are scarce. Being hermaphroditic is thus advantageous as repopulation is possible with every meeting of two individuals of the same species (Appleton, 2002; Dillon, 2000). Self-fertilization rarely occurs with freshwater pulmonates. The Pulmonata morphology favours copulation over self-fertilization (Duncan, 1975). The adaptation of the hermaphrodite duct and carrefour to prevent self-fertilization is not yet clear (Dillon, 2000).

No visible courtship behaviour has been documented for pulmonates that will enhance a suitors' chance for copulation. Mating is initiated when two individuals meet of which one needs an autosperm store (the already conveyed sperm from the carrefour through the male duct into the penis) (Dillon, 2000). If both the snails has recently copulated as a male, no autosperm will be available between them and they would not copulate. However, if autosperm is available during an encounter, the 'male' snail will simply mount the female and move across her shell until he is parallel to her. The male will then evert his penis, and insert it through the vagina into the oviduct (Dillon, 2000; Duncan, 1975; Fretter & Graham, 1964).

The copulation partner may be rejected by the female through jerking and vigorous shaking of the shell in an attempt to dislodge the male. They could also close the opening of the shell with their foot to prevent copulation (Dillon, 2000). *B. tropicus* can breed throughout the year and is not bound to any one season (Stiglingh & van Eeden, 1977).

2.3 Endocrine Disruptive Chemicals

In order to understand the mechanism of endocrine disruptive chemicals, a basic understanding of the endocrine system is necessary.

2.3.1 The endocrine system

The endocrine system is responsible for the production of hormones that act as the body's messengers helping to maintain balance in the internal environment of an individual organism (Rizzo, 2010; Shier *et al.*, 2009). Hormones are secreted by ductless endocrine glands into the circulatory system to be transported to target cells. After binding to the target cell at a specific binding site, an intercellular reaction is induced depending on the type of hormone. Hormones are classified as either steroids, synthesized from cholesterol, or they belong to the amines, peptides, proteins and glycoproteins synthesized from amino acids (Tate, 2009).

Steroid hormones are complex carbon and hydrogen rings. They are differentiated according to the kind, sequence, and number of atoms present on each ring. They are soluble in lipids, making it easy to diffuse through cell membranes into cell. Once inside the target cell, steroid hormones attach to specific protein binding receptors. The hormone-protein complex then binds to a particular region on the cells' DNA, inducing the transcription of that sequence (Rizzo, 2010).

Nonsteroid hormones include the amines, peptides and protein structured hormones (Shier *et al.*, 2009). Unlike steroid hormones, they are not lipid soluble and therefore cannot enter the target cell. There are very specific binding receptors on the membrane of the target cell where these hormones attach (Rizzo, 2010). The receptor activity site then interacts with the other proteins disrupting the usual mechanisms of membrane transport, thus provoking changes in other cellular components (LeBlanc *et al.*, 1998).

The endocrine system of vertebrates is made up of various components including the hypothalamus, pituitary gland, thyroid gland, parathyroid gland, pancreas, pineal gland, testis, and ovaries (Rizzo, 2010; Tate, 2009), each with its own role in the correct functioning of the body. The molluscan endocrine system on the other hand centres primarily on the neurosecretory loci of the central nerve system. This includes the cerebral, pleural, pedal, and abdominal ganglia (Ketata *et al.*, 2008). These nerve cells are able to secrete neuropeptides from the bulbous formation located at the end of the axon. Neuropeptides may act as either a neurotransmitter or a hormone (LeBlanc *et al.*, 1998). Peptide hormones in molluscs include:

- Egg-laying hormone (ELH) synthesized in the abdominal ganglion, responsible for gonad maturation, egg packet production, and the behaviour associated with egg-laying (LeBlanc *et al.*, 1998).
- Dorsal body hormones, synthesized in the cerebral ganglion, responsible for the development of sex organs, gonad maturation, and ovulation (Ketata *et al.*, 2008).
- Molluscan insulin-like peptides (MIP's), also produced by the cerebral ganglion, controls development, growth, and reproduction (Matthiessen, 2008).
- The cardioacceleratory peptide FMRFamide, synthesized by the abdominal ventral ganglion, regulates the contractions of the heart (Broadie *et al.*, 1990).

Molluscs are unique among the protostomes because they are the only phylum which can synthesize complex vertebrate-like steroid hormones from simple molecules, the difference being the absence of the cholesterol side-chain seen in invertebrate steroid hormones (Ketata *et al.*, 2008). These steroid hormones are secreted from true glands (Matthiessen, 2008) and the presence of the sex hormones such as progesterone and androgen has been reported in some species (Wootton *et al.*, 1995).

2.3.2 What are EDCs

Endocrine disruptive chemicals (EDCs) are defined as any exogenous substance that alters the normal functioning of the endocrine system, causing adverse health effects in an individual organism, its' offspring, or a population (Devilliers, 2009; deFur *et al.*, 1999). They have the ability to disrupt hormone-controlled physiological processes including development, growth, reproduction (Duft *et al.*, 2003), sexual differentiation (Schulte-Oehlmann *et al.*, 1995), behaviour (Palanza *et al.*, 1999), and immunity (Segner, 2009). The presence of EDCs has been reported in well water, lakes, the ocean, and rain water (Colborn *et al.*, 1993). They have been found in food products (Guenther *et al.*, 2002) and even breast milk (Bouwman *et al.*, 1992).

EDCs may affect an organism in numerous ways including (Devilliers, 2009; Diamanti-Kandarakis *et al.*, 2009):

- Mimicking hormones produced within the body, e.g. estrogen and androgen;
- Antagonizing hormones;
- Changing the syntheses and the metabolism of hormones;
- The receptor binding site may be altered;
- Or interference in the nervous and immune system, both of which are directly in relationship with the endocrine system, may occur.

Maternal exposure could also have adverse effects on the development of offspring, especially in organs dependant on gonadal hormones. Such organs in females include the mammary glands, the fallopian tubes, uterus and cervix. In male offspring, the prostate, seminal vesicles, epididymus, and testis are vulnerable to deformities. Deleterious effects are also seen in the brain, skeleton, thyroid, liver, kidney, immune system, and external genitalia of both sexes (Colborn *et al.*, 1993).

EDCs are a diverse group of chemicals, differing both structurally and functionally. This group of chemicals includes synthetic hormones, natural hormones, phytoestrogens, chlorinated pesticides, polychlorinated biphenyls (PCBs) and other industrial chemicals. Many of them are persistent with very low vapour pressures (Colborn *et al.*, 1993), and are lipophilic (deFur *et al.*, 1999).

Many EDCs stimulate profound effects at low doses compared to high doses, producing an inverted U-shaped dose-response relationship (Diamanti-Kandarakis *et al.*, 2009). This chemical behaviour challenges risk assessments and poses threats to the environment and human health.

2.4 Conclusion

Molluscs are a diverse phylum, inhabiting almost all corners of the Earth. Their lack of adaptations to break down pollutants has made them popular to use in toxicological studies of very low concentrations. Such as the low concentrations of Cry proteins measured by Tank *et al.* (2010) in water systems around agricultural activities.

The technology for genetically modifying crops has great and novel potentials to lessen the growing world's food crisis. But a new technology can only be used if it is proven safe to non-target organisms. A fact not yet confirmed.

Chapter 3: Materials and methods

This study was conducted using a new and explorative design, since a study of this nature has never been attempted before with either Bt crop residues or *Bulinus tropicus*. The snails used for this study were collected from the Potchefstroom area in the North West province of South Africa (Figure 3.1). Some of the species in the *Bulinus* genus act as intermediate hosts of the *Schistosoma* spp., causing bilharziasis in humans and livestock. Though *B. tropicus* is not a natural intermediate host for *Schistosoma*, the *Schistosoma* cercariae might infect the snails but resulting in a complete inhibition of the cercariae's life cycle. In turn this may also adversely affect *B. tropicus*. Therefore, the collected snails were first placed in a separate aquarium, for multiple generations, to ensure that none of the snails used in during the exposures are infected.

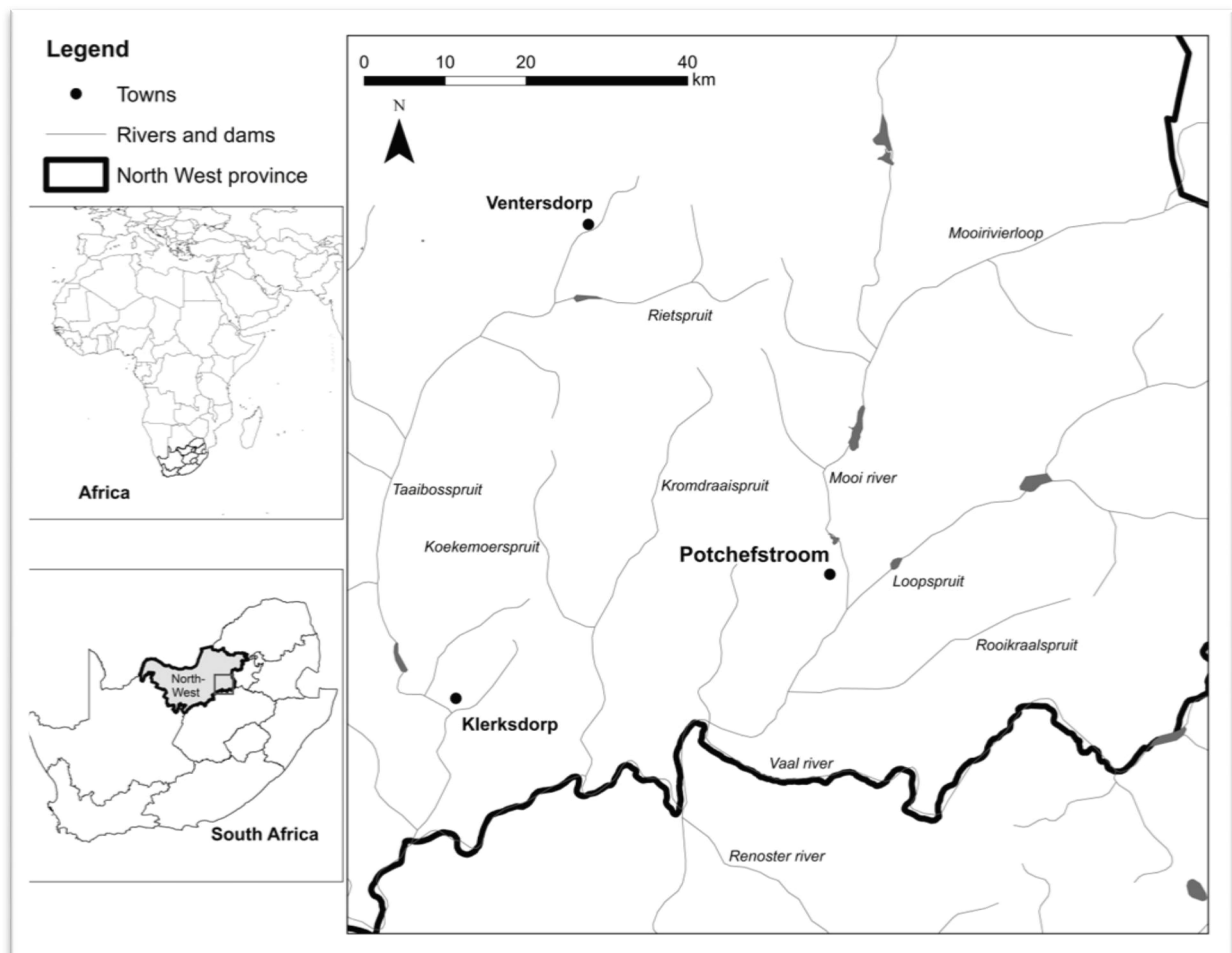


Figure 3.1: Map of Potchefstroom, South Africa, and surrounding areas.

3.1 Test conditions

All treatments were subject to the same laboratory conditions for the duration of exposures. Combrinck & van Eeden (1970) reported that *B. tropicus* inhabits water bodies with a temperature of 21°C, but from our own experience we have found that the snails were more fertile (laying egg packets with more eggs per packet, more frequently) in a constant water temperature of between 25°C and 26°C. We used synthetic freshwater due to the sensitivity of molluscs to chlorides (Valenti *et al.*, 2006; Brungs, 1973) present in tap water. The method used was adopted from the International Organization of Standards' (ISO 3641) report on *Water quality – Determination of the inhibition of the mobility of Daphnia magna Straus (Cladocera, Crustacea) – Acute toxicity test* (2012). Four solutions were made up to 1 L, each, with ultra-pure distilled water (Elga): 11.76 g of calcium chloride dehydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$), 4.93 g of magnesium sulphate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), 2.59 g of sodium bicarbonate (NaHCO_3) and 0.23 g of potassium chloride (KCl). A mixture of 625 mL of each stock solution was filled up to 25 L with ultra-pure distilled water. The water was then stored in white HDPE (high density polyethylene) containers for no more than four days at 27°C. The water in each exposure container was replaced every 96 hours with fresh synthetic water, based on the findings of Chaudhry and Morgan (1986) who reported that the decline in concentration Ca^{+2} , and an increase in ammonia (NH_3) had adverse effects on the growth rate of *B. tropicus*. The water was aerated continuously with filtered air.



Figure 3.2: The set up used in the laboratory during the exposures.

A 12-hour florescent light cycle was maintained to replicate normal day and night cycles. Consol® glass containers were used during the exposures (Figure 3.2). These containers were first washed with Extran® MA 03 (Sigma-Aldrich) phosphate-free soap to remove most

of residues that might have been left on the glass. To ensure that no organic residues were left on the glass, the containers were washed according to the USEPA method 23 (1995). Each glass container was left in chromic acid for 24 hours. The acid was rinsed with tap water and left for another 24 hours filled with tap water, after which they were rinsed with ultra-pure distilled water.

3.2 Exposures

Two sets of exposures were done, one with Bt maize leaves and another with Bt cotton leaves. The cultivars chosen for these exposures included a conventional non-Bt line and its Bt isolines. Isolines are derived from conventional cultivars and has the specific gene encoding for the Cry proteins inserted, ensuring that the only difference between the Bt and non-Bt exposures were the gene encoding for the particular Cry protein.

All the maize leaves were collected from plants growing in a hydroponic fashion inside a controlled environment at EcoRehab, Potchefstroom, where no chemical pesticides were used. The cotton leaves on the other hand were collected from a farm 4 km North East from the town of Potchefstroom. The farmer did not make use of any chemical pesticides.

The leaves were dried in a climate control chamber set to 28°C, for two weeks. The dried leaves were shredded using a multipurpose stick blender (Russle-Hobbs, RHSB 025). 50 cm² (approximately 0.4 g) of shredded leaves were given to each exposure every fourth day as feed. Surface area, and not mass, was used during the exposure because not all the leaves were dried equally. Excess hydration left in some leaves resulting in inconsistent amounts of food each container received.

The cultivars used for the maize exposures were IMP 22-51 (non-Bt) and its isoline IMP 22-51 B (Bt) (Agricol). The effect of different cultivars, in terms of nutritional value, was tested using the non-Bt isoline of CRN 3505 (Monsanto) maize leaves. During this exposure, the effect of the Cry1Ab proteins in solution in the water was measured using additional infusion exposures. Forty-eight hours prior to the water and feed change cycle, 50 cm² of specified leaves were placed in 900 mL of water and aerated. The leaves were then removed and the infused water given to those exposures. The treatments are explained in Table 3.1.

Table 3.1: The treatments used during the maize leaf exposures.

Treatment	Water	Food
Control	Only ISO water	Tetra [®] Pro Algae
CRN	Only ISO water	CRN 3505 leaves
IMP -	Only ISO water	Non-Bt leaves
IMP +	Only ISO water	Bt leaves
IMP -/-	Non-Bt infusion	Non-Bt leaves
IMP +/-	Bt infusion	Non-Bt leaves
IMP +/+	Bt infusion	Bt leaves

The cultivars Delta OPAL (non-Bt) and Delta 12BRF (Bt) (Deltapine) were used for the cotton exposure (Table 3.2). Delta 12BRF is known as a Bollgard I RR Flex isoline. No infusion treatments were used during the cotton leaf experiment, because initial results showed no difference between the maize infusion Bt treatments and IMP-.

Table 3.2: The water and food each treatment received during the cotton leaf exposure.

Treatment	Water	Food
Control	Only ISO water	Tetra [®] Pro Algae
Opal	Only ISO water	Non-Bt leaves
Bol	Only ISO water	Bolgard I

3.2.1 Development and growth

Five adult snails from the stock breeding containers were placed in each of the Consol[®] glass containers, in 900 ml ISO water and fed fish food. When two or more egg packets of the same age were laid the adult snails were removed. If more than two egg packets were found, the extra packets were removed until only two remained. This would keep the population density in each container similar, ensuring that growth inhibition do not take place due to competition and a lack of space (Chaudhry & Morgan, 1986).



Figure 3.3: The ProScope HR microscope/camera used to take the photos of the developing snails.

The embryos were then exposed as indicated (Table 3.1 and 3.2). A photograph of each egg packet was taken daily (24 h time interval) with a ProScope High Resolution microscope/camera (Figure 3.3) and software. After the embryos hatched, measurements were only taken every third day of five snails per container, until sexual maturity was reached (first appearance of an egg packet). Development was determined by measuring the length of the shells, from the edge of the opening to the apex (Figure 3.4). A molluscs' shell consists out of layers of conchiolin, calcium carbonate, and calcareous nacre, which is secreted continuously by the glands in the mantel throughout the molluscs' life (Hickman *et al.*, 2006). Therefore, as the snail develops and grows, the length of its shell should increase. This was measured using tpsDig version 2 free software. Due to the size of the snails after hatching (less than 1 mm in shell length) it was not possible to measure growth in terms of mass (g).

Calibration of each photograph was done using a ruler with 0.5 mm increments. Other endpoints noted included the percentage of hatchlings, the number of days till sexually maturity, and survival of embryos.

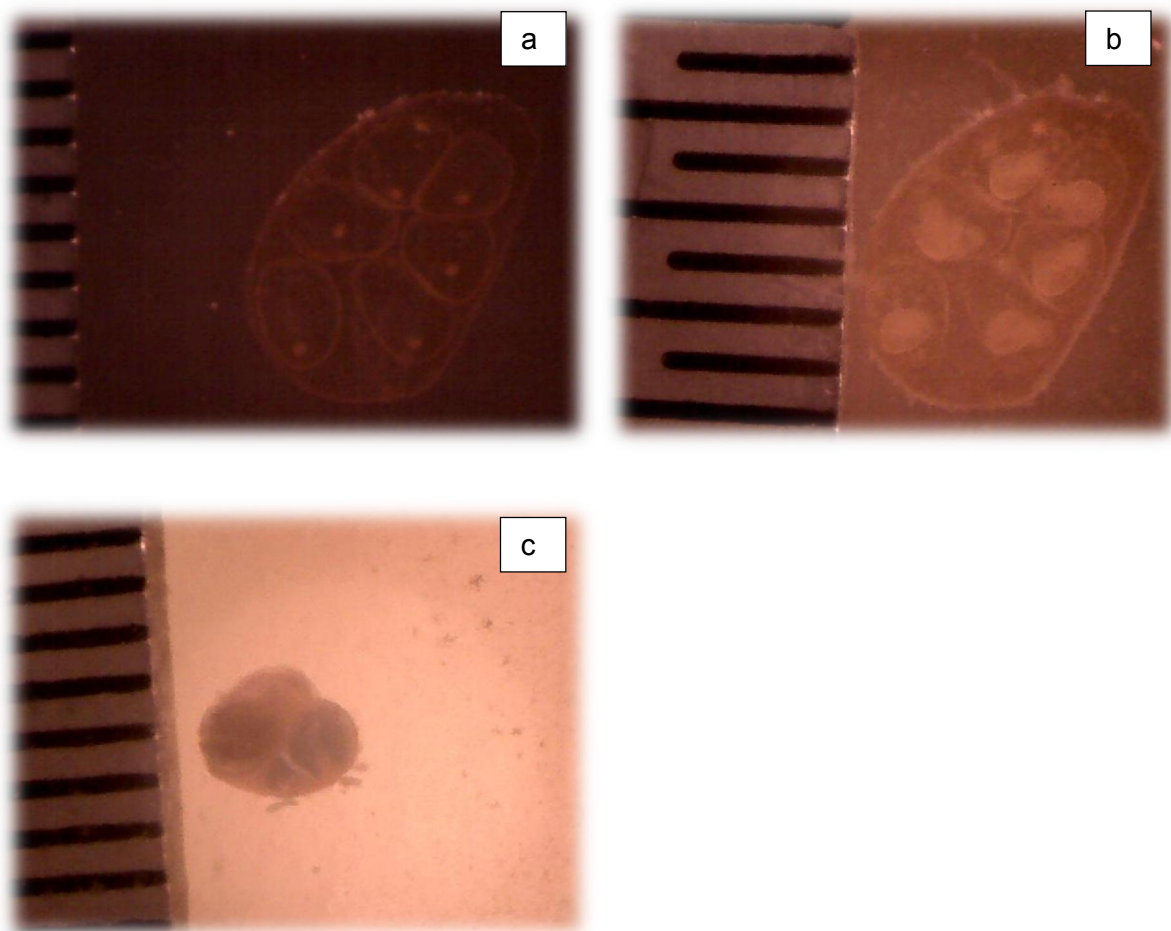


Figure 3.4: The development of the embryos to hatchling. (a) The embryos (small dots) a couple of hours after the egg packet were laid. (b) The embryos on day 5 already look like the adult snail. (c) A young snail just after hatching from the egg capsule.

3.2.2 Reproduction

After the exposed snails reached sexual maturity, daily notes were taken on the number of egg packets per container; as well as the number of eggs per packet. The egg packets were removed from the container every day. This continued for fifteen days.

3.2.3 Dissections

The adult snails were narcotised for four to five hours with a few drops of a chloral hydrate and menthol mixture (6 g menthol and 6.5 g chloral hydrate, ground together into a viscous clear liquid) into their water. During this time, the containers were left alone allowing the snails to protrude from their shells in a relaxed state. After a few hours, the narcotizing agent was decanted (Appleton, 1996).

The relaxed snails were euthanized using 60°C water. They were fixed in a 4 - 10% formalin solution for 24 hours. The formalin was decanted and a 70% ethanol with 5% glycerol solution was used to preserve the snails.

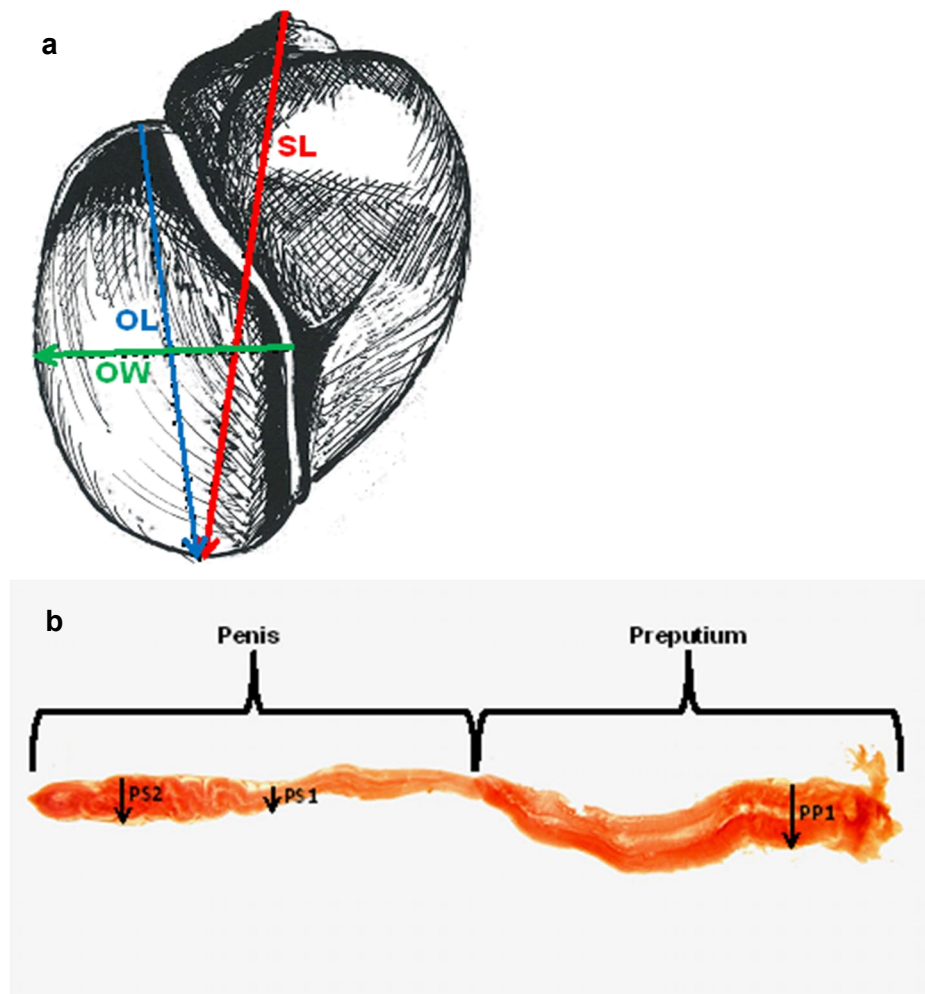


Figure 3.5: The dimensions measured after preservation of the snails. a) Measurements made of the shell length (SL), opening length (OL), and opening width (OW). b) The dimensions measured on the penis-preputium complex, the length of both the penis and preputium, the width of the penis at the narrowest (PS1) and the widest (PS2) sections, and the width of the preputium (PP1).

Before the snails were dissected, measurements of the length of the shell, the length of the opening and the width of the opening (Figure 3.5A) were taken using an electronic calliper (Wilson Wolpert, Digitronic Calliper). The soft body was removed from the shell and the penis-preputium complex (penis, preputium, and vas deference) was excised using Normed forceps under a dissection microscope. Since *B. tropicus* is hermaphroditic, possessing both male and female sex organs, an EDC effect may result in differences in dimensions of the male reproductive organs. The morphometrics of the male penis sheath-preputium complex is an obvious endpoint that can be measured (Figure 3.5B). Therefore the length of the penis

and preputium, and the width of the penis (at it widest and narrowest points) and preputium was measured with a Nikon AZ100 Multi-purpose Zoom microscope using the accompanying software (Figure 3.6).

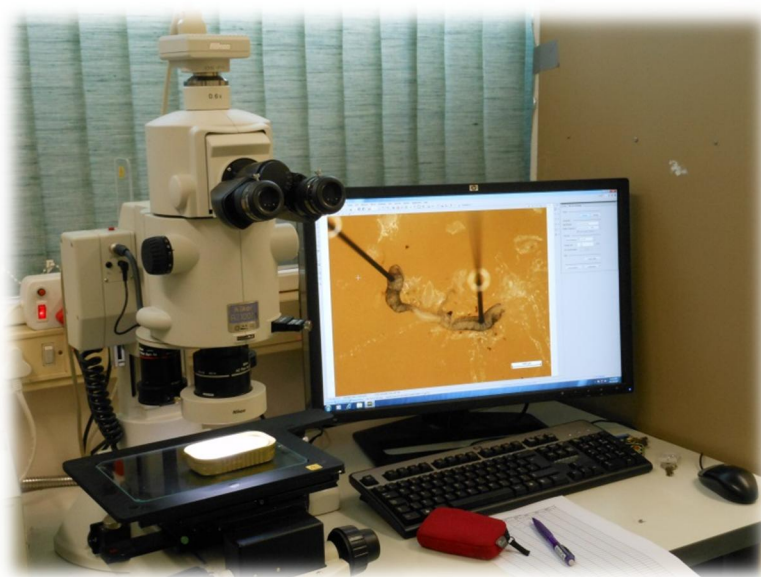


Figure 3.6: The Nikon AZ100 Multi-purpose Zoom microscope used to measure the length of the male reproductive organs.

3.3 Concentrations of Cry1Ab

Water samples from the containers were analysed to determine Cry protein concentrations, but the concentrations were below detection limits. The concentrations displayed in Figure 4.1 were derived from the results attained for maize by Harvey (2013).

Harvey (2013) prepared the water as follows. Three replicates of infusing 12 g () of dried maize leaves in 85 ml of borehole water from the Potchefstroom area were prepared. This is approximately 1124 cm² in 85 ml water equating to 13.2 cm²/ml, while the present experiment used 0.06 cm²/ml, which is 220 times less than in the study of Harvey (2013). Sampling took place at 1h, 2h, 4h, 8h, 24h, 48h, and 96h time intervals. The samples were pooled from all three replicates and frozen at -80°C. Analysis of the samples were done by the GMO Testing Facility located at the University of the Free State. The EnviroLogix QualiPlate Enzyme-linked immunosorbent assay (ELISA) for Cry1Ab/Cry1Ac was used to determine the concentrations of the crystal protein in the samples. During the assay the samples were placed in wells coated with the alkaline-phosphatase-labelled antibodies for both Cry1Ab and Cry1Ac. The proteins present in the sample would bind to the antibodies. This resulted in a change in colour and the plates were read using a microtiter plate reader set to 450 nm. The development of the colour is directly proportional to the concentration

(Table 3.3) of the crystal proteins present in the sample (EnviroLogix, 2013). The limit of detection (LOD) was 1 ng/ml. Calculated from the analytical results, the time-based concentrations the snails were exposed to in the current study was calculated and presented in Table 3.3. The concentrations in the leaves were not measured.

Table 3.3: The concentrations of Cry1Ab proteins that leached into the water from maize leaves over time, as calculated from the results obtained from Harvey (2013).

Sample at specific time interval	Calculated Bt concentration (ng/ml H₂O)
1h	0.665
2h	1.615
4h	1.612
8h	3.856
24h	2.865
48h	4.637
96h	4.897

Harvey (2013) did not repeat this experiment with cotton leaves. Unfortunately no published data concerning the concentration Cry1Ac in water samples, from laboratorial or field studies, could be traced.

3.4 Statistics

The data was analysed using Microsoft® Office Excel (2010) for basic processing. All advanced statistical analyses and visualisation of the data was done using Graphpad Prism (Prism 5 for Windows). Where appropriate, means and standard deviations (SD) were used to display on the graphs. Differences between treatments were determined using either one-way ANOVA, followed by Bonferroni's multi comparison test, or a two-tailed, unpaired t-test. Normality of the data was tested using Shapiro-Wilk normality test; if the data deviated from normal it was transformed to $y=\log(y)$. PCA analyses were done using PC-ORD (Version 5.0). Dimensional data were relativized in columns. Cross-products matrix was calculated from correlation, and scores for dimensions was for a distance-based biplot.

Chapter 4: Results

The cotton and maize experiments were done consecutively. Each experiment therefore, had its own control (Con) to ensure that the experimental setup was valid, comparable, and supportive of hatching, growth, and reproduction during the experiments.

4.1 Concentration of Bt toxins

The concentrations of the Bt protein in the experimental setup used here was too low to allow its measurement with ELISA. The concentration of the Cry1Ab (Figure 4.1) protein, infused from Bt–maize leaves into water was derived from data reported by Harvey (2013) doing similar experiments with tadpoles at exposures 200 times higher. Within the first eight hours that the leaves were placed in the water, a rapid increase in concentration (75% of the eventual concentration) of the protein was seen in the water. After 24 hours, a slower rate of increase in concentrations was observed for up to 96 hours when the water and leaves were changed. Concentrations for cotton (Cry1Ac) were not determined but a similar change in concentration is assumed. Note that with the infusion treatments, 48-hour infusions were used, probably providing an almost constant Bt-protein concentration throughout the infusion experiments.

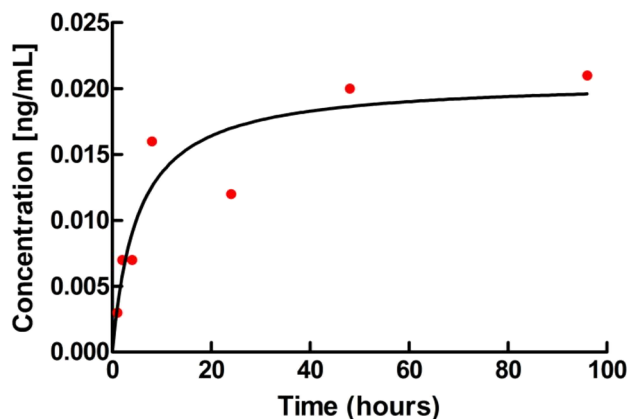


Figure 4.1: The change in concentration of Cry1Ab proteins infused from Bt-maize leaves measured in water, calculated from Harvey (2013). A non-linear (Michaelis-Menten) line was fitted; $R^2 = 0.85$.

4.2 Cotton

4.2.1 Embryo growth

The embryo growth data for the growth of the snail embryos during the cotton exposure had to be split due to the number of embryos that showed apparent growth retardation and eventually did not hatch, and those that eventually did. The changes in length of the embryos that did eventually hatch are represented in Figure 4.2; those that did not are represented in Figure 4.5. Results on percentage hatching will be presented in section 4.2.2.

Figure 4.2 indicates a distinct difference in embryo growth trajectories between the control treatment reared on fish food and those exposed to leaves. Note that lines terminate when no more hatching occurred. Note moreover, that as soon as an embryo has hatched, it no longer contributes to the embryo growth curve, mostly leaving the smaller unhatched embryos in the cohort being measured. After day five when hatching began, the mean length values are therefore artifactual since the larger individuals have left the cohort due to hatching. However, the retarded growth in the leaf treatments is apparent as the embryos took longer to attain a length at which they hatched (see further below).

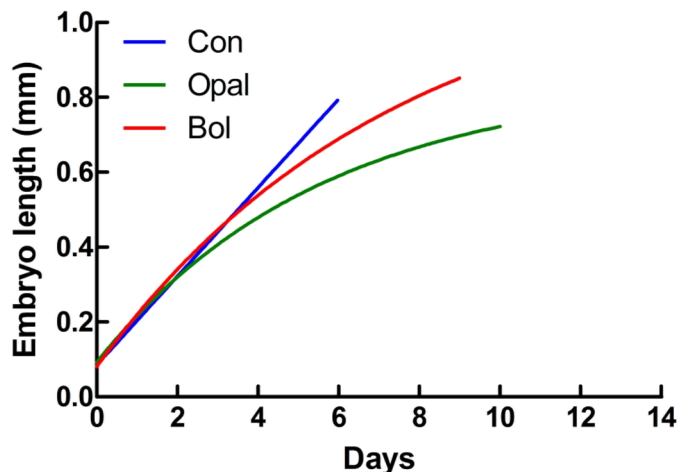


Figure 4.2: Growth, measured as increase in length, of those embryos that eventually hatched.

Con growth was best fitted with a linear regression line (Figure 4.2), but both cotton leave treatments were best fitted with a non-linear curve (one-phase association). The best fit for each data set was chosen as the curve with the best R^2 values as listed in Table 4.1. A delay in hatching time compared with Con seems to be associated with a flattening of the Opal and Bol embryo growth curves, with Opal taking up to five days, and Bol four days, longer to hatch than Con.

Table 4.1: The curve best fitted to the change in length (growth) of embryos that eventually hatched; the goodness of the fit (R^2) and the standard deviation (SD).

	Fit	R^2	SD
Con	Linear regression	0.93	0.26
Opal	One phase association	0.67	0.21
Bol	One phase association	0.87	0.26

On day five, the embryos of Con that have eventually hatched by day seven were significantly larger than those embryos exposed to cotton leaves (Figure 4.3; t-tests results in Table 4.2;). Due to hatching of most of the embryos by day 10, only the smaller embryos were left. This resulted in the significant difference in size between the two treatments ($p=0.0007$, t-test, unpaired, two-tailed). This was the last day of hatching for Bol embryos, but Opal still had one more day left. Though both leaf-fed treatments took longer to hatch than Con, the mean lengths of the embryos the day before each hatched did not differ between the treatments (Figure 4.4). No statistical difference could be found comparing the embryo hatching times of the leaf treatments ($p=0.2388$, two-tailed, unpaired, t-test between Bol and Opal).

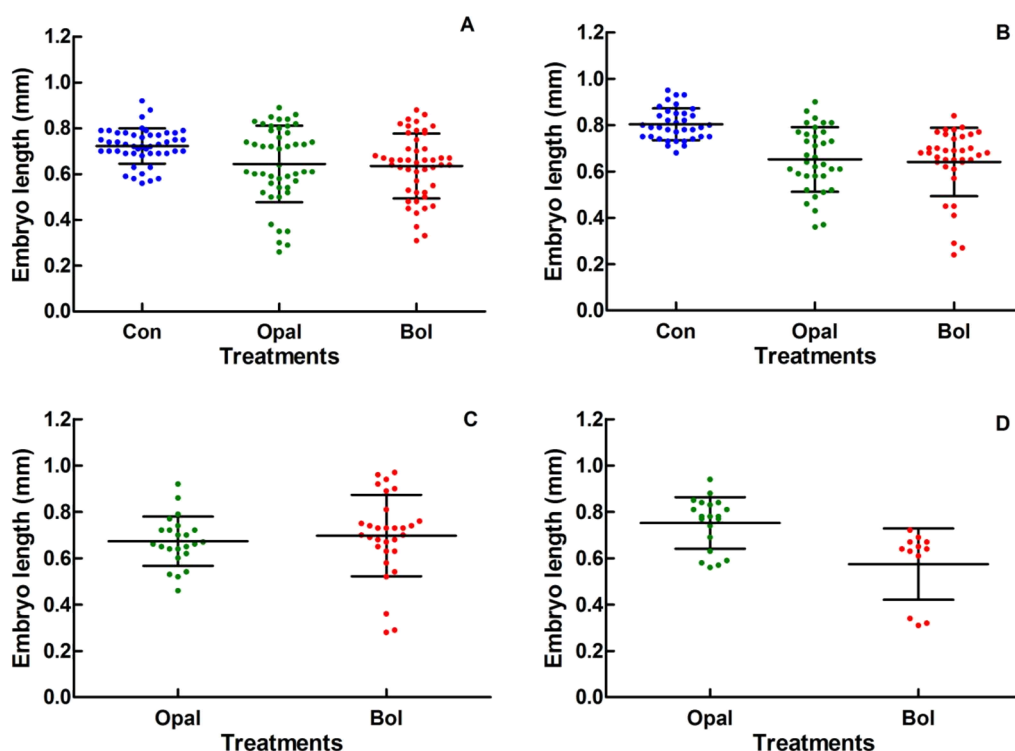


Figure 4.3: The length of the cotton leaf exposed embryos that eventually hatched at specific times; (A) day 5, (B) day 6, (C) day 8, and (D) day 10. Means and standard deviations are indicated.

Table 4.2: T-test (two-tailed, unpaired) p-values of embryo length at different time intervals between each group of the cotton experiment.

	Day 5	Day 6	Day 8	Day 10
Con vs. Opal	0.0038	P<0.0001		
Con vs. Bol	0.0003	P<0.0001		
Opal vs. Bol	0.7863	0.7630	0.5718	0.0007

The retarded growth of the notable number of embryos that did not fully develop and then failed to hatch when exposed to cotton leaves is presented in Figure 4.5. (This will be described further in section 4.2.2.).

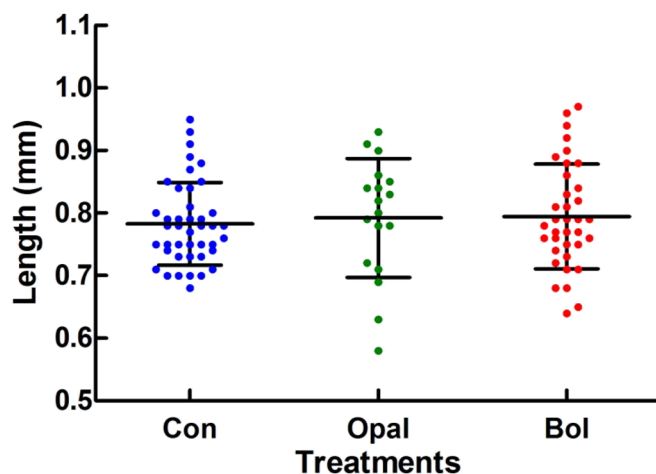


Figure 4.4: The length of the cotton leaf-fed and control embryos the day prior to each of them hatching. Means and standard deviations are indicated.

The growth of Con embryos were not retarded in the same way as seen in the other treatments (they just remained small but apparently alive), and then seem to start rotting, indicated by a whitening of the eggs. A linear regression similar to the growth of hatched embryos within the control group (Figure 4.2) was constructed for the embryos that did not hatch. The delay in growth and complete inhibition of hatching of Opal and Bol is again indicated by the reaching of a plateau on a non-linear regression line (Table 4.3).

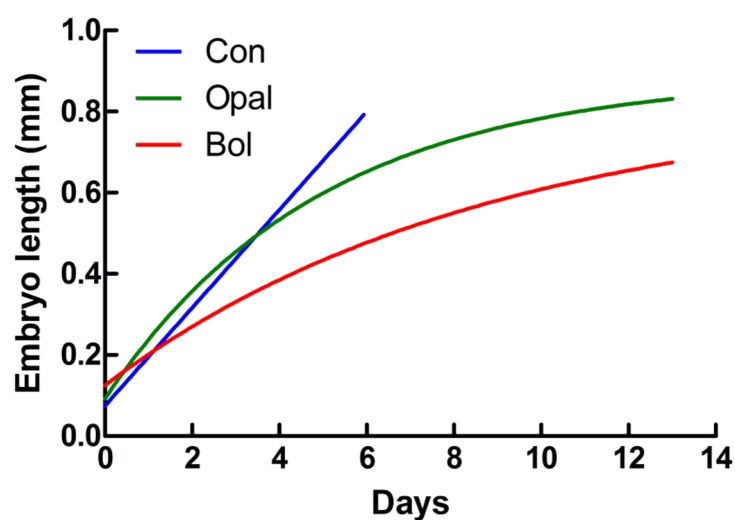


Figure 4.5: Growth in length of snail embryos that failed to hatch after exposure to cotton leaves. Means and standard deviations are indicated.

Table 4.3: The curve best fitted to the retarded growth of embryos exposed to cotton leaves; the goodness of each fit (R^2), and the standard deviation (SD).

	Fit	R^2	SD
Con	Linear regression	0.91	0.26
Opal	One phase association	0.87	0.23
Bol	One phase association	0.71	0.18

4.2.2 Hatching success

The cotton leaf exposures yielded low hatching success of embryos, seen in Figure 4.6. 46% of the non-Bt leaf treatment hatched, and only 53% of the embryos exposed to Bt. Con had an 82% hatching success. Chi-square tests showed significant difference of the high hatching success of Con compared to the leaf treatments, but not between the leaf treatments (Table 4.4).

Table 4.4: Chi-square comparisons of the percentage hatching for the cotton experiment.

	t-test	Chi-square
Con vs. Opal	0.83	0.01
Con vs. Bol	0.93	0.11
Opal vs. Bol	0.88	0.65

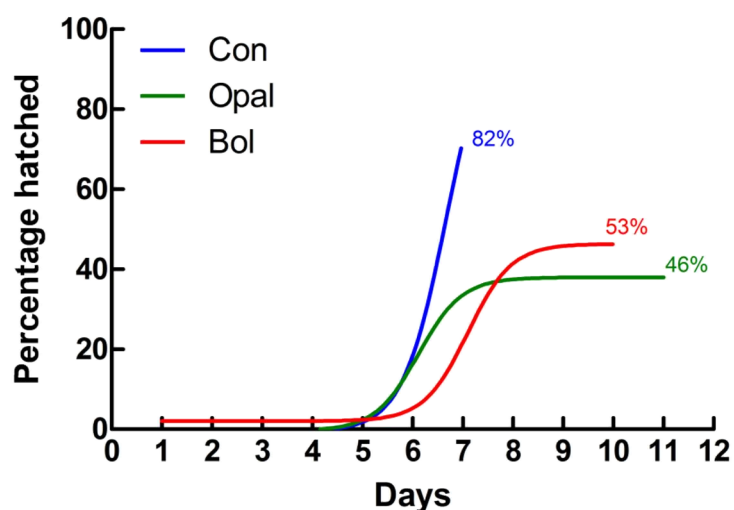


Figure 4.6: The cumulative percentage of eggs that eventually hatched in the cotton leaf experiment. R^2 : Con = 0.99, Opal = 0.97, and Bol = 0.93. Lines terminate when last embryo hatched. Means and standard deviations are indicated.

Con had a significantly shorter embryo period than Bol and Opal (Table 4.5). However, the non-Bt leaf treatment took significantly longer to hatch than Bol (Kruskal-Wallis non-parametric ANOVA, $p < 0.0001$, followed by Dunn's multi comparison of means, $p < 0.05$). The difference in mean embryo period between Bol and Opal was short by about 0.5 days, but the difference was quite strong.

Table 4.5: The mean number of days it took until hatching, and the non-parametric Dunn's multiple comparisons of the means following ANOVA, for the cotton experiment.

	Con	Opal	Bol
Mean	6.741	7.552	7.936
Con		$p > 0.05$	$p < 0.05$
Opal	$p > 0.05$		$p < 0.05$
Bol	$p < 0.05$	$p < 0.05$	

4.2.3 Growth after hatching

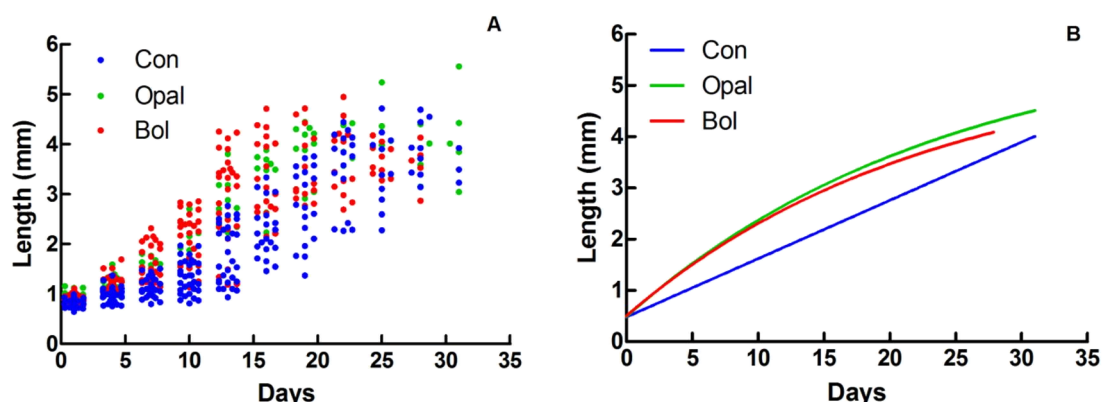


Figure 4.7: The growth in length of *B. tropicus* exposed to cotton leaves, from hatching until sexual maturity is reached (A); and the non-linear regression lines fitted to the data (B). Means and standard deviations are indicated.

The length of the growing snails (representing growth) were measured every third day in each container. Since hatching occurred on different days, the starting times for all measurements of growth were normalised to day one, and therefore normalised as if all have hatched on the same day. Individual snails could not be followed within a container and therefore five random snails were selected per occasion. An initial narrow range in length is observed in Figure 4.7A, widening as the snails age.

Non-linear growth patterns (goodness of fit in Table 4.6) were observed for the growing snails reared on cotton leaves (Figure 4.7B). Measurements were discontinued when sexual maturity was reached within a container (first egg packet detected). The leaf-exposed snails increased in length faster than the control treatment. Con had a steady increase in length over time, but Opal and Bol both grew faster which later slowed as they neared sexual maturity.

Table 4.6: The standard deviations (SD) and the goodness of fit (R^2) of the best-fitted non-linear curves of the growth of snails in the cotton experiment.

	SD	R^2
Con	1.12	0.74
Opal	1.30	0.88
Bol	1.10	0.75

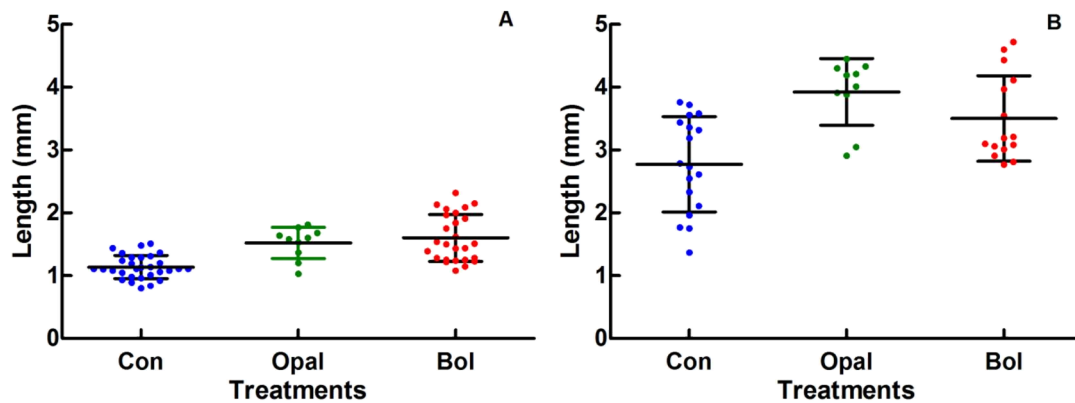


Figure 4.8: The length of the snails at specific time intervals of growth during exposure to cotton leaves; (A) day 7; and (B) day 19. Means and standard deviations are indicated.

The snails reared on cotton leaves, irrespective of the presence of Bt, were significantly larger on day seven (Figure 4.8A and Table 4.7). After 19 days (Figure 4.8B), Opal was the largest, but no significant difference in mean size were found compared to Bol ($p=0.536$, t-test, unpaired, two-tailed).

Table 4.7: T-test (two-tailed, unpaired) p-values comparing the length of the snails exposed to cotton at different ages.

	Day 7	Day 19
Con vs. Opal	$p<0.0001$	0.0002
Con vs. Bol	$p<0.0001$	0.0072
Opal vs. Bol	0.536	0.1116

The remaining snails were euthanized for dissecting purposes at the end of each exposure. The shell of each individual was measured before dissection, therefore the whole surviving population and not only a sample of the population was dissected and is represented in Figure 4.9.

The total shell length, and the length and the width of the opening of *B. tropicus* exposed to cotton leaves are shown in Figure 4.9. The snails reared on cotton leaves (Bol and Opal) were significantly smaller compared to the fish food reared control (Con) (Table 4.8). The smaller mean shell length was also reflected in significantly smaller mean opening length and width (Table 4.8). The snails exposed to Bt cotton were significantly larger than the non-Bt leaf treatment ($p=0.0035$, t-test, unpaired, two-tailed).

Table 4.8: P-values from t-tests (two-tailed, unpaired) comparing the different cotton-exposed treatments' shell dimensions for the cotton experiment. Where SL = shell length; OL = opening length; and OW = opening width.

	SL	OL	OW
Con vs. Opal	p<0.0001	p<0.0001	p<0.0001
Con vs. Bol	0.0030	0.0006	p<0.0001
Opal vs. Bol	0.0035	0.0035	0.2014

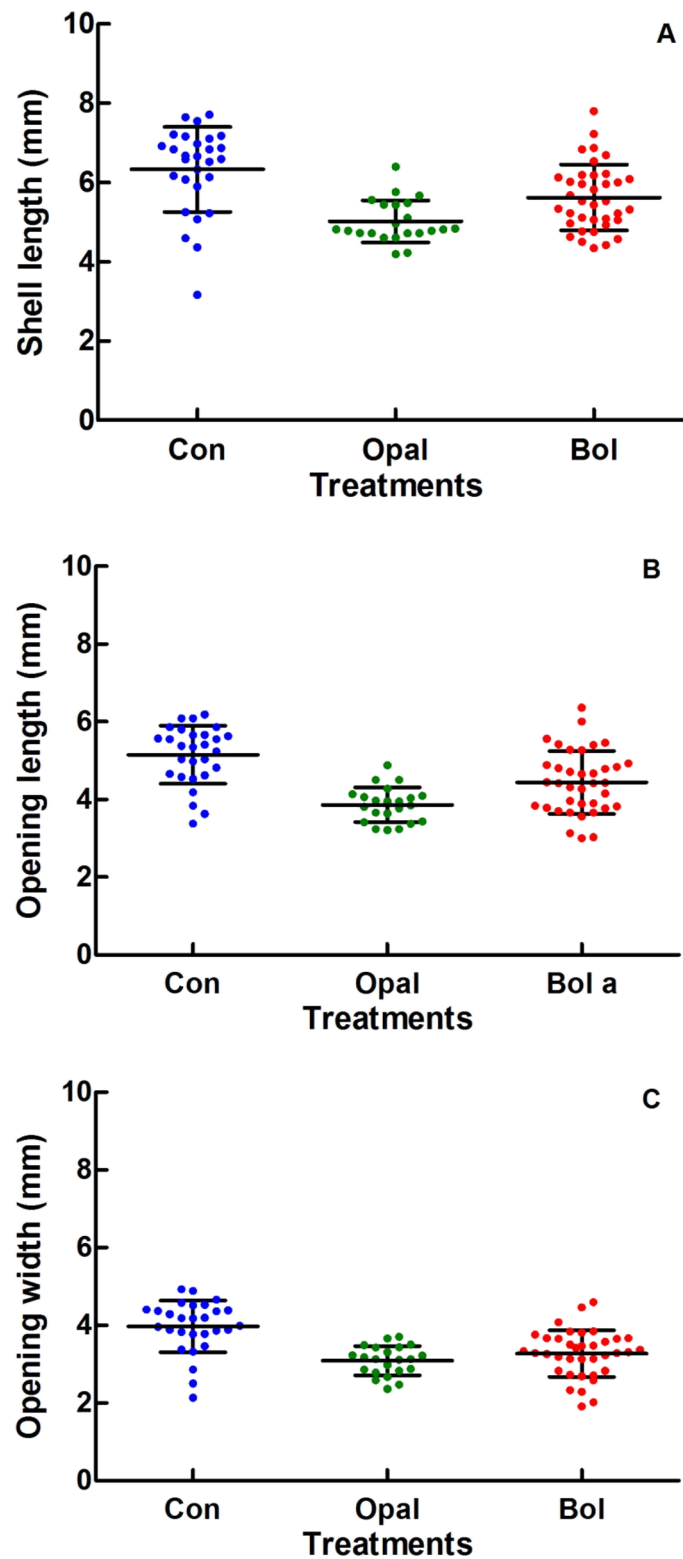


Figure 4.9: The dimensions of the shell of each adult snail after being exposed to cotton leaves. (A) The length of the entire shell, from the opening to the apex. (B) The length of the opening of the shell, and (C) the width of the opening. Means and standard deviations are indicated.

4.2.4 Fecundity

The number of days it took until the first egg packet was noted within each container (denoting at least two snails becoming sexually mature) during the cotton experiment is presented in Table 4.9. It must be noted that only in one of the five containers of the Opal treatment did at least two snails reach sexual maturity (Table 4.9). Unfortunately this resulted in very few data points for the Opal treatment.

Table 4.9: The number of days it took for the first egg packet to appear (therefore at least two snails reaching sexual maturity) in each of five containers of the cotton experiment.

	Days/container			
Con	22	26	32	
Opal	39			
Bol	20	24	33	31

Due to the lack of data for Opal in the cotton leaf exposure the data presented from hereon had to be adjusted for each treatment to represent the same population size.

The number of egg packets found during the cotton leaf exposure is shown in Figure 4.10. Scatterplots were less instructive due to the low numbers of survivors (Table 4.11) in the leaf treatments and box and whisker plots were used. Significant fewer egg packets were observed ($p < 0.0001$, t-test, unpaired, two-tailed) when the snails were exposed to the Cry proteins (Bol) compared to the non-Bt treatment (Opal). Again, the few survivors precluded any conclusive interpretations.

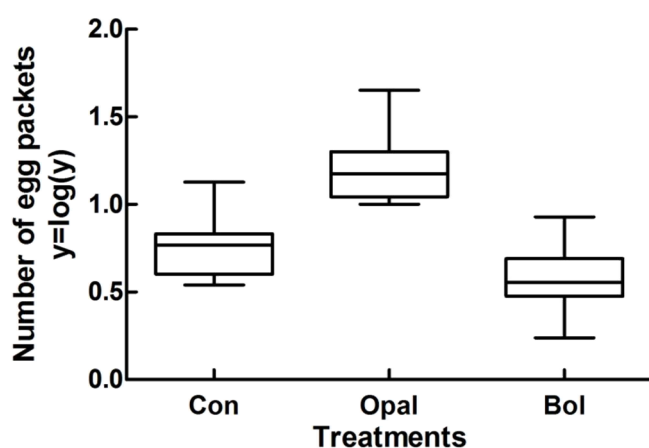


Figure 4.10: The number of egg packets produced during the cotton leaf exposure (ANOVA, $p < 0.0001$). Means, the 5 – 95 percentiles, and range are indicated.

Figure 4.11 shows the total number of eggs (the sum of all individual eggs in all packets) produced for each treatment per container during the cotton leaf exposure. Significantly fewer eggs per container were found in Bol (ANOVA, $p < 0.0001$), than in both Con and Opal.

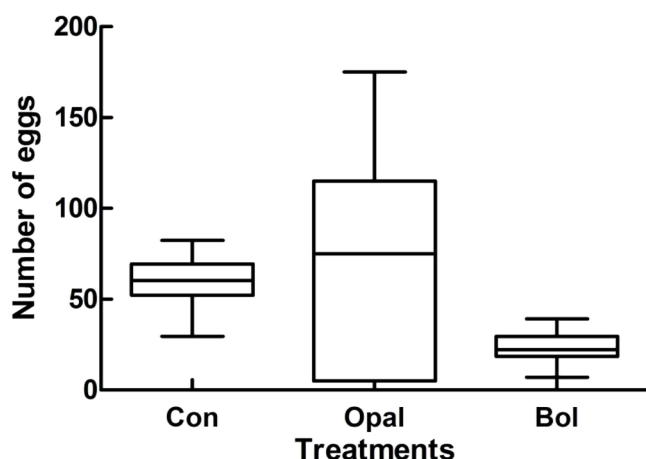


Figure 4.11: The total number of eggs produced in the cotton leaf experiment (ANOVA, $p < 0.0001$). Means, range, and 5 – 95 percentiles are indicated.

The number of eggs per egg packet (represented in Figure 4.12) had a clear leaf-effect. Con had significantly more eggs per packet ($p < 0.0001$, t-test, unpaired, two-tailed) than the treatments fed only cotton leaves, which did not differ significantly with each other (Table 4.10). Opal had a larger range of eggs per egg packet than Bol.

Table 4.10: T-test (two-tailed, unpaired) comparison of treatments and control exposed to cotton leaves, of the number of egg packets, eggs, and eggs per packet.

	Egg packets	Total eggs	Eggs/packet
Con vs. Opal	$p < 0.0001$	0.6970	$p < 0.0001$
Con vs. Bol	0.0039	$p < 0.0001$	$p < 0.0001$
Opal vs. Bol	$p < 0.0001$	0.0106	0.4737

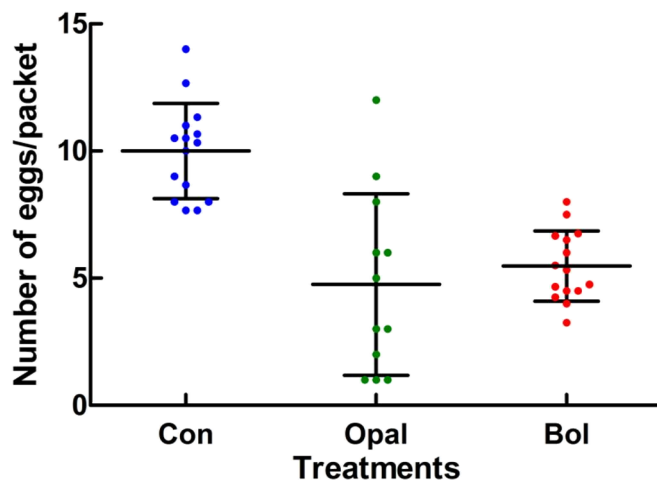


Figure 4.12: The number of eggs per packet in each treatment exposed to cotton leaves (ANOVO, $p < 0.0001$). Means and standard deviations are indicated.

4.2.5 Survival after hatching

Not all snails that hatched made it to the termination of the experiments (Table 4.11 and 4.18). Although it is called 'survival', it is probably mainly due to loss of small individuals during water changes and when leaves were changed. The hatchlings are very small when hatched (less than 1 mm in length), are difficult to detect, and easily dislodged when changing water and leaves. In the treatments with leaves, although care was taken to recover all snails, small snails probably often escaped detection when changing leaves and water. Because of this complication, survival could not be used as an end point. As will be seen later, the carry-over of snails during media changes (water and leaf changes) may unintentionally have resulted in selection of the more visible and larger snails, skewing the data (probably equally for all treatments). Future experiments will need to take this effect into account.

Table 4.11: The number of hatchlings, the number of snails euthanized, and the percentage survival during the cotton experiment.

	<i>n</i> hatched	<i>n</i> dissected	% survived
Con	185	28	15
Opal	105	22	21
Bol	125	37	30

4.2.6 Male reproductive organs

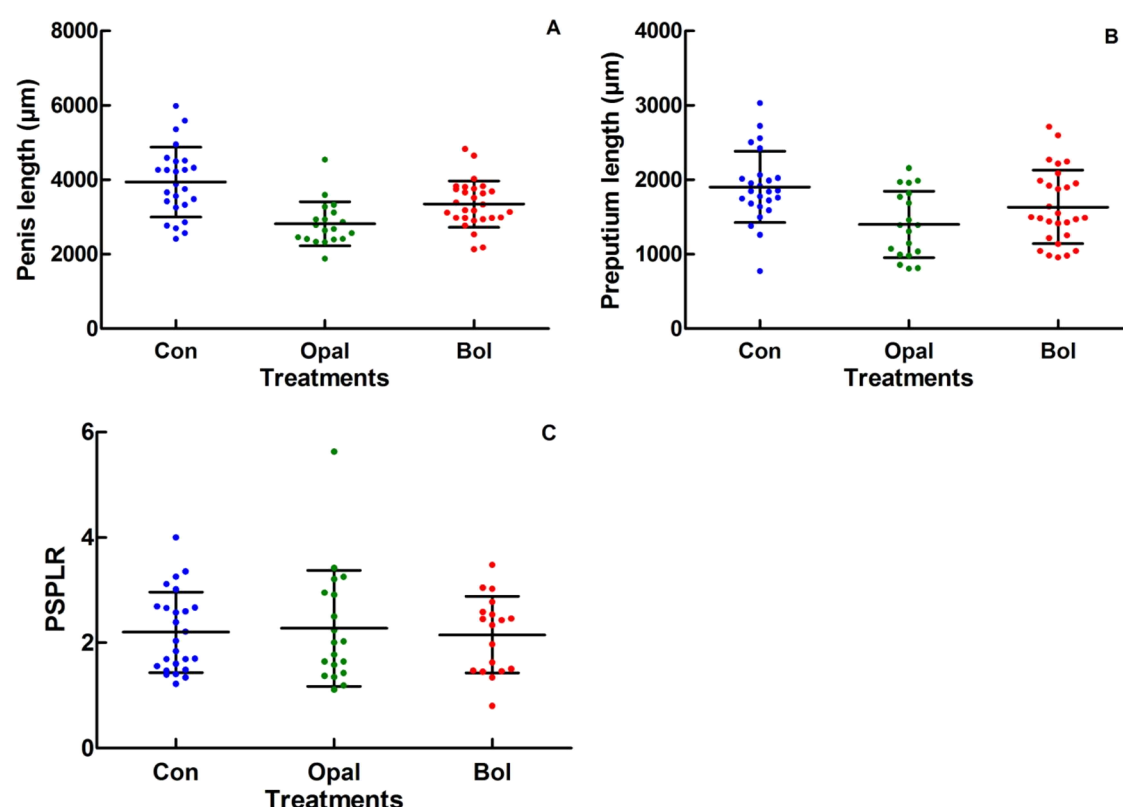


Figure 4.13: The cotton leaf experiments' measured male sex organs measurements. The penis (A) and preputium (B) length is used to calculate the penis sheath-preputium ratio (C). Means and standard deviations are indicated.

The PSPLR (penis sheath-preputium length ratio) is derived from the length of the penis and preputium and the results are represented in Figures 4.13A and B. A significantly shorter penis (Table 4.12) was found for both Opal and Bol compared to Con. Opal also showed a significantly shorter preputium ($p=0.0010$, t-test, unpaired, two-tailed) compared with fish food reared Con. Bol only showed a slightly shorter preputium length, but the ratio of penis sheath and preputium length stayed constant throughout all tree treatments (Figure 4.13C).

Table 4.12: T-tests (two-tailed, unpaired) results comparing different cotton treatments' penis and preputium lengths, and the resulting penis sheath-preputium length ratio.

	PS	PPS	PSPLR
Con vs. Opal	$p<0.0001$	0.0010	0.7869
Con vs. Bol	0.0088	0.0517	0.8435
Opal vs. Bol	0.0055	0.1047	0.6944

Figure 4.14 shows the PCA ordination of the measurements of the penis sheath and preputium complex and the shell dimensions. The distribution of treatments (convex hulls) overlaps quite well. The vectors D1, D2, D3, PS, PS1, PS2, and PP1 are co-variant (all pointing in the same direction). The penis length shows a strong association with the length of the shell (D1), the length of the opening (D2), and the width of the opening (D3). The length of the penis had no association with the length of the preputium (PPS) and vice versa. The length of the preputium did however, had an influence on the PSPLR ratio of each treatment. The opposing PPS and PSPLR vectors indicate that a larger PSPLR is due to a shorter preputium. Differences in preputium length rather than penis length therefore affected the ratio. The PSPLR vector is oriented perpendicular to the covariate shell dimensions, indicating that the ratio is not affected by the shell dimensions. The three convex hulls representing the treatments overlap to quite a large extent, indicating that there were no marked differences between treatments based on the morphometrics of the shell and male reproductive organs. Note should be taken however, that many small snails were lost during the experiment, and that there may have been unintentional selection for the larger snails during media changes.

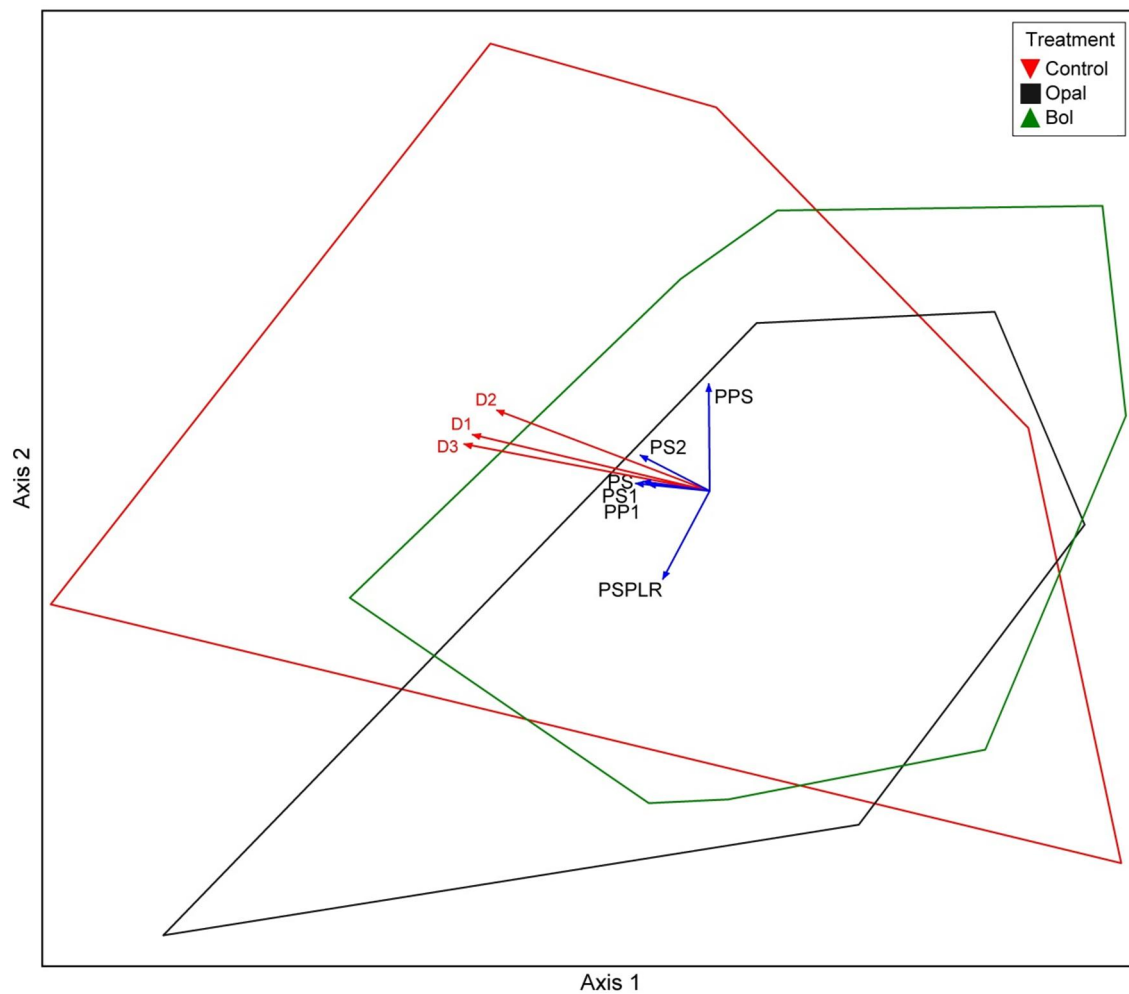


Figure 4.14: PCA bi-plot showing the interactions of all the different (relativised) measurements of the male sex organs and the dimensions of the snails' shells in the cotton leaf experiment. Where PS = Penis Length; PPS = Preputium Length; PS1 = Width of the penis at the thinnest section; PS2 = The width of the penis at the thickest section; PP1 = The width of the preputium at the thickest section; PSPLR = The penissheath-preputium length ratio; D1 = Length of the shell, from the opening to the apex; D2 = The length of the opening of the shell; D3 = The width of the shell opening. Convex hulls for each treatment are indicated. Axis 1 explained 47% of the variation, and axis 2, 28%.

4.3 Maize

4.3.1 Embryo growth

The embryos exposed to maize leaves showed very little retarded growth or hatching for the earlier part of their growth (Figure 4.15). Linear regression growth patterns fitted all treatments, with the regression lines having R^2 of 0.8 or better (Table 4.13, goodness of the fit R^2).

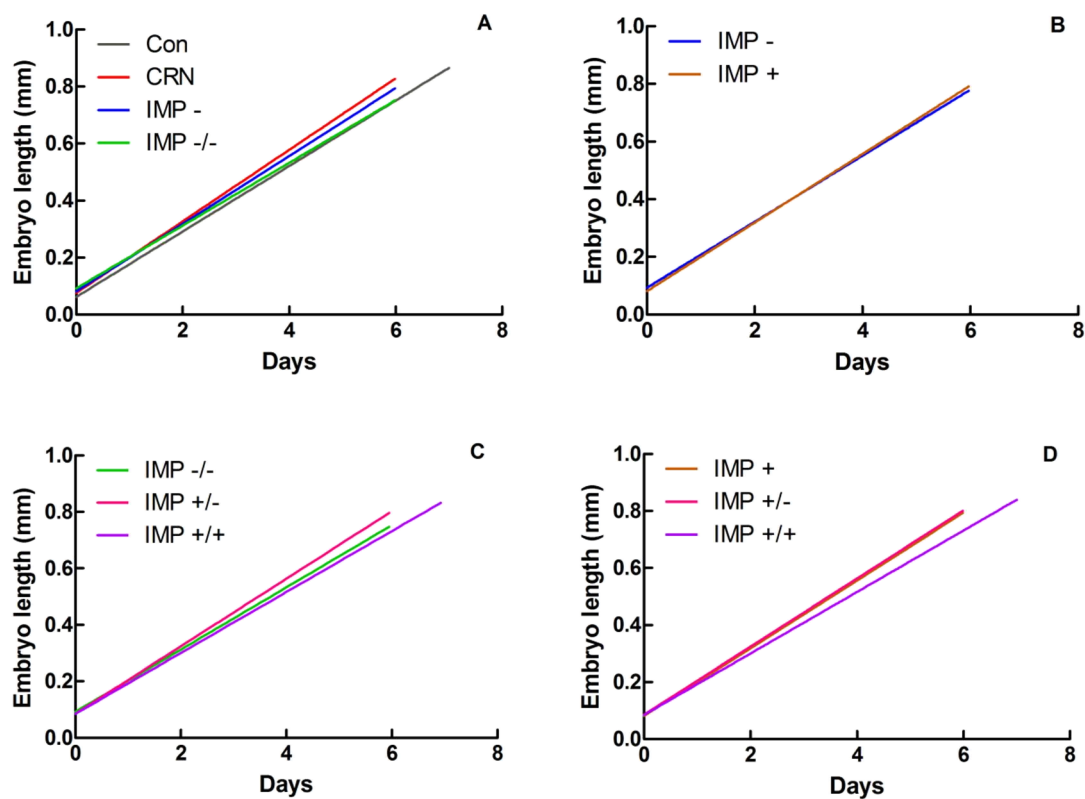


Figure 4.15: Linear regressions of the growth of the embryos exposed to maize leaves. (A) Leaf effect control; (B) low Bt concentrations; (C) high Bt concentration; and (D) Bt-exposed grouping. Means and standard deviations are indicated.

Table 4.13: Standard deviation (SD) and the goodness of fit of the regressions fitted to maize leaf reared embryo growth (R^2).

	SD	R^2
Con	0.29	0.94
CRN	0.28	0.90
IMP-	0.24	0.91
IMP+	0.27	0.91
IMP-/-	0.23	0.82
IMP+/-	0.25	0.91
IMP+/+	0.26	0.92

The leaf-treated embryos were significantly larger by day three (Figure 4.16) compared to Con (ANOVA, $p=0.0003$). CRN further outgrew Con and IMP -/- significantly on day four (ANOVA, $p<0.0001$, followed by Bonferroni's multiple comparison test, $p<0.05$). The embryos exposed to the Cry protein infusions (IMP+/- and IMP+/+) were significantly larger compared to the non-Bt infusion IMP-/- (ANOVA, $p<0.0001$, followed by Bonferroni's multiple comparison test, $p<0.05$) on the fourth day. Hatching began on day six for all treatments, resulting in an artifactual reduction in remaining embryo lengths; all treatments except the embryos in one container of IMP+/+ hatched by the seventh day.

The embryos exposed to the non-Bt maize leaves infusion were significantly smaller on the day prior to hatching (Figure 4.17) compared to the Bt infusions (ANOVA, $p<0.0001$, followed by Bonferroni's multiple comparison test, $p<0.05$), and compared to the control treatments (Con, CRN and IMP-) (ANOVA, $p<0.0001$, followed by Bonferroni's multiple comparison test, $p<0.05$).

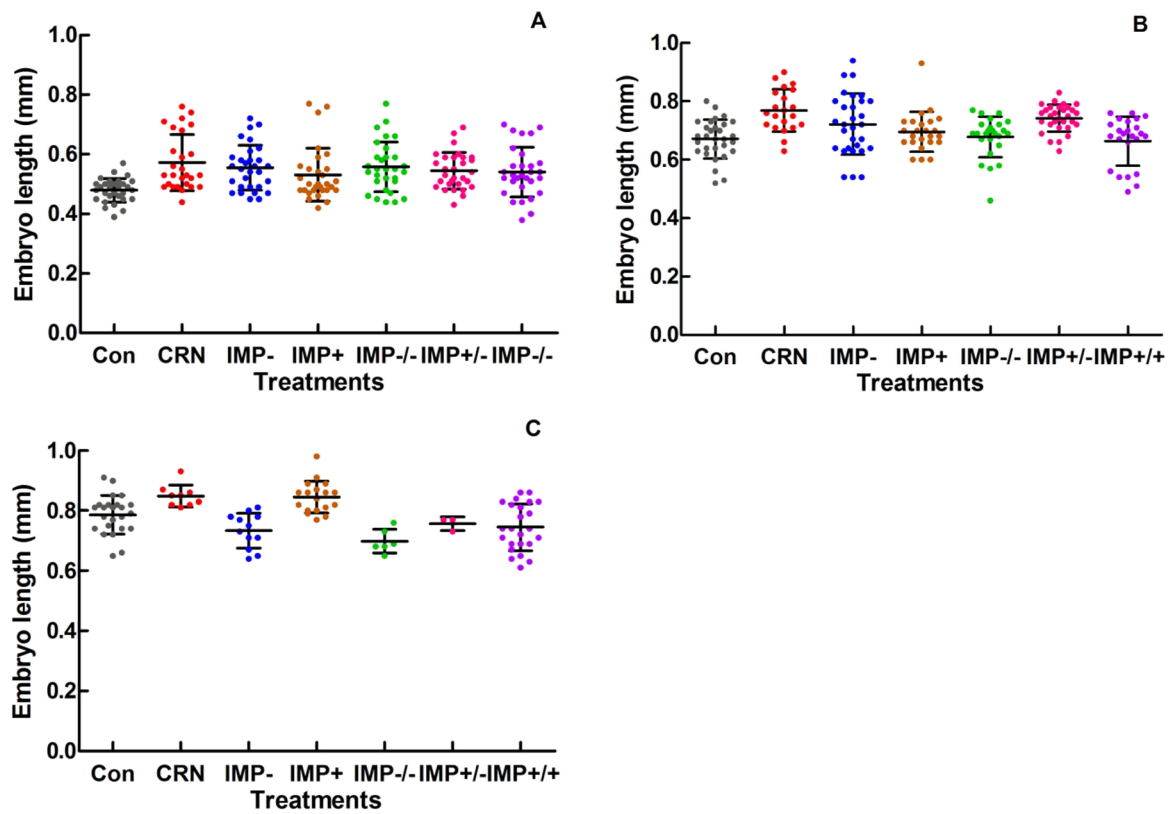


Figure 4.16: Lengths of the maize-fed embryos on a specific day of development; (A) day 3; (B) day 4; and (C) day 5. Means and standard deviations are indicated.

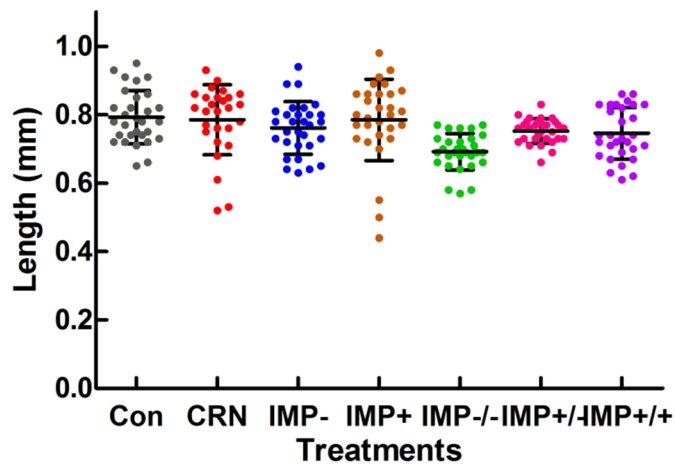


Figure 4.17: The lengths of the embryos in the maize leave experiment one day prior to hatching (ANOVA, $p < 0.0001$). Means and standard deviations are indicated.

4.3.2 Hatching success

The hatching percentages of the embryos exposed to maize leaves were much better than for cotton (Figure 4.18). All treatments had a hatching success of more than 80%. The control treatment had 92% of the embryos hatched, compared to 82% for cotton. The lowest hatching percentage was found in IMP+/- (88%) and IMP+ (89%) respectively. However the treatment with the highest hatching percentage of embryos were also exposed to the highest concentration of Bt (IMP+/+). No significant difference could be discovered between treatments using chi-square tests (Table 4.14).

Table 4.14: Chi-square comparison of the percentage hatched from the maize exposure.

	ANOVA	Chi-square p-value
Leaf effect	0.989	0.828
Low conc.	0.971	0.578
Infusion	0.996	0.805
Bt	0.989	0.615

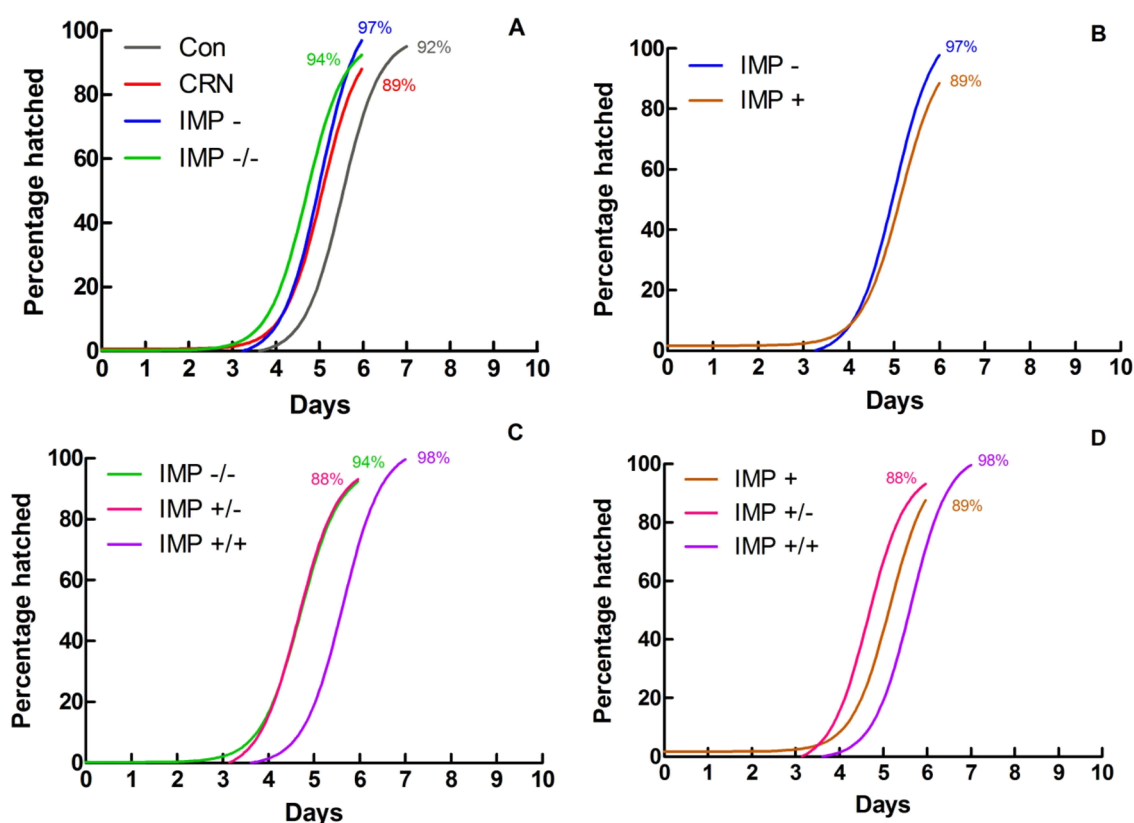


Figure 4.18: The cumulative percentage of eggs that eventually hatched from the maize experiment, fitted to a sigmoidal dose-response curve. (A) Leaf effect control; (B) low concentrations; (C) high concentration; and (D) Bt exposed grouping.

All the leaf treatments, except for IMP+/+, had a significantly shorter embryo period than Con (Table 4.15; Non-parametric Kruskal-Wallis ANOVA, $p < 0.0001$). No significant difference in hatching time were found between Con and IMP+/+ (Table 4.15). Both non-Bt leaf treatments CRN and IMP- hatched significantly faster than the Bt-infusion treatments IMP+/- and IMP+/+. The non-Bt infusion IMP-/- were significantly quicker to hatch than IMP+/+ (Table 4.15).

Table 4.15: The mean number of days it took the embryos to hatch, and the non-parametric Dunn's multi comparison of the means, during the maize experiment.

	Con	CRN	IMP-	IMP+	IMP-/-	IMP+/-	IMP+/+
Mean	6.964	6.35	6.438	6.35	6.109	6.114	7.072
Con		$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p > 0.05$
CRN	$p < 0.05$		$p > 0.05$	$p > 0.05$	$p > 0.05$	$p < 0.05$	$p < 0.05$
IMP-	$p < 0.05$	$p > 0.05$		$p > 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$
IMP+	$p < 0.05$	$p > 0.05$	$p > 0.05$		$p < 0.05$	$p < 0.05$	$p < 0.05$
IMP-/-	$p < 0.05$	$p > 0.05$	$p < 0.05$	$p < 0.05$		$p > 0.05$	$p < 0.05$
IMP+/-	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p > 0.05$		$p < 0.05$
IMP+/+	$p > 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	

4.3.3 Growth after hatching

The growth of the snails (change in length) reared on maize leaves are shown in Figure 4.19. Growth trajectories of Con, IMP-/- and IMP+/+ were best fitted with a linear regression (Table 4.16). The remaining treatments had rapid increasing lengths after hatching which slowed as they neared sexual maturity, resulting in a non-linear regression as best fit.

The non-Bt leaf control treatments IMP- and IMP-/- had slightly slower (but not significant) growth than their Bt iso-line (ANOVA, $p=0.992$). The low concentration Bt treatment (IMP+) also showed a slight reduction in growth compared to the Bt infusion treatments (ANOVA, $p=0.997$).

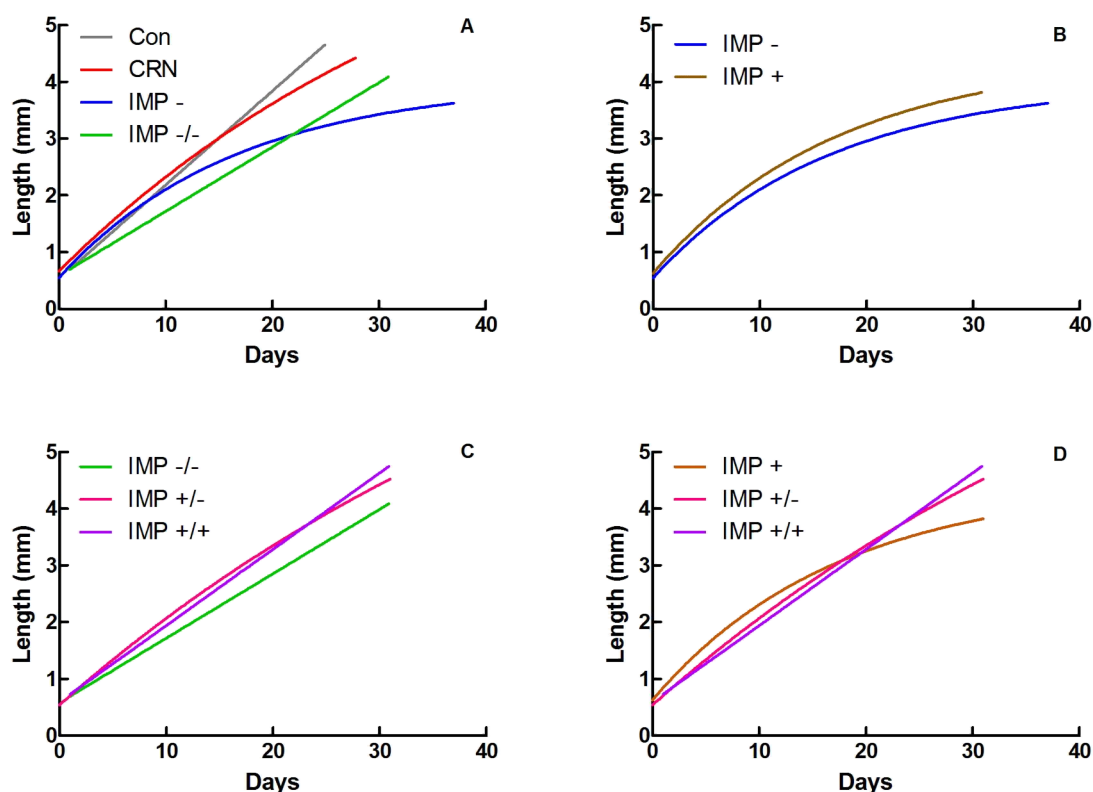


Figure 4.19: Regressions of the change in length of the hatched snails exposed to maize leaves grouped as (A) a leaf effect control; (B) low concentration exposure; (C) high concentration exposure; and (D) Bt-leaf only exposure.

Table 4.16: The best (R^2) curve fitted to the growth of snails exposed to maize leaves, and the standard deviation (SD).

	Fit	R^2	SD
Con	Linear regression	0.84	1.37
CRN	One phase association	0.80	1.16
IMP-	One phase association	0.65	0.90
IMP+	One phase association	0.83	0.97
IMP-/-	Linear regression	0.81	1.13
IMP+/-	One phase association	0.84	1.22
IMP+/+	Linear regression	0.85	1.30

Figure 4.20A shows no differences in the length of the hatched snails between Bt and non-Bt treatments in the first seven days. After 19 days though, both of the Bt infusion treatments were significantly larger than IMP-/- (ANOVA, $p < 0.0001$, followed by Bonferroni's multiple comparison test, $p < 0.05$) (Table 4.17). The initial increased growth of CRN is seen in Figure

4.20A where CRN snails are significantly larger than Con snails (ANOVA, $p < 0.0001$, followed by Bonferroni's multiple comparison test, $p < 0.05$). Later, by day 19, that initial increase (also see Figure 4.19) was replaced by a slower rate of growth allowing Con to catch up.

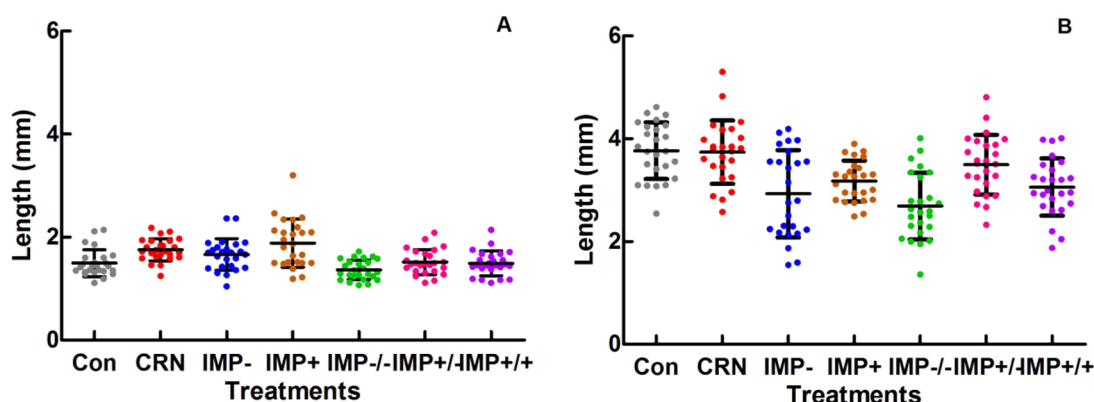


Figure 4.20: The lengths of *B. tropicus* during the maize leaf exposure at (A) day 7, and (B) day 19. Means and standard deviations are indicated.

Table 4.17: One-way ANOVA results comparing the length of the snails exposed to maize at different ages. The groupings are as follows: Leaf effect = Con, CRN, IMP-, and IMP-/. Low conc. = IMP- and IMP+. Infusion = IMP-/-, IMP+/-, and IMP+/+. Bt = IMP+, IMP+/-, and IMP+/+.

	Day 7	Day 19
Leaf effect	$p < 0.0001$	0.164
Low conc.	0.052	0.195
Infusion	0.405	0.011
Bt	$p < 0.0001$	0.195

Figure 4.21 shows the shell dimensions of the snails. A significant smaller size was observed when the snails were reared on maize leaves (ANOVA, $p = 0.0236$), coinciding with the effect seen in Figure 4.9. The low concentration Bt exposed snails (IMP+) showed a significant smaller size compared to IMP- ($p < 0.0001$, t-test, unpaired, two-tailed). This effect in IMP+ was also seen in the mean shell opening dimensions which were significantly smaller than IMP- (opening length, t-test, $p < 0.0001$; opening width, t-test, $p = 0.0005$). Although Con snail shell dimensions were significantly superior in shell length, no difference in the mean opening length or width were found compared to the other non-Bt leaf treatments (opening length ANOVA, $p = 0.6128$; opening width ANOVA, $p = 0.6663$). No significant difference was found between non-Bt treatments Con, CRN, IMP- and IMP-/- (ANOVA). Con had the largest range in any dimension in any of the treatments, but this was probably due to the larger number of survivors of 70 snails (Table 4.18).

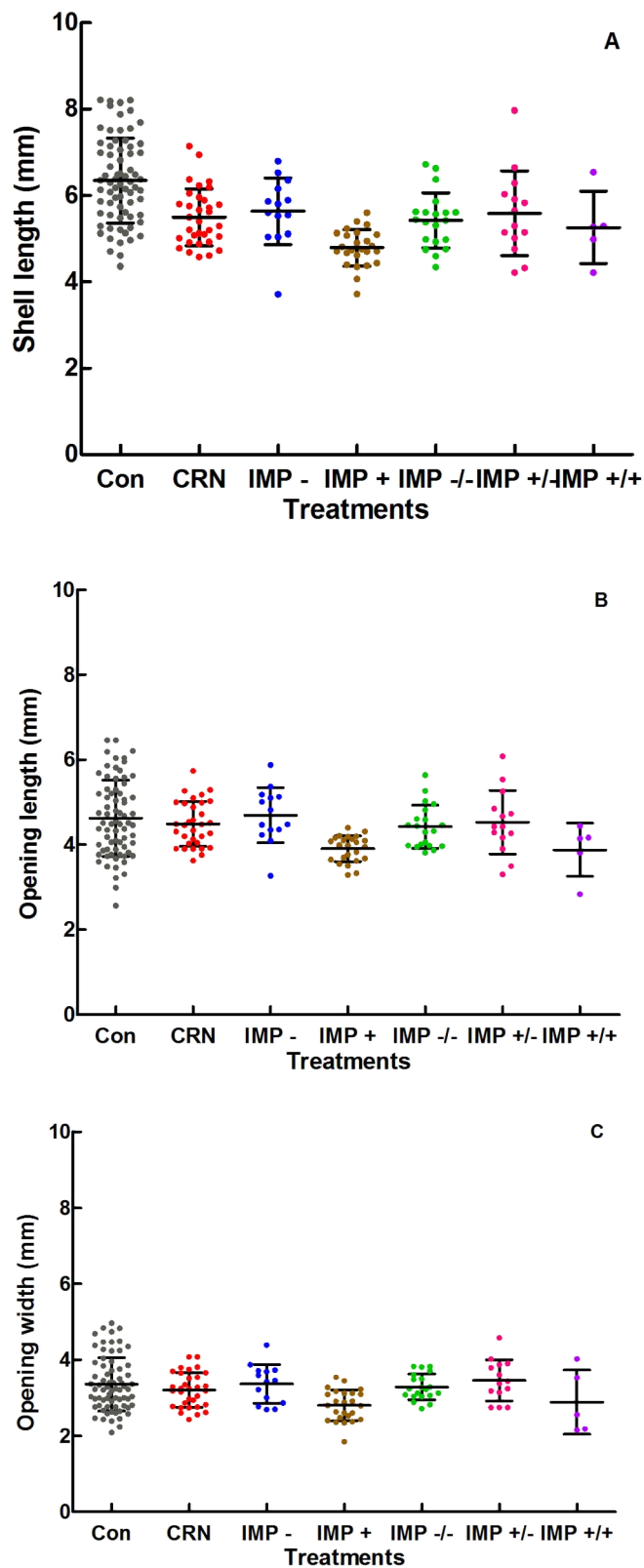


Figure 4.21: The dimensions of the shells of the adult snails after the maize exposure. (A) The length of the shell from the front of the opening to the apex, (B) the length of the opening, and (C) the width of the opening. Means and standard deviations are indicated.

4.3.4 Fecundity

The age at which the first two snails in each container from the maize experiment reached sexual maturity (indicated by the appearance of the first egg packet) is shown in Figure 4.22. The data could be graphically presented because all the treatments had snails in three or more containers that laid egg packets. Con reached sexual maturity well ahead of those fed non-Bt IMP leaves (ANOVA, $p=0.0044$; followed by Bonferroni's multiple comparison test, $p<0.05$). No significant differences were found in age of attaining sexual maturity for Con and CRN, nor for the infusion treatments (ANOVA). IMP+ did reach sexual maturity significantly faster than IMP- ($p=0.038$, t-test, unpaired, two-tailed). The small number of remaining snails in some cases makes interpretation difficult.

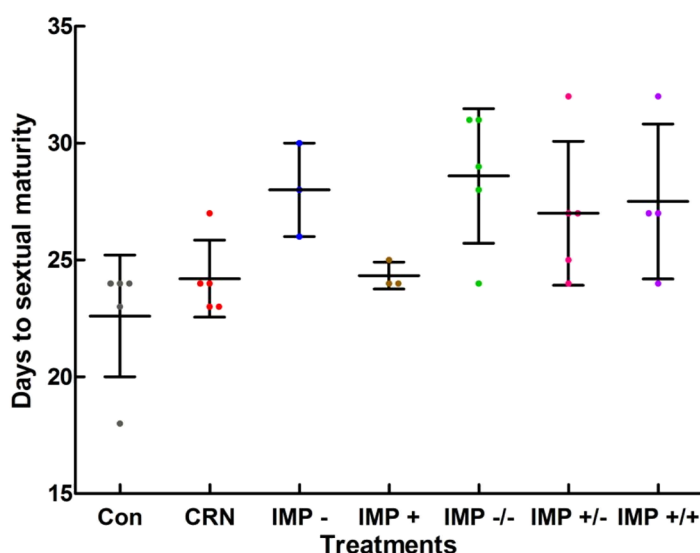


Figure 4.22: The age of the snails when sexual maturity was reached in the maize experiment. Means and standard deviations are indicated.

The number of egg packets found within each treatment (Figure 4.23) showed significantly fewer egg packets per container for the Bt-exposed IMP+ and IMP+/+ when compared to the non-Bt isoline IMP- and IMP-/- (ANOVA, $p<0.0001$, followed by Bonferroni's multiple comparison test, $p<0.05$). Although not statistically significant, there were also fewer egg packets produced in the IMP+/- treatment when compared with the water infusion control IMP-/- ($p=0.1569$, t-test, unpaired, two-tailed). CRN had significantly fewer egg packets than IMP- ($p<0.0001$, t-test, unpaired, two-tailed).

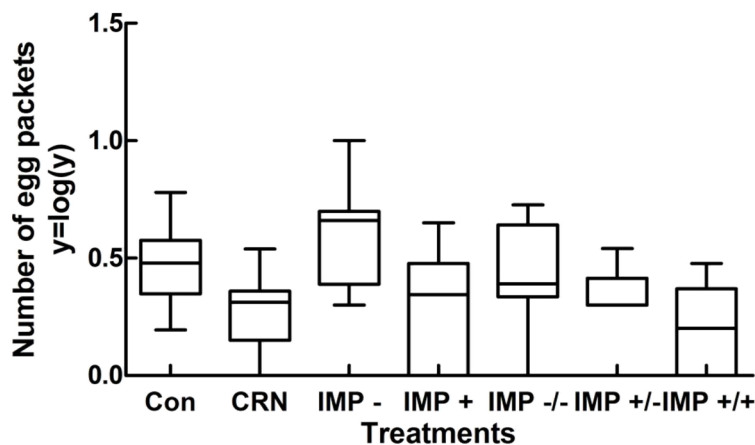


Figure 4.23: The number of egg packets produced in each maize treatment (ANOVA, $p < 0.0001$). Means, range, and 5 – 95 percentiles are indicated.

The total number of eggs produced in each treatment (Figure 4.24) of the maize leaf exposure showed a trend similar to the cotton leaf experiment (Figure 4.11). Fewer eggs were observed in both Bt-exposed treatments, IMP+ and IMP+/, compared to their corresponding non-Bt treatments, IMP- and IMP-/- (ANOVA, $p < 0.0001$). The same tendency was visible in the number of egg packets found for each of these treatments shown in Figure 4.25.

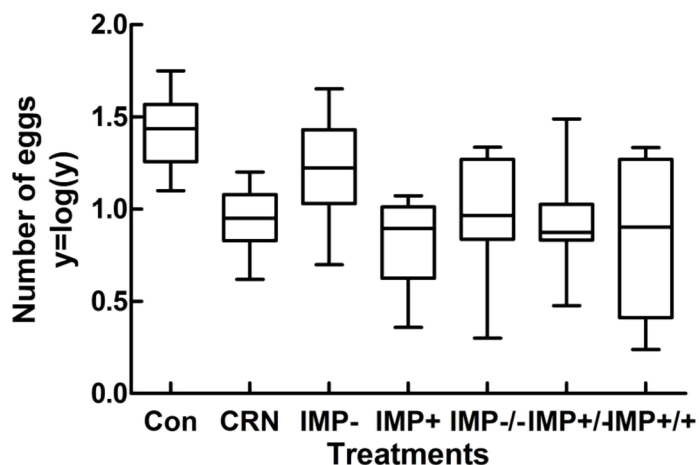


Figure 4.24: The total number of eggs produced in each treatment in the maize exposure (ANOVA, $p < 0.0001$). Means, range, and 5 – 95 percentiles are indicated.

The number of eggs per packet, in Figure 4.27, shows a clear effect of the presence of the leaves in the water; a leaf-effect that negated the comparative power of the control

treatment. The control (Con) treatment, fed only on high quality fish food, had more eggs per packet than any of the treatments reared on maize leaves only (ANOVA, $p < 0.0001$). However, there were no differences (using t-tests) in the number of eggs per packet for any logical pairings (e.g. IMP- and IMP+) of the leave treatments. It should be noted though, that IMP+/+ (the treatment with notionally the largest exposure to Bt from both leaves and infusion) had a noticeably larger range in the numbers of eggs per packet.

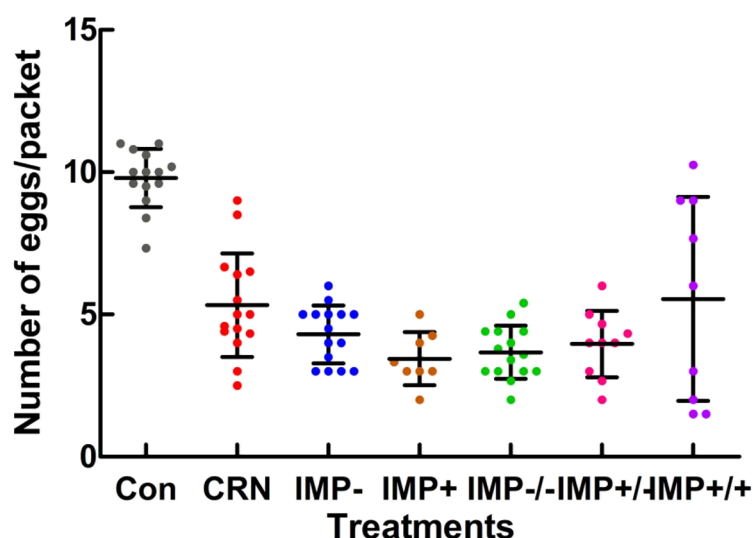


Figure 4.25: The number of eggs per egg packet yielded per treatment in the maize experiment. Means and standard deviations are indicated.

4.3.5 Survival after hatching

Table 4.18: The number of hatchlings, the number of snails euthanized, and the percentage survival during the maize experiment.

	<i>n</i> hatched	<i>n</i> dissected	% survived
Con	169	70	41
CRN	117	33	28
IMP-	144	14	10
IMP+	143	25	17
IMP-/-	137	20	15
IMP+/-	123	14	11
IMP+/+	138	5	4

As mentioned in section 4.2.4, survival could not be measured as an endpoint due to the experimental maintenance constraints. There was only five survivors for IMP+/+, and only 14

snails for both IMP+/- and IMP- (Table 4.18). The percentage of the population lost due to the water change cycle is uncertain.

4.3.6 Male reproductive organs

A slightly shorter penis length can be observed for IMP+ compared to IMP- (Figure 4.26), but a longer penis is seen in the Bt infusion treatments compared to IMP-/-. The length of the preputium in CRN, IMP- and IMP+ were significantly shorter than Con (ANOVA, $p=0.0066$, followed by Bonferroni's multiple comparison test, $p<0.05$).

No significant differences were found for the PSPLR, but it must be stressed that the numbers of survivors available to determine the PSPLR was very small in some treatments (Table 4.18).

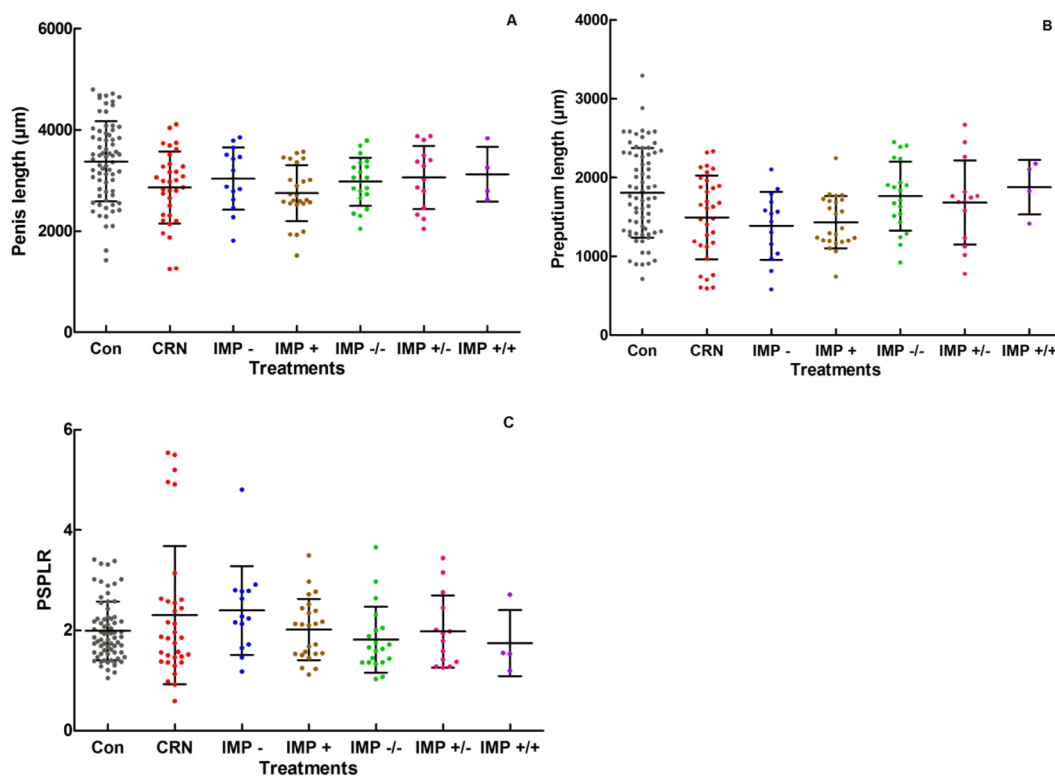


Figure 4.26: The male sex organs proportions from the snails exposed to maize leaves. The penis sheath-preputium length ratio (C) is calculated from the length of the penis (A) and the preputium (B). Means and standard deviations are indicated.

The PCA ordination for the shell and male reproductive organ morphometric data (Figure 4.27 using relativised data) showed overlapping convex hulls for all treatments. The effect of the small data set for IMP+/+ is clearly seen in its resulting small convex hull. The treatments therefore had no effect on the relative shell dimensions. The same pattern of dimensional

associations as with the cotton data can be seen. Again, as in the cotton experiment, only PPS did not co-vary with the other morphometrics (PPS vector perpendicular to the other morphometric vectors). The length of the preputium again had a greater influence on the PSPLR than the length of the penis. As for the cotton experiment, the snails eventually reaching the end of the experiment may have been the larger surviving snails.

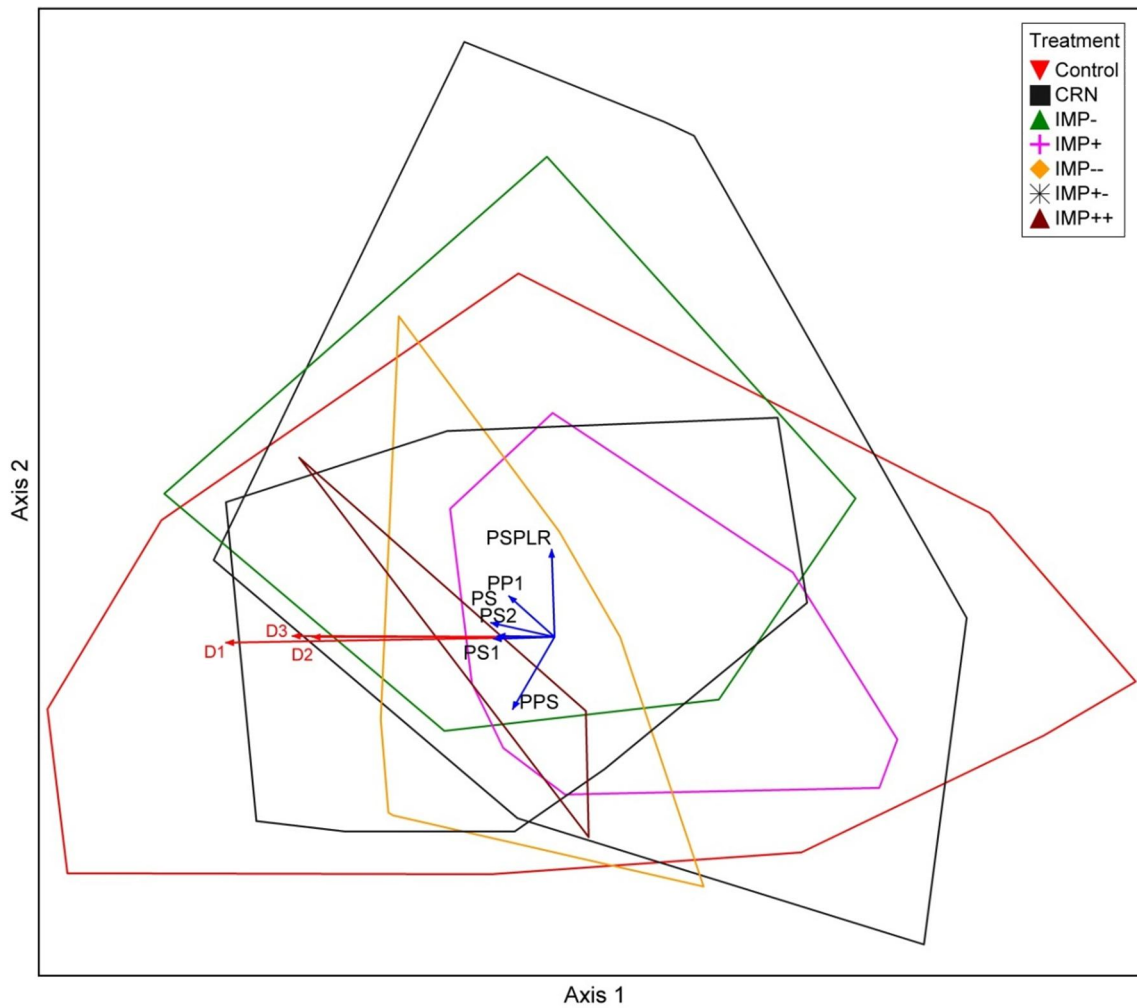


Figure 4.27: PCA bi-plot of the interactions of the (relativised) morphometrics of the male sex organs and the dimensions of the snail's shells from the maize leaf experiment. Where PS = Penis Length; PPS = Preputium Length; PS1 = Width of the penis at the thinnest part; PS2 = The width of the penis at the thickest section; PP1 = The width of the preputium at the thickest section; PSPLR = The penis sheath-preputium length ratio; D1 = Length of the shell, from the opening to the apex; D2 = The length of the opening of the shell; D3 = The width of the shell opening. Convex hulls for each treatment are indicated. Axis 1 explained 41%, and axis 2, 30% of the variation.

4.4 Supplemental fecundity metrics for both experiments

Although it is possible to calculate the number of egg packets and eggs per snail (Figure 4.28 and 4.29), the results suggest that not all snails reached sexual maturity and the basis of the calculation, the number of snails irrespective of sexual maturity, does not produce valid results. Because the eventual number of snails per container was not recorded, only one value per treatment is possible and presented here for sake of completeness and illustrating experimental improvement options in the discussion.

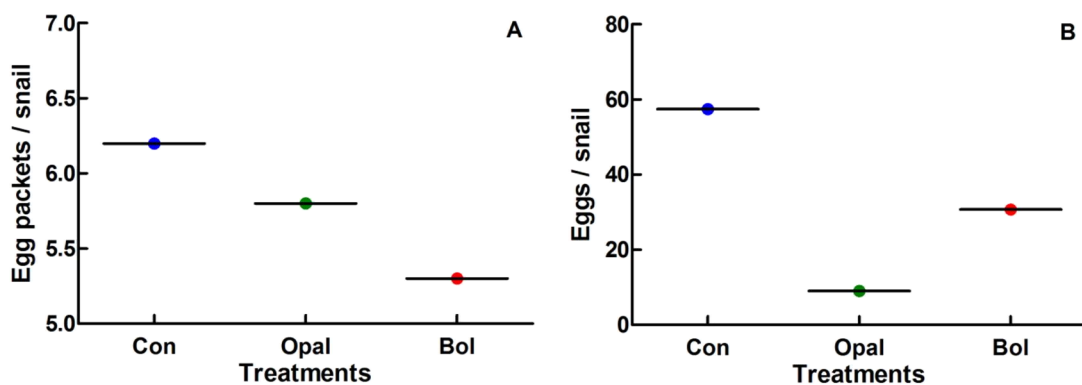


Figure 4.28: The number of egg packets (A) and the number of eggs (B) per snail in the cotton experiment.

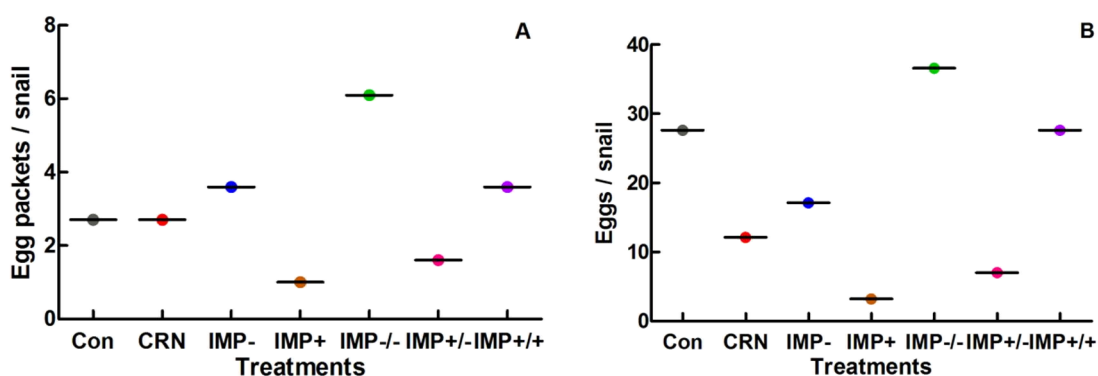


Figure 4.29: The number of egg packets (A) and the number of eggs produced per snail during the maize experiment, for each treatment.

4.5 Flow diagram summaries

Because of the complex nature of interpreting different metrics over a generation, the results are summarised in flow diagrams (Figures 4.30 and 4.31).

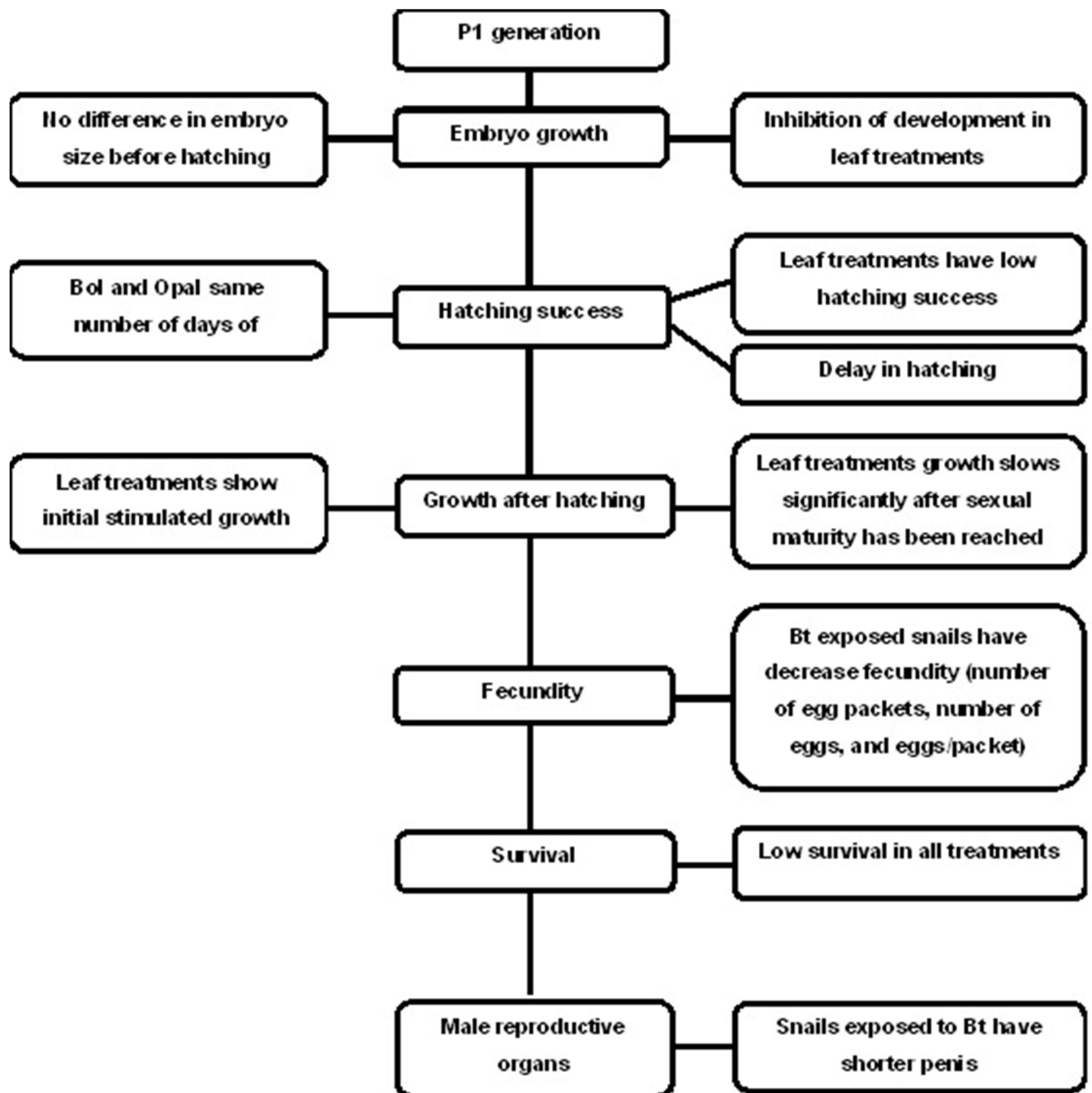


Figure 4.30: The summarized flow diagram of all Bt effects on the generation *B. tropicus* used in the cotton exposure.

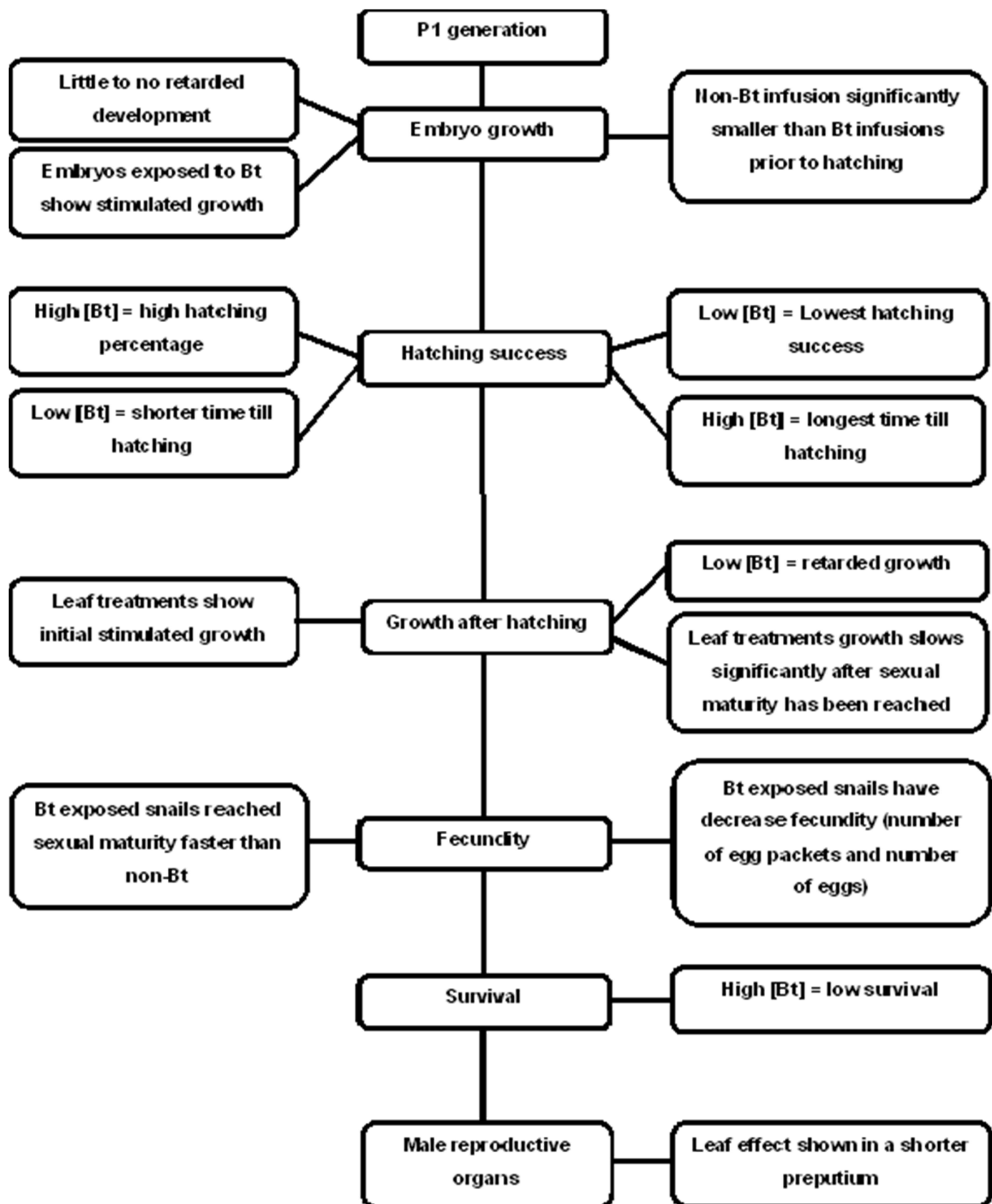


Figure 4.31: A summary of the major effects of Bt on the snails in the maize exposure.

Chapter 5: Discussion

This study was the first to explore the effects of Bt crop residues on freshwater snails. No other study has used any aquatic molluscs as bio-indicator for GM crop residues. This was also the first time *B. tropicus* was used for ecotoxicological exposure studies. The results from this study are therefore difficult to compare to earlier published work. A higher level integration with theory will be done in Chapter 6.

5.1 Concentration of Bt toxins

The water samples taken during the course of the experiments were analyzed using an ELISA kit (see section 3.3). Unfortunately, the detection limit (LOD) of the ELISA kit was 1 ng/ml, and the samples sent for analyses were all below the LOD. Therefore, it was necessary to calculate the concentrations from the results that Harvey (2013) reported on the Cry1Ab protein in maize leaves, the same leaves as used in the present study, and determined in the same year and laboratory. The rate and concentration of the Cry1Ac protein leaching from Bt into water has, to our knowledge, not yet been determined. No mention of it could be found in the literature and future studies based on exposure would have to include Bt concentration determinations. The average concentration of the Cry1Ab protein in stream water near farmlands reported by Tank *et al.* (2010) was 0.014 ng/ml (see section 2.1.4). Within eight hours of placing 50 cm² of maize leaves in 900 ml water for the present experiment, the same concentration (calculated) of Cry1Ab proteins as measured by Tank *et al.* (2010) for their environmental samples was reached (Figure 4.1). After eight hours, the concentration of Cry proteins in laboratory water reached steady state (a flattening of the concentration curve) maintaining a concentration close to an environmentally relevant concentration, making the results of this study relevant to real world scenarios.

The water in each container was changed on a 96 hour cycle (see section 3.1). Thus a near-continuous exposure concentration for the duration of this study corresponded with environmentally relevant concentrations. Very few studies dealing with the effects of Bt reported any data on the concentration of Cry proteins the test organisms were subjected to, making it difficult to compare the effects seen from this study to literature. However, due to the concentrations found in this study being so close to that found in the environment, any effect seen here could be present in the natural systems as well.

The experimental design also had infusion treatments of maize leaves. This means that Bt concentrations in the water would be higher in some treatments. Since these treatments had two day-old water infusions with each water change, a concentration of Bt would at least

have been roughly continuous in $+/+$ (Bt leaves/Bt leaf infusion) treatments. Therefore the Bt $+/+$ embryos and snails would have been exposed for longer to plateau Bt concentrations than Bt $+/-$. There were no $-/+$ treatments as a scenario where non-Bt maize leaves would enter water that contains Bt in solution was deemed too occur seldom.

In the absence of data for cotton, a similar release pattern and an approximate Cry1Ac protein concentration in water was assumed. There were no infusion treatments for cotton.

It must also be noted though, that Cry proteins probably remain in the cotton and maize leaves which forms the sole source of the snail's diet in the present experimental setup. Exposure during the embryo stage is therefore via water only, but after hatching, Bt from both water and leaves will contribute towards exposure and effects.

5.2 Cotton

5.2.1 Embryo growth and hatching success

The parental P1 generation was not exposed to any leaves and were fed fish food in the same manner as the controls of the experiments. The exposures (by adding the leaves) only started after two egg packets of the same age in a container were laid (see section 3.2). Exposure of eggs and subsequent embryos therefore started within hours after the egg packets were laid. The exposure to cotton leaves, irrespective of the presence of Bt, resulted in a high percentage of retarded growth and low survival of the embryos (Figures 4.2 and 4.5). Due to such low hatching percentages for both leaf treatments (Opal and Bol), the data collected had to be divided into two groups. The growth of the embryos that hatched (Figure 4.2) and the graph presenting the growth of the embryos that did not (Figure 4.5). Both revealed similar growth patterns. Clear leaf effects were noticed, as both Opal and Bol deviated from the linear growth pattern Con showed. The growth of Opal and Bol in Figure 4.2 (the surviving embryos) were at first the same as for Con. However, on the third day, Opal started to show retarded growth, and Bol followed a day later. By day five, embryos of both leaf treatments were already significantly smaller than Con (Figure 4.3 and Table 4.2). Figure 4.5 (the embryos that did not hatch) also shows this flattening of the growth curve in both Opal and Bol. The Bt leaf treatment Bol showed growth inhibition much quicker than the non-Bt leaf treatment Opal.

The retarded growth seen in the leaf treatments resulted in an extended embryo development time as seen in Figures 4.2 and 4.3. The mean lengths of the embryos at the time of hatching were not significantly different between the treatments as shown in Figure

4.4. The smaller embryos of the leaf treatments needed to obtain a certain length before they could hatch, resulting in a delay in hatching. Thus, exposure to cotton leaves resulted in developmental toxicity (delaying development but do not result in anatomical defects) (Newman, 2010). The fish food reared control treatment finished hatching on the sixth day, significantly quicker than Opal and Bol (Table 4.5). The embryos exposed to Bt (Bol) hatched significantly quicker, and had a higher percentage hatchings than Opal. A prominent leaf effect was thus observed during the embryo developmental stage of the experiment. There exists the possibility of a chemical pesticide playing a role in the low survival and the retarded growth. The cotton leaves used during this study were gathered from a farm outside of Potchefstroom (see section 3.2). Although the farmer repeatedly stated that he did not use any pesticides, the possibility exists that pesticides originating from neighbouring farms may have been deposited on the Bol and Opal leaves (see section 1). These pesticides could easily enter the egg packets through the freely permeable capsule membrane and mix with the albumin (see section 2.2.3.3), which exposed the embryos to these chemicals.

The effects on embryo growth in the cotton leaf treatments can be contextualised as follows:

- In the initial two to three days, neither the leaf effect nor the Bt in the water had any noticeable effect on embryo growth.
- After day three, both leaf treatments, started to experience a leaf effect (slowing down in growth), probably due to leaf exudates and decomposing products.
- Because the Bt in the water took some time to reach a steady-state concentration, the early developing embryo might have been shielded due to the albumin of the egg packet.
- Embryos of Bol (Bt) that eventually hatched grew slightly quicker than for Opal, possibly due to a stimulatory response, or the artifactual effect of selection of stronger individuals when challenged with Bt.
- Embryos of Bol that did not hatch were affected by the leaf effect and possibly Bt in the water, while Opal experienced only the leaf effect.
- However, the combined effects on Bol embryos did not result in a significant difference from those exposed to Opal, although different selection pressures may have been in play.

The effects seen on the embryos were more likely a product of exposure to the cotton leaves than to the Cry proteins. It should be taken into account therefore, that the measured endpoints for the rest of this experiment are subject to the adverse effects, stresses, or selection pressures already seen during the embryo growth and hatching phase.

5.2.2 Growth after hatching

In Figure 4.7A, a narrow range in length of the snails indicates that the snails were of a similar length after hatching, coinciding with the findings in Figure 4.4 (the length of the embryos a day before hatching). The narrow range in length however, widened over time in all the treatments. The widening in range of lengths attained during different periods after hatching within a treatment suggests that not all of the smaller snails were lost during the water change as will be discussed later.

Compared to Con, enhanced growth was observed for both treatments reared on cotton leaves, Opal and Bol (Figure 4.7B). Both these treatments were significantly larger than Con on day seven and day 19 (Figure 4.8 and Table 4.7), but no statistical difference could be found between the leaf treatments. This could possibly be due to increased stress placed on the snails fed cotton leaves. They may have responded to the stressor by growing faster.

However, this growth stimulation could not be sustained throughout their lifetime. After sexual maturity was reached, measuring of the snails ceased as it was not possible to distinguish between those snails that did or did not reproduce, because reproduction may have affected growth of those reproducing. Fecundity was measured for 15 days, after which, the snails were euthanized and preserved (see section 3.2.3). Before dissecting, the snail's shell dimensions were measured (Figure 3.5). Figure 4.9A shows that although stimulated growth for the snails exposed to cotton leaves were seen in Figure 4.7 and 4.8, Con snails were significantly larger after 15 days than both Opal and Bol treated snails (Table 4.8). The growth curve of Figure 4.7B shows a clear flattening as the snails neared sexual maturity; however, Con showed no indication of this happening. Therefore, Con might have displayed retarded growth at first, but these snails were able to sustain continuous growth afterwards.

Assimilated energy, obtained from an organism's diet, is allocated to functions such as somatic growth, maintenance or storage, and reproduction (Heino & Kaitala, 1999). The amount of energy allocated to such functions may change over the course of an individual's life. The allocation of energy is dependent on the fitness cost or benefits gained for favouring a certain bodily function due to environmental factors experienced at any point of the organism's life (Perrin & Sibly, 1993). Continuous growth after maturation is deemed indeterminate growth. Therefore a trade-off exists between energy allocated to growth, reproduction, and survival. It is assumed that survival takes priority, and only in the case of 'surplus' energy is it directed to growth and reproduction (Heino & Kaitala, 1999). An optimal food source would provide sufficient nutrients and energy. Thus, due to the presumed sub-optimal food quality the snails in the leaf treatments received, the 'surplus' energy was

probably low, resulting in reduced growth rate after maturation. This will be further discussed in section 5.3.3.

The control treatment Con had a larger significant mean shell opening length (OL) and width (OW) compared to the leaf-exposed treatments (Figure 4.9B and C). The mean shell length (SL) of the Bt-exposed snails (Bol) was also significantly larger than the non-Bt leaf treatment Opal (Figure 4.9A and Table 4.8). Proportionately, the OL of Bol was significantly larger than Opal (Figure 4.9B). Conversely, no difference was found in the OW between the two leaf treatments (Figure 4.9C). Potentially, this could result in developmental instability (DI) where the presence of Bt may have influenced the development of an inconsistent phenotype, resulting in fluctuating asymmetry (FA) within the same environment (Newman, 2010).

A phenotype is the observable characteristics of an organism. The appearance of a phenotype depends on the correct expression of a certain gene (Dugatkin, 2013). The expression of a phenotype within a population, exposed to the same environmental factors, would be expected to be consistent. This is referred to as developmental stability. Conversely, DI suggests the variation of a specific phenotype, in this case the proportional dimensions of the shell of *B. tropicus*, within the population. FA describes any random nonconformity in the phenotype. FA is therefore a useful tool to measure DI (Lajus, 2010). The OW of Bol was narrower, disproportionately to its shell length over Opal, indicative of FA in the phenotype expressed by the control (Con). According to Newman (2010), developmental instability may in some cases result in decreased fecundity and survivorship.

Briefly, the effects seen on the growth of the snails included:

- Exposure to the cotton leaves seemed to have placed increased pressure on the snails in a way that enhanced growth.
- This stimulation in growth was unsustainable, and after sexual maturity was reached, more energy was allocated to reproduction and less to growth, thus the growth slowed.
- The final measurement of shell dimensions showed that the snails in Bol were larger than those in Opal, with a larger shell opening but no difference in the shell width.
- Exposure to Bt leaves may have resulted in fluctuating asymmetry (FA) of the shell opening. This could imply developmental instability (DI) which could result in decreased fitness.

The growth and development of the snails were adversely effected by Bt.

5.2.3 Fecundity

The non-Bt cotton leaves used for Opal resulted in high mortality of the snails (review section 5.2.1). Although it was not possible to measure this endpoint due to the setup of the experiment, only one container in Opal reached sexual maturity (survival will be discussed in section 5.2.4). This unavoidably weakened the statistical analysis.

Even though no test for statistical differences could be performed on the number of days it took the treatments in the cotton experiment to reach sexual maturity, it was quite apparent from Table 4.9 that Opal took much longer than both Con and Bol. Again, this effect may be due to the possible effects of chemical pesticides used within the surrounding area of the farm (review section 5.2.1).

Bol snails had significantly fewer egg packets (Figure 4.10) and total eggs (Figure 4.11) than the non-Bt Opal treatment. However, the number of eggs per packet was not different between the two leaf treatments (Figure 4.12). Thus, the number of egg packets being laid decreased when the snails were exposed to the Cry1Ac proteins from Bt cotton leaves, but the egg packets did not have fewer eggs. As mentioned above, fluctuations in a phenotype may lead to decreased fecundity (Newman, 2010). This does not necessarily indicate endocrine disruption but may be due to decreased opportunity to mate. Although there is no documented courting behaviour for *B. tropicus*, 'female' snails have exerted behaviour to rid themselves of an unwanted suitor (Dillon, 2000). In many organisms, symmetry is an indication that a potential mate possesses the genetic foundation to withstand developmental distress. In turn, this will result in stronger offspring which will also be able to endure any developmental distress (Dugatkin, 2013). The enhanced asymmetry of the shells of the Bol snails could have affected mating.

Con had significantly more eggs per packet (Figure 4.12) and more eggs in total (Figure 4.11) than Opal and Bol, indicating that the cotton leaves may be a sub-optimal nutritional substrate for *B. tropicus*. It should be noted that the snails used to measure fecundity had been exposed since their embryo stage, probably culminating in an accumulation of adverse effects.

The effects of the cotton leaves on fecundity briefly included:

- Opal had very few snails reaching sexual maturity, and it took longer to achieve, compared to the other treatments.
- There is a possibility that chemical pollution on the leaves affected the snails more than the Bt toxins.

- However, the Bt exposed treatment Bol had significantly less egg packets, but the egg packets had the same mean number of eggs as Opal.
- Developmental instability (DI) may have caused reduced opportunities for mating, resulting in decreased fecundity.
- The sub-optimal nutritional value of the leaves might have influenced fecundity.

Regarding fecundity, Bt exposure from cotton seems to have had a deleterious effect.

5.2.4 Survival after hatching

Unfortunately, survival was not an endpoint that could accurately be measured or kept track of due to the constraints of the experimental setup, as mentioned previously. Figure 4.4 shows that the length of the snails when they hatched ranged between 0.7 – 0.8 mm, and they were very difficult to see. The water was decanted through a sieve every 96 h and replaced with fresh water (see section 3.1). Great care was taken to retrieve any of the snails that were dislodged and decanted with the water. Due to the small size and large pieces of leaf detritus of the same colour, this was a difficult task.

Survival was calculated from the known number of embryos that hatched and the number of snails eventually dissected from each treatment (Table 4.11). The percentage survival was very low for all treatments with Con having only 15%, and Bol the highest survival of 30%. The percentage of the snails lost versus the actual deaths due to the exposure is not clear. Therefore no conclusions could be made regarding survivorship.

5.2.5 Male reproductive organs

The dimensional proportions of the male reproductive organs were used as a biomarker to determine any morphological changes to the sex organs of *B. tropicus* due to exposure to the Cry proteins derived from Bt crops.

The mean lengths of the penis in both leaf treatments were significantly shorter than the fish food reared Con (Figure 4.13A and Table 4.12). Opal had a shorter preputium than Con, but no difference in length with Bol (Figure 4.13B). No difference in the PSPLR was found between all treatments.

The lengths of the penis and preputium needs to be considered in conjunction with the shell dimensions of the snails at the time of dissection (see section 5.2.2). The PCA ordination shown in Figure 4.14 considers the dimensions of both the shells and the penis-preputium complex. All the parameters of the shells and penis-preputium complex, except for the length of the preputium, were co-variant. Thus, as the snail increased in length, the shell opening,

the width of the opening, and the penis length would increase in length proportionally. The width of the penis and preputium were also co-variant with the length of the snail. The length of the preputium seemed to be independent from the size of the snail. The preputium length was also the major parameter influencing the PSPLR. A shorter preputium in this case therefore results in a larger PSPLR. However, the convex hulls, representing each treatment, overlapped (Figure 4.14) indicating no significant difference of proportions of these dimensions measured between treatments.

The shorter penis and preputium seen in Figure 4.13 were due to the shorter shell length shown in Figure 4.9. Therefore no difference in proportions, represented by the PSPLR, could be found in any of the treatments. This, in turn, means no effect of Bt on male reproductive organ morphometrics as biomarker was seen.

Contextualizing the results on the dimensions of the sex organs:

- The penis length was shorter when snails were reared on cotton leaves, but the shells were correspondingly shorter as well.
- The length of the penis was proportional to the length of the shell.
- The length of the preputium was independent of all of the dimensions measured.
- No differences in the PSPLR meant neither the cotton leaves nor the Bt toxins, influenced the dimensions of the male sex organs.

No effect of Bt on the sex organs of *B. tropicus* were found.

5.3 Maize

The maize were planted in a controlled environment (see section 3.2), and there was no chance of pesticide pollution. Therefore, any effects seen on the snails may be related to the Bt toxins they were exposed to.

5.3.1 Embryo growth and hatching success

The embryos exposed to maize leaves, unlike the embryos exposed to cotton leaves, showed little to no retarded growth (Figure 4.15). The embryo growth trajectories of the different treatments regressed closely together (Table 4.13). The presence of the maize leaves in the water, irrespective of the presence of Bt, did however stimulate growth. Snails of all treatments reared on maize leaves were significantly larger (based on means) on the third day of development than Con (ANOVA, $p=0.0003$. Figure 4.16A), but no differences between the leave treatments were found. Con did not receive any fish food during embryo development; these containers only started receiving fish food after hatching. This indicates

the possibility of the snail embryos responding to or sensing a 'food cue' in the surrounding water. The presences of a 'food cue' in the water (from the maize leaves) might have stimulated the growth of the embryos in the leaf treatments. If there was a food cue, this was probably transmitted via the egg capsule to the embryos. Conversely, the lack of any food cue in the Con containers may have resulted in slower growth.

The fourth day of development did show significantly larger embryos in the Bt-infusion treatments (IMP+/- and IMP+/+) compared to their non-Bt control treatment (IMP-/-) (ANOVA, $p < 0.0001$. Figure 4.16B). On day five, IMP+/- was still significantly larger than IMP-/- (ANOVA, $p < 0.0001$. Figure 4.16C). Although the lengths were not significantly different, larger embryos were observed for IMP+ compared to IMP- ($p = 0.307$, two-tail, unpaired, t-test). Figure 4.17 shows the length of the embryos a day prior to hatching, irrespective on which day the embryos hatched. Here too, IMP-/- was significantly smaller than both the Bt-infusion treatments (IMP+/- and IMP+/+) on the day prior to hatching, implying that Bt had a clear growth stimulatory effect on the embryos.

The apparent stimulation in growth did not result in shorter embryo developing cycles. As indicated by Table 4.15, all the non-Bt leaf treatments hatched significantly earlier than the highest Bt concentration treatment IMP+/+ (Kruskal-Wallis, $p < 0.0001$). IMP +/+ did however have the highest percentage of eggs that hatched (98%), although these percentages were not significantly different from each other (Table 4.14). IMP+/- on the other hand, had the lowest percentage hatched eggs (88%), but hatched significantly quicker than IMP+/+ (Dunn's multi comparison of means, $p < 0.05$). The same trend is seen for IMP+; a lower percentage of eggs hatched, but had a significantly quicker hatching time than the high Bt concentration treatment IMP+/+ (Dunn's multi comparison of means, $p < 0.05$). The lesser concentration of Cry1Ab proteins therefore seemed to have stimulated hatching but probably at the cost of embryo survival. The snails exposed to the highest concentration of Bt (IMP+/+) had the highest percentage embryo survival, but the time it took the embryos to develop and hatch were prolonged. It should be mentioned that no difference in hatching time was found between Con and IMP+/+ (Table 4.14).

Table 5.1 shows the relationship between the time it took the eggs to hatch and the percentage of the embryos that survived. The rest of the leaf treatments did have significantly shorter hatching times compared to Con (Kruskal-Wallis, $p < 0.0001$). This could be due to the possible lack of a food cue in the Con containers, as mentioned above.

Table 5.1: A summary of each treatment's increase in time until hatching of embryos started, ranged from the shortest to the longest time, and the ascending order of the percentage embryos hatched in each treatment.

Mean number of days to hatching		Percentage hatched	
Increase in time until hatching ↓	IMP-/-	IMP+/-	Increase in percentage hatched ↓
	IMP+/-	CRN / IMP+	
	CRN / IMP+	Con	
	IMP-	IMP-/-	
	Con	IMP-	
	IMP+/+	IMP+/+	

The effects of the maize leaf experiment of embryos growth were as follows:

- No retarded growth was observed in any of the treatments.
- Initial stimulation in growth occurred within all the treatments exposed to maize leaves, probably due to the stimulus (or cue) of food in the environment.
- The treatments exposed to Bt leaves had quicker growth compared to the non-Bt leaf treatments, resulting in larger embryos in Bt treatments prior to hatching.
- Low concentrations of Bt resulted in faster hatching but lower hatching success.
- High concentrations of Bt showed lengthened embryo development times and the highest hatching percentage.

Exposure to Bt maize leaves negatively impacted the development of the embryos.

5.3.2 Growth after hatching

Con, IMP-/- and IMP+/+ were the only treatments in the maize leaf experiment to have a linear regression line representing the growth of the hatched snails (Figure 4.19 and Table 4.16). Snails in all the other treatments, at some point in their development started to show retarded growth. Both IMP+ and the equivalent non-Bt treatment IMP-, showed retarded growth from an early onset. The reasons for this growth inhibition are unknown.

In both Figures 4.19B and C, it can be seen that the snails in the treatments exposed to Bt were larger than their non-Bt control treatments. Though no significant differences in lengths for any of the treatments were seen seven days after hatching, as presented in Figure 4.20A, both Bt infusion treatments were significantly larger than IMP-/- by day 19 (ANOVA, $p=0.011$; Figure 4.20B). However, no significant differences between IMP- and IMP+ were observed. As mentioned in section 5.2.2, after the snails reached sexual maturity, growth measurements were terminated. Only after fecundity was measured and the snails were

prepared for dissection, were the dimensions of the shells measured. Figure 4.21A indicates stronger growth retardation for IMP+ during the 15 days while fecundity was measured, resulting in the mean length of the snails from IMP- being significantly larger than IMP+ ($p < 0.0001$; t-test, two-tail, unpaired). Proportionally, the length and width of the opening of the shell showed the same significant difference between IMP- and IMP+ (opening length, t-test, $p < 0.0001$; opening width, t-test, $p = 0.0005$. Figures 4.20B and C). No significant differences in length were observed for the infusion treatments. Where the Bt infusion treatments were significantly larger than IMP-/- 19 days after hatching, all three infusion treatments were the same in length when the snails were euthanized. Thus the accelerated growth caused by Bt after hatching slowed when fecundity monitoring started.

The increased stress placed on the snails exposed to the Cry proteins may have resulted in the increased initial growth seen in both the maize and cotton data (Figures 4.8 and 4.20). In both cases, the growth spurt was not sustained and the measurements of the shell length taken 15 days later indicated retarded growth. In the only other study published on Bt crops and molluscs, the land snail *C. asperses* fed on plant material of Bt maize, resulted retarded growth compared to the snails fed non-Bt maize (Kramarz *et al.*, 2009). Retarded growth was also noticed in the predatory green lacewing *C. carnea* after ingesting lepidopteron prey reared on Bt maize (Hilbeck *et al.*, 1998). The initial increased growth induced by the presence of the Cry proteins implies enhanced stress placed on the organisms. In turn they respond with growth spurts to adulthood, and a stronger body. General adaptation syndrome (GAS) consists of three stages in response to a stressor; the alarm reaction, adaptation, and exhaustion (Newman, 2010). The alarm reaction is an initial short-term response to the stressor, but this is a high-energy response and cannot be maintained over long periods. The second response stage, adaptation, augments tissue level compensation. The increased rate of growth seen in the snails when exposed to Bt is probably at stage two in response to the stressor. If the stressor persists, such is the case in this experiment, the third and final stage of exhaustion is entered. The growth of the snails slowed, and fecundity decreased (discussed below) (Newman, 2010).

The intertidal Limpet *Fissurella crassa* responded to increased parasitism by increasing their growth (measured as shell size) (Aldana *et al.*, 2013). This increase in body size as response to a stressor, representing adaptation as part of GAS, means a better and stronger body condition. Shortened embryonic development was reported for *Octopus vulgaris* in 3°C warmer water conditions (Repolho *et al.*, 2013). Quicker development of embryos also indicated the need for a stronger body. Since during the developing stages an organism normally is at increased risk from the effects of pollutants (Newman, 2010), increased (stimulated) growth would result in a higher chance of survival. Thus the increase in growth

seen as a response to Bt exposure may have important implications for energy budget allocations and population dynamics (Aldana *et al.*, 2013).

The effects on growth of the snails in the maize leaf treatments could be contextualised as follows:

- Initially, no inhibition or stimulation in growth of the snails was observed.
- At day 19, the Bt infusion treatments were larger than the non-Bt infusion control treatment. Increased presence of stressors could have resulted in the stimulation of growth.
- The response to the stressors, increased growth, was not sustained during reproduction, possibly because energy was needed to mate and produce egg packets.

The growth of the snails was affected by the Bt maize leaves.

5.3.3 Fecundity

The number of snails reaching sexual maturity was far greater than during the cotton experiment, but because of the survival issues due to water and food changes, further comparisons were not possible. As expected, the fish food fed control treatment reached sexual maturity well ahead of the leaf reared treatments (Figure 4.22). When exposed to the Cry1Ab proteins, the snails reached sexual maturity quicker than for the near transgenic isoline controls of IMP- and IMP-/- . This is consistent with the stimulated growth seen in Figure 4.20; where the Bt treatments grew quicker and therefore reached sexual maturity earlier.

Bøhn *et al.* (2008) reported quicker maturation in the water crustacean *D. magna* when exposed to the Cry1Ab proteins. It seems that the cost of reaching maturity quicker in *D. magna* was a smaller proportion of the population that were able to reproduce. This endpoint was not explicitly measured during this study but can be taken into account for future studies. The non-Bt cultivar control treatment CRN followed the same pattern as that of the Bt treatments, and matured quicker than the other non-Bt leaf treatments, though it was not significant (Bonferroni's multiple comparison test, $p > 0.05$).

Even with the Bt treatments reaching maturity quicker, Figure 4.23 indicates significantly fewer egg packets laid compared to non-Bt leaf treatments (ANOVA, $p < 0.0001$). The same decline in reproduction was seen in the number of eggs laid by the Bt treatments (Figure 4.24). The number of eggs per packet did not differ between the Bt treatments and the non-Bt leaf treatments. The snails exposed to the Cry1Ac proteins via cotton exhibited the

same comparatively lower number of egg packets and total number of eggs. The number of eggs per packet for the cotton-exposed snails did not differ from the other non-Bt treatments, compared with the effects seen with the maize leaf experiment. Fecundity was lower when snails were exposed to Bt, but the number of eggs per packet stayed the same. CRN displayed the same trend as the Bt treatments. This treatment matured faster than their non-Bt equals; they laid significantly fewer eggs (ANOVA, $p < 0.0001$) and had fewer total eggs than IMP-.

Saxena and Stotzky (2001) reported significantly higher lignin content (33 - 97%) in Bt maize leaves than in near-transgenic isolines. Lignin strengthens the cell walls of plants and coats the cellulose (Nabors, 2004). The modification of the lignin content has the potential of effects on ecological systems. The higher lignin content would result in lower digestion by defoliators (Halpin *et al.*, 1994; Saxena & Stotzky, 2001), and lowering the nutrients test organisms reared only on maize leaves would be able to sustain.

The decrease in fecundity, for both Bt maize leaves and CRN3505 non-Bt maize leaves, may be due to leaves being a sub-optimal food source. Waring *et al.* (1985) for instance reported a significant difference in the leaf chemistry of a willow clone dependant on the amount of light and nutrients available. Three separate treatments were used during the willow experiment, influenced by the amount of light and nutrients in the water supplement received during growth; high light – high nutrients; low light – high nutrients; and high light – moderate nutrients. The highest percentage lignin was found in the high light – moderate nutrients treatment, but very low total nitrogen (N) levels. A high level of total N and a moderate percentage lignin was reported for the high light – high nutrients treatment (Waring *et al.*, 1985).

Differences in the nutritional value of the different maize cultivars may result in a sub-optimal nutrient source, at least as far as dried leaves would be the sole source of food to snails. Canhoto and Graça (1995) found a positive correlation between leaf utilization in stream detritivores and the nutrient content of leaves, from exotic and native trees in Portugal. The survival of the crane fly, *Tipula lateralis* decreased when fed only sub-optimal leaves. An experiment on the feeding preferences of the trichopteran, *Phylloicus* spp. on different leaves found that low lignin and high nutrition (percentage phosphate, nitrogen and total polyphenols) content was favoured (Rincón & Martinez, 2006).

Briefly, the effects seen on the fecundity of the snails included the following:

- Sexual maturity was reached quicker in the Bt-exposed treatments probably due to the stimulated growth discussed in section 5.3.2.

- Fewer egg packets and total number of eggs were associated with snails exposed to Bt.
- The number of eggs per packet stayed constant within all leaf treatments. Con had more eggs per packet but this may be due to a more nutritionally optimal fish food.

The number of eggs laid per snail, measured by Kramarz *et al.* (2009) decreased for the land snail *C. asperses* when they were exposed to the Cry1Ab protein as feed, though the concentrations used during the study were four orders of magnitude higher than the concentrations used in the present study. However, Kramarz *et al.* (2009) was not the only study to find adverse effects of Bt on fecundity. Clark and Coats (2006) exposed the springtail *F. candida* to 2.19 µg/g Bt in maize leaves and also found a significant decrease in the number of offspring produced. Both studies used maize litter as feed, corresponding with a lower nutritional value in Bt maize litter than non-Bt maize. Evidence therefore show that Bt decreases an individual's Darwinian fitness, the ability to contribute offspring to the future population. This in turn can be interpreted as ecological mortality caused by the Cry proteins. The functioning of an exposed individual within the ecosystem is nearly equivalent to somatic death (Newman, 2010).

5.3.4 Survival

The fish food control seemed to have had better survival in the maize experiment than the cotton experiment (Table 4.18). Low survival was again seen in all the treatments partly due to the 96 h water change cycle maintained throughout the experiments. One of the biggest problems was that newly hatched and grazing snails would crawl onto the leaf fragments and were decanted with the water and food. Future studies would need to consider ways where this effect can be avoided.

Though a percentage of the snails lost were probably due to the experimental setup, it was not the only source of loss. Other studies have found that Bt had an deleterious effect on the survival of several non-target species. The first study published on adverse effects of Bt on non-target organisms found decreased survival in a population of monarch butterflies after Bt maize pollen was ingested (Losey *et al.*, 1999). The isopod *C. communis* showed significantly lower survival when exposed to Bt maize leaves (only 31% compared to non-Bt treatments 55%) (Jensen *et al.*, 2010). Bøhn *et al.* (2008) reported a reduced mean life expectancy for *D. magna* daily exposed to 0.4 mg of dry mass Bt maize leaves in 60 ml of artificial medium (ADAM), from 45 days (for non-Bt maize leaf treatment) to a mere 28.2 days.

5.3.5 Male reproductive organs

The penis-preputium complex, as mentioned earlier, was used as a tool to determine whether the Cry proteins could act as EDC's. The advantage of using a hermaphrodite species is that the whole population can be used for this measurement. Due to IMP+/+'s low percentage survival this was quite helpful as all survivors could be sampled.

No difference could be found between the non-Bt and the Bt leaf treatments regarding the length of the penis-preputium complex (Figure 4.26). Con snails however, had a significantly longer mean preputium than CRN, IMP- and IMP+ (AVOVA, $p=0.0066$), but no differences in the PSPLR were found.

As before, with the cotton data, a PCA analysis with all the dimensions measured of the shells and the penis-preputium complex was performed (Figure 4.27). The length of the shell was again co-variant with the length of the penis, meaning that as the shell increases in length so did the penis. Again, the length of the preputium seemed to be independent, and was not influenced by the length of the shell or any of the other parameters measured. The preputium determines the PSPLR because the PSPLR is calculated by dividing the penis length with the preputium. Thus, a longer preputium equals a smaller PSPLR and *vice versa*. However no endocrine disruption was noted.

As with cotton, no effects of exposure to Bt was found in the dimensions of the male sex-organ's.

Chapter 6: Synthesis, conclusions, and recommendations

The purpose of this study was to establish whether Cry proteins, present in the leaves of maize and cotton plants, would affect the freshwater pulmonate *B. tropicus*. The distribution of *B. tropicus* (Figure 2.4) coincides with areas associated with crop farming in South Africa (Figure 2.3), establishing the likelihood of the snails being exposed to Cry proteins from crop residues entering water.

The causes of the differences seen in the endpoints measured compared between treatments, although already referred to in the previous chapter, will be explored further below.

6.1 Nutritional differences

A nutritional difference was expected between the fish food and the cotton or maize leaves. Fish food reared Con showed no signs of stimulation or retarded growth and had high numbers of egg packets and total number of eggs laid. The non-Bt treatments (Opal, IMP- and IMP-/-) were smaller than Con but showed no reduced fecundity. CRN in the maize leaf experiment effectively served as a leaf-effect treatment (see section 3.2). Unfortunately no additional cotton cultivars were available to use in the same manner in the cotton experiment. Snails exposed to CRN did not differ from those exposed to IMP- or IMP-/- during growth of the embryos or growth after hatching. However, CRN revealed a similar pattern to the Bt-exposed treatments during reproduction.

Snails in all treatments exposed to Bt, in both the cotton and the maize experiments, showed decreased fecundity. A lignin content of 33 - 97% higher (Saxena & Stotzky, 2001), depending on the cultivar, than the non-Bt isolines could, as mentioned in section 2.1.5, prolong the digestion and breakdown of the leaf material (Nabors, 2004). It has repeatedly been reported that higher lignin content was rejected by various invertebrates as an optimal food source (Rincón & Martinez, 2006; Canhoto & Graça, 1995). The fact that both earthworm species, *E. Andrei* and *L. terrestris*, showed significant decreased body mass when given Bt leaves as their sole food source (van der Merwe *et al.*, 2012; Zwahlen *et al.*, 2003), may be indicative of a higher lignin content corresponding to lower nutritional value. A direct relationship between nutritional value of the food source and fecundity has been described in copepods (Carli *et al.*, 1995) and fish (Izquierdo *et al.*, 2001). Leaves with a comparatively better nutritional value would, conversely, result in an increase in fecundity.

Thus the high-spirulina diet for the Con treatment increased the snail's fitness, while the exposure to sub-optimal nourishment in Bt leaves reduced their fitness. The effects of

different cultivars were also seen in CRN, indicating reduced nutrients in the leaves of the cultivar CRN3505 compared to IMP 22-51. However, the effects of the combination of both reduced nutrition and the presence of the Cry proteins had far greater adverse effects (seen in Bol, IMP+, IMP+/- and IMP+/+) that cannot be ascribed solely to a sub-optimal nutrition source (found in CRN). This suggests that the Cry proteins probably had effects on reproduction, possibly through endocrine disruption. If endocrine disruption was involved, the pathways should be further explored.

6.1.1 Energy allocation

The energy to sustain an animal is mostly acquired through its diet. A sub-optimal diet would therefore result in less energy available to distribute to different bodily functions. This is especially true for animals with indeterminate growth.

Before maturation is reached, all 'surplus' energy is allocated to growth. During the maize leaf experiment, the Bt-exposed treatments showed enhanced embryo growth compared to non-Bt exposures. In addition, during the cotton experiment, Bt-exposed snails also exhibited enhanced growth after hatching before sexual maturity was reached. These growth 'spurts' were a response to the stress placed on them in the presence of the Cry proteins. The physiological processes during these stress enhanced growth spurts should be investigated in future studies. Critical life-stage tests would be able to provide a more clear indication of the snails' response to the Cry proteins in each individual life stage without the influence of prior fitness reductions (Newman, 2010).

As sexual maturation is reached the 'surplus' energy have to be divided between growth and reproduction. The energy available to the snails in the Bt treatments could not sustain both functions at an optimal level, resulting in comparatively lower fecundity and growth during those 15 days.

6.2 Developmental instability

Except for the low level of nutrition available in Bt leaves, DI also could have played a role in the decreased fecundity of Bol (Bt cotton fed treatment). According to the genetic model of mate choice, symmetry is an honest indicator of good genes. The symmetry of an organism is directly related to the organism's ability to adapt to changing environments as it matures (Dugatkin, 2013). This trait, if used by a female, would most likely be passed on to her own generation, increasing the offspring's fitness. This, in turn, increases the female's fitness.

Fluctuating asymmetry (FA) is a practical tool to measure DI (Newman, 2010). FA was possibly involved in the Bt cotton leaf treatment with Bol. Bol snails were significantly larger than Opal (SL) snails, with a larger OL, but no difference could be found in the OW. This indicates the proportions of the opening of the shells of the snails exposed to the Cry1Ac proteins deviated from the norm. The maize experiment on the other hand showed no sign of FA, and therefore no association with DI. The deviation in phenotype for Bol would have resulted in decreased opportunities or mate acceptance for mating. The DI, nutritional differences, and energy allocation most likely had a combined effect on the reproduction of Bol, creating a concatenation of effects.

The results of this study therefore support the possibility that Bt leached from cotton and maize had effects on a freshwater snail, although the effects differed between the crops. The concentrations the snails were exposed to were very similar to what can be expected in natural waters. However, the impact on snail populations and associated ecology needs further investigation.

6.3 Final remarks

The fitness (growth, fecundity, and survival) of many non-target organisms are reduced when they are exposed to Bt crops in various forms, whether the organisms are exposed to Bt directly, through pollen (Zwahlen *et al.*, 2003), crop litter (Jensen *et al.*, 2010; Kramarz *et al.*, 2009; Bøhn *et al.*, 2008), or get exposed indirectly through bioaccumulation in its prey (Hilbeck *et al.*, 1998). This study added to the body of knowledge that the mere presence of the Cry proteins in solution in the water reduces the fitness of the non-target organism *B. tropicus*.

Due to the lack of adaptations to metabolise the pollutants in their environment, molluscs are good bio-indicators of pollutants at very low concentrations (Oehlmann *et al.*, 2007), such as the concentrations found in environment (Tank *et al.*, 2010). The Bt crop residues in water systems significantly reduced fecundity in *B. tropicus*, compared to non-Bt crop residues. The first indications of deleterious effects on the snails exposed to Bt was found during its embryonic development. Any further effects seen during this study likely were additive effects, escalating to reduced fecundity and survivorship. The ecological implications of reduced fitness of the snails should be further investigated using multi-generational studies. *B. tropicus* lends itself very well to such studies.

6.4 Recommendations

The experimental setup used during this study was new and developmental, but showed great promise. Some shortcomings were identified and recommendations are made to address them for future studies:

- *Measuring survival as an endpoint.* The leaves in the water, as well as decantation of water, caused an unknown percentage of the population to be lost. The candidate propose that the water should not be decanted but rather siphoned out and replaced. This will prevent the snails from being dislodged and flushed away. The leaves should not be used as feed but rather compartmentalized so that the Cry protein can still leach into the water, exposing the snails to the infusion but with more sustainable food added. This would more closely represent natural conditions as maize leaves are not normally part of the diet of aquatic snails, nor would they constitute a significant proportion of the food normally utilized by snails.
- *Separate the hatched snails.* Separating the hatched snails into smaller containers with just three or four snails in each will make it possible to follow the growth of the whole population, and not just a random sample. This will also allow for better observation of growth, reproduction, and mortality.
- *Eliminate unknown pollutants.* It would be advisable to grow all plants used for the experiments in a controlled environment to decrease the probability of chemical pesticides influencing the results of the experiment.

Appendix A

Classification of *Bulinus tropicus*:

Kingdom: Animalia

Phylum: Mollusca

Class: Gastropoda

Subclass: Pulmonata

Order: Basommatophora

Family: Planorbidae

Subfamily: Bulininae

Genus: Bulinus

Species: tropicus

(Hickman *et al.*, 2006; Brown, 1981)

References

- ALAVANJA, M.C.R. 2009. Pesticides use and exposure extensive worldwide. *Reviews on Environmental Health*, 24(4):303-309.
- ALDANA, M., PULGAR, J.M., ORELLANA, N., OJEDA, F.P. & HUIDOBRO, M.R.G. 2013. Increased parasitism of Limpets by a trematode meatcercaria in fisheries management areas of central Chile: effects on the host growth and reproduction. *Ecology and Health*, DOI: 10.1007/s10393-013-0876-9
- APPLETON, C.C. 1996. Freshwater molluscs of southern Africa. Pietermaritzburg: University of Natal Press.
- APPLETON, C.C. 2002. Mollusca. *In: Guides to the freshwater invertebrates of Southern Africa. Volume 6: Arachnida & Mollusca*, Pretoria: Water Research Commission: TT182/02. 42-125 p.
- ARJÓ, G., PORTERO, M., PIÑOL, C., VIÑAS, J., MATIAS-GUIU, X., CAPELL, T., BARTHOLOMAEUS, A., PARROTT, W. & CHRISTOU, P. 2013. Plurality of opinion, scientific discourse and pseudoscience: an in depth analysis of the Séralini et al. study claiming that Roundup Ready corn or the herbicide Roundup cause cancer in rats. *Springer Link Perspectives*, DOI: 10.1007/s11248-013-9692-9
- BAUMGARTE, S. & TEBBE, C.C. 2005. Field studies on the environmental fate of the Cry1Ab Bt-toxin produced by transgenic maize (MON810) and its effect on bacterial communities in the maize rhizosphere. *Molecular Ecology*, 14:2539-2551.
- BAUR, M.E. & BOETHEL, D.J. 2003. Effect of Bt-cotton expressing Cry1A(c) on the survival and fecundity of two hymenopteran parasitoids (*Braconidae*, *Encyrtidae*) in the laboratory. *Biological Control*, 26:325-332.
- BENNET, R., MORSE, S. & ISMAEL, Y. 2006. The economic impact of genetically modified cotton on South African smallholders: yield, profit and health effects. *Journal of Development Studies*, 42(4):662-677.
- BØHN, T., PRIMICERIO, R. & HESSEN, D.O. 2008. Reduced fitness of *Daphnia magna* fed a Bt-transgenic maize variety. *Archives of Environmental Contamination and Toxicology*, 55(4):584-592.
- BORNMAN, M.S., VAN VUUREN, J.H., BOUWMAN, H., DE JAGER, C., GENTHE, B. & BARNHOORN, E.J. 2007. *Endocrine disruptive activity and the potential health risk in an*

urban nature reserve. South Africa: Water Research Commission: WRC report no. 1505/1/07.

BOUWMAN, H., BECKER, P.J., COOPAN, R.M. & REINECK, A.J. 1992. Transfer of DDT used in malaria control to infants via breast milk. *Bulletin of the World Health Organization*, 70(2):241-250.

BRETSCHNEIDER, L.H. & RAVEN, C.P. 1954. Structural and topochemical changes in the egg cells of *Limnaea stagnalis* during oogenesis. *Archives Neerlandaises de Zoologie*, 10(1):1-31.

BROADIE, K.S., SYLWESTER, A.W., BATE, M. & TUBLITZ, N.J. 1990. Immunological, biochemical and physiological analyses of cardioacceleratory peptide 2 (CAP2) activity in the embryo of the tobacco hawkmoth *Manduca sexta*. *Development*, 108:59-71.

BROWN, D.S. 1981. Generic nomenclature of freshwater snails commonly classified in the genus *Bulinus* (Mollusca: Basommatophora). *Journal of Natural History*, 15(6):909-915.

BRUNGS, W.A. 1973. Effects of residual chlorine on aquatic life. *Journal of the Water pollution control federation*, 45(10):2180-2193.

BYRNE, P.A. & O'HALLORAN, J. 2001. The role of bivalve molluscs as tool in estuarine sediment toxicity testing: a review. *Hydrobiologia*, 465:209-217.

CAMPANELLA, L., GATTA, T. & RAVERA, O. 2005. Relationship between anti-oxidant capacity and manganese accumulation in the soft tissues of two freshwater molluscs: *Unio pictorum* *mancus* (Lamellibranchia, Unionidae) and *Viviparus ater* (Gastropoda, Prosobranchia). *Journal of Limnology*, 64(2):153-158.

CANHOTO, C. & GRAÇA, M.A.S. 1995. Food value of introduced eucalypt leaves for a Mediterranean stream detritivore: *Tipula lateralis*. *Freshwater Biology*, 34:209-214.

CARLI, A., MARIOTTINI, G.L. & PANE, L. 1995. Influence of nutrition on fecundity and survival in *Tigriopus fulvus* (Fischer) (Copepoda: Harpacticoida). *Aquaculture*, 134(1-2):113-119.

CASTILLO, L.E., RUEPERT, C. & UGALDE, R. 2010. Ecotoxicology and pesticides in Central America. In: NEWMAN, M.C., ed. *Fundamentals of Ecotoxicology*, 3rd ed. New York: CRC Press. 47 - 54 p.

- CHAMBERS, C.P., WHILES, M.R., ROSI-MARSHALL, E.J., TANK, J.L., ROYER, T.V., GRIFFITHS, N.A., EVANS-WHITE, M.A. & STOJAK, A.R. 2010. Response of stream macroinvertebrates to Bt maize leaf detritus. *Ecological Applications*, 20(7):1949-1960.
- CHANG, K.W., FULTON, M.H. & SCOTT, G.I. 2007. Use of the juvenile clam, *Mercenaria mercenaria*, as a sensitive indicator of aqueous and sediment toxicity. *Ecotoxicology and Environmental Safety*, 67:333-340.
- CHAUDHRY, A.M. & MORGAN, E. 1986. Factors affecting the growth and fecundity of *Bulinus tropicus* (Krauss) (Gastropoda). *Journal of Molluscan Studies*, 53(1):52-61.
- CLARK, B.W. & COATS, J.R. 2006. Subacute effects of Cry1Ab Bt corn litter on the earthworm *Eisenia fetida* and the springtail *Folsomia candida*. *Environmental Entomology*, 35(4):1121-1129.
- CLARK, B.W., PHILLIPS, T.A. & COATS, J.R. 2005. Environmental fate and effects of *Bacillus thuringiensis* (Bt) proteins from transgenic crops: a review. *Journal of Agricultural and Food Safety*, 53(12):4643-4653.
- CLEMENT, A.C. 1938. The structure and development of centrifuged eggs and egg fragments of *Physa heterostropha*. *Journal of Experimental Zoology*, 79(3):435-460.
- COLBORN, T., VOM SAAL, F.S. & SOTO, A.M. 1993. Developmental effects of endocrine-disruptive chemicals in wildlife and humans. *Environmental Health Perspective*, 101:378-384.
- COLGAN, D.F. & MANLEY, J.L. 1997. Mechanism and regulation of mRNA polyadenylation. *Genes and Development*, 11:2755-2766.
- COMBRINCK, C. & VAN EEDEN, J.A. 1970. A natural habitat for the laboratory investigation of freshwater snails. *Wetenskaplike Bydraes van die P.U. vir C.H.O. Beeks B: Natuurwetenskappe*, 27:1-13.
- CONTI, M.E., STRIPEIKIS, J., FINOIA, M.G. & TUDINO, M.B. 2012. Baseline trace metals in gastropod mullusks from Beagle channel, Tierra del Fuego (Patagonia, Argentina). *Ecotoxicology*, 21:1112-1125.
- CRICKMORE, N., ZEIGLER, R.D., FEITELSON, J., SCHNEPF, E., VAN RIE, J., LERECLUS, D., BAUM, J. & DEAN, D.H. 1998. Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiology and Molecular Biology Reviews*, 62(3):807-813.

- CURL, C.L., FENSKE, R.A. & ELGETHUN, K. 2003. Organophosphorus pesticide exposure of urban and suburban preschool children with organic and conventional diets. *Environmental Health Perspectives*, 111(3):377-382.
- DALE, P., CLARKE, B. & FONTES, E.M.G. 2002. Potential for the environmental impact of transgenic crops. *Nature Biotechnology*, 20:567-843.
- DAY, J.A. & DE MOOR, I.J. 2002. *Guides to the freshwater invertebrates of Southern Africa. Volume 6: Arachnida & Mollusca*. South Africa: Water Research Commission.
- DE MAAGD, R.A., BRAVO, A. & CRICKMORE, N. 2001. How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. *Trends in Genetics*, 17(4):193-199.
- DEFUR, P.L., CRANE, M., TATTERFIELD, L.J., INGERSOLL, C.G., STAHL, R.G. Jr., MATTHIESSEN, P. & LEBLANC, G.A. 1999. Workshop on Endocrine Disruption in Invertebrates: Endocrinology, Testing, and Assessment (EDIETA). In: DEFUR, P.L., CRANE, M., INGERSOLL, C.G. & TATTERFIELD, L.J., eds. *Endocrine Disruption in Invertebrates: Endocrinology, Testing, and Assessment*, Pensacola, Florida: SETAC publication. 1-21 p.
- DEVILLIERS, J. 2009. In Silico Methods for Modeling Endocrine Disruption. In: DEVILLIERS, J., ed. *Endocrine Disruption Modelling*, New York: CRC Press. 1-9 p.
- DIAMANTI-KANDARAKIS, E., BOURGUIGNON, J.P., GIUDICE, L.C., HAUSER, R., PRINS, G.S., SOTO, A.M., ZOELLER, R. & GORE, A.C. 2009. Endocrine-disrupting chemicals. An endocrine society scientific statement. *Endocrine reviews*, 30(4):293-342.
- DILLON, R.T. Jr. 2000. *The Ecology of freshwater Molluscs*. Cambridge: Cambridge University Press.
- DONAVAN, W.P., RUPAR, M.J., SLANEY, A.C., MALVAR, T., GAWRON-BURKE, M.C. & JOHNSON, T.B. 1992. Characterization of two genes encoding *Bacillus thuringiensis* insecticidal crystal proteins toxic to Coleoptera species. *Applied and Environmental Microbiology*, 58(12):3921-3927.
- DOUVILLE, M., GAGNÉ, F., MASSON, L., MCKAY, J. & BLAISE, C. 2005. Tracking the source of *Bacillus thuringiensis* Cry1Ab endotoxin in the environment. *Biochemical Systematics and Ecology*, 33:219-232.

- DUFT, M., SCHULTE-OEHLMANN, U., WELTJE, L., TILLMANN, M. & OEHLMANN, J. 2003. Stimulated embryo production as a parameter of estrogenic exposure via sediment in the freshwater mudsnail *Potamopyrgus antipodarum*. *Aquatic toxicology*, 64:437-449.
- DUGATKIN, L.A. 2013. Principles of animal behaviour. Third edition. New York: W.W. Norton & Company.
- DUNCAN, C.J. 1975. Reproduction. In: FRETTER, V. & PEAKE, J., eds. *Pulmonates. Volume 1. Functional anatomy and physiology*, London: Academic Press inc. 309-366 p.
- FIUZA, L., NIELSON-LEROUX, C., GOZE, E., FRUTOS, R. & CHARLES, J. 1996. Binding of *Bacillus thuringiensis* Cry1 toxins to the midgut brush border membrane vesicles of *Chilo suppressalis* (Lepidoptera: Pyralidae): Evidence of shared binding sites. *Applied and Environmental Microbiology*, 62(5):1544-1549.
- FLORES, S., SAXENA, D. & STOTZKY, G. 2005. Transgenic Bt plants decompose less in soil than non-Bt plants. *Soil Biology and Biochemistry*, 37:1073-1082.
- FORACADA, C., ALCÁ CER, E., GARCERÁ, M.D. & MARTINEZ, R. 1996. Differences in the midgut proteolytic activity of two *Heliothis virescens* strains, one susceptible and one resistant to *Bacillus thuringiensis* toxins. *Archives of Insect Biochemistry and Physiology*, 31(3):257-272.
- FRETTER, V. & GRAHAM, A. 1964. 1 vols. Reproduction. In: WILBUR, K.M. & YONGE, C.M., eds. *Physiology of Mollusca*, New York: Academic Press inc. 127-164 p.
- FRUTOS, R., RANG, C. & ROYER, M. 1999. Managing insect resistance to plants producing *Bacillus thuringiensis* toxins. *Critical Reviews in Biotechnology*, 19(3):227-276.
- GARRET, R.H. & GRISHAM, C.M. 2013. Biochemistry, Fifth Edition. Canada: Cengage Learning.
- GARRETT, R.H. & GRISHAM, C.M. 1997. Principles of Biochemistry with a human focus. London: Thomsom Learning.
- GOUSE, M., KRISTEN, J.F. & JENKINS, L. 2003. Bt cotton in South Africa: Adoption and the impact on farm incomes amongst small-scale and large scale farmers. *Agrekon*, 42(1):15-28.
- GRECO, L., PELLERIN, J., CAPRI, E., GARNEROT, F., LOUIS, S., FOURNIER, M., SACCHI, A., FUSI, M., LAPOINTE, D. & COUTURE, P. 2011. Physiological effects of

- temperature and herbicide mixture on the soft-shell clam *Mya arenaria* (Mollusca, Bivalvia). *Environmental toxicology and chemistry*, 30(1):132-141.
- GROCHULSKI, P., MASSON, L., BORISOVA, S., PUSZTAI-CAREY, M., SCHWARTS, J., BROUSSEAU, R. & CYGLER, M. 1995. *Bacillus thuringiensis* CryIA(a) insecticidal toxin: crystal structure and channel formation. *Journal of Molecular Biology*, 254(3):447-464.
- GUENTHER, K., HEINKE, V., THIELE, B., KLEIST, E., PRAST, H. & REACKER, T. 2002. Endocrine disrupting nonylphenols are ubiquitous in food. *Environmental Science and Technology*, 36(8):1676-1680.
- HALPIN, C., KNIGHT, M.E., FOXON, G.A., CAMPBELL, M.M. Boudet, A.M., BOON, J.J., CHABBERT, B., TOLLIER, M. & SCHUCH, W. 1994. Manipulation of lignin quality by downregulation of cinnamyl alcohol dehydrogenase. *The Plant Journal*, 6(3):339-350.
- HARVEY, A. 2013. *Laboratory assessment of the presence of Bt proteins in maize leaves and possible effects thereof on the development of Xenopus laevis tadpoles*. Potchefstroom.
- HEINO, M & KAITALA, V. 1999. Evolution of resource allocation between growth and reproduction in animals with indeterminate growth. *Journal of Evolutionary Biology*, 12:423-429.
- HELLMICH, R.L., SIEGFRIED, B.D., SEARS, M.K., STANLEY-HORN, D.E., DANIELS, M.J., MATTILA, H.R., SPENCER, T., BIDNE, K.G. & LEWIS, L.C. 2001. Monarch larvae sensitivity to *Bacillus thuringiensis* purified proteins and pollen. *PNAS*, 98(21):11925-11930.
- HICKMAN, C.P. Jr, ROBERTS, L.S., LARSON, A., L'ANSON, H. & EISENHOUR, D.J. 2006. *Integrated principles of Zoology*, Thirteenth edition. New York: McGraw-Hill.
- HILBECK, A., BAUMGARTNER, M., FRIED, P.M. & BIGLER, F. 1998. Effects of transgenic *Bacillus thuringiensis* corn-fed prey on mortality and development time of immature *Chrysoperla carnea* (Neuroptera: Chrysopidae). *Environmental Entomology*, 27(2):480-487.
- HOEKEMA, A., HIRSCH, P.R, HOOYKAAS, P.J.J. & SCHILPEROORT, R.A. 1983. A binary plant vector strategy based on separation of vir- and T-region of the *Agrobacterium tumefaciens* Ti-plasmids. *Nature*, 303:179-180.
- HÖFTE, H. & WHITELEY, H.R. 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiological Reviews*, 53(2):242-255.

HUANG, J., HU, R., PRAY, C., QIAO, F. & ROZELLE, S. 2003. Biotechnology as an alternative to chemical pesticides: a case study of Bt cotton in China. *Agricultural Economics*, 29:55-67.

HUBENDICK, B. 1978. 2A vols. Systematics and comparative morphology of the Basommatophora. In: FRETTER, V. & PEAKE, J., eds. *Pulmonates: Systematics, Evolution and Ecology*, New York: Academic Press inc. 1-48 p.

IBRAIMOV, B. 2014. Central Asia Online. http://centralasiaonline.com/en_GB/articles/caii/features/main/2014/04/01/feature-01 Date of access: 02 April 2014.

ISMAEL, Y., BENNET, R. & MORSE, S. 2001. Biotechnology in Africa: The adoption and economic impacts of Bt cotton in the Makhathini Flats, Republic of South Africa. (In Biotechnology conference for Sub-Saharan Africa. Johannesburg.)

ISO, International Organization for Standardization. 2012. *Water quality - Determination of the inhibition of the mobility of Daphnia magna (Straus) (Cladocera, Crustacea) - Acute toxicity test*.

IWATA, H., TANABE, S., SAKAI, N. & TATSUKAWA, R. 1993. Distribution of persistent organochlorines in the oceanic air and surface seawater and the role of ocean on their global transport and fate. *Environmental Science and Technology*, 27:1080-1098.

IZQUIERDO, M.S., FERNANDEZ-PALACIOS, H. & TACON, A.G.J. 2001. Effects of broodstock nutrition on reproductive performance of fish. *Aquaculture*, 197:25-42.

JAMES, C. 2003. *Global status of commercialization transgenic crops: 2003. ISAAA Brief no. 30*. Ithaca, NY.

JAMES, C. 2004. *Preview: Global status of commercialized biotech/ GM crops: 2004, executive summary*. Ithaca: NY.

JAMES, C. 2006. *Global status of commercialized biotech/GM crops: 2006. ISAAA Brief no. 35*. Ithaca: NY.

JAMES, C. 2008. *Global status of commercialized biotech/GM crops: 2008. Brief no. 39*. Ithaca, NY.

JAMES, C. 2009. *Global status of commercialized biotech/GM crops: 2009, executive summary*. Ithaca: NY.

JAMES, C. 2012. *Global status of commercialized biotech/GM crops: 2012*. ISAAA Brief no. 44. Ithaca: NY.

JENSEN, P.D., DIVELEY, G.P., SWAN, C.M. & LAMP, W.O. 2010. Exposure and nontarget effects of transgenic Bt corn debris in streams. *Environmental Entomology*, 39(2):707-714.

JOUBERT, P.H., VAN EEDEN, J.A., PRESTORIUS, S.J., DE KOCK, K.N., VENTER, J.M., WOLMARANS, C.T. & RYKE, P.A.J. 1983. Varswatermolluske in die Magaliesbergreeks tussen Pretoria en Rustenburg. Potchefstroom: Wetenskaplike bydraes van die PU vir CHO, Reeks B: Natuurwetenskappe, 98.

KETATA, I., DENIAR, X., HAMZA-CHAFFAI, A. & MINIER, C. 2008. Endocrine-related reproductive effects in molluscs. *Comparative Biochemistry and Physiology, Part C*, 147:261-270.

KLEE, H.J., WHITE, F.F., IYER, V.N., GORDON, M.P. & NESTER, E.W. 1983. Mutational analysis of the virulence region of an *Agrobacterium tumefaciens* Ti plasmids. *Journal of Bacteriology*, 153(2):878-883.

KLEIN, T.M., FROMM, M., WEISSINGER, A., TOMES, D., SCHAAF, S., SLETTEN, M. & STANDFORD, J.C. 1988. Transfer of foreign genes into intact maize cells with high-velocity microprojectiles. *Proceedings of the National Academy of Science*, 85:4305-4309.

KNECHT, S. & NENTWIG, W. 2010. Effects of Bt maize on the reproduction and development of saprophagous Diptera over multiple generations. *Basic and Applied Ecology*, 11:346-353.

KNOWLES, B.H. & ELLAR, D.J. 1987. Colloid-osmotic lysis is a general feature of mechanism of action of *Bacillus thuringiensis* δ -endotoxin with different insect specificity. *Biochimica et Biophysica Acta*, 924:509-518.

KNOWLES, B.H., FRANCIS, P.H. & ELLAR, D.J. 1986. Structurally related *Bacillus thuringiensis* δ -endotoxins display major differences in insecticidal activity in vivo and in vitro. *J. Cell Sci.*, 84:221-235.

KRAMARZ, P., DE VAUFLEURY, A., GIMBERT, F., CORTET, J., TABONE, E., ANDERSEN, M.N. & KROGH, P.H. 2009. Effects of Bt-maize material on the life cycle of the land snail *Cantareus aspersus*. *Applied Soil Ecology*, 42:236-242.

KUMAR, P.A. 2003. Insect pest resistant transgenic crops. In: UPADHYAY, R.K., ed. *Advances in microbial control of insect pests*, New York: CRC Press. 71-82 p.

LAJUS, D.L. 2010. Developmental instability and fluctuating asymmetry. *In*: NEWMAN, M.C., ed. *Fundamentals of Ecotoxicology. Third edition*, Baco Raton: CRC Press. 227-231 p.

LEBLANC, G.A., CAMPBELL, P.M., DEN BESTEN, P., BROWN, R.P., CHANG, E.S., COATS, J.R., DEFUR, P.L., DHAHIALLA, T., EDWARDS, J., RIDDIFORT, L.M., SIMPSON, M.G., Snell, T.W., THORNDYKE, M. & MATSUMURA, F. 1998. The Endocrinology of Invertebrates. *In*: DEFUR, P.L., CRANE, M., INGERSOLL, C.G. & TATTERSFIELD, L.J., eds. *Endocrine disruption in invertebrates: Endocrinology, testing and assessment*, Pensacola, Florida: SETAC Technical Publications Series. 23-106 p.

LEE, R.F. 1985. Metabolism of tributyltin oxide by crabs, oysters and fish. *Marine Environmental Research*, 17:147-148.

LEGIERSE, K.C.H.M., SIJM, D.T.H.M., VAN LEEUWEN, C.J., SEINEN, W. & HERMENS, J.L.M. 1998. Bioconcentration kinetics of chlorobenzenes and the organophosphorus pesticide chlorthion in the pond snail *Lymnaea stagnalis* - a comparison with the guppy *Poecilia reticulata*. *Aquatic Toxicology*, 41:301-323.

LOSEY, J.E., RAYOR, L.S. & CARTER, M.E. 1999. Transgenic pollen harms monarch larvae. *Nature*, 399:214.

LUSHCHAK, O.V., KUBRAK, O.I., STOREY, J.M. & LUSHCHAK, V.I. 2009. Low toxic herbicide Roundup induces mild oxidative stress in glodfish tissue. *Chemosphere*, 76:932-397.

LUTZ, W. & SAMIR, K.C. 2010. Dimensions of global population projections: what do we know about future population trends and structures? *Philosophical Trnasactions of The Royal Society B*, 365(1554):2779-2791.

MANCEVA, S.D., PUSZTAI-CAREY, M., RUSSO, P.S. & BUTKO, P. 2005. A detergent-like mechanism of action of the cytolytic toxin Cryt1A from *Bacillus thuringiensis* var. *israelensis*. *Biochemistry*, 44(2):589-597.

MARROQUIN, L.D., ELYASSNIA, D., GRIFFITTS, J.S., FEITELSON, J.S. & AROIAN, R.V. 2000. *Bacillus thuringiensis* (Bt) toxin susceptibility and isolation of resistance mutants in the nematode *Caenorhabditis elegans*. *Genetics Society of America*, 155:1693-1699.

MATTHIESSEN, P. 2008. An assessment of endocrine disruption in mollusks and the potential for developing internationally standardized mollusks life cycle test guidlines. *Intergrated Environmental Assessment and Management*, 4(3):274-284.

MAVIER, M., MCCREEDY, C., REGETZ, J. & KAREIVA, P. 2007. A meta-analysis of effects of Bt cotton and maize on nontarget invertebrates. *Sciences*, 316:1475-1476.

MODESTO, K.A. & MARTINEZ, C.B.R. 2009. Roundup causes oxidative stress in the liver and inhibits acetylcholinesterase in muscle and brain of the fish *Prochilodus*. *Chemosphere*, 78:294 - 299.

NABORS, M.W. 2004. Introduction to Botany. San Francisco: Pearson Education.

NARANJO, S.E. 2005. Long-term assessment of the effects of transgenic Bt cotton on the abundance of nontarget arthropod natural enemies. *Environmental Entomology*, 34(5):1193-1210.

NEWMAN, M.C. 2010. Fundamentals of Ecotoxicology. Third Edition. Boca Raton: CRC Press.

OEHLMANN, J., BENEDETTO, P.D., TILLMANN, M., DUFT, M., OETKEN, M. & SCHULTE-OEHLMANN, U. 2007. Endocrine disruption in prosobranch molluscs: evidence and ecological relevance. *Ecotoxicology*, 16:29-43.

OPPERT, B., KRAMER, K.J., BEEMAN, R.W., JOHNSON, D. & MCGAUGHEY, W.H. 1997. Proteinase-mediated insect resistance to *Bacillus thuringiensis* toxins. *The Journal of Biological Chemistry*, 272(38):23473-23476.

OPPERT, B., KRAMER, K.J., JOHNSON, D.E., MACINTOSH, S.C. & MCGAUGHEY, W.H. 1994. Altered protoxin activation by the midgut enzymes from a *Bacillus thuringiensis* resistant strain of *Plodia interpunctella*. *Biochemical and Biophysical Research Communications*, 198(3):940-947.

OWEN, G. 1966. 2 vols. Feeding. In: WILBUR, K.M. & YONGE, C.M., eds. *Physiology of mollusca*, New York: Academic Press inc. 1-51 p.

OWINO, O. 2012. Scientists torn over Kenya's recent GM food ban. <http://www.scidev.net/global/nutrition/news/scientists-torn-over-kenya-s-recent-gm-food-ban.html> Date of access: 02 April 2014.

PALANZA, P., PARMIGIANI, S., LUI, H. & VOM SAAL, F.S. 1999. Prenatal exposure to low dose of estrogenic diethylstilbestrol and o,p'-DDT alters aggressive behavior of male and female house mice. *Pharmacology Biochemistry and Behavior*, 64(4):665-672.

PASTOR, S., CREUS, A., PARRÓN, T., CEBULSKA-WASILEWSKA, A., SIFFEL, C., PIPERAKIS, S. & MARCOS, R. 2003. Biomonitoring of four European populations

- occupationally exposed to pesticides: use of micronuclei as biomarkers. *Mutagenesis*, 18(3):249-258.
- PERRIN, N. & SIBLY, R.M. 1993. Dynamic models of energy allocation and investment. *Annual Review of Ecology, and Systematics*, 24:379-410.
- PERUZZO, P.J., PORTA, A.A. & RONCO, A.E. 2008. Levels of glyphosate in surface waters, sediments and soils associated with direct sowing soybean cultivation in north pampasic region of Argentina. *Environmental Pollution*, 156:61-66.
- PIGOTT, C.R., KING, M.S. & ELLAR, D.J. 2008. Investigating the properties of *Bacillus thuringiensis* Cry proteins with novel loop replacements created using combinatorial molecular biology. *Applied and Environmental Microbiology*, 74(11):3497-3511.
- PILCHER, C.D., OBRYCKI, J.J., RICE, M.E. & LEWIS, L.C. 1997. Preimaginal development, survival, and field abundance of insect predators on transgenic *Bacillus thuringiensis* corn. *Environmental Entomology*, 26(2):446-454.
- PURCHON, R.D. 1968. International series of monographs in pure and applied biology. The biology of the Mollusca. Volume 40. Hungary: Pergamon Press Ltd.
- QUIST, D. 2007. 1 vols. Vertical (trans)gene flow: Implications for crop diversity and wild relatives. In: TRAAVIK, T. & CHING, L.L., eds. *Biosafety first*, Trondheim: Tapir Academic Press. 205-220 p.
- QUIST, D.A., HEINEMANN, J.A., MYHR, A.I., ASLAKSEN, I. & FUNTOWICS, S. 2013. *Hungry for innovation: pathways from GM crops to agroecology*. Denmark.
- RAMIREZ-ROMERO, R., DESNEUX, N., CHAUFAX, J. & KAISER, L. 2008. Bt-maize effects on biological parameters of the non-target aphid *Sitobion avenae* (Homoptera: Aphididae) and Cry1Ab toxin detection. *Pesticide Biochemistry and Physiology*, 91:110-115.
- RAVEN, C.P. 1964. Development. In: WILBUR, K.M. & YONGE, C.M., eds. *Physiology of Mollusca. Volume 1*, New York: Academic Press Inc. 165-195 p.
- RAVEN, C.P. 1975. Development. In: FRETTER, V. & PEAKE, J., eds. *Pulmonates. Volume 1: Functional anatomy and physiology*, New York: Academic Press. 367-398 p.
- REPOLHO, T., BAPTISTA, M., PIMENTEL, M.S., DIONISIO, G., TRUBENBACH, K., LOPES, V.M., LOPES, A.R., CALADO, R., DINIZ, M. & ROSA, R. 2013. Developmental

and physiological challenges of octopus (*Octopus vulgaris*) early life stage under ocean warming. *Journal of Comparative Physiology B*, DOI: 10.1007/s00360-013-0783-y

RINCÓN, J. & MARTINEZ, I. 2006. Food quality and feeding preferences of *Phylloicus* spp. (Trichoptera: Calamoceratidae). *Journal of the North American Benthological Society*, 25(1):209-215.

RISSLER, J. & MELLON, M. 2000. The ecological risks of engineered crops. The MIT Press.

RIZZO, D.C. 2010. Fundamentals of Anatomy and Physiology. NewYork: Delmar, Cengage Learning.

ROSI-MARSHALL, E.J., TANK, J.L., ROYER, T.V., WHILES, M.R., EVANS-WHITE, M., CHAMBERS, C., GRIFFITHS, N.A., POKELSEK, J. & STEPHEN, M.L. 2007. Toxins in transgenic crop byproducts may affect headwater stream ecosystems. *PNAS*, 104(41):16204-16208.

RUNHAM, N.W. 1975. Alimentary canal. *In*: FRETTER, V. & PEAKE, J., eds. *Pulmonates. Volume 1: Functional anatomy and physiology*, London: Academic Press Inc. 53-104 p.

RUNHAM, N.W. 1988. Mollusca. *In*: ADIYODI, K.G & ADIYODI, R.G., eds. *Reproductive Biology of Invertebrates. Volume III: Accessory sex glands*, Chichester: John Wiley & Sons. 113-188 p.

SAXENA, D. & STOTZKY, G. 2000. Insecticidal toxin from *Bacillus thuringiensis* is released from roots of transgenic Bt corn in vitro and in situ. *FEMS (Federation of European Microbiological Societies) Microbiology-Ecology*, 33:35-39.

SAXENA, D. & STOTZKY, G. 2001. Bt corn has a higher lignin content than non-Bt corn. *American Journal of Botany*, 88(9):1704-1706.

SCHNELL, M.J., BUONOCORE, L., WHITT, M.A. & ROSE, J.K. 1996. The minimal conserved transcription stop-start signal promotes stable expression of foreign gene in vesicular stomatitis virus. *Journal of Virology*, 70(4):2318-2323.

SCHNEPH, E., CRICKMORE, N., VAN RIE, J., LERECLUS, D., BAUM, J., FEITELSON, J., ZEIGLER, D.R. & DEAN, D.H. 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiology and Molecular Biology*, 62(3):775-806.

- SCHULTE-OEHLMANN, U., BETTIN, C., FIORONI, P., OEHLMANN, J. & STROBEN, E. 1995. *Marisa cornuarietis* (Gastropoda, Prosobranchia): a potential TBT bioindicator for freshwater environments. *Ecotoxicology*, 4:372-384.
- SEARS, M.K., HELLMICH, R.L., STANLEY-HORN, D.E., OBERHAUSER, K.S., PLEASANTS, J.M., MATTILA, H.R., SIEGFRIED, B.D. & DIVELY, G.P. 2001. Impact of Bt corn pollen on monarch butterfly populations: A risk assessment. *PNAS*, 98(21):11937-11942.
- SEGNER, H. 2009. Zebrafish (*Danio rerio*) as a model organism for investigating endocrine disruptions. *Comparative Biochemistry and Physiology, Part C*, 149:187-195.
- SÉRALINI, G., CLAIR, E., MESNAGE, R., GRESS, S., DEFARGE, N., MALATESTA, M., HENNEQUIN, D. & DE VENDÔMOIS, J.S. 2012. Long term toxicity of Roundup herbicide and a Roundup-tolerant genetically modified maize. *Food and Chemical Toxicology*, 50(11):4221-4231.
- SHALAT, S.L., DONNELLY, K.C., FREEMAN, N.C.G., CALVIN, J.A., RAMESH, S., JIMENEZ, M., BLACK, K., COUTINHO, C., NEEDHAM, L., BARR, D. & RAMIREZ, J. 2003. Nondietary ingestion of pesticides by children in an agricultural community on the US/Mexico border: Preliminary results. *Journal of Exposure analysis and Environmental Epidemiology*, 13:42-50.
- SHELTON, A.M., ZHAO, J.Z. & ROUSH, R.T. 2002. Economic, ecological, food safety, and social consequences of the deployment of Bt transgenic plants. *Annual review of Entomology*, 47:845-881.
- SHIER, D., BUTLER, J. & LEWIS, R. 2009. Hole's essentials of Human anatomy and physiology. Tenth ed. NewYork: McGraw-Hill.
- SIMS, S.R. & HOLDEN, L.R. 1969. Insect bioassay for determining soil degradation of *Bacillus thuringiensis* subsp. *kurstaki* CryIA(b) protein in corn tissue. *Environmental Entomology*, 25:659-664.
- SIMS, S. R., REAM & J.E. 1997. Soil inactivation of the *Basillus thuringiensis* subsp. *kurstaki* CryIIA insecticidal protein within transgenic cotton tissue: laboratory and field studies. *Journal of Agricultural and Food Chemistry*, 45:1502-1505.
- STANFORD, J.C., KLEIN, T.M., WOLF, E.D. & ALLEN, N. 1987. Delivery of substances into cells and tissues using a particle bombardment process. *Particulate Science and Technology*, 5(1):27-37.

STENERSEN, J. 2004. Chemical pesticides. Mode of action and toxicology. New York: CRC Press.

STIGLINGH, I. & VAN EEDEN, J.A. 1970. Notes on the feeding behaviour of *Bulinus* (*Bulinus*) *tropicus* (Krauss). Basommatophora: Planorbidae. *Wetenskaplike bydraes van die P.U. vir C.H.O.*, 22:1-14.

STIGLINGH, I. & VAN EEDEN, J.A. 1976. Functional morphology and histology of the reproductive system of *Bulinus tropicus* (Mollusca: Basommatophora). *Wetenskaplike bydraes van die PU vir CHO. Reeks B: Natuurwetenskappe* Nr.86:1-14.

STIGLINGH, I. & VAN EEDEN, J.A. 1977. Population fluctuations and ecology of *Bulinus tropicus* (Mollusca: Basommatophora). *Wetenskaplike bydrae van die P.U. vir C.H.O. Reeks B: Natuurwetenskappe*, 87:1-37.

STOTHARD, J.R., HUGHES, S. & ROLLINSON, D. 1996. Variation within the Internal Transcribed Spacer (ITS) of ribosomal DNA genes of intermediate snail hosts within the genus *Bulinus* (Gastropoda: Planorbidae). *Acta Tropica*, 61:19-29.

SUN, M., ZHANG, L. & ZINIU, Y. 2003. Molecular biology of *Bacillus thuringiensis*. In: UPADHYAY, K., ed. *Advances in microbial control of insect pests*, New York: Kluwer Academic / Plenum Publishers. 71-82 p.

SWAN, C.M., JENSEN, P.D., DIVELEY, G.P. & LAMP, W.O. 2009. Processing of transgenic crop residue in stream ecosystems. *Journal of Applied Ecology*, 46:1304-1313.

TANG, J.D., A.M., Shelton., VAN RIE, J., DE ROECK, S., MOAR, W.J., ROUSH, R.T. & PEFEROEN, M. 1996. Toxicity of *Bacillus thuringiensis* spore and crystal protein to resistant Diamondback moth (*Plutella xylostella*). *Applied and Environmental Microbiology*, 62(2):564-569.

TANK, J.L., ROSI-MARSHALL, E.J., ROYER, T.V., WHILES, M.R., GRIFFITHS, N.A., FRAUENDORF, T.C & TREERING, D.J. 2010. Occurrence of maize detritus and a transgenic insecticidal protein (Cry1Ab) within the stream network of agricultural landscape. *Proceedings of the National Academy of Science*, 107(41):17645-17650.

TAPP, H. & STOTZKY, G. 1998. Persistence of the insecticidal toxin from *Bacillus thuringiensis* subsp. *kurstaki* from soil. *Soil Biology and Biochemistry*, 30(4):471-476.

TATE, R. 2009. Seelye's Principles of Anatomy and Physiology. New York: McGraw-Hill.

- THOMAS, W.E. & ELLAR, D.J. 1983. *Bacillus thuringiensis* var *israelensis* crystal δ -endotoxin: effects on insect and mamalian cells in vitro and in vivo. *J.Cell Sci.*:181-197.
- TIAN, J., LONG, L., WANG, X., NARANJO, S.E., ROMEIS, J., HELLMICH, R.L., WANG, P. & SHELTON, A.M. 2014. Using resistant prey demonstrates that Bt plants producing Cry1Ac, Cry2Ab, and Cry1F have no effects on *Geocoris punctipes* and *Orius insidiosus*. *Transgenic Plants and Insects*, 14:242-251.
- TOJO, A. & AIZAWA, K. 1983. Dissolution and degradation of *Bacillus thuringiensis* δ -endotoxin by gut juice protease of the silkworm *Bombyx mori*. *Applied and Environmental Microbiology*, 45(2):576-580.
- TRAAVIK, T., NIELSEN, K.M. & QUIST, D. 2007. Genetic engineering of living cells and organisms. In: TRAAVIK, T. & CHING, L.L., eds. *Biosafety first - Holistic approaches to risk and uncertainty in genetic engineering and gentically modified organisms*, Trondheim: Tapir Academic Press. 65-93 p.
- UNSWORTH, J.B., WAUCHOPE, R.D., KLEIN, A.W., DORN, E., ZEEH, B., YEH, S.M., AKERBLOM, M., RACKE, K.D. & RUBIN, B. 1999. Significance of the long range transport of pesticides in the atmosphere. *Pure and Applied Chemistry*, 71(7):1359-1383.
- VALENTI, T.W., CHERRY, D.S., CURRIE, R.J., NEVES, R.J., JONES, J.W., MAIR, R. & KANE, C.M. 2006. Chlorine toxicity to early stages of freshwater mussels (Bivalvia: Unionidae). *Environmental Toxicology and Chemistry*, 25(9):2512-2518.
- VAN AARD, W.J. & VAN EEDEN, J.A. 1976. Aspekte van die respirasiefisiologie by die varswaterslak *Bulinus* (*Bulinus*) *tropicus*. *Wetenskaplike bydraes van die P.U. vir C.H.O. Reeks B: Natuurwetenskappe*, 83:1-13.
- VAN DER MERWE, F., BEZUIDENHOUT, C., VAN DEN BERG, J. & MABOETA, M. 2012. Effects of Cry1Ab transgenic maize on lifecycle and biomarker responses of the earthworm, *Eisenia andrei*. *Sensors*, 12:17155-17167.
- VAN DER OOST, R., HEIDA, H. & OPPERHUIZEN, A. 1988. Polychlorinated Biphenyl congeners in sediment, plakton, molluscs, crustaceans, and eel in freshwater lake: Implications of using reference chemicals and indicatro organisms in bioaccumulation studies. *Archieves of Envrionmental Comtamination and Toxicology*, 17:721-729.
- VAN NOORDEN, R. 2013. Monsanto drops GM in Europe. *Nature. News in focus*, 25 July, p.387.

- WARING, R.H., MCDONALD, A.J.S., LARSSON, S., ERICSSON, T., WIREN, A., ARWIDSSON, E., ERICSSON, A. & LOHAMMAR, T. 1985. Differences in chemical composition of plants grown at constant relative growth rates with stable mineral nutrition. *Oecologia*, 66:157-160.
- WHITEHOUSE, M.E.A., WILSON, L.J., DAVIES, A.P., CROSS, D., GOLDSMITH, P., THOMPSON, A., HARDEN, S. & BAKER, G. 2014. Target and nontarget effects on novel "Triple-stacked" Bt-transgenic cotton 1: Canopy arthropod communities. *Transgenic plants and insects*, 14:218-241.
- WILLEY, J.M., SHERWOOD, L.M. & WOOLVERTON, C.J. 2008. Prescott, Harley and Klein's Microbiology. New York: Mc-Graw Hill.
- WOLFENBARGER, L.L., NARANJO, S.E., LUNDGREN, J.G., BITZER, R.J. & WATRUD, L.S. 2008. Bt crop effects on functional guilds of non-target arthropods: A meta-analysis. *PLOS one*, 3(5):e2118.
- WOLSON, R.A. 2007. Assessing the prospects for the adoption of biofortified crops in South Africa. *The Journal of Agribiotechnology Management and Economics*, 10(3):184-191.
- WOOTON, A.N., HERRING, C, SPRY, J.A., WISEMAN, A., LIVINGSTON, D.R. & GOLDFARB, P.S. 1995. Evidence for the existence of cytochrome P450 gene families (CYP1A, 3A, 4A, 11A) and modulation of gene expression (CYP1A) in the mussel *Mytilus* spp. *Marine Environmental Research*, 39(1-4):21-26.
- YU, S.J. 2008. The toxicology and biochemistry of insecticides. New York: CRC Press.
- ZALDIBAR, B., RODRIGUES, A., LOPES, M., AMARAL, A. & MARIGOMEZ, I. Soto, M. 2006. Freshwater molluscs from volcanic areas as model organisms to assess adaptation to metal chronic pollution. *Science of the Total Environment*, 371(1-3):168-175.
- ZHANG, W., WANG, W. & ZHANG, L. 2013. Arsenic speciation and spatial and interspecies differences of metal concentrations in mollusks and crustaceans from a South China estuary. *Ecotoxicology*, 22:671-682.
- ZWAHLEN, C., HILBECK, A., HOWALD, R. & NENTWIG, W. 2003. Effects of transgenic Bt corn litter on the earthworm *Lumbricus terrestris*. *Molecular Ecology*, 12:1077-1086.