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Possible endocrine disruption in molluscs from the Limpopo Province

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Acknowledgments

Dedicated to Oupa Aster Viljoen and the late Oupa Lucas Viljoen
“The shortest trees, to the tallest trees, know where their roots lie”

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List of abbreviations

Abbreviation	Meaning
A	
ACTH	adrenocoticotropic hormone
ADH	antidiuritic hormone
Ah	arylhydrocarbon
APs	alkyl phenols
B	
BPA	bisphenol-A
C	
CDCH	caudodorsal cell hormone
D	
DBH	dorsal body hormones
DDT	1,1,1-Trichloro-2,2-bis(*p*-clorophenyl)ethane
DES	diethylstilbestrol
DHEA	dehydroepiandrosterone
DHT	dihydrotestosterone
DOH	South African Department of Health
E	
ED	endocrine disruption
EDC	endocrine disrupting compound/chemical
ELH	egg-laying hormone
F	
FR	Limpopo farm reference site
FEN	fenarimol
FSH	follicle-stimulating hormone
G	
GH	growth hormone
H	
HCB	hexachlorobenzene
K	
KNP	Kruger National Park
L	
LC	laboratory control
LD_{50}	acute lethal dose
LH	luteinizing hormone
LimpR	Limpopo reference sites (non-DDT sprayed area)
LimpT	Limpopo test sites (DDT-sprayed area)

LTH	prolactin or luteotropic hormone
M	
MIPs	molluscan-insulin-like peptides
MIS	Müllerian-inhibiting substance
MT	methyltestosterone
N	
NWU	North-West University
O	
OB	breadth of shell opening
OL	length of shell opening
P	
PAHs	polycyclic aromatic hydrocarbons
PCBs	polychlorinated biphenyls
POPs	persistent organic pollutants
Potch	Potchefstroom sites
PP1	widest part of preputium
PPS	preputium length
PS	penis sheath length
PS1	narrowest part of the penis sheath
PS2	widest part of the penis sheath
PSPLR	penis sheath/preputium length ratio
rT_3	reverse T_3
S	
SA	South Africa
SADC	Southern African Development Community
SL	total length of shell
SRY	sex determining region Y
T	
T_3	triiodothyronine
T_4	thyroxine
TBT	tributyl-tin
TCDD	tetrachlorodibenzo-*p*-dioxin
TPT	tryphenyltin
TSH	thyroid-stimulating hormone

Abstract

Possible endocrine disruption in molluscs from the Limpopo Province

With parts of SA in a malaria endemic area, a preventative way of fighting malaria is with the use of pesticides such as 1,1,1-Trichloro-2,2-bis(*p*-clorophenyl)ethane, also known as DDT. DDT is listed under the persistent organic pollutants (POPs) and considered an endocrine disruptive compound (EDC) under the Stockholm Convention. SA registered an exemption to use DDT as means to fight malaria. DDT and its isomers are, however, known EDCs. Combined with their ability to persist in the environment while not being target specific motivates further studies into possible detrimental effects.

The present study aimed to establish if ED was present by comparing the male reproductive organs from snails from an area currently sprayed with DDT (for malaria control) to an area not sprayed with DDT in the Limpopo Province. A possible endpoint (the penis sheath/preputium length ratio or PSPLR) was identified for the freshwater snail *Bulinus tropicus.*

B. tropicus and sediment samples were collected from DDT-sprayed and non-sprayed areas located close together. The snails were dissected and various morphometric parameters measured. Sediments from the sites where the snails were collected were analysed for DDT using GC-MS.

Statistical analysis showed significant differences in PSPLR (and therefore possible ED) between snails from the two areas. The difference in PSPLR values was mainly due to a relatively shorter preputium for the snails from the DDT-sprayed area. Even though the sediment samples showed that DDT was present in most of the DDT-sprayed sites and not in the non-DDT sprayed sites, causality of the possible ED could not be established from this field study. This study indicated the possibility of using the PSPLR as endpoint for ED. Recommendations are made for further development of the PSPLR and *B. tropicus* as biological indicators for endocrine disruption, but causality must first be established.

Key words: Persistent organic pollutants, Mollusc, Endocrine disruption, Endocrine system, DDT, Limpopo Province, Morphometrics, Penis sheath, Preputium, Malaria control

Opsomming

Moontlike endokriene versteuring in slakke van die Limpopo Provinsie

'n Voorkomende maatreël vir die teenkamping van malaria in sekere streke van Suid-Afrika (SA) is die gebruik van insekdoders soos DDT (1,1,1-Trichloro-2,2-bis(*p*-clorophenyl)ethane). DDT is in die Stockholmkonvensie gelys onder die persisterende organiese besoedelstowwe (POBs) en geag as a endokriene versteurder (EV). SA het aansoek gedoen om DDT te gebruik in die stryd teen malaria. DDT en die DDT isomere is egter bekende EVs en besit die vermoë om in die omgewing te persisteer en is nie teiken-spesifiek nie. Hierdie eienskappe dien as motivering tot verdere ondersoek na moontlike negatiewe gevolge.

Die huidige studie was daarop gemik om vas te stel of endokriene versteuring teenwoordig is, deur die manlike geslagsorgane van slakke te vergelyk van twee gebiede in die Limpopo Provinsie. In die een gebied word DDT gespuit en in die ander word geen DDT gespuit nie. 'n Moontlike eindpunt wat geïdentifiseer is, is die penis skede/preputium lengte verhouding of PSPLR, van die varswater slak *Bulinus tropicus*.

B. tropicus en sediment monsters was versamel in twee nabygeleë gebiede. Die slakke is gedissekteer en verskeie morfometriese aspekte was gemeet. Sediment afkomstig van die persele waar slakke versamel was, was vir DDT geanaliseer met 'n GC-MS.

Statistiese analises het getoon dat daar 'n betekenisvolle verskil in die PSPLR waardes was (dus moontlike endokriene versteuring) tussen die slakke van die twee gebiede. Die verskil in PSPLR waardes was hoofsaaklik toegeskryf aan 'n relatiewe korter preputium in die slakke van die gebied wat met DDT gespuit word. Daar was DDT in die meeste van die sediment monsters gevind van die gebied waar DDT gespuit word en geen DDT in die sediment wat uit die gebied kom waar geen DDT gespuit word nie. Die oorsaak van die moontlike endokriene versteuring kon egter nie vasgestel word uit die veldwerk nie. Die studie het wel die moontlikheid om die PSPLR te gebruik as 'n eindpunt vir endokriene versteuring aangedui. Aanbevelings word gemaak vir die verdere ontwikkeling van die PSPLR en *B. tropicus* as biologiese indikators vir endokriene versteuring, maar oorsaaklike verbande moet eers vasgestel word.

Sleutel woorde: Persisterende organiese besoedelstowwe, Slak, Endokriene versteuring, Endokriene stelsel, DDT, Limpopo Provinsie, Morfometriese afmetings, Penis skede, Preputium, Malaria beheer

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Chapter 1: Introduction

If one lives in South Africa, you are constitutionally entitled to many a necessity for life. Two of these entitlements or rights are health and clean water (RSA, 1996). For the government or any other governmental body to provide for just these two rights places them in a "Catch 22" situation, especially in places where malaria is present. In order to help the people not to contract malaria, pesticides are used to combat the insect vector for malaria. However, many of these pesticides are not just persistent, but also not-target specific. Now, the means by which the people are protected becomes another means by which people can be negatively affected. This is then a point of fierce debate with people on both sides of the coin fighting and lobbying against each other, for apparently the same cause, and that is a better life for all (Berenbaum, 2009; Herren & Mbogo, 2010; Noluthungu, 2010; Roberts & Tren, 2010; Urbach, 2009; Wells & Leonard, 2006).

In this bid for a better quality of life for every citizen, there are a number of drivers. Ignoring the fact that there are people driven by personal gain, the following can be considered key drivers in the use of insecticides in malaria control.

1.1 Malaria

Malaria is an infectious disease of humans, birds, and reptiles caused by the *Plasmodium* spp. of parasites from the protozoan group of organisms (Phylum: Apicomplexa, Class: Sporozoasida, Order: Eucoccidiorida). Four species of *Plasmodium* infect humans of which *P. falciparum* is the most common in Africa (Hickman e*t al*., 2004a; MRC, 2010). According to Walker (2002) malaria is exclusively transmitted by the female *Anopheles* mosquitoes. However, only certain species of *Anopheles* are vectors of malaria. In sub-Saharan Africa, the species responsible for transmitting malaria are *An. gambiae*, *An. complex*, *An. funestus*, and *An. pharoensis* (Walker, 2002).

This deadly human disease is widespread and difficult to control and occurs throughout the tropics and subtropics. (See Fig. 1 for the areas in South Africa where there is a risk of contracting malaria.) Malaria is responsible for the deaths of more than one million people and more than 300 million clinical cases every year (MRC, 2010). Additionally, malaria is also seen as a limiting factor in the economic growth of many of the poor, third-world countries (MRC, 2010; Noluthungu, 2010).

A preventative way of fighting malaria is by preventing the transmission of the parasite by the vector. This is done with the use of insecticides such as synthetic

pyrethroids and DDT (1,1,1-Trichloro-2,2-bis(*p*-clorophenyl)ethane), with the latter being the most effective (DOH, 2004; Wells & Leonard, 2006).

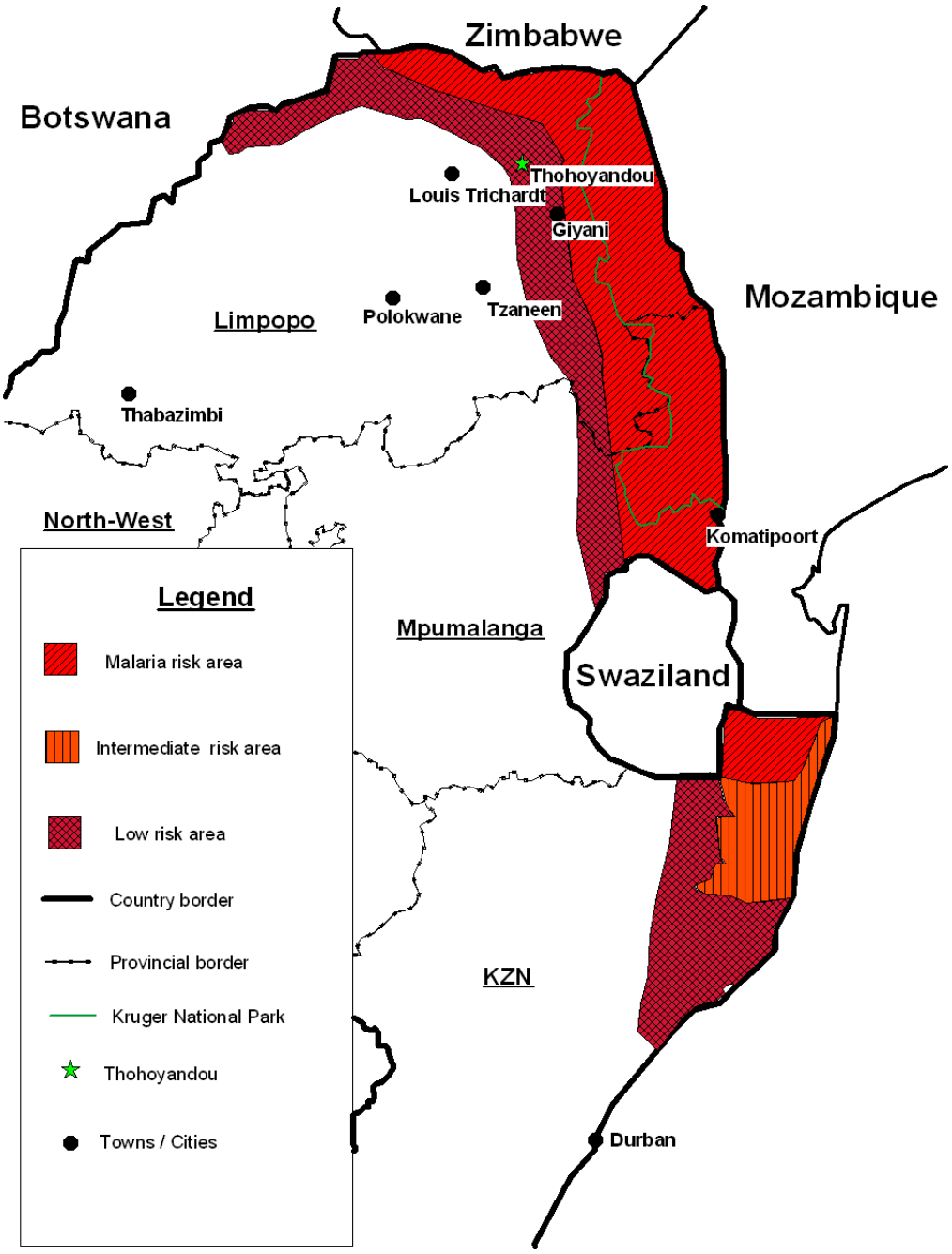

Figure 1: Areas in South Africa generally susceptible to malaria. Adapted from a map published by the South African Department of Health (DOH, 2004).

1.2 DDT

1,1,1-Trichloro-2,2-bis(*p*-clorophenyl)ethane (Fig. 2) also known as DDT, is a derivative of diphenylethane (Gosselin e*t al*., 1976). It was used extensively as an insecticide against the insect vectors of diseases such as malaria and sleeping sickness (Aneck-Hahn e*t al*., 2007; Burger, 2005; Lintelmann e*t al*., 2003). In 1972 DDT was banned in most industrial developed countries and has recently been

identified as an endocrine disrupting compound/chemical (EDC), and listed under the persistent organic pollutants (POPs) in the Stockholm Convention (Aneck-Hahn e*t al*., 2007; Bouwman, 2004; Burger, 2005; Wells & Leonard, 2006). It is, however, still used in developing countries, including South Africa (SA), to fight malaria (Aneck-Hahn e*t al*., 2007; Burger, 2005; Gosselin e*t al*., 1976; Lintelmann e*t al*., 2003). DDT has been detected in water sources in many parts of South Africa, including the Western-Cape, Gauteng, the Free State, and the eastern parts of the country (Barnhoorn e*t al*., 2009; Barnhoorn e*t al*., 2010; Bouwman e*t al*., 2006; Burger, 2005; Marchand e*t al*., 2008; Mlambo e*t al*., 2009).

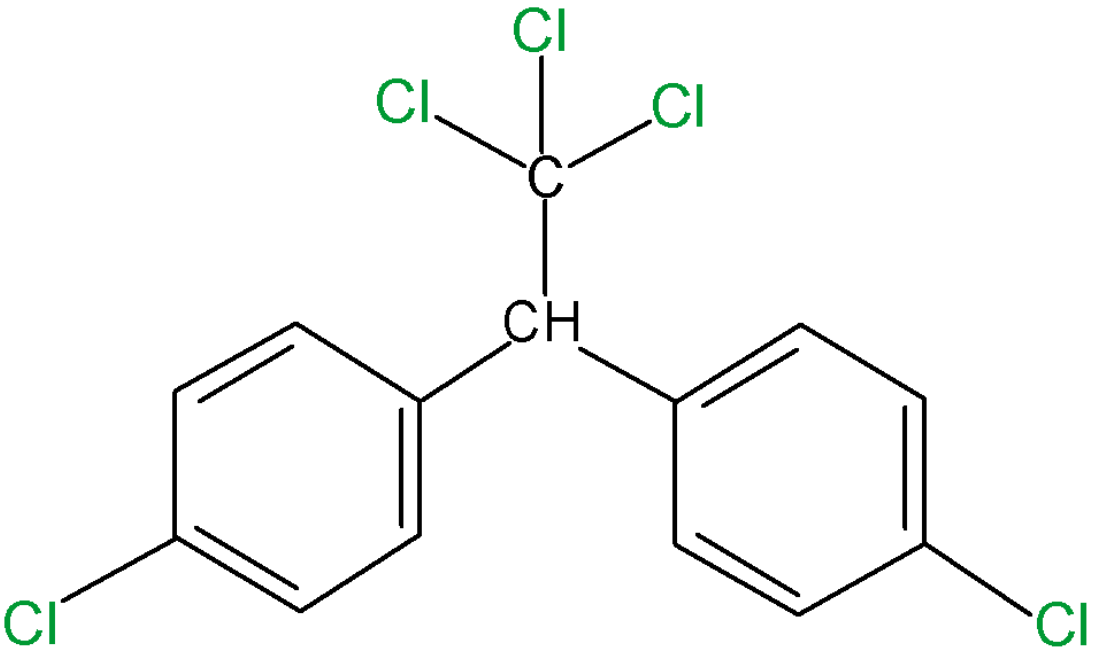

Figure 2: 1,1,1-trichloro-2,2-bis(*p*-clorophenyl)ethane also known as DDT. Adapted from (Harrison, 1997).

1.3 Water

All ecosystems are dependent on water in one form or another. Furthermore, countries need water for both social and economic development. In SA, as with many other Southern African Development Community (SADC) countries, the reliability of this resource is uncertain due to climatic conditions and climate change (SADC, 2008). Water sources are also under threat from inappropriate management practices resulting in pollution which then leads to limited access to safe drinking water and sanitation (SADC, 2008). Due to factors such as those mentioned above and the rapid development within southern and South Africa, it is expected that by 2025, SA will be facing absolute water scarcity (SADC, 2008).

Furthermore, one of the main routs by which terrestrial and aquatic organisms can be exposed to EDC's, such as DDT, is by contact to contaminated water (Rodriguez-Mozaz e*t al*., 2004). Additionally, an increase in the prevalence of malaria and bilharzia can be linked with water wastage, associated with improper irrigation practices (SADC, 2008).

1.4 Research

Throughout history, questions about nature arose in one form or another. In order to answer these questions scientists and researchers undertake numerous studies and research projects. Because of research it is now generally accepted that EDC's are at least partially responsible for the disruption of normal reproductive and developmental function of wildlife species (Bornman *et al*., 2007; Jobling & Tyler, 2006; Lintelmann *et al*., 2003).

Information on the effects of EDC's are, however, limited to a few species of vertebrates and invertebrates. There are many gaps in knowledge since the effects of EDC's on different species have not been comprehensively compared between and within the different taxa.

Additionally, invertebrate models for assessing endocrine effects are much needed in order to gain knowledge of the effects of EDC's within the invertebrate group as well as to compare effects between vertebrates and invertebrates, and has until recently been neglected (Jobling e*t al*., 2003; Ketata e*t al*., 2008).

Differences in ecosystems, sources of pollution, and species of pollutants might have a range of possible methodologies by which screening and testing for endocrine disruption (ED) activity can be done. Finding an effective way to do this is of utmost importance.

1.4.1 Molluscs

Next to the phylum Arthropoda, the Mollusca have the second largest number of living species known to science. In Africa, there is about 350 species of freshwater gastropods, with some of these species having almost pan-African ranges (Appleton, 2002; Brown, 1980). For instance, the genus *Bulinus*, when considered as a whole, show a tolerance for a wide range of temperatures; they can survive in stagnant waters, times of drought, grow rapidly, and breed profusely (Brown, 1980).

Similarly, there are other species and genera of freshwater snails that show a remarkable ability in colonising and inhabiting water bodies that are otherwise uninhabitable by freshwater vertebrates (Appleton, 2002; Brown, 1980).

Globally, some research has been done on marine and freshwater molluscs and the effects of EDC's on them (for example (Gagné e*t al*., 2002; Gagné e*t al*., 2004; Ketata e*t al*., 2007; Ortiz-Zarragoitia & Cajaraville, 2006; Wang & Croll, 2006)). In South Africa, very little has been done on the effects of EDC's on molluscs, either marine or freshwater. What has been done is still at the beginning of this type of research for South Africa. Only one study from South Africa is known; imposex was found in the marine snail *Nassarius kraussianus* from three harbours along the eastern seaboard (Marshall & Rajkamur, 2003). In addition, requests for more knowledge on the endocrine systems of wildlife (vertebrates and invertebrates) are made globally, as well as the need for more knowledge on the effects of EDC's on wildlife (Jobling & Tyler, 2006; Ketata e*t al*., 2008; van Wyk e*t al*., 2005).

1.5 Aim of Study

The main aim of this study was to determine if the pulmonate snail *Bulinus tropicus* shows symptoms of endocrine disruption in DDT-sprayed areas when compared to animals from adjacent non DDT-sprayed areas. This will then help to determine if this species can be used as a biological indicator.

Chapter 2: Literature review

In order to understand the effects of EDCs on gonadal development, an understanding of the endocrine system is required. Because much more is known about the human endocrine system, a short overview of this will be given first, and then contrasted by what is known about the same systems in invertebrates, with a particular emphasis on molluscs.

2.1 The endocrine system; an overview

The endocrine system is one of a number of regulatory systems in the human and animal body. In conjunction with the nervous and immune systems, it controls many pivotal functions (Lintelmann e*t al*., 2003). The structure and functioning of the endocrine system consist of the following basic components:

- The hypothalamus – Part of the brain that stimulates or inhibits the production of hormones by the pituitary gland (Porterfield, 1997).
- The pituitary gland – Produces hormones that regulate and guide the functioning of the other endocrine glands in the body (Benson e*t al*., 1995c; Porterfield, 1997).
- The endocrine glands – An array of glands situated throughout the body that, through hormonal secretion, regulates growth, homeostatic, and developmental mechanisms (Benson e*t al*., 1995c; Lintelmann e*t al*., 2003; Porterfield, 1997).
- Hormones – The chemical messages, transported in the blood in bound or free form (Porterfield, 1997), that relay and inherently causes the different reactions dictated by the hypothalamus.
- Hormone receptors – Situated in the cell or on the cell surface. They interact with the hormones, which then effects cell or organ function (Lintelmann e*t al*., 2003).

As mentioned before, this is a basic list of the components of the endocrine system. The following sections will elaborate on each of these components and its functions.

2.1.1 Human endocrine glands

2.1.1.1 Hypothalamus and pituitary gland

The pituitary gland receives its hormonal queues from the hypothalamus (Lintelmann *et al*., 2003). The pituitary gland is also known as the hypophysis and is connected to the base of the brain by a stalk called the infundibulum. The hypophysis consists of two parts, the anterior adenohypophysis and the posterior

neurohypophysis. The neurohypophysis and the infindibulum are considered to be part of the hypothalamus because they share the same embryological origin as the brain (Benson e*t al*., 1995a).

The pituitary gland(s) plays a central role in the endocrine system. Most of the other endocrine glands are regulated by hormones originating from either the adenohypophysis or the neurohypophysis (Benson e*t al*., 1995b).

2.1.1.1(a) Adenohypophysis (anterior pituitary gland)

The adenohypophysis is part of the hypophysis. It consists of glandular cells that secrete the hormones used in endocrine regulation (Benson e*t al*., 1995b). Releasing or inhibiting hormones produced in the hypothalamus regulates the production and secretion of the adenohypophysis hormones. The regulating hormones are transported to the target cells via the hypophyseal portal system and are produced in various nuclei in the hypothalamus (Porterfield, 1997). Regulation of the regulating hormones and hormone production occurs through the use of a negative feedback systems (Porterfield, 1997). A number of hormones are produced in the adenohypophysis. A general overview of these hormones follows:

- Luteinizing Hormone (LH): One of the functions of LH is to stimulate the conversion of the ovarian follicle to a corpus luteum and then also to maintain the corpus luteum. In women the main target organ is the ovary and in men the interstitial cells in the testis (Benson e*t al*., 1995b; Porterfield, 1997). LH stimulates the corpus luteum to produce estrogen and progesterone. Ovulation is also entirely dependant on LH. In the testis LH stimulates steroidogenesis (production of steroids) by the interstitial cells (Benson e*t al*., 1995b; Porterfield, 1997). Regulation of LH production is done by estrogen and progesterone feedback mechanisms (Benson e*t al*., 1995b).
- Follicle-stimulating hormone (FSH): FSH and LH are both produced by gonadotrope cells in the anterior pituitary gland and is regulated by the gonadotropin releasing hormone produced in the hypothalamus (Porterfield, 1997). In females, FSH stimulates follicular growth and in males it promotes the maturation of spermatozoa in the testis (Benson e*t al*., 1995b). In addition to this, FSH act on the granulosa cells in the ovaries to stimulate aromatisation of thecal androgens to estrogens. In males FSH act on the Sertoli cells to stimulate estrogen formation from androgens and works with testosterone in stimulating the production of androgen-binding protein. Androgen-binding protein is used to maintain high levels of androgen in the testis in close vicinity to the developing germ cells (Porterfield, 1997). FSH

production is regulated by estrogen and testosterone feedback mechanisms (Benson e*t al*., 1995b).

- Thyroid-stimulating hormone (TSH): TSH is a large glycoprotein hormone similar to LH and FSH (Porterfield, 1997). TSH is responsible for the regulation of the iodine uptake rate and the production of the thyroid hormone by the thyroid gland. TSH production is regulated by the hypothalamic tyrotropin-releasing hormone, which in its turn is regulated by a thyroid hormone feedback mechanism (Benson e*t al*., 1995b; Porterfield, 1997).
- Adrenocoticotropic hormone (ACTH): ACTH is mainly responsible for stimulating growth and steroid production in the adrenal gland. It is also suspected to have extra-adrenal actions one of which is to increase skin pigmentation (Porterfield, 1997). ACTH secretion is regulated by corticotropin-releasing hormone from the hypothalamus (Benson e*t al*., 1995b; Porterfield, 1997). This is however not the only stimulant. Many types of stress also stimulate ACTH production and this can also be mediated through the central nervous system (Porterfield, 1997).
- Growth hormone (GH): GH stimulates growth and has metabolic actions. It conserves carbohydrate and proteins by shifting metabolism to lipids for energy production. GH also stimulate cellular lipid uptake and protein synthesis (Porterfield, 1997).
- Prolactin or luteotropic hormone (LTH): In humans, the main purpose of LTH is to initiate and maintain lactation. The functioning of LTH is aided and regulated by the presence of estrogens and progesterone. At the different stages of pregnancy and after childbirth the combined reaction and regulation changes (Benson e*t al*., 1995b; Porterfield, 1997). Some of the LTH actions in humans include mammogenesis (the growth and development of the mammary gland), lactogenesis (preparation of the mammary gland for lactation), and galactopoiesis (maintenance of milk production). Some of the other hormones working in concert with LTH in these action are; estrogens, progesterone, GH, and thyroid hormones (Porterfield, 1997). LTH can have behavioural effects on other species, including non-mammalian species, and is also known to have reproductive actions in many species. Excessive LTH production in humans inhibits reproductive function in both men and women (Porterfield, 1997).

2.1.1.1(b) Neurohypophysis (Posterior pituitary gland)

The neurohypophysis differs from the adenohypophysis in that it is not made up of glandular cells. The neurohypophysis in essence is merely an extension of the

hypothalamus. The nuclei responsible for hormone production is situated in the hypothalamic region, the axons of these nerve cells make up the hypothalamohypophyseal tract and the peripheral terminals of these cells are located in the infindibular process or pars nova (Benson e*t al*., 1995b; Porterfield, 1997). The two hormones, antidiuretic hormone and oxytocin, are synthesised in the cell bodies located in the hypothalamic region which is then secreted by the peripheral terminals situated in the infindibular process (Benson e*t al*., 1995b; Porterfield, 1997). The following is an overview of these two hormones:

- Antidiuritic hormone (ADH): The main function of this hormone is to control the permeability of the collecting tubules in the nephrons for water absorption. ADH also functions to increase arterial blood pressure (Benson e*t al*., 1995b). Other actions of ADH include the stimulation of renal mesangial cell contraction, inhibition of renin secretion, and stimulating adrenocorticotropic hormone secretion. It is also suspected to have influences on behaviour, learning and memory (Porterfield, 1997).
- Oxytocin: Oxytocins main functions are to stimulate milk expulsion in the mammary gland and to stimulate contraction of the uterine myometrium (Porterfield, 1997). Oxytocin increases the strength of uterine contractions and is therefore essential for childbirth (Benson e*t al*., 1995b). Stimulation for the secretion of oxytocin is neurological and stimuli can be received from pressure on the uterine cervix by the unborn child as well as from stimulation through sight and sound from seeing and hearing a hungry infant. Secretion of oxytocin can however be blocked by pain, fear or stress (Benson e*t al*., 1995b; Porterfield, 1997).

Figure 3 gives a pictorial overview for some of the hormones mentioned above.

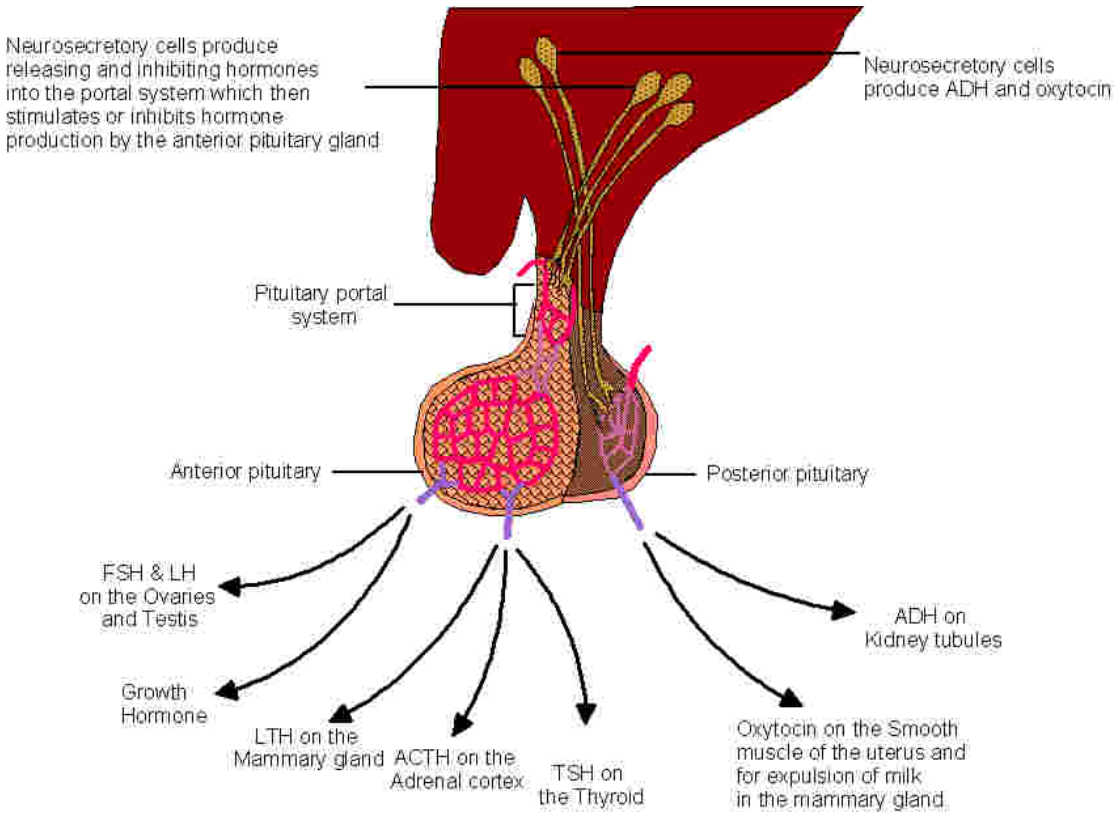

Figure 3: Schematic summary of the main pituitary hormones. Adapted from (MedicaLook, 2007)

2.1.1.2 Thyroid gland

The thyroid gland consist of two lobes connected by an isthmus (Benson e*t al*., 1995b; Porterfield, 1997). It extends across the ventral surface of the lower trachea (Porterfield, 1997). The thyroid is responsible for the production of the thyroid hormones and calcitonin. The three dominant thyroid hormones are; thyroxine (T_4), triiodothyronine (T_3), and reverse T_3 (rT_3) (Benson e*t al*., 1995b; Porterfield, 1997). Thyroid hormones are responsible for actions such as increased metabolic activity of most tissues in the body, acceleration of bone growth in children, enhancement of carbohydrate metabolism, increased heart rate and cardiac output, increased respiratory rate, and increased mental activity (Benson e*t al*., 1995b). Calcitonin together with the parathyroid hormone works in on the levels of calcium in the blood.

Four parathyroid glands are located on the posterior surface of the thyroid gland (Benson e*t al*., 1995b). As mentioned earlier, TSH produced in the adenohypophysis regulates thyroid hormone production (Benson e*t al*., 1995b; Porterfield, 1997). Regulation of calcitonin is controlled by the calcium levels in the blood (Benson e*t al*., 1995b).

2.1.1.3 Endocrine pancreas

The pancreas has both exocrine and endocrine functions. The exocrine portion is responsible for the production of digestive enzymes. Islets of Langerhans constitutes for the endocrine portion (Porterfield, 1997). These islets are made up of alpha, beta, and delta cells, each performing a certain endocrine role.

- The beta cells are responsible for secreting Insulin, an anabolic hormone that promotes the storage of glucose, fatty acids, and amino acids.
- Glucagon is secreted by the alpha cells and is responsible to mobilise glucose, fatty acids, and amino acids. Glucagon also stimulates the production of the growth hormone, insulin, and pancreatic somatostatin.
- The delta cells are responsible for the production of somatostatin. Somatostatin is a growth inhibitor in that it inhibits the release of the growth hormone by the adenohypophysis. It also inhibits the production of insulin and glucagon (Benson e*t al*., 1995b; Porterfield, 1997).

2.1.1.4 Adrenal Gland

Located bilaterally, immediately above the kidneys are the adrenal glands. As with the pituitary gland, the adrenal gland is unique in that it actually consists of two types of endocrine tissues within one organ. The two regions have different functions and produce structurally different hormones (Benson e*t al*., 1995b; Porterfield, 1997). The following is a description of these two regions concerning structure and function.

- The (outer) adrenal cortex: The adrenal cortex forms the bulk of the adrenal and is responsible for the production of many types of steroid hormones. It is formed relatively early in the developmental stages of the embryo and is actively involved with steroidogenesis by the 8th week of gestation (Porterfield, 1997). It consists of three layers. The outermost layer is the zona glomerulosa. It is responsible for the secretion of the mineralcorticoid hormones such as aldosterone, mainly responsible for the regulation of sodium levels in the body and also has an effect on the potassium and chloride levels (Benson e*t al*., 1995b; Porterfield, 1997).

  The middle layer is the zona fasciculata. It is the thickest of the three layers and is mainly responsible for the production of glucocorticoids, hormones responsible for the regulation of blood glucose levels, with the most potent of the natural clucocorticoids being cortisol(Benson e*t al*., 1995b; Porterfield, 1997).

  The third and innermost layer is the zona reticularis. It also produces some of the cortisol but is mainly responsible for the production of the sex hormones. The predominant sex steroids produced by the zona reticularis are the weak

androgens dehydroepiandrosterone (DHEA) and androsterone. These compounds can be converted to more potent androgens, such as testosterone, as well as to estrogens. The androgens produced by the adrenal cortex are not adequate to replace the testicular androgens. However, they are the major source of androgens in woman and are responsible for pubic and axillary hair growth in women (Porterfield, 1997).

Both the zona fasciculate and the zona reticularis are capable of producing cortisol and sex steroids due to the presence of the enzyme 17α-hydroxylase. It is also because of the lack of this enzyme that the outermost layer cannot produce cortisol or the sex steroids(Porterfield, 1997). It should also be noted that mineralcorticoids and glucocorticoids can interact with each other's receptors and therefore an overlap in biological activity is present (Porterfield, 1997).

- The (inner) adrenal medulla: The adrenal medullas embryological origin is similar to that of the ganglionic cells of the sympathetic nervous system. It takes up approximately 20% of the total mass of the adrenal gland (Benson e*t al*., 1995b; Porterfield, 1997). It is responsible for the secretion of two catecholamines, namely norepinephrine and epinephrine. Cells of the autonomic nervous system can also produce norepinephrine. Rather than being secreted into synapses, as is the case in the postganglionic terminals, the catecholamines are secreted into the blood and therefore act as hormones rather than neurotransmitters (Benson e*t al*., 1995b; Porterfield, 1997).

  Damage to the adrenal cortex is life threatening, whereas damage of the adrenal medulla is not. This is mainly due to the importance of the cortex in maintaining the homeostasis of body fluids and minerals. In the case of the medulla, it only supplies 30% of the circulating epinephrine, while the remaining 70% is derived from nerve terminals from which it is diffused into the vascular system (Benson e*t al*., 1995b; Porterfield, 1997).

2.1.1.5 Reproductive glands

2.1.1.5(a) Male reproductive glands

In an adult male, the primary reproductive glands are the testes. In addition to the testes, other tissues associated with the reproductive tract are also responsible for the secretion of sex hormones - they are the male prostate, the penis, the penile urethra, and the scrotum(Porterfield, 1997).

The testes are mainly responsible for the production of sperm for reproduction and for the secretion of the hormone testosterone. The prostate and other associated tissues secrete androgens such as dihydrotestosterone (DHT)

(Benson e*t al*., 1995b; Porterfield, 1997). Excretions from the pituitary gland regulates hormonal production and other functions of the testes (Benson e*t al*., 1995b). More detail on the reproductive glands and the processes concerned will follow in later sections.

2.1.1.5(b) Female reproductive glands

The female reproductive glands are the ovaries. They are responsible for the production of a magnitude of hormones that can be grouped into two main groups, namely estrogens and progesterones. The ovaries also serve the function of releasing the egg cells required for reproduction. Hormone production is regulated by the pituitary gland while the hormones produced regulate and maintain secondary female reproductive characteristics(Benson e*t al*., 1995b; Porterfield, 1997).

2.2 The reproductive endocrine system

As mentioned earlier, the main glands present in the reproductive endocrine system are the testes in the male organism and the ovaries in the female organism. Regulation of these glands as well as the regulatory effects caused by these glands are, however, more complex than was described in the previous section. The following section will be devoted to a more detailed description of the workings of the reproductive endocrine system and associated glands, organs and tissues. Even though some aspects are similar in the male and female, the overall differences justifies a separation of the two sexes whilst describing the systems in more detail.

Before we look into the specifics of each of the sexes, it is worthwhile to first describe the gonads and the genitalia of the foetus at the beginning of the gestation period.

In the case of humans, the sex of an individual is determined by the sex chromosomes. The two chromosomes regulating sex are the X - and Y-chromosomes. For a female, it is a XX pair of chromosomes; for a male, it is a XY chromosomal pair. It is the Y-chromosome that contains the gene responsible for the differentiation of primitive gonad into testis (Porterfield, 1997).

Before six weeks of gestation, indifferent gonads have formed on the gonadal ridge in the foetus. The SRY (sex determining region Y) gene, that is located in the Y-chromosome, is at this stage responsible for coding the putative testicular-determining factor that regulates the development of the testis. If the SRY gene is not present, the gonads will develop into the ovaries. Ovary development only occurs after 9 weeks of gestation (Porterfield, 1997).

Even though the genetic sex of an individual is determined by the lack of or presence of the SRY gene, hormones regulate the sexual phenotype. It is therefore possible for a genotypical male to show as a phenotypical female (Porterfield, 1997).

As mentioned earlier hormones regulate phenotypic sex characteristics and therefore regulate the development of the internal and external genitalia. Originally, the foetus develops with multi-potential internal and external genitalia. There are two Wolffian ducts that can develop into the internal male genitalia, and two Müllerian ducts that can develop into the internal female genitalia. The development of the external genitalia is also regulated hormonally (Porterfield, 1997). The hormonal regulation hereof is described next.

2.2.1 Male

Within 6 to 8 weeks of gestation, the male testes have developed. The foetal testes are responsible for the production of two hormones - testosterone, and Müllerian-inhibiting substance (MIS). Testosterone is produced by the interstitial cells while MIS is produced by the Sertoli cells of the seminiferous tubules (Benson e*t al*., 1995b; Porterfield, 1997).

The testosterone acts in a paracrine manner to stimulate the development and growth of the epididymis, vas deferens, seminal vesicles, and the ejaculatory ducts from the Wolffian ducts. MIS produced by the Sertoli cells stimulate the regression of the Müllerian ducts (Porterfield, 1997). The enzyme 5α-reductase is responsible for the conversion of testosterone to the hormone DHT. DHT, an androgen, is in its turn responsible for the differentiation of the external genitalia of the foetus between 9 and 12 weeks of gestation. In the presence of DHT, the undifferentiated genital tubercle, genital fold, genital swelling, and urogenital sinus develop into the penis, scrotum, penile urethra, and prostate. These tissues then continue to produce DHT. DHT serves as the most potent androgen within these tissues (Porterfield, 1997).

The embryological development of the testes occurs inside the body cavity. During the last two months of development, the testes descend into the scrotum via the inguinal canals. The descend is controlled by testosterone (Benson e*t al*., 1995b).

During embryological development, a hormone produced by the placenta, chorionic gonadotropin, regulates the release of testosterone by the testes. Testosterone production is minimal during early childhood until the onset of puberty at the age of 10 or 11 years. Testosterone production is then regulated by the LH. LH is produced by the adenohypophysis and stimulates the interstitial cells to produce

testosterone. The adenohypophysis also produces the FSH that is responsible for the maturing of the spermatozoa in the testes (Benson e*t al*., 1995b).

During puberty, the testes become larger and produce more testosterone. This causes an enlargement of the penis and scrotum as well as the development of the male sexual characteristics such as the appearance of hair on the face, axillae, chest, and pubic region, the enlargement of the larynx, increased skin thickness, baldness, and increased bone thickness and roughness (Benson e*t al*., 1995b).

2.2.2 Female

In the case of the female, the development of the genitalia is not so much governed by hormones rather than by the lack of certain hormones. As mentioned earlier, the presence of the SRY gene governs whether the gonads develop into testes or ovaries. If the SRY gene is not present, the gonads will, after nine weeks of gestation, develop into ovaries. Unlike the testes, the ovaries do not produce any hormones during embryological development. It is due to the lack of testosterone and MIS that the Müllerian ducts develop into the internal genitalia known as the fallopian tubes, uterus, and upper vagina. The exterior genitalia, namely the labia, clitoris, and lower two thirds of the vagina develop out of the genital folds, genital swelling, and genital tubercle, also due to the lack of testosterone and DHT androgen (Porterfield, 1997).

In the foetus, there are some peaks of LH an FSH *in utero* and 2 to 3 months postpartum, but it remains relatively low until adolescence (Porterfield, 1997).

During foetal development, small groups of cells move inward from the germinal epithelia of each ovary to form the primordial follicles. Some of these primordial follicles develop into Graafian follicle during puberty. Every 28 days a maturing Graafian follicle is expelled during the process of ovulation. The development of these follicles and the subsequent corpus luteum results in the production of the principal female sex hormones, namely estrogens and progesterone (Benson e*t al*., 1995b).

2.2.2.1 Estrogens

At puberty, the production of estrogens is initiated by the release of FSH by the adenohypophysis. In addition to being produced by Graafian follicles, estrogens are also produced by the corpus luteum, placenta, adrenal cortex, and even by the testes in males. Estrogens are responsible for the growth of specific cells in the body and for the control and development of secondary female sex characteristics

(Benson e*t al*., 1995b). The secondary female sex characteristics that are regulated are the following:

- The female sex organs become fully developed
- The vaginal epithelium changes from cuboidal epithelium to stratified epithelium
- The endometrium (uterine lining) becomes more glandular in preparation for the implanting of the fertilised ovum
- The breast form
- Osteoblastic activity increases with more rapid bone growth
- The pelvic bones enlarge and change shape so that the pelvic outlet size is increased
- Calcification of the epiphyses in long bones is hastened
- Fat deposition under the skin, in the hips, the thighs, and buttocks is increased
- Skin vascularisation is increased.

2.2.2.2 Progesterone

After ovulation, the Graafian follicle is replaced by the corpus luteum. The principle hormone produced by the Graafian follicle is progesterone. This hormone is responsible for the promotion of secretory changes in the endometrium in preparation for the implanting of the fertilised ovum. Additionally it also causes mucosal changes in the uterine tubes and promotes proliferation of alveolar cells for milk production if pregnancy occurs. Following pregnancy, the corpus luteum enlarges and produces extra progesterone. Conversion of the Graafian follicle to the corpus luteum is governed by LH production (Benson e*t al*., 1995b).

In addition to these two primary hormones produced, the ovary is also responsible for the production of another progestin namely 17α-hydroxyprogesterone, as well as some weak androgens. The ovaries are also capable of producing minimal amounts of testosterone and dehydrotestosterone. Other hormones produced by the ovaries are the peptides inhibins, activins, and relaxin as well as numerous growth factors (Porterfield, 1997).

2.2.3 Hormonal problems

Because of the complexity of the endocrine system, problems can occur if just one of the parts does not function properly. In Porterfield (1997), numerous examples are given for what happens to individuals when they have problems with the normal functioning of their endocrine system. However, most of these examples are of genetic causes, effects due to the lack of certain endocrine glands, or the incorrect functioning of the glands. Other problems/effects can also occur due to

external factors. This could be in the form of controlled hormonal applications or due to uncontrolled hormonal exposure through the environment. This can also be due to the exposure to chemicals that are synonymous to hormones or that act in on the receptors of the hormones (Burger, 2005; Escamilla e*t al*., 1967; Ghosh & Bhattacharyya, 1967).

The first part of this section covered the human endocrine system. This was to give a baseline and general understanding of the endocrine system and the major groups of hormones and their functioning. In the following section, we look more closely at the endocrine system of the molluscs and more specifically that of the informal (the taxonomy of the molluscs is in flux) gastropod group, the Pulmonata.

2.3 The reproductive endocrine system of molluscs

As mentioned earlier, the endocrine system makes use of chemical messengers that are secreted to coordinate an array of mechanisms and physiological processes in humans and other higher multi-cellular organisms. This allows organisms to react to both environmental and physiological stimuli with the nervous system playing a central role in this coordinating process. With evolution, this coordination has grown in complexity (Ketata e*t al*., 2008). Additionally, the molluscan hormonal system is to a large part comparable to that of vertebrates (Janer & Porte, 2007; LaFont & Mathieu, 2007; Oehlmann e*t al*., 2007).

Molluscs have an organised nervous system with cerebroid ganglia and a ventral nerve chain. Within this framework, neurosecretory cells are present in neurohemal organs or within true endocrine glands such as the cerebral, pleural, pedal, and abdominal glands (Ketata e*t al*., 2008). It is generally accepted that endocrine glands first appeared in molluscs, depending on the definition of endocrine glands one adheres to, since endocrine cells are also found in annelids (Ketata e*t al*., 2008; LaFont, 2000).

Generally, the endocrine system involves several organs and chemical mediators acting in cascades. In the higher order organisms, first, second and third order control systems are defined according to the amount of endocrine glands and target tissues present in such a control system. Within the molluscs, third order systems have only been described in cephalopods. Mostly the molluscan endocrine system, especially the reproductive axis, only comprise of neurosecretory cells and other endocrine glands such as the gonads (Ketata e*t al*., 2008).

The phylum Mollusca has a range of body forms and sizes included in it. Similarly, they have an extremely varied sexuality with a range of forms of the reproductive system. This can range from a simple sack-like gonad that releases ripe

gametes, through a short non-glandular duct into the external medium, to a complex, separate, but interconnected duct system with glands along their lengths that conduct the movement of both male and female gametes and receive and transport exogenous sperm after copulation (Runham, 1988). Due to this variety within the classes and major groups of molluscs, it may be considered that the mollusc endocrine system is one of the most diverse hormonal systems within the invertebrate phyla (Ketata e*t al*., 2008; Oetken e*t al*., 2004).

Runham (1988) gives a detailed account of the variety within the group in the book, “Reproductive Biology of Invertebrates”.

The fresh water snail, *B. tropicus*, is classified in the class and informal group Gastropoda: Pulmonata, informal group Basomatophora (Bouchet & Rocroi, 2005) (See Appendix 1). Therefore, for the purpose of this project I will concentrate on this class and informal group. Many, purely technical, difficulties arose when the classical methods of endocrine research were tried out on the pulmonate gastropods. Methods such as castration or removal of the gonad cannot be done successfully in snails with shells. This is because the gonad is situated in the apical whorls of the shell and is surrounded by the digestive gland. Castration cause severe damage and high mortality. However, in this respect, species of the planorbid family had the advantage that their gonad is situated anterior to the digestive gland in the apical whorls and can therefore be removed experimentally (Boer & Joosse, 1975).

In an effort to avoid technical difficulties, organ culture techniques were introduced to molluscan research and opened opportunities for *in vitro* studies of hormonal effects (Boer & Joosse, 1975).

In terms of the reproductive endocrine system of molluscs, a distinction can be made between the differentiation of the gametes and the further development of these cells, also known as gametogenesis. It is also worth looking at the control of the growth and differentiation of the reproductive tract and the chemistry of the hormones (Boer & Joosse, 1975).

2.3.1 Differentiation of the sex cells in molluscs

In the hermaphrodite gonad of the Pulmonata, the male and female cells differentiate from the germinal epithelial cells (Boer & Joosse, 1975). There is some proof that endocrine control systems are present in molluscs. In the Pulmonata, varying protandric periods are found in all species and a seasonally determined (early spring) phase of protandry was found in several. The protandric phase was then followed by a simultaneous hermaphrodite stage in the Basomatophora. Additionally, sex reversal was observed in stylommatophoran species (Boer &

Joosse, 1975). Morton (1955) also mentioned respective periods of pronounced sperm and egg production for some pulmonate species.

Sex cell differentiation research done with tissue culture techniques also confirmed the presence of endocrine control systems by showing that the presence or absence of the cerebral ganglia, optic tentacles, and haemolymph (taken from animals in different seasons) had an effect on the type of cell differentiation that occurred. For instance, the absence of any of the above mentioned tissues resulted in autodifferentiation of the cells into female cells (Boer & Joosse, 1975). This is similar to what is seen in vertebrates in the sense that when androgenic factors are absent, female characters develop.

2.3.2 Gametogenesis in molluscs

Boer and Joosse (1975) reviewed experimental work done on the endocrine control of gametogenisis. They mention experiments with the slugs *Arion ater* and *A. subfuscus*. In these species, it was found that the optical tentacles produced a hormone that inhibits oogenesis and stimulated spermatogenesis. A hormone produced by the cerebral ganglia stimulated oogenesis. The review by Boer and Joosse (1975) lists experiments conducted by other authors with other species (*Helix pomatia, Vaginula berelliana colosi, Achantina filica,* and *Ariolomax californicus*) that could not obtain the same results, but they did observe changes in the ovotestis and in the production of oocytes and sperm. Boer and Joosse (1975) ascribe these contradictory and varied results to the fact that experimentation was done on different species, at different times of the year, and under different lab circumstances. Furthermore, it is difficult to quantify the exact number of sex cells in an ovotestis and thus to assess the changes in numbers of these cells. Standardisation of techniques used and confirmation of results are needed.

2.3.2.1 Spermatogenesis

In *Helix aspersa,* the androgenic factor produced in the cerebral ganglia is not only involved in the differentiation of the male cells, but is also necessary in the mitotic activity of the various stages of spermatogenesis. In an anhormonal culture medium, it was found that the stages following the spermatogonial stage degenerated. This means that the androgenic factor is necessary for the maintenance of the entire spermatogenic activity. In *A. ater rufus*, all stages of spermatogenesis survived in an *in vitro* anhormonal culture (Boer & Joosse, 1975).

Studies done on *Lymnaea stagnalis* found that spermatogenesis was temperature dependant (Boer & Joosse, 1975); the authors suggest that

spermatogenesis was controlled by a hormone which exerts its effect at the meiotic stage since they found that spermatogenesis was arrested at meiosis at temperatures below 10^{o}C.

2.3.2.2 Vitellogenesis

Autodifferentiation of female cells in *in vitro* cultures is not followed by vitellogenesis; it would seem that some sort of regulation is required in order for this to happen. In the case of prosobranch snails, the addition of the cerebral ganglion of a specimen with a gonad during the active vitellogenesis phase induced vitellogenesis in autodifferetiated *in vitro* cultures. This was however not the case for *H. aspersa* oocyte *in vitro* cultures (Boer & Joosse, 1975).

Studies done on *L. stagnalis* showed that when the dorsal bodies (associated with the cerebral ganglia) were removed in juvenile snails' vitellogenesis and later stages, processes such as oviposition were hampered. Subsequent implantation of the dorsal bodies restored vittelogenesis and later oviposition. These results could not be determined from data collected from adult snails. It was, however, concluded that the dorsal bodies are endocrine organs that produce a hormone that stimulates vittelogenesis (Boer & Joosse, 1975).

Even though the activity of the dorsal bodies increases in spring, just before and during the reproductive period, it was found that this was not dependent on temperature. It was not clear which factors were responsible for the regulation of the activity of the dorsal bodies for *L. stagnalis*. In the case of *H. Aspersa,* vittelogenesis did not continue at low temperature (Boer & Joosse, 1975).

2.3.3 Ovulation and oviposition

To induce ovulation in *L. stagnalis,* oxygen can be supplied to the water they are living in. It could not be concluded whether the direct stimulus for ovulation had a hormonal or nervous character. However, results obtained for the opistobranch species *Aplysia californica*, strongly suggest that gastropod ovulation is under neuroendocrine control. In this species, a special cell type in the abdominal ganglion produce an ovulation hormone (Boer & Joosse, 1975).

2.3.4 Control of the growth and differentiation of the reproductive tract of molluscs

The influence of the gonad on the development and differentiation of the reproductive tract of molluscs was first demonstrated in several gastropod species. In gastropods, it was found that two blood-borne hormones were responsible for the

development of the prostate gland and the female glands, respectively (Boer & Joosse, 1975).

In the case of the planorbid pulmonate snails, similar results were found; the work from various authors confirmed that two types of factors controlled the growth and differentiation of the reproductive tract. The dorsal bodies and neurosecretory cells in the cerebral ganglia produce the hormones that control the factors produced by the gonad. It is believed that the dorsal body produce the hormone controlling the female factor produced by the gonad, while the neurosecretory cells in the cerebral ganglia produce the hormone controlling the male factor (Boer & Joosse, 1975).

2.3.5 Chemistry of the molluscan hormones

The chemical messengers of the endocrine system can be divided into two main categories when considering their chemical properties and mode of action (Ketata e*t al*., 2008). From a comparative point of view it would be expected that all hormones produced by the neurosecretory cells are peptides (Boer & Joosse, 1975; Lintelmann e*t al*., 2003). Up to 1975, nothing was known about the size and amino acid composition of the hormones produced by molluscs (Boer & Joosse, 1975). Among the molluscs, the peptidic messengers are now known to be the most common type of hormone (Ketata e*t al*., 2008).

Boer and Joosse (1975) mentioned several studies that tried to elucidate the effects of well-known vertebrate steroid hormones on pulmonates. This included the injection of oestradiol, testosterone, and progesterone into *Lymnaea* and *Helix*. Similarly, mixtures of FSH and LH were injected into *Agriolimax reticulatus.* It was however suggested that this type of study on invertebrates has not as a rule provided any clear information. Other studies that made use of labelled cholesterol to investigate the synthesis of steroids *in vivo* and/or *in vitro* in *A. ater rufus*, as well as studies on the modifying effect of steroids on the influence of optical tentacle removal needed further investigation to clarify the data obtained in these studies (Boer & Joosse, 1975). However, in 2008, Ketata *et al*. mention some known molluscan neurohormones that are specifically involved in the reproductive system. This includes APGWamide, the caudodorsal cell hormone (CDCH), the dorsal body hormones (DBH), the egg-laying hormone (ELH), and molluscan-insulin-like peptides (MIPs).

When looking at reviews such as by Boer & Joosse (1975), Ketata *et al.* (2008), and Oetken *et al.* (2004), it is clear that the variation within the molluscs as well as, in some instances, between species within the same genus, is large and not yet sufficiently documented or categorised. There are studies however, that show

that molluscs do react to various forms of endocrine disruption (ED), even though the responses differ between taxa.

Furthermore, molluscs have the unique ability to *de novo* synthesise vertebrate type steroid hormones, which may then have specific physiological roles. It is however notable that the key enzyme used to synthesise hormones from cholesterol in vertebrates, has not yet been identified in molluscs. Molluscs are also known to have the ability to bioconcentrate lipophilic compounds in their tissues. Considering this bioconcentrating ability, causes questionability of the endogenous origin of vertebrate type steroids in molluscs (Ketata e*t al*., 2008). From the review by Oetken et al. (2004), it can be deduced that up to now, most of the research pertaining to ED effects in molluscs focused on prosobranchs and bivalves, while little research has yet been done on pulmonates.

Molluscs have varied roles in the environment and ecosystems. Ecologically, molluscs together with the rest of the invertebrates are also food for many terrestrial and aquatic invertebrate and vertebrate species; they are a trophic link in many ecosystems, play an important role in biogeochemical cycling, and can also act as ecosystem engineers (Ketata e*t al*., 2008).

Because of all this, it is worth investing effort in molluscs and other invertebrates even though the exact mechanisms of endocrine disruption are not known. This study will therefore concentrate on the responses to ED, rather than the mechanisms. If there are no worthwhile responses, there would be less urgency to investigate specific mechanisms and how they correspond with the endocrine systems of vertebrates.

2.4 Endocrine disrupting compounds

2.4.1 What is an endocrine disrupting compound?

Endocrine disrupting compounds (EDCs) is a collective name for any chemical that interferes with the normal structure and/or function of hormone-receptor complexes (Burger, 2005). They are able to mimic or antagonise the effects of endogenous hormones such as androgens and estrogens. They can also disrupt the synthesis and metabolism of these hormones and their receptors (Rodriguez-Mozaz e*t al*., 2004). The characterisation of these chemicals as EDCs is not based on their chemical class but rather by their biological effect. Therefore a wide range of chemicals is considered as EDCs (Bouwman, 2004; Rodriguez-Mozaz e*t al*., 2004).

Obviously, one would only associate chemicals used in oral contraceptives or hormone replacements with possible ED effects. However, these pharmaceuticals are not the only chemicals with such effects. Chemicals used in everyday life also

produce ED effects with some capable of inducing detrimental effects at very low doses, up to a million times lower than concerning carcinogen exposure levels (Burger, 2005).

Within the EDCs (as a generic term for a host of compounds), there is a subgroup of chemicals that take a long time to break down or do not break down naturally. These compounds are called persistent organic pollutants (POPs). They can accumulate in fatty tissue and become more concentrated higher up in the food chain over time. They are toxic and can have an array of detrimental effects such as death, disease, and birth defects in animals and humans. Some specific effects that are included are cancer, damage to the central and peripheral nervous system, allergies and hypersensitivity, disruption of the immune system, and reproductive disorders. Children are especially in danger because the developing foetus is exposed through the placenta and babies are exposed through breast milk (Aneck-Hahn e*t al*., 2007; Bouwman e*t al*., 1992; Burger, 2005; Rodriguez-Mozaz e*t al*., 2004).Because of their persistent nature they can cross international borders via rivers, wind, and ocean currents (Aneck-Hahn e*t al*., 2007; Bouwman, 2004; Burger, 2005).

According to Bouwman (2004), these POPs can be grouped in three categories:

1. Pesticides: aldrin, chlordane, DDT, dieldrin, edrin, heptachlor, mirex, toxaphene, alpha hexachlorocyclohexane, beta hexachlorocyclohexane, chlordecone, lindane, pentachlorobenzene.
2. Industrial chemicals: hexachlorobenzene (HCB) and polychlorinated biphenyls (PCBs), hexabromobiphenyl, hexabromodiphenyl ether and heptabromodiphenyl ether (commercial octabromodiphenyl ether), perfluorooctane sulfonic acid, its salts and perfluorooctane sulfonyl fluoride, tetrabromodiphenyl ether and pentabromodiphenyl ether (commercial pentabromodiphenyl ether).
3. Unintended by products: dioxins and furans.

As mentioned earlier, a well-known EDC is DDT, an organochlorine pesticide. More attention will be given to DDT later on. Other substances such as phthalates, plasticisers, alkyl phenols (APs), herbicides, fungicides, organophosphate pesticides, pharmaceuticals, brominated flame retardants, natural and synthetic hormones, parabens, polycyclic aromatic hydrocarbons (PAHs), and other industrial chemicals are also EDCs (Burger, 2005; Rodriguez-Mozaz e*t al*., 2004).

Organisms can be exposed to EDCs through a wide range of pathways. In most cases, exposure occurs through contact with contaminated surface water

(Rodriguez-Mozaz e*t al*., 2004). Exposure can also occur through the ingestion of contaminated food sources. This can be plant material with EDCs present on the surfaces, or animal tissue with stores of EDCs and more specifically POPs, stored mostly in the fatty tissue of animals.

Furthermore, EDCs can act in on the endocrine system various ways. The following subsection will look at the different modes how EDCs affect organisms.

2.4.2 Modes of EDC action

As was seen earlier, the endocrine system is very complex and important for normal functioning and the regulating within an organism. Therefore, any substance that works in on this system in whatever way may have an effect not only on the system but also on the organism itself. This effect can occur at cellular, tissue, organ, body, and behavioural level and can therefore influence the individual organism's fitness as well as the fitness of a group of animals within a community. Therefore, it is worth having a look at the different modes of action by which an EDC interacts with the endocrine system (see sections 2.4.2.1 and 2.4.2.2 for description). There are two main modes of interaction; direct interactions with hormone receptors, and indirect interactions with the endocrine system (Lintelmann e*t al*., 2003). The following looks at these interactions in more detail.

2.4.2.1 Direct interactions of EDCs with hormone receptors:

2.4.2.1(a) Agonistic action:

Agonistic actions occur when an exogenous substance (EDC in this case) binds to a hormone receptor and activates it. This then leads to a reaction similar to what would happen if the normal endogenous ligand would bind to the receptor. The potency of the reaction is dependent on the affinity of the exogenous agonist to the receptor as well as on the ability of the agonist to activate the receptor.

However, different species have different hormone receptor structures (evident from the explanation on mollusc endocrinology in section 2.3) and therefore the agonist's affinity and resultant ED effects can differ between species. For example, well-known estrogen 'copycats' are the synthetic estrogens diethylstilbestrol (DES) and ethinylestradiol. It is also worth mentioning that most of the xenoestrogenic EDCs have an agonistic effect on the hormone receptors (Lintelmann e*t al*., 2003).

2.4.2.1(b) Antagonistic action:

Antagonists are ligands that inhibit or hamper the normal agonistic response of the hormone receptors because the receptor cannot be activated as usual

(Lintelmann e*t al*., 2003). Inhibition of the receptor can be competitive or non-competitive. Competitive inhibition happens when the exogenous antagonist competes with the endogenous agonist for the active binding site and can lead to total deactivation of the receptor. Non-competitive inhibition happens when the antagonist binds at the receptor or the receptor-hormone complex but not at the active binding site, resulting in slower or reduced reactions performed by the receptor (Lintelmann e*t al*., 2003).

Typical antagonists are the herbicides linuron, vinclozolin, their metabolites, or the pharmaceutical tamoxifen, which respectively compete for the binding sites at the androgen and estrogen receptors (Lintelmann *et al*., 2003). For both agonistic and antagonistic reactions between exogenous ligand and hormone receptors, the concentration of the exogenous substance often plays an important role. Endogenous hormones are usually at very low concentrations. Therefore, if the xenobiotic (EDC) concentrations in the organism are high, an ED effect can still occur even if low binding affinity of the exogenous ligands with the hormone receptors are exhibited (Lintelmann e*t al*., 2003).

2.4.2.2 Indirect interactions with the endocrine system

Indirect interactions occur when a exogenous EDC has an effect on the availability and concentration of the natural hormones or the hormone receptors within an individual (Lintelmann e*t al*., 2003).

2.4.2.2(a) Hormone concentration

Hormone metabolism can be influenced in different ways. This can happen by either the induction and/or inhibition of hormone-metabolising enzymes as well as influencing the transport of the hormones in the blood stream (Lintelmann e*t al*., 2003). Induction of the hormone-metabolising enzymes of the P450-group in the liver can for example influence the synthesis and degradation of steroid hormones. PCB congeners and dioxins are examples of chemicals that have an influence here.

Inhibition of for example the enzyme aromatase can lead to higher testosterone levels and lower estrogen concentrations, since this enzyme catalyses the conversion of estrogen from testosterone. The resulting effects can then be termed as either antiestrogenic or -androgenic. A possible example of this is the phenomenon of imposex (females with typical male sex characteristics) in marine neogastropods exposed to TBT compounds (Ketata e*t al*., 2008; Lintelmann e*t al*., 2003).

2.4.2.2(b) Hormone receptor concentration

In receptor-mediated processes, the endogenous ligand as well as the receptor have key functions and are in a sensitive balance that can be shifted by exogenous influences (Lintelmann e*t al*., 2003). An example of a compound that acts in this way is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). TCDD is an exogenous agonist for the arylhydrocarbon (Ah)-receptor. Even though this receptor is not directly involved with hormone metabolism, its activation has different influences on the endocrine system. It can increase the degradation rate of estrogen receptors, induce estradiol metabolising enzymes, and inhibit the gene expression controlled by growth promoting hormones or estradiol (Lintelmann e*t al*., 2003).

Lintelmann et al. (2003) concludes by mentioning that the above-mentioned interactions of exogenous substance and the endocrine system are only a small part of possible modes of action and that further research in this area will most likely illuminate more of the complex relationships between exposed organisms, endocrine disruption, and xenobiotics.

For the purpose of this study, a detailed account of DDT as an endocrine disrupting compound follows.

2.4.3 DDT as EDC

DDT (Fig. 1) came into production nearly 70 years ago (Harrison, 1997). It was used extensively as an insecticide in agriculture, as well as against the insect vectors of diseases like malaria and sleeping sickness (Aneck-Hahn e*t al*., 2007; Kidd e*t al*., 2001; Lintelmann e*t al*., 2003). In 1972, it was banned in most industrial developed countries. It is however still being used in developing countries, including SA, to fight malaria (Aneck-Hahn e*t al*., 2007; Burger, 2005; Gosselin e*t al*., 1976; Lintelmann e*t al*., 2003). Up to its ban, approximately 2 million tons of DDT has entered the environment (Lintelmann e*t al*., 2003). In SA, DDT has been detected in water sources in the eastern parts of the country as well as in the Western-Cape, the Freestate, and Gauteng (Barnhoorn e*t al*., 2009; Barnhoorn e*t al*., 2010; Bouwman e*t al*., 2006; Burger, 2005; Marchand e*t al*., 2008; Mlambo e*t al*., 2009).

In Gosselin *et al*. (1976), they gave a detailed account of what the symptoms of acute DDT poisoning are. Even though DDT has been known since 1968, to be estrogenic (Lintelmann e*t al*., 2003), it was at that stage not known what the long-term effects of DDT were on the endocrine system. Gosselin *et al.* (1976) even mentioned that no harmful effects could be associated as yet with the stores of DDT and its metabolites in the body. Since then, much has changed and DDT is now

considered as an EDC, and as a POP under the Stockholm Convention (Aneck-Hahn e*t al*., 2007; Bouwman, 2004).

2.4.3.1 DDT and its metabolites

The acute lethal dose (LD_{50}) of DDT in rats is 250 mg/kg (Gosselin e*t al*., 1976). Technical grade DDT is a mix of p,p'-DDT, o,p'-DDT and some of the isomers such as DDD and DDE (Burger, 2005; Lintelmann e*t al*., 2003). It is highly lipophilic, nearly insoluble in water, has a high adsorption coefficient, and is very persistent with a half-life of 3 to 20 years (Aneck-Hahn e*t al*., 2007; Burger, 2005; Lintelmann e*t al*., 2003). Because of its lipophilic nature, oils, fats, and solvents of a lipid nature, enhances the uptake of DDT from the gastrointestinal tract, the respiratory tract, and even through intact skin. (Gosselin e*t al*., 1976).

A major step of biotic and abiotic transformation of DDT is through dehydroclorination. This leads to the transforming of DDT to DDE. p,p'-DDE is a principle metabolite of p,p'-DDT (Lintelmann e*t al*., 2003). Of all the DDT isomers and metabolites, DDE is the most persistent (Aneck-Hahn e*t al*., 2007; Barnhoorn e*t al*., 2009; Lintelmann e*t al*., 2003; Nhan e*t al*., 2001). p,p'-DDE and p,p'-DDD shows no estrogenic activity. p,p'-DDE however acts as an antiandrogen (Barnhoorn e*t al*., 2009; Turusov e*t al*., 2002) and has the same effect as the antiandrogenic drug, hydroxyflutamide (Lintelmann e*t al*., 2003). Furthermore, other studies found deleterious effects on the reproductive systems of male animals that were exposed *in utero* to DDT or DDE. These effects included, amongst others, development of ovarian tissue, reduced penis size, hypospadias, and cryptorchidism (Barnhoorn e*t al*., 2009; Veeramachaneni e*t al*., 2007).

DDT and its metabolites accumulate in the organisms' fat tissues. The contribution to the total DDT burden increases with each trophic level (Kidd e*t al*., 2001; Lintelmann e*t al*., 2003; Nhan e*t al*., 2001; Turusov e*t al*., 2002). One example of such an accumulation burden is where it accumulates in the mother and the foetus is then exposed in the womb (Burger, 2005). After birth, exposure of the infant occurs through the breast milk (Bouwman e*t al*., 1992). In addition, it is also suggested that, in an aquatic environment, organisms at the top of littoral and limnetic food webs are more prone to bioaccumulation of persistent pesticides (such as DDT) than organisms at the top of a benthic food web (Kidd e*t al*., 2001).

It has also been suggested that DDT and other pesticide compounds might act in a synergistic way on endocrine function (LeBlanc e*t al*., 1996). DDE has also been hypothesised to act in a multiplicative way with other EDCs (Aneck-Hahn e*t al*., 2007; Turusov e*t al*., 2002).

2.4.3.2 Routes of exposure

Various routes by which an organism can be exposed exist. One of the most common routes for terrestrial and aquatic organisms to be exposed to these pollutants is by coming in contact with contaminated surface water and the associated ecosystems (Lintelmann e*t al*., 2003; Rodriguez-Mozaz e*t al*., 2004). Ingestion of contaminated food sources is also a route of exposure (Lintelmann e*t al*., 2003; Van Dyk e*t al*., 2010). Another route by which an organism, especially foetuses and infants of humans, can be exposed, is through the placenta of the mother and via breast milk (Bouwman e*t al*., 1992; Burger, 2005).

As mentioned earlier, DDT and its metabolites are classified as POPs. Due to its persistent character, lipophilic nature, ability to bio-accumulate, insolubility in water, and its high adsorption coefficient to particles and surfaces, various types of organisms in a wide range of ecosystems can be exposed to DDT and its isomers. Because of all these characteristics these compounds can accumulate not only in the ecosystems but also bio-accumulate in the organisms living in these systems (Burger, 2005; Lintelmann e*t al*., 2003).

Bioaccumulation and bio-concentration cannot be used as synonyms. Both cases refer to an accumulation of chemical concentrations in organisms that is higher than that of the chemical concentrations present in the organisms' environment. In the case of bio-concentration it more specifically refers to chemical concentration via gills and other membranes from an aquatic environment, and not through the ingestion of food that have chemicals in them. Both mechanisms can be in effect at the same time (USGS, 2010a; USGS, 2010b).

In the case of molluscs, they have a general limited ability to metabolise exogenous organic chemicals and to eliminate them via excretory systems. This then causes the chemicals to bio-accumulate in them (Oehlmann e*t al*., 2007). When higher trophic organisms utilise molluscs as food source they will receive the accumulated levels of chemicals. This then causes a greater bioaccumulation of the chemicals in the higher trophic organisms. With each higher trophic level, the bioaccumulation is enhanced. Higher body lipid contents also contribute to a higher bio-concentration directly from water (Kidd e*t al*., 2001).

2.5 Biomarkers of endocrine disruption in natural systems

Knowing what an EDC is and what it is capable of doing across generations, with very little indication that endocrine disrupting is in progress at any specific time, makes it necessary to identify possible EDC's or environments/systems in which ED

is present. Because natural systems are complex and variable, it would be advantageous to find ways to identify affected systems quickly and as cost-effective as possible.

Apart from the fact that chemical analyses are expensive, they are also not sufficient to identify all the environmental chemicals, predict synergistic actions, or show bioavailability. Another way would be to make use of relatively inexpensive bioassays and biomarkers (Aneck-Hahn e*t al*., 2005; Duft e*t al*., 2007), with the disadvantage that the causative EDCs may not be known.

A biomarker is a biological end-point that reacts in a known manner to specific stimuli. Bioassays can be done *in vitro* or *in vivo.* This can be with a whole body, organ, tissue, or cell response system. Even sub-cellular genomic systems can be incorporated into a bio-monitoring system (van Wyk e*t al*., 2005). Integrating *in vitro* and *in vivo* bioassays with the identification of chemicals can further help to elucidate ecotoxicological impacts of contaminated sub parts on a system (Mazurová e*t al*., 2008; Minier e*t al*., 2006). It is also possible to distinguish between exposure biomarkers that indicate if an organism has been exposed to pollutants, and effect biomarkers that indicate the way and magnitude in which an organism reacted to the exposure (Ortiz-Zarragoitia & Cajaraville, 2006). It is not surprising then that in the past decades much effort has been spend on identifying possible biomarkers and ways to implement them. (Duft e*t al*., 2003; Gibbs e*t al*., 1987; Gray, Jr. e*t al*., 2002; Ortiz-Zarragoitia & Cajaraville, 2006; Wepener e*t al*., 2005).

As a class, the Gastropods show promise as possible biomarkers. This is due to their ecological relevance in aquatic (marine and freshwater) and terrestrial ecosystems, their sensitivity to EDC's, and for a large part the general comparability of their hormonal system to that of vertebrates (Duft e*t al*., 2007; Gagnaire e*t al*., 2009; Hall e*t al*., 2009; Janer & Porte, 2007; LaFont & Mathieu, 2007; Oehlmann e*t al*., 2007).

2.6 Documented EDC activity in molluscs

In 1975, Boer and Joosse, documented that a general picture of endocrinology in molluscs was not yet available, even though the number of papers concerning this were increasing. They ascribed it to species specificity of the endocrine control systems as well as to the lack of uniformity of research techniques employed. Matthiessen (2008) reported similarly that there were no internationally standardised tests with molluscs available at that time to assess the long-term exposure to EDC's. Lintelmann et al. (2003) also suggested that several modes of action must be examined carefully before any conclusions can be drawn about the

ED mechanisms of chemicals on molluscs. The review by Ketata et al. (2008) is supportive to this and gives further suggestions to what can be done in this field of study. Contradictory results generated in an effort to elucidate the molluscan endocrine system and endocrine disruption within the Mollusca is generally ascribed to species specific modes of action, and a general lack of knowledge of the endocrine system of molluscs as a whole (Boer & Joosse, 1975; Czech e*t al*., 2001; Ketata e*t al*., 2008; Lintelmann e*t al*., 2003; Matthiessen, 2008).

According to Ketata et al. (2008), imposex is considered to be the most relevant example of chemically induced ED in wildlife, especially invertebrates, and has also been confirmed by many other authors(Gagnaire e*t al*., 2009; Ketata e*t al*., 2008; Oehlmann e*t al*., 2007; Oetken e*t al*., 2004; Quin e*t al*., 2006; Sánchez & Tarazona, 2002; Santos e*t al*., 2005). This is however mainly seen in the prosobranch species of molluscs. Further examples of ED in molluscs are rather limited, but can be ascribed to the lack of knowledge of the molluscan endocrine system. Recent data show that other mechanisms of ED could arise for many other species from exposure to environmental contaminants (Ketata e*t al*., 2008). Additionally, molluscs are also suspected of bio-accumulating EDC's to a greater extent than other invertebrates due to their poor metabolising capacity of especially xenobiotic organic compounds. This could then contribute to a greater bio-magnification of pollutants higher up in the food chain (Hall e*t al*., 2009).

2.6.1 Imposex and intersex in molluscs

- *Definition of imposex: A virilisation phenomenon or a pseudohermaphroditic condition characterised by the development and superimposition of non-functional male sexual characteristics in female individuals (Ketata et al., 2008; Oetken et al., 2004).*
- *Definition of intersex: The transformation of the female pallial organs towards a male morphological structure or the development of male reproductive characters that obstruct normal reproductive capabilities (Ketata et al., 2008; Oetken et al., 2004).*

Imposex is a specific response to organotin compounds and is almost exclusively induced by tributyl-tin (TBT) (Gooding e*t al*., 2003; Janer e*t al*., 2006; Ketata e*t al*., 2008; Oberdörster e*t al*., 2005; Rodriguez e*t al*., 2009; Sternberg e*t al*., 2010; Takeda, 2000). In the environment, effects can be seen at concentration levels as low as 1 ng/l TBT-Sn. The intensity of the effect is concentration dependent and can ultimately lead to sterility. However, not all prosobranchs are susceptible to imposex from TBT. The intensity of the effect can vary between species exposed at

the same concentration. Some species, such as the dog-whelks and the periwinkle show no effect at low TBT levels, but at higher concentrations (5-10 TBT-Sn ng/L) develop intersex conditions (Ketata e*t al*., 2008). In *Marisa cornuarietis*, a freshwater ramshorn snail, imposex was induced by TBT, methyltestosterone (MT), tryphenyltin (TPT), and fenarimol (FEN) (Janer e*t al*., 2006; Schulte-Oehlmann e*t al*., 2000).

2.6.2 Egg and embryo production

A comparative study by Jobling et al. (2003) between fish and a species of snail (*Potamopyrgus antipodarum*) using treated sewage effluent, xenoestrogens, and 17α-ethinylestradiol, showed similar results with enhanced egg and embryo production at lower doses, and inhibitory effects at higher concentrations, resulting in an inverted U-shaped dose-response curve. However many other invertebrates, including snail species such as *L. stagnalis*, showed only a little, if any, effect on reproduction when exposed to estrogens (Jobling e*t al*., 2003). The review by Ketata et al. (2008) showed varied accounts of different molluscan species and their responses according to different and sometimes similar EDCs. Again, it becomes apparent that the responses to EDCs by invertebrates can differ, and in this case within the Mollusca.

The above two sections looked at the two major effects accredited to endocrine disruption in molluscs. In addition to this, some other effects are also observed (see next section). This can be due to a lack of knowledge of the endocrine system of molluscs or just due to the absence of research done on these effects.

2.6.3 Other effects

In a test with *Nucella lapillus*, increased embryo production was observed with exposure to bisphenol-A (BPA). Additionally, a drop in male sperm production as well as a reduction of the prostate and penis was observed (Oehlmann e*t al*., 2000; Schulte-Oehlmann e*t al*., 2001).

In a similar study with antiandrogens, reduction of penis length and of accessory male sex organs was observed. For the freshwater snail *M. cornuarietis*, this was only observed in sexually immature specimens and was reversed as the males matured. For the two marine species (*N. lapillus* and *Nassarius (Hinia) reticulatus*), the adult specimens also showed reduction of the male sex organs (Tillmann e*t al*., 2001). A significant difference in the penis sheath/preputium length ratio (PSPLR) between a site with known vertebrate ED and a control site was also ascribed to possible ED in the pulmonate snail *Bulinus tropicus* (Bornman e*t al*.,

2007). The PSPLR is a ratio calculated by deviding the penis sheath length (PS) with the preputium length (PPS). Therefore, PS:PPS = PSPLR.

In the clam *M. arenaria* and the mussel *M. edulis,* skewed sex ratios towards males were observed after being exposed to contaminated rivers (Ketata e*t al*., 2008). Additionally, a study concerning the effects of TPT on *M. cornuarietis* showed, in addition to imposex in females, adverse effects on the extension of the pallial sex organs of the males. This included a 25% reduction in penis size, when compared to the control group, for males in the 250 and 500 ng/l TPT-Sn exposure groups (Schulte-Oehlmann e*t al*., 2000). Similarly, effects on the prostate gland and penis were observed for *N. lapillus* exposed to 100 ng/l TPT-Sn with a reduction of up to 18 - 21% in mean length when compared to control groups (Schulte-Oehlmann e*t al*., 2000).

Another area worth looking at and that might also be more widespread in mollusc ED research and more relevant in the determination of ED effects is histological alterations. Some of the more common examples include delayed oogenesis or spermatogenesis, tubule necrosis, inflammatory reaction, and conjunctive tissues (Ketata e*t al*., 2008). Further reports show induction of hormone receptors, the enhancement of enzymes, and effects on tissues and processes not necessarily associated with the reproductive endocrine system (Canesi e*t al*., 2007; Ketata e*t al*., 2008).

A study done in South African harbours showed imposex due to TBT pollution in the indigenous neograstropod *Nassarius kraussianus*. This study also showed through the assessment of imposex measurements that the shell length of the snails were apparently not correlated with contamination level (Marshall & Rajkamur, 2003).

An experiment on *L. stagnalis*, a freshwater hermaphroditic snail, using TBT, β-sitosterol, 4-nonylphenol, and t-methyltestosterone also found no positive correlation between shell length and contaminant concentrations (Czech e*t al*., 2001).

Natural causes that may have effects similar to ED by EDCs (confounders) should be noted. Even though the exact mechanism is not known, it was confirmed that maturation of the ovotestis was hampered in *Schistosoma mansoni* infected *Biomphalaria glabrata*, a pulmonate mollusc (Sullivan e*t al*., 1998). This is a well-known phenomenon, termed parasitic castration, where infections with larval digenetic trematodes interfere with host's gametogenisis (Morley, 2006; Sullivan e*t al*., 1998). A possible reason why the parasites interfere with the host's endocrine system is in order to be able to obtain the energy needed for its own metabolism and

to reduce attack by the host's immune system (Rato e*t al*., 2009a). Therefore, only non-parasitized snails should be used to observe chemically induced EDC effects (Morley, 2010). Turning this observation around, one might consider the possibility that ED-affected snails might be more susceptible to parasite infection.

2.7 Conclusion

The Mollusca is a diverse group of organisms with a diversity of body forms, reproductive systems, and endocrine systems. Endocrine mechanisms of control can differ between species within the same taxonomic group for example the Pulmonata. In order to use mollusc species as biological indicators of observed ED effects, it would eventually be necessary to describe the endocrine mechanisms, in order to understand what is observed in the laboratory or in field studies. The major endocrine disruptive effects reported in molluscs suggests that research aimed at elucidating the underlying effects should not only focus on the steroid hormones and their function, but should also explore the whole endocrine system including its regulatory processes (Ketata e*t al*., 2008).

Chapter 3: Materials and Methods

3.1 Species selection

A previous study done at Rietvlei Nature Reserve, close to Pretoria, showed a possible positive correlation between EDC activity and the PSPLR of the freshwater snail *B. tropicus* (Pulmonata). Other indications of possible ED were also identified in the same study. This included intersex in the sharptooth catfish (*Clarias gariepinus*), and gonodal abnormalities in the striped mouse (*Rhabdomys pumilio*). Further studies into using *B. tropicus* were suggested (Bornman e*t al*., 2007), hence the choice of species for this project.

When compared to prosobranch snails, examples of endocrine disruption in the pulmonates are rather limited (Lagadic e*t al*., 2007). The review by Lagadic *et al.* (2007) further emphasises the possible value pulmonates could have in endocrine studies as well as the need for further studies on this group. Some of these aspects include their position in the food chain, their cosmopolitan distribution, and the fact that they are probably one of the most endangered groups of freshwater invertebrates due to human activities (Lagadic e*t al*., 2007).

B. tropicus in the family Planorbidae - the largest family within the pulmonates (Lagadic e*t al*., 2007) - is the species of freshwater snail with the largest number of samples in the National Freshwater Snail Collection, North-West University (NWU), Potchefstroom, South Africa. It has the most extensive geographical distribution as well as a wide range of types of water bodies (14) in which it is found (de Kock e*t al*., 2002). However, even though species such as *B. globosus* and *B. truncatus* are mentioned in Legadic *et al's* (2007) review, *B. tropicus* is not mentioned. Nor is there any mention of the possible endocrine effects of DDT on any pulmonate.

3.1.1 Notes on the PSPLR

As mentioned previously some of the adverse affects associated with endocrine disruption in molluscs concerned changes in the mean lengths of the penis, the preputium, or both (Bornman e*t al*., 2007; Oberdörster e*t al*., 2005; Oehlmann e*t al*., 2000; Schulte-Oehlmann e*t al*., 2000; Schulte-Oehlmann e*t al*., 2001; Tillmann e*t al*., 2001). Additionally, morphometrics (such as measurements of the copulatory organ) of some pulmonate species have been used to successfully distinguish between different species and subspecies within the same subfamilies (Brown & Rollinson, 1996; Pointier e*t al*., 2006). The study by Brown & Rollinson

(1996) focused on the *Bulinus africanus* complex, congeneric with *B. tropicus* (Appleton, 2002).

3.2 Site selection

This project was done in conjunction with the WRC project (K5/1674) undertaken in the Limpopo Province, due to the current and continuous spraying of DDT since 1945. Even though DDT was restricted in Annex B of the Stockholm Convention in 2001, some countries notified exemptions in order to use DDT in the control of the mosquito vectors of malaria. DDT is still being sprayed in the Limpopo Province (Aneck-Hahn e*t al*., 2007).

The WRC project concentrated mainly on the Vhembe Municipal District in the north-eastern part of the Limpopo Province (Fig. 4). The road (R524) between the city of Louis Trichardt and the Pafuri entrance gate to the Kruger National Park (KNP) has a general west-east orientation and follows the Luvuvhu river catchment. The Luvuvhu River joins the Limpopo River in the northern part of the KNP, which then flows through Mozambique into the Indian Ocean. The R524 road was used as the general reference for the transect, because of its orientation and traversing both reference (non-DDT sprayed) and DDT-sprayed areas.

B. tropicus occurs in natural as well as manmade water bodies and permanent as well as seasonal water, therefore sample sites were selected using a GPS map (Garmin, Colorado 300) and locating water bodies within 6 km from the R524. A total of 56 sites were visited between Feb and Dec 2008 of which only 10 sites (Fig. 5) had *B. tropicus*. Two additional sites, close to Potchefstroom, were also sampled for additional reference material.

In addition, a lab control culture was maintained in the laboratories of the School for Environmental Sciences and Development (Zoology) at the Potchefstroom campus of the NWU in South Africa.

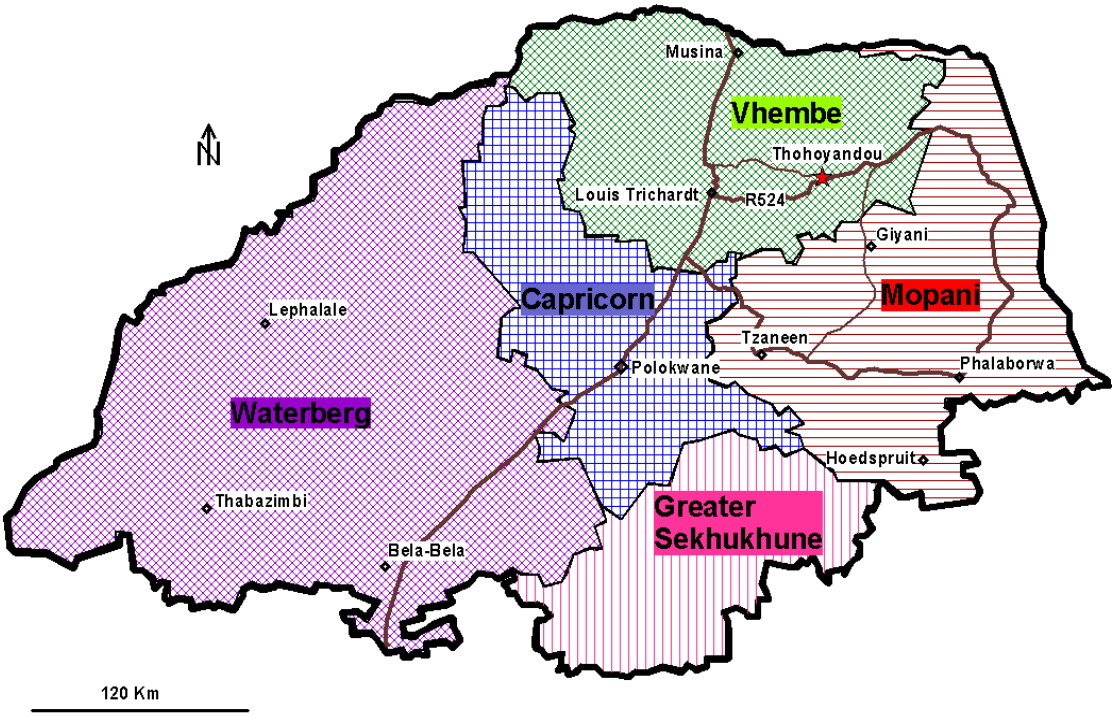

Figure 4: The main municipal districts in the Limpopo Province.

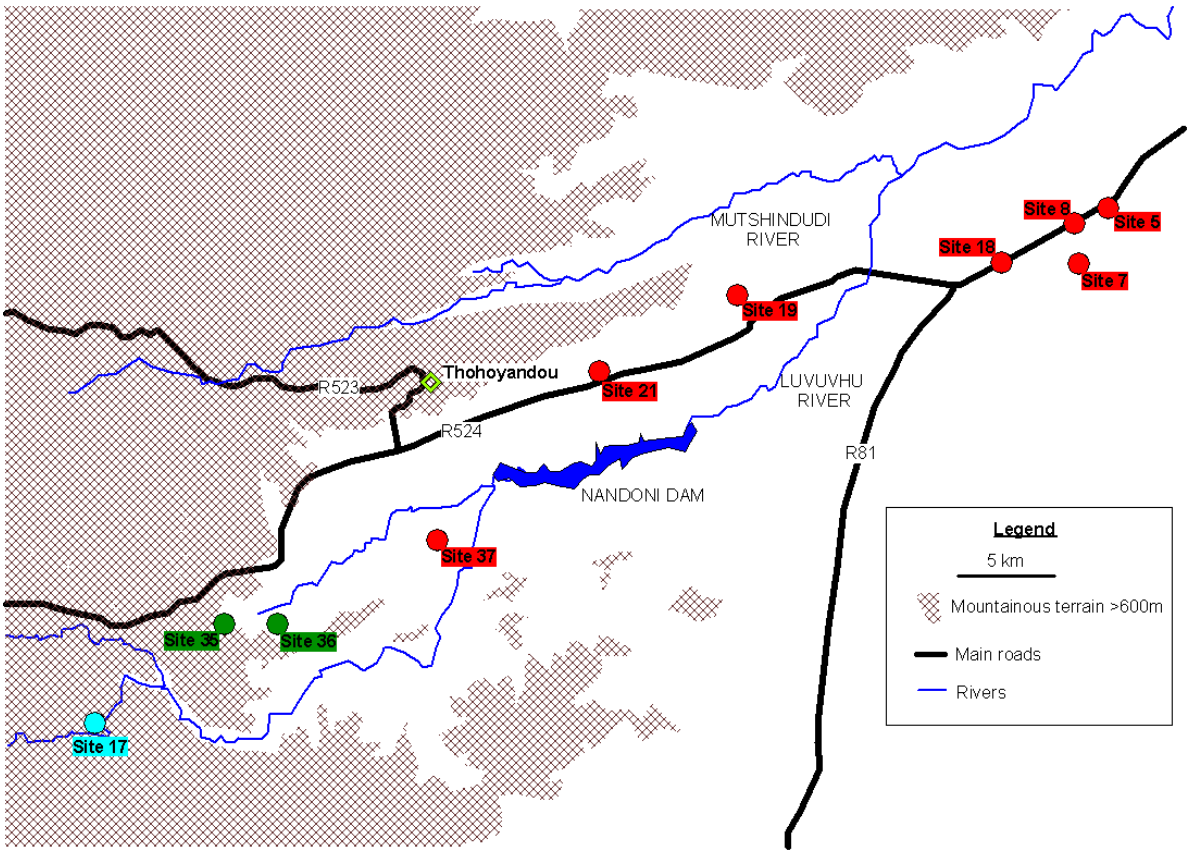

Figure 5: The study area where *B. tropicus* were found. Sites in the DDT-sprayed area are red and sites in the reference area are green and blue.

3.2.1 Site description

As mentioned before, only 10 sites in Limpopo had *B. tropicus*. Three were in the reference area; Sites 17, 35, and 36 (Fig. 5 and Fig. 6-8). Sites 37, 21, 19, 18, 8, 7, and 5 are in the DDT-sprayed area (Fig. 5 and Fig. 9-15).

Tables 1 and 2 give an account of each of the Limpopo sites.

Table 1: Site descriptions of the reference sites in Limpopo where *B. tropicus* were found.

	Site 17 (Fig.6)	**Site 35 (**Fig.7)	**Site 36** (Fig.8)
Coordinates	S23 06.613 E30 18.217	S23 03.835 E30 22.178	S23 03.870 E30 23.745
Type of water body	Quarry hole	Quarry hole	Quarry hole
Manmade (yes/no)	Yes	Yes	Yes
Seasonal / permanent	Semi seasonal	Seasonal	Semi seasonal
Catchment area	Runoff water from the direct surroundings as well as the runoff from a road very close by.	Runoff water from the direct surroundings.	Runoff water from the direct surroundings.
Uses by population	None (Stray cattle may sometimes drink water here).	Watering hole for cattle.	Watering hole for cattle, washing of clothes and children sometimes swim.
Notes of interest	This site is situated on privately owned land. A tar road with regular traffic runs within 20 meters of this site. The direct surroundings are abandoned orchards.	There are some houses in the direct surroundings. Probably more significant is the informal dumping site on one of the surrounding inclines. This results in a watering hole polluted with plastics and other man made substances including soiled diapers. Gravel roads run within 50 meters from this site. The one is situated just behind the dumping site and the other is situated downhill from the quarry hole.	There are no houses in the direct vicinity. There is some visible pollution. A gravel road runs within 50 meters uphill from this site.

Figure 6: Site 17 is situated on a privately owned farm close to a road as well as some abandoned orchards. This farm is in an area renowned in South Africa for the production of tropical fruits.

Figure 7: Site 35 when dry (top) and wet (bottom). Note the informal dumping site on the top left hand side of both pictures.

Figure 8: Site 36 during the wet season. This is an old quarry. Some excavations are going on just west from this site.

Table 2: Site descriptions of the sites in Limpopo on the DDT-sprayed side where *B. tropicus* were found.

	Site 37 (Fig.9)	**Site 21** (Fig.10)	**Site 19** (Fig.11)	**Site 18** (Fig.12)
Coordinates	S23 01.565 E30 28.597	S22 56.825 E30 33.511	S22 54.723 E30 37.647	S22 53.801 E30 45.710
Type of water body	Unknown (Possible quarry)	Stream	Quarry hole	Quarry hole
Manmade (yes/no)	Most likely (Does not look natural)	No	Yes	Yes
Seasonal / permanent	Seasonal	Seasonal	Semi permanent	Semi seasonal
Catchment area	Runoff water from the direct surroundings.	The stream finds its origin in a residential area. It also flows under the R524 and past land used for agricultural purposes.	Runoff water from the direct surroundings.	Runoff water from the direct surroundings as well as the runoff from a road (R524) very close by.
Uses by Population	Watering hole for cattle.	None	Watering hole for cattle, washing of clothes and children sometimes swim.	None (Stray animals may sometimes drink water here).
Notes of interest	There is a scrap metal yard in close proximity from this site. A gravel road passes within 20 meters from this site. The green toilets on the photo were only installed after the snails were collected.	This site is probably more periodic than seasonal. A year after Site 21 was sampled, the landowner started with construction work in this area and in the process completely destroyed the natural flow of the stream.	There is some visible pollution present. This is a favoured washing site for the local population. There are some houses close by.	This site is within 30 meters of the R524. It is almost completely fenced off.

Table 2 Continued

	Site 8 (Fig.13)	**Site 7** (Fig.14)	**Site 5** (Fig.15)
Coordinates	S22 52.730 E30 47.899	S22 53.812 E30 48.032	S22 52.311 E30 48.917
Type of water body	Quarry hole	Dam	Quarry hole
Manmade (yes/no)	Yes	Yes	Yes
Seasonal / permanent	Semi seasonal	Permanent	Seasonal
Catchment area	Runoff water from the direct surroundings.	Two streams feed this dam. Even though the streams do not originate or flow through residential areas, runoff water from residential areas flows into the dam as well as the streams.	Runoff water from the direct surroundings
Uses by population	Watering hole for cattle. (Unknown if people wash clothes or swim here.)	Watering hole for cattle. (Unknown if people wash clothes or swim here)	Watering hole for cattle
Notes of interest	Very little visible pollution. The site is situated within 100 meters of the R524	The local woman warned us of a crocodile in the dam. I therefore suspect that no swimming takes place in this dam. The dam is big enough for fishing but no fishing was observed during any of the visits.	The only human activity in close vicinity to this hole is subsistence farming. There are no houses within the catchment.

Figure 9: Site 37 almost at its fullest. This was after a week of constant rain at the beginning of the wet season.

Figure 10: Site 21 when wet (left, Feb 2008) and dry (right, Dec 2008).

Figure 11: Site 19. Note the women washing clothes to the right while cattle drink water to left.

Figure 12: Site 18 in the wet season (left) and end of the dry season (right). The R524 is visible in the photo on the right.

Figure 13: Site 8 at the beginning of the wet season. The photos were taken after a week of almost constant rain.

Figure 14: Site 7. The donga in the foreground is where runoff water from a nearby road and residential area wash into the dam.

Figure 15: Site 5. This site is probably one of the most isolated sites with no major gravel or tar roads within a 200m radius of it.

3.3 Sample collection

3.3.1 Snails

3.3.1.1 Collection

Snails were collected with scoop nets. This is an aluminium and stainless steel scoop with a handle of approximately 2 meters and a concave sieve at the end with dimensions of approximately 30 cm x 30 cm. At each possible site, the water vegetation close to the edge was inspected for snails. Using the scoop net, the vegetation was bumped repeatedly causing any snails to dislodge and collect in the sieve. All the snails, big enough for dissection, were collected and placed in glass bottles with water from their natural habitat in order to transport them back to the camp. (Fig. 16 -18)

Figure 16 and 17: Making use of the scoop net the snails are bumped of the vegetation and then collected by hand from the sieve.

Figure 18: Sorting and placing of the snails into the transport containers for storage until able to narcotise and fixate at the base camp.

3.3.1.2 Narcotising, fixing, and preserving

At camp, the snails were narcotised for 24 hours using a chloral hydrate and menthol mixture (6 g Menthol and 6.5 g Chloral hydrate, mixed and ground to a viscous liquid) added drop-wise to water. During this time, the containers were not agitated so that the snails would relax and protrude from their shells and then become narcotised in this relaxed state. The narcotising agent was then decanted and the snails were fixed.

Fixing was done by pouring warm water (±60 ^{o}C) over the snails in order to euthanase them. The water was then decanted and the snails were placed in 4% - 10% formalin for a period of 24 to 48 hours.

After fixation, the snails were preserved in 70% ethanol and stored for later dissection. This method was adapted from the method used for obtaining snail specimens for the National Snail Collection (Bornman e*t al*., 2007; de Kock, 2007).

3.3.1.3 Dissecting

Only *B. tropicus* were selected for dissection. Before the snails were dissected, measurements were made of the shell proportions. An electronic calliper (Wilson Wolpert, Digitronic Caliper) was used to measure the length of the shell, the length of the opening, and the breadth of the opening. (Fig. 19)

The soft body tissue was then removed from the shell and the penis-preputium complex was dissected out with the use of watchmaker forceps (Size no. 5) under a dissection microscope (Carl Zeiss, Model: Stemi DV 4). Using a calibrated eyepiece (Carl Zeiss) in a dissection microscope (Kyowa Optical, Model: SDZ-PL), the dimensions of the penis-preputium complex were measured (penis length = PS, preputium length = PPS, narrowest part of penis = PS1, widest part of penis = PS2, widest part of preputium = PP1; Fig. 20). The units reported are measurement units only, and were not calibrated to SI. The measurement unit was, however, constant for all dimensions measured. The same microscope and eyepiece was used for all measurements. The data was captured on a sampling sheet and then transferred to Excel. Additionally, it was also noted whether the snails had trematode infections such as the intermediate stage of *Calicophoron microbothrium*, a conical fluke of cattle (Appleton, 2002).

Dissections was done using a double-blind method in order to eliminate any prejudice that might have occurred if it was known from which site the snail might have come. In total, four different people were involved with the dissections. Spot checks were done to ensure that the differences between dissectors were kept to a minimum and to maintain data quality.

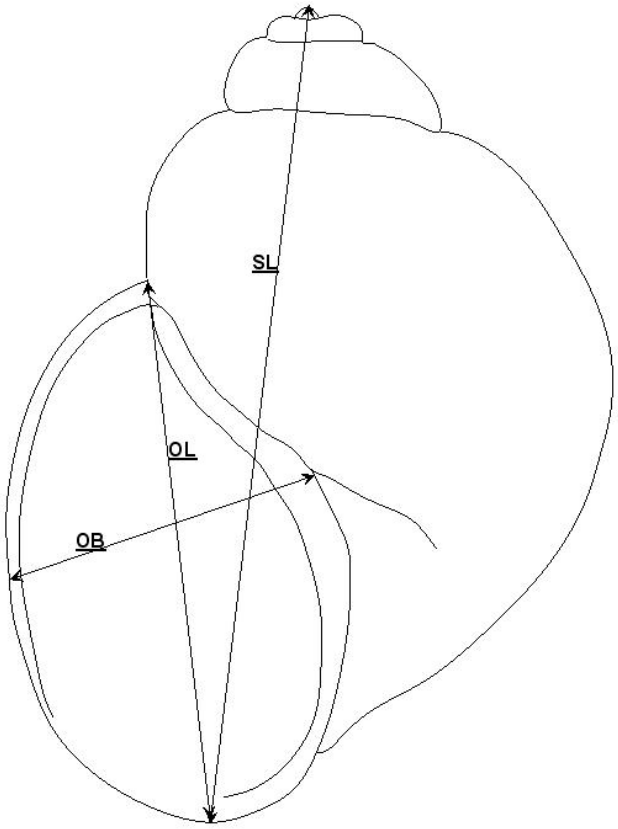

Figure 19: Representation of a *B. tropicus* shell and indication of measurements: SL = total length of shell, OL= length of shell opening, OB = breadth of shell opening. Adapted from (Appleton, 2002)

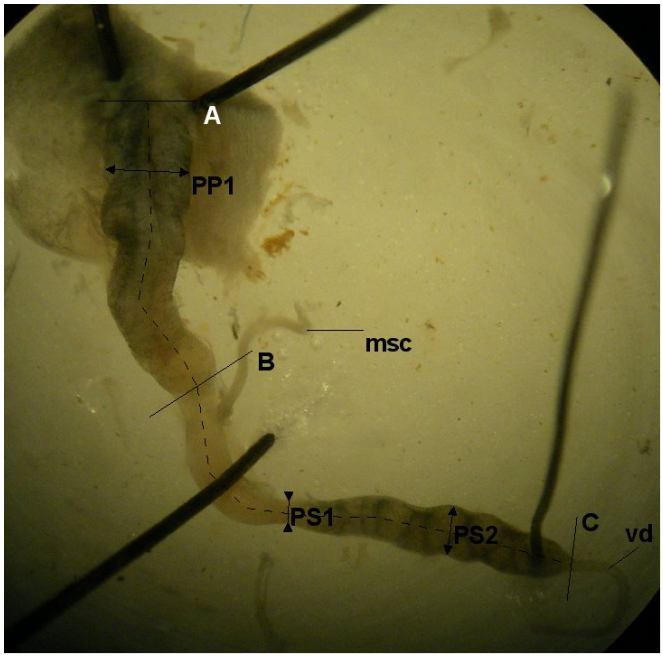

Figure 20: Photo of penis-preputium complex. PPS (preputium length from A-B). PS (penis sheath length from B-C). msc (retractor muscle, also used as an indication of where the preputium and penis meets), vd (vas deferens), PP1 (widest part of preputium), PS1 (narrowest part of the penis sheath), PS2 (widest part of the penis sheath).

3.3.1.4 Statistical analyses

Data was entered into Excel (Microsoft® Excel 2000). Statistical analyses were done with Excel, PCord (PC-ORD. Version 5.0), Statistica (StatSoft®, Statistica , Release 8), and Graphpad Prism (Prism 4 for Windows, Version 4.03). Statistical analysis was done;

- To visualise the data and determine normality (Statistica, PCord, Graphpad Prism, and Excel), and
- To investigate and compare snails morphometrics between the main areas where samples were collected (Statistica, Graphpad Prism, and Excel).

3.3.2 Sediment collection

Sediment was collected from all the sites where *B. tropicus* were sampled and then analysed for DDT and isomers/metabolites of DDT.

The Luvuvhu River flows east with its origin outside of the DDT-sprayed area. It first flows through the reference (hereafter named LimpR) and then the DDT-sprayed area (hereafter named LimpT), into the KNP, where it joins the Limpopo River and flows through Mozambique into the Indian Ocean. The river, as well as the dams in the river, are used for a variety of recreational as well as subsistence purposes. It was not confirmed, but through general observation, it would seem that the further one travels eastwards, the more dependant the local population become on the river, which serves as a means of survival. Therefore, additional sediment samples were also collected from five points along the Luvuvhu River and analysed for DDT. Two of these points are located in the reference area, one in the transitional area (more towards the DDT-sprayed area) and the other two in the DDT-sprayed area (See Fig. 21 and Table 3). Sediment from a secluded site, close to Potchefstroom was used as a blank sample for the analyses.

3.3.2.1 Sediment sampling

All sampling apparatus and containers were washed and then rinsed with acetone and hexane before being used. At five different spots in an area of approximately 10 m^2, two cups of sediment were taken and placed into a steel mixing-bowl. The sediment from the five points was then mixed with a steel rod in order to obtain a homogenous mixture. From this mixture, three glass honey jars were filled. The sediment was then kept frozen until analyses for DDT and the metabolites DDD and DDE. Analysis was done by the FDA Laboratories (Pty) Ltd in Pretoria, South Africa by means of a GC-MS.

Table 3: Description of the five additional sediment samples sites from the Luvuvhu River.

	LVB01T	**LVB02T**	**LVB03T**	**LVB04R**	**LVB05R**
Coordinates	S22 48.381 E30 47.954	S22 54.113 E30 41.732	S22 59.887 E30 30.646	S23 06.990 E30 18.712	S23 06.420 E30 06.167
Type of water body	River	River	Dam	River	Dam
Manmade (yes/no)	No	No	Yes	No	Yes
Seasonal / permanent	Permanent	Permanent	Permanent	Permanent	Permanent
Notes of interest	This site is just downstream from the Xikundu weir. People wash their clothes just upstream from this site and just downstream from the weir.	This sample was taken in the shallows of the inside bend of the river. It is close to the R524. This is also a place where sand is harvested for construction purposes. Just downstream from this point is a place where the people wash their cars as well as clothes.	This sample was taken close to the flow in of the Nandoni dam. The dam is fished extensively. Sediment from the banks of the river and the shore of the dam is harvested for the production of "homemade" bricks. This is a site of man-made disturbances.	This site is close to Site 17. This is also sediment collected from the shallows of an inside bend in the river. This site is however relatively undisturbed by humans. About one kilometre upstream a relatively busy road crosses the river.	This site is situated on the premises of the Shiluvari Lakeside lodge on the shores of the Albasini dam. This dam is not fished as extensively as Nandoni. Generally, the dam also serves as a recreational area for the surrounding population. There is also more privately owned land around the dam.

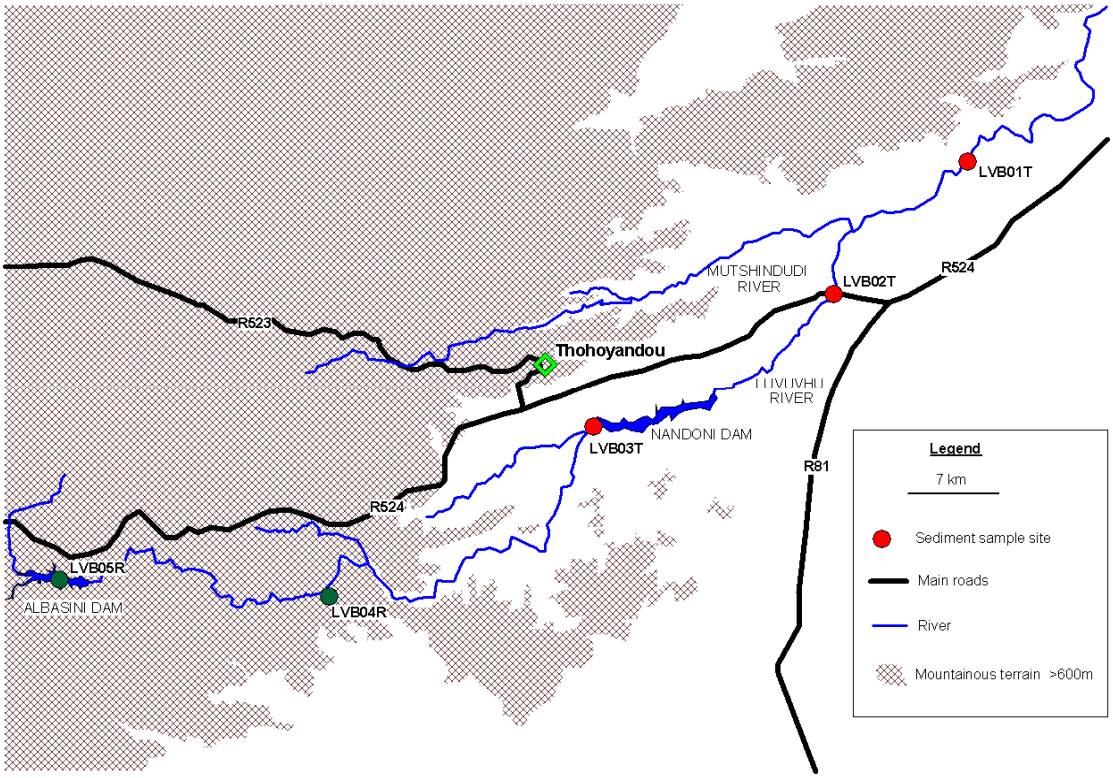

Figure 21: The locations of the five additional sediment sample sites along the Luvuvhu River. The sites on the DDT-sprayed side are represented by red dots and the sites on the reference side by green.

Chapter 4: Results

4.1 Sediment samples

Table 4 gives the results for the sediment analysed for DDT and its metabolites of the *B. tropicus* sites. None of the sites had detectable levels of DDT. Most of the LimpT sites did however have detectable levels of *p,p'*-DDE, with the highest detection in Site 18. Site 18 also had the most DDT metabolite pollution. Of the LimpT sites, only Sites 21 and 37 had no detectable levels of DDT or its metabolites. The FR site also had detectable levels of *p,p'*-DDE. None of the LimpR sites had detectable levels of DDT or its metabolites.

Table 4: DDT and metabolite residue levels (µg/kg, dry mass) in sediment from the Limpopo *B. tropicus* sites.

		***o,p'*-DDT**	***p,p'*-DDT**	***o,p'*-DDE**	***p,p'*-DDE**	***o,p'*-DDD**	***p,p'*-DDD**	**Σ DDT**
	Site	**(µg/kg)**	**(µg/kg)**	**(µg/kg)**	**(µg/kg)**	**(µg/kg)**	**(µg/kg)**	**(µg/kg)**
DDT-sprayed	5	<1.0	<1.0	<1.0	1.0	<1.0	<1.0	1
DDT-sprayed	7	<1.0	<1.0	<1.0	7.4	<1.0	1.9	9.3
DDT-sprayed	8	<1.0	<1.0	<1.0	1.3	<1.0	<1.0	1.3
DDT-sprayed	18	<1.0	<1.0	1.5	28.5	<1.0	1.9	31.9
DDT-sprayed	19	<1.0	<1.0	<1.0	6.2	<1.0	<1.0	6.2
DDT-sprayed	21	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
DDT-sprayed	37	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
Commercial farm	17	<1.0	<1.0	<1.0	1.0	<1.0	<1.0	1
Reference	35	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
Reference	36	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0

The total DDT burden for the sediment of the Luvuvhu River sites can be seen in Table 5. Only LVB 01 T from the DDT-sprayed area had detectable levels of the DDT metabolites *p,p'*-DDE, *o,p'*-DDD, and *p,p'*-DDD. The two samples from the non-sprayed area both had detectable levels of DDT and its metabolites. LVB 04 R had detectable levels of *o,p'*-DDT and *p,p'*-DDT while LVB 05 R had detectable levels of *p,p'*-DDD.

Table 5: DDT and metabolite residue levels (µg/kg, dry mass) in sediment from the Luvuvhu River sites.

Site	*o,p'*-DDT (µg/kg)	*p,p'*-DDT (µg/kg)	*o,p'*-DDE (µg/kg)	*p,p'*-DDE (µg/kg)	*o,p'*-DDD (µg/kg)	*p,p'*-DDD (µg/kg)	Σ DDT (µg/kg)
LVB 01 T	<1.0	<1.0	<1.0	6.1	10.7	38.4	55.2
LVB 02 T	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
LVB 03 T	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
LVB 04 R	1.0	1.0	<1.0	<1.0	<1.0	<1.0	2.0
LVB 05 R	<1.0	<1.0	<1.0	<1.0	<1.0	1.9	1.9

4.2 Bulinus tropicus

The main aim of this study was to compare snails from a DDT-sprayed area (LimpT) with snails from an area not sprayed with DDT (LimpR) in the same geographical region. The snails from Potchefstroom and the laboratory control were additional and will only be used to gain a clearer picture of the Limpopo snails. Snails sourced from Potchefstroom are from an entirely different climatic situation, and served only as an additional reference, due to the complete lack of data on this topic.

Table 6 shows a summary of the sampling effort. “Parasitized” refers to snails that had trematodes, most likely the intermediate larval stage of the nematode *C. microbothrium*. These snails were then excluded because of the effects parasitic infections might have on normal gametogenesis (Morley, 2006; Sullivan e*t al*., 1998). On a percentage basis, more snails were infected with trematodes in the DDT-sprayed area than in the reference area. When the reference snails were compared to the Potchefstroom sites, the prevalence of trematode infections was lower in the snails from Potchefstroom. In both cases, the infection incidences were not significantly different ($p > 0.05$; Kruskal-Wallis nonparametric one-way ANOVA).

Table 6: Summary of the collection effort for all the sites. Numbers of *B. tropicus* collected, dissected, as well as infected with trematode parasites, are given.

	Limpopo Reference (LimpR)	Limpopo Test (LimpT)	Potchefstroom (Potch)	Lab Control (LC)	Total
Sites visited	18	17	2	1	38
Sites with *B. tropicus*	3	7	2	1	13
***B.tropicus* collected**	278	1077	189	30	1574
***B.tropicus* dissected**	144	192	53	29	418
Parasitized	30	48	7	0	85
% Parasitized	20.8	25.0	13.2	0.0	20.3
N used	111	142	46	29	328

A summary of the data obtained from the snails used is presented in Table 7. The means, ranges, medians, and standard deviations (SD) are given. The PSPLR of the LimpR sourced snails did not differ significantly (two-tailed, unpaired t-tests), although borderline, from LimpT sourced snails with $p = 0.0523$. None of the other parameters differed significantly.

When the Potchefstroom (Potch) sourced snails and the laboratory control (LC) sourced snails were taken into account, the PSPLR of snails of LimpR vs. LC differed significantly with $p < 0.01$ (Kruskal-Wallis nonparametric one-way ANOVA). The PSPLR of the LimpR sourced snails did, however, not differ significantly from Potch, nor did any of the other sources' snail PSPLRs differ significantly from each other.

Figure 22 shows a PCA ordination using shell length (SL), penis-preputium sheath length ratio (PSPLR), and the narrowest part of the penis-sheath (PS1). Relatively clear groupings are visible for each of the sources from where the samples originated. When the snails sourced from Potchefstroom and from the Lab Control are removed and each collection site is plotted in a different colour, a PCA of the same parameters produces Figure 23. The PCAs were done to investigate the distribution of each factor, site, and snail, as it became clear that there were unanticipated interactions.

Table 7: Means, ranges, medians, and standard deviations for the data collected from the dissected snails from all the sources.

	LimpR (n=111)				LimpT (n=142)			
	Mean	**Range**	**Median**	**SD**	**Mean**	**Range**	**Median**	**SD**
SL (mm)	10.19	5.56 - 14.46	10.37	1.61	9.46	5.93 - 13.32	9.41	1.36
OL (mm)	7.30	3.82 - 11.01	7.48	1.32	6.48	4.62 -9.17	6.31	1.01
OB (mm)	4.89	2.02 - 8.1	5.05	1.06	4.22	2.7 - 6.38	4.19	0.72
PS	41.4	10 - 72	44	12.3	37.5	9 - 72	39	12
PPS	31.5	11 - 57	32	10.5	26.4	5 - 45	27	8.2
PS:PPS (PSPLR)	1.37	0.76 - 3.32	1.31	0.37	1.46	0.33 - 2.48	1.42	0.35
PS1	2.4	1 - 4.0	2.0	0.7	2.2	1 - 4.0	2	0.6
PS2	4.9	2 - 15	5.0	1.6	5.1	1 - 10	5	1.7
PP1	7.5	2 - 14	8.0	3.0	6.6	1 - 12	6.3	2.5
	Potch (n=46)				**Lab Control (n=29)**			
	Mean	**Range**	**Median**	**SD**	**Mean**	**Range**	**Median**	**SD**
SL (mm)	7.90	6.45 - 11.89	7.66	0.99	6.53	5.23 - 7.86	6.48	0.68
OL (mm)	5.34	4.15 - 8.48	5.40	0.68	4.84	3.43 -5.83	4.84	0.48
OB (mm)	3.5	2.68 - 5.48	3.5	0.5	3.05	2.21 - 3.98	2.95	0.43
PS	30	7 - 53	30	9.4	27.2	19 - 35	27	4.1
PPS	21.5	7 - 41	22	7.2	17.7	12 - 28	17	3.7
PS:PPS (PSPLR)	1.45	0.33 - 2.33	1.39	0.38	1.57	1.21 - 2	1.59	0.26
PS1	2.2	1 - 4	2	0.6	1.9	1 - 3.0	2	0.4
PS2	4.7	1 - 9	5	1.2	4.4	3 - 6	4	0.7
PP1	5.5	1 - 11	5	1.7	5.0	4 - 6	5	0.6

SL = total length of shell; OL= length of shell opening; OB = breadth of shell opening; PPS = preputium length; PS = penis sheath length; PP1 = widest part of preputium; PS1 = narrowest part of the penis sheath; PS2 = widest part of the penis sheath; PSPLR = penis-preputium sheath length ratio.

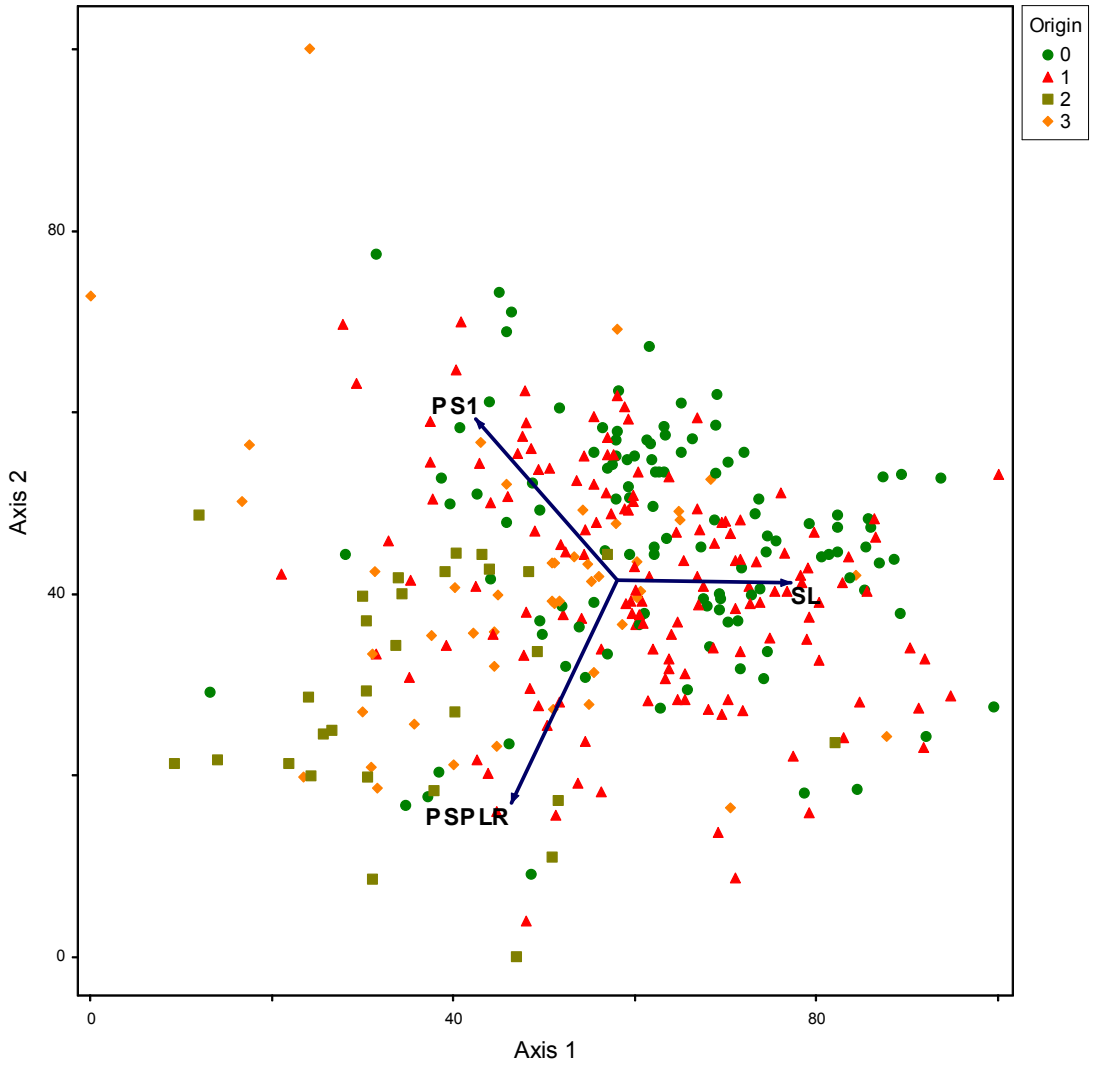

Figure 22: PCA bi-plot of shell length (SL), penis sheath at its narrowest (PS1), and penis sheath/preputium length ratio (PSPLR) of all sites with regards to their origin. Green dots (0=Limpopo Reference), red triangles (1=Limpopo Test), gold squares (2=Lab Control), and orange diamonds (3=Potchefstroom). Data were relativised in rows.

In Figure 23, it would seem that Site 17 (From the Limpopo reference area) shows a distribution of points similar to that from the sites in the DDT-sprayed area. Additionally, when Site 17 was excluded from LimpR an unpaired two-tailed t-test between the PSPLR of LimpR and LimpT snails, yielded a p-value of 0.003 compared to p = 0.0523 when Site 17 was included with LimpR (Fig. 24). The removal of Site 17 also caused the PSPLR of LimpR and Potch snails to differ significantly in a two-tailed, unpaired t-test, with a p-value of 0.0366, compared to p = 0.2310 when Site 17 was included with LimpR. Because of this, it was decided that Site 17 would be treated as a separate source, named FR. Briefly, it was deemed likely that Site 17 snail gonadal parameters were affected by ED from farm

chemicals, and therefore not a proper reference. This separate treatment is further supported when looking at the percentage snails parasitized from Site 17, in Table 8.

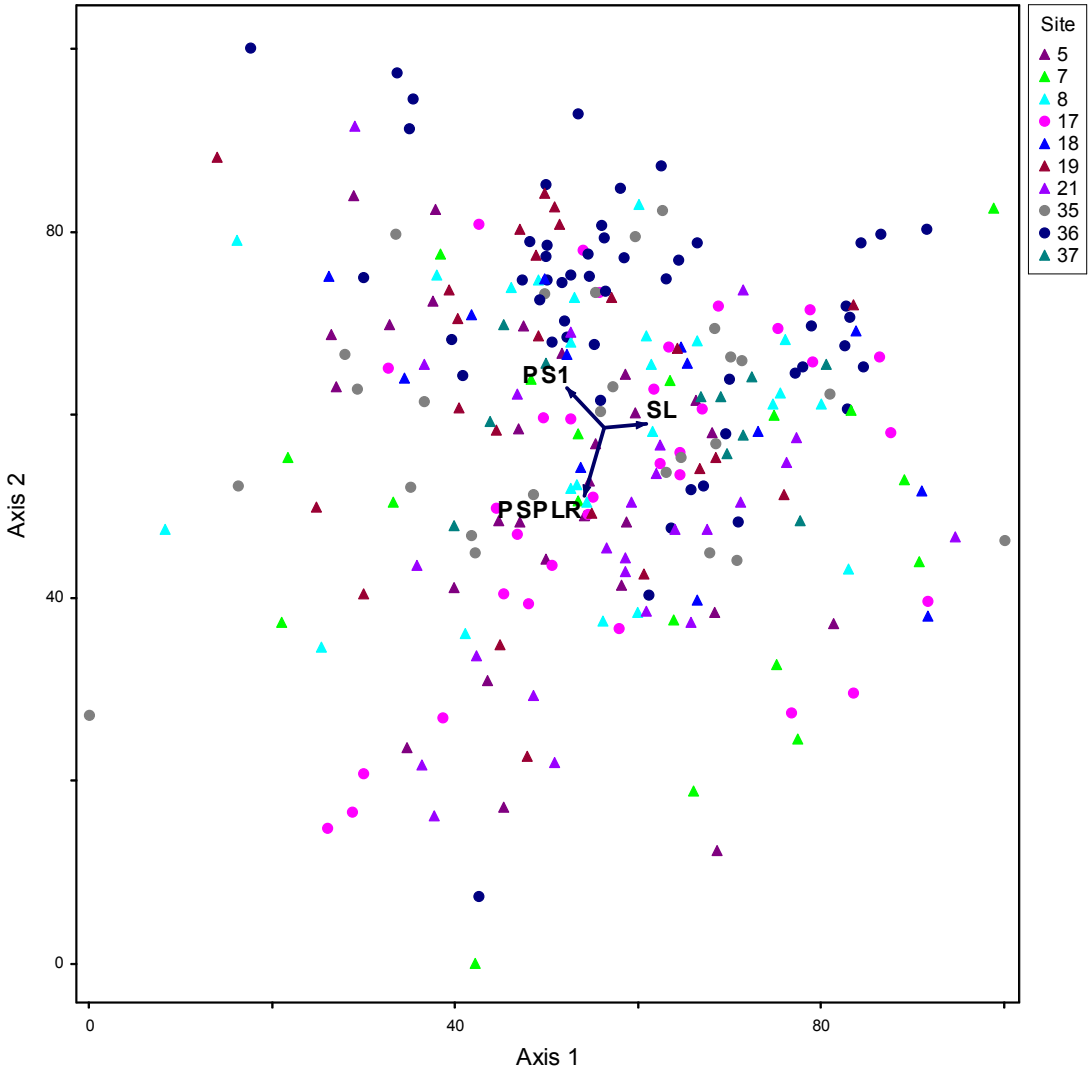

Figure 23: PCA bi-plot of shell length (SL), penis sheath at its narrowest (PS1), and penis sheath/preputium length ratio (PSPLR) of all the Limpopo sites. Site numbers are indicated in the legend. Data was relativised in rows. Sites 17, 35, and 36 are reference sites; the rest were from the DDT-sprayed area.

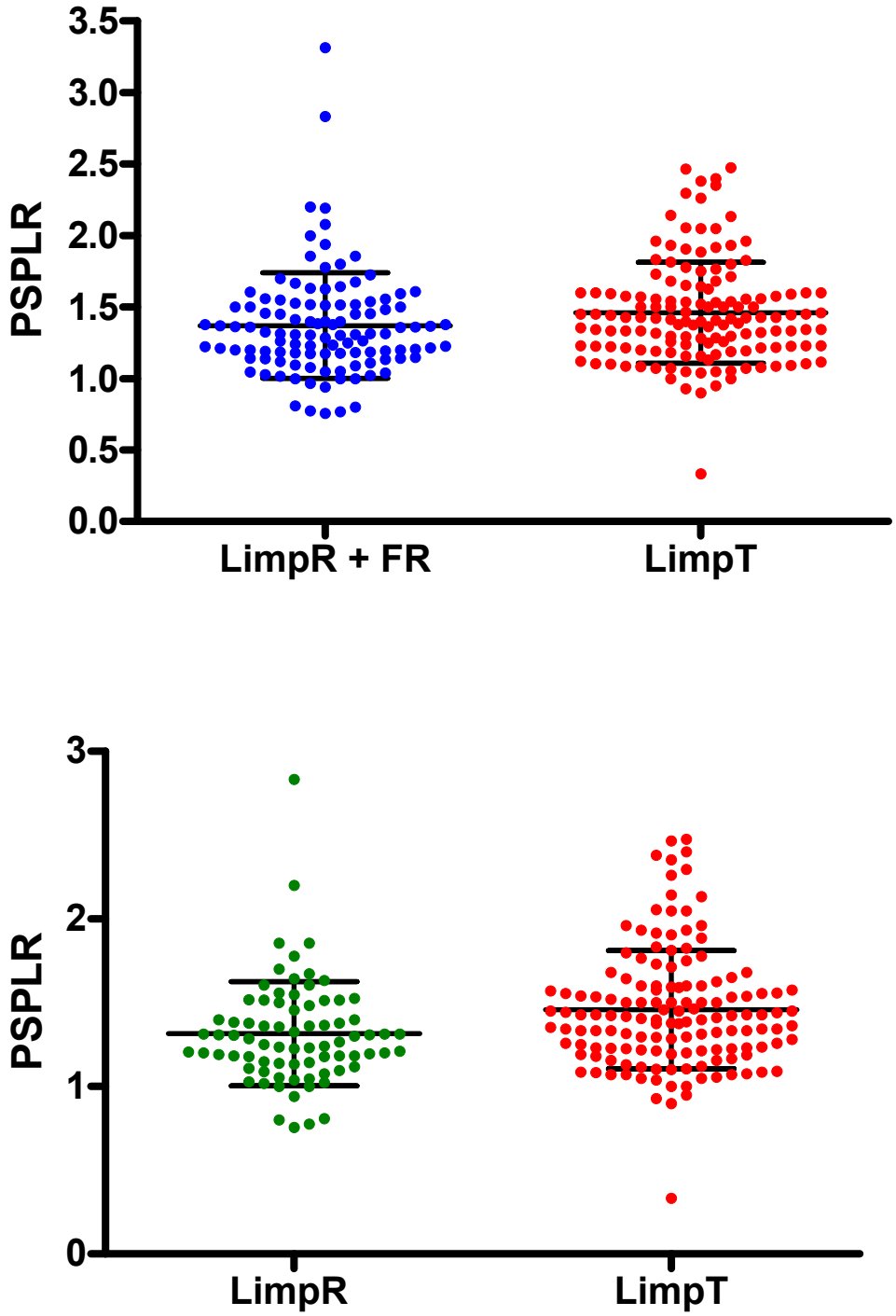

Figure 24: Scatterplots of the PSPLR. Top - Site 17 (FR) is included with LimpR (two-tailed, unpaired, t-test p=0.0523). Bottom - Site 17 is excluded from LimpR (two-tailed, unpaired, t-test p=0.003). Means and standard deviations are indicated.

Table 8 presents the summary statistics with Site 17 (FR) considered separately. Only 9.2% of the LimpR snails were now parasitized by trematodes. This proportion differed highly significantly from FR with $p < 0.001$, as well as from LimpT with $p < 0.05$ (Kruskal-Wallis nonparametric one-way ANOVA). When the prevalence of parasites in LimpR snails were compared to Potch snails, $p > 0.05$ indicated no significant difference (Kruskal-Wallis nonparametric one-way ANOVA).

Table 8: Summary statistics for all the sites. Numbers of *B. tropicus* collected, dissected, as well as infected with trematode parasites, are given. Site 17 is now considered separately (FR).

	Site 17 (FR)	Limpopo Reference (LimpR)	Limpopo Test (LimpT)	Potchefstroom (Potch)	Lab Control (LC)	Total
Sites visited	1	17	17	2	1	38
Sites with *B. tropicus*	1	2	7	2	1	13
***B.tropicus* collected**	99	179	1077	189	30	1574
***B.tropicus* dissected**	57	87	192	53	29	418
Parasitized	22	8	48	7	0	85
% Parasitized	38.6	9.2	25.0	13.2	0.0	20.3
N used	33	78	142	46	29	328

Table 9 shows the percentage of parasitized snails for each of the sites. Sites 7, 18, and 37 had the highest percentage of parasitized snails for the DDT-sprayed area, while Site 17 had the highest percentage for the reference area. In the case of the Potchefstroom sites, site 24 had the highest percentage of parasitized snails.

Table 9: The number of dissected snails that were infected with trematode parasites for each site with *B. tropicus.*

Parasites				
Origin	**Site**	**n dissected**	**n parasitized**	**% parasites**
FR	17	57	22	38.6
LimpR	35	26	0	0.0
LimpR	36	61	8	13.1
LimpT	5	29	1	3.4
LimpT	7	27	8	29.6
LimpT	8	27	3	11.1
LimpT	18	26	13	50.0
LimpT	19	27	3	11.1
LimpT	21	27	2	7.4
LimpT	37	29	18	62.1
Potch	24	28	5	17.9
Potch	25	25	2	8.0
Lab Control	LC	29	0	0.0

Because it was decided to exclude Site 17 from LimpR and rather treat it as a separate source (FR), the data in Table 7 can now be represented as in Table 10. Table 10 gives the mean, range, median, and SD values of the data collected from the non-parasitized snails. From Table 10 it is clear that LimpR generally had the largest snails while the smallest came from LC; the small size was due to early harvesting of the snails, and not a constraint of any other factor – generally, we are able to culture snails to the same size as in nature.

In Figure 26, the SLs of the various sources are represented as scatter-plots. Here also the general tendency of larger snails for LimpR can be seen. Table 10 also shows that LimpR stands out in almost all the measured as well as derived data. LimpR had the highest PS and PPS means, as well as the lowest mean PSPLR. A nonparametric (Kruskal-Wallis test) one-way ANOVA of SL shows that all except LimpT vs. FR and Potch vs. LC were significantly different from each other (Table 14).

In order to compare only the Limpopo sourced snails, a nonparametric (Kruskal-Wallis test) one-way Anova was done for PS, PPS, PSPLR, and SL (Table 11). FR and LimpT did not differ significantly for any parameter. LimpR differed significantly from both LimpT and FR for all parameters.

Table 10: Means, ranges, medians, and standard deviations for the data collected from the dissected snails for all of the sources.

	* FR (n=33)				LimpR (n=78)				LimpT (n=142)			
	Mean	**Range**	**Median**	**SD**	**Mean**	**Range**	**Median**	**SD**	**Mean**	**Range**	**Median**	**SD**
SL (mm)	9.50	6.94 - 13.09	9.40	1.48	10.48	5.56 - 14.46	10.82	1.59	9.46	5.93 - 13.32	9.41	1.36
OL (mm)	6.73	4.45 - 9.52	6.48	1.24	7.54	3.82 -11.01	7.67	1.29	6.48	4.62 - 9.17	6.31	1.01
OB (mm)	4.34	2.02 - 6.01	4.35	0.88	5.12	2.58 - 8.1	5.28	1.05	4.22	2.7 - 6.38	4.19	0.72
PS	33	10 - 63	29	12.1	44.9	16 - 72	46	10.6	37.5	9 - 72	39	12
PPS	22.7	11 - 42	21	8.1	35.2	13 - 57	35.5	9.2	26.4	5 - 45	27	8.2
PS:PPS (PSPLR)	1.50	0.77 - 3.32	1.45	0.46	1.32	0.76 - 2.83	1.28	0.31	1.46	0.33 - 2.48	1.42	0.35
PS1	2.1	1 - 3	2	0.6	2.6	1 - 4	3	0.6	2.2	1 - 4	2	0.6
PS2	4	2 - 6	4	1.3	5.2	2 - 15	5	1.6	5.1	1 - 10	5	1.7
PP1	5.2	2 - 12	4	2.7	8.5	3 - 14	9	2.6	6.6	1 - 12	6.3	2.5

	Potch (n=46)				Lab Control (n=29)			
	Mean	**Range**	**Median**	**SD**	**Mean**	**Range**	**Median**	**SD**
SL (mm)	7.90	6.45 - 11.89	7.66	0.99	6.53	5.23 - 7.86	6.48	0.68
OL (mm)	5.34	4.15 - 8.48	5.40	0.68	4.84	3.43 -5.83	4.84	0.48
OB (mm)	3.5	2.68 - 5.48	3.5	0.5	3.05	2.21 - 3.98	2.95	0.43
PS	30	7 - 53	30	9.4	27.2	19 - 35	27	4.1
PPS	21.5	7 - 41	22	7.2	17.7	12 - 28	17	3.7
PS:PPS (PSPLR)	1.45	0.33 - 2.33	1.39	0.38	1.57	1.21 - 2	1.59	0.26
PS1	2.2	1 - 4.0	2	0.6	1.9	1 - 3	2	0.4
PS2	4.7	1 - 9	5	1.2	4.4	3 - 6	4	0.7
PP1	5.5	1 - 11	5	1.7	5	4 - 6	5	0.6

* Site 17 is now handled as a separate source (FR).SL = total length of shell; OL= length of shell opening; OB = breadth of shell opening; PPS = preputium length; PS = penis sheath length; PP1 = widest part of preputium; PS1 = narrowest part of the penis sheath; PS2 = widest part of the penis sheath; PSPLR = penis-preputium sheath length ratio.

Table 11: Summary of nonparametric (Kruskal-Wallis test) one-way ANOVA's for PS, PPS, PSPLR, and SL between the different Limpopo sources.

Anova					
		PS	**PPS**	**PSPLR**	**SL**
Kruskal-Wallis test	**P-Value**	P<0.0001	P<0.0001	0.003	P<0.0001
Dunn's Multiple	**LimpR vs LimpT**	P < 0.001	P < 0.001	P < 0.01	P < 0.001
Comparison Test	**LimpR vs FR**	P < 0.001	P < 0.001	P < 0.05	P < 0.001
	LimpT vs FR	P > 0.05	P > 0.05	P > 0.05	P > 0.05

Figure 25 gives a visual representation of the relationships between PS, PPS, and PSPLR with SL of snails for LimpR, LimpT, and FR. In all three cases, the slopes of the linear regressions did not differ significantly from each other. In PS vs. SL and PPS vs. SL, all the slopes were significantly not zero (or flat). However, in the PSPLR vs. SL regression, only the slope for LimpT was significantly not zero, while the slopes for LimpR and FRs' PSPLR did not change significantly with an increased SL (Table 12). Additionally, Table 12 shows that the slopes of LimpT and FR were in close proximity for PPS vs. SL and PSPLR vs. SL. In the case of PS vs. SL, the slopes of LimpR and LimpT had a closer proximity. Considering Table 13, PS and PPS correlated (Spearman correlation) with SL for all the sources. However, the PSPLRs of all the sources, except for LimpT, did not correlate (Spearman correlation) with SL.

In Figure 26, the SLs of the various sources are represented as scatter-plots. Here also the general tendency of larger snails from LimpR can be seen. Because of the variation in size between the different sources, the snails were grouped (binned) into two overlapping size ranges. One group consisted of snails with a SL of between 8 and 10.99 mm; the next were snails with a SL of between 10 and 12.99 mm. Figure 27 shows the scatter-plots for SL of the 8 -10.99 mm and 10 -12.99 mm snails. Due to the mean small size of LC snails, they were excluded from this investigation. Similarly, the Potch snails were excluded from the 10 -12.99 mm group as there were too few. Narrower ranges caused the number of snails per source present in a grouping to not be enough for statistical analysis. An example of this can be seen in Table 13 where correlations could not be done with the FR snails due to a too small sample. Additionally groupings in the smaller size ranges would exclude many snails of the Limpopo sources.

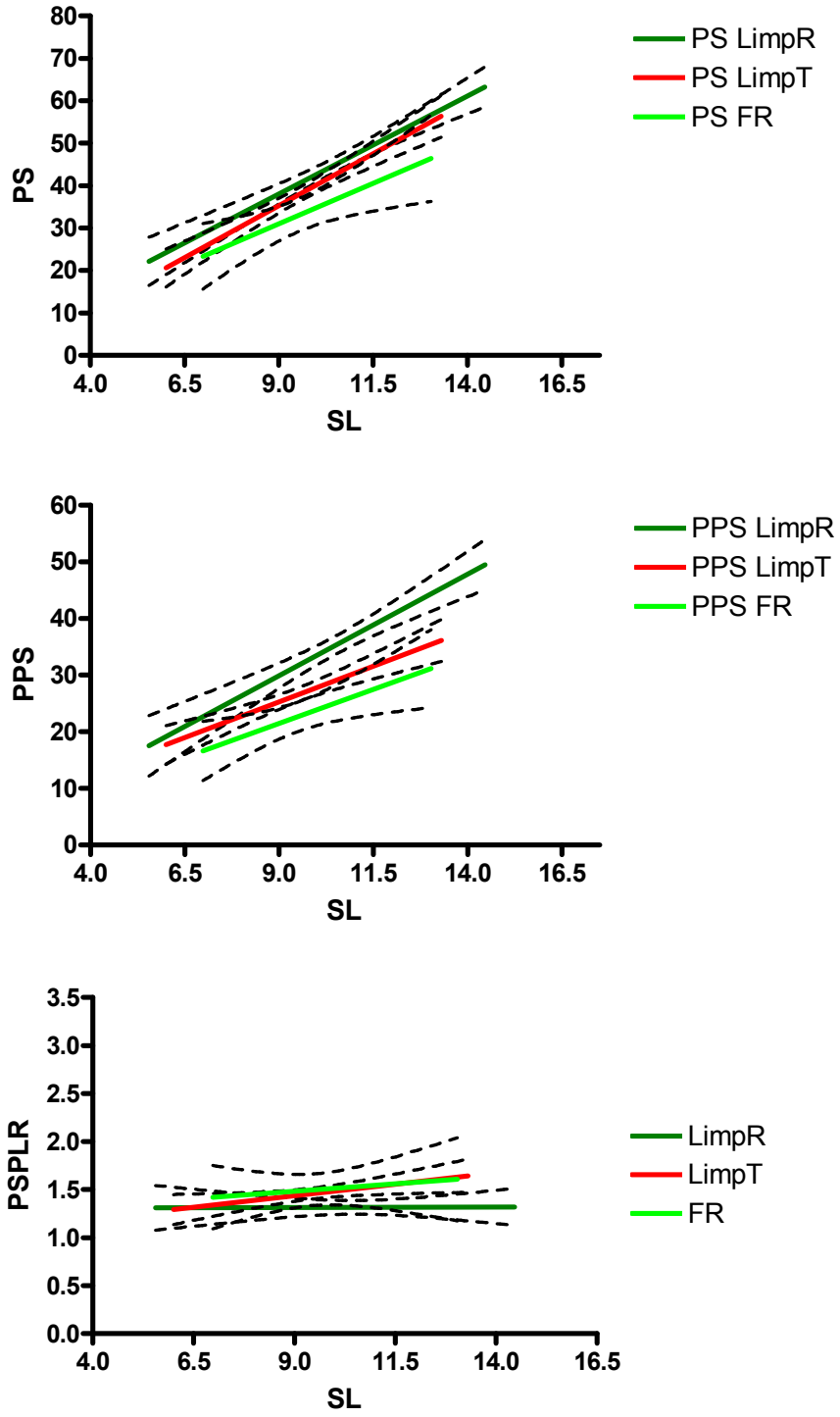

Figure 25: Linear regressions for PS (top), PPS (middle), and PSPLR (bottom) vs. SL of LimpR, LimpT, and FR. The 95% confidence intervals are shown.

Table 12: Summary of linear regressions for PS, PPS, PSPLR vs. SL of LimpR, LimpT, and FR.

Linear regression	LimpR		LimpT		FR	
	Do slopes differ from each other?	Best fit slope	Slope significantly not zero?	Best fit slope	Slope significantly not zero?	Best fit slope	Slope significantly not zero?
All snails							
PS vs. SL	p=0.6925	4.617 ± 0.5516	p< 0.0001	4.894 ± 0.6163	p< 0.0001	3.806 ± 1.301	p=0.0064
PPS vs. SL	p=0.2622	3.592 ± 0.5188	p< 0.0001	2.522 ± 0.4637	p< 0.0001	2.404 ± 0.8821	p=0.0105
PSPLR vs. SL	p=0.3762	0.001073 ± 0.02257	p=0.9622	0.04822 ± 0.02154	p=0.0267	0.03070 ± 0.05546	p=0.5838
8-10.99 mm snails							
PS vs. SL	p=0.081	5.525 ± 1.309	p=0.0002	5.789 ± 1.202	p< 0.0001	-1.393 ± 3.208	p=0.669
PPS vs. SL	p=0.5967	4.320 ± 1.470	p=0.0057	3.389 ± 0.9158	p=0.0003	1.380 ± 2.580	p=0.5989
PSPLR vs. SL	p=0.364	0.02894 ± 0.06966	p=0.6803	0.02231 ± 0.04205	p=0.5968	-0.1395 ± 0.08530	p=0.1185
10-12.99 mm snails							
PS vs. SL	p=0.079	2.556 ± 1.561	p=0.1072	-0.6102 ± 1.998	p=0.7614	8.964 ± 5.317	p=0.1357
PPS vs. SL	p=0.2962	3.036 ± 1.544	p=0.0544	-0.3274 ± 1.582	p=0.8369	3.259 ± 3.502	p=0.383
PSPLR vs. SL	p=0.3412	-0.06658 ± 0.06829	p=0.3338	-0.02547 ± 0.08038	p=0.7528	0.2123 ± 0.3409	p=0.5532

SL = total length of shell; PPS = preputium length; PS = penis sheath length; PSPLR = penis-preputium sheath length ratio.

Table 13: Summary of correlations (Spearman correlation) for PS, PPS, and PSPLR vs. SL of all the sources. A p-value of p<0.05 indicates correlation.

Spearman Correlation	LimpR		LimpT		FR		Potch		LC	
	r	P-value (two-tailed)	r	P-value (two-tailed)	r	P-value (two-tailed)	r	P-value (two-tailed)	r	P-value (two-tailed)
All snails										
PS vs. SL	0.5322	P<0.0001	0.5814	P<0.0001	0.3681	p=0.0351	0.3962	p=0.0064	0.4169	p=0.0244
PPS vs. SL	0.4983	P<0.0001	0.4188	P<0.0001	0.4031	p=0.02	0.4008	p=0.0058	0.5215	p=0.0037
PSPLR vs. SL	-0.00908	p=0.9371	0.1768	p=0.0353	-0.1488	p=0.4087	-0.0716	p=0.6363	-0.2086	p=0.2775
8-10.99 mm snails										
PS vs. SL	0.4217	p=0.0084	0.4929	P<0.0001	-0.1144	p=0.6215				
PPS vs. SL	0.3232	p=0.0478	0.3652	p=0.0001	0.002604	p=0.9911				
PSPLR vs. SL	0.03699	p=0.8255	0.03056	p=0.757	-0.4222	p=0.0566				
10-12.99 mm snails										
PS vs. SL	0.2245	p=0.0932	-0.1532	p=0.288	0.6025	ns				
PPS vs. SL	0.1944	p=0.1473	-0.1151	p=0.426	0.2167	ns				
PSPLR vs. SL	-0.08761	p=0.517	0.01239	p=0.9319	0.3	ns				

SL = total length of shell; PPS = preputium length; PS = penis sheath length; PSPLR = penis-preputium sheath length ratio.

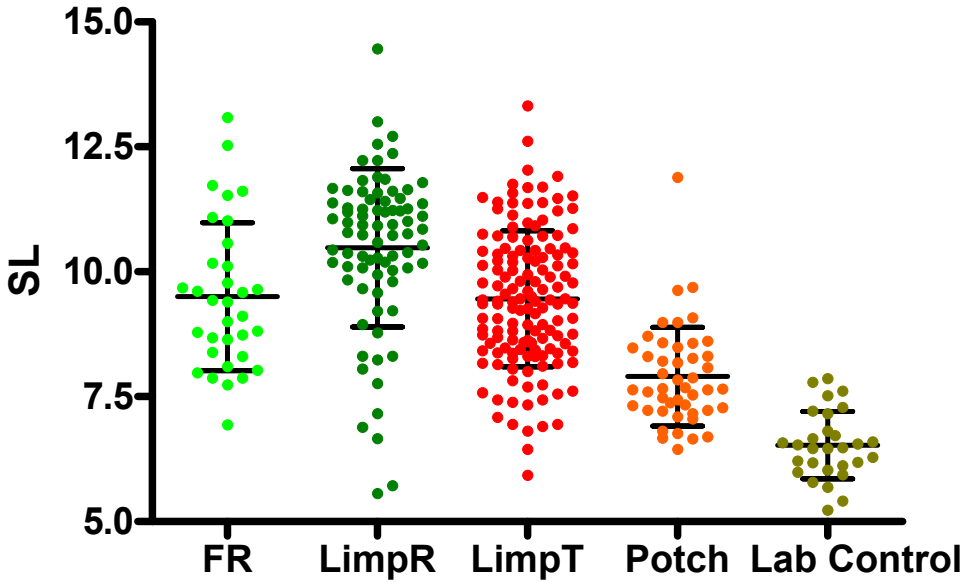

Figure 26: Scatter-plots comparing the shell lengths (SL) of *B. tropicus* from the various sources. Means and standard deviations are indicated.

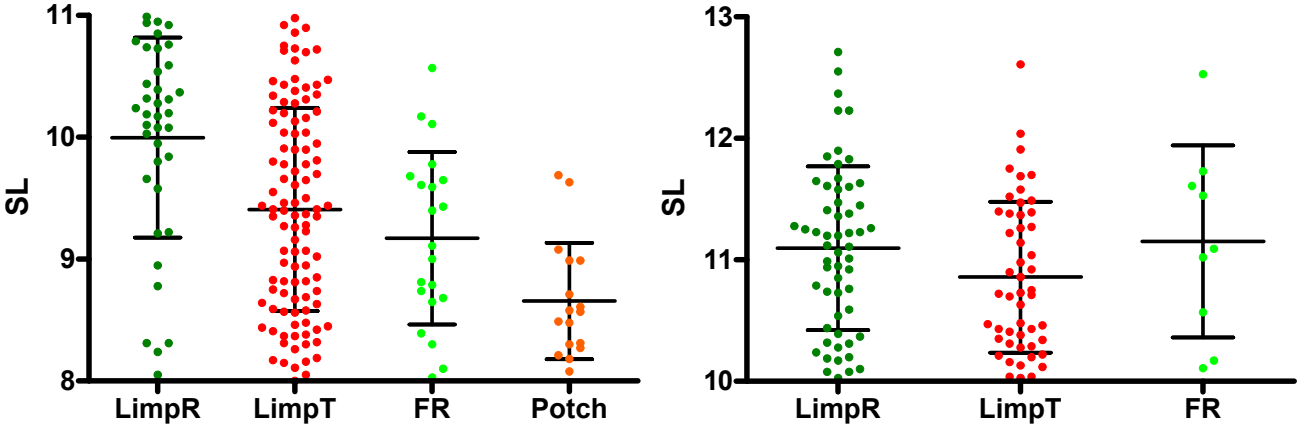

Figure 27: Scatter-plots for snails of 8 -10.99 mm (left) and snails of 10 -12.99 mm (right). Means and standard deviations are indicated.

In order to compare the two SL groupings, linear regressions were drawn of PS, PPS, and PSPLR vs. SL for LimpR, LimpT, and FR snails (Figs. 28,29, and 30). A summary of the linear regressions and the correlations for the two groups can be

seen in Tables 12 and 13. In both size groupings, the slopes for snail PS, PPS, and PSPLR did not differ significantly between the sources. Table 12 shows that for both the 8 -10.99 mm snails and the 10 -12.99 mm snails, none of the sources' slopes for PSPLR vs. SL was significantly not zero. Additionally, only FRs' slopes were not significantly not zero, for PS and PPS vs. SL. In the 10 -12.99 mm SL group, none of the slopes were significantly not zero. In Table 13 it can be seen that the snail PSPLRs of LimpR, LimpT, and FR did not correlate (Spearman correlation) with SL in the 8 -10.99 mm SL grouping. Additionally, only FRs' PS and PPS did not correlate (Spearman correlation) with SL in the 8 –10.99 mm SL grouping. In the 10 -12.99 mm SL group, no correlation (Spearman correlation) was found between the SL and PS, PPS, or PSPLR of LimpR and LimpT snails. As mentioned before, FR did not have enough samples in the 10 -12.99 mm grouping to determine correlation (Spearman correlation).

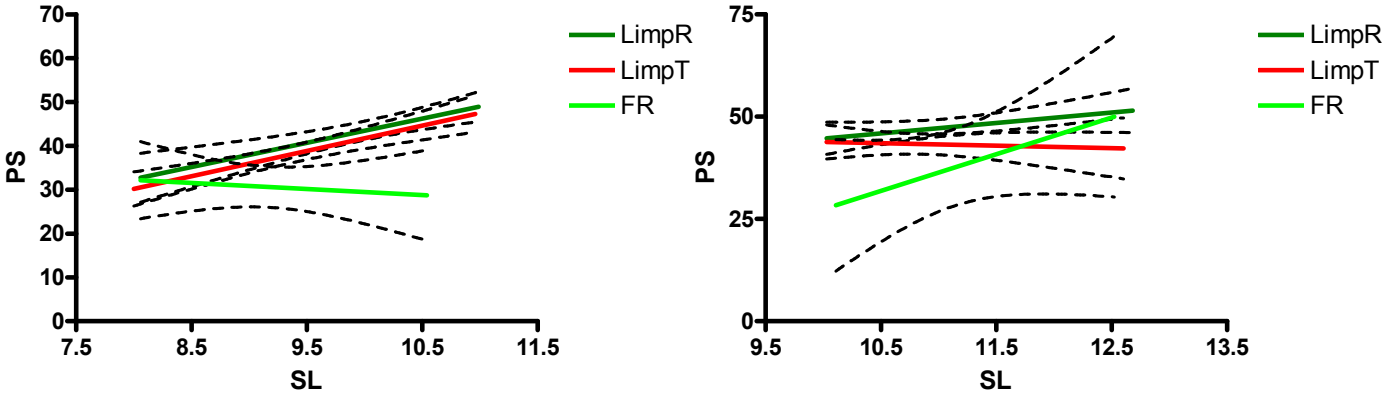

Figure 28: Linear regression of PS vs. SL for 8 –10.99 mm Snails (left) and 10 –12.99 mm Snails (right). The 95% confidence intervals are shown.

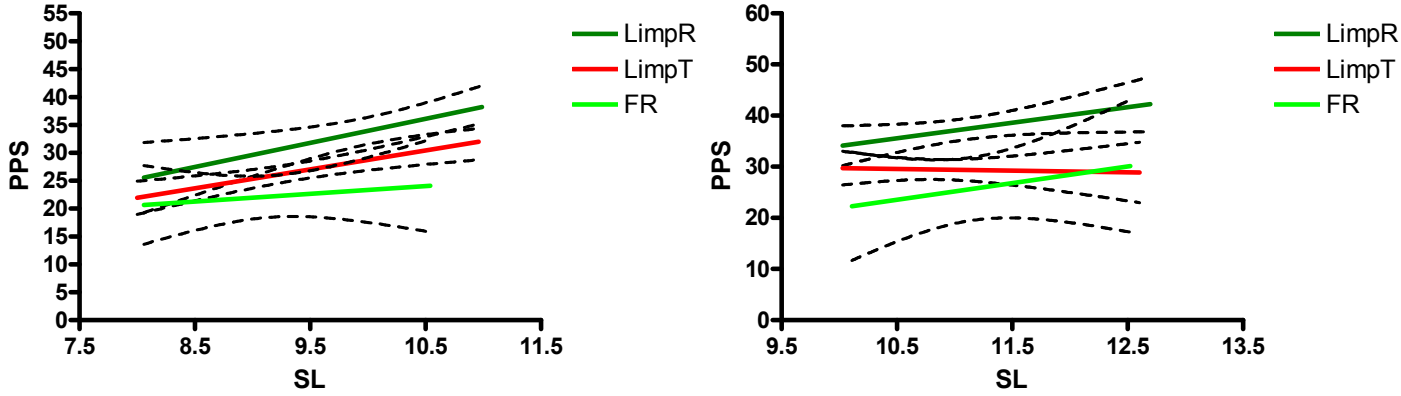

Figure 29: Linear regression of PPS vs. SL for 8 –10.99 mm Snails (Left) and 10 –12.99 mm Snails (Right). The 95% confidence intervals are shown.

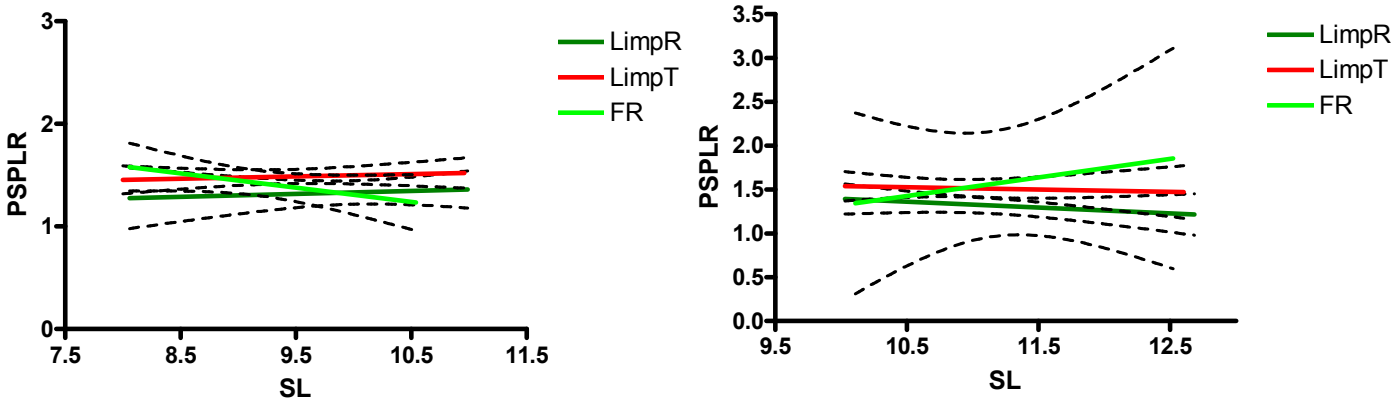

Figure 30: Linear regression of PSPLR vs. SL for 8 –10.99 mm Snails (Left) and 10 –12.99 mm Snails (Right). The 95% confidence intervals are shown.

In order to compare the data from Limpopo with the data from the additional sources, a nonparametric (Kruskal-Wallis test) one-way ANOVA was done for PS, PPS, PSPLR, and SL between the different sources (Table 14). Only LimpT vs. FR, and Potch vs. LC did not show any significant difference for any of the parameters. LimpR snails differed significantly in all parameters from LimpT as well as from LC.

Because the aim of the project was to compare snails from two adjacent areas differing in DDT use, unpaired t-tests were done (Table 15) for PS, PPS, PSPLR and SL from LimpT and LimpR. Additionally, just for information purposes but acknowledging that the assumptions for t-test are violated, comparisons with all sources were done in order to simulate what would happen when any combination of two sources would be selected. Given these assumptions, LimpR differed significantly from all the other sources for all four parameters. The only significant difference between Potch and FR was for SL. Except for LimpR, none of the other sources differed significantly in snail PSPLR.

Table 14: Summary of nonparametric (Kruskal-Wallis test) one-way ANOVA's for PS, PPS, PSPLR, and SL between the different sources.

Anova					
		PS	**PPS**	**PSPLR**	**SL**
Kruskal-Wallis test	**P-Value**	P<0.0001	P<0.0001	0.0005	P<0.0001
Dunn's Multiple	**LimpR vs LimpT**	P < 0.001	P < 0.001	P < 0.05	P < 0.001
Comparison Test	**LimpR vs FR**	P < 0.001	P < 0.001	P > 0.05	P < 0.05
	LimpR vs Potch	P < 0.001	P < 0.001	P > 0.05	P < 0.001
	LimpR vs LC	P < 0.001	P < 0.001	P < 0.001	P < 0.001
	LimpT vs FR	P > 0.05	P > 0.05	P > 0.05	P > 0.05
	LimpT vs Potch	P < 0.001	P < 0.01	P > 0.05	P < 0.001
	LimpT vs LC	P < 0.001	P < 0.001	P > 0.05	P < 0.001
	FR vs Potch	P > 0.05	P > 0.05	P > 0.05	P < 0.001
	FR vs LC	P > 0.05	P > 0.05	P > 0.05	P < 0.001
	Potch vs LC	P > 0.05	P > 0.05	P > 0.05	P > 0.05

Table 15: Summary of two-tailed, unpaired t-tests for PS, PPS, PSPLR, and SL between the different sources.

t-test p-values				
Unpaired, two-tailed	**PS**	**PPS**	**PSPLR**	**SL**
LimpR vs LimpT	P<0.0001	P<0.0001	0.003	P<0.0001
LimpR vs FR*	P<0.0001	P<0.0001	0.0158	0.003
LimpR vs Potch*	P<0.0001	P<0.0001	0.0366	P<0.0001
LimpR vs LC*	P<0.0001	P<0.0001	0.0002	P<0.0001
LimpT vs FR*	0.0514[$]	0.0193	0.5793	0.8654
LimpT vs Potch*	0.0001	0.0004	0.8661	P<0.0001
LimpT vs LC*	P<0.0001	P<0.0001	0.1108	P<0.0001
FR vs Potch*	0.2269	0.5103	0.5955	P<0.0001
FR vs LC*	0.0169	0.0035	0.4673	P<0.0001
Potch vs LC*	0.1279	0.01	0.1379	P<0.0001

*For simulation purposes only

[$]Because the SL of LimpT and FR did not differ significantly, it makes sense that the PS alsow did not differ significantly. However, the fact that the PPS did differ significantly could indicate that between LimpT and FR there is an effect on the PPS.

In order to compare between the PSPLR sample sets of the different sources, scatter-plots and relative frequency histograms were drawn. Figure 31 compares the PSPLRs of the Limpopo sources, and Figure 32 compares the PSPLRs of the additional sources to that of LimpR and LimpT.

Figure 31 shows a scatter-plot and frequency histogram of the PSPLR for LimpR, LimpT, and FR snails. A nonparametric Kruskal-Wallis one-way ANOVA (Table 11) indicated that LimpR differed significantly from LimpT as well as FR with $P < 0.01$ and $P < 0.05$ respectively. LimpT and FR did not differ significantly ($P > 0.05$). The histogram also shows that LimpR had more snails towards the lower end of the PSPLR spectrum. LimpT and FR looked very similar with both peaking at a PSPLR value of 1.4.

In Figure 32, the scatter-plots of the PSPLR for LimpR, LimpT, Potch, and LC snails is presented. A nonparametric (Kruskal-Wallis test) one-way ANOVA (Table 14) indicated that LimpR and Potch did not differ significantly ($P > 0.05$), however a two-tailed, unpaired t-test between LimpR and Potch gave a p-value of 0.0366. When LimpT was compared with Potch, a p-value of $P > 0.05$ (Kruskal-Wallis one-way ANOVA) was obtained. The relative frequency histogram shows that LimpR had more snails in the lower end of the PSPLR spectrum. Both Potch and LimpT peaked at a PSPLR value of 1.4.

A nonparametric (Kruskal-Wallis test) one-way ANOVA (Table 14) between LimpR and LC gave a highly significant p-value of $P < 0.001$. When LimpT and LC snails were compared, a p-value of $P > 0.05$ (Kruskal-Wallis one-way ANOVA) was obtained. The relative frequency histogram shows that LC did not have a Gaussian distribution with peaks at PSPLR values of 1.2 and 1.8. As mentioned before, LC was not a natural population, and harvested early due to time constraints.

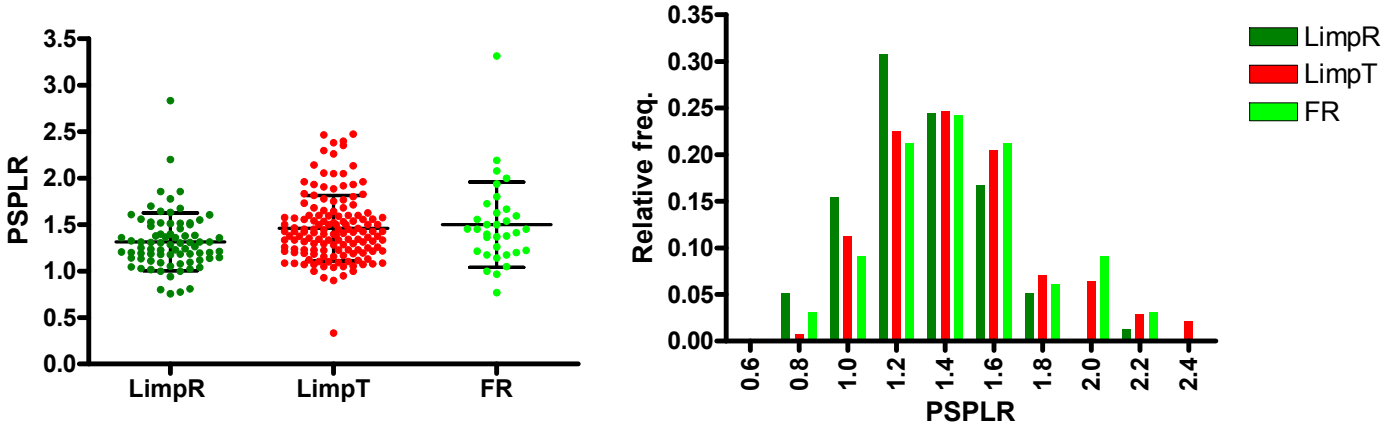

Figure 31: Scatter-plot and relative frequency histogram of the PSPLR for LimpR, LimpT, and FR.

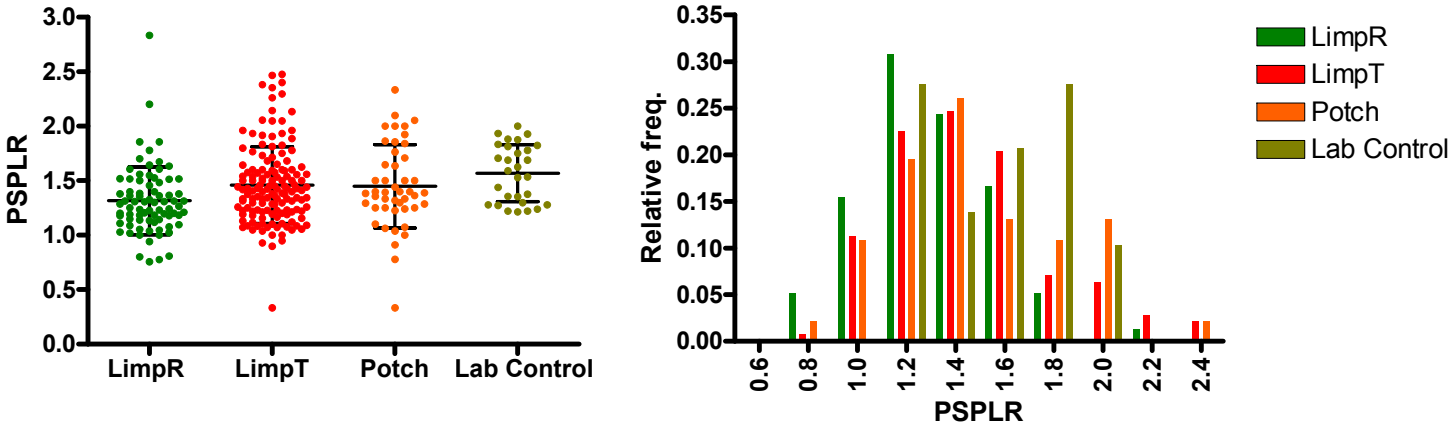

Figure 32: Scatter-plot and relative frequency histogram of the PSPLR for LimpR, LimpT, Potch, and Lab Control.

In order to compare the Potch and LC sourced snails with LimpR, linear regressions and spearman correlations were done. Figure 33 gives the linear regressions of PS, PPS, and PSPLR vs. SL for LimpR, Potch, and LC. Table 16 gives a summary of the linear regression data, while a summary of correlations (Spearman correlation) for the same parameters can be seen in Table 13.

For all three sources, the slopes of the PS, PPS, and PSPLR vs. SL did not differ significantly from each other (Table 16). The slopes of PS and PPS vs. SL for LimpR, Potch, and LC were significantly not zero (Table 16). None of the slopes for PSPLR vs. SL was significantly not zero (Table 16).

In Table 13, PS, and PPS correlated (Spearman correlation) with SL for LimpR, Potch, and LC. The PSPLRs of LimpR, Potch, and LC did, however, not correlate (Spearman correlation) with SL. Compared to Potch and LC, LimpR's PSPLR correlated (Spearman correlation) the worst with SL, while the PS and PPS correlated (Spearman correlation) the best with SL.

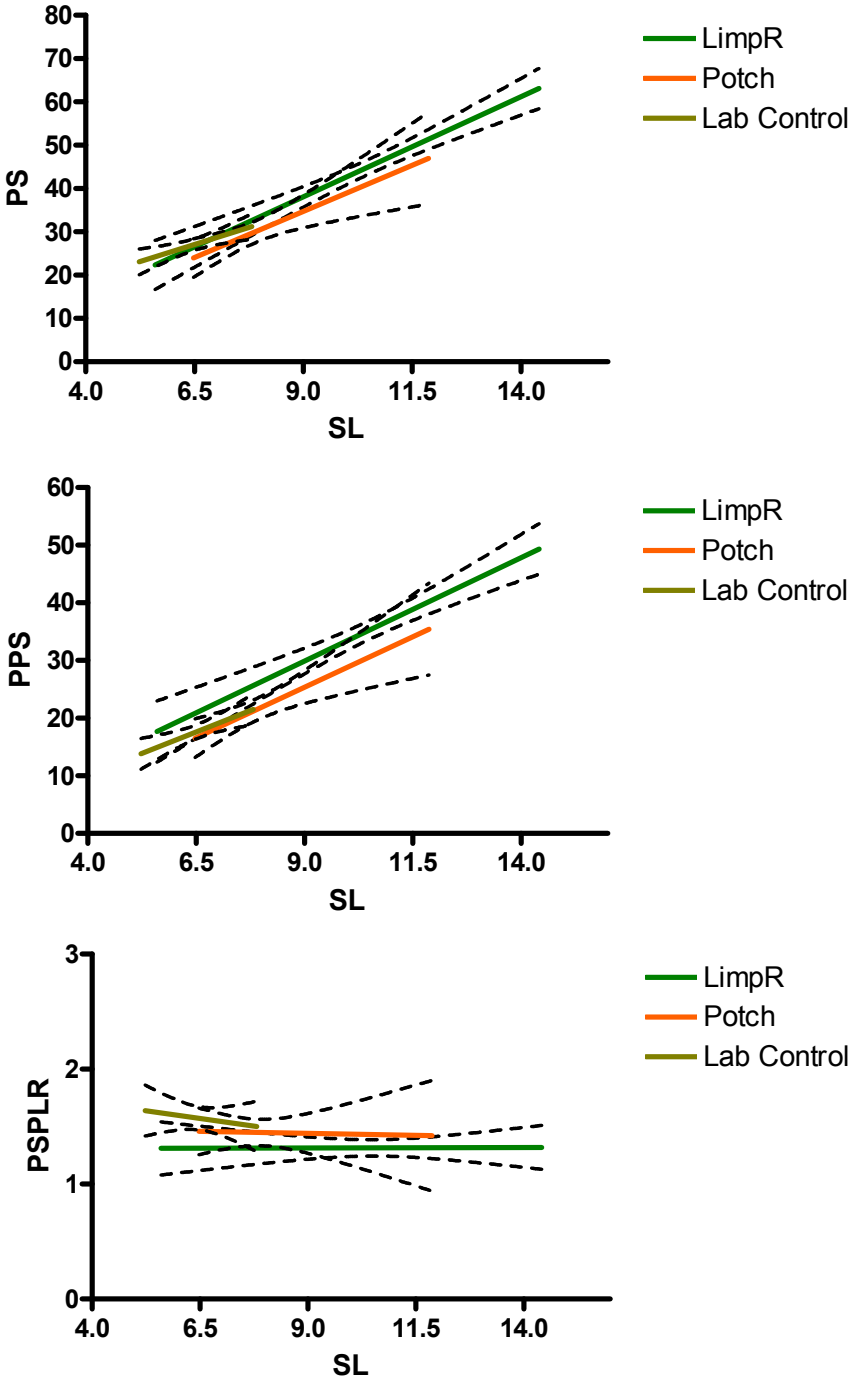

Figure 33: Linear regressions for the PS, PPS, and PSPLR vs. SL of LimpR, Potch, and Lab Control. The 95% confidence intervals are shown.

Table 16: Summary of linear regressions for PS, PPS, PSPLR vs. SL of LimpR, Potch, and LC.

Linear regression		LimpR		Potch		LC	
	Do slopes differ from each other?	Best fit slope	Slope significantly not zero?	Best fit slope	Slope significantly not zero?	Best fit slope	Slope significantly not zero?
All snails							
PS vs. SL	p=0.7726	4.617 ± 0.5516	p< 0.0001	4.267 ± 1.282	p=0.0018	3.153 ± 0.9961	p=0.0038
PPS vs. SL	p=0.9454	3.592 ± 0.5188	p< 0.0001	3.502 ± 0.9653	p=0.0007	2.978 ± 0.8942	p=0.0025
PSPLR vs. SL	p=0.504	0.001073 ± 0.02257	p=0.9622	-0.007062 ± 0.05832	p=0.9042	-0.05313 ± 0.07385	p=0.4781

SL = total length of shell; PPS = preputium length; PS = penis sheath length; PSPLR = penis-preputium sheath length ratio.

To establish which parameter (PS or PPS) is responsible for the variation in snail PSPLR between sources, the mean values for PS, PPS, PSPLR, and SL were used to calculate the relative percentage values of these parameters concerning LimpR. LimpR was chosen because it was the original reference site, had the largest snails, and showed the best correlations (Spearman correlation) for PS and PPS to SL as well as the worst correlation (Spearman correlation) of PSPLR to SL. Table 17 shows the mean PS, PPS, and SL values for the different sources as well as the relative percentage values. Only LC showed a similar reduction in SL and PS size. The rest of the sources showed varied magnitudes of reduced parameter sizes. However, for all sources the greatest percentage-based reduction was found for preputium sheath length (PPS).

Table 17: The mean values of PS, PPS, and SL for the different sources as well as the relative percentage of the values for LimpR.

	Mean PS	Mean PPS	Mean SL	PS% of LimpR	PPS% of LimpR	SL% of LimpR
LimpR	45	35	10.48	100	100	100
LimpT	38	26	9.46	84	75	90
FR	33	23	9.50	73	64	91
Potch	30	22	7.90	67	61	75
LC	27	18	6.53	61	50	62

Chapter 5: Discussion

From the review by Colborn *et al*.(1993), it is clear that detrimental effects of EDCs on wildlife have been known from as early as 1973 (the negative effect of DDT on embryonic survival of bald eagles due to eggshell thinning and cracking). At the time of the review, concerns for and indications that adverse effects of the human reproductive system could be associated with environmental chemical exposure, was growing (Colborn e*t al*., 1993). Relative certainty was, however, available for some cases in marine molluscs, fish, reptiles, birds, and mammals (Harrison e*t al*., 1997).

Recently, associations between chemicals and adverse effects (with regards to endocrine disruption) on humans, wildlife and domestic animals were made (Aitken e*t al*., 2004; Hamlin & Guillette, 2010; Hotchkiss e*t al*., 2008; Matthiessen, 2003). As a result, the importance of endocrine disruptive studies on wildlife has gained ground, not only to protect the environment, but also to protect human health (Aitken e*t al*., 2004; Barnhoorn e*t al*., 2009; Hotchkiss e*t al*., 2008; Hutchinson e*t al*., 2006; Matthiessen, 2003).

DDT and the DDT isomers have been implicated in many studies to either be present in organisms or to show ED effects on an array of organisms, in varying taxa (Barnhoorn e*t al*., 2010; Bornman e*t al*., 2010a; Bouwman e*t al*., 2006; de Jager e*t al*., 2009; de la Cal e*t al*., 2008; Guillette Jr. e*t al*., 1996; Hamlin & Guillette, 2010; Kidd e*t al*., 2001; Marchand e*t al*., 2008; Metcalfe e*t al*., 2000; Mlambo e*t al*., 2009; Nhan e*t al*., 2001; Veeramachaneni e*t al*., 2007). Not much has, however, been done in line with the effects of DDT on molluscs and nothing has been done on the effects of DDT on *B. tropicus*. There is now evidence that link ED effects in molluscs to ED effects in fish (Jobling e*t al*., 2003), causing the gap between invertebrates and vertebrates to be narrowed.

This narrowing of the gap between invertebrates and vertebrates, together with the suggestion that the molluscan hormonal system is somewhat comparable to that of vertebrates (see section 2.3 and 2.5), and the ecological relevance of molluscs (see sections 2.3.5 and 6.3), further emphasise the propability that any ED effects observed in molluscs, (and in this case *B. tropicus*), could serve as an indication of possible ED effects in other wildlife species, domestic animals, and humans.

5.1 Sediment samples

DDT is highly lipophilic, nearly insoluble in water and has a high adsorption coefficient to solid materials (Aneck-Hahn e*t al*., 2007; Burger, 2005; Lintelmann e*t al*., 2003). These aspects contribute to the presence of DDT in the sediment. After particles to which DDT has bound settles to the bottom sediment, the adsorbed DDT can still be accumulated by benthic organisms and thus re-enter the food chain (Barnhoorn e*t al*., 2010; Nhan e*t al*., 2001).

In some instances, the presence of DDT in sediment could be linked to the presence of DDT in organism tissue samples (Barnhoorn e*t al*., 2010; de la Cal e*t al*., 2008; Nhan e*t al*., 2001). This was also the case for some other EDC's in a study where snails were exposed to EDC-impregnated sediment in laboratory (Duft e*t al*., 2007). A certain degree of contradiction arise however, in studies where the DDT levels in sediment were not quantifiable, but were found quantifiable in water and fish from the same sites (Barnhoorn e*t al*., 2009). Another study found that DDT levels in water samples from the same site, differed from one survey to the next (Barnhoorn e*t al*., 2010). These are natural variations, and point towards assays that include effects that integrate exposures over time, rather than grab samples of environmental media.

Sediment samples were taken from all of the Limpopo sites where *B. tropicus* were collected (Fig. 5, Tables 1 and 2) and analysed for DDT in order to ascertain whether endocrine effects in the snails could be associated with the presence of DDT. Additionally, five more sites (Fig. 21, Table 3) were sampled along the Luvuvhu River and analysed for DDT.

5.1.1 *B. tropicus* sites

As expected, most of the LimpT sites (Table 4) were polluted with the very persistent DDT metabolite *p,p'*-DDE. Only two of these sites contained the metabolite *p,p'*-DDD, and only Site 18 also had the metabolite *o,p'*-DDE. Interestingly Site 21 and Site 37 were not polluted with quantifiable levels of DDT.

In the case of Site 37, the reason for no DDT could be because this pond is not situated close to any residential areas from which it can receive runoff water. It also does not receive runoff water from the road that passes close by. The reason no DDT was found at Site 21 could only be guessed at. It is possibly due to the periodicity of the stream as well as to the many anthropogenic disturbances close to the site.

On the other hand, Site 17, on the reference side, was polluted with *p,p'*-DDE while none of the other LimpR sites contained any quantifiable DDT. A possible

reason for this might be that dust and mud that originate in the DDT-sprayed area are carried by vehicles and then deposited on the road surface of the tarred road that passes close to Site 17. The runoff from this road flows into Site 17 but there is no outflow from Site 17. Therefore, accumulation of trace amounts of DDT could be possible over many years of depositing. Historic use of DDT on the farm, as well as possible use of old stock might also be possible. For the main comparison between LimpR and LimpT, the reference LimpR sources did not contain quantifyable levels of DDT while most (5 out of 7) of the LimpT sources from the DDT spayed area contained quantifyable levels of DDT.

5.1.2 Luvuvhu sites

It was not expected to observe DDT pollution (Table 5) in both of the reference sites while DDT was only found in one of the three sites from the DDT-sprayed area. A similar situation was observed by Barnhoorn *et al*. (2010) and Marchand *et al*. (2008). What the reasons for this might be can only be speculated at this stage, but long-range air transport or illegal use cannot be ruled out. In addition to the fact that DDT can travel long distances via air (Aneck-Hahn e*t al*., 2007; Marchand e*t al*., 2008), the following is observed. The presence of DDT in LVB04R might be ascribed to the road crossing the river upstream from this site, as in the case of Site 17. However, different isomers of DDT were in both sites, therefore, different sources should be suspected. It could therefore be assumed that the reasons for the presence of the DDT metabolites are not the same for LVB04R and Site 17, even though they are in close proximity to each other and the same road.

LVB03T and LVB05R are similar in that both samples were taken from the shores of dams. LVB03T from the Nandoni Dam and LVB05R from the Albasini Dam. The Nandoni Dam was completed in 2005 while the Albisini Dam was completed in 1952 (DWA, 2009). Furthermore, the site of sample collection in the Nandoni Dam showed recent anthropogenic disturbances on the shore. It is unlikely, however, that these differences between the sites were the key factors in the different levels of DDT metabolites.

The only similarity between LVB02T and LVB03T were that they seem to be more often disturbed by humans with the means of earth moving equipment. This type of disturbance was also seen at Site 21.

5.2 Bulinus tropicus samples

5.2.1 Parasites

Molluscs as a group serve as the intermediate host for a variety of trematode parasites. Trematodes are present in both marine and freshwater habitats. The adult parasite resides in vertebrate hosts from which the eggs are shed into the aquatic medium. From the egg, a free-living stage (miracidia) emerges and infects the molluscs. In the mollusc, the parasite goes through asexual cycles (complexity varies between species) after which another free-living form emerges that will then infect the next invertebrate or vertebrate host (Morley, 2006; Rato e*t al*., 2009a).

A variety of trematodes can infect *Bulinus* sp. (de Kock, 2007). One such trematode is *C. microbothrium*, a conical fluke of cattle. The intermediate host for this parasite is the snail *B. tropicus* (Appleton, 2002; de Kock e*t al*., 2002). Therefore, in order for snails to be infected with this parasite, it is necessary for the water body that they are in, to encounter cattle that are infected with *C. microbothrium.*

Even though parasitic trematode infections have been shown to have detrimental effects on the molluscs' endocrine system (see section 2.6.3) and render infected snails useless for studying ED effects of toxicants, infection prevelance might still be usefull in describing toxicant effects on snail populations.

Except for Site 17, all sites were on communal or public land. The cattle owners in this area of Limpopo generally do not fence in their cattle and cattle herds are regularly herded to the open water to drink. It is therefore possible for cattle to gain access to all of these sites. Although Site 17 is on privately owned land, signs (cattle faeces within 10 meters from the waters edge) that cattle does come in contact with this water were observed.

Looking at Table 8 it would seem that snails in the DDT-sprayed area are more prone to infection by trematodes than snails from the reference area. The fact that a DDT metabolite was present at Site 17 could most likely cause one to assume that, snails in habitats with DDT pollution are more prone to infections by trematodes. When looking at Table 9 as well as Table 4, it becomes apparent that this may not be the case. Site 37 had the highest percentage of parasitized snails for the LimpT side, while at the same time not having any detectable DDT present. Both Sites 21 and 36 had parasitized snails, although no DDT was present. Site 36 from the LimpR side actually had a greater percentage of parasitized snails than Site 21. It must be realised though, that DDT levels seem to be very variable in aquatic environments. That most sites with detectable levels of DDT were found on the DDT-sprayed side

indicates runoff from applications, but also possible involvement with parasitic infection. More research on this topic is needed.

5.2.2 Site 17 (FR)

The initial analysis showed that the PSPLR of LimpR snails did not differ significantly (two-tailed, unpaired t-test), although borderline, from LimpT (Fig. 24). When the snails from LC and Potch were also taken into consideration, the PSPLR of LimpR and LC differed significantly (Kruskal-Wallis nonparametric one-way ANOVA, data not shown). None of the other sources snail PSPLRs differed significantly from each other. It seemed as though the Site 17 data, at this stage, included with the reference sites, behaved differently from the rest of the reference data. An initial PCA (Fig. 22) of the data (data classified in accordance with its origin) showed relatively definite groupings of the snails from the various sources. The snails from the LC as well as from Potch were in a similar grouping, while the snails from LimpR and LimpT were grouped separately.

The data from Potch and the LC was then removed and a PCA (Fig. 23) was drawn using only the data from Limpopo and categorizing it according to the specific site of origin. This picture showed mainly snails from Site 17 distributed to the lower part of the plot, together with the snails from the DDT-sprayed side. Because Site 17 differed from the rest of the Limpopo sites in terms of its location relative to human population, close proximity to commercial farming, and the presence of detectable DDT residues, it was decided that the Site 17 data should rather be treated as a separate source named FR. When this was done, the PSPLR of LimpR differed significantly (Fig. 24) from LimpT with p=0.003 (two-tailed, unpaired t-test) compared to when Site 17 was included in LimpR when the p-value was 0.0523 (two-tailed, unpaired t-test).

A comparison between the PSPLR of LimpR and FR snails yielded a p-value of 0.0158 (two-tailed, unpaired t-test). A nonparametric (Kruskal-Wallis) one-way ANOVA (Table 11) between the Limpopo sites showed that the PSPLRs of LimpR differed significantly from both LimpT and FR snails, while LimpT did not differ significantly from FR. This then further confirmed that Site 17 should be treated as a separate source from LimpR. It also provided the first indication that the DDT-sprayed sites seem to have ED effects as represented by the differences in PSPLR.

5.2.3 Effect of size

A previous study done on *B. tropicus* suggested that the PSPLR of snails could be used as an indicator of endocrine disruption and that it should be further

investigated (Bornman e*t al*., 2007). Studies on other snail species also implicated penis size as a possible endpoint for ED effects (Oehlmann e*t al*., 2000; Schulte-Oehlmann e*t al*., 2000; Schulte-Oehlmann e*t al*., 2001; Tillmann e*t al*., 2001). Furthermore, estrogen receptors were identified on the penis sheath complex of *M. cornuarietis* (Bannister e*t al*., 2007). Together with the fact that vertebrate type estrogen receptors are known to be associated with the male reproductive system, and the possibility that these receptors may be affected by EDC's (Bannister e*t al*., 2007), a cause is established for further studies that would help to better understand effects of EDC's on wildlife and man.

Therefore, the focus was directed at measuring the penis sheath length (PS) as well as the preputium sheath length (PPS) and establishing the PSPLR. In addition to the PS and PPS, other parameters (such as in Table 7 and Table 10) were also measured with the aim of assisting in the quantification of the PSPLR method. In the end, the focus returned to the PS, PPS, and PSPLR with the addition of shell length (SL), but more attention may be needed on the other morphometric parameters in the future.

The snails SL can however only be used as a general indication of size and not as an indicator of condition or of contamination levels since the shell length and other shell proportions can vary between snails within the same population (Czech e*t al*., 2001; de Kock, 2007; Marshall & Rajkamur, 2003; Stiglingh & van Eeden, 1977). The SL is also not indicative of age. This was observed when obtaining the LC snails. The snails reared in captivity grew at different rates. The growth rate was in a large part determined by the concentration of snails in a container – the more snails per container, the slower the growth rate.

From Table 10 and Figure 26 it becomes evident that almost none of the sites had snails with comparable SL ranges. Only the LimpT and FR, and Potch and LC snails had similar SLs (Kruskal-Wallis nonparametric one-way ANOVA).

In accordance with the different snail SLs, the mean PS and PPS also differed between the sources (Table 10). However, it would seem that the PSPLRs did not react in a similar way as the PS and PPS. This was then confirmed in the linear regressions (Figs. 25 and 33, and Table12) and Spearman correlations (Table 13). The PS and PPS of the snails from the different sources correlated with the respective SLs, while the PSPLRs did not. However, only the LimpT snails PSPLR correlated with SL. The degree of correlation, however, differed between the sources. Generally, it would seem, except for LimpT, that the PSPLR was not coupled with the SL. The constant PSPLR ratio found indicates that the PS and PPS increase proportionally to SL. PSPLR is a ratio. Therefore, as the snails grow, the absolute

sizes of PS and PPS grow proportionally, but the ratio of the lengths remains constant. This is a strong indication that the use of the PSPLR ratio may be a very useful indicator of ED is the ED effect would be more pronounced on one of the two components of the PSPLR. This should be confirmed by controlled laboratory studies. Discussion that follows however, indicate that there are additional factors to be considered.

For the main comparison between LimpR and LimpT it would seem that snails in the DDT-sprayed area are smaller than the snails in the reference area. However, as mentioned before, in field conditions the SL of snails cannot be used as an indication of condition or contamination levels. Future laboratory investigations might shed more light.

5.2.3.1 Two SL groups

In a further effort to eliminate any effect that size may have, the snails were divided into two groups. The first group included snails ranging in size from 8 mm to 10.99 mm. The second group ranged in size from 10 mm to 12.99 mm. This grouping however caused the LC snails to be excluded from the 8-10.99 mm interval, as they were all too small. In the case of the 10-12.99 mm snails, the Potch snails as well as the LC snails were excluded. In the 10-12 mm snail group, FR was only represented by 9 snails. (See Fig. 27)

Any narrower groupings also resulted in one or more of the sources being represented by a small number of snails. Never the less, when linear regressions (Figs. 28, 29, and 30) and Spearman correlations (Table 13) of these groupings were done for PS, PPS, and PSPLR versus SL, the picture of the LimpR and LimpT 8-10.99 mm snails were consistent with the picture created when all the snails were used. However, in the 10-12.99 mm snails, LimpT and LimpR showed no correlation (Spearman) for the snails PS and PPS with SL, while FR did not have enough samples to establish correlation. For LimpR, LimpT, and FR the PSPLR of the snails also did not correlate (Spearman correlation) with SL in both the 8-10.99 mm snail and 10-12.99 mm snail groupings.

It is therefore conclusive that the absolute length of PS and PPS are coupled to the size of the snail. When these two parameters are however placed in relation to each other (the PSPLR as a ratio of the two), the effect of snail size (SL) is lost. Therefore, any differences between the PSPLR of different populations could be regarded as an indication of ED effects on the PS or PPS and not as differences due to size.

This is further confirmed in Tables 14 and 15, where one-way ANOVA's and two-tailed, unpaired t-tests showed significant differences between many of the sources when the snails PS, PPS, or SL were compared. When the PSPLR's were compared, only LimpR still differed significantly from the other sources. The mean PSPLR was highly significantly different between LimpT and LimpR, the two sites pertinent to the main aim of the study. However, the comparisons with other sources indicate a more complex scenario that needs further investigation. In addition, a difference in ratio indicates a relative shortening or lengthening of one of the two components – an aspect addressed in the following section.

5.2.4 PSPLR

The PSPLR is the ratio obtained when PS of the snail is divided by PPS of the snail. As proved in the previous section, this ratio is not under the influence of absolute size, but the relative lengths of PS and PPS. If for arguments sake the measurements were made in cm in one study and in inches in another, the ratios of the different studies will still be comparable. Though the ratio may be indicative of whether there are differences between populations or if an endocrine effect is present, it lacks the ability to show whether the observed effect is on the penis, the preputium, or both. An illustration of this follows in Table 18.

Table 18: Theoretical PS and PPS values to indicate the effect on the PSPLR when either one of them change.

	Scenario 1	Scenario 2	Scenario 3	Scenario 4
PS	10	10	13	13
PPS	8	5	8	5
PSPLR	1.25	2	1.63	2.6

From Table 18 the following can be deduced.

1. A longer PS results in a higher PSPLR value
2. A shorter PPS results in a higher PSPLR value
3. A Longer PPS results in a lower PSPLR value
4. A shorter PS results in a Lower PSPLR value

Considering Tables 14 and 15, it becomes clear that the biggest significant difference in snail PSPLR values are between LimpR and LC and then between LimpR and LimpT. LimpR then also differs significantly from FR and Potch. None of the other sources differed significantly from each other with regards to the PSPLR.

Looking at Figures 31 and 32 it seems that the relative frequencies for the snails PSPLR's of LimpT, LimpR, and Potch were very similar while they differed from LimpR. LC (Fig. 32), however, was different from all the other sources. It also did not show a Gaussian distribution in the relative frequency histogram. Even though this was the case, LC did not differ significantly (Kruskal-Wallis nonparametric one-way ANOVA) from LimpT, FR, and Potch with $p > 0.05$ in all three cases.

From Tables 12 and 16 it became clear that the slopes of PS and PPS vs. SL were the most significantly not zero for the LimpR and LimpT snails. Additionally the slope for PSPLR vs. SL was the least significantly not zero for the LimpR snails. This means that effectively the PSPLR stayed constant with an increase in SL. This, in addition to Table 13 that showed that PSPLR of LimpR correlated (Spearman correlation) the least with SL and that the PS and PPS for LimpR and LimpT correlated (Spearman correlation) the most with SL, indicated that the different PSPLR values obtained were likely due to possible ED effects in the other sources and not in LimpR. The possibility that it was the other sources, instead of LimpR that was affected, was to a lesser degree, supported by the visual interpretation of the linear regressions (Figs. 25 and 33). In Figures 25 and 33, the regression lines for PS vs. SL looked similar for LimpR, LimpT, and Potch. However, Potch and FR also shared some similarity. For the PPS vs. SL regression lines, LimpT, FR and Potch looked similar. This inconsistency of similarities for the regression lines, to a lesser degree, supports the hypothesis that the different PSPLR values are due to effects in the other sources, rather than effects in LimpR. An attempt to quantify the different PSPLR values follows.

5.2.4.1 Is the effect seen in the PSPLR due to changes in the PS or PPS or both?

As illustrated from Table 18, a difference in the PSPLR does not indicate if either the PS or the PPS is affected for whatever reason. In addition, previous studies mainly refer to a reduction in penis size (Oehlmann e*t al*., 2000; Schulte-Oehlmann e*t al*., 2000; Schulte-Oehlmann e*t al*., 2001; Tillmann e*t al*., 2001) or a different PSPLR value (Bornman e*t al*., 2007) when test specimens are compared to control/reference specimens.

It is not enough to establish if there is merely a difference in PSPLR values between populations, one must also determine and describe the difference. Since the PSPLR of LimpR snails differs significantly from all the other sources, the following

section will aim to describe the observed difference of the PSPLR's using LimpR snails as the base of comparison.

In order to do so, the mean PS and PPS values of the different sources will be given as a percentage value of the mean PS and PPS values of LimpR, the *ab initio* reference source of snails for this study. Since PS and PPS are positively correlated to size, the percentage values of SL will also be considered. A summary of the results can be seen in Table 17

5.2.4.1(a) LimpR vs. LimpT

LimpR was the *ab initio* reference source for this study. Subsequently, FR was removed from this set for reasons explained in section 5.2.2. For the original comparison of the snail PSPLR between LimpR and LimpT, a p=0.003 (two-tailed, unpaired t-test) was obtained. Visually the difference between the regression lines was greater for PPS vs. SL than for PS vs. SL (Fig. 25). Additionally, in Table 17, The PPS of LimpT was 75% of LimpR compared to the LimpT PS that was 84% of the LimpR PS. It can therefore be concluded that the difference in PSPLR was due to a relatively shorter PPS of LimpT.

Additionally it should be noted that from Figures 28 and 29 it seems that the differences between the regression lines of LimpR and LimpT for both the PS and PPS were bigger in the 10 -12.99 mm snails, but not in the smaller snails. This effect needs to be further investigated with controlled laboratory studies. One study (Tillmann e*t al*., 2001) did find effects in younger snails that disappeared with getting older. A possible age dependence of ED effects (if for the moment it is assumed that age equates with SL in field snails) might indicate sensitive development stages, not an uncommon phenomenon of ED. One such example is were prenatal exposure to *o,p'*-DDT and diethylstilbestrol resulted in male and female mice behaving more aggressive in adulthood(Palanza e*t al*., 1999).

It should also be mentioned that *B. tropicus* is a simultaneous hermaphrodite that shows a brief protandric phase and an earlier development of the male reproductive tract (Brackenbury & Appleton, 1991). It is unknown if this phase or any other phases that might be present, have an effect on the components of PSPLR. If this protandric phase has an effect on the PSPLR, it might be the reason why the smaller LC snails differed from LimpR and the other sources. The LC snails were all younger than 6 weeks when sampled. In the light that *B.tropicus* matures in 13 weeks at 15^{o}C and 2.3 weeks at 29^{o}C (Brackenbury & Appleton, 1991), it could mean that the LC snails were still in the protandric phase. If this is similar to the effect

observed in the prosobranch snail, *M. cornuarietis* is unknown. For *M. cornuarietis*, juvenile males (exposed to antiandrogens) showed an effect on the penis, but a reversal of the effect was seen in adults (Tillmann e*t al*., 2001). This aspect of development could support an age-dependant sensitivity (assuming that SL and age correspond in field snails) to ED effects, an aspect that needs further clarification.

The following subsections consider comparisons between sources of snails in addition to the main aim of the study. It should be seen as interrogative (based on what-if scenarios) rather than substantial, with the aim of broadening the scope for further investigations.

5.2.4.1(b) LimpR vs. FR

FR was originally part of LimpR. However, it became clear from initial analysis, that there were unanticipated effects at play, and the site was hereafter considered a separate source. The additional comparison of the snail PSPLR between LimpR and FR gave a $p=0.0158$ (two-tailed, unpaired t-test) and $p < 0.05$ (Kruskal-Wallis one-way ANOVA for LimpT, LimpR, and FR). From the linear regressions in Figure 25, it can be deduced that both the PS and PPS of FR snails were relatively shorter than that of LimpR, with a greater difference for the PPS vs. SL regression line. This is also confirmed in Table 17, where the reduction in PPS is much larger than the reduction in PS. However, the magnitude by which PS is reduced when compared to the reduction in SL leads one to believe that there is also an effect on the PS.

Even though the presence of DDT in this site (Table 4) might be a possible explanation for the differences observed (Tables 11, 12, 17, and Fig. 25) in the PS and PPS of FR snails, when compared to LimpR snails, it does not explain why LimpT and FR snails differed from each other in the linear regression for PS vs. SL (Fig. 25) as well as the percentage values for PS and PPS (Table 17). A study done on the terrestrial gastropod *H. aspersa* suggested airborne pollution from vehicular traffic as cause for effects seen (Regoli e*t al*., 2006). In addition, the fact that some chemicals originating from vehicle pollution are also EDCs (Aitken e*t al*., 2004; Matthiessen, 2003; Owens Jr. e*t al*., 2006), could possibly explain the results obtained for FR, since this site receives runoff water directly from a road passing by within a 20 meter radius. The migration of other pesticides and herbicides from the surrounding farms into FR should not be excluded. FR therefore probably had multiple sources of various EDCs, in contrast to LimpT sites that had mostly subsistence farming, domestic activities, and DDT as possible contributors. The effects seen in PSPLR indicate that this parameter, together with PS and PPS, can be developed as a strong detection tool for ED in freshwaters.

5.2.4.1(c) LimpR vs. Potch and LimpR vs. LC

For these two additional sources, a one-way ANOVA (Kruskal-Wallis) of the PSPLR for LimpR, LimpT, FR, Potch, and LC snails gave a $p > 0.05$ for LimpR vs. Potch and a $p < 0.001$ for LimpR vs. LC. Two-tailed, unpaired t-tests however gave a $p=0.0366$ for LimpR vs. Potch and a $p=0.0002$ for LimpR vs. LC. In both cases, the regression lines for PPS vs. SL showed a greater difference than the PS vs. SL regression lines, when compared to LimpR. From Table 17 it became clear that the higher Potch PSPLR value is due to a relatively shorter PPS (PPS value of 61% compared to a PS value of 67%). In the case of LC, the PPS showed the greatest reduction with a value of 50% compared to a PS value of 61%. LC also differed from the rest of the sources in magnitude of PS reduction. In the case of LimpT, FR, and Potch, the PS values differed from the SL values by 6%, 18%, and 8% respectively. The LC PS, however, differed only by 1% from SL.

The Potchefstroom sites (Sites 24 and 25) presumably could have served as alternative reference sites. The difference in PSPLR values could possibly be ascribed to differences between populations. It should however also be noted that the Potchefstroom sites are situated within 10 meters of a very busy road that connects Potchefstroom to Carletonville. Additionally one of the two sites was also polluted with plastic materials. When the two sites' PSPLR values were compared using an unpaired t-test, a p-value of 0.2237 indicated no significant difference between the two Potchefstroom sites. It also indicates that for field assessments, reference localities for sites being investigated for ED effects need to be selected as close as possible to the sites of interest.

Chapter 6: Conclusion

The main purpose of this study was to establish if the freshwater pulmonate snail *Bulinus tropicus* shows effects of possible endocrine disruption in an area that is actively sprayed with DDT when compared to an area that is not sprayed with DDT.

6.1 Possible endocrine disruption

According to the literature, an endpoint indicative of endocrine disruption in gastropod snails is the reduction of male reproductive organs, specifically the penis (Oehlmann e*t al*., 2000; Schulte-Oehlmann e*t al*., 2000; Schulte-Oehlmann e*t al*., 2001; Tillmann e*t al*., 2001). In this study, the main difference observed between LimpR and LimpT snails was a significant reduction of preputium length in the DDT-sprayed (LimpT) sites.

Varying but similar results were found concerning the PSPLR, PS, and PPS of the snails from other sources. In the case of the Limpopo snails, differences in PSPLR were observed when comparing LimpT and FR sourced snails. Looking at the presence of DDT and its metabolites in these sources, one might draw the conclusion that DDT is the cause of the change in PSPLR values. This would however not be completely true, due to the probable but unknown presence of other pollutants in these sources. Making a mistake in this conclusion is also emphasised by the fact that snails in FR also showed a reduction in PS together with a reduction in PPS. Taking into consideration all of the known and unknown variables that might play a role, a more correct conclusion would probably be that the differences in PSPLR values between the Limpopo sources is due to possible endocrine disruption. For the present case it is likely, but not proven, that DDT and its metabolites act in an additive or multiplicative way (Aneck-Hahn e*t al*., 2007; Turusov e*t al*., 2002) with other unknown pollutants to cause the differences seen.

In the case of the LC and Potch snails, the differing PSPLR values when compared to LimpR snails was due to, in the case of LC, a relatively larger PS together with a relatively smaller PPS, and in the case of the Potch snails, a relatively smaller PPS. The P1 snails of the F2 generation LC snails came from the Potch sites. The differences in PSPLR values from that of LimpR could therefore possibly be ascribed to geographical population differences. That would imply that the populations in these two geographical regions might in fact be subspecies when taking into account the results of morphometric studies on other pulmonate species (Brown & Rollinson, 1996; Pointier e*t al*., 2006). If, however, they were not

subspecies, it would imply that some form of endocrine disruption is present in the Potch sites. It is however not possible to deduce the same for the LC snails, since these snails were all less than 6 weeks old and it is unknown what effects the age and/or the protandric phase of the snails might have on the PSPLR and more specifically the PS.

When considering the interpretation dilemma arising with the Potch and LC snails, it would be advisable, when doing field assessments, to select reference localities – for sites being investigated for ED effects - as close as possible to the sites of interest.

6.2 Parasites

This study confirmed (results not given) that infections with the intermediate stages of trematode parasites have a negative effect (similar to endocrine disruption) on the host snail (Morley, 2006; Rato e*t al*., 2009b; Sullivan e*t al*., 1998). Additionally it was noted that more of the LimpT snails were infected with trematode parasites than snails from LimpR and FR, collectively. This can however not be coupled to the presence of DDT since individual sites where no DDT was present showed the highest percentage of trematode infections. Therefore, in this study, no correlation can be made between DDT and the likelihood of trematode infections. The possibility still exists though, that ED might compromise the defences of the snails, even though the source of ED is not known.

6.3 Ecological relevance

Similar to other documented freshwater and marine snail species (Hall e*t al*., 2009; Lagadic e*t al*., 2007) *B. tropicus* fills an ecological relevant role in its position in the aquatic food web. Its detritivorous feeding behaviour (Lagadic e*t al*., 2007) place it in a position to be able to take up pollutants through bioconcentration and bio-magnification, and then making it available to the higher trophic levels (Hall e*t al*., 2009). Even though this study did not look into these aspects, this is definitely a point worth looking into, especially taking into account the ability of DDT to bio-accumulate.

6.4 Some pitfalls to watch out for in future studies and possible ways to avoid them

6.4.1 Obtaining of specimens

A difficulty that arises with the use of *B. tropicus* is the habitat selection of the species. Obtaining of reference specimens from sites that are undisturbed is relatively difficult since the snails tend to prefer habitats that are disturbed or have some form of organic enrichment (Bornman e*t al*., 2007). It therefore becomes difficult to obtain wild specimens that are truly undisturbed. On the other hand, using pollution sensitive snail species will result in none of them being present in polluted areas. Using *B. tropicus* with a short generation time and tolerance to organic pollution seems to be the best compromise.

6.4.2 Fixating, dissecting, and measuring procedure

A very important aspect that will need to be looked at is the actual measuring of the penis-preputium complex. The measuring procedure can be much improved. The breadth as well as the length of the penis or the preputium after fixation could be affected by an array of factors before, during, and after fixation. Some of the effecting factors are the following:

- *The level of relaxation of the snail during narcotising and at the point of fixation.* The level of relaxation of the snail can have an effect on the penis-preputium complex. In this study, the complex was measured in the relaxed, completely retracted state. However, in some instances the penis sheath was slightly imbedded in the preputium because the penis was not fully retracted, preventing measurement. Where possible compensation was made for the overlapping effect, but it is not sure as to what effect this could have on the data.
- *The ability and precision of the dissector while dissecting out the penis-preputium complex, unfolding and pinning the complex and then measuring it.* This differs between individuals within a lab. Spot-checks were made during the dissections for this study, and although not documented, there were no major measurement deviations that would have affected the conclusions.
- *The plasticity of the complex due to the degree of fixation.* The longer time spent in the fixation medium the less placid the complex becomes. This together with the previous point has an effect on the measurements, since

plasticity, together with force applied during the unfolding and pinning down process has an effect on how the complex is then observed and measured. Fortunately, these factors were constant during this investigation, but the absolute results may not be comparable with future studies. A proposed way of eliminating some of these problems would be to make microscopic slide preparations of the complex and then to take pictures and take measurements by making use of microscope-camera hardware and software.

6.4.3 Standardising and interpreting parameters

Assuming that the PSPLR of *B. tropicus* is indicative of endocrine disruption, the following can be seen as ways for future studies to better utilise the species and the data as well as to gain better insights into results and causality.

- If the aim is to compare wild populations of *B. tropicus*, the following is proposed. It would be advantageous to identify measurable morphometric standards that stay constant in the snails irrespective of the age, size, locality, health, or any other influence. This can then be used to determine if the observed difference between the PSPLR values is due to an effect in the PS or the PPS. The effected PS or PPS can then more easily be ascribed to what ever the influencing factor might be.
- Another option, and more likely solution to the problem of too many unknown variables associated with sites, is to look into the development of a laboratory-based bioassay. Snails that are raised under controlled conditions are free from any differences in PSPLR that might be caused by demographics, water quality, or population traits. Furthermore, age will then become a known variable and size can be standardised. Once reactions to known EDC are quantified, snails can then be exposed to sediment or extracts from sites under question. The bioassay is currently being developed.

6.5 Final remarks

Possible endocrine disruption was observed in *B. tropicus* from an area in the Limpopo Province where DDT is sprayed when compared with the same species from an adjacent area not sprayed with DDT. This was done by making use of the PSPLR. Yet, causality with DDT has not been established. However, there are strong indications from literature (Barnhoorn e*t al*., 2009; Barnhoorn e*t al*., 2010; Mlambo e*t al*., 2009; Turusov e*t al*., 2002; Veeramachaneni e*t al*., 2007) and fieldwork

suggesting that DDT could be implicated in this matter, if only in a synergistic way (Aneck-Hahn e*t al*., 2007; Turusov e*t al*., 2002). Initial trails under controlled conditions in the aquarium at NWU showed susceptibility of *B. tropicus* to known EDCs, including the DDTs, especially on the male gonadal parameters. Furthermore, due to its position in the food web, geographical distribution, and likely bioaccumulation and bio-magnification, *B.tropicus* should be further investigated as a possible biological indicator in the field and laboratory (de Kock e*t al*., 2002; Duft e*t al*., 2007; Gagnaire e*t al*., 2009; Hall e*t al*., 2009; Janer & Porte, 2007; LaFont & Mathieu, 2007; Lagadic e*t al*., 2007; Oehlmann e*t al*., 2007). Causality, however, must first be established with rigorous controlled experiments.

It is also informative that studies on humans and fish from the same area in the Limpopo Province have also found ED effects associated with DDT. Some of the associated effects were; a higher chance for mothers exposed to DDT to have boys with urogenital birth defects (Bornman e*t al*., 2010b), intersex in the freshwater fish *Oreochromis mossambicus* possibly due to the presence of DDT (Barnhoorn e*t al*., 2010). Other studies looking at the levels of DDT in human breast milk is underway, while a study (Van Dyk e*t al*., 2010) concerning levels of DDT in human serum, indoor air, floor dust, outside soil, potable water, leafy vegetables, and chicken samples (muscle, fat and liver) were also done.

Finally, it is in the opinion of the candidate and in light of this study, that further studies in quantifying endocrine disruption as well as identifying the EDCs in the Limpopo Province and rest of South Africa is much needed in order to maintain environmental integrity and to promote human health.

Appendix 1

Old clasification

Kingdom: Animalia

Phylum: Mollusca

Class: Gastropoda

Subclass: Pulmonata (Euthyneura)

Order: Basommatophora

Suborder:

Family: Planorbidae

Subfamily: Bulininae

Genus: *Bulinus*

Species: *tropicus*

(Compiled from(Brown, 1980; Hickman e*t al*., 2004b)

New classification

Kingdom: Animalia

Phylum: Mollusca

Class: Gastropoda

Clade: Heterobranchia

Informal group: Pulmonata

Informal group: Basommatophora

Clade: Hygrophila

Super family: Planorboidea

Family: Planorbidae

Subfamily: Bulininae

Tribe: Bulinini

Genus: *Bulinus*

Species: *tropicus*

(Bouchet & Rocroi, 2005)

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