RESEARCH OUTPUT

SEASONAL VARIATIONS IN THE GROWTH AND REPRODUCTION OF *HELCION CONCOLOR* (KRAUSS, 1848) LIMPET ALONG THE WILD COAST OF SOUTH AFRICA

By

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ABSTRACT

Growth and reproduction are crucial in understanding the biology and ecology of rocky intertidal limpet species. It is inevitable that seasonal fluctuations affect the different reproductive stages of each limpet species in the wild. This led to this research project investigating the seasonal effects on the reproduction and growth rate of the colour variable limpet *Helcion concolor* (Krauss, 1848). Growth was quantified by comparing the shell dimensions (i.e. shell length, shell width and shell height) and weight (i.e. total body mass, shell weight, somatic weight and gonad weight) of both male and female individuals across the seasons. Histological studies were conducted to quantify the reproductive variation of both male and female individuals during different seasons. Shell conicity (SC), shell ellipticity (SE) and gonad somatic index (GSI) were then calculated to determine seasonal variability. The results revealed that *H. concolor* females were longer, broader, taller and heavier than the males. Daily incremental shell length analysis detected that the limpets gained more shell length and width in autumn while they gained more shell height in spring and autumn. The ratio of males to females fluctuated on a monthly basis resulting in more females (51.67 ± 4.41 %) than males (48.33 ± 4.41 %) in this study. Males had a larger GSI than females. GSI for females was lowest in summer and highest in spring. For males, the GSI was highest during the winter season. Regression results revealed that shell length and width were useful in determining GSI in both male and females. Histological analysis detected five stages of germ cells for females (Oogonia, Previtellogenic oocyte, Vitellogenic oocyte, Mature oocyte and Atresic oocyte) and four stages for males (Spermatogonia, Spermatocytes, Spermatids and

Spermatozoa). Mature oocytes were the most abundant stage for females, mostly abundant in autumn and winter. For males, spermatozoa increased from summer to winter and decreased during spring. Histological studies further revealed that *H. concolor* was a partial spawner as the gonads always contained sperm and eggs within them. Overall, this study highlighted that seasons had more effect on the reproduction than the growth rate of this limpet.

Keywords: histology, gonad somatic index, oocytes, patellid, shell dimensions, spawning.

DECLARATION

I, Vuyokazi Nibe, student number, solemnly declare that this research paper entitled "Seasonal variations in the growth and reproduction of *Helcion concolor* limpet along the Wild Coast of South Africa" is my original work. All sources used or quoted in the study have been indicated and acknowledged by way of complete references.

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DECLARATION TO PLAGIARISM

1. I am aware that plagiarism is defined at Walter Sisulu University (WSU) as the inclusion of another's ideas, writings, works, discoveries and inventions from any source in an assignment or research output without the due, correct and appropriate acknowledgement to the author'(s) or source(s) in breach of the values, conventions, ethics and norms of the different professional, academic and research disciplines and includes unacknowledged copying from intra- and internet and peers/fellow students.
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CHAPTER 1: GENERAL INTRODUCTION

Limpets are common singled shelled marine gastropods found inhabiting intertidal rocky shores worldwide. They show a broad variation in shell size, the shape of the aperture, and the architectural features on the shell's surface (Khuow 2006, Paulo Cabral 2007, Harley *et al* 2009). This has led to some limpets being given common names due to their shell shapes. For example, the limpet *Scutellastra cochlear* (Born, 1778) is referred to as the pear limpet due to its pear-like shape. *Lottia gigantea* (G. B. Sowerby I, 1834) is also the called owl limpet because of its brownish grey color with pale markings. *Cymbula oculus* Born, 1778 is commonly known as the goat's eye limpet as it resembles the eye of a goat due to its colour pattern. The limpet *Helcion pruinosus* Krauss, 1848 is commonly referred to as the rayed limpet because of the colourful rays of its shell which radiate from a central point. All members of the Fissurellidae family are referred to as keyhole or slit limpets because they possess a small hole at the apex of their shells. However, all limpets have a conically shaped shell which is not spiral compared to snails.

Limpets are molluscan gastropods which comprise four super-families Fissureloidea (Aktipis & Giribet, Hewerda & Wesselingh 2014.), Patelloidea (Ridgway *et al* 1998, Nakano & Ozawa 2004; 2007), Nacelloidea (Bouchet 2005) and Siphonarioidea (White *et al* 2011, White & Dayrat 2012). The Patelloidea are regarded as true limpets since they contain a modified pallial gill cordon and are subdivided into eight families (Ridgway *et al* 1998, Nakano & Ozawa 2007, Nakano & Sasaki 2011). These families are Eoacmaeidae, Patellidae, Nacellidae, Pectinodontidae, Lepetidae, Neolepetopsidae, Lottiidae and Acmaeidae.

Earlier workers (e.g. Powell 1973) taxonomically treated the order Patellogastropoda as three families (Acmaeidae, Patellidae and Lepetidae) based on their relatively featureless simple conical limpet shells. Cladistic analyses based on morphological characters were then developed in the 1990s (see Lindberg 1988, Jamieson *et al* 1991, Ridgway *et al* 1998, Sasaki 1998) and the patellogastropod limpets were then reclassified into six families: Patellidae, Nacellidae, Lepetidae, Acmaeidae, Lottiidae and Neolepetopsidae. However, the classification system based on morphological characters proved to be problematic as it was found to show convergence and a new classification system using molecular phylogenetics (e.g. Koufopanou *et al* 1999, Simison & Lindberg 2003, Nakano & Ozawa 2007, Nakano & Sasaki 2011) has since been used to try resolve the phylogenetic relationships within Patellogastropoda. Molecular phylogenetics can now classify the Patellogastropoda from the level of family to species. As such, there are currently at least eight families (Eoacmaeidae, Patellidae, Nacellidae, Pectinodontidae, Lepetidae, Neolepetopsidae, Lottiidae and Acmaeidae) and an estimate of 36 genera residing under this Patellogastropoda order.

The family Eoacmaeidae includes only the genus *Eoacmaea* and there are about 14 species assigned under the *Eoacmaea* genus. The Lottiidae family is the most diverse within Patellogastropoda as it is composed of numerous genera (*Lottia*; *Patelloida*; *Nipponacmea*;*Tectura*; *Notoacmea*; *Scurria*; *Discurria*; *Potamacmaea*; *Yayoiacmea*;

Atalacmea and *Asteracmea*) and about 130 species (Nakano & Ozawa 2007). On the other hand, the family Acmaeidae is composed of three genera *Acmaea*; *Niveotectura* and *Erginus*, while eight genera (*Lepeta, Propilidium, Lothia, Cryptobranchia*, *Sagamilepeta*, *Maoricrater*, *Bathylepeta* and *Limalepeta*) are currently recognized within Lepetidae (Sasaki 1998). The Neolepetopsidae family was proposed by McLean (1990) and consists of *Neolepetopsis*; *Eulepetopsis* and *Paralepetopsis* genera. The Pectinodontidae family includes the *Pectinodonta*, *Bathyacmaea* and *Serradonta* genera (Sasaki *et al* 2005, Sasaki *et al* 2007).

The family Patellidae is the most studied group among Patellogastropoda. This is because Patellogastropod limpets represent the basal branch of the extant Gastropoda as they are considered to be the most primitive group (Haszprunar 1988, Lindberg 1988, Ponder & Lindberg 1997). Based on the cladistic analysis of morphological characters review of Patellidae by Ridgway *et al* (1998), 38 species in four genera are recognized throughout the world, with southern African shores having the greatest diversity. The genus *Patella* comprises nine species occurring in the north-eastern Atlantic. The two genera *Cymbula* and *Helcion* are endemic to Southern Africa with nine *Cymbula* species and four *Helcion* species found throughout this region. The genus *Scutellastra* genus is paraphyletic, comprising sixteen species, and can be subdivided into three sub clades corresponding to geographical distribution in South Africa, South Australia and the Indo-Pacific (Koufopanou *et al* 1999, Nakano & Ozawa 2004; 2007, Lindberg 2007).

Limpets are further grouped into two broad generic groups known as true limpets and false limpets. True and false limpets' shells differ in many ways, particularly in terms of vascular cavity morphology (White *et al* 2011). For example, true limpets require water as they use gills for gaseous exchange. On the other hand, false limpets have a combination of gills and a pulmonary cavity that enable them to respire both in and out of water (Hodgson 1999). All limpets under the family Patellidae are regarded as true limpets (Ridgway *et al* 1998) whereas false limpets fall under the genera *Siphonaria*, *Trimusculus*, and *Williamia*. In South Africa, most false limpets fall under the *Siphonaria* genus.

The research question was: Does seasonal variation influence the reproduction and growth of *Helcion concolor* along the Wild Coast? The growth rate and reproduction of *H. concolor* may be more pronounced in certain seasons than others. The aim of this dissertation was to quantify the effects of seasons on the reproduction and growth rate of this species. The first objective was to compare the growth of males and females during different seasons. The second aim was to conduct histological studies on the reproduction of this species during different seasons. This dissertation was structured such that chapter 2 synthesized on the reproduction and growth of limpets. Chapter 3 was designed to compare growth rate of *H. concolor* males and females during different seasons in Nqabara. Growth parameters such as shell length, width, height and body weight were measured. Chapter 4 identified and compared the reproductive stages

during different seasons using histological techniques. This was done for male and female individuals of *H. concolor*. The purpose of the general discussion was to synthesize the findings of this project in relation to other studies conducted elsewhere.

The study was conducted in Nqabara (32° 20' S, 28° 47' E), a non-reserve rocky shore area situated outside Dwesa Marine Protected Area (32° 18' S, 28° 50' E) on the east coast of southern Africa in the Eastern Cape (Figure 1). Near to the rocky shore area, there is a village and an Eco River Lodge with cottages for tourists. Since this rocky shore is unprotected, it experiences high levels of exploitation (Nakin *et al* 2012, Nakin & McQuaid 2014). The fishing ground spots available are often used by the villagers and tourists and the exploitation of limpets for food and bait is always high.

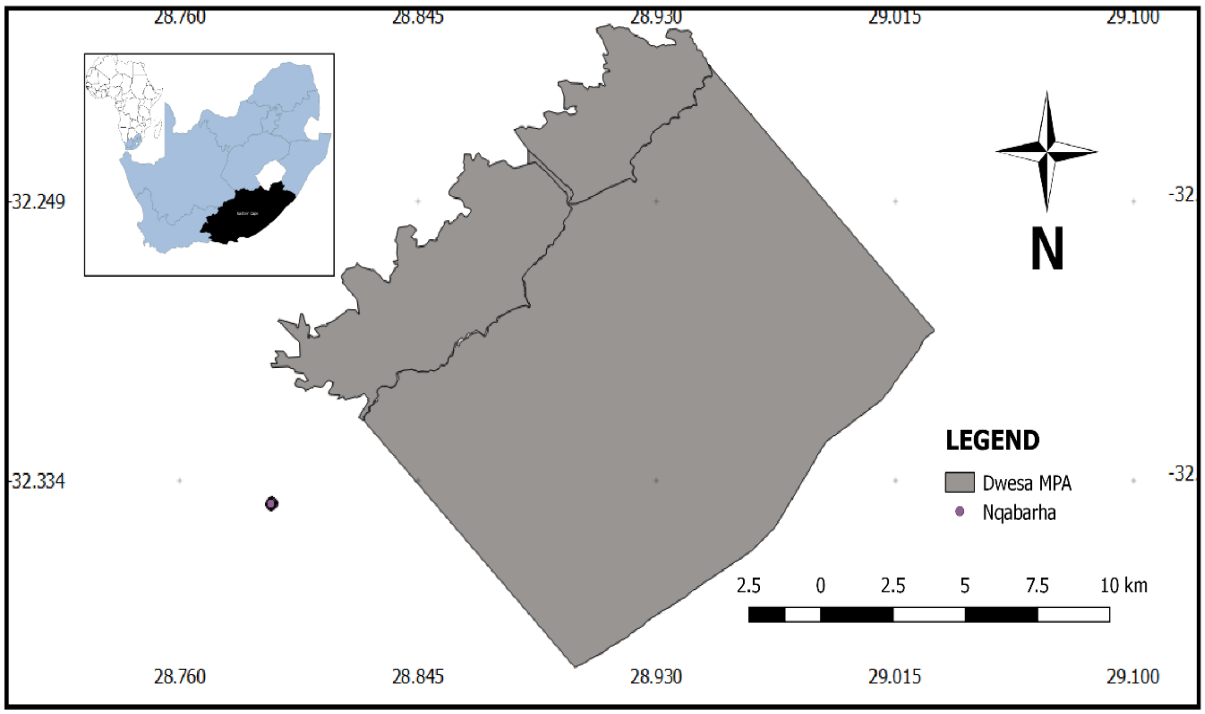

Figure 1: A map showing the situation of Nqabarha rocky shore in relation to Dwesa MPA along the Wild Coast of the Eastern Cape Province in South Africa.

CHAPTER 2: LITERATURE REVIEW

Many studies on patellid limpets have focused on their systematic and evolutionary relationship of these limpets (Hodgson *et al* 1996, Ridgway *et al* 1998, Weber *et al* 1997, Nakano & Ozawa 2004; 2007, Nakano & Sasaki 2011). The abundance and diversity of patellid limpets around the coast of South Africa has also led to a number of studies being done on the biology and ecology of these limpets (Branch 1981; 1985, Creese 1981, Vat 2000, Henninger & Hodgson 2001, Gray & Hodgson 2003). However, the majority of these studies focused on West Coast species and neglected species that are limited to the East Coast of South Africa.

Limpets are sexually reproducing organisms and can either be dioecious or hermaphroditic (Branch 1981). The limpets which are dioecious possess only one sex per individual, either male or female, whereas hermaphroditic limpets possess both sexes during their lifecycle. The hermaphrodites are further divided into two forms i.e. synchronous and sequential hermaphrodites (Leonard 2013). The synchronous hermaphrodites have both the male and female reproductive organs active simultaneously whereas the sequential hermaphrodites possess both sexes but only one sex is active at a given period. Sequential hermaphroditism in limpets is often called protandry which means that the limpet species first becomes male and then changes to be a female during its lifecycle (Collin 2006; 2013).

In the Patellidae family, protandrous hermaphroditism was first suggested by Dodd (1956). Orton *et al* (1956) observed a predominance of males in smaller size classes and females in larger size classes suggesting that males change into females at a later stage of their lives. Branch (1981) later explained protandry in patellids differently, as he found no hint of protandry in seven Patella species he examined. A recent study by Henninger & Hodgson (2001) raises an argument that protandric hermaphrodism cannot be explained within some Patellidae limpets as some individuals appear to be functionally male and female at the same time. Reasons for suggesting protandry in patellids may be due to external environmental factors. On the other hand, limpets from the Siphonariidae family are all hermaphroditic and most are protandrous (Branch 1981, Chambers & McQuaid 1994, Hodgson 1999, Pal & Hodgson 2004).

External fertilization occurs in the form of spawning in limpets (Morriconi 1999). This spawning occurs in three forms: the deposition of eggs and sperm directly into the water column (broad cast), scattering of gametes on top of an open substrate and brood hiding (hiding or burying fertilized eggs). All Patellidae limpets are broadcast spawners that release eggs and sperm into the water column for external fertilization (Branch 1981, Henninger & Hodgson 2001, McCarthy *et al* 2008, Ribiero 2009). Siphonariid reproduction differs from patellid in that fertilization is internal, and all the fertilized eggs are laid embedded in mucous ribbons and in almost all cases are attached to the substrate, in the Siphonariidae family (Hodgson 1999, Pal & Hodgson 2004). Furthermore, the sperm are transmitted by a penis to a sperm receiving structure called a *bursa copulatrix*

(spermatheca) in the siphonariids. The siphonariids have a glandular region containing albumen and mucous glands, which is why the eggs laid by most siphonariids are found embedded in mucous ribbons (Chambers & McQuaid 1994, Hodgson 1999).

Biogeographic locality has been observed to have an influence on the timing and pattern of spawning in limpets. In South African studies, reproductive patterns have revealed that cool temperate species from the West Coast tend to have a single spawning period, usually around late autumn/early winter for the West Coast region (Branch 1974). On the other side, limpets from the warm temperate waters of the East to Southeast coastline either have a biannual spawning periodicity with the main reproductive period occurring in twice a year during summer and in autumn to early winter (Foster 1997, Vat 2000). This pattern also occurs in many North American acmaeid limpets (Fritchman 1962). There are also relatively few species exhibiting extended seasons with multiple spawning episodes and little or no resting stage (Branch 1981, Creese & Ballantine 1983).

In addition, there are also physical and biological factors that can influence the growth of limpets. These include tidal height and wave exposure (Hodbay 1995, Tanaka *et al* 2002), high temperature and desiccation stresses (Denny *et al* 2006, Paulo Cabral 2007, Harley 2009), habitat and position of the limpet on the rocky shore (Branch 1981, Hodbay 1995) and food availability or competition for food within and/or between a species (Lasiak & White 1993, Gray & Hodgson 2003).

The growth rate of shelled marine molluscs is usually measured as an increase in shell length since these intertidal limpets exhibit great morphological plasticity (Branch 1981; Denny 2000). Morphological features such as the shape and texture of the shell are of importance as they show correlation with physical conditions (Clarke *et al* 2004). These morphological features help to regulate factors such as heat stress, water loss and tenacity in limpets. The growth rate and shell shape of limpets is affected by physical and biological factors such as position on the shore, wave action, latitude, seasonal and habitat-associated differences in food availability, intra- and inter-specific interactions, and seasonal fluctuations in temperature.

Limpets either grow allometrically, where shell height increases more rapidly than shell length or isometrically, where shell length increases more rapidly than shell height. A variety of methods has been used to measure and monitor growth rates of limpets. These include the mark and recapture method (Vat 2000, Gray & Hodgson 2003, Clarke *et al* 2004); allometric versus isometric growth studies (Vat 2000, Gray & Hodgson 2003); morphometric analysis of the shell shape and form (Vat 2000); age determination using micro-growth band analysis (Gray 1996, Gray & Hodgson 2003, Vat 2000); and estimates of seasonal growth rates using similarly sized limpets (Vat 2000).

In South Africa, there are many different biogeographic regions occurring along the coast. As a result, the South African coastline is influenced by differing oceanographic

conditions. The decrease in primary productivity from the west to east direction results in individual species being smaller along the south and east coasts when compared to those of the west coast is due to differences in temperature of currents. The northward flowing cold Benguela current keeps the west coast cool while the southward flowing warm Agulhas current keeps the southeast coastline warm.

CHAPTER 3: SIZE VARIATION AND SHELL MORPHOMETRY OF *HELCION CONCOLOR* IN NQABARA ROCKY SHORES ALONG THE WILD COAST OF SOUTH AFRICA

3.1 Introduction

Helcion is one of the four genera under the Patellidae family of gastropods (Nakano & Ozawa 2004; 2007, Nakano & Sasaki 2011). It comprises five species which have been identified globally. According to Ridgway *et al* (1998), four out of the five species under this genus are indigenous to southern African waters. *Helcion pruinosus* (Krauss, 1848) and *Helcion pectunculus* (Gmelin, 1791) are sister species distributed throughout southern Africa (Webber *et al* 1997, Gray & Hodgson 2003). They have a broad geographic distribution, ranging from rock platforms of southern KwaZulu Natal extending to the northern coast of Namibia. The only non-southern African *Helcion* species, *Helcion pellucidum* (Linnaeus, 1758), has a north eastern Atlantic origin (Weber *et al* 1997).

Helcion concolor Krauss 1848 is found in the Indian Ocean from the east coast of South Africa to Mozambique, where it is regarded as the variable limpet due to its colour variation (Branch 2002). The colour of this species ranges from bright yellow to reddish orange to bluish black. According to Etter (1988) as well as Sokolovo & Berger (2000), this shell colour polymorphism is a common feature of both intertidal and terrestrial gastropods. Furthermore, colour variation within a species is based on natural selection and the conditions associated with the type of habitat occupied by the species (Lindberg

& Pearse 1990, Miura *et al* 2007). As a result, the colour variation in *H. concolor* benefits this species as a means of camouflage against predation on the rocky shores.

Helcion concolor is distributed throughout the rocky intertidal shore but commonly found in the mid-shore where it often inhabits intertidal rock pools. The ability to survive submergence under water is due to the existence of an incomplete pallial gill cordon in *H. concolor* resulting in this species being regarded as a true limpet (Christiaens 1973). This increases the distribution range of *H. concolor* allowing it to cover various zones throughout the rocky intertidal zone.

The growth and shell shape of limpets such as *H. concolor* are affected by various physical and biological factors. These include wave action, latitude, food availability, habitat, intra- and inter-specific interactions, the position of limpet on the shore, as well as seasonal temperature fluctuations (Branch 1981, Lisiak 1993, Gray & Hodgson 2003). Other factors such as substrate and epibiosis also influence the growth rate of limpets. To date, there has been one published study conducted on the growth of *Helcion* species in South Africa. Gray & Hodgson (2003) studied the growth rate of *H. pectunculus* from a southeast coast and southwest coast population in South Africa. Nakin *et al* (2012) compared growth and mortality rates within two non-reserve and two reserve areas along the Wild Coast using *Helcion concolor* as one of the investigated species. However to my knowledge, there have been no published studies on the seasonal size and morphological variation

of *H. concolor* thus far. Hodbay (1995) and Tanaka *et al* (2002) state that within the marine intertidal environments, interspecific zonation patterns result from variation in physical conditions and that the same physical conditions causing interspecific zonation give rise to intraspecific zonation patterns. These physical conditions differ from one shore level to the next. In addition during different seasons of the year, the vertical distribution patterns are likely to change due to fluctuation of factors affecting distribution pattern. As a result, this chapter aimed to determine the degree to which seasons influence the shell size and morphology of *H. concolor* in Nqabara rocky shores. The main objective was to compare the size of males to females during each season.

3.2 Materials and Methods

3.2.1 Sampling

Sampling of *H. concolor* was carried out monthly during low spring tide between July 2015 and June 2016. Since this species is threatened and protected in South Africa, only a total of 10 individuals were randomly collected once monthly during low spring tide and transported to the laboratory. Shell length (SL), height (SH) and width (SW) were measured to the closest 0.01 mm using digital Vernier calipers. Each limpet was then weighed, to the nearest 0.001 g, for total body weight (BW). Total body weight therefore included both the somatic mass (SM) and shell weight. To determine the condition index, the somatic tissue was excised out so that the shell weight and somatic tissue were weighed independently.

3.2.2 Data analysis

The shell conicity and base shell ellipticity were then calculated to determine whether *H. concolor* grows allometrically or isometrically, using the following equation:

Shell conicity $=\left[\frac{Shell\ height}{shell\ length}\right]$*100 and Shell ellipticity = $[\frac{shell\ width}{shell\ length}] * 100$

Condition index (CI) was calculated to determine the proportion of somatic mass and shell weight to total body weight. As such, the condition index was calculated as follows using the equation from Paulo Cabral (2007).

$$CI(\%) = \left(\frac{Somatic\ mass}{Shell\ weight}\right) \times 100$$

Data was tested for normality using Bartlett test. Thereafter, t-test was used to compare means of data that was not normally distributed. A parametric one-way ANOVA was then used to compare means of normally distributed data before Tukey test was performed to detect sources of significance between the measured and weighed variables throughout the seasons. Tukey test is one of several multiple comparison tests that can be used to determine which means amongst a set of means differ from the rest as it compares the difference between each pair of means with appropriate adjustment for the multiple testing. In comparison with other multiple comparison tests like both the t-test and ANOVA, it assumes that the data from the different groups come from populations where the observations have a normal distribution and that the standard deviation is the same for each group. For data that was not normally distributed, a nonparametric Kruskal-Wallis test was used. A pairwise t-test was then used to compare the growth rate between

males and females of *H. concolor* for each season. Linear regression analysis was used to measure the relationship between the mean value of one measured/weighed variable and corresponding value of other measured/weighed variables. Regression analysis is a set of statistical processes for used for estimating the relationships among variables. While it incorporates many techniques for modelling and analyzing several variables, its main focus is on the relationship between a dependent variable and one or more independent variables.

3.3 Results

3.3.1 GENDER SIZE COMPARISON

The two sample t-test detected that females were significantly longer than the males in summer (t = -2.3635, df = 28, p = 0.01264), autumn (t = -2.5124, df = 28, p = 0.009014) and in spring (t = -3.3973, df = 28, p = 0.001028) (Figure 2). On the other hand, no significant differences (t = 0.21694, df = 28, p = 0.5851) were detected between males and females in winter.

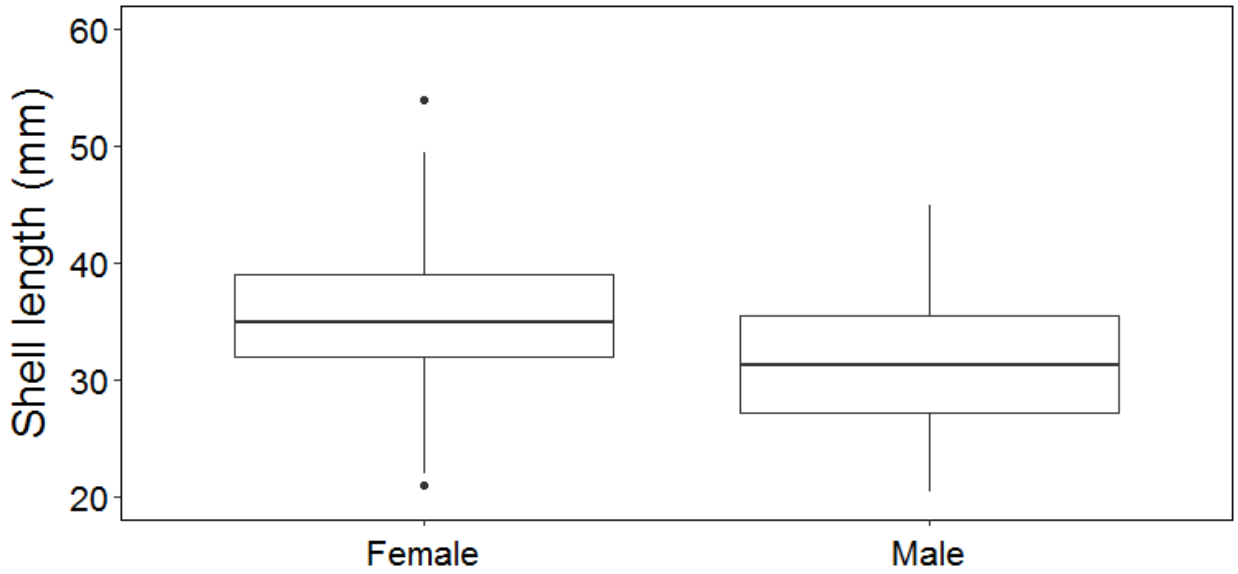

Figure 2: Comparison of shell length between males and females of *H. concolor* from Nqabara rocky shores. The solid lines represent the median while the box represents the distribution range of the 50% of the data and the whiskers reflect the lower and upper quantiles.

There was no statistical difference (t = 2.9258, df = 118, p = 0.9979) between male and female shell widths (Figure 3). The t-test detected significant differences between males and females in summer (t = -2.1559, df = 28, p = 0.01991), autumn (t = -2.2651, df = 28, p = 0.01572) and spring (t = -2.8725, df = 28, p = 0.003841) but no significant difference in winter (t = -0.059865, df = 28, p = 0.4763).

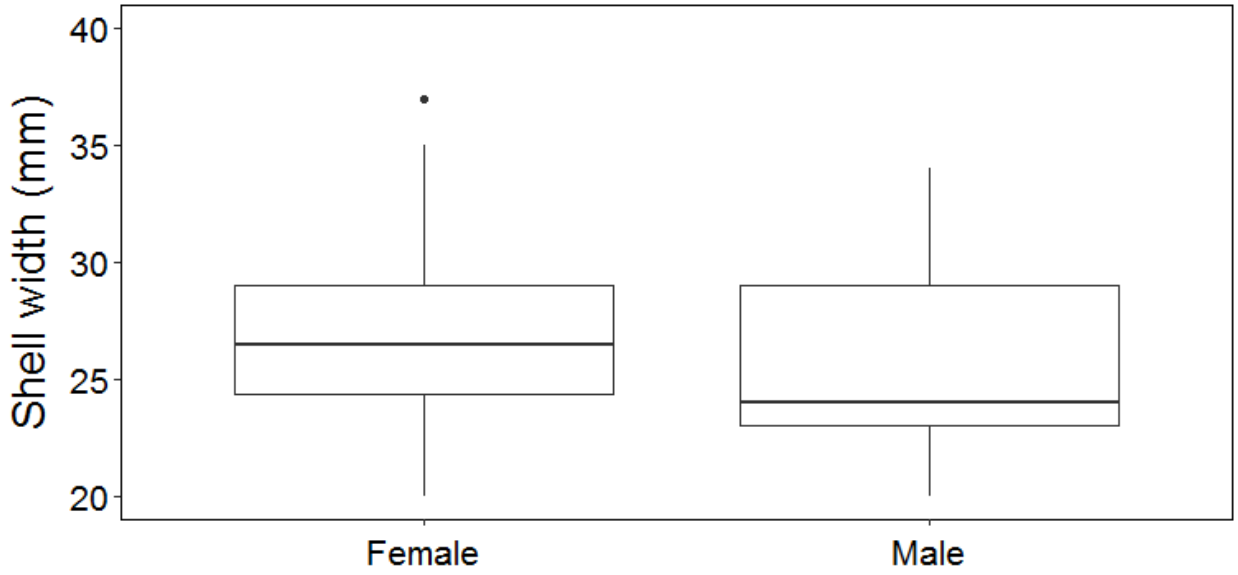

Figure 3: shell width comparison between *H. concolor* males and females irrespective of month-to-month and seasonal variations.

No significant differences (t = 1.6307, df = 118, p = 0.9472) were detected when comparing the height of males and females (Figure 4). A similar trend was detected between the two groups in summer (t = -0.63601, df = 28, p = 0.265) and winter (t = -0.13303, df = 28, p = 0.4476). However, significant differences were detected in autumn (t = -1.9988, df = 28, p = 0.02771) and spring (t = -2.4335, df = 28, p = 0.0108) whereby females were bigger than males.

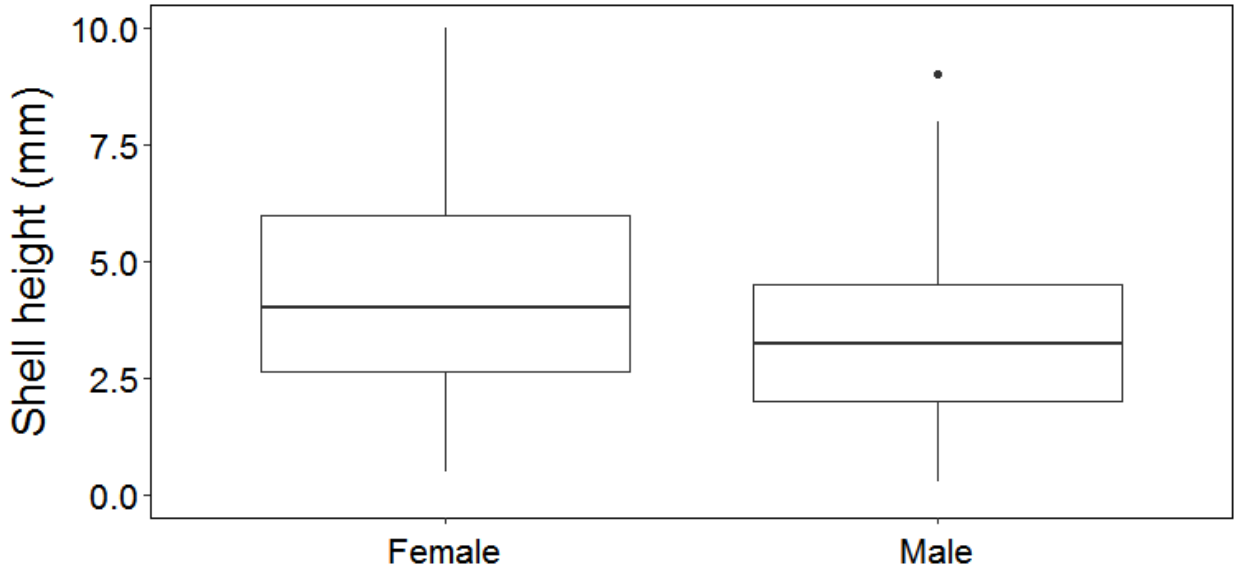

Figure 4: Distribution of shell height measurements for males and females of *H. concolor*.

The two sample t-test detected no significant differences (t = 2.7289, df = 118, p = 0.9963) between the body mass of males and females (Figure 5). The body mass was also not significantly different in summer (t = -1.4919, df = 28, p = 0.07346) and winter (t = -0.068301, df = 28, p = 0.473). However, females were significantly heavier than males in autumn (t = -2.5406, df = 28, p = 0.008448) and spring (t = -2.9181, df = 28, p = 0.003436).

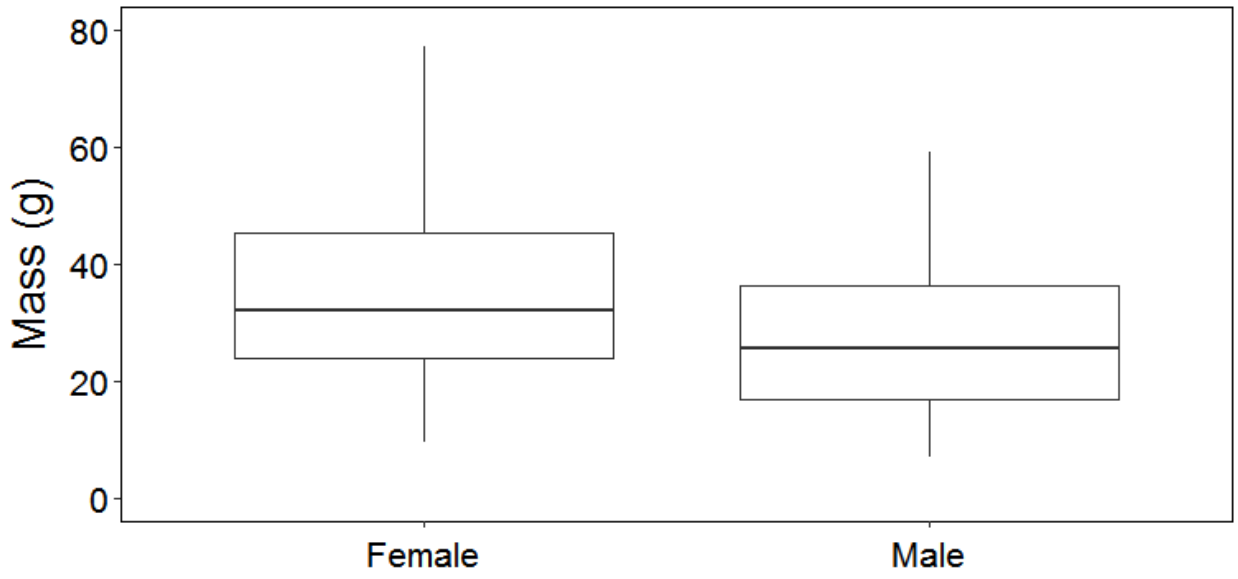

Figure 5: A comparison of body mass between *H. concolor* males and females.

3.3.2 SEASONAL GROWTH VARIATIONS

Daily incremental shell length (Figure 6) was not significantly different between females ($H = 19.249$, $df = 17$, $p = 0.3144$) and males ($H = 15.579$, $df = 17$, $p = 0.5538$). No significant growth rate differences were detected between the two groups (summer: $t = -1.2156$, $df = 13$, $p = 0.1229$; autumn: $t = 0.5333$, $df = 13$, $p = 0.6986$; winter: $t = 0.53479$, $df = 12$, $p = 0.6987$ and spring: $t = -0.76209$, $df = 12$, $p = 0.2304$).

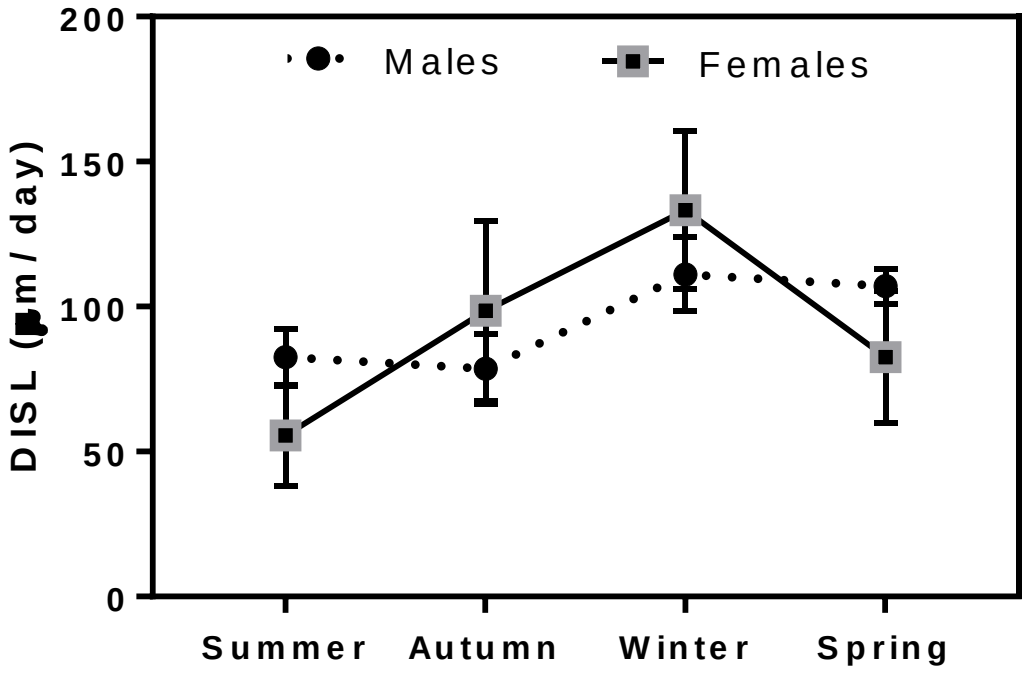

Figure 6: Mean daily incremental shell length (DISL) of both male and female *H. concolor* individuals throughout the seasons. The vertical bars represent the standard error of the mean.

Kruskal tests detected no seasonal differences for females (H = 4.3087, df = 10, p = 0.9323) and males (H = 14.014, df = 14, p = 0.4486) in limpet shell width (Figure 7). T-test analysis revealed that there were no significant differences between males and females in spring (t = 0.42064, df = 16, p = 0.6602), summer (t = -0.30134, df = 17, p = 0.3834), and winter (t = -1.5492, df = 16, p = 0.07044). However, females gained more width (t = -1.9297, df = 17, p = 0.03525) than the males in autumn.

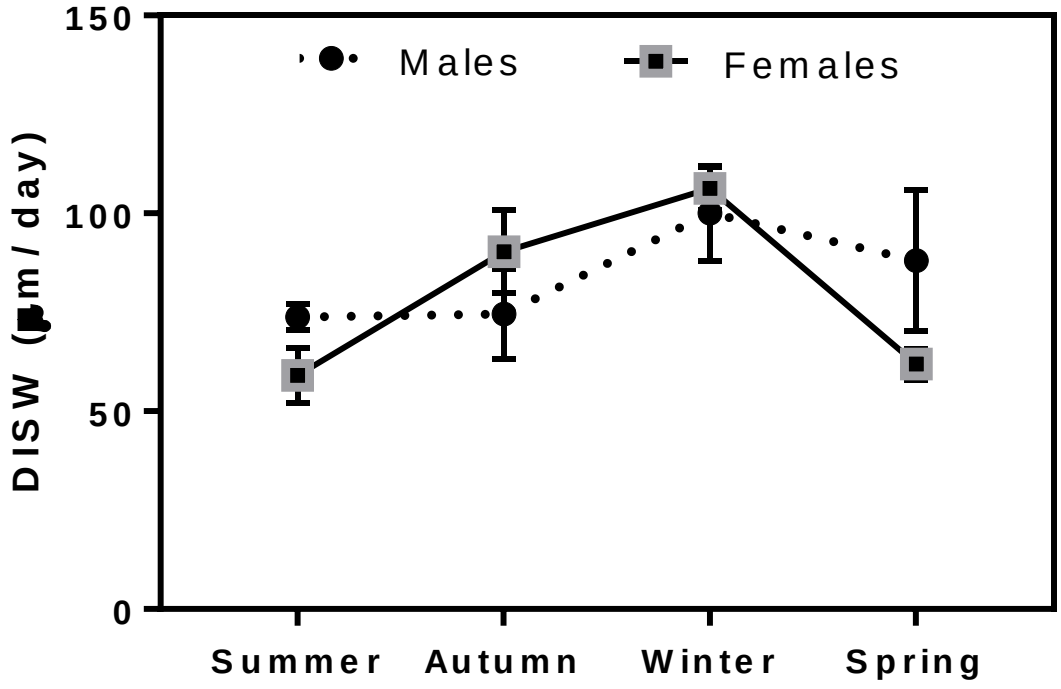

Figure 7: Daily incremental shell width (DISW) across seasons.

There was no significant height gain differences between seasons for both males (H = 6.4593, df = 8, p = 0.5959) and females (H = 2.6741, df = 6, p = 0.8485) (Figure 8). As a result, gender comparison detected that both males and females gained the same height in summer (t = 0.075263, df = 13, p = 0.5294), autumn (t = 1.8966, df = 13, p = 0.9598), winter (t = -0.28098, df = 12, p = 0.3918) and spring (t = -0.58095, df = 12, p = 0.286).

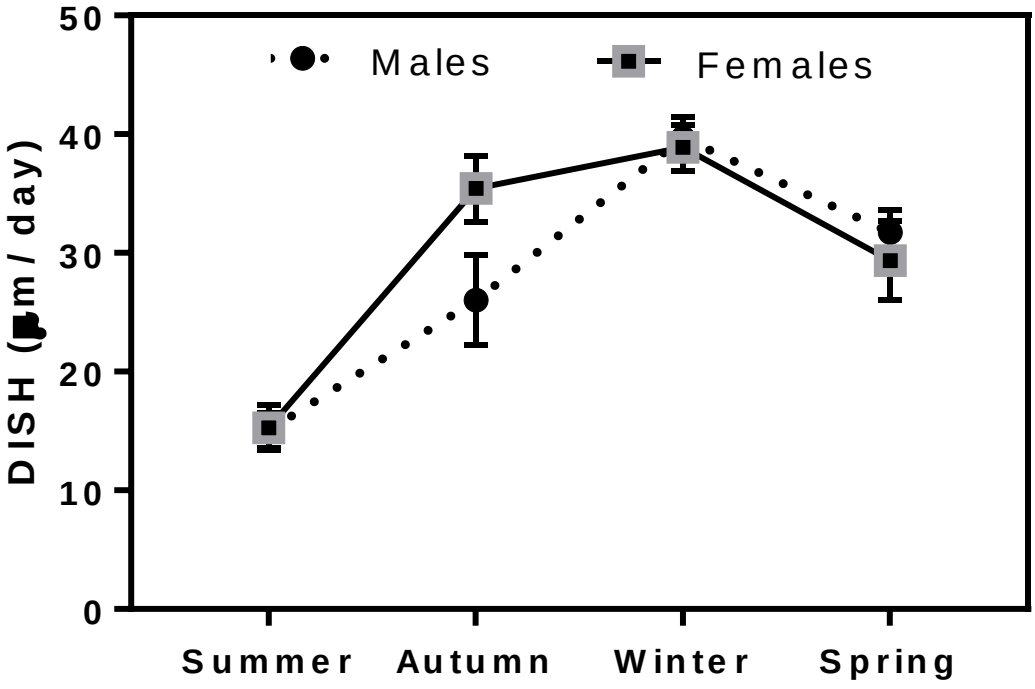

Figure 8: Seasonal variations in daily incremental shell height (DISH) of *H. concolor* throughout all seasons.

The female shells were slightly more conical (12 ± 0.64 %) than the males (11 ± 0.66 %). Female shells were also more elliptical (74 ± 0.69 %) than males (73 ± 0.61 %). The effects of shell length on the shell height was similar for both small males and females (Figure 9), and there were no significant differences ($F = 1.736$, $df = 116$, $p = 0.1903$) in the slopes of males and females. The intercepts of males and females were also not significantly different ($F = 1.339$, $df = 117$, $p = 0.2495$). Regression analysis detected that there was a significant influence of shell length on shell height of both males ($R^2 = 0.72$, $F = 147.3$, $df = 56$, $p < 0.0001$) and females ($R^2 = 0.48$, $F = 54.58$, $df = 60$, $p < 0.0001$).

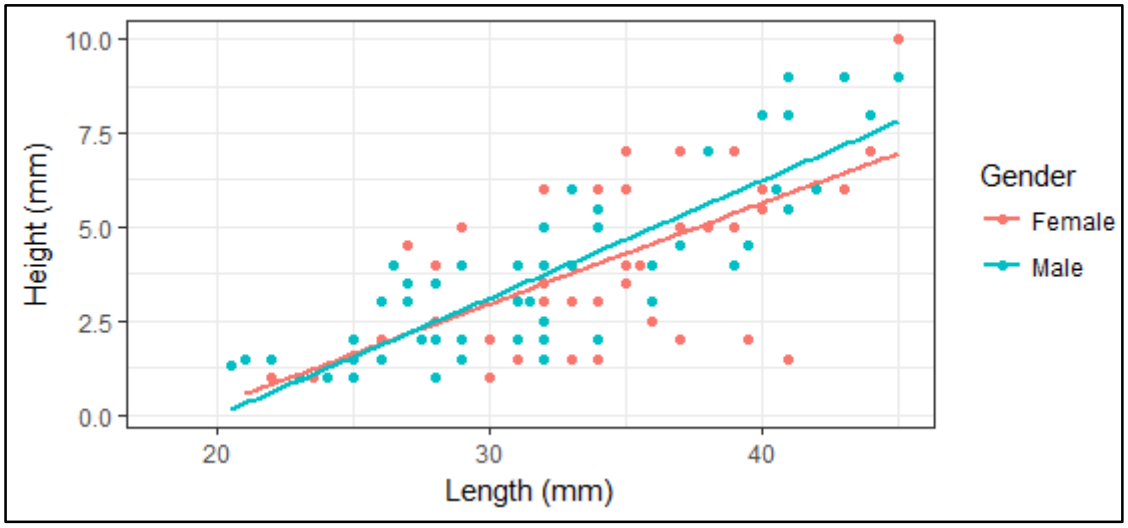

Figure 9: The influence of shell length on the shell height of both male and female *H. concolor* individuals throughout the study period.

There was a positive influence of shell length on the shell width of both male and female *H. concolor* individuals (Figure 10). Regression analysis revealed that shell width significantly influenced the shell length of males (R^2 = 0.94, F = 886.3, df = 56, p < 0.0001) and females (R^2 = 0.83, F = 303.2, df = 60, p < 0.0001). As a result, slope analysis revealed that there were no significant differences (F = 0.9511, df = 116, p = 0.3315) between the slopes of males and females. No significant differences were also detected between the intercepts of males and female (F = 0.02166, df = 117, p = 0.8832).

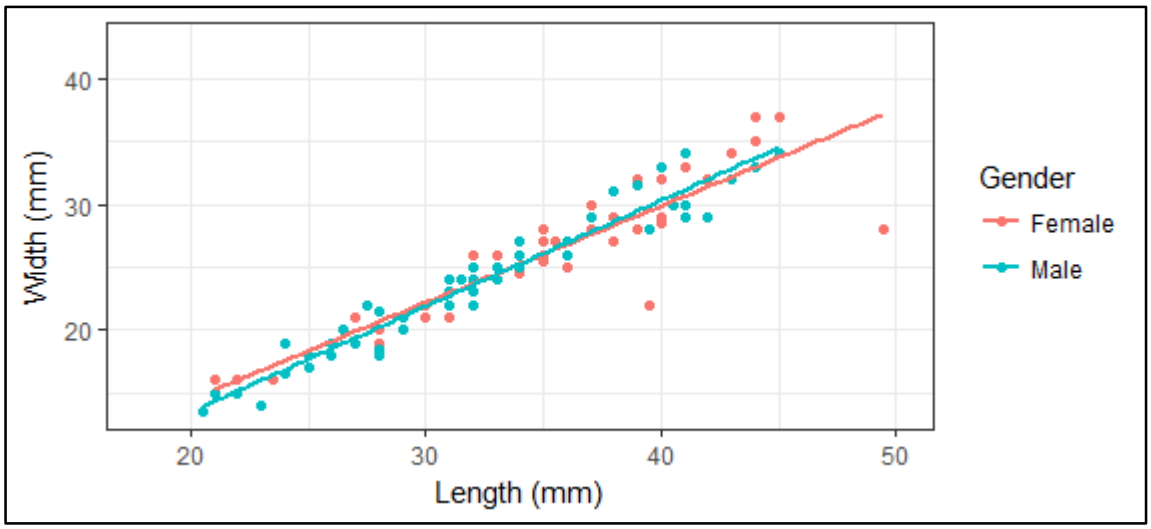

Figure 10: Relationship between shell length and shell width of male and female *H. concolor* individuals.

Kruskal-Wallis test detected no significant differences between seasons for male (H = 57, df = 57, p = 0.4751) and female (H = 61, df = 61, p = 0.4759) condition index (Figure 11). When comparing male-to-female condition index, the t-test detected no significant differences in summer (t = 1.3865, df = 28, p = 0.9117), autumn (t = -1.0873, df = 28, p = 0.1431), winter (t = 0.804, df = 28, p = 0.7859) and spring (t = 1.8373, df = 28, p = 0.9616).

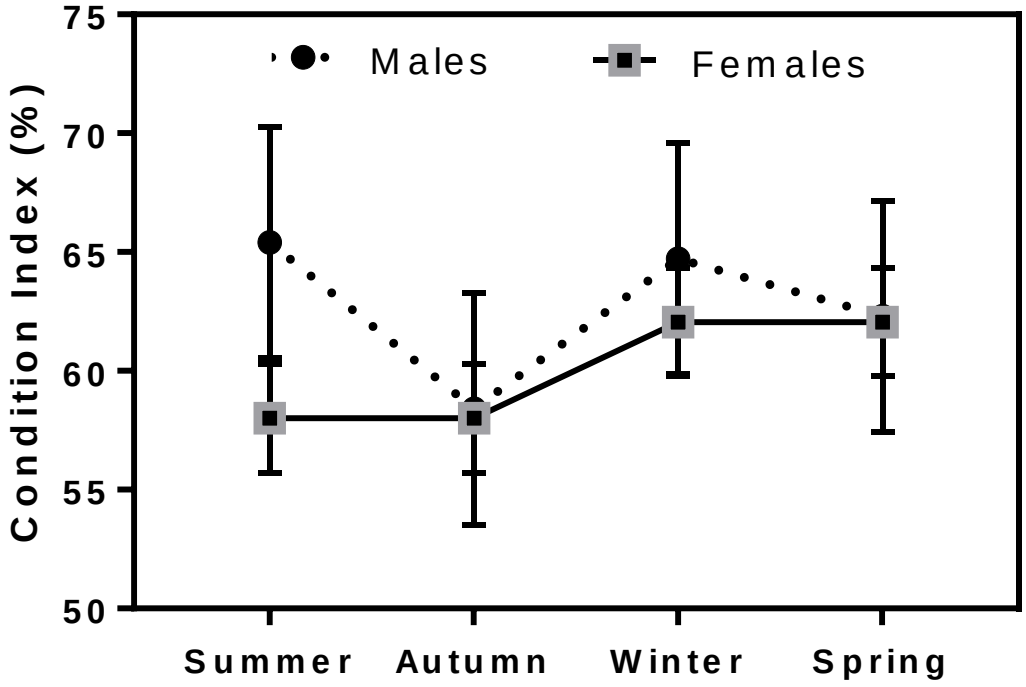

Figure 11: Seasonal variations in the condition index of both male and female *H. concolor* individuals.

3.4 Discussion

Since South African sea surface temperatures (SST) are lower in winter (Gray & Hodgson 2003, Dufois & Rouault 2012), the high growth rate in winter recorded in this study may be attributed to lower water temperatures during this season. This was similar to a study by Kenny (1983) who found the related *Cellana conciliata* Iredale 1940 and *C. tramoserica* Holten 1802 growing faster in low temperatures compared to warm water. McGrath (1992) also recorded a pattern similar to the current study whereby the highest growth for *Helcion pellucidum* was recorded during the winter season (December to January). While comparing reserve and non-reserve, Nakin & McQuaid (2014) also recorded the growth rate of *H. concolor* to be high in winter. This might therefore mean that less energy was utilized for regulating temperature.

Habitat gradients and recruitment densities also strongly influence size distribution (Thompson 1980, Hodbay 1995). Janson (1982) stated that exposure to stronger wave action plays a major role in reducing the effect of turbulence and increasing the tenacity of an organism. Since *H. concolor* is a common mid shore rock pool limpet, it is less exposed to strong waves and spends less energy on resisting turbulence and increasing tenacity during windy seasons. In addition to this, even though food production is affected by seasons, rock pools have a diversity of food. Thompson (1980) stated the availability of suitable microhabitat for limpet growth and survival depends both on the population dynamics of the organisms and the rates of physical disturbances on the population. Therefore, it might be possible that this species consumes more than one

food source and the competition for food within or between other species is of little importance. The results also exhibited that the height of *H. concolor* was more elevated with no significant differences throughout the seasons (Figure 4). The winter season has violent winds which can cause more wave action. In a number of studies, shell height to length ratios have been found to correlate with tidal levels (Vermej 1973, Branch 1981). Wave action is an important factor that determines allometry (Paulo Cabral 2007). This was consistent to Vermeij (1980) who reported that many marine gastropods exhibit seasonal variation in growth rates both within and between species. The rapid growth rate in limpets coincides with early maturation, high mortality rate and a short lifespan (Branch 1981, Gray 1996).

The larger and bigger body size shown by *H. concolor* females suggested that this species may be a protandric hermaphrodite since protandric limpet species change sex from male to female as they grow older. This was in agreement with Dodd (1956) who reported protandric hermaphroditism for the limpet *Patella vulgata* L. 1758. Later Branch (1974) reported that the limpet *Scutellastra longicosta* Lamarck 1819 is a protandric species. Gray and Hodgson (2003) also found that the larger size of females *H. pectunculus* was not a result of rapid growth rates in females but was rather due to female individuals being older than the males.

The difference in growth rate across seasons may also be due to differences in food abundance since Valiela *et al* (1992), Morand & Merceron (2005), Fox *et al* (2008) and Teichberg *et al* (2010) highlighted that nutrient loading into coastal waters results in increased algal abundance. Although Olsen *et al* (1986), Elser *et al* (1988) and Kahlert (2002) assumed that nutrients are constantly supplied to the water column, Emanuel *et al* (1992) and Wheeler & Björnsäter (1992) pointed out that nutrients are frequently supplied in pulses with sudden influxes of nutrients. This results in high nutrient loading due to high summer rainfalls leading to nutrient loading that enhanced algal growth in autumn and subsequently resulting in high winter growth rates for the herbivorous *H. concolor*.

Morphological features such as shell shape and texture of limpets are of importance and have often been found to correlate with physical conditions (Branch 1985). Dehydration and tenacity are major challenges that face limpet species and isometric growing limpet species are more prone to dehydration than dislodgement. This is because flattened shells are less prone to forces exerted by water (Denny 2000). Generally, gastropods exhibit two intra specific size distribution patterns, in which there is an increase in size for species inhabiting the high shore and a decrease in size for species living in the lower intertidal (Vermeij 1973). In the higher shore, species occur at low densities with less completion and this favours the occurrence of larger individuals. High shore species undergo active migration (Hobday 1995) and differential growth rate patterns (Creese 1980).

The mean monthly percentages of shell ellipticty were greater than that of shell conicity. Meaning that in terms of shape *H. concolor* is a broader flattened species. The shell weight, total body weight and somatic weight all indicated a clear pattern of mean monthly variation. On top of that, the monthly pie charts displayed that the shell weight proportion was always greater than the somatic mass even though the proportions varied across the months.

The regression results revealed that shell width was more affected by the shell length than shell height was (Figure 10). This was due to limpets generally needing to be flatter and broader in order to maximize surface area for adhesion while reducing wave drag (Denny 1988). As such, *H. concolor* fits in the asymmetrical group of limpets in Denny (2000) that have a stronger relationship between shell length and width than shell length and shell height. This group of limpets is sometimes regarded as growing isometrically, where shell length and width increases more rapidly than shell height. According to Branch (1981), limpets that undergo this growth inhabit the high shore and lower latitude and often possess textured shells. *Helcion concolor* fits this characteristic since it also has fine ribs running outward from the apex of the shell. Vermeij (1973) had suggested that these adaptations may help reduce water loss and improve thermoregulation. This was due to these species having water loss that is proportional to shell volume (Branch 1981).

This flattened morphology also contributes towards *H. concolor* being abundant in rock pools and is therefore less challenged by dehydration due to thermal stress. This is because species on emergent rocks tend to have taller and narrow shells to avoid thermal stress from the hot rocks, whereas in the rock pools limpets can be flatter and broadened because the rock is cooler (Denny 2000).

The condition index was low in autumn and high in winter and spring although not significant. Seasonal variations in the condition of a limpet may reflect variations in food abundance and the reproductive stage. According to King (2007), a greater wet weight of a limpet, results in a greater condition index and vice-versa. In our study, the condition factor data was not significant. The *H. concolor* gonads were in the mature stage during the winter and spring seasons which may have resulted in the higher condition index because of the increase in gonad tissue. According to numerous studies, limpets are seasonal breeders with a cycle of gamete production and release (Branch 1981, Hodgson 1991, Chambers & McQuaid 1994, Vat 2000, Henninger & Hodgson 2001, Pal & Hodgson 2004). The increase in body size in limpets has been found to correlate with an increase in fecundity (Creese 1980, Branch 1981).

CHAPTER 4: HISTOLOGICAL STUDIES OF SEASONAL VARIATIONS ON GAMETES OF *H. CONCOLOR*

4.1 Introduction

Reproductive modes found in limpets differ from species to species. Limpets under the family Siphonariidae are all hermaphroditic with internal fertilization and in almost all cases the fertilized eggs are laid on the rocky shore substrate (Hodgson 1999, Pal & Hodgson 2004). Limpets within the family Fissurellidae have separate sexes even though the processes of reproduction vary from species to species (Ward 1966, Gonzalez 1999, Perez 2007). Some species are broadcast spawners, other species produce benthic egg masses whereas some species brood their young. However, the Patellidae limpets are dioecious broadcast spawners that release eggs and sperms outside into the water column for external fertilization (Branch 1981, Henninger & Hodgson 2001, McCarthy *et al* 2008 and Ribiero 2009).

Typical of many other South African molluscs, limpets are also seasonal breeders and have an annual reproductive cycle with definite spawning periods (Branch 1974, Gray 1996, Vat 2000, Henninger & Hodgson 2001, Gray & Hodgson 2003, Pal & Hodgson 2004). As such, the differing geographic locations in South Africa influences the timing of gamete formation and spawning in limpets (Branch 1981, Vat 2000, Gray & Hodgson 2003). Cool temperate species of the West Coast tend to have a single spawning period

in winter (Branch 1974) and warm temperate species of the East and South East Coast either have a biannual (summer and autumn) or a prolonged spawning period (Lasiak 1990, Gray 1996, Vat 2000).

As limpets grow in body size their fecundity also increases (Greese 1978 NIR, Branch 1981). The rocky shores of the West Coast of South Africa have high primary productivity compared to east and southeast coast (Bustamante *et al* 1995, Vat 2000). The growth and spawning of limpets is affected by various physical and biological factors such as the action of waves, latitude, the availability of food in a habitat, intra- and inter-specific interactions, seasonal temperature fluctuations, and the position of limpet on the shore (Branch 1981, Lisiak 1993, Foster & Hodgson 1995).

Conventionally, histological analysis of gonads is the best way of determining the sex and gamete development in limpets. Many histology studies have been done on limpets (Nui & Fuji 1989, Morriconi 1999, Vat 2000, Henninger & Hodgson 2001, Rocha-Barreira 2002, Gray & Hodgson 2003, Pal & Hodgson 2004, McCarthy *et al* 2008, Prusina *et al* 2014). Only two studies on the reproduction histology of *Helcion* genus have been done in South Africa. Henninger & Hodgson (2001) conducted a comparison study on the reproductive seasonality two *H. pruinnosus* populations from the south-east coast and south-west coast regions of South Africa. Gray & Hodgson (2003) compared the annual reproductive cycle of *H. pectunculus* populations from a cool and warm temperate environment and

also looked into geographic differences in the growth rate. To date there have been no published studies on the reproductive histology of *H. concolor*. The aim of this study was subsequently to determine seasonal variation in the gonadal structures and spawning season for *H. concolor* along the Wild Coast of South Africa. Studying reproductive biology is essential, especially for marine exploited species. The classification of sexual patterns are fundamental for the implementation of new conservation strategies since some limpet species are often prone to size-selective poaching. Therefore, in order to come up with the correct management strategies for any species certain aspects of reproductive biology must be considered.

4.2 Materials and Methods

4.2.1 Sampling

Since there is a maximum bag limit for this species, 10 individuals were randomly collected once monthly during low spring tide, preserved in 10 % formalin and prior to transportation to the Zoology laboratory of Walter Sisulu University. The somatic weight (SM), visceral mass (VM) & gonadal tissue (GM) were excised out of the shell & weighed to the nearest 0.001 g.

4.2.2 Histology

The gonads were fixed in Davidson's fixative for 48 hours, washed in tap water and kept in 70 % ethyl alcohol. After following dehydration and clearing, the gonads were embedded in Paraplast using the Thermo Scientific embedding machine, sectioned (7 µm) using Leica microtone and counterstained with haematoxylin-eosin. The sex and the gonadal stages were identified based on macroscopic characteristics which include colour, transparency, texture and enlargement based on studies done by (Orton *et al* 1956; Branch 1974; Niu & Fuji 1989). Female gonad developmental stages were based on the presence and frequency of different oocytes stages within the gonads throughout the months. Male gonad developmental stages were based on the presence or absence of gametogenic cells within the gonads throughout the months.

4.2.3 Data analysis

The Data were presented as means and standard error of means. The gonadal somatic index (GSI) of *H. concolor* was calculated for males and females (Morriconi 1999, Henninger & Hodgson 2001) and to assess the reproductive status of the invertebrate relative to its total mass. Based on Gray's (1996) findings there are no significant differences between preserved and unpreserved limpets which makes it possible to compare the gonad indices of the animal.

$$GSI = \left(\frac{gonadmass}{totalbodymass}\right) \times 100$$

Two-sample t-test was used to compare males and females. Seasonal differences were tested for males and females using the parametric single factor analysis of variance. To detect the source of variance, Tukey HSD multiple comparison test was used. Predictive analysis were then performed using multiple regression analysis.

4.3 Results

4.3.1 Sex ratio

The ratio of males to females fluctuated on a monthly basis as such there were more male for five months, more females for four months and equal numbers for three months (Figure 12). As such, males were more than females in March; June; July and September. Contrary, females were more than males in February; May; August and October. Similar numbers of males and females were only recorded in April; November and December. Overall, there were more females (51.67 ± 4.41) than males (48.33 ± 4.41) for this study although the Two Sample t-test detected no significant differences ($t = -0.53452$, $df = 22$, $p = 0.2992$) between the number of males and females. The Pearson's product-moment correlation also revealed a significantly negative correlation ($r = -1$, $p < 0.0001$) between males and females over the different months. ANOVA revealed no significant influence of seasons on the number of both males ($F = 0.035$, $p = 0.991$) and females ($F = 0.035$, $p = 0.991$).

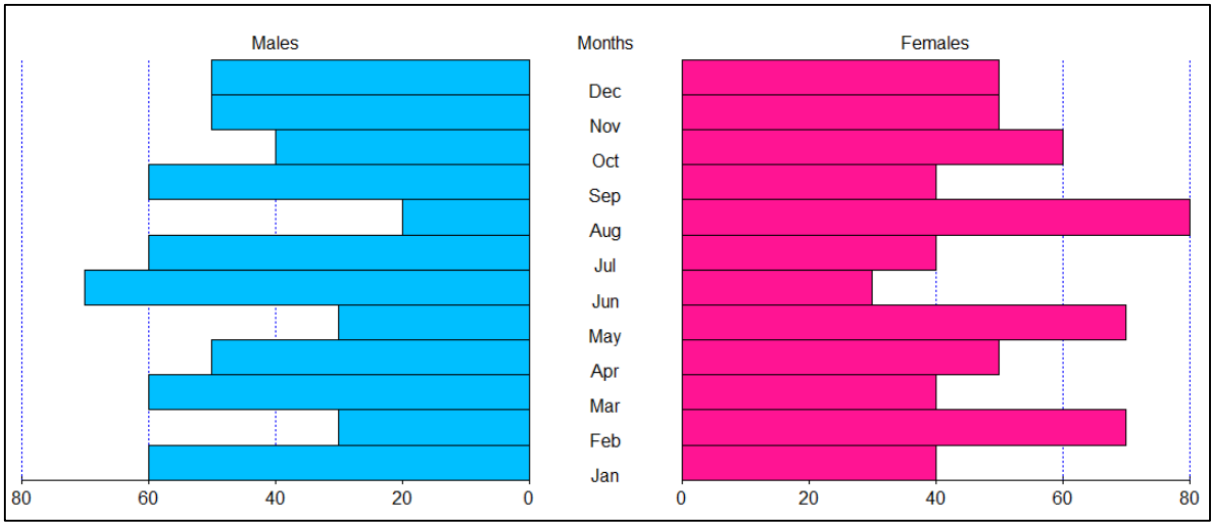

Figure 12. *H. concolor* male – to – female ratio (%) during the different sampling months of this study.

4.3.2 Female gonad characteristics and developmental stages

In female gonads of *Helcion concolor*, five layers of germ cells or oocytes were identified within the photomicrographs (A) in (Figure 13). Oogonia (OG) were identified as the smallest stage in size in all ovarian sections. They were attached to the tubular wall (TW) and contained a nucleus with a single heavily stained nucleolus. Previtellogenic oocytes (PO) were the second stage. They had a pear shape, with a nucleus larger than that of oogonia. The nucleus contained one or two intensely stained nucleoli. Vitellogenic oocytes (VO) were recognised as the third stage. In this stage the oocytes were losing the pear shape and appeared a little oval, having a single nucleolus inside the nucleus. The nucleolus was seen located closely to the nuclear membrane. The mature oocytes (MO), which formed stage four, were oval shaped and had no visible nucleolus. However, the nuclei were present in some cells and in others it could not be clearly seen. The last stage for females were the atresic oocytes (AO) which were characterised by an abnormal

shape and the cells had completely lost the nuclei. For the early active stage (**A**), there were many OG attached to the tubular wall. Previtellogenic oocytes varied in sizes and a few VOs were visible. The germ cells were spaced and the connective tissue was in abundance. During the late active stage (**B**), the OG were scarce, VOs increased in number while POs were in the inter-oocyte space and had started decreasing. During the ripe stage (**C**), the ovary contained mainly VOs and MOs while POs were very few. Some MOs were already showing signs of atresia at this stage. During the atresic stage (**D**), the MOs became tightly packed and atresia increased drastically while only a few VOs were present. , Although the MO were still dominant during the early spawning stage (**E**), the MOs and AOs started separating from each other and the early stages of germ cells, i.e. OG and POs, started appearing in between the MOs. During the partial spent stage (**F**), few degenerative oocytes (DO) and MO were present. There were many OG attaching to the tubular wall and the connective tissue was present around the inter-oocyte space.

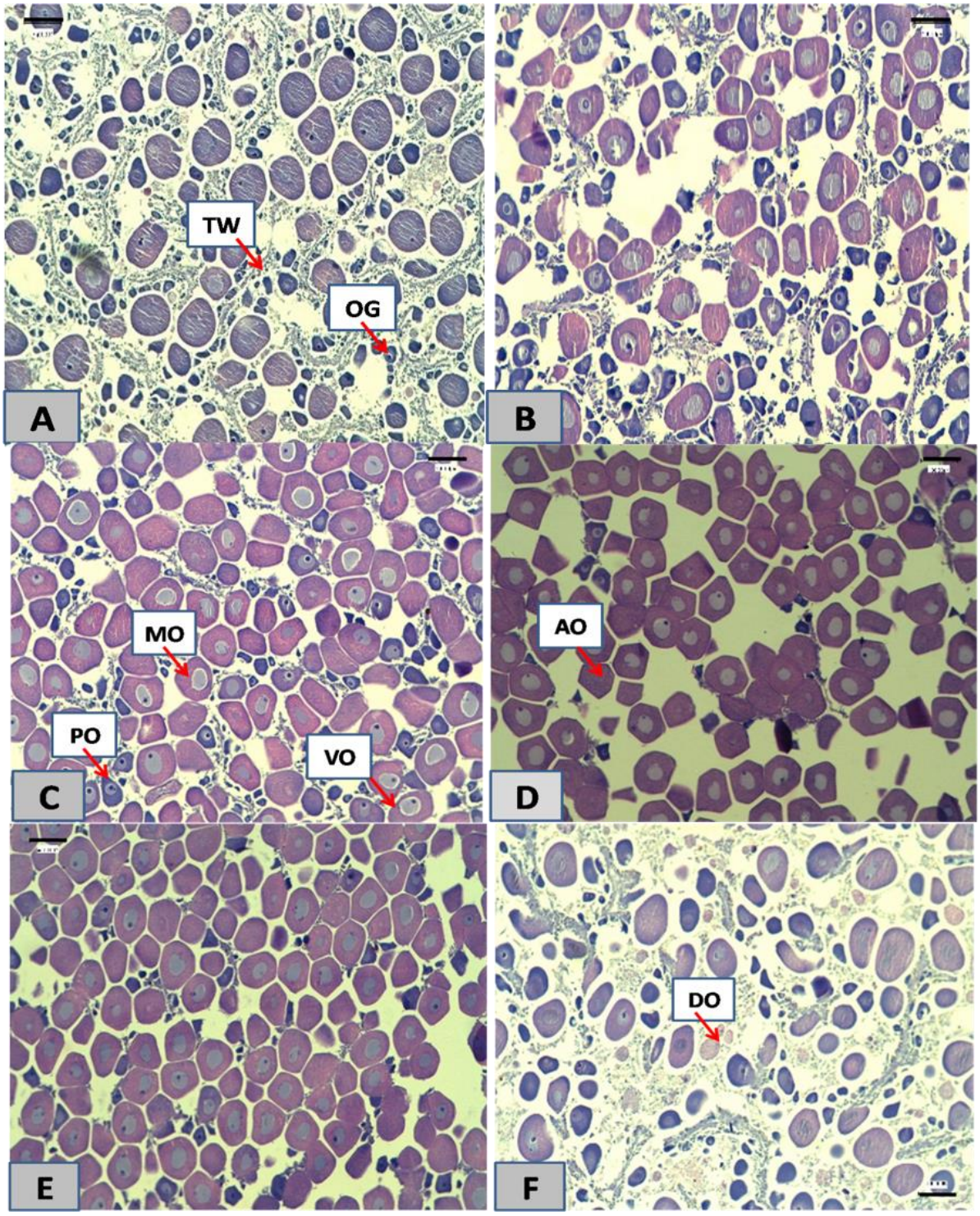

Figure 13: Female gonad development stages of *Helcion concolor*: **A -** Early active stage; **B -** Late active stage; **C -** Ripe stage; **D -** Atresic stage; **E -** Early spawning stage; **F -** Partial spent stage. OG = oogonia; PO = previtellogenic oocyte; VO = vitellogenic oocyte; MO = mature oocyte; AO = atresic oocyte; DO = degenerative oocyte; TW = tubular wall. Scale bar = 100 µm.

4.3.3 Male gonad characteristics and developmental stages

In males, four gonadal stages were identified (Figure 14). The first stage was Spermatogonia (SPG) which were found attached to the tubular wall (TW). Inside each SPG there was a nucleus with one or two heavily stained nucleoli. The SPG were the larger male germ cells. Spermatocytes (SPC) were the second layer of germ cells resulting from the mitotic division of SPG. They were round purple dark dots due to haematoxylin staining. The SPC were also grouped together and intermingled with spermatids (SPDs). The SPDs thus constituted the third stage and were derived from the meiotic division of the SPC. They were spherical and had a reddish colour. The last stage was made up of Spermatozoa (SPZ). Haematoxylin staining resulted in the SPZ having heavily stained triangular heads and were located in the tubular lumen containing pink tails due to eiosin staining. As a result, the lumen had a pink colour. Spermatozoa were the result of spermatid metamorphosis. During the early active stage (**A**), layers of SPG were found lining the TW and the sperm were very scarce. During the late active stage (**B**), different phases of germ cells in this stage could be seen as the SPG, SPC and SPD formed a pattern from the tubular wall to the lumen. Sperms started increasing in number. In the ripe stage (**C**), sperms were the main germ cells. The SPZ filled the lumen and the pink colour of the SPZ tails became more visible. During the spawning stage (D), the SPZ decreased drastically even though few residual spermatozoa (RS) remained within the tubules. The gonad tissue had spaces and the connective tissue was apparent while new walls for the formation of new germ cells could be seen.

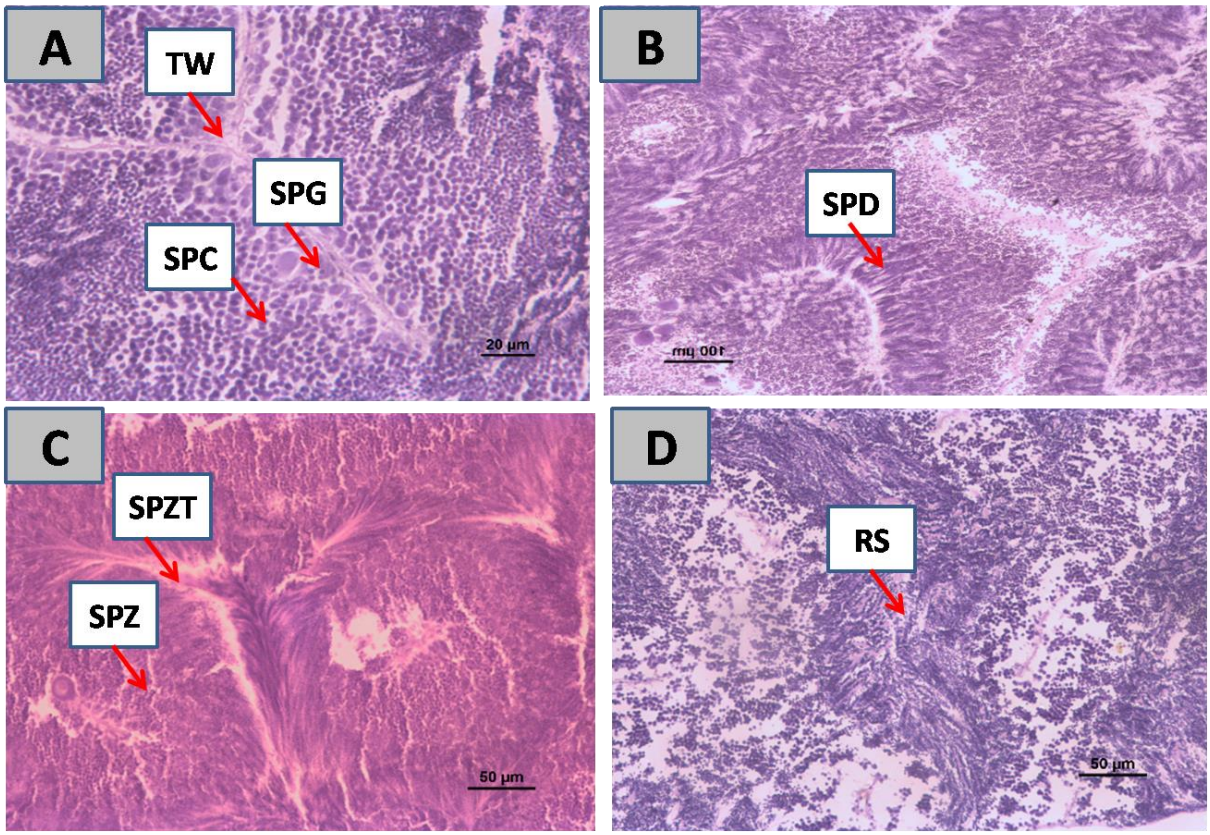

Figure 14: Male gonad development stages of *Helcion concolor*: **A =** Early active stage; **B =**Late active stage; **C =** Ripe stage; **D =** Spawning stage. SPG = spermatogonia; SPC = spermatocytes; SPD = spermatids, SPZ = spermatozoa; SPZT = spermatozoa tails, RS = residual spermatozoa. Scale bar: 20-100 µm.

4.3.4 Abundance of germ cell stages

Female mature oocytes were the dominant stage through all seasons (Figure 15), but were found in more abundance during winter and autumn. The atresic stage was only dominant in autumn and no inequal proportions during the other seasons. Oogonia were abundant in summer and least in winter. Previtellogenic oocytes were absent in autumn and highest in spring. Vitellogenic oocytes increased from summer towards winter and decreased from winter towards summer. For males, spermatids increase from winter to summer and decrease in autumn. The same pattern was recorded for the spermatocytes with highest abundance in summer and lowest levels in winter. The spermatozoa were more abundant in autumn and spring while being found in less abundance during summer and winter. Spermatozoa increased from summer to winter and decreased during spring.

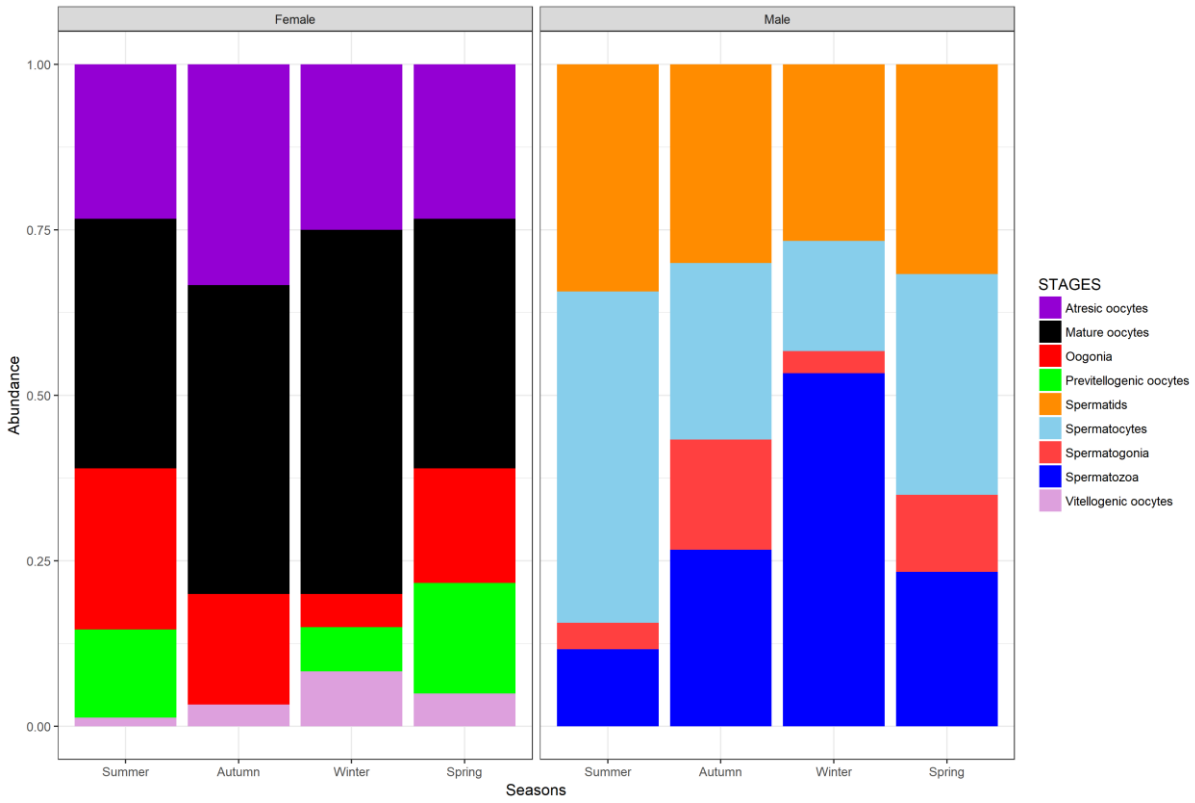

Figure 15: stacked bar graph highlighting the abundance of germ cell stages for both males and females across all seasons.

T test revealed that there was no significant difference between the GSI of males and females (p = 0.13) (Figure 16).

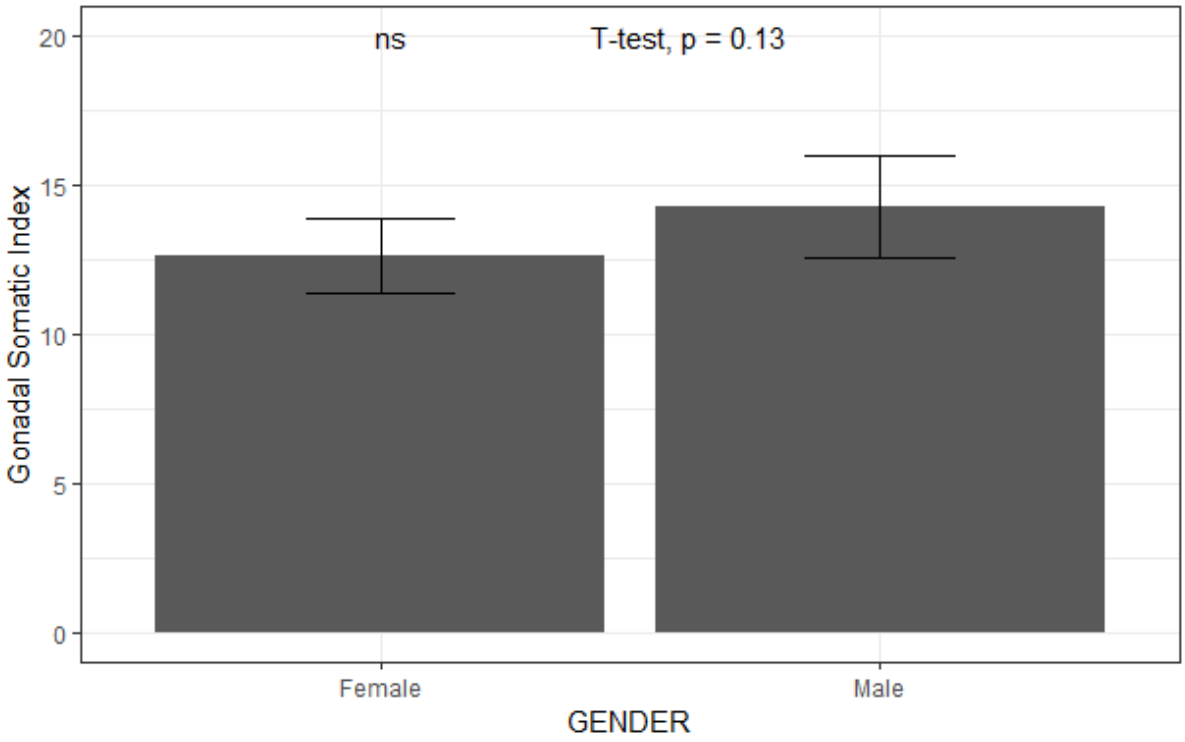

Figure 16: graph comparison of the GSI between males and females of *H. concolor*.

4.3.5 Variations in GSI

The two-sample t-test detected no significant differences (t = -1.3105, df = 118, p = 0.09629) between the mean GSI of the two genders (Figure 17). When overall seasonal analyses were done, seasonal changes significantly influenced GSI (F = 6.675, df = 3, p<0.0001) resulting in higher GSI during winter. However, ANOVA detected no significant difference (F = 1.449, df = 3, p = 0.238) in female GSI across the seasons. For males, ANOVA results showed that there were significant GSI differences between the seasons (F = 12.91, df = 3, p < 0.0001) with Tukey HSD detecting winter GSI to be significantly higher (p < 0.05) than the other seasons.

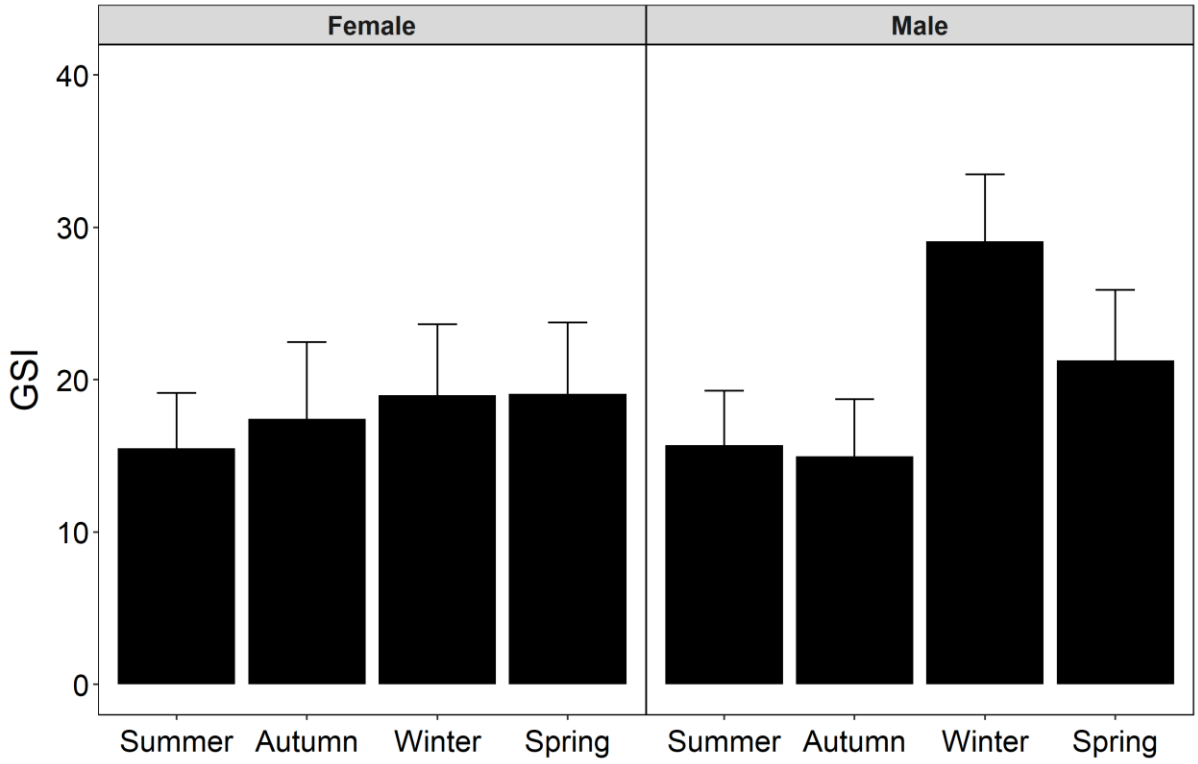

Figure 17: Variation in gonadal somatic index of both male and female *H. concolor* individuals through different seasons

Multiple regression analysis reflected that shell length was useful in predicting GSI in *H. concolor* (R^2 = 0.2014, F = 29.76, df = 119, p < 0.0001). However, shell length was more effective in males (R^2 = 0.4026, F = 37.74, df = 57, p < 0.0001) compared to females (R^2 = 0.1406, F = 9.9816, df = 61, p = 0.0027). Seasonal analysis (Figure 18) revealed that only autumn shell length could be used in predicting GSI (R^2 =0.4053, F =19.09, df = 29, p < 0.0001) compared to winter (R^2 = 0.1045, F = 3.268, df = 29, p = 0.0814), spring (R^2 =0.01915, F = 0.5466, df = 29, p = 0.4659) and summer (R^2 =0.01879, F =0.5362, df = 29, p = 0.4701).

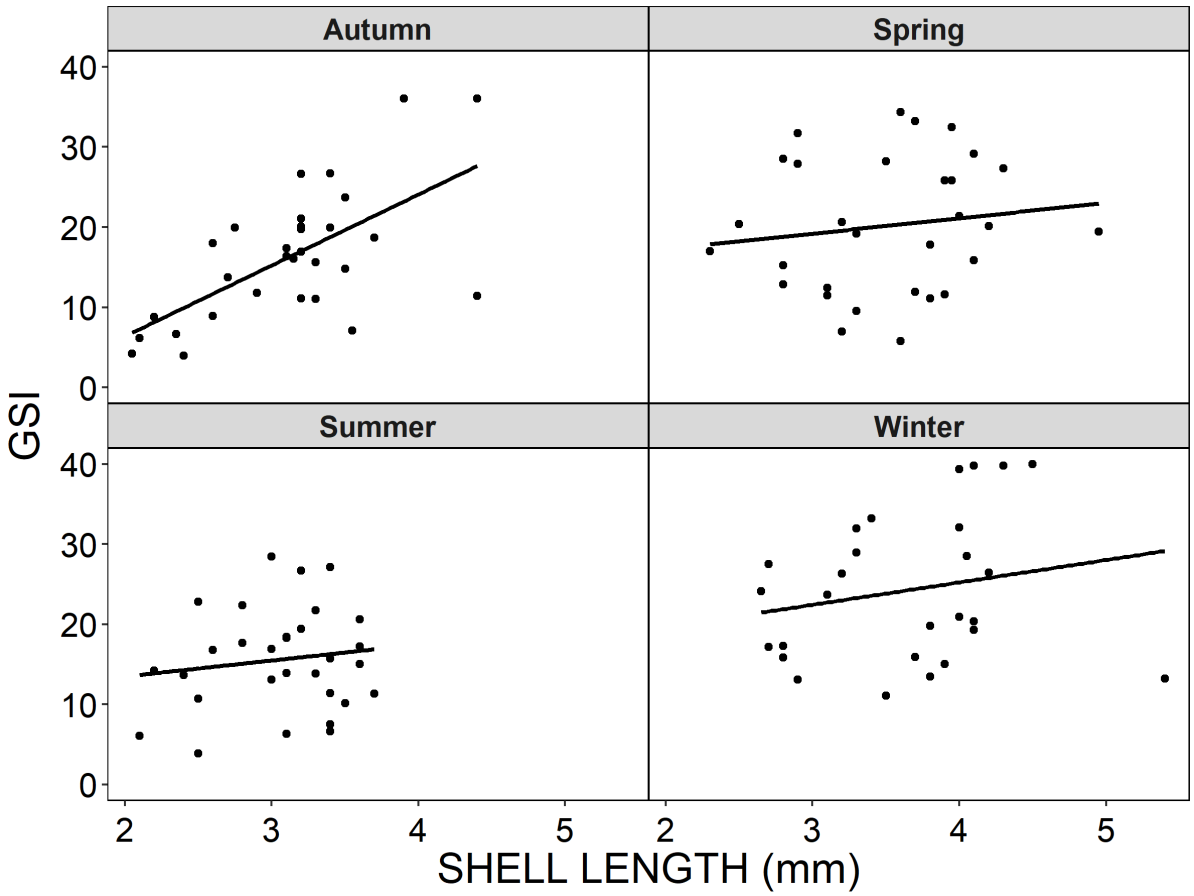

Figure 18: Seasonal variation in GSI with regards to shell length of *H. concolor*.

Linear regression analysis proved that shell width can be used to determine GSI (R^2 = 0.1714, F = 24.4, df =119, p < 0.0001). The width of both males (R^2 = 0.3972, F= 36.9, df = 57, p < 0.0001) and females (R^2 = 0.3972, F = 36.9, df = 57, p < 0.0001) can be equally used to predict the GSI. Winter (R^2=0.1018, F= 3.173, df = 29, p = 0.08574). Spring (R^2 = 0.007585, F = 0.214, df = 29, p = 0.6472). Summer (R^2 = 0.005771, F = 0.1625, df = 29, p = 0.6899). Autumn (R^2 = 0.3298, F = 13.78, df = 29, p = 0.0009055).

Male nucleus size showed positive correlation with both the shell length and shell height (Figure 19). The linear regression analysis revealed significant differences for length (R^2 = 0.9188, F = 373.4, df = 34, p < 0.0001) and width (R^2 = 0.8166, F = 147, df = 34, p < 0.0001).

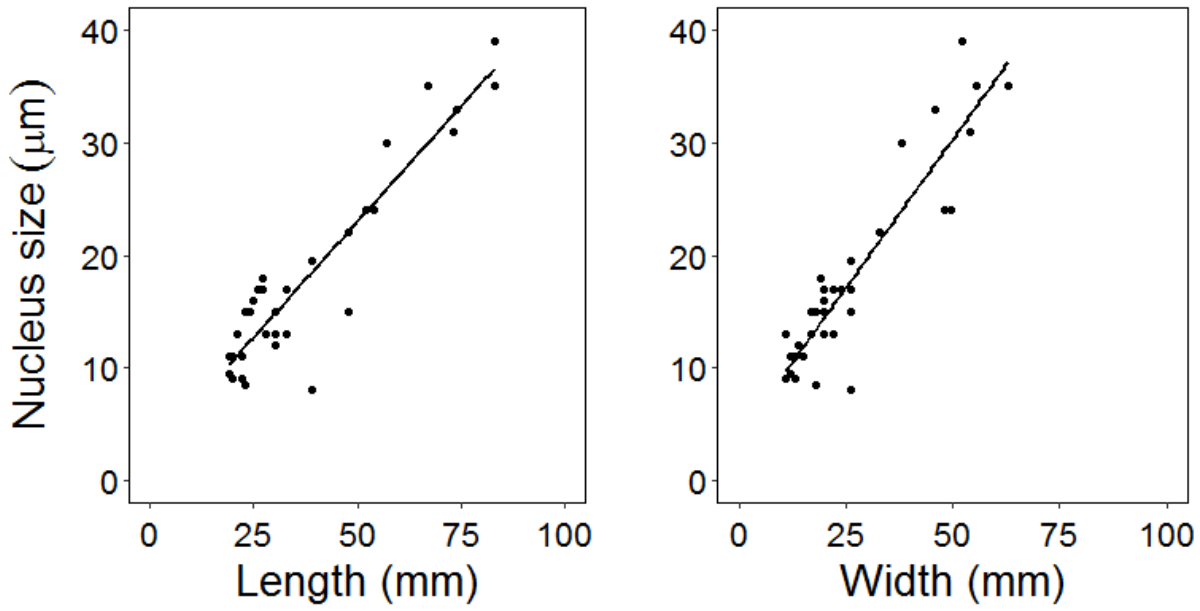

Figure 19: Correlation of nucleus size against shell length and width for males

4.3.6 Male germ cell sizes

Spermatogonia length ranged from 19 to 141 µm while the width ranged from 11 to 65 µm. The nucleus ranged from 8 to 60 mm while the maximum nucleolus size was 24 mm. Although spermatogonia length was not affected by seasonal changes (ANOVA: $F_{(3,31)}$ = 2.794, p = 0.0567) seasons significantly influenced spermatogonia width (ANOVA: $F_{(3,31)}$ = 6.471, p = 0.00157). Seasons also significantly affected the nucleus diameter (ANOVA: $F_{(3,31)}$ = 3.515, p = 0.0265) while there were no seasonal effects (ANOVA: $F_{(3,31)}$ = 0.442, p = 0.724) on the nucleolus diameter. TukeyHSD test for seasons detected that winter significantly increased spermatogonia length (p = 0.0410), spermatogonia width (p = 0.008) and nucleus diameter (p = 0.0318) in males. Multiple regression analysis revealed that spermatogonia dimensions were significantly useful in predicting nucleus diameter (R^2=0.92, $F_{(2,32)}$ = 191.4, p<0.0001) although spermatogonia length (p<0.0001) was the best predictive variable than spermatogonia width (p = 0.2028). Seasons were also significant predictors of nucleus diameter (R^2 = 0.25, $F_{(3,31)}$ = 3.515, p = 0.02655) although summer (p = 0.205) was not a predictor. Contrary, spermatogonia dimensions could not explain nucleolus diameter (R^2 = 0.11, $F_{(2,32)}$ = 1.896, p = 0.1666) as both spermatogonia length (p = 0.0765) and width (p = 0.0608) were not significantly influential. The different seasons were also not useful predictors of the nucleolus diameter (R^2 = 0.04, $F_{(3,31)}$ = 0.4422, p = 0.7245) as winter (p = 0.271), spring (p = 0.851) and summer (p = 0.611) were not statistically significant.

4.3.7 Female germ cells sizes

4.3.7.1 Oogonia

The smallest oogonia was 20 µm long and 8 µm wide while the largest oogonia was 120 µm long and 100 µm wide. Nucleus diameter inside oogonia ranged from 6 to 40 µm while the smallest nucleolus was 3 µm while the largest nucleolus was 30 µm. Significant seasonal effects were detected for oogonia length (ANOVA: $F_{(3,79)} = 11.1$, $p < 0.0001$), width (ANOVA: $F_{(3,79)} = 11.51$, $p < 0.0001$), nucleus (ANOVA: $F_{(3,69)} = 27.21$, $p < 0.0001$) and nucleolus (ANOVA: $F_{(3,69)} = 11.61$, $p < 0.0001$). Regression detected that oogonia dimensions were strong enough ($R^2 = 0.54$, $F_{(2,70)} = 41.66$, $p < 0.0001$) to predict female nucleus diameter as both oogonia length ($p = 0.0516$) and width ($p = 0.0127$) were significant. Both oogonia length ($R^2 = 0.50$, $F_{(1,71)} = 71.23$, $p < 0.0001$) and width ($R^2 = 0.52$, $F_{(1,71)} = 76.27$, $p < 0.0001$) were also significant for predicting nucleolus diameter.

4.3.7.2 Pre-vitellogenic oocytes

The smallest previtellogenic oocyte was 60 µm long and 45 µm wide while the largest was 205 µm long and 141 µm wide. The smallest nucleus was 10 µm while the largest was 85 µm meanwhile the smallest nucleolus was 4 µm and the largest was 78 µm. Previtellogenic oocytes were significantly longer ($t = -6.5388$, $df = 48$, $p < 0.0001$) and significantly wider ($t = -6.2471$, $df = 48$, $p < 0.001$) in spring compared to summer. Spring season also had a significantly larger nucleus ($t = -4.817$, $df = 48$, $p < 0.0001$) and nucleolus ($t = -8.7546$, $df = 48$, $p<0.0001$) than summer.

4.3.7.3 Vitellogenic oocytes

Vitellogenic oocytes length ranged from 60 to 205 µm and there was a length increase from autumn through winter, with spring having the longest vitellogenic oocytes, before a decrease in summer. As a result, ANOVA detected significant differences ($F_{(3,69)}$ = 4.467, p = 0.00632) between seasons with spring differing significantly from summer (p = 0.0056). The width of vitellogenic oocytes ranged from 45 to 141 µm. Significant differences ($F_{(3,69)}$ = 3.58, p = 0.0181)were recorded in Vitellogenic oocytes width over the seasons, with spring having significantly broader oocytes (p = 0.01118) compared to summer. The nucleus ranged from 10 to 85 µm while the nucleolus ranged from 4 to 78 µm, resulting in the nucleolus occupying an average 66 % of the nucleus. There were significant seasonal differences in nucleolus ($F_{(3,69)}$ = 3.403, p = 0.0224) and nucleus ($F_{(3,69)}$ = 8.925, p < 0.0001) diameter, with the smallest nucleolus (p = 0.0162) and nucleus (p = 0.0103) being detected in winter.

4.3.7.4 Mature oocytes

The smallest mature oocyte was 85 µm long and 65 µm wide while the largest was 225 µm long and 165 µm wide. The mature oocytes were significantly longer ($F_{(3,86)}$ = 38.7, p < 0.0001) and wider ($F_{(3,86)}$ = 13.52, p < 0.0001) in spring compared to the other seasons. The diameter of the nucleus was also significantly larger ($F_{(3,86)}$ = 29.44, p < 0.0001) in spring compared to other seasons.

4.4 Discussion

The fluctuating number of males to females was expected since Liu (1994) found unequal male-to-female ratio in related patellid species, with *Cellana grata* (Gould, 1859) having more females than males and *Patelloidea pygmaea* (Dunker, 1860) having more males than females. Henninger & Hodgson (2001) later found the sex ratio in *H. pruinosus* to comprise more males in Gonubie while more females were recorded in Kommetjie. Thereafter, Gray & Hodgson (2003) found a male-to-female ratio of 2:1 in *H. pectunculus* at Port Elizabeth and Bloubergstrand. This means that the sex ratio in *Helcion* species is site specific.

Male and female *H. concolor* individuals also differed in their number of gonad development stages. The uneven number of gonad developmental stages between males and females was similar to Morriconi (1999), who found that male gonads of related patellid, *Nacella (P.) deaurata* (Gmelin, 1791), contained five developmental gonad stages whereas females had eight stages. Recently, Prusina *et al* (2014) also found seven female gonad developmental stages and five male gonad stages for the related patellid species *Patella rustica* (L. 1758). However, previous studies by Niu & Fuji (1989) on *Collisella heroldi* (Dunker, 1861), Liu (1994) on *Cellana grata* and *Patelloidea pygmaea* and Rocha-Barreira (2002) on *Collisella subrugosa (*Orbigny, 1846) found an even number of gonad developmental stages between males and females. This shows that the classification of

gonad developmental stages in limpet species depends upon the limpet species and the characteristic features found within the gonad structures.

The dominance of the mature stage in *H. concolor* was similar to that recorded in *Cellana grata* where the mature stage was more dominant during winter and autumn seasons. Morriconi (1999) also recorded the dominance of mature stage in *Nacella (P.) deaurata* occurring in winter and autumn seasons. Prusina *et al* (2014) only found the abundance of mature stage during the winter season in *Patella rustica*. Contrary, Liu (1994) found that the mature stage was dominant throughout the year for the related *Patelloida pygmae.* Niu & Fuji (1989) also found that the mature stage dominated throughout the year in *Collisella heroldi.* The presence of mature stage throughout the year for *H. concolor* highlights that this limpet species has no resting phase and is therefore a partial spawner.

The larger GSI exhibited by *H. concolor* males over females is common among the South African *Patella* species such as *Cellana capensis* (Lasiak 1990), *Patella granularis* (Vat 2000), *Helcion pruinosus* (Henninger & Hodgson 2001) and *H. pectunculus* (Gray & Hodgson 2003). For example, Henninger & Hodgson (2001) recorded a larger GSI (35 %) in males compared to females which had a smaller GSI (30 %) for *H. pruinosus* in Kommetjie whereas a maximum GSI was recorded for males (25 %) and females (22 %) in Gonubie for the same species. Gray & Hodgson (2003) found significant differences

between males and females of *H. pectunculus* from Bloubergstrand with the GSI of males always being approximately 5 % greater than that of females. However, no significant differences were found between the GSI of males and females of *H. pectunculus* population in Port Elizabeth. This is because males have to contain large quantities of sperm in order to compensate for better chances of fertilizing the female eggs during spawning as sperms face many limitations in the wild.

Seasonal variations in GSI have been reported for many limpets such *Collisella heroldi* (see Niu & Fuji 1989), *Nacella (P.) deaurata* (see Morriconi 1999), *Cellana ornata* Dillwyn 1817 (see Dunmore & Schiel 2000), *Scutellastra* (*Patella) granularis* (see Vat 2000) and *Patella rustica* (see Prusina *et al* 2014). For *Collisella heroldi,* the developmental process of the gonads revealed an annual breeding cycle which was composed of resting period from winter (November to March); developing period from mid-spring to mid-summer (April to July); and a spawning period in late summer into mid-autumn (August to October). However, there was no resting phase in the reproductive cycle of *H. concolor*. The analyses of the variation of the gonadal stage percentages for *Nacella (P.) deaurata* showed the number of mature males and females increased from late winter to spring (August to November) while spawning was highest in spring (September to November). *Cellana ornata* had a single spawning period annually during summer, with the greatest gonad sizes in January and February. The two spawning seasons recorded in *H. concolor* was similar to that of *Scutellastra* (*Patella*) *granularis* and *H. pectunculus.* In *S. granularis,* both the gonad indices and detailed histological examination, it appeared that

the limpets from the south-east cost spawned twice a year, once in summer and once in winter with mature oocytes being present for most of the year. Meanwhile *H. pectunculus* spawned in summer and in autumn similarly to *H. concolor* in this study. Contrary, the related *Patella rustica* only has one reproductive cycle per year with a spawning peak between late autumn (November) and early winter (December and January).

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

This study was undertaken to quantify seasonal variations in the growth rates and reproduction of *H. concolor*. This dissertation also performed monthly histology on the gonads of *H. concolor* in order to determine the various stages and their seasonal abundance. Correlations were therefore drawn regarding the genders of the species to determine differences in growth rate and reproduction.

Literature revealed that South Africa has a rich diversity of *Helcion* species and that physical and biological factors affect the growth rate and reproduction of *Helcion* species. Literature also revealed that reproduction studies had been done for only two South African *Helcion* species. Literature further revealed that most growth studies have been conducted on West Coast limpet species, some South Coast and East Coast species (see Branch 1974, Vat 2000, Gray & Hodgson 2003, Nakin *et al* (2012) in South Africa. Vat (2000) compared seasonal growth rates of *Scutellastra* (*Patella*) *granularis* residing in different types of substrata; Gray & Hodgson (2003) conducted a comparison study on the growth rate of *H. pectunculus* populations from differing biogeographical regions; whereas the study by Nakin *et al (*2012) focused on comparing growth rates of different limpet species within and outside reserves. During their study, Gray & Hodgson (2003) did not even mention *H. concolor* once despite it being one of the four *Helcion* species found in South Africa. Although Nakin *et al* (2014) measured the growth of *H. concolor*, they lumped all individuals and did not investigate males separately from females. Furthermore, there is very limited literature on *H. concolor* regarding any aspect of this

species and this poses a serious challenge as there is very little literature to compare the current study results with.

Many studies on the histology of limpets have been conducted on species from other regions of the globe (e.g. Dodd & 0rton *et al* 1956, Ward 1966, Niu & Fuji 1989, Liu 1994, Morriconni 1999, Pérez *et al* 2007, McCarthy 2008, Ribeiro 2009, Prusina *et al* 2014) and only a few studies in South Africa (e.g. Branch 1974, Vat 2000, Henninger & Hodgson 2001, Gray & Hodgson 2003, Pal & Hodgson 2004) with Branch (1974) covering only *Patella* species for the West Coast region. Some of these studies (e.g. Dodd 1956, 0rton *et al* 1956, Branch 1974, Pal & Hodgson 2004) focused on determining limpet species hermaphroditism, while others (e.g. Liu 1994, Pal & Hodgson 2004, Ribeiro 2009) compared the reproductive cycle seasonality of different limpet species in a region. Other studies (e.g. Henninger & Hodgson 2001, Gray & Hodgson 2003) compared reproductive cycle seasonality of the same limpet species from different biogeographical regions and yet other studies (e.g. Vat 2000) focused on determining differences in reproductive seasonal cycles within one species residing in different types of substrata. Gender based reproduction studies are still lacking for limpet species inhabiting the Wild Coast of South Africa.

The results from this study are therefore interesting as they revealed that seasonal variations affected the growth rate *H. concolor*. Winter was the season of highest growth

rates and summer season yielded the slowest growth rate for both males and females. Females were frequently bigger than males throughout the study period. Contrary, the GSI of males was bigger than that of females. *Helcion concolor* was found to be a partial spawner as females had mature oocytes and the male spent stage was never recorded throughout the year. Furthermore, this species spawned twice a year and the nucleus sizes increased with oocyte maturity.

The research question was successfully answered as the objectives were met through seasonal monthly field sampling and histological analysis of both male and female *H. concolor* individuals. The null hypothesis stating that seasonal changes would not influence reproduction and growth of *H. concolor* was rejected as seasons showed marked differences. The use of histology in this study was significant since very few histological studies have been conducted on South African limpets. This has revealed the reproductive stages that would otherwise not be visible using conventional methods.

Gender based histological studies of the other *Helcion* species found along the wild Coast are needed in order to compare with *H. concolor*. Laboratory manipulated experiments of *Helcion* species must be done in order to make accurate assessments on the influences of environmental factors which trigger the various life history stages of these intertidal organisms.

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APPENDIX A

1 a. Histology procedure

1. Preparation of Davidson's Fixative (AFA)

- 330ml 95% ethyl alcohol
- 220ml 100% Formaldehyde (Sigma-Aldrich) solution 37% stabilized
- 115ml glacial acetic acid (Saarchem)
- 335ml tap water or distilled water
- Stored at room temperature

2. Tissue Processing

- Fix in AFA for 48hours
- Wash with tap water
- 70% ethanol (wash for 15 minute and preserve)
- 80% ethanol for 15 minutes
- 90% ethanol for 15 minutes
- 100% ethanol for 15 minute and 30 minutes
- Xylene for 20 minutes X2 (clearing agent)
- Mix tissue in Xylene with Wax for 30 minutes at 60°C
- Pour pure wax and store in the oven at 60°C for 30 minutes (infiltration sequence)

3. Embedding

- Trim tissue into a desirable size
- Put it at the centre of the embedding cassette (steel)
- Pour wax
- Put it on ice

4. Aqueous lithium carbonate preparation

- 1.54g of lithium carbonate powder

- Dissolve in 100ml of distilled water
- Stir with magnetic stirrer

5. Preparation of working Solution

Haematoxylin

- Haematoxylin=1g
- Ethyl alcohol=10ml
- Ammonia alum=20g
- H2O = 200ml
- Mercuric oxide=0.5g
- Acetic acid=few drops

Eosin

- Eosin Y (C.I.45380) = 1g
- 70% ethyl alcohol=1000ml
- Glacial acetic acid =5ml
- Acetic acid 2-3 drops

6. Staining Procedure (with modified Mayer's Haematoxylin)

- Slides with cut tissues stored at 37°C for 6hours
- Then baked in oven at 60°C for 10 minutes
- Xylene (1) for 3 minutes
- Xylene (2) for 3 minutes
- 100% ethanol (1) and 100% ethanol (2) for 3 minutes

95% ethanol for 3 minutes

90% ethanol for 3 minutes

70% ethanol for 3 minutes

- Water for 3 minutes
- Haematoxylin for 3 minutes
- Running water for 3 minutes

- Scott solution for 3 minutes
- Eosin 2 minutes
- 90% ethanol with eosin drops 2 dips

95% ethanol for 3 minutes

- 100% ethanol 3 for 3 minutes
- 100% ethanol 4 for 3 minutes
- Xylene (3) and (4) for 3 minutes
- Mount cut tissue with DPX & cover with slip