THE USE OF *ULVA* AS A FEED SUPPLEMENT IN THE DEVELOPMENT OF AN ARTIFICIAL DIET AND FEEDING REGIMES TO PRODUCE EXPORT QUALITY ROE FROM THE SEA URCHIN *Tripneustes gratilla* (LINNAEUS)

By

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UNIVERSITY OF CAPE TOWN
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DECLARATION

I declare that this thesis is my own, unaided work, except for amino acid and nutrient analysis that was carried out by the University of Stellenbosh, Department of Food and Animal Science.Stable Carbon and Nitrogen isotope analysis was carried out by the University of Cape Town, Archaeology Department. This thesis has not been submitted in this or any form to another University. Where use has been made of the research of others, it has been duly acknowledged in the text. Experimental work discussed in this thesis was carried out under the supervision of Prof. J. J. Bolton of the Department of Biological Sciences, University of Cape Town and Dr. Brett M. Macey, Department of Agriculture, Fisheries and Forestry.

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DEDICATION

This thesis is dedicated to all those that have supported me throughout this adventurous journey... But particularly to my parents Digby and Rose Cyrus for their loving support, and Stacey Jordaan for her continues encouragement, motivation and love.....I love you all.

"We are here to laugh at the odds and live our lives so well that death will tremble to take us."
Charles Bukowski

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Abstract

Tripneustes gratilla is a fast growing, shallow water echinoid, which occurs across the Indo-Pacific, with its south-western limit in warm, temperate waters of South Africa. The success of *T. gratilla* cultivation depends, in part, on the development of a high quality, cost-effective, gonad-conditioning diet that can produce large, marketable quality gonads. The aim of this research was to determine whether *Ulva* supplementation would improve palatability, consumption and digestibility of an artificial feed administered to *T. gratilla* and optimise gonad production and quality. At an inclusion level of 20% (20U), *Ulva* was shown to significantly ($p < 0.05$) improve the attractiveness and palatability of a formulated feed, compared to a nutritionally equivalent feed that had not been supplemented with dried *Ulva* (0U). Food consumption rates (FCR) and apparent digestibility coefficients (ADC %) for protein and energy, using insoluble ash as an indigestible marker, were measured for all experimental diets. FCR was significantly higher ($p < 0.05$) for urchins fed artificial feeds supplemented with *Ulva,* when compared to urchins fed non-supplemented feeds, suggesting that the inclusion of *Ulva* into the artificial diets acts as a feeding stimulant.Increased palatability and consumption subsequently led to significantly increased protein retention in urchins fed the 20U diet. The addition of *Ulva* to artificial feeds also significantly improved gonad colouration duringgonad enhancement trials. Gonad lightness (L*), redness (a*) and yellowness (b*) were quantified using a hand-held reflected-light, fibre-optic spectrophotometer and showed that, at an inclusion level of 20%, *Ulva* produced gonads that did not differ in colouration from those fed a natural diet of fresh *Ulva* (FU), and significantly improved gonad colour, particularly gonad yellowness (b*), compared to the same artificial diet without *Ulva* supplementation. This response was likely related to *Ulva's* high β-carotene content. The *Ulva* content of artificial feed significantly improved gonad colouration, while the formulated portion of the feed produced significantly larger gonads than those produced using a natural diet of fresh *Ulva*. The 20% *Ulva* inclusion diet, in particular, increased the gonad somatic index (GSI) by 205%, in just nine weeks (7.6 to 23.3%), compared to a 57% increase in the control group (fresh *Ulva*).

Full life-cycle growth trials were also conducted using two of theformulated feeds (20U & 0U), as well as fresh *Ulva,* to establish appropriate feeding regimes thatcould produce a

harvestable product in the shortest time. During grow out, juvenile somatic growth needs to be maximised until an individual reaches marketable size and sexual maturity, which is followed by gonad enhancement, through nutritive cell development,aimed to maximise gonad yield and quality before harvest. The effects of 5 different feeding regimes on somatic and gonadal growth of juvenile *T. gratilla* were investigated, over a 32 week period. The feeding regimes used were: fresh *Ulva* (FU) only; fresh *Ulva* for 20 weeks and the 20U diet for 12 weeks (FU-20U); fresh *Ulva* – 0% *Ulva* (FU-0U); 20% Ulva – fresh *Ulva* (20U-FU) and 0% *Ulva* – fresh *Ulva* (0U-FU). Somatic growth was largely effected by the presence of *Ulva* within a diet. Similar growth, in diameter, of juvenile urchins was achieved using either an artificial diet containing *Ulva* (20U) or fresh *Ulva* (FU), during the somatic growth phase.An artificial diet with the same nutritional properties but without *Ulva* supplementation (0U) produced urchins that were significantly ($p > 0.05$) smaller. Gonad production in the somatic growth phaseof the trial was higher for urchins fed with artificial diets (0U & 20U), but the reduced size of urchins in the 0U diets significantly reduced gonad mass. After the diets were changed from artificial feeds (0U & 20U) to fresh *Ulva* (FU) and vice versa (week 20), both gonad size and colour were affected, with artificial diets promoting gonad growth, while FU improved gonad colour. At the end of the study, all feeding regimes produced similar amounts of gonad, except for those individuals that were fed the 0U feed during the somatic growth phase (which were significantly ($p > 0.05$) smaller). Gonad colourationof all treatments at the end of the study was of marketable quality. From these results it is recommendedthat FU be fed inthe somatic growth phase, while the 20U artificial diet should be used to increase gonad size, and optimise gonad colour, in the gonad enhancement phase.

Through the use of stable isotope mass spectrometry and IsoSource, a mixing and mass balance model, the relative contribution and importance of specific feed ingredientsto gonad production was determined. *Ulva* was shown to be an important isotopic source for gonad production, accounting for an average of 33% of the isotopic signal across all *Ulva*-containing, diets at the end of the trial.

The final section of work focused on efforts to manipulate the gametogenic cycle of *T. gratilla*, by altering daylength, to attempt to reduce the production of large amounts of gametes, which would decrease gonad value. Histology indicated that urchins exposed to a Short day (8:16 h) were significantly more advanced reproductively, with the majority of urchins in a mature or spent state compared to urchins exposed to a Long day (16:8 h), which were mostly premature. Nutritive phagocyte (NP) density within the gonads supported the

findings from histology, as gonads from urchins exposed to a short day had significantly less NP's (21.58 ± 4.35%), compared to the Long day treatment (65.26 ± 3.09%). The results from this study suggest that urchins of this species exposed to Long days progress through gametogenesis more slowly than those exposed to Short days, which, appeared to mature more rapidly. These findings could allow for the production of a high quality product for longer periods of the year, without the onset of gonad maturation.

The research in this thesis clearly shows that the use of the macroalga *Ulva* as a feed, or feed additive, to artificially formulated, high protein feeds can have a number of significant benefits in echinoculture. The use of the artificial feeds and feeding regimes developed in this work, along with the ability to manipulate daylenth to suspend gametogenisis, could greatly facilitate the success of the newly developing *T. gratilla* industry, both in South Africa and worldwide.

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CHAPTER 1:

General Introduction

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1.1 Sea urchin taxonomy and anatomy

Sea urchins are members of the phylum Echinodermata, which includes the sea stars, sea cucumbers, brittle stars, and crinoids (Nicholson, 1880; Barnes *et al.,* 2001). Currently, sea urchins are allocated to approximately 19 orders and 70 families consisting of a total of 850 living species (Kroh & Smith, 2010). Edible sea urchins are, however, only distributed among four of these orders consisting of seven families (Table 1.1) (Lawrence, 2007). The body parts of sea urchins are arranged along five rays of symmetry (Fig. 1.1) and thus they are pentaradially symmetrical, like most echinoderms. The outer casing of a sea urchin, known as the test, is a hard spherical structure made up of an internal mesodermal skeleton,composed of fused plates of calcium carbonate (calcite),covered by a thin dermis and epidermis. The test is divided into five alternating ambulacral and inter-ambulacral areas. Each area consists of two rows of plates, so that the test consists of 20 rows in total. The plates are covered in rounded tubercles to which spines are attached (Nicholson, 1880; Barnes *et al.,* 2001). Spines are generally longer and more numerous on the inter-ambulacral plates, whereas tube feet are more prominent on the ambulacral plates. The inner surface of the test (Fig. 1.2) is lined by theperitoneum and is dominated by five, usually brightly-coloured orange/red/yellow gonads, which can comprise up to 25% of the body cavity (Cyrus *et al.,* 2014). The remaining internal structures of a sea urchin include coelomic fluid, the gut, and the Aristotle's lantern, which is formed by five teeth-like structures which extend through the peristome surrounding the mouth and make up the feeding apparatus (Nicholson, 1880; Barnes *et al.,* 2001).

Table 1.1: Classification of edible sea urchins (Lawrence, 2007, adapted from Smith, 1984).

Class	Subclass	Infraclass	Cohort	Superorder	Order	Family	Genus
Echinoidea							
	Perischoechinoidea						
	Cidaroidea						
	Euechinoidea	Echinothurioidea	Echinothuriacea		Echinothurioida	Echinothuriidae	*Echinothuria*
		Acroechinoidea	Diadematacea		Diadematoida	Diadematidae	*Centrostephanus*
							Diadema
			Echinacea	Stirodonta	Phymosomatoida	Arbaciidae	*Arbacia*
				Camarodonta	Echinoida	Echinidae	*Echinus*
							Loxechinus
							Paracentrotus
							Psammechinus
						Echinometridae	*Anthocidaris*
							Echinometra
							Evechinus
							Heliocidaris
						Strongylocentrotidae	*Hemicentrotus*
							Strongylocentrotus
						Toxopneustidae	*Lytechinus*
							Pseudoboletia
							Pseudocentrotus
							Toxopneustes
							Tripneustes
			Irregularia				

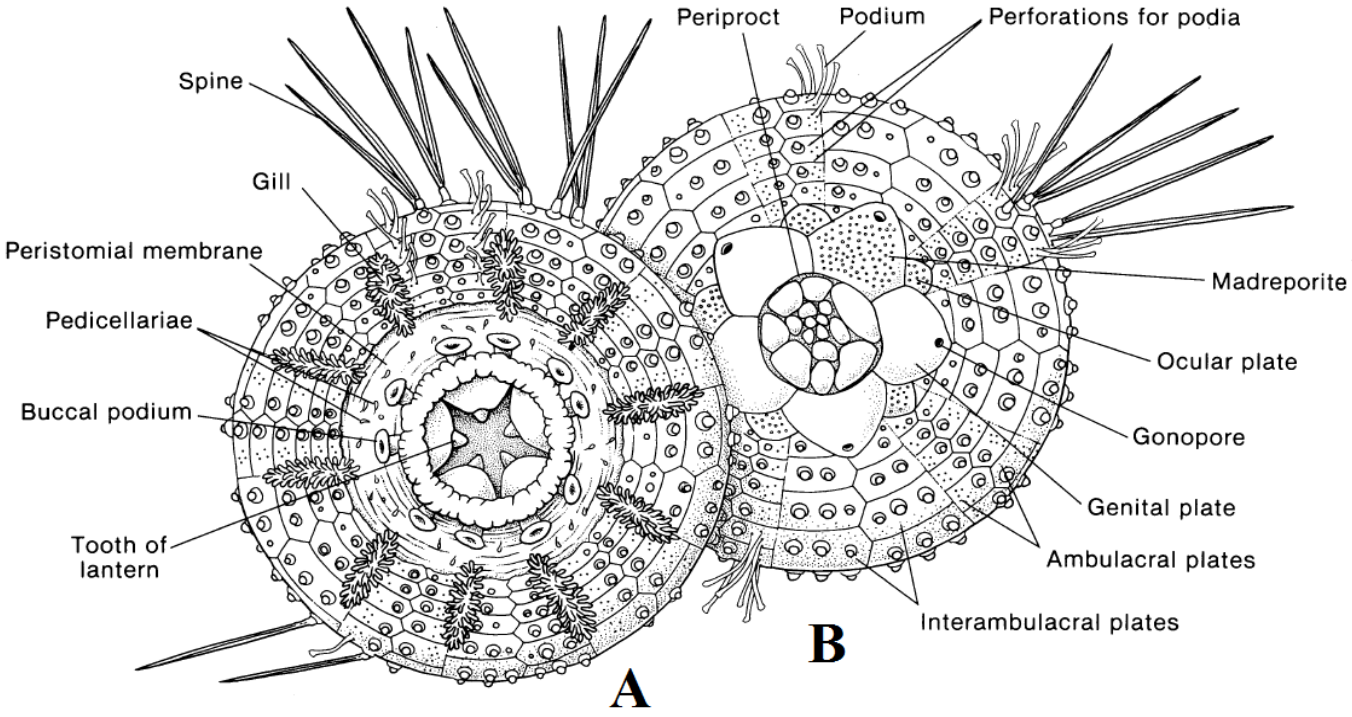


Figure 1.1: The external anatomy of a regular sea urchin. (A) Oral view. (B) Aboral view. (After: Reid W.M. In: Ruppert & Barnes, 1994).

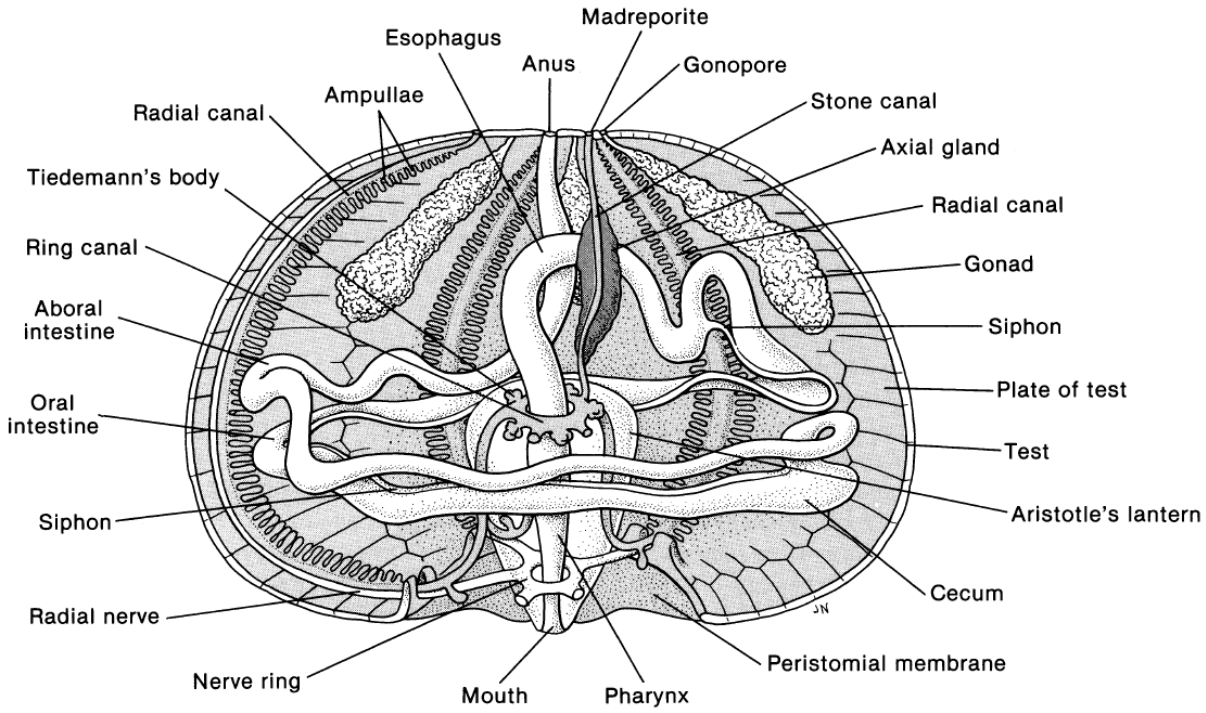


Figure 1.2: The internal anatomy of a regular sea urchin. (After: Reid, W.M., In: Ruppert & Barnes, 1994)

1.2 Edible sea urchins

The gonads from both sexes represent the edible part of a sea urchin (Fig. 1.3). Of the 850 species identified by Kroh & Smith (2010), only 19 genera are harvested for food worldwide (Lawrence, 2007), an increase from the 16 edible species recorded by Keesing & Hall (1998). Edible sea urchins are distributed among a number of common echinoid orders (Lawrence & Bazhin, 1998; Robinson *et al.*, 2002). Although relatively few species are commercially consumed, Lawrence (2007) states that there should be no *a priori* reason why any urchin species would be inedible. Three possible explanations for why only particular species are eaten have been suggested: (1) accessibility: most consumed urchin species are found within shallow water; (2) palatability: many urchin species are found in abundance but are not consumed as they are not very palatable (Lawrence & Bazhin, 1998); and (3) historical /cultural preferences: there is a long tradition of consuming sea urchin gonads of specific

species in many cultures, particularly in Asia, Polynesia, the Mediterranean and Chile (Andrew *et al*. 2002). The maximum size of a species may also influence their selection as small urchins with limited gonad content may not be worth the effort of harvesting.

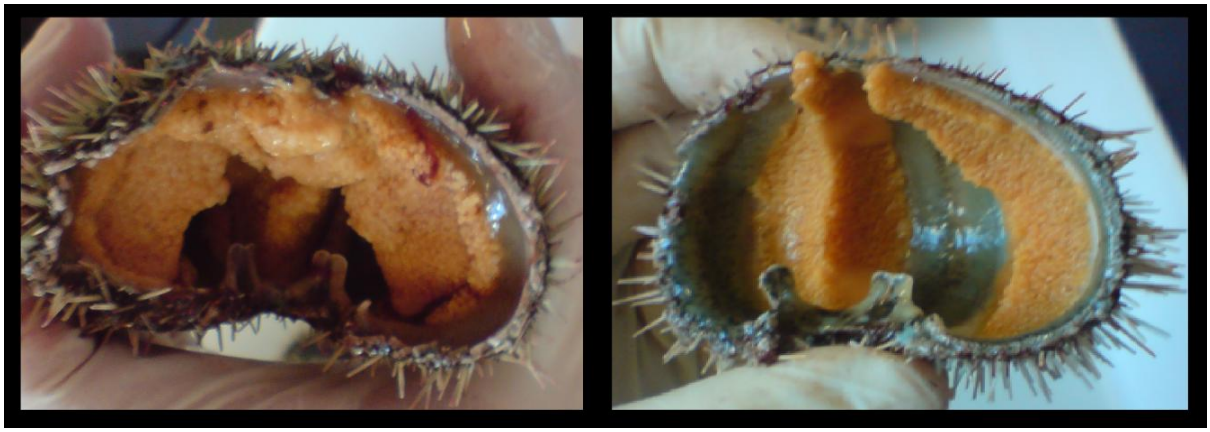


Figure 1.3: Brightly coloured gonads observed in sexually mature *Tripneustes gratilla* (photos: Mark Cyrus)

1.3 The international sea urchin trade

The international sea urchin trade is based on the production of marketable quality gonads, which are sold largely for consumption in the sushi/shashimi restaurant trade. Due to the high degree of importance placed on product presentation in the sushi/shashimi market, the appearance of sea urchin gonads, otherwise known as "roe" or "uni" is very important and influences the price of the product (Robinson *et al.*, 2002). Although the term roe is generally reserved for egg-bearing ovaries, in the sea urchin industry roe is synonymous with both male and female gonads. Gonads that are large in size, contain few to no gametes, have a firm texture, and are bright yellow to orange in colour are regarded as the most commercially valuable (Robinson *et al*., 2002; Shpigel *et al.*, 2004). Sea urchin roe is a highly valued and prized delicacy in many countries, particularly in Japan, where it is regarded (along with tuna, lobster and abalone) as premium seafood. At present, Japan consumes more than 80% of the world's total production of sea urchin roe (Sonu, 1995; 2003; Hagen, 1996; Keesing & Hall, 1998; Andrew *et al*., 2002; Yokota, 2002; Agatsuma, 2010), followed by France

(Hagen, 1996; Explorations Unlimited Inc. 2006). It is easy to see that Japan is the world's leading market for sea urchin products. However, this market is continuously growing around the world as the popularity of sushi and shushimi increases. The rapid growth of modern markets, particularly in Japan, has led to the development and expansion of new sea urchin fisheries mainly around the Pacific Ocean and Mediterranean Sea (Williams, 2002). In 1999, the Japanese sea urchin market imported US$ 216 million of product, in contrast to the USA, which was the second largest importer at that time, and purchased only US$ 19 million worth of product (FAO, 2002).

Sea urchin roe is marketed in several different forms which are generally determined by the gonad quality. These forms are: fresh (*namauni*), frozen (*reitouni*), baked and frozen (*yakiuni*), steamed (*mushiuni*), and salted (*shiouni*). The latter two forms are generally reserved for the lower grades of uni. Uni is also prepared as *neriuni* or *tsubiuni*, which are blended urchin pastes (Explorations Unlimited Inc. 2006). The most popular and highest valued use for this product, however, is chilled fresh roe that is used to make sushi and sashimi. In Japan uni is considered one of the most valuable seafood products, which in some cases, can have a wholesale value in excess of $850 per kg (Explorations Unlimited Inc., 2006). Most fresh sea urchin roe is auctioned through the Tokyo Central Wholesale Market and in 2002 fresh roe was the most valuable sea urchin product imported into Japan, representing almost 49% in value, but only 16% in volume (Sonu, 2003). Live sea urchin imports accounted for 23% in value and 64% in volume, however it should be noted that edible roe generally makes up only about 10-25% of total live weight (Sonu, 2003). Imported frozen roe accounted for 19% in value and 14% in volume, followed by prepared roe with 6% in value and 4% in volume. Salted sea urchin roe imports were minimal in 2002, with 3% in value and only 1% in volume (Sonu, 2003). During months of January and September

Japanese roe fetches the highest prices, which reflects the low availability of the product during these times (Sonu, 2003).

1.3.1 History of the world sea urchin fishery

In just 20 years (1975 – 1995)**,** total world landings of sea urchins increased by 370%, from 31 000 metric tons (mt) to 117 000mt.Thereafter sea urchin landings have been on a downward trend, decreasing to 87 000 mt in 2001 (Fig. 1.4). During the last three decades there has been increasing demand for high quality sea urchin roe (Hammer *et al.,* 2006; Siikavuopio *et al.,* 2007), resulting in extensive exploitation of natural sea urchin populations throughout the world (Lawrence *et al.,* 1997; Hammer *et al.,* 2006; Siikavuopio *et al.,* 2007). Consequently, many sea urchin fisheries such as Japan (Saito, 1992), France (Sloan, 1985), Ireland (Byrne, 1990), California, USA (Kalvass & Hendrick, 1997), Maine, USA (Lessing &Walker, 1998), Washington, USA (Pfister & Bradbury, 1996), the Caribbean (Scheibling & Mladenov, 1987) and British Columbia, Canada (Keesing & Hall, 1998), have recorded marked declines in their harvests throughout history. These declines have generally resulted in the collapse and subsequent closure of the commercial fishery. In the Philippines, for example, poor fisheries management, along with unregulated harvesting of the sea urchin *Tripneustes gratilla*, led to the collapse of the multi-million peso (USD $225 000 – 386 000) fishery (Juinio-Meñez *et al*., 2008).

The high initial production capacity of new fisheries during the developmental stages is due to the phenomenon of “fishing-down” accumulated biomass of older and larger animals. Yields during the initial “fishing down” phase are generally far greater than those that can be sustained in the long term. The potential for “fishing-down” in most sea urchin fisheries is almost completely exhausted and future production will rely on the annual recruitment and growth of the remaining populations (Williams, 2002). The general trend in most sea urchin

fisheries thus far is that of rapid development, the depletion of stocks within different areas of the fishery, followed by marked declines in production and sometimes the collapse of the fishery over a period of years (up to a decade). One exception to this short-term pattern of `boom and bust' is the Japanese sea urchin fishery, which suffered a much longer-term 50 year decline (Kelly, 2005), despite efforts to enhance stocks by releasing juveniles, setting minimum legal size limits and even closures of particular fishing grounds (Saito, 1992, Hagen, 1996, Agatsuma, 1998). The decline of Japan's local urchin populations is a major contributor to the increasing demand for imported roe (Keesing & Hall, 1998) and has led to dramatic changes in supplier profiles throughout the globe. Subsequently, there has been increased pressure on urchin populations from around the world, with catches either declining or reaching their peak in most producing countries (Andrew *et al*., 2002).

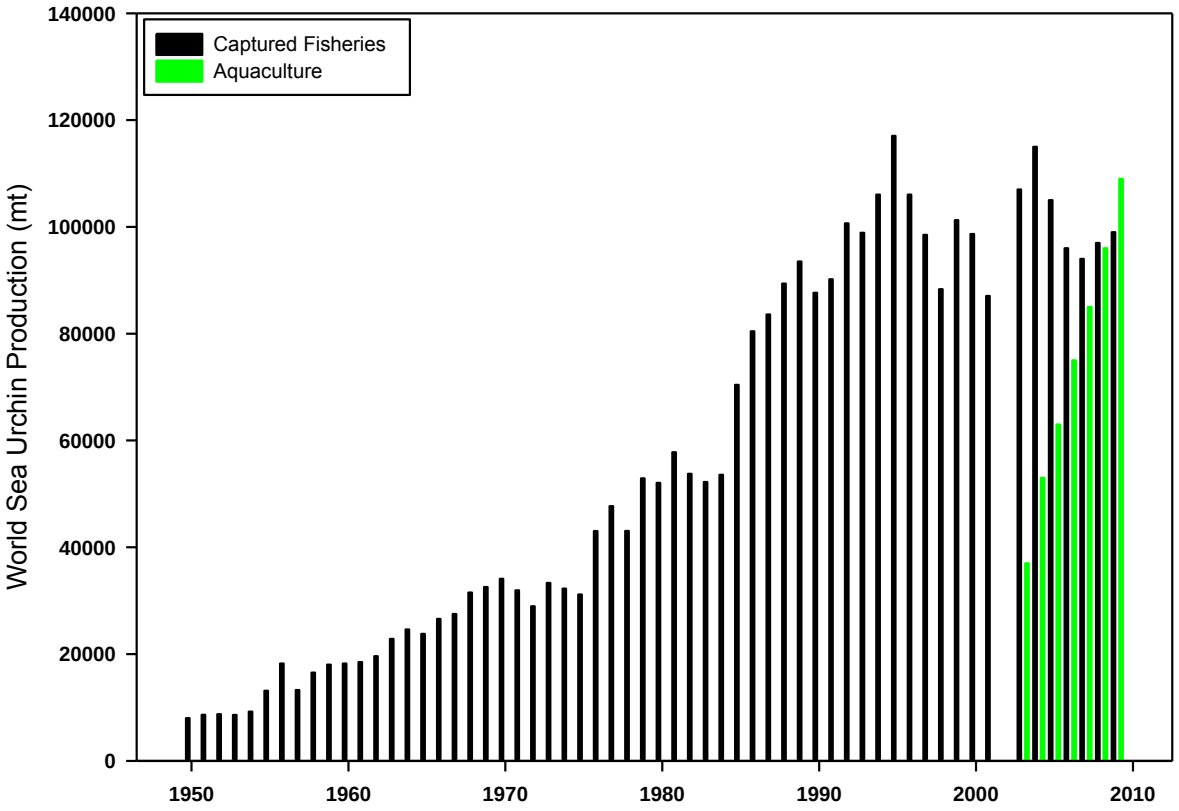


Figure 1.4: World sea urchin production from capture fisheries and aquaculture from 1949 - 2009 (Adapted: FAO, 2009; 2012)

The decrease in global landings has been largely due to lower harvests of the five major sea urchin producing countries, namely: Chile, the United States, Japan, Canada, and Mexico. In 1995, these five countries had a combined harvest of 108 130 mt. However, by 2001 their combined harvest declined to 79 839 mt, a decrease of 26% in just 6 years. A total of 29 nations reported sea urchin landings in 2001 (Fig. 1.4). Of these, Chile, the United States and Japan recorded the highest landings, accounting for 54%, 14% and 13%, respectively, of the total landings in 2001 (Shinbun Sha, 1993). Canada and Mexico's contributions to world landings in 2001 were 8% and 3% of the world total respectively, while the Republic of Korea and Russia each contributed about 2% towards the world total. The remaining 22 countries accounted for only 2% of the total world production. World production in the past few years has been maintained by compensatory increases in production from Chile. These catches are being sustained by the harvesting of undersized urchins (Explorations Unlimited Inc., 2003) and the discovery of new fishing grounds (Andrew *et al*., 2002; Kelly, 2005), a trend that is unlikely to continue. If Chile is excluded from the world's sea urchin harvests, it is clear to see that the world production has been steadily declining for the last 20 years (Andrew *et al*., 2002). According to Williams (2002), the Chilean fishery is likely to suffer large reductions in production and, as the fishery currently contributes more than half of global sea urchin supply, this is likely to have a significant effect on world output.

World sea urchin landings are dominated mainly by two taxa: *Loxechinus albus* and *Strongylocentrotus* spp. *(S. intermedius, S. nudus, Mesocentrotus (S.) franciscanus* and *S. droebachiensis*) and between 1991 and 2001 they accounted for 24-55% and 38-68% of total worldwide sea urchin landings, respectively (Hagen, 1996; Keesing & Hall, 1998; Andrew *et al*., 2002; Agatsuma, 2007). Expansive growth of the *L. albus* fishery in Chile has meant that this species now accounts for the largest proportion of world production (Andrew *et al*., 2002). Landings of other species during this time were relatively small and only accounted

for 5 to 11% of total world landings, however many of these species are still commercially valuable and are listed in Table 1.2.

Table 1.2: A list of commercially valuable sea urchin species accompanied by the country of origin and when they are commonly harvested (adapted from: Williams, 2002 and Krause, 2003)

Scientific name	**Commercial name**	**Country of harvest & season**
Strongylocentrotus intermedius	Ezobafun (horse dung) Urchin	Japan: June - September
		Russia: May - August/May-June
		North Korea: May - Augustus
		Kuriles Island: October - June
S. nudus	Kita murasaki (Northern purple) urchin	Japan: December – May
		South Korea: April – June
		China: May-July
		North Korea: May – Augustus
Pseudocentrotus depressus	Aka (red) urchin	Japan: March – December
Hemicentrotus pulcherrimus	Bafun (horse dung) urchin	Japan: March – December
Anthocidaris crassispina	Murasaki (Purple) urchin	Japan: March – December
Tripneustes gratilla	Shirahige (white beard) urchin	Japan: March – December
S. droebachiensis	Green sea urchin	North America: September – April
		: October - March
S. fransiscanus	Red sea urchin	North America: September - March
S. pallidus	Pale Urchin	Russia:
S.polyacanthus		Russia:
S.purpuratus	Purple sea urchin	North America:
Loxechinus albus	Erizo	Chile: Febuary - October
Evechinus chloroticus	Kina	New Zealand: October - February
Heliocidaris erythrogramma	Purple sea urchin	Australia: May - December
Lytechinus variegatus		West Atlantic & Caribbean:
Psammechinus miliaris		Northeast Atlantic:

More recently world production of sea urchins alone has become difficult to accurately estimate as FAO statistics (FAO, 2002 – 2009) report combined echinoderm production. This poses an issue as some countries in the tropics have significant holothurian fisheries (Sloan, 1985; Conand & Bryne, 1993; Kelly, 2005). Contributions from a growing sea urchin aquaculture sector have also helped meet recent growing market demands, as production increased from 25 mt in 2002 to 60 852 mt in 2004 (Fig. 1.3). This is an average annual growth of 4833.6% (FAO, 2006), making urchins and other echinoderms number one on the "FAO top ten species in terms of growth in production" in aquaculture from 2002-04. In 2009 sea urchins and other echinoderms totalled 99 000 mt worth of capture fisheries valued at US$ 266 million while contributions from the growing aquaculture sector totalled 109 000 mt for sea urchins and other echinoderms and was valued at US$ 378 million (FAO, 2009).

Global production of sea urchin roe from wild fisheries will, in all likelihood, decline in the future and the major contributions to world production will be from fisheries that have supported active management strategies that provide long-term sustainability, as well as aquaculture operations. Since current world production is unlikely to be sustained at current levels, the cultivation of important urchin species is becoming necessary to fufill market demands, and given that this demand is unlikely to decline, the economic value of future production will increase (Williams, 2002).

1.4 Sea urchin aquaculture (echinoculture)

As wild populations continue to decline and demand for urchins increases, sea urchin aquaculture will become increasingly economically viable. This is supported by the recent increase in research and interest into this field (Kelly, 2005), as well as the growing global echinoculture industry. In 2009 contributions from sea urchins and other echinoderms to

world aquaculture production totalled 109 000 mt, a 179% increase from 2004, further indicating the expansion of this fairly new industry (FAO, 2009). Echinoculture has been practiced in Japan on a large scale for many decades and Japan has well established methods for cultivating sea urchins within its waters (Kelly, 2005). However, echinoculture remains a relatively recent practice in most other countries (Pearce *et al*., 2002a; Cook & Kelly, 2007a). The major producing countries are currently exploring a variety of production methods, including stock enhancement through reseeding of natural habitats with juveniles (Yokota, 2002a; Sakai *et al.*, 2004), roe enhancement of wild caught individuals to increase gonad yield and quality (Keesing & Hall, 1998, Fernandez & Caltagirone, 1994; Klinger *et al*., 1997; Lawrence *et al*., 1997; Kelly *et al*., 1998; Robinson & Colborne, 1998; Vardas *et al*., 2000; Olave *et al*., 2001; Pearce *et al*., 2002a,b,c.; Mortensen *et al*., 2003; James, 2006b; Hammer *et al*., 2006; Cyrus *et al.*, 2014), and full life-cycle sea urchin echinoculture, where larvae are produced in hatcheries and juveniles are grown to commercial size either at sea, in some kind of containment systems, or in land-based tanks (Le Gall, 1990; Grosjean *et al*., 1998; Keesing & Hall, 1998; Devin, 2002; Daggett *et al.*, 2006; James, 2006a; Cook & Kelly, 2007a).

In order for echinoculture to remain successful commercially, feed types and feeding regimes that can produce high quality gonads cost effectively are required. Although the major sea urchin species currently under investigation for echinoculture are predominantly grazers of macroalgae or seagrasses, the use of natural diets alone for either "gonad enhancement" or "full life-cycle production" is unlikely to be commercially viable on a large commercial scale. The reasons for this are: (1) limited natural stocks of suitable macroalgal species: e.g. Troell *et al.*(2006) stated that kelp harvesting has greatly increased in South Africa with the expansion of the abalone industry and that kelp populations are now becoming increasingly impacted; (2) restrictions on harvesting of algal populations, with permits allocated to

selected individuals; (3) temporal variation in quality and/or quantity of algae; (4) negative environmental effects of harvesting large quantities of natural algae; and (5) problems with storing commercial-scale amounts of algae. These issues are, however, negated if an alga such as *Ulva* is grown in aquaculture on the farm, as is the case on some South African abalone farms (Bolton *et al*., 2009). The main reason, however, that macroalgae diets alone are not suitable for use in echinoculture is due to the relatively low protein and energy content of naturally occurring seaweeds, which are generally not high enough to support maximal gonad growth. For example, the South African kelp *Ecklonia maxima* [Osbeck] Papenfuss, has a crude protein content of 11-12% (DW) throughout the year (Smith, 2007). According to a study by Akiyama *et al*. (2001) the optimum dietary protein level in a purified diet for young red sea urchins, *Pseudocentrotus depressus* is 20%, while Kuroki & Tashiro (1991) suggest that protein requirements for juveniles of both *Pseudocentrotus depressus* and *Tripneustes gratilla* are closer to 30%. In South Africa the abalone *Haliotis midae* is cultured using the artificial feed Abfeed®, which has a protein content of 34.6% (Naidoo *et al*., 2006).The use of artificial diets as feed for sea urchins has proven to be a viable alternative to natural algae (Olave *et al.*, 2001).

1.4.1 Artificial feeds and components

Artificial or prepared feeds are considered better than natural foods as they can be formulated to a standard composition, which will promote maximal growth and production (Lawrence *et al.*, 1997), and eliminate dependency on wild-harvested feed. Artificial diets are also recognized as being less expensive than natural foods (Lawrence *et al.*, 1997). According to Troell *et al.* (2006), based on figures from Hahn (1989) and Britz (1996), feeding abalone naturally-occurring kelp is actually more expensive than feeding artificial feeds due to differences in food conversion ratios between seaweeds and formulated diets. Artificial diets are also much easier to work with, as they can be easily transported and are of a standard

composition, which makes them very well suited to large-scale commercial operations. It should be noted, however, that there are both advantages and disadvantages to using artificial feeds and it has been shown that there are benefits to feeding cultured organisms a mix diet (both artificial and natural feeds) and/or changing the diets over the course of the life-cycle.

The development of suitable prepared feeds for gonad enhancement in sea urchins has, in the last decade, been an area of considerable scientific research, with a multitude of studies showing that artificial diets can greatly increase the gonad yield of adult sea urchins (Lawrence *et al*., 1997; Olave *et al.,* 2001; Pearce *et al.,* 2002a, b; Pearce *et al*., 2004; Shpigel *et al.,* 2005; Cyrus *et al*., 2014). Since the marketability and price of roe is linked to quality factors such as gonad colour, firmness, and taste, gonad quality must be at least as important as gonad yield in the commercial sea urchin industry (Pearce *et al.,* 2002a). Components and factors of an artificial feed which affect gonad yield and quality, and make a feed suitable for commercial use, include protein, carotenoid pigments, feeding stimulants and feed stability.

1.4.1.1 Protein

In nature sea urchins must ingest and process large quantities of protein-poor food in order to meet their nutritional requirements for protein (Hammer *et al.,* 2006). This may explain why macroalgal diets used in echinoculture have typically produced urchins with small sized gonads when compared to urchins fed animal-derived diets (Cook *et al.,* 1998; Fernandez & Boudouresque, 1998; Fernandez & Boudouresque, 2000). As protein is one of the most expensive components of an artificial feed, it is essential to determine the optimal levels of protein required to maximize growth while still utilizing the protein efficiently. Data suggest that dietary protein levels less than 10% do not generally support maximal gonad growth in sea urchins, although it would appear that high protein diets do not support maximal growth either (Kuroki & Tashiro, 1991; Akiyama *et al.,* 2001; Hammer *et al.,* 2006). Hammer *et al.* (2006) suggest this may be due to the energetic cost of protein utilization. From previous

studies it can be inferred that a moderate dietary protein level between 20 – 40% is most efficiently used by sea urchins when trying to produce gonads of a commercial standard.

1.4.1.2 Carotenoid pigments

Carotenoid pigments are the source of the red, orange and yellow colouration that is so sought after in sea urchin gonads (Agatsuma *et al.,* 2005). Although sea urchins contain a number of carotenoids (Symonds *et al.,* 2007), echinenone, which is synthesised from β-carotene, has been recognised as being particularly important in the production of market quality gonads (Pearce *et al*., 2002a; Robinson *et al*., 2002; McBride *et al.,* 2004). Shpigel *et al.* (2005) showed a positive relationship between echinenone levels and gonad colouration in the urchin *Paracentrotus lividus*. Sea urchins obtain carotenoid pigments through their diets, which are generally composed of fleshy macroalgae or seagrass. Feeding artificial diets with no pigments to sea urchins in aquaculture results in large but pale coloured gonads that are commercially unacceptable (Robinson *et al.,* 2002; Shpigel *et al.,* 2005).

The addition of β-carotene to artificial feeds would seem the most appropriate means of improving the gonad colour of culture organisms. Robinson *et al.* (2002) and Shpigel *et al.* (2005) showed that pigment origin affects gonad colour, with naturally-derived β-carotene producing better colouration that that derived from synthetic sources. Pearce *et al.* (2003) also found that algal-derived β-carotene produced a better tasting gonad compared to synthetic β-carotene.Aside from the obvious commercial benefits, there are several other good reasons for including carotenoid pigments in prepared sea urchin diets. Pigments within actively growing tissues may: (1) act as cellular antioxidants; (2) stabilise proteins; and (3) provide ultraviolet protection for sensitive tissues (Robinson *et al.,* 2002).

1.4.1.3 Feeding stimulants

Delivery of dietary components (e.g. protein & pigments) to a cultured organism is usually manipulated by varying the concentration or digestibility of the particular components within the feed itself, but this may also be achieved by changing the amount of a feed actually consumed by an organism (Jobling *et al.*, 2001).

Increasing the palatability of artificial diets, by giving them desirable chemosensory characteristics through the addition of feeding stimulants can increase both consumption and digestibility (Kasumyan & Døving, 2003; Dworjanyn *et al.,* 2007). Sea urchins are considered generalist herbivores, although they do display a hierarchy of preferences when offered a choice of natural diets (Dworjanyn *et al.,* 2007; Stimson *et al.,* 2007; Scholtz, 2008). Preferences have been attributed to factors such as the nutritive value and physical properties of the food, as well as the presence/absence of attractant/deterrent compounds within the algae (Stimson *et al.,* 2007). Preferences displayed by sea urchins are thought to be adaptive, as urchins generally grow faster when fed a highly preferred algae species compared to when fed low preference ones (Dworjanyn *et al.,* 2007). Inclusion of small quantities of macroalgae in artificial diets fed to *T. gratilla* has been shown to enhance palatability and subsequently increase protein consumption (50%) and growth rate (30%), compared to individuals feed a control diet containing no algae (Dworjanyn *et al.,* 2007).

1.4.1.4 Feed stability

Another important factor in developing a suitable artificial diet is feed stability. Sea urchins are predominantly grazers and therefore a prepared feed that breaks down soon after immersion in saltwater may not be consumed as readily by urchins as a feed that remains intact (Pearce *et al.,* 2002b). Lowe (1974) states that manipulation of food by different appendages is an important step in feeding, and plant species that are easily caught and transferred to the mouth would be preferred. In addition to reducing consumption rates, artificial feeds that are less stable in solution leach important vitamins, minerals, protein and

lipids, making them less nutritious than originally formulated. Leaching may also lead to decreases in culture water quality.

1.5 Marine aquaculture in South Africa

The major marine species produced in South Africa in 2010 included *Haliotis midae* (abalone), *Crassostrea gigas* (oysters), *Mytilus galloprincialis*, *Choromytilus meridionalis* (mussels) and *Argyrosoumus japonicas (*Dusky Kob). The fin fish *Argyrosoumus indorus* (Silver Kob) and *Seriola lanandi* (Yellowfin Tuna) are also currently being farmed at a pilot scale. Due to high demand and the popularity of these particular species, as well as declining yields of many marine fisheries world-wide, aquaculture in South Africa has been identified as an important industry and is part of the key industries for promotion, in line with the country's Industrial Policy Action Plan II (IPAP II). Furthermore, aquaculture is currently being promoted to help create employment opportunities in historically disadvantaged communities, particularly those that have been impacted by the closure of wild fisheries.Marine aquaculture production (excluding seaweeds) totalled 1992 mt in 2010, and had an estimated value of R 378 million, an increase of 11.2% from the previous year. Of the major aquaculture subsectors, abalone accounted for 93.9% of the total production and had a monetary value of R 355 million, currently making South Africa the largest producer of cultured abalone outside Asia (Troell *et al*., 2006; Robertson-Andersson *et al*., 2008). The next major contributor to total aquaculture production was the oyster subsector, which accounted for 3.8% and was valued at R 14.4 million. Finally, the mussel subsector accounted for 2.4% and had a production value of R 9.1 million (DAFF 2012). Of the total aquaculture production a total of 1 039 mt was exported, with the majority of the exports (96.7%) made up by abalone, followed by oysters (3.17%) and mussels (0.96%).

Feed usage in the abalone subsector in 2010 totalled 6 926 mt, which comprised of 979mt of artificial feed and 5 937 mt of seaweed (*Ecklonia maxima* (kelp), *Ulva* and *Gracilaria*). South African seaweed production in 2010 was made up almost entirely of the species *Ulva* spp. and *Gracilaria* spp and totalled 2015 mt, a 6% increase from the previous year.

It is clear from the above statistics that the South African aquaculture industry is dominated by the mollusc *Haliotis midae,* with oysters and mussels accounting for only 6.2% of the total production. For this reason there has been considerable effort into the development of new aquaculture crops and culture technology to help diversify South Africa's aquaculture sector. Focus has been placed on developing high value indigenous species which have a global demand. Some examples of such species in research stages include: Atlantic salmon (*Salmo salar*), White stumpnose (*Rhabdosargus globiceps*), South African scallop (*Pecten sulcicostatus*) and the short spined sea urchin (*Tripneustes gratilla*).

1.6 The biology of *Tripneustes gratilla*

The culture species under investigation for commercial aquaculture in this study is *Tripneustes gratilla,* also commonly known around the world as the "collector urchin", "bearded urchin" or the "short spined urchin".

1.6.1 *Classification*

Kingdom: Animalia

Phylum: Echinodermata

Class: Echinoidea

Order: Temnopleurioda

Family: Toxopneustidae

Genus: *Tripneustes*

Species: *T. gratilla*

The genus *Tripneustes* has a pantropical distribution that extends into the subtropics. The genus previously included three species (Fig. 1.5) with non-overlapping distributions: (A) *T. ventricosus,* the "white sea urchin", (Lamarck 1816) found in the Western parts of Atlantic Ocean; (B) *T. depressus*, the "brown sea urchin", (Agassiz 1863), found in the eastern Pacific Ocean only; and (C) *T. gratilla,* the "collector sea urchin", (Linnaeus1758), found in the central and western Pacific Ocean as well as the Indian Ocean. The three species are morphologically very similar and it has been suggested that they may constitute a single species (Clark, 1912; Mortensen, 1943; Zigler & Lessios, 2003). Work by Zigler & Lessios (2003) using mitochondrial DNA analysis (cytochrome oxidase I gene region) has shown that the Eastern Pacific and Indo-West Pacific *Tripneustes* do not differ from one other, but that they do differ from the Caribbean and Brazilian *Tripneustes.* Lessios *et al.* (2003) stated that the major barriers responsible for *Tripneustes* evolution and distribution are (1) the long stretch of deep water separating the eastern from the western Atlantic, (2) the Isthmus of Panama, (3) the cold water upwelling occuring close to the tip of South Africa and (4) the freshwater plume of the Orinoco and the Amazon rivers between the Caribbean and the coast of Brazil.

Figure 1.5: Species of *Tripneustes: T. depressus* (A), *T. gratilla* (B), and *T. ventricosus* (C) [(A) Teresa Zubi, www.starfish.ch (B) R.Scholtz, (C) Derek Otto, www.pegndereksnorkeling]

1.6.2 *Geographic distribution*

Tripneustes gratilla has a circumtropical distribution which extends into the sub-tropics. Mortensen (1943) reported *T. gratilla* throughout the Indo-West Pacific from East Africa (Red Sea to Natal), to the South Sea Islands (from the Norfolk and Kermadec Islands to the Marquesas and Hawaii), and from Australia (to Port Jackson on the east coast and Sharks Bay on the west), to southern Japan (with the Bonin Islands). In South Africa, *T. gratilla* distribution extends northward from the eastern regions of the Eastern Cape province (Marshall *et al*., 1991), with the most southern distribution recorded at Haga Haga, in the Eastern Cape, just north of East London: 32°45′4.23″S, 28°16′41.30″E (personal observation). Modelled inshore temperature data (AJ Smit, JJ Bolton & RJ Anderson, *in prep*.) show that this population occurs in a 50km coastal section where the maximum and minimum monthly means are 20.0 and 17.9°C respectively, but not immediately south, where the corresponding means are 19.4° and 17.9°C. *T. gratilla* distribution is limited mainly by seawater temperature (Lawrence 2007) and salinity (Lessios *et al.*, 2003). Tokioka (1966) found that *T. gratilla* almost completely disappeared from shallow waters at Seto, Japan after water temperatures decreased below 20°C from mid-November to mid-April 1965 (Tokioka 1966).

1.6.3 *Habitat and behaviour*

Tripneustes gratilla is most common in shallow, inshore waters although it has been found at depths up to 75m (Mortensen, 1943). They are found on a wide range of substrates from seagrass and algal beds (Klumpp *et al.,* 1993; Lyimo *et al.,* 2011), coral reef flats (de Loma *et al.,* 2002; Byrne *et al.,* 2004), and sand with rubble and rock (Schumacher, 1974). Elbert (1971) suggested that *T. gratilla* thrives in protected habitats, although it is capable of surviving a wide range of hydrodynamics.

In Madagascar, Maharavo (1993) found that *T. gratilla* behaviour led them to have a random distribution, whereas Nojima & Mukai (1985) found a tendency for pairing amongst individuals of this species. Covering behaviour, whereby urchins cover themselves with algae or seagrass, is observed in this species and is believed to have a number of functions. Petit (1930) believed that this behaviour could be photo-defensive, while Nojima & Mukai (1985) report that this behaviour could be linked to the collection of material for consumption, as they reported that inactive *T. gratilla* held material close to their test while active urchins, feeding or moving, did not cover themselves. It may also be linked to predator avoidance.

1.6.4 *Life cycle and reproduction*

Tripneustes gratilla generally reach sexual maturity within one year. The sexes are impossible to separate by their external morphology, unless the gonads are mature and gametes are released. In this instance, sexes maybe determined by looking at the colour of the gametes released, as the male semen is a light yellow to white in colour (Fig. 1.6A) while females usually release bright orange eggs (Fig. 1.6B).

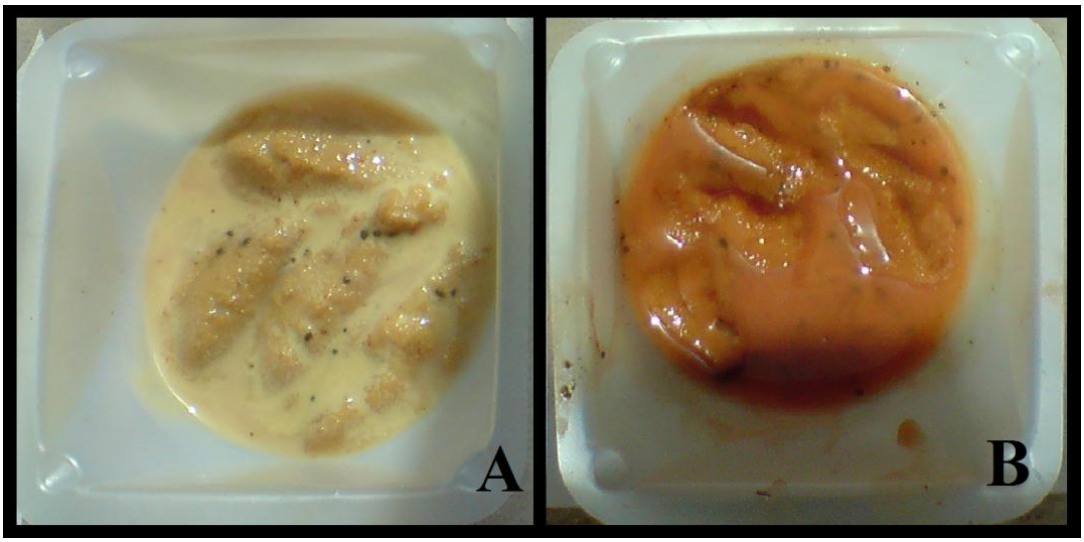


Figure 1.6: Male **(A)** and female **(B)** gonads of *T. gratilla* exhibiting the phenomenon known as melting: where gametes are release from the gonad.

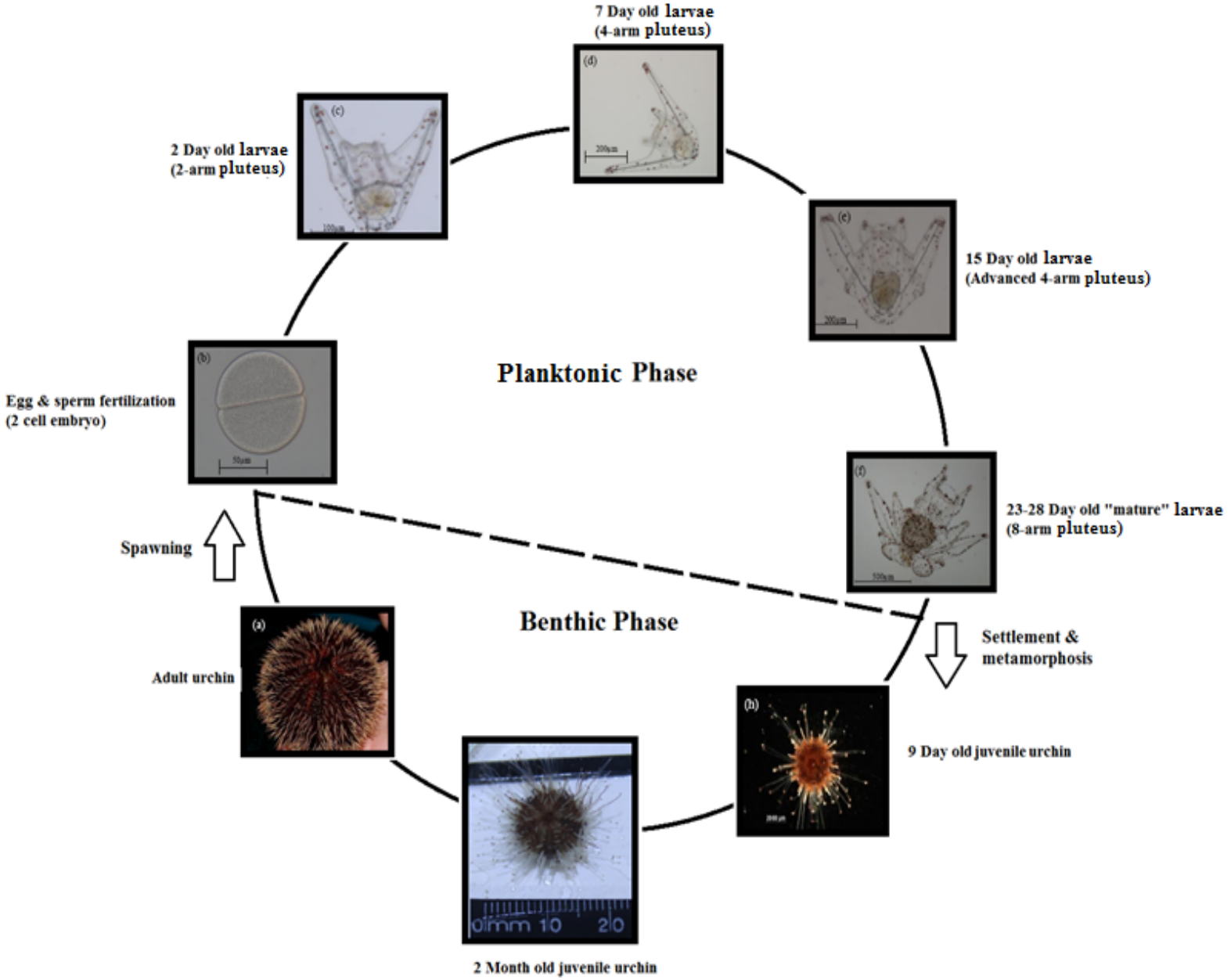


Figure 1.7: The life cycle of *T.gratilla (*Images adapted from: Scholtz, 2011)

Under natural conditions, the sex ratio of *T. gratilla* in the wild has been recorded as 1:1 (Fouda & Hellal, 1990; Muthiga, 2005). In the wild, gonads start to develop when urchins reach about 50mm in diameter, however this can vary markedly between populations, depending primarily on their geographic location and food availability. *Tripneustes gratilla* is a broadcast spawner, meaning that both eggs and sperm are released directly into the sea water column where fertilization and embryonic development occur (Hyman, 1955; Bruce,

1988). Spawning is largely synchronised, so that as many urchins as possible release eggs and sperm together to maximise their chance of achieving fertilisation. Sea urchin larvae and all developmental stages are pelagic and planktonic up until settlement and metamorphosis, whereafter urchins become benthic (Fig. 1.7). Sea urchins have a complex life cycle (Fig. 1.7) that is divided into 5 stages, namely (1) fertilization of the egg; (2) development through blastula and gastrula; (3) development to a pluteus larvae; (4) growth and development of feeding pluteus to a mature larva; and finally (5) metamorphosis and growth to a juvenile urchin (Hinegardner, 1969; Scholtz *et al*., (2012).

Studies dealing with the reproductive cycle of *T. gratilla* document variable spawning periods related to geographical distribution (O'Connor *et al.,* 1976; Chen & Chang, 1981; Lawrence & Agatsuma, 2001). A study by Maharavo (1993) in the Indian Ocean, on the north coast of Madagascar, reported year-round gametogenic activity with a peak spawning period in late winter. This is a common occurrence in some tropical echinoid species located near the equator, where the breeding season is spread throughout the year. Farther away from the equator, however, spawning occurs over a restricted period (Pearse & Cameron, 1991). The spawning season of *T. gratilla* is varied and dependent on location, it has been reported from summer to autumn off the coast of Kenya (Muthiga, 2005), winter in the northern Red Sea (Pearse, 1974), summer off the coast of Japan (Kobayashi, 1969), spring to autumn on the Great Barrier Reef, Australia (Stephenson, 1934) and in autumn in Taiwan (Chen & Chang, 1981). This variability in the reproductive cycle is generally explained by reference to environmental factors such as photoperiod, temperature, food availability and hydrodynamism, which are themselves subjected to periodic cycles which vary in intensity according to geographical location. Higher seasonal variability in temperate regions results in annual spawning which appears to become extended/year round as in more tropical regions. On the temperate east coast of South Africa, Baliwe (2010) reported that reproduction of *T.*

gratilla at Tshani (31^{o} 56, 765'S; 29^{o} 12,312'E) was seasonal, with recovery of gonads occurring from late spring to mid-autumn and spawning starting in early winter to spring.

1.6.4 *Gametogenesis*

Tripneustes gratilla gonads are made up of two main cell types, the nutritive phagocytes and germ cells. Germ cells in females consist of the ovum, oogonium and oocytes. In males, the germ cells comprise the spermatocyte, spermatogonium, spermatozoon and spermatid (Yokota *et al*., 2000). Nutritive phagocytes are somatic cells which store nutrients necessary for gametogenesis, such as proteins, carbohydrates and lipids (Walker, 1982). The proportion of nutritive phagocytes and germ cells present in the gonad varies throughout the reproductive cycle, and has a significant effect on the size and quality of gonads (Yokota *et al*., 2000; Brewin, 1994; Buisson, 2001; Fell, 2002). A clear understanding of the reproductive cycle of *T. gratilla* and how it can be manipulated by diet, photoperiod and/or feeding regimes is important for successful cultivation of this species, as the gonad or roe is the only part of the urchin that is of any commercial value. Gonad development of *Paracentrotus lividus*was described and allocated six different maturity stages by Byrne (1990) and Spirlet *et al*. (1998), and this classification has subsequently been used in a number of studies (Vaïtilingon *et al.,* 2005; Cyrus *et al.,2014*) to quantify the maturity of *T. gratilla* gonads. Maturity stages are based on oocyte size in females, the thickness of peripheral spermatocyte layer in males and the amount of non-germinal nutritive tissues in both males and females and are categorized as follows: (1) recovery; (2) growing; (3) premature; (4) mature; (5) partly spent and (6) spent (Fig. 1.8).

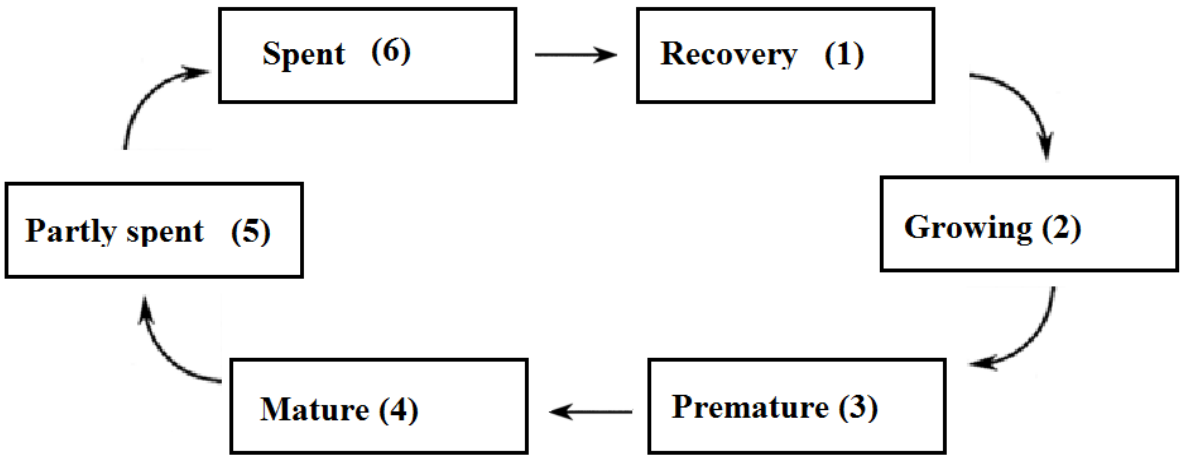


Figure 1.8: Gonad maturity cycle as classified by Byrne, 1990

The reproductive stage of the gonads affects both their size (represented by gonad somatic index or GSI) and quality and subsequently their value (Fig. 1.9). Gonads that are in a late recovery, growing or early premature stage are the most valuable commercially. The cues that stimulate gametogenesis are not fully understood, but the primary cues are believed to be nutrition, changing photoperiod (Pearse *et al.,* 1986; McClintock & Watts, 1990; Walker & Lesser, 1998; Shpigel *et al.,* 2004; Böttger *et al.,* 2006; Harrington *et al.*, 2007) and/or temperature (Spirlet *et al.,* 2000; Shpigel *et al.,* 2004; James & Heath, 2008). Although sexually mature animals are required for the induction of spawning and the subsequent farming of juvenile offspring, the large majority of adult urchins cultivated commercially are harvested in the initial stages of gonad development prior to the onset of gametogenesis, when they are considered to be the most desirable for human consumption (Shpigel *et al.,* 2004).

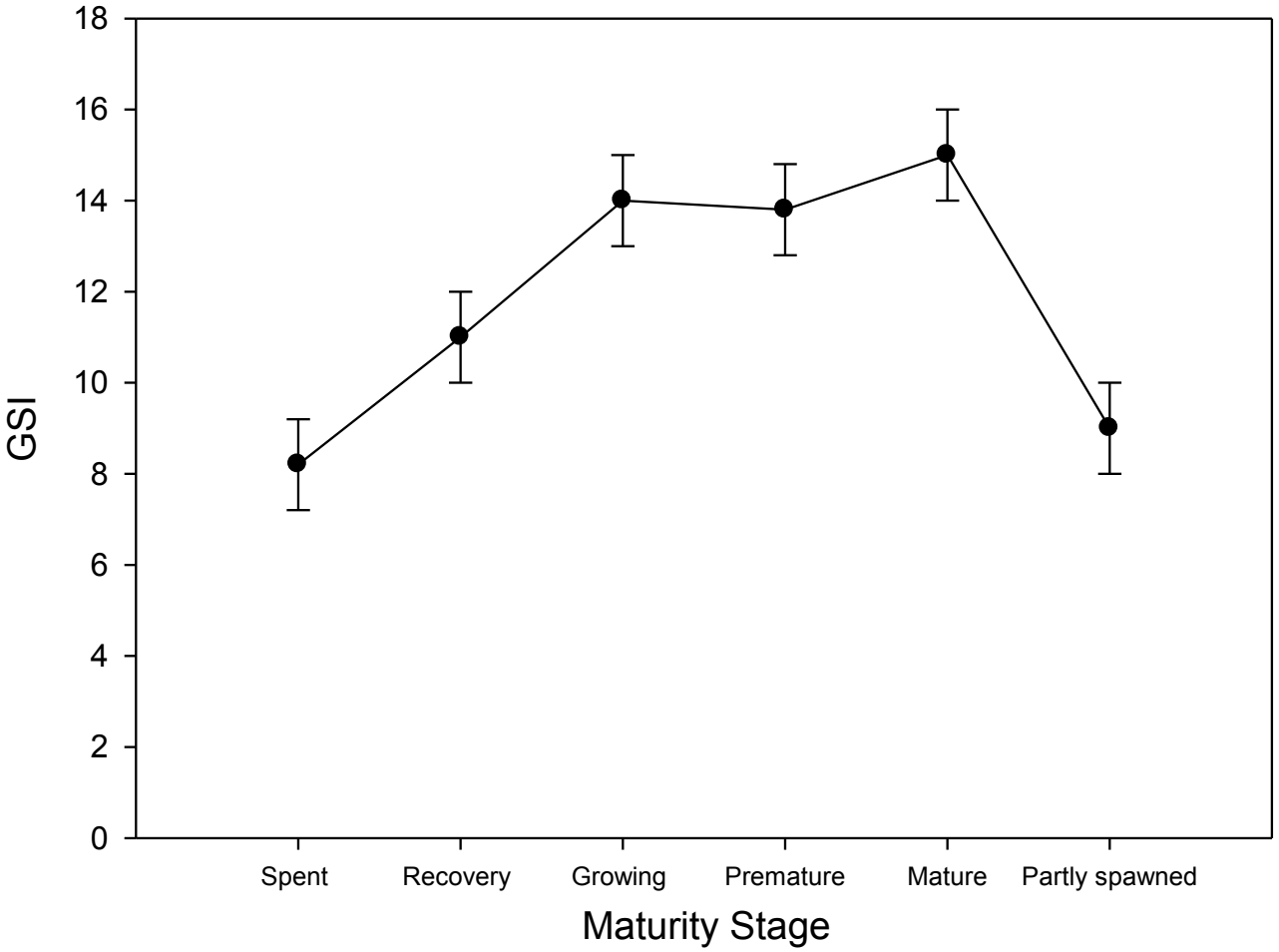


Figure 1.9: The relationship between gonad size (GSI) and maturity stage of wild *T. gratilla* from Tshani (31°56, 765'S; 29°12, 312'E) on the eastern coast of South Africa (Baliwe, 2010).

1.6.5 *Characteristics of* T. gratilla *suited to aquaculture*

According to Lawrence & Bazhin (1998) there are specific life-history traits that make certain sea urchin species more suitable for aquaculture than others. These include (1) growth rate: high growth rates mean a shorter time to reach maturity and increased production; (2) longevity: most sea urchins have a negative correlation between longevity and growth rate meaning short lived species tend to grow faster; (3) reproductive effort: a species that has a high reproductive effort is advantages in aquaculture, compared to a species that allocates most of it energy to maintenance, resulting in low assimilation efficiency; (4) body structure:

allocation of resources to structural defences is characteristic of an urchin species with a low potential for aquaculture production; (5) capacity to feed: allocations of processed energy to growth and maintenance depend upon the amount of energy consumed; and (6) palatability: palatable eggs without secondary metabolites would be expected from species with greater production capacity.

If production alone is used as the criterion for choosing a sea urchin species for aquaculture, the most appropriate species would be those that have the shortest time to maturity, highest fecundity and short life span (Lawrence & Bazhin, 1998). For sea urchin aquaculture to be successful, selection of the right species of urchin for cultivation with the correct traits is required. The reasons why *T. gratilla* is well suited to echinoculture are numerous and the specific traits are listed below:

(1) High growth rate: According to Lawrence & Bazhin (1998), toxopneustids, the family to which *T. gratilla* belongs, have high growth rates in nature and therefore are better suited for aquaculture compared to the other two urchin groups, the echinids and strongylocentrotids. In a study by Lawrence (2007), three different species of urchins *Echinometra mathaei*, *Diadema setosum* and *T. gratilla* were fed the same algal turfs for a period of 6 months during which time the growth rates of the different species were measured. Growth rates between the different species revealed that *T. gratilla* increase in size by 10 347% compared to *E. mathaei* and *D. setosum* which increased by 996 and 2 982% respectively. *T. gratilla* is also considered to have substantial advantages for, because it only takes 8-9 months from fertilization for urchins to reach marketable size and therefore readiness for roe harvesting (Williamson, 2002).

(2) Short lived species: Elbet (1982) showed that *T. gratilla* was essentially an annual species with a life span of generally one year. Bacolod and Dy (1986) found that annual

mortality of *T. gratilla* in the central Philippines was 99%, while Shimabukuro (1991) found that *T. gratilla* seldom lived more than 2 years at Okinawa, Japan. Personal observations of this species under brood stock conditions tend to indicate that this species can, however, live for much longer periods, surviving for more than 5 years in captivity.

(3) High reproductive effort: O'Connor *et al.* (1976) found that *T. gratilla* in the Solitary Islands off the coast of Australia had mature ova and sperm throughout the year. In Madagascar, gametogenic activity is seen in *T. gratilla* populations throughout the year as well (Maharavo, 1993).

(4) Body structure: *Tripneustes* spp. appear to allocate more energy to reproduction than to protection and maintenance (Pena *et al.,* 2010). Therefore, they grow rapidly and have roe production at an early stage, making them more suitable candidates for aquaculture.

(5) Capacity to feed: Lawrence & Bazhin (1998) consider it probable that feeding rate differences are responsible for the greater productivity of species such as *P. lividus*, *Tripneustes* spp. and *Strongylocentrotus* spp. Schumacher (1974) observed that *T. gratilla* was a diurnal grazer, feeding during the entire day, which is in agreement with Klumpp *et al.*, (1993), who demonstrate that the feeding of *T. gratilla* was continuous.

(6) Palatability: Families such as the toxopneustids and strongylocentrotids are more ruderal and competitive and therefore more likely to have gonads with palatable eggs without secondary metabolites (Lawrence & Bazhin, 1998).

(7) Market Acceptance: Roe from *T. gratilla* is of high quality and has excellent market acceptance (Dworjanyn *et al.,* 2007). In Japan *T. gratilla* is known as "white uni" and is considered to be among the best quality roe at the Tokyo Market (Williamson, 2002).

1.7 Objectives of this thesis

The aim of this study was to increase our understanding of the mechanisms whereby large, well-pigmented gonads, of a high market acceptance, are produced by the sea urchin *T. gratilla*. A greater understanding of these mechanisms would facilitate the development of appropriate artificial feeds, feeding regimes and culture technology for the production of this species in aquaculture operations. One of the primary aims of most aquaculture operations is to maximise the growth rates of the organism(s) being cultured; however since the gonads of a sea urchins are the harvested product, there are two different stages of growth that need to be considered when developing a feed(s). The first is juvenile somatic growth, which should be maximised until an individual reaches marketable size. The second is to promote the production of the gonads via the accumulation of nutritive cells in gonadal tissues, in order to maximise gonad yield and quality after sexual maturation. It is well established that natural and artificially formulated diets can have significantly different effects on growth and gonadal development of cultured urchins. Natural macroalgal diets typically promote development of brightly coloured gonads which are small in size, while high protein artificial diets generally promote increased gonad mass but poor gonad colour. It is hypothesized that the addition of a preferred macroalgal species (*Ulva*) to an artificial diet could significantly improve feed palatability and enhance gonad quality factors such as colour, texture and firmness. It is further hypothesized that *Ulva*-supplemented artificial diets will enhance somatic and gonadal growth of urchins under aquaculture conditions. The specific objectives of this study were formulated to test these hypotheses under controlled laboratory conditions and were to:

(1) Investigate the effects of *Ulva*-supplemented artificial feeds on feed palatability, consumption and digestibility;

(2) Determine whether *Ulva*-supplemented feeds promote uniform gonad development, yield and quality of adult wild-caught urchins (gonad enhancement);

(3) Investigate the effects of different feeds and feeding regimes on juvenile somatic growth and gonad development to establish cost effective feeding strategies for full life-cycle production;

(4) Investigate the impacts of Short and Long daylength on gonad development, quality and maturity in an attempt to suspend gametogenesis in adult urchins; and finally

(5) Track the incorporation of important dietary ingredients into the gonad through the use of stable carbon and nitrogen isotopes.

CHAPTER 2:

The advantages of using *Ulva* (Chlorophyta) as an additive in sea urchin formulated feed: Effects on consumption and digestibility

Contents

2.1 Abstract

In this study four protein-rich artificial diets supplemented with varying amounts of the macroalga *Ulva* (0%, 5%, 15% and 20% weight/weight (w/w); designated 0U, 5U, 15U and 20U, respectively) were formulated.*Ulva* was selected as it was the most preferred algal species for the sea urchin *Tripneustes gratilla,* in pair-wise consumption trials conducted with *Ulva rigida, Ecklonia maxima, Porphyra capensis* and *Gigartina polycarpa.* The aim of the study was to determine whether the addition of *Ulva* to artificial diets could act as a feeding attractant and affect the consumption and digestibility of the artificial diets over a 20 day experimental period. Findings from this study indicated that the inclusion of *Ulva* at a level of 20% significantly improved the chemosensory characteristics of the feed, making it equally as attractive as fresh *Ulva* (FU) and significantly more attractive than the basal diet that had not been supplemented with *Ulva* (0U). Improved palatability of the formulated feeds resulted in increased consumption and acceptability of the diets and, in turn, boosted protein intake. These findings will be extremely useful in commercial sea urchin operations where a large proportion of artificial feed ingredients are not components of an urchin's natural diet and thus may not invoke a foraging response, reducing feed intake and ultimately growth rates.

2.2 Introduction

For echinoculture to be economically viable, high-quality feeds need to be formulated to promote fast growth rates and improve the market acceptability of urchin gonads. Over the last decade, numerous studies have demonstrated that artificial diets can enhance somatic growth and gonad yield of sea urchins (Lawrence *et al.*, 1997; Olave, *et al.*, 2001; Shpigel *et al.*, 2005; Cyrus *et al.*, 2014), with protein (Pearce *et al.*, 2002a, b; Pearce *et al.*, 2004) and energy (Schlosser *et al.*, 2005) identified as two of the main contributing factors. The total protein or energy content of an algal or prepared diet may not, however, represent the true quantity of nutrients that are actually available to and absorbed by an organism, and it is, therefore important to calculate the nutrient digestibility of dietary components within a feed to formulate feeds that are biologically and economically suitable.

Nutrient digestibility or absorption efficiency represents the difference between the amount of feed consumed and the amount of faeces excreted, or the proportion of a substance that a living organism absorbs across exchange boundaries (e.g. gastrointestinal tract). The delivery of dietary components, such as protein and energy, is usually manipulated by varying the digestibility or concentration of particular ingredients within the feed, although this can also be achieved by changing the amount of feed actually consumed by a cultured organism (Jobling *et al.*, 2001). One way in which this can be achieved is by increasing the palatability of an artificial diet through the addition of feeding stimulants or attractants (Dworjanyn *et al.*, 2007). In studies on fish, Kasumyan & Døving (2003) showed that by giving artificial feeds desirable chemosensory characteristics, both the consumption and digestibility of the feed can be increased. The addition of feeding stimulants and attractants have also been identified as being potentially useful for increasing the palatability of diets that utilise sources of protein that are not normally consumed by the cultured organisms (Dworjanyn *et al.*, 2007).

Most prepared sea urchin diets contain 20 - 40% fishmeal proteins and generally have absorption efficiencies which are greater than 60% (Fuji, 1967; Klinger *et al.,* 1998; McBride *et al.,* 1998; Akiyama *et al.,* 2001). Akiyama *et al.* (2001) showed that a dietary protein level of 20% was optimal for growth in a purified diet for young red sea urchins, *Pseudocentrotus depressus.* Kuroki & Tashiro, (1991), however suggest that protein requirements for juveniles of both *Pseudocentrotus depressus* and *Tripneustes gratilla* should be closer to 30%. Increasing consumption and digestion of aquaculture feeds is thus particularly desirable as feeds can account for more than half of the variable operating costs, with protein being the most expensive nutrient in prepared diets (Fleming *et al.,* 1996). The high cost of fishmeal and shortages due to its increasing requirement in formulated feeds in general poses problems (Deutsch *et al.,* 2007; Olsen & Hasan, 2012) and therefore improving feed consumption and digestion under intensive aquaculture conditions is a high priority.

Sea urchins are considered to be generalist herbivores in their feeding habits, although it has been demonstrated that certain echinoids display a hierarchy of preferences when offered a choice of natural diets, strongly preferring some algae species over others (Prince & LeBlanc, 1992; Solandt & Campbell, 2001; Dworjanyn *et al.,* 2007; Stimson *et al.,* 2007). These preferences have been attributed to factors that include the nutritive value and physical properties of the food as well as the presence of attractant and/or absence of deterrent compounds within a feed (Dworjanyn *et al.,* 2007; Stimson *et al.,* 2007). Dworjanyn *et al.* (2007) simultaneously offered *Tripneustes gratilla* seven different species of seaweed, to determine whether this species displayed any major consumption preferences, and found that *T. gratilla* strongly preferred *Ecklonia radiata*, but also readily consumed *Sargassum linearifolium* and *Ulva lactuca.* The same authors went on to incorporate 5% (dry weight) of each of the above mentioned algal species into three separate artificial feeds to act as a feeding stimulant, and demonstrated that *T. gratilla* consumed twice the mass of the diet

containing *S. linearifolium* compared to the control diet, which did not contain a feeding stimulant. The addition of algae as a feeding stimulant did not significantly affect the nutritional value of the artificial diet, but instead encouraged feeding behaviour of the sea urchins.

The aim of the current study was to investigate whether incorporating a dried alga into an artificial diet would affect *Tripneustes gratilla*'s preference for and consumption of the diet, as well as the protein and energy digestibility of the formulated diet. To identify the most appropriate algal species to add to the diet, the preference of *T. gratilla* for four, locally abundant seaweed species (*Ulva rigida, Ecklonia maxima, Porphyra capensis* and *Gigartina polycarpa*) was tested, before drying and incorporating the most preferred species into an artificial feed, previously formulated for *T. gratilla* (Cyrus *et al.*, 2014). Different inclusion levels (0, 5, 15 and 20% weight/ weight (w/w)) of the most preferred algal species were then tested on apparent protein and energy digestibility, consumption and the attractant properties of the feed.

2.3 Materials and methods

2.3.1 Collection and maintenance of Tripneustes gratilla

Adult *Tripneustes gratilla* (50–70 mm test diameter) were collected during low tide from shallow rock pools near Haga-Haga, Eastern Cape, South Africa (32°45′4.23″S, 28°16′41.30″E) on 15–17 September 2008 (Fig. 2.1). Haga-Haga has a warm temperate marine regime (Stephenson, 1948), with a mean annual seawater temperature of 17.7°C, with mean temperatures for the coldest and warmest months of the year being 15.8 and 21°C respectively (Bolton 1986). These temperatures represent the lower limits of the species range and are, in fact, below the preferred range for this species, which is from 18 to 29°C

(Rahman *et al*., 2009). *Tripneustes gratilla* larvae, juveniles and adults are typically cultured at a temperature of 24–25°C (Dworjanyn *et al*. 2007; Dworjanyn & Pirozzi, 2008).

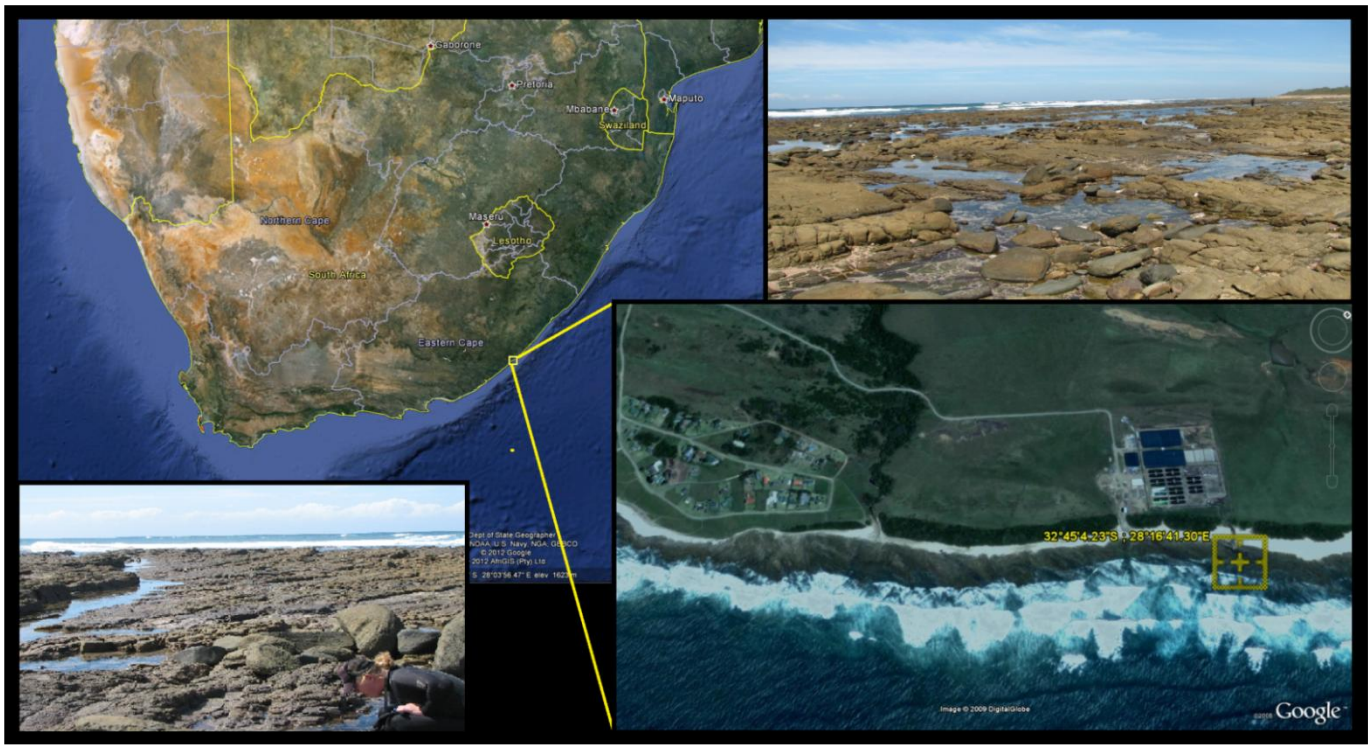


Figure 2.1: Location of collection site and surrounding area. (Google Maps 2009)

Following collection, animals were transferred to the Department of Agriculture, Forestry and Fisheries (DAFF) Aquaculture Research Facility in Cape Town, South Africa. Sea urchins at this facility were held in plastic crates (L x W x H: 60 x 40 x 20 cm; with W x L: 3 x 30 mm slits along the sides and bottom) suspended in four large fibreglass tanks (L x W x H: 282 x 182 x 50 cm), supplied with heated, recirculating seawater maintained at a salinity of 35 and at 24 - 25°C under fluorescent lights set to provide a 12:12 day. Seawater flowed through each tank at a rate of 10 $L.min^{-1}$ and the tanks were constantly aerated. The recirculating seawater systems were also equipped with a common sand filter, bio-filter and protein skimmer to maintain optimal water quality conditions. To reduce differences in gonad development and standardize nutritional condition, sea urchins were acclimated for at least 3 months and fed a diet of fresh kelp, *Ecklonia maxima*, every second day until they were used for the feed preference, chemosensory, consumption and digestibility trials.

2.3.2 Water quality monitoring

Water quality parameters were monitored weekly during each of the experimental studies. Dissolved oxygen (>90%) and pH (8.0 ± 0.2, mean ± SE) were recorded using a Cyberscan PD300 waterproof hand-held pH and dissolved oxygen meter (Eutech Instruments Pte/Oakton Instruments, Vernon Hills, IL, USA), while temperature and salinity were recorded using a hand-held WTW LF340 salinity and temperature meter (Wissenschaftlich-Technische Werksta¨tten, Weilheim, Germany). Ammonia and nitrates were measured weekly using Sera® ammonium/ammoniak- and nitrit-test kits, respectively, and values remained within the parameters for a healthy recirculation system (<0.003 and <0.001 mg L^{-1} for ammonia and nitrate respectively).

2.3.3 Paired preference test

Four species of macroalgae were used in the pair-wise preference tests: *Ulva rigida, Ecklonia maxima*, *Porphyra capensis* and *Gigartina polycarpa*. These species were chosen because they are abundant, accessible (collected during low tide from shallow rock pools outside of the DAFF Aquaculture Research Facility) and have previously been shown to be attractive to *T. gratilla* based on pair-wise touch preference tests that utilized these four macroalgal species as well as *Grateloupia capensis* (Scholtz, 2008). For the latter tests, touch preferences were established once urchins chose to touch an algal species with their lantern teeth.

The pair-wise preference tests performed in this study used the wet weight of the algae consumed as a measure of preference. Fresh algal material was offered in each pair-wise preference test. All algal species were thoroughly rinsed and cleaned with 0.4 µm filtered seawater to remove any epiphytes or organisms present on the algae prior to feeding. Algae were then allowed to drip dry in a crate for 10 minutes, before being placed into a salad spinner and spun for 30 seconds to remove excess water. Each algal offering was attached to

a rubber band which was subsequently attached to a flattened weight (± 115 g) to prevent any species from floating and thus becoming less accessible. Individual sea urchins (80-100 mm TD), which had been starved for 2 days prior to the start of the experiment, were placed into rectangular tanks (L × W × H: 51.4 × 35.8 × 41.2 cm) supplied with running seawater (salinity of 35 at 24 - 25°C) and constant aeration. Pre-weighed fresh algae (± 50g fresh weight) was offered to each urchin in all possible pair-wise combinations (N=3 for each combination) and the weight of each alga consumed was measured 48 hours later.

2.3.4 Preparation of experimental feeds

Five dietary treatments were investigated in this study. Four of the dietary treatments were artificially formulated and consisted of a semi-purified (contain natural ingredients in as pure a form as is available) 'basal' formula (of which all dry ingredients were milled to a constant particle size) supplemented with different amounts of dried macroalga *Ulva* spp. to achieve final concentrations of 0, 5, 15 and 20% (w/w). *Ulva* used in this study was produced in paddle raceway aquaculture systems at Irvine & Johnson (I&J) Cape Abalone farm (34^{o}34'60 S; 19^{o}21'0 E) and consisted of a mixture of predominantly *Ulva rigida* with some '*Ulva lactuca*' (Robertson-Andersson *et al.*, 2008; Bolton *et al.*, 2009; Shuuluka, 2011). South African *U. lactuca* (*sensu* Stegenga, Bolton & Anderson, 1997) is, however, not the same species as the *U. lactuca* known elsewhere in the world and currently has no valid name (L. Kandjengo, University of Namibia pers. comm).*Ulva* collected from the facility was dried for several days in ovens at 60°C before being ground to a fine powder using a hammer mill. Alternatively, a portion of the fresh algae was transferred to the DAFF Aquaculture Research Facility, where a live culture was maintained in a large cylindrical fibreglass tank (W×H*:* 130×100 cm) supplied with continuously flowing (300 L.h^{-1}) natural seawater at 15-18°C with natural light and with strong aeration to keep the *Ulva* suspended (Fig. 2.2). This culture was then used as a control diet of Fresh *Ulva* (FU), throughout the trial. The basal diets

supplemented with and without *Ulva* were formulated and manufactured at the Division of Aquaculture, University of Stellenbosch, South Africa. Diets were produced in the form of semi-moist extruded chips (L X W: 2 x 4 cm), which were dried to a constant weight in a drying oven at 70^{o}C and then frozen. To ensure that the diets remained functionally equivalent, an artificial "*Ulva* Additive", designed to have the same proximate composition as *Ulva* (Table 2.1), was created using different dietary ingredients (Table 2.2). Thus varying amounts of *Ulva* spp. could be substituted out of the treatments without significantly changing the nutritional value and characteristics of the feed. This was done by conducting a complete nutrient analysis on *Ulva* (Table 2.1) and using this analysis to formulate a suitable additive. The formulated dietary treatments (Fig. 2.3) were abbreviated as follows: 20% *Ulva* = 20U; 15% *Ulva* = 15U; 5% *Ulva* = 5U; 0% *Ulva* = 0U and Fresh *Ulva* = FU.

Figure 2.2: Tank in which *Ulva* was cultivated and maintained over the course of the study

Figure 2.3: Formulated feeds produced at the Division of Aquaculture, University of Stellenbosch, South Africa for the purpose of this study, ordered in decreasing *Ulva* content from left to right. Treatment groups: FU = fresh *Ulva*; 20U = 20% *Ulva*; 15U = 15% *Ulva*; 5U = 5% *Ulva*; 0U = 0% *Ulva*. Basal formula = FB.

Table 2.1: Nutrient composition (per g dry matter) of the *Ulva* collected from Irvine & Johnson (I&J) Cape Abalone farm in the Western Cape Province of South Africa for the preparation of the experimental diets utilized in this study.

	% Dry matter
Crude protein	18.310
Ash	32.660
Crude fibre	6.024
Crude fat	0.380
Nitrogen free extract (calc)	30.259
Phosphorus (P)	0.172
Potassium (K)	1.897
Calcium (Ca)	1.034
Magnesium (Mg)	4.310
Sodium (Na)	5.172
Iron (Fe)	0.007
Copper (Cu)	0.001
Zinc (Zn)	0.001
Manganese (Mn)	0.001
Bromine (Br)	0.006
Aluminium (Al)	0.006
Total	**100**

Table 2.2: The dietary ingredients and ratios at which they were combined to formulate the *Ulva* additive.

Ingredient	**Ration (%)**
Maize	45.89
Wheat bran	22.00
Maize gluten 60	10.00
Hamlet protein 300	8.30
Lucerne meal 15%	5.00
Potassium chloride	2.48
Dicalcium phosphate	2.16
Oil - sunflower	1.50
Limestone	1.00
Salt	1.00
Sodium bicarbonate	0.67
Total	**100.00**

2.3.5 Nutrient analysis

All nutritional analysis was conducted at the Department of Food and Animal Science, University of Stellenbosch, South Africa. The four artificial diets, as well as *Ulva*, were analysed to determine their crude protein, fat, moisture, ash, fibre, carbohydrate and gross energy contents, using protocols described by AOAC International (2002). This analysis was then used to ensure that the formulated diets were similar in nutritional content, even though they contained varying amounts of *Ulva* and/or *Ulva* Additive. The results of the nutrient analysis are presented in Table 2.3.

Table 2.3: Nutrient analysis of fresh *Ulva*, the *Ulva* additive, the basal formula (no *Ulva* or *Ulva* additive included) and the four prepared diets fed to *Tripneustes gratilla* during the experiments (per g dry matter).Treatment groups: FU = fresh *Ulva*; 20U = 20% *Ulva*; 15U = 15% *Ulva*; 5U = 5% *Ulva*; 0U = 0% *Ulva*.

	FU	**20U**	**15U**	**5U**	**0U**	***Ulva* additive**	**Basal formula**
Protein (%)	18.31	25.69	26.38	26.58	26.48	18.88	28.60
Fat (%)	0.38	2.31	3.52	3.16	2.72	0.68	3.77
Moisture (%)	15.30	9.61	9.45	8.52	8.55	9.61	8.52
Ash (%)	32.66	13.89	11.44	8.69	7.57	9.12	7.46
Gross energy ($MJ.kg^{-1}$)	9.44	15.49	16.22	16.83	17.18	15.89	17.36
Fibre (%)	6.02	4.75	5.44	5.32	6.07	4.61	4.2
Carbohydrate (%)	27.33	43.75	43.76	47.73	48.61	57.10	47.45

2.3.6 Feed stability

As prepared feeds may leach organic material upon immersion in seawater (Caltagirone *et al.,* 1992), which may impact consumption and digestibility estimations, feed stability tests were conducted. Triplicate samples of each formulated feed (1.13 ± 0.055 g) were added to baskets (L×W×H: 40 × 30 × 16 cm), lined with oyster mesh (8 mm^2 slits) along the bottom,

that were suspended in 100 L cylindrical black tanks (*W*× *H:* 48 × 56 cm). The tanks were supplied with heated re-circulating seawater maintained at a salinity of 35 and 24-25°C and fine aeration, as per the consumption and digestibility tests described below. The stability of each feed was assessed as the percentage dry matter lost from feed in seawater over 24 h. Feed removed from the baskets was dried in a drying oven at 60°C until a constant weight was reached. The dry weight was subsequently recorded to the nearest 0.001 g using an electronic balance and the dry matter loss calculated according the formula from Senaratna *et al.* (2005):

$$\text{Dry matter loss (\%)} = [(DM_0\text{-}DM_t) \div DM_0] \times 100$$

where DM_0 is the initial dry matter of the feed and DM_t is the dry matter of the same feed after being immersed in seawater for 24 hours.

2.3.7 Consumption and digestibility trial

In order to determine daily feed consumption and apparent digestibility coefficients for protein and energy for each feed (20U, 15U, 5U, 0U & FU), a 20 day consumption and digestibility trial was conducted from 8 – 28 October 2009.

Adult *T. gratilla* that were acclimated for 3 months on a diet of fresh *E. maxima* were transferred to plastic baskets (L×W×H: 40×30×16 cm; 8 urchins per basket) that were suspended in 100 L cylindrical black plastic tanks (Fig. 2.4). The base of each plastic basket was lined with 8 mm^2 oyster mesh, which retained uneaten food and allowed faecal pellets to fall through. There were a total of 10 experimental tanks available, thus allowing for each feed treatment to be duplicated. The experimental tanks were supplied with heated re-circulating seawater maintained at a salinity of 35 and 24 - 25°C, with a 12h light cycle. Seawater flowed through each tank at a rate of 2 L min^{-1} and each tank was supplied with fine aeration. The re-circulating seawater systems were also equipped with a common sand filter,

bio-filter, protein skimmer and UV light treatment to maintain optimal water quality conditions. Sea urchins in each tank were fed a measured amount (≈ 20 g) of food at 10:00 am once every second day. Food was available in excess of what urchins would consume at all times. The remaining uneaten food was carefully removed after 24 h from each basket and placed into individual pre-weighed drying trays. Excess water was removed from the algae by placing it into a salad spinner and spinning for 30 seconds before recording the wet weight to the nearest 0.001 g using an electronic balance. All feeds were subsequently dried to constant weight at 60°C.

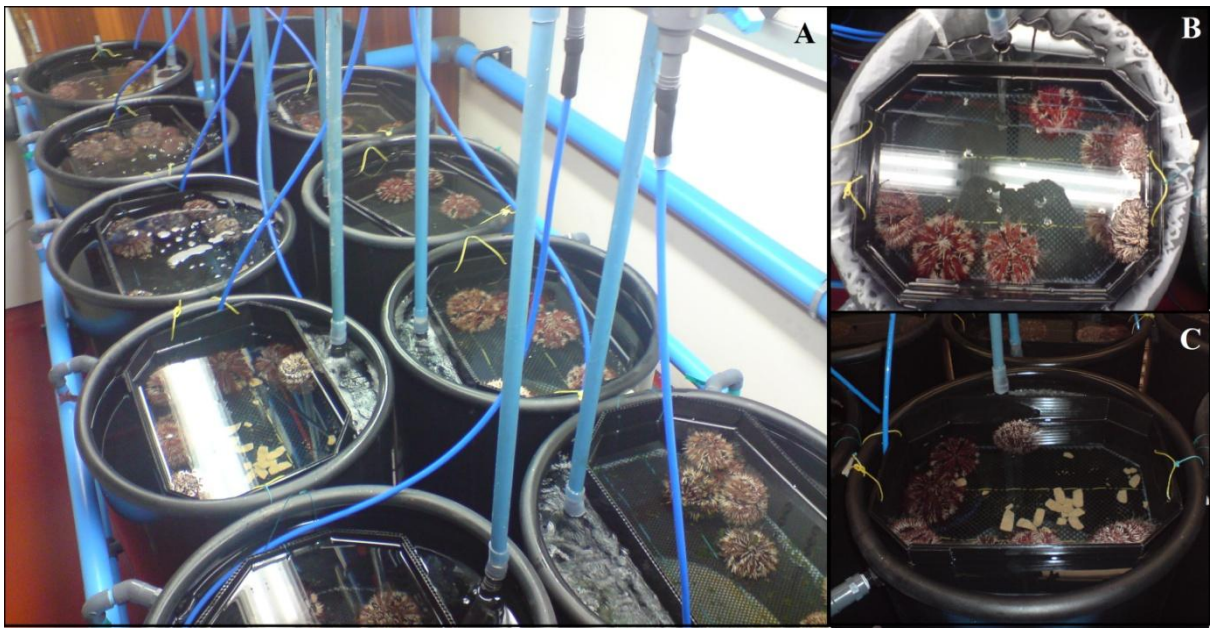


Figure 2.4: (A) Experimental design of the consumption and digestibility trial, showing **(B)** tanks fitted with 200µm nylon mesh bags for faecal collection and **(C)** with bags removed to prevent the collection of uneaten food.

The dry weights (DW) were recorded as described above and daily feed consumption per individual urchin was calculated according to the following formula:

$$\text{Daily Feed Consumption (DFC)} = (F_g - F_u) \div (\#\text{Urchins}_t)$$

where F_g is the weight of the food supplied, F_u is the weight of the uneaten food and $\#\text{Urchins}_t$ indicates the number of urchins in a basket at a specific sampling time (t). Mean

daily feed consumption rates per individual urchin for each treatment group were then determined by calculating the average consumption over the 20 day experimental period (N= 9 sampling dates per duplicate treatment group). Mean daily feed consumption (DFC) is reported as grams of dry food consumed per animal per day.

To allow for the collection of faeces for determining the absorption efficiency of the five tested feeds, faecal collection bags made from 200µm nylon mesh were placed around each basket immediately after the removal of uneaten feed, as described above. This practice avoided contamination of faeces with uneaten food that could be dislodged during feeding. Although these pieces are not large enough to significantly affect consumption measurements, they could potentially influence digestibility results. Faecal material was collected for 24 h before removing the collection bags, immediately prior to the next feeding event. Faeces collected in each bag were then transferred to individual drying trays and dried in an oven at 60°C to constant weight. Following drying, any urchin spines that may have been lost during feeding were carefully removed from each sample to prevent possible effects on digestibility values. To obtain sufficient faecal material for analysis, the approach of Lares (1999), Hammer *et al.* (2004) and Schlosser *et al.* (2005) was adopted and faecal samples from each tank were pooled after every third sampling day, resulting in six pooled samples per treatment group. Pooled dried samples were subsequently transferred to separate sealed glass vials and stored at room temperature until needed for further analysis.

Apparent digestibility coefficients for protein and energy for pooled samples (N = 6) from each feed treatment were calculated indirectly based on the formulas described by Lowe and Lawrence (1976), where ash was used as an indigestible marker. The use of inert markers, particularly ash, to determine apparent nutrient digestibility coefficients of feed ingredients are favoured over total faecal collection or gravimetric methods as they do not require total faecal collection or evacuation (Lares, 1999; Shipton & Britz, 2001a,b). These methods do

however assume that the selected marker is indigestible, is accumulated in the faeces, and passes through the gut at the same rate as any other components entering it (Arrontes, 1989). In this study, ash content was determined gravimetrically after combustion of samples in a muffle furnace at 400°C for 24 h (Paine, 1971).The weight of each sample was subsequently recorded to the nearest 0.001 g using an electronic balance. Crude protein and gross energy contents of the diets and faecal matter were determined using the protocols described by AOAC International (2002). Pooled oven-dried samples from each tank were analysed in triplicate. All analyses were conducted at the Department of Food and Animal Science, University of Stellenbosch.

Apparent Digestibility Coefficients (ADC) were calculated according to the following formula (Schlosser *et al.*, 2005):

$$ADC\ (\%) = 100 - [100 \times (DIA_{food} \div DIA_{faeces}) \times (\text{energy or protein}_{faeces} \div \text{energy or protein}_{food})]$$

Where DIA_{food} is the dietary insoluble ash in the food and DIA_{faeces} is the dietary insoluble ash in the faeces. Digestible protein (DP) and digestible energy (DE) in each diet was then calculated using the ADC values and diet compositions according to the following two formulae:

$$\text{Digestible protein (DP)} = (mg.g^{-1} \text{ total protein}) \times (ADC_{protein})$$

$$\text{Digestible energy (DE)} = (kJ.g^{-1} \text{ gross energy}) \times (ADC_{energy}).$$

DP and DE intake (DPI & DEI) were calculated as:

$$\text{DPI or DEI} = \text{DP or DE} \times (\text{dry feed consumed animal}^{-1} day^{-1}).$$

2.3.8 Chemosensory trial

To determine whether the dried *Ulva* incorporated into the artificial feeds tested in this study acted as a feed attractant, the 20U and 0U diets were tested against fresh *Ulva* (FU) in pair-wise chemosensory trials using a Y-shaped maze (Fig. 2.5A). The Y-shaped maze (L × W × H: 71× 30 × 15 cm) was constructed out of clear Perspex and modelled after the designs of Castilla & Crisp (1970) and Vadas (1977). Each branch of the Y-maze was supplied with heated seawater (25 ± 1°C) at a rate of approximately 8 $L.min^{-1}$. Water entering the branches first flowed into a small compartment at the top of each branch, which was separated from the remainder of the system by a piece of Perspex drilled with a row of 4 mm holes, approximately 2 cm lower in height than the remainder of the system. Feeds (≈ 5 g) to be tested in the chemosensory trial were added to this compartment once they were secured in 120µm mesh bags, thus allowing for chemical cues released from the feeds to enter the seawater and prevent feeds from being washed down the maze. Basic flow visualization experiments were conducted to assess plume dynamics within the maze, using food colouring as a dye. A Sony Ericson digital camera (Vivaz) was used to capture overhead images of the dyed plume (Fig. 2.5B). The dye tests clearly demonstrated that mixing of water/ chemical cues between the two arms only took place approximately mid-way along the length of the trunk section of the Y-maze.

Chemosensory trials were conducted on a total of 24 urchins (80 -100 mm test diameter) over the course of several days, with each urchin tested only once for a single trial on any given day. For each pair-wise chemosensory test, a single urchin was placed in the drained end (bottom) of the Y-shaped maze and their movements monitored and recorded over a 15 min period. Preliminary experiments indicated that urchins were capable of moving from the drained end of the maze into an arm within 5min. A choice was recorded when an urchin moved from the bottom of the Y-shaped maze and more than 10 cm up one of the arms

within 15 min. A 'no choice' was recorded when an urchin remained stationary or did not perform this task within the 15 min test period. Feeds (20U, 0U and FU) were tested in pairs or individually against a blank, where no feed was added to test for Y-maze arm preferences.

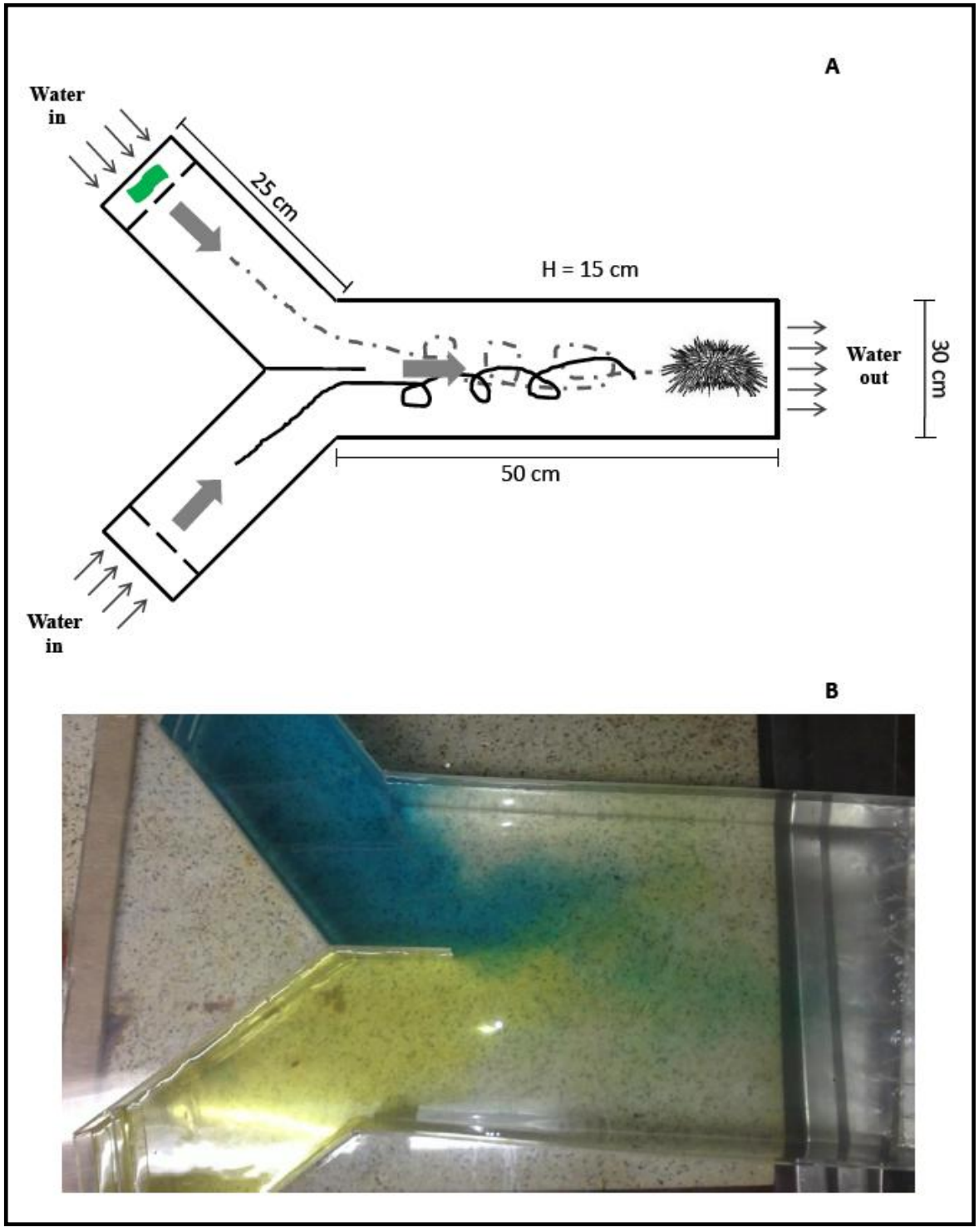


Figure 2.5: (A) Diagram of the Y-maze, used to test the chemosensory characteristics of the different feeds, **(B)** A photograph showing the mixing of the two water bodies from their separate arms; the two sources have been dyed with food colouring to demonstrate that the two water sources mix together uniformly.

After every 6th pair-wise chemosensory trial, the Y-shaped maze was emptied, washed out with seawater and then refilled to prevent urchins from following any physical or chemical signals left by urchins of previous trials. Fresh feeds were then returned to the short-arms, but were added to the opposite arm used in the previous 6 trials. Water was then allowed to flow through the maze for 10min prior to the start of a new trial.

2.3.9 Statistical analysis

SigmaPlot version 12 software was used to perform all statistical analysis. Student t-tests were used to test for significant differences in consumption between urchins offered pair-wise choices of the four macroalgal species tested. One-way analysis of variance (ANOVA) was then performed to test for significant differences in the average consumption of each macroalgal species over a 48 h period for all pair-wise preference tests combined. To test for significant differences in the percentage dry matter loss (feed stability) following 24 h immersion in seawater, paired t-tests were performed. One-way ANOVA was subsequently performed to test for significant differences between feed treatments. One-way ANOVA was also used to determine whether dry feed consumption differed between dietary treatment groups. To determine whether ADC values for protein and energy, DP, DE, and the DP & DE intake differed significantly between the prepared diets (FU treatment excluded), a one-way ANOVA was performed. The Holm-Sidak method was used for all post hoc multiple comparisons between individual treatment groups. Significant differences in the DP/ DE ratio between prepared diets were determined using one-way ANOVA. Tests for equal variance (Levene's test) failed for these data sets; therefore, a Kruskal-Wallis ANOVA on Ranks test was used to test for significant differences. The Tukey method was used for all post hoc multiple comparisons between individual treatment groups in the latter tests. Significance was assigned to p-values of < 0.05 for all analyses. For the chemosensory trial data analysis,

each trial was treated as a Bernoulli trial having one of only two possible outcomes. The exact probability of the outcome of a series of trials was then calculated.

2.4 Results

2.4.1 Paired preference test

Tripneustes gratilla displayed some significant preferences when offered pair-wise choices of four different macroalgal species (Fig. 2.6 A). Each macroalgal species was consumed at least once and significant preferences always involved *Ulva* as the most preferred species (paired t-test, $p < 0.05$). The average consumption of each macroalgal species over a 48 h period for all pair-wise preference tests combined (Fig. 2.6 B) showed that the *Ulva* spp. was significantly preferred (one-way ANOVA, $p < 0.001$) over all other macroalgae offered, with 10.61 ± 0.63 g (mean ± SE) of *Ulva* being consumed per urchin within a 48 hour period. *T. gratilla* consumed almost 40% less *Ecklonia maxima* (6.7 g) over the same time period and consumed much less of the remaining two species, *Gigartina polycarpa* (2.3 g) and *Porphyra capensis* (3.9 g). There was no difference in consumption between the latter two species (one-way ANOVA, $p = 0.148$) however *Ecklonia maxima* was consumed in significantly higher amounts.

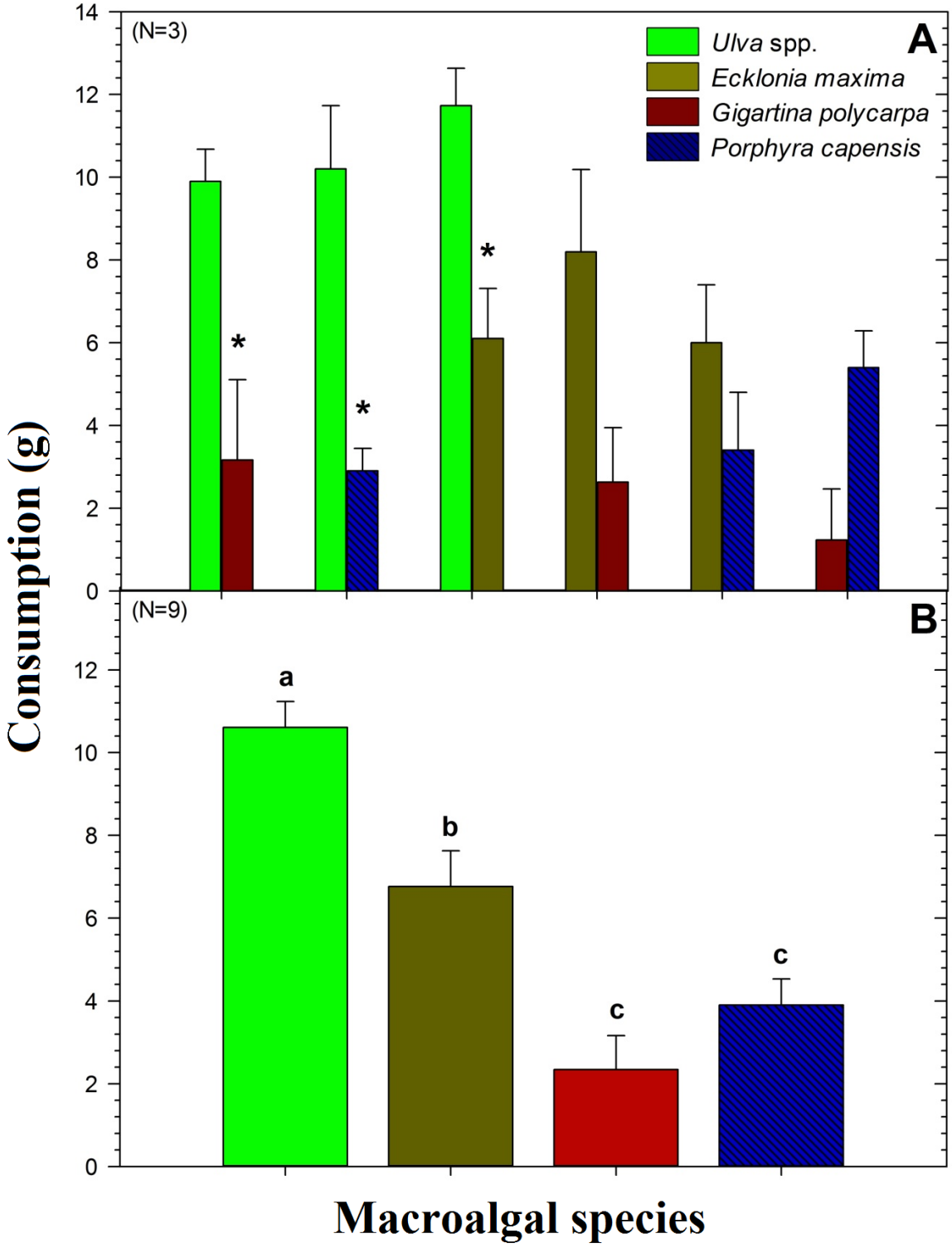


Figure 2.6: (A) Mean consumption of *T.gratilla* during pair-wise preference tests. Data represents mean ± SE of three replicates of each pair-wise combination. *($p < 0.05$, Tukey test) represents a significant difference in the means of urchins fed the different algal diets: *Ulva rigida, Ecklonia maxima,, Porphyra capensis* and *Gigartina polycarpa.***(B)** Mean consumption of the different algal diets (*Ulva rigida, Ecklonia maxima, Porphyra capensis* and *Gigartina polycarpa*) across all pair-wise preference tests. Data represents mean ± SE of nine replicates. Letters a, b & c ($p < 0.05$, Tukey test) represents a significant difference in the means of urchins fed the different algal diets.

2.4.2 Feed stability

The average dry matter loss for each prepared feed over a 24 h period (0.286 ± 0.016g) was significant (Student T-test, $p = 0.0003$). There was, however, no significant difference (One-way ANOVA, $p < 0.05$) in the percentage dry matter loss between any of the artificial diets tested (data not shown). All prepared feeds were readily accepted and consumed by *T. gratilla* within a few hours of being introduced into the tanks and handling of feeds did not appear to affect the stability of the feed. Fresh *Ulva* did not experience any significant weight gain due to growth over feeding periods and was not affected by handling.

2.4.3 Consumption and digestibility trial

Feed consumption on a dry matter basis differed significantly (One-way ANOVA, $p < 0.001$) among diets, with *T. gratilla* consuming significantly more of the formulated feeds than fresh *Ulva* (Fig. 2.7A).Of the prepared diets tested in this study, diets containing higher *Ulva* concentrations were more readily consumed. *T. gratilla* consumed significantly higher amounts of the 20U diet (1.96 $g.urchin^{-1}.day^{-1}$) than the 5U (1.68 $g.urchin^{-1}.day^{-1}$; $p=0.007$) and 0U (1.64 $g.urchin^{-1}.day^{-1}$; $p=0.002$) diets. Mean dry feed consumption rates were also significantly higher ($p = 0.03$) in urchins fed the 15U diet (1.88 $g.urchin^{-1}.day^{-1}$) compared with those fed the 0U diet. No significant differences were, however, observed between any other dietary treatment groups.

Table 2.4: Apparent Digestibility Coefficients (ADC%, Mean ± SE) for protein and energy, and the Digestible Protein (DP) and Digestible Energy (DE) content of diets fed to *Tripneustesgratilla* during the feed trials.

	Artificial diets				
	20U	**15U**	**5U**	**0U**	**Fresh *Ulva***
ADC (%)					
Protein	71.20±0.99[a]	65.83±0.58[b]	74.88±0.25[c]	70.24±0.97[a]	-165.91±3.08[†]
Energy	65.58±1.54[a]	64.98±0.32[a]	73.28±0.27[b]	70.66±0.78[b]	-77.08±2.32[†]
DP (mg.g^{-1})	182.89±2.56[a]	173.96±1.36[b]	199.06±0.66[c]	186.00±2.56[a]	†
DE (kJ.g^{-1})	10.12±0.24[a]	10.53±0.05[a]	12.28±0.04[b]	12.15±0.13[b]	†
DP/ DE ratio (mg.kJ^{-1})	18.09 ± 0.2[a]	16.53 ± 0.14[ab]	16.21± 0.04[ab]	15.31 ± 0.06[b]	†

Data are presented as the mean (± SE). Different letters indicate a significant difference (One-way ANOVA, $p < 0.001$) between values. A dagger (†) represents data that were not included in the statistical analysis.

Urchins fed fresh *Ulva* had negative absorption efficiencies for both protein and energy, when using ash as a marker, in the present study (Table 2.4). The percentage ash in the faeces of urchins fed fresh *Ulva* (27.04 ± 0.5) was similar to that recorded in the faeces of urchins fed the prepared diets (27.34 ± 1.54). In contrast, the percentage protein in the faeces of animals fed fresh *Ulva* (40.24 ± 0.17) was significantly higher (Mann-Whitney Rank Sum Test, $p < 0.001$) compared with urchins fed the prepared diets (20.46 ± 0.36), resulting in negative absorption efficiencies for both protein and energy for animals fed fresh *Ulva* (Fig. 2.7 B & C). As a result, data from this treatment group (FU) was excluded from the one-way ANOVA analysis used to determine whether ADC values for protein and energy (DP, DE) and the DP & DE intake differed significantly between the dietary treatment groups.

Apparent Digestibility Coefficient (ADC) values for protein differed significantly among the artificial diets (One-way ANOVA, $p < 0.001$) and ranged from 65 – 75% (Table 2.4). The 15U diet had a significantly lower ($p < 0.001$) ADC for protein compared with all other

prepared diets, whereas the highest value was recorded for the 5U diet. ADC values for protein did not differ significantly ($p = 0.386$) between the 20U and 0U diets. Since dietary protein values did not vary between prepared diets (Table 2.3), the above mentioned differences in ADC had a direct effect on the amount of Digestible Protein (DP) in each feed (Table 2.4). Daily DP intake (Fig. 2.7 B) of urchins fed the prepared diets also differed significantly (One-way ANOVA, $p < 0.001$), with urchins fed the 20U diet having a significantly higher DP intake compared with the 15U, 5U and 0U treatments.

The absorption efficiency and digestible energy contents of the artificial diets differed significantly (One-way ANOVA, $p < 0.001$), with diets containing less *Ulva* (5U & 0U) recording significantly higher absorption efficiencies and DE values compared with the 20U and 15U diets (Table 2.4). There was, however, no significant difference in these values between the 5U and 0U diet.

Furthermore, daily DE intake of urchins did not vary significantly between the prepared diets (Fig. 2.7 C). Energy efficiency (DP/ DE ratios) increased with increasing *Ulva* content in the prepared feeds (Table 2.4). Most notably, the 20U diet had a significantly (One-way ANOVA, $p < 0.05$) higher DP/ DE ratio compared with the 0U diet, indicating that more protein in relation to energy is available in the 20U diet.

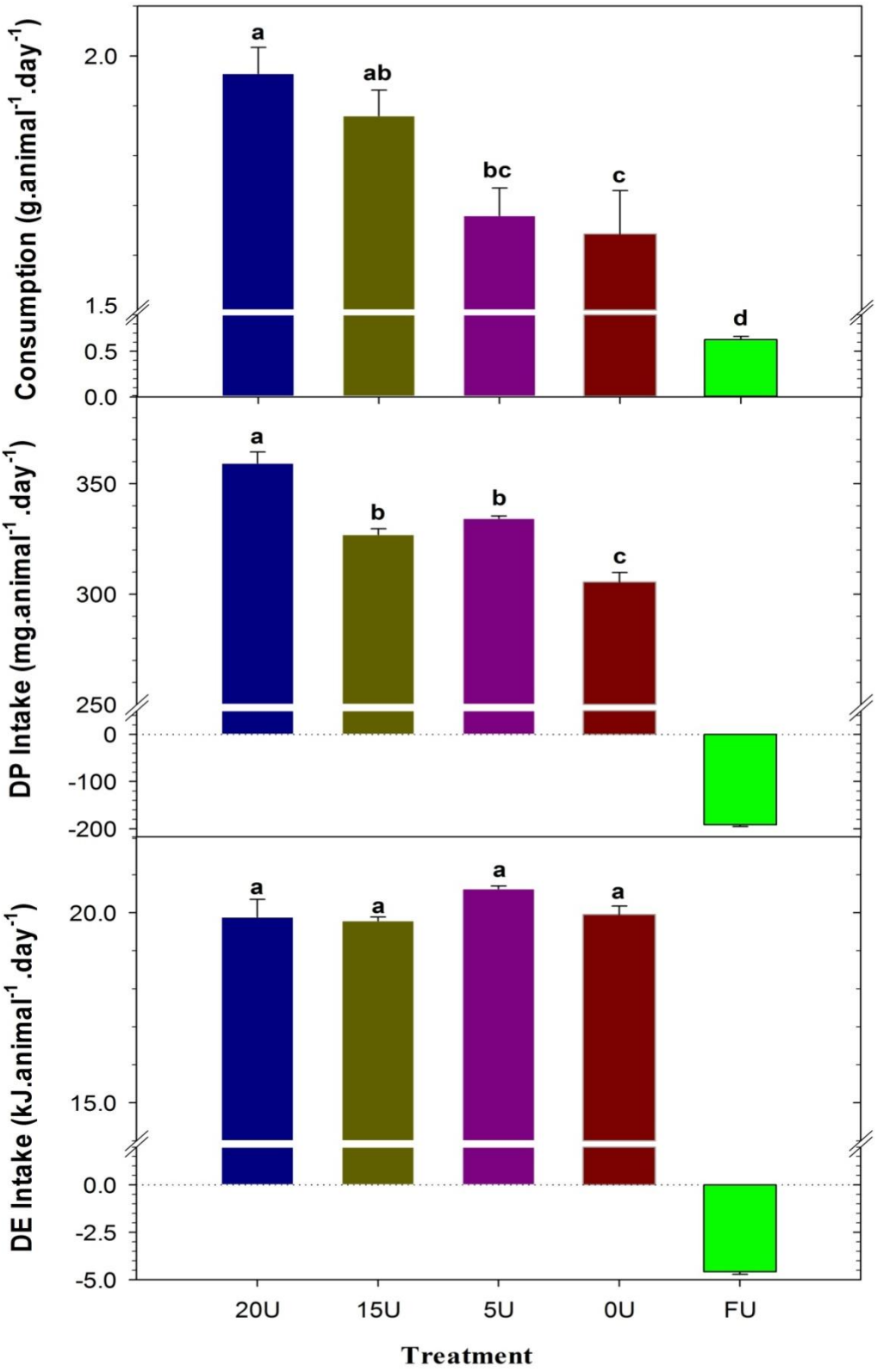


Figure 2.7: Mean **(A)** daily consumption, **(B)** daily digestible protein (mg) and **(C)** digestible energy (kJ) per animal for *Tripneustes gratilla* fed with four artificial diets or fresh *Ulva* over a 20 day consumption and digestibility trial in a recirculating seawater systems. Data represents mean ± SE. Letters a, b, c, d ($p < 0.05$, Tukey test) represent a significant difference in the means of urchins fed the artificial or fresh *Ulva* diets. Treatment groups: FU = fresh *Ulva*;; 20U = 20% *Ulva*; 15U = 15% *Ulva*; 5U = 5% *Ulva*; 0U = 0% *Ulva*.

2.4.4 Chemosensory trial

Tripneustes gratilla showed no preference for either arm of the Y-maze when feeds were tested against a seawater blank in either the right or left arm (Table 2.5).

Table 2.5: Number of sea urchins entering the arm of the Y-maze containing no food (Blank), Fresh *Ulva* (FU), an artificial diets containing 0% *Ulva* (0U) or an artificial diets containing 20% *Ulva* (20U). Expected distribution in each paired test = 50:50.

Paired test	(n)	No. of sea urchins choosing	One-tailed probability
FU **20U**	(12)	9 3	P= 0.0537
20U **0U**	(12)	10 2	P= 0.0161
FU **0U**	(12)	10 2	P= 0.0161
0U **Blank**	(12)	9 3	P= 0.0537
FU **Blank**	(12)	11 1	P= 0.0029
20U **Blank**	(12)	10 2	P= 0.0161

Urchins chose the fresh *Ulva* and the 20U artificial diets over the 0U diet ($p = 0.0161$ for both FU and 20U diets) and the seawater blank ($p = 0.0029$ and $p = 0.016$ for FU and 20U, respectively). Urchins did not, however, show significant preferences between the FU and 20U diets ($p = 0.0537$), although the number of selections (Table 2.5) suggested a preference for FU, with 9 out of 12 individuals selecting it. The same trend was observed for the 0U and seawater blank ($p = 0.0537$), which also indicated only a slight preference for 0U over seawater.

2.5 Discussion

The palatability and chemosensory characteristics of formulated feeds, as well as the bioavailability of nutrients (protein and energy) within them, are essential prerequisites for the preparation of biologically and economically optimized feeds for cultured marine and freshwater organisms (Jobling *et al.*, 2001; Shipton & Britz, 2001a, b; Dworjanyn *et al.*, 2007). Suboptimal feed formulations will results in the loss of nutrients from uneaten/undigested food, which, in turn, will have an adverse effect on water quality, the growth and health of the cultured organism(s), and the overall costs of the farming operation (Olafsen, 2001; Malham *et al.*, 2003; Dworjanyn *et al.*, 2007). In this study the palatability of an artificial diet, specifically formulated for the local production of *T. gratilla* (Cyrus *et al.*, 2014) has been successfully improved, by incorporating 20% weight/weight of a preferred algal species (*Ulva*). Chemosensory experiments conducted in this study showed that the incorporation of *Ulva* acted as a feeding stimulant, and the consumption and digestibility trials demonstrated that dietary *Ulva* supplementation significantly improved both consumption and daily protein intake of the prepared diets.

The South African abalone aquaculture industry has expressed interest in the cultivation of *T. gratilla* and many of these abalone farms are presently cultivating *Ulva* as an alternative food source for the cultured abalone. The palatability of a variety of common algal species was tested to determine the most appropriate algae to use as a feeding stimulant in an artificial feed, previously formulated for this species (Cyrus *et al.*, 2014). All algal species tested in this study were consumed by *T. gratilla*, providing further evidence for the generalist feeding behaviour previously reported for this species. Lyimo *et al.* (2011) showed that, of the 59 micro and macro-algal species found to co-occur with *T. gratilla* in Tanzanian waters, 48 were found in the gut contents of sampled urchins. Numerous other studies have, however, shown that sea urchins will display a hierarchy of preferences when offered a choice of

natural diets, strongly preferring some algae species over others (Prince & LeBlanc, 1992; Solandt & Campbell, 2001; de Loma & Conand, 2002; Dworjanyn *et al.*, 2007; Stimson *et al.*, 2007; Kasim, 2009; Lyimo *et al.*, 2011). Similarly, paired preference tests in the present study revealed that *Ulva rigida* was the most preferred algal species, followed by *Eklonia maxima*, *Porphyra capensis* and *Gigartina polycarpa*. *Ulva* also presents the most difficult challenge to consume out of all the algae tested, as it occurs in thin flat sheets. Meaning many pieces of *Ulva* must be consumed to be equivalent to a single piece of kelp, for example, thus further supporting our findings as Physiognomy did not affect consumption.

Incorporating dried algae into an artificial diet did not adversely affect the chemosensory properties of the algae. These findings are in agreement with Dworjanyn *et al.* (2007), who showed that *T. gratilla* consumed significantly more of an artificial diet supplemented with either dried *Ecklonia radiata* or dried *Sargassum linearifolium*, compared with an algae-free control diet. Since all of the diets in the latter study had similar protein and energy contents and differed only by the presence and/ or species of algae that had been incorporated in the artificial diets, these authors concluded that urchins are attracted to micronutrients or other factors derived from the algae. *Ulva* dried to a constant weight at 60°C and incorporated into an artificial urchin diet (20U treatment) in the present study, was found to be as attractive as fresh *Ulva* (FU), when offered simultaneously to urchins in chemosensory experiments (Table 2.5). Both these diets (20U & FU) were also found to be significantly more attractive to urchins than an artificial diet without added *Ulva* (0U). *Tripneustes gratilla,* thus, responded to the chemosensory properties of the *Ulva* rather than the structural or morphological properties of the feeds. Chemosensory experiments also revealed that urchins did not differentiate between the 0U diet and a blank seawater treatment, indicating that this diet does not contain a chemical attractant and hence *T. gratilla* did not actively forage for the 0U feed. Prince and LeBlanc (1992) reported similar findings, demonstrating that

Strongylocentrotus droebachiensis was unable to detect *Codium fragile*, and suggested that this seaweed does not produce a chemical attractant. However, *S. droebachiensis* consumed *C. fragile* when it came into contact with it, as observed for the consumption of the 0U diet by *T. gratilla* in the present study (Fig. 2.7 A). Leighton (1966; 1971) proposed that sea urchins are selective in conditions where food is abundant, but that this selectivity dissipates when food becomes limiting.

Ulva is known to contain high amounts (up to 6977 $\mu g.g^{-1}$) of dimethylsulfoniopropionate (DMSP) (Smit *et al.*, 2007), which has been shown by Van Alstyne *et al.* (2001) to be a significant feeding stimulant for sea urchins. The inclusion of DMSP in diets fed to crustaceans, fish and amphibians has also been shown by Nakajima (1996) to increase growth rate, stress resistance and metabolism of these cultured organisms. The mechanism involved in inferring these benefits may lie in DMSP yielding methyl groups via methylation reactions which are then available as additional growth substrates (Nakajima, 1996). In the present study, it was assumed that the concentration of DSMP within the artificial diets increased with increasing *Ulva* inclusion. This may explain why the 20U diet was consumed in significantly greater amounts compared to the lowest *Ulva* inclusion diet (0U diet), and why the 20U and the FU diets were more attractive to urchins than the 0U diet in the chemosensory experiments. The transformation of DMSP by the enzyme DMSPlyase to acrylic acid and dimethylsulfide (DMS) occurs once the alga has been damaged by herbivores. These two compounds have the opposite effect to DMSP, and have been shown to strongly deter urchins from feeding (Van Alstyne *et al.*, 2001; Van Alstyne *et al.*, 2003). Even though acrylic acid and DMS may have been released from *Ulva* when consumed by *T. gratilla* in the paired preference trials, these compounds did not appear to deter feeding as *Ulva* remained the most preferred alga of the species offered. Borowsky & Borowsky (1990) showed that heat-killed *Ulva* fed to the amphipod *Gammarus palustris* was consumed twice

as much as fresh *Ulva* of the of the same species. It is hypothesized that heating, through the drying of *Ulva* prior to its addition to feed, reduced, or possibly prevented, DMSP from being converted to acrylic acid and DMS, thus reducing and/or eliminating any negative effects associated with these herbivore deterrents. Chemosensory experiments support this hypothesis, as the 20U and FU diets were shown to be equally attractive. Nonetheless, it is likely that other micronutrients, or constituents other than DMSP, are present in *Ulva* and could have contributed towards the attractiveness of the FU and 20U diets tested in this study.

The apparent digestibility coefficient (ADC) values for protein in the present study ranged from 65 - 75% (Table 2.4). These values are in agreement with other studies investigating protein digestibility in urchins, where diets containing 20 - 40% protein had ADC values which were generally greater than 60% (Frantzis & Grémare, 1992; Klinger *et al.*, 1998; McBride *et al.*, 1998; Akiyama *et al.*, 2001). Although ADC values for protein differed significantly among the artificial diets tested in the present study, there was no apparent correlation with the amount of *Ulva* included in each diet, with the highest ADC value for protein recorded for the 5U diet and the lowest for the 15U diet. In contrast, the higher *Ulva* inclusion diets encouraged increased consumption rates, there by significantly improving protein intake (>15%) in urchins fed the 20U diet (Fig. 2.7). These findings suggest that the amount of protein absorbed by urchins in each treatment group depended primarily on the amount of protein ingested, and to a lesser extent on the absorption efficiencies of the nutrients.

The ADC values for energy also varied significantly among the artificial diets tested in the present study; however, unlike protein, diets with a higher *Ulva* content had significantly lower values. The observed differences in ADC values for energy may be attributed to the varying concentrations of algal polysaccharides within the artificial diets. Algal polysaccharides within *Ulva* and other seaweeds are structurally complex and are

consequently difficult to digest (Lawrence & Lane, 1982; Erasmus *et al.*, 1997). Hence, artificial diets supplemented with increasing amounts of *Ulva* may have less energy available for uptake due to the associated increase in structural polysaccharides, regardless of the improved consumption of artificial diets containing more *Ulva*. The absorption efficiencies for both protein and energy were, however, greater than 65% for all artificial diets tested in the present study. It is postulated that these values could have been lower if the *Ulva* incorporated into the feeds was not dried and ground to a powder prior to its inclusion in the artificial feeds, as this process may have contributed to the breakdown/rupture of polysaccharide-containing cell walls and consequently made protein and energy within the algal cells more accessible than in its natural form (Hiratsuka & Uehara, 2007; Fabbrocini *et al.,* 2012). Indeed, Otero-Villanueva *et al.* (2004) suggested that the high absorption efficiencies recorded for the urchin *Psammechinus miliaris* fed *Laminaria saccharina*, compared with previous reports, was as a results of chopping and homogenising the algae during diet preparation, thus making the cellular components more accessible to the urchin. This consideration, and its low protein content, may also explain why the use of unprocessed/fresh *U. lactuca* fed to *P. lividus* (Shpigel *et al.*, 2005) and *T. gratilla* (Cyrus *et al.,* 2014) in previous studies resulted in poor gonad somatic indices (GSI), when compared to urchins fed artificial feeds.

Negative absorption efficiencies for fresh *Ulva* were recorded for both protein and energy when using ash as an inert marker in the present study (Table 2.4). Negative absorption efficiencies have been recorded previously from sea urchin digestibility studies (Lowe & Lawrence, 1976; Hawkins, 1981; Lane & Lawrence, 1982) and several mechanisms have consequently been proposed for the manifestation of these negative values, including loss of a portion of the ash content from the faeces and the addition of nitrogen to the faeces (Lares 1999). The nitrogen content of faeces can originate from several difference sources, including

undigested protein, addition of organic material, such as mucus and digestive tract cells, and enrichment by parasitic and/ or symbiotic inhabitants of the gut (Fuji, 1967; Lares, 1999). Bjorndal (1980) suggested that protein derived from symbiotic gut microorganisms may contribute towards absorption efficiencies of specialist herbivores (e.g. sea turtles) but not in sea urchins, which are generalist herbivores. She argued that only a long-term constant natural diet would allow for the development of a specialized microflora of sufficient population size and so contributions from this source to faecal nitrogen content would be negligible, particularly for wild urchins. As urchins in this study were fed fresh *Ulva* continuously for 20 days, it may be possible that gut microorganisms did contributed to the increased nitrogen content of the faeces and affected ADC values. The addition of organic material to faecal matter through mucus may have also contributed towards the negative absorption efficiencies recorded from urchins fed fresh *Ulva* in this study. De Ridder & Jangoux (1982) stated that all sea urchins, with the exception of the cidaroids, surround ingested food with mucus in the pharynx to form a pellet that will remain intact through defecation. The selective passage and accumulation of particles of different density has been shown to occur in molluscs (Shipton & Britz, 2001b) as well as in echinoids (Lares, 1999). In the abalone *Haliotis midae*, three distinct faecal pellets are produced and the protein and inert marker (chromium oxide, which was added to the diet) content of each distinct pellet differed significantly (Shipton & Britz, 2001). In *T. gratilla*, food fragments are not organised into pellets, but rather, are loosely formed into agglomerates that are held together by a mucus substance (Vaïtilingon, 2003). Since urchins in the present study were fed artificial diets (pellets) as well as fresh *Ulva*, which are structurally and morphologically distinct feeds, differences in the passage and accumulation of food fragments, and the amount of mucus associated with these food fragments, would be expected and could likely have contributed towards the observed differences in ADC values between artificial and fresh

feeds. Very little is, however, known about the nutritional value, and more specifically the nitrogen and energy content, of *T. gratilla* mucus and further investigation into this is thus needed.

In this study *Ulva* has been successfully incorporated into an artificial feed, previously formulated for *T. gratilla* (Cyrus *et al.*, 2014), which has been shown to significantly improve palatability of the feed and the subsequent consumption and daily protein intake of urchins fed this diet. These findings are of significance to the integrated aquaculture industry of South Africa. With more than 2500 metric tonnes of *Ulva* being produced annually on South African abalone farms alone (DAFF, 2012), there is real potential for the use of this alga as a supplement feed for a South African sea urchin industry.

CHAPTER 3:

The development of a feed containing *Ulva* (Chlorophyta) to promote rapid growth and enhanced production of high quality roe in *Tripneustes gratilla*

Contents

3.1 Abstract

This study investigated growth and gonad production of *Tripneustes gratilla* fed four protein-rich artificial diets supplemented with varying amounts (0%, 5%, 15% and 20% weight/weight (w/w); designated 0U, 5U, 15U and 20U, respectively) of the macroalga *Ulva* over a 12-week period. Gonad size, texture, colour and a number of production performance parameters were quantified and compared with urchins fed fresh *Ulva* (FU) and a combination of FU and artificial feed (FB). All artificial diets significantly ($p < 0.05$) increased gonad somatic indices (GSI) compared with the FU treatment. The 20U treatment increased GSI by 205% by week 9, compared with a 57% increase in the GSI of urchins fed FU. Gonad colour was calculated using three colour parameters, namely L* (lightness), a* (redness) and b* (yellowness). Whereas L* and a* values did not differ significantly between treatments, b* values for all treatments, with the exception of the 20U and FB treatments, were significantly ($p < 0.05$) lower or less yellow than the FU treatment by week 12. These results show that this study has successfully formulated a feed (20U) which can produce commercially acceptable gonads in terms of both size and colour, indicating the potential for this artificial feed to support commercial echinoculture.

3.2 Introduction

Over the last decade numerous studies have demonstrated that artificial diets can enhance somatic growth and gonad yield of sea urchins (Lawrence *et al*., 1997; Olave *et al*., 2001; Shpigel *et al*., 2005), and protein has been identified as the main contributing factor for this enhancement (Pearce *et al*., 2002a,b; Pearce *et al*., 2004). In nature, sea urchins must ingest and process large quantities of macroalgae and/or seagrass to meet their nutritional requirements for protein (Hammer *et al*., 2006), possibly explaining why administration of macroalgal diets alone typically produces urchins with lower growth rates and smaller sized gonads, when compared with urchins fed diets formulated using animal-derived material (Cook *et al*., 1998; Fernandez & Boudouresque, 1998, 2000). Hammer *et al*. (2006) demonstrated that an artificial diet containing a moderate protein level of approximately 20% is used most efficiently by sea urchins, resulting in enhanced consumption rates, survival, specific growth rates and gonad production efficiency, when compared with a diet containing low levels of protein (< 9%). These findings are supported by several other studies, which also indicate that a moderate dietary protein level of approximately 20% is most efficiently utilized by sea urchins (Akiyama *et al*., 2001; Pearce *et al*., 2002a; Schlosser *et al*., 2005).

Other components of an artificial feed which affect gonad yield and quality include pigment concentration (especially carotenoids), feed stimulants, feed binder type and feed shape (Plank *et al*., 2002; Pearce *et al*., 2004; Hammer *et al*., 2006; Cook & Kelly, 2007). Inclusion of small quantities of macroalgae in artificial diets fed to *T. gratilla* has been shown to enhance palatability (Dworjanyn *et al*., 2007). Carotenoid pigments, mainly β-carotene, which is synthesized by macroalgae, are the source of the red, orange or yellow gonad colouration of sea urchin gonads (Agatsuma *et al*., 2005), and addition of β-carotene to artificial feeds has been shown to improve gonad colour of cultured urchins (Pearce *et al*., 2002a; Robinson *et al*., 2002; McBride *et al*., 2004; Shpigel *et al*., 2005). However, despite

these potential benefits, the effects of a protein-rich formulated feed supplemented with varying amounts of a particular macroalga on the somatic and gonadal growth of *T. gratilla* have not yet been investigated. A preliminary study (Chapter 2) demonstrated that *T. gratilla* consumed significantly higher amounts of *Ulva rigida* when offered four seaweed species [*Ulva rigida*, *Ecklonia maxima* (kelp), *Porphyra capensis* and *Gigartina polycarpa*] in paired consumption tests (Scholtz, 2008). As South African *Tripneustes* generally prefer *Ulva* and because farm-grown *Ulva* is available in large quantities from local aquaculture facilities (Bolton *et al*., 2009), *Ulva* was selected as an additive for an experimentally formulated urchin feed. In addition, the inclusion of *Ulva* in artificial feeds has been shown to significantly increase both consumption and digestibility (Chapter 2) at a level of 20%.

The aim of this study was to determine the effects of incorporating varying levels of the algae *Ulva* into an artificially formulated feed, with the specific aim of increasing gonad mass and improving gonad colour and quality of the sea urchin *Tripneustes gratilla*. *Ulva* was incorporated into the formulated diets to act as both a natural feeding stimulant (Dworjanyn *et al*,. 2007), as well as a source of β-carotene, an important carotenoid pigment associated with gonad colouration. *Ulva lactuca* has been shown to contain between 25 and 45 $\mu g.g^{-1}$ [fresh weight (FW)] of β-carotene (Bischof *et al*., 2002), whereas the total carotenoid content of farmed *Ulva rigida* and *Ulva lactuca* has been reported as high as 10% [dry weight (DW)] (Shuuluka 2011). Urchins fed the various diets were then assessed on a monthly basis to record somatic growth, gonadal growth and a number of gonad quality factors, such as colour, texture, gonad somatic index and the dominant maturity stage of urchins in each experimental group.

3.3 Materials & methods

3.3.1 Collection and maintenance of urchins

Adult *Tripneustes gratilla* (50–70 mm test diameter) were collected during low tide from shallow rock pools near Haga-Haga, Eastern Cape, South Africa (32°45′4.23″S, 28°16′41.30″E) on 15 - 17 September 2008 and maintained as described in Chapter 2 (Section 2.3.1.)

3.3.2 Preparation of experimental feeds

The effects of six dietary treatments on gonad production and quality factors, such as colour, texture and firmness of adult *T. gratilla* were assessed in a 12-week feeding trial. Four of the dietary treatments consisted of a semi-purified (contain natural ingredients in as pure a form as is available) 'basal' formula supplemented with different amounts of dried macroalga *Ulva* spp. to achieve final concentrations of 0, 5, 15 and 20% (w/w) (Chapter 2, Section 2.3.4). The proximate nutrient analysis of the six dietary treatments is presented in Chapter 2 (Table 2.3). In addition to the extruded feeds, cultivated Fresh *Ulva* (FU) was included as a dietary treatment to act as a control, and determine the effects of a fresh macroalgal diet alone on gonad yield and quality. A mixed diet of fresh *Ulva* and basal feed (FB the formula used to make the artificial diets prior to the addition of *Ulva* or *Ulva* Additive) was also included to determine the effect of using fresh *Ulva* compared with dried *Ulva* on gonad yield and their characteristics. The treatments were abbreviated as in Chapter 2.

3.3.3 Feeding trial

The feeding trial was initiated on the 11 March 2009 and ran for a total of 90 days, concluding on the 8 June 2009. A total of 12 baskets were suspended in each of the four large fibreglass tanks (Fig. 3.1A) and were stocked at an initial stocking density of 15 animals per basket. Within each tank there were two replicates of each treatment, resulting in an overall experimental design that consisted of eight replicates and six treatments (20U, 15U, 5U, 0U,

FU and FB). These treatments were randomly allocated to baskets within each of the four fibreglass tanks at the start of the feeding trial to account for the potential effects of specific feeding regimes on surrounding baskets within a tank (Fig. 4.1B).

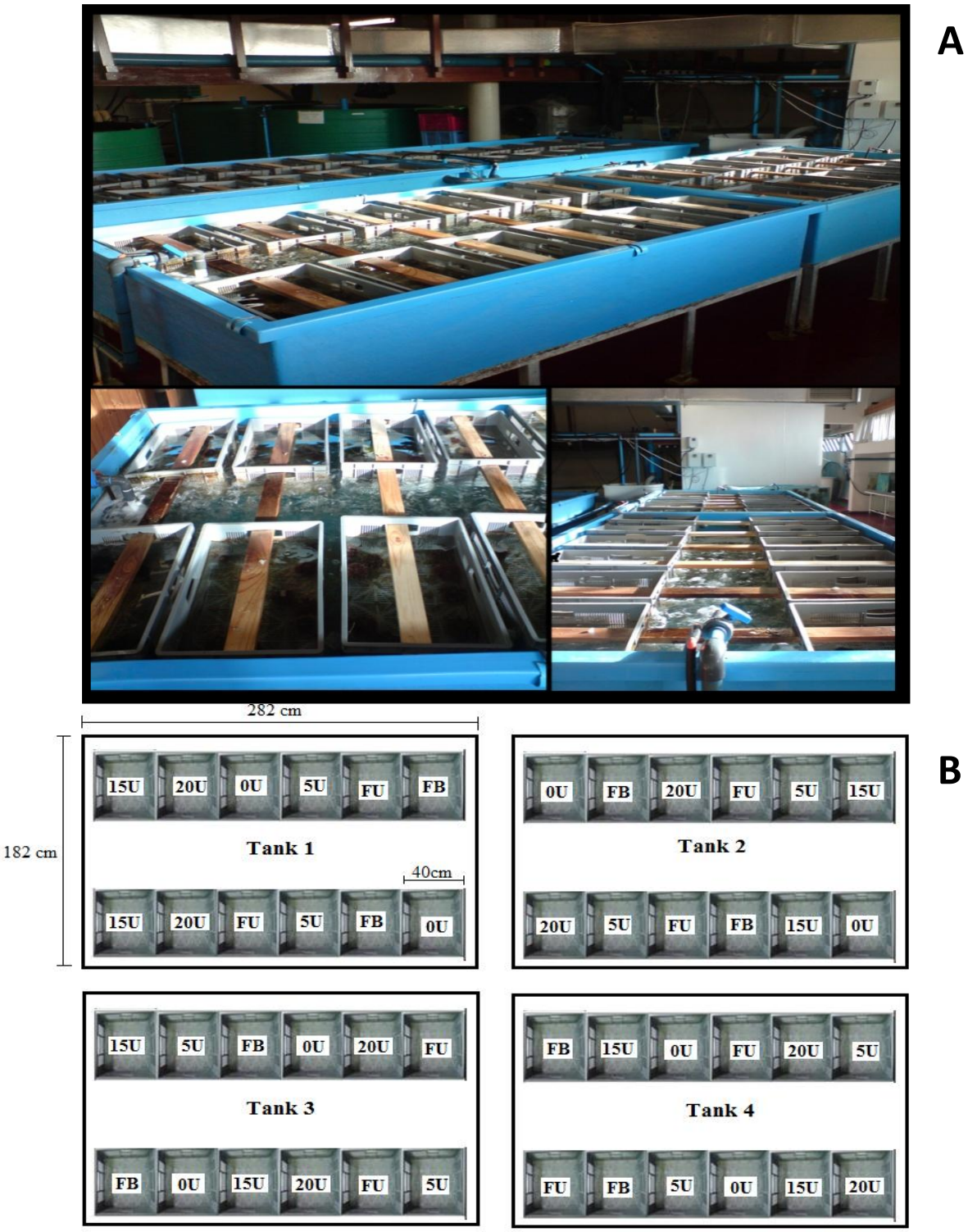


Figure 3.1: Photographs **(A)** the experimental tank design and **(B)** a schematic of the random assignment of basket for each experimental treatment group.

Sea urchins were fed each experimental diet *ad libitum* every second day after the removal of uneaten feed from each basket. This ensured adequate food was always available to urchins and allowed urchins to feed until they reached saturation. The urchins did not reach satiation during this time, as urchins were not observed to stop feeding during the trial. It should be noted that even though the number of urchins within each treatment was decreasing overtime, this did not have an effect on food availability, as an excess of food was always provided throughout the study. Water quality parameters were monitored weekly during the study (as described in Chapter 3, Section 3.3.2) and remained within the parameters for a healthy recirculation system. Immediately before the start of the experiment, 10 sea urchins were randomly selected from individual baskets and dissected to establish the initial state of the animals. Thereafter, one urchin from each basket was sampled at random each month, for the duration of the feeding trial. Each sampled urchin was blotted dry with paper towel, and total body weight, test diameter, test height and urchin drained weight (coelomic fluid removed) were carefully recorded to the nearest 0.01 g or 0.01 mm. The gonads were then carefully dissected out and the gonad wet weight recorded to the nearest 0.01 g using an electronic balance, and the Gonad Somatic Index (GSI) was calculated as described below (Section 3.3.4). Gonad texture, firmness and colour were rated visually and manually, by a single observer according to the procedures described below. A separate gonad from each urchin was transferred into Davidson's Fixative (per litre: 300 mL 95% ethyl alcohol, 200 mL 100% formalin, 100 mL glycerol, 100 mL glacial acetic acid and 300 mL distilled water) immediately following dissection and fixed for 48 h, before being transferred into 70% ethanol, and processed for routine paraffin histology (Bucke, 1989). A separate gonad from each urchin was also removed for isotope analysis (See Chapter 6). These gonads were dried at 70 ^{o}C until a constant weight was reached, and then moved to glass vials and stored for further analysis.

3.3.4 Gonad measurements

3.3.4.1 Calculation of gonad somatic index (GSI)

Gonad indices are indicators of gonad development which are independent of urchin size (James, 2007). All GSI results are presented as wet weight GSI as this is the most common technique used in previous studies, making the results easily comparable with similar bodies of work. It should be noted however that test thinkness may affect results between different species.

The body and gonad wet weights of individual urchins fed the various experimental diets (n = 8 per dissection) were used to calculate gonad somatic index according to the formula used by Pearce *et al*. (2002a):

$$GSI\ (\%) = W_g / W_t \times 100$$

where W_g is the wet weight (g) of the gonad and W_t is the total wet weight (g) of the sea urchin.

The GSI increase per week over the course of the study was calculated using the following formula, to allow comparison of growth rates with similar studies:

$$\text{Gonad yield per a week} = [(GSI_{(end)} - GSI_{(start)})/ (\#days)] \times 7$$

Where $GSI_{(end)}$ is the GSI at the end of the study and $GSI_{(start)}$ is the GSI at the start of the study.

3.3.4.2 Assessment of gonad colour

Eye-rated gonad colour was assessed by visually ranking each gonad (n = 8 per treatment) in categories, ranging from most desirable to unacceptable, according to the rating system described by Pearce *et al*. (2002a). These observations were made by a single observer [Mark D. Cyrus (MDC)] over the course of the study. The categories were allocated numbers which were ranked as follows: (1) bright yellow-orange gonads (excellent quality); (2) yellow-

orange gonads (acceptable quality); (3) pale yellow-orange or dark yellow-orange gonads (low quality) and (4) white or brown gonads (unacceptable). Values from within the different dietary treatments were averaged to produce a single value that could be compared between treatments. The lower the average eye rated colour value, the better the colour and, therefore, the quality of the gonads.

Due to inherent difficulties with the subjective technique of "Eye Rated Colour", Robinson *et al.* (2002) described a more accurate way of evaluating colour, and broke gonad colour down into three measurable components, similar to those of three-dimensional measurement system developed by the Commission Internationele de l'Eclairage in 1976 (Robinson *et al*., 2002). The technique is called 'CIE (L*a*b*) 1976' and uses a spectrophotometer to measure hue (a* - the *X* axis) which extends from green on the negative side to red on the positive side and chroma (b* - the *Y* axis) which extends from blue on the negative side to yellow on the positive side. These two axes define any colour, however the lightness or intensity of the colour is determined by the Lightness (L* - the *Z* axis), which extends from black on the negative side to white on the positive. The three values produced from this technique provide a true measure of gonad colour, as well as the variance within a sample, and therefore gonad colour can be statistically evaluated (Robinson *et al*., 2002).

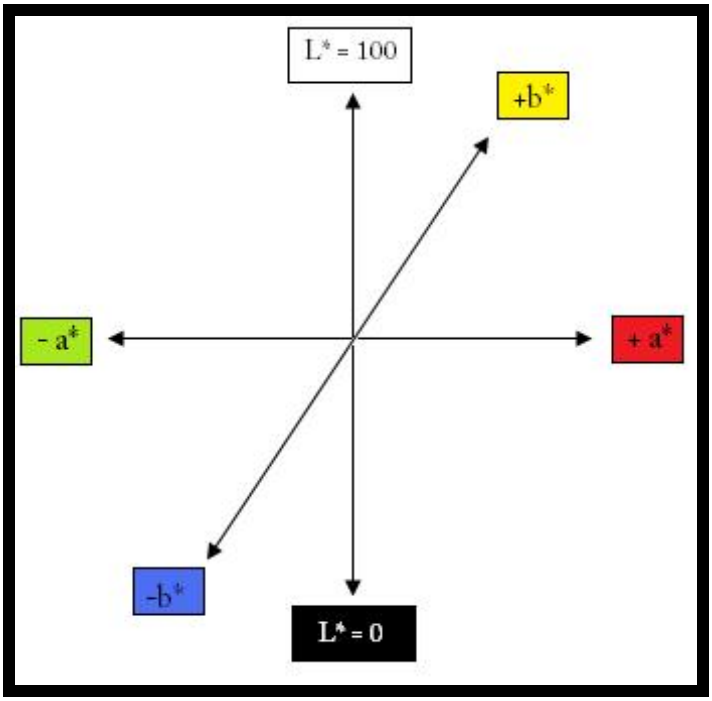


Figure 3.2: Diagram showing the three plans (L*, a* & b*) used by the spectrophotometer to measure colour

Spectrophotometer rated gonad colour was objectively quantified using a hand-held reflected-light, fibre-optic spectrophotometer [Gardner (BYK) Colour Guide, Wesel, Germany]. Three replicate measurements of intensity of lightness (L*), redness (a*) and yellowness (b*) for each gonad were recorded. These values were then used to determine the average L*, a* and b* values for each measured gonad sample. Total difference in gonad colour from 'A grade roe' (the fresh *Ulva* treatment, which was regarded in this study as having the most desirable gonad colour) was calculated for urchins fed the six diets, by comparing gonad colour in these treatments to the average colour of urchin gonads fed the fresh *Ulva* spp. The following formula from McBride *et al*. (2004) was used to calculate these differences:

$$\Delta E_{ab}^* = [(L^*_{Ulva} - L^*_{Sample})^2 + (a^*_{Ulva} - a^*_{Sample})^2 + (b^*_{Ulva} - b^*_{Sample})^2]^{1/2}$$

3.3.4.3 Assessment of gonad texture and firmness

Texture affects gonad appearance and was evaluated according to the visibility of individual gonad follicles (smoothness) and segment halves, where gonads with greater separation

between follicles represented a lower quality product. Individual gonads (n = 8 per treatment/ sampling date) were visually assessed, by a single observer (MDC), using the protocol from Pearce *et al*. (2002a) and were allocated a rating as follows: (1) two distinct gonad segment halves with little to no follicle separation; (2) two distinct gonad segment halves with visible follicle separation; (3) distinction of gonad segment halves possible, but quite granular and (4) distinction of gonad segment halves not possible, gonads rough/granular. The textural properties, and hence the firmness of gonads, are very important becuasue gonads that remain intact during processing and packaging are required to produce a high quality, marketable product. Firmness, is defined as the force required to compress a sea urchin gonad a fixed distance for a specific time. However, equipment to test firmness directly was not available, thus gonads (n = 8 per treatment) were visually rated subjectively by pressing down on the gonad with a finger and scoring each gonad according to one of the following subjective criteria described by Pearce *et al*. (2002a): (1) very firm; (2) firm; (3) soft and (4) very soft. These observations were made by a single observer (MDC) over the course of the study.

3.3.5 Histology

Histological analysis was conducted to determine the amount of gametogenic activity in the gonads of urchins fed the various diets. High quality sea urchin gonads are considered to be those which contain little to no gametogenic activity. Fixed tissues (n = 8/treatment/sampling date) were processed using standard histological techniques described below:

3.3.5.1 Sampling, preparation & staining

Previous research has shown homogeneity in the reproductive/gametogenic state of single gonads, as well as between each of the five gonads within an individual urchin, of the species *T. gratilla* (Vaïtilingon *et al.,* 2005). These findings are in agreement with research on other echinoid species: *E. chloroticus* (Brewin, 1994), *Paracentrotus lividus* (Fuji, 1960; Spirlet *et al.*, 1998) and *Centrostephanus rodgersii* (King *et al*., 1994). Hence, histology in this study

was performed only on the middle portion of one of the five gonads from each sampled individual.

Fixed samples were processed using standard histological techniques (Bucke, 1989). Samples were rinsed, dehydrated and embedded in paraffin wax using a Shandon Citadel 2000 tissue processor, after which embedded samples were sectioned on a LKB 2218 Historange microtome at 7 μm. Histological sections were stained with Harris' haematoxylin and eosin and examined under an Olympus BX 51 light microscope, equipped with a Leica digital camera and Nikon Imaging Systems (NIS) Elements Basic Research (BR) image analysis software (Version 3.1). Individual gonads were categorized into one of six different maturity stages according to published literature on echinoid gametogenesis (Byrne, 1990; Spirlet *et al.,* 1998; Vaïtilingon *et al.,* 2005). These maturity stages were based on oocyte size (females), thickness of peripheral spermatocyte layer (males) and the amount of non-germinal nutritive tissue (males and females), and categorized as follows: (1) recovery; (2) growing; (3) premature; (4) mature; (5) partly spent and (6) spent. Of the six categories, gonads in the recovery and growing phases were regarded as high-quality gonads with good market acceptance.

3.3.5.2 Determination of sex & maturity stage

The sex ratio and reproductive condition of sampled urchins was assessed using the six reproductive stages mentioned previously and described in Byrne (1990), Spirlet *et al*. (1998) and Vaïtilingon *et al.* (2005). The maturity stages within ovaries and testes were then categorised as follows (Fig. 3.4):

Testes

Stage 1: Recovery

The characteristic feature of recovering testes (Fig. 3.4A), are clusters of spermatogonia and the appearance of the first primary spermatocytes lining the acinal walls, forming a thin basophilic layer. In more than 50% of males, this generally occurs at the mid-late spent stage, during the re-absorption of relict spermatozoa. For the rest, the recovery stage is initiated at the late spent stage, when all relict spermatozoa have been reabsorbed and the acini are filled with nutritive phagocytes. During this time the wall of the follicle is generally in a contracted state, having many ripples.

Stage 2: Growing

Growing testes (Fig. 3.4B) are characterised by a rapidly developing layer of primary spermatocytes and spermatogonia along the periphery lining of the acinal walls. The thickness of the spermatocyte layer generally ranges from 10 to 30 μm. From this layer, columns of spermatocytes migrate towards the lumen, which is still filled with nutritive phagocytes.

Stage 3: Premature

In premature testes (Fig. 3.4C), spermatogenesis is vigorous and the number of nutritive phagocytes is greatly reduced, as the tissue is being used for growth, and therefore forms a thin layer on the acinal wall. The lumen is connected to the spermatocyte layer at the acinal wall by columns of spermatocytes, along which sperm differentiation occurs. After differentiation and maturation, spermatozoa detach from the tips of the columns and accumulate in the lumen. In more advanced male follicles, small patches of sperm have begun to form in the centre of the follicle, although the area which they occupy is limited.

Stage 4: Mature

The lumen of mature testes (Fig. 3.4D) is filled with densely packed ripe spermatozoa and may change shape due to the accumulation of gametes. At this stage almost all of the

nutritive phagocytes have been absorbed and along the acinal wall a thin peripheral layer of spermatocytes is observed, which continues spermatogensis, although at a reduced rate.

Stage 5: Partly spawned

The lumen of partly spawned testes (Fig. 3.4E) contains spermatozoa which are less densely packed. On the periphery vacated spaces are observed, which indicate the partial release of spermatozoa. The acinal wall is lined with a spermatocyte layer, which has columns projecting towards the centre of the acini, indicating that spermatogenesis is not complete.

Stage 6: Spent

Testes at the spent stage (Fig. 3.4F) can be characterised most noticeably by the great reduction of spermatozoa and the presence of gaps in the lumen of the follicles. The presence of a developing nutritive phagocyte layer along the acinal wall also indicates the gonad is in a spent state. In early spent testes, relict spermatozoa can still be present and are generally being resorbed by the phagocytes. Late spent testes are characteristically devoid of any germinal cells and contain a dense meshwork of nutritive phagocytes, with no observed spermatogonial proliferation.

Ovaries

Stage 1: Recovery

Recovering ovaries (Fig 3.4G) contain nutritive phagocytes which form a meshwork that projects into the lumen, creating a vacuolated appearance. The appearance of the first small previtellogenic oocytes along the ovary wall, defines the recovery stage, with the diameter of the previtellogenic oocytes generally ranging from 10 to 30 μm. This happens either at mid-late or late spent stage. Relict globules of lysed material and unspawned ova may be present

Stage 2: Growing

This stage is characterised by growth in size of the previtellogenic oocytes due to the onset of vitellogenesis. The growing ovaries (Fig 3.4H) contain numerous vitellogenic oocytes that are tightly packed along the ascinal wall and surrounded by the nutritive phagocytes, which become increasingly dense. The size of vitellogenic oocytes generally ranges from 30 to 60 μm in diameter, with advanced oocytes that protrude inward into the follicle and adhere to the wall, although these are generally restricted to a small number. During this time the ovary expands simultaneously with the growth of the oocytes. Early vitellogenic oocytes with a distinct nucleus can also be present.

Stage 3: Premature

In premature ovaries (Fig 3.4I) gonads display active oogenesis, with vitellogenesis being a continual process. Oocytes of all stages are present, ranging in size from 15-95 μm in diameter. This stage characterized by the large increase in size of individual oocytes. Vitellogenic oocytes surrounded by nutritive phagocytes continue to grow and as they become larger, they detach from the acinal wall and migrate to the centre of the acini.

Stage 4: Mature

Almost all available space within the ovarian lumen is filled with densely packed circular-shaped ova and most of the nutritive tissues have been used up, leaving a thin layer at the periphery of the acini wall. Mature ova (Fig 3.4J) are identifiable by a small nucleus and diameter measuring 65 to 80 μm. Vitellogenic activity is not yet fully complete, indicated by the presence of vitellogenic oocytes in the germinal layer.

Stage 5: Partly spawned

Partly spawned ovaries (Fig 3.4K) are characterised by the presence of ova that are less densely packed than mature ovaries, while vacant spaces are apparent in the acini, due to partial release of ova during spawning. In the germinal layer, a thin layer of nutritive tissue

surrounds the vitellogenic oocytes. Oogenesis continues and as primary oocytes develop, they subsequently replace mature ova as they are shed.

Stage 6: Spent

In this stage ovaries are generally characterized by the presence of an empty space at the centre of the follicles and the presence of a few vitellogenic oocytes and ripe unspawned ova. Ovaries in an early spent stage (Fig 3.4L) have acinal walls which are lined with a thin layer of non-germinal nutritive phagocytes. Re-absorption of germinal cells (ova and vitellogenic oocytes), by phagocytes, occurs throughout the spent stage, resulting in ovaries that are completely deprived of any germinal cells and are filled with dense meshwork of nutritive phagocytes (late spent stage).

3.3.5.3 Determination of nutritive phagocyte (NP) density

Lastly, the density of nutritive phagocytes (NP) within each gonad was calculated to determine whether the density of NP's within each gonad differed between the dietary treatment groups or stages of gonad maturity. Stained histological sections of gonads were examined under an Olympus BX 51 light microscope and photographed using a Leica digital camera. Nikon Imaging Systems (NIS), Elements Basic Research (BR) image analysis software (Version 3.1) was used for all image analysis. Three randomly allocated regions of interest (ROI) were then selected from each image. Allocated regions of interest (ROI) were defined as subsets of a sample of a standard size within an image. They were identified at random, and used to give and overall idea of a gonads current state of maturity. Using Pixel Classifier software, dyed areas within the gonad occupied by NP's, gametes or unoccupied space were accurately separated, making it possible to quantify the relative area that each of these three cell-types occupied within the ROI. The classifier allows for segmentation of the image pixels according to different user-defined classes and is based on pixel features, such as intensity values and RGB values. The Pixel Classifier software accurately records the area

occupied by NP's, gametes and unoccupied space which can then be used to determine NP density. The values obtained from each of the three ROI were then averaged to get a mean ROI for each gonad (Fig. 3.3 A–D)

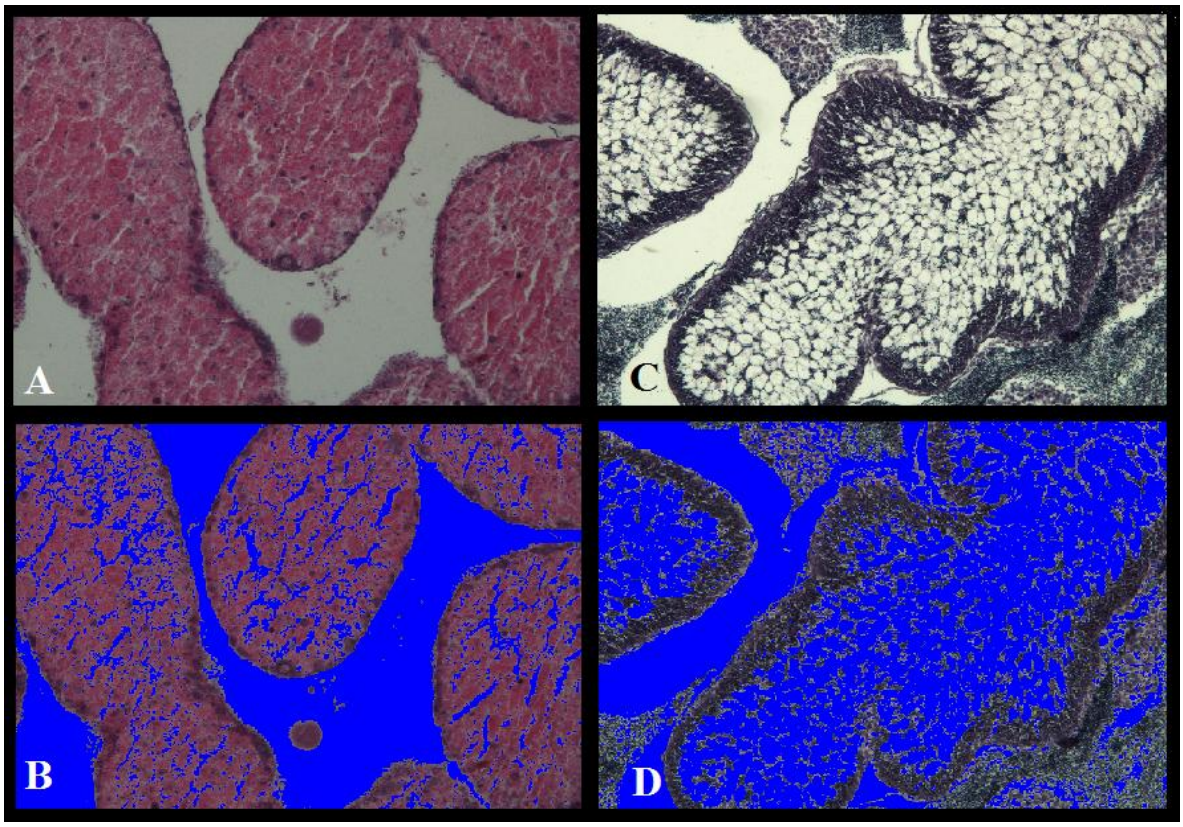


Figure 3.3: Histological section of a male gonad from *Tripneustes gratilla* fed either fresh *Ulva* (A and B) or the 0% *Ulva* supplemented diet (0U treatment, C and D). Images A and C represent the original images (400x magnification), whereas the pixels within images b and d have been subdivided based on pixel intensity values and RGB values, allowing for the separation of areas within the gonad occupied by nutritive phagocytes (NP), gametes or unoccupied space. Sections shown the accumulation of NP's within gonads of urchins fed fresh *Ulva* (A and B) compared with the 0U treatment group (C and D).

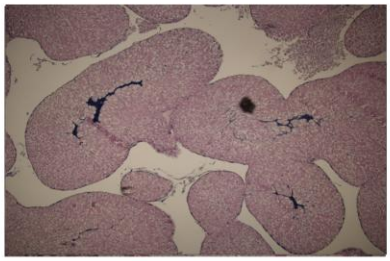
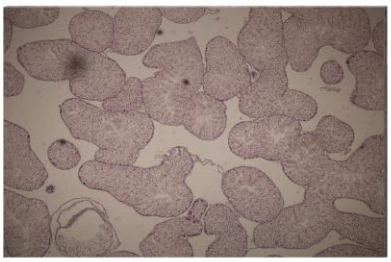
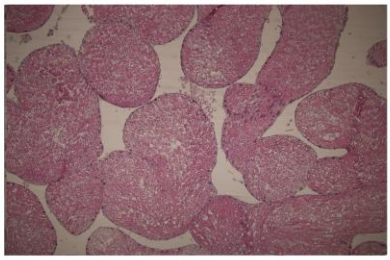
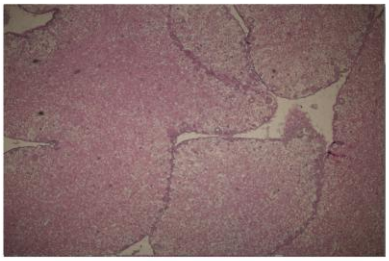
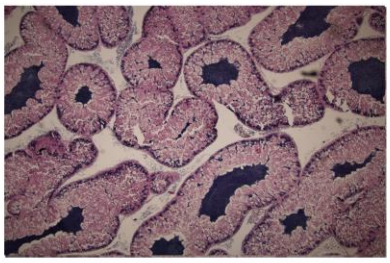
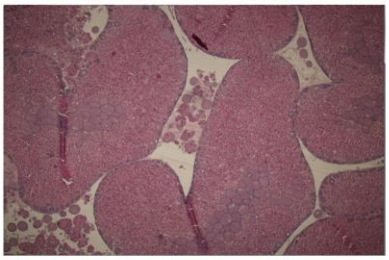
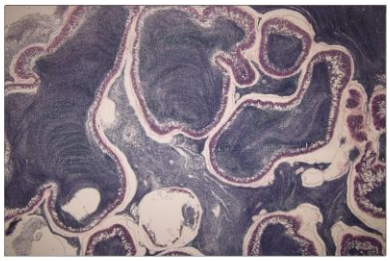
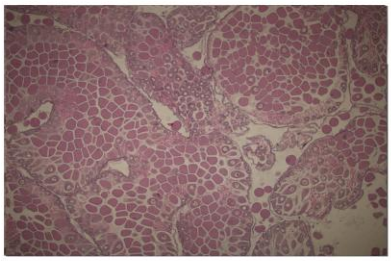
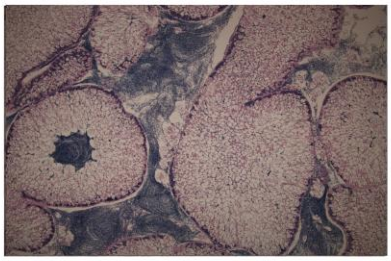
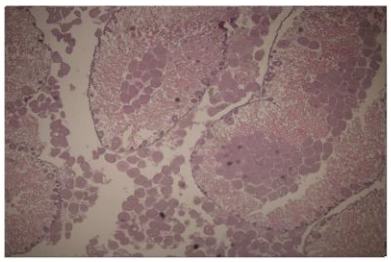
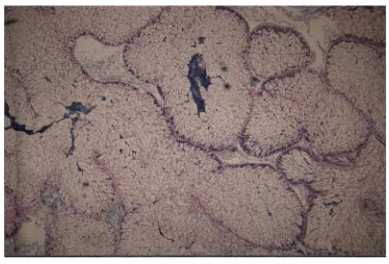
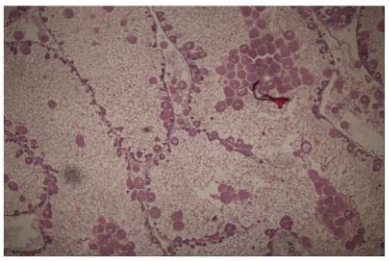
Male Testes	Female Ovaries	Maturity Stage
A 	 G	1. Recovery
B 	 H	2. Growing
C 	 I	3. Premature
D 	 J	4. Mature
E 	 K	5. Partley Spawned
F 	 L	6. Spent

Figure 3.4: Gonad histology of male (A-F) and female (G-L) gonads in different states of gonad development.

3.3.6 Statistical analysis

To determine whether urchin wet weight, mortality, GSI, gonad wet weight and gonad colour (L*, a* and b*) changed as a function of time, within individual treatment groups or as a function of treatment at individual sampling dates, a one-way analysis of variance (ANOVA) was performed using Statistica 8 statistical software. All tests for normality (Kolmogorov Smirnov test) and equal variance (Levene's test) passed for all data sets. One way ANOVA was also used to test for significant differences in eye-rated gonad colour, gonad texture and firmness and gonad maturity (histological data) within individual treatment groups over time and between treatment groups, at specific sampling dates. All tests for normality (Kolmogorov - Smirnov test) and equal variance (Levene's test) in these data sets failed and therefore a Kruskal - Wallis ANOVA on Ranks test was performed to test for significant differences. The Tukey method was used for all post-hoc multiple comparisons between individual time points within a treatment group and between the different treatment groups, at specific sampling dates. Significance was assigned to p-values of < 0.05 for all analysis.

A Fit Generalized Estimating Equations General Linear Model (geeglm) was used to determine variation in the spectrophotometer rated gonad colour values (L*, a*, b*), in relation to diet type, sex of urchin, maturity level and GSI, using the R package geepack for generalized estimating equations (Halekoh *et al.*, 2006). Unlike the General Linear Model, where observations are assumed to be independent, Generalized Estimating Equations (GEE) are particularly useful for observations which are clustered or dependent on one another, such as the image data recorded in this study. All tests for normality and equal variance passed for all data sets. Each geeglm consisted of diet type, sex of urchin, maturity level and GSI as independent variables and the spectrophotometer rated gonad colour values (L*, a*, b*) as dependent variables. All possible combinations between the independent variables were treated as interaction effects for this analysis. A Generalized Linear Model (GLM) was used

to determine variation in the density of nutritive phagocytes within each gonad, in relation to wet gonad weight, diet type, sampling date, gonad maturity and sex of the urchin. For this GLM, wet gonad weight, diet type, sampling date, gonad maturity and sex of the urchin were included as independent variables, and the density of nutritive phagocytes as the dependent variable. As before, all possible combinations between the independent variables were treated as interaction effects for this analysis. Significance was assigned to p-values of < 0.05 for all analysis.

3.4 Results

3.4.1 Urchin survival and somatic growth

Tripneustes gratilla survival rates over the course of the study were high and did not vary significantly between diets, ranging from 92.5% for animals fed the 0U diet to 97.5% for animals fed Fresh *Ulva* (FU, data not shown). Somatic growth, determined using urchin wet weight, increased significantly (one-way ANOVA, $p < 0.05$) within all dietary treatment groups over the 12-week experimental period (Fig. 3.5 A). Overall, the mean wet weight (±SE) of urchins across all treatment groups increased from 99.08 ± 10.15 g at the beginning of the study, to 163.13 ± 4.07 g at the end of the study. Urchins fed the artificial diets showed an average gain in wet weight of 68.2% over the course of the 12 - week study, compared with a 46.7% increase in wet weight for urchins fed fresh *Ulva*. There was, however, no significant difference (one-way ANOVA, $F_{5,42} = 0.813$, $p = 0.546$ for the 12-week sampling date) in urchin wet weight between any of the treatment groups by the end of the study period. Similar results were recorded for urchin test diameter (data not shown).

3.4.2 Gonad growth and quality

5.4.2.1 Gonad growth and yield

Urchins fed the formulated feeds had a significantly greater gonad wet weight and GSI by week 9, and at the end of the experiment, when compared with urchins fed exclusively on a diet of fresh *Ulva* (one-way ANOVA; $p < 0.001$ for gonad wet weight and GSI for the 12-week sampling date) (Fig. 3.5 B and C). By week 9, urchins fed formulated feeds had already achieved a 190 ± 12.09% (mean ± SE) increase in GSI, from the initial starting value, compared with a 57.3% increase in GSI for animals fed exclusively with fresh *Ulva*, over the same time period (Fig. 3.5 C). There was, however, no significant difference between the GSI of urchins fed the different formulated feeds, at any stage of the experiment.

5.4.2.2 Gonad colour

Mean eye-rated gonad colour (Fig. 3.6) ranged from 1.8 to 3.4 for urchins fed the formulated feeds, whereas urchins fed with fresh *Ulva* produced lower colour ratings, which fluctuated between 1.4 and 2. At the end of the feeding trial, urchins fed the 20U diet and fresh *Ulva* had comparable colour ratings that were not significantly different from one another (Kruskal - Wallis, $p > 0.05$). In contrast, the eye rated gonad colour of urchins fed the remaining three artificial diets (0U, 5U, 15U) was shown to be significantly different from FU (Kruskal - Wallis, $H5 = 22.37$, $p = 0.0004$ for the 12-week sampling date), meaning the colour was worse in terms of market acceptance, compared to the fresh *Ulva* treatment at the end of this study.

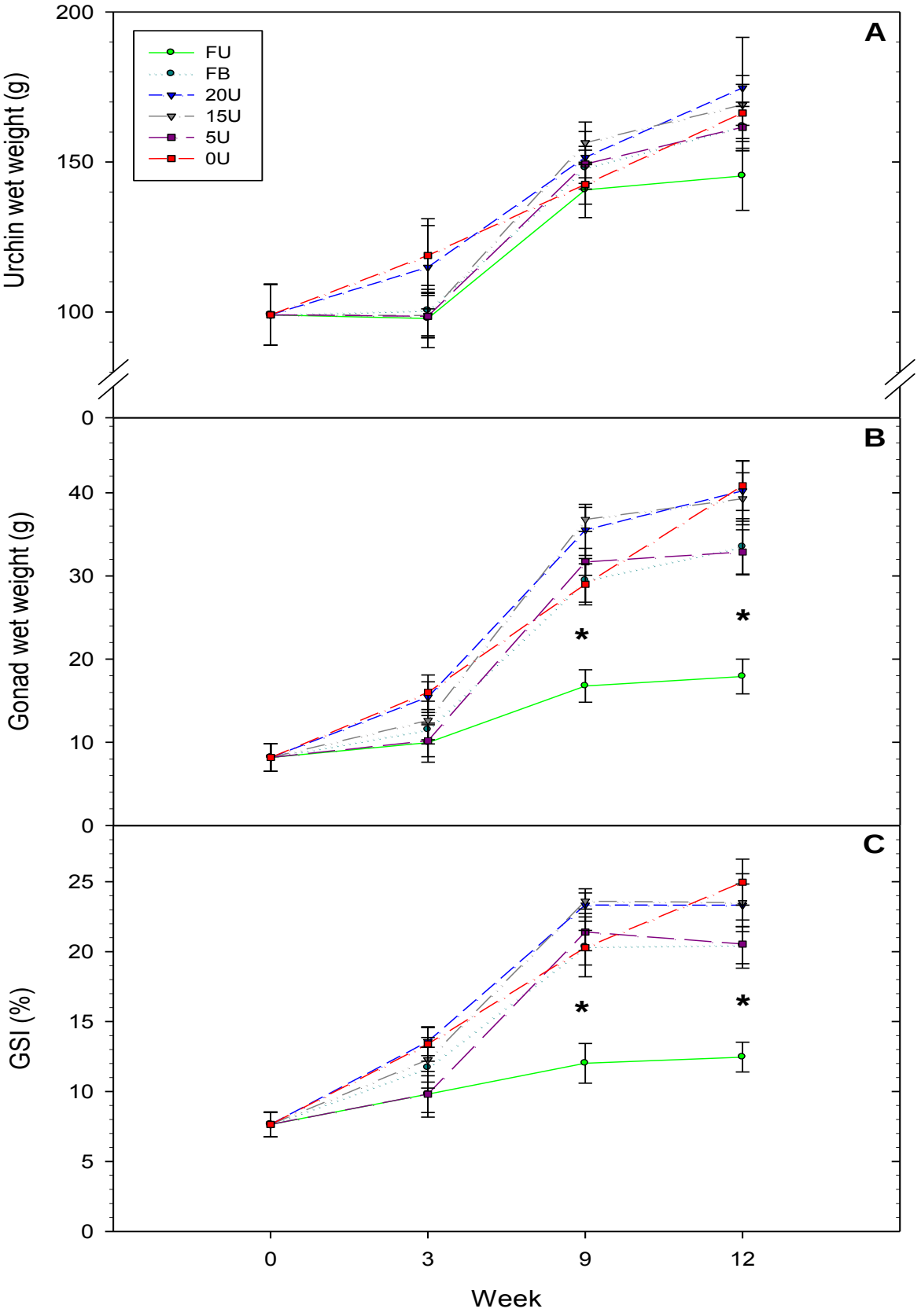


Figure 3.5: Mean **(A)** urchin wet weight, **(B)** gonad wet weight and **(C)** gonad somatic index (GSI) of *Tripneustes gratilla* fed with four artificial diets, fresh *Ulva* or a mixed diet consisting of fresh *Ulva* and a basal feed over a 12-week grow-out period in recirculating seawater systems. Data represents mean ± SE of eight replicates per treatment group. *($p < 0.05$, Tukey test) represents a significant difference in the means of urchins

fed the artificial or mixed diets from the means of urchins fed fresh *Ulva*. Treatment groups: FU = fresh *Ulva*; FB = fresh *Ulva* and the basal diet; 20U = 20% *Ulva*; 15U = 15% *Ulva*; 5U = 5% *Ulva*; 0U = 0% *Ulva*.

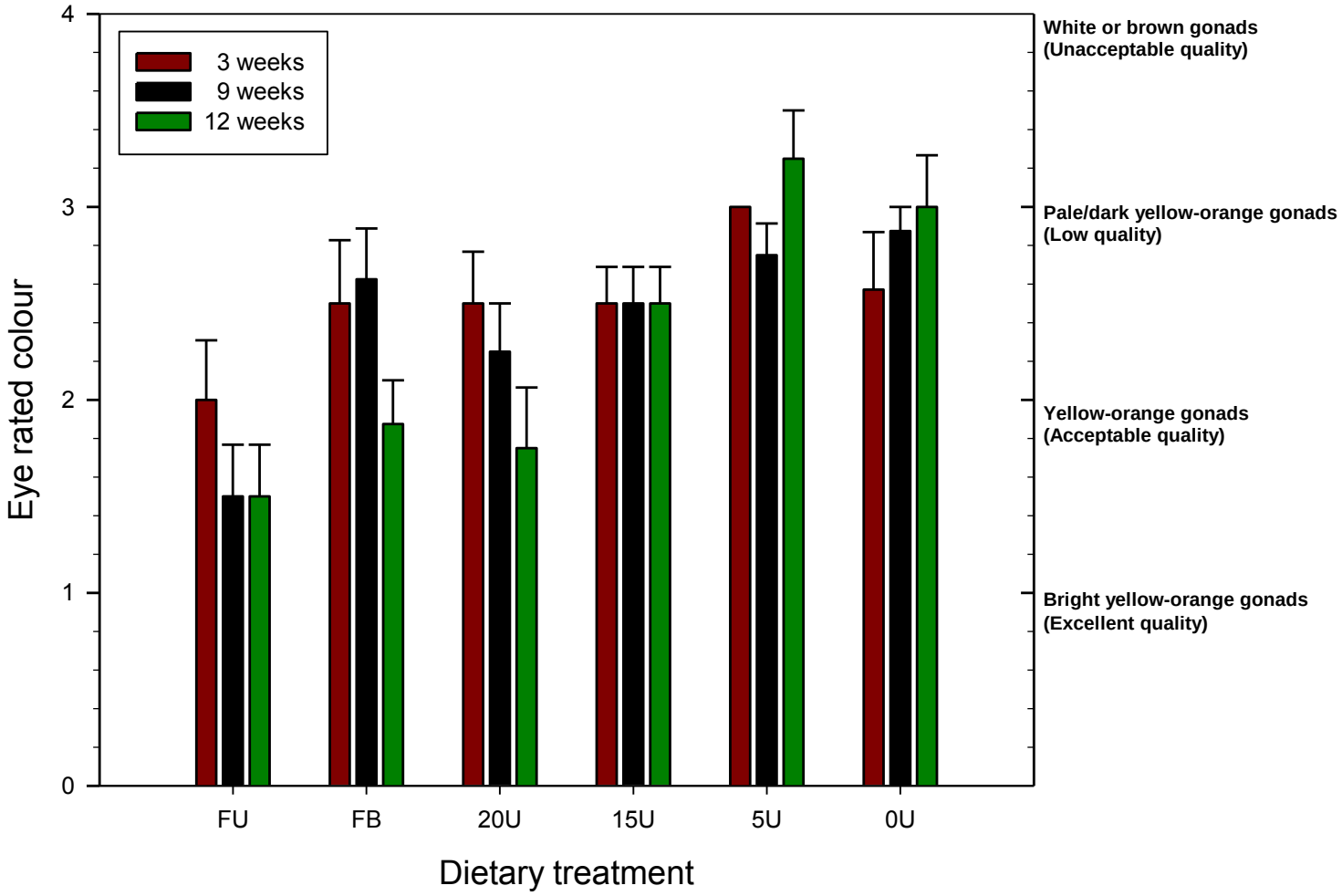


Figure 3.6: Mean eye rated (ER) gonad colour of *Tripneustes gratilla* fed with four artificial diets, fresh *Ulva* or a mixed diet consisting of fresh *Ulva* and a basal feed over a 12-week grow-out period. Data represents the mean ± SE of eight replicates per treatment group. Gonad colour was visually ranked in categories ranging from most desirable to unacceptable. The categories were allocated numbers, which were ranked as follows: (1) bright yellow-orange gonads (excellent quality); (2) yellow-orange gonads (acceptable quality); (3) pale yellow-orange or dark yellow-orange gonads (low quality) and (4) white or brown gonads (unacceptable). Treatment groups: FU = fresh *Ulva*; FB = fresh *Ulva* and the basal diet; 20U = 20% *Ulva*; 15U = 15% *Ulva*; 5U = 5% *Ulva*; 0U = 0% *Ulva*.

Gonad lightness (L*) values ranged from 57.88 to 62.44 and were unaffected by diet, with no significant difference recorded between any of the dietary treatments by the end of this study (Fig. 3.7 A). Likewise, gonad redness (a*) values did not vary significantly between treatment groups over the course of the study and ranged from 11.17 to 15.22 (Fig. 3.7 B). In contrast, mean gonad yellowness (b*) values decreased with the decreasing *Ulva* content of each diet and, by week 9, urchins in the 5U treatment group showed significantly lower gonad b* values, when compared with the FU treatment group (Fig. 3.7 C; one-way

ANOVA; $F_{5,42}$ = 3.891, p = 0.001). By the end of the feeding trial, the 15U, 5U and 0U treatment groups had significantly lower b* values ($F_{5,42}$ = 5.92, p = 0.0003), compared with the FU treatment group. In contrast, urchins in the 20U and FB treatment groups produce gonads with similar yellowness values that were not significantly different from urchins fed a diet of fresh *Ulva*, by the end of the study.

The total difference in gonad colour, calculated using the formula from McBride *et al*. (2004), between urchins fed fresh *Ulva* and the various artificial diets, support the latter findings, with total gonad colour values increasing as the *Ulva* content of each diet decreased (Fig. 3.8). The 20U and FB treatment groups did, however, produce urchins with gonads that were similar in colour to urchins fed fresh *Ulva*, and these two treatments had significantly better colouration (one-way ANOVA, F = 2.901, d.f. = 39, p = 0.035 for the 12-week sampling date), in terms of their market acceptance, compared with the 15U, 5U and 0U treatment groups by week 12.

In addition, a Fitted Generalized Estimating Equations General Linear Model (geeglm) revealed that gonad colour (L*, a*, b*) was significantly influenced by dietary treatment and gonad maturity, as gonads in the FU treatment group were significantly better coloured, in terms of their market acceptance, than those in the 15U (p = 0.016), 5U (p = 0.000031) and 0U (p = 0.0001) treatment groups (Table 3.1). The 20U and FB treatment groups, however, did not vary significantly in terms of gonad colour when compared with the FU diet. Gonad maturity also contributed to and significantly affected gonad colour, with gonads in a recovering stage having similar coloured gonads to those in a spent stage. Whereas gonads in the growing (p = 0.0071), pre-mature (p = 0.0182), mature (p = 0.0005) or partly spent (p = 0.0016) states had significantly better coloured gonads than those in a recovery or spent state (Table 3.2).

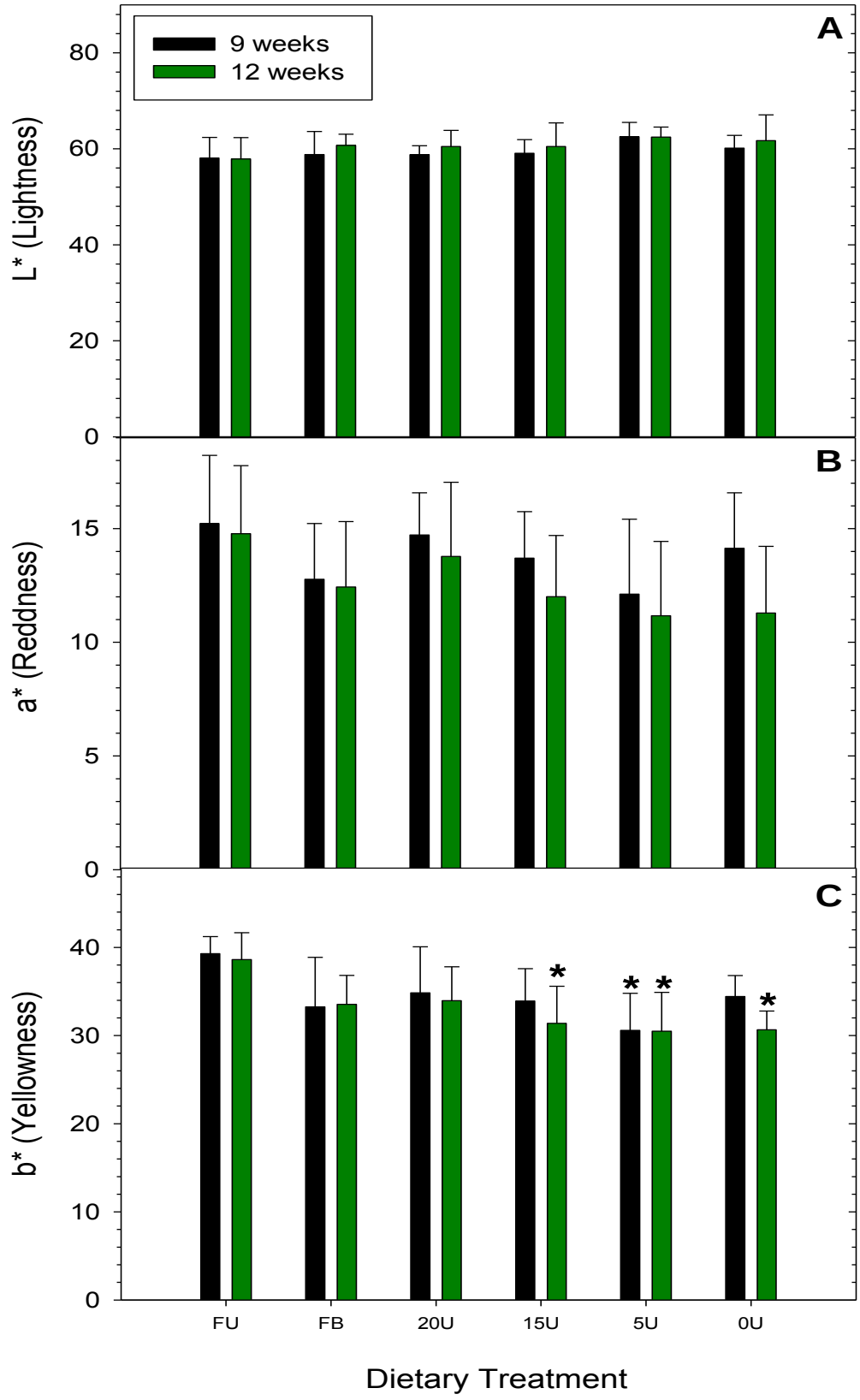


Figure 3.7: Mean gonad (a) L* (lightness), (b) a* (redness) and (c) b* (yellowness) values of *T. gratilla* fed with four artificial diets, fresh *Ulva* or a mixed diet consisting of fresh *Ulva* and a basal feed over a 12- week grow-out period. Data represent the mean ± SE of eight replicates per treatment group. *($p < 0.05$, Tukey test) represents a significance differences in the means of urchins fed the artificial or mixed diets from the means of

urchins fed fresh *Ulva*. Treatment groups: FU = fresh *Ulva*; FB = fresh *Ulva* and the basal diet; 20U = 20% *Ulva*; 15U = 15% *Ulva*; 5U = 5% *Ulva*; 0U = 0% *Ulva*.

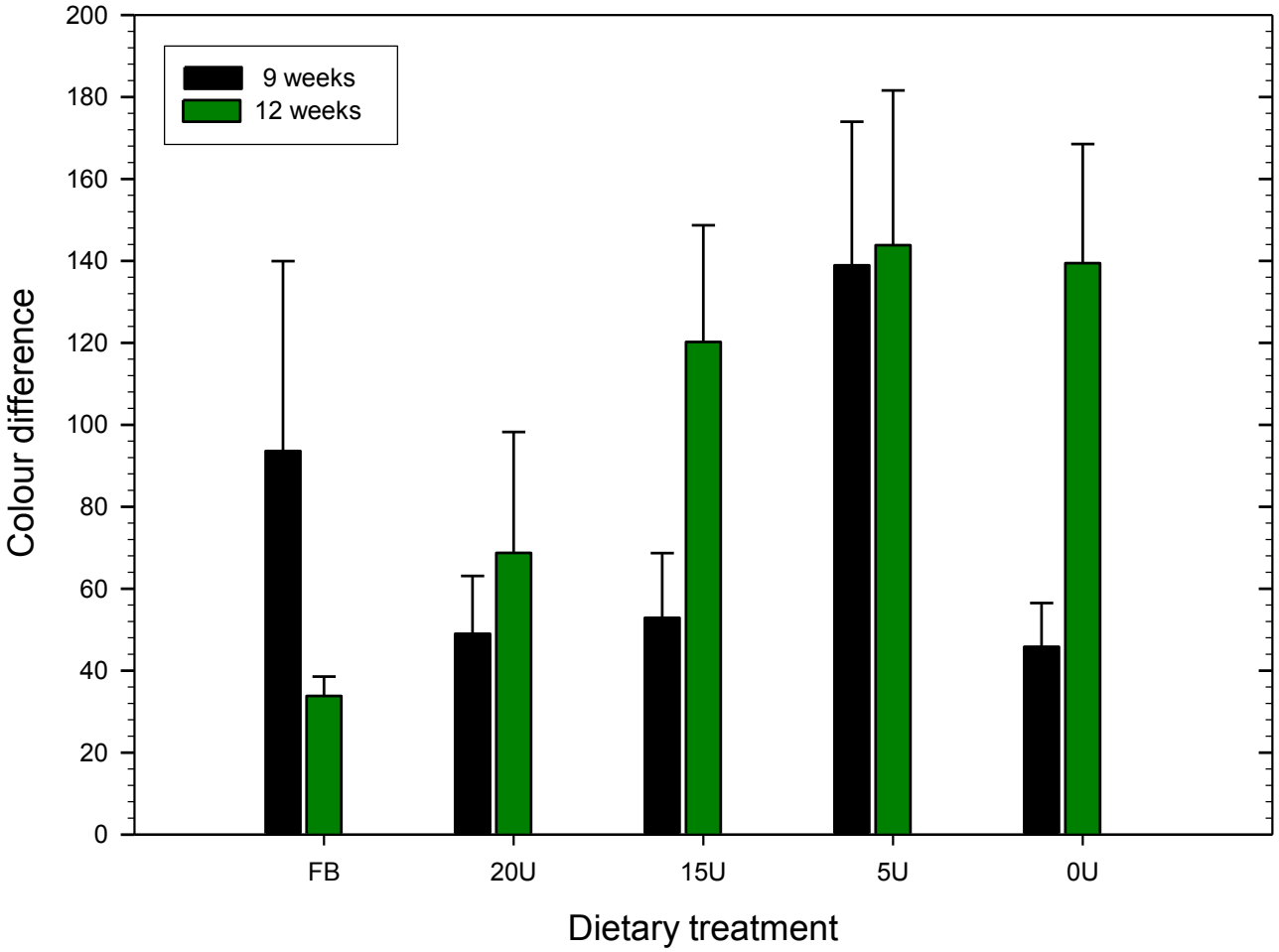


Figure 3.8: The total difference in spectrophotometer rated gonad colour of *Tripneustes gratilla* fed with the four artificial diets or a mixed diet (consisting of fresh *Ulva* and a basal diet) from the gonad colour of urchins fed with fresh *Ulva*. Data represent the mean ± SE of eight replicates per treatment group. Treatment groups: FU = fresh *Ulva*; FB = fresh *Ulva* and the basal diet; 20U = 20% *Ulva*; 15U = 15% *Ulva*; 5U = 5% *Ulva*; 0U = 0% *Ulva*.

Table 3.1: A Fit Generalized Estimating Equations General Linear model (geeglm) analysis showing the significant interactions between dietary treatment, sex of an urchin, certain maturity stages and Gonad Somatic Index (GSI) when predicting spectrophotometer rated gonad colour values (L*, a*, b*) of *Tripneustes gratilla* fed with four artificial diets, fresh *Ulva* and a mixed diet over a 12-week grow-out period. All non-significant interactions were excluded from the table. Treatment groups: FU = fresh *Ulva*; FB = fresh *Ulva* and the basal diet; 20U = 20% *Ulva*; 15U = 15% *Ulva*; 5U = 5% *Ulva*; 0U = 0% *Ulva*

Effect	Est. GLM	SE. GLM	Wald Test Statistic	Pr(>|W|)	Significance
(Intercept)	35.3798	0.7921	1994.65	< 2e-16	***
Treatment with reference to FU					
20U	1.7054	0.9095	3.52	0.0607	
15U	1.9280	0.8028	5.77	0.0163	*
5U	3.1301	0.7514	17.35	0.000031	***
0U	3.1610	0.8332	14.39	0.0001	***
FB	0.7904	1.1891	0.44	0.5062	
Sex of urchin	-0.6120	0.7239	0.71	0.3978	
Maturity stage					
Growing	-2.1599	0.8032	7.23	0.0071	**
Premature	-1.5835	0.6711	5.57	0.0182	*
Mature	-2.5422	0.7354	11.95	0.0005	***
Partly spent	-2.5407	0.8050	9.96	0.0016	**
Spent	-2.0112	1.1217	3.21	0.0729	
GSI	0.06081	0.0354	2.94	0.0865	

Significancecodes*:* *** ($p < 0.001$), ** ($p < 0.01$), * ($p < 0.05$)

5.4.2.3 Gonad texture and firmness

Mean gonad texture ratings at the end of the study period ranged from 1.75 to 2.88 and all treatment groups consisted of gonads with two distinct gonad segment halves with at least some visible follicle separation. There were, however, no significant differences in gonad texture ratings between any of the treatment groups at each of the sampling dates. Similarly, mean gonad firmness ratings did not differ significantly between treatments at the end of the study period and ranged from 2 to 3, indicating that gonads were soft - firm in their appearance.

5.4.2.4 Histology

Histological analysis of sea urchin gonads revealed a significant degree of variability in overall gametogenic development, and therefore gonad maturity, between sampling dates within all treatment groups (Kruskal - Wallis, H = 72.68, d.f. = 3, p = 0.00000066). However, there were no significant differences between the different dietary treatment groups at each sampling date (Fig. 3.9). At the beginning of the feeding trial, the gonads of approximately 40% of urchins were in a recovery stage while another 40% were spent, the remaining 20% were however on partly spent (Fig. 3.9A). Thereafter, there was a fairly consistent progression in gonad maturity, with gonads developing or maturing throughout the study period and accumulating nutritive phagocytes, which were stored and then used to produce gametes. Although gonad maturity did not differ significantly between the treatment groups, a General Linear Model analysis revealed (Table 3.2) that the density of NP is significantly affected by dietary treatment, gonad maturity and sex of the urchin. During week 9, diet type significantly ($F_{5,37}$ =2.743, p = 0.033) affected the storage of nutrients and therefore the density of NP's (Fig. 3.10) within the gonad. Urchins in the 20U treatment group had significantly more densely packed NPs compared with the 5U (p = 0.005) and 0U (p = 0.025) treatment groups, possibly indicating that the addition of *Ulva* to prepared diets may affect cell density. Gonad maturity was also shown to have an effect on NP density with gonads in the recovery stage having significantly denser packed NP's compared with gonads in a partly spent (p = 0.006) or spent stage (p = 0.007) (Table 3.2). Sex was also shown to significantly (p < 0.00001) affect NP density within the gonad, as female gonads had more densely packed NP's compared with male gonads. Nutritive phagocytes density therefore appeared to show a general trend of decreasing as gonads developed gametes and the gonads mature.

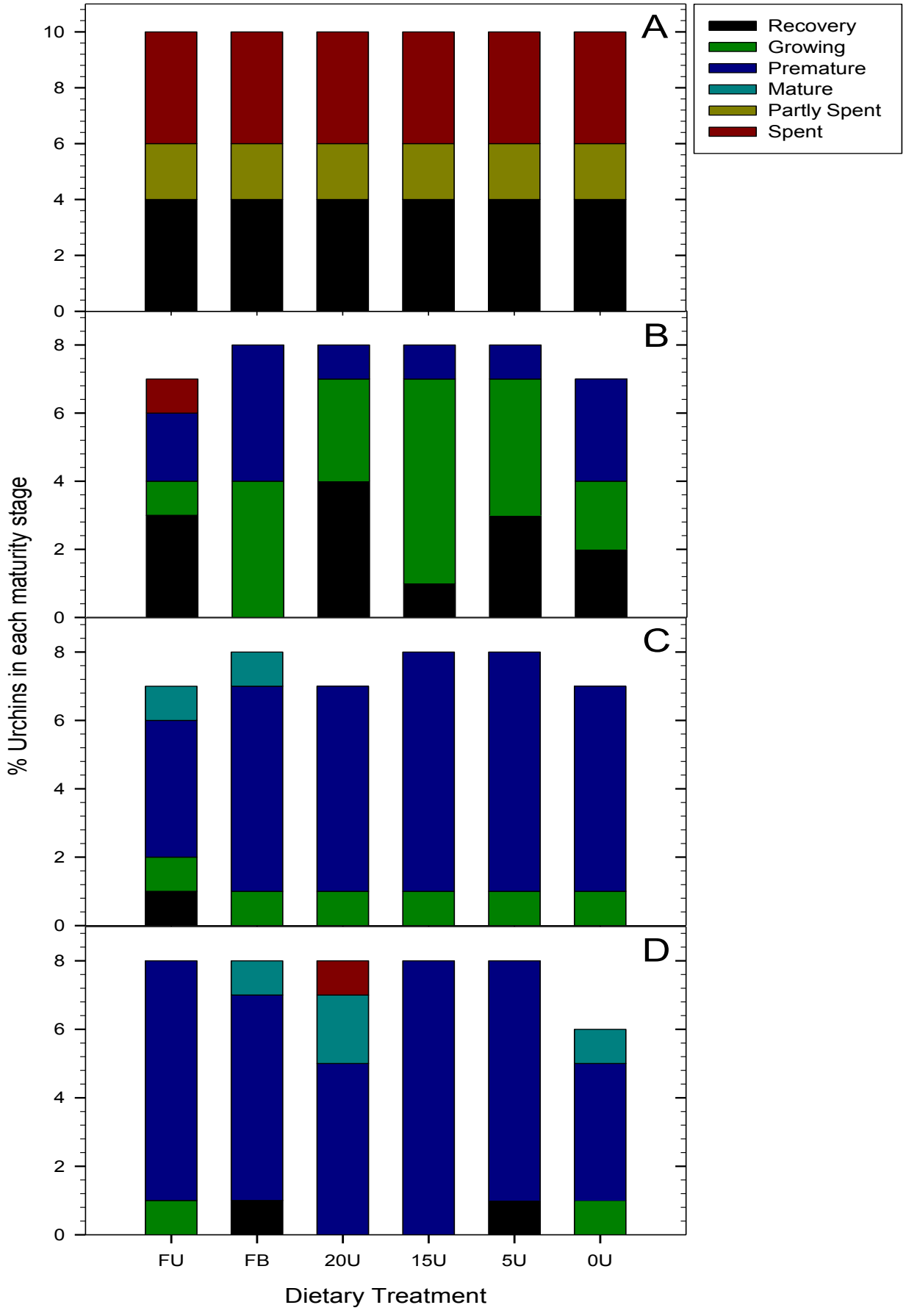

A
B
C
D
Recovery
Growing
Premature
Mature
Partly Spent
Spent
% Urchins in each maturity stage
0
2
4
6
8
10
FU
FB
20U
15U
5U
0U
Dietary Treatment

Figure 3.9: The mean number of *Tripneustes gratilla* (n = 8) of both sexes allocated to each maturity stage over the 12-week grow-out period. Gonads of urchins fed the four artificial diets, Fresh *Ulva* and a mixed diet (consisting of fresh *Ulva* and a basal feed) were processed for histology at **(A)** week 0, **(B)** week 3, **(C)** week 9 and **(D)** week 12 to assess the amount of gametogenic activity within the gonad.

Table 3.2: A Generalized Linear Model (GLM) showing the significant interactions between wet gonad weight, dietary treatment, sampling date, gonad maturity and sex of urchin when predicting the density of nutritive phagocytes within the gonads of *Tripneustes gratilla* fed with four artificial diets, fresh *Ulva* and a mixed diet over a 12-week grow-out period. All non-significant interactions were excluded from the table. Treatment groups: FU = fresh *Ulva*; FB = fresh *Ulva* and the basal diet; 20U = 20% *Ulva*; 15U = 15% *Ulva*; 5U = 5% *Ulva*; 0U = 0% *Ulva*

Effect	**Est. GLM**	**SE. GLM**	**t-value**	**Pr(>|t|)**	**Significance**
(Intercept)	65.49723	3.71022	17.653	< 2e-16	***
Wet gonad weight	-0.1365	0.09236	-1.478	0.14133	
Treatment with reference to 20U					
15U	-4.81675	2.8702	-1.678	0.09519	
5U	-8.14094	2.89657	-2.811	0.00554	**
0U	-6.6041	2.9327	-2.252	0.02564	*
FU	-1.50686	3.3551	-0.449	0.65393	
FB	-3.33947	2.93875	-1.136	0.25745	
Sampling date					
Week 9	-2.24497	2.96187	-0.758	0.44955	
Week 12	0.78831	3.1022	0.254	0.79972	
Maturity stage with reference to Recovery					
Growing	0.51753	3.32856	0.155	0.87663	
Premature	-4.94792	3.35902	-1.473	0.14264	
Mature	-7.31106	4.57093	-1.599	0.11162	
Partly spent	-12.92725	4.67189	-2.767	0.00630	**
Spent	-15.93999	5.82699	-2.736	0.00691	**
Sex of urchin	8.8668	1.69475	5.232	5.00E-07	***

Significancecodes: *** ($p < 0.001$), ** ($p < 0.01$), * ($p < 0.05$)

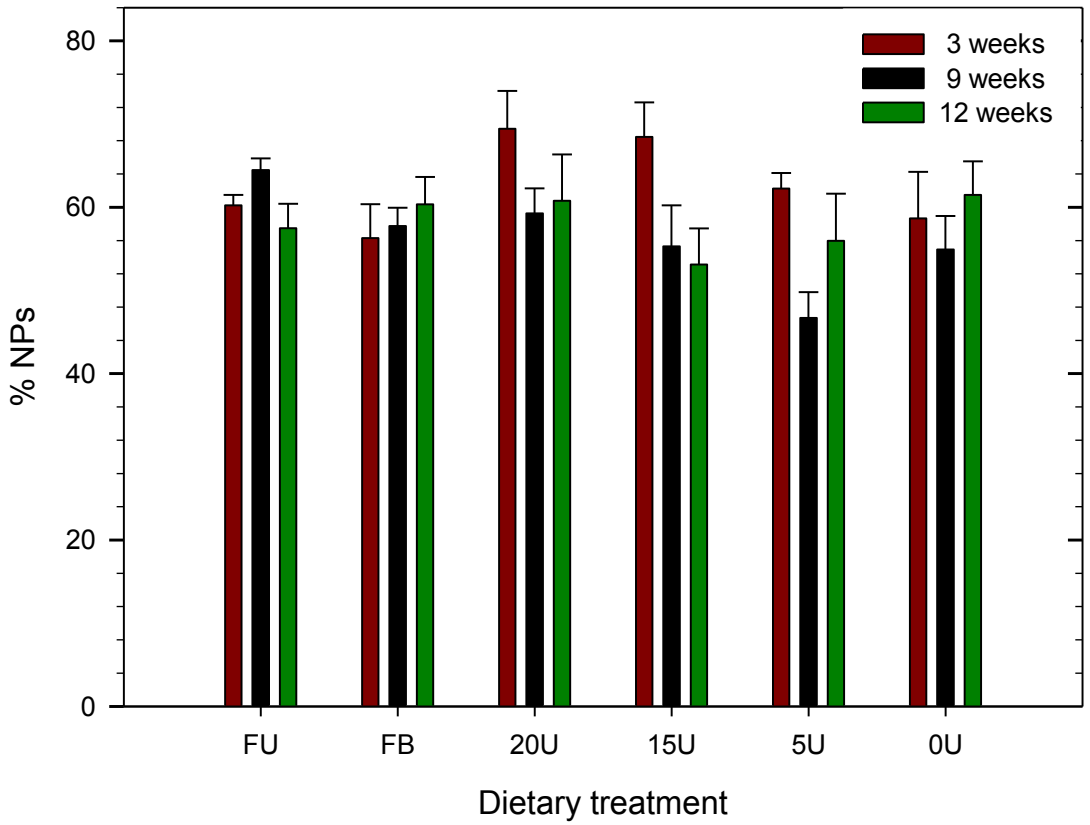


Figure 3.10: Mean nutritive phagocyte density (NP) (percentage ± SE, n = 8) within the gonads of *Tripneustes gratilla* fed with four artificial diets, fresh *Ulva* or a mixed diet consisting of fresh *Ulva* and a basal feed over a 12-week grow-out period. Treatment groups: FU = fresh *Ulva*; FB = fresh *Ulva* and the basal diet; 20U = 20% *Ulva*; 15U = 15% *Ulva*; 5U = 5% *Ulva*; 0U = 0% *Ulva*.

3.5 Discussion

The artificial diets formulated and tested in this study have been shown to significantly increase gonad yield in the sea urchin *Tripneustes gratilla*, when compared with urchins fed a diet of fresh *Ulva*. The GSI of urchins fed artificial diets in this study ranged from 20.05% to 24.96% (≈ 200% increase) at the end of the 12-week study, whereas urchins fed exclusively with a natural algae diet had significantly lower GSI values, reaching a maximum of just 12.5% (64% increase), within the same time period. These findings are consistent with the results of previous studies that have demonstrated the effectiveness of artificial feeds for enhancing the growth and development of sea urchins. Artificial diets have successfully increased the GSI of cultured *Evechinus chloroticus* (James, 2006; Phillips *et al.*, 2009;

Phillips *et al*., 2010), *Psammechinus miliaris* (Pantazis *et al*., 2000), *Paracentrotus lividus* (Spirlet *et al*., 2000; McBride *et al*., 2004; Schlosser *et al*., 2005; Shpigel *et al*., 2005), *Strongylocentrotus droebachiensis* (Walker & Lesser, 1998; Pearce *et al*., 2002a; 2004), *Lytechinus variegates* (Hammer *et al*., 2004; Taylor *et al*., 2009) and *Loxechinus albus* (Lawrence *et al*., 1997; Olave *et al*., 2001) when compared with wild caught individuals or individuals fed natural diets. This study also demonstrated that cultured *T. gratilla* fed *ad libitum* with the macroalga *Ulva* achieved significantly higher GSI values (up to 12.5%) when compared with the GSI values (maximum of 4.9%) reported for *T. gratilla* collected from the wild (Muthiga, 2005). However, even though the artificial diets tested in these studies produced large gonads, many of the gonads were pale and unmarketable. In contrast, this study demonstrates that artificial diets supplemented with 20% (w/w) dried *Ulva* (20U) can produce bright yellow, marketable gonads, which do not differ significantly in colour in the experimental conditions from those produced on fresh *Ulva*.

The gonad growth rates obtained from urchins fed formulated feeds in this study compare quite favourably to those in other studies in the literature for formulated and natural feeds (Table 3.3). On average, *T. gratilla* fed formulated feeds had a 1.2% increase in gonad somatic index, per week, over the course of this study, which is comparable to the growth rates reported by Pearce *et al*. (2004) and Shpigel *et al*. (2005) for *Strongylocentrotus droebachiensis* and *Paracentrotus lividus*, respectively. Moreover, weekly gonad growth rates on the 20U feed formulated in this study were 19 and 48% higher than the growth rates of *Evechinus chloroticus* achieved in a similar study using two different formulated feeds, currently used in commercial echinoculture (Woods *et al.*, 2008). These reports would tend to suggest that some of the feeds currently formulated for commercial echinoculture do not achieve maximum GSI gain and therefore can be improved.

Table 3.3: Gonad growth, expressed as a per cent increase in gonad somatic index (GSI) per week, of a variety of sea urchin species fed with artificial and natural macroalga feeds

Taxon	Diet type	Protein content (%)	Gonad growth (GSI increase.week^{-1})	[a]Reference
Tripneustes gratilla	*Ulva*	18	0.37	1
T. gratilla	Prepared (20U)	26	1.22	1
T. gratilla	Prepared (15U)	26	1.23	1
T. gratilla	Prepared (5U)	26	1.00	1
T. gratilla	Prepared (0U)	26	1.35	1
Evechinus chloroticus	Prepared Feed	N/A	0.73	2
E. chloroticus	Prepared Feed	15	1.01	3
E. chloroticus	Prepared Feed	21	0.64	3
Paracentrotus lividus	Prepared Feed	20	1.20	4
P. lividus	*Ulva lactuca* & *Gracilaria conferta*	N/A	0.00	4
P. lividus	Prepared Feed	N/A	0.87	5
Strongylocentrotus droebachiensis	Prepared Feed	23	1.17	6
S. droebachiensis	*Laminaria longicruris*	13	0.50	6
Loxechinus albus	Prepared Feed	N/A	0.50	7
L. albus	*Ulva*	N/A	-0.30	7

[a]References: [1]This study; [2]James (2006); [3] Woods *et al.*(2008); [4]Shpigel *et al*. (2005); [5]Spirlet *et al.* (2000); [6]Pearce *et al.* (2004); [7] Lawrence *et al.* (1997)

The high GSI values recorded in the literature for urchins fed artificial diets compared with those fed natural algal diets have been attributed, primarily, to differences in protein levels between the feeds. As protein is one of the most expensive components of an artificial feed, it is essential to determine the optimal levels of protein required to maximize growth. Previous studies have determined that a moderate dietary protein level of approximately 20% is most efficient for maximizing the gonad growth of sea urchins (Akiyama *et al*., 2001; Pearce *et al*., 2002a; Schlosser *et al*., 2005). In this study, all artificial diets were formulated to contain roughly equal crude protein concentrations (≈26%), so that the only variable differing between the prepared diets was the inclusion of different amounts of *Ulva* or *Ulva* additive. *Ulva* used in this study had a crude protein content of 18.31%, which is significantly lower than the protein content of the prepared diets (≈26%); possibly explaining the enhanced

gonad growth and development of urchins fed prepared diets compared with fresh *Ulva*. It could, however, also be argued that the enhanced gonadal growth of urchins fed the prepared diets in this study may be attributed to differences in consumption rates. Consumption rates for *T. gratilla* fed the 20U diet were significantly higher compared with animals fed the 5U and 0U diets (Chapter 2). However, no significant differences in urchin wet weight or gonad wet weight were recorded between these treatment groups (Fig. 3.5). Also, each urchin consumed, on average, approximately 3.5 g of *Ulva* (wet weight) per day compared with 1.5–2.0 g of each prepared feed (dry weight) per day (data not shown). As *Ulva* has a moisture content of approximately 80% (Shuuluka, 2011), this equates to approximately 0.7 g of *Ulva* (dry weight) per urchin per day. Previous studies have often indicated greater consumption of algeal diets compared to artificial ones, however as a result of *Ulva's* morphology and high water content this is not the case here. These findings suggest that the high protein content and increased consumption of the prepared feeds may have collectively contributed to the enhanced gonadal growth of urchins fed the prepared diets in the present study, although other factors, such as feed shape and texture, should also be considered. Moreover, consumption data (Chapter 2) also suggests that the inclusion of high quantities of dried *Ulva* into a prepared feed may act as a feeding stimulant.

Schlosser *et al*. (2005) suggest that digestible energy may also be a limiting factor in naturally available diets, such as *Ulva*. They showed that a prepared diet with a crude protein content of 23% produced significantly higher GSI values compared with a natural diet of *Ulva lactuca*, with a crude protein content of 35%. In their study, the prepared feed and fresh *Ulva* had gross energy values of 19.39 and 13.39 $MJ.kg^{-1}$, respectively. Schlosser *et al*. (2005) postulate that the difference in energy content between these two feeds resulted in enhanced growth and gonad development with the prepared feed. The higher energy content of the prepared feed used in that study is similar to the results recorded in the present study,

where the prepared feeds in the current study had gross energy contents between 15.49 and 17.18 MJ kg^{-1}, whereas the natural *Ulva* diet had a gross energy content of 9.44 $MJ.kg^{-1}$. Both protein and energy contents of *Ulva* in this study were, therefore, shown to be suboptimal, as the artificial feeds contained 65% more energy and 44% more protein than *Ulva*. The low gross energy, crude protein content and consumption rate of *Ulva* utilized in this study makes it difficult to determine which of the three factors, if not all, may have contributed to the low GSI values recorded for urchins fed fresh *Ulva,* compared with those fed artificial diets.

Gonads of commercial quality need to be acceptable in terms of colour, which in the past has proven to be more difficult to achieve using artificial diets compared with natural diets (Senaratna *et al*., 2005; Shpigel *et al*., 2005). Differences in colour between animals fed natural and artificial diets have been attributed mainly to the lack of carotenoid pigments contained within artificial diets (Robinson *et al*., 2002; Shpigel *et al*., 2005; Shpigel *et al*., 2006; Symonds *et al.,* 2007). In this study, the addition of *Ulva* to the formulated diets acted as a natural source of carotenoid pigments, with *Ulva rigida* and *Ulva lactuca* from the same aquaculture system having an average total carotenoid content of 6.7% and 7.3% DW, respectively (Shuuluka, 2011). Echinenone, which is synthesized from β-carotene, is responsible for the yellowish-orange colour of high quality roe (Pearce *et al*., 2002a; McBride *et al*., 2004; Shpigel *et al*., 2005). The present study demonstrated that gonad colour was dependent on the inclusion level (0%, 5%, 15% and 20% w/w) of *Ulva* within the diets, with higher inclusion rates promoting better coloured gonads. The FU diet, as expected, proved to be the most successful at producing gonads of marketable colour, and this is most likely due to the high concentration of β-carotene contained within this diet, compared with the artificial ones. Indeed, the FU diet produced gonads which differed significantly in eye-rated colour from all other diets used in this study with the exception of the 20U diet. As eye-rated colour may be quite subjective, a non-subjective colour measure was also employed,

which gave three colour values, namely, lightness (L*), redness (a*) and yellowness (b*). In this study there were no significant differences in gonad lightness (L*) or redness (a*) between any of the dietary treatments. However, the yellowness (b*) values recorded in this study varied significantly between the sampling dates and dietary treatments. By the 9th week, FU gonad b* values varied significantly from only the 5U diet. However, by the 12th week the b* values of the FB and 20U diets did not vary significantly from that of the FU diet, while diets which contained less *Ulva* (15U, 5U and 0U diets) produced gonads which were less yellow. These findings reinforce the importance of carotenoid pigments in sea urchin diets for increasing gonad quality, and suggest that both the 20U and FB diets can successfully produce gonads that are not significantly different, in terms of colour, from gonads of urchins fed a natural diet of *Ulva*. Robinson *et al*. (2002) reported similar results for gonad yellowness (b*) values during the course of their study. Yellowness decreased significantly over time in gonads of animals fed artificial diets, whereas wild urchins (*Strongylocentrotus droebachiensis*), feeding on natural algae diets, had an increase in gonad yellowness over time. Aside from the obvious commercial interests in higher profits obtained from better coloured gonads, it has been suggested that there are several other advantages to adding carotenoid pigments to prepared sea urchin diets. This is becuase pigments within actively growing tissue can act as important antioxidants, are involved in protein stabilization, aid in egg production and provide ultraviolet protection for sensitive tissues (George & Lawrence, 2002; Robinson *et al*., 2002).

In this study, texture and firmness ratings for all treatment groups and sampling dates did not vary significantly over the study period and generally fell within an acceptable range for sale on commercial markets. However, a gonad factor which differed significantly between treatment groups and sampling dates was the density of NPs contained within gonads. There was a trend of decreasing gonad NP density with decreasing *Ulva* content, demonstrating that

the 5U and 0U diets had significantly less densely packed NPs, ranging from 46.8% to 62.2% over the course of the study, compared with the FU, FB, 20U and 15U diets (ranging from 53.1% to 68.7%) which did not differ significantly in NP density from each other. Gonads in a recovery phase were also shown to have significantly more densely packed NPs compared with gonads in a partly spent or spent stage, whereas urchins in a growing, premature or mature stage did not differ significantly. This result is, however, not surprising, as development of gonads from recovery to mature stages is characterized by nutrient accumulation and gamete development, whereas immediately before and after spawning nutrients have been used up and most gametes have been released, resulting in a decrease in gonad NP density. Results from this study support findings by Böttger *et al*. (2006), who found that *S. droebachiensis* in early pre-gametogenesis have an increased density of NP's within the gonad compared with gonads in later maturity stages. Histological analysis of gonads over the course of this study did, however, reveal no significant differences in gonad maturity between the different dietary treatments. Gonad maturation followed a similar trend in all treatment groups, with gonads accumulating nutrients within NPs, followed by the development and storage of gametes.

In conclusion, it has clearly been demonstrated that prepared diets (26% crude protein content) can significantly increase gonad yield of urchins within 9 weeks, compared with urchins fed only a natural diet of Fresh *Ulva*. More importantly, it has been demonstrated that when these artificial diets are supplemented with 20% (w/w) dried *Ulva*, marketable quality gonads can be produced, which do not differ significantly in colouration from the gonads of urchins fed with fresh seaweed (*Ulva*). Therefore, there is clearly potential for artificial diets containing *Ulva* to support the commercial cultivation of *Tripneustes gratilla* in South Africa, and allow for the local industry to become part of an already successful and very lucrative, international echinoculture industry.

CHAPTER 4:

Development of a feeding regime using fresh *Ulva* and artificial feeds containing *Ulva* for full life-cycle grow-out of *Tripneustes gratilla*

Contents

4.1 Abstract

This study investigated the use of 5 different feeding regimes, consisting of different combinations of artificial feeds containing 20% (20U) or 0% *Ulva* (0U) and Fresh *Ulva* (FU), and their effects on somatic and gonadal growth of juvenile *Tripneustes gratilla,* during full life-cycle grow-out in land based aquaculture systems. The aim of the study was to determine whether different feeding regimes tested could affect the production time needed to produce urchins of marketable size and if these regimes affected gonad size and quality over a 32-week period. The trial was divided into two phases, a somatic growth phase (20 weeks) and a gonad enhancement phase (12 weeks), with different combinations of feeds tested for each phase. The five regimes used were FU only, FU for 20 weeks and 20U for 12 weeks (FU-20U), FU-0U, 20U-FU and 0U-FU. Findings from this study indicate that comparable somatic growth of juvenile urchins could be achieved during the first 20 weeks of the trial using either an artificial diet containing *Ulva* (20U) or Fresh *Ulva* (FU), while an artificial diet with the same nutritional properties, but without the addition of *Ulva* (0U) produced urchins that were significantly smaller. Gonad production in the first 20 weeks of the trial was higher for urchins fed with artificial diets (0U & 20U); however, the reduced size of urchins in the 0U diets significantly reduced gonad mass. After the dietary switch at 20 weeks, both gonad size and colour were significantly affected. Artificial diets promoting gonad growth of urchins previously fed FU, while administration of FU improved gonad colour of urchins previously fed the artificial diets. The FU only treatment produced gonads with the most desirable colour. The results from this trial led to the recommend use of FU for the somatic growth phases of this species, while the 20U artificial diet should be used to increase gonad size at the end of grow-out.

4.2 Introduction

Increasing demand for high quality sea urchin products has been the major driving force behind the development of culture technology for many commercially important sea urchin species (Mos *et al.,* 2012). The cultivation of sea urchins can be divided into two distinct types. The first is gonad enhancement where adults are collected from wild populations, maintained in captivity, and fed natural and/or artificial diets to increase gonad mass and quality to market standards (Vadas *et al.,* 2000; Hammer *et al*., 2006). The second is full life-cycle grow-out, where adults are collected from the wild, spawned in captivity, larvae are raised in hatcheries and juveniles are grown to commercial size either at sea, in some kind of containment system, or in land-based tanks (Daggett *et al.,* 2006; James, 2006; Cook & Kelly, 2007a). Since gonad enhancement practices are contributing towards the decline of natural sea urchin populations, full life-cycle grow-out echinoculture is gaining popularity and the industry is growing rapidly (Andrew *et al.,* 2002; Jimmy *et al.,* 2003; Daggett et al., 2005). Full life-cycle grow-out research is also being used in re-seeding programs, to re-populate natural urchin populations in over-exploited areas (Shokita & Yamaguchi, 1991; George *et al.,* 2000; Andrew *et al.,* 2002; Daggett *et al.,* 2005; Dworjanyn & Pirozzi, 2008; Juinio-Menez *et al.,* 2008; Rahman *et al.,* 2009b). However, even though echinoculture of *T. gratilla* has occurred in Japan and the Philippines (since 1994) for many years, published studies describing the full life-cycle grow-out of this species are limited, particularly pertaining to feeds (Juinio-Menez *et al.,* 2008). Determining optimal culture conditions and feed requirements, which can efficiently maximize survival, growth and gonad production, are essential requirements to make echinoculture commercially profitable.

Previous research in echinoculture has focused mainly on larval culture (Dworjanyn & Pirozzi, 2008; Byrne *et al.,* 2009; Rahman *et al.,* 2009a,b; Azad *et al.,* 2010; Sameoto *et al.,* 2010), gonad enhancement (Fernandez & Caltagirone, 1994; Lawrence *et al.,* 1997; Keesing

& Hall, 1998; Kelly *et al.*, 1998; Robinson & Colborne, 1998; Vardas *et al.*, 2000; Olave *et al.*, 2001; Pearce *et al.*, 2002c.; Hammer *et al*., 2006; James, 2006a,b; Cyrus *et al.*, 2014), formulated feed development (Klinger *et al.*, 1997; Pearce *et al.*, 2002a,b; Mortensen *et al.*, 2003; Cyrus *et al.*, 2014; Eddy *et al.*, 2012), and the development of suitable culture systems (Kessing & Hall, 1998; Daggett *et al.*, 2006; James, 2007). One area of research that has not been as thoroughly investigated is the somatic growth of sea urchins post-settlement to market size. Somatic growth during the hatchery and grow-out phase of echinoculture is critical (Basuyaux & Blin, 1998; Pearce *et al.*, 2005) and requires a substantial investment in time and resources, particularly if artificial feeds are to be used. Growth rates ultimately affect the success of any aquaculture industry, since production is mostly limited by the time needed for an individual to reach marketable size. Grow-out is, therefore, responsible for a considerable proportion of production costs, and, although there has been extensive research into developing artificial feeds and feeding regimes that can enhance gonad quality and quantity, far fewer studies have investigated the effects of these parameters on the growth and development rates of urchins throughout the hatchery and grow-out phases. Maximizing somatic growth as well as gonadal growth could potentially minimize production time and, therefore, the production costs associated with complete life-cycle culture. The anatomy and physiology of sea urchins is influenced largely by food quality and quantity, with food availability affecting the distribution and allocation of resources to different components of both somatic and gonadal growth (Beddingfield & McClintock, 1998; Russell, 1998; Guillou *et al.*, 2000). In the last few decades, there has been a global effort to develop formulated feeds that can optimize both the somatic and gonadal growth of cultured urchins (Lawrence *et al.*, 1997; Olave *et al.*, 2001; Shpigel *et al.*, 2005). Although many studies have focused on feed development, very few have investigated the effects of feeding strategies and regimes on gonad and/or somatic growth (James, 2012).

Feeding regimes (defined in this study as: the timing and manner in which feeds and different feed types are administered to cultured animals) are vital to the success of most aquaculture industries, as feed costs account for a significant proportion of total production costs. In order to optimise the use of feeds for aquaculture, feed regimes need to be designed to maximize growth, but their effects on body partitioning and resource allocation should also be accounted for. It is possible that different diets may be required for different developmental stages of an urchin's life, as observed for the abalone *Haliotis midae* (Shipton & Britz, 2001). Should this be true, the use of high protein diets aimed at promoting gonad development may not be required during early juvenile development, as these urchins are not sexually mature and have not yet started to produce gonads or initiate gametogenisis. A number of studies have shown that successful reproduction of sea urchins is directly linked to the quality and quantity of food available to the animals (Vadas, 1977; Minor & Scheibling, 1997). Nutritional factors do not, however, initiate the gametogenesis process (Pearse & Cameron, 1991), although they may result in the preferential development of gonads over somatic growth or vice versa, which could affect their rate of development. Food availability is the most important factor associated with regulating cellular composition, energy storage, and ultimately relative size of sea urchins and their gonads throughout the year (Garrido & Barber, 2001). It is, therefore important that one understands how food availability and quality affect the somatic growth of juvenile urchins, so as to maximize production efficiency.

Previous studies into the growth of *Tripneustes gratilla* have revealed that individuals usually reach a 50 - 70 mm test diameter within the first year of growth (Shimabukuro, 1991; Maharavo, 1993). It has been observed that most of this growth generally occurs early, within the first few months of a juvenile's life. *Tripneustes gratilla* reached 60 mm in just five months in the Philippines (Bacolod & Dy, 1986) and the Gulf of Aqaba (Dafni, 1992).

Knowing when and why urchins develop gonads is extremely important in aquaculture, because the size of an urchin heavily influences the amount of gonad an individual can produce. Muthiga (2005) reported that there was no significant relationship between gonad index and test diameter of *T. gratilla*, but that there was a significant relationship between gonad weight and test diameter. Previous research suggests that *T. gratilla* starts to develop gonads at a diameter of 50 mm (Dafni & Tobol, 1986; Maharavo, 1993; Juinio-Meñez *et al.*, 1998). Field studies have shown that urchins occurring in habitats where there is always abundant food may become reproductively mature at a smaller size and/or an earlier age, compared with urchins in habitats where food is limiting (Buchanan, 1966; Kawamura & Taki, 1965; Dix, 1970; Kawamura, 1973; Sivertsen & Hopkins, 1995). Sea urchin gonads not only act as a reproductive organ but as a storage organ as well, storing excess nutrient reserves before gametogenesis, in the form of nutritive phagocytes (NP's). This is extremely important, as both gonad production and gonadal maturation rate have a significant effect on the commercial value of an individual.

The aim of the current study was to determine the most appropriate feeding regime for enhancing both the somatic and gonadal growth of *Tripneustes gratilla* during full life-cycle grow-out echinoculture. Urchins fed either fresh *Ulva,* or formulated diets with and without dried *Ulva* (20U and 0U diets, respectively) supplementation at specific stages of growth, were assessed on a monthly basis to record somatic growth, gonadal growth and a number of gonad quality factors, such as colour, texture, gonad somatic index, and the dominant maturity stage of urchins in each experimental group. The specific objectives of this investigation were to address the following questions: (1) Do artificial diets promote better somatic growth of juvenile *T. gratilla* compared to the natural macroalgae *Ulva* and (2) Which feeding regimes will produce the most desirable quality product in large scale commercial operations?

In order for the aquaculture of this species to be commercially viable, it will be necessary to develop cost effective feeding regimes, which will maximize growth and production. Dietary treatments were, therefore, used in combination during the 32 week trial to test the effects of formulated feeds on early juvenile development, as well as how dietary regimes can affect gonad conditioning. Dietary regimes were changed after 20 weeks because it only takes 8-9 months from fertilization for *T. gratilla* to reach marketable size (Williamson, 2002), and a previous gonad enchantment trial (Cyrus *et al.,* 2014; Chapter 3) has shown that our formulated feeds are capable of producing large, high quality gonads in just 12 weeks. Thus, this allowed for a 12 week finishing/gonad enhancement period after the initial 20 weeks.

4.3 Materials & methods

4.3.1 Collection and maintenance of urchin broodstock

Adult *Tripneustes gratilla* (65 - 80 mm test diameter) were collected during low tide from shallow rock pools near Haga-Haga, Eastern Cape, South Africa (32°45′4.23″S, 28°16′41.30″E) on 2 - 5 August 2010, and maintained as described in Chapter 2 (Section 2.3.1) until used for experimental trials. Sea urchins were, however, fed a mixed diet of artificial feed 20U and fresh kelp (*Ecklonia maxima*) every second day. The artificial feed (Cyrus *et al.,* 2014) was prepared in the form of a semi-moist extruded feed pellet as described in Chapter 2 (Section 2.3.4), which was dried to a constant weight in a drying oven at 70°C. At each feeding, uneaten food was removed and the baskets cleaned before adding fresh feed.

4.3.2 Laval production

4.3.2.1 Spawning and fertilization

Sea urchins with a mean body diameter of 80.3 ± 6.25 mm (n=10) were spawned artificially, by injecting 1 - 2 mL of 2 Mol KCl through the peritoneum into the coelomic cavity (Shokita

& Yamaguchi, 1991, Dworjanyn & Pirozzi, 2008, Scholtz *et al.*, 2013). Individual sea urchins were only spawned once. Once injected, animals were immediately placed aboral-side down on top of sterile Erlenmeyer flasks (250 mL), filled with 0.2 µm filtered seawater. Samples of sperm or eggs released by each individual were then transferred to a haemocytometer for direct counting under a light microscope. The eggs of three females were fertilized with the sperm of a single male at a sperm: egg ratio of approximately 100:1. Only sperm that were active were used for fertilization. Sperm and eggs were mixed in a 5 L sterile flask filled with 0.2 µm filtered seawater and fertilization was allowed to occur. After 2 minutes, fertilization could be confirmed by the presence of a fertilization membrane, the success of which was almost 100%. Fertilized eggs were transferred to a 53 µm sieve and gently rinsed with 0.2 µm filtered seawater to remove excess sperm, and then placed into a 40 L rectangular container containing 0.2 µm filtered seawater maintained at 25°C with fine aeration, and allowed to develop for 2 days into 2-armed pluteus larvae.

4.3.2.2 Larval rearing and feeding

Larvae were reared in a 1000 L cylindrical polyethylene tank (D × H: 50 × 60 cm), at an initial stocking density of 5 larvae per mL. The tank was supplied with finely aerated re-circulating seawater maintained at a salinity of 35 and at 24 - 25°C. The tank was fitted with an inlet pipe and an overflow perforated stand pipe covered initially with an 80 µm sock sieve to prevent loss of larvae. The mesh diameter of the sieve was eventually increased to 150 µm, once total length of larvae exceeded 200 µm, to prevent frequent clogging of the sieve from feed. Seawater flow rate was carefully maintained at approximately 1100 $mL.min^{-1}$ so that the water exchange within the tank was approximately 1.5 tank volumes per day. The experimental system was equipped with a bio-filter, sand filter and protein skimmer to maintain optimal water quality. Dissolved oxygen, pH, salinity and temperature were measured daily and remained stable throughout the study period.

Larvae were maintained under a 12 h light-dark cycle using cool white fluorescent lamps (40 $\mu mol.m^{-2}.s^{-1}$) and fed a mixed microalgal diet consisting of *Pavlovalutheri, Isochrysis* spp. and *Chaetoceros muelleri* (1:1:1 ratio), at a total concentration of 4000 algal cells per larva, each day (Scholtz *et al.*, 2013). Larvae were allowed to feed for approximately 8 h each day, during which time the flow in the tank was turned off to prevent microalgae loss through the sieve. During feeding, the temperature within the tank was maintained at 24-25°C, using four 300 W aquarium heaters. After each feeding, the flow was immediately turned on to remove all uneaten feed. The bottom of the tank was siphoned once a week to remove any dead larvae and uneaten food. Larvae where maintained under the conditions described above for a period of approximately 25 - 28 days, at which point most larvae were deemed competent to settle and were transferred to settlement tanks. Larvae were deemed competent to settle when the adult rudiment was larger than the gut, one or two pedicellariae were present and/or one could visually see movement of the ambulacral feet in the rudiment (Hinegardner, 1969, Carcamo *et al*., 2005, Dworjanyn & Pirozzi, 2008, Scholtz *et al*., 2013).

4.3.2.3 Larval settlement

A combination of four benthic microalgae cultures, *Amphora* spp., *Cocconei* ssp., *Nitzschia closterium* and *Navicula jeffreyi*, were used for the settlement of competent *T. gratilla* larvae. Each algal species culture was grown separately to a density of approximately 6 x 10^6 $cells.mL^{-1}$, in a total volume of 100 L of sterile seawater supplemented with 1 $mL.L^{-1}$ Walne's growth medium (Walne, 1966) at 18°C, before being transferred to a large rectangular settlement tank (L x D x H: 270 x 90 x 60 cm). Cultures in the 100 L bags were used for a maximum of 7 days from the date of inoculation. The settlement tank was equipped with multiple settling plates, made from clear corrugated PVC, that were suspended vertically in the tank. The seawater in the tank was supplemented with 1 $mg.mL^{-1}$ Walne's growth medium (Walne, 1966) and maintained at 22°C, with constant aeration and no water

exchange for a period of 2 weeks, to facilitate settlement of microalgae on the vertical plates. Thereafter, the flow of seawater to the tank was turned on to flush out excess algae and ensure optimal conditions for the settlement of larvae. Finely aerated re-circulating seawater maintained at a salinity of 35 and temperature of 24 - 25°C was supplied to the settlement tank from an experimental system that was equipped with a bio-filter, sand filter and protein skimmer. Water quality was routinely monitored as described above. Competent larvae were then transferred from the rearing tanks by emptying the entire contents into the settlement tanks. Larvae were allowed to settle, metamorphose and develop into juvenile urchins. Once the larvae were large enough to see and handle without damaging them (± 1 cm test diameter), they were transferred into holding tanks (L x D x H: 85 x 40 x 35 cm) were they could be monitored more closely. Juvenile urchins were fed with fresh *Ulva* until they were used in the feeding trials described below.

4.3.3 Preparation of experimental feeds

The effects of the five feeding regimes (Fig. 4.1) on somatic growth, gonad production and gonad quality factors (colour, texture and firmness) of *T. gratilla* were assessed in a 32-week feeding trial. Two of the dietary treatments consisted of a semi-purified 'basal' formula supplemented with dried *Ulva* (20% w/w; 20U diet) or without dried *Ulva*, but *Ulva* addative. (0U diet) (Chapter 2, Secion 2.3.4). A nutrient analysis of the three dietary treatments is presented in Chapter 3, Table 2.3. The remaining dietary treatment consisted of Fresh *Ulva*. The treatments were abbreviated as follows: 20% *Ulva* = 20U; 0% *Ulva* = 0U and Fresh *Ulva* = FU.

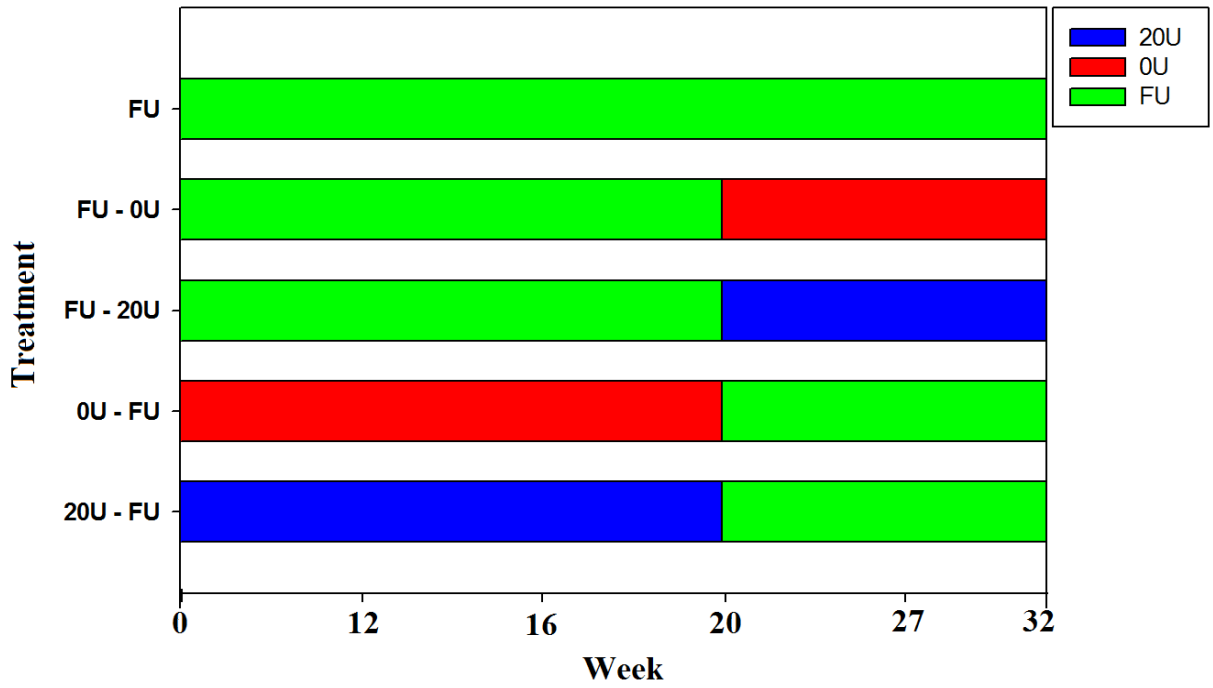


Figure 4.1: The five feeding regimes and the period for which they were fed for over a 32 week trial. The treatments were abbreviated as follows: 20% *Ulva* = 20U; 0% *Ulva* = 0U and Fresh *Ulva* = FU.

4.3.4 Feeding regime trial

Five different feeding regimes were tested during this study (Fig 4.1).The regimes consisted of feeding a single diet of FU, 20U or 0U for a period of 20 weeks, before switching diets according to the feeding regime described in Fig. 4.1 and feeding urchins for a further 12 weeks. Each treatment consisted of 4 replicates, each containing 4 urchins, as the number of urchins in each treatment was limited by the supply of successfully settled juvenile urchins (n = 16 urchins per feeding regime). Urchins were kept in the same experimental setup and maintained as described previously in Chapter 3 (Section 3.3.3). Sea urchins were fed each experimental diet *ad libitum* every second day, after the removal of uneaten feed from each basket. Consumption was not recorded in this study as urchins were food continuously.

Somatic growth of juveniles within each dietary treatment group was recorded once monthly, for the first 12 weeks of the study. Each sampled urchin was blotted dry with a paper towel and total body weight (WW), test diameter (HD) and test height (VH) were carefully recorded to the nearest 0.01 g or 0.01 mm, before returning the animal to its respective

basket. All urchins were measured, and not sacrificed, during this period of the study. After 16 weeks, a single animal was removed from each basket within each treatment group. Sampled animals were weighed, and measured as described above, before being sacrificed for the determination of urchin drained weight (DW) (coelomic fluid removed), and removal of gonads. Gonad wet weight was recorded to the nearest 0.01 g, using an electronic balance, and the gonad somatic index (GSI) was calculated as described below. Gonad texture, firmness and colour were rated visually and manually by a single observer, according to the procedures described below. A single gonad from each urchin was randomly selected and transferred into Davidson's Fixative (per litre: 300 mL 95% ethyl alcohol, 200 mL 100% formalin, 100 mL glycerol, 100 mL glacial acetic acid and 300 mL distilled water) immediately following dissection and fixed for 48 h, before being transferred into 70% ethanol and processed for routine paraffin histology (Bucke, 1989).

4.3.4.1 Calculation of test volume

In order to determine the amount of space available in the test for gonad development, urchins were assumed to be hemispherical. The volume of each sea urchin test was then calculated using the following formula:

$$V = (2/3)\pi r^3$$

Where r is the average radius of the urchin test calculated as follows:

$$r = (HD + VH)/3$$

Where HD is the horizontal test diameter and VH the vertical test height (Fig. 4.2).

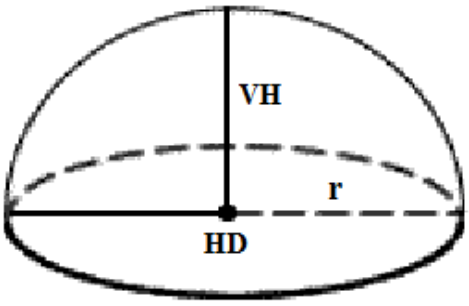


Figure 4.2: Diagram indicating measurements needed to estimate test volume

4.3.4.2 Calculation of gonad somatic index (GSI)

The body and gonad wet weights of individual urchins fed the various experimental diets (n = 4, 8) were used to calculate gonad somatic index (Chapter 3, Section 3.3.4.1).

4.3.4.3 Assessment of gonad colour

Gonad colour was assessed by visually ranking each gonad (n = 8 per treatment) in categories and by measuring the intensity of gonad lightness (L*), redness (a*) and yellowness (b*) with a hand-held reflected-light, fibre-optic spectrophotometer (Chapter 3, Section 3.3.4.2).

4.3.4.4 Histology

Fixed tissues (n = 4, 8 per treatment) were processed and described using standard histological techniques presented in Chapter 3 (Chapter 3, Section 3.3.5).

4.3.5 Statistical analysis

To determine whether urchin wet weight, diameter, height, mortality, GSI, gonad wet weight and gonad colour (L*, a* and b*) changed as a function of time, within individual treatment groups, or, as a function of treatment at individual sampling dates, one-way analysis of variances (ANOVA) were performed using Statistica 8. All tests for normality (Kolmogorov Smirnov test) and equal variance (Levene's test) passed for all data sets. One way ANOVA was also used to test for significant differences in gonad texture and firmness and gonad maturity (histological data) within individual treatment groups over time and between treatment groups, at specific sampling dates. All tests for normality (Kolmogorov–Smirnovtest) and equal variance (Levene's test) in these data sets failed and therefore a Kruskal–Wallis ANOVA on Ranks test was performed to test for significant differences. The Tukey method was used for all post-hoc multiple comparisons between individual time points within a treatment group, and between the different treatment groups, at specific sampling dates. Significance was assigned to p - values of < 0.05 for all analyses.

4.4 Results

4.4.1 Urchin survival

Survival rates of *Tripneustes gratilla* over the course of the study were high (99%) and did not vary significantly between diets, with only one individual (from the 0U treatment) dying over the 32 week trial.

4.4.2 Urchin somatic growth

Total body weight (mean ± SE) of urchins across all treatment groups increased from 14.39 ± 0.54 g at the beginning of the study, to 172.13 ± 15.04 g at the end of the study, while mean diameter and height increased from 33.05 ± 0.5 to 75.18 ± 2.78 mm and from 19 ± 0.33 to 43.7 ± 1.66 mm at the end of the study, respectively.

Tripneustes gratilla somatic growth (Fig. 4.3), determined using urchin wet weight (Fig. 4.3A), test diameter (Fig. 4.3B) and test height (Fig. 4.3C), increased significantly within all dietary treatment groups over the course of the 32 week experimental period. No significant differences in total body weight and test diameter were recorded between any of the dietary treatments groups or feeding regimes over the course of the experiment, with the exception of urchins initially fed the 0U diet and then FU (0U-FU treatment group). Weight gain for urchins in all treatments was rapid for the first 6 weeks of the trial, however, animals within the 0U-FU feeding regime had a significantly lower (one-way ANOVA, $p < 0.05$) total wet weight and test diameter after 12 weeks, compared to animals in all other feeding regimes. After 12 weeks it appeared that urchins being fed 0U had approached a maximum size (until the diets were changed), only increasing their diameter by 1.14 mm over 8 weeks, compared to the 7.06 and 4.46 mm increases seen for urchins fed 20U and FU diets. The overall weight increases from the start of the trial for the FU, FU – 20U, FU - 0U and 20U – FU treatment

regimes were 1307.65, 998.74, 1272.73 and 1100.42%, respectively, and were all significantly greater than the 797.18% increase seen for urchins fed the 0U – FU diet regime.

In contrast, there was no significant difference in test height between any of the treatment groups by week 12. Thereafter, urchins fed Fresh *Ulva* achieved a significantly greater test height compared with urchins in the 0U treatment group. This trend continued for the duration of the experiment and by week 32, urchins within the fresh *Ulva* and FU-0U feeding regimes had significantly greater test heights than the 0U- FU treatment group. The only exception to this trend was urchins in the FU-20U treatment regime. Once urchins in this treatment regime were switched from being fed FU to a 20U diet, there was no further increase in test height.

To determine the potential space available for gonad development, the volume of each sea urchin test was calculated. Although *T. gratilla* is slightly flattened, the same protocol was used on all urchins and thus the data provided does give us some indication of the amount of space available for gonad development. Test volume revealed that urchins fed the 0U diet had significantly smaller test volumes compared to all other dietary treatment groups/regimes after just 12 weeks of being fed this diet. This trend continued throughout the study, while no other significant differences between any of the dietary treatment groups were recorded. At the end of the trial, volumes in all treatments had increased significantly from the start of the trial, reaching 130.74 ± 7.45 (20U - FU); 86.69 ± 7.41 (0U - FU); 154.06 ± 7.66 (FU); 133.54 ± 4.73 (FU - 20U) and 139.91 ± 11.17 cm^3 (FU-0U) (mean ± SE), while the 0U - FU treatment still remained significantly smaller compared to all other treatments.

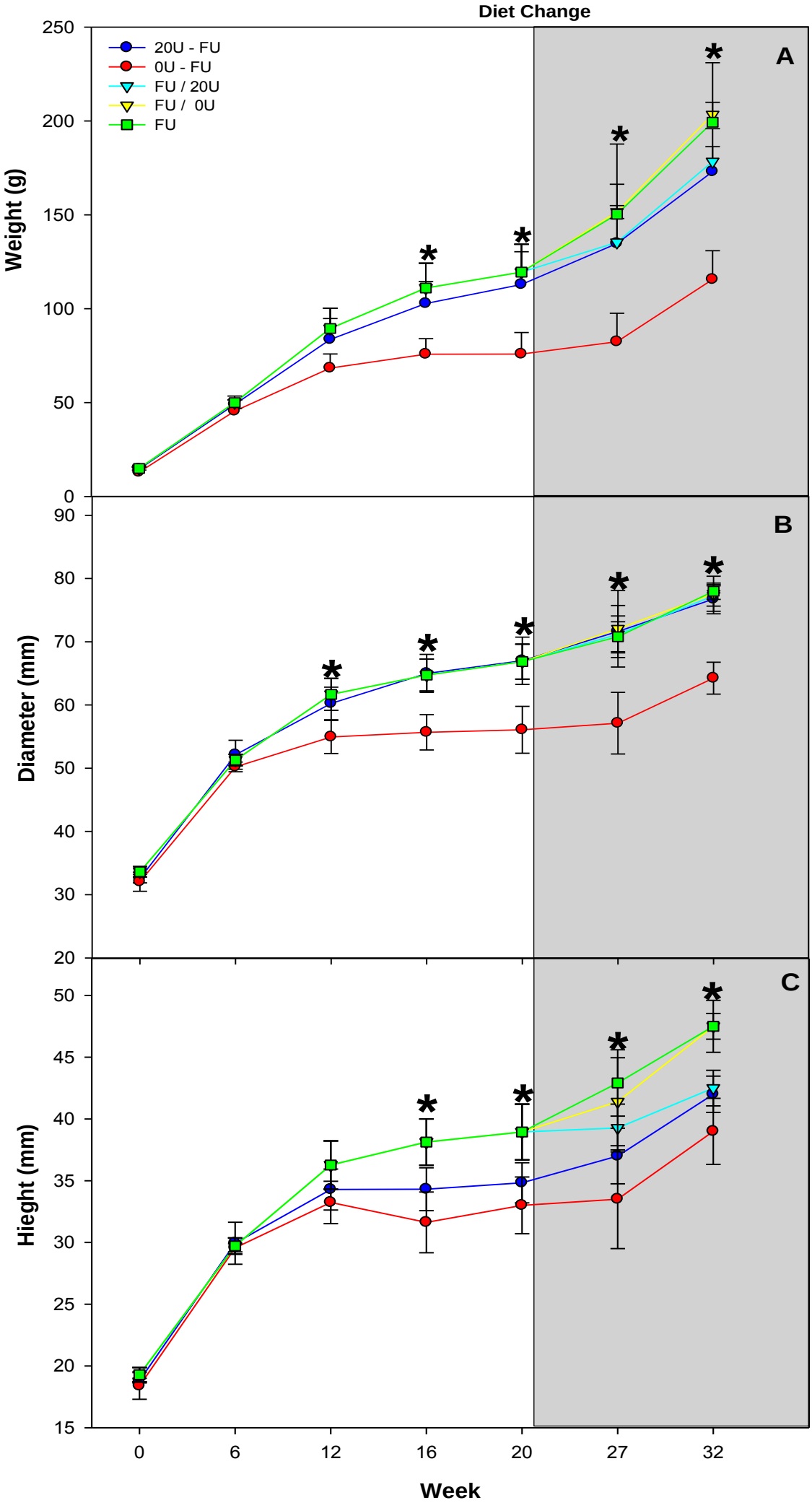


Figure 4.3: Mean **(A)** urchin wet weight, **(B)** diameter and **(C)** height of *Tripneustes gratilla*fed 5 different feeding regimes which include combinations of two artificial diets and/or fresh *Ulva* over a 32-week grow-out period in a recirculating seawater system. Data represents mean ± SE *(P < 0.05, Tukey test) represents a significant difference in the means of urchins fed the different dietary treatments at a particular sampling date. The grey shaded portion of the graph represents the change in diet within the feeding regimes. Treatment regimes: FU = fresh *Ulva*; FU – 20U = fresh *Ulva* to 20% *Ulva*; FU - 0U = fresh *Ulva* to 0% *Ulva*; 20U – FU = fresh *Ulva* to 20% *Ulva*; 0U - FU = 0% *Ulva* to fresh *Ulva*.

4.4.3 Gonad growth and quality

4.4.2.1 Gonad growth and yield

Gonad somatic index and gonad wet weight were recorded for thefirst time at week 16 (Fig. 4.5A) of the experiment. At this point in time, urchins fed the two artificial diets had a significantly greater gonad somatic index (One-way ANOVA, $p < 0.0001$), compared to urchins fed FU. Similar findings were observed for gonad wet weight (Fig. 4.5B). The trend observed for GSI continued untill week 20, immediately prior to the change in diets. Thereafter, seven weeks following the change in diets (week 27), urchins in different feeding regimes had very similar GSI and gonad wet weight values and there were no longer significant differences between any of dietary treatments groups. However, by the end of the study (week 32), urchins initially fed the 0U diets and then FU at week 20 had a significantly lower GSI and gonad wet weight compared with urchins in all other dietary treatment groups. Furthermore, urchins previously fed with FU and then switched to either the 20U or the 0U diets, now had significantly higher GSI and gonad wet weights at week 32 compared with values recorded at week 20. Urchins within these two treatment regimes also produced the largest gonads, as reflected by total gonad wet weight, compared to urchins in the other treatment regimes.

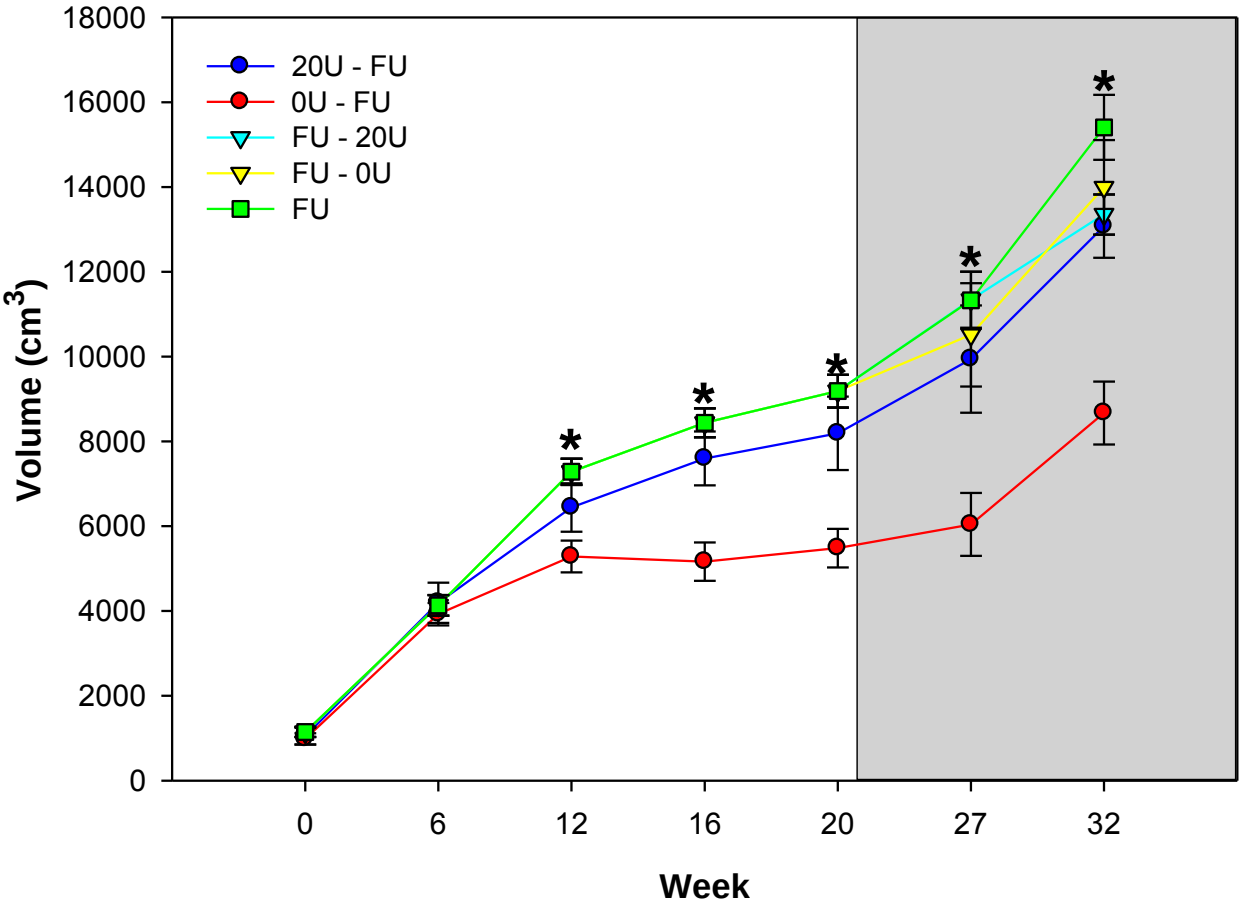


Figure 4.4: Mean test volume of *Tripneustes gratilla* fed five different feeding regimes which include combinations of two artificial diets and/or fresh *Ulva* over a 32-week grow-out period in a recirculating seawater system. Data represents mean ± SE *($p < 0.05$, Tukey test) represents a significant difference in the means of urchins fed the different dietary treatments at a particular sampling date. The grey shaded portion of the graph represents the change in diet within the feeding regimes. Treatment regimes: FU = fresh *Ulva*; FU – 20U = fresh *Ulva* to 20% *Ulva*; FU - 0U = fresh *Ulva* to 0% *Ulva*; 20U – FU = fresh *Ulva* to 20% *Ulva*; 0U - FU = 0% *Ulva* to fresh *Ulva*.

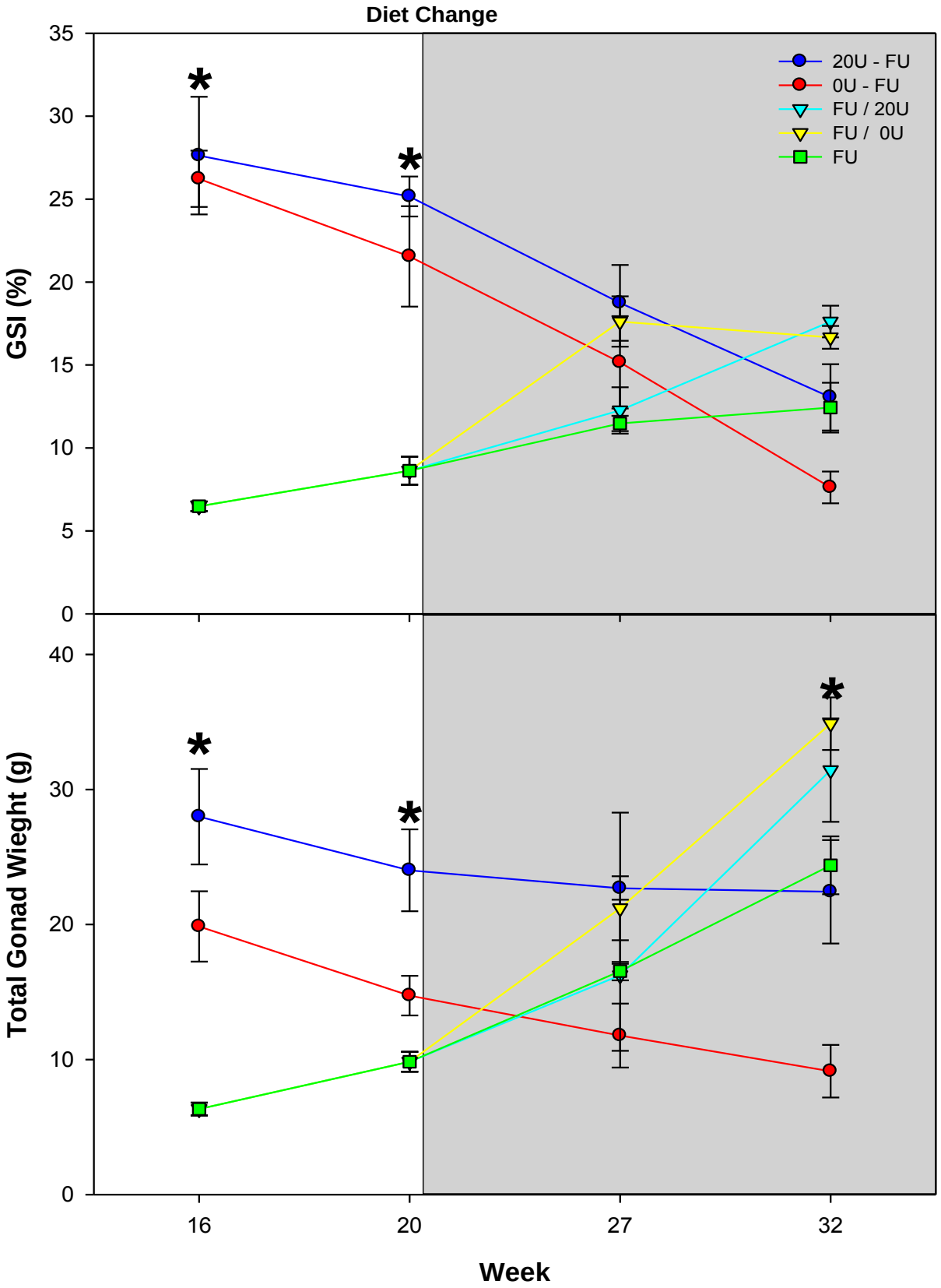


Figure 4.5: Mean **(A)** gonad wet weight and **(B)** gonad somatic index (GSI) of *Tripneustes gratilla* fed five different feeding regimes which include combinations of two artificial diets and/or fresh *Ulva* over a 32-week grow-out period in a recirculating seawater system. Data represents mean ± SE *($p < 0.05$, Tukey test) c The grey shaded portion of the graph represents the change in diet within the feeding regimes. Treatment regimes: FU = fresh *Ulva*; FU – 20U = fresh *Ulva* to 20% *Ulva*; FU - 0U = fresh *Ulva* to 0% *Ulva*; 20U – FU = fresh *Ulva* to 20% *Ulva*; 0U - FU = 0% *Ulva* to fresh *Ulva*.

4.4.2.2 Gonad colour

Mean eye-rated gonad colour (Fig. 4.6) ranged from 1, an excellent quality bright yellow or orange coloured gonad, to 4, an unacceptable white or brown coloured gonad, for urchins fed diets that incorporated either one of the artificial feeds (20U or 0U). However urchins fed exclusively Fresh *Ulva* consistently produced excellent quality gonads, with a colour rating of 1 throughout the study period. Gonad colour ratings of urchins fed the 20U or 0U diets improved significantly from week 20 to 32 once their diets were switched to FU at week 20. At the end of the study period, urchins in the latter two treatment regimens (20U - FU and 0U - FU) produced gonads of acceptable quality (Fig. 4.6) that had a colour rating of 2. In contrast, the gonad colour rating of urchins fed FU deteriorated once their diets were switched to either 20U or 0U at week 20. However, gonads from these urchins were still of acceptable quality at the end of the study period, with colour ratings between 1 and 2. Furthermore, at the end of the trial, the gonads of urchins in the FU and FU – 20U feeding regimes were not significantly different.

Gonad lightness (L*) values (Fig. 4.7A) ranged from 52.37 to 68.32 and were significantly affected by diet. By week 16, urchins fed the 20U and 0U artificial diets had significantly lighter coloured gonads (one-way ANOVA, $p = 0.0002$) compared with the urchins fed FU. However, by week 20, the L* values of urchins fed 20U and 0U diets decreased and there was no longer a significant difference between any of the dietary treatments. Seven weeks following the change in diets (week 27), gonad L* values remained similar and did not vary significantly. However, at the end of the study period (week 32), gonad lightness values of urchins in the 0U - FU treatment decreased further, and were significantly lower than L* values recorded in the FU - 20U and FU - 0U treatments. No other significant differences were recorded at this time point.

Gonad redness (a*) and yellowness (b*) values were both significantly affected by diet (Fig. 4.7 B & C) and ranged from 3.63 to 17.16 and 20.47 to 44.59, respectively. By week 16, sea urchins fed with FU had produced gonads that were significantly redder and yellower than animals fed with both the 20U and 0U diets. Furthermore, urchins fed the artificial feed supplemented with dried *Ulva* (20U diet) produced gonads that were significantly redder than the gonads of urchins fed the 0U diet. A similar trend was observed for gonad yellowness at week 16, and the trends observed for both gonad a* and b* persisted into week 20, at which point the diets were altered. Seven weeks following the change in diets (week 27), all treatments produced gonads of similar a* and b*, with the exception of urchins previously fed 0U, which produced gonads that were significantly less red and yellow in colour than the gonads of urchins fed FU only, for the first 20 weeks of the study. Gonad a* and b* decreased following the switch from FU to artificial feed, and by the end of the study only those animals continuously fed with FU produced gonads that were significantly redder and yellower than gonads produced by urchins in the other treatment groups.

The total difference in gonad colour (Fig. 4.8), calculated using the formula from McBride *et al*. (2004), clearly showed that the gonads produced by urchins fed artificial feeds are significantly different from gonads produced by urchins fed fresh *Ulva*. Furthermore, urchins fed the non-*Ulva* supplemented artificial feed produced gonads that were most dissimilar from the A grade roe, produced by *Ulva*-fed urchins, at week 16. At week 20, urchins being fed 0U produced gonads that were significantly (one-way ANOVA, $F_{2,9}$ = 54.08, $p < 0.05$) different from the "total colour difference" of both FU ($p = 0.00009$) and 20U ($p = 0.0002$) diets. After the dietary change, urchins sampled at week 27 revealed that gonads in the 0U - FU treatment produced a total colour difference that was significantly (one-way ANOVA, $F_{4,14}$= 6.63, $p < 0.05$) higher than urchins fed a regime of FU ($p = 0.004$), FU - 20U ($p = 0.02$), 20U - FU ($p = 0.015$) and FU - 0U ($p = 0.05$), while differences

between these treatments were not significant. At the end of the study (week 32), no significant differences were recorded in total colour difference from fresh *Ulva*, indicating that gonad colours were fairly comparable across all treatments.

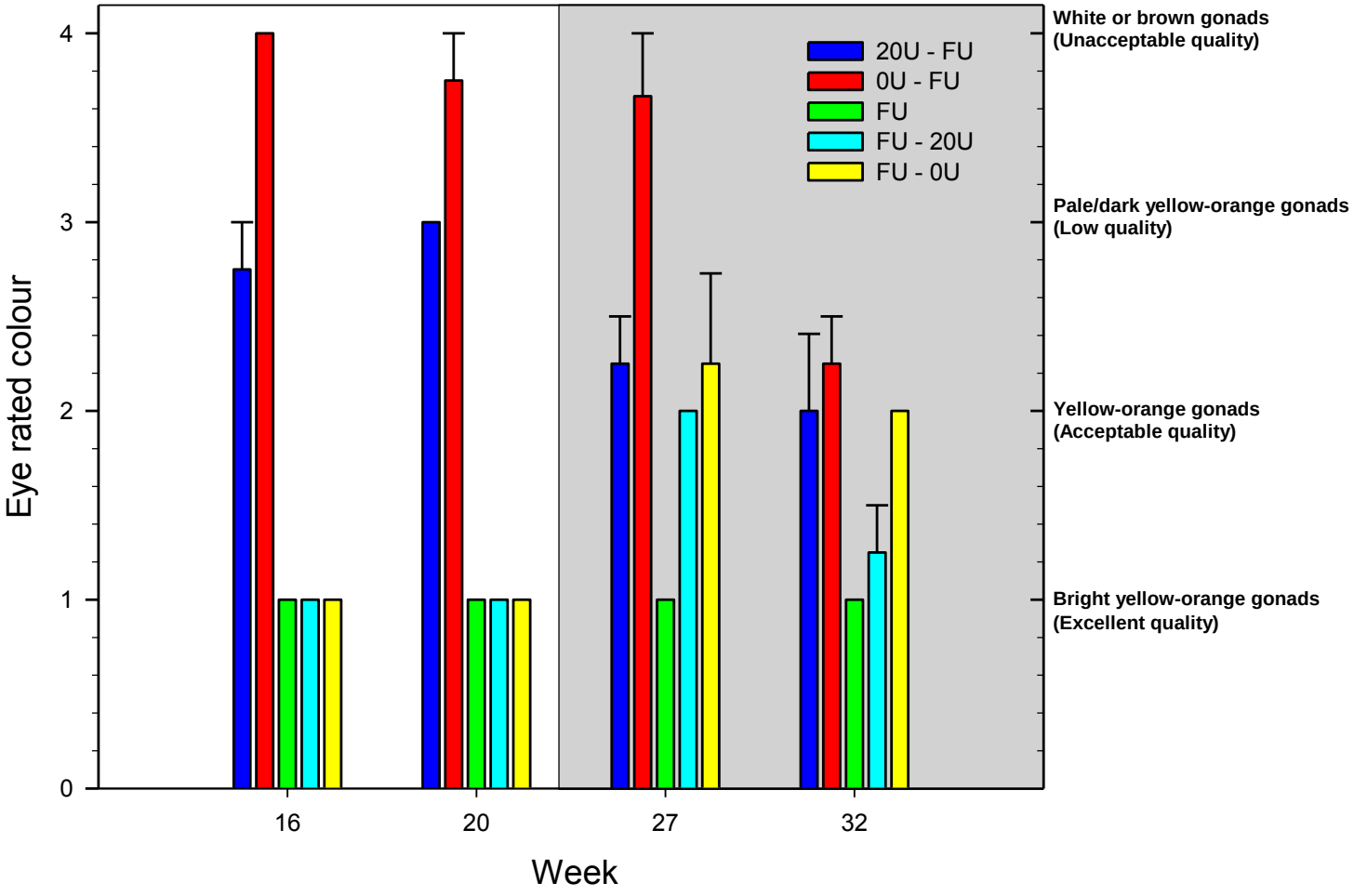


Figure 4.6: Mean eye rated (ER) gonad colour of *Tripneustes gratilla* fed with five different feeding regimes which include combinations of two artificial diets and/or fresh *Ulva* over a 32-week grow-out period. Data represents the mean ± SE. Gonad colour was visually ranked in categories ranging from most desirable to unacceptable. The categories were allocated numbers, which were ranked as follows: (1) bright yellow-orange gonads (excellent quality); (2) yellow-orange gonads (acceptable quality); (3) pale yellow-orange or dark yellow-orange gonads (low quality) and (4) white or brown gonads (unacceptable). The grey shaded portion of the graph represents the change in diet within the feeding regimes. Treatment regimes: FU = fresh *Ulva*; FU – 20U = fresh *Ulva* to 20% *Ulva*; FU - 0U = fresh *Ulva* to 0% *Ulva*; 20U – FU = fresh *Ulva* to 20% *Ulva*; 0U - FU = 0% *Ulva* to fresh *Ulva*.

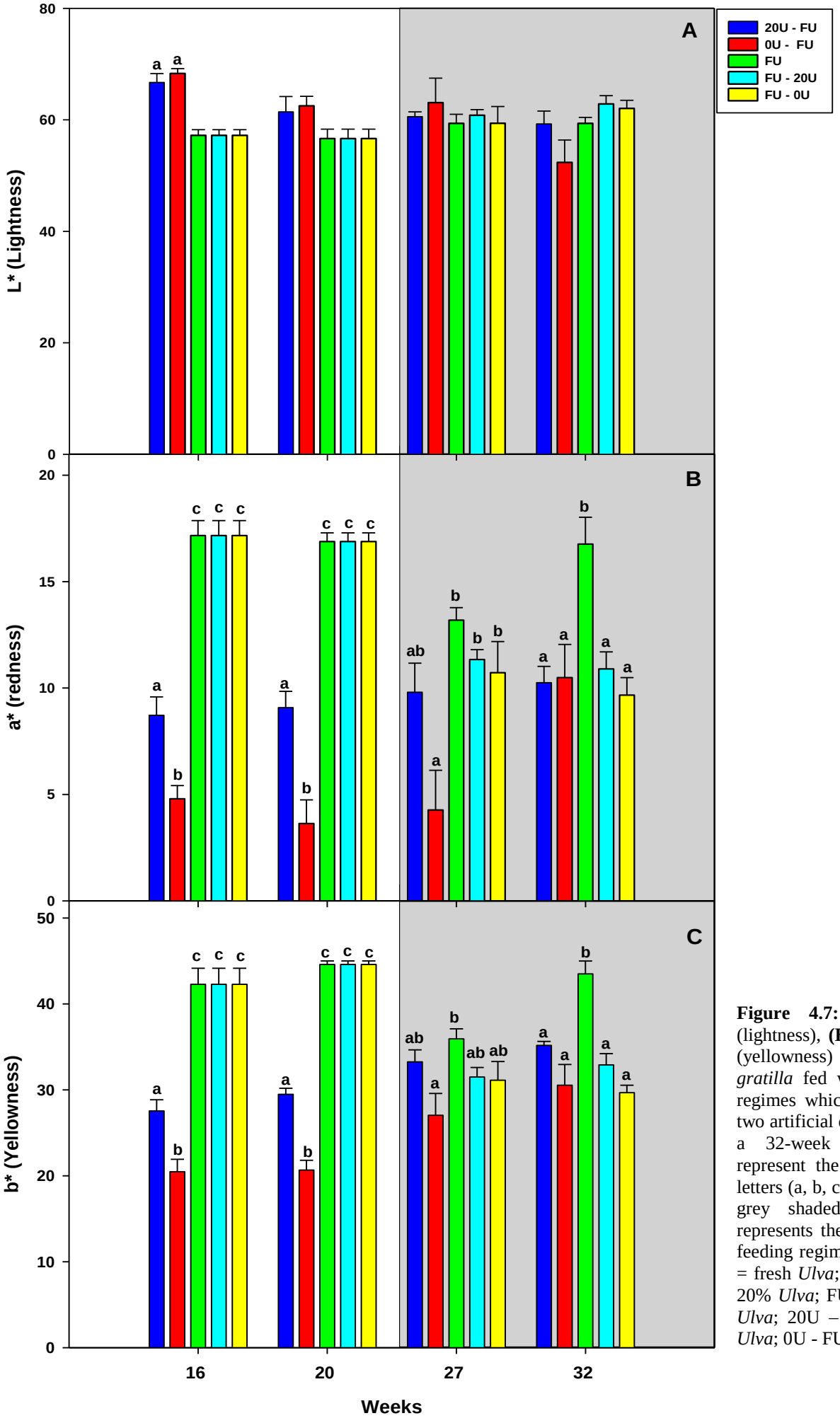


Figure 4.7: Mean gonad **(A)** L* (lightness), **(B)** a* (redness) and **(C)** b* (yellowness) values of *Tripneustes gratilla* fed with five different feeding regimes which include combinations of two artificial diets and/or fresh *Ulva* over a 32-week grow-out period. Data represent the mean ± SE. Lower case letters (a, b, c ;$p < 0.05$, Tukey test). The grey shaded portion of the graph represents the change in diet within the feeding regimes. Treatment regimes: FU = fresh *Ulva*; FU – 20U = fresh *Ulva* to 20% *Ulva*; FU - 0U = fresh *Ulva* to 0% *Ulva*; 20U – FU = fresh *Ulva* to 20% *Ulva*; 0U - FU = 0% *Ulva* to fresh *Ulva*.

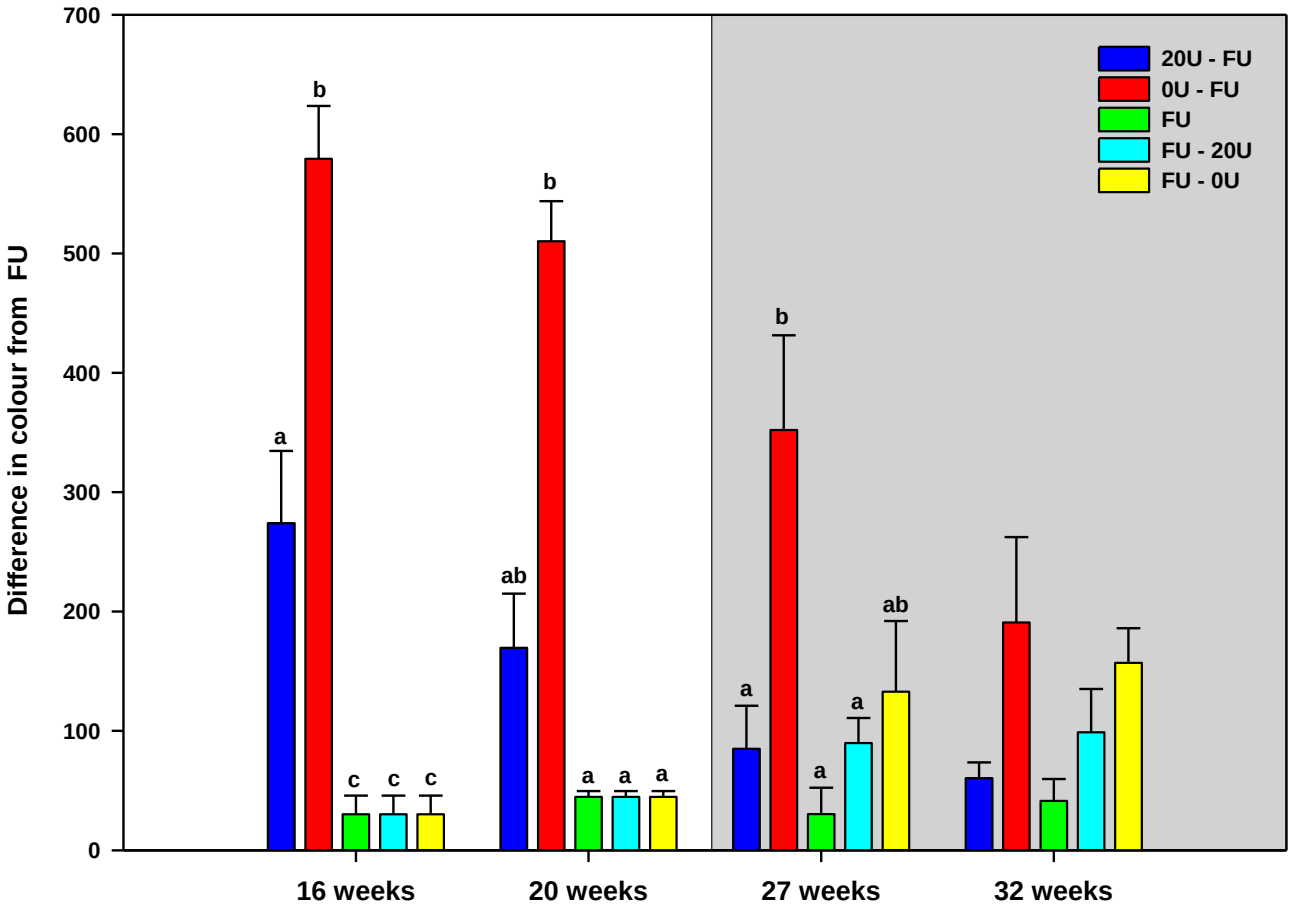


Figure 4.8: The total difference in spectrophotometer rated gonad colour of *Tripneustes gratilla* fed with five different feeding regimes which include combinations of two artificial diets and/or fresh *Ulva* over a 32-week grow-out period. Data represent mean ± SE. Lower case letters (a, b, c ; $p < 0.05$, Tukey test). Treatment regimes: FU = fresh *Ulva*; FU – 20U = fresh *Ulva* to 20% *Ulva*; FU - 0U = fresh *Ulva* to 0% *Ulva*; 20U – FU = fresh *Ulva* to 20% *Ulva*; 0U - FU = 0% *Ulva* to fresh *Ulva*.

5.4.3.1 Histology

Histological analysis of sea urchin gonads across all of the sampling dates (Fig. 4.9) revealed a significant degree of variability in gametogenic development between dietary treatments at individual sampling dates, and within treatments over time (Kruskal - Wallis, $p < 0.05$). At the first and second sampling dates (week 16 & 20, Fig. 4.9A& B) urchins from all dietary treatments were in significantly (Kruskal - Wallis, $p < 0.05$) different states of maturity. Urchins fed the two artificial diets (20U and 0U) produced animals that were in a far more advanced state, compared with urchins in the FU treatment, which produced gonads that were in a growing phase.

After the diet change (week 27, Fig. 4.9C), gonads of urchins that were previously fed artificial diets were reaching the end of the reproductive cycle. Gonads in the 20U - FU treatment were half in recovery and half in a partly spent state, while urchins in the 0U - FU treatment were all in a spent state. Urchins in the FU treatment seemed to follow a more natural progression of gonad development, becoming premature in week 27. Urchins that had been switched from FU to 20U seemed to be slightly further advanced than those being fed only FU , while urchins that were switched from FU to 0U seemed to be slightly delayed in their gonad development and were, predominantly in a growing state. At the end of this trial (Fig. 4.9D), the majority of the treatments produced urchins that were in a premature state, with a few still indicating that they were growing. The only exception to this trend was seen in the 0U - FU treatment, which indicated that the majority of organisms were still in a spent state.

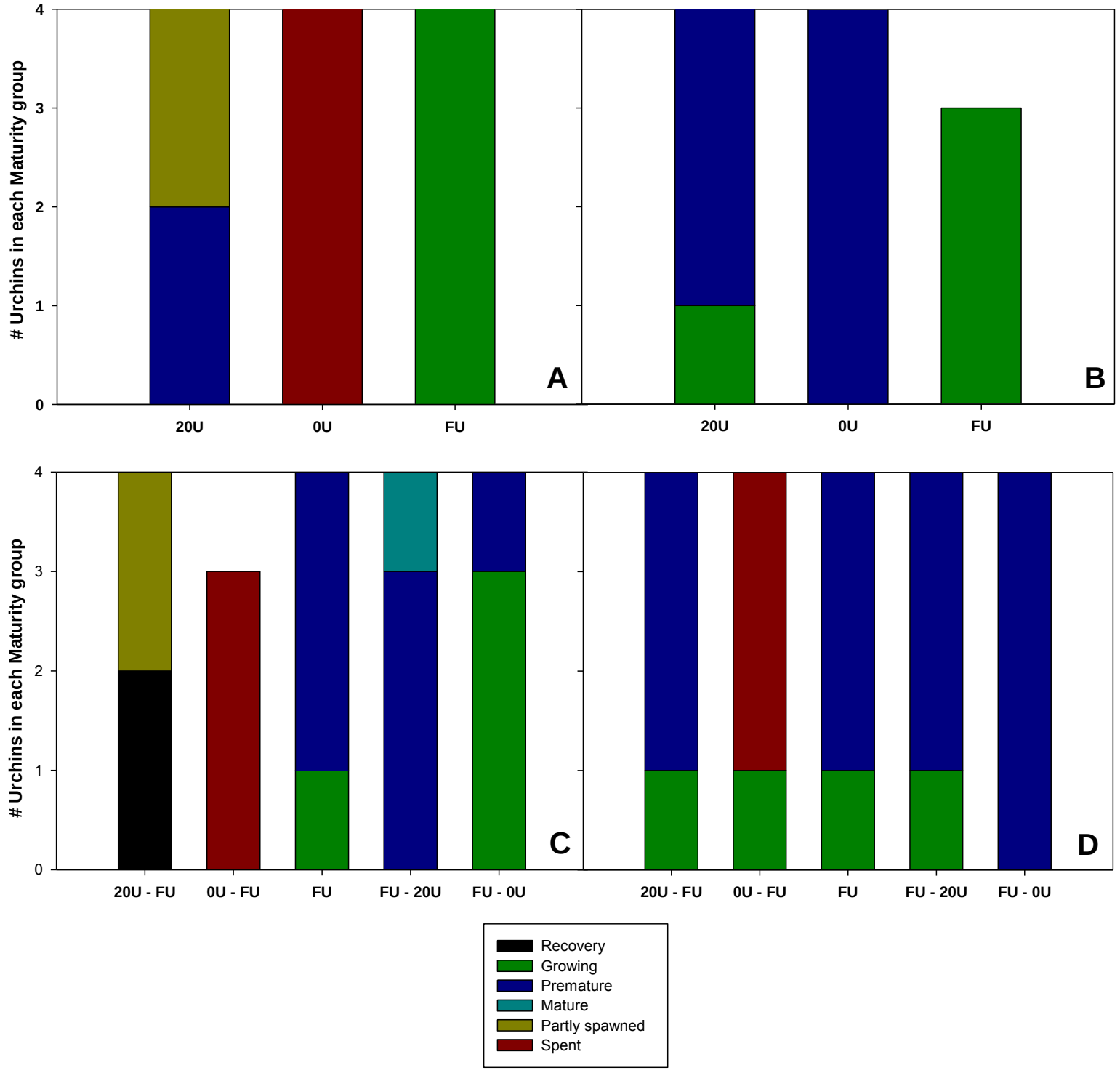


Figure 5.9: The number of *Tripneustes gratilla* of both sexes allocated to each maturity stage over the 32-week grow-out period. Gonads of urchins fed with 5 different feeding regimes which include combinations of 2 artificial diets and/or fresh *Ulva* were processed for routine paraffin histology at **(A)** week 16, **(B)** week 20, **(C)** week 27 and **(D)** week 32 to assess the amount of gametogenic activity in the gonads and categorized as (1) recovery; (2) growing; (3) premature; (4) mature; (5) partly spent or (6) spent. Treatment regimes: FU = fresh *Ulva*; FU – 20U = fresh *Ulva* to 20% *Ulva*; FU - 0U = fresh *Ulva* to 0% *Ulva*; 20U – FU = fresh *Ulva* to 20% *Ulva*; 0U - FU = 0% *Ulva* to fresh *Ulva*.

4.5 Discussion

Fresh *Ulva* (FU) and an artificial feed supplemented with dried *Ulva* (20% w/w) were shown, in this study, to be equally effective at significantly improving the somatic growth of *Tripneustes gratilla,* compared with urchins fed a non-*Ulva* supplemented artificial feed. These findings suggest that *Ulva*, or its constituents, are responsible for the enhanced somatic growth observed in juvenile *T. gratilla.* These findings are consistent with the results of previous studies that have demonstrated that somatic growth of juvenile urchins can be improved by dietary seaweed supplementation. Kennedy *et al*. (2005) demonstrated that juvenile green sea urchins *Strongylocentrotus droebachiensis,* fed the brown kelp *Laminaria longicruris* produced significantly better somatic growth, compared with urchins fed artificial diets without seaweed supplementation. However Dagget *et al.* (2005) showed that *S. droebachiensis* fed either *Porphyra purpurea* or *Ulva linza* produce similar growth rates to urchins fed a high protein artificial diet, without these seaweeds. The somatic growth of small and medium-sized *Evechinus chloroticus* was also significantly better when fed diets consisting of *Macrocystis pyrifera* and *Ulva lactuca,* compared with prepared feeds consisting of different protein sources (soybean; soybean & fishmeal) (Barker *et al*.,1998). More specifically, working with juvenile *T. gratilla,* Asia (2009) and Asia *et al.* (2012) found that fresh *Sargassum* spp. produced comparable somatic growth to either a dry or fresh artificial pelletized diet containing *Sargassum.*

Previous research into the development of artificial diets has identified protein as a major factor influencing growth and production in echinoids (Frantzis & Gremare, 1992; Kelly *et al*., 2000; Hammer *et al*., 2004; 2006). For example, Otero-Villanueva *et al.* (2004) and Eddy *et al.* (2012), demonstrated that high protein artificial diets produce better somatic growth in the urchins *Psammechinus miliaris* and *Strongylocentrotus droebachiensis,* compared to natural diets of macroalgae. These results are in conflict with the findings of the present

study; however, Cook *et al.* (1998) stated that the size of an urchin can influence the effect of the diet. Adult *P. miliaris,* fed an artificial salmon feed, produce significantly higher growth rates compared to urchins fed a natural diet of *L. saccharina*, however, when smaller urchins of either juvenile or intermediate size were used, both diets produced similar somatic growth. In contrast, this study showed that the somatic growth of juvenile *T. gratilla*, when fed either a macroalgal diet or an artificial diet supplemented with 20% macroalgae, was better, compared to a non - *Ulva* supplemented artificial diet. The results of Chapter 3 also showed that adult somatic growth is not significantly different between groups of urchins fed either fresh *Ulva* or one of four artificial feeds supplemented with varying amounts of *Ulva* (0, 5, 15 & 20% w/w), over a 20 week period, although gonad growth does differ.

In the current study, *T. gratilla* fed two artificial diets (0U and 20U) with no significant difference in protein content (≈26%), produced significantly different somatic growth. These results suggest that differences in somatic growth recorded among juvenile *T. gratilla* are not attributable directly to differences in dietary protein. The fact that urchins fed the FU diet (18% protein) had comparable growth to urchins fed the 20U diet (26% protein), and grew significantly better than urchins fed the 0U diet (26% protein), supports this hypothesis. Since the only difference between the artificial diets was the presence of *Ulva,* it follows that one or more of *Ulva's* constituents contributed to improving or supporting somatic growth.

The presence of minerals and/or pigments within a sea urchin diet is regarded as an important factor influencing juvenile urchin somatic growth. In particular, minerals such as calcium and magnesium have been shown to be important elements in the production of the sea urchin test (Okazaki, 1956; Pearse & Pearse, 1975; Grosjean *et al*., 1998; Ebert, 2001) and a lack of these minerals can potentially lead to reduced somatic growth. South African *U. lactuca* and *U. rigida* have been recorded to have a mean calcium and magnesium content of 0.5 and 2.5% DW, respectively, throughout the year (Shuuluka, 2011). As both artificial diets used in

this study included the same concentration of vitamin and mineral premix containing 1% Ca, the only difference in dietary minerals between the diets was from the inclusion of *Ulva*, which resulted in a 0.1 and 0.5% increase in the calcium and magnesium contents, respectively, of the 20U diet. This was, however, corrected for in the 0U diet. The FU diet was not supplemented with vitamin and mineral premix, but urchins fed this diet grew at the same rate as urchins fed the 20U diet. This suggested that sufficient Ca and Mg was available to urchins fed FU and urchins in this treatment group were able to derive minerals like calcium and magnesium from other sources, in addition to their diet, such as from the surrounding seawater (Irving, 1926; Grosjean *et al.,* 1998; Powell *et al.,* 2009).

These findings also suggest that other components, such as pigment(s), within *Ulva* may be responsible for improving somatic growth of *T. gratilla* in this study. Kennedy *et al.* (2007) suggested that pigment influenced the somatic growth of juvenile urchins more that the addition of minerals. This hypothesis supports the results of the present study, in which an artificial diet (0U) containing adequate minerals, but not supplemented with *Ulva* (which contains pigments), produced urchins that had significantly smaller test diameters than those that received diets supplemented with *Ulva*.

The main pigment found within *Ulva* spp. is β-carotene, which can be present in relatively large amounts (0.357 $mg.g^{-1}$) (Abirami & Kowsalya, 2011). β-carotene is a precursor to Vitamin A (Leo & Lieber, 1999; Von Lintig & Vogt, 2000) which, in the form of retinoic acid, has been shown to be an important hormone-like growth factor and may help explain the improved growth seen in these studies. Diameters of urchins fed the 20U and FU diets revealed that there was no significant difference between the two treatments, which would tend to indicate that if pigments or some other constituents within *Ulva,* are responsible for improved growth, higher pigment concentrations than that found in the 20U diet did not support further increased somatic growth. This hypothesis is supported by findings of

Robinson *et al.* (2009), who found that a diet containing 50 $mg.kg^{-1}$ of β-carotene produced significantly better growth than the same diet with 0 $mg.kg^{-1}$ β-carotene, however, increasing β-carotene levels to 100 and 250 $mg.kg^{-1}$ did not improve growth. It should also be noted that the addition of β-carotene in both this study and Kennedy *et al.* (2007) was achieved by the addition of algae, either *Ulva* or *Dunaliella* (Algro^{TM}). Both these algal species have also been shown to be high in vitamin C (42.6 $mg.g^{-1}$) and ascorbic acid (Ortiz *et al*., 2006; García-Casal *et al*., 2007; Abirami & Kowsalya, 2011). Jones (2011) determined that the addition of ascorbic acid to feed for juvenile *Lytechinus variegatus* increased weight gain and organ production, and appeared to increase at higher inclusions. It is thus possible that vitamin C and/ or ascorbic acid may have been responsible for the improved test growth observed in the present study. Further investigations into what constituents are responsible for the improved growth seen in urchins fed fresh *Ulva* or the 20U diet are, therefore, still needed.

By week 16, *T. gratilla* fed the artificial diets (20U & 0U) had significantly increased gonad production (GSI) compared to urchins fed FU. These results are in agreement with those of Chapter 3 and similar research in the literature, showing that formulated feeds containing high protein can significantly increase gonad yield (Fernandez & Caltagirone, 1994; Keesing & Hall, 1998; Lawrence *et al*., 1997; Kelly *et al*., 1998; Robinson & Colborne, 1998; Vardas *et al*., 2000; Olave *et al.*, 2001; Pearce *et al.*, 2002c.; Shpigel *et al.*, 2005; James, 2007a,b; Hammer *et al*., 2006; Cyrus *et al.*, 2014). Although the GSI of urchins fed the artificial diets were not significantly different from each other (weeks 16 and 20), analysis of gonad histology revealed that urchins in the 0U treatment were more advanced reproductively, particularly at week 16. In contrast, gonads of urchins in the FU treatment were in a growing state prior to the diet change. It is well documented that increased food availability or quality can result in increased gonad production (Walker & Lesser, 1998; Lawrence, 2000; Lawrence

et al., 2001; Spirlet *et al.,* 2001; Schlosser *et al.,* 2005). In large mature urchins this result is desirable, as the marketable product is the gonad, whereas, for immature, juvenile urchins that have not yet reached marketable size, the aim is to maximize somatic growth.

Food availability and reproductive condition are generally responsible for determining the composition of gonad biomass, although body size ultimately determines the maximum gonad size (Hagen, 1998). The volume of a sea urchin test is, therefore, an estimate of the amount of space available for gonad development. After just 12 weeks of this study, the test volume of urchins in the 0U treatment were already significantly smaller than those of the other two treatments (20U & FU) and did not increase over the next eight weeks of the trial, compared to urchins fed the 20U and FU diets. Urchins in the 0U treatment, therefore, had significantly less space for gonad development and this was reflected in the difference in gonad production, when comparing the two artificial treatments at week 16. Although the GSI was not significantly different, urchins in the 20U diet had significantly larger gonads, in terms of gonad wet weight than those in the 0U treatment, while urchins fed FU had significantly smaller gonads (as well as lower GSI) than both formulated treatments. At week 20, urchins in the 20U treatment had significantly heavier gonads than both the 0U and FU treatments. The 0U and FU treatments did not differ significantly between each other in terms of gonad mass, however, their GSI values differed by 12.9%.

After changing the diets from artificial feed to fresh *Ulva*, GSI and gonad weight decreased, while those previously fed fresh *Ulva,* started to increase once fed artificial feeds. This indicates the importance of a high protein feed for gonad production and development. By the end of the trial all dietary regimes produced similar gonad weights with the exception of the 0U - FU treatment which had a significantly lower yield, due to the significantly smaller test volume of these urchins. Although FU and 20U diets produced similar somatic growth throughout the trial, the same could not be said for gonad production or maturity. Since the

market value of sea urchin gonads is inherently linked to gonad maturity and gamete numbers, it is important to understand how a diet/feeding regime will affect gametogenisis. The ability to control or suspend gonad maturation or gametogenisis is, therefore, seen as a huge advantage to the echinoculture industry and should be investigated further (see Chapter 5). The use of artificial diets in this study appeared to prolong the reproductive state of urchins, so that, by week 20, urchins were still in a premature state. These results are similar to Cook *et al.* (1998), who found feeding *Psammechinus miliaris* a high protein salmon feed extended the spawning period, suggesting that the transfer of nutrients from NP's to gametes can be continuous, as long as the correct nutrients are available. Urchins that were fed FU appear to develop much slower than those that were fed artificial feeds and this was reflected in both gonad mass, as well as the histological data, as *Ulva* fed animals had smaller gonads that were in a growing state.

Gonad quality during week 16 & 20 revealed that the artificial diets produced gonads which generally had a dull coloration. This was more pronounced in the 0U diet, and was associated with a lack of pigment, namely β-echinenone (Robinson *et al.*, 2002; Shpigel *et al.*, 2005). Urchins fed the FU treatment produced gonads that were brightly coloured, but small in size. Based on eye rated (ER) colour, the 20U and 0U treatments produced gonads of low (20U) or unacceptable (0U) quality, while urchins fed FU had excellent quality gonads. The redness (a*) and yellowness (b*) values of the gonads supported these findings, as artificial treatments had significantly lower values, compared to the FU treatment in the first 20 weeks of the trial. It should be noted that redness (a*) and yellowness (b*) values of gonads between the two artificial diets were also different, with the 20U treatment having significantly better coloured gonads. Overall colour difference from fresh *Ulva* revealed that the 0U diet was significantly different to all other dietary treatments tested during week 16 & 20. Although significantly different at week 16, the 20U diet did not differ from FU at week 20, indicating

that pigment was being accumulated during gonad development in this treatment (Kennedy *et al.,* 2007). After the diet change, the effect of the higher pigment concentration of the FU treatment was clearly apparent, becuase urchins previously fed artificial diets showed a marked improvement in gonad colouration, while FU treatments changed to either artificial diet suffered a reduction in gonad colouration due to lower pigment concentration and rapid gonad development. Gonads of all treatments at the end of the trial were of marketable colour, however, the FU treatment still had significantly better coloured gonads compared with all other feeding regimes.

The results from this study suggest that the diets and feeding regimes tested can produce significantly better growth in terms of both diameter and weight, compared with similar studies on *T. gratilla* published in the literature (Table 5.1; Dworjanyn *et al*., 2007; Asia, 2009; Asia *et al.,* 2012). Weekly weight and diameter increases for the best performing diets in this study were 42.75, 5.92 (FU) and 38.32, 6.23% (20U), respectively, which far exceeds that of previous studies (Table 5.1). The next best performing diet, dried pellets containing *Sargassum* spp., fed at 5% body weight (Asia, 2009) only produced weekly weight and diameter increases of 5.5 and 3.5%, respectively. Comparisons between Asia (2009) and Asia *et al.* (2012) should, however, be treated cautiously, because culture temperatures varied by + 4 ^{o}C compared to this study. Thus, results from the present study indicate that it is possible for a natural diet (*Ulva* spp.) to produce comparable somatic growth to high protein artificial diets. Sinces the true protein content of farmed *Ulva* (determined using a specific nitrogen to protein conversion factor of 5.45, Shuuluka *et al.,* 2012) is even lower than assumed through crude protein estimates, averaging 15.89%, these results further indicate hat protein content is not necessarily correlated with somatic growth. Gonad production, however, seems to be heavily dependent on protein content, because artificial diets significantly increase GSI, compared to a diet of fresh *Ulva*.

These results suggest that the use of high protein artificial diets during the somatic growth phase of juvenile urchins is not necessary, as comparable growth can be produced by feeding *Ulva*. Gonad production on a diet of *Ulva* alone, however, is significantly lower than that obtained using an artificial diet, and, as a result, it is recommended that artificial diets are rather used for gonad development and/or enhancement. For best results and improved profitability, a dietary regime of fresh *Ulva* should be used, until urchins reach the desired size required for market, after which a finishing diet of artificial feed (20U) should be used to enhance gonad production. The use of this feeding regime would, however, depend on a farmer's access to large amounts of *Ulva*, which may not always be feasible or possible. It is, therefore, important to remember that equal somatic growth can be achieved using the 20U diet and, therefore, this diet can be used throughout an urchin's entire culture period. Although not tested in this study, a combination of both artificial feed and *Ulva* during the gonad enhancement phase may provide improved gonad colour, similar to that in the FU treatment, at the end of the trial.

Further research into the effect of dietary regimes on final gonad taste will also need to be completed, as the use of different diets types, during different developmental stages of the gonad, could affect the final flavour of the gonad. These findings could have a dramatic effect on the potential cost of the commercial production of *T. gratilla*, because feed costs account for a large proportion of production costs in aquaculture operations. In South Africa, two abalone farms, Wild Coast Abalone and Irvine and Johnson (I & J) and Cape Cultured Abalone Pty Ltd have integrated Abalone/*Ulva* systems, each producing between 1 and 2 tons of *Ulva* per month (Robertson-Andersson, 2003; 2007; Bolton 2006a, b; Robertson-Andersson *et al*. 2008; Bolton 2009; Shuuluka, 2011; DAFF, 2012) and thus it would therefore appear feasible, as well as beneficial, to do the same on sea urchin farms in the

future. However, research into the growth of *Ulva* in sea urchin effluent, specifically, would need to be investigated.

In conclusion, the combination of sea urchin/*Ulva* systems in South Africa would be very beneficial to the commercial production of *T. gratilla,* in terms of lowering farm operational costs. As has clearly been shown in this study, fresh *Ulva* can produce better/comparable somatic growth to high protein artificial diets (0U and 20U). An artificial feed such as 20U can be used at the end of the trial as a finishing feed, two months prior to harvest, to enhance gonad growth and increase product yield (Chapter 3). The findings from this study will contribute to lowering operating costs and will help to improve the commercial success of echinoculture operations, focused on the production of *T. gratilla,* in South Africa and around the world.

CHAPTER 5:

Investigating the effects of varying daylength in order to manipulate gametogenesis in *Tripneustes gratilla*

Contents

5.1 Abstract

The aim of this study was to investigate the effect of varying daylength over a 12 week period, in an attempt to suspend the onset of gametogenesis and gonad maturation of *Tripneustes gratilla*. The ability to control the reproductive state of commercially cultivated urchins is essential for producing gonads in an acceptable state of maturity, as well as being able to supply markets with gonads during periods considered to be out of season. To investigate the effect of daylength on gonad maturity, urchins where exposed to a Short (8:16 h) and a Long (16:8 h) day light regime. During the trial urchins were fed an artificial diet (20U) that had previously produced large gonads of high quality. At the end of the study, there were significant differences in gonad maturity between the two treatments, however, both somatic and gonadal growth did not varying significantly amongst the different treatments. Histology indicated that urchins exposed to a Short day (8:16 h) were significantly more advanced reproductively, with the majority of urchins being in a mature or spent state compared to those exposed to a Long day (16:8 h), which were mostly premature. Nutritive phagocyte (NP) density within the gonads supported the findings in histology, as gonads from urchins exposed to a Short day had significantly less NP's (21.58 ± 4.35%), compared to the Long day treatment (65.26 ± 3.09%). The results from this study suggest that Long days reduce the rate of gametogenesis in this species, while Short days increase gonad maturation. It is therefore recommended that long day photoperiods be used for the commercial production of the sea urchin *Tripnesutes gratilla*. This will ensure gonads are of marketable quality in terms of maturity, for longer periods of the year, therefore improving the potential success of this newly developing industry.

5.2 Introduction

For sea urchin gonads to be considered of high quality they must contain few or no gametes, contain a high percentage of nutritive cells (phagocytes), be bright yellow-orange in colour and have a firm texture (Robinson *et al.,* 2002; Shpigel *et al.,* 2004; Böttger *et al., 2006*). Seasonal changes associated with both photoperiod and temperatures have been implicated as potential cues for the initiation of gametogenesis. Although food quality and quantity have been shown to have significant effects on both gonad yield and quality (Vadas, 1977; Minor & Scheibling, 1997), nutritional factors do not initiate the gametogenesis process (Pearse & Cameron, 1991). Echinoculture will require a comprehensive understanding of the exogenous factors that may affect gametogenesis (Shpigel *et al.,* 2004; Spirlet *et al*., 2000; McBride *et al.,* 1997; Muthiga, 2005), so that an optimal product can be produced.

Gonad development in sea urchins exposed to natural conditions is generally characterised by seasonal changes in gonad mass, relative to total body mass (Gonad Somatic Index; GSI). In areas with significant seasonal variation in both light and water temperature, a large degree of variation in GSI is observed. In addition to the observed variation in GSI, there are also distinct changes in the cellular composition of the germinal epithelium, in both sexes, that occur during the annual gametogenic cycle. These changes are associated with the accumulation of nutrients within intergonial nutritive phagocytes (NP), and the subsequent transfer of these stored nutrients to gametogenic cells, which is followed by the production and storage of accumulated gametes that are eventually spawned (Harrington *et al., 2007;* Scheibling & Hatcher, 2007; Spirlet *et al*., 2000). The inter-cellular processes responsible for the simultaneous initiation of nutrient mobilization from nutritive phagocytes and gonial mitosis are largely unknown (Walker *et al.,* 2007). It has been suggested that the same environmental cues may be important for both processes found in these two differing gonadal

cell populations, however, these cues may also differ from species to species, and so little is known about what really initiates these cellular processes in *T. gratilla*.

Daylength has been shown to be responsible for controlling the reproductive development of various echinoids, and it has been experimentally shown that gametogenesis in a few species of sea urchins can be controlled by manipulating photoperiodic conditions. In the Gulf of Maine, Walker & Lesser (1998) demonstrated a positive correlation between the initiation of gametogenesis and the onset of shortening day lengths in *Strongylocentrotus droebachiensis* that were experimentally exposed to a photoperiod regime advanced by 4 months, compared to urchins in the field experiencing ambient photoperiod. Similarly, Böttger *et al.* (2006) and Harrington *et al.* (2007) showed that the initiation of gametogenesis in *S. droebachiensis* was governed by shortening day-lengths and decreasing water temperature and that gametogenesis could be suspended by exposing urchins to invariant summer (long-day) photoperiods. Previous studies have also shown that gametogenesis in *Strongylocentrotus purpuratus* (Pearse *et al.,* 1986), *Paracentrotus lividus* (Shpigel *et al.,* 2004) and *Eucidaris tribuloides* (McClintock & Watts, 1990) is initiated by shortening day length. Collectively, these studies demonstrated that Short days initiated, but long days may also inhibit, the onset of gametogenesis in some urchin species. Conversely, Kelly (2001) showed that the gonads of *Psammechinus miliaris,* maintained under a short-day photoperiod regime for 7 months, remained immature, while urchins maintained under a lengthening-day treatment regime progressed normally with their gametogenic cycle, indicating that long-days were an important cue for the initiation of gametogenesis in this species. Other research into the effects of photoperiod has revealed that varying day length has little to no effect on the gametogenic processes of urchins *P. miliaris* (Spirlet *et al.,* 2000) and *Evechinus chloroticus* (James & Heath 2008), however, both of these experiments were relatively short term (10 and 45 days respectively). It should be noted that *T. gratilla* is predominately a tropical species

and so may experiences much less variation in daylength under natural conditions compared to more temperate species mentioned above. Urchins in this study were, however, collected from one of the species most southern distributions (Haga Haga, South Africa, 32°45′4.23″S, 28°16′41.30″E), where daylenght can vary from a maximum of 15 h to a minimum of 10 h through the year. The ability to control the reproductive state of commercially cultivated urchins is essential for producing gonads in an acceptable state of maturity, as well as being able to supply markets with gonads during periods considered to be out of season.

The aim of the current study was to investigate the effects of Long and Short daylength on gonad development and gonad quality in the sea urchin *T. gratilla*. The following questions were addressed: (1) Do Long or Short days influence gametogenesis and prevent gonad development, suspending reproduction to produce gonads ready for marketing over longer periods? (2) Does daylength have a direct effect on gonad quality factors such as colour and texture?

5.3 Materials & methods

5.3.1 *Collection and maintenance of urchins*

Adult *Tripneustes gratilla* (65 - 80 mm test diameter) were collected during low tide from shallow rock pools near Haga-Haga, Eastern Cape, South Africa (32°45′4.23″S, 28°16′41.30″E) on 2-5 August 2010, and maintained as described in Chapter 2 (Section 2.3.1), until used for experimental trials. To reduce differences in gonad development and standardize nutritional condition, sea urchins were starved for 2 months prior to the start of the study, ensuring that gonads were reabsorbed and that most gonad development occurred during the study period (Spirlet *et al.*, 2000).

5.3.2 *Daylength trial*

On the 17 February 2011 adult *T. gratilla* were transferred to plastic baskets (L×W×H: 40 × 30 × 16 cm; 5 urchins per basket) that was suspended in 100 L cylindrical black tanks, with 4 replicate tanks for each treatment. The base of each plastic basket was lined with 8 mm^2 oyster mesh, which retained uneaten food and allowed faecal pellets to fall through. To test the effects of varying photoperiod on *T. gratilla* gonad development and quality, urchins were exposed to either Long days (16:8) or Short days (8:16). To ensure that experimental animals were exposed only to their allocated photoperiod treatments, light-tight lids were fitted to each cylindrical tank. Light was provided by means of a single 20W "cool white" compact fluorescent bulb, which had a light intensity of 3 $\mu mol.m^{-2}.s^{-1}$ at the water surface and was controlled with electronic timers to ensure photoperiods were consistent throughout the study period (Fig. 5.1). The experimental tanks were supplied with heated re-circulating seawater maintained at a salinity of 35 and temperature of 24-25°C. Seawater flowed through each tank at a rate of 2 $L.min^{-1}$ and each tank was supplied with fine aeration. To ensure that feeding and cleaning the tanks did not interrupt the photoperiod treatments, tank lids were only removed during the light period. Urchins were fed an artificial diet containing 20% dried *Ulva* (20U) that has previously been shown to produce marketable quality gonads in *T. gratilla* (Cyrus *et al.,* 2014), *ad libitum* every second day, after the removal of uneaten feed from each basket.

Immediately before the start of the experiment, six sea urchins were randomly selected and dissected to establish the initial state of the gonads. Thereafter, one urchin from each basket was sampled at random, each month, for the duration of the feeding trial. Each sampled urchin was blotted dry with paper towel, and total body weight, test diameter, test height and urchin drained weight (coelomic fluid removed) were carefully recorded to the nearest 0.01 g or 0.01 mm. The gonads were then carefully dissected out and the gonad wet weight was

recorded to the nearest 0.01 g using an electronic balance. The GSI was then calculated as described below. Gonad texture, firmness and colour were rated visually and manually by a single observer, according to the procedures described below. A separate gonad from each urchin was transferred into Davidson's Fixative (per litre: 300 mL 95% ethyl alcohol, 200 mL 100% formalin, 100 mL glycerol, 100 mL glacial acetic acid and 300 mL distilled water) immediately following dissection and fixed for 48 h, before being transferred into 70% ethanol and processed for routine paraffin histology as described below.

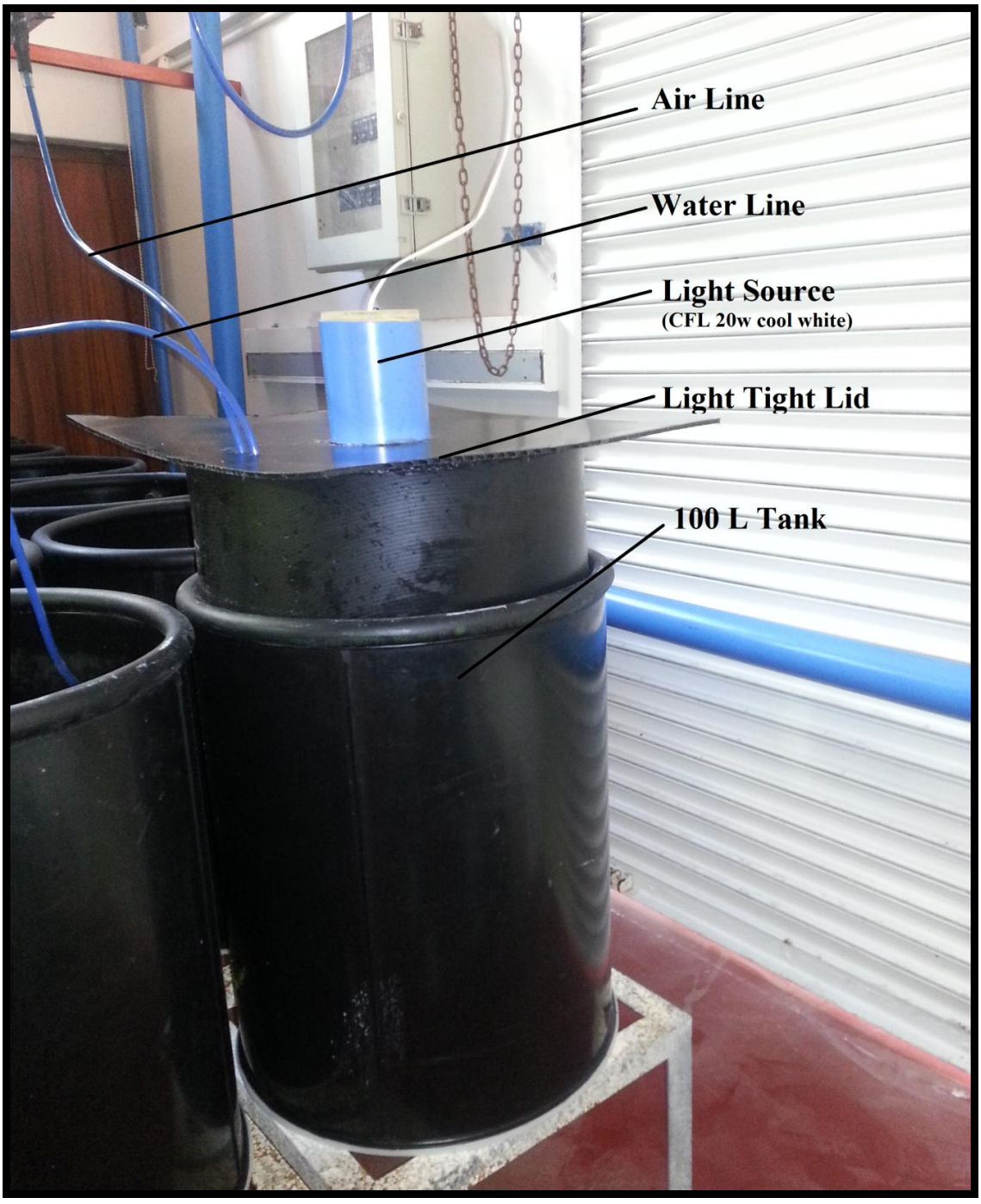


Figure 5.1: Experimental design used to investigate the effects of daylength on gonad conditioning.

5.3.2.1 Calculation of Gonad Somatic Index (GSI)

The body and gonad wet weights of individual urchins fed the various experimental diets (n = 8) were used to calculate gonad somatic index as described in Chapter 3 (Section 3.3.4.).

5.3.2.2 Assessment of gonad colour

Gonad colour was assessed by visually ranking each gonad (n = min 4 per treatment) in categories and by measuring the intensity of gonad lightness (L*), redness (a*) and yellowness (b*) with a hand-held reflected-light, fibre-optic spectrophotometer as described in Chapter 3 (Section 3.3.4.2).

5.3.2.3 Assessment of gonad texture and firmness

Individual gonad texture and firmness (n = min 4 per treatment) were visually assessed by a single observer (MDC), using the protocol described in Chapter 3 (Section 3.3.4.3).

5.3.2.4 Histology

Fixed tissues (n = min 4 per treatment) were processed and described using standard histological techniques presented in Chapter 3 (Section 3.3.5).

The density of nutritive phagocytes (NP) within each gonad was also calculated to determine whether the density of NP's within each gonad differed between the photoperiod treatments and/or stages of gonad maturity as described in Chapter 3 (Section 3.3.5.3).

5.3.3 *Statistical analysis*

To determine whether urchin wet weight, diameter, mortality, GSI, gonad wet weight and gonad Lightness (L*),Redness (a*) or Yellowness (b*) changed as a function of time, within individual treatment groups, or as a function of treatment at individual sampling dates, a one-way analysis of variance (ANOVA) was performed using Statistica 8 statistical software. The Tukey method was used for all post-hoc multiple comparisons between individual time points

within a treatment group. Paired student T-tests were used to test for significant differences between treatments at specific sampling dates. Significance was assigned to p-values of < 0.05 for all analysis. All tests for normality (Kolmogorov Smirnov test) and equal variance (Levene's test) passed for these data sets. A Mann-Whitney U test was used to test for significant differences in ordered data: eye rated gonad colour, texture, firmness and gonad maturity (histological data), within individual treatment groups over time and between treatment groups at specific sampling dates.

5.4 Results

5.4.1 Urchin survival and somatic growth

Survival rates of *T. gratilla* over the course of the study were high and did not vary significantly between the two treatments, with 100% survival for animals held under Short day (8:16) treatments and 95% for animals under Long day (16:8) treatments. Urchin size, determined using urchin wet weight increased significantly (one-way ANOVA, $p < 0.05$) over the 12-week experimental period (Fig 5.2A). Overall, the mean wet weight (± SE) of urchins in both treatments was 113.1 ± 6.07 g at the beginning of the study and 158.78 ± 7.81 g at the end of the study. Similar results were recorded for urchin test diameter (data not shown).

5.4.2 Gonad growth and quality

5.4.2.1 Gonad growth and yield

Gonad growth, represented by gonad wet weight (Fig. 5.2B) and GSI (Fig. 5.2C), increased significantly (one-way ANOVA, $p < 0.05$) in both treatments, over the course of the study. There was, however, no significant difference (pair t-test, $t = 0.746$, $df = 12$, $p = 0.47$) between the treatments at any of the sampling dates. At the end of the trial the final gonad weights were 21.04 and 23.84 g for the short and long day treatments respectively.

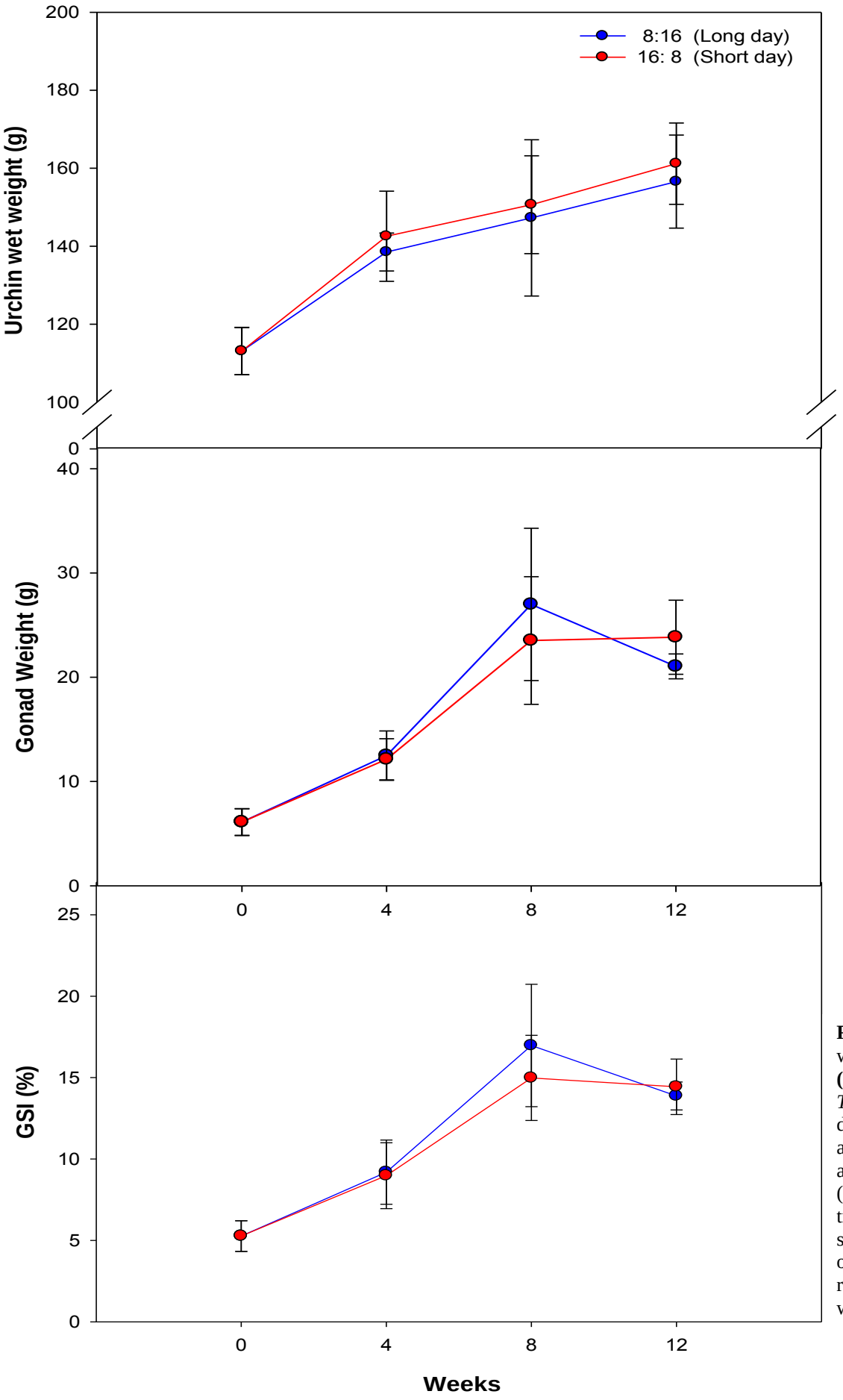


Figure 5.2: Mean **(A)** urchin wet weight, **(B)** gonad wet weight and **(C)** Gonad Somatic Index (GSI) of *Tripneustes gratilla* held under two different photoperiods; long (16:8) and short days (8:16) and fed with a artificial diet containing 20% *Ulva* (20U), over a 12-week photoperiod trial in recirculating seawater systems. Data represents mean ± SE of six, four, four and twelve replicates per treatment group for weeks 0, 4, 8 and 12 respectfully.

5.4.2.2 Gonad colour

Mean eye-rated gonad colour (Fig. 5.3) ranged from 2.5 to 2.75 for urchins held under Short day treatments (8:16), while urchins held in Long day treatments (16:8) produced lower gonad colour ratings, which fluctuated between 1.25 and 2.18 over the course of the trial. A significant difference (Mann Whitney U, U= 1 p = 0.029) in eye-rated gonad colour, between the Short and Long day treatment, was recorded at the Week 4 sampling point, indicating that ER colour was significantly better in gonads from urchins held in long day treatments (16:8). No other significant differences were recorded.

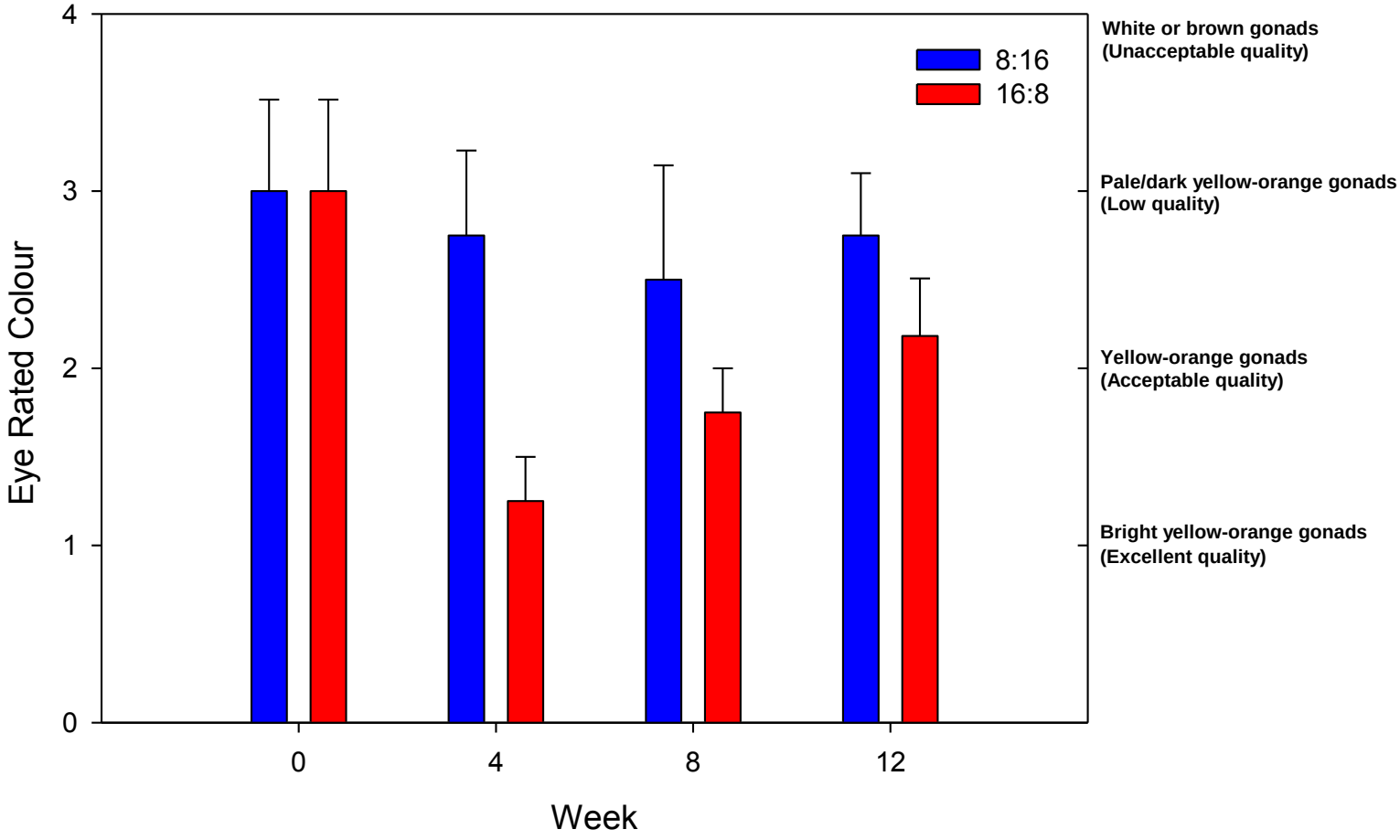


Figure 5.3: Mean eye rated (ER) gonad colour of *Tripneustes gratilla* fed an artificial diet 20U, and maintained under two different photoperiod treatments: Short days (8:16) and Long days (16:8), over a 12-week period. Data represents the mean ± SE of six, four, four and twelve replicates per treatment group for weeks 0, 4, 8 and 12 respectfully.

Gonad lightness (L*) values ranged from 44.66 to 58.98 over the course of the study and significantly lower (paired t-test, t = 2.62, df = 21, p = 0.016) values were recorded from urchins maintained under the Short day treatment, at the end of the study (Fig. 5.4A). Conversely, mean gonad redness (a*) values did not vary significantly between the two treatments, at any of the sampling dates (Fig. 5.4B), whereas mean gonad yellowness (b*) values were significantly higher (pair t-test, t = -6.26, df = 3, p = 0.0082) in animals maintained in the Long day (16:8) treatment, at week 4 only (Fig. 5.4C). Gonad colour over the 12 weeks of the study deteriorated as gonads advanced through their reproductive cycle, however, significant differences within the treatments were only recorded for gonad lightness, with gonads in both treatments being significantly lighter at the end of the study compared to at the start.

5.4.2.3 Gonad texture and firmness

Mean gonad texture ratings at the end of the study period ranged from 1.5 to 2.5 and all treatment groups consisted of gonads with two distinct gonad segment halves, with at least some visible follicle separation. There were no significant differences in gonad texture ratings between treatments at any of the sampling dates. Mean gonad firmness ratings (Fig. 5.5) ranged from 1.5 to 2.6 throughout the trial, indicating that gonads were soft–firm in their appearance, but of acceptable standard. Gonad firmness did not differ significantly between treatments until the end of the study, at which point the Long day treatment group had significantly (Mann Whitney U, U = 30, p = 0.025) firmer gonads compared to the Short day treatments.

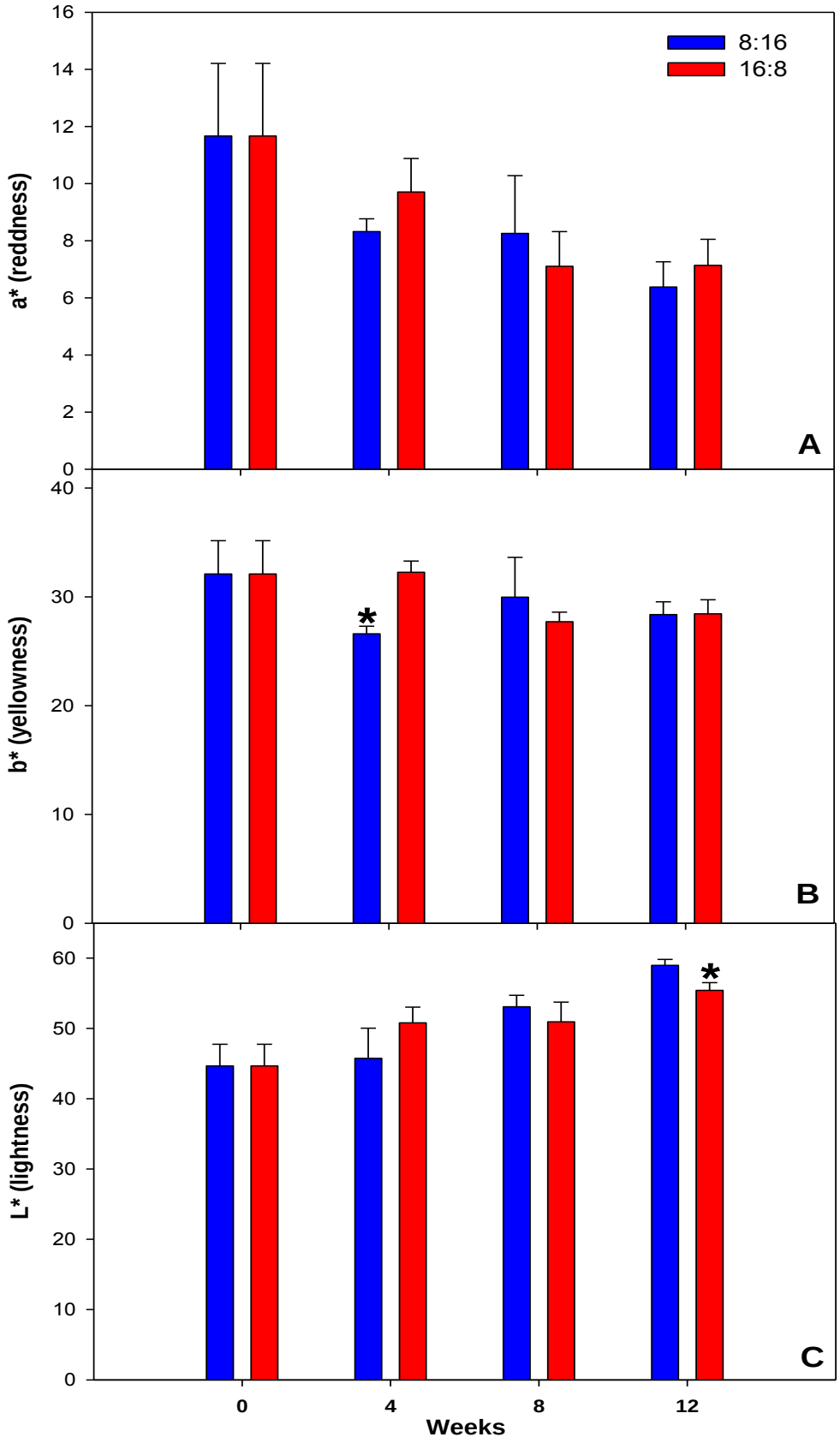


Figure 5.4: Mean gonad **(A)** L* (lightness), **(B)** a* (redness) and **(C)** b* (yellowness) values of *Tripneustes gratilla* maintained under two different photoperiod treatments: Short days (8:16) and Long days (18:6), over a 12-week period and feed an artificial diet containing 20% *Ulva* (20U). Data represent the mean ± SE. *($p < 0.05$, Student T-test) represents a significance differences between the means of urchins held under the two different photoperiod treatments.

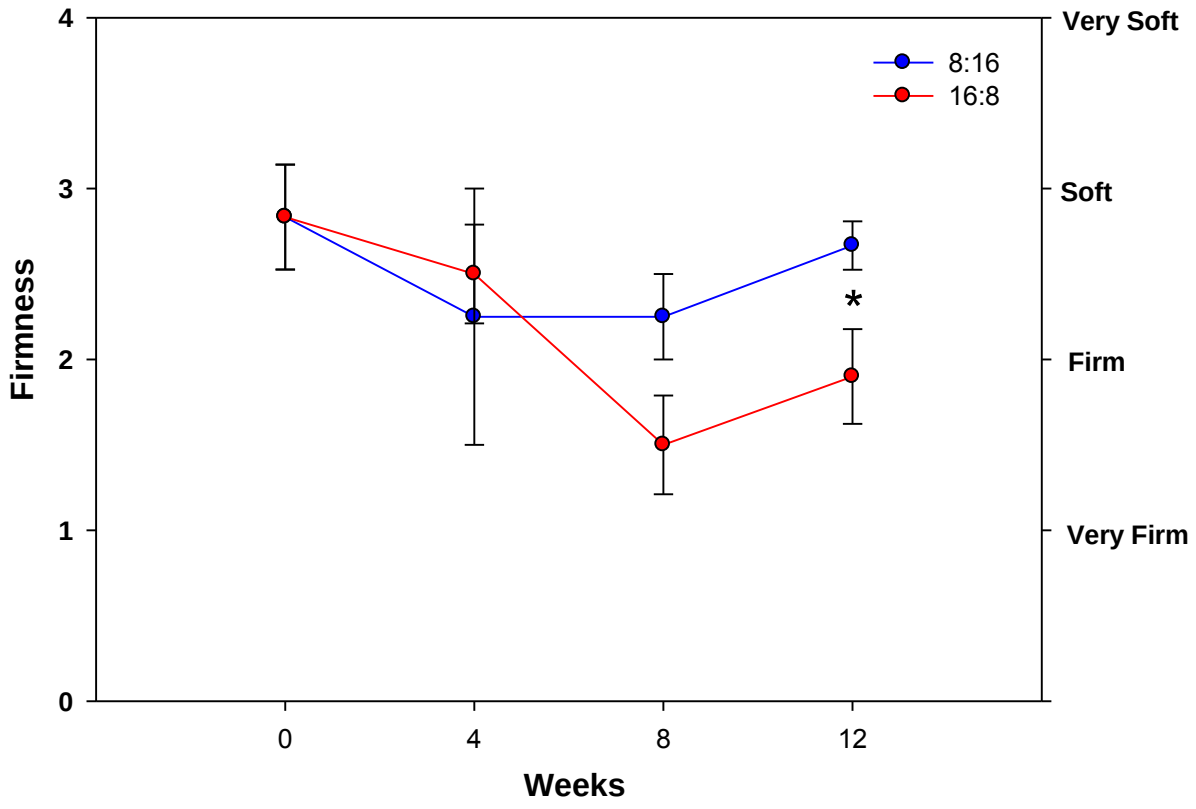


Figure 5.5: Mean gonad firmness values of *Tripneustes gratilla* maintained under two different photoperiod treatments: short days (8:16) and Long days (18:6), over a 12-week period and feed an artificial diet containing 20% *Ulva* (20U). Data represent the mean ± SE. *($p < 0.05$, Student T-test) represents a significance differences between the means of urchins held under the two different photoperiod treatments

5.4.2.4 Histology

Histological analysis of sea urchin gonads within the two photoperiod treatments (Fig. 5.7) revealed a significant degree of variability in overall gametogenic development, and therefore gonad maturity, between the initial histology samples taken at week 0, and those taken after 12 weeks (Short daylength: Mann-Whitney U, $U_{12} = 0$, $p = 0.000054$; Long daylength: $U_{11} = 6$, $p = 0.0024$), indicating progressive gonad development over the study period. Gonads from both treatments did not vary significantly from each other during week 4 and 8 of the trial, with the majority of these gonads found to be in a growing or premature state. Significant differences (Mann-Whitney U, U12= 4, p = 0.0000088) in gonad development were, however, found between treatment groups at the end of the 12 week study, with urchins maintained in the Short day treatments being more advanced (the majority of their gonads in a partly spent and spent state) compared to gonads sampled from urchins in the Long day

treatment (which were either still growing or premature). Within the Short day treatment, gonad maturity progressed significantly (Mann-Whitney U, $U_{12} = 1.5$, $p = 0.0022$) from week 8 to week 12, whereas no significant gonad development took place within the Long day treatment over this time, indicating that the gonad maturity appeared to be affected by photoperiod.

Nutritive phagocyte (NP) density supported the findings in histology, with gonads from both treatments showing no significant differences in the number of NP's until week 12 (Fig. 6.6). By week 12, mean NP density within gonads of urchins maintained in the Short day treatment was 21.58 ± 4.35% and reflected the gonad stage reported above, as most urchins were in a spent or partly spent stage. Conversely, in the Long day treatment, NP density was significantly (Student t-test, t = -8.046, DF= 21 p = 0.00000008) higher (65.26 ± 3.09%) compared with the Short day treatment. The majority of gonads within this treatment were observed to be in a premature state.

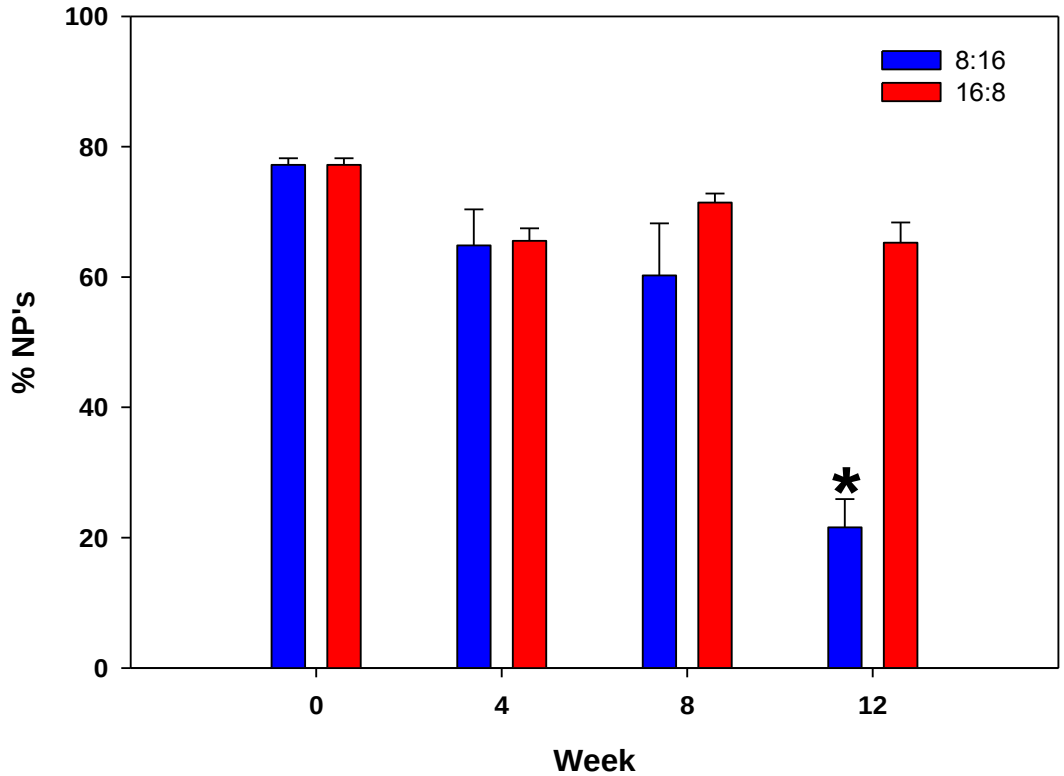


Figure 5.6: Mean nutritive phagocyte density (NP) (percentage ± SE) within the gonads of *Tripneustes gratilla* maintained under two different photoperiod treatments: Short days (8:16) and Long days (18:6), over a 12-week period and feed an artificial diet containing 20% *Ulva* (20U). *($p < 0.05$, Student t - test) represents a significance differences between the means of urchins held under the two different photoperiod treatments.

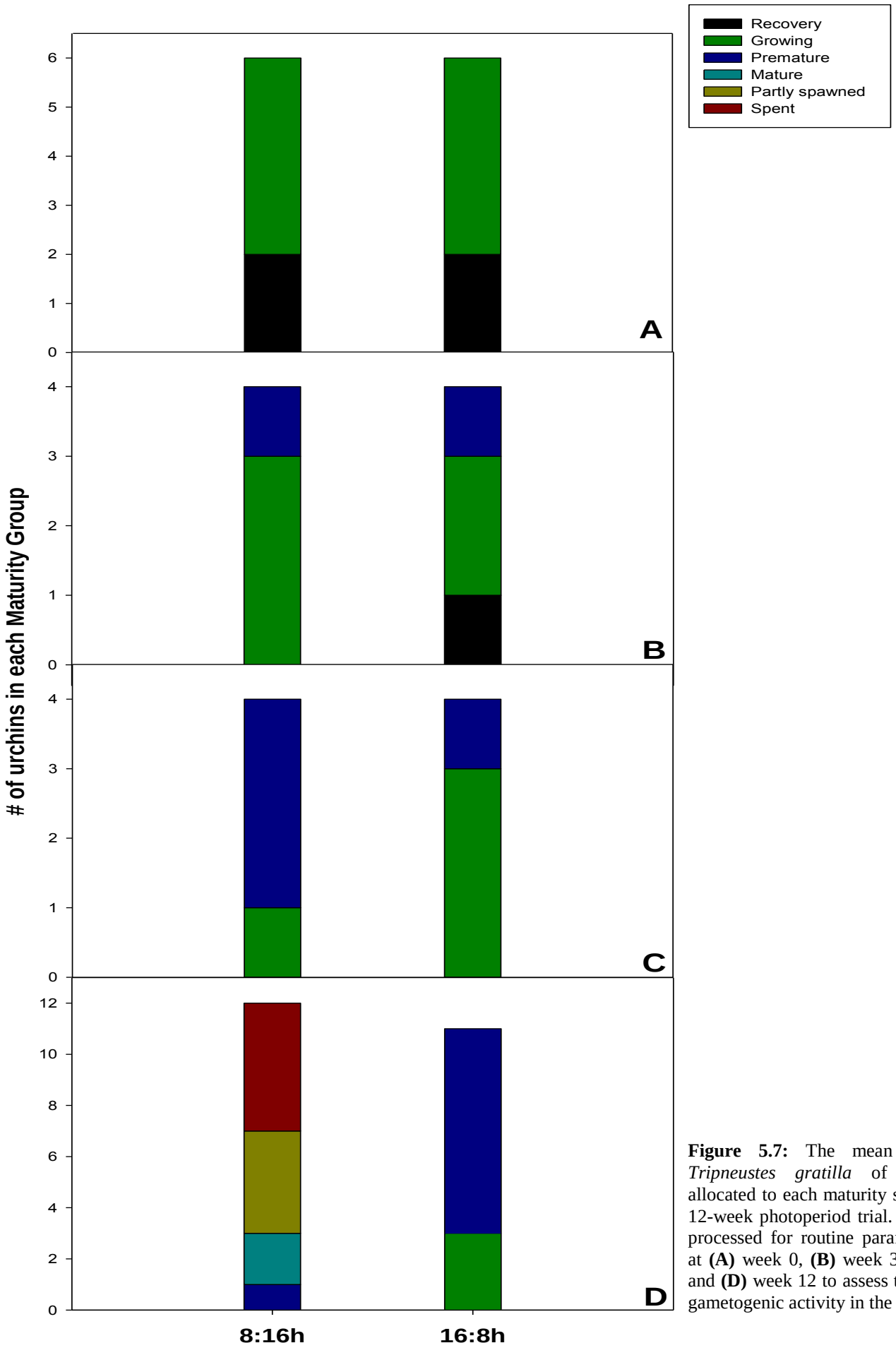


Figure 5.7: The mean number of *Tripneustes gratilla* of both sexes allocated to each maturity stage over the 12-week photoperiod trial. Gonads were processed for routine paraffin histology at **(A)** week 0, **(B)** week 3, **(C)** week 9 and **(D)** week 12 to assess the amount of gametogenic activity in the gonad.

5.5 Discussion

The results of this 12 week study demonstrate that daylength does not affect somatic growth of *T. gratilla,* but does significantly affect gonad development and maturity, with Short days (8:16) initiating and/or Long days (16:8) inhibiting gametogenesis. These findings are consistent with other studies conducted on sea urchins, including *S. droebachiensis* (Walker & Lesser, 1998; Böttger *et al.,* 2006; Dumont *et al.,* 2006), *Eucidaris tribuloides* (McClintock & Watts, 1990), *Paracentrotus lividus* (Shpigel *et al.,* 2004) and *S. purpuratus* (Pearse *et al.,* 1986). However, to the best of our knowledge this is the first recorded study documenting the effect of photoperiod on gonad development of *T. gratilla.*

Even though gametogenesis is dependent on the quality and quantity of food consumed (Vadas, 1977; Minor & Scheibling, 1997), nutrition is not responsible for the initiation of the gametogenetic process (Pearse & Cameron, 1991). In the present study, all animals were fed an artificial feed (20U diet; Cyrus *et al.,* 2014) and so it is unlikely that the observed differences in maturity were associated with differences in nutrition. Previous studies have also shown that photoperiod has no significant effects on the feeding rate of *P. lividus* (Shpigel *et al.,* 2004), *S. purpuratus* (Pearse *et al.,* 1986) and *S. droebachiensis* (Dumont *et al.,* 2006). Thus it was assumed that differences in gonad maturity and development among the treatments in this study were unlikely to be due to variations in feeding and or absorption rates, although these were not recorded. Schumacher (1974) and Klumpp *et al.* (1993) have, however, showed that feeding in *T. gratilla* is continuous and unaffected by light. Our findings would tend to support these claims, because during the study GSI indicated no significant differences between either of the photoperiod treatments, with the Short (8:16) and the Long (16:8) day treatments reaching a peak GSI of 16.97 ± 3.75 and 14.98 ± 2.6%, respectively. These values are similar to those recorded for *T. gratilla* in natural conditions along the east coast of South Africa (Baliwe, 2010). The results also support findings by

Spirlet *et al*. (2000) and Shpigel *et al.* (2004), who observed that photoperiod had no significant effect on GSI in *P. lividus*. Similar observations have also been made for *S. purpuratus* (Pearse *et al*., 1986) and *Psammechinus miliaris* (Kelly, 2001).

Although there were no significant differences in the GSI between the two treatments over the study period, there were significant differences in gonad development. Commercial sea urchin markets require gonads which are in either a growing or premature stage, because this is when gonads are generally large in size and have a high percentage of nutritive phagocytes (NP's) compared to mature gametes (Dumont *et al.*, 2006). As gonads mature, the ratio of NP's to gametes shifts in favour of the gametes and consequently results in a lower valued product (Lee & Haard, 1982). The ability to prolong/prevent the onset of gonad maturity in commercially cultured urchins would be considerable advantages as acceptable products could then be made available all year round.

Results from this study indicate that the rate at which urchins' progress through the various stages of gametogenesis can be affected over a 12 week period by specific photoperiod regimes. Short days (8:16) increaed the rate of gametogenesis, while Long days (16:8) appeared to reduce gametogenic activity. The majority of urchins in the Short day (8:16) treatments had gonads that were either in a partly spent or spent state, meaning that the majority of NP's had been used up to produce gametes. This is compared to those in the Long day (16:8) treatments, which were in a growing or premature state and had not yet used up all stored NP's. The NP density in the two treatments further support these findings, as significant differences in gonad composition were found at the end of the study, with Short day (8:16) treatments containing less NP's compared to Long day (16:8) treatments. It is important to note, however, that one cannot determine whether gametogenesis is initiated by the effects of Short days or long nights, in this case, without conducting a light break in the middle of the dark period. This has not been done for this or any other studies conducted on

sea urchins thus far, and so further investigation is recommended. Gonad firmness was indirectly affected by photoperiod, as it is influenced by gonad maturity. This, in turn, resulted in the production and accumulation of gametes in the Short day treatment, which significantly affected the firmness of the gonads. The advancement of gametogenesis and gonad maturity within the Short day treatment resulted in gonads which were soft, had a fluid texture, and released gametes readily. This runny state causes the product value to decrease and generally results in a product that is unacceptable to consumers (Hickey, 1982; Parker, 2005). By increasing daylength (Long days) in this study, it has been shown that it is possible to successfully suspend the gametogenic process and, therefore, significantly lower the proportion of male and female urchins producing mature gametes at harvest. Reduced gametogenic activity in these treatments also resulted in a significantly increased NP density, which subsequently improved gonad quality. It must be noted that this effect may also be due to the absence of a Short day cue, which may initiate gametogenesis.

Although the exact process of the photoperiodic cue remains unknown (Walker *et al*., 2007), Walker & Lesser (1998) suggested that changing autumn photoperiod, which results in shortening daylength, may lead to the activation of spermatogonial or oogonial mitosis for the urchin *S. droebachiensis,* through direct mitogen induction or, alternatively, it may result in the mobilization of stored nutrients within NP's that encourage gonial cell mitosis. Millott (1986) found that the radial nerve of *Diadema antillarum* is photosensitive and that the external epithelium of this species contained putative photoreceptors. Their findings suggested a relationship between dermal photosensitivity and abundant superficial nerves. Walker *et al.* (2007), however, proposed that, within the tube feet of the sea urchin *S. droebachiensis,* there might remain some ancestral components of the visual system (Pax 6 genes), which could provide a mechanism to sense changes in ambient light, which, in turn, might initiate biochemical and/or molecular responses. Lesser *et al.* (2011) confirmed Walker

et al.'s (2007) hypothesis by determining that tube feet are photosensory organs that detect and respond to changes in the underwater light, through the expression of the Pax 6 gene and the differential expression of opsin, which was dependent on light exposure.

Measurements of gonad colour during this trial indicated that there were significant differences in both yellowness (b*) and lightness (L*) at specific sampling dates. Samples taken during week 4 indicated that the Long day (16:8) treatment produced gonads that were significantly yellower than those of the Short day (8:16) treatments. Additionally gonad lightness (L*) during week 12 indicated that the Short day treatments had significantly lighter gonads. Increasing gonad lightness (L*) in week 12 is consistent with findings by James & Heath (2008), who found that in their study differences in gonad lightness (L*) were due to changes in the reproductive condition of the urchin and that these changes occur due to the presence/absence of gametes. Differences in gonad yellowness (b*) during week 4 could not be explained, and require further investigation.

In conclusion this study has shown that for the sea urchin *T. gratilla*, maintained on an artificial diet of 20U under artificial Long Day (16:8) photoperiod, (1) gametogenesis can be inhibited and thus the progression of gonad development can be prevented, significantly improving gonad quality and (2) Long Day (16:8) photoperiods do not negatively affect any gonad quality factors. Sea urchin reproduction is characterized by two major phases: (1) storage of nutrients in the form of nutritive phagocyte and (2) production of gametes through gametogenesis (Scheibling & Hatcher, 2007; Walker *et al*., 2007). The ability to inhibit or reduce the rate of gonad development and gametogenesis, through Long Day (16:8) photoperiod, allows the maintenance of urchins in a nutrient storage phase, resulting in gonads of marketable quality for longer periods. This knowledge could also potentially be used to promote "out of season" gametogenesis and gonad maturity of commercial species, spawned for larval production (Kelly, 2001), another factor that can limit echinoculture

operations due to seasonal spawning patterns. It is, therefore recommended that Long day photoperiods be used for the commercial production of the sea urchin *Tripnesutes gratilla*, in order to ensure gonads are of marketable quality in terms of maturity, for longer periods of the year, therefore improving the potential success of this newly developing industry.

Chapter 6:

The use of stable δ13C and δ15N isotopes to track the incorporation of dietary ingredients into the gonads of *Tripneustes gratilla*

Contents

6.1 Abstract

This study used stable carbon and nitrogen isotope analysis to investigate the incorporation of specific dietary ingredients from 4 artificially formulated feeds (0U, 5U, 15U & 20U), containing varying amounts of the macroalga *Ulva*, in the production of gonads by *T. gratilla*, over a 20 week period. Stable carbon and nitrogen isotopes are non-hazardous markers, which have been widely used in evaluating the energy flow of aquatic ecosystems, as well as aquaculture operations. By inputting the isotopic values of sampled gonads and individual dietary ingredients into IsoSource, a mixing and mass balance model, it was possible to estimate the relative contribution of each dietary ingredient to gonad production and highlight the importance of specific feed ingredients. Results from this study estimated that the macroalga *Ulva* was an important isotopic source for gonad production, accounting for an average of 33% of the isotopic signal across all *Ulva* containing diets (5U, 15U & 20U), at the end of the trial. It also indicated the importance of macroalgae in gonad production, as a diet without *Ulva* (0U) was estimated to rely heavily on dietary carbon and nitrogen obtained from Kelp, even though this feed was only fed prior to the start of the study. In summary the use of stable isotope analysis can be extremely useful in assessing the effectiveness of particular dietary ingredients, particularly in cases such as this where growth of specific organs is being investigated. In general, feeding trials cannot assess the incorporation of specific dietary ingredients, but through the use of stable isotope analysis and mixing and mass balance models, such as IsoSource, it is possible to estimate their relative contributions to production, which can help to improve diet formulations in the future.

6.2 Introduction

Stable isotopic analysis has in recent years, become a popular tool for studying ecosystem functionality and community structure (Post, 2002). One of the most common applications of stable isotope analysis is the use of δ13C and δ15N to estimate the proportional contribution of sources to a mixture, such as in animal diet reconstruction. It can also be used to infer trophic links and dietary relationships (Peterson & Fry, 1987; Jennings *et al.,* 1997). This is possible because different foods have distinct isotopic signatures (DeNiro & Epstein, 1978, 1981; Vander Zanden *et al*., 1999) and a consumer's signature reflects the isotopic signatures of assimilated material over time (Peterson & Fry, 1987; Beltrán *et al.*, 2009; Martínez-Rocha *et al*., 2013). At each trophic level, heavier isotopes of any given element generally increase in abundance, compared to the lighter forms, which is referred to as isotope discrimination (Caut *et al.,* 2009). Carbon (C) isotopic values between trophic levels are generally very similar (DeNiro & Epstein, 1978a, b), having a discrimination factor of around 1‰ (DeNiro & Epstein, 1981; Peterson & Fry, 1987; France & Peters, 1997). Nitrogen (N), on the other hand, is more enriched than carbon when moving between trophic levels and generally has a discrimination factor of 3 - 4‰, relative to the diet consumed (DeNiro & Epstein, 1981; Minagawa & Wada, 1984; Peterson & Fry, 1987).

With the use of mixing and mass balance models, the relative contribution of each dietary source can be estimated (Phillips & Gregg, 2003; Burford *et al*., 2004; Fry, 2006). In aquaculture, natural stable isotopes have been used to track the fate of nutrients within a culture system and identify areas impacted by aquaculture operations (Schroeder, 1983; Burford *et al*., 2004; Vizzini & Mazzola, 2004; Carballeira *et al.,* 2012). Stable isotopes also provide researchers with a tool to assess ingestion and nutrient utilisation by cultured organisms. Estimates of the relative importance of particular nutrient sources or feed ingredients can thus be made and the use of these methods in marine and, more specifically,

aquaculture studies, has already proven to be a useful tool (Benstead *et al*., 2006; Duffy *et al*., 2011; Le Vay & Gamboa-Delgado, 2011; Sun *et al*., 2013). Previously, methods such as digestive tract analysis (De Barros & Valenti, 2003), serological estimation of prey-protein (Hoyt *et al*., 2000), natural fluorescence of gut contents (Penry & Frost, 1990; Hinz *et al*., 2001), and fluorescently-labelled micro-diets (Soto-Rodriguez *et al*., 2003) have been used. However despite extensive research the nutritional requirements and relative nutritional allocation of many cultured species is not yet fully understood, mostly due to difficulties in assessing feed intake.

The development of cost effective feeds that meet the nutritional requirements of a cultured organism is presently a major focus area in aquaculture research. This is because feed costs can account for a large proportion of operational costs, require substantial resource investment, and poorly designed feeds can have major adverse effects on both animal and ecosystem health. Protein, primarily fish meal, is often the most expensive ingredient of formulated feeds for aquaculture and this has prompted industry and researchers to find alternative dietary protein sources. The anatomy and physiology of sea urchins is, however, influenced largely by food quality and quantity, with food availability affecting the distribution and allocation of resources to different components of both somatic and gonadal growth (Russell, 1998; Beddingfield & McClintock, 1998; Guillou *et al.*, 2000; Chapter 4). Identifying the specific dietary constituents that are incorporated into specific organs (eg. gonads), or an organism as a whole, will provide valuable information that can be used to improve feed formulations and help identify alternative sources of dietary protein

Stable isotopes have been successfully used to investigate sea urchin feeding habits and trophic positioning (Rodríguez, 2003; Yatsuya & Nakahara, 2004; Tomas *et al.*, 2006; Vanderklift *et al.*, 2006; Prado *et al.*, 2012), however, no studies to date have utilized this technique to investigate which ingredients within formulated aquaculture feeds are important

for gonad development. For echinoculture to be economically viable, high-quality as well as cost effective and environmentally sustainable, feeds need to be formulated to produce market quality gonads. With the use of stable isotope analysis, it may be possible to identify the relative importance of each ingredient and its contribution to gonad development.

The aim of this study was to use stable isotopes analysis to evaluate the importance of individual dietary ingredients within four formulated sea urchin feeds, containing varying amounts of *Ulva* (Cyrus *et al.*, 2014). The main objectives were to (1) determine if one could use stable-isotopes to identify the contribution of specific ingredients to gonad development and (2) determine the relative importance/contribution of *Ulva*, the seaweed component of the diet, to gonad development. The study aims to provide new understanding about the relative importance of *Ulva* and other ingredients within these artificial feeds and use these findings to help improve future diet formulation.

6.3 Materials & methods

6.3.1 Collection and maintenance of urchins

Adult *Tripneustes gratilla* (50 - 70 mm test diameter) were collected from shallow rock pools near Haga-Haga, Eastern Cape, South Africa (32°45′4.23″S, 28°16′41.30″E) and maintained on a diet of kelp (*Ecklonia maxima*) as described in Chapter 2 (Section 2.3.1). This, therefore, gave them a unique isotopic signature.

6.3.2 Feeding trial and sampling

Urchin tissue samples utilised for this component of the study were obtained from the urchins sampled during the feeding trial described in Chapter 3 (section 3.3.3). In addition to these samples, urchins were also dissected on the 27 July 2009, 30 days after the conclusion of the feeding trial (during which time they were maintained on the same diets). This resulted in four sampling points (week 3, 9, 12 & 20), from each treatment group (20U, 15U, 5U and 0U). Urchins from the fresh *Ulva* spp. treatment were not sampled as the purpose of this study was to determine the relative contribution of each of the artificial dietary ingredients, with and without dried *Ulva* supplementation, to gonad development.

To establish the initial δ13C and δ15N valuesof experimental urchins before the start of the experiment, 10 sea urchins were randomly selected and their gonads removed. These gonads were rinsed with distilled water and then dried at 70°C until a constant weight was reached (~ 48 h), after which they were stored in glass vials for future isotope analysis. Thereafter, eight urchins from each treatment were sampled at random during week 3, 9, 12 and 20, over the duration of the feeding trial, and stored in the same way. Samples of each dietary ingredient, as well as each of the experimental feeds (20U, 15U, 5U and 0U), were collected at the time of diet formulation and stored in glass vials until further analysis.

6.3.3 Sample processing and isotope analysis

After drying, samples were ground to a fine powder, using a Retsch 200 mm ball mill for 10 minutes, and stored in 20 mL glass vials. Powdered samples of 0.6 - 0.7 ± 0.001 mg were then weighed into foil cups, using a Sartorius micro balance. Once the samples had been weighed into the foil cups, the cups were squashed to enclose the sample.

Isotope analysis was performed at the University of Cape Town, using a Delta Plus XP Isotope Ratio Mass Spectrometer (IRMS) (Themo electron, Germany) coupled to a Flash EA 1112 elemental analyser (Thermo Finnigan, Italy) via a Conflo III gas control unit (Thermo Finnigan, Germany). Carbon and nitrogen were analysed in a dual isotope mode. Samples of reference material (internal standards) were used to calibrate the system and compensate for drift with time. Stable isotope values were then calculated as:

$$\delta X\ (‰) = [(R_{sample}/R_{standard})-1] \times 1000$$

Where X is ^{13}C or ^{15}N value and R is the ratio of the heavy to light isotope ($^{15}N/^{14}N$ or $^{13}C/^{12}C$).

Isotope ratios were expressed relative to a Vienna-PeeDee Belemnite (VPDB) standard for carbon and atmospheric N_2 in air for nitrogen. The deviation from the standard is denoted by the term δ and the results expressed as parts per thousand (‰).

6.3.4 IsoSource analysis

The mixing model software programme IsoSource (Phillips and Gregg, 2003) was used to analyse the contribution of each isotopic source or dietary ingredient to the development of the gonad. IsoSource (www.epa.gov/wed/pages/models.htm) is able to calculate feasible ranges of source contributions using stable isotope data by: (1) iteratively calculating all possible combinations of diet source proportions that sum up to 100%, using user-defined increments (generally 1%); (2) calculating predicted isotopic values for each mixture using

linear mixing model equations that preserve mass balance (Phillips, 2001); and (3) comparing predicted consumer isotope values with observed consumer values, which, if equal or within a small user defined tolerance (e.g.: 0.2%, typical of analytical measurement error for δ 13C and δ 15N), represent feasible solutions that satisfy isotopic mass balance and are recorded as possible solutions.

The isotopic sources or dietary ingredients included in the analysis consisted of fish meal, soya, extruded maize, *Ulva*, *Ulva* additive, wheat, basal formula and kelp. Kelp was included in the IsoSource analysis as it had been used as a maintenance feed prior to the start of the feeding trials (Cyrus *et al.,* 2014) and so would have an influence on the isotopic signal of sampled gonads, particularly at the beginning of the study. The basal formula was also included in the IsoSource analysis to account for the isotopic signals derived from minor ingredients within the basal feed that could not be analysed independently, for various reasons. For analysis of tissues obtained from urchins fed the 0U and 20U diets, *Ulva* and the *Ulva* additive, respectively, were omitted as these ingredients were not present in these diets.

Discrimination factors for each dietary ingredient incorporated into the gonad were not assessed in this study, instead, discrimination factors of δ15N and δ13C determined by Prado *et al.* (2012) for the sea urchin *Lytechinus variegates* fed a similar formulated feeds, were used (δ15N of -0.14 ‰ for nitrogen and a δ13C of 0.15 ‰ for carbon). Discrimination factors represent the amount of change in isotopic ratio when food/prey is incorporated into the tissue of a consumer, and are often cited as a weak link in the application of mass balance and mixing models for stable-isotope analysis for diet reconstruction (Gannes *et al*., 1997, Wolf *et al*., 2009), making it important to use appropriate discrimination factors, as used here. The source increment in IsoSource was set at 1% while the tolerance was set at 0.1 ‰, however if isotope mixture values were out of bounds (outside the delineated polygon of the seven or eight ingredients), tolerance values were incrementally increased by 0.1 ‰, up to a maximum

of 0.2 ‰ (Phillips, 2001). IsoSource outputs for each dietary source were recorded as a mean, as well as ranges (minimum - maximum feasibility) in source contributions (Phillips & Gregg, 2003; Benstead *et al*., 2006; Sun *et al*., 2013). Source contribution ranges are reported from the 1st percentile to 99th percentile, to reduce the inclusion of extreme outliers.

6.3.5 Statistical analysis

SigmaPlot version 12 software was used to perform all statistical analyses. To determine whether δ15Nand δ13C values differed significantly as a function of time within individual treatment groups, or as a function of the treatment (0U, 5U, 15 & 20U) at individual sampling dates, one-way analyses of variance (ANOVA) were performed. All tests for normality (Kolmogorov-Smirnov test) and equal variance (Levene's test) were passed for all data sets. The Tukey method was used for all post-hoc multiple comparisons between individual treatment groups. Significance was assigned to p - values of < 0.05 for all analyses.

6.4 Results

6.4.1 *Isotope analysis*

Nutrient analysis, as well as carbon and nitrogen isotopic signatures of the four prepared diets fed to *Tripneustes gratilla* during the experimental period, are presented in Table 6.1. Mean δ13C and δ15N values recorded from *T. gratilla* gonads ranged from -16.11‰ to -13.08 ‰ and 6.01 to 6.80 ‰, respectively, over the course of the trial (Fig. 6.1). Carbon (C) and nitrogen (N) isotopes provided good discrimination among ingredient sources, with mean values for most sources separated by at least 2 ‰ on one or more axes (Fig. 6.2). Almost all (94%) of the gonads sampled over the course of the study had values which fell within the polygons delineated by the source end members within each diet (Fig. 6.2), confirming that all major ingredient sources in the formulated diets were present.

Table 6.1: Nutrient analysis of fresh *Ulva*, the basal formula (no *Ulva* included) and the four prepared diets fed to *Tripneustes gratilla* during the experiments (% dry matter). Treatment groups: 20U = 20% *Ulva*; 15U = 15% *Ulva*; 5U = 5% *Ulva*; 0U = 0% *Ulva*.

Feed	Protein (%)	Fat (%)	Moisture (%)	Ash (%)	Gross Energy (MJ/kg)	Fibre (%)	Carbohydrates (%)	δ15N	δ13C
20U	25.69	2.31	9.61	13.89	15.49	4.754	43.746	6.45	-18.78
15U	26.38	3.52	9.45	11.44	16.22	5.449	43.761	6.28	-18.97
5U	26.58	3.16	8.52	8.69	16.83	5.324	47.726	5.86	-19.32
0U	26.48	2.72	8.55	7.57	17.18	6.071	48.609	5.47	-19.63
Basal Formula	28.6	3.77	8.52	7.46	17.36	4.199	47.451	5.81	-19.82

No significant differences were noted in gonad δ13C values between any of the dietary treatments, over the course of the study (Fig. 6.1A). The δ13C signatures of all treatments in the first three weeks of the trial were not significantly different from the starting values. Thereafter, values decreased significantly (one-way ANOVA, $p < 0.01$), with all treatments exhibiting a decrease in δ13C of at least 2‰ over the course of the trial.

No significant differences in δ15N values were found between any of the treatments, with the exception of the 20U diet, which had significantly higher δ15N values (One-way ANOVA, $p < 0.01$) compared to the 5U and 0U diets during week 12 (Fig. 6.1B). Within treatments significant differences (One-way ANOVA, $p < 0.05$) were only found within the higher *Ulva* inclusion diets, namely 20U and 15U. It should be noted that a spawning event occurred prior to the final sampling date, between week 12 and 20, which may have affected the isotopic values of the gonads of all urchins across all treatments in this study.

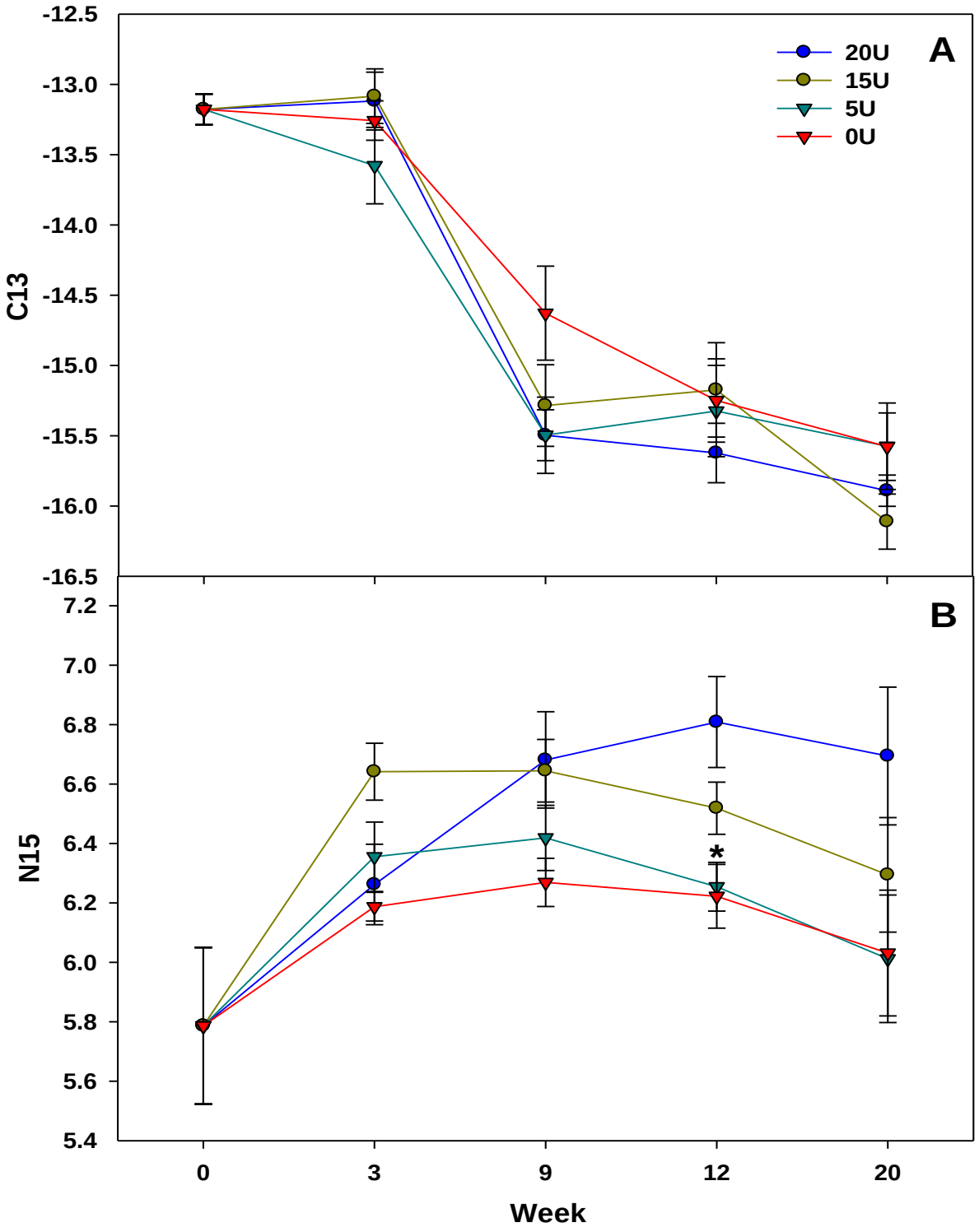


Figure 6.1: Mean (±SE) **(A)** δ13C and **(B)** δ15N stable isotope composition of sea urchin (*Tripneustes gratilla*) gonads of individuals fed on 4 artificially formulated diets (0U, 5U, 15U & 20U) at each sampling date of the experiment.

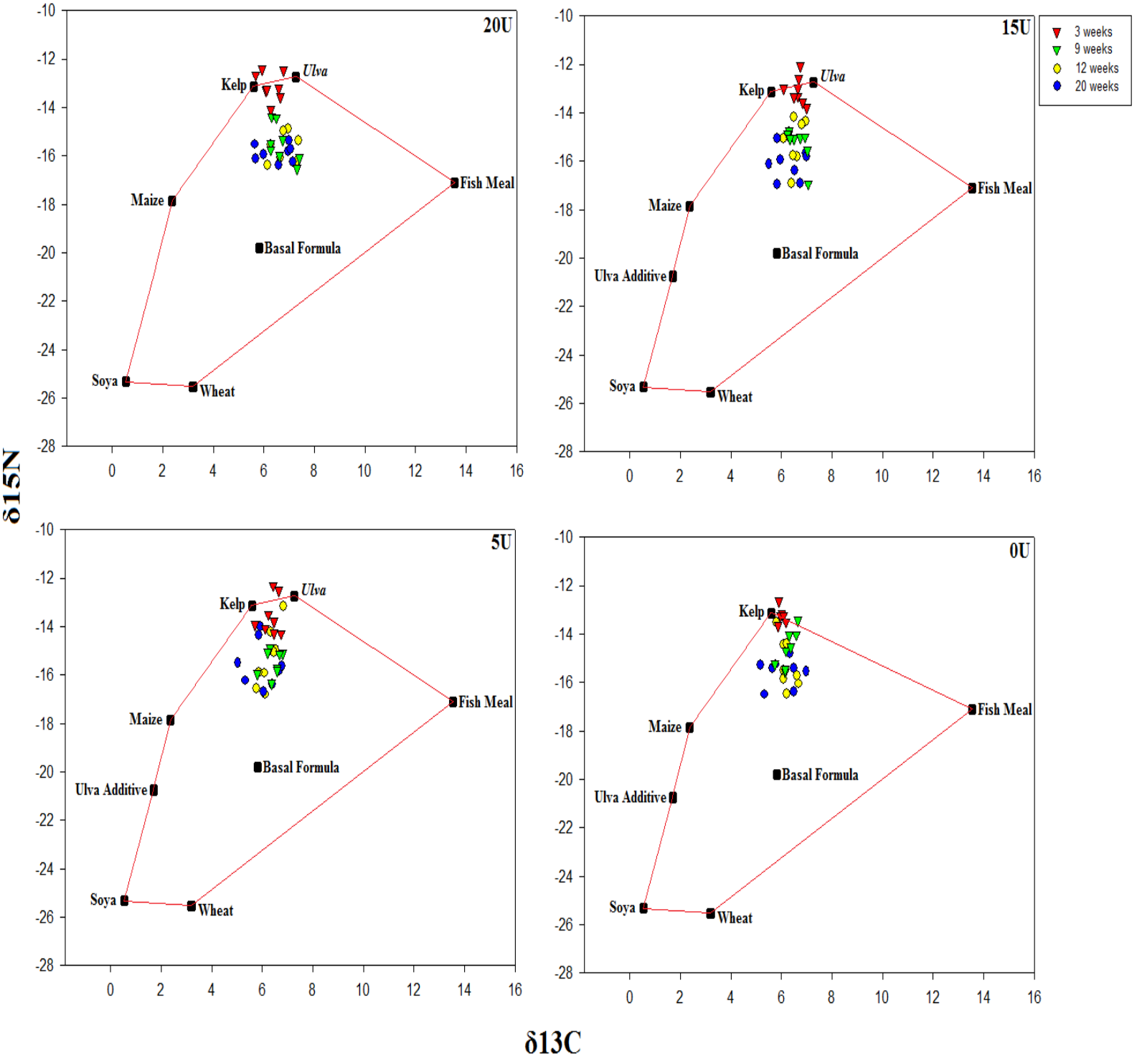


Figure 6.2: Stable carbon and nitrogen isotope ratios of each dietary ingredient (solid squares) and gonads obtained from *Tripneustes gratilla* fed 4 artificially formulated feeds containing varying amounts of dried *Ulva* (20U, 15U, 5U & 0U) ploted against one another (δ15N vs. δ13C) over a 20 week period.

6.4.2 *IsoSource analysis*

With the use of IsoSource, the relative contribution range of individual ingredients to gonad growth was estimated. The estimated contribution of fish meal to sea urchin gonads averaged 1.5% (minima) to 21% (maxima), among all dietary treatment groups (0U, 5U, 15U & 20U), over the course of the study. Estimated contributions from *Ulva* averaged 0% (minima) to 72.3% (maxima) among the 3 *Ulva* inclusion diets (5U, 15U & 20U) (Fig. 6.3), while *Ulva* additive averaged 0% (minima) to 21.33% (maxima) for the 3 diets containing it (0U, 5U & 15U). Feasible contributions of wheat to gonad growth averaged 0% (minima) to 15.75% (maxima), while maize averaged 0% (minima) to 32.25% (maxima), and soya averaged 0% (minima) to 14.25% (maxima). Feasible contributions of Kelp to gonad development averaged 12% (minima) to 78% (maxima) among the different diets, over the course of the study.

The C and N isotope plots for the 4 dietary treatments (0U, 5U, 15U & 20U) at the end of the trial illustrated the estimated mean (± SD) incorporation of specific dietary ingredients into the gonads of *T. gratilla* (Fig. 6.3). Analysis of gonads from urchins fed the *Ulva* supplemented feeds indicated that *Ulva* was incorporated the most out of any of the dietary ingredients, having contributed approximately 32, 31.4 & 36.7% to gonad development of urchins in the 20U, 15U and 5U treatments, respectively. Kelp, which accounted for the largest contribution (61.6%) to gonad development in the 0U treatment, was the 2nd most important ingredient in the remaining three diets tested, according to IsoSource estimates, contributing approximately 23.9, 22.9 and 26.8% to gonad development of animals in the 20U, 15U and 5U treatment groups, respectfully. The only other dietary ingredient that made a significant contribution to gonad composition was the protein source, fishmeal, and its contribution appeared to increase in diets containing higher *Ulva* contents. A similar trend was also seen for the other protein component of this diet, namely, soya. The remaining

dietary ingredients (maize, soya, wheat, basal formula or *Ulva* additive) accounted for less than 10% of gonad composition by the end of the trial.

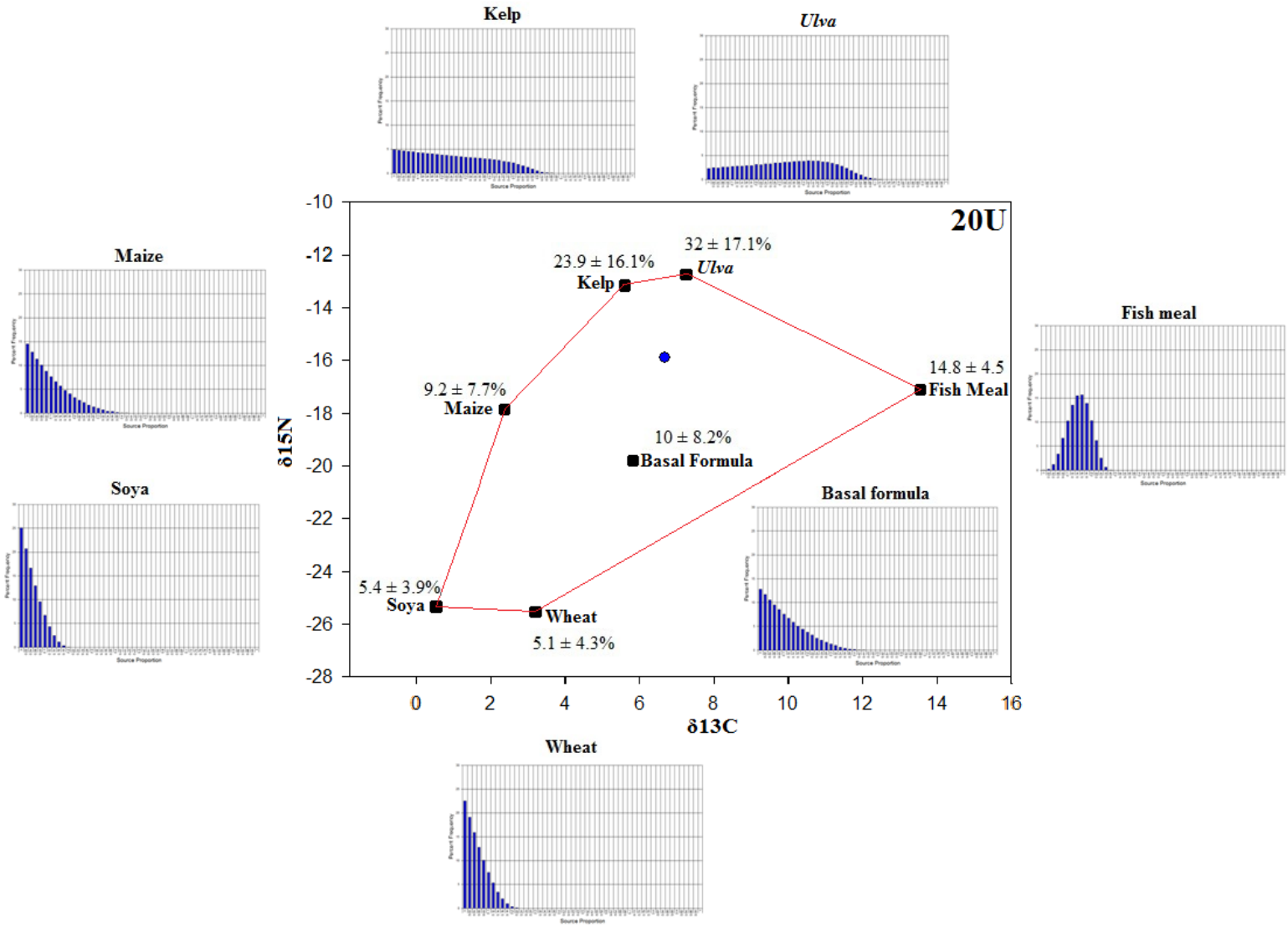


Figure 6.3: Mixing polygons delineating the δ13C and δ 15N signatures of up to eight dietary ingredients and the gonads of the sea urchin *T. gratilla* (after correcting for isotopic discrimination) fed four artificially formulated feeds (0U, 5U, 15U & 20U) containing varying amounts of *Ulva* (0, 5, 15 & 20%) for 20 weeks. Histograms (x axis = source proportion, y-axis = percentage frequency) show the distribution of feasible contributions from each source to the gonad while values represent estimated Means (± Std. Dev).

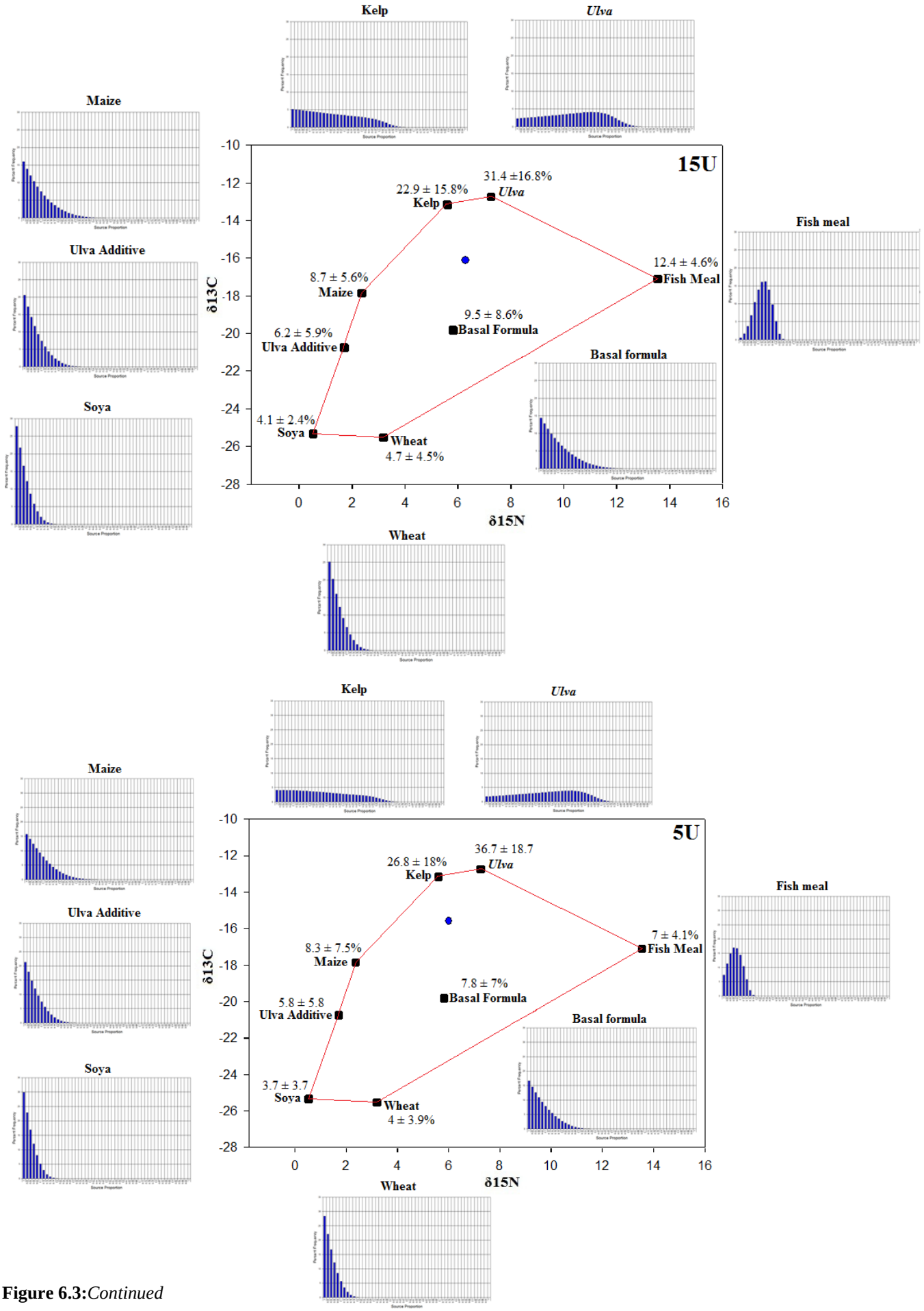


Figure 6.3:*Continued*

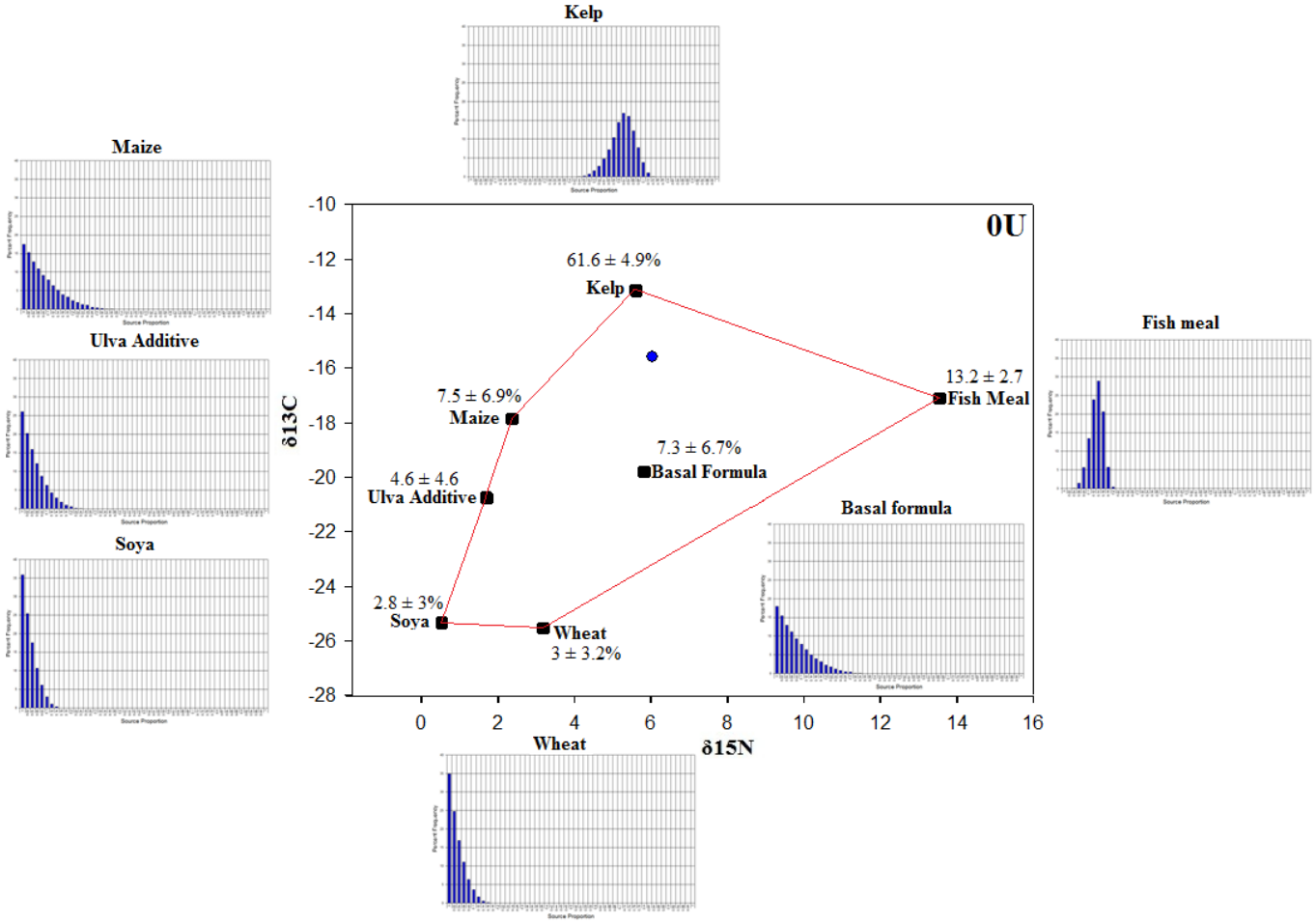


Figure 6.3:*Continued*

6.5 *Discussion*

Stable isotopes have been used previously to provide information about sea urchin diets over extended periods of time (Rodríguez, 2003; Yatsuya & Nakahara, 2004; Tomas *et al.*, 2006; Vanderklift *et al.*, 2006; Prado *et al.*, 2012), however, according to the literature, this is the first time that stable C^{13} and N^{15} isotopes have been used to determine the contribution of individual dietary ingredients to the growth of *T. gratilla* gonads. In addition to *Ulva* improving the palatability of formulated feeds and protein retention (Chapter 2), it is clearly demonstrated here that *Ulva* added to artificial feeds makes a significant contribution (>30%) to the final content of the gonad. Moreover, the incorporation of other proteins, particularly fishmeal, increased with the increasing dietary *Ulva* content of the diets, supporting the

findings of Chapter 2, which demonstrated that dietary *Ulva* inclusion improved protein retention. In this study, stable isotope analysis has been successfully used to estimate the importance of particular dietary ingredients in the production of sea urchin gonads, and this tool can provide useful information, which will help to improve future diet formulations.

The contrasting δ13C and δ15N values of *Ulva* and *Ulva* additive resulted in the experimental diets having different isotopic profiles, while protein and energy content remained similar. If dietary ingredients contributed to gonad development in the ratio at which they were supplied in the diet, mixing models would reflect the relative formulation of each diet. However, if the ingredients are not equally used one would expect to see the preferential uptake of particular ingredients and their incorporation into the gonad. Mixing polygons delineated by the dietary ingredients in this study were broad, with gonad values tending to fall near the top end of the figure (Fig. 6.1 & 6.3), indicating the importance of *Ulva* and kelp in the composition of these gonads. In general, the mixing models estimated that *Ulva* made a significant contribution towards the final tissue content of the gonad in the *Ulva* inclusion treatments (5U, 15U & 20U), at the end of the trial. In these diets, *Ulva* was estimated to account for an average of more than 30% of total gonad production, while it was only included at a maximum concentration of 20%. To my knowledge, this is the first study to demonstrate that *Ulva* is directly incorporated into the gonads of *T. gratilla* and is not just acting as a feeding stimulant (Chapter 2), or gonad colour enhancer (Chapter 3). In Chapter 4, it was shown that the addition of *Ulva* to a protein rich diet can significantly enhance the somatic growth of urchins, relative to non-*Ulva* supplemented feeds, thus resulting in more space for gonad development. In the 0U diet, which contained no *Ulva*, kelp appeared to make the most significant contribution towards nutrients used in gonad production, contributing an estimated average of 61.6% at the end of the trial. These findings tend to further support the notion that seaweeds (*Ulva* and kelp) play an important role in the production of the gonads, contrary to

the prevalent idea that they are a poor source of protein. The other three treatments also indicated that kelp contributed a large portion of nutrients to gonad development. Although it was not fed to urchins during the trial, they were maintained on it for two months prior to its commencement of this study and had accumulated gonad mass (8.17 ± 1.66g), exclusively composed from nutrients obtained from kelp. Contributions of kelp to final gonad production in this study should, in theory, be similar to the percentage contribution of initial gonad mass to the final gonad mass. This can be calculated as a percentage of the final weight and averaged 20% across the different treatments (data not shown). In the *Ulva* inclusion diets, kelp contributions do roughly follow the suggested trend, accounting for an estimated average of 23.9 ± 17.1, 22.9 ± 15.8 and 26.8 ± 18% for the 20U, 15U and 5U diets, respectively (Fig. 6.3), while in the 0U diet Kelp accounts for 61.6 ± 4.9% (a much larger portion). It is hypothesized that as *Ulva* and kelp are dietary ingredients that are regularly encountered by *T. gratilla* in nature, these ingredients could, therefore, potentially play an important role in tissue growth, compared to other terrestrial ingredients (soya, extruded maize, *Ulva* additive, wheat & basal formula) that are unknown to them. For example, seaweeds have generally been reported as having a low lipid content compared with terrestrial plants and vegetables such as Soy (Darcy-Vrillon, 1993), and are thus labelled as a low source of nutritional energy. However, the lipid fraction from seaweeds might contain higher levels of essential polyunsaturated fatty acids (Wahbeh, 1997; Van Ginneken *et al*., 2011; Tabarsa *et al*., 2012), compared with traditional vegetables (Oritz *et al.*, 2006), thus making them more suited and preferentially used and/or stored. *Ulva* additive did not appear to act as a suitable replacement for dried fresh *Ulva* in this study, as it was not utilized to the same extent for gonad production, even though it was formulated to have the same nutritional properties. Fish meal, a major protein source in all diets, was shown to contribute relatively consistently to the isotopic values of the gonads, averaging 12% among the diets, which is consistent with its inclusion (12.23%). Average fish meal inclusion appeared to increase in

urchins feed diets containing higher *Ulva* contents (20U diet), and may be linked to the increased protein digestibility in this feed (Cyrus *et al.,* 2014*;* Chapter 2). This hypothesis could also help to explain the significantly higher δ15N values of the 20U diet during week 12, prior to urchin spawning, which resulted in a loss of gametes and so a decrease in δ15N. Soya, another major protein source within all diets, also appeared to be incorporated more in the higher *Ulva* inclusion diets, but was shown to contribute less to gonad development than would be expected, averaging just 4% at the end of the trial, although it was included in the diets at significantly higher amounts (12%). Maize and wheat each account for almost 25% of all the dietary ingredients used to formulate the feeds, but their relative contribution to gonad production only averaged 8.4 and 4.2%, respectively, among the diets.

Together, both macroalgae (*Ulva* and kelp) contributed, on average more than 50% to gonad production in this study. It is hypothesized that macroalgae may contain important proteins, amino acids, fatty acids, pigments and other compounds that are assimilated in high concentrations during the production of the gonads. Amino acid analysis of both Kelp and *Ulva* indicates that they are high in alanine (data not shown). Alanine is one of the simplest non-essential amino acids, with respect to molecular structure, and so is one of the most widely used in protein construction. It is thus possible that concentrations of amino acids from *Ulva* were more readily available and so used directly for the synthesis of proteins in the gonads. Recently, algae and, in particular *Ulva*, has received increased attention as a possible protein replacement in feeds for many cultured species including finfish (Wassef *et al*., 2001), shrimp (Cruz-Suárez *et al.*, 2010), abalone (Naidoo *et al.*, 2006) and goats (Ventura & Castañon, 1998). Gannes *et al*., (1998) stated that the composition of organisms fed a high protein diet often more closely resembles the isotopic values of the dietary protein sources, compared to the bulk of dietary ingredients. According to this statement, *Ulva* appears to account for a large portion of the protein assimilated into the gonads of urchins fed the *Ulva*

inclusion diets. In the 0U diet, however, gonads have a greater dependence on protein assimilated from kelp. Fisler *et al*. (1982) state that in diets which are protein-deficient, amino-groups are recycled from degraded tissue protein and new amino acids are synthesized, using the carbon derived from dietary carbohydrates and lipids. It should, however, be pointed out that, in almost all diets, the entire Fish Meal portion appeared to be utilized in gonad development, and so it could not be determined whether *Ulva* would be a suitable protein replacement for this ingredient. The inclusion of *Ulva* into formulated feeds positively increases gonad coloration through the storage of important carotenoid pigments, particularly echinenone (which is synthesised from β-carotene) (Cyrus *et al.*, 2014). Gonad colour is a rough indication of carotenoid content, and substandard gonad colour usually results from carotenoid levels either being too low, resulting in unattractive pale gonads, or too high, leading to a dark gonad discolouration (Hagen *et al.*, 2008). In this study, the 20% *Ulva* inclusion (20U) diet had significantly better coloured gonads than the other treatments used (Cyrus *et al.*, 2014; Chapter 3), indicating that pigments and/or their derivatives (e.g. echinenone) from *Ulva* are being incorporated into the gonad at higher concentrations, as its relative inclusion increases.

Previous studies conducted on aquatic species (Lu *et al*., 2006; Johnston *et al*., 2007) have indicated that ingestion, rather than assimilation, of nutrients determines the acceptability of new feed ingredients within formulated feeds. However, in the current study, assimilation of ingested food ingredients does not appear equal and the determination of the precise inclusions of different sources varies greatly. It is, however, important to remember that all solutions identified by IsoSource are consistent with isotopic mass balance; therefore there is no reason to prefer one solution over another on a strictly isotopic basis. However, factors such as availability, palatability, and percentage inclusion will vary greatly among dietary ingredients and these differences will determine the unique solution of what is assimilated

(Benstead *et al.,* 2006). Mean contribution estimates of all source contributions should therefore be treated extremely cautiously. IsoSource provides information about the distribution and range of the possible source contributions when no unique solution exists, because there are too many source contributors (Phillips & Gregg, 2003). By including additional constraints to IsoSource analysis, for example, the protein and energy requirements of the studied organism (Minagawa, 1992) and/or the exact discrimination factors of individual ingredients, it may be possible to confirm or rule out certain ingredients as major contributors, due to infeasible dietary combinations.

In conclusion, the relative assimilation estimates of dietary ingredients from different sources in this study may enable more accurate assessment of the cost-effectiveness of formulated diets for use in echinoculture and particularly in culturing *T. gratilla*. *Ulva* appears to be beneficial as an additive in formulated feeds for the sea urchin *T. gratilla,* particularly by contributing to gonad development. The use of stable isotope analysis is a simple and safe method for estimating the contributions of important dietary ingredients to an organism's development. With the resolution of such studies likely to be enhanced in the future, through the uses of compound specific stable isotope analysis (CSIA), which allows measurement of isotope ratios from individual compounds (Krummen *et al.*, 2004, Sessions, 2006), this method will likely be an important tool in developing cost-effective feeds and identifying important dietary ingredients in the future.

Chapter 7:

Final Discussion

In this study, the inclusion of *Ulva* at a level of 20% weight/weight to an artificially formulated feed was shown to significantly improve the feed's attractant properties and palatability. In turn, increased consumption and protein digestibility were observed when compared to urchins fed diets that were not supplemented with *Ulva*. All artificial diets were shown to significantly increase gonad yield in *T. gratilla,* when compared with urchins fed a diet of fresh *Ulva*, supporting previous studies that have demonstrated the effectiveness of high-protein artificial feeds for enhancing growth and development of urchin gonads. Gonad quality, particularly gonad colour, of adult wild-caught urchins was also improved significantly over a 12 week period for urchins fed the higher *Ulva* inclusion (15% & 20% dried *Ulva*) diets. As outlined in the Introduction, gonad colour is largely determined by the presence of pigments within an urchin's diet. Beta-carotene is known to occur in relatively high amounts (25 - 45 $\mu g.g^{-1}$ fresh weight) (Bischof *et al*., 2002) within *Ulva* and has been shown to improve gonad colour (Pearce *et al*., 2002a; Robinson *et al*., 2002; McBride *et al*., 2004; Shpigel *et al*., 2005). However, its exact concentrations within fresh *Ulva* (FU) and the *Ulva*-supplemented feeds are unknown and future studies should focus on determining the optimal concentration of β-carotene needed to produce an A-grade coloured product. Furthermore, the total carotenoid content of *Ulva* can account for between 5 - 12.5% of its dry weight (Shuuluka, 2011) and β-carotene only makes up part of this. Other carotenoid pigments such as zeaxanthin, astaxanthin, etc. are also present and their specific effects on gonad colouration are not well known and should also be investigated. It may also be beneficial to investigate the genetic selection of an *Ulva* spp. with a high β-carotene contents.

Unlike gonad enhancement, full-life cycle grow-out of urchins comprises two phases, the optimization of somatic growth followed by conditioning of the gonad as the final product (Seymour *et al*., 2013). Using combinations of fresh *Ulva* (FU) and artificial feeds supplemented with (20U) or without *Ulva* (0U), it was shown that both the FU and 20U diets significantly improved somatic growth of juvenile urchins, compared with urchins fed the non-*Ulva* supplemented feed (0U). In support of the previous findings, the artificial diets with or without *Ulva*-supplementation produced the largest gonads, whereas the gonads of urchins fed fresh *Ulva* were significantly smaller. However, when the latter animals were conditioned for a further 6 weeks on an artificial diet (20U or 0U), their gonads attained a similar size as urchins continually fed the 20U diet. In addition to promoting somatic growth and producing large gonads that are bright yellow to orange in colour, it is important to produce marketable gonads, which need to have a firm texture and ideally are in the recovery and/or growing phase. Findings demonstrated that diet alone cannot be used to manipulate the gametogenic cycle, whereas specific diets in combination with a suitable daylength could be used to produce firm gonads that are predominantly in the preffered phases. More specifically, it was demonstrated that Long days (16:8) inhibited and/or Short days (8:16) promoted the progression of gametogenesis in *T. gratilla*. These findings will be particularly useful in a commercial application, as cultured urchins can be maintained in a desirable reproductive state throughout the grow-out period. Further investigations into the length of time for which gametogenesis can be suspended using this technique still need to be undertaken, however, as this may not be indefinite.

To address the final aim of this study, stable C13 and N15 isotope analysis was used, together with mixing and mass balance models to determine the relative contribution of each dietary source/ingredient to the gonadal tissue of urchins in each dietary treatment. *Ulva* was shown to not only improve the chemosensory properties of an artificial feed and improve

consumption, somatic growth and gonad colour, but it was also incorporated into gonadal tissue. Isotopic analysis also provided further support to the observations that *Ulva* facilitates protein retention from diets, with the gonads of urchins fed diets containing more *Ulva* incorporating higher amounts of protein from each dietary source.

Overall, this study has clearly demonstrated the benefits that dietary *Ulva* supplementation could have for the development of *T. gratilla* aquaculture in South Africa. Not only is *Ulva* a preferred seaweed for this species, it also significantly improved the chemosensory properties of the high protein artificial diet that was specifically developed for this study. These findings support a growing body of literature reporting both health and growth benefits of dietary *Ulva* supplementation (Wassef *et al.*, 2001; Michalak & Chojnacka, 2009; Cruz-Suárez *et al.*, 2010; Wang *et al.*, 2010; Wijesekara *et al.*, 2011). Since *Ulva* is the top aquaculture product by weight in South Africa, with more than 2,500 tons produced in 2011 (DAFF, 2012), these findings are of particular interest to the local aquaculture production and/or feed sector. However, even though it was clear that *Ulva* acted as an attractant in this study, it is not clear what seaweed constituents are responsible for the observed response. Preliminary experiments have indicated that an ethonoic extraction of *Ulva* acts as an attractant for *T. gratilla*, however, the compounds within this extract that are responsible for this response are unknown. Further studies should be conducted to determine which compounds are responsible for the observed effects, as their identification may have significant benefits for aquaculture feed development.

Although fresh *Ulva*, or the inclusion of dried *Ulva* into an artificial feed at an optimal level of 20% (weight/weight), significantly enhanced somatic growth of *T. gratilla* and improved gonad colour (resulting in bright yellow to orange gonads), a high protein content in the artificial diets was required to promote the production of large gonads in these animals. The

results suggest that different diets should be utilized for the different growth phases of an urchin, namely urchin somatic growth and the conditioning of the gonads. Either fresh *Ulva*, or the *Ulva* supplemented feed (20U diet), can be utilized to promote rapid somatic growth, particularly during the early juvenile stages. However, since fresh *Ulva* is being grown successfully in large quantities in South Africa, it is recommended that fresh *Ulva* be used as an initial feed. Artificial feeds supplemented with *Ulva* can then be used to promote rapid gonad development, improving gonad size and quality, prior to harvest.

These findings could have a significant effect on the potential costs involved for the commercial production of *T. gratilla* in the future, as feed costs account for a large proportion of production costs in aquaculture operations. Consideration of the production cost of seaweeds is important too, as these can vary hugely depending on the species chosen and the cultivation and/or harvesting system used (Kirkendale *et al.,* 2010). *Ulva* has long been identified as a potential culture species in aquaculture, because some species grow naturally unattached in high nitrogen environments (eg: waste water) and these species show a high capacity to take up nutrients (Neori *et al.,* 1991; Gil *et al.,* 2005), which makes them particularly well-suited to integrated aquaculture operations (Bolton *et al.,* 2009). Biofiltration by algae is assimilative, as solar energy and excess nutrients (particularly C, N and P), are converted, through photosynthesis, into new biomass (Krom, 1986), which can then be harvested as a secondary product (e.g. feed). Algae, and in particular seaweeds, have been identified as the most suitable for biofiltration, because they are said to have the highest productivity of all plants and have the potential to be economically valuable (Gao & McKinley, 1994).

Vandermeulen & Gordin (1990) showed that *Ulva* could efficiently remove up to 85% of the ammonium from fish pond wastewater. Similarly, Cohen & Neori (1991) indicated that *Ulva* (10 m^2 pond) used as a biofilter could remove over 90% of the ammonia within effluent water

produced by 1 kg of daily feed or approximately 75 kg of fish. Sea urchins are ammonotelic in their excretion of nitrogenous waste (Prosser & Brown, 1961), meaning they excrete largely ammonia, making them particularly well-suited to integrated aquaculture operations using *Ulva*, although this area still needs further investigation. Koike *et al.* (1987) found that *T. gratilla* faeces contained large amounts of ammonium (1.7-5.4 mg N individual/day), which enriched the surrounding water column. These findings were supported by Dy & Yap (2000), who reported that *T. gratilla* in the Philippines excretes ammonium at a rate of 1447 ± 310 $nmol.g^{-1}$ $DW.h^{-1}$. In South Africa, two abalone farms (Wild Coast Abalone and Irvine and Johnson (I & J) and Cape Cultured Abalone Pty Ltd.) currently use integrated Abalone/*Ulva* systems (Robertson-Andersson, 2003, 2007; Bolton, 2006a, b; Robertson-Andersson *et al*., 2008; Bolton *et al.*, 2009; DAFF, 2012) and so it would, therefore, appear feasible, as well as beneficial, to do the same on sea urchin farms in the future. According to Folke & Kautsky (1992), a successful sustainable integrated farming system should, as much as possible, mimic the way in which a natural ecosystem behaves. Integrated systems allow for multiple species to be kept in the same culture systems, recreating a "mini-ecosystem" where, if properly balanced, algal autotrophy can counter act organismal (fish, shrimp, sea urchin etc.) and microbial heterotrophy, not only in respect to nutrients, but also with respect to oxygen, pH and CO_2 (Hirata et al., 1994; Chopin *et al.*, 2001; Neori *et al*., 2004). In this way, integrated aquaculture can provide nutrient biofiltration, bioremediation and economic diversification through the production of other marine crops, as well as mutual benefits to the co-cultured organisms. In turn, these benefits all increase the per cultivation unit profitability of the aquaculture operation. Integrated systems are also an extremely efficient use of space, highlighted as one of the largest limiting factors faced by aquaculture of the future (Barrington *et al*., 2009).

Nobre *et al*. (2010) showed that by using an integrated multi-trophic aquaculture system comprised of Abalone and *Ulva,* farm profits could be raised by 1.4 to 5%. They also showed that the overall economic gain was, in fact, several fold higher than the net gain in profit, through a reduction in: (1) nitrogen discharges into the adjacent coastal ecosystem, (2) harvested natural kelp and (3) GHG emissions, and it was estimated between US $1.1- 3.0 million per annum. A recent study by Siskey & Baldwin (2011) provided experimental proof that an integrated aquaculture system comprising of fin fish (*Tautogolabrus adspersus*), sea urchins (*Strongylocentrotus droebachiensis*) and macroalgae (*Ulva lactuca*) could yield higher production than if the organisms were grown separately. It has also recently been suggested that the recycling of waste nutrients through biofiltration by algae is likely the most economical way to improve world mariculture sustainability (Cuomo *et al*., 1997; Blancheton, 2000). The use of *Ulva* in integrated aquaculture systems not only reduces the levels of ammonia and harmful bacteria (Flodin, 2005) within the effluent, but also, it reduces electrical costs due to the partial re-circulation of treated water and the light-absorbing properties of the seaweed, which increase the culture water temperature, while producing a supplementary feed as a by-product. Growing *Ulva* using waste effluent as a fertilizer has also been shown to improve its nutritional attributes. For example, in South African systems, it has been shown to be possible to increase *Ulva's* protein content to over 30% of its dry weight (Robertson-Andersson 2003; 2007). Shuuluka (2011) also showed that the total carotenoid content of effluent grown *Ulva* (7.3 ± 0.2% DW) was significantly higher than that of natural populations (4.8 ± 0.1% DW). These findings could also lead to the selective breeding of favourable traits (e.g. high carotenoid content), allowing for the improvement of *Ulva* as a feed.

Ulva species are known to have useful vitamin and mineral profiles as feed, and are reported to be particularly rich in β-carotene (Bischof *et al.,* 2002) and vitamin C/ascorbic acid (Ortiz

et al., 2006; Garcı´a-Casal *et al*., 2007). It is suggested that catotenoid pigments (such as β-carotene) may be partly responsible for the increased growth, as they are precursors of Vitamin A (Von Lintig & Vogt, 2000). Retinoic acid (Vitamin A in its irreversibly oxidized form) has been shown to be a hormone-like growth factor responsible for modulating cell survival, cellular proliferation, differentiation, regionalization, and organogenesis in the developing embryo in both vertebrates and non-vertebrates (Blomhoff & Blomhoff, 2006; Glover *et al*., 2006; Mark *et al*., 2006). Recent analyses of genomic data suggests that molecular components of the retinoic acid signalling cascade are present in invertebrate groups, such as sea urchins and hemichordates (Campo-Paysaa *et al.,* 2008). Additionally, retinoids have been implicated in the fundamental functioning of gene transcription and, thus, are considered essential for normal cell division and differentiation (Wolf, 1984; Blomhoff *et al*., 1991). The addition of ascorbic acid (Vitamin C) to diets has been shown to increase weight gain and organ production in juveniles, as well as produce adult urchins with firmer tests and an increased organic content (Jones, 2011). It is hypothesized that the addition of *Ulva* to artificial diets in this study may have acted as a significant source of the β-carotene (vitamin A) and ascorbic acid (vitamin C), which resulted in better growth. Further research into the effects of Vitamin A and C on *T. gratilla* somatic growth is suggested to confirm this hypothesis.

Daylength was shown to have a significant effect on the development of gonads, although it should be noted that this study could not determine whether it was Long days (16:8h) that inhibit or Short days (8:16h) that promote gametogenesis and so further research is recommended, perhaps making use of a short light break during the dark period to determine whether it is Short days or Long nights that promote gametogenesis.

In conclusion, the research conducted in this thesis has clearly shown that there are a number of benefits to the addition of *Ulva* in artificial feeds, and that fresh *Ulva* produces comparable somatic growth to these diets. This has led to the recommendation of a cost-effective feeding regime, where fresh *Ulva* is used to promote somatic growth until urchins are of a marketable size, whereafter they are feed a high protein artificial feed containing *Ulva* (20U), to increase gonad size and condition prior to harvesting. With the use of this strategy a harvestable product can be produced from juvenile urchins (± 20 mm test diameter) in just 32 weeks, and with the implementation of a Long day photoperiod, gametogenesis can be delayed. These findings could be of major benefit to the echinoculture industry worldwide, but particularly in South Africa, where *T. gratilla* is under investigation as a potential new species for aquaculture, and where *Ulva* is grown in large amounts on a number of abalone farms as feed, and in integrated systems for nutrient removal.

Chapter 8:

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