MATTHEW ROBERT MAINIERI

NOVEL MICRODIETS FOR THE EARLY DEVELOPMENTAL STAGES OF PURPLE SEA URCHIN (*PARACENTROTUS LIVIDUS*)

UNIVERSIDADE DO ALGARVE
Faculdade de Ciências e Tecnologia
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Declaração de autoria de trabalho

Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Matthew Robert Mainieri

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Resumo

Paralela ao constante crescimento da população mundial, é a crescente pressão exercida nos setores de produção de alimentos à escala global. A indústria alimentar deve responder a este problema com um esforço combinado das indústrias agrícola e pecuária, por exemplo. O papel da aquacultura neste empreendimento é da maior importância uma vez que o interesse em espécies novas e emergentes pode melhorar o mercado e diversificar o leque de espécies que está disponível aos consumidores. Uma espécie em particular pertence à classe Echinoidea e conhece-se como ouriço-do-mar. As civilizações na história do mundo têm utilizado as suas gónadas como um alimento *gourmet*. Relatórios recentes têm mostrado que as populações de ouriço-do-mar estão esgotadas devido a sobrepesca e exploração. Apesar da dizimação da população de ouriços, existem esforços para restabelecer estas populações com a aquacultura.

As primeiras tentativas de produção de ouriços-do-mar em aquacultura, focaram-se maioritariamente nos primeiros estádios de desenvolvimento, até ao estádio de juvenil. Os ouriços deste estádio foram libertados para aumentar a população selvagem. Estudos recentes mostram que há uma procura para ouriços no mercado de frutos do mar e que o seu valor comercial por quilograma é relativamente alto e estável. O interesse na aquacultura de todo o ciclo de vida deste organismo tem vindo a crescer e a necessidade de um protocolo bem definido para o seu cultivo, é cada vez mais evidente. Tradicionalmente, as dietas larvares são constituídas por uma mistura de microalgas. A produção de microalgas pode ser bastante dispendiosa e exaustiva. Depois de muito tempo, o foco foi mudado, dando prioridade ao uso de dietas inertes em larvicultura, eliminando a necessidade de cultivar microalgas. Até à data, as tentativas de formalizar e estabelecer protocolos de produção viáveis das dietas inertes são escassas. A testagem das várias dietas e a comparação destas a dietas com um base de microalgas para provar a sua eficiência é a meta da nutrição de larvicultura de ouriços-do-mar. Uma substituição de dietas de microalgas pelas dietas inertes será um importante avanço neste campo da aquacultura.

O objetivo principal deste trabalho pode ser divido em dois focos: primeiro, a formulação de uma microdieta inerte para as larvas de *Paracentrotus lividus* em aquacultura, e segundo, a promoção do crescimento e sobrevivência durante os estádios larvares. Muitas dificuldades existem para os produtores de ouriços-do-mar. O reduzido crescimento e baixas taxas de sobrevivência são problemas comuns nos estádios iniciais

de vida deste organismo. Se uma dieta inerte bem equilibrada e com boa aceitação promover o crescimento e melhorar a sobrevivência, então o principal objetivo deste trabalho será cumprido. O ensaio desenvolvido no âmbito desta tese foi realizado na estação de investigação do Ramalhete em Faro, Portugal. Os reprodutores foram injetados com 1 mL de cloreto de potássio (KCl (0,5M)) para induzir a libertação de gâmetas. Os ovos fertilizados foram distribuídos entre nove tanques de 100 L e foram observados para determinar a taxa de eclosão. Três tratamentos foram usados (A, B e C), cada um correspondendo a uma dieta: uma mistura de microalgas congeladas (A: CTRL), uma dieta inerte à base de algas (B: ALGAE), e uma dieta inerte de origem marinha (C: MARINE). A alimentação foi completada uma vez ao dia, duma maneira cuidadosa. Os parâmetros da água (i.e., temperatura, oxigénio, salinidade, amónia, etc.) foram medidos e registados diariamente e anomalias relacionadas com a qualidade da água foram resolvidas de imediato. As amostragens ocorreram no dia de eclosão e nos dias 4, 8, 12, 16, e 21 depois da mesma. No final do estádio larvar, a metamorfose ocorreu em alguns tratamentos e os dados foram recolhidos para calcular as taxas de competência e assentamento. Foram feitas observações 21 dias depois da eclosão por 96 horas adicionais para calcular a taxa de assentamento das pós-larvas.

Os parâmetros medidos no ensaio para determinar a eficácia das dietas inertes foram comparados aos parâmetros medidos da mistura de microalgas, utilizada como dieta controlo. O *comprimento total (TL)* foi medido no momento da eclosão e a cada amostragem até ao 16º dia depois da eclosão. A largura *(W)*, o comprimento do estômago *(ST)*, a largura do estômago *(SW)*, o número de braços *(AN)*, e o comprimento do braço pós-oral *(POAL)* foram medidos durante a amostragem das larvas ao dia 4 após a eclosão até ao dia 16. O comprimento do rudimento *(RL)* e a largura do mesmo *(RW)* foram medidos apenas ao 21º dia após a eclosão. Medições das proporções corporais foram consideradas para determinar a eficiência de cada dieta. Outros parâmetros tais como as taxas de sobrevivência *(SR)*, competência *(CR)*, e assentamento *(STR)* foram determinados para cada tratamento de modo a determinar qual a que promoveu um melhor desenvolvimento larvar e metamorfose.

As dietas inertes promoveram um maior crescimento e desenvolvimento das larvas, comparativamente à dieta controlo. As dietas não tiveram um impacto significativo em termos da taxa de sobrevivência em cada tratamento. Os resultados dos tratamentos usando dietas inertes foram semelhantes durante o ensaio, porém, a dieta inerte formulada

com ingredientes marinhos possibilitou uma taxa mais elevada de competência e de assentamento. Os resultados mostram que dietas inertes podem substituir as dietas tradicionalmente feitas com uma mistura de microalgas frequentemente utilizadas em aquacultura de ouriços-do-mar. O uso de dietas inertes pode também reduzir os custos altos de produção de microalgas e reduzir a necessidade de trabalho manual para o seu cultivo.

Da mesma forma, estudos semelhantes concluem que dietas inertes usadas nos estádios larvares do ouriço-do-mar podem ser uma boa alternativa para produzir larvas com bom crescimento, promovendo também a sua metamorfose e assentamento. Estas conclusões dão promessa à possibilidade de produzir ouriços-do-mar em cada estádio de vida e, por sua vez, aliviar as pressões de sobre-exploração das populações selvagens e também fornecer um produto comercializável ao mercado alimentar. O campo de nutrição de larvas de equinóides é pouco estudado e incerto, porém, os resultados deste ensaio e de outros análogos podem ser uma guia para formular dietas nutricionalmente adequadas. Porém, são necessários mais estudos no campo de nutrição de larvas de ouriço-do-mar que assim como esta tese, ajudem a colmatar a lacuna de conhecimento que ainda existe nesta área.

Palavras-chave: aquacultura, larvicultura, microdietas, nutrição, ouriço do mar

Abstract

Locating an alternative inert diet to a microalgae-based feeding regime in sea urchin larvae rearing is an area of research that is gaining interest. A trial was carried out to test two inert diets during the larval stages of production. Rearing tanks (100 L) were filled with static, filtered seawater with 25-50% daily water renewal. Fertilized eggs were added to each tank and observed for hatching. After hatch, the larval diets fed consisted of ingredients of algal origins (Treatment B) (55.55% protein and 11.65% lipid content) while the other inert diet consisted of marine based ingredients (Treatment C) (56.86% protein and 12.75% lipid content). Both inert diets were compared to the control microalgal diet which consisted of a blend of *Skeletonema sp.*, *Isochrysis galbana*, and *Nannochloropsis sp.* (Treatment A).

Various biometric parameters were measured throughout the trial to determine the efficacy of the inert diets. The larvae from the inert diet treatments were larger and more developed than the larvae from the microalgae blend treatment. However, at the final sampling 16DAH, larvae fed the marine based inert diet (Treatment C) showed more robust growth in length (~ 623.87 µm), width (~ 214.83 µm), stomach area, and rudiment size among other measurements. Additional results from the inert diet treatments were fairly comparable throughout the trial, however, the inert diet with marine based ingredients (Treatment C) produced larvae with a higher competency rate (~ 4.58%) which in turn promoted the highest settlement rate (~ 27.38%) of all treatments. The diets had no major impact on the survival of the larvae from each treatment. The results show that inert diets can replace the traditional microalgal diets that are commonly used in sea urchin aquaculture which in turn can reduce high production costs and manual labor of microalgae cultivation.

Keywords: aquaculture, larviculture, microdiets, nutrition, purple sea urchin

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List of Abbreviations

AN	Arm Number
BA	Body Area
CR	Competency Rate
DAH	Days After Hatching
DM	Dry Matter
FI	Feeding Incidence
FR	Fertilization Rate
GI	Gonad Index
GSI	Gonadosomatic Index
HR	Hatching Rate
POAL	Post Oral Arm Length
RL	Rudiment Length
RW	Rudiment Width
SA	Stomach Area
SD	Standard Deviation
SR	Survival Rate
ST	Stomach Length
STR	Settlement Rate
SW	Stomach Width
TL	Total Length
USD	United States Dollar
W	Width

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1. Introduction

1.1 *Paracentrotus lividus* (Purple Sea Urchin)

Echinoidea have fossil records dating to at least the Permian era of about 300 million years ago. Sea urchins are an echinoderm species and currently there are upwards of 1,000 known species (Mongiardino Koch, 2018). *Paracentrotus lividus*, (Lamarck, 1816) pertaining to the Parechinidae family, are one such species with growing economic and conservation interests. *P. lividus* appears as a spherical ball and is covered in appendages known as spines which extend from the hard exoskeleton called the test as seen in Figure 1.1. Along with the spines, the test also contains the podia which with the spines aid in locomotion, entrapment of prey, and protection from predators (McBride, 2005).

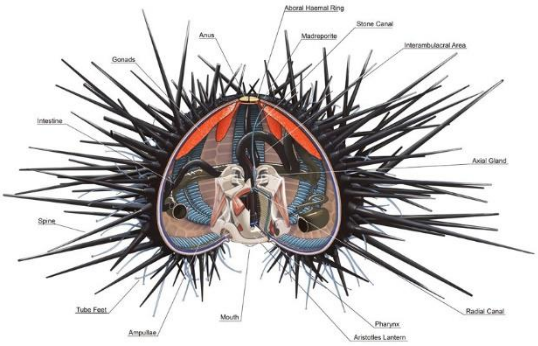


Figure 1.1. Sea urchin anatomy (Ries, 2011).

The organism is primitive in nature and has no specialized circulatory or respiratory system. Urchins have been known to be able to survive for long periods of time without feed and can lower their metabolism rate in harsh environmental conditions or during periods of feed scarcity (James and Siikavuopio, 2012).

Known as a sentinel species, urchins can indicate potential harmful fluctuations in water quality before affecting other cohabitating species. Therefore, interest in scientific research pertaining to urchins and other echinoderms has been gaining attention to further study the state of marine habitats and the importance of their role in ecosystems around the globe (McBride, 2005). Sea urchins are benthic and play a major role in the health and stability of kelp forests and coral reefs (Harrold, 1987; Hughes, 1994; Edmunds, 2001). They can be found in intertidal zones and up to about 20 m in depth. *P. lividus* may be found in many parts of the world such as from the Northeast Atlantic to Scotland, to Southern Morocco and the Mediterranean basin

(Boudouresque and Verlaque, 2013). Sea urchins are opportunistic feeders and in and around the Mediterranean, this species can be found mostly in rocky habitats and/or in seagrass beds among *Posidonia oceanica*. In the rocky locations, *P. lividus* feeds mainly on algae accumulations and in *P. oceanica* beds it feeds mainly on epiphytes and on the entire leaf (Benedetti-Cecchi *et al*., 1998; Hereu, 2006; Nédelec and Verlaque, 1984; Shepherd, 1987; Tomas *et al*., 2005, 2006). Many studies claim *P. lividus* becomes more active at night to avoid diurnal predators (Dance, 1987; Hereu, 2005). When this species looks for a suitable habitat, they search for two main factors: adequate shelter for protection from predators and a reliable source of feed.

The reproduction cycle of *P. lividus* consists of four stages (I-IV) (Walker *et al*., 2007) with a spawning event 1-2 times per year depending on habitat and geographic location. Generally, a 1:1 male to female ratio is believed to exist in the wild. Most spawning events occur while the GI (gonad index) is relatively high (~20% of body weight and at stage IV) and takes place during the late winter to early spring. The entire sea urchin life cycle can be seen in Figure 1.2.

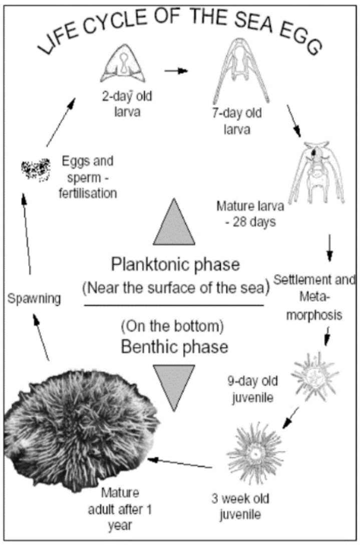


Figure 1.2. Sea urchin life cycle (Mahon and Parker, 1999)

Fertilization occurs in the water column when males release the sperm and females release the ova. Photoperiod is thought to influence gonad maturation while temperature and environmental factors are thought to influence the spawning events (Spirlet *et al*., 2000). Due to *P. lividus'*s primitive digestive system, the gonads store nutrients obtained from prey and utilize them for reproduction and metabolism purposes (James and Siikavuopio, 2012).

1.2 Sea Urchin Larval Biology

The larval stages are the most delicate phases in the life of a sea urchin and most mortalities occur during these stages. Before the larval stages, the gametes are fertilized and successful fertilization creates a fertilization membrane surrounding the newly formed embryo. Fertilized embryos undergo many series of cellular divisions and morphological changes as seen in Figure 1.3. Sea urchin embryos undergo radial holoblastic cleavage which in turn divides the embryo into animal and vegetal tiers. The animal tier contains mesomeres and equal cellular divisions are carried out (Gilbert, 2000).

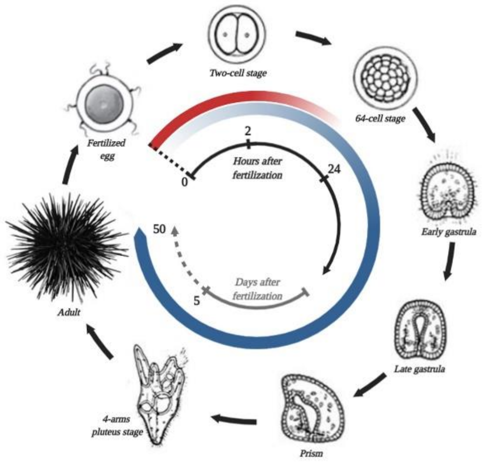


Figure 1.3. Sea urchin cellular stages until adulthood (Adonin *et al*., 2021).

The vegetal tier contains macromeres and micromeres in which unequal cellular divisions occur. Each tier will play a major role in differentiation later in the developmental process. After seven cellular divisions, the embryo enters the blastula stage and contains 128 cells (Gilbert, 2000).

The blastula stage contains cells of uniform size and a hollow sphere known as a blastocoele develops. Once 9-10 divisions occur, new genes are expressed and the blastula becomes ciliated (Masuda and Sato, 1984). The cells in the animal tiers produce an enzyme that digests the fertilization membrane and in turn becomes a free-swimming hatched blastula (Lepage *et al.*, 1992). Following the blastula stage, the gastrula stage is initiated. During the early gastrula stage, the vegetal side of the zygote begins to thicken and flatten whilst primary mesenchyme cells begin to form. These cells will ultimately form the larval skeleton and are referred to as skeletogenic mesenchyme cells. The larval skeleton and spicules, or legs, are composed primarily of calcium carbonate ($CaCO_3$) (Wilt, 1999). Furthermore, the internal cells in each of the animal and vegetal tiers begin to differentiate and become specialized. On the ventral side of the gastrula, a blastopore is formed and begins to invaginate. During the late stages of gastrulation, the blastopore continues to invaginate creating the archenteron and the short gut rudiment begins to form which appears as a long, slender tube. As the gastrula goes through this metamorphic change by the aid of secondary mesenchyme cells and filopodia, the gastrula becomes a prism-stage larva. After further cellular differentiation and morphological changes, the prism-stage larva finally becomes a larvae pluteus with a mouth, continuous digestive tract, and an anus (Gilbert, 2000).

Many studies categorize the larval phases in seven stages which, as opposed to other species, the stages are more determined by morphological features rather than age (Davidson *et al.*, 1995; Arenas-Menas *et al.*, 1998, 2000). The larval stages occur once the larva enters the pluteus phase.

It should be noted that development within the same fertilization cohort can vary drastically and are by no means synchronous (Smith *et al.*, 2008). The first phase following the pluteus phase is known as the four-arm stage and the first feeding occurs during this stage as seen in Figure 1.4 The arms begin to grow and extend to a length of 1/3-1/4 of the total body length. Furthermore, there are two coeloms, a right and a left, which begin at the mouth area and start to extend towards the scheitel, or the posterior areas of the body rods (Smith *et al.*, 2008).

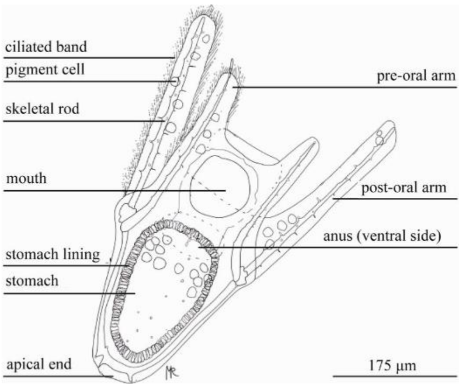


Figure 1.4. Sea urchin larval anatomy (Rock *et al*., 2011).

Following the four-arm phase is the eight-arm phase which involves further development. In *P. lividus*, larvae can sometimes exhibit six arms before the final pair of arms begin to grow. During the eight-arm stage the intestines begin to elongate and the esophagus begins to extend in size. The left coelom transforms into the hydrocoele which eventually becomes the rudiment which is crucial for competency to metamorphosize in a later stage. The next phase can be considered as vestibular invagination which in this stage the larvae begin to change in shape.

The epaulettes begin to form during this phase while the stomach begins to enlarge. The larvae begin to widen and become rounder in shape while the rudiment growth further progresses (Smith *et al*., 2008).

The following phase can be referred to as the rudiment initiation stage which prepares the larvae for a change from being pelagic in nature to a benthic state. The preoral arms begin to elongate and the vestibule and coelom begin to converge and fully form the rudiment (Smith *et al.*, 2008). The pentagonal disc phase follows which includes the first signs of metamorphosis. The tube-foot primordia begin to grow in the forming rudiment which will be the apparatus used in settlement in the stages to follow. The epaulettes grow to their maximum size during this phase and are easily visible. Furthermore, the initial formation of the dental sacs and spines occurs during this phase. The advanced rudiment phase follows and includes the further growth and development of the dental sacs and spines. The adult plate begins to form and structures form at the distal ends of the tube feet which will attach to a substrate during settlement. The final phase pertains to the actual protrusion of the tube feet from the rudiment. Many studies agree competency occurs around 18 days after hatching (DAH) and determined when the rudiment is as large or larger than the stomach and when spines and tube feet are clearly visible in the rudiment (Kelly *et al*., 2000; Kelly *et al*., 2015). The larval arms begin to decrease in size and the rudiment occupies most of the interior space. At this stage the larvae are ready to settle and become benthic and the larval phase is concluded (Smith *et al.*, 2008). The aforementioned phases can be seen in Figure 1.5.

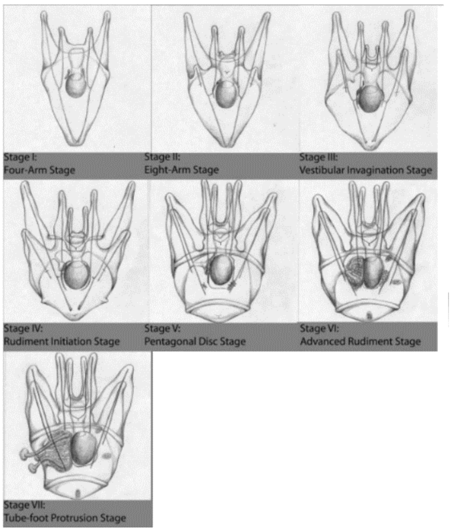


Figure 1.5. Sea urchin larval stages with rudiment formation and protrusion of tube feet (Smith *et al*., 2008).

The length of the entire process varies greatly between species and the environment in which a species inhabits can greatly influence the duration of the process. In *P. lividus* this entire process on average takes between 17 and 26 days to complete (Pinna, 2014). Proper nutrition can play a major role in the duration and success of these major morphological changes and may determine the survival and long-term health of a single larva.

1.3 Larval Echinoderm Feeding and Nutrition

Echinoderm larvae feed on phytoplankton and other suspended particles. Stomach contents of larval echinoderms have been studied and remains of dinoflagellates, diatoms, small flagellates, and other unrecognizable particles among other have been found within the stomach (Lebour, 1922; Strathmann, 1971). However, echinoderm larvae can be selective during feeding as to which feed particles to consume (Liu *et al.*, 2007a) and has also been speculated to ingest almost any particle of the appropriate size (Strathmann, 1975). Sea urchin larvae have ciliated bands located anterior and posterior to the mouth which creates a current allowing feed particles to be directed towards the mouth (Strathmann, 1975). Most feed particles are transported from the body of the larvae to the mouth by means of the ciliated bands. The mouth and esophagus both have cilia as well which allow feed to accumulate in the mouth until the particles are swallowed by muscular contraction.

In the adult sea urchin, an odor-guided rheotaxis is thought to be used for chemtrail navigation and odor source localization (Atema, 2012). Perhaps in larvae a similar mechanism is utilized but further studies must be carried out. In high concentrations of feed particles with varying types of feed, *P. lividus* will ingest the particles of the highest concentration first (Rassoulzadegan and Fenaux, 1979). If a particle is to be rejected, the larva will contract the esophageal muscles and reverse beat the cilia to expel the particle (Strathmann, 1975). The larvae also use the ciliated bands for locomotion as well as for feeding. The larvae can direct itself toward a feed particle and change speed and direction by moving the post oral arms, the epaulettes, and the posterodorsal arms.

The digestive tract is ciliated and a mucus film containing large amounts of mucopolysaccharides coats the esophagus and the feed itself encourages mucus production for swallowing (Ryberg and Lundgren, 1975). Feed absorption takes place in the stomach where a network of mesenchymatic cells is present (Runnström, 1912). The feed is broken down by digestive enzymes and carbohydrate metabolism is thought to be brought about by β-1,3 glucanase and α-amylase. In *P. lividus* larvae, alkaline phosphatase has been found to play a role in the digestion process (Evola-Maltese, 1957). Finally, acetylcholinesterase has been described to aid in digestion in regards to the regulation of intestinal movements (Augustinnson and Gustafson, 1949).

In regards to feeding, microalgae are almost always the utilized feed source in larval trials in an aquacultural setting. Nutritionally, microalgae provide essential lipids and essential fatty acids such as docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and arachidonic acid (ARA) (Liu *et al*., 2007a). Often a mixture of microalgae is used to ensure a balanced diet and to optimize necessary nutrients for larval growth. *Chaetoceros calcitrans* is one such microalgal species which is often used in conjunction with other microalgae species to promote growth and increase survival (Martínez-Fernández and Southgate, 2007; Castilla-Gavilán *et al*., 2018; Carbonara *et al*., 2019). More specifically *Dunaliella tertiolecta* or *Chaetoceros spp*. are used as a standard and fed about 48 h after fertilization when the stomach is formed (Kalam Azad, 2010; Kelly *et al*., 2000; Kelly *et al*., 2015; Liu *et al*., 2007a). The production costs of microalgae are often high and rather labor intensive. Furthermore, batch crashes and contaminations can occur causing a plethora of issues for a producer or researcher therefore, an alternative feed must be investigated.

A relatively small number of research trials have been performed which tested the efficacy of manufactured inert diets in sea urchin larviculture. To date, a single specific sea urchin diet has never been formulated as the inert diets used are intended for shrimp or shellfish such as Frippak™ and Lansy™ which are manufactured by INVE, Belgium (Liu *et al*., 2007a). The lack of sea urchin specific diets is directly correlated with the lack of knowledge of specific dietary requirements of the organism. The inert diets however did not support rudiment development in the trial and produced a lower growth rate compared to the sole use of a microalgal feed (Liu *et al*., 2007a). However, a combination of the inert diet and the microalgal (*Dunaliella tertiolecta*) feed (50:50) did prove to show promising results pertaining to increased growth and survival. *Lytechinus variegatus* (green sea urchin) fed *Dunaliella tertiolecta* (microalgal diet) produced larger larvae and produced a higher survival rate than the inert diet of 13.2% protein and 6.3% lipid content used (E-Z Larva, Ziegler Bros., Gardners, PA, USA) (George *et al*., 2004). Major issues with inert diets intended for fish and shellfish pertain to lack of acceptance, nutritional inadequacies, and lack of buoyancy and stability. It has been speculated that sea urchin larvae are selective to various feedstuffs and therefore a specific formulated diet for *P. lividus* could be essential for successful larvae growth (Liu *et al*., 2007a). By comparing mixtures of microalgae and analyzing their combined nutrient composition, it has been concluded that a successful diet would include a high dietary lipid content and *n*-3 long-chain polyunsaturated fatty acids as diets with these attributes produced larger and more developed larvae (Carboni *et* al., 2012). The essential goal of a species-specific inert diet in sea urchin larviculture would be to produce fast and efficient growing larvae, reduce mortalities, and to promote metamorphosis/settlement all while reducing production and labor costs of microalgae cultivation and in turn streamlining the production process.

1.4 Sea Urchin Aquaculture

The global population is expected to increase to over nine billion inhabitants between the years 2040 and 2050 (UN, 2019). An increasing population also illustrates an increasing demand and need for aquatic products. Capture fisheries are causing the exploitation of many marine resources in order to meet this growing demand. Many species have already been exploited to the brink of collapse and echinoderms are no exception.

Sea urchins have been used in culinary practices since ancient times. The ancient Greeks may have been some of the first to appreciate sea urchins' gonads as a delicacy (Sartori et al., 2016). In the present era, sea urchin roe is still considered a delicacy in many countries such as Japan, China, Korea, and France (Kalam Azad, 2010). Japan leads the world in sea urchin consumption representing 90% of the global market (Rocha *et al.,* 2019) and in Europe the principal market is France, with the target species being the purple sea urchin *P. lividus* (James *et al.,* 2017). This highly prized species dominates roe markets and proves to be a good candidate to diversify the European aquaculture industry (Grosjean *et al,* 1998). As recently as December 6th, 2021, according to a supplier of real-time foodstuffs market information known as Tridge, urchin values in the Tokyo market reached as high as $38.15 (USD) per kilogram. It is no surprise that Japan leads the world in sea urchin imports with an annual value of $102 million followed by Hong Kong with a mere value of $17.9 million compared to Japan. In regards to exports, surprisingly Russia leads the world with a value of $78.25 million followed by Japan and the United States (Tridge, 2021). It should be noted that the vast majority of the aforementioned values come directly from capture fisheries. In regards to the geographical distribution of the species, overfishing and exploitation have taken a toll on local populations. A process must be developed which caters to each life stage of the species. After achieving said process, hopes to rely on an aquaculture-based system will relieve the current exploitations on natural populations (Grosjean *et al.*, 1998).

Aquaculture can trace its roots to many ancient societies such as China, Egypt, and Rome (Beveridge and Little, 2002), however, sea urchin aquaculture is in its infancy across the globe and promising attempts to establish a reliable protocol are on the rise. Modern sea urchin aquaculture can be divided into the various life stages of the animal. These stages include: broodstock, egg fertilization, larval culture, metamorphosis, juvenile growth, and adult growout/conditioning.

Japan leads the way in sea urchin research as studies have been carried out as early as the 1960s to cultivate urchins to replenish natural stocks, develop spawning techniques, and enhance fisheries (Hagen, 1996; McBride, 2005). The first documented attempts at sea urchin aquaculture occurred in the Yamaguchi Prefecture of Japan in 1968 with three main species, *Pseudocentrotus depressus* (pink sea urchin), *Hemicentrotus pulcherimmus* (bafun sea urchin), and *Heliocidaris crassispina* (purple sea urchin, Pacific) (McBride, 2005). The initial attempts of sea urchin aquaculture in Japan placed no emphasis on the complete production cycle of the organism. Instead, the focus entailed growing them large enough for wild stock repopulation after rearing in a land-based facility (Kawamura, 1973). On the European continent, Le Gall as recent as the late 1980s, developed a recirculating, closed-system operation which would encompass the entire life cycle of the organism (Le Gall and Bucaille, 1989). Sea urchin production and research also has taken afoot in other European countries such as Scotland, Ireland, and Norway. Efforts in Israel show promising results in regards to the culture of *P. lividus* (McBride, 2005). In the Americas, four main countries are the driving force behind sea urchin research and production: Canada, the United States, Mexico, and Chile.

The major area of sea urchin aquaculture research pertains to the conditioning of mature organisms and the attempt to improve the gonadal somatic index (*GSI*) and to improve roe color and quality. It is estimated that a marketable sea urchin has a *GSI* of about 20% (McBride, 2005). Many operations collect urchins and feed them an *ad libitum* diet. They are maintained under optimal conditions for gonadal growth and many studies have been carried out to locate proper nutrition, temperature, oxygen levels, and density (Lawrence *et al.*, 1992; Gago *et al.*, 2001; Sartori *et al.*, 2015; Spirlet *et al.*, 2000, 2001). Studies have aimed to establish diets to improve the *GSI* and roe color as these two parameters are of utmost importance in the sea urchin market (McBride, 2005). Recent findings have concluded that diets lower in protein (30% dry matter, DM) and lower in lipids (6% DM) which are fed to sexually mature adults produce a higher gonad yield in both sexes (Baião *et al.*, 2019). Many feeding regimes have been designed using an inert feed or a mixture of microalgae in order to improve gonadal color to a yellow or dark orange which is the market standard, however, more research is needed in this area of roe enhancement because results have not provided the desired results (Baião *et* al., 2019; Barker *et al*., 1998; Grosjean *et al*., 1998; Robinson *et al*., 2002; Walker *et al*., 1998; Watts *et al*., 1998). Due to the growing interest in the species, producers and scientists speculated to establish production protocols of sea urchins at earlier life stages.

A large portion of sea urchin research pertains to the juvenile life stage. It has been noted that a decline in juvenile populations occurs from premature harvesting which further contributes to the total decline of sea urchins due to the juveniles' missed opportunity to reach sexual maturation (Pinna, 2014). Much like studies pertaining to adult conditioning, studies regarding juveniles pertain to nutrition and growth. However, the early juvenile stages are critical periods in the life cycle and survival rates tend to be low (Kalam Azad, 2010). Urchin growth is relatively slow in the juvenile stages and the digestive tract is still in formation. Efforts are currently underway to formulate inert and/or micro/macro algal blend diets, optimize rearing conditions, and increase the rate of survival. The juvenile stage usually occurs around one month of age and the juveniles are transferred to a flow through system (Mendes *et al*., 2019) which follows the delicate larval stages where the aim of this work attempts to contribute to the sparse number of publications pertaining to this crucial life stage.

Larval research for sea urchins does exist, however, far fewer publications exist than the aforementioned areas of research. Many studies pertain to areas in reproduction such as induced spawning techniques and larval growth patterns. The majority of research use a common approach to the induction of spawning. Typical procedures include injecting 0.5-1 mL of 0.5-1.0 M KCl into the peristomal membrane of the sea urchin (Kelly *et al*., 2000; Liu *et al*., 2007a). Most studies suggest inverting the urchins over a collecting container and allow them to set for 20-30 min or until either sperm or ova exit through the gonopores.

Many trials have used different system layouts for the larval stage. However, most use a 50-200 L tank with thermoregulated static seawater with varying degrees of water exchanges per day. One study tested a flow through system for the larval stage and compared it to a static system. The study claims that the flow through system can obtain higher larval survival rates than a static system due to decreased stress during cleaning and water exchange (Carboni *et al*., 2012). The flow through system also had an inflow of water with high oxygen levels to the tanks. It should be noted that the researchers found no degradation in water quality between the two treatments. The vast majority of sea urchin larval trials use a static system as the flow through system requires higher production costs and a lower margin of error due to a possible system failure. All previously performed trials stress the importance of delicacy whilst performing water exchanges and tank cleaning due to the fragile state of sea urchin larvae.

The aim of this study was to determine if sea urchin larvae fed with inert diet at mouth opening would perform better than larvae fed with a microalgae blend by promoting growth and survival. A major objective for using inert diets pertains to formulating a balanced diet for the delicate phases of sea urchin larvae that will produce a high-quality juvenile.

2. Materials and Methods

2.1 Broodstock Management

Broodstock sea urchins were kept in two tanks in a recirculating aquaculture system at Ramalhete Field Station (CCMAR/UAlg, Faro, Portugal). The broodstock was collected in January 2022 from Praia do Evaristo in Albufeira, Portugal (N 37° 04.459, W 008° 18.134). The tanks were kept at 20º C ± 2º C and the sea urchin broodstock were fed *ad libitum* an inert diet (SPAROS Lda., Olhão, Portugal) and the macroalgae *Ulva lactuca* produced on site. Animals were maintained under a photoperiod of 12 h of light and 12 h of darkness. Water abiotic factors were monitored to ensure optimal conditions for sea urchin broodstock. Tanks were cleaned by use of a siphon to maintain optimal water quality. The sea urchins were left to acclimatize and mature for a period of two months before spawning inductions were carried out.

2.2 Spawning Induction

Once the selection of the broodstock had been completed, the selected specimens were cleaned with filtered seawater before spawning induction. A syringe filled with 1 mL of 0.5 M KCl (Leighton, 1995) was injected into the peristomal membrane of the sea urchin and shaken vigorously for around 20 seconds (Figure 2.1). The injected urchins were then placed inverted over a receptacle so that the gonopores released sperm in the case of a male and ova in the case of a female directly into the collecting vessel. In the case of a male, the urchin was then placed upright for semen collection. Following a short period, of about 20-30 min, the sperm was collected using a micropipette and placed on ice until ready for use. The ova were collected, diluted, and then counted using a Burker chamber.

Figure 2.1. Spawning induction of *Paracentrotus lividus* broodstock.

Once an estimation of the number of ova collected was made, the fertilization took place. The ova and semen were added to a 5 L beaker filled with filtered seawater and fertilization was carried out. The ratio of ova to semen was 1,000,000:20 µl for this trial. The fertilized eggs were kept in static conditions at a temperature of 20º C ± 1º C until hatching. Eggs were viewed under a stereoscope and in order to locate the fertilization membrane (Figure 2.2). If cellular divisions were observed (Figure 2.3) the eggs were incubated.

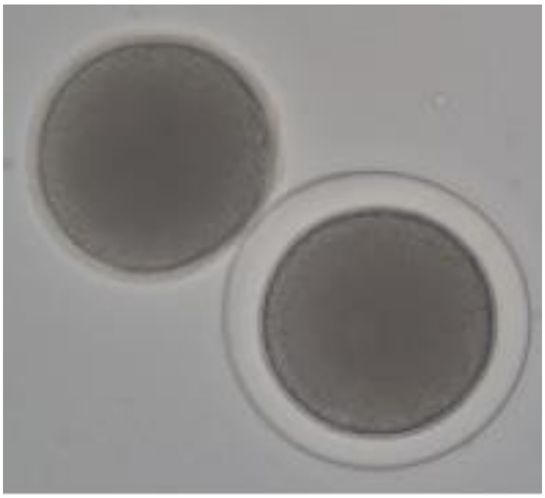


Figure 2.2. Unfertilized and fertilized zygote in *S. purpuratus* with a fertilization envelope (Invertebrate Embryology, 2010).

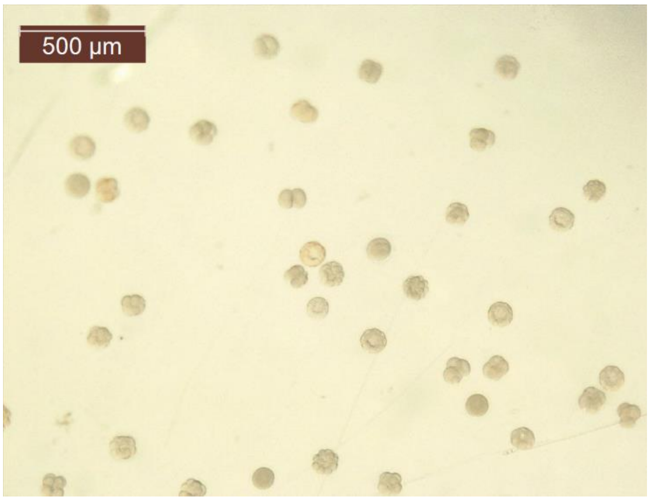


Figure 2.3. Various stages of initial cellular development post-fertilization in *P. lividus.*

Fertilization rate (FR) was calculated using the following formula:

$$FR = \frac{Number\ of\ fertilized\ eggs}{Number\ of\ eggs} \times 100$$

2.3 Hatching Rate

Hatching blastocysts generally occurs within 24 h for *P. lividus* (Kelly *et al*., 2015). Once a hatching rate was calculated, the larvae were divided equally and transferred into the experimental tanks. The hatching rate (HR) of every tank was calculated and the overall average was used in further calculations. The following equation was used to calculate the HR of *P. lividus*:

$$HR = \frac{Number\ of\ larvae}{Number\ of\ total\ eggs} \times 100$$

2.4 Larval Rearing

P. lividus larvae were raised at Ramalhete Field Station during the month of March 2022 for a period of 21 days with an additional four days of settlement observations following the larval rearing period.

2.4.1 Experimental Conditions

The larval rearing conditions consisted of a static system of nine 100 L tanks (Figure 2.4). A density of 13.86 fertilized eggs ml^{-1} was used for a total initial number of 1,386,000. The initial density of larvae per tank was 10.46 larvae ml^{-1}, or 1,046,792 larvae per tank. Tanks were filled with filtered seawater and kept at a temperature of 20º C ± 1º C using aquarium thermostats and monitored daily.

Figure 2.4. Assembled larval rearing tanks at Ramalhete Field Station.

A target salinity of 35 ppt ± natural fluctuations was sustained throughout the trial. A dissolved oxygen level in water of >95% of saturation was targeted and measured daily and corrected if conditions were not met. In order to maintain the desired level of dissolved oxygen, small air tubes were installed in each tank and adjusted to release a few bubbles per second so that the delicate larvae not be disturbed. Nitrogenous compounds were measured daily which included NH_4 and NO_2 to ensure that no tank exceeded 0.75 mg/L. A photoperiod of 12 h of light and 12 h of darkness was used with a light intensity of 115 lux.

2.4.2 Tank Cleaning and Water Renewal

In order to prevent the accumulation of nitrogenous compounds and to promote optimal rearing conditions, seawater was renewed daily. Every morning during the trial, around 25% of the water in each tank was siphoned through a 53 µm mesh filter as to not disturb the growing larvae. However, every few days, 50% of the seawater was renewed to raise the dissolved oxygen levels and eliminate ammonia and nitrite levels. The seawater to replace the effluent was maintained in a separate tank and held at 20º C to ensure a sustained temperature between the tanks during water renewal. The rate of renewal administered to each tank was at a rate of 0.20 L min^{-1}. This process lasted between 1-2h and performed in a delicate manner as to not inflict damage or mortalities on the larvae.

2.4.3 Feeding Regime

A total of three dietary treatments were done in triplicate; a microalgae blend (Treatment A) of three species including, *Skeletonema sp.*, *Isochrysis galbana*, and *Nannochloropsis sp.*; two experimental inert diets formulated with algal ingredients (Treatment B) or marine ingredients (Treatment C). Each dietary treatments were randomly assigned the rearing tanks (Figure 2.5).

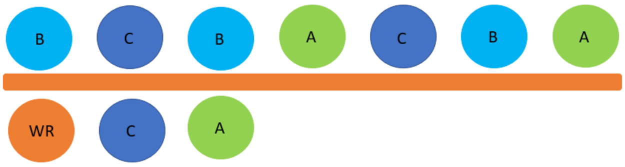


Figure 2.5. Tank layout with treatments A, B, and C. WR refers to the water renewal tank.

The microalgae were produced by Necton SA, (Olhão, Portugal) and the experimental diets manufactured by Sparos, Lda. (Olhão, Portugal). The proximate composition of the dietary treatments is exhibited in Table 2.1.

Table 2.1. Proximate composition of the diets.

DIET	MICROALGAE (TREATMENT A)	ALGAL INERT (TREATMENT B)	MARINE INERT (TREATMENT C)
Protein (%)	Data not available	55.55	56.86
Fat (%)	Data not available	11.65	12.75
Ash (%)	-	9.23	5.45
Energy (KJ/G)	-	21.55	22.32

The feeding regime timeline and amounts administered to the sea urchin larvae can be seen in Table 2.2

Table 2.2. Feeding regime of the inert diets and microalgae treatments during purple sea urchin larval development.

Treatment	0-9DAH	10-14DAH	15DAH-End
A Microalgae (cells/ml/day)	120,000	200,000	300,000
B Inert (Algal) (g/day/tank)	0.4	0.4	0.4
C Inert (Marine) (g/day/tank)	0.4	0.4	0.4

DAH: Days after hatching.

The microalgae blend was slowly administered in the same area of the tank as the water inlet. The blend was administered after thawing from a frozen state and blended until a homogenous mixture was attained. The inert diets were dispersed from a sieve and distributed uniformly among the tanks while ensuring that clumps did not form.

2.5 Sampling

Sampling occurred throughout the trial to analyze various parameters such as the following: fertilization rate (FR), hatching rate (HR), growth, survival (*SR*), feeding incidence (*FI*), competency rate (*CR*), and settlement rate (*STR*). Table 2.3 defines the occurrence of the samplings and the corresponding larval stage of *P. lividus*.

Table 2.3. The samplings and the corresponding larval stage of *Paracentrotus lividus*. DAH: Days after hatch

STAGE	EGG	LARVAE PLUTEUS	4 ARMS	4 ARMS	6 ARMS	8 ARMS	METAMORPHOSIS
Code	Egg	0DAH	4DAH	8DAH	12DAH	16DAH	21DAH
DAH	-2	0	4	8	12	16	21

Sampling was performed according to the relative growth stage of the larvae in conjunction with the number of arms present at a specific day after hatching as seen in Figure 2.6 (Mendes *et al*., 2019). The sampling methodology entailed the collection of larvae from each tank from four sites corresponding to the four cardinal directions on a compass. Samples were collected at the surface and mid-depth to ensure a representative sample. The collection device was a plastic tube with a 1 cm diameter opening and the analyzations will be explained in the following sections.

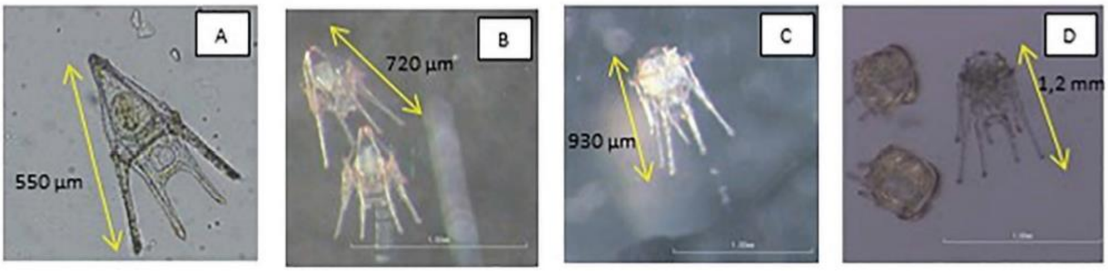


Figure 2.6. Larval development of purple sea urchin (*Paracentrotus lividus*). (A) Larvae *pluteus* 0 days after hatching (DAH), (B) The 4-arm phase from 0-12DAH, (C) The 6-arm phase begins 12DAH, and (D) The 8-arm phase begins 16DAH (Mendes, *et al*., 2019). DAH: Days after hatching.

2.5.1 Fertilization Rate and Hatching Rate

FR and *HR* were sampled at -2 d before hatching and at 0 d respectively. The method of calculating each rate was performed by using the equations previously presented in sections 2.2 and 2.3.

2.5.2 Growth

Various quantitative measurements can show each dietary treatment's efficacy on growth. The sea urchin larva has several anatomical features which can be measured for length and width as these parameters are ideal indications for growth. Measurements were taken at 0, 4, 8, 12, and 16 days after hatching (DAH; Table 2.6). It should be noted that measurements were taken at 21DAH, however, only until 16DAH will the results be included due to the loss of entire replicate tanks, unclear photos, and due to some larvae lacking the various structures needed for sampling. A total of 50 (*n*=50) (when applicable) larvae were sampled from each replicate tank (*n*=3) during each sampling for a total of 150 individuals for each treatment. All viable larvae from the sampling were included in the survival rate as described in the following section. After collection, the larvae were immobilized with distilled water in a Petri dish. The larvae were photographed using Leica i9 and LAS software. Photos were analyzed and measurements were taken using Image J software. The measurements recorded were adapted from Kelly *et al.* (2000) and included the following: total length (*TL*), width (*W*), arm number (*AN*), post oral arm length (*POAL*), stomach length (*ST*), and stomach width (*SW*), rudiment length (*RL*), and rudiment width (*RW*). Correlations were made between *TL* and *W* at 4, 8, 12, and 16DAH. A visual description can be seen in Figure 2.7.

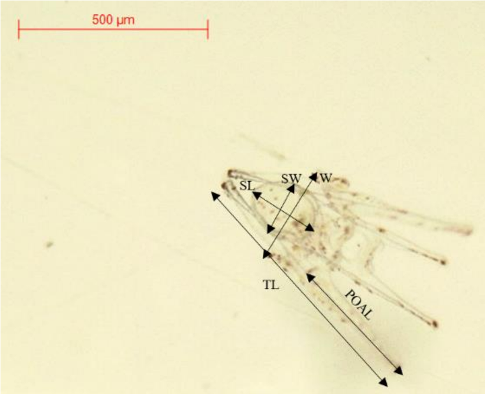


Figure 2.7. Measuring parameters for *Paracentrotus lividus* (12DAH) larvae including *TL* (total length), *W* (width), *POAL* (post oral arm length), *ST* (stomach length), and *SW* (stomach width).

Beginning at 4DAH until 16DAH, the following formula shows a body measurement ratio involving *AN* and the *TL* and *W*.

$$Body\ ratio = \frac{W}{TL} \times AN$$

This ratio compared the *AN* in regards to the total size of the larvae. A higher ratio value signified a larger larva that also had a higher *AN*. Once the stomach became more prominent at 8DAH, a ratio was calculated on this sampling day until 16DAH to compare the body area (*BA*) to the stomach area (*SA*). A lower ratio value indicated that the larval stomach size was more proportional to the body size. Also beginning at 8DAH and lasting until 16DAH, a ratio between *TL* and *POAL* was calculated. A higher ratio value indicated a greater *TL* and *POAL* and in turn a larger larva. The rudiment will appear next to the stomach and was measured similarly to *ST* and *SW*. However, it should be noted the *RL* and *RW* were only measured 21DAH due to its initial visibility around this stage. Furthermore, on 0DAH only *TL* was recorded. The *AN* was counted at each sampling after 0DAH by counting the number of visible arms of each larva.

2.5.3 Feeding Incidence

Feeding incidence (*FI*) data was collected at 8 and 16DAH by analyzing photos of larvae taken from the sampling points. Photos were analyzed to determine feed content in the stomach. The number of larvae with content in the stomach was calculated and a ratio was determined between larvae with and without content. Any visible feed within the stomach was considered as a positive feeding incident (Figure 2.8).

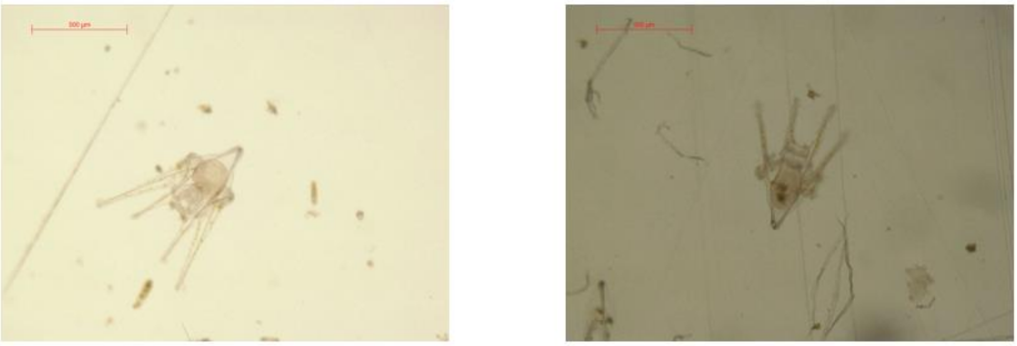


Figure 2.8. Four-arm purple sea urchin larvae exhibiting feeding incidence. The larvae in the photo on the left photo exhibits a larva with no content within the stomach while the photo on the right exhibits a larva with gut content.

2.5.4 Survival

Volumetric data collection for survival occurred on 4, 8, 12, and 16DAH (Table 3) and calculations were based on larval density. Survival at 21DAH was not included due to the loss of multiple tanks and inconsistent data. A sample was taken from each of the nine tanks and diluted to an appropriate volume if necessary for easier counting. Values were extrapolated to determine the survival rate (*SR*) in each tank as described by the following equation. The initial number of larvae used in the below equation was based on the hatching rate calculated previously. A total of six counts were made for each tank from a sample of 100 ml and averaged to determine the surviving number of larvae per ml. A glass pipette of 1 mL was used to take a smaller sample from the 100 ml sample and observed under a stereoscope. In the case of low numbers of larvae in the samples, a sample with a fixed volume was filtered through a 53 µm mesh filter and its contents were transferred to a Petri dish for counting under the stereoscope. If 100 ml was not sufficient to achieve a representative sample then a larger sample was taken from each tank.

$$SR = \frac{Surviving\ number\ of\ larvae}{Initial\ number\ of\ larvae} \times 100$$

2.5.5 Competency

Competency occurs in sea urchin larvae just prior to metamorphosis. Therefore, competency sampling was performed at 21DAH. The rudiment was measured and determined competent if it was in fact about the size or larger than the stomach (Figure 2.9). This was achieved by using the Image J software to analyze photos acquired from the Leica i9 and LAS software. The same larvae that were sampled for growth were also used for the rudiment analysis for a total of 50 larvae per replicate tank if possible.

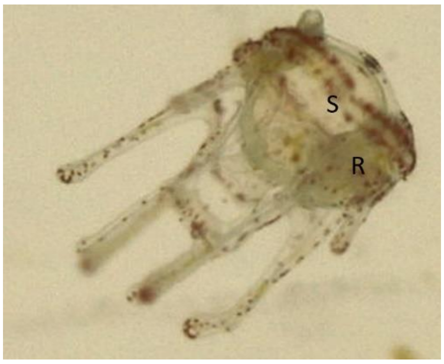


Figure 2.9. The Rudiment (*R*) and stomach (*S*) of a sea urchin larva 21 days after hatching.

Larvae were assigned either a 0 for not competent or a 1 for competent based on the stomach area to rudiment area ratio. After the determination of competency, the following equation was used to determine competency rate (*CR*) within each replicate tank.

$$CR = \frac{Competent\ larvae}{Incompetent\ larvae} \times 100$$

2.5.6 Settlement

Data collection for settlement was based on procedures put forth by Kelly *et al*. (2000). The initial sampling took place on 21DAH and final settlement observations occurred four days following the conclusion of the larval rearing period. Petri dishes were preconditioned in the water renewal tank using filtered seawater for 5 days prior to use to allow for a natural biofilm development. The biofilm will act as a substrate for the larvae which encouraged metamorphosis and settlement. Once a biofilm was established, 50 larvae from each replicate tank (if applicable) were then transferred to the preconditioned Petri dish and 50 ml filtered seawater was added. The competent larvae were allowed to settle and observations were made after 24, 48, 72, and 96 h to determine settlement rate. Settlement was determined by observing the competent larvae settling and attaching on the bottom of the Petri dish (Figure 2.10). The following equation was used to calculate the settlement rate (STR).

$$STR = \frac{Settled\ larvae\ in\ dish}{Initial\ larvae\ in\ dish} \times 100$$

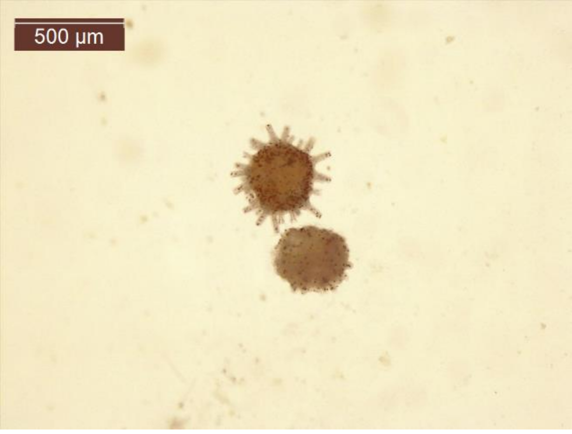


Figure 2.10. Settled post larvae from Treatment C at 21DAH.

2.5.7 Proximate Composition

Proximate analysis was also performed on each of the three diets to determine nutrient content through means of dry matter (*DM*) and ash. Diet A from Treatment A chemical analysis was carried out with a method for both protein and lipid content. The protein analysis was performed according to the Lowry method with modifications (Rutter, 1967). The analysis was done in triplicate. The method was based on the Biuret reaction in which peptide bonds of the proteins react with copper under alkaline conditions to produce Cu^+. This ion reacts with the Folin reagent where phospomolybdotungstate is reduced to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic amino acids. The lipid analysis was also done in triplicate. The analysis was a modification of SINTEF of the Bligh and Dyer method (Bligh and Dyer, 1959) and altered for non-animal content. Diet samples were weighed and diluted and later homogenized using chloroform, methanol, and distilled water. Centrifugation followed and the chloroform was extracted with the lipid content and allowed to evaporate leaving only the lipid content to be weighed.

The experimental diets' (Diet B and C) chemical analyses followed standard procedures of the Association of Official Analytical Chemists (AOAC, 2006) and was done in duplicates. Before analysis, the diets were finely ground. Dry matter was determined by drying the samples at 105°C for 24 h and the ash content was determined by incineration in a muffle furnace at 550°C for 6 h. Freeze-dried diet samples were analyzed for crude protein (N x 6.25) using a Leco nitrogen analyzer (Model FP-528; Leco Corporation, St. Joseph, MI, USA). Crude fat was calculated by a petroleum ether extraction using a Soxtherm Multistat/SX PC (Gerhardt, Germany). Gross energy was determined using combustion in an adiabatic bomb calorimeter (Werke C2000; IKA, Staufen, Germany) that is calibrated with benzoic acid.

2.5.8 Statistical Analysis

Statistical analysis was performed to determine statistical significances between the three dietary treatment groups. The collected data was tested by the Shapiro-Wilk test to determine a normal distribution and the Levene’s test was used to check for homogeneity of the variances. If these assumptions were met, the one-way ANOVA (analysis of variance) statistical test was used. Furthermore, the post-hoc Tuckey’s test was applied to further explain statistical differences between the pairwise comparisons. However, if assumptions were not met, the non-parametric Kruskal-Wallis test was used (Zar, 2010). In the case of comparing frequencies, a chi-squared (χ^2) test was used to determine statistical significances between groups. A post-hoc analyzation was performed following the χ^2 test using the Bonferroni pairwise comparison. All statistics used were calculated with a 95% confidence interval ($p<0.05$). In instances with percentages, the percentage values were calculated and transformed into an arcsine value for further statistical analysis. All statistical analyses were performed in the Statistica™ software program developed by Stat Soft, Inc., Tulsa, OK, USA.

3. Results

The results will be conveyed in sections beginning with the hatching rate (*HR*) and fertilization rate (*FR*) followed by growth measurements. Total length (*TL*) will follow *HR* and *FR* and contain every sampling day. The sections that will follow, respectively, are data for width (*W*), arm number (*AN*), *TL/W* to *AN* ratio, post oral arm length (*POAL*), *TL* to *POAL* ratio, stomach length (*ST*), stomach width (*SW*), body to stomach area ratio, rudiment length (*RL*), and rudiment width (*RW*). Data for survival will follow the aforementioned findings which in turn will be followed by gut fullness data, competency data, and settlement data.

3.1 Fertilization Rate and Hatching Rate

The fertilization rate was 88.57% ± 21.46%. The hatching rate varied between the replicate tanks with the maximum being 100% and the minimum calculated at 21.24%.

3.2 Growth

3.2.1 Total Length (*TL*)

The first total length (*TL*) sampling occurred at day 0 and measurements were taken on 4, 8, 12, and 16DAH to determine the impact of dietary treatments on larval growth in length (Figure 3.1).

Figure 3.1. Growth of *P. lividus* from each treatment at 4, 8, 12, and 16DAH. Treatments are oriented horizontally.

At 0DAH, an average of the sea urchin larvae *TL* was 315.5 ± 66.9 µm. This average was used to determine the growth pattern for the entire trial as seen in Figure 3.2. At 4DAH, larvae from Treatment C showed the largest *TL* (457.5 ± 49.7 µm) when compared to larvae fed Diet A and B (398.0 ± 45.2 µm and 408.6 ± 54.5, respectively, p <0.05). The maximum *TL* at 4DAH came from Treatment C (590.5 µm) and the minimum came from Treatment A (237.4 µm).

At 8DAH, statistical significances were found between all larvae (p <0.05). The largest *TL* was found in larvae from Treatment C (505.0 ± 68.5 µm). Sea urchin larvae from treatments A and B presented a *TL* of 475.8 ± 66.1 µm and 379.8 ± 49.8 µm respectively. The maximum *TL* at 8DAH came from Treatment C (748.61 µm) and the minimum came from larvae from Treatment A (257.5 µm).

At 12DAH, statistical significances were found between Treatments B and C compared to Treatment A (p <0.05). The largest *TL* mean was found in larvae from Treatment B which was calculated at 568.8 ± 138.3 µm while larvae from Treatments A and C were calculated at 372.5 ± 82.9 µm and 530.0 ± 97.3 µm respectively. The maximum *TL* of larvae at 12DAH came from Treatment B (972.9 µm) and the minimum came from larvae from Treatment A (246.2 µm).

At 16DAH, statistical significances were similar to 12DAH (Non-parametric, Kruskal-Wallis, p <0.05). The largest *TL* mean was observed in larvae from Treatment C which measured 623.9 ± 128.3 µm while larvae from Treatments A and B presented 373.6 ± 100.2 µm and 601.6 ± 117.7 µm respectively. The maximum larval *TL* came from Treatment B at 16DAH (877.8 µm) and the minimum was observed in larvae from Treatment A (221.3 µm). Larvae from Treatments B and C consistently grew throughout the trial while larvae growth from Treatment A remained relatively stagnant and even decreased over time as seen in Figure 3.3 which shows the average growth rates of all treatments throughout the course of the trial. Treatments B and C are statistically significant from Treatment A (p <0.05).

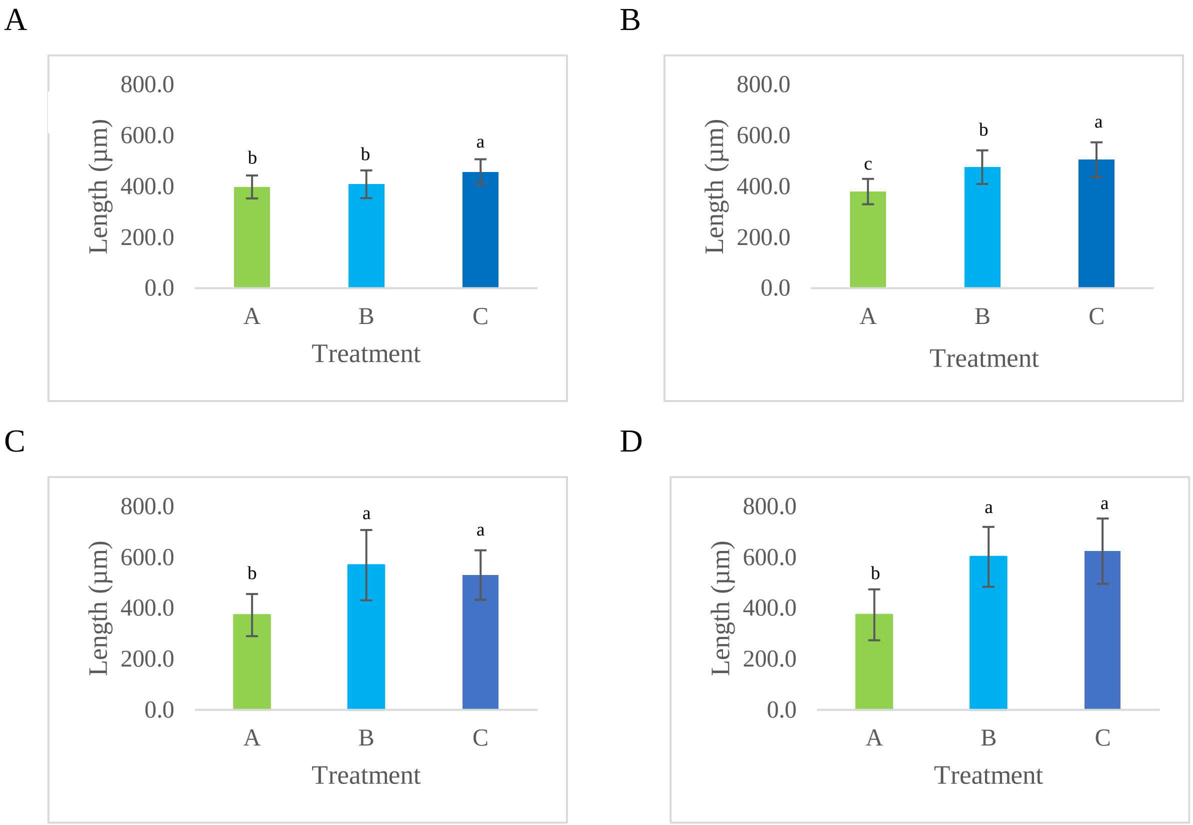


Figure 3.2. Sea urchin larvae *Total Length* at 4 (A), 8 (B), 12 (C), and 16DAH (D). Differing letters indicate statistical significance between the treatments (p <0.05). Values are presented as means ± standard deviation (n=3) for 4, 8, and 12DAH for all treatments and (n=2) for treatments A and C and (n=3) for treatment B at 16DAH.

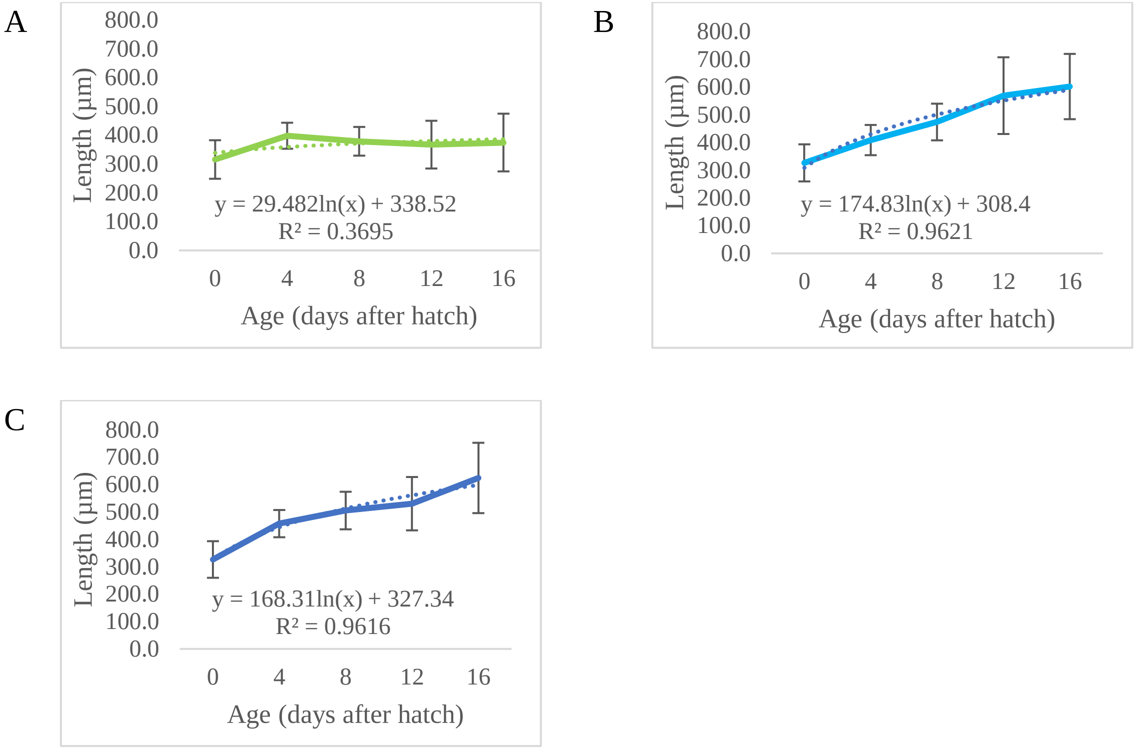


Figure 3.3. Sea urchin larvae *Total Length* average growth rate for the course of the trial. Treatments are shown in order, Treatment A (A), Treatment B (B), and Treatment C (C). Included in each graph is the logarithmic growth rate and R^2 value.

3.2.2 Width (*W*)

The first sea urchin larval width (*W*) measurements were taken at 4DAH and also sampled at 8, 12, and 16DAH. Figure 3.4 shows the *W* data between the larvae on the respective sampling days. At 4DAH statistical significances were present between the treatments while Treatment A differed from the other treatments ($p < 0.05$). The largest mean *W* came from larvae of Treatment C (126.9 μm ± 19.4). Treatments A and B had mean larval widths calculated at 115. 8 μm ± 15.6 and 122.3 μm ± 21.8 respectively. The largest *W* was measured at 183.2 μm in larvae from Treatment C and the smallest larvae were observed in Treatment A at 68.1 μm

At 8DAH, statistical significances were found between all treatments ($p < 0.05$). The largest mean was found in larvae from Treatment C (150.7 μm ± 28.7). Mean larval width from Treatments A and B were calculated at 123.0 μm ± 24.2 and 143.3 μm ± 66.1 respectively. The maximum *W* was found in larvae from treatment A which measured 240.1 μm and the minimum was from larvae of Treatment B measuring 69.4 μm.

At 12DAH, statistical significances were found between Treatments B and C compared to Treatment A ($p < 0.05$). It was at this point in the trial where larval growth from Treatment A became stagnate compared to the other treatments. The largest mean *W* was found larvae from Treatment B (191.8 µm ± 59.3). Means of the larvae from Treatment A and C were calculated at 125.9 µm ± 35.4 and 186.6 µm ± 49.4 respectively. The maximum measured *W* was found in larvae from Treatment C which measured 432.6 µm and the minimum was found in larvae from Treatment A at 47.3 µm.

At 16DAH, statistical significance was relatively similar to 12DAH ($p < 0.05$). The largest mean *W* was found in larvae from Treatment C (214.8 µm ± 67.7). Means of the larvae from Treatments A and B were calculated at 124.4 µm ± 41.2 and 188.3 µm ± 49.3 respectively. The maximum *W* was found in larvae from Treatment C which measured 466.5 µm and the minimum was again found in larvae from Treatment A at 60.0 µm.

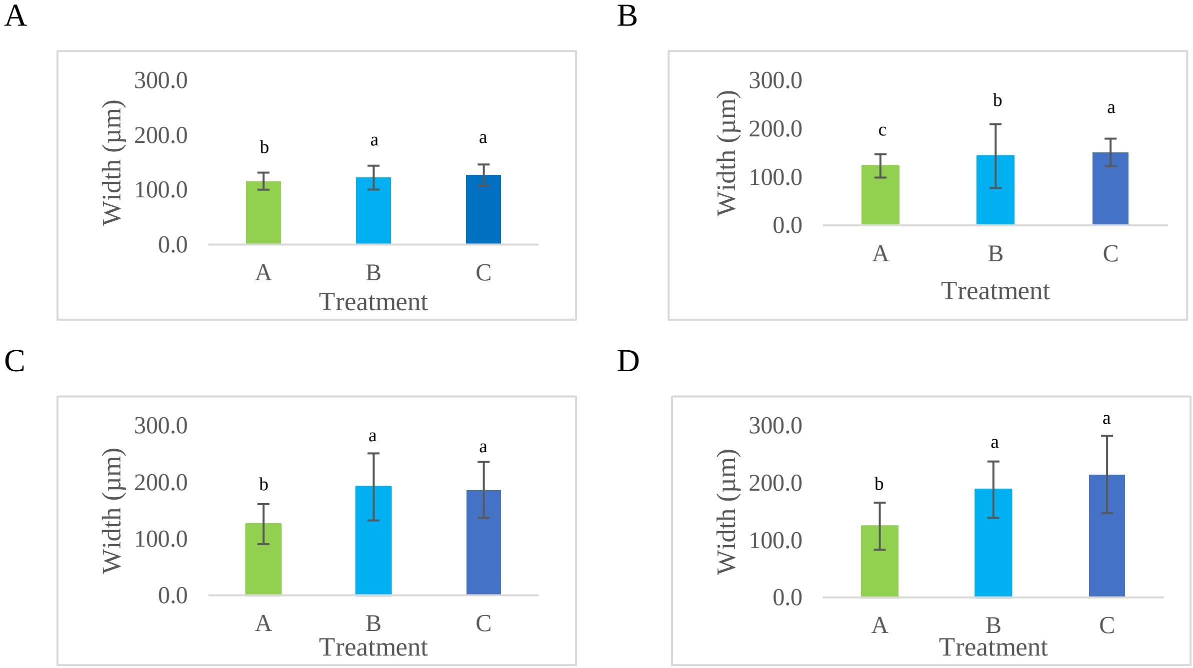
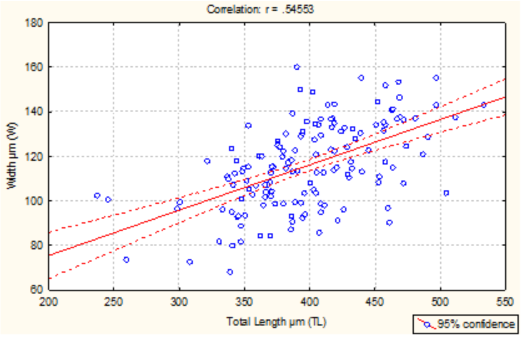


Figure 3.4. Sea urchin larvae W*idth* at 4 (A), 8 (B), 12 (C), and 16DAH (D). Differing letters indicate statistical significance between the treatments ($p < 0.05$). Values are presented as means ± standard deviation (n=3) for 4, 8, and 12DAH for all treatments and (n=2) for treatments A and C and (n=3) for treatment B at 16DAH.

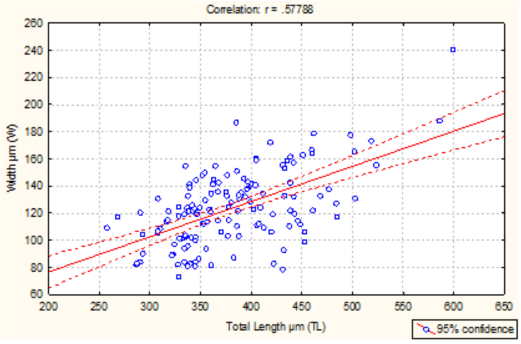
Correlations were made, as shown in Figure 3.5, between the *TL* and *W* at each sampling day for each treatment. After statistical analysis, no statistical significances were found between the treatments regarding the correlations. The correlations show that relatively strong and positive correlations existed between the *TL* and *W* of larvae from all treatments.

A

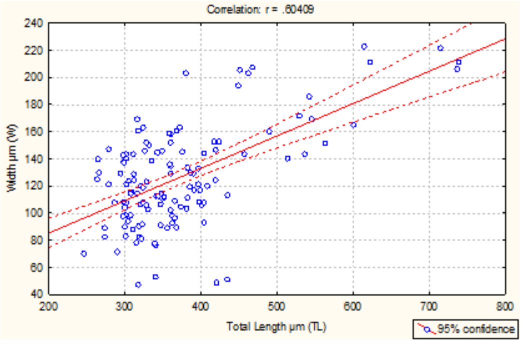
4DAH



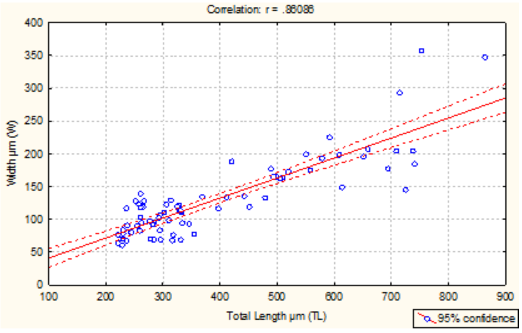
8DAH



12DAH

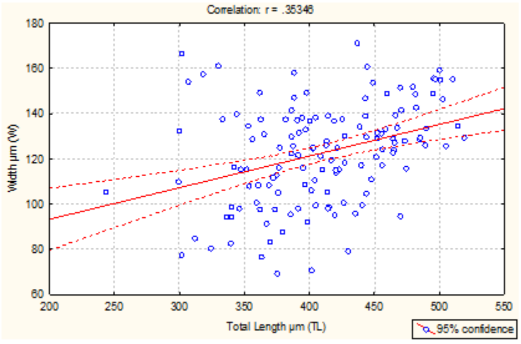


16DAH

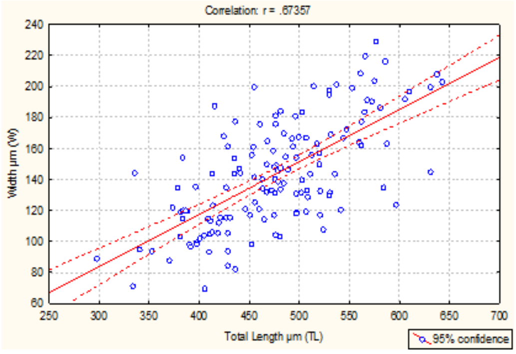


B

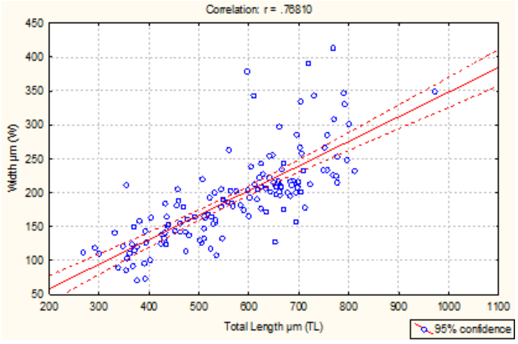
4DAH



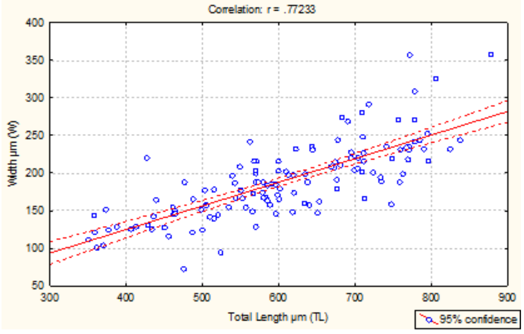
8DAH



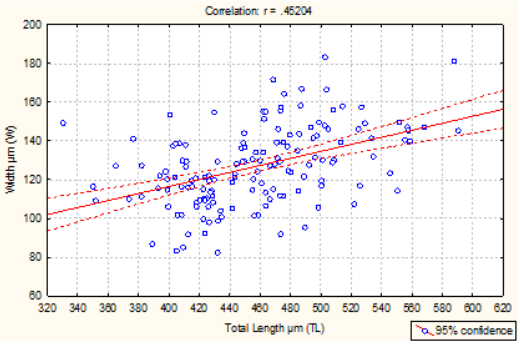
12DAH



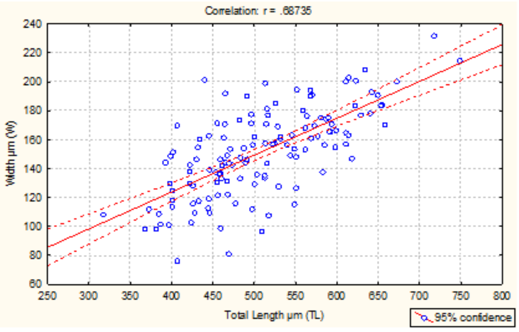
16DAH



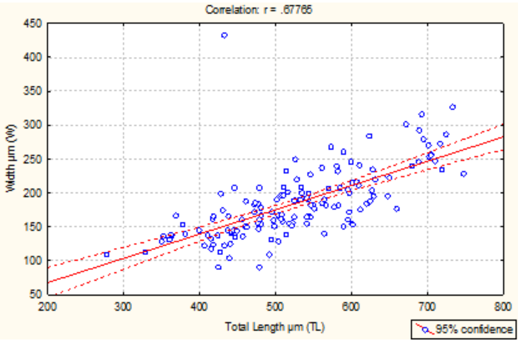
C 4DAH



8DAH



12DAH



16DAH

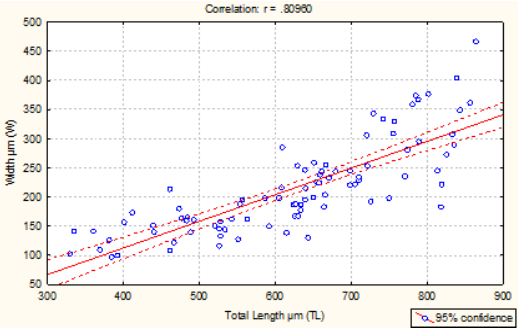


Figure 3.5. Correlations between *Width* and *Total Length* at 4, 8, 12, and 16DAH for treatments A (A), B (B), and C (C) with R^2 values. No statistical significances found between the data.

3.2.3 Arm Number (*AN*)

The *AN* was counted during each sampling at 4, 8, 12, and 16DAH. Statistical significances existed between the treatments which can be seen in Table 3.1 and Figure 3.6. Starting at 8DAH, sea urchin larvae fed diets B and C showed a higher *AN* than larvae fed diet A.

Table 3.1. χ^2 analysis showing statistical significances between treatments at 4, 8, 12, and 16DAH. Statistical significances were considered when $p<.05$.

DAH	***n***	**χ^2 value**	**χ^2 *p*-value**	**df**
4	436	21.2881	0.000278	4
8	413	15.1616	0.004377	4
12	417	43.9258	<0.00001	6
16	298	42.2579	<0.00001	6

At 12DAH, larvae fed diet B had a larger *AN* than larvae from treatment A and C, respectively. At 16DAH, larvae from treatment A showed more of a prevalence of 1-2 armed larvae than at 8DAH while larvae from treatments B and C had a greater number of larvae with 5-6 arms than were present at 12DAH. Larvae from Treatment C had a greater prevalence of larvae with more than five arms than larvae from any other treatment at 16DAH.

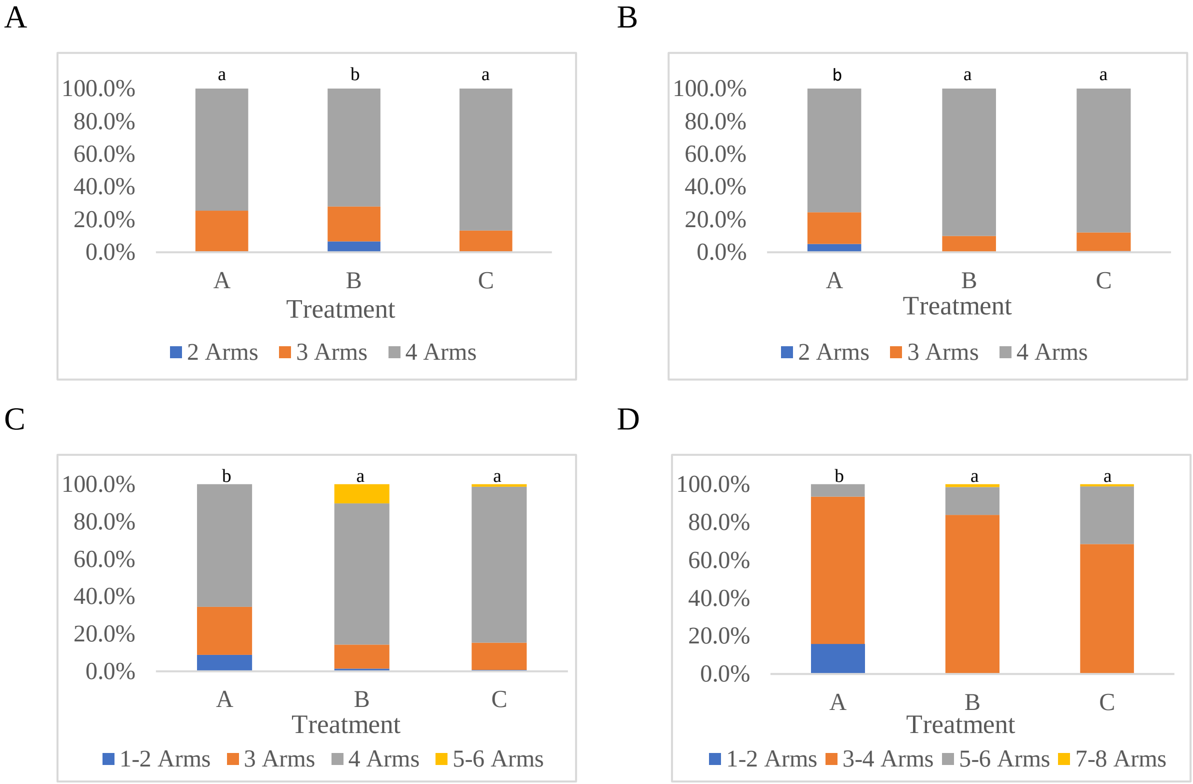


Figure 3.6. *Arm number* frequency at 4 (A), 8 (B), 12 (C), and 16DAH (D). Replicates were (*n*=3) for 4, 8, and 12DAH for all treatments and (*n*=2) for treatments A and C at 16DAH. Following a χ^2 statistical analysis, a Bonferroni post-hoc analysis was performed. Statistical significance (p <0.05) is indicated by differing letters between treatments.

3.2.4 Post Oral Arm Length (*POAL*)

The post oral arm length (*POAL*) was measured beginning at 8DAH and measured at every sampling which followed. It can be seen in Figure 3.7 that the data for *POAL* follows a similar pattern as both *TL* and *W*. Treatments B and C are not statistically significant after 8DAH whereas treatment A is consistently statistically significant from the other treatments (p <0.05) for the duration of the trial. Larvae from treatment B and C had a much larger *POAL* than larvae fed diet A at all sampled ages.

At 8DAH, larvae from Treatment C had the largest *POAL* mean and was calculated at 266.5 µm ± 52.3. The *POAL* means for larvae from Treatments A and B measured 156.4 µm ± 40.7 and 237.2 µm ± 52.8 respectively. At 12DAH, larvae from Treatment B had the largest mean calculated at 300.7 µm ± 107.7 and larvae from Treatments A and C had means of 144.6 µm ± 68.2 and 273.8 µm ± 77.2 respectively. At 16DAH, larvae from Treatment C had the largest *POAL* mean of 340.5 µm ± 97.9 and larvae from Treatments A and B had means of 149.8 µm ± 87.0 and 333.5 µm ± 95.9 respectively.

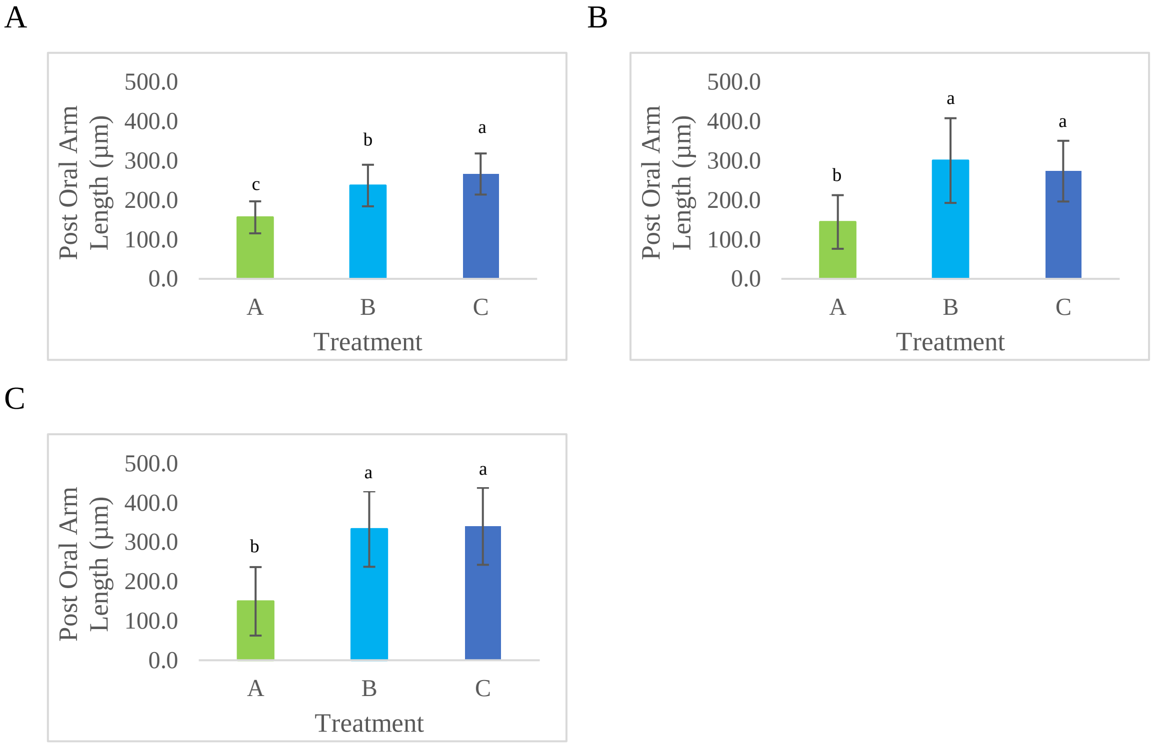


Figure 3.7. *Post oral arm length* at 8 (A), 12 (B), and 16DAH (C). Differing letters indicate statistical significance between the treatments (p <0.05). Values are presented as means ± standard deviation (n = 3) for 4, 8, and 12 DAH for all treatments and (n=2) for treatments A and C and (n=3) for treatment B at 16 DAH.

3.2.5 Stomach Length (*ST*) and Stomach Width (*SW*)

Stomach length (*ST*) and stomach width (*SW*) were both measured simultaneously beginning at 8DAH and was sampled at every sampling point thereafter until 16DAH. In Figure 3.8, *ST* was consistently longer in larvae from treatments B and C and statistically significant from treatment A (p <0.05) for the entirety of the trial. *ST* increased as the trial progressed in the larvae from treatment B, however, larvae from treatment C showed a slight decrease in *ST* which was observed from 12DAH to 16DAH. In larvae from treatment A, *ST* was rather stagnant in growth.

SW followed a similar pattern as *ST* and again treatment A was statistically significant from treatments B and C (p <0.05) and the larvae showed a decrease in width as the trial progressed as seen in Figure 3.8. Larvae from treatments B and C showed an increase in *SW* at each sampling point up until 16DAH. Figure 3.9 shows the *ST* and *SW* over the course of the trial and demonstrates the growth and/or decline according to the treatments.

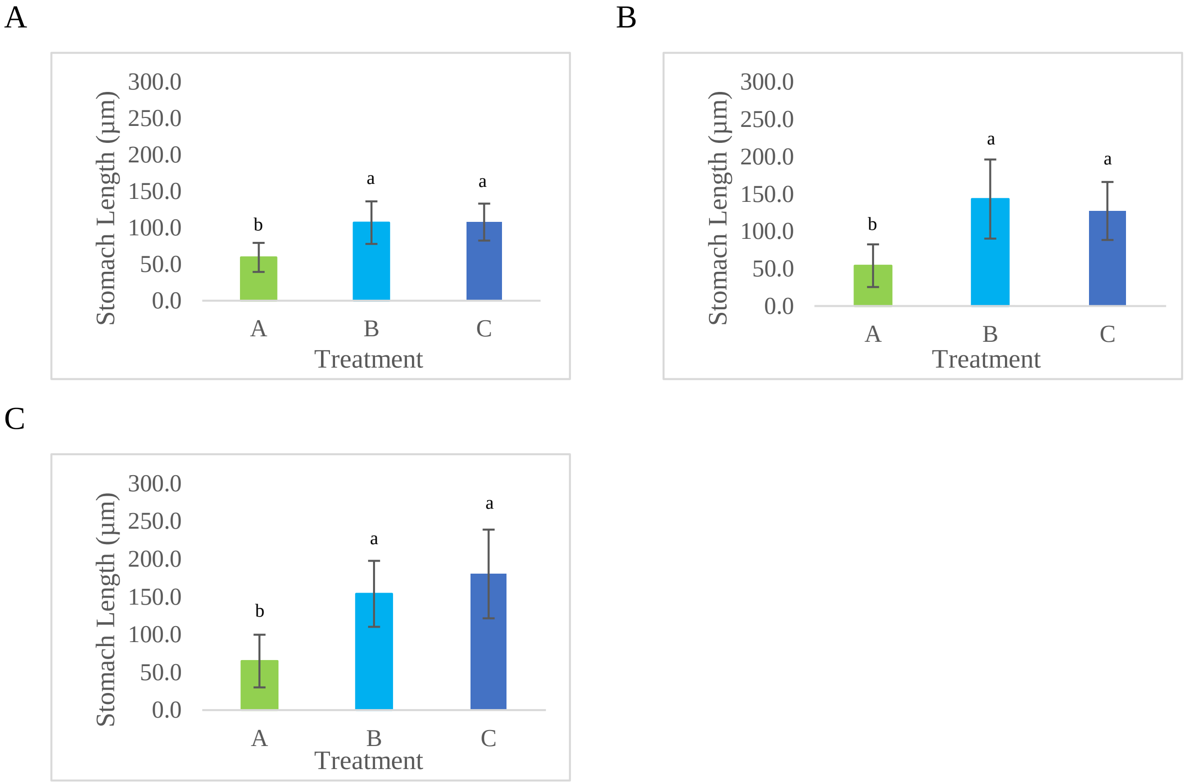


Figure 3.8. *Stomach length* at 8 (A), 12 (B), and 16DAH (C). Differing letters indicate statistical significance between the treatments ($p < 0.05$). Values are presented as means ± standard deviation ($n = 3$) for 4, 8, and 12 DAH for all treatments and ($n=2$) for treatments A and C and ($n=3$) for treatment B at 16 DAH.

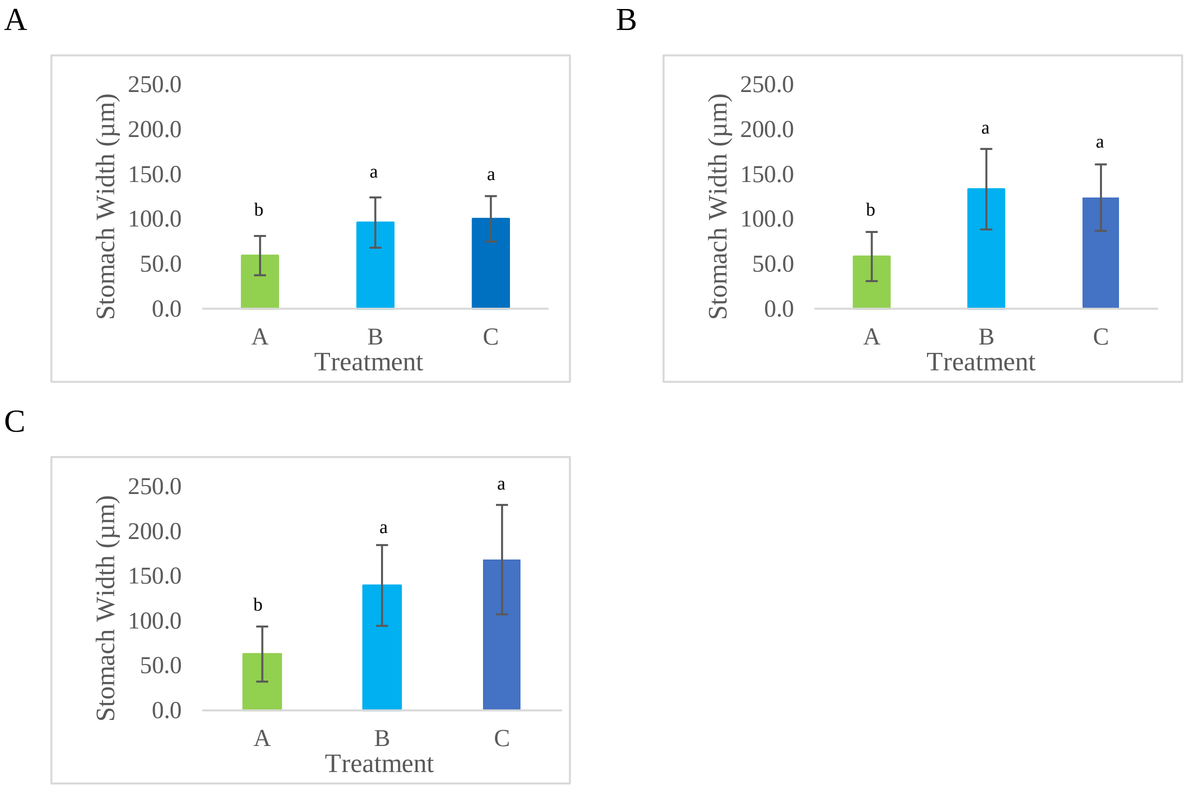
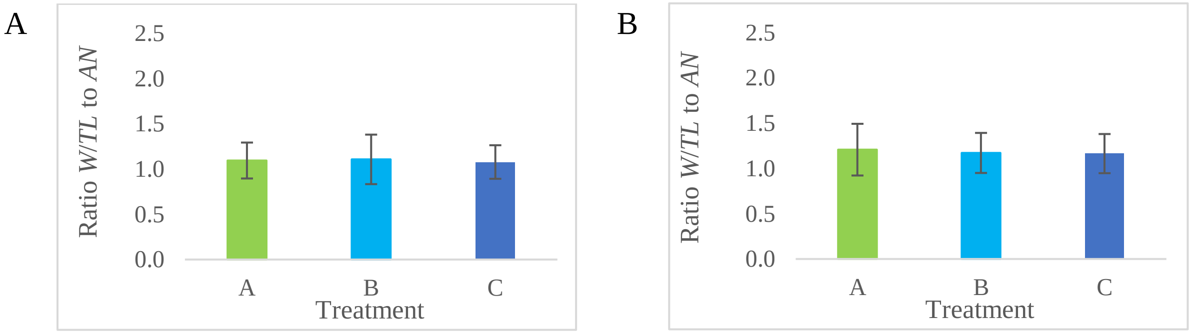


Figure 3.9. *Stomach width* at 8 (A), 12 (B), and 16DAH (C). Differing letters indicate statistical significance between the treatments (p <0.05). Values are presented as means ± standard deviation (n = 3) for 4, 8, and 12 DAH for all treatments and (n=2) for treatments A and C and (n=3) for treatment B at 16 DAH.

3.2.6 Body Measurement Ratios

Various body measurement ratios were taken starting at 4DAH and at every sampling until 16DAH. The body to stomach area and the body to *POAL* ratios were determined beginning at 8DAH and until 16DAH. Figure 3.10 shows the ratio between larval body length and width to the number of arms for each treatment. No statistical significances were found except for at 16DAH where treatment C showed statistical significance (p <0.05). This finding implicates that at 16DAH, the *AN* was greater in treatment C based on the *W*:*TL* ratio compared to the other treatments.



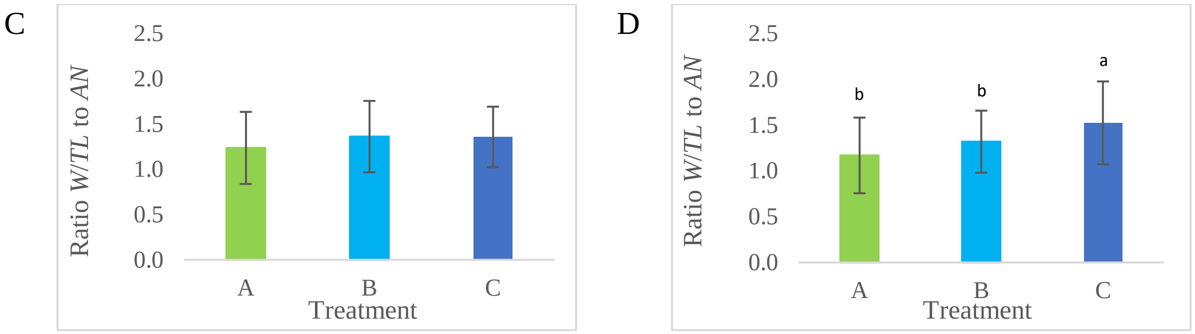


Figure 3.10. The ratio of *Width/Total Length* to *Arm Number* 4 (A), 8 (B), 12 (C), and 16DAH (D) larvae for treatments A, B, and C. Values are presented as means ± standard deviation. Differing letters indicate statistical significance between the treatments (p <0.05) while an absence of letters indicates no statistical significances present. Replicates were (n=3) for 8, and 12DAH for all treatments and (n=2) for treatments A and C and (n=3) for treatment B at 16DAH.

In Figure 3.11, the ratio of the larva body size (*TL* and *W*) to stomach area (*ST* and *SW*) are displayed in each sampling. Larvae from treatment A consistently achieved a higher ratio value signifying that the larva stomach area was much smaller than the body size. Larvae from Treatments B and C achieved similar ratios until 16DAH where treatment C was statistically significant (p <0.05).

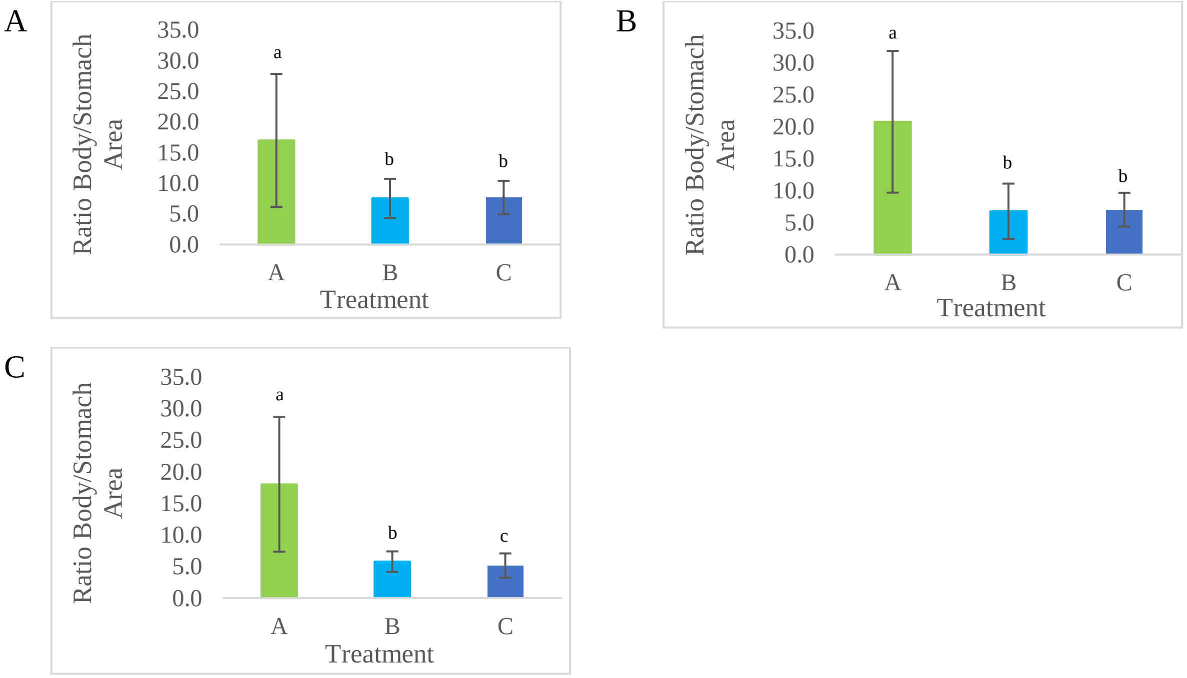


Figure 3.11. The ratio of body size to stomach area at 8 (A), 12 (B), and 16DAH (C) larvae for treatments A, B, and C. Values are presented as means ± standard deviation. Differing letters indicate statistical significance between the treatments (p <0.05). Replicates were (n=3) for 8, and 12DAH for all treatments and (n=2) for treatments A and C and (n=3) for treatment B at 16DAH.

The ratio between *TL* and *POAL* is shown in Figure 3.12 which shows that larvae from treatment A had a higher ratio value signifying that the *TL* was much longer than the *POAL* and was statistically significant at every sampling point (p <0.05).

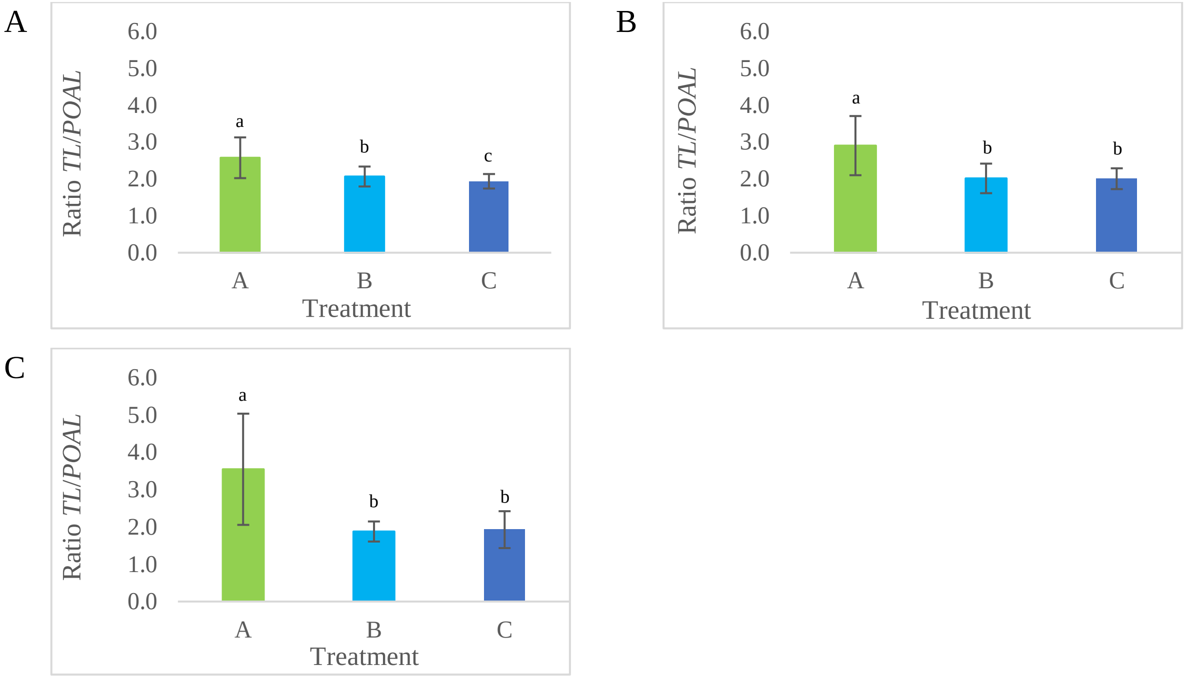


Figure 3.12. The ratio of *Total Length* to *Post Oral Arm Length* at 8 (A), 12 (B), and 16DAH (C) larvae for treatments A, B, and C. Values are presented as means ± standard deviation. Differing letters indicate statistical significance between the treatments (p <0.05). Replicates were (n=3) for 8, and 12DAH for all treatments and (n=2) for treatments A and C and (n=3) for treatment B at 16DAH.

3.2.7 Rudiment Length (*RL*) and Rudiment Width (*RW*)

The rudiment length (*RL*) and the rudiment width (*RW*) were measured at 21DAH. Larvae from treatment A had no visible rudiment formation and was excluded from analysis. Treatments B and C differed statistically (p <0.05) (Figure 3.13) with larvae from treatment C having more visible and developed rudiments.

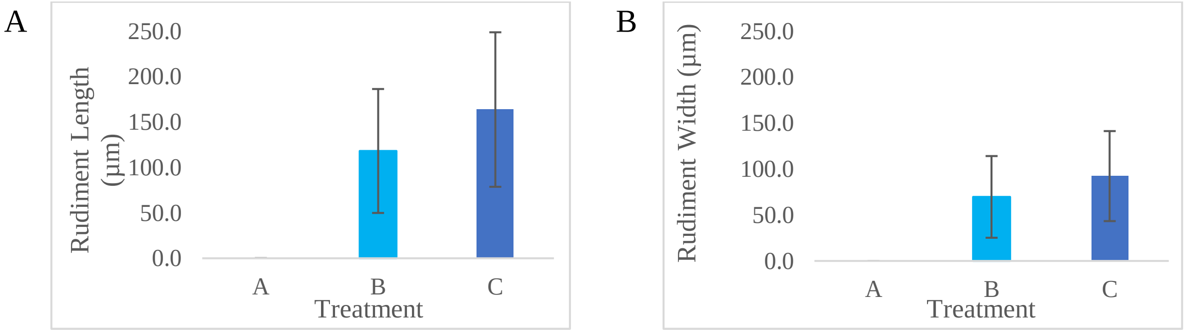


Figure 3.13. *Rudiment Length* (A) and *Rudiment Width* (B) mean measurements at 21DAH larvae for treatments B and C while treatment A was excluded. Values are presented as means ± standard deviation. The absence of letters indicates that no statistical significances were found between the two treatments (B and C) (p >0.05). Replicates were (n=2) for the treatments.

3.3 Feeding Incidence (*FI*)

Data collected for feeding incidence was collected at 8 and 16DAH. In both sampling days, and as seen in Figure 3.14, the prevalence of feed within the stomach of the larvae was significantly different ($p < 0.05$) between dietary treatments. More larvae from diet A presented an empty stomach when compared with larvae fed diet B or C. Larvae fed diets B and C were fairly consistent and varied slightly between sampling days with no statistical significance. However, sea urchin larvae feeding incidence increased between 8 and 16 DAH.

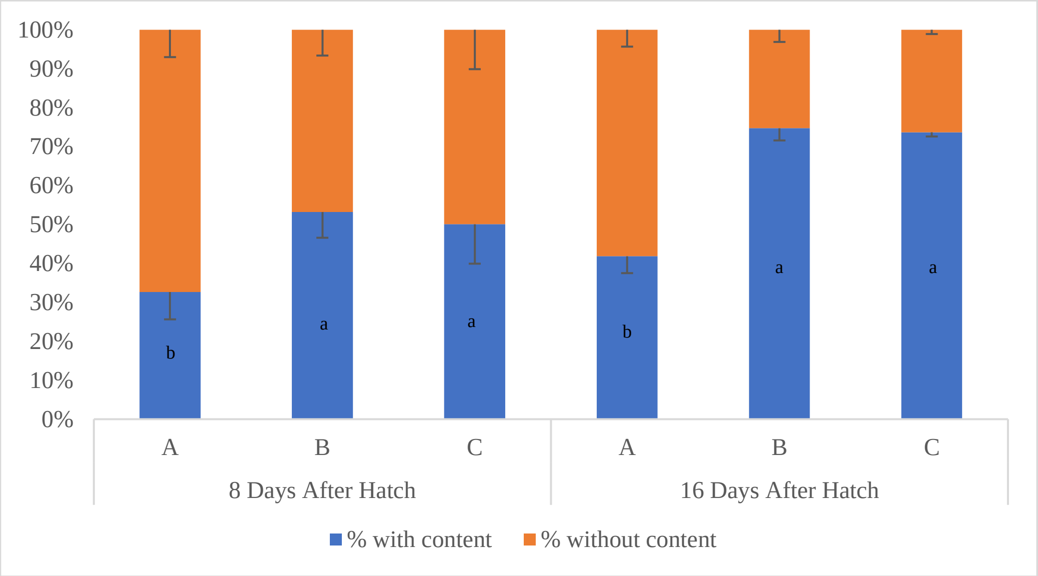


Figure 3.14. *Feeding Incidence* at 8 and 16 DAH between each treatment. Values are represented as means between treatments ± SD. All treatments at 8 DAH were n=3 where at 16 DAH Treatments A and C were (n=2) and Treatment B was (n=3). A χ^2 analysis was performed for each sampling day (8 DAH, X^2 (2, N=442) =14.8548, p= 0.000595 and at 16 DAH X^2 (2, N=325) = 30.8741, $p < 0.00001$, where ($p < 0.05$) is considered statistically significant. A Bonferroni post-hoc pairwise comparison was performed following the χ^2 analysis. Differing letters indicate statistical significance between the treatments ($p < 0.05$).

3.4 Survival

Survival measurements were acquired volumetrically at 4, 8, 12, and 16DAH. All treatments exhibited a decrease in the *SR* as the trial progressed. At 16DAH two tanks were lost from treatments A and C due to possible contamination by opportunistic organisms as many copepods and artemia were found within the tanks. All treatments followed a similar trend in *SR* and larvae from Treatment A concluded the trial with a *SR* of 8.0% ± 6.75%, larvae from Treatment B with 10.7% ± 15.64%, and larvae from Treatment C with 8.8% ± 5.63% at 16DAH. Figure 3.15 shows the *SR* trend between the treatment groups. No statistical significances were found between the groups at each sampling point ($p < 0.05$).

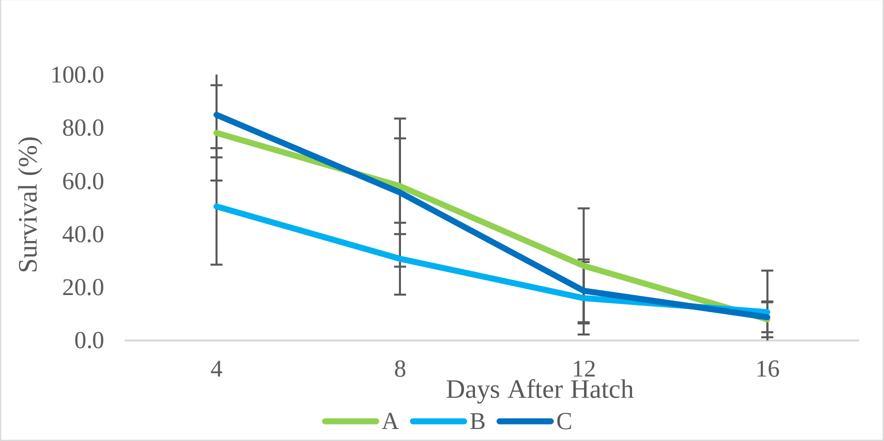


Figure 3.15. *Survival Rate* for all treatments at 4, 8, 12, and 16DAH. Values are presented as means ± standard deviation. An absence of letters indicates that no statistical significances were found between the treatment groups ($p > 0.05$). Replicates were ($n=3$) for 8, and 12DAH for all treatments and ($n=2$) for treatments A and C and ($n=3$) for treatment B at 16DAH.

3.5 Competency (*CR*)

Competency results were gathered at 21DAH which were based on the size of the rudiment compared to the stomach. Only Treatments B and C produced viable larvae, Treatment A had no larvae that were considered competent and for that reason was excluded. A ratio of the stomach area to rudiment area was used to determine larval competence. If the rudiment area was larger than the stomach area, the larvae was considered competent. No statistical significance was observed between the two treatments (X2 (1, N=141) = 0.898, p=.343316), probably due to the low number of competent larvae in both treatments. The *CR* for larvae from Treatment B was calculated at 1.14% ± 1.61% and the *CR* for larvae from Treatment C was calculated at 4.58% ± 2.75% (Figure 3.16) 21DAH.

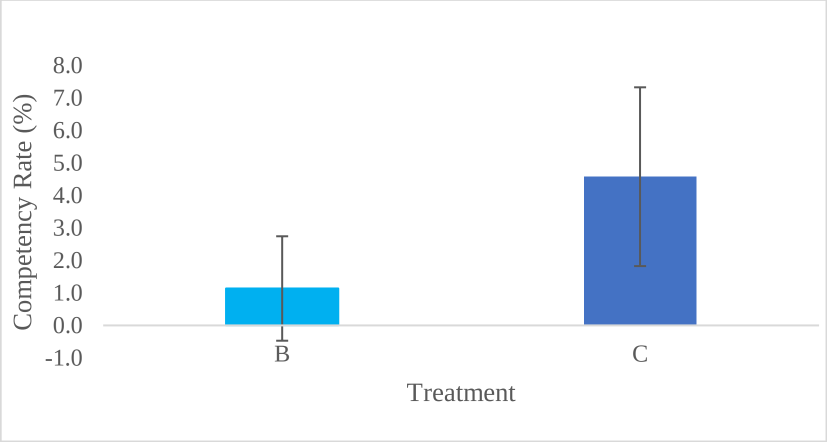


Figure 3.16. Sea urchin larval *Competency Rate* at 21DAH. Absence of letters indicate no statistical significances between the treatments ($p < 0.05$). Data was collected from the remaining viable tanks ($n=2$) from each treatment.

3.6 Settlement (*STR*)

Settlement data was gathered at 24, 48, 72, and 96 h after 21DAH to determine the settlement rate (*STR*). Treatment A was excluded from the data due to no larvae being competent at 21DAH. The *STR* of larvae from Treatment C was significantly higher ($p < 0.05$) compared to larvae fed diet B during each observation until 96 h post 21DAH (Figure 3.17). The final *STR* of the larvae from Treatment C after 96 h was calculated at 27.38% ± 5.66% whereas larvae from Treatment B only reached as high as 1.0% ± 0.71%.

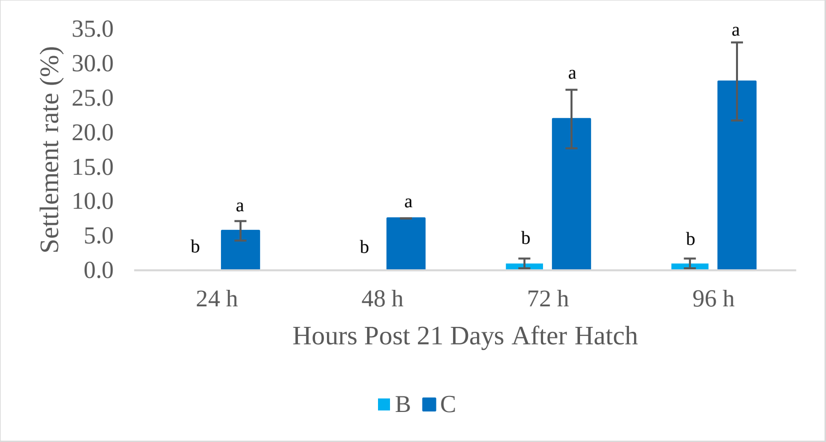


Figure 3.17. *Settlement Rate* for Treatments B and C (n=2) at 24, 48, 72, and 96 h following the 21DAH sampling. Values presented are means between the treatments ± SD. Differing letters indicate statistical significance between treatments where ($p < 0.05$) is considered statistically significant.

4. Discussion

The various sea urchin aquaculture studies that have been carried all have produced fertilization (FR) and hatching rate (HR) data. Survival and fertilization rate are usually quite high in this species and a good proxy for high quality larvae. Previous studies (Repolho, 2012; Kelly, 2015) calculated the *FR* at 99% and 98% respectively. In the present study the *FR* of 89% was slightly lower than comparative studies, however, the rate was still considered to be a high *FR*. Many factors can contribute to varying *FR*s and perhaps different rearing conditions and the gonadosomatic index GSI of the broodstock may have influenced the slightly lower *FR* (Levitan *et al.*, 1991).

The *HR* of the present study varied significantly between the tanks and therefore the average *HR* of the entire population was considered while calculating various measures such as survival. The *HR* of this study was consistently lower than similar studies. One study produced a *HR* of 90% ± 1% (Lie *et al*., 2007a) and another study observed values over 85% (Kelly *et al*., 2015). One further study reported a *HR* of an even lower value of 82% (Carboni *et al*., 2012). A *HR* of 75% in this trial was considerably lower in the trial at hand and various fluctuations in water temperature and/or dissolved oxygen may have influenced these lower rates possibly due to daily and nocturnal fluctuations as daily oxygen and temperature readings were well within the target values during the incubation (Rahman, *et al*., 2009).

Few studies have been carried out to test the impacts of formulated inert diets in sea urchin larval growth and survival. Two studies by Liu *et al*. (2007a; 2007b), tested the efficacy and plausibility of formulated larval diets in two sea urchin species; *Paracentrotus lividus* and *Psammechinus miliaris*. In each study, the researchers tested various diets which included two formulated inert diets and a microalgal paste. In those studies, the inert diets were originally formulated for shrimp larvae as a sea urchin specific larval feed has, to date, never been formulated. Perhaps the most conducive data collected in which to determine the efficacy of a feed are the growth parameters. A microalgal diet blend was most often used as a control as it was in the present study. Throughout the trial and until 16DAH the two experimental inert diets had more of an impact on larval total length (*TL*) as larvae from Treatments B and C outperformed the larvae fed the microalgae blend (Treatment A) which in turn promoted larger larvae. For instance, the *TL* of *P. lividus* was measured at various stages of the larval period and the inert diets promoted a larger larval *TL* than the microalgal paste in a similar fashion to the present study (Liu *et al*., 2007a). Both inert diets (Treatment B and C) promoted a higher larval *TL* than the diet fed to the larvae of Treatment A. The final recorded *TL* in this work was recorded at 16DAH. Comparing the average *TL* from this study to the study by Liu *et* al. (2007a) shows similarities where the average larval *TL* was around 600 µm in each trial for the microencapsulated feeds.

Another study examined the efficiency of the same microencapsulated diets as the study by Liu *et al*. (2007a) but using *Psammechinus miliaris*. Again, in a similar aspect to the present study and the aforementioned study, larvae fed the microalgal paste showed a much slower growth than the larvae fed inert diets. The *TL* of the larvae from another study followed a similar pattern as the present study where the inert diets showed larger lengths and no statistical significances between them (Liu *et al*., 2007b). The first study to ever utilize a microencapsulated feed for sea urchin larvae found that the inert diets used, despite encouraging smaller growth compared to a microalgal diet still produced sizeable and normally developed larvae (George *et al*, 2004). This study used *Lytechinus variegatus* which further confirms that the microencapsulated diets, although not specifically formulated for sea urchin larvae, can be used as an adequate feed for positive larval growth and development (George *et al*., 2004). A study claims that although the inert diet alone can be used solely as an adequate feed for growth and development, the feeding of a microalgae along with the inert diet showed the largest larval *TL* of *P. lividus* compared to the sole use of microalgae or a microencapsulated feed (Repolho, 2012). Although the production of each feed separately would need to be required, a diminished amount of each would be necessary which could in turn reduce production costs. The possibility of a co-feeding regime may be further studied in future trials not only for efficiency but for economic feasibility as well. Based on findings from this trial, the use of a frozen microalgal blend and an inert diet may promote even better growth, survival, and metamorphosis, however, the use of different species of microalgae for feedings should also be explored further to determine the most effective species to be included in a diet.

The aforementioned studies also measured the larval width (*W)* between the dietary treatments. Much like the data for *TL* in this study, the *W* was higher in the larvae fed microencapsulated feeds (Treatments B and C) as opposed to the microalgal blend (Treatment A). In a study regarding both *P. lividus* and *P. miliaris*, the larval *W* showed no differences between the inert diet treatments and proved to be much wider than larvae fed the microalgal paste (Liu *et al*., 2007a, 2007b). A positive correlation between *TL* and *W* was observed between all treatments in every trial performed indicating uniform growth. Comparing the *W* of *P. lividus* in this trial and the larvae from a study by *Liu et* al. (2007a) with the same species, the *W* in this study of larvae fed with the microencapsulated diets proved to be much less than the larvae in the compared study. The average *W* was around 200 µm for the inert diets whereas in the aforementioned trial, the average *W* was around 400 µm at the same age (Liu *et al*., 2007a). The larval weight for the microalgal paste blend was not included in the aforementioned trial for comparison. Perhaps a difference in diet composition or a rearing technique may have caused the discrepancy between the vastly differing *W* measurements between the trials. Another trial shows the average *W* at 16DAH comparable to this study however, as with *TL*, the diets containing a mixture of the inert diet and microalgal diet (*D. tertiolecta*) produced larvae with the average *W* of more than 350 µm (Repolho, 2012). Perhaps larval nutritional requirements change within the short 21-day larval period and providing an adequate diet can produce larger larvae. Nonetheless, the microencapsulated diet alone can promote good growth rates and development in larvae as this trial and other trials have exhibited.

Arm number (*AN*) is a good indicator of larval growth and can indicate the onset of metamorphosis and, if adequately fed, can roughly indicate the age of the larvae (Mendes *et al*., 2019). In this trial, larvae fed the inert diets of Treatments B and C had more developed arm pairs than Treatment A. Other studies also found that larvae fed with the algal paste had less developed arms than larvae fed with the inert diets (Liu *et al*., 2007a, 2007b). It has been speculated that at about 16DAH, *P. lividus* larvae are commonly found with four pairs of legs (Mendes *et al*., 2019). However, in the present study, only a fraction of the sampled larvae was found to have six legs. The majority of larvae from each treatment had four legs and larvae from Treatment C were the most developed of the treatments with more larvae having at least six legs. The discrepancy between the performed trial and other studies indicates that a feeding regime discrepancy or a difference in diet size and composition could be the culprit as George *et al.* (2004) claims that larger and more arm pairs indicate a more nutritionally balanced diet. A reduction in *AN* has been identified as a normal event in sea urchin larvae but just prior to metamorphosis (Liu *et al*., 2007a). Since the samplings in the current trial ended at 16DAH for *AN*, a reduction in *AN* was not seen across the treatments indicating that at this point in the trial, metamorphosis had not yet begun.

The post oral arm length (*POAL*) is also a good indicator for larval growth and development much like *AN*. Diet can have a major effect on the growth and overall length of the *POAL* (Hart and Scheibling, 1988). *POAL* generally increased among all treatments throughout the trial. Larvae from Treatments B and C had much larger *POAL*s than larvae fed diet A. Studies have shown that just prior to metamorphosis, a reduction in the *POAL* occurs as was seen in data regarding *AN*. Measurements of *POAL* stopped at 16DAH and no decrease in length was found between the treatments. Trials using *P. lividus* and inert diets have seen *POAL*s reach lengths of around 225 µm and around 175 µm when fed an algal diet (Liu *et al*., 2007a). The marine inert diet treatment (Treatment C) in the performed trial produced larvae with the highest *POAL*s of over 300 µm which is larger than the findings of the mentioned study. Treatment A produced larvae with the lowest average *POAL* of about 180 µm. In both studies, the algal treatment produced larvae with a smaller *POAL*. The arms are directly involved in the locomotion and capture of feed and as seen in the feeding incidence (*FI*), larvae from the microalgae blend treatment had a much lower *FI* than those of the inert diets which could show a relationship between a shorter *POAL* and decreased *FI*.

A study has been carried out regarding the *POAL* in pre-feeding larvae which could explain why the *POAL* was in fact shorter in the microalgae treatment as opposed to the inert diet treatments. Larvae exposed to algae in the early stages often exhibited a shorter *POAL* due to feed-induced dopamine signaling. It is believed that during times of feed abundance, the larvae can reduce their feed acquisition potential and henceforth the *POAL* may be reduced (Adams *et al*., 2014). The larvae fed inert diets did not show a decrease in *POAL* but were also fed an abundance of feed. The shortening of the *POAL* could be a sign of overfeeding (Kelly *et al*., 2000). The feeding of each diet was performed just once daily and it was observed that the microalgae diet seemed to stay in the water column until the following feeding whereas the inert diets did not. Perhaps this movement of feed may have influenced the actual abundance of feed within the tanks over a period of time and may have rendered the inert diets less abundant than the microalgae treatment.

FI is a good indicator of acceptance of feed and utilization. At 8 and 16DAH, the *FI* was analyzed and recorded and it was found that larvae from Treatments B and C had a much higher *FI* than larvae from Treatment A. The *FI* across all treatments increased from 8 to 16DAH although the largest increase came from the treatments using the microencapsulated diets. In other trials, the poor acceptance of feed may be caused by a lack of and/or poor chemical cues, nutritionally deficient in essential nutrients, feed availability, particle size, biotic factors, and/or a lack of buoyancy in the microencapsulated feeds (Kelly *et al*., 2000; Liu *et al*., 2007a). Although the microencapsulated feeds in the present trial had good buoyancy, the feed did fall out of the water column after some time. Despite the sinking feed the inert diets were much more readily accepted than the microalgal blend as seen in the data for the *FI*. Another possibility is due to the fact that the microencapsulated feeds were more nutritionally favorable in the early stages of development. The particle size may have been easier to digest and more readily assimilated in the gastrointestinal tract (Liu *et al*., 2007b). As *FI* was higher in the larvae fed the inert diets, the larvae grew to larger sizes and larval structures were much larger. This could indicate that the inert diets were more nutritionally balanced and appropriate to the larval stages than the microalgae blend despite the abundance of feed available. Selectivity of the feed may also be a factor. Despite the high quantity of microalgae feed available, the larvae seem to have more of an affinity to the inert diets due to chemical cues and/or palatability issues.

A major growth indicator for sea urchin larvae pertains to the stomach length (*ST*) and stomach width (*SW*). In the performed trial, *ST* and *SW* followed a similar growth pattern between the treatments. However, Treatments B and C showed a much larger *ST* and *SW* than Treatment A for the duration of the trial. It seems that in Treatment A, the stomach size remained relatively stagnant in growth and did not increase in width until after 16DAH. The stagnant growth could be due to a nutrient deficiency in the microalgal blend. In another trial using *L. variegatus*, it was found that larvae fed an inert diet showed larger stomachs (George, 2004). This finding is in accordance to the findings observed in *P. lividus* larvae. In two studies, the microalgal paste treatment showed a constant stomach shrinkage in both trials (Liu *et al*., 2007a, 2007b). The larvae from Treatments B and C in the present study showed a stomach shrinkage after 16DAH however still they were considered quite large. This was around the time of the onset of metamorphosis and perhaps the stomach shrinkage was the result of metamorphological changes occurring in the larvae as the *TL* and *W* were relatively large during this larval stage. The stomach is the only larval organ retained by sea urchins through metamorphosis and into adulthood and it serves as a primordium to the juvenile gut (Burke 1981; Miner 2005). A large stomach, as seen in Treatments B and C during the larval stages is important for the ability to metamorphosize into the next life stage (Strathmann *et al*., 1992; Cárcamo *et al*., 2005).

Various body measurement ratios were calculated throughout the trial. No other study to date has done a comparative analysis on the body ratios studied during this trial. The ratios calculated aid in explaining the growth of the larvae between the treatments. The ratio of *W* and *TL* to *AN* (*W*/*TL*:*AN*) describes the growth and development of the larvae biometrically while taking into account the number of arms the larvae possess. Up until 12DAH, no differences were seen between the treatments. At 16DAH, the only differing treatment was Treatment C which had a higher ratio value. This value indicates that compared to the other treatments, this treatment on average had a greater *AN* when the *W*/*TL* ratio was larger. Therefore, the larger larvae had more pairs of arms. This finding indicates that perhaps the microencapsulated diet of Treatment C was more suitable than the other diets for larval growth and consequently produced larger and more developed larvae. Evaluating the results from additional studies, the treatments fed the inert diets produced larvae with a larger *TL* and *W* while at the same time had more arm pairs than the treatment fed the microalgal paste (Liu, *et al*., 2007a, 2007b).

The ratio of body area (*BA*) to stomach area (*SA*) (*BA*:*SA*) took into account the *TL* and *W* for the *BA* and the *ST* and the *SW* for the *SA*. Consistently throughout the trial, Treatment A had a much higher ratio value indicating that the stomach was considerably smaller than the body size which in turn signified a less developed larva. Treatments B and C only differed at 16DAH where Treatment C had a smaller *BA*:*SA* ratio indicating that the stomach and body size were proportional. Both of these treatments had rather large stomachs when the photos were analyzed and the stomach itself occupied a large area within the body. The larger stomach of the larvae allowed for an improved feed utilization throughout the trial and allowed the larvae to develop more mature larval structures such as the rudiment. Due to the larger *SA* and larger *BA* to allow for a larger stomach, the formation of the rudiment itself may indicate that some diets are better utilized than others (George *et al*., 2004). This allotment for the allocation of the essential nutrients from the inert diets, which had higher protein and lipid levels than the microalgal blend, may have promoted the growth of adult larval structures in Treatments B and C as opposed to the continued growth of basic larval structures as was seen in Treatment A.

The *TL*:*POAL* ratio essentially measured symmetrical growth between these two growth parameters. Treatment A consistently had a high ratio between *TL* and *POAL* throughout the trial whereas Treatments B and C had fairly low ratios. A lower ratio indicated that the length of the body was proportional to the *POAL*. Taking all aforementioned parameters into account thus far, these findings further confirm that the larvae from Treatment A not only were smaller in size than the other two treatments, but they were also much less developed in terms of more mature larval structures such as the number of pairs of arms, the stomach size, and the rudiment size. However, comparing the two inert diets, larvae from Treatment C were more developed and had better growth than larvae from Treatment B.

In regards to rudiment size and development, the previously discussed data confirms as to why larvae from Treatment A did not develop a rudiment. The rudiments in Treatments B and C mostly developed around 21DAH. Rudiments have been seen to commonly develop in *P. lividus* around 18DAH (De la Uz, 2013). The formation of the rudiment, however, signifies that the inert diets do contain the nutrients essential for this process of development, but the delay in rudiment formation compared to other findings may indicate that the inert diets are not as nutritionally inclusive as they could be. Various sea urchin species begin to form the rudiment at different periods of the larval stage. Therefore, comparing the findings from this study to the study by Liu *et al*. (2007a) using *P. lividus* is most appropriate. Larvae from the treatment fed the algal paste in the aforementioned study did not promote rudiment development much like the results from Treatment A in this study (Liu *et al*., 2007a). Larvae from the inert diets from the study had developed a rudiment as early as 14DAH compared to 21DAH in this study. Perhaps differing rearing conditions were utilized between the trials and the diet compositions most definitely varied. For example, the inert diets in the study had higher protein and lipid contents than the microalgal diet and algal paste diet (Liu *et al*., 2007a). The protein and lipid contents of the inert diets in the present trial also were higher than in the microalgal blend.

Rudiment size varied between Treatments B and C in this study. The larger rudiment length (*RL*) and rudiment width (*RW*) in Treatment C is congruent with the other findings related to growth and development. The diet of Treatment C outperformed all of the other diets in terms of growth and development. Findings by George *et al*. (2004), found that the rudiment size in *L. variegatus* did not vary between the microalgal diet and the inert diets. Furthermore, in *P. miliaris* the live cultured microalgae diet of *D. tertiolecta* produced larvae with larger rudiments than those fed on inert diets, however, no rudiment formed in larvae fed the microalgal paste (Liu *et al*., 2007b). In regards to *P. lividus,* larvae fed the inert diets were shown to have larger rudiments since larvae fed the microalgal paste did not form a rudiment (Liu *et al*., 2007a). Due to the vast differences in regards to rudiment sizes and diets between species, it can be speculated that different species of sea urchins, although closely related, may require a completely different diet to meet nutritional needs to optimally support growth and development into metamorphosis.

The survival rate (*SR*) of the larvae between the treatments in this trial did not vary significantly. In comparison to other studies, whether using inert diets or solely microalgae, the *SR* was much lower in this trial than other findings. In the early stages of the trial, *SR*s were rather high but a steep and consistent decline occurred after 4DAH. The highest *SR* in this trial at 16DAH was 11%. *SR* at 21DAH was excluded due to losses of entire tanks and *SR*s well below 1%. *SR* up until metamorphosis in other trials using sea urchin larvae have been recorded at 72% (George *et al*., 2004), 68-76% (Liu *et al*., 2007a), 60-68% (Saito, 1992), and 47% (Jimmy *et al*., 2003). In each of these aforementioned trials, *SR*s did not differ statistically depending on the diet fed whether inert or microalgal. Since no statistical significances were present between the treatments in this trial, the diet, although having had a major impact on growth and development, did not have the same impact on survival. The vast differences in *SR* between this trial and other trials using sea urchin larvae may not be due to diet but rather to the larval rearing method used. Most sea urchin larval studies include static water systems; however, one study saw an increase of 15% when a flow through system was used as opposed to the traditional static system (Carboni *et al*., 2012). Contamination may have also drove the *SR* down in this trial as copepods and other zooplankton were found in the majority of the tanks at the end of the trial and may have contributed to the total loss of two of the tanks at 16DAH. Another study also lost two tanks due to contamination during the trial, however, a high *SR* of 72% was still attained (George *et al*., 2004).

Various feeding frequencies have been tested in trials from various studies (Gomes *et al*., 2021; Liu *et al*., 2007a, 2007b). Perhaps testing various feeding frequencies with the same diets would affect the *SR* since only a daily feeding was performed in this trial. Furthermore, the tanks utilized had a flat bottom and accumulated uneaten feed which altered the water quality and may have in turn lowered the *SR* despite daily water exchanges. Since the larvae are microns in size, an effective cleaning method was difficult to propose. Cylindroconical tanks may prove to be easier to clean and a more efficient tank to use (George *et al*., 2004). Seawater contamination was an issue throughout the trial as the water used was only filtered and thermally regulated prior to use. Perhaps the sterilization of the seawater prior to use and the combined aforementioned factors would decrease zooplanktonic contamination and in turn produce higher *SR*s.

Competency rate (*CR*) and settlement (*STR*) are closely related parameters which are good indicators of nutritionally adequate feeds. In the present trial, the *CR* was determined at 21DAH based on the rudiment size compared to the stomach size. In Treatment A, no larvae were considered competent and therefore no larvae had settled. In Treatments B and C larvae were considered competent and had developed rudiments. Similar results were found in a study where the larvae were fed inert diets (Liu *et al*., 2007a) in which larvae had not developed a rudiment until at least 20DAH. In the study, *CR*s reached as high as 18% compared to 6% found in the present study. Despite the low *CR* between Treatments B and C, the *STR* in Treatment C was rather high (28%) compared to Treatment B (1%). This finding may indicate that the diet used in Treatment C provided the essential nutrients to promote settlement as opposed to the diet in Treatment B despite an almost identical composition apart from the raw ingredients used. The inert diets and the *D. tertiolecta* diet in another study produced *STR* as high as 66% (Liu *et al*., 2007b). In each study, the *STR* increased dramatically between 48-72 h after placing the larvae on the conditioned plates. The difference in *STR* between the two trials could be due to different rearing conditions, diets, and the actual conditioning method of the settlement plates. As the present trial concluded following the larval stage, it would be interesting to see if the experimental diets had an effect on juvenile growth and survivorship into adulthood.

Improvement of the feed and feeding protocol can be studied in further trials with sea urchin larvae. A combination of microencapsulated feeds and small amounts of microalgae may be used in conjunction to promote better larvae growth and metamorphosis. Nutritional requirements of sea urchin larvae are understudied and offering a variety of diets may influence the success of the diet. In *Tripneustes gratilla*, adding algae feeding stimulants to inert diets increased the palatability and perhaps the addition of small amounts of a microalgal diet may increase feed intake and acceptance of the diet (Dworjanyn *et al*., 2007). Feeding frequency may have an impact on larval growth as well and perhaps offering more than one feeding per day could have a positive influence on growth, metamorphosis, and survival (Strathmann, 1971).

5. Conclusions

The findings from this study of the novel microdiets for the larvae of *Paracentrotus lividus* conclude that microencapsulated diets can be a replacement for the traditional microalgal diets normally utilized in zooplanktonic sea urchin larvae. A microencapsulated diet can promote good growth rates, promote metamorphosis and settlement, and can sustain the larvae until the pre-juvenile stages. A formulated diet allows for the manipulation and precise inclusion of specific nutrient compounds. The use of microdiets in sea urchin larvae rearing can reduce labor and production costs and can streamline and standardize the production process which in turn would be more economically and logistically beneficial for producers.

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