Research and development of hatchery techniques to optimise juvenile production of the edible Sea Urchin, *Paracentrotus lividus*

CARBONI STEFANO

FEBRUARY 2013

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

INSTITUTE OF AQUACULTURE

UNIVERSITY OF STIRLING

DECLARATION

This thesis has been composed in its entirety by the candidate, except where specifically acknowledged. The work described in this thesis has been conducted independently and has not been submitted for any other degree.

CANDIDATE NAME: ……...

SIGNATURE: ……...

DATE: ……...

SUPERVISOR NAME: ……...

SIGNATURE: ……...

DATE: ……...

Abstract

Research and development in aquaculture has supported the knowledge-based development of the sector over the last decades. In particular, species diversification is playing an important role to ensure sustainability of the industry and helping to reduce pressure on wild stocks of those aquatic species for which farming technology is still at the early stages. Due to the increasing pressures on more traditional carnivorous marine finfish species (aquafeed reliance on fishmeal and fish oil, environmental impact, market price) low trophic organisms are receiving more attention to provide sustainable alternatives and integrate production activities with the aim of reducing environmental impacts and to provide secondary high value crops. Integrated Multi-Trophic Aquaculture (IMTA) systems are therefore at the forefront of innovation in the industry. Several invertebrate species have been investigated and tested as integral part of IMTA (mussels, oysters, abalone and macroalgae) and echinoderms have also been considered as good candidates for the future development of this technology. In order to allow for a more widespread uptake of integrated aquaculture, several technical and biological challenges need to be overcome, including a reliable supply of juveniles. In recent years, this has prompted investigation on Echiniculture as a whole and on hatchery technologies in particular. This PhD investigated key constraints in edible sea urchin (*Paracentrotus lividus*) juvenile production with the aim to improve commercial sea urchin hatchery outputs.

The research firstly focused on larval nutrition (Chapter 3 and 4) and specifically tested the hypothesis that larvae required higher dietary inputs of long chain fatty acids than those provided by *Dunaliella tertiolecta*, a microalgae species widely used in echinoderm larval rearing. Fatty acid composition of *P. lividus* eggs, investigated in Chapter 3, supported this hypothesis, which was further confirmed by the results

obtained in Chapter 4 where microalgae (*Cricosphaera elongata*, *Pleurochrisis carterae* and *Tetraselmis suecica*) with a more balanced fatty acid profile, in particular richer in long chain fatty acids, were employed. This resulted in a significantly improved larval development and survival. Results also indicated that these alternative microalgae species could be successfully grown without modification of the microalgae production protocols in the hatchery where the experimentation had taken place.

The third experimental chapter compared static and flow through systems which provides more stable water quality through constant water exchange and reduces larval handling and associated stress. Results indicated that larval survival was significantly improved by the flow-through system and the need for tank cleaning was reduced (three versus seven times per larval cycle when using flow-through and static rearing systems respectively). However, water quality, based on the parameters assessed (NH_4, PO_4^{-3}, NO_2 and NO_3), did not show any significant differences between systems. Reduced handling could have therefore played the most important role in promoting larval survival. Both these trials resulted in a significant 5 to 20 % increased survival. A follow-up study, combining flow-through with more suitable microalgae, should be carried out and could result in even further enhanced survival.

Then, chapters 6 and 7 focused on broodstock nutrition and subsequent improvement of gamete quantity and quality. These two trials aimed to explore and describe the biological effects that some important nutrients, such as proteins, lipids, fatty acids and carotenoids, have on urchins' somatic and gonadal growth, gonad biochemical composition during gametogenesis, fecundity and maternal provisioning to developing embryos. Results from the experiment described in Chapter 6 indicated that higher protein content can improve somatic growth in *P. lividus* adults and that more expensive, protein-, lipid- and energy-rich diets do not significantly enhance fecundity

or offspring performance. Results, moreover, highlighted the need for a specifically formulated broodstock diet and gave some insights into what its composition should be, especially in relation to carotenoids. In Chapter 7, fatty acid profiles of *P. lividus* gonads throughout gametogenesis were studied for the first time. It was observed that, among Long Chain Polyunsaturated Fatty Acids (LC-PUFAs), Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA) are primarily accumulated during gametogenesis, whilst Arachidonic acid (ARA) appears to be independent of dietary input. In addition, it was clearly shown that ARA is the only LC-PUFA accumulated in the eggs along with Non Methylene Interrupted Fatty Acids (NMI FAs). As well as looking at the biological effects of different diets on fatty acid profiles of gonadal and larval tissues, the work also expanded on a more fundamental level to explore the metabolic pathway through which precursors could be used by sea urchins for the endogenous production of long chain fatty acids (Chapter 8). Three Expressed Sequence Tags (ESTs) for putative fatty acyl desaturases, one of which was closely related to *Octopus vulgaris* Δ5-like fatty acyl desaturase, were identified. The newly cloned putative desaturase of *P. lividus* possessed all typical features of other fatty acyl desaturases. However, because of time constraints, functional characterisation, originally planned, of the new protein could not be performed and further research effort is needed to investigate this important aspect of sea urchin physiology.

Overall, the aim of this research project has been achieved as it provided a set of exploitable results and protocols to improve hatchery practices for the production of *P. lividus* juvenile. However, more research is required to investigate some of the underlying mechanisms behind the observed biological effects such as delay in larval development when *T. suecica* was used as larval feed, increased broodstock fecundity,

improved larval survival in the flow-through system and higher gonadal concentration of some fatty acids (mainly DHA) than provided in the feed.

Acknowledgments

I thank my wife, Marilisa, for her restless support, for the coffee in the morning, for her patience in all my outbursts, for listening, for talking and for silence… for our daughter, Eloisa. I thank my parents and brother Mila, Andrea and Claudio for the encouragement and for believing in me every time I couldn't.

I am thankful for the constant support and direction from my two academic supervisors Prof. Herve Migaud and Prof. Douglas Tocher who had showed me both ends of perfection.

I thank Adam Hughes, colleague and friend, for many stimulating conversations and for listening in difficult times. I am grateful to Julien Vignier, he knows why, but mostly for reading the gospel in Italian at my wedding.

I will always have eternal debt of gratitude with Tim Atack, mentor, and to all the staff at the Ardtoe Marine Laboratory: Jim, Roman, Duncan, Less, Hazel, RossAmy and Mark. To Jon Sherwood for all I know about larval rearing I've learned it from you and to Monte for endless table tennis.

I would also like to thank Mariachiara Chiantore and all the other ENRICH partners, in particular: Muki Shpigel and Hanna Rosenfeld.

I thank all who I had the pleasure to meet over the last four years: Beppe, Andy, Benedict, Bridie, Rogelio, Eric, John, Andrew, Elena, Kat, Debby, Fiona and James and those I have always known: Marcello, Nana and Daniele.

I have known most of you half as well as I would like and liked half of you half as much as you deserved.

Table of Contents

“NOT ALL
THOSE
WHO
WANDER
ARE LOST”

J.R.R. Tolkien

To Amelia…

List of Figures and Tables

Figures:

Figure 1.7. Diagrammatic representation of the sea urchin reproductive system in aboral side view; (b) Diagrammatic representation of the tissues in the sea urchin gonad wall, after Strenger (1973); (c) Lobe of a sea urchin gonad stained with phalloidin to show muscles on the exterior surface of the GHS of the inner sac (white strips). Abbreviations: A – anus; CTL – connective tissue layer; GL – gonad lumen; M – madreporite; NP – nutritive phagocytes; GCS – genital coelomic sinus; GHS – genital hemal sinus; TF – tube feet; VP – visceral peritoneum. (Extracted from Walker et al., 2007)

Figure 1.8. Histology of ovary (a) Stage I: cross-section through ascinus of recovering ovary showing periodic acid Schiff-positive globules (arrowheads) derived from lysis of relict oocytes; extensions of nutritive phagocytes (NP) project into lumen; small previtellogenic oocytes (PO) occur along ovary wall. Testirs (b), Stage I: crosssection through ascinus of recovering testis containing relict spermatozoa (R) and nutritive phagocytes (NP) which form an eosinophilic meshwork. (Extracted from Byrne, 1990)

Figure 1.9. Histology of ovary (a) Stage II: growing ovary with early vitellogenic oocytes (EV) and nutritive phagocytes; N: nucleus. Testis (b) Stage II: columns of spermatocytes project centrally (arrowheads) in growing testes, nutritive phagocytes fill ascini. (Extracted from Byrne, 1990)

Figure 1.10. Histology of ovary (a) Stage III: premature ovary with oocytes at all stages of development; nutritive phagocytes surround vitellogenic oocytes (VO) which detach from ascinal wall, and ova (O) accumulate in the lumen. Testis (b) Stage

III: premature testis with spermatozoa (S) in centre and nutritive phagocytes around periphery. (Extracted from Byrne, 1990)

Figure 1.11. Histology of ovary (a) Stage IV: mature ovary packed with ova, nutritive phagocytes are reduced to a thin layer along ascinal wall.Testis (b) Stage IV: mature testis filled with spermatozoa and largely devoid of nutritive tissue. (Extracted from Byrne, 1990)

Figure 1.12 Histology of ovary (a) Stage V: partly spawned ovary but still in Stage III condition, with oocytes at different stages of development and nutritive phagocytes; most vitellogenic oocytes will eventually mature and move to the lumen. (b) Ovary at Stage V: partly spawned ovary with loosely packed ova and a scarcity of nutritive material; except for the empty spaces left by spawned ova, ovary is similar to Stage IV. Testis (c) Stage V: partly spawned testis with spaces left empty by spawned spermatozoa. (Extracted from Byrne, 1990)

Figure 1.13. Histology of ovary Stage VI: spent ovary largely devoid of ova and nutritive phagocytes; all vitellogenic oocytes and relict ova will be resorbed (a). Ovary intermediate between Stages VI and I, with relict ova undergoing lysis (L); lysed material is taken up by nutritive phagocytes. Testis (c) Stage VI: spent testis largely devoid of spermatozoa or nutritive material. (Extracted from Byrne, 1990)

Figure 1.14. Reproductive endocrine axis in vertebrates and sea urchin. The lack of a central nervous system in sea urchin makes it difficult to investigate the middle steps of the pathway transducing environmental cues into sex steroids production, which control reproduction. (H. Rosenfeld pers. Comm.)

Figure 1.15. Morphology of sea urchin sperm before and after acrosome reaction (extracted from Santella et al., 2012).

Figure1.16. Schematic representation of the four arms stage. Stomach, Anterolateral and Postoral arms in evidence. Modified from Smith et al., 2008

Figure 1.17. Early and late eight arms stage, with main features highlighted. Modified from Smith et al., 2008.

Figure1.18. Schematic drawing of the main characteristics of the Initial vestibule invagination stage (a) and initial development of the epaulettes structures (b).

Figure 1.19. Tissues forming the rudiment. Three tube-foot primordia project from the hydrocoel, pressing into the vestibular floor. The somatocoel is extended between hydrocoel and stomach. Modified from Smith et al., 2008

Figure1.20. View from left side of the larvae. Close-up of rudiment during the pentagonal disc stage; tube-foot primordia surrounds the radial canal. Modified from Smith et al., 2008

Figure 1.21. Schematic general appearance of the pentagonal disc stage pluteus. Modified from Smith et al., 2008

Figure 1.22. *P. lividus* behavioural sequence shown by competent larvae in rearing conditions .A) Swimming phase (near the air/water interface). B Sinking phase. C Control phase. D Ascending phase. (Extracted from Gosellin and Jangoux, 1998)

Figure 1.23. *P. lividus* Metamorphosis. After a positive recognition of the substratum during a control phase (A), the larva opens its vestibule, allowing the protrusion of the echinoidrudiment's spines and primary podia that hold in to the substratum (B), and transforms in less than 1 h into a spherical postlarva (C). (Extracted from Gosellin and Jangoux, 1998)

Figure 1.24. Schematic representation of the digestive system of *Paracentrotus lividus*. S= Stomach; I= Intestine; E= Esophagus; R= Rectum; S= Siphon. (Modified from Claerebout and Jangoux, 1985)

Figure 1.25. The biosynthesis pathway of long-chain and very long-chain polyunsaturated fatty acids from α-linolenic (18:3n−3) and linoleic (18:2n−6) acids. Enzymatic activities shown in the scheme are predicted from heterologous expression in S. cerevisiae of the Δ5 fatty acyl desaturase (Fad), the three Δ6 Fad (Hastings et al., 2005: Zheng et al., 2005; Monroig et al., 2010), and Elovl2 and Elovl5 elongases (Hastings et al., 2005; Morais et al., 2009). Steps catalysed by the newly characterised Atlantic salmon Elovl4 are also shown. Extracted from Carmona-Antoñanzas et al. (2011)

Figure 1.26. Echinenone molecular stracture.
http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?sid=99437372&loc=es_rss

Figure 1.27. Proposed metabolic conversion of dietary all-trans ß Carotene into 9'-cis-Echinenone according to Symonds et al., (2007). 1: all-trans ß Carotene; 2: all-trans isocryptoxanthin; 3: 9'-cis- ß Carotene; 4: all-trans Echinenone; 5: 9'-cis-

isocryptoxanthin; 6: 9'-cis-echinenone. A: Hydroxylation; B: Isomerisation; C: Oxidation.

Figure 3.1. Calibration curves used to correlate optical density and microalgae cell density

Figure 3.2. Survival rate of *P. lividus* larvae fed *D. tertiolecta*, *P. carterae* and *C. elongata* diets. Data are expressed as mean ± SD (n=2). Superscripts indicate significant differences.

Figure 4.4.2.1. Survival rate of *P. lividus* larvae fed *Pleurochrysis*, *Dunaliella* and *Cricosphaera diets*. Data are expressed as mean ± SD (n=3). Superscripts indicate significant differences.

Figure 4.1. Larval length (a), Post-oral arm length (b) and Body width (c) of sea urchin larvae fed different microalgae diets from 5 to 23 days after fertilization. Data are expressed as mean ± SD (n=3). Superscripts indicate significant differences between treatments at each time point.

Figure 4.2. The appearance of third pair of arms and larval rudiment for *P. lividus* larvae fed different diets. Data are expressed as mean ± SD (n=3). Superscripts indicate significant differences between treatment at each time point.

Figure 4.3. Total lipid content of *P. lividus* larvae fed four microalgae diets throughout development. Data are expressed as mean ± SD (n=3). Asterisks indicate significant differences between treatments.

Figure 4.4. Percentage of total saturated (A) and total monounsaturated (B) fatty acids of *P. lividus* larvae fed different diets throughout development. Values are given as percentage of total fatty acids (mean ± SD, n=3). Superscripts indicate significant differences between treatments at each time point.

Figure 4.5. Total n-3 (A) and n-6 PUFA (B) contents of *P. lividus* larvae fed different diets throughout development. Values are given as percentage of total fatty acids (mean ± SD, n=3). Superscripts indicate significant differences between treatments at each time point.

Figure 5.1. Survival rates of *P. lividus* larvae reared in either static (control) of flow through systems over the trial period up to competence for settlement. Data are expressed as mean ± SD (n=3). Superscripts indicate significant differences.

Figure 5.2. NH4, PO4-3, NO2 and NO3 concentrations in the microalgae culture used to feed P. lividus larvae over the trial period. Data are expressed as mean ± SD (n=3).

Figure 5.3. NH4, PO4-3, NO2 and NO3 concentrations in the rearing water taken during three following days after each feed over the trial period. Data are expressed as mean ± SD (n=3).

Figure 6.1. Growth expressed as Test Diameter (TD) over the trial period in the four diet treatments. Superscripts indicate significant differences (Mean ± SD, n = 2).

Figure 6.2. Gonadal Index (GI) measured at the end of the trial and expressed as the percentage of body wet weight in the four diet treatments. Superscripts indicate significant differences (Mean ± SD, n = 2).

Figure 6.3. Relative fecundity expressed as number of eggs per gram of female body wet weight in the four diet treatments. Superscripts indicate significant differences (Mean ± SD, n = 2).

Figure 6.4. Total Pigments and Echinenone content in gonads of urchins fed the four diets over the trial period. Superscripts indicate significant differences (Mean ± SD, n = 2).

Figure 6.5. Survival rate of urchins' larvae (up to competence) from broodstock fed the four dietary treatments (Mean ± SD, n = 3).

Figure 7.1. Gametogenic stages of the individuals under experimental conditions as observed during the trial period (n=2, 5 individuals/replicate/time point). Individuals from the two treatments were pooled.

Figure 7.2. Non-metric Multi Dimensional Scale (nMDS) plot for fatty acid composition of sea urchin mature gonads (n=2; 3 individuals/replicate), egg (n=2; 3 females/replicate) and embryos (n=3, approximately 5000 embryos/replicate) in the two treatments.

Figure 7.3. EPA (a) and DHA (b) content in gonads of urchins fed kelp or pellet diets throughout gametogenesis. Values of EPA and DHA are given as percentage of total fatty acids (mean ± SD, n=2; 10 individuals/treatment/stage). Superscripts indicated significant differences between treatments.

Figure 8.1. The evolutionary history was inferred using the Neighbor-Joining method (Saitou & nei 1987). The optimal tree with the sum of branch length =

13.13555584 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. The analysis involved 42 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 329 positions in the final dataset.

Figure 8.2. Image of a 1% agarose gel in which PCR products for *P. lividus* desaturase-like products 5a-c and 4 are shown.

Figure 8.3. The full-length nucleotide sequence of the putative Δ5-like desaturase of *P. lividus* with single letter translation of the presumed translated protein sequence.

Figure 8.4. Alignment of the deduced amino acid (aa) sequence of the newly cloned putative Δ5-like desaturase of *P. lividus*. Identical residues are shaded black. The cytochrome b5-like domain is dot-underlined and the three histidine boxes (HXXXH, HXXHH and QXXHH) are highlighted. The asterisks mark the heme-binding motif, HPGG

Tables:

Table 1.1. Classification of echinoids with representative genera (modified from Lawrence, 2007)

Table 1.2. Review of the proximate composition of urchin's feed used in recent growth trials.

Table 5.1. Microalgae *Dunaliella tertiolecta* ration given to the two treatments per each feed.

Table 6.1. Proximate compositions of the four diets tested. Ash, carbohydrate, fibre, protein and lipids are expressed as a percentage of the diet dry weight. Energy content is given per gram of diet dry weight. Pigments are expressed as ug/g of diet (mean ± SD, n = 3). Superscripts indicate significant differences whilst asterisks indicate values below detection limit.

Table 7.1 Formulated feed ingredients

Table 7.2. GLM outputs for analyses done on EPA and DHA data showing degrees of freedom, F and P values.

Table 7.3. Each fatty acid is expressed as a percentage of total fatty acids (mean ± SD, n=3). Ash, carbohydrates, fibre, protein and lipids are expressed as a percentage of the diet dry weight. Energy content is given per gram of diet dry weight. Asterisks indicate non-detected values and superscripts indicate significant differences between treatments.

Table 7.4. LC-PUFAs and NMI FAs in mature gonads, egg and embryos. Each FA is expressed as percentage of total fatty acids. Superscripts indicate significant differences between treatments within tissues (mean ± SD, n=2).

Table 7.5 Fatty acid profiles of the gonads during gametogenesis of urchins fed the two diets. Each fatty acid is expressed as a percentage of total fatty acids (mean ± SD; n=2; 10 individuals/treatment/stage). Asterisks indicate non-detected values.

Samples were taken at 30 days intervals. Superscripts indicate significant differences between stages within treatments (mean ± SD, n=2)

Table 8.1. Summary of *P. Lividus* specific EST's identified with high identity to reference vertebrate and invertebrate desaturase sequences.

Table 8.2. Primer sequences, and details of the PCR reactions employed

Chapter 1 - Introduction

1.1 Phylogeny and taxonomy of invertebrate deuterostomes.

The invertebrate deuterostomes consist of all deuterostomes outside Subphylum Vertebrata namely: Cephalochordates, Urochordates and Echinoderms.

The Cephalochordates, commonly known as amphioxus, include 28 species, grouped into two genera both relatively sedentary and sand dwelling filter feeders. Most developmental studies have been carried out on three species of the genus Branchiostoma.

The Urochordates or tunicates, are represented by 3000 species usually grouped into three classes: Ascidiacea, Thaliacea and Larvacea. The group of ascidians is the most numerous (2300 species) and has been most studied from a developmental prospective. They are benthic sessile filter feeders that develop via bilaterally symmetrical non-feeding larva. Unlike ascidians, thalaceans and larvaceans remain free-swimming throughout their life cycle (Bone, 1998).

Most present day species of invertebrate deuterostomes are members of the Phylum Echinodermata (7000 species). Adult echinoderms are bottom dwelling, radially symmetrical animals, but their larvae are free-swimming and bilaterally symmetrical.

Although exclusively marine, they were able to colonise their environment extremely successfully, have been well established in the oceans since the start of the Cambrian period (600 million years ago) and are now found at all depths and in all climates.

Modern echinoderms are grouped into five classes: Echinoidea (sea urchins, heart urchins and sand dollars), Holothuroidea (sea cucumbers), Asteroidea (starfish), Ophiuroidea (brittle stars), and Crinoidea (feathers stars and sae lilies).

Until recently, morphological and molecular data suggested that, among the invertebrate deuterostomes, Echinoderms and Hemichordates were closely related to one another and represent a distinct clade, that the Urochordates were monophyletic and could be considered as a separate phylum, and that Cephalochordates are most closely related to vertebrates (Bromham and Degnan, 1999; Cameron et al., 2000; Swalla, 2001). More recent data obtained by mitogenomic techniques, however, demonstrates that each deuterostome phyla is monophyletic and that a common ancestor diversified rapidly soon after appearance in the early Cambrian period and generated all major deuterostome lineages during a short historical period, which is consistent with "Cambrian explosion" revealed by paleontologists (Zhong et al., 2009). Moreover, new evidence based on multiple nuclear gene/protein sequences provided the evidence that Urochordates, not Cephalochordates, represent the closest living relatives of vertebrates (Blair and Hedges, 2005; Bourlat et al., 2006; Delsuc et al., 2006; Vienne and Pontarotti, 2006).

Among invertebrate deuterostomes, only echinoderms (Echinoidea and Holothuroidea) are commercially exploited by fisheries and aquaculture and commercial interest has contributed to the intense investigation of their reproductive biology and nutrition over the last 20 years.

1.2 Echinoids

1.2.1 Classification and anatomical features

The Class Echinoidea is separated into four cohorts (Table 1.1) of which, three (Echinothuriacea, Diadematacea and Echinacea) includes regular Echinoids (sea urchins) and one (Irregularia) includes irregular Echinoids (sand dollars and heart urchins). Irregular body shape with a marked bilateral symmetry characterises sand dollars and heart urchins. The first presents a flattened body shape, with a round or oval shape, whereas the second are indented on one side (the anterior ambulacrum) and present a more evident bilateral symmetry. Both present soft spines that facilitate their "life style" as sand and mud borrowing animals. Regular sea urchins, instead, present a "pseudo-spherical" radially symmetric body and hard prominent spines and prefer a hard substratum.

Common feature of all echinoids is the Aristotele's lantern associated with the mouth. This is a complex pentasymmetric structure consisting of more than 40 interconnected ossicles and moved by powerful muscles that allow protraction and retraction of the teeth and all structures associated with them. The rear part of the lantern is used for respiration and muscles force water currents through the peristomial gills. The echinoid body is entirely enclosed in a shell, or test. In regular echinoids the test is typically built by 20 rows of plates, which can be distinguished in larger interambulacral and smaller ambulacral plates. These are paired and interconnect by a V-shaped end, whilst connections between interambulacral and ambulacral plates lie on a flat end in order to increase test strength. Interambulacral plates host the spines whilst one pairs of podia emerge from each ambulacral plate.

In the uppermost part of the test, the aboral region, a membrane (the periproct) surrounds the anus, enclosed by the five genital plates each pierced by one gonopore. Although all echinoids could be considered edible (Lawrence, 2001), only a few species belonging to the regular cohort and grouped in the order Echinoida are subject of fishery or aquaculture and will, therefore, be the focus of this work.

Table 1.1. Classification of echinoids with representative genera (modified from Lawrence, 2007)

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

1.2.2 Echinoids life cycle

Most echinoids are free spawners and produce large numbers of small, yolk-poor eggs that develop into planktonic, obligate planktotrophic larvae known as echinoplutei (McEdward and Miner, 2001). After the planktonic stages, larvae seek a suitable substrate to settle on and undergo metamorphosis. The development from post-larvae to juvenile and adult and consequent sexual maturation will give start to a new cycle (Fig 1.1).

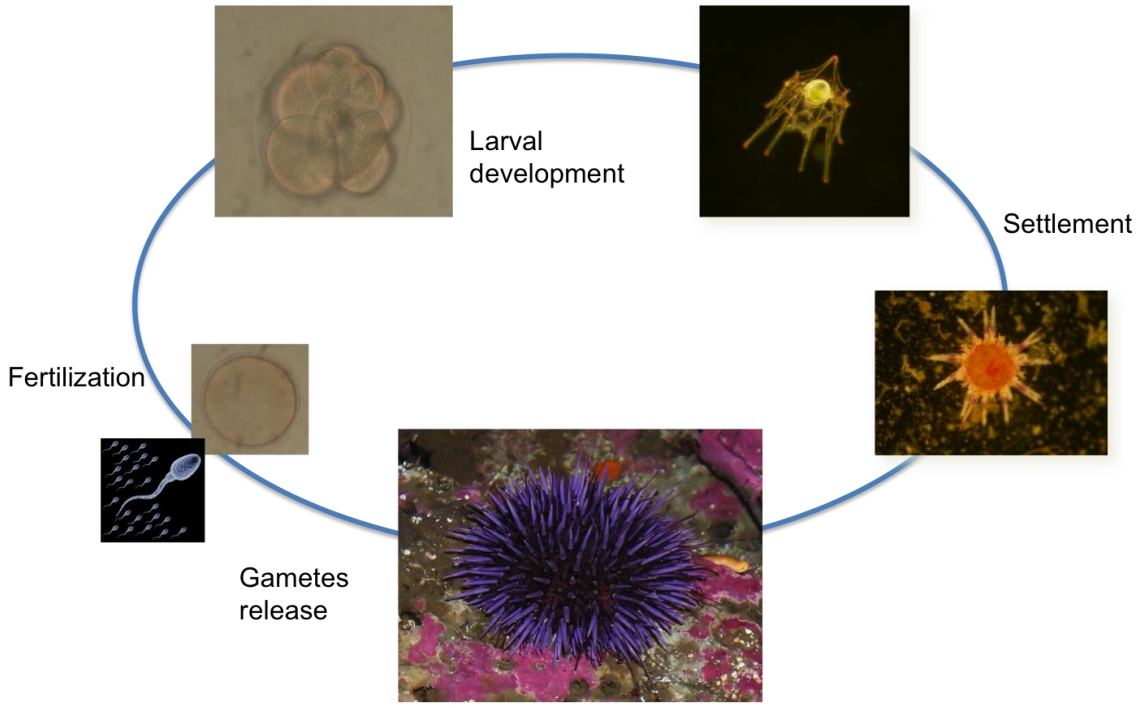

Figure 1.1. Echinoids life cycle

This pattern, although occurring in the vast majority of species (McEdwards and Miners, 2001), is considered ancestral for echinoids (Strathman 1978; Wray, 1995). The duration of the planktonic stage is commonly in the order of four weeks even though this varies among species. Planktotrophic larvae such as *P. lividus* echinoplutei grow substantially during the different development stages. On average,

juvenile diameters are 4 fold greater than egg diameters and juvenile test volumes are 94.5 fold greater than egg volume (McEdward and Miner, 2001). However the range is considerable. Initially, growth increases the size and complexity of the body through addition and elongation of the larval arms in order to improve swimming and feeding ability (Smith et al., 2008). Although the geometric growth of the larva is considerable, the increase in biomass, protein, energy content and metabolic rate are modest, in the order of 2 to 4 fold. Growth of the juvenile rudiment is energetically expensive; involving a 9 to 14 fold increase in protein and metabolic activity (McEdwards, 1984).

This developmental mode is, however, not adopted by all sea urchin species and other life cycle patterns have been described (McEdward and Miner, 2001). Facultative planktotrophy has been documented in only two species: the Clypeasteroid *Clypaster rosaceus* (Emlet 1986) and the Spatangoid *Brisaster latifrons* (Hart 1996). Species producing non-feeding larvae (lecitotrophic) have very large eggs, large juveniles and a relatively short larval period, in the order of 1-2 weeks (Emlet et al., 1987). Lecithotrophy involves development via a yolk-rich larva and occurs in only 9 species. Not all species release larvae in the water column and brooding has been indeed observed in 49 species (McEdwards and Miners, 2001) in which adults retain offspring among spines (e.g. *Cassidus caribearum*, Gladfelter 1978), under the body near the mouth (e.g. *Goniocidaris umbraculum*, Barker 1985), or in depressions of the test (e.g. *Abatus nimrodi* and *Abatus shackletoni*, Schinner and McClintock, 1993). Brooders produce large eggs but there is little information on the sizes of the juveniles and duration of development (Emlet et al., 1987; McEdwards and Miners, 2001). Non-feeding brooded larvae, should not add biomass during development, and utilisation of metabolic fuel should result in a decrease in

biomass until onset of feeding. However, the few data available for brooding species show increase in biomass or energy content (8-40%) between the egg and juvenile stage (Lawrence et al., 1984; McClintock and Pearse, 1986). McEdwards and Miners (2001) suggested that this has two potential explanations: the maternal transfer of nutrients could have occurred during brooding or juvenile feeding might have begun before the analysis of juvenile biomass. In contrast pelagic non-feeding larvae undergo no net change in biomass or energy content during development or metamorphosis (Hough-Guldberg and Emlet, 1997). Direct development has been documented in only one species of echinoid, the Antarctic spatangoid *Abatus cordatus* (Shatt and Feral, 1996). In this species eggs are large and juveniles are very large in comparison with other echinoids and development is extremely slow.

The most common development mode does not seem to present any specific advantage *per se* but it is most likely selected by the circumstances in which the species has evolved. Indeed, the comparison of metabolic costs of larval development in congeneric sea urchins, *Heliocidaris tuberculata* (planktotrophic) and *Heliocidaris erythrogramma* (Lecitotrophic), illustrates that the energy required to complete larval development is very similar (McEdward and Miner, 2007) although the organic dry matter content of lecitotrophic eggs is 100 fold greater than in planktotrophic species (Hoegh-Guldberg and Emlet, 1997).

1.3 Echinoid fishery

1.3.1 Status and management practices

Echinoids are commercially relevant and the object of intense investigation by aquaculture industry and researchers as urchin wild stocks are subject to increasingly unsustainable global scale fisheries activities (Andrew et al., 2002).

Sea urchin fisheries are localized in temperate regions of the globe and are generally focused on few genera, mainly Strongylocentrotidae and Echinidae.

The majority of sea urchin fisheries around the world share a common pattern: after an initial period characterised by low fishing effort and limited landings, fishery evolves into a rapid expansion period typified by the highest production peaks, this is generally followed by a third phase of full exploitation where, usually, catches level off. After these three phases landings often start declining, initiating a depauperation phase. The extent of the depauperation period, and its consequences on wild stocks, seem variable between countries and often depends on management policies and conservation activities undertaken by each country. This trend is also reflected in world production records and, as presented by Andrew et al., (2002) and further updated here, world sea urchin production significantly increased during the latter half of last century following the expansions of new fisheries grounds in Japan, USA and Chile and, more recently, China and the Russian Federation. From the mid nineties onwards, however, this trend was inverted and today a 30% loss in landings is observed (Fig. 1.2).

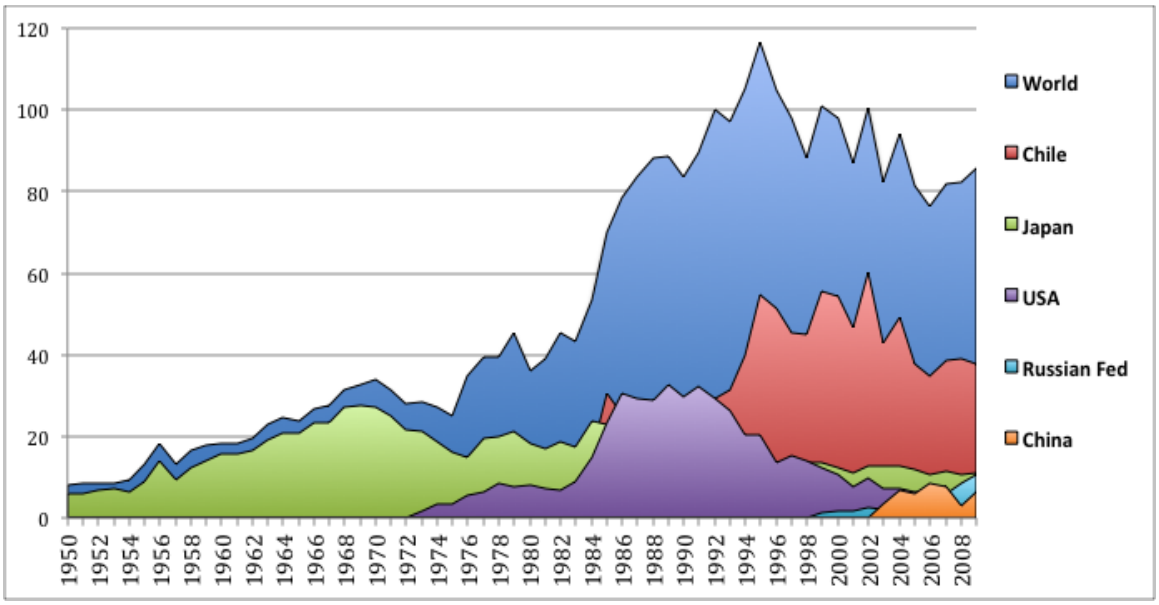

Figure 1.2. World Echinoids landings from 1950 to 2009. Source Fishtat (FAO)

This was mainly caused by the sharp productivity decline in some of the most important fishery grounds in USA and Japan where 75% and 50% loss were respectively recorded over the last 15-20 years. This was, however, compensated in the same years by the dramatic increase of Chilean production, which led world production from that point onwards. Nonetheless, between 1999 and 2009, Chilean production experienced a significant drop, as described in the book chapter "The history of the Chilean urchin fishery: Chronicle of an announced death" by Stotz (2009). Nonetheless, during mid 2000 China and the Russian Federation made their entry into world market increasing their production by more than ten fold, together compensating for the reduced outputs from Chile.

1.3.2 Fishery technology

The main driver of sea urchin exploitation is the high return on investment achievable by this specialized and relatively small-scale fishery industry. This is

mainly due to the low capital and running costs of the venture and to the relatively high market price of the product. Fishing activities are, in fact, carried out mainly in shallow coastal water, using small fishing vessels often owned and operated by divers, either SCUBA or surface-supplied, who also hand harvest the urchins (Andrew et al., 2002). The use of more mechanised gear, such as drags, has also been reported in Maine (USA) where they account for less than 20% of total catch (Andrew et al., 2002). The primitive design of this fishing tool, called "chain sweep", was a modified scallop dredge that, because of the damage it produced on sea urchin, was rapidly substituted by a lighter gears purposely designed (Pipe drag and Green drag) (Baron-Taltre, 2005). The main factor limiting profitability of the fishing industry is its very seasonal nature. Sea urchin roes are considered to be of unacceptable quality during spawning season; therefore, fishery can only take place during relatively short period of the year. Knowledge of the reproductive biology of all different species and stocks subject to fishing is sometimes used by fishery managers to define fishing grounds and seasons, allowing for better stock management and to potentially extend fishery activities for the whole year (Andrew et al., 2002; Matzui et al., 2008, Hernandez et al., 2011).

1.3.3 Enhancement strategies

Considering the ecology (habitat and distribution), reproductive biology and recruitment efficiency (discussed later) of the species subject to fishery activities, it is clear that sea urchins wild stocks can be very susceptible to overexploitation. Indeed, in many producing countries such as Ireland and France in Europe, and in the states of Maine, Northern California and Washington in the USA, landings are reduced to about 10-20% of what they used to be at their peak (Andrew et al., 2002).

In order to mitigate fishing pressure, enhancement practices have been established with mixed success in several regions. As reviewed by Andrew et al. (2002), management strategies can be divided into three categories:

- Reseeding,
- Habitat enhancement, and
- Transplantation.

Reseeding is chosen as enhancement practice on the assumption that low recruitment is limiting population size and that population size is below environmental carrying capacity (Doherty, 1999). Recruitment can be limited by poor fertilisation success, predation of larvae before and during settlement and larvae dispersal mechanisms. Due to the difficulties in tagging large numbers of restocked juveniles, very few studies were able to reliably measure reseeding success (Andrew et al., 2002). Discrimination between hatchery-reared juveniles and wild individuals becomes particularly difficult in fishery grounds where wild stocks are still present and indistinguishable from restocked individuals. Invasive tags such as stainless steel or monofilament wire, colour/code labels drilled or plunged through the test have been used in laboratory and field trials with poor survival results (Nelson and Vance, 1979; Hur et al., 1985) whilst passive integrated transponder (PIT) tags have resulted in higher survival (Hagen, 1996) and seemed not to interfere with growth (Kalvass and Hendrix. 1997) in laboratory tests and, therefore, represent a valid possibility to evaluate restocking success. However, contrasting results have being obtained from field studies and some interference with growth and survival has been reported (Lauzon-Guay and Scheibling 2008).

Moreover practical complications of using PIT tag underwater still need to be addressed, mainly because no submersible reading device is available. More recently, aluminium tags inserted through the peristomial membrane were successfully used to identify restocked individuals using submersible metal detectors (Duggan and Miller 2001). Chemical tagging is a less invasive option and two chemicals (Tetracycline and Calcein) have been used so far to monitor growth in laboratory scale and field trials (Russell et al., 1998; Ebert et al., 1999; Lamare and Mladenov, 2000). Two studies conducted in California by Ebert et al., (1992) and Schroeter, et al. (1998), measured reseeding success marking *Strongylocentrotus franciscanus* juveniles with Calcein. Survival of restocked individuals varied widely depending on locations (between 0 and 20% of restocked animals) with bigger animals (>15mm) generally resulting in higher survival. The limited survival performances reported in all these studies and the costs involved in culturing animals to an effective seeding size, suggests that reseeding with hatchery-reared juveniles is probably not economically feasible. Nonetheless, reseeding was still very popular in Japan in 2002 and more than 70 million juveniles are transplanted every year (Andrew et al., 2002). Survival of restocked juveniles is however not monitored and positive effects on fisheries are still to be conclusively demonstrated. Nontheless, positive results were reported by Sakai et al., (2004) who had marked hatchery reared *Strongylocentrotus intermedius* with the red fluorescent dye alizarin complexone (ALC). Work from Juinio-Menez et al., (1998) and reported by Andrew et al., (2002) seem to suggest that some success was also obtained when juvenile of *Tripneustes gratilla* were introduced into marine protected areas in the Philippines. These individuals, sheltered from fishery activities, could act as reproductive reserves significantly increasing population size in the area in which they were restocked

(Juinio-Menez et al., 1998). This seems, however, to be contradicted by numerical larval dispersal models developed by the same author in the same region which indicate that sea urchin populations are not self-seeding but, instead, are regularly mixed (Juinio-Menez and Villanoy, 1995) causing a high level of gene flow between geographically discreet populations (Malay et al., 2000). Moreover, rapid variation in population densities have been repeatedly observed over the years (Boudouresque et al., 1989; Delmas, 1992; Turon et al., 1995; Benedetti-Cecchi and Cinelli, 1995; Sala, 1996; Sala and Zabala, 1996; Sala et al. 1998), and no clear conclusion as what the main cause for such variation is could be made. It is likely that rapid increase and/or decrease in population size is multifactorial rather than dependent on increased recruitment only.

Differences in the genetic structure of wild and hatchery-reared populations could provide useful information on the effectiveness of restocking program and on the potential consequences of cross-breeding between wild and domesticated stocks (Addison and Hart, 2002, 2004; Calderon et al., 2009; González-Wangüemert et al., 2012). Beside the controversial efficacy of restocking programmes, reseeding practice was the initial incentive to develop bigger scale hatchery facilities and rearing protocols, paving the way for the development of echinoid aquaculture.

Habitat enhancement aims at expanding areas of suitable habitat for sea urchins and promotes colonisation of algae as food (Morikawa, 1999). This practice has been extensively applied in Japan (Taki and Higashida, 1964; Kawamura, 1973; Agatsuma 1991) and South Korea (Andrew et al., 2002). Despite the 30 years history of this activity, the effects on coastal fisheries and ecosystems have, however, not been investigated in any detail (Andrew et al., 2002).

Transplantation involves the collection and subsequent relocation of adult sea urchins from areas at lower gonad productivity (attributed to food limitation) to areas where gonad yield is generally higher (kelp forests or other macroalgae beds). Roe enhancement in wild populations has been successfully conducted on the west coast of Ireland (Moylan, 1997), while in most other fisheries, such as California (Tegner, 1989) and Mexico (Andrew et al., 2002), this work remains at research scale and has not been commercialised. This practice clearly has management and ecological consequences as broodstock density reduction in lower productivity areas might disrupt fertilisation success of local stocks with consequences on recruitment success; on the other hand, increasing adult density in more productive areas, such as kelp forests, could have deleterious effects on macroalgal assemblages with the associated risks of converting a productive area into a barren ground. This same concept has been recently exploited by aquaculture ventures where urchins collected from the wild go through few months of roe's enhancement period in tanks inland before commercialisation, *de facto* increasing yield and reducing the above-mentioned ecological consequences. Collection from the wild and enhancement in tanks, however, still poses serious questions on possible effects on reproduction success of wild populations especially considering that juvenile sea urchins can be caught to be grown to commercial size, potentially reducing recruitment to adulthood with relative consequences on broodstock population size and structure. Moreover, wild macroalgae will have to be harvested to feed the urchins, still putting pressure on wild macroalgae stocks.

1.3.4 Ecological impacts of sea urchin fishery

As mentioned before, most sea urchins are harvested by hand, thus the effect of harvesting is mainly evident through the changes in benthic communities due to the extraction of the urchins rather than direct damage to the sea bottom (Dayton et al., 1998; Tegner and Dayton 2000). The greatest ecological impact of the large-scale removal of sea urchins is the rapid development of large brown algae beds and consequent changes in the relative abundance of fish and benthic invertebrates usually associated with it (Lawrence, 2001). This has direct negative consequences on sea urchin recruitment. McNaught and colleagues have, in fact, demonstrated that post-settlement mortality caused by predation is significantly higher within Kelp forests (McNaught and Steneck, 1998; McNaught, 1999; Balch and Scheilbling 2001) simply due to the increased presence of sea urchin natural predators. Therefore, the rapid expansion of macroalgae beds as consequences of sea urchin over exploitation, although promoting biodiversity, significantly reduces urchins' recruitment and ultimately reduces productivity of the fishery.

1.4 Echinoid Aquaculture

Finfish and shellfish aquaculture research has supported the continuous development of the different industry's sectors over many years, attaining important results on environmental sustainability, economic profitability and rearing protocols of new species, enhancing the knowledge based development of what is rapidly becoming the most important food production industry at global level. Given the constantly rising demand on urchin products on the global market and the proven unsustainability of commercial fisheries, it is predicted that aquaculture will be required to fill the gap between supply and demand (Pearce, 2010).

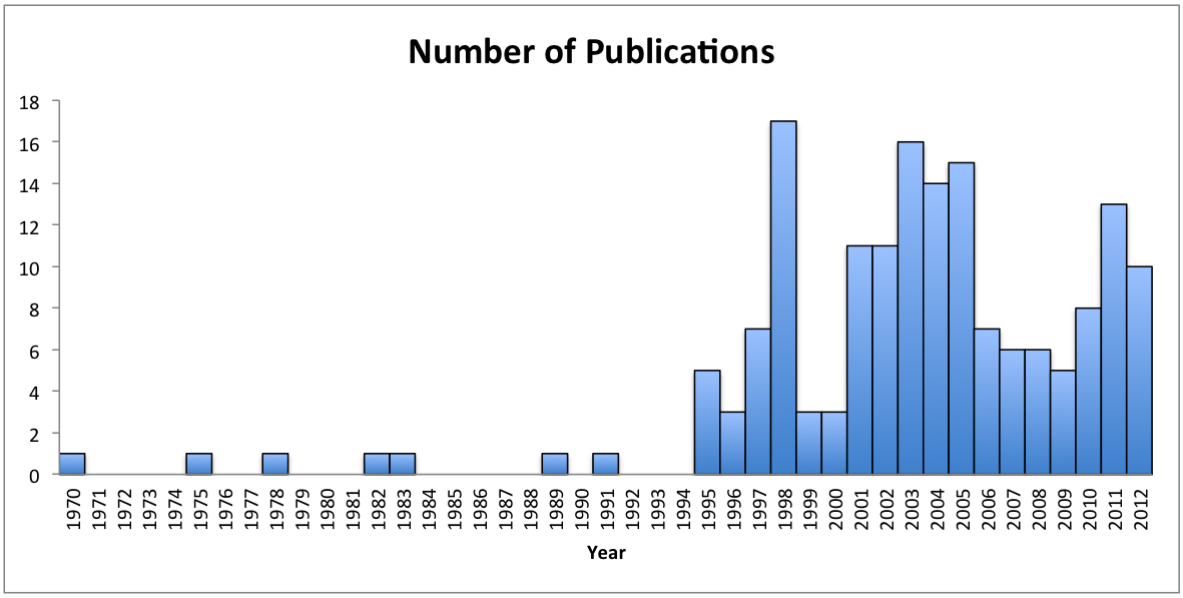

Figure 1.3. Number of publications on sea urchin aquaculture by year. Literature search was conducted using Web of Knowledge with "urchin" AND "aquaculture" as search parameters. (Updated from Pearce 2010)

To this end several research groups around the world have focused on the solution of the different bottlenecks limiting echiniculture development as testified by the increased number of papers published on the topic during the last 15 years (Fig. 1.3). Beside the discrepancy between supply and demand, aquaculture can also solve the important problem of seasonality in the supply. As gonads are the only edible part of

sea urchin, two important parameters are of utmost importance to determine fishing seasons: size of the gonads, or yield, and reproductive stage. As mentioned in the previous section, urchin gonads grow during the year and seasonal reproductive cycle affects their palatability and therefore market acceptance, this commodity can only be consumed during certain months. More specifically, gonads are of bitter taste and unpleasant texture during spawning seasons, while gonadal index is too low during some parts of the year. This leaves only a narrow window for commercially viable exploitation (Fig. 1.4.). Aquaculture, especially inland systems, could however bring good quality gonads to the market all year round, by control of those parameters (temperature, photoperiod and nutrition) influencing gonadal growth and reproduction.

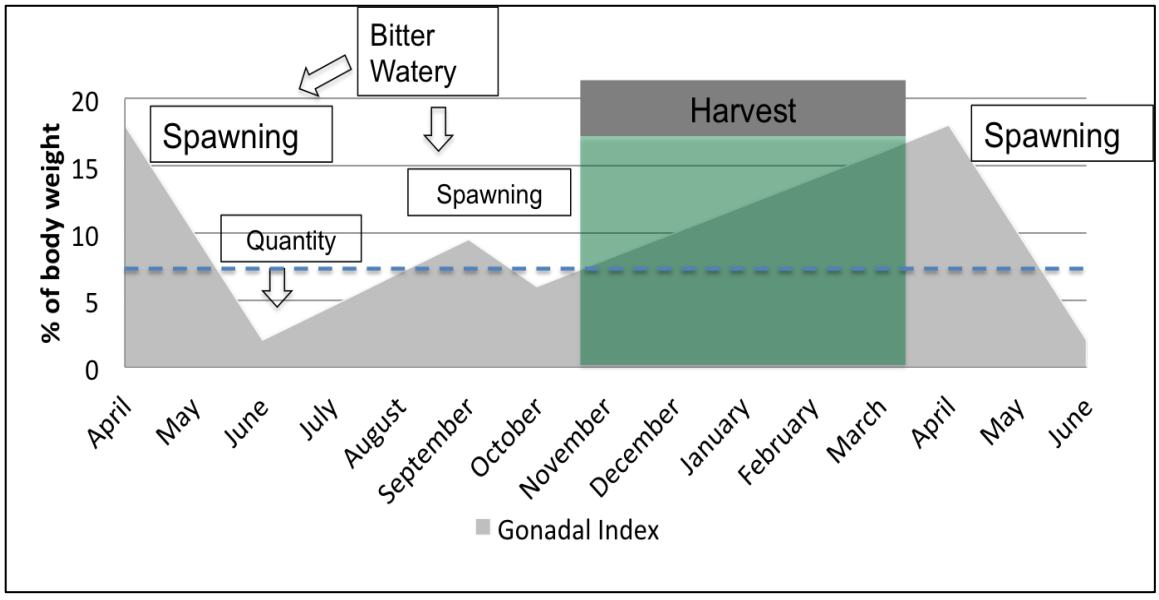

Figure1.4. Generic Sea urchin sp. gonadal index throughout the year. Harvesting takes place only when quality and yield are considered acceptable by the market and economically sensible by the fisherman. Modified from: www.gourmetmarine.ie

Grosjean et al., (1998), after seven years investigation and modifying the original protocol developed by Le Gall and Bucaille (1989), gave a comprehensive description of the main steps for the land-based cultivation of sea urchin that, with some

adaptation, is currently adopted for all farmed echinoid species around the globe. The cycle can be summarized in six steps: Fertilisation, larval rearing, metamorphosis, juvenile growth, sub–adults growth and broodstock conditioning (Fig 1.5).

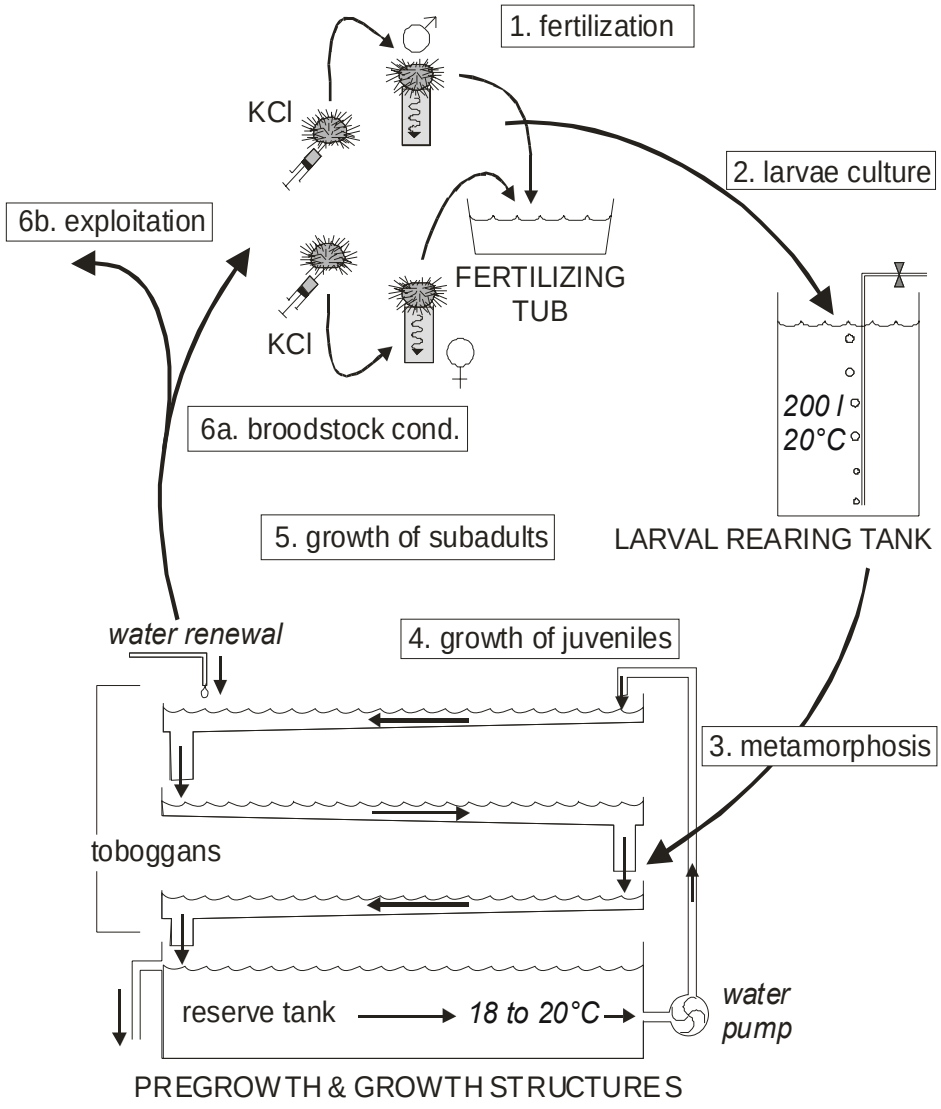

Figure 1.5 Overview of the closed-cycle processes and devices used to produce sea urchins in land based facilities. (Extracted from Grojean et al., 1998).

1.4.1 Fertilisation technique

Under laboratory or hatchery conditions spawning is obtained by injection of KCl (1 molar) via the peristomial membrane commonly at dosage of 40µl to 50µl gram^{-1} of

body weight. Ova and sperms are collected in separate containers and mixed at 5:1 ratio in filtered (1µm) seawater. The mix is kept overnight at 18-20 ˚C and then fertilisation rate is evaluated and the fertilized eggs volumetrically counted; fertilisation rate often exceeds 90% (Grojean et al., 1998; Kelly et al., 2002; Liu et al., 2007). It is common procedure to use more than one male per female during spawning event as it has been demonstrated that fertilisation rate is increased when eggs are exposed to sperms from different males although this process is also mediated by male x female interaction and gamete compatibility can enhance or depress fertilisation success (Evans and Marshall, 2005).

1.4.2 Larval rearing

Larval rearing is usually conducted in cylindrical polyethylene tanks, usually terminating in a conical bottom, in gently aerated and filtered (1-4 µm) seawater. UV sterilization (approximately 280 mJ $cm^{-1}s^{-1}$) is also often employed. Different stocking densities are reported in the literature, from 0.25 larvae ml^{-1} (Grojean et al., 1998) to 4 larvae ml^{-1} (Kelly, 2000; Liu et al., 2007). When the lower stocking densities are employed water exchange is usually not required, however at higher stoking densities a complete water change is carried out every third day (Kelly et al., 2000; Liu et al., 2007). Flow through systems could bring several advantages to echinoid larval rearing allowing for higher stocking densities and reduced need for complete water exchange and consequent stress to the larvae.

Live microalgae are usually fed to echinoplutei and different investigators have employed several species to successfully rear sea urchin larvae, in general the larvae readily accept motile microalgae of 200 to 400 μm^3 in volume. According to published literature one of the most common microalgae used for echinoid larval

rearing is the green flagellate *Dunaliella tertiolecta*, however questions about suitability of its fatty acids profile have recently arisen (Liu et al., 2007). Indeed, larval commercial production in Dunmanus Seafood Ltd. (Ireland) is based on *Pheodactylum tricornutum* and *Pleurochrysis carterae* and not on *D. tertiolecta*.

Research on alternative diets (microcapsules or pastes) has been conducted in recent years and indicates that such diets have the potential to fulfil echinoplutei nutritional requirements although palatability issues and practical considerations such as price and availability need to be taken into account (George et al., 2004; Liu et al., 2007).

Larval rearing ends after 18-21 days post fertilisation, during which larvae undergo considerable morphological changes, which terminates with the development of the larval rudiment when competence for settlement is achieved (see later for details on larvae development stages and morphology).

1.4.3 Metamorphosis

From the sixteenth day onward, competence to *metamorphosis* is checked daily (Gosselin and Jangoux, 1998) and competence is considered achieved when rudiment size is equal or bigger than the larval stomach. A sub-sample of the larvae (20 – 40) is transferred in small plastic container, usually a Petri dish, where a diatom biofilm have been allowed to develop as metamorphosis stimulating factor. The percentage of metamorphosed larvae is determined 24 h later (see later for details on metamorphosis). If the number of metamorphosed individuals is about 80% of the larvae used for the competence test, the whole batch is transferred into the settlement tanks. Batches containing large amounts of larvae exhibiting poor development, abnormalities or too low metamorphosis rates are discarded (Grosjean et al., 1998).

1.4.4 Juvenile and sub–adults grow out

The settlement tanks contain PVC wave plates coated with a marine biofilm, which are held vertically by plastic racks. Flow through or re-circulated systems can be employed and temperature can be maintained between 15 and 30 °C. After metamorphosis, juveniles (0.5 - 1 mm) will start grazing on the biofilm and are soon able to ingest small quantities of macroalgae (*Enteromorpha linza*). Once juveniles of approximately 3 – 4 mm diameter are easily visible on the plates and tanks walls, soft pieces of *Saccharina latissima* or *Ulva lactuca* are added to supplement the juveniles' diet. The juveniles will remain in the settlement tank until they reach at least 5mm in test diameter. At this point individuals are graded and kept in separate growing tanks and fed *ad libitum* with macroalgae or formulated feeds until market size (20mm for juveniles or 40mm for adults) is achieved. Stocking density during the juvenile grow-out stage is usually 400 individuals per m^2.

At Dunmanus Seafood Ltd juvenile urchins are grown to market size by ranching them in intertidal pools and subtidal areas. Water in the intertidal pools is totally exchanged every tide and feed is supplemented with additional macroalgae. The juveniles reach market size (50 to 55 mm) in 2 to 4 years. Recent interest from mussel farmers in obtaining juvenile urchins for on-growing has arisen as the industry seeks to diversify to avoid some of the restrictions on harvesting periods resulting from harmful algal blooms (Kelly and Chamberlain, 2010).

1.4.5 Market conditioning

In aquaculture settings the seasonal environmental cues regulating gametogenesis such as photoperiod, temperature and food abundance and quality are often lacking.

Under these circumstances, urchins tend to by-pass the growth stage of the gonads and present permanent gametogenesis during which gonads are soft and deprived of nutritive phagocyte and therefore unacceptable for the market. For this reason market conditioning is often required prior to commercialization. During market conditioning urchins are starved at a temperature of 12 to 14 °C at a 12h light / 12h dark photoperiod. This leads to consumption of all reserves in the gonads and synchronizes gametogenic phase. After two months of starvation, sea urchin are fed *ad libitum* and temperature is increased to at least 16 °C, this stage usually lasts for two to three months (Grosjean et al., 1998). At the end of this phase gonads are ready for the market. It has been proven that in some sea urchin species requiring shortening day length as cue to initiate gametogenesis such as *S. droebachiensis* (Walker et al., 2005), gametogenesis suppression can be achieved in aquaculture settings by long day invariant photoperiod treatment (Bottger et al., 2006). It is therefore possible that short days invariant photoperiod regime might have a similar effect on species that require increasing day length as a gametogenesis trigger such as *P. lividus* (Shpigel et al., 2004).

Another important aspect to be considered for urchins' marketability is the shelf life of the product and the packaging and transport techniques to be employed to retain acceptable quality. As part of the SPIINES2 project conducted at the Scottish Association for Marine Science (SAMS, Oban, Scotland, UK) urchins were harvested and packed at different temperature regimes (4, 8, 16 and 21 °C) to determine the best methods for ensuring urchins arrive at their destination fresh and fit to eat. A measure of 'spoilage' was also made: this was defined as the point at which bacterial and enzymatic activity had affected the edible tissue so as to produce 'off' flavours and smells and render the product unsuitable for consumption. Spoilage rate was

assessed by quantifying the build-up of bacteria in the coelomic fluid and roe. Resulting bacterial colonies were then counted. The study showed urchins should be transported under refrigerated conditions. Urchins packed at 4°C and 8°C showed signs of life (spine movement) after 96 hrs. Assessment of spoilage, bacterial numbers in the coelomic fluid, showed that it was unchanged from the initial values. Urchins stored at 16 °C and 21 °C were visibly deteriorating (spine loss) after 48 and 24 hours respectively and had significantly more bacteria in the coelomic fluid than the initial groups and those at lower temperatures. Starving the urchins prior to transport reduced the spoilage rate significantly. Packing the sea urchins in macroalgae was not a good option, as the macroalgae itself degraded accelerating the spoilage process. A slightly damp environment within the packaging prolonged survival. The best way to transport urchins was refrigerated (4 – 5 °C) and, ideally, transport for urchins to be sold as whole / live should not take longer than 48 hours (SPIINES 2 final Report).

1.4.6 Broodstock conditioning

In land based aquaculture systems, all year round maintenance of mature broodstock is achieved by keeping individuals at relatively high temperature (between 18 and 20 °C) and under an invariant photoperiod, which leads to the disruption of the reproductive cycle. Under such circumstances food becomes the most important factor regulating gonadal cycle and large quantities of good quality gametes can be obtained by individuals fed to satiation using brown kelp such as *Laminaria digitata* (Grosjean et al., 1998). Alternatively, the set up of distinct populations under different photoperiod regimes will provide hatchery operations with synchronous populations that can be induced to spawn according to their photoperiod regime as

long as individuals are fed to satiation with fresh macroalgae (Pers. obs.). In the largest (3 million individuals in 2006) commercial hatchery currently operating in Europe (Dunmanus, Ireland) broodstock is selected from intertidal populations considered to perform better under hatchery conditions and a selective breeding program to select fastest growing individuals is currently in place (Kelly and Chamberlain, 2010)

1.4.7 Triploidy

Triploidy technology has been employed in aquaculture practice primarily in order to increase growth performance by suppression of sexual maturation. The technique is relatively straight forward in animals that are fertilized as primary oocytes (molluscs) or as secondary oocytes arrested at metaphase of second meiotic division (fish) and involves the treatment of oocytes with chemical or physical shock to prevent release of the second polar body. Triploidy in sea urchins cannot be achieved using these methodologies since haploid (1n) ova are released from the gonopores and fertilized in the water column. Nonetheless, a very recent study (Böttger et al., 2011) illustrates a new methodology that overcomes this bottleneck. Böttger and colleagues have exploited results from previous studies indicating that fusion of the haploid ova of sea urchins (*S. droebachiensis*) can be accomplished by a variety of means (Wilson, 1953; Bennett and Mazia, 1981a,b; Richter et al., 1981; Sekirina et al., 1983; Vassetzky and Sekirina, 1985; Vassetzky et al., 1986). This study revealed that the removal of the jelly coat and vitelline membrane by mechanical means allows both successful fusion of two haploid ova (2n) and subsequent fertilisation by haploid (1n) sperm generating triploid (3n) gastrulas first and prism stage plutei later. Development to the gastrula stage depends upon the correct expression of

genes or use of proteins in the oocytes during oogenesis and before meiosis (Davidson, 1987). Malfunction of gene expression are potentially fatal at gastrulation. The fact that triploid embryos did gastrulate successfully confirms that "maternal stored RNA" remains and is accessible during early development. Moreover, the observation that triploids developed successfully to prism stage suggest that the equivalence of zygotic RNA transcribed from the triploid cell nucleus was available and translated successfully during development. Nonetheless, as no triploid juveniles were produced during this study, it is impossible to evaluate how the triploid status would affect gametogenesis or the incorporation of nutrients in the nutritive phagocyte fraction of the gonads. Moreover, questions about market acceptance of triploid sea urchin might arise potentially hampering the commercial viability of this technique.

1.5 *Paracentrotus lividus*

1.5.1 Biogeography and Ecology

Paracentrotus lividus is distributed throughout the Mediterranean Sea and in the north-eastern Atlantic, from Scotland and Ireland to Southern Morocco and the Canary Islands. Temperature is the main factor influencing geographical distribution and abundance is higher where winter water temperatures range from 10 to 15 °C, and summer temperatures from 18 to 25□°C such as western Mediterranean, Portugal and Biscayne Bay (Boudouresque and Verlaque, 2007). It is typically a sub-tidal species, however individuals can be found above the highest tide margin in rock pools (Crook et al. 2000) and isolated individuals have been found as deep as 80 meters (Cherbonnier 1956; Tortonese 1965). Evidence suggests that, as larvae can be found at considerable depth and pressure and as temperatures commonly encountered in deep areas of the Mediterranean sea do not seem to limit settlement, vertical distribution is influenced more by biotic factors, such as predation and/or food availability, than physical conditions (Young et al. 1997). Preferred habitats are those formed by rocks or boulders (Verlaque 1987) where displacement by waves and predation is avoided by digging cup-shaped cavities in the substratum (sandstone, limestone, granite and basalt) where individuals find shelter (Boudouresque and Veralque 2007). Nonetheless, *P. lividus* populations can be also found in shallow coastal lagoons living on more mobile substratum such as sand or mud (Fernandez et al. 2003). A considerable size difference exists between open sea and coastal lagoon populations with the first presenting significantly bigger individuals than the second. This difference is probably due to the higher stress

derived by more fluctuating temperature and salinity in the shallower coastal lagoons (Le Gall et al., 1989; Fernandez et al., 2003). Moreover, it has been long recognized that a good percentage of sea urchin diet is composed of drifting macroalgae and aquatic plants detritus that some urchin species, including *P. lividus*, are able to capture and feed on (Rodriguez and Farina, 2001; Kelly et al., 2012). It has been recently shown that in exposed regions of the coastline the likelihood of urchin getting in contact with this food source is greatly increased promoting both growth and gonadal index (Livore and Connell, 2012). This is, however, in contrast with data reported by Guettaf et al. (2000) and by Gianguzza et al. (2013), where gonadal index was found to be higher in *P. lividus* populations living in low hydrodynamic conditions. This discrepancy highlights the very localized nature of food supplies and hence somatic and gonadal growth, also reflected in the high variability of spawning seasons across *P. lividus* geographical distribution.

1.5.2 Predation, competition and densities

In the Mediterranean Sea, common predators of *P. lividus* are crustaceans (Bernandez et al., 2000), fish and gastropods (Sala and Zabala, 1996) (see Boudouresque and Verlaque, 2007 for complete predators list). Predation seems to be the main factor affecting *P. lividus* diel activity. Like many other sea urchins, *P. lividus* in fact, exhibits a predominant nocturnal activity. This behaviour, documented over 50 years of research (Kempf 1962; Sala 1996; Miyamoto and Koshima, 2006; Young and Bellwood, 2011), can be however reversed by local conditions such as localized presence of nocturnal predators (Kitching and Thain, 1983), this observation is supported by the documented ability of echinoderms to perceive body fluids of injured individuals and potentially relate this predation cue with light level

(Lawrence, 1975; Nance and Braithwaite, 1979). Very little is known about what kills echinoid larvae during their planktonic life, predation is the most likely cause of larval mortality. Under laboratory conditions, in fact, invertebrate and vertebrate predators (Rurill and Chia 1985; Pennington et al., 1986), and suspension feeders (Tegner and Dayton 1981) consume echinoid larvae and mortality rates have been estimated from field studies to be in the range of 6 - 27% per day (Rumrill 1990; Lamare and Barker 1999).

Echinoplutei, however, have several defences that can potentially reduce predation (Young and Chia 1987; Rumrill 1990). As reviewed by McEdwards and Miner (2001), embryos and early larval stages are more readily consumed by predators than later larval stages (Rumrill and Chia 1985; Pennington et al., 1986) and this difference could be explained by the presence of more articulated structure of the skeletal rods, bigger size or defensive behaviour adopted by older larvae.

Starvation could be a cause of death in obligate planktotrophic larvae such as *P. lividus*, however is not known if echinoid larvae starve to death in the sea (McEdward and Miner, 2001). Indeed, larvae develop slower when food is scarce (Herrera 1998) and this would increase the risk of predation (Pearse and Cameron 1991; Strathmann 1996). Extreme temperature, salinity and ultraviolet radiation levels have potentially lethal or sub-lethal effects on echinoid larvae (Pennington and Emlet 1986; Metaxas 1998). Echinoid larvae use behavioural means and chemicals to protect against UVR. It has been, in fact, demonstrated that some sea urchin larvae can migrate downward to reduce risk of UVR damage (Pennington and Emlet 1986).

Competition with other organisms, partially or completely overlapping *P. lividus* ecological niche, might influence adults and juvenile abundance and behaviour. As reviewed by Boudouresque and Verlaque (2007), the species most commonly

associated with *P. lividus* are the echinoid *Arbacia lixula* in the Mediterranean Sea and *Psammechinus miliaris* along the Atlantic coasts. *A. lixula* and *P. lividus* feeding niche, although overlapping, are nevertheless distinct; the first prefers to feed on erect macroalgae and utilize drifting material, whereas the second is mainly a grazer of incrusting coralline algae. However, when resources are limited and erect algae are no longer present (e.g. in barren-grounds) the feeding niche of *P. lividus* shifts and largely overlaps that of *A. lixula* (Frantzis et al., 1988; Delmas 1992). More recently, it has been shown that *A. lixula* influences *P. lividus* vertical distribution with the former being prevalent in the upper layers and the latter at lower ones (Ruitton et al., 2000). The other competitor with potential to influence *P. lividus* distribution, densities and behaviour is the limpet *Patella caerulea*. When this species is present, in fact, *P. lividus* individuals must increase their feeding ground areas to satisfy feeding requirements (Verlaque et al., 1987). Of minor importance is the competition with herbivorous fish such as the Sparids *Salpa sarpa* (Sala, 1996) and *Diplodus puntazzo* (Verlaque, 1990).

Densities of *P. lividus*, ranging from a few to a dozen individuals per meter square, are quite common over its whole geographical distribution and depths. Very high densities (>50–100 individuals per square meter) usually occur in shallow habitats, on rocks, pebbles or boulders and in intertidal rock pools (Kempf 1962; Pastor 1971; Crapp and Willis 1975; Torunski 1979; Harmelin et al. 1981; Delmas and Régis 1986; Delmas 1992). Localized aggregations, often forming barren-grounds, correspond with much higher densities (>1600 individuals per square meter). Although the basis for this phenomenon remains unclear (Mastaller 1974; Keegan and Könnecker 1980), it is hypothesized that such behaviour may be a defence strategy against predators, a feeding strategy or a spawning behaviour (Boudouresque and Verlaque,

2007). However several other causes have been proposed to explain this phenomenon such as organic pollution (Harmelin et al., 1981) and the over-fishing of natural predators which might increase juvenile recruitment (Boudouresque et al., 1992; Sala et al., 1998a).

1.5.3 Immune system, diseases and parasites

For a long time it has been thought that invertebrates did not possess a recognizable immune system, however it was in 1893 that Metchnikoff described the mechanism of cellular encapsulation in invertebrates using echinoderm larvae of the species *Astropecten pentacanthus* (Smith et al., 2006). Only seventy years later Hildemann and colleagues were able to demonstrate the ability of several echinoderm species to distinguish between endogenous and exogenous tissues (Hildemann and Dix, 1972; Karp and Hildemann, 1976) and, between 1977 and 1993, several authors have identified the coelomocytes, categorized as amoeboid phagocytes, as the cells responsible for defence responses to injury and infections and clearance of exogenous substances in adults sea urchin (Coffaro and Hinegardner, 1977; Reinisch and Bang, 1971; Bertheussen, 1981; Yui and Bayne, 1983; Plytycz and Seljelid, 1993). As reviewed by Smith et al. (2006) embryos and larvae also live in environments abuntandt in potential pathogens, consequently, they must have protective mechanisms against microbial colonization and invasion at the two major potential sites of pathogen entry: the ectodermal surfaces and along the internal surfaces of the gut (Smith, 2005). In 2000, Silva demonstrated that *L. variegatus* embryos possessed phagocytic activity towards yeast cells injected into the blastocoelar cavity. The cells involved in this mechanism were however not identified and the current hypothesis is still that of Gibson and Burke (1985, 1987) who hypothesized

the involvement of cells within the blastocoelar space and pigment cells near the ectoderms (Fig. 1.6). Both these cell types have been recently shown to be capable of bacterial recognition and phagocytosis; thus, they may protect the larval ectoderm and gut from microbial colonization and invasion (Smith et al., 2006).

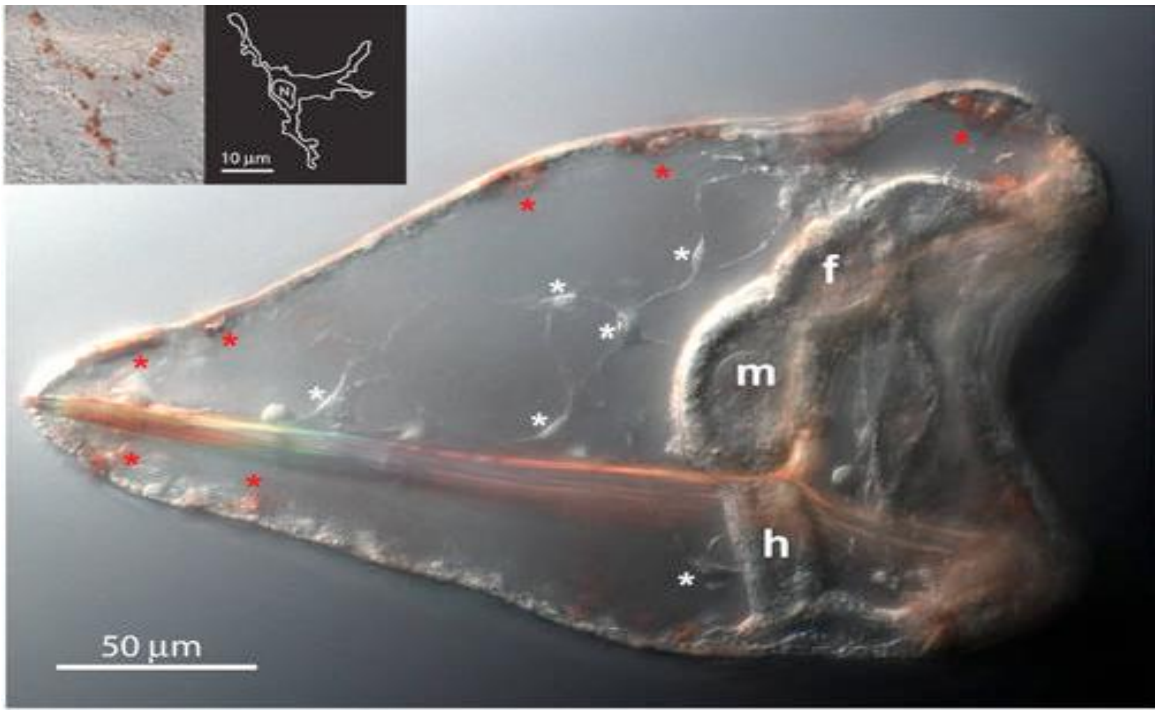

Figure 1.6 Cells with immune function in the sea urchin pluteus larva. Blastocoelar cells, marked with white asterisks, occupy the blastocoel and surround the gut. Pigment cells, marked with red asterisks, are in close apposition to the aboral ectoderm. A single pigment cell with vesicles is shown among cells of the aboral ectoderm (left inset). The same pigment cell is shown in outline in the right inset. Subdivisions of the gut are indicated for orientation. f, fore-gut; m mid-gut; h, hind-gut. Extracted from Smith et al., 2006

Providing there is adequate husbandry and water quality, there have been few health issues associated with the culture of echinoderms. Sea urchins held in tanks for prolonged periods (years) and subjected to some degradation in water quality have been known, on occasions, to show spine loss and/or to develop lesions of the test (Hughes pers. Com.). The spine loss is primarily of the longer spines. The lesions can appear as green or black. These conditions appear chronic and even a return to good

conditions does not seem to promote recovery (pers. obs.) and mortalities might occur. During the 70's and 80's a disease outbreak responsible for mass mortalities was reported in the Mediterranean region (Spain, Italy, Croatia and France) and in the Atlantic coast of France (Boudrouesque and Verlaque 2001). The disease was named "bald-sea-urchin disease" as its main symptom caused sea urchins to lose their spines (Jangoux, 1987). The known pathogens responsible for this disease are bacteria of the genera *Aeromonas*, *Vibrio* (Jangoux, 1990) and *Flexibacter* (Takeuchi et al., 1999). A recent study demonstrates that bacterial communities observed on diseased individuals of the same echinoid species but originating from distinct locations are not similar and thus supports the hypothesis that bacteria involved in this worldwide disease are opportunistic and not specific (Becker et al., 2008). The parasitic trematode *Metacercaria crassigula* infects the water vascular system limiting sea urchin ability to move and grip on the substratum and therefore escape predation (Boudouresque and Verlaque, 2007). There is only one report of parasite related mortality within aquaculture systems, the Amphipod *Elasmopus levis*, which was considered responsible for epithelium lesions and subsequent death of *Lythechinus variegatus* (Gibbs et al., 2011).

1.6 Reproduction

Sea urchins gonads increase in volume during the reproductive cycle not only because the size and number of germinal cells increase during gametogenesis, but also because somatic cells within the gonads store extensive nutrient reserves before gametogenesis begins (Walker et al., 2007). A thorough understanding of urchin reproductive cycle is necessary for the sustainable exploitation of wild stocks, to provide all year round spawning in the hatcheries and supply the markets with good quality products.

1.6.1 Sex ratios and Gonadal Index cycle

In *P. lividus*, like in the majority of echinoid species, sexes are separate although some degree of hermaphroditism has been observed (Byrne, 1990). Sex ratio in the wild seems difficult to establish and it changes between locations and regions, during the year and from one year to the next (Guetaff, 1997). Biotic factors such as selective predation of one gender over the other, such as the case of preferential female predation by the starfish *Marthasterias glacialis,* can modify sex ratio (Gianguzza et al., 2009). Both skewed sex ratios in favour of female and even sex ratios have, however, been reported in other cases (Guetaff, 2000).

The annual cycle of the gonadal index (gonad weight / whole weight x 100) of *P. lividus* has one or two seasonal peaks, which can differ conspicuously between neighbouring localities (Lozano et al. 1995; Guetaff 1997; Sánchez-España et al. 2004). Contradictory results have been obtained from field data as what parameters favour gonadal growth (Byrne, 1990; Fernandez, 1990, 1996; San Martin, 1995; Guetaff and San Martin, 1995; Lonzano et al., 1995; Fernandez and Boudouresque,

1997; Guetaff, 1997), however from laboratory studies high food availability seems to be the main requirement for gonad production (Lawrence et al. 1992; Gago et al. 2003). This observation is in accordance with the dual function (reproductive organ and nutrients store) of urchin gonads suggested by Lonzano et al. (1995) and corroborated by Hughes (2005). A drop in the mean gonadal index of a population in often used to estimate when a spawning event has occurred. Gonadal index, however, is not a direct measure of the gametogenic status and no clear relationship between gonadal index and reproductive stage has been established, this method is therefore to be considered with caution (Boudouresque and Verlaque, 2007).

Despite the multiple gonadal index peaks, often reported from field data (Crapp and Willis, 1975; Byrne, 1990; Lonzano et al., 1995; Guetaff, 1997), histological studies indicate only one or two annual gametogenic cycles (Byrne 1990; Lozano et al., 1995; Guetaff, 1997; Spirlet et al., 1998; Martínez et al., 2003). Nonetheless, different sub-populations or individuals within the same population can be unsynchronized and specimens with mature gonads can be effectively present year-round (Sánchez-España et al., 2004). This might represent a strategy whereby the risks associated with planktonic larval loss are spread over time (Boudouresque and Verlaque, 2007).

In summary, Gonadal index and gametogenic stage could be relatively independent, the first regulated by food availability and the second regulated by abiotic factors such as temperature and photoperiod. It is however clear that seasonal cues also affect food availability, in principle establishing the basis for an indirect relationship between gametogenic stage and gonadal index in the wild.

1.6.2 Gonad anatomy

Five gonad branches adhering to the aboral portion of the test constitute the reproductive organ in sea urchin. Its functional unit is the ascinus where all gametogenic processes occur. A single gonoduct exits each gonad and emerges through the test through via a pore on each of the genital plates (Fig. 1.7). In both sexes, the gonadal wall is composed by an outer and inner sacks, each comprised of several characteristic layers. The genital coelomic sinus (GCS) separates the two sacks. The outer sac includes a visceral peritoneum (VP) facing the perivisceral coelom, which is attached to a connective tissue layer (CTL). On the other side, towards the GCS, epithelial cells flank the CTL.

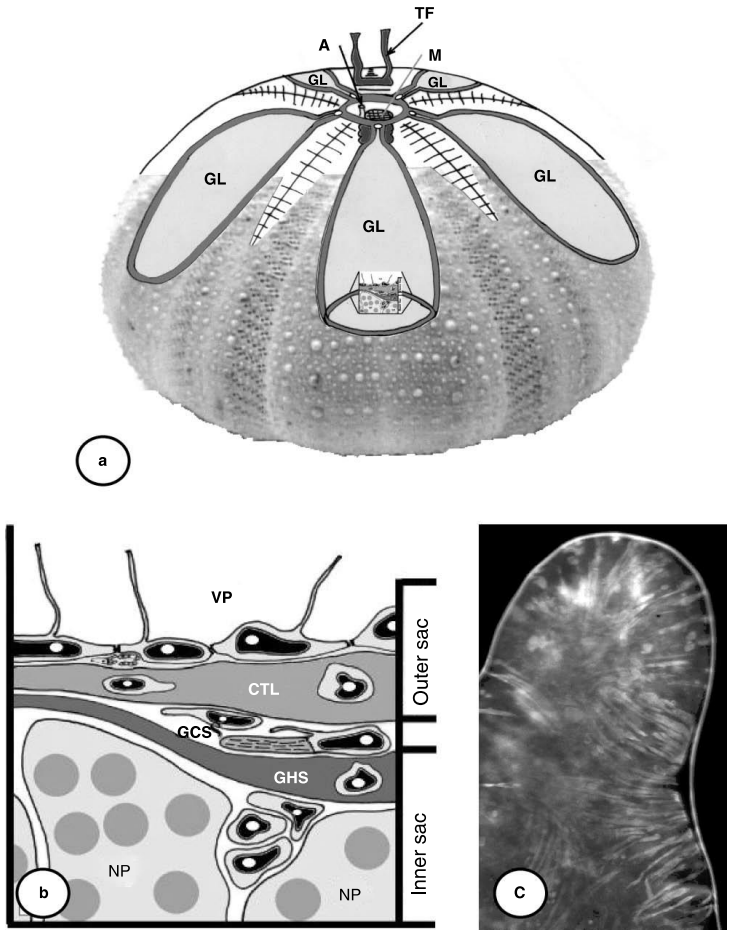

Figure 1.7. Diagrammatic representation of the sea urchin reproductive system in aboral side view; (b) Diagrammatic representation of the tissues in the sea urchin gonad wall, after Strenger (1973); (c) Lobe of a sea urchin gonad stained with phalloidin to show muscles on the exterior surface of the GHS of the inner sac (white strips). Abbreviations: A – anus; CTL – connective tissue layer; GL – gonad lumen; M – madreporite; NP – nutritive phagocytes; GCS – genital coelomic sinus; GHS – genital hemal sinus; TF – tube feet; VP – visceral peritoneum. (Extracted from Walker et al., 2007)

The inner sac is a genital hemal sinus (GHS) bears ciliated myoepithelial cells on its outer face, similar to those observed in sea stars gonads (Walker 1979, 1982), whilst, on its internal side, the GHS supports the germinal epithelium. Myoepithelial cells act as muscles and rhythmically contract during gamete release (Okada et al. 1984; Okada and Iwata 1985) (Fig. 1.7) and nerves synchronize contraction of the gonads during spawning (Walker et al., 2007). The principal function of the inner sac is gametogenesis with very limited nutrient storage activity, whilst nutrient storage takes place in the nutritive phagocytes (NP) portion. About 80% of the protein within

NP is a glycoprotein that was originally identified as the predominant component of yolk granules in sea urchin eggs and termed major yolk protein (MYP) (Harrington and Easton, 1982).

MYP is predominately synthesized in the intestine of the adult sea urchin (Shyu et al., 1986). It is then secreted into the coelomic fluid, transported through the two epithelial layers of the ovarian capsule, and is absorbed by the NPs where it is stored in large granules (Brooks and Wessel, 2003). Unlike other oviparous animals where the yolk protein is female-specific (Vitellin) both male and female sea urchins produce MYP prior to gametogenesis (Unuma et al., 1998). The cloning and characterization of MYP (Brooks and Wessel, 2002) suggests that it is an iron binding protein similar to transferrin rather than a vitellin-like protein. This classification seems to better fit its physiological role and suggests that its function is to transport iron to support gametogenesis and embryogenesis activities. After gametogenesis begins, MYP in NPs is utilized to synthesize new proteins and other components for eggs and sperm (Unuma et al., 2003; Unuma and Walker, 2010).

1.6.3 Gametogenesis

Reproductive cycles can be monitored by histological observation of the gonads. Changes within the gonads can be classified according to the relative proportions of the two major cell populations composing the germinal epithelium: the germinal cells (oogonia and spermatogonia) and somatic cells or Nutritive Phagocytes (NP).

According to Byrne (1990) gametogenic cycle can be divided in six stages:

- *Stage I: Recovery stage*
- *Stage II: Growing stage*
- *Stage III: Premature stage*

- *Stage IV: Mature stage*
- *Stage V: Partially spawned stage*
- *Stage VI: Spent stage*

According to Byrne (1990) the Recovery stage (Stage I) begins after spawning with the resorption of the majority of residual gametes. In the ovary (Fig. 1.8 a) it is characterized by the presence of small droplets surrounding the lumen (arrows), which result from lysis of relict oocytes.

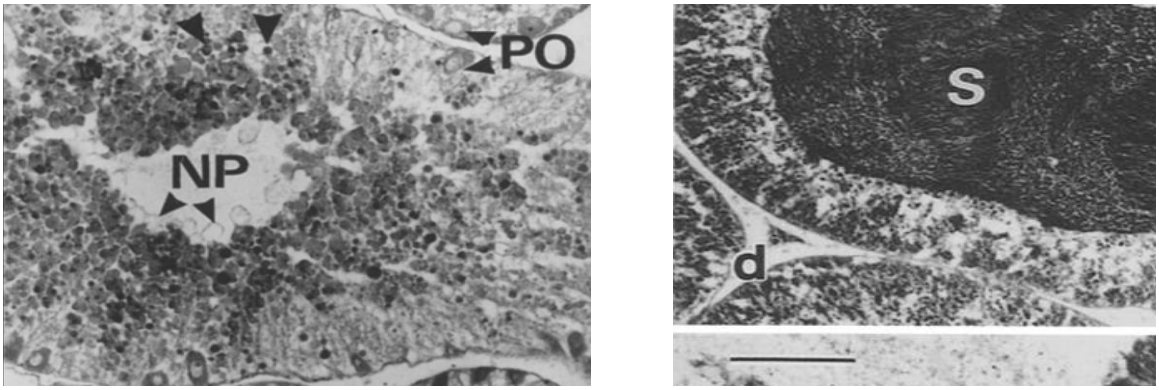

Figure 1.8. Histology of ovary (a) Stage I: cross-section through ascinus of recovering ovary showing periodic acid Schiff-positive globules (arrowheads) derived from lysis of relict oocytes; extensions of nutritive phagocytes (NP) project into lumen; small previtellogenic oocytes (PO) occur along ovary wall. Testirs (b), Stage I: crosssection through ascinus of recovering testis containing relict spermatozoa (R) and nutritive phagocytes (NP) which form an eosinophilic meshwork. (Extracted from Byrne, 1990)

Few NPs project towards the lumen and in this stage they are at their smallest size of the entire cycle as a consequence of the mobilization and release of most of the nutrients they originally contained (Walker et al., 2007). Pre-vitellogenic oocytes (PO, 5-30 μm) begin to form on the ovary wall and will participate in the next gametogenic event.

In the testes (Fig. 1.8 b), a thin layer of spermatogonia and primary spermatocytes adhere to the ascinal wall and nutritive phagocytes (NP) form a meshwork across the gonad and relict spermatozoa (R) may still be present in the lumen.

Onset of vitellogenesis (Stage II) takes place during the growing stage when early vitellogenic oocytes (EV) increase in size (10 to 50μm) and nuclei (N) become visible. They remain attached to the ascinal wall and are generally surrounded by nutritive phagocytes, which, in the meantime, have increased in numbers and size and now fill the lumen. Groups of primary oocytes and delayed relict oocytes may still be visible (Fig. 1.9 a). In testes (Fig. 1.9 b), the germinal cell layer increases in depth as spermatocytes move towards the lumen (arrows). Like for the ovaries, growing NPs form a compact meshwork within the ascinus.

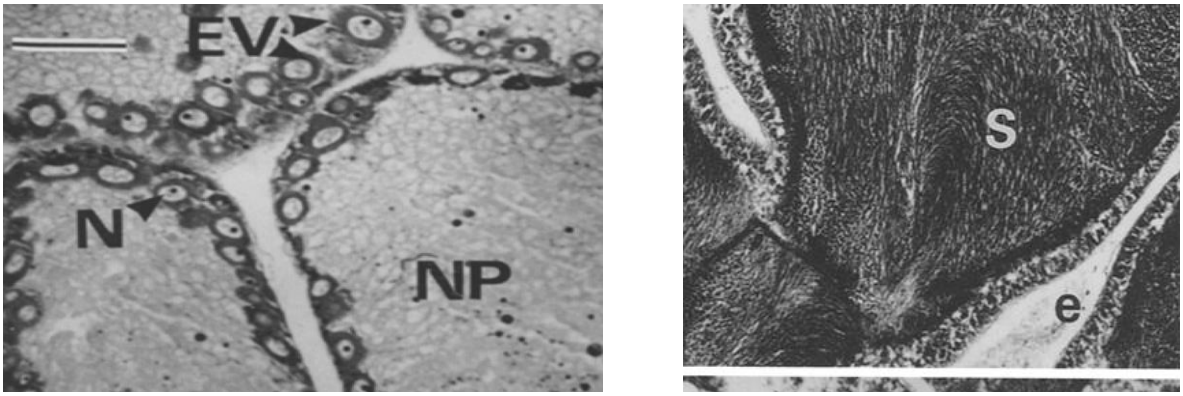

Figure 1.9. Histology of ovary (a) Stage II: growing ovary with early vitellogenic oocytes (EV) and nutritive phagocytes; N: nucleus. Testis (b) Stage II: columns of spermatocytes project centrally (arrowheads) in growing testes, nutritive phagocytes fill ascini. (Extracted from Byrne, 1990)

In the ovaries, vitellogenesis continues during the premature stage (Stage III), and oocytes at all stages of development are present in the ovary (Fig. 1.10 a). The large primary oocytes detach from the ascinal wall and begin to move towards the centre of the lumen. As vitellogenesis proceeds oocytes increase in size and the NPs are displaced from their central position. Once the primary oocytes have reached

maximum size (90 μm) they undergo maturation and ova (O) accumulate in the lumen. A very similar dynamic can be observed in the testes (Fig 1.10 b), where columns of spermatocytes move towards the lumen as maturation proceeds, displacing NPs and filling the lumen with spermatozoa (S).

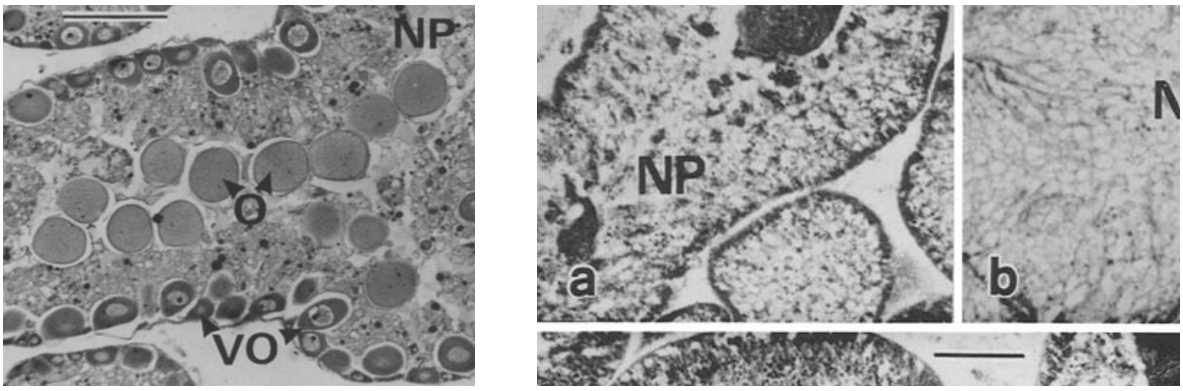

Figure 1.10. Histology of ovary (a) Stage III: premature ovary with oocytes at all stages of development; nutritive phagocytes surround vitellogenic oocytes (VO) which detach from ascinal wall, and ova (O) accumulate in the lumen. Testis (b) Stage III: premature testis with spermatozoa (S) in centre and nutritive phagocytes around periphery. (Extracted from Byrne, 1990)

The mature stage (stage IV), in both ovaries (Fig. 1.11 a) and testes (Fig. 1.11 b) features the small NPs that form a loose network between the walls and the lumen, which is now filled with densely packed ova and spermatozoa. In the ovary, few small (10-60μm) oocytes may still be present close to the ascinal wall.

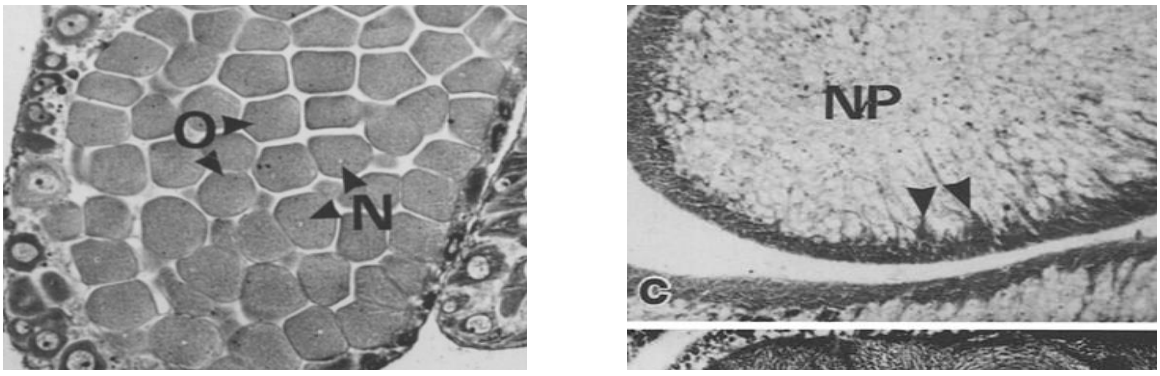

Figure1.11. Histology of ovary (a) Stage IV: mature ovary packed with ova, nutritive phagocytes are reduced to a thin layer along ascinal wall.Testis (b) Stage IV: mature testis filled with spermatozoa and largely devoid of nutritive tissue. (Extracted from Byrne, 1990)

At spawning (Stage V), ova and spermatozoa are released in the gonoduct and, consequently, the ascinus lumen appears more loosely packed than in the previous stage (Fig. 1.12 a, b, c).

In ovaries, however, a significant amount of developing oocytes might still be present and undertake maturation to replace spawned ova. Vitellogenic oocytes are still present on the ascinal walls and NPs are still visible although significantly reduced in numbers. In partially spawned ovaries with these characteristics, vitellogenesis will continue for the first part of the breeding season and gonads might still appear similar to those at stage III (Fig. 1.12 a). In other cases, however, developing oocytes are absent and only a few vitellogenic oocytes are present on the ascinal wall and the ovaries appear devoid of nutritive phagocytes (Fig. 1.12 b). In this second case, vitellogenesis stops and only one cohort of ova will be produced.

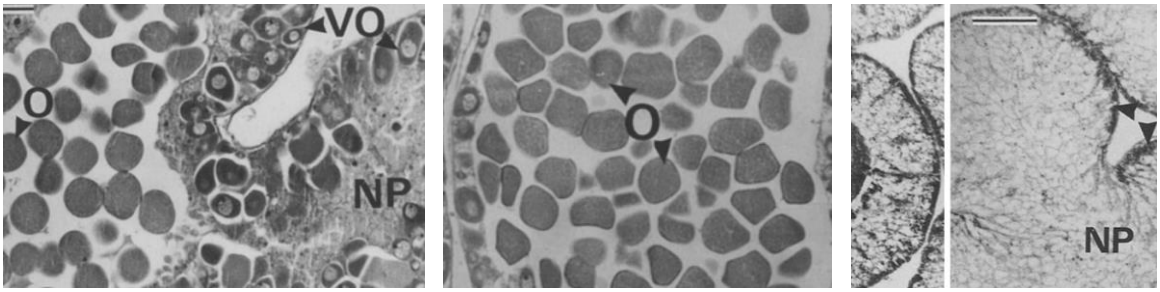

Figure 1.12 Histology of ovary (a) Stage V: partly spawned ovary but still in Stage III condition, with oocytes at different stages of development and nutritive phagocytes; most vitellogenic oocytes will eventually mature and move to the lumen. (b) Ovary at Stage V: partly spawned ovary with loosely packed ova and a scarcity of nutritive material; except for the empty spaces left by spawned ova, ovary is similar to Stage IV. Testis (c) Stage V: partly spawned testis with spaces left empty by spawned spermatozoa. (Extracted from Byrne, 1990)

After spawning (Stage VI), residual ova and vitellogenic oocytes undergo a phagocytotic process and the remaining material (L) is taken up by NPs that now start regaining their size and preparing for the next cycle (Fig. 1.13 a, b). Testes

lumen appears empty but spermatozoa might still be present in the gonoduct (Fig. 1.13 c)

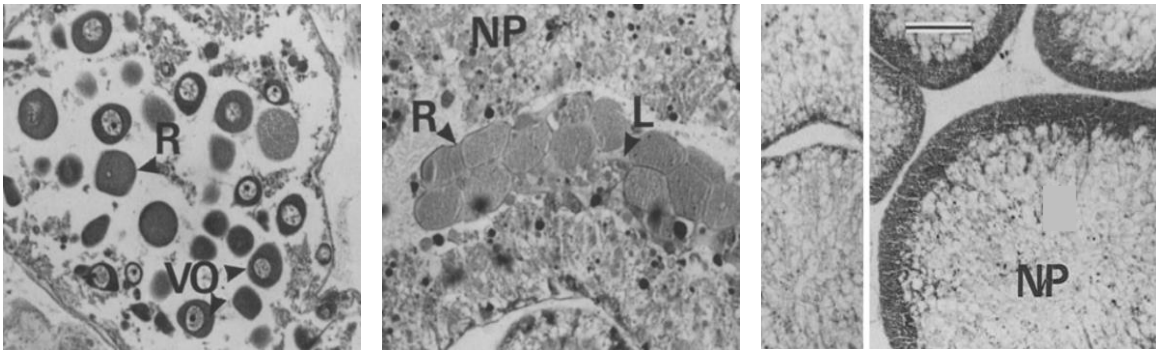

Figure 1.13. Histology of ovary Stage VI: spent ovary largely devoid of ova and nutritive phagocytes; all vitellogenic oocytes and relict ova will be resorbed (a). Ovary intermediate between Stages VI and I, with relict ova undergoing lysis (L); lysed material is taken up by nutritive phagocytes. Testis (c) Stage VI: spent testis largely devoid of spermatozoa or nutritive material. (Extracted from Byrne, 1990)

1.6.4 Environmental regulation of reproduction

In echinoids, photoperiod has been shown to primarily influence the gametogenic cycle (McClintock and Watts, 1990; Pearse and Cameron, 1991; Shpigel et al, 2004). Kelly (2001) suggested that for the cold-water sea urchin such as *Psammechinus miliaris*, lengthening days are an important cue for the completion of gametogenesis in both males and females and low temperature is an important cue for completion of vitellogenesis. Pearse et al. (1986) showed that photoperiod controlled both gametogenesis and growth in the echinoid *S. purpuratus* by influencing energy allocation between somatic growth and reproductive effort. On the Pacific coast of North America, this species spawns mainly in January and February, suggesting that either long days inhibit or short days enhance gametogenesis. Pearse and Eernisse (1982) obtained similar results for the sea star (*Pisaster ochraceus*) and Shpigel et al. (2004) for *P. lividus*. The recent discovery of photosensory organs in the tube feet provides solid evidence on the ability of sea urchins to perceive light (Lesser et al.,

2011) and corroborates the hypothesis that photoperiod is a strong environmental cue for onset of gametogenesis. Contrary to these results, Spirlet et al. (2000) found that photoperiod has less influence than temperature on the reproductive cycle and growth of *P. lividus* in a land-based cultivation system, however the trial was conducted for 45 days only whilst complete gonadal cycle requires at least 120 days (Shpigel, 2004).

1.6.5 Endogenous regulation of reproduction

Regardless of which environmental cues are mainly responsible for gonad maturation, their effects on the physiology and morphology of the gonads suggest that exogenous cues are translated to endogenous factors regulating reproduction in adult individuals (Wasson and Watts, 2007). Figure 1.14 illustrates a comparison between the known endogenous factors and their interactions in vertebrates and in sea urchins.

Several bioactive compounds, such as sex steroids (Colombo and Belvedere, 1976; Varaksina and Varaksin, 1991; Watts et al., 1994; Wasson et al., 2000a, b), proteins and peptidergic factors (Harrington and Ozaki 1986; Cochran and Engleman, 1972), catecholaminergic, and cholingeric factors (Khotimchenko 1983; Khotimchenko and Deridovich 1991) have been ascribed a role in gonad functions. However, no data exist on the chemical messengers required to promote nutrient translocation from the gut to the gonads, the mobilization of nutrients within the nutritive phagocytes, or translocation of nutrients to developing gametes.

Nonetheless, paracrine/autocrine and endocrine mechanisms are likely to be responsible for regulation of gamete maturation and spawning. In the paracrine

mechanisms, responses in the target cells are induced by a chemical messenger produced by neighbouring cells.

Nutritive phagocytes surround developing gametes and provide the required local environment to promote gamete proliferation and growth. In male sea urchins, the role of nutritive phagocytes has been considered similar to that of Sertoli cells in mammals (Pearse and Cameron 1991). Studies by Varaksina and Varaksin (1991) demonstrated the ability of endogenous steroids to convert enzymes in nutritive phagocytes, whilst Wasson et al., (2000) reported the involvement of estradiol and progesterone in mature gonad growth and gametogenesis, suggesting that steroids may be produced by the nutritive phagocytes and influence gametogenesis or nutrient translocation to developing gametes via tight junctions. The promotion of gonad growth, gametogenic activity, and nutrient storage by sex steroids and/or their derivatives suggest that sex steroids receptors are present in echinoid gonads (Wasson et al. 2000a). The presence of steroid receptors in either nutritive phagocytes or germinal cells has not been, however, investigated yet in echinoids. Histochemical studies indicate that developing oocytes and spermatids possessed the ability to convert Androstendione and Estrone into Testosterone (T) and Estradiol (E2) (Varaksina and Varaksin 1991), suggesting that developing gametes also synthesize steroids that may act in autocrine mechanisms (Wasson and Watts, 2007).

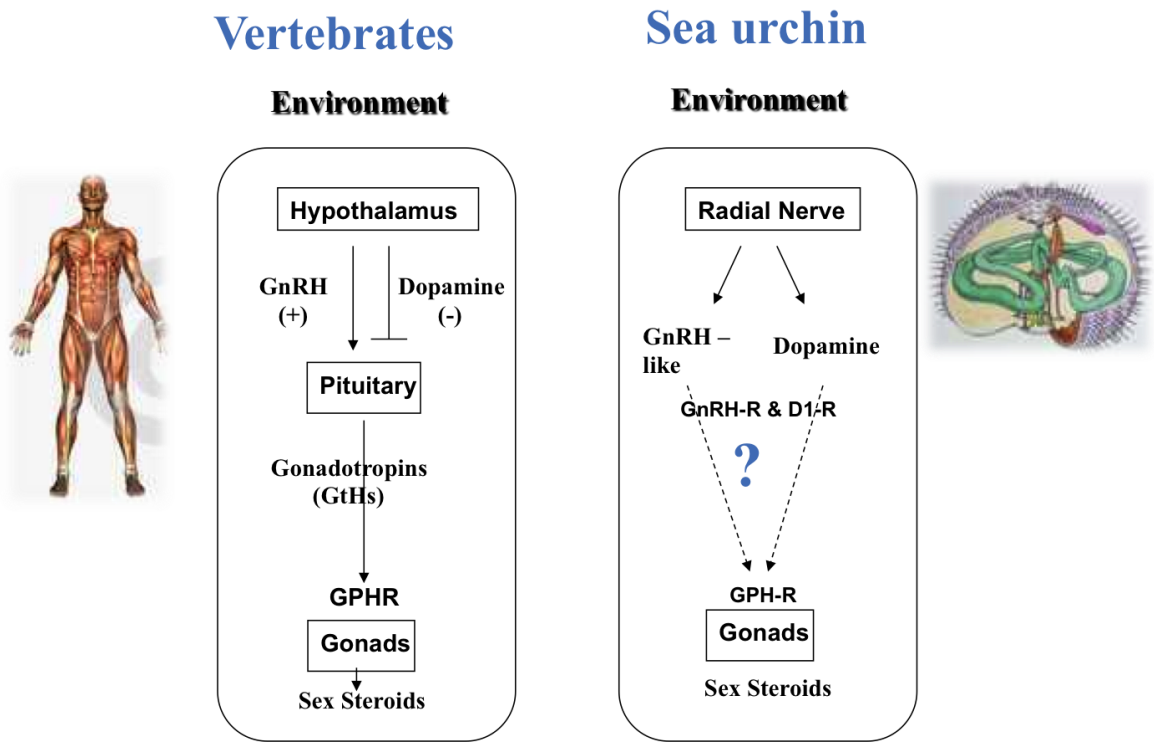

Figure1.14 Reproductive endocrine axis in vertebrates and sea urchin. The lack of a central nervous system in sea urchin makes it difficult to investigate the middle steps of the pathway transducing environmental cues into sex steroids production, which control reproduction. (H. Rosenfeld pers. Comm.)

The identification of a spawning inducing factor, a neuropeptide, from the radial nerve of *S. purpuratus* by Cochran and Engelmann (1972) was believed to be the first demonstration of the existence of an endocrine system in sea urchin. The "radial nerve factor", in fact, induced the production of a "gonad factor" which itself induced spawning in *S. purpuratus* testis fragments. It is possible, however, that this factor might only induce muscular contraction during gamete release and play no role in oocyte or spermatocyte maturation (Wasson and Watts, 2007). The mechanism of action and mode of transfer of the radial nerve factor is moreover still unknown (Wasson and Watts, 2007).

Several levels of endocrine regulation can however be hypothesized when examining sea urchin reproduction. The required synchrony of spawning among individuals within a population indicates that some factors, maybe a pheromone, released into

the water by an individual induce spawning in other individuals. The influence of the environment on gametogenesis and nutrient storage suggests, moreover, the existence of a communication system between the environment and the organs involved in reproduction. Finally, coordination of gametogenic and nutrient translocation activities among and within each gonad branch indicates the existence of a physiologically controlled mechanism that coordinates these activities (Wasson and Watts, 2007).

1.6.6 Spawning

In *P. lividus* spawning events do not involve all individuals of a population (Allain, 1975). More frequently, groups of 10 to 20 individuals aggregates on elevated stones or on the top of sea grass leaves for a few hours and release gametes in the water column (Boudouresque and Verlaque, 2007). Spawning can be triggered by a suspension of gametes that could potentially provide a cue for synchronization (Keckes et al., 1966). As spawning involves only a small proportion of the population and very localized conditions promote gonadal maturation, spawning events are occurring all year round over the geographical distribution of the species. Indeed, all year round presence of the larvae in the plankton, although at different concentrations, seems to corroborate this conclusion. No clear environmental cue can be identified as sole responsible event for triggering spawning in all echinoid species. Rising (Fenaux, 1968; Byrne 1990; Pedrotti 1993) or dropping (Agatsuma et al., 2004) seawater temperature, or increase (Spirlet et al., 1998) or decrease (Bay-Schmith and Pearse, 1987) in day length have been considered alternatively responsible for spawning induction in several echinoid species. Furthermore, phytoplankton blooms have been considered responsible for spawning induction in

Strongylocentrotus droebachiensis (Starr et al., 1992, 1994). No evidence of lunar cycle influence on spawning has been established for *P. lividus*, however several other echinoid species such as *Echinometra*, *Anthocidaris crassipina*, *Evechinus chloroticus*, seem to be able to perceive lunar cycle and spawning is reported to occur near full moon (Iliffe and Pearse, 1982) or new moon (Agatsuma et al., 2004). In captivity, spawning is usually induced by intracoelomic injection of 0.5 – 1.0 M KCl, however several other cues have been recently investigated such as temperature, salinity and mechanical shocks, emersion and suspension of gametes in the adults' rearing water (Gago, 2009). Temperature and salinity shock did not yield gametes although extreme treatments led to varied degree of mortality. Mechanical shock did induce spawning and it seems that shocking times as short as 1 minute are able to induce mass gamete release with no mortality being reported. Emersion for short period also yielded gametes although long exposures (6 to 12 hours) to dry conditions were usually required. Very high concentrations of conspecific gametes suspension triggered spawning and this seemed to be gender specific, clearly no mortality was observed with these techniques, although the need to get gametes in the first place somewhat limits practical utilisation of this methodology. Overall, according to the observations by Gago, injection with KCl produced reliable spawning although a positive correlation between gametes release and KCl concentration was observed.

1.6.7 Fertilisation

There are five sequential events in the process of sea urchin fertilisation: The chemo-attraction of sperm to egg-released peptides, the induction of the Acrosome Reaction (AR) of sperm by egg jelly, the binding (adherence) of sperm to the egg vitelline

layer, the fusion of the plasma membranes of sperm and egg, the fusion of haploid egg and sperm pro-nuclei in the egg cytoplasm that restores the diploid genome.

The high CO_2 pressure and consequent low pH within the testis prevents the activation of dynein ATPase in the flagella, effectively keeping spermatozoa immotile (Santella et al., 2012).

Once exposed to seawater, the increment in pH and the concomitant activation of adenylyl cyclase prompts cAMP-dependent protein kinase (PKA) to phosphorylate proteins essential for flagella motility (Trimmer and Vacquier, 1986, Santella et al., 2012). Spermatozoa swimming trajectory is directed towards the target by the presence of small diffusible activating peptides (SAPs) present in the egg jelly and by a cycle of hyperpolarization and depolarization of the membrane potential mediated by Ca^{2+} fluctuations in the sperm head and $Na^{+}/Ca^{2+}/K^{+}$ exchanger in the flagellum (Neill et al., 2004; Jayantha et al., 2007; Darszon et al., 2008, 2011; Santella et al., 2012).

Once the sperm head contacts the vitelline layer of the egg the acrosome vesicle is projected forward via exocytosis. The kinetic of the sperm acrosome reaction (AR) was discovered by Dan (1954) in starfish sperm. She found that increased permeability to Ca^{2+} was the triggering event leading to exocytosis of the acrosomal vesicle and to the consequent formation of a filament process from the tip of the sperm head (Fig. 1.15). In sea urchin, the acrosomal filament extends for 1 μm and is covered with bindin, the adhesive protein responsible for the species-specific attachment of sperm to the egg's vitelline layer.

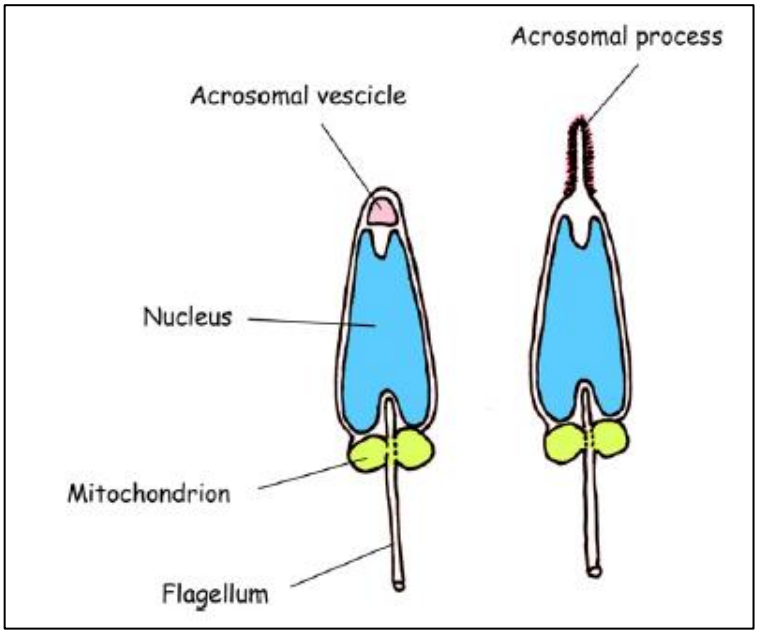

Figure 1.15 Morphology of sea urchin sperm before and after acrosome reaction (extracted from Santella et al., 2012).

Fertilized eggs require a mechanism to prevent the formation and development of the polyspermic zygotes. In echinoderms, the elevation of the fertilisation envelope serves as a mechanical block to polyspermy, however this mechanism seems to be too slow to justify the fact that the rate of re-fertilisation of sea urchin eggs is much lower than that of the initial fertilisation, and therefore a more rapid block to polyspermy mechanism must be present after the interaction of the first successful sperm (Santella et al., 2012).

Intracellular recordings from sea urchin eggs have shown that the first detectable electrical event at fertilisation is the step-like depolarization, which is accompanied by an increase in voltage noise about 13 s (the latent period) before the long depolarization of the membrane potential (Santella et al., 2012). Jaffe (1976) first suggested that this rapid shift of the membrane potential to a positive level causes the fast block to polyspermy, which renders the egg plasma membrane refractory to supernumerary spermatozoa. The hypothesis of electrical fast block to polyspermy

has been reported in several other species, but was not supported unanimously (Dale and Monroy, 1981).

A turning point in the studies on the egg activation was the pioneering observation of a Ca^{2+} increase in the eggs of sea urchin following fertilisation (Mazia, 1937). However, it took 40 years to conclusively demonstrate that Ca^{2+} was responsible for sea urchin egg activation. The Ca^{2+} specific luminescent protein aequorin visualized the explosive rise of free Ca^{2+} during fertilisation of sea urchin eggs (Steinhardt et al., 1977). The Ca^{2+} signals in fertilized eggs initiated at the point of sperm entry and spread over like a wave, reaching the opposite pole in about 20 s (McDougal et al., 2000).

After fertilisation, the zygote begins cleavage. Sea urchins undergo radial cleavage, as do typical deuterostomes, such as chordates, ascidians, and other echinoderms. Cleavage proceeds through blastula stage when the embryo "hatches" from the fertilisation envelope. The cells bear cilia, allowing the embryos to swim. Embryogenesis proceeds with gastrulation and the formation of the primitive gut, the archenteron. At the same time, the mesenchyme cells move to eventually line up in positions where they will begin to secrete the calcium-phosphate skeleton of the sea urchin. As the skeleton grows, the embryo changes shape into the larval form.

1.7 Larval development

Larval development involves significant changes in the larval body structure, and terminates with the formation of the rudiment and the achievement of competence for settlement. The echinopluteus is a pelagic larva showing a variable number of arms (depending on species and development stage), which possess the ciliated feeding structures supported by calcareous skeletal rods (Okazaki, 1975; McEdward and Miner, 2001). Echinoplutei feed on suspended particulate food, normally microalgae, and possess a fully functional gut (Burke, 1981). Nerves have been found along the ciliated band and at the oesophagus and have a coordination role in the production of feeding and swimming currents. The larval blastocoelic space is mostly filled with gel (Strathmann, 1989) and presents three pairs of coelomic sacs: anterior axocoels, hydrocoels, and posterior somatocoels. The body forms of echinoid larvae are very diverse because of variation in the numbers (from 2 to 13, 8 in *P. lividus*) and relative sizes of the larval arms (Onoda, 1936; Mortensen, 1938; Pearse and Cameron 1991; Wray, 1992).

A detailed description of larval development for planktotrophic echinoids larvae has been proposed by Smith et al. (2008) and the following scheme has been suggested:

- Stage I, four-arm stage;
- Stage II, eight-arm stage;
- Stage III, vestibular invagination stage;
- Stage IV, rudiment initiation stage;
- Stage V, pentagonal disc stage;
- Stage VI, advanced rudiment stage;

- Stage VII, tube-foot protrusion stage.

1.7.1 Stage I: Four-Arm Stage

Larvae at this stage begin to feed and both anterolateral (a) and the postoral arms (po) slightly project anteriorly passing the oral hood. During this stage, the anterolateral arms extend to about one third of a body length, while the postoral arms extend slightly further and become thicker than the anterolateral arms (Fig. 1.16).

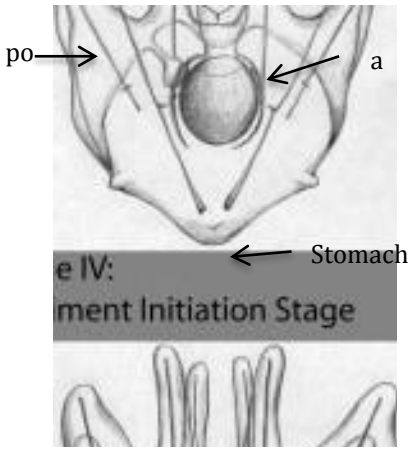

Figure1.16. Schematic representation of the four arms stage. Stomach, Anterolateral and Postoral arms in evidence. Modified from Smith et al., 2008

1.7.2 Stage II: Eight Arms Stage

The eight-arm stage is characterized by the further development of the skeletal elements and two new pairs of arms appear during this stage. The first are the posterodorsal arms (pdr) in the anterior portion of the body and positioned closer to the anterolateral arms

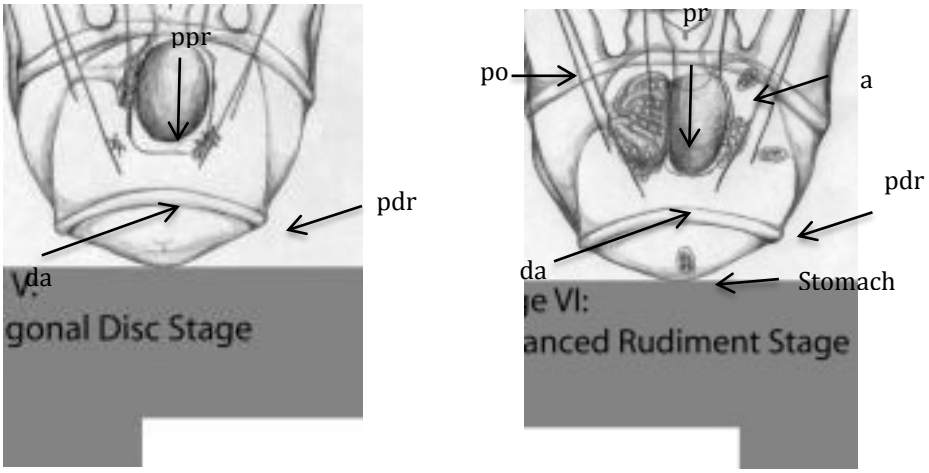

Figure 1.17. Early and late eight arms stage, with main features highlighted. Modified from Smith et al., 2008.

The next skeletal element appearing during this stage is the tri-radiate precursor of the dorsal arch (da). This begins to form above the stomach (Fig. 1.17). At this stage the structure of the oral region becomes more elaborate, as two projections (the pre-oral arms, ppr) start to develop between the anterolateral arms. At the beginning of stage II these new structures, are often not evident and larvae thus appear to have 3 pairs of arms, this stage is therefore often called six arms stage in aquaculture literature (Liu et al., 2007). During the latest part of this stage and in the next one the spicules of the dorsal arch enters the newly developed structure sustaining it and forming the pre-oral arms (pr).

1.7.3 Stage III and IV: Vestibular invagination stage and rudiment initiation stage

The most important morphological event occurring during stage III is the formation of the vestibule (v) that will form part of the adult rudiment. The ectoderm begins to invaginate from the left side towards the left side of the stomach (fig. 1.18 a). During this stage larvae become wider and new swimming and feeding apparatus, the

anterior and posterior (aep, pep) epaulettes, develop between the posterodorsal and postoral arms (Pearse and Cameron, 1991, Smith et al., 2008).

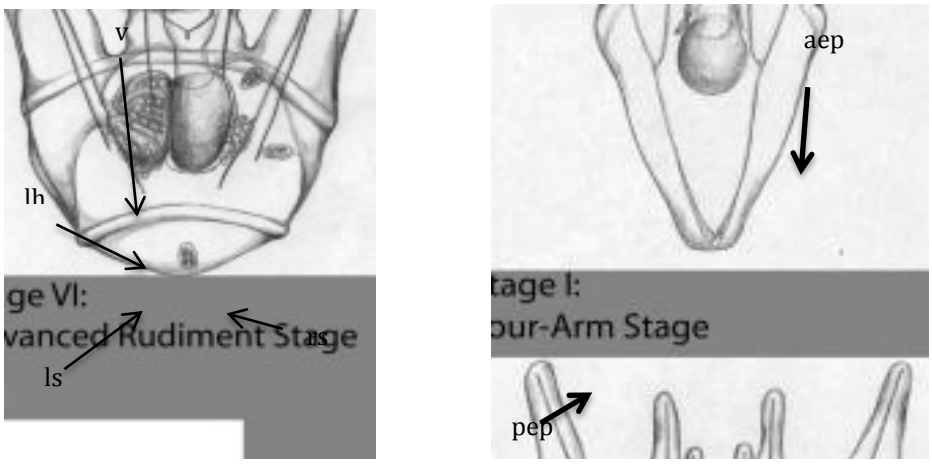

Figure1.18. Schematic drawing of the main characteristics of the Initial vestibule invagination stage (a) and initial development of the epaulettes structures (b).

During the early stage IV the invaginating vestibule contacts the left hydrocoel, lh (Fig. 1.18 b). The layer of ectoderm that contacts the hydrocoel is called vestibular floor, derivatives of which are destined to form the nervous system of the adult urchin (MacBride, 1903).

As the vestibule meets the left hydrocoel, adult skeletal elements begin to form at the proximal ends of the posterodorsal and postoral arm spicules. Each structure forms an initial tri-radiate that elaborates many branches typical of the adult body wall skeleton. The resultant calcareous networks will become the genital and terminal plates of the adult test (Smith et al., 2008).

The epithelial floor of the vestibule flattens against the hydrocoel and thickens, continuing the process begun in the vestibular invagination stage. After the vestibule and hydrocoel meet, left and right somatocoels (ls) begins to extend posteriorly along the stomach, and, by the end of stage IV, meet at the most posterior pole of the stomach.

1.7.4 Stage V: Pentagonal Disc Stage

At this stage the vestibular wall (vw) is attached to the hydrocoel via the vestibular ceiling (vc) and five hydrocoel projections (tfp) becomes visible as they press against the vestibular floor and the left somatocoel (ls)(Fig. 1.19).

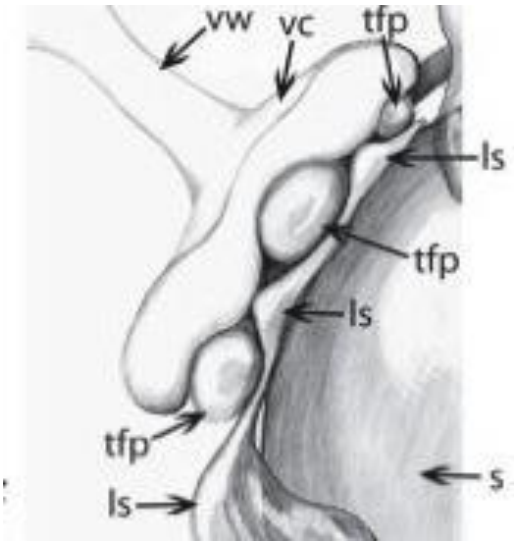

Figure 1.19. Tissues forming the rudiment. Three tube-foot primordia project from the hydrocoel, pressing into the vestibular floor. The somatocoel is extended between hydrocoel and stomach. Modified from Smith et al., 2008

These five projections establish the primordia of the radial canals and the terminal podia that will lie at the dorsal-most extent of the body wall of the adult urchin (MacBride, 1903; Smith et al. 2008). When the rudiment is observed from the left side of the larva, the hydrocoelar projections resemble a pentagonal disc presenting one tube-foot at each vertex (Fig. 1.20). The projections extend into the vestibular space from these points and form bilayered radial canals (rca), with an outer layer constituted by the vestibular floor and an inner by the hydrocoel. The distal ends of these canals terminate in the primary tube feet, which will protrude into the vestibular space as they develop (Smith et al., 2008).

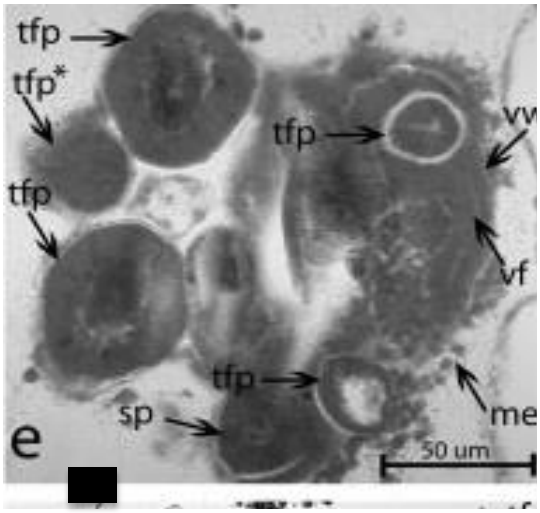

Figure1.20. View from left side of the larvae. Close-up of rudiment during the pentagonal disc stage; tube-foot primordia surrounds the radial canal. Modified from Smith et al., 2008

Once the five tube feet are formed, the left somatocoel extends five projections up against the vestibular floor, which will form the five dental sacs that will originate the teeth of Aristotle's lantern (MacBride, 1903; von Ubisch, 1913; Smith et al., 2008).

By the end of the pentagonal disc stage, the posterior and anterior epaulettes form two complete circles around the larval body (Fig. 1.21)

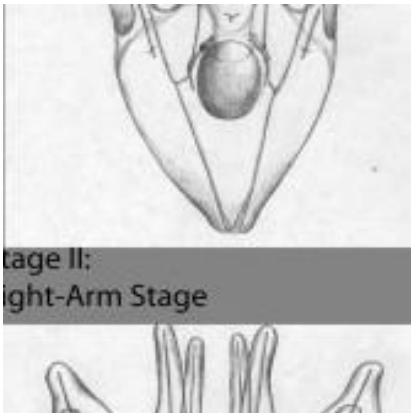

Figure 1.21. Schematic general appearance of the pentagonal disc stage pluteus. Modified from Smith et al., 2008

1.7.5 Stage VI and VII, advanced rudiment stage and tube-foot protrusion stage.

During the advanced rudiment stage, the tube feet primordia and dental sacs continue to grow and, as observed by Smith et al., (2008) in *S. purpuratus* skeletal elements begin to develop within the rudiment. According to Gordon (1926) and as cited in Smith et al., (2008), the first skeletal elements observed within the rudiment are three terminal plates; followed by three pre-ambulacral plates. Soon after the appearance of the plates primordia, juvenile spines can be observed on the perimeter of the rudiment. As spines begin to develop the tube feet develop sucker-shaped structures on their ends, which will eventually enclose a calcified ring in the centre, and will become the sensory organs of the tube feet. These organs have been proposed to be receptors for tactile stimuli involved in the induction of metamorphosis (Burke, 1980) and both chemo and photo receptors in juveniles and adults (Pisut, 2004; Ullrich-Luter et al., 2011).

During stage VII the tube feet and spines can emerge from the vestibular opening, larval arms become shorter and the epidermis starts to degenerate. During this period larvae behaviour also change as they actively look for suitable substrates to undertake metamorphosis.

1.8 Settlement and metamorphosis

1.8.1 Perimetamorphic period

In the Echinoids' life cycle the period immediately before and after metamorphosis is called perimetamorphic period. It is an essential stage in the life history as it links the planktonic larval period with the benthic juvenile stage (Gosselin and Jangoux, 1998). These events may be grouped into three distinct stages:

- Competent stage, at the beginning of which the larva acquires the ability to metamorphose and during which it searches for a suitable substratum
- Metamorphic stage, during which pelagic larvae settle on the substrate and transform their body plan
- Postlarval stage, where the post-metamorphic individual prepares its passage to juvenile life.

Morphological changes occurring during the perimetamorphic period in *P. lividus* have been described by Gosselin and Jangoux (1998) and are here briefly reported.

When competence is achieved the rudiment is fully formed, the larvae are still feeding exogenously and are pelagic (Fig.1.22 a). At this point and in presence of adequate environmental stimuli, larvae have the ability to "swim" towards the bottom to make contact with the substratum and test its suitability for benthic life (Fig 1.22 b). Recently, histamine leaching from algae and sea grasses has been suggested as a habitat marker and metamorphic cue for larvae of several sea urchin species (Swanson et al., 2012).

Just after coming in contact with the substratum, the larvae pull the epaulets' cilia down to the bottom, bend their preoral lobe forwards and spread out their postoral

and posterodorsal arms located on the left side. This allows the opening of a hole in the vestibular wall (vestibular pore) and the partial protrusion of the primary podia, which make contact with the substratum (Fig. 1.22 c). Without the appropriate stimulation, primary podia are folded back into the vestibule, the arms are repositioned in their original location and larvae swim back up to the surface (Fig. 1.22 d). However, if the plutei meet a suitable substrate, they will start metamorphosis.

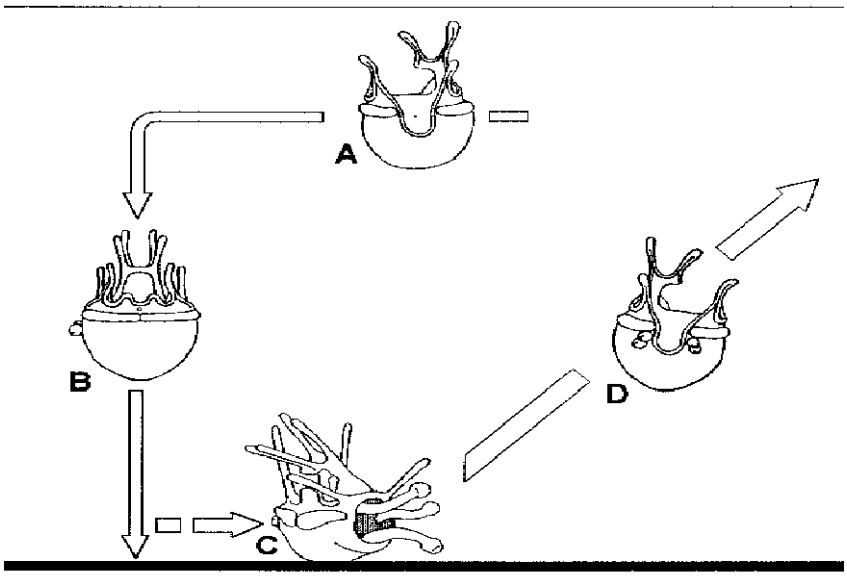

Figure 1.22. *P. lividus* Behavioural sequence shown by competent larvae in rearing conditions .A) Swimming phase (near the air/water interface). B Sinking phase. C Control phase. D Ascending phase. (Extracted from Gosellin and Jangoux, 1998)

1.8.2 Metamorphosis

Metamorphosis occurs in about one hour and follows a predetermined pattern common to all regular sea urchins (Burke, 1987): the echinoid rudiment evaginates from the vestibular pore on the left larval flank while most of the old larval structures move to the right side where they will be either lost, as for larval arms, or

progressively integrated by the vestibular wall (Fig 1.23), as for the epidermis associated with the genital plates (Gosselin and Jangoux, 1998).

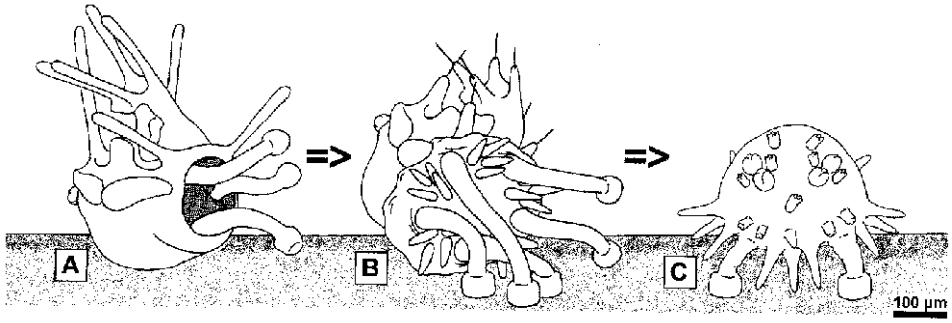

Figure 1.23. *P. lividus* Metamorphosis. After a positive recognition of the substratum during a control phase (A), the larva opens its vestibule, allowing the protrusion of the echinoidrudiment's spines and primary podia that hold in to the substratum (B), and transforms in less than 1 h into a spherical postlarva (C). (Extracted from Gosellin and Jangoux, 1998)

1.8.3 Postlarval morphology

During this period of about 8 days, postalarvae are sessile and autotrophic and measure about 350 µm in diameter. The postlarval body can be divided in three main regions: an upper coronal region, an equatorial midcoronal region and a lower infracoronal region. The supracoronal region includes the genital plates with the associated pedicellariae and the spines, and the hydropore (single and heavily ciliated orifice of about 10 µm diameter). The coronal region is subequatorial and it includes ten zones: five interambulacral areas, each presenting a group of four spines, and five ambulacral areas each presenting one central primary podium and two spines in aboral positions (Gosselin and Jangoux, 1998). The infracoronal region covers the rest of the postlarval body and, in the young postlarva, it is free of appendages and surrounded by a ciliary band. The main morphological changes involve the formation of the skeletal ambulacral and oral systems. Genital plates grow medially and distally to fuse with a central anal plate on the top of the test and

with the more external terminal plates. These connect with ambulacral and interambulacral plates where primary and secondary podia and spines are forming. On the infracoronal region, five pairs of oral plates define the location where the mouth of the exotrophic juvenile will appear (Gosselin and Jangoux, 1998).

1.8.4 Juvenile development

Transition to the juvenile stage is marked by two major morphological and functional changes: the development of a functional digestive system and the regression of the primary podia. After the opening of the oral orifice the mouth becomes functional and the five teeth of Aristotle's lantern are visible and mobile. Simultaneously, the anal plate rises preceding the appearance of the anus. The regression of the primary podia starts with the reduction of their stem followed by the regression of the terminal disc and the resorption of their skeletal support. As opposed to the postlarva, the juvenile is mobile and capable of "walking" and graze on the substratum. After 3 months, the diameter of the juvenile's test has increased to 5–20 mm and the morphological structures have assumed more complex features typical of the adult stage. Complexity of the oral region has also increased as new pedicellaria and buccal podia increase in numbers and develop to improve feeding efficiency.

1.8.5 Cues for settlement and metamorphosis

Beside larval survival, the second most important bottleneck in the reliable supply of juvenile to the aquaculture industry is post-settlement survival. Indeed, high mortality (>90%) is often reported during the first week post metamorphosis (Grosjean et al., 1998; Rahim et al., 2004; Buitrago et al., 2005) and the underlying

biochemical processes involved in substrate recognition and successful exogenous feeding initiation remain unclear. It is recognized that competent larvae undergo metamorphosis only in the presence of settlement cues (Mos et al., 2011). Environmental cues, such as temperature, are considered important to promote post-larval survival, however it does not *per se* promote metamorphosis. Indeed it has beneficial effects only when kept within the ranges most suitable for the investigated species (Mos et al., 2011). In several echinoid species metamorphosis rate is enhanced by a number of cues such as the presence of bacterial and/or diatom films (biofilms), presence of macroalgae and presence of adult conspecifics (Pearce and Scheibling, 1991; Gosselin and Jangoux, 1996; Hugget et al., 2006). Although many species positively react to all these cues without a clear preference, species-specific responses have been identified between some sea urchin species (*Heliocidaris erythrogramma* and *Centrostephanus rodgersii*) and their "host" macroalgae or sea grass species, or bacteria isolated from them, suggesting that some degree of specialization is present. The positive response to the presence of these substrates and the observed species-specificity, suggests that biochemical compounds are likely to play a key role in substrate recognition by competent larvae, however a single factor or co-factor has yet to be clearly identified (Mos et al., 2011). Nonetheless, Histamine has been very recently suggested as a mediating factor between some sea urchins (mainly lecitotrophic species) and their preferred substrate. Indeed, in two of the tested species (*H. purpurascens* and *H. inflatus*) it was able to promote metamorphosis in absence of any substrate. Histamine concentration in macroalgae has been measured and leaching of this compound has been suggested to act as a habitat marker and metamorphic cue for sea urchin competent larvae (Swanson et al., 2012).

1.9 Nutrition

As major bottlenecks in sea urchin aquaculture hatchery operations are resolved it has become clear that the development of formulated feeds for mantainance and grow out will be the next turning point in the successful and sustainable development of Echiniculture.

In 2007 Lawrence et al., reviewed the available information on sea urchin feeding and digestion. They have highlighted that current knowledge is inadequate and that *"…much opportunity exists for study of the complex behaviour associated with attraction to food and feeding. More information on digestion is needed. A major gap in our knowledge is information about proteases and lipases. Systematic, comparative studies of digestive enzymes and conditions in the gut during digestion are imperative. The role of microorganisms in the digestive processes of sea urchins must be clarified."* (Lawrence et al., 2007).

A summary of their review is reported here and updated, moreover content not covered in that review such as protein, carbohydrates, carotenoids and lipid requirements will be included here and discussed.

1.9.1 Ingestion

Ingestion is an important aspect of nutrition. Adult sea urchins feeding behavior can be strongly influenced by the food chemical composition and physical properties (Lowe 1974; Lilly 1975; Himmelmann and Carefoot, 1975; Klinger 1982). Abiotic factors such as temperature and light regime, and the physiological status of the animals such as their nutritional status (well fed *vs.* starved; Lawrence et al., 2003),

the reproductive stage (gonads in growing, spawning or recovery phases; Klinger 1997, Fernandez and Boudouresque 1997), can alter feeding frequency.

The response of sea urchin to the presence of food is complex. Individuals, in fact, may be attracted to food but not stimulated to eat it or they may take the food but avoid ingestion. Movement of sea urchins toward food indicates that positive chemotactic mechanisms are in place. Experimental demonstration of aggregation of sea urchins on food in the field has been shown for *S. droebachiensis* (Himmelman and Steele 1971; Scheibling and Hamm 1991; Vadas et al. 1986), *S. franciscanus* (Mattison et al. 1977), *Tetrapygus niger* (Rodríguez and Ojeda 1998; Rodríguez and Fariña 2001) and *L. variegatus* (Beddingfield and McClintock 2000; Vadas and Elner 2003). Moreover, there has been some evidence of the increased attraction of sea urchins towards food when stimulants were employed (Nagi and Kaneko, 1975; Dworjanyn et al., 2007). Despite the few data available on the chemical nature of the feeding stimulants for sea urchins, their preference for natural food and factors affecting this preference have long been explored (Vadas, 1977; Wright et al., 2005). Sea urchins display a hierarchy of preferences when offered a choice of natural diets, strongly preferring some seaweed species to others (Steinberg and van Altena, 1992). This preference has been attributed to factors including physical properties of the food and presence/absence of attractant/deterrent compounds (Nagi and Kaneko, 1975; Vadas, 1977; Sakata et al., 1989; Hay and Steinberg, 1992; Hay, 1996; Wright et al., 2005). Nonetheless, sea urchins will eat less preferred foods and even consume algae that contain toxic compounds when given no choice (Wright et al., 2005).

Diel rhythm in food consumption has been recorded for many species such as *P. lividus* (Kemp, 1962) *Diadema antillarum* (Lewis 1964), *D. setosum* (Lawrence and

Hughes-Games 1972), *T. gratilla* (Lison de Loma et al. 1999; Vaïtilingon et al. 2003) and *Echinometra. mathaei* (Mills et al. 2000). Light is the obvious stimulus (Lawrence et al., 2007). This probably is a response to a diurnal activity of predators with light as a learned conditioned stimulus for the sea urchins (Kemp, 1962). Most differences in feeding regime were associated to diurnal change in light intensity rather than photoperiod (Fuji, 1967; Lison de Loma et al., 1999).

The effect of temperature on food consumption rate has been described in several sea urchin species. Results indicate that short-term decreases in temperature depress feed intake in *L. variegatus* and *S. franciscanus*, (Watts et al., 2011; McBride, 1997) whilst no difference in feed intake of *Eucidaris tribuloiedes* were observed at different temperatures (Lares and McClintock, 1991). Long-term change in temperature appears to result in acclimation of food consumption rate for some species. Moore and McPherson (1965) reported complete seasonal acclimatization to temperature for *T. ventricosus* and Fuji (1967) found food consumption of *S. intermedius* was similar in summer and in winter. In contrast, higher food consumption in summer was reported for *S. droebachiensis* (Larson et al. 1980) and *S. nudus* (Machiguchi 1993). Moreover, food consumption rate of *E. tribuloides* was significantly less at 18 °C than at 27 °C for 5 months (Lares and McClintock 1991) and *P. lividus* held at temperature from 16 to 24 °C showed similar consumption rate, however this species showed lack of acclimation as consumption rate remained significantly higher at these temperatures than at 12 °C (Spirlet et al. 2000). A recent study by Hasegawa and colleagues, highlighted the importance of temperature for digestion ability of the sea urchin *S. intermedius* demonstrating that digestive

enzymes activity ceased when individuals were held at temperature above their optimum (Hasegawa et al., 2012).

A wide range of feeding behaviour is also displayed by the larval stages. Echinoplutei feed on particles that are suspended in the surrounding water. The feeding capacity of the pluteus larva is proportional to the total length of the ciliated band because of the mechanics of current production and particle capture (Strathmann, 1971; Hart, 1991). Currents are produced by the action of the cilia on the ciliated band. Water flows across the band and away from the mouth and circum-oral field of the larva at the velocity of 1300-1700 um per second. In response to a food particle, a transient (0.1 sec) and localised (100um of band) ciliary beat is induced and the particle is retained on the upstream side of the ciliated band. Captured particles are transported to the mouth and ingested. At low particle concentration, larvae feed at maximum rate, however at very high concentration feeding rate decrease or seems to cease altogether (Strathmann, 1971).

The natural diet of echinoid larvae is not well known, but larvae in the laboratory eat unicellular algae ranging from 5 to 50 um in diameter or length (McEdward and Miner, 2001). Echinoid larvae have also the capacity to acquire dissolved organic matter (DOM), especially neutral amino acids, directly from sea water (Manahan 1990). In the early stages of development, DOM uptake can provide up to 79% of estimated metabolic requirements of *S. purpuratus* larvae (Manahan et al., 1983). However, there is no evidence that planktotrophic larvae can successfully complete development and metamorphosis solely on DOM. Moreover, marine bacteria involvement in echinoid larvae nutrition is unknown (McEdward and Miner, 2001).

The effect of several natural and artificial diets on larval survival, development rate, growth and metamorphic success has been tested in several echinoid species. Even

though many species of cultured algae support growth and development through metamorphosis, such as *Rodhomonas lens*, *Dunaliella tertiolecta*, *Pheodactylum tricornutum*, *Isochrysis galbana*; *Cricosphera elongata* (Lawrence et al., 1977) not all of them promotes development and survival in the same way. Palatability of the diet and an adequate nutritional value are the main characteristics playing a critical role in echinoplutei nutrition. The first has been extensively studied and it is influenced by food particle size (Rassoulzadegean and Fenaux 1979; Rassoulzadegean et al., 1984), food particles concentration (Strathmann 1971; Pedrotti 1995; Miloslavich et al. 2007) and, indirectly, their ability to stay in the water column. Flavour might also play a role in plutei selectivity (Rassoulzadegean et al., 1984; Pedrotti 1995) as when microencapsulated diets are coated with algae exudates echinoplutei show significant preference towards them (McEdward and Miner. 2001). Not many papers have been published on the latter, it is however likely that palatability will be influenced by nutritional properties of the diets such as lipid, protein and fatty acid content and composition.

In most of the studies food particle concentration has been the main variable tested, and when different microalgae were used as diet and provided in the same amount the specific nutritional characteristics of each species were ignored (Pedrotti, 1995).

Only one study has been conducted on the relationship between Fatty Acid composition of live microalgae used as feed and larvae performances in *P. lividus* (Liu et al., 2007). In this study no significant differences were observed between larval performances of larvae fed a live microalgae species (*Dunaliella tertiolecta*) and micro-encapsulated diets, while algae paste was unable to sustain larval development. This study suggests that even if nutritional characteristic of the diets were different a significant biological effect wasn't observed. However differences in

the physical structure and hydrodynamic behaviors of the different diets might have seriously compromised palatability of the microencapsulated diets. As microcapsules might have failed to be ingested (as the authors observed, larvae selectively avoided micro-encapsulated diets) or algae paste might have failed to remain mixed in the water column and therefore *de facto* reducing food particles concentration.

To date, the correlation between nutritional properties of the diets and biological effects on the larvae still need to be understood. Echinoplutei specific nutritional requirements are still unknown and the best way to fulfil them is still to be identified.

1.9.2 Digestion

Gut structure and histology are similar in all echinoids (De Ridder and Jangoux, 1982) and consist of a pharynx, esophagus, stomach, intestine, and rectum (Fig. 1.24). Mucus cells are found in the pharynx and esophagus in most regular echinoids, with the exception of some diadematoids, and they extend throughout the entire gut in cidaroids (Holland and Ghiselin 1970). Mucus surrounds ingested food to form pellets (De ridder and Jangoux, 1982) that remains intact through defecation. Observations on these pellets are poor and their role is not clear (Lawrence et al., 2007). Nonetheless, mucus cover must be porous as digestion of their contents and absorption of the digestive products occurs. Buchanan (1969) suggested that pellets are involved in internal volume control or in packaging the feces. De Ridder and Jangoux (1982) implied a role in the digestion of plant material, pointing out that pellets are not formed in carnivorous species.

The stomach and intestine have specialized functions, the first possess exocrine cells consistent with presumptive enzyme production and secretion (Anisimov 1981; Fuji

1961; Holland and Lauritis 1968; Powis in De Ridder and Jangoux 1982; Sweijd 1990; Tokin and Filimonova 1977), whilst the intestine has numerous enterocytes and well-developed mitochondria consistent with absorption functions (De Ridder and Jangoux 1982; Tokin and Filimonova 1977). It is believed that the siphon has the function of removing excess water from stomach to intestine and this would avoid dilution of digestive enzymes in the stomach (Lawrence et al., 2007).

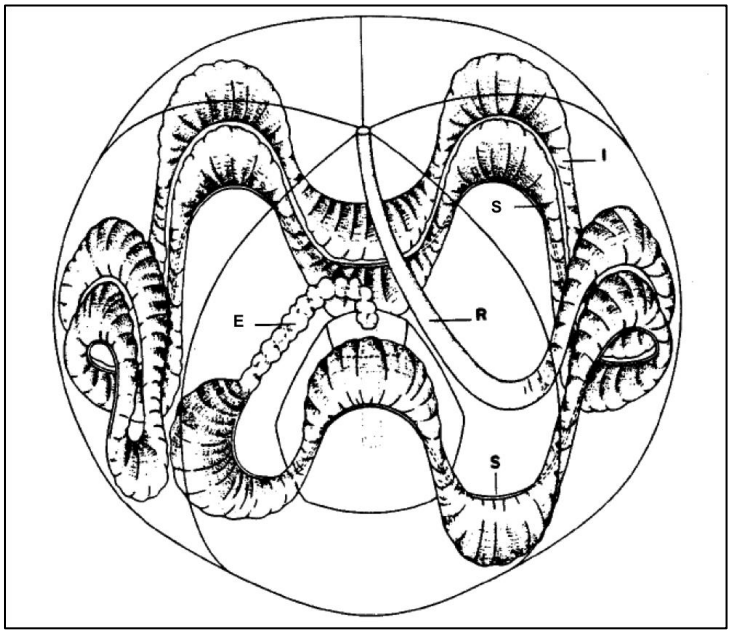

Figure 1.24. Schematic representation of the digestive system of Paracentrotus lividus. S= Stomach; I= Intestine; E= Esophagus; R= Rectum; S= Siphon. (Modified from Claerebout and Jangoux, 1985)

During digestion nutrients will pass through the intestinal wall to coelomic or hemal system where they will be distributed throughout the organism and undigested materials are discarded through the rectum.

Digestive systems in animals have been classified as batch reactors, plug-flow reactors, continuous-flow, and stirred-tank reactors (Penry and Jumars 1987). Sea urchins digestive system seems similar to the continuous-flow, stirred tank reactor in which material within the gut flows and is continuously mixed (Lawrence et al.,

2007). The continuous mix implies that the first appearance of a meal in feces does not indicate the transit time of the entire meal (Lares and McClintock 1991) with consequent challenges associated with studies on digestion and gut transit time (Lawrence et al., 2007).

One of the unknown factors in urchin digestion is the action of the gut flora; indeed, it is still unclear whether bacteria can provide support to sea urchin's digestion. Nonetheless, two roles for gut bacteria in sea urchin digestion have been hypothesized: digestion of algal cells (Lasker and Giese, 1954; Claereboudt and Jangoux, 1985; Sawabe et al., 1995) and nitrogen fixation and production of amino acids essential to sea urchins (Fong and Mann 1980; Guerinot and Patriquin 1981). The first hypothesis is supported by the identification of bacteria isolated from sea urchin gut able to degrade macroalgae cells by digestion of a variety of algal polysaccharides *in vitro* (Garcia-Tello and Baya, 1973; Prim and Lawrence, 1975). However, in a review by De Ridder and Foret (2001) it is concluded that the bacteria in sea urchin's gut are usually opportunistic in the utilization of marine plant polysaccharides, and that there is little direct evidence of an effective contribution to digestion *in vivo*. The second hypothesis is supported by observation of radioactive carbon in all of the body tissues protein amino acids when urchins were fed labelled glucose or cellulose but only in some amino acids (non-essential) when radioactive glucose was injected in the coelom after suppression of gut flora (Fong and Mann, 1980).

1.9.3 Protein

As highlighted by Hammer et al., (2006), an increased understanding of sea urchin protein requirements will contribute to the development of formulated feeds for the

aquaculture of commercially important sea urchin species. However, the specific nutrient requirements for optimal sea urchin somatic growth in aquaculture remain obscure (Eddy et al., 2012) and essential amino acid requirements have not yet been identified (Heflin et al., 2012). Protein is an essential but expensive component in the diet; knowledge of the optimum sources and level of protein is therefore necessary for the formulation of cost-effective feeds.

Many of the studies to date on the nutrition of urchins in culture conditions have examined the role of nutrients in promoting gonad yield and quality (Barker et al. 1998; Meidel and Scheibling 1999; Robinson et al. 2002; Shpigel et al. 2005; Siikavuopio et al. 2007). A number of other studies have compared somatic and gonad growth of urchins fed various formulated feeds with macroalgal diets, or compared the use of different algal species as feed (Cook et al. 1998; Russell 1998; Spirlet et al. 2001; Chang et al. 2005; Daggett et al. 2005; Lyons and Scheibling 2007). Other studies have begun defining the gross levels of protein and carbohydrates required for somatic growth by urchins. McBride et al. (1998) for instance, observed no significant differences in growth of *S. franciscanus* fed prepared diets with protein levels of 30, 40, and 50%, but they did see a decrease in feeding rate with increased protein levels.

Fernandez and Boudouresque (2000) measured *P. lividus* growth when individuals were fed three feed types varying in quality ("vegetable," "mixed," and "animal"), and found that the higher protein feeds ("mixed" and "animal") gave better results than the less protein rich "vegetable" feed. Akiyama et al. (2001) concluded that a dietary protein level of 20% was the optimum for *P. depressus* and Hammer et al. (2006) came to the same conclusion after a feeding study with *L. variegatus*, where they

determined that a 20% protein diet was more efficient than either a 9% protein or a 31% protein diet.

The faster growth associated with the consumption of relatively protein rich feed is also coupled with increased feed conversion ratio (FCR) as highlighted in several studies (Fernandez and Boudouresque, 2000; McBride et al., 1998; Hammer et al., 2004) and it has been suggested that sea urchins may consume large amounts of carbohydrate rich (protein poor) material in order to process and obtain necessary dietary protein, only to later discharge carbohydrates as dissolved organic material (Miller and Mann, 1973). These observations suggest that adult sea urchins consume more food of low protein quality in an attempt to compensate for the lack of available protein in the diet. An interesting contrast is the study of Akiyama et al. (2001) in which *P. depressus* fed prepared diets of 10% to 51% protein had significantly greater daily consumption when fed the 10% or 51% protein diets compared to the intermediate (21% or 31%) protein diets. Consumption of low protein feed (below 10%) has moreover been associated with poorer survival performances in several urchin species such as *L. variegatus* (Hammer et al., 2004) or *S. droebachiensis* (de Jong-Westman et al., 1995). Results from these studies are, however, in contrast with other observations and Akiyama et al. (2001) found 100% survival in small *P. depressus* held for 8 weeks on prepared diets of 10% to 51% protein. This discrepancy might be explained by the shorter duration of the latter trial.

It is not clear whether dietary protein content affects growth of different body compartments in different ways. Test growth, especially dry weight, is significantly negatively affected by low dietary protein content (below 10%) as shown in several studies (Hofer et al., 2002; Hammer et al., 2004; Eddy et al., 2012). Interestingly, however, it appears that excessive protein density also negatively affects test growth

(dry weight) and it has been recently suggested by Eddy et al., (2012) that this is potentially related to the metabolic cost of processing excess protein. Gut weight seems to be more independent from dietary protein content as long as minimum requirements are met, this has been highlighted in a trial conducted by McBride et al., (1998) were this parameter did not change in urchins fed diet with protein contents from 30 to 50% but it is not known whether the function of the gut was altered.

Despite of being the focus of the majority of the trials, gonadal growth dependency on dietary protein content is still not clear. Noise in the data due to potential species specific response, different age or size of the experimental individuals, dissimilar or not reported maturation stage at the beginning of the trials, duration of the trials and often undisclosed ingredients composition, make it very difficult to be conclusive. In *L. variegatus*, for instance, gonad weight gain was directly dependent on dietary protein during a nine week trial (Hammer et al., 2012). In contrast, Fernandez et al. (1995) reported no significant difference in gonadal index in *P. lividus* fed different protein levels after 6 months trial. Moreover, Pearce et al. (2002) found no significant differences in gonad index with adult *S. droebachiensis* fed diets containing 19%, 24% and 29% protein. Similar results were observed by McBride et al. (1998), which found no significant differences in gonad index with juvenile *S. droebachiensis* fed diets containing 30%, 40% or 50% protein diets for 10 months. This might be due to protein requirements for the two species being already met at the lowest tested protein content. Hammer et al. (2004) observed that juvenile *L. variegatus* fed a 9% protein diet had gonad weights that were significantly smaller than urchins fed 15%, 21% or 33% protein diets. This might be explained by a potentially high protein requirement of this species but also by the young age of the

individuals that might require higher protein content. Moreover, Akiyama et al. (2001) reported no significant differences in gonad index with juvenile *P. depressus* fed diets containing 10%, 21%, 31%, 41% or 51% protein for 8 weeks. This again might disconfirm the hypothesis of younger individuals requiring more protein but it might also depend on the relatively short length of the trial or, indeed, on the species specific requirements.

1.9.4 Carbohydrates

With the exception of most carnivorous fish, especially marine species, where lipids are the main source of energy, carbohydrates seem to be the preferential energy source of sea urchins (Marsh and Watts, 2007; Hammer et al., 2012). Formulated diets should, therefore, supply enough energy from dietary carbohydrates to fulfil the energetic requirements of sea urchins so that more expensive nutrients like protein will be spared (Hammer et al., 2012). Almost all studies on digestive enzymes in sea urchins concern carbohydrates (Lawrence et al., 2007). No study has shown cellulase activity (Lewis 1964; Suzuki et al. 1984) and the appearance of intact algal and seagrass cells in sea urchin feces indicate that cellulase activity, if present, might be minimal. Cabral de Oliveira (1991) and Klinger (1984) concluded that sea urchins are not able to digest structural plant polysaccharide. Nonetheless, the more soluble carboxymethylcellulose is digested (Claereboudt and Jangoux 1985; Obrietan et al. 1991, Gómez-Pinchetti and García-Reina 1993; Trenzado et al., 2012). Moreover, reserve polysaccharides such as starch form of plant origin are digested. Amylase occurs in *P. lividus* (Claereboudt and Jangoux 1985), *S. purpuratus* (Lasker and Giese 1954), *S. droebachiensis* (Obrietan et al. 1991), and glycogenase in *S. purpuratus*

(Lasker and Giese 1954), *D. antillarum* (Lewis 1964), and *Parechinus angulosus* (Sweijd 1990).

Various levels of hydrolysis of algal polysaccharides have been found. Agarase activity has been reported for *S. purpuratus* (Farmanfarmaian and Phillips 1962), *D. antillarum* (Gómez-Pinchetti and García-Reina 1993; Lewis 1964), and *Anthocidaris crassispina* (Yamaguchi et al. 1989). Hydrolase activity for algin, the main structural polysaccharide of brown algae, is variable. Huang and Giese (1958) did not find alginase in *S. purpuratus*, but Eppley and Lasker (1959) did.

Recent studies on the sea urchin nutritional requirements have highlighted the importance of the relative abundance of protein and carbohydrates. Eddy et al., (2012) investigated requirements of *S. droebachiensis* juvenile finding that diets with lower protein levels (16-22%) and higher carbohydrates level (>40%) produced the fastest growth. In contrast Hammer et al., (2012) found that diets with higher protein content (36%) and lower carbohydrates content (21%) produced the highest growth in *L. variegatus*, and have associated their observation with the highest protein/energy ratio obtained with this diet. A possible explanation of these apparently contrasting results could be found in species specificity of protein and carbohydrates requirements (omnivorous *vs* herbivorous) or in the different life stage of the individuals used in the two experiments (juveniles *vs* adult). It is in fact possible that during early stages more digestible carbohydrates could enhance growth whilst more complex physiological functions occurring in adults such as sexual maturation might require a higher protein input.

1.9.5 Lipids

The main difference between how lipids and proteins are utilized by animals consists in the relationship between the dietary inputs and the animals' body composition. Regardless of amino acid composition of a given diet, body protein composition of, for example, a trout will always be that of a trout; this is however not true for its body fat composition (Sargent et al., 2002). Therefore, to define the dietary requirements of essential fatty acids in animals requires considering the animals' innate ability to metabolize fatty acids (Sargent et al., 2002). Lipids are the favoured source of metabolic energy in fish, especially marine carnivorous fish where carbohydrates play a little role as energy source (Watanabe, 1998). With the only exception of cholesterol, all lipid classes (Triacylglycerols, Wax Esters, Phosphoglycerides, Sphingolipids, Sterols) contain fatty acids. Major role of these lipid classes is to generate energy through β-oxidation of the acyl chains producing acetyl-CoA and NADH that are further metabolised via the tricarboxylic acid cycle and oxidative phosphorylation, respectively (Tocher, 2010).

Triacylglycerol (TAG) is the major lipid class supplying energy to fuel larval development in many echinoderm and mollusc eggs (Podolsky et al., 1994; Sewell and Manahan, 2001; Villinski et al., 2002; Sewell, 2005; Prowse et al., 2008). As major components of most lipids, fatty acids (FA) have functional roles as a source of metabolic energy (as in TAG), as structural components (as in membrane Phospholipids), and as precursors of bioactive molecules (Sargent et al., 2002; Tocher, 2003). In biochemistry, fatty acids are carboxylic acid with an aliphatic chain, which is either unsaturated or saturated depending on the presence or absence of double bonds. Fatty acids also differ by the carbon chain length, and are categorised

as short chain, with fewer than six carbons, long chain with more that 14 carbons to very long chain presenting more than 22 carbons. The concomitant presence of several double bonds and more than 14 carbons gives the name of a particularly interesting category of fatty acids known as Long Chain Polyunsaturated Fatty Acids (LC-PUFAs). Among these, docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:5n-3), and arachidonic acid (ARA, 20:4n-6) have important physiological functions acting as ligands for transcription factors and nuclear receptors, influencing gene expression (Izquierdo and Koven, 2011) and regulating cortisol production possibly playing a role in stress response (Ganga et al., 2006). Dietary ARA, EPA and DHA compete for acylation and incorporation into the membrane phospholipids and also as substrates for the eicosanoid enzyme systems (Bell et al., 1991). Therefore the ratio of these fatty acids in the diet is at least as important as their absolute values.

Essential Fatty acids are those for which most animals have absolute requirements and need to be incorporated in the diet. If a dietary deficiency occurs, the animal stops growing and reproducing, develops various pathologies and eventually dies. EFAs include members of both n-6 and n-3 series typified by linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3). In animals capable of converting 18-carbon PUFA to LC-PUFA, the first step is the action of fatty acyl Δ6-desaturase, which converts 18:2n-6 to 18:3n-6 and 18:3n-3 to 18:4n-3 (Tocher, 2003). The second step in the LC-PUFA biosynthesis pathway is the action of fatty acyl elongase, which converts 18:3n-6 to 20:3n-6 and 18:4n-3 to 20:4n-3. These elongated products are then further desaturated (Δ5 fatty acyl desaturase) to 20:4n-6 (ARA) and 20:5n-3 (EPA), respectively. EPA is also substrate for further elongation and desaturation to produce 22:6n-3 (DHA). Further elongation to very long chain fatty acids (VLC-FA)

has been recently demonstrated by Carmona-Antoñanzas et al. (2011) in Atlantic salmon and illustrated in figure 1.25.

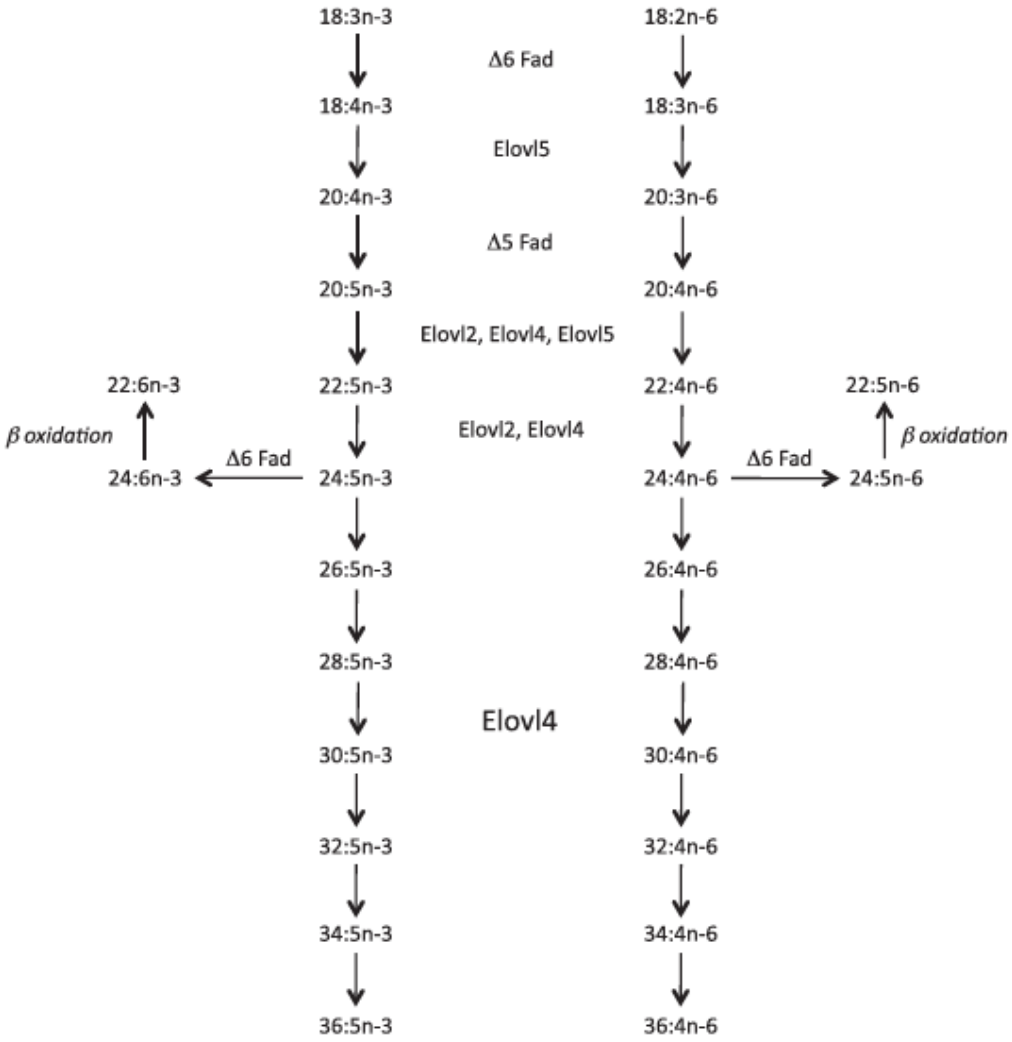

Figure 1.25. The biosynthesis pathway of long-chain and very long-chain polyunsaturated fatty acids from α-linolenic (18:3n−3) and linoleic (18:2n−6) acids. Enzymatic activities shown in the scheme are predicted from heterologous expression in *S. cerevisiae* of the Δ5 fatty acyl desaturase (Fad), the three Δ6 Fad (Hastings et al., 2005: Zheng et al., 2005; Monroig et al., 2010), and Elovl2 and Elovl5 elongases (Hastings et al., 2005; Morais et al., 2009). Steps catalysed by the newly characterised Atlantic salmon Elovl4 are also shown. Extracted from Carmona-Antoñanzas et al. (2011)

In species that cannot perform these conversions such as the vast majority of marine teleosts, the C_{20} and C_{22} PUFA themselves are, therefore, dietary EFA and their C_{18} do not satisfy EFAs requirements. This is the result of adaptation to a combination of the

predominant PUFAs in the marine food webs (marine microalgae are primary producers of these fatty acids).

The fatty acid composition of different tissues, eggs or larvae has been described for several sea urchin species including *P. lividus*, *P. miliaris*, and *S. droebachiensis*, (Hughes et al. 2005; Liu et al. 2007; Gonzales-Duran et al., 2008). It is generally recognized that the FA composition of sea urchin tissues reflects dietary inputs especially in the gut (Gonzales-Duran et al., 2008). However, LC-PUFAs such as ARA, EPA and DHA in gonads and test have often been reported to be in higher concentration than in the diets (Cook et al. 2000; Pantazis et al. 2000; Spirlet et al., 2000; Bell et al. 2001; Gonzales-Duran et al., 2008). The same has also been observed in *P. lividus* larvae (Liu et al., 2007). It has been therefore speculated that desaturase and elongase activities may be present in sea urchins, which may suggest the ability for *de novo* synthesis of LC-PUFA.

However, FA profiles of each larval development stage and therefore a description of how these change during development or the FA profiles of gonads at each gametogenic stage have not yet been reported in any sea urchin species. Moreover, beside experimental observation and relative speculations, molecular investigation on the presence and activity of the required genes encoding for the enzymes needed to complete these bioconversions is yet to be conducted in sea urchins. Nonetheless, genes encoding for fatty acyl elongase and desaturase and their functional characterisation in recombinant yeast has been carried out in other invertebrates such as octopus (Monroig et al., 2010)

Other peculiar fatty acids such as Non-Methylene Interrupted Fatty Acids have been reported to be present in sea urchin body tissues although being absent in the diet (González-Durán et al., 2008). The biological role and function of NMI FA are not well

understood, although it has been suggested that they might play a structural and functional role in biological membranes (Tunnicliffe et al., 1998; Pirini et al., 2007; Ventrella et al., 2008). It has been moreover suggested that NMI FAs might provide increased resistance against oxidative process and microbial lipases and can therefore represent a biochemical adaptation of benthic organisms to their specific habitats (Barnathan, 2009). Increased structural fluidity has also be attributed to cell membrane rich in NMI FAs; the ability of synthetising them is therefore an important assets for organisms exposed to challenging and rapidly changing thermic environment such as hydrothermal vents.

1.9.6 Carotenoids

Carotenoids are pigments usually red, orange, or yellow in color found in the chloroplasts and chromoplasts of plants and algae, bacteria and fungi. There are over 700 known carotenoids (Britton et al., 2004) and all share the same chemical structure and are classified as tetraterpenoids (formed by 8 isoprene molecules and contain 40 carbon atoms). They are, however, divided in two classes: the Carotenes containing no oxygen in their molecules such as ß-Carotene, and their oxidative derivates such as Astaxanthin, Lutein, Zeaxanthin, and Fucoxanthin commonly known as Xanthophylls.

Carotenoids generally cannot be synthetized by animals, but are instead obtained from the diet. Main roles of carotenoids in organisms are provitamin A activity, photoprotection, radical quenching, pigments, and immunological modulation (Bendich 1994; Krinsky 1994; Matsuno 1991).

In sea urchins most of the carotenoid pigmentation occurs in the gonads. This is of particular importance as colour is one of the most important criteria indicating the

quality of the product. Indeed, only when colour is bright yellow or orange market acceptance and higher price can be achieved. Formulated feeds have so far failed to consistently and reliably produce gonads of acceptable colour, mainly because synthetic pigments do not seem to affect gonad colouration in the same way natural pigments do (Goebel and Barker, 1998; Robinson et al., 2002) and "finishing" diets of natural macroalgae must be employed prior commercialization in order to achieve market acceptance (Shpigel et al., 2005). However, as colour is successfully manipulated by the use of dietary carotenoids in other well-established seafood products, such as salmon and trout, scope exists to keep investigating carotenoids requirements in sea urchin and the pathway leading to their absorption in the gut, metabolic conversion and deposition in the gonads. This would not only increase business profitability, but also increase sustainability reducing reliance on large amount of fresh macroalgae from the wild.

Significant differences exist in gonad carotenoid profiles between edible (Echinoids), and non-edible sea urchin species (Cidaroids and Echinothurioids); in the first group, in fact, the most represented carotenoid is Echinenone (Fig. 1.26) accounting for 50 to 60% of total pigments present in the gonads, whilst in the other groups ß-Carotene, α-Carotene, Canthaxanthin and Astaxanthin are the predominant carotenoids.

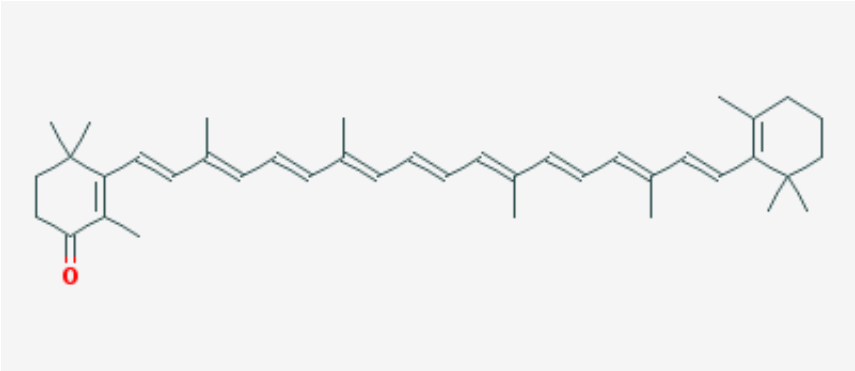

Figure 1.26. Echinenone molecular stracture. http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?sid=99437372&loc=es_rss

In sea urchins Echinenone can be derived from dietary ß-carotene via hydroxylation into all-*trans* isocryptoxanthin followed by oxidation to all-*trans* echinenone and final isomerization to 9'-*cis* Echinenone, as exemplified by the left half of figure 1.27 based on speculations from the results of carotenoid analysis by Griffiths and Perrott (1976). However, Symonds et al., (2007) did not find all-*trans* isocryptoxanthin during their analysis and therefore suggested an alternative pathway for the bioconversion of ß-carotene into 9'-*cis* Echinenone (right half of figure 1.27). In this new suggested pathway ß-carotene is first isomerized into its 9'-*cis* form, then hydroxylised into 9'-*cis* isocryptoxanthin and finally oxidized into 9'-cis Echinenone. Figure 1.27 illustrates the two proposed pathways.

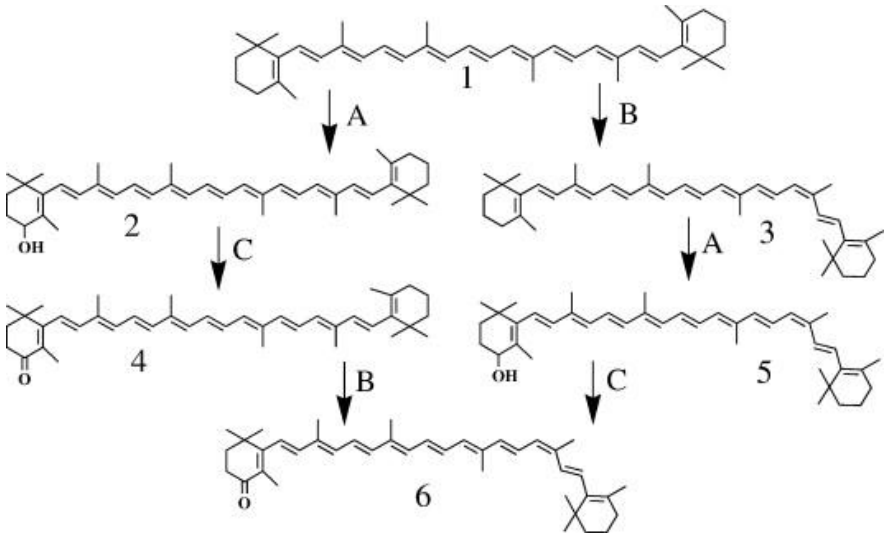

Figure1.27. Proposed metabolic conversion of dietary all-*trans* ß Carotene into 9'-*cis*-Echinenone according to Symonds et al., (2007). 1: all-*trans* ß Carotene; 2: all-*trans* isocryptoxanthin; 3: 9'-*cis*- ß Carotene; 4: all-*trans* Echinenone; 5: 9'-*cis*- isocryptoxanthin; 6: 9'-*cis*-echinenone. A: Hydroxylation; B: Isomerisation; C: Oxidation.

In the gut, ß -carotene, fucoxanthin, and fucoxanthinol are the most common carotenoids (Matzuno and Tsushima, 2001). The carotenoid patterns of the gut of sea urchins are influenced by the carotenoid profiles in the diets and metabolic products of fucoxanthin from the natural diet (brown algae and diatoms) of herbivorous sea urchins have not been found in carnivorous species (Tsushima, 2007). In addition to these carotenoids, Symonds et al., (2007) found presence of Echinenone in *P. lividus* gut wall and noted that neither the all-*trans* nor 9'-*cis* forms of Echinenone occur in their natural diet. Therefore, the presence of both forms of echinenone in the gut wall demonstrates their formation *in situ* indicating that this organ acts as a major site of carotenoid metabolism.

The most common pigment in test and spines is naphthoquinone which derivatives have significant pharmacological properties. They are cytotoxic, have significant antibacterial, antifungal, antiviral, anti-inflammatory, and antipyretic properties.

Carotenoids are also present in test and spines and their profile seems to follow that of the gonads (Matsuno and Tsushima, 2001).

Relatively higher concentration of carotenoids in the gonads than in any other body compartments led to the hypothesis that carotenoids might play a role in sea urchin reproduction. Indeed, broodstock fed higher concentration of the xanthophyll Fucoxanthin produced more eggs than broodstock fed ß–carotene in an experiment conducted on *P. depressus* by Kawakami et al., (1998). Moreover, other xanthophylls such as Lutein, and Zeaxanthin enhanced egg production when fed to *L. variegatus* broodstock (George et al., 2001). This is important for sea urchin aquaculture, as a diet containing xanthophylls would enable production of larger numbers of juveniles (Tsushima, 2007).

1.9.7 Minerals

Kennedy et al. (2007) have presented evidence that a lack of appropriate dietary minerals is a likely factor contributing to the shortcomings of prepared feeds in those cases where natural kelp diets have produced better somatic growth than prepared diets.

1.9.8 Formulated feeds

Artificial diets have been successfully used for edible sea urchin species maintenance and somatic and gonadal growth and to examine the impact of specific ingredients such as fatty acids (FA) (Bell et al. 2001, Cook et al, 2007) or pigments (Suckling et al. 2011).

These diets have contained a selection of terrestrial plant materials (potato starch, soybean meal and cereals), either with or without animal origin proteins and lipids,

and they have ranged from simple moist or agar bound diets to pellets extruded in commercial processing equipment (Cook et al., 1998; Pantazis et al., 2000; McLaughlin and Kelly, 2001; Otero-Villanueva et al., 2004).

The proximate compositions of formulated diets used in recent feeding trials is reported in the table below:

Table 1.2. Review of the proximate composition of urchin's feed used in recent growth trials.

Nutrients	Hammer et al., 2006	Eddy et al., 2012	Helfin et al., 2012	Hammer et al., 2012
Protein %	14-50%	16-40%	12-36%	17-31%
Carbohydrates %	18-56%	29-47%	21-39%	32-47%
Fiber %	6.6%	*	4.5%	2.5%
Ash %	8.3-8.9%	25-45%	24%	23.2%
Lipids %	9.8%	4.7-5.3%	7%	7.5%
Energy Kcal/g	3.9-4.5	*	3-3.7	3.6-3.8

Overall, the vast majority of published feeding trials have included an experimental diet that mimicked the proximate composition of the most common natural diet for sea urchin species, brown kelp. It is therefore not surprising that artificial diets producing better performances presented a proximate composition similar to that of fresh brown kelp with protein level close to 20%; carbohydrates level around 45-50% and with lipids level between less than 1 and 7% (Eddy et al., 2012). Surprisingly, however, the relationship between nutrition and reproduction, and especially the role of dietary protein, carbohydrates, and lipids contents and fatty acid profile in promoting onset of gametogenesis, fecundity or enhancing larval and juvenile survival has been so far neglected. This is of particular importance when the seasonal variations in proximate composition of natural kelp are taken into account. These variations, which for some nutrients such as lipids can be as large as ten folds or for some ratio such as carbohydrates: lipids can vary from about 3:1 in winter to 30:1 during the Summer (Eddy et al., 2012), are in fact likely to be influenced by the

same seasonal cues influencing reproduction in sea urchin. It could be therefore hypothesized that a specific Protein/Carbohydrate/Lipid ratio might be advantageous for gamete production and/or quality, or indeed that the shift in diet proximate composition might provide a synchronizing cue for the onset of gametogenesis. This aspect might play a critical role in the development of tailor made broodstock diets able to increase broodstock and larval performances and, therefore, hatchery outputs. However, species specificity or even area of origin of the broodstock in case of widely distributed species such as *P. lividus*, will have to be considered as different sub-populations or, indeed, different species might have different requirements in this respect.

1.10 Aims and context of the study

In 2009 the European Union funded the ENRICH project (Project number: n°222492), which aimed to play a role within the worldwide effort to preserve edible sea urchin natural stocks via improvements of aquaculture production. The main objectives of the ENIRCH project were to:

1. Optimise *Paracentrotus lividus* grow-out techniques both inland and offshore
2. Optimise diets and rearing conditions to improve quantity and quality of larvae and juveniles
3. Set up Integrated Multi-Trophic Aquaculture demonstration sites

At that time the Ardtoe Marine Laboratory – Viking Fish Farm Ltd. had a keen interest in scaling up and further developing the hatchery technology for the production of *P. lividus* and was therefore involved in the project. Subsequent discussion with the Institute of Aquaculture of the University of Stirling and in

agreement with the project coordinator and all partners, this PhD project was initiated with the specific aim to improve the urchin hatchery output in Ardtoe.

In order to achieve this overarching aim, in line with the Objective 2 of the ENRICH project, specific tasks were identified as objectives of this PhD:

- Investigation of alternative live algae to improve larval survival;
- Design and testing of alternative larval rearing systems;
- Investigation of the effects of formulated feeds on broodstock reproduction, gonads biochemical composition and maternal provisioning to embryos and larvae;
- Investigation of the relationship between adults' nutrition and offspring performances.
- Identification of the genes and sequencing of the proteins involved in the desaturation and elongation process of Essential Fatty Acid synthesis.

The link between these objectives, and main theme of this work, has been the investigation of the role of fatty acids in *P. lividus* physiology. More specifically, emphasis has been placed on their evolution during gametogenesis, embryogenesis and larval development, their transfer from diet to gonads and from gonads to eggs and embryos and, ultimately, their potential ability to promote larval survival.

Last but not least, and from a more personal viewpoint, the main goal of this PhD has been to provide the author with the opportunity to undertake a learning journey into the scientific method offering the unique chance to curiously explore and enjoy the process of thorough investigation of a completely new topic.

Chapter 2 - General Materials and Methods

2.1 Sea urchin production at the Ardtoe Marine Laboratory

The gametes are obtained by stimulating the parents to spawn with an injection of 1 M potassium chloride (KCl) at a dose of 40ug per gram of body weight. As there is no external means of differentiating the sexes, several individuals have to be injected at once to ensure the production of both gametes. Each individual is kept separately, and then gametes are mixed for the fertilisation (5 ml of diluted sperm per litre of diluted eggs, number of sperms and eggs are not counted). Cleavage and embryos development is very fast and fertilised eggs, kept in the dark in static filtered sea water, reach the gastrula stage after 24 hours at 20°C. Now swimming gastrulae are volumetrically counted and transferred to the larval rearing unit.

The larval rearing is carried out in continuous light photoperiod, in white 80 litres conical bottom polyethylene tanks using filtered and sterilised seawater at 18 ±2 °C. Embryos are introduced at a density of 4/ml. The tanks are constantly aerated and under static regime, without a flow of seawater. Hence, every third day, the cultures are siphoned through a sieve (25µm mesh size). The culture bins are then drained, cleaned and brushed with freshwater and diluted hydrogen peroxide as detergent, and refilled with clean 1 µm filtered and UV treated seawater. When ready for the first feeding (48 hours post hatch) and every 3rd day thereafter, larvae are given the microalgae *Dunaliella tertiolecta*. Feeding ration is adjusted depending on developmental stage (pairs of larval arms) as illustrated in the table below:

Table2.1.1. Feeding regime for larval *P. lividus*. Cell numbers describe *Dunaliella tertiolecta* algal cell density in the larval culture.

Pairs of Larval Arms	2	3	4
Dunaliella tertiolecta cells ml $^{-1}$	3000	9000	15000

Microalgae cultures are grown in 100 L polyethylene bags in sterilized seawater enriched with the f/2 medium and used as larval feed during exponential growth phase. Microalgae culture densities are determined by spectrophotometry (DR/2000 Direct Reading Spectrophotometer, Hach Lange Ltd, UK) using standard concentration curves prepared using light microscopy and cell counting via hemocytometer.

After planktonic life (21 and 25 days), larvae are ready for metamorphosis. This is termed “settlement” or “recruitment” and, at this stage, larvae are transferred to the settlement unit consisting of 0.5 m^3 tanks kept at 20 °C employing a recirculation system, in continuous light photoperiod regime in which plastic corrugated plates of 0.5 m^2 surface are placed to provide metamorphosis substrate. The plates are “marinated” in natural sea water for 4 weeks before deployment to allow marine-biofilm to develop, they are then transferred to the settlement unit. Once juveniles are approximately 1-2mm in diameter they are easily visible on the plates and tank walls, at which point soft pieces of seaweed are added to supplement the juveniles’ diet.

When juveniles reach 4-5 mm in test diameter, they are slowly acclimatised (1 °C / day) to ambient temperature and then transferred to the nursery area, which consists of 4 race-ways (3 m^3 each) under natural photoperiod and water temperature located inside a polytunnel. Throughout the on-growing period, they

are fed with a mixture of macroalgae collected from the wild, predominantly *S. lattissima*, *L. digitata*, and *P. palmata*. From 4-6 months old, juveniles are graded by size at regular intervals to maximize growth.

Modifications from the protocols described in this section were required in each of the trials undertaken within the course of the PhD project and specific rearing methods and conditions are described in detail in each experimental chapter.

2.2 Histological sampling and analysis

During the experiment described in Chapter 7 histological analysis of the gonads was conducted. Gonad samples were obtained by cutting around the peristomial membrane and exposing the viscera, gonads were separated from the other organs and blotted dry with paper towel. Samples were stored in 10% neutral buffered formalin (NBF) and then dehydrated, embedded in paraffin and sectioned at 5 µm. The sections were stained with haematoxylin and eosin (H/E) and analyzed under the binocular microscope (Olympus, BH2). One volume of tissue per ten volumes of fixative was used to store the samples until sectioning.

Fixed samples were trimmed and placed individually into cassettes that were then placed in an automated tissue processor (Shandon citadel 2000, Thermo shandon Cheshire, UK) which then dehydrated, cleared and impregnated each sample with paraffin wax as follows:

1.	50 % methylated spirit	30 min
2.	80 % methylated spirit	90 min
3.	100 % methylated spirit	90 min(x3)
4.	Chloroform	50 min(x2)

5.	Molten wax	105 min
6.	Molten wax	90 min (x2)

Samples were then embedded using a histoembedder (Leica UK Ltd, Milton Keynes, UK), after hardening the wax blocks were trimmed, before three serial sections of 5µm thickness were sliced using a rotary microtome (Leica UK Ltd, Milton Keynes, UK) and placed on glass slides. Before section cutting, each block of urchin's gonads was soaked in water for approximately 30 minutes, then were blotted dry and cooled on a cold-plate prior to sectioning.

In order to examine sections effectively under the microscope they require to be stained. The sections were stained with Mayer's haematoxylin and eosin Y using modified procedure of Bancroft and Stevens (1991). The haematoxylin component stains the cell nuclei blue to blue/black, whilst the eosin counterstain demonstrates the general histological architecture. Eosin distinguishes between different types of connective tissues, by staining in varying shades of orange, pink and red.

The following staining protocol was used:

Table 2.2.1. Protocols for staining as used during histological analysis of urchin gonads.

Step	Duration
Xylene	3 mins
Xylene	2 mins
Absolute Alcohol I	2 mins
Methylated spirit	1.5 mins
Tap water	30 sec
Haematoxylin Z	5mins
tap water	30 sec
1% Acid Alcohol	3 quick dips
tap water	30 sec
Scott's tap water substitute	1min
tap water	30 sec
Eosin	5 mins
tap water	30 sec
Methylated spirit	30 sec
Absolute alcohol II	2 mins
Absolute alcohol III	1.5 mins
Xylene (Clearing)	5 mins

Slides were kept in xylene until the cover-slip was positioned and mounted using Pertex. Identification and characterization of the different germ cell types was carried out according to Byrne (1990).

2.3 Lipid extraction, Fatty Acids Methyl Esters (FAME) preparation and data analysis

Lipid extraction was carried out following the protocol outlined by Folch (1957). Sampled tissues were weighed and added to 5ml Chloroform:Methanol (2:1, v/v) containing 0.01 % butylated hydroxytoluene (BHT) as antioxidant in glass vial and stored in the freezer at -20 °C. Once in the lab, samples were transferred to test tubes

kept on ice and 20ml of Chloroform:Methanol (C:M) was added. Samples were then homogenised using Ultra Turrax™ in a fume cupboard rinsing the probe in C:M (2:1 v/v) between samples. Samples were then left on ice for one hour, after which 5ml of 0.88% (w/v) KCl was added to the homogenised samples. Treated tissues were then centrifuged (Jouan C 412 bench centrifuge) at 1500 rotation per minute (rpm) for five minutes. Using a water pump under fume cupboard, the top layer (KCl) was then removed by aspiration and the bottom layer (solvent with lipids) transferred to pre-weighed tubes through a Whatman number 1 filter paper washed with C:M (2:1 v/v). Solvent was then evaporated from each tube and samples were dessicated *in vacuo* overnight. Tubes were then re-weighed and total lipid weight recorded. Samples were then re-dissolved in C:M (2:1 v/v) with BHT at a concentration of 10mg/ml and transferred to 2ml glass vials to be stored under nitrogen in a freezer at -18 °C.

Fatty acid analysis was carried out following the protocol described by Christie (2003) and detailed here. Total lipids extracted with Folch protocol are transferred from the 2ml vials into test tubes and the Heptadecaenoic acid (17:0) fatty acids standard is added at 10% of the total lipid mass. Solvent is then evaporated on Nitrogen evaporator. 1ml of Toluene is added to the tubes in order to dissolve neutral lipids and then 2ml of methylation reagent (1% solution of sulphuric acid in methanol) is added. After thorough wirlimix, tubes are left overnight in hot block at 50 °C. Tubes are then cooled down to room temperature and 2ml of 2 % $KHCO_3$ and 5 ml of iso-hexane : diethyl ether (1:1 v/v) plus 1% BHT are added, tubes are mixed and then centrifuged at 1500 rpm for two minutes. The upper organic layer is then transferred to another test tube and 5ml of iso-hexane : diethyl ether (1:1 v/v) is added and the same procedure is repeated.

Solvent is then evaporated in the Nitrogen evaporator and Fatty Acid Methyl Esters (FAME) are re-dissolved in 100 µl of iso-hexane.

FAME are then purified by Thin Layer Chromatography (TLC) plates (20 x20 cm) loading the samples using a 50µl Hamilton syringe, plates are chromatographed in iso-hexane: diethyl ether: acetic acid (90:10:1 v/v). After evaporation under nitrogen in a fume cupboard of the excess solvent, plates are sprayed with 1% (w/v) of iodine in chloroform to visualise the FAME on the silica surface of the plates, which is then scraped from the TLC plate into test tubes using a straight edge scalpel blade. FAME are eluted from the silica with 10 ml of iso-hexane: diethyl ether (1:1 v/v) plus 0.01% (v/v) of BHT and silica is sedimented via centrifugation. The solvent layer is then transferred to test tubes and evaporated on the nitrogen evaporator. FAME are then resuspended in iso-hexane at a concentration of 1mg/ml and transferred to 2ml glass vials to be stored under nitrogen at -20 °C before Gas Liquid Chromatography (GLC) analysis. Fisons instruments GLC 8160 gas chromatograph was used with 30 m x 0.32 mm internal diameter x 0.25µm capillary column CP Wax 52CB with hydrogen as a carrier gas. Individual FAMEs were identified by comparison with known standards and by reference to published data (Ackman, 1980; Tocher and Harvie, 1988). Data were collected and processed using Chromcard for Windows (version 1.19), and FAMEs quantified through comparison with a Heptadecaenoic acid (17:0) internal standard.

2.4 Proximate analysis of urchin diets

2.4.1 Moisture content

Approximately 1.0 g of the wet samples (diets and tissues) were weighed into a pre-weighed aluminium tray and placed into a drying oven at 110°C for 24 h or until constant weight was achieved. The samples were quantified gravimetrically by reweighing the samples after cooling in a desiccator for 15 min (AOAC 2000).

$$\text{Moisture, \%} = \frac{\text{Sample weight (g)} - \text{Dried sample weight (g)}}{\text{Sample weight (g)}} \times 100$$

2.4.2 Protein content

Crude protein content was determined from the nitrogen content of each sample, which assumes that protein contains 16% nitrogen, using automated Kjeldahl analysis (Tecator Kjeltec TM 2300 analyser, Foss, Warrington, U.K). Briefly, 200 mg of sample was accurately weighed and placed into a digestion tube. Then two Mercury Kjeltab catalyst tablets and 5 ml conc. sulphuric acid were added and the samples subsequently digested in a digestion block at 400°C for 1 h. After cooling for 20 min, 20 ml of de-ionised water and 5 ml of sodium thiosulphate solution (330 g/L) were added to the digestion tubes and mixed. The digestion tubes were placed into the Kjeltec Autoanalyser prior to distillation and titration. 50 mg of ammonium sulphate was used as a positive standard and background titre effects (blank) were taken into consideration by measuring tubes without samples. The percentage of nitrogen/protein content was calculated using the following formulas:

$$\text{Nitrogen in standard, \%} = \frac{(\text{Sample titration} - \text{Blank titration}) \times 280.14}{\text{Standard weight (mg)}}$$

$$\text{Protein, \%} = \frac{(\text{Sample titration} - \text{Blank titration}) \times 1750.875}{\text{Sample weight (mg)}}$$

Where 280.14 is derived from 14.007 (N_2) × 0.2 (normality of HCl) ×100 (%)

Where 1750.875 is derived from 14.007 × 0.2 × 100 × 6.25 (protein factor).

2.4.3 Lipid content

Crude lipid content was determined according to the Soxhlet method (Tecator Soxtec system 2050 Auto Extraction apparatus, Foss, Warrington, U.K). Approximately 1.0 - 3.0 g of dry samples were weighed into extraction thimbles and covered with a cotton wool. Pre-weighed extraction cups containing 6-10 glass beads followed by 80 ml of petroleum ether (40 – 60°C boiling point) were prepared. The extraction thimbles and extraction cups were placed into the Soxtec extraction unit and the samples boiled at 135°C for 20 min, rinsed for 2 h and the solvent evaporated over 15 min. The extraction cups were placed in an oven at 105°C for 1 h to complete drying and were gravimetrically re-weighed after being allowed to cool in a desiccator for 15 min. The percentage of lipid was calculated according to the following formula:

$$\text{Lipid, \%} = \frac{W2\,(g) - W1\,(g)}{\text{Sample Weight (g)}} \times 100$$

Where W1= weight of sample and aluminium cup before extraction

Where W2= weight of sample and aluminium cup after extraction

2.4.4 Fibre content

The fibre content was determined by removing all the digestible materials from the sample by sequential boiling with acid and alkali solutions. After drying, the organic fibre is incinerated in a furnace so that the fibre content can be calculated. Around 0.5 - 1 g of sample was weighed into a pre-weighed fibercap capsule before de-fatting with petroleum ether. The capsules were agitated in a beaker containing 120 ml of petroleum ether for 30 s and then the solvent allowed to drain out of the capsules. The process was repeated three times after which the capsules were dried in a fume cupboard and placed into the carousel. The samples in the carousel were boiled with 350 ml of 1.25 % sulphuric acid solution in the extraction vessel for 30 min, and then washed three times with hot deionised water for 1 min. The samples in the carousel were then boiled in 350 ml of 1.25 % sodium hydroxide solution for 30 min and washed with hot deionised water as above. The samples in the capsules were dried for at least 5 h in an oven at 105^{o}C and allowed to cool in a desiccator for 15 min. The capsules were re-weighed, placed into pre-weighed crucibles and the samples incinerated for at least 4 h at 600^{o}C and the crucible reweighed.

$$\text{Fiber, \%} = \frac{\text{Dried capsule + residues wt (mg)} - \text{Initial capsule wt - ash wt (mg)} \times 100}{\text{Sample weight (mg)}}$$

2.4.5 Energy content

The gross energy contents of the diets were determined by Bomb Calorimetry (Gallenkamp Autobomb System) where dry samples are completely combusted in an oxygen filled container, and the heat released measured and energy content calculated. Approximately 1 g of ground diet was accurately weighed and pressed to

make a firm pellet, which contained a cotton thread inside, before placing into a crucible. The crucible was placed onto the supporting ring. Nickel firing wire was fixed between the electrodes and then a cotton thread was wound from the pellet around the firing wire and the shorter electrode. The electrode assembly was then put into the bomb calorimeter, the bomb cap hand-tightened, and filled with oxygen to ~30 bar and the sealing cap fitted. The water jacket of the bomb was filled with water and the calorimeter vessel was filled with water at 21 - 23°C. The calorimeter vessel was weighed to exactly 3 kg and then placed into the water jacket. Before firing the calorimetry bomb, the thermometer reading was recorded as the initial temperature. The bomb was then fired and when the temperature stabilised it was again recorded (final temperature).

$$\text{Gross Energy} = \frac{\lfloor(\text{Final Temp.} - \text{Initial Temp.})' \ 10.82\rfloor - 0.0896}{\text{Sample Weight (g)}}$$

Where 10.82 is the heat capacity factor and 0.0896 represented the combined energy values of the cotton thread and wire.

2.5 Carotenoids

In order to assess carotenoid content and composition, a precise amount of total lipid was resuspended in a known volume of iso-hexane and the absorbance measured spectrophotometrically at 450 nm before transfer to 1 ml amber glass auto-sampler vials. High performance liquid chromatography (HPLC) was carried out using a stainless steel column (Luna 5 µm silica 2, 125 x 4 mm). Mobile phase was delivered using a mono piston pump (Waters 501, Waters) and consisted of iso-hexane/acetone (82:18, v/v) at a flow rate of 1.2 mL min^{-1} at room temperature with

a maximum pressure of 1000 psi. Samples (50 µL) were injected via auto-sampler (Waters 717plus) and individual carotenoids were detected using a multi-wavelength UV-VIS detector (Waters 486, Waters) at 450 nm. Identification was made using retention time (Rt) in comparison to standards of β-carotene. Quantification of carotenoids was carried out by integration of peak areas using Clarity Data System software and calculated according to the following formula:

Calculation of total carotenoid concentration

$$[C] = \frac{1}{\text{wt.(g)}} \times [\text{Abs @470 nm}] \times \frac{10{,}000}{2{,}100} \times DF$$

Calculation of the standard concentration from the spectrophotometer

$$SC = [\text{Abs@470 nm}] \times \frac{10{,}000}{2{,}100}$$

Where C is the concentration (µg/ml), SC is the standard concentration (µg/ml) and DF is the dilution factor.

Where $\frac{10{,}000}{2{,}100}$ is the extinction co-efficient of astaxanthin in iso-hexane.

Calculation total and individual pigment concentration from the HPLC

$$\text{Concentration (mg/kg)} = \frac{SC \times \text{peak area} \times DF}{\text{std area} \times \text{wt of sample (g)}}$$

2.6 Water quality analyses

Water quality analyses were performed and NH^{4+}, NO_2, NO_3 and PO_4^{-3} were measured. Ammonia is excreted directly by living organisms as well as being the primary product of urea mineralisation and forms a central part of the nitrogen cycle and it is toxic at levels >0.1mg/l. Ammonia was measured employing the indophenol

method, measuring the intensity of the colour development when reacted with sodium hypochlorite and phenol, in an alkaline medium, using sodium nitroprusside as a catalyser. The colour is then measured on a spectrophotometer (Hach DR 2000) at 640nm. Before analysis, 25ml of oxidising reagents were prepared by adding 5ml of sodium hypochlorite to 20 ml of alkaline reagent (50g of sodium citrate in 250ml of deionised water with addition of 2.5g of sodium hydroxide) and mixing thoroughly. 1ml of phenol, 1ml of sodium nitroprusside and 2.5ml of oxidizing reagent were added to each sample swirling between additions. The solutions were left to develop for 50 minutes and then each sample was read in the spectrophotometer after selection of the appropriate method (method 954). With this method total ammonia nitrogen (TAN) is returned and ammonia is calculated as 2% of TAN at 10°C and pH8.

Ammonia is converted by denitrifying bacteria into nitrite (NO_2) and nitrate (NO_3). Nitrite is therefore present as natural product of this conversion and is toxic at levels >1mg/l. As for ammonia, nitrite is measured by the intensity of the colour development when reacted with sulphanilamide in an acid medium. This reacts with naphtyethylenediamine dihydrochloride (NED) to form an azo dye, which is pink/purple in colour. Water samples (25ml) are transferred to conical flasks and deionised water is used as blank. To each sample flask 1ml of sulphanilamide reagent (25ml of Hydrochloric acid added to 225ml of deionised water plus 2.5g of sulphanilamide) is added and swirl mixed, followed by 1ml of NED (250mg of N-naphthyl-ethylenediamine dihydrochloride dissolved in 250ml of deionised water). The appropriate method (method 952) is then selected in the spectrophotometer and concentrations are recorded as mg/l.

While nitrate is much less toxic than ammonia, levels over 30 ppm of nitrate can inhibit growth, impair the immune system and cause stress in aquatic species. NO_3 was measured with the cadmium reduction method. The appropriate program was selected in the spectrophotometer (355) and the wavelength adjusted to 500nm. The vials were filled with 25ml of samples and one Nitraver 5 Nitrate reagent Powder Pillow was added to the sample. Samples were then shacken vigorously for one minute and left to react for five minutes. The sample was then read in the spectrophotometer and the result returned in mg/l.

Orthophosphate was measured using the Acid Persulfate Digestion Method. One potassium persulfate powder pillow was added to 25 ml of sample. 2ml of sulphuric acid solution (5.2 N) was then added and samples placed on hot plate to boil for 30 minutes. Samples were then returned to room temperature and 2ml of sodium hydroxide solution (5.0 N) was added. Samples were then poured into a 25ml-graduated cylinder and the volume returned to 25ml and transferred into the spectrophotometer vial. The appropriate program (490) was selected and wavelength set at 890 nm. A blank was used to zero the spectrophotometer. One Phosver 3 Phosphate Powder Pillow was added to each sample and 2 minutes were allowed for the reaction to take place before inserting the sample vial into the hatch and read. Concentrations were returned in mg/l. All results were then converted into µmols.

Chapter 3 - Preliminary experimental work

3.1 Abstract

This preliminary work aimed at establishing the operational protocols for the commercial production of alternative microalgae species potentially suitable for *P. lividus* larval rearing, test the experimental designs and verify that laboratory analyses could be successfully performed on the selected samples. Moreover, the hypothesis that the microalgae species (*Dunaliella tertiolecta*) commonly used as larval feed might present an unsuitable fatty acid profile was tested comparing it with *P. lividus* eggs.

Standard operating procedures used at the Ardtoe Marine Laboratory for microalgae production have been confirmed to be adequate for the production of *Pleurochrysis carterae* and *Cricosphaera elongata*. The significantly lower EFA content in *D. tertiolecta* than in *P. lividus* egg suggested that an alga with more balanced FA profile richer in LC-PUFAs might prove useful to increase larvae survival. Indeed, when *P. carterae* and *C. elongata* were used as larval feed, survival up to competence was significantly improved.

This investigation has also proven to be of fundamental importance for the validation of the standard sampling and analysis protocols for eggs, larvae and micro/macro algae that were used throughout the thesis.

3.2 Suitability of *Pleurochrysis carterae* and *Cricosphaera elongata* as sea urchins larval diet: Production methods and preliminary feeding trial

3.2.1 Introduction

In order to design the experimental work described in the following chapters, some preliminary investigations had to be carried out to establish protocols and rearing methods and to answer some basic and practical questions. In particular, the feasibility to commercially producing other microalgae than *D. tertiolecta* in Ardtoe had to be investigated. Moreover, the theoretical unsuitability of this species for *P. lividus* larval rearing on the basis of its fatty acid profile had to be tested by comparison with *P. lividus* eggs fatty acid profile. In order to answer these questions two preliminary trials were carried out. The first consisted in identifying the protocol required to produce alternative microalgae species. The second aimed to compare *P. lividus* egg fatty acid profile with that of *D. tertiolecta.*

Microalgal cultures that reached suitable density within reasonable time and, more importantly, responded to the production protocols already in place in the hatchery were used as larval feed. Acceptance of the alternative microalgae species by *P. lividus* larvae and plutei survival were then monitored and compared with that observed when *D. tertiolecta* was used.

3.2.2 Materials and methods

Alternative microalgae species were selected among those presenting sizes within the range accepted by *P. lividus* larvae. The choice fell on two species: *Pleurochrysis carterae* and *Cricosphaera elongata*.

Stock cultures of these species were kept in 250 ml borosilicate glass, flat-bottomed conical flasks fitted with a cotton wool plug at the neck and Guillard's F/2 was used as culture medium (Table 3. 1). Flasks were positioned on a bench illuminated by 8-watts fluorescent lamps at a light intensity of about 450 lux measured at the culture surface.

Each species stock was sub-cultured at monthly intervals to maintain a vigorous and healthy state. Following removal of the cotton wool plug from a stock culture flask and flaming the neck of the flask with a Bunsen burner, an inoculum of 50 ml was transferred into another sterile flask containing autoclaved medium. The plug was inserted after flaming of the neck of the new flask. The original stock culture was kept for a few weeks in the event that the new stock culture failed to grow.

Table 3.1. Guillard's F/2 medium used for culturing microalgae

Nutrients	**Quantity (g per l)**
Nitrate ($NaNO_3$)	75.0
Phosphate ($NaH_2PO_4.H_2O$)	5.0
Silicate ($Na_2SiO_3.9H_2O$)	30.0
Trace Metals $FeCl_3.6H_2O$ Na_2EDTA $CuSO_4.5H_2O$ $ZnSO_4.7H_2O$	 3.5 4.35 9.8 22.0

The semi-continuous method was employed for the intermediate culture and 20 litres carboys filled with autoclaved culture medium were inoculated with each species. Cultures were left to grow until a further increase in cell density was inhibited by the failure of the light to adequately penetrate the culture. At this point

Carboys were partially harvested and the volume was replaced with freshly prepared culture medium. Harvested volume was used to inoculate 100 litres polyethylene bags filled with sterile seawater and nutrients and used for mass production. Microalgae density was measured using "improved Neubauer" haemocytometer chamber (B.S. 748; Weber, England and Gallenkamp) under light microscope. Moreover, absorbance at set densities was measured using spectrophotometer and calibration curve were calculated for each species.

Larval rearing for this preliminary work was conducted in accordance with production protocols at the Ardtoe Marine Laboratory (described in the general materials and methods section) and fertilised eggs from three different females were mixed and then divided between three treatments (*D. tertiolecta*, *P. carterae* and *C. elongata*) in duplicates. Larvae were reared in static systems with feeding and complete water exchange occurring every 3rd day. Feeding in the different treatments was equal in terms of number of cells per ml of rearing water. Survival was volumetrically assessed at competence.

3.2.2.1 Statistical analysis

Survival rates (described as a percentage of eggs stocked) were analysed using analysis of proportions where 95% confidence limits were calculated for the respective proportions (Fowler and Cohen, 1987). When the upper and lower confidence limits of the respective proportions were not found to overlap the proportions were considered to be statistically different at the 5% level (p=0.05).

3.2.3 Results

The protocol described above produced reliable and stable growth at each culture stage and no contamination from protozoa was observed during normal counting operation in the haemocytometer under the microscope. Densities in the mass production at exponential phase for each microalgae tested are shown in the table 3.2.

Table 3.2. Maximum densities achieved for each species

Species	**Density (10^6 cells / ml)**
Dunaliella tertiolecta	4.8
Pleurochrysis carterae	2.5
Cricosphaera elongata	1.5

Moreover, calibration curves for each species were produced in order to reduce labour in microalgae culture production (Figure 3.1). The graphs below show counted microalgae cells against measured absorbance and return a useful forecasting tool to quickly assess microalgae density avoiding tedious counting under the microscope.

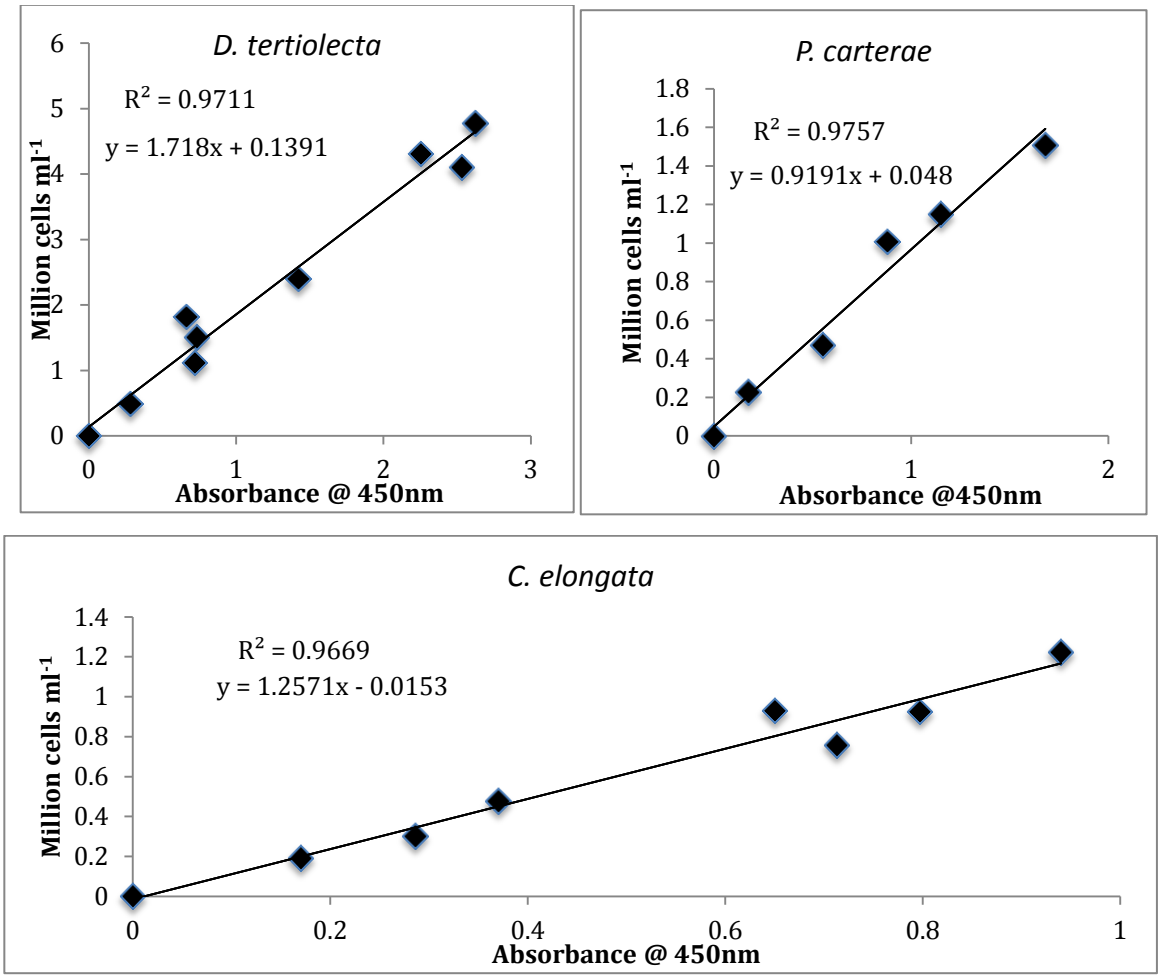

Figure 3.1. Calibration curves used to correlate optical density and microalgae cell density

All treatments supported *P. lividus* larval growth up to competence and no difference in microalgae consumption was noticed between the treatments. When checked under the microscope before water change, microalgae residual was very low (about 50 cells ml^{-1}) in all rearing tanks.

Larvae fed *P. carterae* and *C. elongata* showed significantly higher survival at competence than larvae fed *D. tertiolecta* (Figure 3.2). Although not measured, it seemed that larvae fed the first two diets were more homogenous in size and more synchronised in terms of developmental stages. By looking at the larvae under the microscope it also appeared that arm length was shorter in larvae fed *P. carterae* and

C. elongata than in the control although gut fulness did not appear to be different between treatments. These larvae also achieved competence few days in advance compared to larvae fed *D. tertiolecta*.

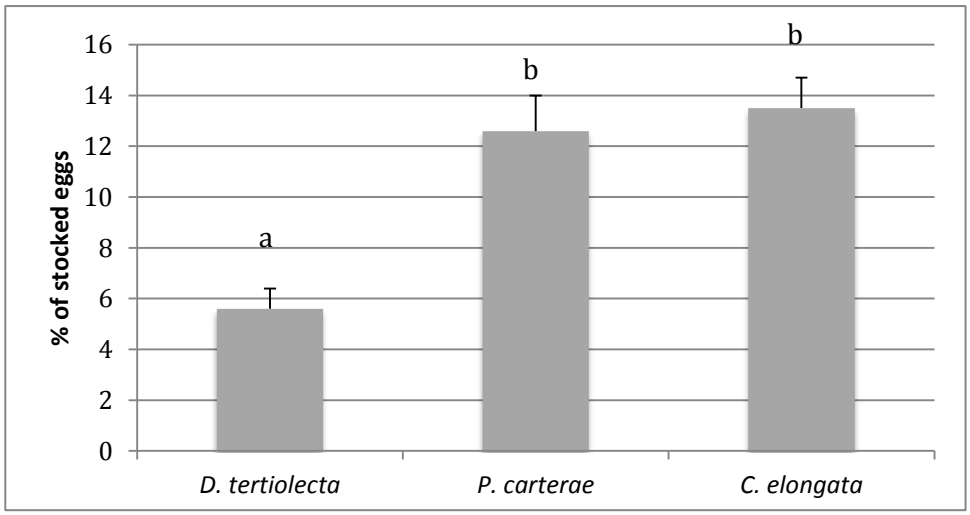

Figure 3.2. Survival rate at competence of *P. lividus* larvae fed *D. tertiolecta*, *P. carterae* and *C. elongata* diets. Data are expressed as mean ± SD (n=2). Superscripts indicate significant differences.

3.3 *Paracentrotus lividus* gamete quality: Essential Fatty Acid

3.3.1 Introduction

The hypothesis behind the observed difference in survival between larvae fed *D. tertiolecta* and those fed *P. carterae* and *C. elongata* observed in the previous trial and reported in the literature (Pedrotti and Fenaux, 1993) was the low Essential Fatty Acids (EFA) content of *D. tertiolecta*, which might have been insufficient to satisfy larvae requirements. In this second trial, we have attempted to confirm this by comparing *P. lividus* egg fatty acid profile with that of *D. tertiolecta* (Kreissig et al., 2009). The FA profiles of the broodstock diet with that of the eggs were also compared to see whether similarities existed and to trial the sampling protocols and analytical procedures. For the same reasons, larvae were sampled throughout development.

3.3.2 Materials and Methods

The purple sea urchin (*P. lividus*) broodstock were between 5 and 6 years old and were originally collected on the west coast of Ireland. They were held at Ardtoe for 1.5 years prior to the experiment, fed with kelp (*Laminaria digitata*,) collected from the shore at Ardtoe. The broodstock used for this experiment were an out of season stock (4 months advanced photoperiod), held at 14 ± 1 °C.

Five sea urchins (three females and two males) were placed separately in glass bowls filled with seawater. They were injected through the peristomial membrane as described in the materials and methods section. Each female was fertilised with the diluted sperm of both males. The fertilised eggs were left to hatch undisturbed for 24

hrs. Three batches of replicate larvae per female were stocked into 80 L cylindrico-conical white polythene tanks at a density of 4 larvae mL^{-1}. Larval rearing methods were as described in the materials and method section. Water temperature, salinity, development stage, gut content (full or empty) and residual algae from feeding were monitored daily.

The samples for lipid analysis were taken prior to feeding with *D. tertiolecta*. Fifty eggs at day 0 and larvae thereafter, were counted and transferred in seawater into small vials, containing 5 mL chloroform/methanol (2:1, v/v) and stored at −30 °C. Samples of *D. tertiolecta* were filtered through glass fibre filters and preserved the same way as sea urchins eggs and larvae. An overview of the sampling schedule is given in Table 3.3 below.

Table 3.3. Sampling schedule and materials (modified from Kreissig et al., 2009)

Culture day	Development stage	Sample type
0		Unfertilised eggs
2	Pyramid stage	Larvae
5	4-arm stage	Larvae and *D. tertiolecta*
11	6-arm stage	Larvae and *D. tertiolecta*
17	8-arm stage	Larvae and *D. tertiolecta*
20	Rudiment	Larvae and *D. tertiolecta*

Samples of *Laminaria digitata* (broodstock diet) were collected at low tide from the shore at Ardtoe, and stored in seawater. They were processed for lipid analysis within 12 h. The kelp was washed to remove the seawater. Subsequently it was blotted dry, weighed and 30 g were chopped. It was frozen at −70 °C for 3 hours and then transferred to a freeze-drier to remove the water, followed by vacuum desiccation for 50 h. The dry samples were ground with mortar and pestle and 0.5 – 1 g of the resulting powder transferred to glass boiling tubes. Thereafter, the

procedure was carried out on ice as described in the general materials and methods chapter. Minitab 15.0 was used for statistical analysis and one-way ANOVA followed by the Tukey multiple comparison test was employed to assess significant differences.

3.3.3 Results

Fatty acid compositions of the broodstock diet, eggs and *D. tertiolecta* are shown in Table 3.4. Kelp fronds were rich in palmitic acid (16:0), oleic acid (18:1n-9) and the Polyunsaturated Fatty Acids, ARA, stearidonic acid (18:4n-3) and EPA (all above 10 %). However, they did not contain any fatty acids greater than C20 chain length. The microalgae *D. tertiolecta* displayed a high content of C16 fatty acids (16:0 as well as mono- and polyunsaturated components) and was rich in C18 PUFA, particularly α-linolenic acid 18:3n-3, but only relatively low levels of ARA, EPA and DHA. The sea urchin eggs exhibited high proportions of ARA and EPA and relatively lower level of DHA. Their overall composition was generally similar to that of the broodstock diet, however the high 18:4n-3 concentration was not reflected in the sea urchin egg lipids which, also, contained 18:0 Dimethylacetale (DMA) and 20:0 and 22:2 NMID. Moreover, the comparison of EFA profiles between *D. tertiolecta* and *P. lividus* eggs showed that their content in the eggs were significantly higher that in the larval diet. FA profiles of the larvae at each developmental stage was impossible to evaluate due to the small number of larvae collected at each sampling point.

Table 3.4. Fatty acids profile of broodstock diet, *P. lividus* eggs, and larval diet. Data are expressed as mean ± SD (n=3). Superscripts indicates significant differences. Nd: Not detected (modified from Kreissig et al., 2009).

Fatty acids	*L. digitata*	Eggs	*D. tertiolecta*
14:0	7.0 ±1.5	7.1 ±0.8	0.7 ±0.1
15:0	Nd	1.1 ±0.6	16.8 ±1.8
16:0	17.9 ±1.1	17.1 ±1.1	1.7 ±1.0
18:0	0.7 ±0.1	3.6 ±0.6	4.6 ±2.5
Tot. Sats	26.2 ±0.3	29.9 ±3.4	23.9 ±4.6
16:1	8.4 ±1.2	4.8 ±0.9	6.5 ±1.5
18:1n-9	13.1 ±3.0	5.0 ±1.2	7.1 ±1.9
18:1n-7	Nd	2.8 ±0.8	1.9 ±1.1
20:1	Nd	9.8 ±0.5	0.8 ±0.6
22:1	Nd	3.4 ±0.5	0.6 ±0.4
Tot. Monos	21.7 ±2.1	26.1 ±0.4	16.8 ±3.1
18:2n-6	6.0 ±0.1	1.7 ±0.7	5.0 ±0.8
20:2n-6	Nd	5.1 ±1.5	3.4 ±0.3
20:4n-6 (ARA)	12.4 ±4.4 [a]	9.0 ±0.7 [a]	1.2 ±1.0 [b]
22:4n-6	Nd	1.1 ±0.5	Nd
Tot. n-6 PUFA	19.4 ±4.8	16.9 0.1	9.8 ±1.8
18:3n-3	6.5 ±1.1	1.3 ±0.4	31.2 ±3.3
18:4n-3	13.0 ±0.5	2.5 ±0.8	1.4 ±0.3
20:3n-3	Nd	0.9 ±0.2	Nd
20:5n-3 (EPA)	12.1 ±2.3 [a]	9.9 ±1.7 [a]	0.5 ±0.2 [b]
22:6n-3 (DHA)	Nd	1.1 ±0.6 [a]	0.3 ±0.1 [b]
Tot. n-3 PUFA	19.4 ±4.8	16.8 ±2.9	33.9 ±2.7
18:0 (DMA)	Nd	3.6 ±0.2	Nd
Tot. (NMI FAs)	Nd	6.6 ±1.4	Nd
Tot. PUFA	51.7 ±1.8	40.4 ±3.2	58.4 ±4.4

3.4 Discussion

The first preliminary trial demonstrated the feasibility of producing *C. elongata* and *P. carterae* at the Ardtoe Marine Laboratory. Although the cell densities achieved for both species were lower compared with *D. tertiolecta,* production was still sufficient to provide enough feed for the developing larvae. Moreover, acceptance of the

alternative microalgal species was comparable with that of the control diet and overall larval performances were superior.

Main limitations of this trial were, however, the low replication (larval rearing was only in duplicated design) and the absence of morphometric data able to properly describe larval growth and development. Moreover, feeding ration were the same in all treatments and difference in microalgae cell size was not taken into account. As *P. carterae* and *C. elongata* are significantly larger than *D. tertiolecta*, this resulted in differences in the biomass provided to the larvae at each feed, which might have affected larval performances. Nonetheless, the promising results obtained prompted the decision to design a new trial (Chapter 4) where all the mentioned flaws were corrected and one more microalgae treatment (*Tetraselmis suecica*) was added.

The second preliminary trial has demonstrated that a significant difference exists between the fatty acid profile of *P. lividus* eggs and that of *D. tertiolecta*; in particular EFA concentration is significantly lower in the latter indicating that nutritional deficiencies might occur during larval development when *D. tertiolecta* is used as larval feed. Egg fatty acid composition, moreover, resembled that of broodstock diet; DHA, however, was not detected in the latter whilst it was present, although in relatively low concentration, in the eggs. This indicates that this fatty acid might be synthetised by sea urchin and transferred to the eggs, also suggesting that it might play an important role during larval development. Interestingly, other FA not present in the diet were recorded in the eggs such as DMA and NMID and roles of these fatty acids in sea urchins and other marine invertebrates are still to be clarified. As already mentioned, sample size for larval fatty acid analysis was too small in this study and constitutes its main flaw. For this reason more larvae were collected

during the follow-up study (Chapter 4) and FA analysis of each development stage of larvae fed all the microalgal diets has been successfully carried out.

This preliminary work has been of paramount importance in training the author in the larval rearing and microalgae production techniques as well as lipid extraction and fatty acid methyl esters preparation. Furthermore, this initial period allowed for a better understanding of the main critical factors influencing larvae performances and provided useful insights on what parameters (morphology, gut-fulness, time of appearance of larval arms, residual microalgae) should be carefully measured in the follow-up trials. Moreover, via a methodic trial and error approach, a firm grasp of the methodologies to be used to standardise rearing conditions across treatments and prevent replicate loss was acquired and implemented in all larval related trials during the course of the PhD.

Chapter 4 - Effects of dietary microalgae on growth, survival and fatty acid composition of sea urchin, *Paracentrotus lividus*, throughout larval development.

Published in:

Aquaculture. (2012). Vol. 324-325 p.250-258

Authors:

Stefano Carboni[a], Julien Vignier[b], Mariachiara Chiantore[c], Douglas R. Tocher[a], Herve Migaud[a]

Affiliations:

[a] Institute of Aquaculture, Stirling University, Stirling, FK9 4LA, Scotland, UK

[b] Ardtoe Marine Laboratory, Acharacle PH36 4LD, Scotland, UK

[c] DipTeRis, Genova University, Viale Benedetto XV, Genova, IT

Key words:

Paracentrotus lividus; sea urchin; larvae; nutrition; microalgae; fatty acids

Contribution

The author has designed and conducted the trial, collected the samples and carried out all laboratory and statistical analysis and wrote the manuscript.

4.1 Abstract

This study investigated the growth, survival and fatty acid composition of sea urchin *Paracentrotus lividus* larvae fed four microalgal diets: *Cricosphaera elongata*, *Pleurochrysis carterae*, *Tetraselmis suecica* and *Dunaliella tertiolecta* (control). Larvae were successfully raised to competence for metamorphosis when fed *C. elongata*, *P. carterae* and *D. tertiolecta* diets but significant differences were found in survival rate and development.

Larvae fed *C. elongata* showed 3 times higher survival and 20% faster development than larvae fed the other two microalgae diets that supported development. In contrast, *T. suecica* failed to fully support development and larvae stalled at the four arms stage for more than 30 days. The urchin larvae could accumulate long-chain polyunsaturated fatty acids (LC-PUFA) such as docosahexaenoate (DHA; 22:6n-3), eicosapentaenoate (EPA; 20:5n-3) and arachidonate (ARA; 20:4n-6), either by assimilation and retention of dietary fatty acids, and/or synthesis from α-linolenic acid 18:3n-3 and linoleic acid 18:2n-6. Moreover, an accumulation of n-3 LC-PUFA and higher EPA/DHA and EPA/ARA ratios appeared to be associated with improved larval performance. The results indicate that live microalgae species, with appropriate fatty acid profiles are able to improve *P. lividus* larval performance, ultimately increasing hatchery profitability.

4.2 Introduction

Sea urchin gonads (roe) are considered a delicacy worldwide and especially in Japan where they are called “uni” and valued as sushi (Lawrence, 2001). As natural stocks

are declining due to fishing pressure (Andrew et al., 2002; Boudouresque and Verlaque, 2001), mariculture has an important role to play to supply high quality seeds, juveniles and adults (Guidetti et al., 2004; Pais et al., 2007). Due to its high roe content, the purple sea urchin *Paracentrotus lividus* (Lamarck, 1816) has been identified as an ideal candidate to satisfy increasing demand in Europe but wild stocks generally cannot sustain a fishery (Watson and Stokes, 2000). *P. lividus* is widely distributed in south-western Europe, and reaches the northern limits of its range in Scotland. As there are only a few isolated populations in Scotland, a fishery is not viable and so a fully-farmed approach is the only option for its production. The first commercial production of this species in the UK started at the Ardtoe Marine Laboratory (AML) in 2007. Research on sea urchin culture was initiated more than 100 years ago (Koehler, 1883) and more recently studies have focused on larval feeding (Fenaux et al., 1985, 1994; George et al., 2004; Liu et al., 2007; Pedreotti and Fenaux, 1993), culture methods for both larvae (Kelly et al., 2000; Leighton, 1995) and adults (Grosjean et al., 1998). However, many aspects of *P. lividus* biology including control of sexual maturation and the larvae and adults nutritional requirements still require investigation to support a successful intensive commercial production. Importantly, most research trials performed to date have used laboratory scale culture methods that often result in survival rates far greater than those achieved by the industry and so are not always applicable to commercial scale ventures (Fenaux et al., 1985; George et al., 2004; Kelly et al., 2000; Liu et al., 2007; Pedreotti and Fenaux, 1993).

High mortalities during larviculture of marine fish and shellfish remain a major bottleneck in aquaculture that limits production (Dhert et al., 2001). Larval nutrition and the provision of optimal feeds are known to be a key factor in overcoming these

problems (Rainuzzo et al., 1997). It is well known that marine organisms require certain essential fatty acids (EFA), specifically n-3 and n-6 polyunsaturated fatty acids (PUFA), for their normal development. Many studies have shown that the absolute and relative amounts of dietary EFA have direct effects on larval development and survival of aquaculture species (Coutteau et al., 1997; Sargent et al., 1999, 2002; Tocher, 2003). Furthermore, each echinoderm species has specific dietary requirements and may require specific PUFA at given developmental stages (Castell et al., 2004; Cook et al., 2000). The importance of PUFA, especially linolenic acid (18:3n-3) and the long-chain PUFA (LCPUFA), docosahexaenoic acid (DHA; 22:6n-3), has been demonstrated in the sand dollar *Dendraster excentricus* larvae where dietary provision of these fatty acids led to better survival and growth (Schiopu et al., 2006).

The use of artificial feeds has been investigated in *P. lividus*. The aim was to enhance larvae performance (survival and growth) through the provision of higher levels of total lipid (energy) and specific EFA, including DHA, eicosapentaenoic acid (EPA, 20:5n-3) and arachidonic acid (ARA, 20:4n-6), than those present in the control diet *Dunaliella tertiolecta* (Liu et al., 2007). Results from that trial indicated that the artificial feed could support development, but larvae showed better growth performance when fed *D. tertiolecta*, mainly due to poor acceptance of the artificial diet. Nonetheless, the fatty acid profile of *D. tertiolecta* appears to be suboptimal as it contains only a very low level of EPA and essentially no DHA (Kelly et al., 2000; Liu et al., 2007) that could lead to nutritional deficiencies in the larvae. Artificial feeds that are commercially available to date are not likely to fully support the needs of sea urchin hatcheries due to a number of reasons including palatability issues, as larvae have the ability to select feed prior to ingestion (Liu et al., 2007), lack of information

on diet formulations and the prohibitive costs of producing feeds with very fine particles (micro-encapsulated) for a relatively small market. For these reasons *D. tertiolecta* is one of the most common live microalgae used for the rearing of many echinoderms larval stages. However, previous studies suggested that other microalgae species including *Cricosphaera elongata* and *Pleurochrysis carterae* could be used for sea urchin larviculture (Pedreotti and Fenaux, 1993; Leighton 1995; Grosjean et al., 1998).

The present study revealed, for the first time, the developent of larvae fatty acid profile and the effects of dietary fatty acid composition on urchin larval fatty acid composition throughout development, it also provides a possible solution to improve the production output of a commercial hatchery. To this end, we compared sea urchin larvae growth performance when fed different live microalgae species that are easy to culture and readily accepted by the larvae, and with more balanced fatty acid profiles, especially, LC-PUFA. The hypothesis being that these microalgae with more appropriate levels and ratios of LC-PUFA will better support development and enhance growth of *P. lividus* larvae relative to those raised on EPA/DHA deficient *D. tertiolecta*. Furthermore, this work will help to further understand physiological requirements of echinoplutei and enable optimisation of *P. lividus* larval rearing protocols to increase hatchery profitability.

4.3 Materials and methods

4.3.1 General methods

Three year old *P. lividus*, raised at the Ardtoe Marine Laboratory (AML; 56 N 46' -5W52'), and fed on brown algae *Palmaria palmata*, *Laminaria digitata* and

Saccharina latissima (20:40:40, wet weight) over the culture period, were induced to spawn in February 2010 by injection of 1 M KCl (40 µl per g of body weight) into the coelom via the peristomial membrane. Three females (51.0±1.3 g) and three males (49.0±1.3 g) were spawned. Each female spawned approximately 2 million eggs that were fertilized by adding few drops of diluted sperm. Fertilization rate, assessed 2 h post fertilization, was 98.5±1.0%. The fertilized eggs were left to hatch in static seawater without aeration for 24 h in the dark. Hatching rate was 85.0±1.0%.

Seawater used during the process of spawning, hatching, and larval rearing was filtered (4 µm) and UV treated, and room temperature was maintained at 18±2 °C throughout the larval cultivation period. Larvae were stocked at a density of 4 per ml in 80 L conical plastic tanks and cultivated in aerated static water in continuous light. A complete water exchange and thorough cleaning of the tanks was carried out every 3rd day. Age at competence was defined as the number of degree-days required for at least 75% of the larvae fed each treatment to reach competence for settlement, which was considered achieved when the rudiment was equal in size or larger than the stomach. As temperature differences between treatments could affect larval rate of development Degree days (°C d^{-1}) unit was used to compare age at competence. The larval culture methods were adapted from Kelly et al. (2000).

4.3.2 Experimental diets

The experimental design involved four triplicated treatments. *Dunaliella tertiolecta* (7µm Equivalent Spherical Diameter (ESD), 180µm3 Volume) (control), *Tetraselmis suecica* (7µm ESD; 180µm3 Volume), *Pleurochrysis carterae* (8.9 µm ESD; 380µm3 Volume) and *Cricosphaera elongata* (8.9µm ESD; 380µm3 Volume) were used as diets. The first three species were sourced from the Culture Collection for Algae and

Protozoa (CCAP: Oban, Scotland) while the last was isolated at Dip.Te.Ris (Genova University, Italy) and shipped to AML. Microalgal cultures were grown in 100 L polyethylene bags in sterilized seawater enriched with the f/2 medium. Meta-silicates were added to media for *C. elongata* and *P. carterae*. Microalgae during their exponential growth phase were fed to the larvae every 3rd day and ration was standardized between treatments according to microalgae species cell size. For larvae with two, three and four pairs of arms, the daily feeding rate was 1500, 4500, and 7500 cells ml^{-1} respectively when using *D. tertiolecta* or *T. suecica*, as adapted from Kelly et al. (2000) and Jimmy et al. (2003), and 750, 2250 and 3250 cells ml^{-1} respectively when using *C. elongata* or *P. carterae*. Microalgae culture densities were determined by spectrophotometry (DR/2000 Direct Reading Spectrophotometer, Hach Lange Ltd, UK) using standard concentration curves for each species prepared using light microscopy and cell counting via hemocytometer. Accuracy was checked via regression analysis (R-sq ≥0.8).Whilst particle counting is often used in small scale trials to assess microalgae culture density, spectrophotometry is widely used in commercial aquaculture and has been considered of good precision when compared with other commonly used methods including cell counting via hemocytometer (Butterwick et al., 1982).

The equation used to calculate feed ration was:

$$Volume\ of\ Algae\ given = \frac{\text{(number of algae cells x rearing volume)}}{\text{Algae culture concentration}}$$

4.3.3 Larval growth, morphology and survival

Larval morphology can be drastically influenced by feeding regime (Fenaux et al., 1994; Kelly et al., 2000; Strathmann et al., 1992) and, therefore, the major larval body features were measured. At 5, 11, 17 and 23 days post fertilisation (DPF), 20 larvae were randomly sampled from each rearing tank and larval length, body width and postoral arm length were measured using an image analysis software (Image Pro PlusTM, Media Cybernectics, Silver Spring, Maryland, USA). The development of additional larval arms was also recorded.

Larval survival was assessed volumetrically every 3rd day and when competence for settlement was achieved by at least 75% of the larvae in each rearing tank.

4.3.4 Lipid and fatty acid analyses

Microalgae samples (20 x 10^6 cells) were collected by filtration onto a GF/F filter (Whatman Ltd, Maidstone, UK) before being placed in glass vials containing 5 ml of chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene (BHT) as antioxidant. Algal culture concentration was assessed as described above and the volume filtered recorded to assess the number of cells present in each sample.

Urchin eggs were collected from the five gonopores immediately after spawning using a pipette and placed in glass vials containing 5 ml chloroform/methanol plus BHT as above. Urchin larvae samples (about 1200 larvae) from each replicate at each developmental stage (pyramid, 4 arms, 6 arms, 8 arms and rudiment) were collected after filtration through a 40 µm sieve and stored in glass vials as above. All samples were stored at −20 °C for 5 weeks prior to analyses.

Total lipid of microalgae, urchin eggs and larvae was extracted following the method of Folch et al. (1957). Samples were homogenized in the chloroform/methanol (as above) using a tissue disrupter (Ultra Turax™, IKA Werke Gmbh & Co. KG, Staufen, Germany), and 1 ml 0.88% KCl was added and the homogenates mixed before centrifugation at 600 g for 5 min (Jouan C412, Pegasus Scientific Inc., Rockville, USA). The upper aqueous phase was aspirated and the solvent evaporated under a stream of oxygen-free nitrogen (OFN). Lipid content was determined gravimetrically after desiccation overnight. The total lipid extracts were re-dissolved at a concentration of 10 mg/ml in chloroform/methanol (2:1, v/v) plus BHT.

Fatty acid compositions of total lipid were determined by gas chromatography according to Christie (2003). Fatty acid methyl esters (FAME) were prepared from total lipid by acid-catalyzed transesterification at 50 °C for 16 h with extraction and purification by thinlayer chromatography as described previously (Tocher and Harvie, 1988). The FAME were separated and quantified by gas–liquid chromatography using a Fisons 8160 instrument (ThermoFisher, UK) equipped with a 30 m×0.32 mm i.d., 0.25 µm capillary column (CP

Wax 52CB, Chrompak, London, U.K.) and on-column injection. Hydrogen was used as carrier gas and temperature programming was from 50 to 150 °C at 40 °C min^{-1} and then to 230 °C at 2.0 °C min^{-1}. Individual methyl esters were identified by comparison with known standards and by reference to published data (Ackman, 1980; Tocher and Harvie, 1988). Data were collected and processed using Chromcard for Windows (version 1.19), and FAME quantified through a comparison with a heptadecanoic acid (17:0) internal standard.

4.3.5 Statistical analysis

All analyses were carried out using the statistical package of Minitab 15.0 (Minitab Ltd., UK). Larval morphometric measurements were analysed using a General Linear Model (Zar, 1999). Normality and homogeneity of variance were improved where necessary by either log or reciprocal transformations. Development of arms and rudiment, age at competence and fatty acids were analysed using one-way ANOVA followed by the Tukey multiple comparison test to assess where significant differences occurred. Survival rates (described as a percentage) were analysed using analysis of proportions where 95% confidence limits were calculated for the respective proportions (Fowler and Cohen, 1987). When the upper and lower confidence limits of the respective proportions were not found to overlap the proportions were considered to be statistically different at the 5% level (p=0.05).

4.4 Results

4.4.1 Lipid content and fatty acid composition of microalgae

The total lipid (TL) content varied between the four microalgal diets. *D. tertiolecta* had the highest TL content (0.14 mg per million cells) followed by *C. elongata* (0.09 mg per million cells), by *T. suecica* (0.06 mg per million cells) and by *P. carterae* (0.05 mg per million cells). *P. carterae* contained the highest proportion of total PUFAs, followed by *C. elongata*, *T. suecica* and *D. tertiolecta* (Table 4.1).

Table 4.1. Fatty acid profiles of microalgae live feeds. Values are given as % of total fatty acids. [1]Total saturated contain 15:0 and 22:0; [2]predominantly 16:1n-7; [3]predominantly 20:1n-9; [4]Totals contain 20:2n-6. ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentanoic acid; PUFA, polyunsaturated fatty acid.

Fatty acids	*D. tertiolecta*	*P. carterae*	*C. elongata*	*T. suecica*
14:0	0.8	0.6	0.3	0.5
16:0	18.6	17.4	21.6	20.9
18:0	6.8	1.8	2.0	0.5
20:0	0.4	2.2	5.0	0.2
∑ saturated[1]	30.1	22.2	29.4	22.3
16:1[2]	5.5	7.6	3.8	9.4
18:1n-9	7.7	5.8	3.9	12.4
18:1n-7	1.5	0.9	1.5	1.6
20:1[3]	0.9	0.3	1.3	2.3
22:1n-11	0.3	0.2	0.0	0.1
24:1n-9	0.3	0.0	0.2	0
∑ monounsaturated	16.4	14.8	10.7	25.8
18:2n-6	4.5	14.4	8.0	4.6
18:3n-6	3.2	1.8	1.0	1.2
20:3n-6	0.7	0.0	2.6	0.1
20:4n-6	0.9	0.1	0.2	1.1
22:5n-6	0.0	0.1	0.6	0.0
∑ n-6 PUFA[4]	9.1	16.6	12.7	7.1
18:3n-3	28.3	9.8	8.3	11.9
18:4n-3	1.2	16.9	16.9	7.3
20:4n-3	0.0	0.0	0.2	0.5
20:5n-3	0.7	3.5	3.5	7.5
22:5n-3	0.0	0.2	0.1	0.1
22:6n-3	1.4	8.6	10.3	0.6
∑ n-3 PUFA	31.7	38.9	39.5	28.0
16:2	1.3	4.8	3.4	1.0
16:3	2.3	0.4	2.3	1.6
16:4	8.9	0.5	0.5	12.0
Total C16 PUFA	12.4	5.8	6.2	14.6
Total PUFA	45.2	61.3	58.4	49.7
EPA/ARA	0.8	31.5	16.1	6.8
DHA/EPA	1.9	2.5	2.9	0.1

Similarly, total n-3 and n-6 PUFA were highest in *P. carterae*, followed by *C. elongata*, *D. tertiolecta* and *T. suecica*. However with regard to LCPUFAs, *T. suecica* showed the highest proportion of EPA followed by *C. elongata* and *P. carterae*, with *D. tertiolecta* showing only a very low level. In contrast, *C. elongata* was rich in DHA followed by *P. carterae*, with *T. suecica* and *D. tertiolecta* showing low levels. *D. tertiolecta* on the other hand showed the highest proportion of ARA, followed by *T. suecica*, whereas both *P. carterae* and *C. elongata* showed low level of this fatty acid (Table 4.1).

4.4.2 Survival and age at competence

Sea urchin larvae fed *C. elongata* showed significantly higher survival at 24 DPF and competence (14.4%) compared with larvae fed *P. carterae* (5.7%) or *D. tertiolecta* (5.2%) (Fig. 4.1).

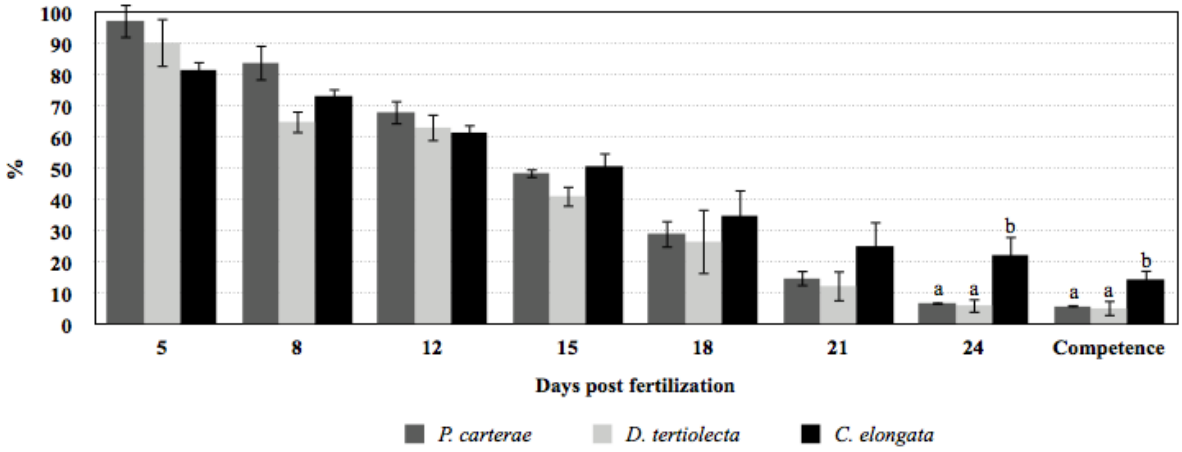

Figure 4.1. Survival rate of P. lividus larvae fed *Pleurochrysis*, *Dunaliella* and *Cricosphaera* diets. Data are expressed as mean ± SD (n=3). Superscripts indicate significant differences.

Competence for settlement was first reached by larvae fed *C. elongata* (462±30 °C d^{-1}), followed by larvae fed *P. carterae* (519±30 °C d^{-1}) and by larvae fed *D. tertiolecta* (578±29 °C d^{-1}). Sea urchin larvae fed on *T. suecica* never reached competence and after 600 °C d^{-1} were still at the four arms stage.

4.4.3 Growth and development

The sea urchin larvae growth rate was reflected in larval body indices, such as post-oral arm length, larval length and body width (Fig. 4.2 a, b and c). Post-oral arm length did not increase in larvae fed *T. suecica* and it was significantly smaller than all the other treatments at 11 and 17 DPF. Post-oral arm length in urchin larvae fed *D. tertiolecta*, *P. carterae* and *C. elongata* increased over the first 11 DPF, and decreased

thereafter. Arm shortening continued in larvae fed *D. tertiolecta*, *P. carterae* and *C. elongata* and at competence the arms of larvae fed these treatments were significantly shorter than in larvae fed *T. suecica* (Fig. 4.2 a).

Larval length increased over the first 11 DPF when significant differences were observed between treatments with larvae fed *P. carterae* significantly longer than larvae fed the other treatments, and larvae fed *D. tertiolecta* and *C. elongata* significantly longer than larvae fed *T. suecica* (Fig. 4.2 b). Length of larvae fed *P. carterae*, *C. elongata* and *D. tertiolecta* decreased between 11 and 23 DPF but, at the end of the trial, they were still significantly longer than larvae fed *T. suecica* (Fig. 4.2 b).

No significant difference in larval width was observed between larvae fed *D. tertiolecta*, *P. carterae* and *C. elongata* up to 11 DPF (Fig. 4.2 c).

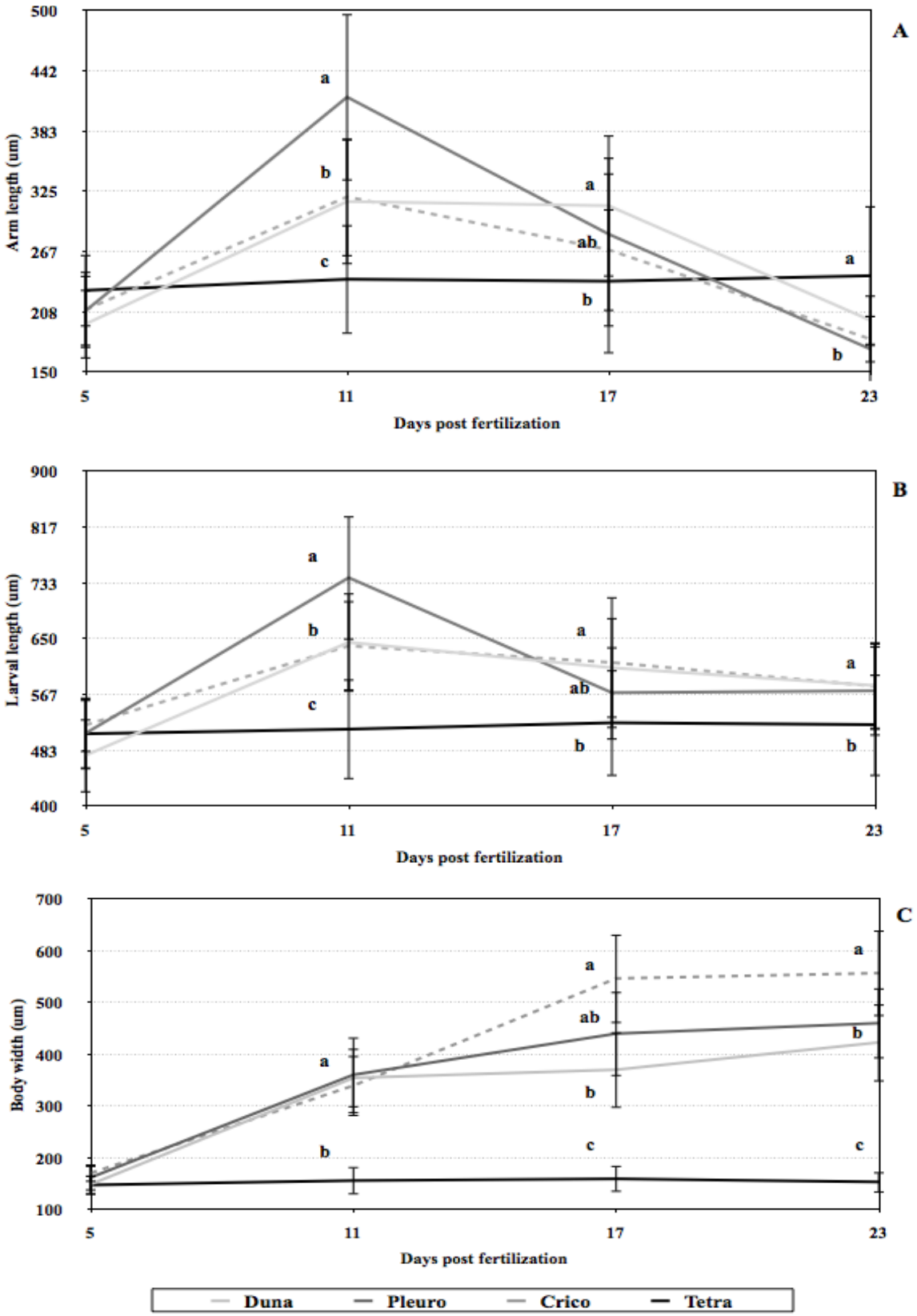

Figure 4.2. Larval length (a), Post-oral arm length (b) and Body width (c) of sea urchin larvae fed different microalgae diets from 5 to 23 days after fertilization. Data are expressed as mean ± SD (n=3). Superscripts indicate significant differences between treatments at each time point.

However, from 17 DPF onwards, significant differences between treatments were observed. Larvae fed *C. elongata* were the largest, followed by *P. carterae*, *D. tertiolecta* and *T. suecica*. No significant change in body width was observed in larvae fed *T. suecica* treatment during the whole period of larval culture (Fig. 4.2 c). The timing of development of new pairs of arms and rudiment varied between urchin larvae fed the different microalgae treatments (Fig. 4.3).

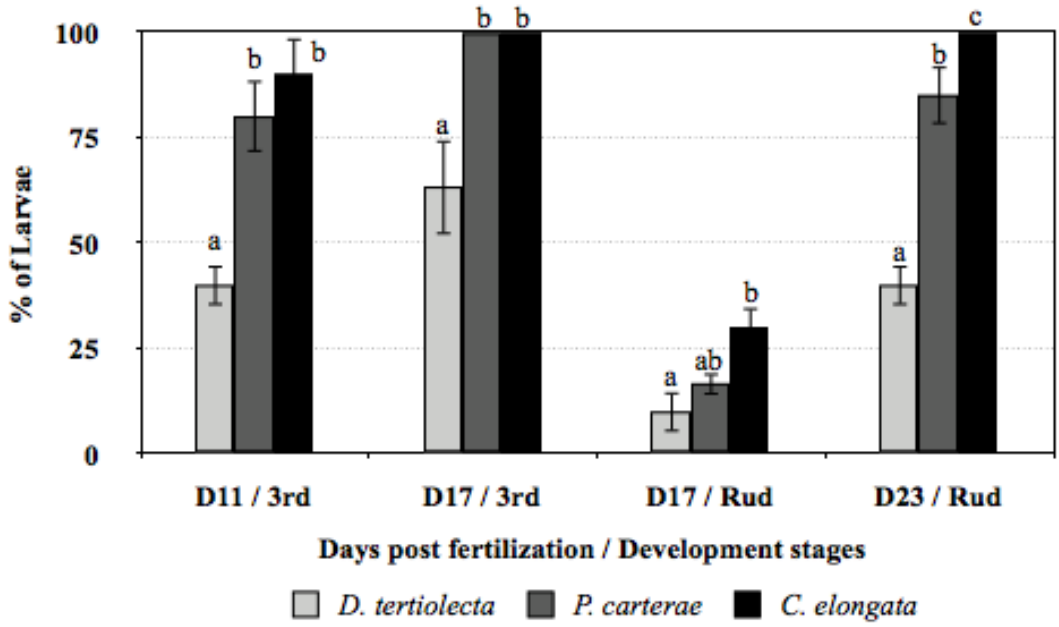

Figure 4.3. The appearance of third pair of arms and larval rudiment for P. lividus larvae fed different diets. Data are expressed as mean ± SD (n=3). Superscripts indicate significant differences between treatment at each time point.

As shown above for age at competence, larvae fed *C. elongata* and *P. carterae* had the fastest rate of development, with a greater average percentage of larvae having the 3rd pair of arms by 11 DPF and the rudiment by 23 DPF compared to urchin larvae fed *D. tertiolecta*.

By 17 DPF, 100% of the larvae in the *C. elongata* and *P. carterae* treatments had developed the 3rd pair of arms compared with an average of 63.3% in the *D.*

tertiolecta treatment. A significant difference in rudiment development was observed at 23 DPF when 100% and 85% of the larvae in *C. elongata* and *P. carterae* treatments, respectively, had developed rudiment, whereas only 40% of the larvae with rudiments were observed in larvae fed *D. tertiolecta*. Larvae fed *T. suecica* showed shrinkage of the stomach from 10 DPF onwards and did not develop the third pair of arms or show any signs of rudiment development.

4.4.4 Lipid content and fatty acid composition of *P. lividus* larvae

Total lipid content of the urchin larvae increased during the trial period regardless of the dietary treatment, however no significant differences were observed until the rudiment stage (Fig. 4.4).

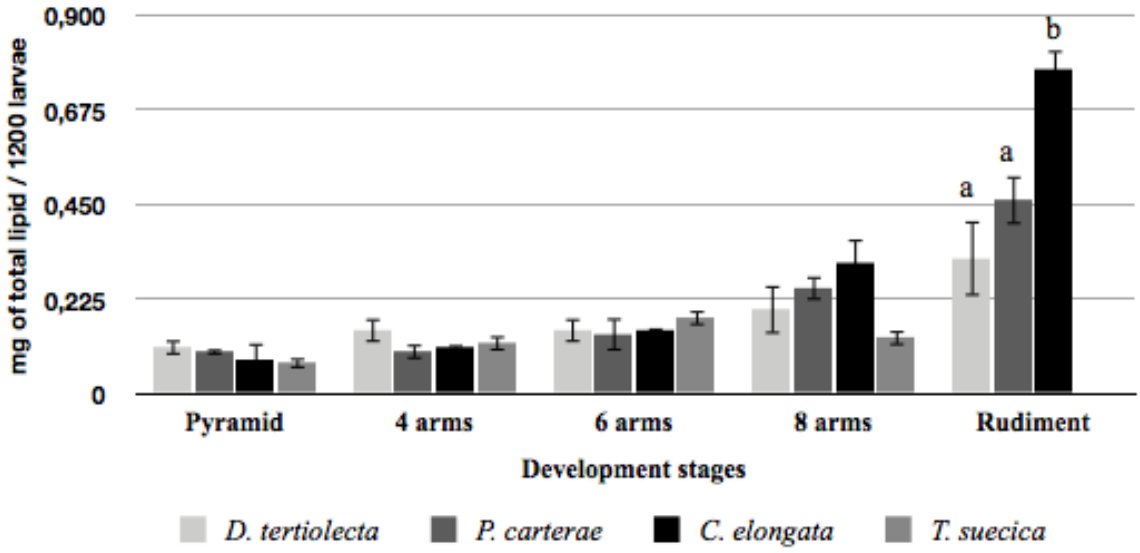

Figure 4.4. Total lipid content of P. lividus larvae fed four microalgae diets throughout development. Data are expressed as mean ± SD (n=3). Asterisks indicate significant differences between treatments.

By the end of the trial larvae fed *T. suecica* had significantly lower lipid content compared with the other treatments. Significant differences between *C. elongata*, *P.*

carterae and *D. tertiolecta* treatments were observed at the rudiment stage with lipid content in larvae fed *C. elongata* being significantly higher than larvae fed the other two treatments.

The fatty acid profiles of the urchin larvae throughout development showed the clear effects of the onset of exogenous feeding (4 arms stage) and differences between treatments increased from the six arms stage onwards (Figs. 4.5, 4.6, Table 4.2). Total saturated and monounsaturated fatty acids of larvae fed *C. elongata*, *P. carterae* and *D. tertiolecta* showed a slight increment during embryonic development (from eggs to pyramid stage). From onset of feeding onwards however they showed significant decreasing trends regardless of the treatment (Figs. 4.5 a, b).

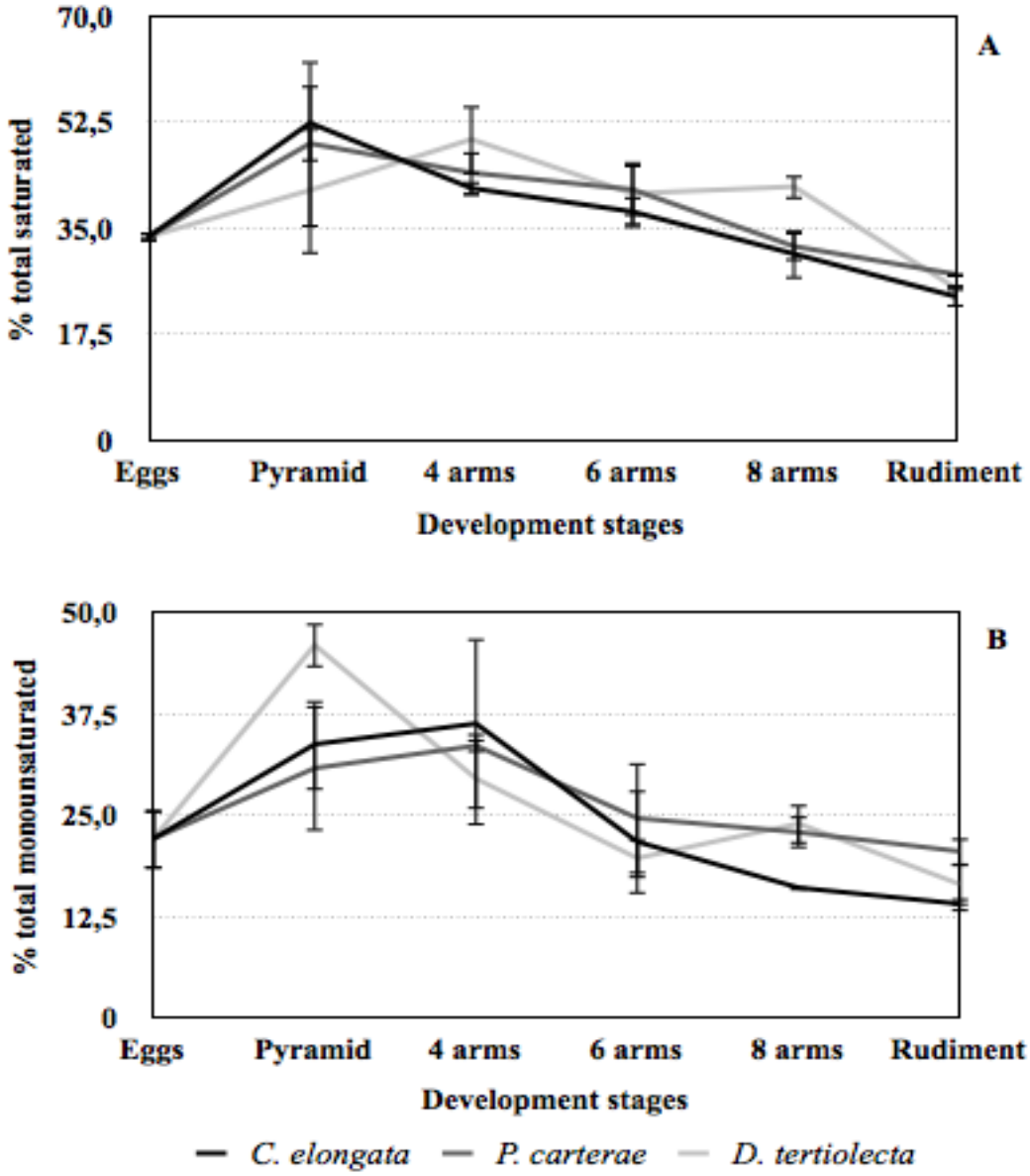

Figure 4.5 Percentage of total saturated (A) and total monounsaturated (B) fatty acids of P. lividus larvae fed different diets throughout development. Values are given as percentage of total fatty acids (mean ± SD, n=3). Superscripts indicate significant differences between treatments at each time point.

Relative abundance of total n-3 PUFA decreased during embryonic development, but increased from the pyramid stage onwards when larval body structures begin to form (Fig. 4.6 a). Significant differences were observed between treatments from the 8 arms stage onwards when larvae fed *C. elongata* showed the highest relative n-3 PUFA content followed by *P. carterae* and *D. tertiolecta* treatments. At 8 arms, larvae

fed *C. elongata* still showed the highest n-3 PUFA content but now followed by larvae fed *D. tertiolecta* and then *P. carterae*.

Total n-6 PUFA relative abundance did not change significantly up to the 8 arms stage when larvae fed *P. carterae* showed significantly higher n-6 PUFA content than *D. tertiolecta* and *C. elongata* (Fig. 4.6 b).

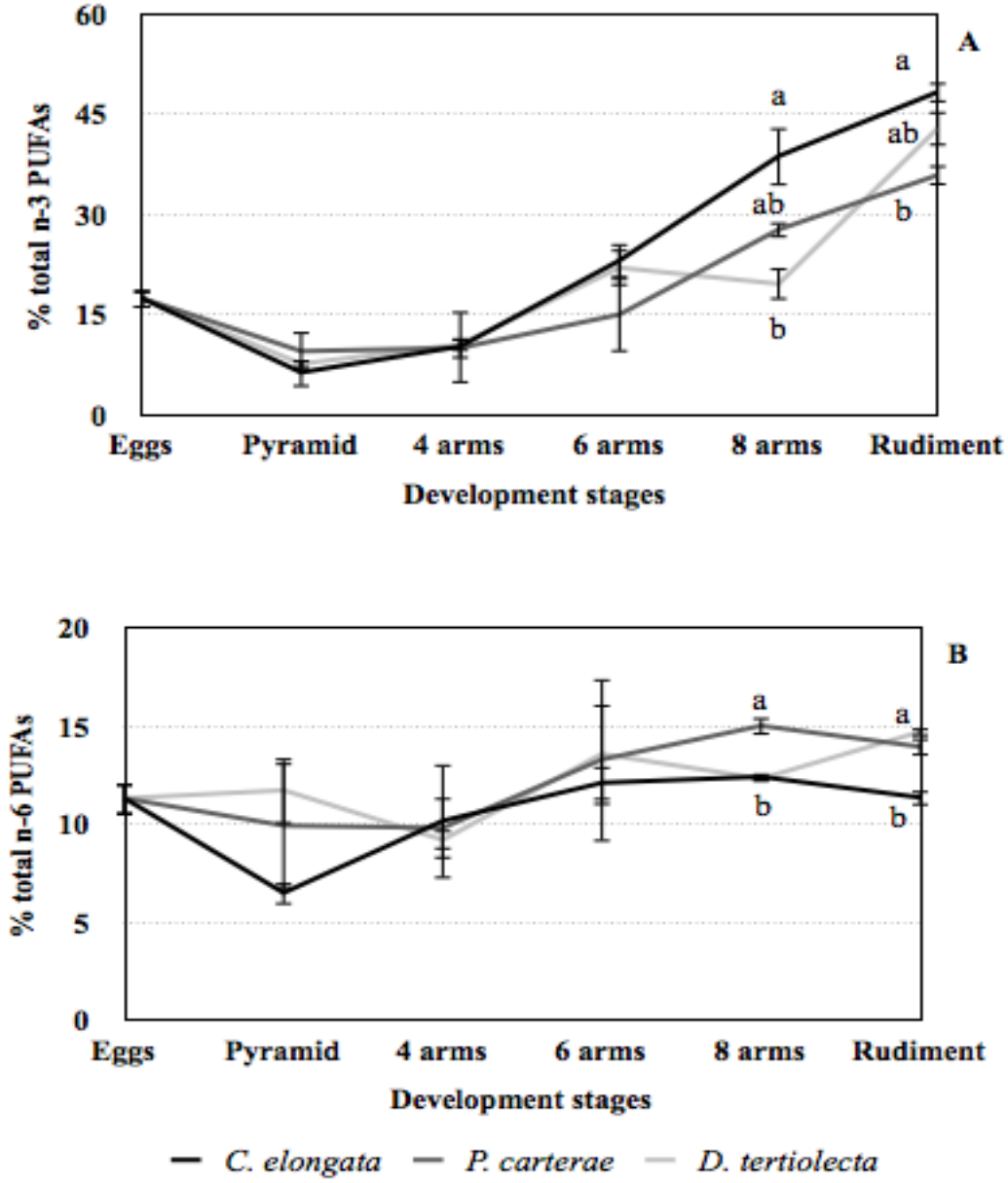

Figure 4.6. Total n-3 (A) and n-6 PUFA (B) contents of P. lividus larvae fed different diets throughout development. Values are given as percentage of total fatty acids (mean ± SD, n=3). Superscripts indicate significant differences between treatments at each time point.

At the rudiment stage D. tertiolecta and P. carterae fed larvae showed higher n-6 PUFA content than *C. elongata*. The proportion of EPA in the larvae decreased significantly during embryonic development, but increased after onset of feeding to reach, at 6 arms stage, comparable levels to eggs (Table 4.2).

Table 4.2 Essential fatty acid relative ratios of P. lividus larvae fed different diets throughout development.

Fatty acid	EPA			ARA			DHA			EPA/ARA			DHA/EPA		
Treatment	***C. elongata***	***P. carterae***	***D. tertiolecta***	***C. elongata***	***P. carterae***	***D. tertiolecta***	***C. elongata***	***P. carterae***	***D. tertiolecta***	***C. elongata***	***P. carterae***	***D. tertiolecta***	***C. elongata***	***P. carterae***	***D. tertiolecta***
Egg	10.7 ±0.9	10.7 ±0.9	10.7 ±0.9	8.6 ±1.1	8.6 ±1.1	8.6 ±1.1	0.3 ±0.2	0.3 ±0.2	0.3 ±0.2	1.2	1.2	1.2	0.03	0.03	0.03
Pyramid	2.9 ±1.5	2.2 ±1	1.9 ±0.9	1.7 ±0.6	1.2 ±0.5	1.0 ±0.9	1.6 ±0.7	1.5 ±1.2	2.9 ±0.5	1.7	1.8	1.8	0.6	0.7	1.5
4 arms	2.6 ±0.9	3.2 ±0.6	3.5 ±0.4	1.7 ±0.6	1.7 ±0.7	2.0 ±0.8	2.3 ±1.2	2.9 ±0.7	2.9 ±0.9	1.6	1.8	1.7	0.9	0.9	0.8
6 arms	9.0 ±1.1	7.1 ±2.5	7.9 ±1.6	3.4 ±0.2	4.1 ±1.3	4.3 ±0.2	**6.0 ±0.5a**	**4.9 ±1.7ab**	**1.9 ±0.1b**	2.6	1.7	1.8	0.7	0.7	0.2
8 arms	**12.6 ±1.1a**	**9.1 ±0.8ab**	**6.0 ±0.5b**	3.9 ±0.1	3.9 ±0.1	4.3 ±0.2	**8.6 ±0.3a**	**7.1 ±0.2b**	**1.5 ±0.2c**	3.3	2.3	1.4	0.7	0.8	0.3
Rudiment	**13.4±1.1a**	**9.13 ±0.6b**	**12.3±1.6a**	**3.0±0.4a**	**2.9±0.3a**	**5.2±0.2b**	**8.0 ±0.7a**	**7.1 ±0.2a**	**0.7 ±0.1b**	4.5	3.1	2.4	0.6	0.8	0.05

Until the 6 arms stage, no significant differences were observed between treatments, whereas at the 8 arms stage larvae fed *C. elongata* showed higher EPA levels than larvae fed *D. tertiolecta*, and larvae fed *P. carterae* showed no significant difference with either *C. elongata* or *D. tertiolecta* treatments. At competence for settlement, the level of EPA in larvae fed *C. elongata* and *D. tertiolecta* were significantly higher than larvae fed *P. carterae*. The proportion of ARA in larvae also decreased during embryonic development, but only increased slightly after initiation of exogenous feeding (Table 4.2). The only significant difference between treatments was observed at competence, when larvae fed *D. tertiolecta* had higher ARA than larvae fed *C. elongata* and *P. carterae*, reflecting the algae fatty acid profiles. The DHA content was negligible in urchin eggs but its proportion increased during all phases of development in larvae fed *C. elongata* and *P. carterae* whereas it declined after onset of exogenous feeding in larvae fed *D. tertiolecta* (Table 4.2).

Ratios between EPA/ARA and DHA/EPA in larvae also varied between dietary treatments. The EPA/ARA ratio increased during larval development in all treatments although it started to increase earlier in larvae fed *C. elongata* (4 arms stage) than in larvae fed *P. carterae* (6 arms stage) and *D. tertiolecta* (8 arms stage) treatments. Therefore, at competence, this ratio was higher in larvae fed *C. elongata* and *P. carterae* than in larvae fed *D. tertiolecta*. The DHA/EPA ratio was very low in the eggs and increased in larvae fed *C. elongata* and *P. carterae* up to 4 arms stage, but it did not change significantly thereafter. A different trend was observed for this ratio in larvae fed *D. tertiolecta* where it peaked at the pyramid stage to then sharply decrease during larval development (Table 4.3).

Table 4.3. Relative essential fatty acid ratios of P. lividus larvae fed different diets during development. Data are expressed as mean ± SD (n = 3).

Fatty acid	EPA/ARA			DHA/EPA		
Treat.	*C. elongata*	*P. carterae*	*D. tertiolecta*	*C. elongata*	*P. carterae*	*D. tertiolecta*
Egg	1.2	1.2	1.2	0.03	0.03	0.03
Pyramid	1.7	1.8	1.8	0.6	0.7	1.5
4 arms	1.6	1.8	1.7	0.9	0.9	0.8
6 arms	2.6	1.7	1.8	0.7	0.7	0.2
8 arms	3.3	2.3	1.4	0.7	0.8	0.3
Rudiment	4.5	3.1	2.4	0.6	0.8	0.05

4.5 Discussion

4.5.1 Effect of dietary treatments on larval growth, morphology and survival

The age at competence observed in the present trial was greater than previously observed (Gosselin and Jangoux, 1996; Kelly et al., 2000; Liu et al., 2007), but within the timeframe of previous observations at AML when using out of season broodstock. The different dietary microalgae showed significant effects on development that may be attributable to the nutritional properties of the diets. Thus, development was more rapid in larvae fed *C. elongata*, followed by *P. carterae* and *D. tertiolecta* and this was also reflected in the appearance of the third pair of arms and rudiment. The shorter cycle of larvae fed the *C. elongata* treatment was reflected in a significantly higher survival. At a hatchery level, higher survival and a shorter culture period means significant economic advantages such as increased juvenile output and savings in labour, space and consumables. More specifically, the major practical

implications of this result was the increased number of settled post larvae produced by the hatchery (50,000 competent larvae per rearing tank versus 16,000 usually obtained with the standard rearing protocol) and in a 20% shorter time, allowing for a better economic return when *C. elongata* was used as larval feed. Although this result brings considerable advantages it is still significantly lower than what is reported in smaller scale trials, suggesting that better larval survival could be achievable, for instance, by reducing stocking density. However, production output relies on numbers of individuals produced per cycle and not on percentage of survived individuals. Commercial operations, in fact, often trade survival percentage for overall production output and are therefore reluctant in reducing stocking densities.

Significant differences in post-oral arm length and larval length during development were observed between larvae fed the dietary treatments. However, by the end of the trial, there were no significant differences between those treatments that supported development but they all were significantly different from larvae fed *T. suecica*. Morphometric data and their trend over time shown in the present study agree well with previous observations (Liu et al., 2007). Shortening of the post-oral arms was suggested to be a sign of over-feeding (Kelly et al., 2000), or homeostatic response of the larvae to allocate energy towards earlier metamorphosis when food was abundant (Fenaux et al., 1994; Strathmann et al., 1992). Moreover, programmed cell death (PCD) process was identified in the sea urchin *Hemicentrotus pulcherrimus* arms concomitantly with metamorphosis (Sato et al., 2006). It seems possible, although not yet known, that PCD might already be active during late larval developmental stages and be responsible for the observed arms shortening. These suggestions led to the conclusion that the observed general shortening of arms

during development can probably be explained as a natural phenomenon accompanying larval development. For this reason, total larval length and post-oral arm length cannot be considered as good indicators of larvae growth or condition. On the contrary, larval body width in the *C. elongata* treatment was significantly greater than all the other treatments and this correlated well with the overall better performance (higher survival and faster development) of larvae fed this diet. We therefore suggest the latter as amore reliable body feature for the evaluation of growth and general larvae condition.

This study also shows that a majority of the *P. lividus* larvae fed *T. suecica* did not develop the 3rd pair of arms by the end of the experimental period, whereas the larvae fed all the other treatments had already achieved competence by this time. Larvae fed *T. suecica* also showed a significantly reduced body width compared to larvae fed the other diets. Although most larvae had settled by 27 DPF the experiment was continued until 31 DPF to allow extra time for the development of larvae in the *T. suecica* treatment, but no sign of further development in this treatment group was observed. This suggests that either the nutritional value of *T. suecica* was insufficient for normal development or that the larvae were unable to feed effectively on this microalga. Observations of stomach content and feeding activity indicated that larvae had consumed *T. suecica* cells but signs of stomach shrinkage occurred from 10 DPF onwards as previously reported for *P. lividus* larvae fed commercial algae paste that contained 20% of *T. suecica* (Liu et al., 2007). These observations suggest that this microalgae species may disrupt digestive functions possibly resulting in some damage to the stomach. Nonetheless, signs of starvation, such as elongation of the arms (Miner, 2005), were not observed and larvae were able to survive for a long time without developing beyond the four arms stage. This

has previously been observed by Liu et al. (2007), but direct correlation with a specific component of the diet (*T. suecica*) was not reported.

4.5.2 Larval development and fatty acid composition

The four microalgae diets used in the present trial differed in both lipid contents and fatty acid profiles. Although *D. tertiolecta* showed the highest total lipid content, it had only very low levels of the n-3 LC-PUFA, DHA and EPA, whereas these EFA were fairly abundant in the other microalgae. However, *D. tertiolecta* showed the highest percentage of ARA, which was not detected in either *C. elongata* or *P. carterae* and only in small amounts in *T. suecica*. *P. carterae* showed the highest proportions of total n-3 LC-PUFA and DHA with a high DHA/EPA ratio. In contrast, *T. suecica* and *C. elongata* both showed higher EPA levels with low DHA/EPA ratios. Therefore it was surprising that *T. suecica* and *C. elongata* showed such contrasting effects on larval development. This suggested that larvae perform better when fed microalgae that show a low DHA/EPA (high EPA) but with low levels of ARA. The ARA level recorded in *T. suecica*, however, represents exception to this, but digestion issues are preventing larvae to thrive when fed this particular microalgae.

Although it is difficult to compare fatty acids profile of microalgae between studies due to the influence of culture conditions, comparable fatty acid profiles were reported for *D. tertiolecta* (Liu et al., 2007; Mendoza Guzman et al., 2011), *T. suecica* (Gonzales-Araya et al., 2011; Mendoza Guzman et al., 2011), and *P. carterae* (Kato et al., 1996).

It has been shown that the ratio between specific LC-PUFA (DHA/EPA and EPA/ARA) is as important, or more, than their absolute levels (Liu et al., 2007; Reitan et al., 1997; Schiopu et al., 2006). However contrasting results were reported for sea

urchins as Schiopu et al. (2006) reported that larvae of *D. excentricus* develop better when fed a higher ratio of DHA/EPA whilst Liu et al. (2007) suggested that *P. lividus* larvae develop better when fed lower DHA/EPA ratio. In the present study, larvae performed better when their dietary DHA/EPA ratio was lower and EPA/ ARA higher, confirming the observation of Liu et al. (2007) and highlighting a possible species-specific response to dietary EFA proportions. High EPA alone did not support development. Whether low DHA or high ARA could be the limiting factor in *T. suecica* is not clear. *P. carterae* had a high DHA content and DHA/EPA ratio when compared to *C. elongata*, but also had significantly lower lipid content that may also affect its nutritional quality for urchin larvae. Therefore the present results suggest that high dietary lipid content and n-3 LC-PUFA, with low DHA/EPA and high EPA/ARA ratios were the best combination for promoting growth, development and survival of *P. lividus* larvae. Of course, other physical or biochemical characteristics of *C. elongata* other than lipid content and fatty acid composition, such as protein and micronutrient content, may also be important factors in promoting larval growth and survival. Further research in this area is therefore required. The fatty acid profiles of the urchin larvae throughout embryonic development (eggs to pyramid) may indicate that specific fatty acids are consumed during embryonic development. This appears to be a consequence of the maternal urchin diets as the macroalgae used are generally rich in EPA and ARA and deficient in DHA (Jamieson and Reid, 1972), which was reflected in the egg fatty acid composition. As a consequence, EPA and ARA could have been relatively more utilized for energy (oxidation), although EPA may also be converted (desaturated and elongated) to DHA during embryogenesis. Conversely, it is also possible that the reduced proportions of EPA and ARA in the pyramid stage could be a consequence of increased proportions of saturated and

monounsaturated fatty acids due to possible increased lipogenic activity. In contrast, the relative proportions of LC-PUFA generally increased during larval development. This accumulation may be a direct result of exogenous feeding and selective retention of LC-PUFA relative to saturated and monounsaturated fatty acids whose proportions declined during larval development probably reflecting preferential oxidation. Detailed study of the levels of LC-PUFA in the *P. lividus* larvae fed the various microalgae feeds, which differed in LC-PUFA compositions, revealed some interesting suggestions on endogenous fatty acid metabolism of the larvae and notably conversion of dietary fatty acids and possibly biosynthesis from precursors. The data from larvae fed *D. tertiolecta*, showing substantial increased EPA, despite relatively low dietary EPA, suggest biosynthesis of EPA from the high level of dietary 18:3n-3. The activity of 18:3n-3 to EPA pathway in *P. lividus* larvae was supported by the fact that ARA levels increased in larvae fed all microalgae feeds, including *C. elongata* and *P. carterae* that had essentially no ARA, suggesting active biosynthesis of ARA from 18:2n-6 and other n-6 precursors (18:3n-6/20:3n-6).

The elongation and desaturation of 18:2n-6 and 18:3n-3 to ARA and EPA, respectively, was also observed in the larvae of *D. excentricus* (Schiopu et al., 2006), juvenile *Strongylocentrotus droebachiensis* (Castell et al., 2004), and to some extent in the adult sea urchin *P. miliaris* (Bell et al., 2000; Pantazis et al., 2000). Although several digestive enzymes have been found in echinoderm larvae, such as peptidase (Doyle, 1956), esterase (Ryberg, 1973), and alkaline phosphatase (Evola-Maltese, 1957), there have only been indirect reports of desaturase activity in adult sea urchins (*P. miliaris*) to date (Bell et al., 2000). Further study in this area is required to better understand sea urchin nutrition and lipid metabolism. However, the decreasing level of DHA in the larvae fed *D. tertiolecta* reflects the low dietary input

of DHA and suggests little conversion of EPA to DHA. Thus the increasing levels of DHA in the larvae fed both *C. elongata* and *P. carterae* must reflect selective retention of dietary DHA rather than conversion of dietary EPA, which was also accumulated in larvae fed these microalgae. This appears to be supported by the DHA/EPA data as larvae fed *C. elongata* and *P. carterae* maintained relatively high ratios throughout development, while it sharply decreased in larvae fed *D. tertiolecta* supporting the conclusion that DHA levels in *P. lividus* larvae were more dependent upon dietary DHA than biosynthesis from EPA.

Among LC-PUFA, the n-3 series was particularly used during embryonic development and then accumulated in larvae tissues during development. Similar or slightly higher levels of n-3 LC-PUFA were found in larvae at competence compared with the respective diets, which indicated that larvae actively accumulated these fatty acids from the feed and retained them in their tissues. This observation is in contrast to that observed in previous trials (Liu et al., 2007) where the opposite trend was observed. Liu et al. (2007) also suggested that higher level of 20:3n-3 and 20:4n-3 in the larvae was beneficial for larval development, however no correlation between these fatty acids and performance of the larvae was found in the present study. Larvae fed *C. elongata* that showed the best survival and greatest body width had contents of 3.5% and 4.1% for these fatty acids respectively, whereas larvae fed *D. tertiolecta* had the worst performance but the highest 20:3n-3 content and an intermediate 20:4n-3 content, and larvae fed *P. carterae* showed intermediate survival and body width but with the lowest contents of 20:3n-3 and 20:4n-3. Our data on fatty acid profile of the early development stages differ from that reported by Gago et al. (2009). However, the only likely comparable treatment used in that study was the "wild" diet but, unfortunately, details of that diet were not reported.

Although it is likely that adult *P. lividus* were fed wild *Laminaria sp.*, it would be different in its nutritional properties from the diet used in the present study due to seasonal effects as the previous study was carried out in summer whereas the present study was performed over winter. A previous study conducted on *P. lividus* fed wild *Laminaria sp*. in winter, and spawned in winter (out of season) confirmed our data (Kreissig, 2009). Moreover EFA content of late larval stages in the present study agreed well with that reported for larvae fed *D. tertiolecta* (Liu et al., 2007).

4.6 Conclusions

Normal development of *P. lividus* larvae was observed in *Cricosphaera elongata*, *Pleurochrisys carterae* and *Dunaliella tertiolecta* treatments.

Larvae fed *Tetraselmis suecica* did not show any sign of development and stalled at the four arms stage, and also showed signs of stomach shrinkage although no culture crash was observed until 31 DPF. The mechanisms regulating this highly plastic response to adverse conditions remain to be identified. The data suggest that relatively high dietary lipid content and n-3 LC-PUFA, with low DHA/EPA and high EPA/ARA ratios were the best combination for promoting growth, development and survival of *P. lividus* larvae, and so microalgae species or blends that can supply this combination would be preferable feeds.

Although other nutritional qualities of the test diets such as protein and/or amino acid, vitamins and mineral contents and compositions will vary between live feeds and almost certainly play important roles in larval nutrition, it was beyond the scope of the present work to measure and quantify all potential factors affecting echinoplutei performances. Nonetheless, the present data clearly show increased production output and a shorter production cycle when *C. elongata* is used as larval

diet providing production cost reduction and increased revenue thus increasing hatchery profitability.

Chapter 5 - Evaluation of flow through culture technique for sea urchin (*Paracentrotus lividus*) larvae commercial production

Published in:

Aquaculture Research 1-5 (doi: 10.1111/are.12019)

Authors:

S. Carboni[a] , M. S. Kelly[c] , A. D. Hughes[c], J. Vignier[b], T. Atack[b] & H. Migaud[a]

Affiliations:

[a]Institute of Aquaculture, Stirling University, Stirling, FK9 4LA, Scotland, UK

[b]Ardtoe Marine Laboratory, Acharacle, PH36 4LD, Scotland, UK

[c]Scottish Association for Marine Science, Oban, PA37 1QA, Scotland, UK

Key words:

Sea Urchin, *Paracentrotus lividus*; Larval rearing, Flow through

Contribution

The author has designed and conducted the trial, collected all samples, carried out statistical analysis and wrote the manuscript.

5.1 Introduction

Sea urchin gonad is a valuable food product. The world urchin harvest increased from 48,000 tonnes in 1982 to a peak of 120,000 tonnes in 1995 mainly due to the expansion of the Chilean, Canadian and US (Californian and Maine) fisheries (Andrew et al., 2002). However, this was followed by a sharp decline during the late 90's and first decade of the new millennium (Pearce, 2010) mainly due to overfishing. Japan is considered as the main sea urchin consumer with in 1995 over 6,000 metric tonnes of sea urchin gonads imported for a value of 251 million dollars. By 2007 Japanese import increased by 300% (Pearce, 2010). The retail price of unprocessed fresh sea urchin in Europe varies greatly depending on the season, country, species and retailers type, and can vary between 0.30 and 3 € per individual while the price of processed gonads can reach 150 € per kilogram.

Research on sea urchin culture has been developing rapidly, especially in Asia and Europe to develop methods for intensive cultivation and roe enhancement of *Strongylocentrotus droebachiensis*, *Paracentrotus lividus* (Grosjean et al., 1998, Botterg et al., 2006) and *Psammechinus miliaris* (Kelly et al., 2000; Cook et al., 2007). Among these species, *P. lividus* is the most consumed and is considered as a good candidate for the diversification of aquaculture. However, the expansion of the industry is dependant on a reliable supply of juveniles. *P. lividus* is highly prolific with up to 10 million eggs per female however larval survival, which is at best 5 % of the eggs stocked under standard commercial conditions (Pers. Obs), remains a key commercial bottleneck limiting the potential development of echinoculture. While a number of studies have investigated larval diets to improve echinoplutei survival (Pedreotti and Fenaux 1993; Kelly et al., 2000; Jimmy et al., 2003; Carcamo et al.,

2005; Liu et al., 2007, Carboni et al., 2012), there has been less effort on the optimisation of rearing protocols to improve larval survival. Rearing systems used for sea urchin larviculture usually involve a static system with complete water exchange every 2 or 3 days (Pedrotti and Fenaux 1993; Kelly et al., 2000; Liu et al., 2007). However, as already demonstrated in many other marine species such as Abalone, *Haliotis sp.* (Olin and McBride, 2000), Lobster *Hommarus gammarus* (Olin, 2000) and bivalves such as pearl oyster, *Pinctada margaritifera*, and clams (Southgate and Ito, 1998; Braley, 1992), flow through systems can be very beneficial to larval rearing. The present study aimed to compare *P. lividus* survival when reared in a flow through incubation system as opposed to static conditions.

5.2 Materials and methods

A flow through system for rearing of *P. lividus* larvae up to the stage of metamorphosis was tested against static conditions at the Ardtoe Marine Laboratory (AM, Scotland; 56N 46' - 5W 52') in May 2009. The static treatment (control), commonly used in AML for *P. lividus* commercial production, was adapted from Liu et al. (2007). In this system, filtered (4 µm) and UV (approximately 280 mJ cm-1s-1) treated water was exchanged every 3rd day after siphoning out the plutei over a plankton mesh (40 µm). Fresh live microalgae was added and the plutei were returned to the rearing tank. The second treatment consisted in a continuous flow through of treated seawater dripping into the rearing tanks, and daily feeding with live microalgae. Water exchange was set at 55 ml/min corresponding to 100% exchange rate per day. Both systems were aerated using a slow stream of air delivered at the bottom of the conical rearing tank using a 3 mm diameter glass tube with a plastic pipette tip to minimize bubble size. The airflow was set in such a way

that 1-2 bubbles reached the surface every second. The larvae were retained in the rearing tank by a 40 µm filter (Banjo filter) on the outflow (standpipe) situated at the centre of the rearing tank. Microalgae cultures were grown in 100 L polyethylene bags in sterilized seawater enriched with the f/2 medium (Guillard, 1975). *Dunaliella tertiolecta* culture (CCAP code: 19/27) was fed to the larvae in both treatments. *D. tertiolecta* is the most common live feed utilised for larviculture of several echinoid species (Kelly et al., 2000; George et al., 2004; Liu et al., 2007). The amount of algal cells given to the plutei was changed according to larval developmental stages (Table 5.1).

Table 5.1. Microalgae Dunaliella tertiolecta ration given to the two treatments per each feed.

Larval development stage	***4 arms***	***6 arms***	***8 arms***	***Rudiment***
Flow through	1000 cells/ml	3000 cells/ml	5000 cells/ml	6000 cells/ml
Static	3000 cells/ml	9000 cells/ml	15000 cells/ml	18000 cells/ml

Adult *P. lividus*, four males (47.3 ± 1.4 g) and one female (51.1 g), were induced to spawn by injecting 1 M KCl (40 µl per g of body weight) into their coelom. Sperm from the four males were pooled and few drops (2 ml) of the mix were used to fertilise the eggs. Fertilised eggs were stocked in a 10 L container of static seawater and left in the dark for 24 hrs to hatch. Fertilisation rate, assessed 2 hrs after artificial fertilisation by observation of cell cleavage under microscope, was 96.4 ± 2.0 %, and hatching rate was 82 ± 1 %.

After hatching, active and floating embryos were skimmed from the surface, volumetrically counted and stocked into 6 x 80 L polyethylene cylindrical-conical tanks (3 / treatment) at a density of 4 larvae/ml. Seawater used during the process

of spawning, hatching, and larval rearing was filtered to 4 μm and UV treated as described above. Water temperature was maintained at 18 ± 2 °C using a room thermostat and photoperiod set at 24 hours continuous light. In the static treatment, larval survival was assessed volumetrically every 3rd day coinciding with feeding and water exchange and at the end of the trial. In the flow through treatment, survival was volumetrically assessed only when a new developmental stage was reached and at the end of the trial. Water samples were collected for three consecutive days starting from day 4, 10, 16 and 22 according to the feeding schedule of the static system. Each time, three samples were collected by filtering 150 ml of the rearing water through glass fibre filters (Whatman). Nitrite, nitrate, phosphate and ammonia analyses were performed according to standard methods using spectrophotometer (AOAC, 2000). At day 4, 16 and 22 three replicate samples of the microalgae culture were taken and analysed in the same way. All statistical analyses were carried out using Minitab 15.0 (Minitab Ltd., UK). Water quality data were tested by two way repeated measures ANOVA. Analysis of proportion was used to evaluate significant differences in survival between the two treatments.

5.3 Results and discussion

Results showed that larval survival up to competence for settlement was significantly higher, from the 8 arms stage onwards, in the flow through treatment compared to the static treatment (Fig. 5.1). At competence the average survival was 6.7 ± 1.9 % and 21.2 ± 3.3 % in the static and flow through systems respectively. No differences were observed in growth or development rate between the two treatments and the plutei reached the competent stage at 27 days post-fertilization.

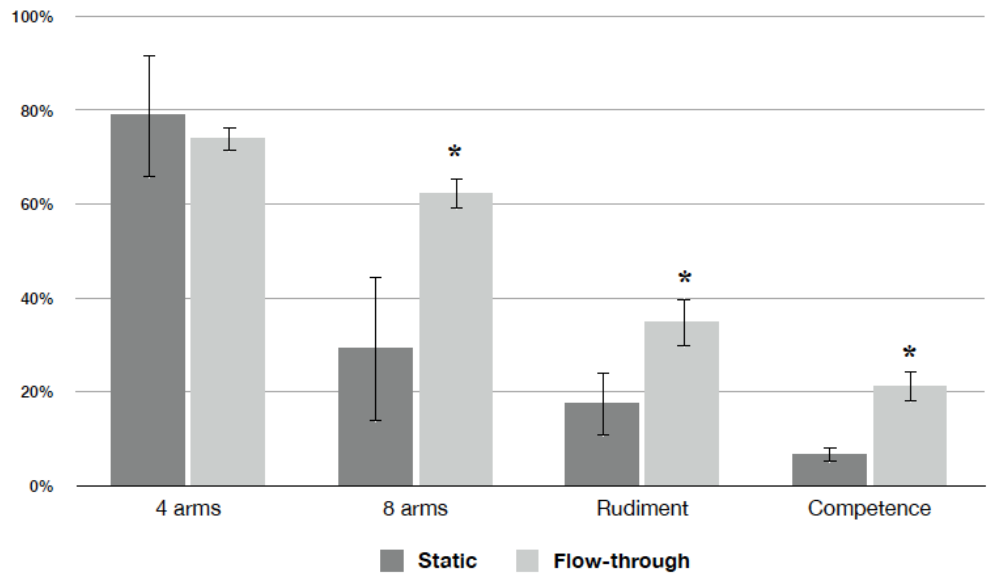

Figure 5.1. Survival rates of *P. lividus* larvae reared in either static (control) of flow through systems over the trial period up to competence for settlement. Data are expressed as mean ± SD (n=3). Superscripts indicate significant differences.

Survival recorded in this trial under static conditions was consistent with previous commercial and experimental results obtained at AML (Carboni et al., 2012) while being much lower than previously published trials (Liu et al., 2007) for trials carried out at laboratory scale where stocking densities are usually lower and rearing conditions can be better controlled. In a similar trial, Leighton (1995) observed that all the *P. lividus* larvae fed *D. tertiolecta* died by day 15 post fertilization. This difference could be explained by the different spawning protocol used (KCl injection versus dissection) or by differences in feeding regime or aeration system unfortunately not described in Leighton`s work.

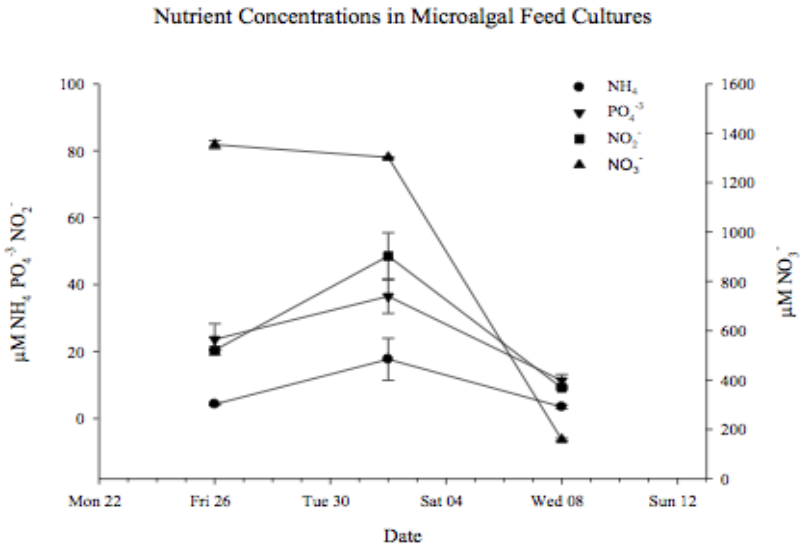

Figure 5.2. NH4, PO_4^{-3}, NO_2 and NO_3 concentrations in the microalgae culture used to feed *P. lividus* larvae over the trial period. Data are expressed as mean ± SD (n=3).

NO_2, NO_3 and PO_4^{-3} concentrations in the rearing water correlated well with the microalgae culture nutrients concentration (Fig. 5.2), suggesting that feed inputs and not larvae catabolites had a major role in the concentration of these ions in the rearing water for both treatments (Fig. 5.3). No significant differences between treatments were observed in the analysed water quality parameters (Fig. 5.3) due to large variability at any given time, except for PO_4^{-3} at the end of the trial, although this clearly did not affect larval survival.

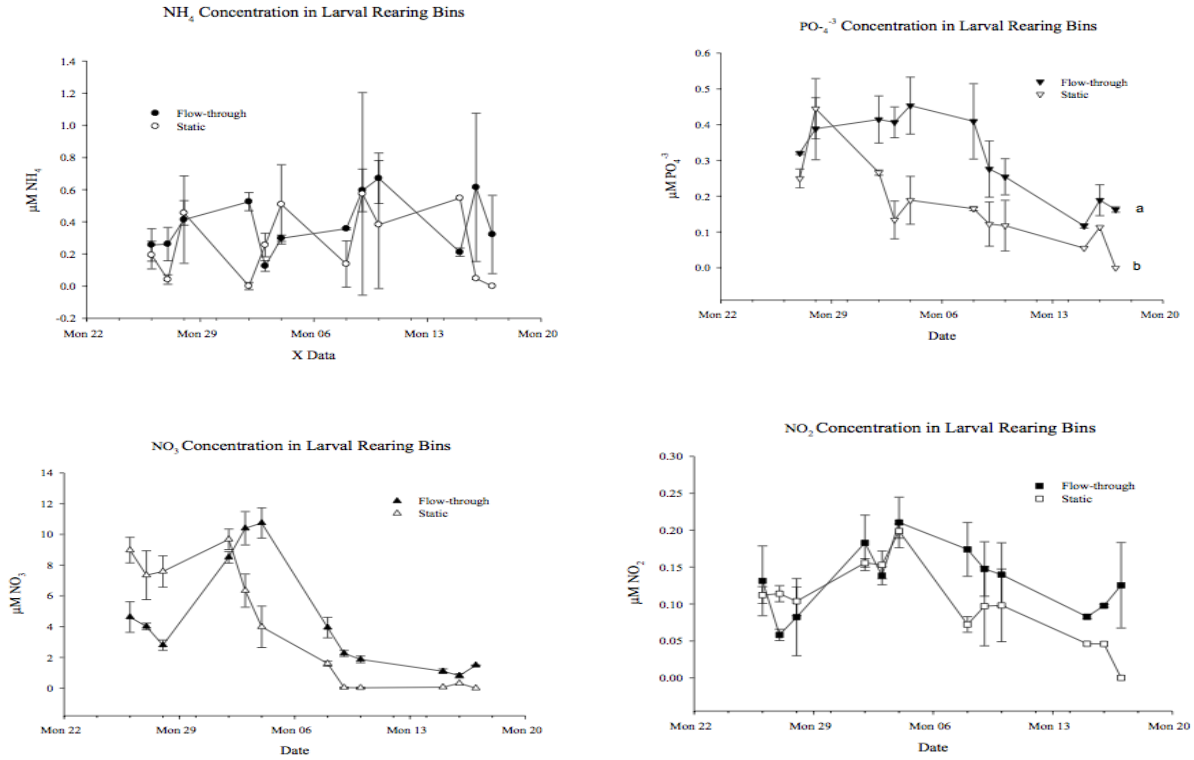

Figure 5.3. NH4, PO4-3, NO2 and NO3 concentrations in the rearing water taken during three following days after each feed over the trial period. Data are expressed as mean ± SD (n=3).

However, PO_4^{-3} concentrations measured in the flow through treatment consistently appeared to be higher than in the static system. This could be due to the fewer cleaning operations undertaken in the rearing tank under flow-through conditions and by the accumulation of algae debris on the tank walls.

As no significant difference in water quality parameters were observed in this trial, the most likely explanation for the observed reduced mortality in the flow through system is the reduction of stress, and possibly physical damage to the larvae due to handling during manual water changes. Inspection of the culture bins revealed that the microalgae density had visibly reduced by day three in the static system, it is

therefore likely that regular feeding (daily vs. every 3rd day) providing a constant food supply in the tanks would have improved survival.

5.4 Conclusion

The present study showed that larger numbers of *P. lividus* larvae could be reared through to settlement using a flow through culture system instead of standard static conditions. This method offers a number of advantages when compared to conventional static water culture systems including reduced physical handling of the larvae leading to increased plutei survival up to competence and reduced labour. The flow through system described here is therefore a viable method for the hatchery production of *P. lividus* larvae.

Chapter 6 - Influence of broodstock diet on somatic growth, fecundity, gonadal carotenoids and larval survival of sea urchin *Paracentrotus lividus*

Accepted in:

Aquaculture Research

Authors:

Stefano Carboni[a,b]*, Adam D. Hughes[b], Tim Atack[c], Douglas R. Tocher[a], Herve Migaud[a]

Affiliations:

[a]Institute of Aquaculture, Stirling University, Stirling FK9 4LA, Scotland UK

[b]Scottish Association for Marine Science, Oban, Argyll, PA37 1QA, Scotland UK

[c]Ardtoe Marine Laboratory, Acharacle PH36 4LD, Scotland, UK

Key words:

Sea Urchin, *Paracentrotus lividus*; larval survival, formulated feeds, carotenoids, fecundity, growth

Contribution

The author has designed and conducted the trial, collected all samples, carried out statistical analysis and wrote the manuscript.

6.1 Abstract

The effects of broodstock diets on growth, gonadal index, fecundity and carotenoid composition in the sea urchin *Paracentrotus lividus* were investigated. Performance of offspring derived from each dietary treatment was compared to determine whether maternal provisioning of nutrients affected offspring development and survival under commercial culture conditions. Urchins were fed three new formulated diets in comparison to fresh brown Kelp (*Laminaria digitata*) for a period of three months during which they were sampled for somatic growth and gonadal index. At the end of the experimental period urchins were induced to spawn, relative fecundity measured, and offspring derived from each treatment were reared independently and survival up to competence assessed. Carotenoid composition of the gonads was also measured over the trial period. The data showed that the highest dietary protein content significantly improved somatic growth whilst higher lipid content increased Gonadal Index (GI) consistent with the use of gonads as nutrient storage in sea urchins. Nonetheless, relative total lipid content of the gonads was not significantly different between treatments indicating that when lipids were available in higher quantity in the diet they were stored via increased gonadal size rather than increased lipid content of the gonadal tissue. Interestingly, GI and fecundity were not correlated, whilst the latter may have been enhanced by dietary xanthophyll. The results clearly indicated the importance of including carotenoids in broodstock diets in order to enhance hatchery outputs. The present study also indicated that, although broodstock diet can influence fecundity, it had no significant

impact on larval survival, which was instead likely to be significantly influenced by rearing conditions such as feed quantity and quality, temperature and salinity.

6.2 Introduction

The common European sea urchin, *Paracentrotus lividus* (Lamarck) inhabits European coastlines of the North Atlantic (Scotland, Brittany and Spain) and coastal areas of the Mediterranean Sea (Boudouresque & Verlaque, 2007). It is the most exploited sea urchin in the Mediterranean and Atlantic Europe, where both males and females of the species are harvested for their gonads, generally referred to as "roe" in the fishery and catering market (Lawrence, 2007). The world's sea urchin fisheries have had a poor record of sustainability, with a lack of appropriate stock assessments and ineffective management (Andrew et al., 2002). Nonetheless, new techniques such as geostatistic are now showing great potential to evaluate more precisely the status of wild stocks (Addis et al., 2009). In this context aquaculture is recognized as a potential solution to fill the gap between the growing demand and a falling supply. Most studies on sea urchin nutrition so far have focused on improvement of gonadal index (GI), gonad palatability and flavour, and/or colour enhancement for human consumption (Shpigel et al., 2007; Suckling et al., 2011). In contrast, there are few available data on the effects of broodstock diet on gamete quality (Gago etal., 2009), and no information on the effects of maternal nutrition on *P. lividus* larval and juvenile performance.

In sea urchin, the energy for embryo and larval development up to the onset of feeding is provided by the reserves deposited in the eggs (Strathmann et al., 1992; Prowse et al., 2008). Maternal provisioning of nutrients is therefore important for the development of the embryo and plays a significant role on offspring performance. As already shown in previous studies, gonad and egg biochemistry can be altered by

broodstock diet (Gago et al., 2009). However, it is not yet clear if this can be exploited to improve the commercial production of sea urchin juveniles. Moreover, culture conditions such as feeding regime, temperature and salinity can also affect larval and juvenile growth, development and survival (Fenaux et al., 1994; Kelly et al., 2000; Liu et al., 2007).

The importance of carotenoid content in sea urchin gonads has been recognized as a major factor influencing roe coloration (Shpigel et al., 2006; Symonds et al., 2007). Carotenoids, however, also play important roles in promoting immune responses in sea urchins (Kawakami et al., 1998) and in reproduction in other echinoid species such as in *L. variegatus* where carotenoids (especially lutein and zeaxanthin) can impact fecundity (George et al., 2001), and in *S. droebachiensis* where β-carotene increases both gonad growth and the rate of larval development (de Jong-Westman et al., 1995). However, no information is yet available on the effects of dietary carotenoids on the fecundity and offspring performance of the common European sea urchin *P. lividus*.

The aim of the present study was thus to determine the effects of broodstock diets with varying proximate and carotenoid compositions on *P. lividus* somatic growth, fecundity, GI, and larval survival up to competence. To do so, three new diet formulations with varying levels of lipids and pigments have been tested.

6.3 Materials and Methods

6.3.1 Culture conditions and experimental design

After a starvation period of three weeks, urchins (27.4 mm ± 0.3 test diameter) reared at the Ardtoe Marine Laboratory (AML, Argyll, Scotland; 56N 46’ / 5W 52’)

were randomly divided into eight groups corresponding to four treatments in duplicate (50 individuals/group). Three experimental diets (Diets A, B and C), formulated to produce a range of protein, lipid, carbohydrates, energy content and carotenoids (Table 1), and fresh brown algae (kelp, *Laminaria digitata*, Diet K) were fed to adult *P. lividus* over a period of three months (May - July 2011). Urchins were kept under ambient temperature and photoperiod within plastic baskets suspended in 100 L tanks with the water exchange set at 1 L min-1. Daily temperature and photoperiod rose gradually from 9°C/15 h in May to 16 °C/17 h in June. The urchins were fed daily and, every third day, uneaten pellets were siphoned from the bottom of the tank. As the urchins were kept in suspended baskets, crumbs of uneaten pellets were depositing on the bottom of the tank and were thus not available to the urchins, thereby ensuring that only fresh pellets were ingested during the trial period. Uneaten kelp was also removed as required and replaced with fresh fronds. At the end of the trial period three females and three males from each replicate were induced to spawn by injection of 1 M KCl (40 µL per g of body weight) into the coelom via the peristomial membrane. The released eggs were fertilized by addition of 10 ml of diluted sperm in 5 L of filtered seawater. Fertilization rate was assessed under the microscope 2 h post fertilization and fertilized eggs were incubated in the dark in static seawater without aeration for 24 h. Hatching rate was estimated for each batch as the proportion of swimming larvae (counted volumetrically) over the number of incubated eggs.

The seawater used during the process of artificial fertilization, egg incubation and larval rearing was filtered (4 µm) and UV treated, whilst room temperature was maintained at 18 ± 2 °C throughout the larval cultivation period. Triplicate groups of offspring from each treatment (approximately 320,000 larvae per replicate) were

stocked at a density of 4 mL-1 in 80 L conical plastic tanks and reared under continuous light in aerated static water. A complete water exchange and thorough cleaning of each of the tanks was carried out every 3rd day. All groups of larvae were fed with *Dunaliella tertiolecta* originally sourced from the Culture Collection for Algae and Protozoa (CCAP: Oban, Scotland, UK) according to the feeding ration adopted by Kelly et al. (2000). Larval survival was assessed volumetrically every third day and at the end of the trial. Competence was considered as achieved when at least 75 % of the observed larvae showed that the rudiment was at least equal in size to the stomach.

6.3.2 Proximate composition

Diets were ground prior to determination of proximate compositions, moisture and ash contents according to standard procedures (AOAC, 2000). The crude protein level of each diet was calculated from the nitrogen content (N × 6.25) using automated Kjeldahl analysis (Tecator Kjeltec Auto 1030 analyser, Foss, Warrington, UK) according to Lynch & Barbano (1999). Crude lipid contents were determined using the Soxhlet method according to the standard procedure (AOAC, 2000) with extraction in petroleum ether at 120 ^{o}C (Avanti Soxtec 2050 Auto Extraction apparatus, Foss, Warrington, UK). Fibre level was analysed using the Foss fibrecap system in which 1.0 g of sample was de-fatted with petroleum ether, boiled for 30 min in 350 mL 1.25 % sulphuric acid, and then washed in boiling distilled water. Samples were then boiled for a further 30 min in 350 mL 1.25 % NaOH and washed again in water. Samples were incinerated at 600 °C for 4 h and finally re-weighed. Dietary fibre content was calculated as a percentage of the initial weight of the sample. Energy content of the diets was measured by bomb calorimetry using a Parr

6200 calorimeter according to standard procedures. The carbohydrate content was calculated as the difference between dry weight and the sum of protein, lipid, ash and fibre.

6.3.3 Somatic growth, gonadal index and fecundity

At the beginning of the feeding trial (Day 0) and every 30 days thereafter, all individuals from each replicate were measured at two perpendicular points across the ambitus (test diameter, TD) using calipers. At each time point, five individuals from each replicate were also weighed to the nearest mg (wet weight), and the gonads dissected out by cutting outside the peristomial membrane, separating the gonads from the other organs, blotting them with a dry paper towel and then weighing them to the nearest mg. Gonadal index was measured as a percentage of the total wet body weight. After spawning, the number of eggs from each female was assessed volumetrically and relative fecundity was recorded as number of eggs per g of female total wet body weight.

6.3.4 Total lipid and carotenoids

After dissection, gonadal samples comprising of two branches per individual from 5 individuals per replicate, were placed in 5 ml chloroform/methanol (2:1 by vol.) containing 0.01 % butylated hydroxytoluene (BHT) as an antioxidant and stored at -20 °C prior to analyses. Diet and gonad total lipids were extracted and quantified according to Folch et al., (1957).

In order to assess carotenoid content and composition, a precise amount of total lipid was resuspended in a known volume of iso-hexane and the absorbance measured spectrophotometrically at 450 nm before transfer to 1 ml amber glass auto-sampler

vials. High performance liquid chromatography (HPLC) was carried out using a stainless steel column (Luna 5 µm silica 2, 125 x 4 mm). Mobile phase was delivered using a mono piston pump (Waters 501, Waters) and consisted of iso-hexane/acetone (82:18, v/v) at a flow rate of 1.2 mL min-1 at room temperature with a maximum pressure of 1000 psi. Samples (50 µL) were injected via auto-sampler (Waters 717plus) and individual carotenoids were detected using a multi-wavelength UV-VIS detector (Waters 486, Waters) at 450 nm. Identification was made using retention time (Rt) in comparison to authentic standards of β-carotene. Quantification of carotenoids was carried out by integration of peak areas using Clarity Data System software.

6.3.5 Statistical analysis

All analyses were carried out using the statistical package Minitab 15.0 (Minitab Ltd., UK). Normality and homogeneity of variance were confirmed using Kolmogorov-Smirnov test. Growth, GI and gonad carotenoid content were analyzed using a General Linear Model with all interactions being analysed by Tukey post hoc test to identify significant differences. Fecundity and total lipid content was analyzed using one-way ANOVA followed by the Tukey multiple comparison test to assess where significant differences occurred. Larval survival rates (described as a percentage) were analyzed using analysis of proportions where 95 % confidence limits were calculated for the respective proportions (Fowler & Cohen, 1987). When the upper and lower confidence limits of the respective proportions were found not to overlap the proportions were considered to be statistically different at the 5 % level ($p = 0.05$). In all cases, significant differences were determined at $p < 0.05$.

6.4 Results

6.4.1 Diet composition

The proximate compositions of the new formulated feeds were similar for most of the measured parameters except for higher proportions of protein and carbohydrate, and lower ash, in diet C compared to the other diets. As expected, moisture content was highest in the fresh kelp (diet K) and its lipid, protein and energy contents were the lower than new formulated diets (Table 6.1). The only carotenoid detected in the artificial diets was β-carotene and its content was significantly lower in diet A compared to diets B and C. Diet K showed significantly higher β-carotene content compared to the other diets and xanthophylls (lutein and zeaxanthin) were identified only in this diet (Table 6.1).

Table 6.1. Proximate compositions of the four diets tested. Ash, carbohydrate, fibre, protein and lipids are expressed as a percentage of the diet dry weight. Energy content is given per gram of diet dry weight. Pigments are expressed as ug/g of diet (mean ± SD, n = 3). Superscripts indicate significant differences whilst asterisks indicate values below detection limit.

Nutrients	K	A	B	C
Moisture %	^{a}75.4 (± 0.08)	^{b}9.5 (± 0.21)	^{b}6.5 (± 0.49)	^{b}10.2 (± 3.72)
Ash %	^{a}5.7 (± 0.01)	^{b}10.3 (± 0.18)	^{b}13.3 (± 0.06)	^{c}3.6 (± 0.04)
Carbohydrate %	^{a}79.3 (± 0.2)	^{b}65.1 (± 0.2)	^{b}61.0 (± 0.3)	^{c}67.2 (± 1.02)
Fibre %	2.9 (± 0.6)	1.9 (± 0.4)	2.4 (± 0.2)	2.0 (± 0.5)
Protein %	^{a}11.9 (± 1.2)	^{b}16.9 (± 0.04)	^{b}17.9 (± 0.04)	^{c}22.4 (± 0.07)
Lipid %	^{a}0.2 (± 0.02)	^{b}6.8 (± 0.05)	^{b}5.4 (± 0.04)	^{b}4.8 (± 0.04)
Energy (KJ/g)	^{a}3.1 (± 0.01)	^{b}14.9 (± 0.19)	^{b}14.4 (± 0.28)	^{b}16.1 (± 0.19)
β-carotene	^{a}73.6 (±2.0)	^{b}7.0 (±0.7)	^{c}18.3 (±2.0)	^{c}26.8 (±3.0)
Xanthophylls	312	*	*	*

6.4.2 Growth, gonadal index and fecundity

No significant differences were found in TD during the first 60 days of the trial, however, urchins fed diet A and B were significantly smaller ($p < 0.05$) than urchins fed diets C at day 90 (Fig. 6.1).

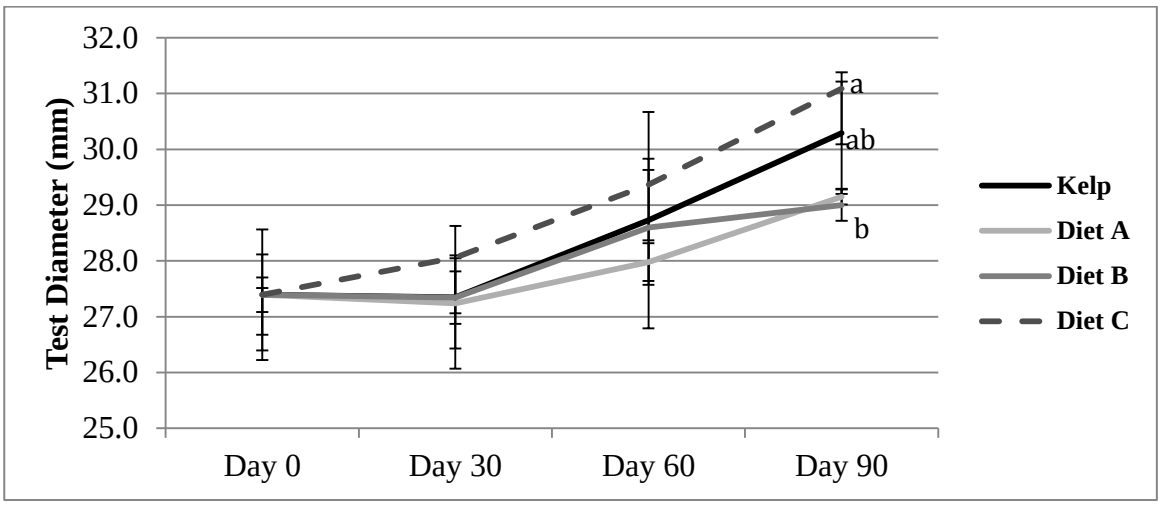

Figure 6.1. Growth expressed as Test Diameter (TD) over the trial period in the four diet treatments. Superscripts indicate significant differences (Mean ± SD, n = 2).

All dietary treatments promoted gonadal growth, but there were significant differences between treatments with urchins fed diet A showing higher GI than urchins fed diet K ($p < 0.05$) at the end of the trial (Fig. 6.2).

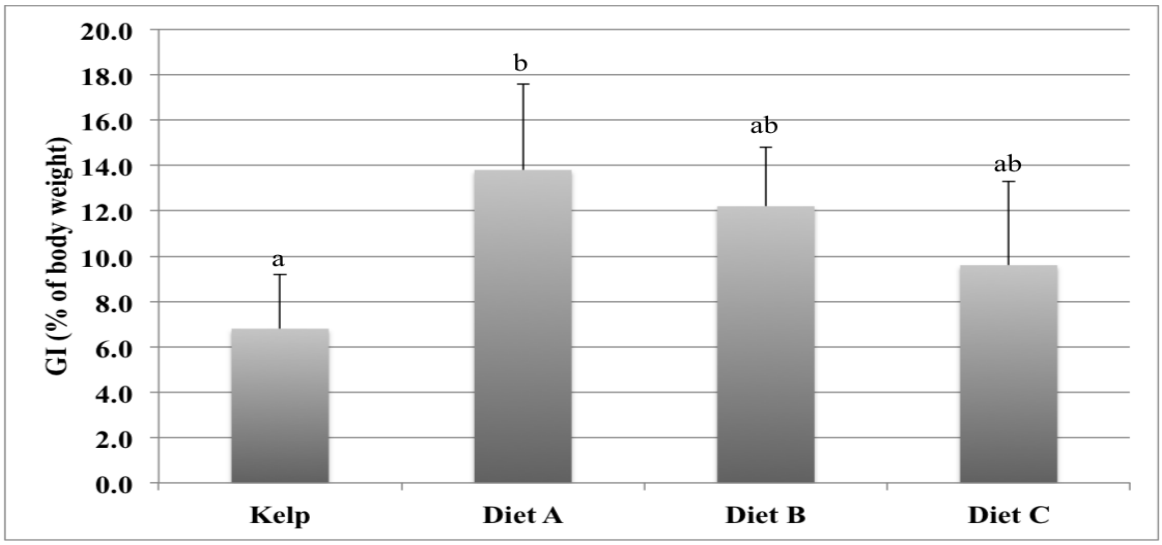

Figure 6.2. Gonadal Index (GI) measured at the end of the trial and expressed as the percentage of body wet weight in the four diet treatments. Superscripts indicate significant differences (Mean ± SD, n = 2).

Although urchins fed the kelp diet had the lowest GI, their relative fecundity was significantly higher than urchins fed the other diets ($p < 0.05$) , whilst urchins fed diet A, which had the highest GI, showed the lowest relative fecundity (Fig. 6.3)

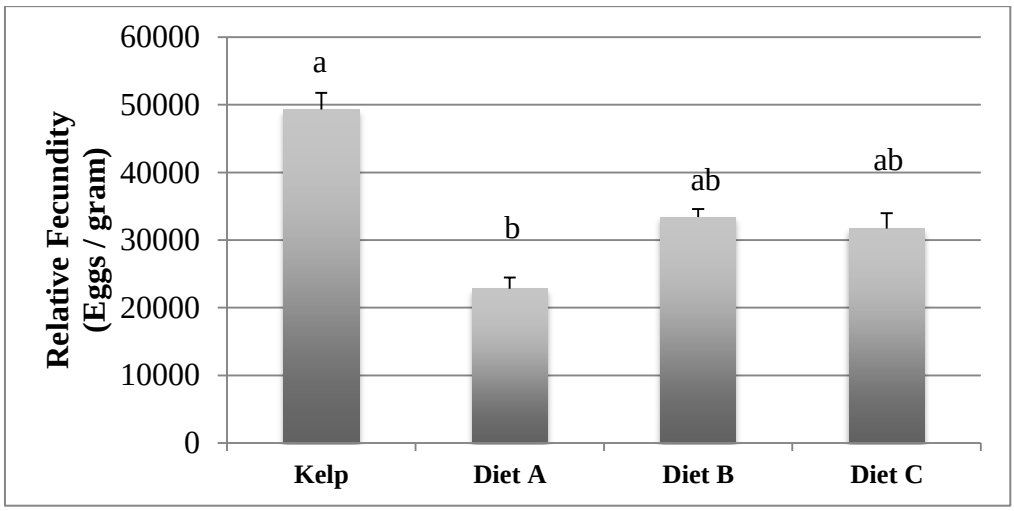

Figure 6.3. Relative fecundity expressed as number of eggs per gram of female body wet weight in the four diet treatments. Superscripts indicate significant differences (Mean ± SD, n = 2).

6.4.3 Gonad total lipid and carotenoid contents and composition

Total lipid content of the gonads, expressed as mg lipid per g of gonadal tissue, did not change significantly during the trial and no differences were observed between treatments. Total carotenoid content of gonad decreased significantly during gametogenesis ($p < 0.05$) in urchins fed diets A and C, but not in urchins fed kelp. At the end of the trial a significant difference in gonad carotenoid content was found between urchins fed the kelp diet and those fed diet A (Fig. 6.4).

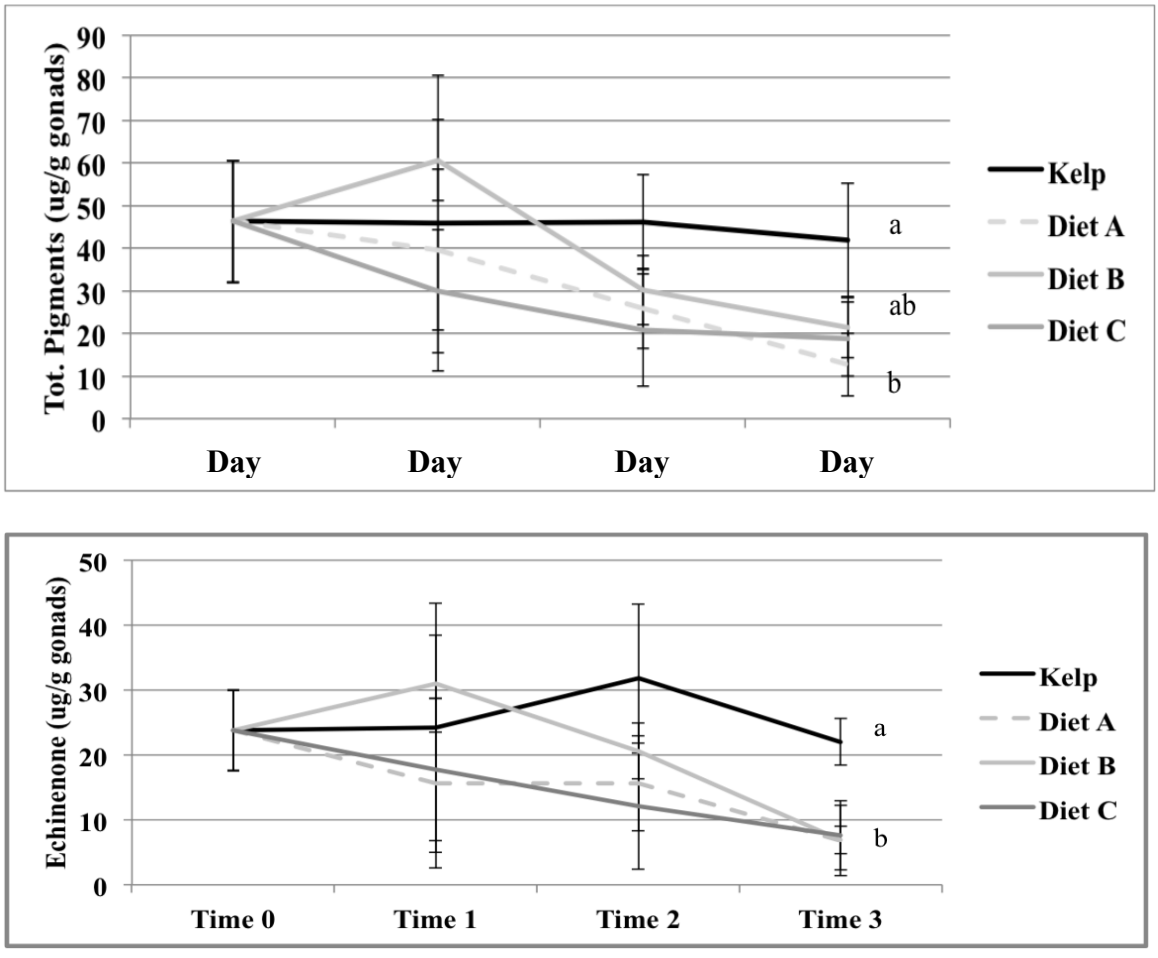

Figure 6.4. Total Pigments and Echinenone content in gonads of urchins fed the four diets over the trial period. Superscripts indicate significant differences (Mean ± SD, n = 2).

Echinenone was the most abundant carotenoid in the gonads, and during gametogenesis its content reflected the total carotenoid pattern. By the end of the

trial, echinenone was significantly higher in gonads of urchins fed kelp than in those fed the other diets (Fig. 6.4.3.1). No differences were observed between treatments for β-carotene content, which did not change during the trial period.

6.4.4 Fertilization rate, hatching rate and Larval survival

The average fertilization rate was 98 ± 1 % with no significant differences between treatments. The average hatching rate was 85 ± 2 % and no significant differences between treatments were observed. No significant differences in larval survival were observed although survival in diet K treatment (5.1 %) appeared lower, although not significantly so, than in the other treatments (8.0 to 11.5%) (Fig. 6.5).

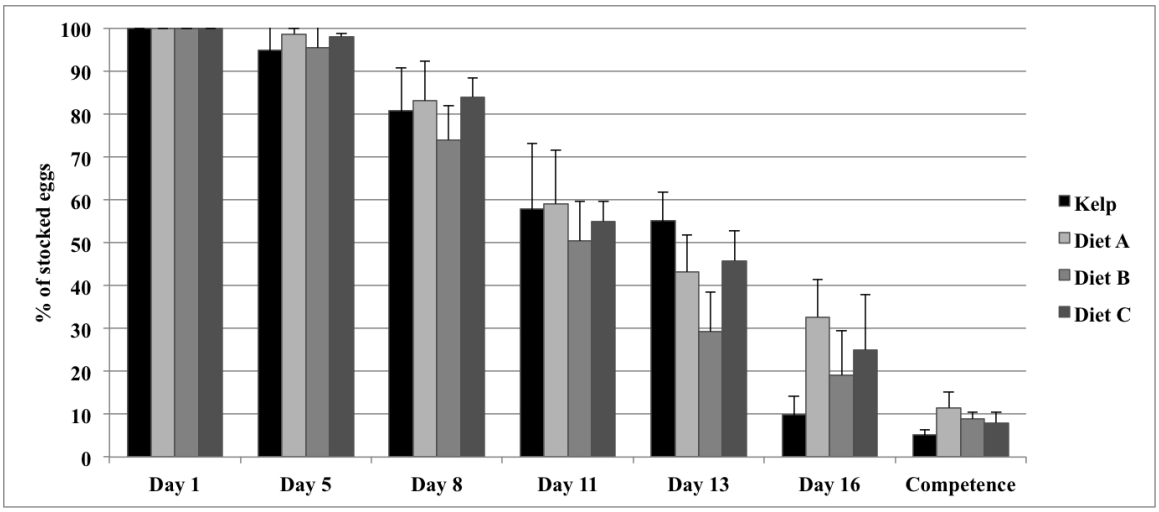

Figure 6.5. Survival rate of urchins' larvae (up to competence,) from broodstock fed the four dietary treatments (Mean ± SD, n = 3).

6.5 Discussion

Formulated feeds must be developed to substitute wild collected macroalgae for *P. lividus* aquaculture to become commercially and environmentally sustainable. The primary objective of the present study was to evaluate the effects of high protein, energy-rich diets on *P. lividus* reproduction, somatic growth, gonadal index and, ultimately, on off-spring performance.

The data showed that the most protein-rich and energetic diet (diet C) positively affected somatic growth of *P. lividus* broodstock. This finding is in contrast to that observed by other authors who suggested that dietary protein content negatively influenced somatic growth in *S. droebachiensis* promoting instead gonadal growth (Pearce et al., 2004; Daggett et al., 2005; Kennedy et al., 2005). The present data, however, corroborates previous observations in *P. lividus* growth trials (Fernandez, 1997; Schlosser et al., 2005) and further highlights the species-specific growth response to protein content described by Cook & Kelly (2007). Intermediate protein contents (14.5 % diet DW) in diets A and B resulted in the smallest individuals. This might be due to possible differences in protein and energy digestibility between these two diets and diets K and C (Schlosser et al., 2005), or on the higher ash content in diets A and B (2-3 times higher than diet K, and 3-4 times higher than diet C) that might have interfered with digestion and absorption processes. Or indeed by the possible presence of anti-nutritional factors that might be present in these two diets. However, precise ingredients of the test diets cannot be disclosed for confidentiality reasons. Interestingly, the gonads of urchins fed all the diets showed similar total lipid content relative to gonad unit weight suggesting that, although

more available in some of the diets than in others, lipids were not accumulated in the cells but, instead, increase gonadal growth, which agreed with previous observations on the dual role (reproduction and reserve storage) of sea urchin gonads (Hughes et al., 2006). Higher dietary lipids resulted in higher GI in urchins fed diet A. Nonetheless, it is possible that other parameters also influenced GI. Overall, GIs reported in the present study were comparable to those reported in a previous study (Fabbrocini & D'Adamo, 2010). However, GI and fecundity were apparently not related, which can be explained by the fact that somatic cells with an extensive nutrients storage role (the nutritive phagocytes) also contribute to the overall gonad size. Fecundity might have been instead enhanced by the presence of lutein and zeaxanthin in the Kelp diet as previously suggested in other echinoid species (de Jong-Westman et al., 1995).

As hatchery output is ultimately dependent on the availability of good quality fertilized eggs, increased relative fecundity can be considered as a major step forward for the profitable production of sea urchins. The present results highlighted the importance of carotenoids in broodstock feed formulations. Echinenone was the most abundant carotenoid in the gonads and its content has been shown to be positively correlated with acceptable roe coloration (Suckling et al., 2011). Echinenone is synthesized in the gonads from β-carotene via β-isocryptoxanthin and, therefore, it is not surprising that urchins fed Kelp (with a high β-carotene content) produced gonads with the highest echinenone content. Echinenone concentrations observed in the present study were comparable with those recorded by Shpigel et al. (2006) under culture conditions, but lower than the contents observed in sea urchins captured in the wild (Symonds et al., 2007). This suggests that the animals might

require more diverse food sources than that provided in a hatchery setting to reach the highest echinenone concentrations.

Although the present study confirmed that gonad biochemistry can be manipulated by broodstock diet, this had no statistically significant impact on larvae survival, despite mean survival in the kelp diet appeared lower. This would suggest that biotic and abiotic factors during larval rearing such as feeding, temperature, salinity and water exchange might have a more important role in influencing larval survival. Survival rate achieved in the present experiment was similar to that commonly observed at AML during commercial production when *D. tertiolecta* is used as larval feed, and it was comparable with previous observations made under commercial conditions (Carboni et al., 2012). However, it was lower than the survival rate commonly reported in laboratory scale trials (Liu et al., 2007) where initial larval density was lower.

In conclusion, all the diets tested supported somatic growth and gonadal growth as expected. Whilst more expensive, protein-, lipid- and energy-rich diets appeared to have positive effects for the adult stages, they failed to promote increased fecundity or enhance offspring performance compared with the less protein and energy dense brown kelp. The present study highlighted the need for a specifically formulated broodstock diet and gave insights to what its composition should be, especially in relation to carotenoids. However, an ideal sea urchin broodstock diet, able to improve hatchery outputs beyond that obtained with brown kelp, remains to be developed.

Chapter 7 - Fatty acid profiles during gametogenesis in sea urchin (*Paracentrotus lividus*): Effects of dietary inputs on gonad, egg and embryo profiles

Paper published in:

Comparative Biochemistry and Physiology A. (2012). 164:2, pp. 376-382

Authors:

Stefano Carboni[a], Adam D. Hughes[b], Tim Atack[c], Douglas R. Tocher[a], Herve Migaud[a]

Affiliations:

[a]Institute of Aquaculture, Stirling University, Stirling FK9 4LA, Scotland UK

[b]Scottish Association for Marine Science, Oban, Argyll, PA37 1QA, Scotland UK

[c]Ardtoe Marine Laboratory, Acharacle PH36 4LD, Scotland, UK

Key words:

Paracentrotus lividus; Sea urchin; Nutrition; Fatty acids; Diets; Gonads

Contribution

The author has designed and conducted the trial, collected all samples, carried out the parametric statistical analysis and laboratory analysis and wrote the manuscript.

7.1 Abstract

The effects of dietary fatty acids (FA) on the composition of *Paracentrotus lividus* gonads were investigated to determine whether dietary inputs affect their relative abundance during gametogenesis. Egg and embryo FA compositions were compared with that of mature gonads to understand how maternal FA are transferred to the offspring. Urchins were fed an experimental pelleted diet in comparison to brown Kelp (*Laminaria digitata*). FA profiles of diets, gonads, eggs and embryos revealed the presence in gonads of FA that were absent in the diets and/or higher contents of some long-chain polyunsaturated fatty acids (LC-PUFA). Moreover, some unusual FA, such as non-methylene interrupted dienes (NMI), were found in gonads, eggs and embryos, but not in the diets, suggesting that *P. lividus* may be capable of synthesizing these FA and accumulating them in the eggs. A description of gonad FA profiles during gametogenesis is reported for the first time and data suggest that eicosapentaenoic and docosahexaenoic acids are accumulated during gametogenesis, while arachidonic acid is highly regulated and is the only LC-PUFA clearly accumulated into the eggs along with NMI. Further studies are required to determine if maternal provisioning of FA has the potential to influence sea urchin production outputs and increase hatchery profitability.

7.2 Introduction

Larvae of marine organisms initiate their development supported by nutrients provided by the egg. In species that have planktotrophic feeding larvae, nutrients within the egg fuel development of the feeding larva ultil on-set of feeding. In invertebrates, including echinoderms with indirect development, the feeding stage is achieved only after the differentiation of the digestive tract, ciliary feeding apparatus and enzyme systems (Gallager et al., 1986; Strathmann et al., 1992; Pernet et al., 2004), and the duration of the facultative feeding period varies among species and with the availability of maternal provisions (Byrne et al., 2008). Maternal provisioning of nutrients, including essential fatty acids, is therefore important for the normal development of the embryo and plays an important role in offspring performance.

In echinoids the duration of development and general larval condition are also strongly influenced by environmental factors (Fenaux et al., 1994; Miller and Emlet, 1999; Schiopu et al., 2006; Liu et al., 2007). In favorable conditions, development time is shortened, minimizing the duration of the vulnerable planktonic stage usually characterized by high mortality (López et al., 1998; Lamare and Barker, 1999; Liu et al., 2007). In addition to supporting the planktonic stage, nutrients accumulated by the larvae provide energy for the metamorphosis and development of the early juvenile (George et al., 1997; Moran and Emlet, 2001; Schiopu et al., 2006). The size, growth and survival of early juveniles are in fact strongly influenced by the nutrients accumulated and stored by the larvae (Vaïtilingon et al., 2001; Pechenik, 2006; Pernet et al., 2006). Thus in echinoids, larval culture conditions affect metamorphic success and juvenile performance.

Triacylglycerol (TAG) is the major lipid class supplying energy to fuel larval development in many echinoderm and mollusc eggs (Podolsky et al., 1994; Sewell and Manahan, 2001; Villinski et al., 2002; Sewell, 2005; Prowse et al., 2008). TAGs are metabolized during pre-feeding development, while egg phospholipid (PL) and protein remain relatively stable as these nutrients are used primarily as structural components of the developing larval body (George et al., 1997; Sewell, 2005; Meyer et al., 2007; Prowse et al., 2008). As major components of most lipids, fatty acids (FA) have functional roles as a source of metabolic energy (as in TAGs), as structural components (as in membrane PL), and as precursors of bioactive molecules (Sargent et al., 2002; Tocher, 2003). In particular, long-chain polyunsaturated fatty acids (LC-PUFA) such as docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:5n-3), and arachidonic acid (ARA, 20:4n-6) have important physiological functions. In growing larvae LC-PUFA can act as ligands for transcription factors and nuclear receptors, influencing gene expression (Izquierdo and Koven, 2011). Furthermore, it has been shown that LC-PUFA, through cyclooxygenase and lipoxygenase-derived eicosanoids, regulate cortisol production by modulating ACTH-stimulated inter-renal cells in sea bream, *Sparus aurata*, possibly playing a role in stress response (Ganga et al., 2006). Dietary ARA, EPA and DHA compete for acylation and incorporation into the PL membrane of cells and also as substrates for the eicosanoid enzyme systems (Bell et al., 1991a, b). Therefore, the overall impact of LC-PUFA on larval physiology is directly related to the level and ratio of these compounds in tissue phospholipids. For these reasons these FA are the main focus of the present work.

Fatty acid composition of gonads, eggs or larvae have been described in several echinoid species such as *Paracentrotus lividus*, *Psammechinus miliaris*,

Strongylocentrotus droebachiensis, *Dendraster excentricus* and *Lythechinus variegatus* (Cook et al., 2000; George et al., 2000; Castell et al., 2004; Hughes et al., 2006; Schiopu et al., 2006; Gago et al., 2009; Suckling et al., 2011; Carboni et al., 2012). It is generally recognized that FA compositions of sea urchin gonads reflect dietary inputs although reproductive status could alter relative FA abundance in *P. lividus* (Hughes et al., 2005, Martinez-Pita et al., 2010). However, a complete description of the FA profile of each gametogenic stage is not currently available. Marine vertebrates cannot synthesize PUFA de novo although they can have limited ability to further elongate and desaturate dietary PUFA (Sargent et al., 2002). It has been suggested that PUFA desaturase and elongase activities may also be present in some marine invertebrates such as adult sea urchins (Cook et al., 2000; Bell et al., 2001; Castell et al., 2004) and larvae (Schiopu et al., 2006; Liu et al., 2007; Carboni et al., 2012).

Most studies on *P. lividus* nutrition have focused on gonadal index (GI) improvement or gonads' flavour and/or colour enhancement for human consumption (Shpigel et al., 2006; Symonds et al., 2007). In contrast, there are few available data on the effects of dietary FA on maternal provisioning to *P. lividus* larvae. The aims of the present work were 1) to determine the effects of broodstock diet on gonad fatty acid composition during the various gametogenic stages, and 2) to evaluate how egg and embryo fatty acid composition were influenced by the maternal diet.

7.3 Materials and methods

7.3.1 Culture conditions and experimental design

After a starvation period of three weeks, 200 individuals (27.4 mm ± 0.3 test diameter) reared at the Ardtoe Marine Laboratory (AML, Argyll, Scotland; 56N 46' /

5W 52') were randomly divided into four groups corresponding to two treatments in duplicate (50 individuals/group). One experimental pelletized diet (Table 1 for ingredients), provided by the Scottish Association for Marine Science (Pellet diet, P), and a diet of fresh brown algae (*Laminaria digitata*, Kelp diet, K) were fed to adult *P. lividus* over a period of three months (May - July 2011).

Table 7.1 Formulated feed ingredients

Ingredients %	
Soybean meal	21.27
Wheat meal	22.85
Canola meal	21.27
Potato starch	19.84
Gelatine	5.60
Sodium alginate	2.24
Linseed oil	2.24
Lecithin (plant based)	2.24
Vitamin premix	0.56
Mineral premix	0.34
Inositol	0.01
Stabilised vitamin C	0.09
Paradigmox (antioxidant)	0.22
Algro®	1.25

Urchins were kept under ambient temperature and photoperiod within plastic baskets suspended in 100 L tanks with water exchange set at 1 L min^{-1}. Temperature, recorded daily, rose gradually from 9 to 16 °C and day-length increased from 15 h in May to 17 h in June 2011. The urchins were fed daily and, every third day, uneaten pellets were siphoned from the bottom of the tank. As the urchins were kept on suspended baskets, crumbs of uneaten pellets were depositing on the bottom of the tank and therefore were not available to the individuals. Only fresh pellets were therefore ingested during the trial period. Uneaten kelp was also removed as required and replaced with fresh fronds.

At the end of the trial, individuals from each replicate were induced to spawn by injection of 1 M KCl (40 µl per g of body weight) into the coelom via the peristomial membrane (Kelly et al., 2000; Liu et al., 2007; Carboni et al., 2012) and gametes from three females and three males from each replicate were collected and mixed before fertilization. Eggs were fertilized by addition of 10 ml of diluted sperm. Fertilization rate was assessed under the microscope 2 h post fertilization. Fertilized eggs were

incubated in static seawater without aeration for 24 h in the dark (Liu et al., 2007). Hatching rate was estimated for each batch as the proportion of swimming larvae (counted volumetrically) over the number of incubated eggs. A proportion of the offspring from each treatment (approximately 100,000 larvae per replicate) were reared in isolation in triplicate glass tanks of 2 L volume. Developing embryos from each treatment were sampled 48 hours post-fertilization for fatty acid analyses. Seawater used during the process of artificial fertilization, egg incubation and embryos rearing was filtered (4 μm) and UV treated, and water temperature was maintained at 18±2 °C throughout the cultivation period.

7.3.2 Proximate composition

Diets were ground prior to determination of proximate composition, moisture and ash contents according to standard protocols (AOAC, 2000). Crude protein contents were measured by determining nitrogen content (N × 6.25) using automated Kjeldahl analysis (Tecator Kjeltec Auto 1030 analyzer, Foss, Warrington, UK) according to Lynch and Barbano (1999). Crude lipid contents were determined using the Soxhlet method according to standard procedures (AOAC, 2000) with extraction in petroleum ether at 120 °C (Avanti Soxtec 2050 Auto Extraction apparatus, Foss, Warrington, UK). Fibres were analysed using the Foss fibrecap system with 1 g samples being de-fatted with petroleum ether then boiled for 30 min in 350 ml 1.25 % sulphuric acid and washed in boiling distilled water. Samples were then boiled for another 30 min in 350 ml 1.25 % NaOH and washed again in water. Samples were incinerated at 600 °C for 4 h and finally re-weighed. Dietary fibre content was calculated as a percentage of the initial weight of the sample. Energy content of the diets was measured by bomb calorimetry using a Parr 6200 calorimeter according to

standard procedures. The carbohydrate content was calculated as the difference between dry weight and the sum of protein, lipid, ash and fibre.

7.3.3 Histology

At the beginning of the feeding trial (Day 0) and every 30 days, five sea urchins per replicate were cut around the peristomial membrane and gonads separated from the other organs, blotted dry with paper towel. Samples were stored in 10% neutral buffered formalin and then dehydrated, embedded in paraffin and sectioned at 5 µm. Three branches from each individual were analysed in order to confirm synchronization between branches. The sections were stained with haematoxylin and eosin (H/E) and analysed under the binocular microscope (Olympus, BH2). Gametogenic stages were identified according to Byrne (1990). The two remaining branches from each individual were stored at -20 °C in chloroform/methanol (2:1 by vol.) containing 0.01 % butylated hydroxytoluene (BHT) for lipid extraction and fatty acid analysis.

7.3.4 Total lipid and fatty acid contents and composition

After dissection, gonadal samples from each individual were independently stored in 5 ml chloroform/methanol (2:1 by vol.) containing 0.01 % butylated hydroxytoluene (BHT) as an antioxidant at -20 °C prior to analyses. Gonad samples from each individual were analyzed separately. FA profiles of gonad samples from individuals showing the same gametogenic stage within each treatment were averaged to describe the FA profiles characteristic of each observed gametogenic stage.

Urchin eggs were collected from the five gonopores immediately after spawning using a pipette and stored in glass vials as above. Embryos from each replicate were

collected by filtration onto a GF/F filter (Whatman Ltd, Maidstone, UK) before being placed in glass vials and stored as above.

Total lipids were extracted and quantified according to Folch et al. (1957). Fatty acid compositions were determined by gas chromatography of FA methyl esters (FAMEs) essentially according to Christie (2003) all as described in detail by Carboni et al. (2012). Individual FAMEs were identified by comparison with known standards and by reference to published data (Ackman, 1980; Tocher and Harvie, 1988). Data were collected and processed using Chromcard for Windows (version 1.19), and FAMEs quantified through comparison with a heptadecanoic acid (17:0) internal standard.

7.3.5 Statistical analysis

Statistical analysis was performed with MINITAB ® version 15.0. Normality and homogeneity of variance were confirmed using Kolmogorov-Smirnov test. Gonadal LC-PUFA contents were compared using a General Linear Model (GLM) with all interactions being analysed by Tukey post hoc test to identify significant differences (Table 7.2).

Table 7.2. GLM outputs for analyses done on EPA and DHA data showing degrees of freedom, F and P values.

Source	EPA			DHA		
	DF	**F**	**P**	**DF**	**F**	**P**
Time	3	0.90	0.453	3	4.17	0.015
Treat	1	84.63	0.000	1	51.93	0.000
Time*Treat	3	27.20	0.000	3	10.29	0.000
Rep (Treat)	2	0.03	0.973	2	1.68	0.204

SS and MS were ≤0.01

Egg, embryo and diet LC-PUFA, expressed as percentages of total fatty acids, and moisture, ash, carbohydrate, fibre, protein and lipid as well as fertilization and hatching rates were arcsine transformed and analysed by one-way ANOVA. The non-

parametric multivariate analysis ANOSIM (Analysis of similarities) was used to identify significant differences in FA profiles between gametogenic stages in the gonads, eggs and embryos. SIMPER (Similarity percentage) test was used to identify which FAs were primarily responsible for the observed differences (Clarke and Warwick, 1994). Data were untransformed and Euclidian distance was used as the metric. In all cases, significant differences were determined at $p < 0.05$.

7.4 Results

7.4.1 Diets composition

The proximate compositions of the two diets showed significant differences for all the measured parameters. Moisture ($F=422$; $p= 0.002$), ash ($F=225$; $p=0.004$) and carbohydrate ($F=140$; $p=0.007$) contents were higher in the Kelp diet whilst its lipid ($F= 680$; $p=0.001$), protein ($F=57,6$; $p=0.02$) and energy ($F=1265$; $p=0.001$) contents were lower (Table 7.3).

Table 7.3. Each fatty acid is expressed as a percentage of total fatty acids (mean ± SD, n=3). Ash, carbohydrates, fibre, protein and lipids are expressed as a percentage of the diet dry weight. Energy content is given per gram of diet dry weight. Asterisks indicate non-detected values and superscripts indicate significant differences between treatments.

Fatty Acids	Kelp	Pellet
14:0	6.7 (±1.3)	2.0 (± 0.3)
16:0	16.0 (±2.0)	15.5 (±1.0)
18:0	*	[a]3.4 (±0.1)
∑ saturated	23.9 (±3.5)	21.6 (±1.3)
16:1n-9	2.8 (±0.2)	*
16:1n-7	2.1 (±0.2)	3.4 (±0.01)
18:1n-9	[b]14.0 (±1.2)	[a]19.1 (±0.6)
18:1n-7	*	[a]4.1 (±0.03)
20:1n-9	*	[a]4.7 (±0.9)
22:1n-11	*	[a]4.4 (±0.1)
∑ monounsaturated	20.9 (±1.6)	38.4 (±1.0)
18:2n-6	[b]5.6 (±0.5)	[a]26.9 (±0.3)
18:3n-6	0.8 (± 0.1)	*
20:3n-6	0.4 (± 0.0)	*
20:4n-6 (ARA)	[a]9.3 (±1.5)	*
∑ n-6 PUFA 1	16.2 (±2.1)	27.3 (±0.3)
18:3n-3	6.2 (±0.5)	3.5 (±0.1)

18:4n-3	[a]11.4 (±1.7)	[b]0.9 (±0.1)
20:5n-3 (EPA)	[a]13.8 (±1.9)	[b]2.9 (±0.3)
22:5n-3	*	0.6 (±0.04)
22:6n-3 (DHA)	*	[a]4.2 (±0.2)
∑ n-3 PUFA	32.0 (±4.2)	12.4 (±0.1)
Total C16 PUFA	20.9 (±1.6)	19.3 (±1.1)
Total PUFA	48.3 (±6.3)	40.0 (±0.3)
EPA/ARA	1.5 (±0.04)	13.2 (±2.2)
DHA/EPA	*	1.5 (± 0.2)
Moisture %	[a]75.4 (± 0.08)	[b]10.2 (± 3.72)
Ash %	[a]5.7 (± 0.01)	[b]3.6 (± 0.04)
Carbohydrate %	[a]79.3 (± 0.2)	[b]67.2 (± 1.02)
Fibre %	[a]2.9 (± 0.06)	[b]2.0 (± 0.05)
Protein %	[b]11.9 (± 1.2)	[a]22.4 (± 0.07)
Lipid %	[b]0.2 (± 0.02)	[a]4.8 (± 0.04)
Energy (KJ/g)	[b]3.1 (± 0.01)	[a]16.1 (± 0.19)

Moreover, multivariate analysis revealed that there was a significant difference in overall FA signature of the diets (p=0.01), FA compositions are shown in Table 7.4.3. Monounsaturated FAs were the most represented group in the Pellet diet, although 18:2n-6 was the most abundant FA in this diet accounted for the vast majority of n-6 PUFA. In the Kelp diet, n-3 PUFA was the most abundant class with EPA and 18:4n-3 being the most represented FA. ARA and EPA contents were significantly higher in Kelp than in the Pellet diet (ARA: F=70.3 p=0.01; EPA: F=63.2 p=0.01) whilst no DHA was found in Kelp. Therefore, only individuals fed the Pellet diet received dietary DHA input. The differences in EPA, DHA and ARA contents between the diets were reflected in the relative proportion of these LC-PUFAs with the EPA/ARA ratio being significantly higher in the Pellet diet than in Kelp, whilst the absence of DHA in the Kelp diet did not allow for DHA/EPA ratio calculation for this diet.

7.4.2 Fertilization and hatching rates

The average fertilization rate was 98 ± 1% with no significant difference between treatments. The average hatching rate was 85 ± 2% and no significant differences

between treatments were observed. These values are similar to those observed in previous studies where the same spawning trigger was used (Liu et al., 2007).

7.4.3 Histology

Both diets supported development of ovaries and testes over the trial period with no significant differences between treatments and, at the end of the trial, all induced-individuals produced viable gametes as demonstrated by the high fertilization rate obtained in both treatments. As no significant difference between treatments was recorded, histology data from individuals in the two treatment groups were pooled in order to describe gametogenesis of the entire population under the experimental conditions. At the beginning of the trial the majority of individuals (90%) were found to be at the recovery stage (stage I) with a few individuals still in the spent stage (stage VI). At day 30 the population was less homogeneous and 10 % of the individuals were still at stage I, whereas the rest were equally divided between stage II and III. By day 60 most of the urchin population (75 %) was in stage III with the remainder at stage II and at day 90 only 30 % were still at stage III while 70 % progressed to stage IV (Fig. 7.1).

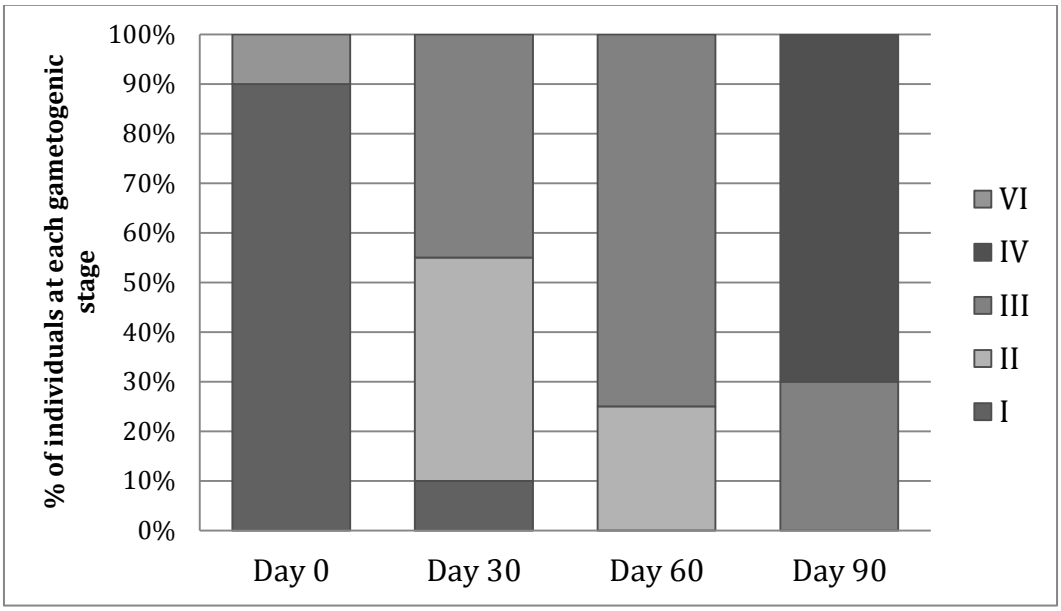

Figure 7.1. Gametogenic stages of the individuals under experimental conditions as observed during the trial period (n=2, 5 individuals/replicate/time point). Individuals from the two treatments were pooled.

7.4.4 Fatty acid composition of eggs, embryos and gonads during gametogenesis

ANOSIM analysis revealed significant differences between treatments when all the tissues (gonads, egg and embryos) were pooled (P = 0.01), and this is clearly shown in the nMDS plot in Fig. 7.2. Nonetheless, when tissues within each treatment were analyzed by one-way ANOSIM followed by pair-wise test, only overall FA signatures of gonads and embryos within the Pellet diet were found to be significantly different (P = 0.02).

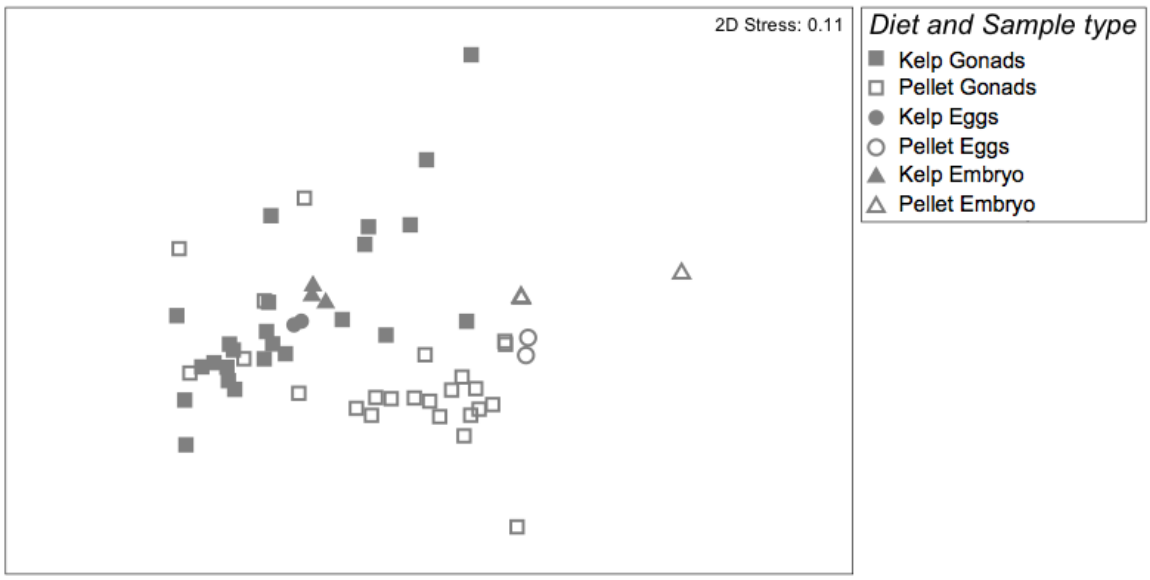

Figure 7.2. Non-metric Multi Dimensional Scale (nMDS) plot for fatty acid composition of sea urchin mature gonads (n=2; 3 individuals/replicate), egg (n=2; 3 females/replicate) and embryos (n=3, approximately 5000 embryos/replicate) in the two treatments.

The SIMPER test showed that the only LC-PUFA involved in the observed difference was ARA, which was clearly accumulated in the eggs and further retained during embryo development in the Pellet treatment (Table 7.4).

Table 7.4. LC-PUFAs and NMI FAs in mature gonads, egg and embryos. Each FA is expressed as percentage of total fatty acids. Superscripts indicate significant differences between treatments within tissues (mean ± SD, n=2).

Diet	**Kelp**			**Pellet**		
Sample	**Gonad**	**Egg**	**Embryos**	**Gonad**	**Egg**	**Embryos**
ARA	^{a}7.4±1.0	^{a}10.3±0.8	^{a}11.3±1.6	^{a}6.9±1.0	^{a}11.6±0.7	^{a}13.0±1.2
EPA	^{a}12.4±0.9	^{a}12.6±0.9	^{a}12.0±2.0	^{b}5.8±1.2	^{b}5.9±0.2	^{b}6.6±0.7
DHA	^{b}0.2±0.1	^{b}0.6±0.1	^{b}0.8±0.2	^{a}2.8±0.4	^{a}3.1±0.04	^{a}3.0±0.4
20:2 NMI	^{b}4.9±0.8	^{b}6.5±0.01	^{b}6.0±0.2	^{a}6.7±0.6	^{a}7.9±0.1	^{a}7.8±0.1
20:3 NMI	^{b}3.9±0.6	^{b}5.8±0.8	^{b}5.2±0.3	^{a}6.6±1.4	^{a}8.5±0.2	^{a}8.0±0.5

Moreover EPA was significantly higher in eggs and embryos produced by urchins fed Kelp than in those derived from the broodstock fed the Pellet diet, whilst the opposite was true for DHA, following a common pattern observed in the gonads. Interestingly 20:2 and 20:3 NMI FA were also accumulated in the eggs from urchins fed both dietary treatments although eggs and embryos derived from the Pellet treatment had significantly higher 20:2 and 20:3 NMI compared with those of the Kelp treatment (Table 7.4).

As significant differences between males and females were not observed, individuals of both genders were pooled according to gametogenic stage. Fatty acid profiles of all gametogenic stages observed during the trial are shown in Table 7.5.

One-way ANOSIM for FA signatures of gonads at different maturity stages highlighted a significant difference between Stage I and all the other stages for urchins in both treatments, however the only significant difference beyond this point was observed between Stage I and Stage III in gonads of urchins fed the Kelp diet. The SIMPER test is of particular interest in this case as it highlighted a continual increment of saturated FA (14:0) as gametogenesis progressed in gonads of urchin fed Kelp whilst it did not change significantly in urchins fed the Pellet diet (Table 7.5). Interestingly, the FA primarily responsible for the observed differences between maturity stages were not the same in the two treatments: 18:1n-9 for Kelp diet and18:2n-6 for Pellet diet. More specifically 18:1n-9 significantly decreased after Stage I in the Kelp treatment whereas it increased in the Pellet treatment, and 18:2n-6 significantly increased after Stage I in the Pellet treatment whereas it did not change in urchins fed the Kelp treatment.

As for LC-PUFA, EPA increased significantly during gametogenesis in urchins fed Kelp whilst its relative content decreased significantly in urchins fed the Pellet diet

establishing a major difference between the two treatments at gametogenic Stage II, III and IV (Fig. 7.3 a). DHA exhibited an opposite pattern, significantly increasing in gonads of urchins fed the Pellet diet, whilst it did not change in gonads of urchins fed Kelp (Fig. 7.3 b).

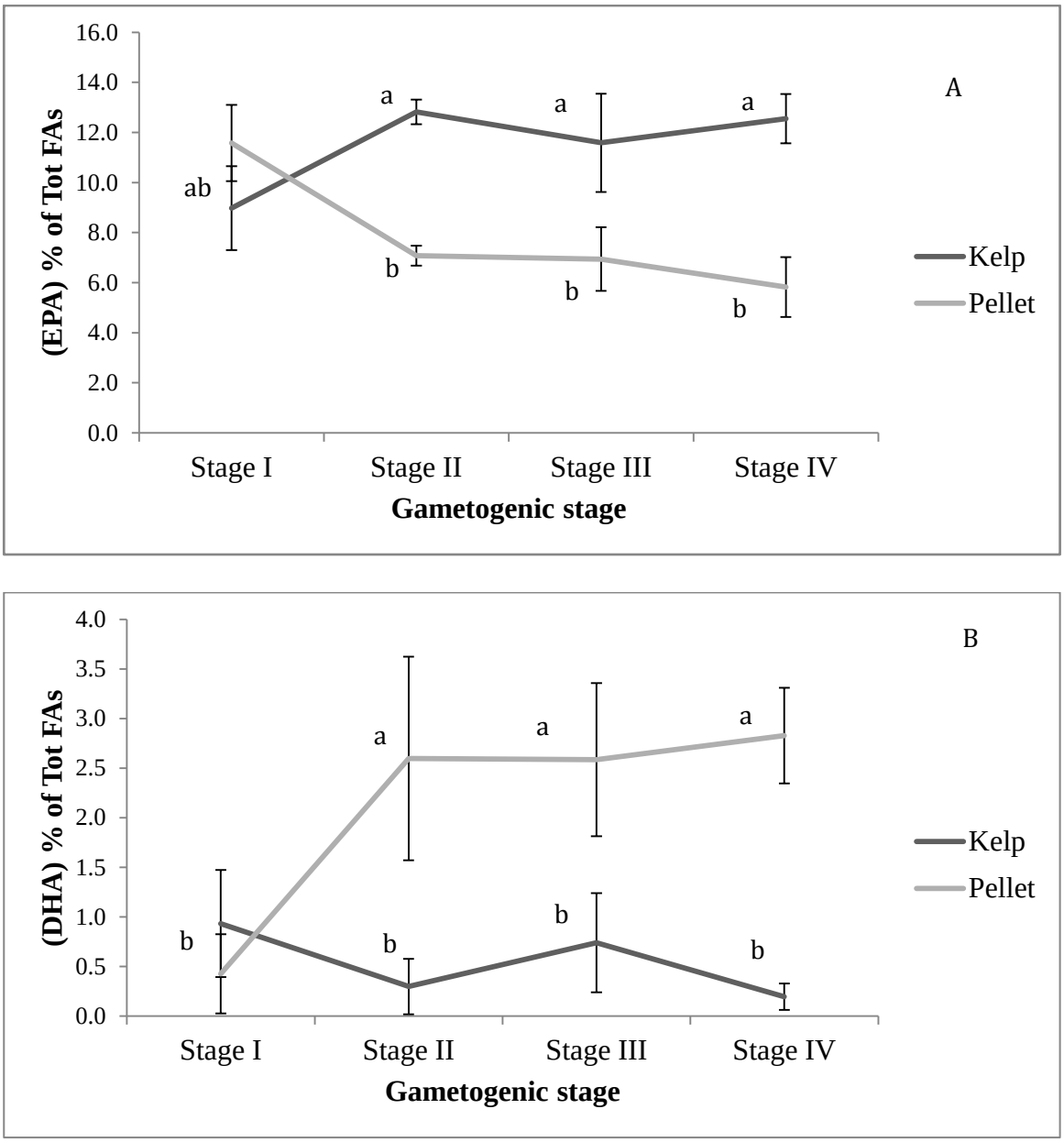

Figure 7.3. EPA (a) and DHA (b) content in gonads of urchins fed kelp or pellet diets throughout gametogenesis. Values of EPA and DHA are given as percentage of total fatty acids (mean ± SD, n=2; 10 individuals/treatment/stage). Superscripts indicated significant differences between treatments.

Despite being absent in the Pellet diet, ARA content in the gonads of urchins fed the two treatments was not significantly different and it did not change during gametogenesis (Table 7.5).

Table 7.5. Fatty acid profiles of the gonads during gametogenesis of urchins fed the two diets. Each fatty acid is expressed as a percentage of total fatty acids (mean ± SD; n=2; 10 individuals/treatment/stage). Asterisks indicate non-detected values. Samples were taken at 30 days intervals. Superscripts indicate significant differences between stages within treatments (mean ± SD, n=2)

Diet	**Kelp**				**Pellet**			
Fatty Acids	**Stage I**	**Stage II**	**Stage III**	**Stage IV**	**Stage I**	**Stage II**	**Stage III**	**Stage IV**
14:0	^{b}5.7 (± 2.0)	^{a}11.4 (± 1.2)	^{a}10.8 (± 4.6)	^{a}13.7 (± 2.1)	10.5 (± 2.0)	7.1 (± 2.8)	7.4 (± 2.1)	8.1 (± 1.4)
16:0	14.7 (± 3.2)	14.2 (± 0.5)	14.1 (± 2.4)	15.1 (± 1.1)	14.4 (± 2.2)	13.2 (± 2.7)	13.6 (± 1.5)	15.7 (± 1.2)
18:0	^{a}4.4 (± 1.3)	2.6 (± 0.2)	2.5 (± 1.3)	^{b}2.3 (± 0.3)	2.1 (± 0.5)	2.6 (± 0.6)	2.4 (± 0.4)	2.4 (± 0.3)
∑ saturated	26.4 (± 5.5)	29.2 (± 1.7)	28.5 (± 5.7)	32.1 (± 2.9)	28.5 (± 3.6)	23.8 (± 5.0)	24.1 (± 3.2)	26.9 (± 1.8)
16:1n-7	3.2 (± 0.5)	3.2 (± 0.5)	3.5 (± 1.2)	4.0 (± 0.8)	4.8 (± 1.9)	3.5 (± 1.3)	3.2 (± 0.9)	3.3 (± 0.7)
18:1n-9	7.2 (± 3.2)	3.2 (± 0.5)	4.0 (± 1.3)	4.2 (± 0.6)	3.6 (± 0.9)	7.3 (± 0.8)	6.3 (± 0.9)	6.1 (± 0.3)
18:1n-7	2.2 (± 0.5)	2.3 (± 0.2)	2.3 (± 0.3)	2.3 (± 0.1)	2.7 (± 0.9)	2.6 (± 0.4)	2.8 (± 0.4)	2.9 (± 0.1)
20:1n-11	5.5 (± 1.7)	4.0 (± 0.7)	4.2 (± 1.0)	3.8 (± 0.5)	3.8 (± 2.2)	3.9 (± 0.4)	3.9 (± 0.5)	3.6 (± 0.4)
20:1n-9	5.4 (± 0.8)	6.3 (± 0.4)	6.4 (± 1.0)	6.4 (± 1.1)	6.1 (± 0.6)	6.5 (± 0.7)	6.4 (± 0.6)	5.9 (± 0.9)
22:1n-9	2.9 (± 0.8)	2.6 (± 0.2)	2.8 (± 0.8)	2.7 (± 0.3)	3.6 (± 0.4)	2.3 (± 0.6)	2.2 (± 0.4)	1.8 (± 0.1)
∑ monounsaturated	29.5 (± 5.2)	22.4 (± 1.4)	23.3 (± 1.9)	23.6 (± 0.5)	24.7 (± 4.6)	27.1 (± 1.9)	25.8 (± 1.3)	24.8 (± 1.1)
18:2n-6	1.9 (± 1.0)	1.9 (± 0.9)	3.0 (± 2.4)	1.8 (± 0.3)	^{b}2.3 (± 1.5)	^{a}10.9 (± 2.6)	^{a}10.1 (± 2.3)	^{a}10.7 (± 1.0)
18:3n-6	0.1 (± 0.1)	0.2 (± 0.0)	0.3 (± 0.1)	0.2 (± 0.0)	0.3 (± 0.1)	0.1 (± 0.0)	0.1 (± 0.0)	0.1 (± 0.0)
20:3n-6	0.4 (± 0.4)	0.6 (± 0.3)	0.6 (± 0.3)	0.5 (± 0.1)	^{b}0.3 (± 0.1)	^{ab}0.8 (± 0.3)	^{b}1.3 (± 0.4)	^{b}1.7 (± 0.5)
20:4n-6	9.8 (± 3.3)	8.5 (± 0.8)	8.4 (± 2.0)	7.4 (± 1.3)	9.3 (± 2.1)	6.8 (± 1.9)	7.3 (± 1.4)	7.0 (± 0.3)
∑ n-6 PUFA	12.4 (± 4.0)	11.4 (± 1.8)	13.0 (± 4.1)	10.0 (± 1.5)	12.4 (± 2.3)	18.8 (± 3.8)	19.6 (± 3.0)	19.6 (± 0.9)
18:3n-3	1.2 (± 0.4)	2.2 (± 0.3)	1.8 (± 0.5)	2.0 (± 0.2)	2.0 (± 0.5)	1.9 (± 0.3)	1.8 (± 0.3)	1.7 (± 0.3)
18:4n-3	^{b}2.1 (± 1.1)	^{a}5.1 (± 0.3)	^{ab}3.7 (± 1.9)	^{ab}4.2 (± 0.9)	^{a}4.1 (± 1.1)	^{b}1.4 (± 0.9)	^{b}1.3 (± 0.7)	^{b}1.0 (± 0.8)
20:5n-3	^{b}9.0 (± 0.5)	^{a}12.8 (± 0.5)	^{a}11.6 (± 2.0)	^{a}12.6 (± 1.0)	^{a}11.6 (± 1.5)	^{b}7.1 (± 0.4)	^{b}6.9 (± 1.3)	^{b}5.8 (± 1.2)
22:5n-3	0.4 (± 0.2)	0.3 (± 0.1)	0.4 (± 0.3)	0.4 (± 0.2)	0.4 (± 0.1)	0.7 (± 0.4)	0.6 (± 0.2)	0.5 (± 0.1)
22:6n-3	*	*	*	*	*	2.6 (± 1.0)	2.6 (± 0.8)	2.8 (± 0.5)
∑ n-3 PUFA	15.8 (± 3.4)	23.2 (± 0.8)	20.5 (± 3.8)	21.4 (± 0.7)	20.4 (± 2.8)	14.6 (± 0.5)	14.3 (± 1.9)	12.7 (± 2.0)
Total PUFA	37.3 (± 6.2)	44.9 (± 2.5)	44.3 (± 3.6)	41.3 (± 2.3)	43.2 (± 2.3)	46.2 (± 6.2)	47.2 (± 3.1)	45.5 (± 0.5)
18:0 DMA	6.1(± 2.1)	3.2 (± 0.6)	3.5 (± 1.5)	3.0 (± 0.5)	3.2 (± 2.0)	2.6 (± 0.6)	2.7 (± 0.7)	2.2 (± 0.4)
20:2 NMI	4.5 (± 1.1)	5.3 (± 0.7)	5.3 (± 0.9)	5.2 (± 0.8)	5.8 (± 1.9)	6.5 (± 0.7)	6.4 (± 0.5)	6.8 (± 0.6)
20:3 NMI	3.8 (± 1.8)	4.4 (± 0.4)	5.0 (± 1.8)	4.2 (± 0.5)	4.0 (± 0.9)	5.9 (± 2.4)	6.4 (± 1.7)	6.4 (± 1.3)

7.5 Discussion and conclusions

Artificial feeds (diets) must be developed to substitute wild collected macroalgae for *P. lividus* aquaculture to become commercially and environmentally sustainable. Early studies investigated diet formulations with higher protein, lipid and energy contents compared to natural macroalgae via inclusion of protein-rich fish or plant meals and fish oil. These diets were able to promote good somatic and gonadal growth. Nonetheless, no data are currently available on the effects of broodstock diets on the FA profiles of the different gametogenic stages of *P. lividus* gonads, and on the maternal provisioning of fatty acids to eggs and embryos.

During the present trial, gametogenesis was not affected by differences in the diets suggesting that environmental conditions such as temperature and photoperiod regulate this process as long as minimal nutritional requirements are met. Stage V gonads were not observed and this might be due to the relatively low maximum temperature (16 °C) achieved during this trial. This is supported by the fact that natural spawning was never observed during commercial production at AML, suggesting that a higher daily average temperature is required to meet the Effective Accumulated Temperature (EAT) to trigger natural spawning as previously suggested (Liu et al., 2002).

In animals capable of converting 18-carbon PUFA to LC-PUFA, the first step is the action of fatty acyl Δ6-desaturase, which converts 18:2n-6 to 18:3n-6 and 18:3n-3 to 18:4n-3 (Tocher, 2003). The second step in the LC-PUFA biosynthesis pathway is the action of fatty acyl elongase, which converts 18:3n-6 to 20:3n-6 and 18:4n-3 to 20:4n-3. These elongated products are then further desaturated (Δ5 desaturase) to ARA and EPA, respectively. EPA is also substrate for further elongation and

desaturation to produce DHA. The presence in the gonads of some fatty acids that are not detected in the diets such as 18:3n-6 and 20:3n-6, not found in the Pellet diet, or 22:5n-3 and DHA, not found in the Kelp diet, or the much higher content of ARA in the gonads of individuals fed the Pellet diet than in the diet itself, suggests that sea urchin may have the ability to synthesize LC-PUFA. The positive correlation between dietary inputs of FA substrates (18:2n-6 and 18:3n-3) of the LC-PUFA biosynthesis pathway and their content in the gonads clearly shows that tissue levels of these two fatty acids are influenced by their contents in the diets. The very nature of a multistep enzymatic pathway where the fatty acid product of one step becomes substrate for the following step makes it difficult to be conclusive about endogenous metabolism based simply on relationships between tissue levels of the fatty acids. This is further complicated in the present study, by significant dietary inputs of some of the pathway intermediates such as 18:4n-3, 20:4n-3 or indeed EPA.

The fact that NMI FA are not present in either of the diets used in the present study also suggests that P. lividus may be capable of synthesizing these fatty acids. Cook et al. (2000) and Castell et al. (2004) found similar NMI FA in *P. miliaris* and *S. droebachiensis* and both suggested that these species of sea urchin were capable of de novo synthesis of NMI FA. Moreover Zhukova (1986, 1991) used 14C-labeled acetate to show that mussels were capable of de novo synthesis of the same NMI FA identified in sea urchins. Our data also showed that NMI FAs are selectively accumulated in the eggs and embryos suggesting that they may be important for larval development. Results of the present experiment agree with a relationship between NMI FA and essential FA previously suggested for *S. droebachiensis* for which, under specific conditions, primitive taxa such as echinoids could use NMI FA to provide physiological functions commonly associated with LC-PUFA (Castell et al.,

2004; Gonzalez-Duran et al., 2008). Indeed, the ability to biosynthesize 20:3 NMI FA from the precursor 18:2n-6 and to substitute ARA in the membrane phospholipids may represent an important advantage for organisms exposed to fluctuating temperatures which requires membrane fluidity adaptations. Indeed, it was shown that the unusual double bond position in 20:3 NMI FA causes a melting point shift of about 10 °C lower (Zakhartsev et al., 1998; Pirini et al., 2007) and this could partly explain the wide geographical distribution of *P. lividus*.

The significant differences in 20:2 and 20:3 NMI FA in eggs and embryos observed between dietary treatments might be explained by the higher levels in the Pellet diet of their precursors (20:1n-9 and 18:2n-6), which are converted via Δ5 desaturase into 20:2 and 20:3 NMI FA respectively, as suggested by Zhukova (1991).

In stage I gonads of urchins fed the Kelp diet, 18:1n-9 was present although it decreased during gametogenesis and its contents in eggs and embryos were lower than those of urchins fed the Pellet diet. This could be explained by elongation of 18:1n-9 to 20:1n-9 which, in turn, might be converted to 20:2 NMI if the metabolic pathway identified by Zhokova (1991) in bivalves was also operating in sea urchin. On the other hand, 18:2n-6 is a precursor for both 20:3 NMI and ARA (Barnathan, 2009) and both of these FA were not present in the Pellet diet. However, it is not possible to be conclusive on how this FA was utilized, as end products of both pathways were present in the gonads, eggs and embryos of urchins fed the Pellet diet.

In conclusion, the present study provides, for the first time, a detailed description of the evolution of fatty acid profiles of P. lividus gonads during gametogenesis. Although no definitive conclusions can be made, it seems that, among LC-PUFA, EPA and DHA are primarily accumulated during gametogenesis when available in the diet.

In contrast, ARA appears to be more constant throughout gametogenesis and more independent of dietary input. ARA is the only LC-PUFA clearly accumulated in the eggs along with NMI FA.

As already suggested by Gago et al. (2009), we confirmed that FA profiles of sea urchin eggs and embryos can be controlled through broodstock nutrition. This could play a role in the development of new feeds and protocols for first feeding of sea urchin larvae. Further studies on the effects of maternal provisioning of LC-PUFA on larvae performance are required to determine if broodstock nutrition has indeed the potential to be used to influence sea urchin production output. Moreover, it is important to confirm the capacity of sea urchins for endogenous production of LC-PUFA and if so, identify and functionally characterize the genes involved in LC-PUFA production. Finally, in depth investigation should be carried out to better understand NMI FA metabolism and interactions between these quantitatively minor FA and LC-PUFA in echinoids species.

Chapter 8 - Identification of fatty acyl desaturases from *Paracentrotus lividus*.

8.1 Abstract

Several observations in previous studies and data collected during this experimental work suggest that the potential ability of sea urchin to endogenously synthesise long chain fatty acids from precursors might indeed be present in these marine invertebrates. During this short experiment we interrogated public database in search of *Paracentrotus lividus* expressed sequence tags (ESTs) using available desaturases sequences isolated from other marine invertebrates and vertebrates. After the identification of fragments showing a high degree of identity, *P. lividus* RNA was extracted, cDNA was synthesised, desaturase-like fragments cloned and full-length sequences obtained. Results indicate that one of the isolated sequences was phylogenetically coherent with other invertebrates' desaturase and possessed all features characteristics of this group of proteins. Functional characterisation by heterologous expression will be now required in order to identify whether the newly cloned protein has the ability to perform fatty acids desaturation.

8.2 Introduction

Evidence of the beneficial effects of provisioning LC-PUFA to sea urchin larvae was described in Chapter 4 where the potential ability of sea urchin to biosynthesise LC-PUFA was also suggested. This possibility could also be inferred from previous studies on larval nutrition of *P. lividus* (Liu et al., 2007) and from results obtained by Bell et al., (2001) on the echinoid *P. miliaris*. Moreover, indication of this biosynthetic mechanism being active in *P. lividus* could be observed analysing FA profiles of broodstock diets and gonadal tissue as described in Chapter 7 of this thesis.

The specific FAs able to satisfy an organism's EFA requirements depend on the ability of each species to endogenously synthesise LC-PUFA through bioconversion of dietary FA, which is dependent on the presence of enzymes capable for such conversions (Bell and Tocher 2009). In vertebrates, the fatty acyl desaturases (Fads) and elongases (Elovls) have been identified as the key enzymes involved in these conversions. Fads enzymes are classified based on the position in which they insert a double bond (unsaturation) such as positions C6 or C5 in the fatty acyl chains counted from the carboxyl group. In the first case the enzyme is known as Δ6 Fad and in the latter as Δ5 Fad. In vertebrates, the LC-PUFA biosynthetic pathway has been extensively investigated and a number of genes encoding either Fad or Elovl proteins have been characterised, particularly from fish. Among non-vertebrates, the eukaryotic protist *Thraustochytrium sp.* (Qiu et al. 2001), the nematode *Caenorhabditis elegans* (Beaudoin et al. 2000; Watts and Browse 2002) and the Cephalopod *Octopus vulgaris* (Monroig et al., 2011) represent some of the few examples where Fad- and Elovl- genes have been studied. As far as we are aware,

however, neither desaturases nor elongases have been previously isolated from Echinoderms.

8.3 Materials and Methods

8.3.1 *In silico* search for *P. Lividus* desaturase genes

A tblastx search of *P. Lividus* EST's was performed on publically available databases (http://www.ncbi.nlm.nih.gov/BLAST/) using the available Delta 5/6 desaturase sequences from Atlantic salmon (*Salmo salar*) and common octopus (*Octopus vulgaris*). This search identified four fragments with apparent high identity (Table 8.1).

Table 8.1: Summary of P. Lividus specific EST's identified with high identity to reference vertebrate and invertebrate desaturase sequences.

Accession number	Fragment size	Nominal designation
AM559332	834bp	P. Lividus Delta 5a
AM220309	792bp	P. Lividus Delta5b
AM571147	794bp	P. Lividus Delta5b
AM537908	751bp	P. Lividus Delta 5c

Thereafter, the phylogenetic relationship of the fragments to themselves and to registered desaturase genes was examined using MEGA version 5 (Tamura et al., 2011) to deduce and bootstrap phylogenetic trees using the neighbour joining method (Saitou & Nei, 1987).

8.3.2 RNA Extraction & cDNA synthesis

P. Lividus totRNA was extracted from tube feet collected from individuals produced at the Ardtoe Marine Laboratory. Approximately 100 mg of tissue was homogenised in 1 ml tri reagent, (TRIzol®, Invitrogen UK), according to manufactures instructions. The homogenised samples were centrifuged at 12,000 g at 4 °C for 10 min in order to separate tissue debris. The supernatant was removed into a clean DNA- and RNA-free eppendorf tube and incubated at room temperature for 5 min. 100 µl of 1-

bromo-3-chloropropane (BCP) (Sigma-Aldrich, Gillingham, UK) was added, samples mixed, and incubated at room temperature for 10 min. RNA extractions were then centrifuged for 15 min at 4 °C at 12,000 g. The clear aqueous layer was removed into a new eppendorf and totRNA was precipitated by adding 500 µl of isopropanol to the samples and vortexed to mix. Extractions were incubated at room temperature for 10 min to precipitate RNA and then centrifuged at 4 °C, 12,000 g for 10 min. The RNA pellet was washed in 75 % ice-cold ethanol and the pellet air dried before being rehydrated in an appropriate volume of nanopure H_2O to achieve a concentration ≤ 1000 µg/µl. Total RNA concentration and quality was determined using a ND-1000 Nanodrop spectrophotometer (Labtech Int., East Sussex, UK). When the 260/280 ratio was between 1.8 and 2.0 the RNA is considered to be pure.

8.3.3 cDNA synthesis

cDNA was reverse transcribed from totRNA using high-capacity reverse transcription kit without RNase inhibiter (Applied biosystems, UK). RNA was diluted to 10 µl and 10 µl of master mix (see below) then added to each reaction.

cDNA master mix

- 4.2 µl H_2O
- 2.0 µl 10 x Buffer
- 0.8 µl 25 x DNTP mix (100 mM)
- 2.0 µl 10 x RT Random primers
- 1.0 µl MultiScribe™ reverse transcriptase 50 U/µL

The reaction is then incubated in a thermocycler at 25 °C for 10 min followed by 37 °C for 120 minutes and 85 °C for 5 min. All cDNA samples were then stored at -20 °C.

8.3.4 PCR cloning of *P. lividus* desaturase-like fragments and full-length construction of desaturase 5 sequences

Bioinformatic analysis resolved the four *P. lividus* desaturase-like EST fragments to three consensus sequences against which three PCR primer pairs were designed (Table 8.2).

Table 8.2. Primer sequences, and details of the PCR reactions employed

Target	Primer sequence	Annealing temperature	Product size
P. lividus Delta 5a F	tcacgcagtgggccaagagaca	60°C	672bp
P. lividus Delta 5a R	acagaagagggggtccaatgagga		
P. lividus Delta 5b F	accgcgcttcgacatcgctccg	64°C	477bp
P. lividus Delta 5b R	ggtcgcgatgagacaggctgcca		
P. lividus Delta 5c F	ggacgtcatgccatcgccttgc	64°C	596bp
P. lividus Delta 5c R	tcggaccaccgcgtaggcca		
P. lividus 5a 5' inR	tctttggcgatctgcccaatgtgga	60°C	N/A
P. lividus 5a 5' outR	ggaaggtccaccaacagaatccata	60°C	N/A
P. lividus 5a 3' inF	tcattggacccccctcttctgtttc	60°C	N/A
P. lividus 5a 3' outF	gcatggcacactacaggctcaggt	60°C	N/A

These primer pairs were used with the *P. lividus* cDNA produced as described above. Partial cDNA sequences were generated by PCR using 0.5 µM of primers (Eurofins MWG Operon, Edersberg, Germany) (Table 8.2.4.1.) one fortieth of the original cDNA synthesis reaction, Klear Taq polymerase with supplied buffer (Kbiosciences, UK), and 1 mM $MgCl_2$ in a final volume of 20 µl using a PCR strategy: 15 min at 95 °C followed by 30 cycles of 95 °C for 20 s, X °C for 20 s, and finally 72 °C for 1 min. The annealing temperature is denoted as X °C in the description as it varied with the different primer pairs (Table 8.2.4.1). Full-length construct for Delta 5a was generated using Rapid Amplification of cDNA Ends (RACE)-PCR with the RACE cDNAs generated from 1 µg of tube feet sample total RNA as described in the manual using the SMART™ RACE kit (Clontech, USA). The 5' and 3' RACE amplicons were

generated by two rounds of PCR using nested primers as listed in Table 8.2.4.1. All PCRs were run at an annealing temperature as listed in Table 8.2.4.1 and extension time of 3 min was applied for unpredictable RACE PCR products. All primers were designed using Primer Select Ver. 6.1 program (DNASTAR, www.dnastar.com). All generated products were purified using illustra GFX PCR DNA and GelBand Purification Kit (GE Healthcare) according to manufacturer's instructions and then cloned into the pGEM®-T Easy vector system (Promega, Southampton, UK). Plasmids were harvested from discrete colonies using a GenElute™ Plasmid Miniprep Kit (sigma Aldrich, Gillingham, UK) and the presence of an insert checked by enzymatic digestion (ECoR1, Invitrogen Paisely, UK). Plasmids with the correct sized insert were sequenced using a Beckman 8800 auto sequencer and sequence assembled and analysed using SEQman as part of Lasergene software package (DNASTAR, Madison, Wisconsin, USA). Identity of all sequences was confirmed *in silico* by performing a BLAST analysis. MEGA Ver.4.1 (http://www.megasoftware.net/) was used to deduce a phylogenetic tree using the neighbour joining method.

8.4 Results

8.4.1 PCR confirmation of *P. lividus* desaturase-like ESTs

Phylogenetic analysis resolved the four identified *P. lividus* ESTs into three separate desaturase-like consensus sequences (designated d5a-cFads) located within the delta 5/6 fatty acyl desaturase cluster (Fig 8.1)

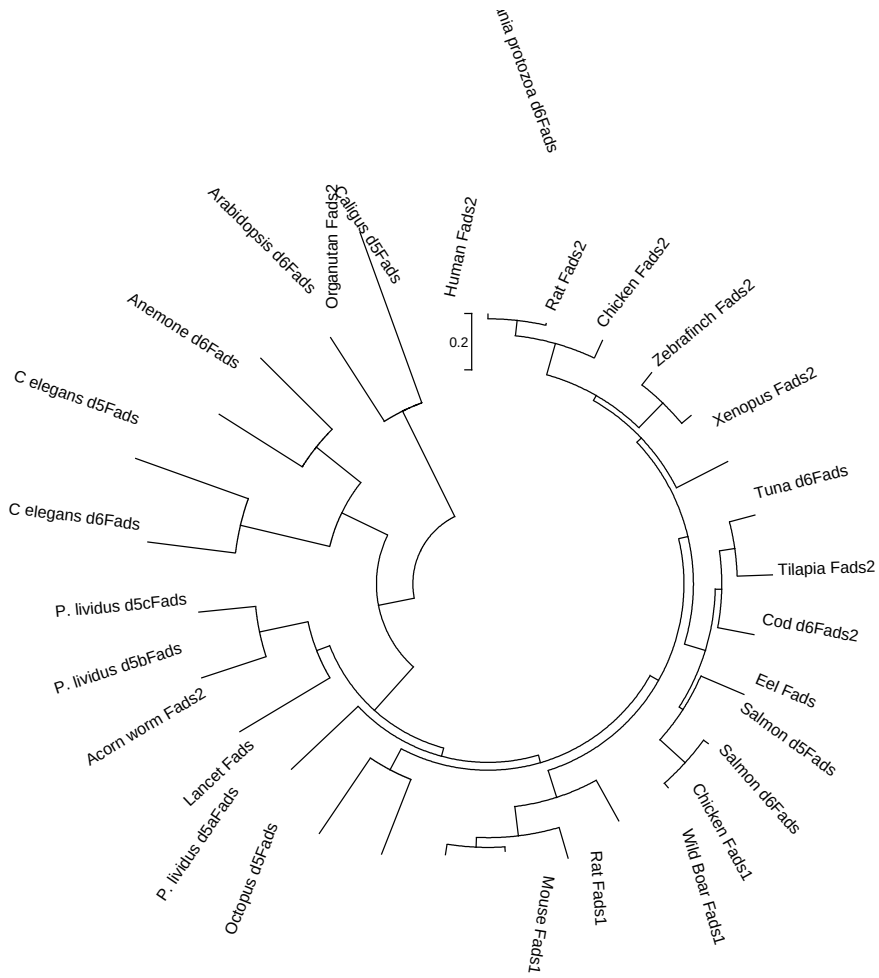

Figure 8.1. The evolutionary history was inferred using the Neighbor-Joining method (Saitou & nei 1987). The optimal tree with the sum of branch length = 13.13555584 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. The analysis involved 42 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 329 positions in the final dataset.

A routine PCR test of *P. lividus* cDNA generated PCR products of expected size for all desaturase contigs (Fig 8.2).

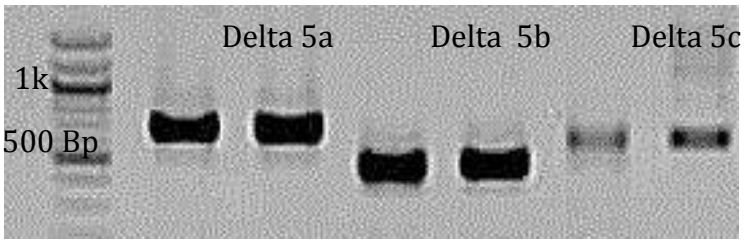

Figure 8.2. Image of a 1% agarose gel in which PCR products for P. lividus desaturase-like products 5a-c and 4 are shown.

8.4.2 Full length construction of the putative Δ5-like desaturase of *P. lividus*

A full-length sequence was constructed for the *P. lividus* putative d5a Fads cloned from the 5a EST fragment. The full-length sequence of 2398bp contained a 1365bp coding sequence (CDS), which translates into a 455 amino acid protein (Fig 8.3).

```
         10         20         30         40         50         60         70         80
90
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AAAATCAGCTGAGTCTAGGCTGTGCTGATAAGTTCGCTGGATCTGGAACCTCCCTTGGCAGCTTGAAAAAAGTAGCGGAGTGCAACGATG
                                                                                  M

        100        110        120        130        140        150        160        170
180
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GGTCTGGGAGCAAACCAGCAAGGTGATGGAGAGGTGCAGGGTGCTATCCACAAAGCAAAGAGGTTTGTGGGTGATGTGACCTGGGAGGAA
 G  L  G  A  N  Q  Q  G  D  G  E  V  Q  G  A  I  H  K  A  K  R  F  V  G  D  V  T  W  E  E

        190        200        210        220        230        240        250        260
270
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GTAAAGAAGCATGATGGGAAGGTGGTCAAGGACAAGTGGTTGGTGATAGATAACCAGGTGTATGACATCACACAGTGGGCCAAGAAACAT
 V  K  K  H  D  G  K  V  V  K  D  K  W  L  V  I  D  N  Q  V  Y  D  I  T  Q  W  A  K  K  H

        280        290        300        310        320        330        340        350
360
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CCAGGAGGTTTCAAAGTTATCACTCACTATGCTGGTCAAGATGGATCTGAAGCCTTTACTGCTTTCCACAATGATGAGCAATATGTAAGA
 P  G  G  F  K  V  I  T  H  Y  A  G  Q  D  G  S  E  A  F  T  A  F  H  N  D  E  Q  Y  V  R
        370        380        390        400        410        420        430        440
450
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AAGTTTATGAAAGCAATCCACATTGGGCAGATCGCCAAAGAACACGAGGAGCATAAAGATGTCGTCAAAGACTTTGCAGAGCTTCGTAAA
 K  F  M  K  A  I  H  I  G  Q  I  A  K  E  H  E  E  H  K  D  V  V  K  D  F  A  E  L  R  K
        460        470        480        490        500        510        520        530
540
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ACAGCTGAAAAAATGGGTCTGTTCAATGCCAACTTCCTCTTCTTCTTCCTGCACATGTCTCATATCATTGGTCTTGAGATAGCTTCTTAT
 T  A  E  K  M  G  L  F  N  A  N  F  L  F  F  F  L  H  M  S  H  I  I  G  L  E  I  A  S  Y
        550        560        570        580        590        600        610        620
630
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TTTGTCATGAGGACGTATGGATTCTGTTGGTGGACCTTCCTTGTCTGTATGGCAATGCATGGCACACTACAGGCTCAGGTAGGGTGGTTT
 F  V  M  R  T  Y  G  F  C  W  W  T  F  L  V  C  M  A  M  H  G  T  L  Q  A  Q  V  G  W  F

        640        650        660        670        680        690        700        710
720
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CAACATGATCTTGGTCATCTCTCCTGCTTTAAGTCATCAAAATGGAACCATGTGTTTCATTATGTTTTCATGTCTACAGTCAAGGGTGCA
 Q  H  D  L  G  H  L  S  C  F  K  S  S  K  W  N  H  V  F  H  Y  V  F  M  S  T  V  K  G  A
```

```
        730         740         750         760         770         780         790         800
810
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TCAGCTAAATGGTGGAATCATATGCACTACCAGCATCATGCAAAGCCGAATGTGATGAACAAGGATCCTGATGTGCGTCTTGAAAAGCTG
 S   A   K   W   W   N   H   M   H   Y   Q   H   H   A   K   P   N   V   M   N   K   D   P   D   V   R   L   E   K   L

        820         830         840         850         860         870         880         890
900
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TTTGTAGTTGGAGAGAAGATGCCTAAGATGGTTGCTGAGAGTGGACAGTCAACAATGCCATACCAACATCAACACAAGTATTTCTTCCTC
 F   V   V   G   E   K   M   P   K   M   V   A   E   S   G   Q   S   T   M   P   Y   Q   H   Q   H   K   Y   F   F   L

        910         920         930         940         950         960         970         980
990
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ATTGGACCCCCTCTTCTGTTTCCTCTCTACTTCCAGTTCATGATTTACCGTCATATGATCAAGCAGAAGGAATGGGTCGATCTGGCCTTC
 I   G   P   P   L   L   F   P   L   Y   F   Q   F   M   I   Y   R   H   M   I   K   Q   K   E   W   V   D   L   A   F

        1000        1010        1020        1030        1040        1050        1060        1070
1080
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AACTCATTATACTACATAAGGTTTTTAGCCTGCTATGTTCCTCTCATGAAATGGTGGCAACTTCTCCTCTTCTATGAATCCTTCAGAGTA
 N   S   L   Y   Y   I   R   F   L   A   C   Y   V   P   L   M   K   W   W   Q   L   L   L   F   Y   E   S   F   R   V

        1090        1100        1110        1120        1130        1140        1150        1160
1170
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ATGGAGAGTATCTGGTTCACATGGGTTTCTCAGTCTAACCACATACCAATGGAGATTGATGAAGATTTAGCACGTCCTTGGCTAGAGCTA
 M   E   S   I   W   F   T   W   V   S   Q   S   N   H   I   P   M   E   I   D   E   D   L   A   R   P   W   L   E   L

        1180        1190        1200        1210        1220        1230        1240        1250
1260
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CAGCTTCGAGCTACCTGCAATCTGGAACAAAGTTTCTTCAATGACTGGTTTACTGGACATCTCAATTTCCAAATTGAACATCATCTATTC
 Q   L   R   A   T   C   N   L   E   Q   S   F   F   N   D   W   F   T   G   H   L   N   F   Q   I   E   H   H   L   F

        1270        1280        1290        1300        1310        1320        1330        1340
1350
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CCAACAATGCCTCGTCATAACTATGTGCATATCGCACCATTGGTGAAGACCCTGTGTAAAAAGCATGGCATCGATTACAAGACCAAGACC
 P   T   M   P   R   H   N   Y   V   H   I   A   P   L   V   K   T   L   C   K   K   H   G   I   D   Y   K   T   K   T

        1360        1370        1380        1390        1400        1410        1420        1430
1440
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TTGTTGCAGGGATTTGCTGATATAGTCCATTCCCTGAAGTCCTCTGGTGAGATCTGGTACGATGCCTACTATCATCTTCAGGACCAGTAT
 L   L   Q   G   F   A   D   I   V   H   S   L   K   S   S   G   E   I   W   Y   D   A   Y   Y   H   L   Q   D   Q   Y

        1450        1460        1470        1480        1490        1500        1510        1520
1530
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TCAACGGACTAACCCTTCTGCTCTCGTTAATCTGCAGAGCCTACATGACTCGCTCTTGATATTTATCTAATGGCATTCTAGGAGTTAATT
 S   T   D   *

        1540        1550        1560        1570        1580        1590        1600        1610
1620
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ACATGAATTAGATACATCAACATAGTCCCAAATGAGTTCATATCTTTTTCTGTCATGATAGAGTATAATTCTTTGTATGTAACAATCATT

        1630        1640        1650        1660        1670        1680        1690        1700
1710
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TGCTTTCATTTGTTTTTTAGGGTAACATTAACAAGCTTCCTCTATGTGTTAATGTCCTGTCAATGTCTTTTAAATCTAAAACTTTATAGA

        1720        1730        1740        1750        1760        1770        1780        1790
1800
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AAGTAAACCTGTTTTAAAAAGATTTTCATTTCAAATGTTAAATTATGTTGTGATTTTGTAGTAAACTGATAGGCCTAAAGGGTAAATATC

        1810        1820        1830        1840        1850        1860        1870        1880
1890
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TGAGGAAGTGTGATGTATATAGCTGATGAAATTTATTTTGCACATTTATTGAATTGTTTTTTTTTTAATGGGGATGTGTCATGTAAATAT

        1900        1910        1920        1930        1940        1950        1960        1970
```

```
1980
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ATATGTTTTTGAGCAGTGTGATTTTTGGTGATTAGGTTTGAAGCACTTAAAGTGCCATGATCTGAAAGTAGATCTTATAAAGTCCAAATC

        1990        2000        2010        2020        2030        2040        2050        2060
2070
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CTTCAGAAGTGATTTAGACCTACAATTTTAAGCTATTATGATTGGCAAAGAAACAACTTATTCTATTTCTAATGACATCTTTATCTCAAC

        2080        2090        2100        2110        2120        2130        2140        2150
2160
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TCAAAACCATACTATGACACTATGGTAATGAAATAATAAAGTCTATGTAGGAAGGATCAGCTCGTATTGTAGGGCAATATAGTTGTAGTA

        2170        2180        2190        2200        2210        2220        2230        2240
2250
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TGATAGGTAGTGTATATCTAAACTTTTTGGGGAATACTGGGAAATATGATTATGGATGCAAAGCTGTCTCTATCTTTTATCATGGAGTAG

        2260        2270        2280        2290        2300        2310        2320        2330
2340
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ATATGTTTATTACATGTATTGGTTTGGTATATGTTTCCATTTTGATTAGTAATGAAATAGTTTTGAATTGTTATCCTGCCTTTTGTGAAT

        2350      2360      2370      2380      2390
....|....|....|....|....|....|....|....|....|....|...
TGGAATACCTTTGGTATTTCACAAGTTTAGAAAATCATGCAGACTAAAAAAAAAAAAA
```

Figure 8.3. The full-length nucleotide sequence of the putative Δ5-like desaturase of *P. lividus* with single letter translation of the presumed translated protein sequence.

The deduced amino acid sequence contained a cytochrome b5-like domain, three histidine boxes and the heme-binding motif commonly reported in other vertebrate and invertebrate delta5/6 fatty acyl desaturases (Fig 8.4).

```
10        20        30        40        50        60        70
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
P. lividus          MGLGANQQGD-GEVQGAIHKAKRFVG---DVTWEEVKKHDGKVVKDKWLVIDNQVYDIXQWAKXHPGGFK
Abalone Fads1       MGKGGQG------TQNGISKVKDFS-------MDDVKHHDKR--DDKWLVIQGEVYNITDWARRHPGGSK
Octopus d5Fads      MGRGGENSDTTSPNPNTDSSQRYYS-------WEEIKKHNQK--EDRWLVIDRQVYNITNWSQRHPGGSK
A. salmon d5Fads    MGGGGQQTESSEPAKGDGLEPDGGQGGSAVYTWEEVQRHSHR--SDQWLVIDRKVYNITQWAKRHPGGIR
Tuna d6Fads         MGGGGQLTEPGEPGSG---RPGS------VYTWEEVQRHCSR--NDQWLVIDRKVYNITHWAKRHPGGFR
S. aurata d6Fads    MGGGGQLTEPGEPGSR---RAGG------VYTWEEVQSHSSR--DDQWLVIDRKVYNVTKWAKRHPGGFR
A. salmon d5Fads    MGGGGQQNDSGEPAKGDRGGPGGGLGGSAVYTWEEVQRHSHR--GDQWLVIDRKVYNITQWAKRHPGGIR
                                                                                    ****

                              80        90       100       110       120       130       140
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
P. lividus          VITHYAGQDGSEAFTAFHNDEQYVRKFMKAIHIGQIAKEHEEHK-----DVVKDFAELRKTAEKMGLFNA
Abalone Fads1       VISHYAGQDATDAFRAFHNDLSFVKKYLKPIHIG-SLREETKET-----EVERDFRELRATAEKMNLFKP
Octopus d5Fads      VIGHYAGQDATEAFRAFHNDLSFVRKFLKPLHIGSSCSDEFPVT-----EMSADFEELRRMAEKMGLFKP
A. salmon d5Fads    VISHFAGEDATEAFSAFHLDANFVRKFLKPLLIGELAPTEPSQDHGKNAALVQDFQALRDHVEREGLLRA
Tuna d6Fads         VISHYAGEDATDAFAAFHPDPKFVQKFLKPLLIGELAATEPSHDRNKNAAIIQDFHTLRAHAEREGLFQA
S. aurata d6Fads    VINHYAGEDATEAFTAFHPDLKFVRKFLKPLLIGELAATEPSQDRNKNAAVIRDFHTLRVQAESDGLFRA
A. salmon d5Fads    VISHFAGEDATDAFVAFHPNPNFVRKFLKPLLIGELAPTEPSQDHGKNAVLVQDFQALRNRVEREGLLRA

                              150       160       170       180       190       200       210
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
P. lividus          NFLFFFLHMSHIIGLEIASYFVMRTYGFCWWTFLVCMAMHGTLQAQVGWFQHDLGHLSCFKSSKWNHVFH
Abalone Fads1       NALFFVLHLGHILLLEYMAYAVMAYFGTGWIPYILSLVFYSTVQAQVGWLQHDFGHLSVFHISKWDHLLH
Octopus d5Fads      SYCFFFLNVGYVLILEVFAYLTLKYLGTGWLPYFLSVLFYSIVQAQTGWIQHDFGHLSVFKRSKFDHFWH
A. salmon d5Fads    RLLFFSLYLGHILLLEALALGLLWVWGTSWSLTLLCSLMLATSQAQAGWLQHDYGHLSVCKKSSWNHKLH
Tuna d6Fads         RPLFFCFHLGHILLLEVLAWLIVWLWGTSWMLTLLCSVILATAQAQAGWLQHDFGHLSVFKKSSWNHVLH
S. aurata d6Fads    QPLFFCLHLGHILLLEALAWLIIWLWGTSWTLTFLISIILATAQAQAGWLQHDFGHLSVFKKSSWNHILH
A. salmon d5Fads    RPLFFSLYLGHILLLEALALGLLWVWGTSWSLTLLCSLMLATSQSQAGWLQHDYGHLSVCKKSSWNHVLH
                                                                      HXXX
```

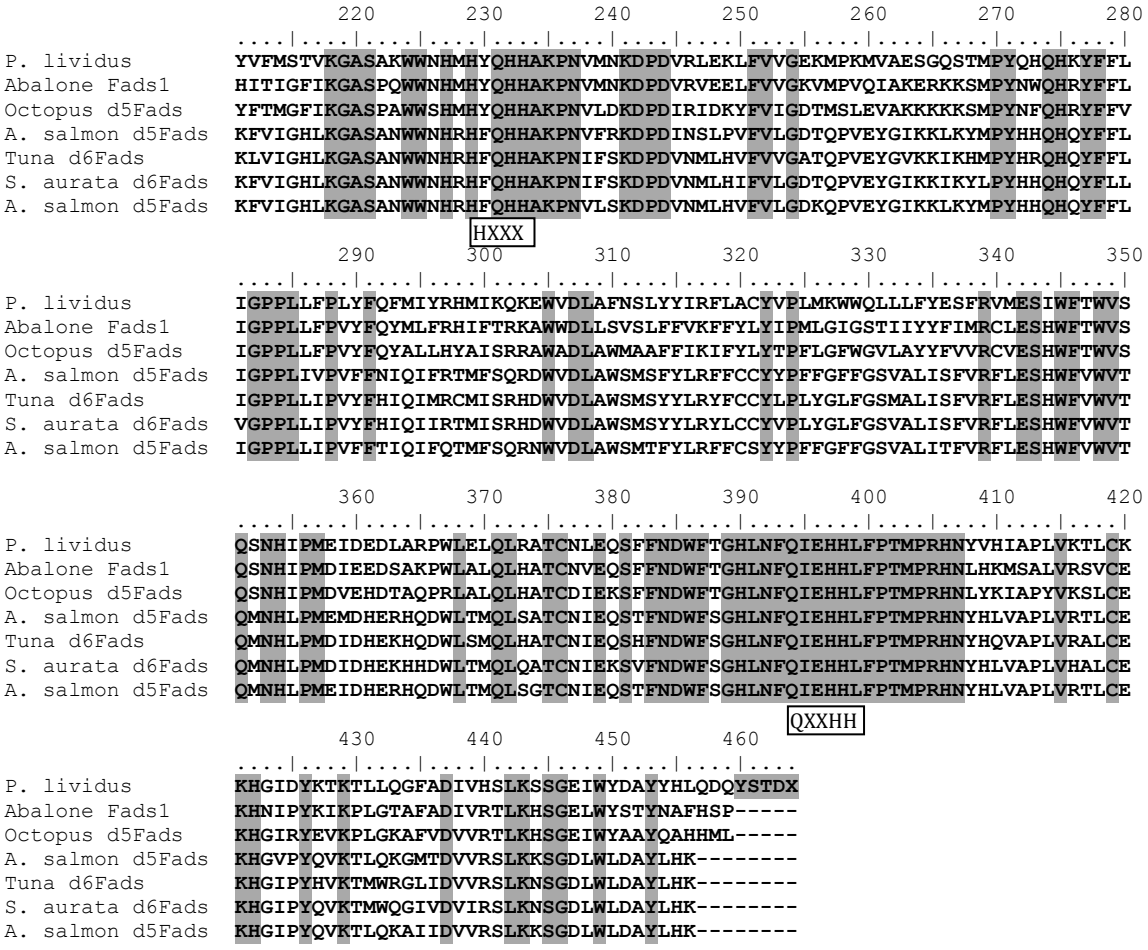

Figure 8.4. Alignment of the deduced amino acid (aa) sequence of the newly cloned putative Δ5-like desaturase of *P. lividus.* Identical residues are shaded black. The cytochrome b5-like domain is dot-underlined and the three histidine boxes (HXXXH, HXXHH and QXXHH) are highlighted. The asterisks mark the heme-binding motif, HPGG

8.5 Discussion

Three ESTs for putative Δ5-like fatty acyl desaturases were identified in *P. lividus* by interrogation of databases. Phylogenetic analysis showed that one of them (*P. lividus* d5fads a) was closely related to *Octopus vulgaris* Δ5-like fatty acyl desaturase, which was shown to be capable of the desaturation of both saturated fatty acids and PUFA (Monroig et al., 2012). This EST was therefore chosen for the generation of full-length sequences. The newly cloned putative desaturase of *P. lividus* possessed all typical features of fatty acyl desaturases, reflecting that these enzymes have conserved functional domains during evolution (Sperling et al. 2003). 5b Fad was composed by two ESTs, which have shown high similarity with differences ascribed to sequencing errors and this is, therefore, here considered as a single putative Fad. 5c Fad, instead, presented a single EST, which did not align with neither of 5b ESTs. These are here considered as two distinct fatty acyl desaturases. Both fads (5b and 5c) of *P. lividus* are, moreover, closely related to Acorn worm fads2 and have not been investigated here.

Because of time constraints this investigation had to be terminated at this point. However, functional characterisation by heterologous expression in *Saccharomyces cerevisiae* will be required in order to identify whether the newly cloned protein has the ability to perform desaturation of fatty acids and, by doing so, identify whether *P. lividus* has the ability to utilise shorter chain C18 fatty acids to satisfy LC-PUFA requirements. The identification of a functional fatty acyl desaturase in sea urchin will provide important information of the evolution of this metabolic function in the marine environment and potentially clarify the metabolic pathway through which LC-PUFA are synthesised by marine invertebrates.

Chapter 9 - Summary of the main findings

In this section the main findings of each research chapter are summarised:

Chapter 4: *Effects of dietary microalgae on growth, survival and fatty acid composition of sea urchin, Paracentrotus lividus, throughout larval development*

- Urchin larvae showed improved survival and enhanced growth when fed *C. elongata*
- Urchin larvae fed *T. suecica* stalled at the four arms stage
- LC-PUFA accumulated in larvae
- Higher EPA/DHA and EPA/ARA ratios may be associated with improved larval performance

Chapter 5: *Evaluation of flow through culture technique for sea urchin (Paracentrotus lividus larvae commercial production*

- Urchin larvae showed improved survival when reared under flow-through conditions
- Water quality was not responsible for the observed difference in survival rate
- It is still not clear if reduced handling or daily feeding had produced the observed improvement in larval performances

Chapter 6: *Influence of broodstock diet on somatic growth, fecundity, gonads carotenoids and larval survival of sea urchin*

- Higher protein content improved somatic growth in *P. lividus* adults

- Expensive, protein-, lipid- and energy-rich diets failed to increase fecundity or enhance offspring performance
- The present study highlighted the need for a specifically formulated broodstock diet and gave insights to what its composition should be, especially in relation to carotenoids.
- However, an ideal sea urchin broodstock diet, able to improve hatchery outputs beyond that obtained with brown kelp, remains to be developed.

Chapter 7: *Fatty acid profiles during gametogenesis in sea urchin (Paracentrotus lividus): Effects of dietary inputs on gonad, egg and embryo profiles:*

- Detailed description of the evolution of fatty acid profiles of *P. lividus* gonads during gametogenesis is given for the first time.
- Among LC-PUFA, EPA and DHA are primarily accumulated during gametogenesis.
- ARA appears to be independent of dietary input.
- ARA is the only LC-PUFA clearly accumulated in the eggs along with NMI FA.

Chapter 8: *Identification of fatty acyl desaturases of Paracentrotus lividus:*

- Three ESTs for putative Δ5-like fatty acyl desaturases were identified
- One was closely related to *Octopus vulgaris* Δ5-like fatty acyl desaturase
- The newly cloned putative desaturase of *P. lividus* possessed all typical features of other fatty acyl desaturases

Chapter 10 - General Discussion

Depletion of wild stocks of echinoids due to over-fishing and the constantly growing market demand for good quality sea urchin products, highlight the importance of echiniculture in bridging the gap between demand and supply of this niche but valuable commodity. Nonetheless, several technical bottlenecks need to be overcome before aquaculture can significantly contribute to sea urchin world production.

The overarching aim of the present project was the identification of operational protocols that would improve *P. lividus* juvenile production within a commercial setting. To this end, two main research lines were investigated. The first focused on larval rearing procedures and the identification of more suitable larval feed and rearing methods (Chapters 4 and 5). The second attempted to identify the most important characteristics that a formulated feed should possess in order to improve broodstock performance (fecundity) and enhance off-spring survival (Chapter 6). Fatty acid profiles of gonads during the gametogenic cycle were also investigated in order to understand how fatty acids are utilised and transferred to the eggs and developing embryos (Chapter 7). Finally, results from these experiments prompted an attempt to identify the genes involved in LC-PUFA biosynthesis in order to clarify whether sea urchin have the ability to utilise shorter chain fatty acids to satisfy essential fatty acid requirements (Chapter 8).

The context in which this research project was conducted was deliberately limited to a focus on hatchery operations, and by no means intended to suggest that no other challenges exist in the scaling up of sea urchin aquaculture operations. It is, however, considered highly relevant in tackling challenges related with a key component of the production cycle and the work was carried out within a commercial environment.

Nonetheless, although often providing practical solutions to the identified problems, the main limitation inherent to such experimentation was the limited focus on the underlying physiological mechanisms responsible for the observed biological effects, or to verify how these effects could impact the production of market size juveniles. A clear example of this is represented by the experiments described in Chapter 4, published in the scientific literature (Aquaculture, 2012, 324-325: 250-258) where it was demonstrated that larvae performances could be significantly improved providing alternative microalgae species readily accepted by the larvae and with a more balanced fatty acid profile. It was, moreover, demonstrated that the new microalgae positively affected development significantly reducing the delicate larval period. Although this study clearly demonstrated improved performance of plutei fed *C. elongata* and *P. carterae*, it could only suggest that fatty acids were responsible for the observed differences. Moreover, the study was unable to conclusively identify the reasons behind the failed development of larvae fed *T. suecica*. A new study, which characterises the other nutritional properties of the tested microalgae, should be carried out in order to identify other potential positive effects of their use and to understand the reasons for the observed lack of development in larvae fed *T. suecica.* As this species is commonly used for larval rearing of other invertebrates such as the Native oyster (*Ostrea edulis*) and the pacific oyster (*Crassostrea gigas*), it would be interesting to determine whether this microalga species contains anti-nutritional factors that would affect sea urchin growth and development or whether its relatively low buoyancy would limit availability to the feeding larvae. Furthermore, the design of new microencapsulated diets able to overcome palatability issues, possibly via to the incorporation of microalgal exudates, may open up the possibility of designing diets aimed at investigating specific nutritional requirements of larvae.

In addition, microalgae mixtures able to provide a more balanced nutritional profile than mono-species feeds surely represent the next step in the identification of refined larval rearing protocols.

Likewise, results obtained in the experiment described in Chapter 5 and published in the peer-reviewed literature (Aquaculture Research 2012, 1-5; DOI: 10.1111/are.12019), showed the advantages of a flow-through system for commercial rearing of echinoplutei and clearly indicated a practical route to improve hatchery output. However, as no significant differences were observed in water quality between the two treatments, doubts still remain concerning the role that the feeding regime (every 3rd day vs. daily) might have played in the observed results. Further studies should be carried out to clarify whether other water quality parameters affected larvae performance or, in contrast, whether the more regular feed supply indeed did significantly contribute to the observed difference in larval survival.

Overall, the main limitation of these two studies was the fact that it was not possible to follow the post-metamorphic development of the larvae produced in all the different treatments as juveniles and not competent larvae are the commercial output of a hatchery. It is entirely possible, in fact, that whilst a given microalgae species might promote survival up to competence for settlement, another one might promote post-metamorphic survival or early juvenile growth. Too little is known about the specific nutritional requirements promoting survival post-metamorphosis and future studies should focus on this important aspect of the production cycle.

The vast majority of studies on *P. lividus* nutrition focused on the effects of formulated feeds on the gonad characteristics with the aim to improve product marketability. At the start of this PhD project, very little was known about the

nutritional requirements of broodstock and the relationship between broodstock nutrition and offspring performance. The availability of broodstock diets is a recurrent problem in most aquaculture species and the experiment described in Chapter 6 (Accepted by Aquaculture Research) evaluated the effects of high protein, energy-rich diets on *P. lividus* reproduction, somatic and gonadal growth and, ultimately, on off-spring survival. Data showed that, although such diets improved somatic growth, they had a limited or no effect on the characteristics that would be important for broodstock such as fecundity and gamete quality. Fecundity was in fact lower than in the urchins fed the control diet (brown kelp), and larval survival was not significantly improved. It should be emphasised that the diets used in this experiment were not specifically developed to improve broodstock or offspring performances. Indeed, the study described the effects of diets formulated for the grow-out stage on broodstock fecundity and larval survival, and compared them with urchins fed the main item constituting their natural diet (kelp). As such, this experiment could not properly investigate the nutritional requirements of *P. lividus* broodstock. Indeed the use of commercial feed, although still in the developing phase, prevented the investigation of the effects of each nutrient on broodstock performances and this represented the main limitation of this trial. More than one nutrient, in fact, varied between the formulated feeds used in this experiment. Nonetheless, its very applied nature allowed us to conclude that formulated feeds, designed to maximise growth are not ideal for broodstock performances improvement highlighting, therefore, the need for the development and production of broodstock-specific diets. Indeed, the complete absence of Xanthophylls and the significantly reduced β-carotene content in the formulated feeds is potentially the main responsible for the observed reduced fecundity in *P. lividus* broodstock as

suggested by George et al. (2001). This should therefore be taken in consideration in the formulation of broodstock-specific diets.

In order to understand fatty acid metabolism of *P. lividus* and the relative importance of particular fatty acids for gonad maturation and gamete development, a further study investigated the FA profiles of the gonads of urchin fed an experimental and a natural diet during gametogenesis. The study also evaluated the transfer of fatty acids from diet to gonads and from gonads to eggs. Some indication of FA utilisation during embryogenesis was also provided and discussed in Chapter 7, and published (Comparative Physiology and Biochemistry Part A. 2012. 164: 376-382). During this study, it was observed that, when available in the diet, EPA and DHA were accumulated during gametogenesis, whilst ARA levels appeared to remain constant throughout gametogenesis and more independent of dietary input. The latter observation suggested that the level of ARA contained in the formulated feed was possibly greater than the physiological requirements of *P. lividus* and that the lower ARA content of the kelp diet did not affect its final concentration in the gonads. In contrast, the accumulation of DHA during gametogenesis in urchins fed a relatively high DHA diet may suggest that higher dietary inputs of this FA might be beneficial in broodstock feeds. However, a low level of DHA was observed in urchin fed the kelp diet despite this FA being absent or below instrumental detection limit in kelp. This may simply reflect a selective and highly efficient retention and/or accumulation of this fatty acid in the gonads, but may also suggest that *P. lividus* has some capability for endogenous biosynthesis of LC-PUFA from shorter chain dietary precursors. From a practical point of view, further trials should contain feeds with serially increased DHA concentrations to levels higher than that used in the present trial. This would enable the upper threshold, above which this fatty acid is not further

accumulated in the gonads, to be determined. The hypothesis of endogenous elongation and desaturation of shorter chain fatty acids in sea urchins to produce LC-PUFA is very interesting and warrants further confirmation. It would be very relevant to verify if this endogenous activity was indeed present in *P. lividus* and determine its stoichiometry in order to properly evaluate if cheaper, more sustainable ingredients providing LC-PUFA precursors could be employed in formulated feeds instead of using more expensive and unsustainable marine origin ingredients providing high concentration of LC-PUFA.

This thesis also confirmed that urchin gonad biochemistry and composition could be influenced by the diet. This information, combined with the indication that LC-PUFA might promote larval survival (Chapter 4), could be of particular relevance in developing broodstock diets able to improve gonad and egg LC-PUFA profiles, especially DHA. Furthermore, these data will help to verify whether off-spring derived from broodstock fed such diets could indeed benefit from increased DHA content in very early developmental stages. Interestingly, this thesis also reported the presence of unusual fatty acids (NMI FA) in *P. lividus* tissues. As previously mentioned, we do not know the precise biological role(s) of these FAs and further studies to investigate the biosynthesis and functions of NMI FA should be carried out.

Some initial studies were performed in the latter stages of this PhD project in an effort to investigate the more fundamental question concerning endogenous ability of LC-PUFA biosynthesis in *P. lividus* (Chapter 8). This preliminary work resulted in the identification of three ESTs for putative fatty acyl desaturases that possessed all structural sequence features of this group of proteins and were phylogenetically related with Δ5- fatty acyl desaturase homologues of other invertebrates (*O. vulgaris* and *Haliotis* sp.). The full-length cDNA and amino acid sequence was obtained for one

of the EST fragments. Unfortunately, there was no time available to take this investigation further to its logical conclusion and functionally characterise the newly cloned protein. The isolation of desaturases encoding genes and functional characterisation of the protein involved in this bioconversion has, so far, been confirmed in only one commercially relevant marine invertebrate, *Octopus vulgaris* (Monroig et al., 2011). One preliminary study has also suggested that the bivalve *O. edulis* might be able to biosynthetise LC-PUFA (Ross et al., 2010). Moreover, Bell et al. (2001) conclusively showed that adults *P. miliaris* could convert 18:3n-3 to 20:5n-3 and strongly suggested that this species possesses the required genetic apparatus to perform this biosynthesis. The confirmation of sea urchins being able to biosynthetise LC-PUFA via the isolation and functional characterisation of the genes involved, and the investigation of the related metabolic pathways, will help to clarify how this important physiological mechanism has evolved in the marine environment. Moreover, if this hypothesised ability was confirmed, it will have important implications for the understanding of the near shore biogeochemical cycling of fatty acids in which sea urchins play a critical role in re-distributing fatty acids from the benthic to the pelagic environment (Hughes et al., 2011). This would be particularly true in the case of DHA. This FA is, in fact, not measured in kelp fronds but it is regularly observed in sea urchin gonads, gametes and larvae. If the ability of sea urchin to synthetise DHA was to be proven it would mean that these organisms do not only act as passive fatty acids vehicles from the benthic to the pelagic environment and up the food web, but also actively contribute in the production of this extremely important fatty acid in the marine environment.

Several other research lines could have been further explored during this PhD thesis although time restrictions did not allow. For instance, it would have also been

interesting to investigate the effects of different environmental cues on reproduction and their complicated interactions with nutrition. The background knowledge on this topic is confusing and often contradictory and therefore some clarity would be beneficial in order to understand and prioritise the main environmental cues responsible for the onset and/or suppression of gametogenesis or for spawning initiation and synchronisation. Moreover, almost nothing is currently known about the mechanisms by which environmental cues are transduced within the "endocrine/paracrine" system in echinoderms.

From a practical view point, unsynchronised gametogenesis in adult populations held in a commercial environment poses two main problems: the first related with unreliable prediction of which individuals should be used as parents and therefore injected to trigger spawning, and the second related with marketability of the gonads which are of variable market value depending on the gametogenic phase. In *Paracentrotus lividus* culture, these problems are further enhanced in those countries where natural spawning occurs in captivity such as Israel or Italy (H. Rosenfeld and C. Falugi, pers. comm.) and less of a problem in Scotland where lower mean temperatures normally prevents the Effective Accumulated Temperature to be achieved and consequently natural spawning to take place.

It is known that sea urchins can perceive light (Lesser et al., 2011) and there is strong indication that photoperiod is an important cue for maturation. Indeed, day length has been demonstrated to be able to strongly influence gametogenesis in several echinoids species (McClintock and Watts, 1990; Pearse and Cameron, 1991; Kelly, 2001; Shpigel, 2004) and constant long-day photoperiod in combination with formulated feeds has been successfully used to prevent gametogenesis in the echinoid *S. droebachiensis* held in commercial environment (Bottger et al., 2006). It

is, however, not known what effects light intensity or spectrum may have and the potential economic advantages achievable by tailoring the photo-environment on the specific requirements of each species within an aquaculture set-up.

From an ecological viewpoint, it is moreover not clear whether seasonal cues have a direct effect on gametogenesis or if their effect is somehow mediated through the biochemical composition of sea urchin primary feed items (macroalgae). If nutritional composition of the natural diet was indeed able to provide a trigger for the onset of gametogenesis, the cue may be localised in nature and operate on the biochemical composition of macroalgae rather than directly on the sea urchin reproductive axis. Hydrodynamism, for instance, may have the required localised characteristics and still be, by and large, related with seasonality. It could operate through enhanced or depressed gas exchange through kelp blade (Peteiro and Freire, 2011) possibly favouring the shift in biochemical composition of kelp, as large as ten-fold, between winter and summer (Eddy et al., 2012), that in turn might trigger the onset of gametogenesis of those sea urchins feeding on that specific seaweed patch. It is tempting to speculate that such a mechanism could explain the presence of synchronised sub-populations of *P. lividus* as small as a few dozen individuals, or the difference in spawning time between individuals occupying the same geographical area (Lozano et al., 1995; Guetaff, 1997; Sánchez-España et al., 2004). Trials designed to test this hypothesis, firstly investigating the biochemical composition of macroalgae under different hydrodynamic conditions, followed by trials using formulated feeds mimicking the switch in composition, would provide some insight on whether this process is at work and whether it could be a fruitful line of research.

In conclusion, this thesis provides a significant contribution to the ongoing development of sea urchin hatchery techniques. It clearly proposes easily applicable

solutions potentially able to significantly increase juvenile output and, more widely, it contributes in overcoming one of the bottlenecks currently preventing a more extensive utilisation of echinoids in Integrated Multi-Trophic Aquaculture systems such as the reliable supply of sea urchin juveniles. Moreover, it provides an insight into the physiology of the species, describing the evolution of fatty acids profile of the gonads during gametogenesis and their transfer from the diets to the gonads, eggs and embryos. It also further highlights echinoplutei requirements for specific fatty acids and indicates a simple route to meet these, effectively overcoming palatability issues related to micro-encapsulated diets suggested in other studies. This work also opens new interesting research lines into the development of tailor made broodstock diets and, on a more basic level, on the fatty acid metabolism of the species and on its potentially important ecological role as LC-PUFAs provider to the higher levels of the marine food web.

Many questions still remain unanswered; nonetheless the continued effort into the investigation of new aquaculture species, especially those occupying the lower trophic levels, will help to significantly improve the overall economic and environmental sustainability of the whole industry.

References

Ackman, R.G. (1980) Fish lipids. In: Connel, J.J. (ed.) *Advances in Fish Science and Technology*. Farnham: Fishing News Books, p.87-103.

Addis, P., Secci, M., Manunza, A., Corrias, S., Niffoi, A., Cau, A. (2009) A geostatistical approach for the stock assessment of the edible sea urchin, *Paracentrotus lividus*, in four coastal zones of Southern and West Sardinia (SW Italy, Mediterranean Sea). *Fish Res,* 100, p.215-221.

Addison, J.A. and Hart, M.W. (2002) Characterization of microsatellite loci in sea urchins (*Strongylocentrotus spp.*). *Mol Ecol Notes*, 2 (4), p.493–494.

Addison, J.A., and Hart, M.W. (2004) Analysis of population genetic structure of the green sea urchin (*Strongylocentrotus droebachiensis*) using microsatellites. *Mar Biol*, 144, p.243-251.

Agatsuma, Y. (1991) *Strongelocentrotus intermedius* (A. Agassiz). In: Nagasawa, K. and Torisawa, M. (ed.). *Fishes and invertebrates of Hokkaido: Biology and Fisheries*. Sapporo: Kita-Nihon Kaiyo Center Co. Ltd., p.324-329.

Agatsuma, Y., Sakai, Y., Andrew, N.L. (2004) Enhancement of Japan's sea urchin fisheries. In: Lawrence, J.M. and Guzmán, O. (ed.) *Sea urchins: fisheries and ecology*. Lancaster: DEStech Publications, p.18–36.

Akiyama, T., Unuma, T., Yamamoto, T. (2001) Optimum protein level in a purified diet for young red sea urchin, *Pseudocentrotus depressus*. *Fisheries Science*, 67, p.361–363.

Allain, J.Y. (1975) Structure des populations de *Paracentrotus lividus* (Lamarck) (Echinodermata, Echinoidea) soumises à la pêche sur les côtes Nord de Bretagne. *Rev Trav Inst Pêches Marit*, 39 (2), p.171–212.

Andrew, N.L., Agatsuma, Y., Ballesteros, E., Bazhin, A.G., Creaser, E.P., Barnes, D.K.A., Botsford, L.W., Bradbury, A., Campbell, A., Dixon, D.J., Einarsson, S., Gerring, P.K., Hebert, K., Hunter, M., Hur, S.B., Johnson, C.R., Juinio-Menez, M.A., Kalvass P., Miller, R.J., Moreno, C.A., Palleiro, J.S., Rivas, D., Robinson, S.M.C., Schroetr, S.C., Steneck, R.S., Vadas, R.L., Woodby, D.A., Xiaoqi, Z. (2002) Status and management of world sea urchin fisheries. *Oceanogr Mar Biol Annu Review,* 40, p.343-425.

Anisimov, A.P. (1981) Morphological and cytochemical characteristics of the alimentary canal epithelium of sea urchin *Strongylocentrotus intermedius* and *Strongylocentrotus nudus* (Echinodermata: Echinoidea). *Biol Morya*, 3, p.32–42.

AOAC (2000) Official methods of analysis. Association of official analytical chemist. EUA

Axelsson, M. and Farrell, A.P. (1993) Coronary blood flow in vivo in the coho salmon (*Oncorhynchus kisutch*). *Am J Physiol*, 264 (R), p.963 - 971.

Balch, T., Scheibling, R.E. (2000) Temporal and spatial variability in settlement and recruitment of echinoderms in kelp beds and barrens in Nova Scotia. *Mar Ecol Prog Ser*, 205, p.139-154.

Barker, M., Keogh, J., Lawrence J., Lawrence A. (1998) Feeding rate, absorption efficiencies, growth and enhancement of gonad production in the New Zealand sea urchin, *Evechinus chloroticus* Valeniennes (Echinoidea), fed prepared and natural diets*. J Shellfish Res*, 17 (5), p.1582–1590.

Barker, M.F. (1985) Reproduction and development of *Goniocidaris unmraculum*, a brooding echinoid. In: Keegan, B.F. and O'Connor, B.D.S. (ed.) *Echinodermata*., Rotterdam: A. A. Balkema, p.207-214.

Barnathan, G. (2009) Non-methylene-interrupted fatty acids from marine

invertebrates: Occurrence, characterization and biological properties. *Biochimie*, 91, p.671–678.

Baron-Taltre., T. (2005) *The Rise and Fall of the Maine Sea Urchin Industry: A Failure of Management and Institutions?* Master of Science (MS) Theses. Available from: http://digitalcommons.library.umaine.edu/etd/1488. Orono, Maine: University of Maine.

Bay-Schmith, E. and Pearse, J.S. (1987) Effect of fixed day lengths on the photoperiodic regulation of gametogenesis in the sea urchin *Strongylocentrotus purpuratus*. *Int J Invert Repro Develop*, 11, p.287–294.

Becker, P.T., Egea, E., Eeckhaut, I. (2008) Characterization of the bacterial communities associated with the bald sea urchin disease of the echinoid *Paracentrotus lividus. J Invertebr Pathol*, 98 (2), p.136–147.

Beddingfield, S.D. and McClintock, J.B. (2000) Demographic characteristics of *Lytechinus variegatus* (Echinoidea: Echinodermata) from three habitats in a North Florida Bay, Gulf of Mexico. *Mar Ecol PSZN*, 21, p.17–40.

Bell, J.G., McVicar, A.H., Park, M.T., Sargent, J.R. (1991a) High dietary linoleic acid affects the fatty acid compositions of individual phospholipids from tissues of Atlantic salmon (Salmo salar): association with stress susceptibility and cardiac lesions*. J Nutr*, 121, p.1163-1172.

Bell, J.G., Raynard, R.S., Sargent, J.R. (1991b) The effect of dietary Linoleic acid on the fatty acid composition of individual phospholipids and lipoxygenase products from gills and leucocytes of Atlantic salmon (*Salmo salar*). *Lipids,* 26, p.445-450.

Bell, M.V., Dick, J.R., Kelly, M.S. (2001) Biosynthesis of eicosapentaenoic acid in sea urchin *Psammechinus miliaris*. *Lipids,* 36, p.79-92.

Bell, M.V., Tocher R.D. (2009) Biosynthesis of Polyunsaturated Fatty Acids in Aquatic Ecosystems: General Pathways and New Directions. M.T. Arts et al. (eds.), *Lipids in Aquatic Ecosystems*. p. 211-236

Bendich, A. (1994) Recent advances in clinical research involving carotenoids. *Pure Appl Chem*, 66, p.1017–1024.

Benedetti-Cecchi, L. and Cinelli, F. (1995) Habitat heterogeneity, sea urchin grazing and the distribution of algae in littoral rock poolson the west coast of Italy (western Mediterranean). *Mar Ecol Prog Ser*, 126, p.203-212.

Bennett, J., Mazia, D. (1981a) Interspecific fusion of sea urchin eggs. Surface events cytoplasmic mixing. *Exp Cell Res*, 131, p.197–207.

Bennett, J., Mazia, D. (1981b) Fusion of fertilized and unfertilized sea urchin eggs. Maintenance cell surface integrity. *Exp Cell Res*, 134, p.494–498.

Bernárdez, C., Freire, J., González-Gurriarán, E. (2000) Feeding of the spider crab *Maja squinado* in rocky subtidal areas of the Ría de Arousa (north-west Spain). *J Mar Biol Ass UK*, 80, p.95–102.

Bertheussen, K. (1981) Endocytosis by echinoid phagocytes in vitro. II. Mechanisms of endocytosis. *Dev Comp Immunol*, 5, p.557-564.

Blair, J.E., and Hedges, S.B. (2005) Molecular phylogeny and divergence times of deuterostome animals. *Mol Biol Evol*, 22, p.2275-2284.

Bone, Q. (1998) *The biology of pelagic tunicates*. Oxford: Oxford University Press.

Bottger, S.A., Devin, M.G., Walker, C.W. (2006) Suspension of annual gametogenesis in North American green sea urchins (*Strongylocentrotus droebachiensis*) experiencing invariant photoperiod - Applications for land-based aquaculture. *Aquaculture*, 261 (4), p.1422-1431.

Böttger, S.A., Devin, M.G., Walker, C.W. (2006) Suspension of annual gametogenesis in North American green sea urchins (*Strongylocentrotus droebachiensis*) experiencing invariant photoperiod - Applications for land-based aquaculture. *Aquaculture,* 261, p.1422-1431.

Böttger, S.A., Eno, C.C., Walker, C.W. (2011) Methods for generating triploid green sea urchin embryos: an initial step in producing triploid adults for land-based and near-shore aquaculture*. Aquaculture*, 318, p.199–206.

Boudouresque, C.F., Caltagirone, A., Lefèvre, J.R., Rico, V., Semroud, R. (1992) Macro-zoobenthos de la reserve naturelle de Scandola (Corse, Méditerranée nord-occidentale). Analyse pluriannuelle de "l'effet réserve". In: Olivier, J., Gerardin, N., Jeudy de Grissac, A. (ed.) *Economic impact of the Mediterranean coastal protected areas*. Medpan, Fr: Hyeres, p.15–20.

Boudouresque, C.F., Veralque, M., Azzolina, J.F., Meinesz, A., Nedelec, H., Rico, V. (1989) Evolution des population de *Paracentrotus lividus* et d'*Arbacia lixula* (Echinoidea) le long d'un transect permanent a Galeria (Corse). *Trav Sci Parc Nat Reg Nat Corse*, 22, p.65-82.

Boudouresque, C.F. and Verlaque, M. (2008) Ecology of *Paracentrotus lividus*. In: Lawrence, J. M. (ed.) *Sea Urchin: Biology and Ecology*. Amsterdam: Elsevier science B.V, p.243-285.

Boudrouesque, C.F. and Veralque, M. (2007) Ecology of *Paracentrotus lividus*. In: Lawrence, J.M. (ed.) *Edible sea urchin biology and ecology*. Amsterdam – London - New York – Oxford- Paris- Shannon-Tokyo: Elsevier Science, p.243-285.

Bourlat, S.J., Juliusdottir, T., Lowe, C.J., Freeman, R., Aronowicz, J., Kirschner, M., Lander, E.S., Thorndyke, M., Nakano, H., Kohn, A.B., Heyland, A., Moroz, L.L., Copley, R.R., Telford, M.J. (2006) Deuterostome phylogeny reveals

monophyletic chordates and the new phylum Xenoturbellida. *Nature*, 444, p.85-88.

Braley, R.D. (1993) Pros and cons of methodologies used in the hatchery and land-based nursery phase of giant clam culture. In: Fitt, W.K. (ed.) *Biology and Mariculture of Giant Clams*. Canberra: ACIAR, p.87-93.

Branthan, G. (2009) Non-methylene-interrupted fatty acids from marine invertebrates: Occurrence, characterization and biological properties. *Biochimie*, 91, p.671-678.

Britton, G., Liaaen-Jensen, S., Pfander, H. (2004) *Carotenoids: Handbook.* Birkhäuser, Basel.

Bromham, L.D., Degnan, B.M. (1999) Hemichordates and deuterostome evolution: robust molecular phylogenetic support for a hemichordate + echinoderm clade. *Evol Dev*, 1 (3), p.166-171.

Brooks, J.M. and Wessel, G.M. (2002) The major yolk protein in sea urchins is a transferrin-like, iron binding protein. *Dev Biol*, 245, p.1–12.

Brooks, J.M. and Wessel, G.M. (2004) The major yolk protein of sea urchins is endocytosed by a dynamin-dependent mechanism. *Biol Reprod*, 71, p.705–713.

Buchanan, J.B. (1969) Feeding and the control of volume within the tests of regular sea urchins. *J Zool Lond*, 159, p.51–64.

Buitrago, E., Lodeiros, C., Lunar, K., Alvarado, D., Indorf, F. (2005) Mass production of competent larvae of the sea urchin *Lytechinus variegatus* (Echinodermata: Echinoidea). *Aquacult Int*, 13, p.359–367.

Burke, R.D. (1980) Podial sensory receptors and the induction of metamorphosis in echinoids. *J Exp Mar Biol Ecol*, 47, p.223–234.

Burke, R.D. (1981) Structure of the digestive tract of the pluteus larva of *Dentraster*

excentricus (Echinodermata, Echinoidea). *Zoomorphology*, 98, p.209-225.

Burke, R.D. (1987) Echinoderm metamorphosis: comparative aspects of the change in form. In: Jangoux, M. and Lawrence, J.M. (ed.) *Echinoderm studies, Vol.3*. Rotterdam: Balkema, p.81–108.

Byrne, M. (1990) Annual reproductive cycles of the commercial sea urchin *Paracentrotus lividus* from an exposed intertidal and a sheltered habitat on the west-coast of Ireland. *Mar Biol*, 104, p.275–289.

Byrne, M. (1990) Annual reproductive cycles of the commercial sea urchin *Paracentrotus lividus* from an exposed intertidal and a sheltered subtidal habitat on the west coast of Ireland. *Mar Biol,* 104, p.275-289.

Byrne, M., Sewell, M.A., Prowse, T.A.A. (2008) Nutritional ecology of sea urchin larvae: influence of endogenous and exogenous nutrition on echinopluteus growth and phenotypic plasticity in *Tripneustes gratilla*. *Funct Ecol* 22, p.643–648.

Cabral de Oliveira, M. (1991) Survival of seaweeds ingested by three species of tropical sea urchins from Brazil. *Hydrobiologia*, 222, p.13–17.

Calderón, I., Turon, X., Pascual, M. (2009) Isolation of nine nuclear microsatellites in the common Mediterranean sea urchin, *Paracentrotus lividus* (Lamarck). *Mol Ecol Resour*, 9 (4), p.1145-1147.

Cameron, C.B., Garey, J.R. and Swalla, B.J. (2000) Evolution of the chordate body plan: new insights from phylogenetic analyses of deuterostome phyla. *Proc Natl Acad Sci USA*, 97, p.4469–4474.

Carboni, S., Vignier, J., Chiantore, M., Tocher, D.R., Migaud, H. (2012) Effects of dietary microalgae on growth, survival and fatty acid composition of sea urchin,

Paracentrotus lividus, throughout larval developemnt. *Aquaculture*, 324-325, p.250-258.

Carcamo, P.F., Candia, A.I., Chaparro, O.R. (2005) Larval development and metamorphosis in the sea urchin *Loxechinus albus* (Echinodermata: Echinoidea): Effects of diet type and feeding frequency. *Aquaculture*, 249, p.375–386.

Carmona-Antoñanzas, G., Monroig, Ó., Dick, J.R., Davie, A., Tocher, D.R. (2011) Biosynthesis of very long-chain fatty acids (CN24) in Atlantic salmon: Cloning, functional characterisation, and tissue distribution of an Elovl4 elongase. *Comp Biochem Physiol*, *Part B*, 159, p.122–129.

Castell, J.D., Kennedy, E.J., Robinson, S.M.C., Parsons, G.J., Blair, T.J., Gonzalez-Duran, E. (2004) Effect of dietary lipids on fatty acid composition and metabolism in juvenile green sea urchins (*Strongylocentrotus droebachiensis*). *Aquaculture*, 242 (1-4), p.417-435.

Chang, Y.Q., Lawrence, J.M., Cao, X.B., Lawrence, A.L. (2005) Food consumption, absorption, assimilation and growth of the sea urchin *Strongylocentrotus intermedius* fed a prepared feed and the algal *Laminaria japonica*. *J World Aquacult Soc*, 36 (1), p.68–75.

Cherbonnier, G. (1956) Les Echinodermes de Tunisie. *Bull Stat Océanogr Salammbô*, 53, p.1–23.

Christie, W.W. (2003) Preparation of derivates of fatty acids. In: W.W. Christie (ed.), *Lipid Analysis: isolation, separation and structural analysis of lipids*, 3rd ed. Bridgewater: J. Barnes and Associates, p.205-225.

Claereboudt, M. and Jangoux, M. (1985) Conditions de digestion et activité de quelques polysaccharidases dans le tube digestif de l'oursin *Paracentrotus*

lividus (Echinodermata). *Biochem Syst Ecol*, 13, p.51–54.

Clarke, K.R. and Warwick, R.M. (1994) Change in Marine Communities: an Approach to Statistical Analysis and Interpretation. Plymouth Marine Laboratory, Plymouth.

Cochran, R.C. and Engelmann, F. (1972) Echinoid spawning induced by a radial nerve factor. *Science*, 178, p.423–424.

Coffaro, K.A. and Hinegardner, R.T. (1977) Immune response in the sea urchin *Lytechinus pictus*. *Science*, 197, p.1389-1390.

Colombo, L., Belvedere, P. (1976) Gonadal steroidogenesis in echinoderms. *Gen Comp Endocrinol*, 29, p.255–256.

Cook, E.J. and Kelly, M.S. (2007) Effect of variation in the protein value of the red macroalga *Palmaria palmata* on the feeding, growth and gonad composition of the sea urchins *Psammechinus miliaris* and *Paracentrotus lividus* (Echinodermata). *Aquaculture*, 270, p.207–217.

Cook, E.J., Bell, M.V., Black, K.D., Kelly, M.S. (2000) Fatty acid composition of gonadal material and diets of the sea urchin, *Psammechinus miliaris*: trophic and nutritional implications. *J Exp Mar Biol Ecol*, 255, p.261-274.

Cook, E.J., Kelly, M.S., Mckenzie, J.D. (1998) Somatic and gonadal growth of the urchin *Psammechinus miliaris* (Gmelin) fed artificial salmon feed compared with a macroalgal diet. *J Shellfish Res*, 17 (5), p.1549–1555.

Crapp, G.B., Willis, M.E. (1975) Age determination in the sea urchin *Paracentrotus lividus* (Lamarck) with notes on the reproductive cycle. *J Exp Mar Biol Ecol*, 20, p.157–178.

Crook, A.C., Long, M., Barnes, D.K.A. (2000) Quantifying daily migration in the sea urchin *Paracentrotus lividus. J Mar Biol Assn UK*, 80, p.177–178.

Daggett, T.L., Pearce, C.M., Tingley, M., Robinson, S.M.C., Chopin, T. (2005) Effect of prepared and macroalgal diets and seed stock source on somatic growth of juvenile green sea urchins (*Strongylocentrotus droebachiensis*). *Aquaculture*, 244, p.263–281.

Daggett, T.L., Pearce, C.M., Tingley, M., Robinson, S.M.C., Chopin, T. (2005) Effect of prepared and macroalgal diets and seed stock source on somatic growth of juvenile green sea urchins (*Strongylocentrotus droebachiensis*). *Aquaculture*, 244, p.263–281.

Dale, B. and Monroy, A. (1981) How is polyspermy prevented? *Gam Res.*, 4, p.151–169.

Darszon, A., Guerrero, A., Galindo, B.E., Nishigaki, T., Wood, C.D. (2008) Sperm-activating peptides in the regulation of ion fluxes, signal transduction and motility. *Int J Dev Biol*, 52, p.595–606.

Darszon, A., Nishigaki, T., Beltran, C., Trevino, C.L. (2011) Calcium channels in the development, maturation, and function of spermatozoa. *Physiol Rev*, 91, p.1305–1355.

Davidson, E.H. (1987) *Gene Activity in Early Development, 3rded*. New York: Academic Press, p.670.

Dayton, P.K., Tegner, M.J., Edwards, P.B., Riser, K.L. (1998) Sliding baselines, ghosts and reduced expectations in kelp forest communities. *Ecol Appl*, 8, p.309-322.

De Jong Westman, M., Qian, P.Y., March, B.E., Carefoot, T.H. (1995) Artificial diets in sea urchin culture: Effects of dietary protein level and other additives on egg quality, larval morphometrics, and larval survival in the green sea urchin, *Strongylocentrotus droebachiensis. Canadian Journal of Zoology - Revue Canadienne de Zoologie*, 73 (11), p.2080-2090.

de Jong-Westman, M., Qian, P., March, B.E., Carefoot, T.H. (1995) Artificial diets in sea urchin culture: effects of dietary protein level and other additives in the green sea urchin, *Strongylocentrotus droebachiensis*. *Can J Zool*, 73, p.2080–2090.

De Ridder, C. and Foret, T. (2001) Non-parasitic symbioses between echinoderms and bacteria. *Echino Stud*, 6, p.111–169.

De Ridder, C. and Jangoux, M. (1982) Digestive systems: Echinoidea. In: Jangoux, M. and Lawrence, J.M. (ed.) *Echinoderm nutrition*. Rotterdam: Balkema, p.213–234.

Delmas, P. (1992) Etude de population de *Paracentrotus lividus* (Lam.) (Echinodermata: Echinoidea) soumises a une pollution complexe en Provence nord-occidentale: densites. structure, processus de detoxication (Zn, Cu, Pb, Fe). These Doct Univ Aix-Marseille III.

Delmas, P. and Régis, M.B. (1986) Données préliminaires sur le contenu digestif de l'oursin comestible *Paracentrotus lividus* (Lamarck) soumis à l'influence d'effluents domestiques. *Mar Environ Res*, 20, p.197–220.

Delsuc, F., Brinkmann, H., Chourrout, D., Philippe, H. (2006) Tunicates and not cephalochordates are the closest living relatives of vertebrates. *Nature*, 439, p.965-968.

Doherty, P.J. (1999) Recruitment limitation is the theoretical basis for stock enhancement in marine populations. In: Howell, B.R., Moksness, E., Svasand, S. (ed.) *Stock enhancement and sea rancing.* Oxford: Fishing News Books, p.9-21.

Duggan, R.E. and Miller R.J. (2001) External and internal tags for the green sea urchin. *J Exp Mar Biol Ecol*, 258 (1), p.115-122.

Dworjanyn, S.A., Pirozzi, I., Liu, W. (2007). The effect of the addition of algae feeding stimulants to artificial diets for the sea urchin *Tripneustes gratilla*. *Aquaculture*, 273, p.624–633.

Ebert, T.A., Dixon, J.D., Schroeter, S.C., Kalvass, P.E., Richmond, N.T., Bradbury, W.A., Woodby, D.A. (1999) Growth and mortality of red sea urchins *Strongylocentrotus franciscanus* across a latitudinal gradient. *Mar Ecol-Prog Ser*, 190, p.189-209.

Ebert, T.A., Russell, M.P. (1992) Growth and mortality estimates for red-sea urchin S*trongylocentrotus-franciscanus* from San Nicolas Island, California. *Mar Ecol-Prog Ser*, 81 (1), p.31-41.

Eddy, S.D., Brown, N.P., Kling, A.L. Watts, S.A., Lawrence, A. (2012) Growth of Juvenile Green Sea Urchins, *Strongylocentrotus droebachiensis*, Fed Formulated Feeds with Varying Protein Levels Compared with a Macroalgal Diet and a Commercial Abalone Feed. *J World Aquacult Soc*, 43 (2), p.159-173.

Emlet, R.B. (1986) Facultative planktotrophy in the tropical echinoid *Clypeaster rosaceus* and a comparison with obligate planktotrophy *Clypeaster subdepressus*. *J Exp Mar Bio Ecol*, 95, p.183-202.

Emlet, R.B., McEdward, L.R., Strathmann, R.R. (1987) Echinoderm larval ecology viewed from the egg. *Echinoderm Stud,* 2, p.55-136.

Eppley, R.W. and Lasker, R. (1959) Alginase in the sea urchin, *Strongylocentrotus purpuratus*. *Science*, 129, p.214–215.

Evans, J.P., Marshall, D.J. (2005) Male-by-female interactions influence fertilization success and mediate the benefits of polyandry in the sea urchin *Heliocidaris Erythrogramma*. *Evolution*, 59 (1), p.106-112.

Fabbrocini, A. and D'Adamo, R. (2010) Gamete Maturation and Gonad Growth in Fed and Starved Sea Urchin *Paracentrotus lividus* (Lamarck, 1816). *J Shellfish Res*, 29 (4), p.1051-1059.

Farmanfarmaian, A. and Phillips, J.H. (1962) Digestion, storage, and translocation of

nutrients in the purple sea urchin (*Strongylocentrotus purpuratus*). *Biol Bull*, 123, p.105–120.

Fenaux, L. (1968) Maturation des gonades et cycle saisonnier des larves chez *A. lixula*, *P. lividus* et *P. microtuberculatus* (Echinides) à Villefranche-sur-Mer. *Vie Milieu*, 19 (A1), p.1–52.

Fenaux, L., Strathmann, M.F., Strathmann, R.R. (1994) 5 tests of food-limited growth of larvae in coastal waters by comparisons of rates of development and form of echinoplutei. *Limnol Oceanogr*, 39, p.84-98.

Fernandez, C. (1990) *Recherches preliminaires a la mise en place d'un pilote d'aquaculture de l'oursin Paracentrotus lividus dans un etang corse*. Dipl Etudes approve Oceanol, Univ. Aix-Mareilles II.

Fernandez, C. (1996) *Croissance et nutrition de Paracentrotus lividus dans le cadre d'un project aquacole avec alimentation artificielle*. These Doct Oceanol, Univ. Corse.

Fernandez, C. (1997) Effect of diet on the biochemical composition of *Paracentrotus lividus* (Echinodermata: Echinoidea) under natural and rearing conditions. *Comp Biochem Physiol*, 118, p.1377-1384.

Fernandez, C. and Boudouresque, C.F. (1997) Phenotypic plasticity of *Paracentrotus lividus* (Echinodermata: Echinoidea) in a lagoonal environment. *Mar Ecol-Progr Ser*, 152, p.145–154.

Fernandez, C. and Boudouresque, C.F. (2000) Nutrition of the sea urchin *Paracentrotus lividus* (Echinodermata: Echinoidea) fed different artificial food. *Mar Ecol-Prog Ser*, 204, p.131–141.

Fernandez, C., Pasqualini, V., Johnson, M., Ferrat, L., Caltagirone, A., Boudouresque, C.F. (2003) Stock evaluation of the sea urchin *Paracentrotus lividus* in a

lagoonal environment. In: Féral, J.P., David, B. (ed.) *Echinoderm research 2001*. Lisse: AA Balkema, p.319–323.

Folch, J., Lees, M., Sloane-Stanley, G.H. (1957) A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem*, 226, p.497-509.

Fong, W. and Mann, K.H. (1980) Role of gut flora in the transfer of amino acids through a marine food chain. *Can J Fish Aquat Sci*, 37, p.88–96.

Fowler, J. and Cohen, L. (1987) Statistics for ornithologist. BTO Guide 22. Tring, UK.

Frantiz, A., Berthon, J.F., Maggiore, F. (1988) Relations trophiques entre les oursins *Arbacia lixula* et *Paracentrotus lividus* (Echinoidea regularia) et le phytobenthos infralittoral superficiel dans la baie de Port-Cros (Var, France). *Sci Rep Prot-Cros Nation Park*, 14, p.81-140.

Fuji, A. (1961) Studies on the biology of the sea urchin IV. Histological observation of the food canal of *Strongylocentrotus intermedius*. *Bull Fac Fish Hokkaido Univ*, 11, p.195–202.

Fuji, A. (1967) Ecological studies on the growth and food consumption of Japanese common littoral sea urchin, *Strongylocentrotus intermedius* (A. Agassiz). *Mem Fac Fish*, Hokkaido Univ, 15, p.83–160.

Gago, J., Range, P., Luís, O. (2003) Growth, reproductive biology and habitat selection of the sea urchin *Paracentrotus lividus* in the coastal waters of Cascais, Portugal. In: Féral, J.P., David, B. (ed.) *Echinoderm research 2001*. Lisse: AA Balkema, , p.269–276.

Gago, J.A.E. (2009) Sea urchin *Paracentrotus lividus* (Lamarck 1816) eggs and endotrophic larvae: Potential of their use as marine larval fish first-feeding. Doctoral Thesis, Faculdade De Ciências Departamento De Biologia Animal, Universidade de Lisboa.

Gago, J.M., Luis, O.J., Repolho, T.R. (2009) Fatty acid nutritional quality of sea urchin *Paracentrotus lividus* (Lamarck, 1816) eggs and endotrophic larvae: relevance for feeding of marine larval fish. *Aquacult Nutr*, 15, p.379-389.

Gallager, S.M., Mann, R., Sasaki, G.C. (1986) Lipid as an index of growth and viability in three species of bivalve larvae. *Aquaculture*, 56, p.81-103.

Ganga, R., Tort, L., Acerete, L., Montero, D., Izquierdo, M.S. (2006) Modulation of ACTH-induced cortisol release by polyunsaturated fatty acids in interrenal cells from gilthead seabream *Sparus aurata*. *J Endocrinol*, 190, p.39-45.

García-Tello, P. and Baya A.M. (1973) Acerca de la posible función de bacterias agaroliticas aisladas del erizo blanco (*Loxechinus albus* (Mol.)). *Mus Nac Hist Nac Pub Oc*, 15, p.3–8.

George, S.B., Lawrence, J.M., Lawrence, A.L. (2004) Complete larval development of the sea urchin *Lytechinus variegatus* fed an artificial feed. *Aquaculture*, 242, p.217-228.

George, S.B., Lawrence, J.M., Lawrence, A.L., Ford, J. (2000) Fertilization and development of eggs of the sea urchin *Lythechinus variegatus* maintained on an extruded feed. *J World Aquacult Soc*, 31, p.232-238.

George, S.B., Lawrence, J.M., Lawrence, A.L., Smiley, J., Plank, L. (2001) Carotenoids in the adult diet enhance egg and juvenile production in the sea urchin *Lytechinus variegatus*. *Aquaculture*, 199 (3-4), p.353-369.

George, S.B., Young, C.M., Fenaux, L. (1997) Proximate composition of eggs and larvae of the sand dollar *Encope michelini* (Agassiz): the advantage of higher investment in plankotrophic eggs. *Invertebr Repro Dev*, 32, p.11–19.

Gianguzza, P., Bonaviri, C., Guidetti, P. (2009) Crushing predation of the spiny star *Marthasterias glacialis* upon the sea urchin *Paracentrotus lividus*. *Mar Biol*, 156

(5), p.1083-1086.

Gianguzza P, Bonaviri C, Prato E, Fanelli G, Chiantore M, Privitera D, Luzzu F, Agnetta D. (2013) Hydrodynamism and its influence on the reproductive condition of the edible sea urchin *Paracentrotus lividus*. Mar Environ Res. http://dx.doi.org/101016/j.marenvers.2012.12.007

Gibbs, V.K., Brown, K.A., Powell, M.L., Watts, S.A. (2011) Opportunistic Predation of the Sea Urchin, *Lytechinus variegatus*, by the Amphipod, *Elasmopus levis*, in an Intensive Inland Culture System. *J World Aquacult Soc*, 42 (3), p.364–369.

Gibson, A.W. and Burke, R.D. (1985) The origin of pigment cells in embryos of the sea urchin *Strongylocentrotus purpuratus*. *Dev Biol*, 107, p.414-419.

Gibson, A.W. and Burke, R.D. (1987) Migratory and invasive behaviour of pigment cells in normal and animalized sea urchin embryos. *Exp Cell Res*, 173, p.546-557.

Gladfelter, W.B. (1978) General ecology of cassiduloid urchins *Cassiduls caribearum*. *Mar Biol*, 47, p.149-160.

Goebel, N. and Barker, M.F. (1998) Artificial diets supplemented with carotenoid pigments as feeds for sea urchins. In: Mooi, R. and Telford, M. (ed.) *Echinoderms: San Francisco*. Rotterdam: Balkema, p.667–672.

Gómez-Pinchetti, J.L. and García-Reina, G. (1993) Enzymes from marine phycophages that degrade cell walls of seaweeds. *Mar Biol*, 116, p.553–558.

González-Durán, E., Castell, J.D., Robinson,, S.M.C., Blair, T.J. (2008) Effects of dietary lipids on the fatty acid composition and lipid metabolism of the green sea urchin *Strongylocentrotus droebachiensis*. *Aquaculture*, 276 (1-4), p.120-129.

González-Wanguemert, M., Vega-Fernandez, T., Perez-Ruzafa, A., Giacalone, M., D'Anna, G., Badalamenti, F. (2012) Genetic considerations on the introduction

of farmed fish in marine protected areas: the case of study of white seabream restocking in the Gulf of Castellammare (Southern Tyrrhenian Sea). *J Sea Res*, 68, p.41-48.

Gordon I. (1926) Development of the calcareous test of *Echinus miliaris*. Phil Trans R Soc B 214, p.259–312.

Gosselin, P. and Jangoux, M. (1996) Induction of metamorphosis in *Paracentrotus lividus* larvae (Echinodermata: Echinoidea). *Oceanol Acta*, 19, p.293–296.

Gosselin, P. and Jangoux, M. (1998) From competent larvae to exotrophic juveniles: a morphofunctional study of the perimetamorphic period of *Paracentrotus lividus* (Echinodermata, Echinoida). *Zoomorphology*, 118, p.31-43.

Griffiths, M. and Perrott, P. (1976) Seasonal changes in the carotenoids of the sea urchin *Strongylocentrotus droebachiensis*. *Comp Biochem Physiol*, 55 (B), p.435–441.

Grosjean, P., Spirlet, C., Gosselin, P., Vaitilingon, D., Jangoux, M. (1998) Land-based, closed-cycle echiniculture of *Paracentrotus lividus* (Lamarck) (Echinoidea: Echinodermata): A long-term experiment at a pilot scale. *J Shellfish Res*, 17, p.1523–1531.

Grosjean, P., Spirlet, C., Gosselin, P., Vaitilingon, D., Jangoux, M. (1998) Land based closed cycle echiniculture of *Paracentrotus lividus* Lamarck (Echinodermata: Echinoidea): a long term experiment at a pilot scale. *J Shellfish Res*, 17, p.1523–1531.

Guerinot, M.L. and Patriquin, D.G. (1981) The association of N_2-fixing bacteria with sea urchins. *Mar Biol*, 62, p.197–207.

Guettaf, M. (1997) *Contribution a l'etude de la variailite du cycle reproductive (indice gonadique et histologie des gonads) chez Paracentrotus lividus en Mediterranee*

sud-occidentale (Algerie). These Doct. Univ. Aix-Marseille II.

Guettaf, M. and Saint Martin, G.A. (1995) Etude de la variabilite de l'indice gonadique de l'oursin comestible *Paracentrotus lividus* (Echinodermata: Echnoidea) en Mediterranee nord-occidentale. *Vie Milieu*, 45, p.129-137.

Guettaf, M., San Martin, G.A., Francour, P. (2000) Interpopulation variability of the reproductive cycle of *Paracentrotus lividus* (Echinodermata : Echinoidea) in the south-western Mediterranean. *J Mar Biol Assoc UK*, 80 (5), p.899-907.

Guillard, R.R.L. (1975) Culture of phytoplankton for feeding marine invertebrates. In: Smith, W.L. and Chanley, M.H. (ed.) *Culture of Marine Invertebrate Animals*. New York: Plenum Press, p.26-60.

Gunaratne H.J. and Vacquier, V.D. (2007) Sequence, annotation and developmental expression of the sea urchin Ca2+ -ATPase family. *Gene*, 397, p.67–75.

Hagen, N. (1996) Echinoculture: from fishery enhancement to closed cycle cultivation. *World Aquacult*, 27, p.6-19.

Hammer, B.W., Hammer, H.S., Watts, S.A., Desmond, R.A., Lawrence, J.M., Lawrence, A.L. (2004) The effects of dietary protein concentration on feeding and growth of small *Lytechinus variegatus* (Echinodemata: Echinoidea). *Mar Biol*, 145, p.1143–1157.

Hammer, H.S., Powell, M.L., Jones, W.T., Gibbs V.K., Lawrence A.L., Lawrence J.M., Watts, S.A. (2012) Effect of feed protein and carbohydrate levels on feed intake, growth, and gonad production of the Sea Urchin, *Lytechinus variegatus. J World Aquacult Soc*, 43 (2), p.145-158.

Hammer, H.S., Watts, S.A., Lawrence, A.L., Lawrence, J.M., Desmond, R. (2006) The effect of dietary protein on consumption, survival, growth and production of the sea urchin *Lytechinus variegatus*. *Aquaculture*, 254, p.483–495.

Harmelin, J.G., Bouchon, C., Hong, J.S. (1981) Impact de la pollution sur la distribution des échinodermes des substrats durs en Provence (Méditerranée Nord-occidentale). *Téthys*, 10 (1), p.13–36.

Harrington, F.E. and Easton, D.P. (1982) A putative precursor to the major yolk protein of the sea urchin. *Dev Biol*, 94, p.505–508.

Harrington, F.E. and Ozaki, H. (1986b) The major yolk glycoprotein precursor in echinoids is secreted by coelomocytes into the coelomic plasma. *Cell Different*, 19, p.51–57.

Hart, M.W. (1991) Particle captures and methods and the method of suspension feeding by echinoderm larvae. *Am Nat*, 146, p.415-426.

Hart, M.W. (1996) Evolutionary loss of larval feeding: development, form and function in a facultatively feeding larva, *Brisaster latifrons*. *Evolution,* 50, p.174-187.

Hasegawa, S., Ura, K., Tanaka, H., Ojima, T., Takagi, Y. (2012) Purification and biochemical characterization of a cellulase from the digestive organs of the short-spined sea urchin *Strongylocentrotus intermedius*. *Fisheries Sci*, 78 (5), p.1107-1115.

Hay, M.E. (1996) Marine chemical ecology: what's known and what's next? *J Exp Mar Biol Ecol*, 200, p.103–134.

Hay, M.E. and Steinberg, P.D., (1992) The chemical ecology of plantherbivore interactions in marine versus terrestrial communities. In: Rosenthal, J.A. and Berenbaum, M.R. (ed.) *Herbivores: Their Interaction with Secondary Metabolites, Evolutionary and Ecological Processes*. San Diego: Academic Press, p.371–413.

Heflin, L.E., Gibbs, V.K., Powell, M.L., Makowsky, R., Lawrence, J.M., Lawrence, A.L., Watts, S.A. (2012) Effect of dietary protein and carbohydrate levels on weight

gain and gonad production in the sea urchin *Lytechinus variegatus. Aquaculture*, 358, p.253-261.

Hernández, J.C., Clemente, S., Brito, A. (2011) Effects of seasonality on the reproductive cycle of *Diadema aff. antillarum* in two contrasting habitats: implications for the establishment of a sea urchin fishery. *Mar Biol*, 158 (11), p.2603-2615.

Herrera, J.C. (1998) Nutritional strategies of echinoplutei. PhD dissertation. University of Florida, Gainesville, FL.

Hildemann, W.H. and Dix, T.G. (1972) Transplantation reactions of tropical Australian echinoderms. *Transplantation*, 14, p.624-633.

Himmelman, .JH., Carefoot, T.H. (1975) Seasonal changes in calorific values of three Pacific coast seaweeds, and their significance to some marine invertebrate herbivores. *J Exp Mar Biol Ecol*, 18, p.139–151.

Himmelman, J.H. and Steele, D.H. (1971) Foods and predators of the green sea urchin *Strongylocentrotus droebachiensis* in Newfoundland waters. *Mar Biol*, 9, p.315–322.

Hoegh-Guldberg, O., Emlet, R.B. (1997) Energy use during the development of a lecithotrophic and a planktotrophic echinoid. *Biol Bull*, 192, p.27–40.

Holland, N.D. and Ghiselin, M. (1970) A comparative study of gut mucous cells in thirty-seven species of the class Echinoidea (Echinodermata). *Biol Bull*, 138, p.286–305.

Holland, N.D. and Lauritis, J.A. (1968) The fine structure of the gastric exocrine cells of the purple sea urchin, *Strongylocentrotus purpuratus*. *Trans Amer Microsc Soc*, 87, p.201–209.

Huang, H. and Giese, A.C. (1958) Tests for digestion of algal polysaccharides by some

marine herbivores. *Science*, 127, p.475.

Huggett, M.J., Williamson, J.E., de Nys, R., Kjelleberg, S., Steinberg, P.D. (2006) Larval settlement of the common Australian sea urchin *Heliocidaris erythrogramma* in response to bacteria from the surface of coralline algae. *Oecologia*, 149, p.604–619.

Hughes, A.D., Catarino, A.I., Kelly, M.S., Barnes, D.K.A., Black, K.D. (2005) Gonad fatty acids and trophic interactions of the echinoid *Psammechinus miliaris*. *Mar Ecol-Prog Ser*, 305, p.101-111.

Hughes, A.D., Kelly, M.S., Barnes, D.K.A., Catarino, A.I., Black, K.D. (2006) The dual function of sea urchin gonads are reflected in the temporal variation of their biochemistry. *Mar Biol*, 148, p.789-798.

Hughes A.D., Cook E.J., Orr H., Kelly M.S., Black K.D. (2011) The transformation of long chain polyunsaturated fatty acids in benthic food webs: The role of sea urchins. J Exp Mar Biol Ecol 409. P.229–234

Hur, S.B., Yoo, S.K., Rho, S. (1985) Laboratory tagging experiment of sea urchin, *Hemicentrtus pulcherrimus*. *Bull Korean Fish Soc*, 18, p.363-368.

Iliffe, T.M. and Pearse, J.S. (1982) Annual and lunar reproductive rhythms of the sea urchin *Diadema antillarum* (Philippi) in Bermuda. *Internat J Invert Reprod*, 5, p.139–148.

Ichinose H, Tokuda K, Kudo T, Agatsuma Y. 2005. Char ac ter is tics of an nual bands for mation as age marker of the hatch ery-raised and wild sea urchin *Strongylocentrotus intermediusin* Northern Hokkaido, Japan. Suisan Zoshoku 53:75-82. [in Japanese with English abstract]

Izquierdo, M. and Koven W. (2011) Lipids. In: Holt, J. (ed) *Larval fish nutrition*. Wiley-Blackwell, p 47-81.

Jangoux, M. (1987) Diseases of Echinodermata. I. Agents microorganisms and protistans. *Dis Aquat Org*, 2, p.147–162.

Jangoux, M. (1990) Disease of Echinodermata. In: Kinne, O. (ed.) Disease of marine animals, Vol. 3. *Biologische Anstalt Helgoland*, p.439-567.

Jimmy, R.A., Kelly, M.S., Beaumont, A.R. (2003) The effect of diet type and quantity on the development of common sea urchin larvae Echinus esculentus. *Aquaculture*, 220, p.261-275.

Juinio-Menez, M.A. and Villanoy, C. (1995) Sea urchin recruitment studies for management considerations. Proceedings of the 4th LIPI-JSPS Joint Seminar in Marine Science. Jakarta, Indonesia, p.15-20.

Juinio-Menez, M.A., Macawaris, N.N.D., Bangi, H.G.P. (1998) Community-based sea urchin (*Tripneustes gratilla*) grow-out culture as a resource management tool. *Can spec Publn Fisha aquat Sci*, 125, p.393-399.

Kalvass, P. and Hendrix, J.M. (1997) The California red sea urchin, *Strongilocentrotus franciscanus*, fishery: catch, effort and management trends. *Mar Fish Rev*, 59, p.1-17.

Karp, R.D. and Hildemann, W.H. (1976) Specific allograft reactivity in the sea star *Dermasterias imbricata*. *Transplantation*, 22, p.434- 439.

Kawakami, T., Tsushima, M., Katabami, Y., Mine, M., Ishida, A., Matsuno, T. (1998) Effect of ß, ß -carotene, ß -echinenone, astaxanthin, fucoxanthin, vitamin A and vitamin E on the biological defense of the sea urchin *Pseudocentrotus depressus*. *J Exp Mar Biol Ecol*, 226, p.165–174.

Kawakami, T., Tsushima, M., Katabami, Y., Mine, M., Ishida, A., Matsuno, T. (1998) Effect of β, β-carotene, β-echinenone, astaxanthin, fucoxanthin, viatmin A and

vitamin E on the biological defense of the sea urchin *Pseudocentrotus depressus*. *J Exp Biol Ecol*, 226, p.165-174.

Kawamura, K. (1973) Fishery biological study on a sea urchin, *Strongylocentrotus intermedius*. Scientific reports of the Hokkaido Fisheries Experimental Station. 16, p.1-54.

Keckeš, S., Ozretic, B., Lucu, C. (1966) About a possible mechanism involved in the shedding of sea-urchins. *Experientia*, 22, p.146–147.

Keegan, B.F. and Könnecker, G. (1980) Aggregation in echinoderms on the West coast of Ireland. An ecological Harmelin perspective. In: Jangoux, M. (ed.) *Echinoderms: present and past*. Rotterdam: AA Balkema, p.199.

Kelly, J.R., Krumhansl, K.A., Scheibling, R.E. (2012) Drift algal subsidies to sea urchins in low-productivity habitats. *Mar Ecol Prog Ser*, 452, p.145-157.

Kelly, M.S. (2001) Environmental parameters controlling gametogenesis in the echinoid *Psammechinus miliaris*. *J Exp Mar Biol Ecol*, 266 (1), p.67 – 80.

Kelly, M.S. (2002). Survivorship and growth rates of hatchery-reared sea urchins. *Aquacult Int*, 10 (4), p.309-316.

Kelly, M.S., Chamberlain J. (2010) Recent Advances in Sea-Urchin Aquaculture and Enhancement in Scotland and Ireland. In: Bull Aquacul Assoc. Canada 108-1, p.23-30

Kelly, M.S., Hunter, A.J., Scholfield, C.L., McKenzie, J.D. (2000) Morphology and survivorship of larval *Psammechinus miliaris* (Gmelin) (Echinodermata: Echinoidea) in response to varying food quantity and quality. *Aquaculture*, 182, p.223-240.

Kelly, J.R., Krumhansl, K.A., Scheibling, R.E. (2012) Drift algal subsidies to sea urchins in low-productivity habitats. *Mar Ecol Prog Ser,* 452, p.145-157

Kemp, M. (1962) Recherches d'écologie compare sur *Paracentrotus lividus* (Lmk) et *Arbacia lixula* (L). *Rec Trav St Mar Endoume*, 25, p.47–116.

Kennedy, E.J., Robinson, S.M.C., Parsons, G.J., Castell, J.D. (2005) Effect of protein source and concentration on somatic growth of Juvenile green sea urchins *Strongylocentrotus droebachiensis. J World Aquacult Soc*, 36 (3), p.320-336.

Kennedy, E.J., Robinson, S.M.C., Parsons, G.J., Castell, J.D. (2007) Effect of dietary minerals and pigment on somatic growth of juvenile green sea urchins, *Strongylocentrotus droebachiensis*. *J World Aquacult Soc*, 38 (1), p.36–48.

Khotimchenko, Y.S. (1983) Effect of adrenotropic and cholinotropic preparations on growth, maturation, and spawning of the sea urchin *Strongylocentrotus nudus*. *Biol Morya*, 2, p.57–63.

Khotimchenko, Y.S. and Deridovich, I.I. (1991) Monoaminergic and cholinergic mechanisms of reproduction control in marine bivalve molluscs and echinoderms: a review. *Comp Biochem Physiol*, 100 (C), p.311–317.

Kitching, J.A. and Thain, V.M. (1983) The ecological impact of the sea urchin *Paracentrotus lividus* in Lough Ine, Ireland. *Phil Trans Roy Soc London, B*, 300, p.513-552.

Klinger, T.S. (1982) Feeding rate of *Lytechinus variegatus* (Lamarck) (Echinodermata: Echinoidea) on differing physiognomies of an artificial food of uniform composition. In: Lawrence, J.M. (ed.) *Echinoderms: proceedings of the international conference, Tampa Bay.* Rotterdam: Balkema, p.29–32.

Klinger, T.S. (1984) Activities and kinetics of digestive α-and β -g1ucosidase and β -galactosidase of five species of echinoids (Echinodermata). *Comp Biochem Physiol*, 78 (A), p.597–600.

Klinger, T.S., Lawrence, J.M., Lawrence, A.L. (1997) Gonad and somatic production of

Strongylocentrotus droebachiensis fed manufactured feeds. *Bull Aquacul Assoc Canada*, 97 (1), p.35–37.

Kreissig, K., Tocher, D.R., Carboni, S. (2009) *Assessment of quality of purple sea urchin gametes (Paracentrotus lividus) through analysis of Essential Fatty Acids.* MSc Thesis Institute of Aquaculture, Stirling University.

Krinsky, N.I. (1994) The biological properties of carotenoids. *Pure Appl Chem*, 66, p.1003–1010.

Lamare, M.D. and Barker, M.F. (1999) In situ estimates of larval development and mortality in the New Zealand sea urchin *Evechinus chloroticus* (Echinodermata: Echinoidea). *Mar Ecol-Prog Ser*, 180, p.197-211.

Lamare, M.D., Mladenov, P.V. (2000) Modelling somatic growth in the sea urchin *Evechinus chloroticus* (Echinoidea: Echinometridae). *J Exp Mar Biol Ecol*, 243 (1), p.17-43.

Lares, M.T. and McClintock, J.B. (1991) The effects of food quality and temperature on the nutrition of the carnivorous sea urchin *Eucidaris tribuloides* (Lamarck). *J Exp Mar Biol Ecol*, 149, p.279–286.

Larson, B.R., Vadas, R.L., Keser, M. (1980) Feeding and nutritional ecology of the sea urchin *Strongylocentrotus droebachiensis* in Maine, USA. *Mar Biol*, 59, p.49–62.

Lasker, R., Giese, A.C. (1954) Nutrition of the sea urchin, *Strongylocentrotus purpuratus*. *Biol Bull*, 106, p.328–340.

Lauzon-Guay, J.S. and Scheibling, R.E. (2008) Evaluation of passive integrated transponder (PIT) tags in studies of sea urchins: caution advised. *Aquat Biol*, 2 (2), p.105-112.

Lawrence, A.L, Watts, S.A., Lawrence, J.M. (2007) Feeding, digestion, and digestibility. In: Lawrence, J.M. (ed.) *Edible Sea urchins: Biology and Ecology*. Elsevier Press, p. 135-158.

Lawrence, J., Fenaux, L., Corre, M.C., Lawrence, A. (1992) The effect of quantity and quality of prepared diets on production in *Paracentrotus lividus* (Echinodermata: Echinoidea). In: Scalera-Liaci, L., Canicatti, C. (ed.) *Echinoderm research 1991*. Rotterdam: AA Balkema, p.107–110.

Lawrence, J.M. (1975) On the relationship between marine plants and sea urchins. *Oceanogr Mar Biol Ann Rev*, 13, p.213-286.

Lawrence, J.M. (2001) The edible sea-urchins. In: Lawrence, J.M. (ed.) *Edible sea urchin biology and ecology*. Amsterdam – London - New York – Oxford- Paris-Shannon-Tokyo: Elsevier Science.

Lawrence, J.M. (2007) Edible Sea Urchins: Use and Life-History Strategies. In: Lawrence, J. M. (ed.) *Sea Urchin: Biology and Ecology*. Amsterdam: Elsevier science B.V., p.243-285.

Lawrence, J.M. (2007) The edible sea-urchins. In: Lawrence, J.M. (ed.) *Edible sea urchin biology and ecology*. *2nd Ed*. Amsterdam – London - New York – Oxford-Paris- Shannon-Tokyo: Elsevier Science.

Lawrence, J.M. and Hughes-Games, L. (1972) The diurnal rhythm of feeding and passage of food through the gut of *Diadema setosum* (Echinodermata: Echinoidea). *Isr J Zool*, 21, p.13–16.

Lawrence, J.M., Fenaux, L., Jangoux, M. (1977) Qualitative nutritional requirements of echinoderm lavae. In: Recheige, M. (ed.) *CRC handbook series in nutrition and food, section D. Nutritional requirements*. Cleveland: CRC press, Inc., p.401-407.

Lawrence, J.M., McClintock, J.B., Guille, A. (1984) Organic level and caloric content of eggs of brooding asteroids and an echinoid (Echinodermata) from Kerguelen (South Indian Ocean). *Int J Invert Reprod Dev*, 7, p.249–257.

Lawrence, J.M., Plank, L.F., Lawrence, A.L. (2003) The effect of consumption frequency on consumption of food, absorption efficiency, and gonad production in the sea urchin *Lytechinus variegatus*. *Comp Biochem Physiol*, 134 (A), p.69–75.

Le Gall, P. and Bucaille, D. (1989) Sea urchins production by inland farming. (Abstract) In: De Pauw, N. *et al*. (ed.) *Aquaculture: a biotechnology in progress*, 1, p.53-59.

Le Gall, P., Bucaille, D., Dutot, P. (1989) Résistance aux variations de salinité chez *Paracentrotus* et *Psammechinus*. *Vie Mar HS*, 10, p.83–84.

Leighton, P., 1995. Contributions towards the development of echinoculture in North Atlantic waters with particular reference to Paracentrotus lividus (Lamarck). PhD Thesis submitted to the National University of Ireland for the degree of Doctor of Philosophy.

Lesser, M.P., Carleton, K.L., Böttger, S.A., Barry, T.M., Walker, C.W. (2011) Sea urchin tube feet are photosensory organs that express a rhabdomeric-like opsin and PAX6. *Proc Biol Sci*, 278(1723), p.3371-9.

Lewis, J.B. (1964) Feeding and digestion in the tropical sea urchin *Diadema antillarum*, Philippi. *Bull Mar Sci*, 16, p.151–158.

Lilly, G. (1975) *The influence of diet on the growth of the tropical sea urchin Tripneustes ventricosus (Lamarck).* Doctoral dissertation. University of British Columbia, Vancouver.

Lison de Loma, T., Harmelin-Vivien, M.L., Conand, C. (1999) Diel feeding rhythm of

the sea urchin *Tripneustes gratilla* (L.) on a coral reef at La Reunion, Indian Ocean. In: Candia Carnevali, M.D. and Bonosoro, F. (ed.) *Echinoderm research 1998.* Rotterdam: Balkema, p.87–92.

Liu, H., Kelly, M.S., Cook, E.J., Black, K., Orr, H., Zhu, J. X., Dong, S. L. (2007) The effect of diet type on growth and fatty-acid composition of sea urchin larvae, *Paracentrotus lividus* (Lamarck, 1816) (Echinodermata). *Aquaculture*, 264, p.247-262.

Liu, H., Kelly, M.S., Cook, E.J., Black, K., Orr, H., Zhu, J.X., Dong, S.L. (2007) The effect of diet type on growth and fatty acid composition of the sea urchin larvae, II. P*samechinus miliaris* (Gmelin). *Aquaculture*, 264, p.263-278.

Liu, M.Q., Zhong, W., Wang, Q. (2002) Study on the spawning before hand technology on sea urchin, *Strongylocentrotus intermedius*. *Fish Sci*, 21(5), p.4-7.

Livore, J.P. and Connell, S.D. (2012) Reducing per capita food supply alters urchin condition and habitat. *Mar Biol*, 159 (5), p.967-973.

Lonzano, J., Galera, J., Lopez, S., Turon, X., Palacin, C., Morera, G. (1995) Biological cycles and recruitment of *Paracentrotus lividus* (Echinodermata: Echinoidea) in two contrasting habitats. *Mar Ecol-Prog Ser*, 122, p.179-191.

López, S., Turon, X., Montero, E., Palacín, C., Duarte, C.M., Tarjuelo, I. (1998) Larval abundance, recruitment and early mortality in *Paracentrotus lividus* (Echinoidea), inter-annual variability and plankton-benthos coupling. *Mar Ecol-Prog Ser*, 172, p.239-251.

Lowe, E. (1974) *Absorption efficiencies, feeding rates, and food preferences of Lytechinus variegatus (Echinodermata: Echinoidea) for selected marine plants*. Master's thesis. University of South Florida, Tampa.

Lynch, J.M. and Barbano, D.M. (1999) Kjeldahl Nitrogen Analysis as a reference method for protein determination in dairy products. *J AOAC Int*, 82 (6), p.1389-1398.

Lyons, D.A. and Scheibling, R.E. (2007) Differences in somatic and gonadic growth of sea urchins (*Strongylocentrotus droebachiensis*) fed kelp (*Laminaria longicruris*) or the invasive algae *Codium fragile ssp*. *tomentosoides* are related to energy acquisition. *Mar Biol*, 152, p.285–295.

MacBride, E.W. (1903) The development of *Echinus esculentus*, together with some points on the development of *E. miliaris* and *E. acutus*. *Phil Trans R Soc, B*, 195, p.285–330.

Machiguchi, Y. (1987) Feeding behaviour of sea urchin *Strongylocentrotus intermedius* (A. Agassiz) observed in Y-shaped chamber. *Bull Hokkaido Reg Fish Res Lab*, 51, p.33–37. (in Japanese, English abstract)

Malay, M.C.D., Juino-Meñez, M.A., Villanoy, C. (2000) Population genetic structure of the sea urchin *Tripneustes gratilla* from selected sites in western Luzon and eastern Philippines. In: *Proceedings of the 9th International Coral Reef Symposium*. Bali, Indonesia, October 23 – 27, 1, p.107-111.

Manahan, D.T. (1990) Adaptations by invertebrate larvae for nutrient acquisition from sea water. *Am Zool*, 30, p.147-160.

Manahan, D.T., David, J.P., Stephen, G.C. (1983) Bacteria free sea urchin larvae: selective uptake of neutral aminoacids from sea water. *Science*, 220, p.204-206.

Marsh, A.G. and Watts, S.A. (2007) Energy metabolism and gonad development. In Lawrence, J.M. (ed.) *Edible sea urchins: biology and ecology, 2nd edition*. Amsterdam, the Netherlands: Elsevier Science B. V., p.35–54.

Martínez, I., García, F.J., Sánchez, A.I., Daza, J.L., del Castillo, F. (2003) Biometric

parameters and reproductive cycle of *Paracentrotus lividus* (Lamarck) in three habitats of southern Spain. In: Féral, J.P., David, B. (ed.) *Echinoderm research 2001*. Lisse: AA Balkema, p.281–287.

Martinez-Pita, I., Garcia, F.J., Pita, M.L. (2010) Males and Females gonad fatty acids of the sea urchin *Paracentrotus lividus* and *Arbacia lixula* (Echinodermata). *Helgol Mar Res*, 64, p.135-142.

Mastaller, M. (1974) Zerstörung des Makrophytals an der nordadriatischen Küste durch intensives Abweiden durch Seeigel; eine Untersuchung über pipulationsstrukturen, nahrung und Fressverhalten bei dem Echinoiden *Paracentrotus lividus* (Lamarck). Dipl Arbeit, Zool Inst Univ München.

Matsui, T., Agatsuma, Y., Ogasawara, M., Taniguchi, K. (2008) Coincidence in reproduction of the sea urchin *Strongylocentrotus intermedius* in Hirota Bay, on the Pacific Ocean off northern Honshu, and in the sea of Japan off Hokkaido, Japan. *J Shellfish Res*, 27 (5), p.1283-1289.

Matsuno, T. (1991) Xanthophylls as precursors of retinoids. *Pure Appl Chem*, 63, p.81–88.

Matsuno, T. and Tsushima, M. (2001) Carotenoids in sea urchins. In: Lawrence, J.M. (ed.) *Edible sea urchins: biology and ecology*. Amsterdam: Elsevier Science B.V., p.115–138.

Mattison, J.E., Trent, J.D., Shanks, A.L., Akin, T.B., Pearse, J.S. (1977) Movement and feeding activity of red sea urchins (*Strongylocentrotus franciscanus*) adjacent to a kelp forest. *Mar Biol*, 39, p.25–30.

Mazia, D. (1937) The release of calcium in Arbacia eggs on fertilization *J Cell Comp Physiol*, 10, p.291–304.

McBride, S.C., Lawrence, J.M., Lawrence, A.L., Mulligan, T.J. (1998) The effect of

protein concentration in prepared feeds on growth, feeding rate, total organic absorption, and gross assimilation efficiency of the sea urchin, *Strongylocentrotus franciscanus*. *J Shellfish Res*, 17 (5), p.1563–1570.

McBride, S.C., Pinnix, W.D., Lawrence, J.M., Lawrence, A.L., Mulligan, T.M. (1997) The effect of temperature on production of gonads by the sea urchin *Stronglycentrotus franciscanus* fed natural and prepared diets. *J World Aquacult Soc* ,28, p.357–365.

McClintock, J.B. and Watts, S.A. (1990) the effects of photoperiod on gametogenesis in the tropical sea-urchin *Eucidaris tribuloides* (lamarck) (Echinodermata, Echinoidea). *J Exp Mar Biol Ecol*, 139 (3), p.175-184.

McClintock, J.B., Pearse, J.S. (1986) Organic and energetic content of eggs and juveniles of Antarctic echinoids and asteroids with lecithotrophic development. *Comp Biochem Physiol A*, 85, p.341–345.

McDougall, A., Shearer, J., Whitaker, M. (2000) The initiation and propagation of the fertilization wave in sea urchin eggs. *Biol Cell*, 92, p.205–214.

McEdward, L.R. (1984) Morphometric and metabolic analysis of the growth and form of an echinopluteus. *J Exp Mar Biol Ecol*, 82, p.259-287.

McEdward, L.R. and Miner, B.G. (2001) Echinoid larval ecology. In: Lawrence, J.M. (ed.) *Edible sea urchin biology and ecology*. Amsterdam – London - New York – Oxford- Paris- Shannon-Tokyo: Elsevier Science.

McLaughlin, G. and Kelly, M.S. (2001) Effect of artificial diets containing carotenoid-rich microalgae on gonad growth and colour in the sea urchin *Psammechinus miliaris* (Gmelin). *J Shellfish Res*, 20, p.377–382.

McNaught, D.C. (1999) The indirect effects of macroalgae and micropredation on the post-settlement success of the green sea urchin in Maine. PhD thesis, University

of Maine.

McNaught, D.C. and Steneck, R.S. (1998) Settlement and survival of the green sea urchin in Maine: effects of algal habitat. Final report to the Maine Department of Marine Resources, School of Marine Sciences, University of Maine.

Meidel, S.K. and Scheibling, R.E. (1999) Effects of food type and ration on reproductive maturation and growth of the sea urchin, *Strongylocentrotus droebachiensis*. *Mar Biol*, 134, p.155–166.

Meyer, E., Green, A.J., Moore, M., Manahan, D.T. (2007) Food availability and physiological state of sea urchin larvae (*Strongylocentrotus purpuratus*). *Mar Biol*, 152, p.179-191.

Miller, B.A. and Emlet, R.B. (1999) Development of newly metamorphosed juvenile sea urchins (*Strongylocentrotus franciscanus* and *S. purpuratus*): morphology, the effects of temperature and larval food ration, and a method for determining age. *J Exper Mar Biol Ecol*, 235, p.67-90.

Miller, R.J. and Mann, K.H. (1973) Ecological energetics of the seaweed zone in a marine bay on the Atlantic coast of Canada. III. Energy transformations by sea urchins. *Mar Biol*, 99, p.99–114.

Mills, S.C., Peyrot-Clausade-Peyrot, M., Fontaine, M.F. (2000) Ingestion and transformation of algal turf by *Echinometra mathaei* on Tiahura fringing reef (French Polynesia). *J Exp Mar Biol Ecol*, 254, p.71–84.

Miloslavich, P., Lawrence, J.M., Schiopu, D., Klein, E. (2007) Effect of food concentration on protein and carbohydrate production during larval development of the sea urchin *Lytechinus variegatus*. *J Shellfish Res*, 26 (4), p.1177-1182.

Miyamoto, K. and Kohshima, S. (2006) Experimental and field studies on foraging

behaviour and activity rhythm of hard-spined sea urchin *Anthocidaris crassispina. Fish Sci*, 72 (4), p.796-803.

Monroig, O., Navarro, J.C., Dick, J.R., Alemany, F., Tocher, D.R. (2010) Identification of a Delta 5-like Fatty Acyl Desaturase from the Cephalopod *Octopus vulgaris* (Cuvier 1797) Involved in the Biosynthesis of Essential Fatty Acids. *Mar Biotechnol*, 14 (4), p.411-422.

Moore, H.B. and McPherson, B.F. (1965) A contribution to the study of the productivity of the urchins *Tripneustes esculentus* and *Lytechinus variegatus*. *Bull Mar Sci,* 15, p.855–871.

Moran, A. and Emlet, R.B. (2001) Offspring size and performance in variable environments: Weld studies on a marine snail. *Ecology*, 82, p.1597-1612.

Morikawa, T. (1999) Status and prospects on the development and improvement of coastal fishing groud. In: Marine ranching: global perspective with emphasis on the Japanes experience. FAO Fisheries Circular 943, p.136-239.

Mortensen, T. (1938) Contributions to the study of the development and larval forms of echinoderms. IV, D. *Kgl. Danske Vidensk Slesk. Biol. Skr., Naturvidensk. og Mathem.* Afd, 9, 7.

Mos, B., Cowden, K.L., Nielsen, S.J., Dworjanyn, S.A. (2011) Do cues matter? Highly inductive settlement cues don't ensure high post-settlement survival in sea urchin aquaculture*. PLoS One*, 6 (12): e28054.

Moylan, E. (1997) Gonad conditioning and wild stock enhancement of the purple sea urchin *Paracentrotus lividus* on the west coast of Ireland. *Bull Aquaculture Assoc Can*, 97, p.38-41.

Nagi, Y. and Kaneko, K. (1975) Culture experiments on the sea urchin *Strongylocentrotus pulcherrimus* fed an artificial diet. *Mar Biol*, 29, p.105–108.

Nance, J.M. and Braithwaite, L.F. (1979) The function of mucous secretions in the cushion star *Pteraster tesselatus* Ives. *J Exp Mar Biol Ecol*, 40 (3), p.259–266.

Neill, A.T., Moy, G.W., Vacquier V.D. (2004) Polycystin-2 associates with the polycystin-1 homolog, suREJ3, and localizes to the acrosomal region of sea urchin spermatozoa. *Mol Reprod Dev,* 67, (4), p.472–477.

Nelson, B.V. and Vance, R.R. (1979) Diel foraging patterns of the sea urchin *Centrostephanus coronatus* as a predator avoidance strategy. *Mar Biol*, 51 (3), p.251-258.

Obrietan, K., Drinkwine, M., Williams, D.C. (1991) Amylase, cellulase and protease activities in surface and gut tissues of *Dendraster excentricus*, *Pisaster ochraceus* and *Strongylocentrotus droebachiensis* (Echinodermata). *Mar Biol*, 109, p.53–57.

Okada, Y. and Iwata, K.S. (1985) A substance inhibiting rhythmic contraction of the gonad in the shedding sea urchin. *Zool Sci*, 2, p.805–808.

Okada, Y., Iwata, K.S., Yanagihara, M. (1984) Synchronized rhythmic contractions among five gonadal lobes in the shedding sea urchin: coordinative function of the aboral nerve ring. *Biol Bull*, 166, p.228–236.

Okazaki, K. (1975) Normal development to metamorphosis. In: Czihak, G. (ed.) *The sea urchin embryo. Biochemistry and morphogenesis*. Berlin: Springer-Verlag, p.177-232.

Olin, P. (2000) Lobster culture. In: Stickney, R.R.(ed.) *Encyclopedia of Aquaculture*. Wiley-Interscience Publication, p.501-506.

Olin, P. and McBride, S. (2000) Abalone culture. In: Stickney, R.R. (ed.) *Encyclopedia of Aquaculture*. Wiley-Interscience Publication, p.1-7.

Onoda, K. (1936) Notes on the development of some Japanes echinoids with special reference to the structure of the larval body. *Jap J Zool*, 8, p.1-13.

Otero-Villanueva, M., Kelly, M.S., Burnell, G. (2004) How diet influences energy partitioning in the regular echinoid *Psammechinus miliaris*; constructing an energy budget. *J Exp Mar Biol Ecol*, 304, p.159-181.

Pantazis, P.A., Kelly, M.S., Connoly, J.G., Black, K.D. (2000) Effect of artificial diets on growth, lipid utilization and gonad biochemistry in the adult sea urchin *Psammechinus miliaris*. *J Shellfish Res*, 19, p.995–1001.

Pastor, R. (1971) Distribución del erizo de mar, *Paracentrotus lividus* (Lmk), en la Ría de Vigo. *Publ Técn Dir Gen Pesca Marít, Spain*, 9, p.255–270.

Pearce, C.M. (2010) Introduction. In: Waddy, S. (ed.) Sea-Urchin Aquaculture. *Bull Aquacult Assoc Can*, 108 (1), p.1-2.

Pearce, C.M. and Scheibling, R.E. (1991) The effect of macroalgae, microbial films, and conspecifics on settlement and metamorphosis of the green sea urchin, *Strongylocentrotus droebachiensis* (Muller). *J Exp Mar Biol Ecol*, 147, p.147-162.

Pearce, C.M., Daggett, T.L., Robinson, S.M.C. (2004) Effect of urchin size and diet on gonad yield and quality in the green sea urchin (*Strongylocentrotus droebachiensis*). *Aquaculture*, 233, p.337-367.

Pearce, J.S. and Cameron, R.A. (1991) Echinodermata: Echinoidea. In: Giese, A.C, Pearce, J.S., Pearce, V.B. (ed.) *Reproduction of marine invertebrates. Volume VI. Echinoderms and Lophophorates.* Pacific Grove, California: The Boxwood Press, p.513-662.

Pearse, J.S. and Eernisse, D.J. (1982) Photoperiodic regulation of gametogenesis and gonadal growth in the sea star *Pisaster ochraceus*. *Mar Biol*, 67 (2), p.121-125.

Pearse, J.S., Pearse, V.B., Davis, K.K. (1986) Photoperiodic regulation of gametogenesis and growth in the sea urchin *Strongylocentrotus purpuratus*. *J Exp Zool*, 237, p.107-118.

Pechenik, J.A. (2006) Larval experience and latent effects, metamorphosis is not a new beginning. *Integr Comp Biol*, 46, p.323–333.

Pedrotti, M.L. (1993) Spatial and temporal distribution and recruitment of echinoderm larvae in the Ligurian Sea. *J Mar Biol Ass UK*, 73, p.513–530.

Pedrotti, M.L. (1995) Food selection (size and flavour) during development of echinoderm larvae. *Invert Reprod Dev*, 27, p.29-39.

Pedrotti, M.L. and Fenaux, L. (1993) Effects of food diets on the survival, development and growth rates of two cultured echinoplutei (*Paracentrotus lividus* and *Arbacia lixula*). *Inver Rep Dev*, 24 (1), p.59-70.

Pennington, J.T. and Emlet, R.B. (1986). Ontogenic and diel vertical migration of the planktonic echinoid larvae, *Dendraster excentricus*: occurrence, causes, and probable consequences. *J Exp Mar Biol Ecol*, 104, p.69-95.

Pennington, J.T., Rumrill, S.S., Chia, F.S. (1986) Stage-specific predation upon embryos and larvae of the Pacific sand dollar, *Dendraster excentricus*, by 11 species of common zooplanktonic predators. *Bull Mar Sci*, 39 (2), p.234-240.

Penry, D.L. and Jumars, P.A. (1987) Modeling animal guts as chemical reactors. *Amer Nat*, 129, p.69–96.

Pernet, F., Bricelj, V.M., Cartier, S. (2006) Lipid class dynamics during larval ontogeny of sea scallops, *Placopecten magellanicus*, in relation to metamorphic success and response to antibiotics. *J Exper Mar Biol Ecol*, 329, p.265-280.

Pernet, F., Tremblay, R., Langdon, C., Bourget, E. (2004) Effect of additions of dietary triacylglycerol microspheres on growth, survival, and settlement of mussel (*Mytilus sp.*) larvae. *Mar Biol*, 144, p.693-703.

Pirini, M., Manuzzi, M.P., Pagliarani, A., Trombetti, F., Borgatti, A.R., Ventrella, V. (2007) Changes in fatty acid composition of *Mytilus galloprovincialis* (Lmk) fed

on microalgal and wheat germ diets. *Comp Biochem Physiol Part B*, 147, p.616-626.

Pisut, D. P. (2004) *The distance chemosensory behavior of the sea urchin Lytechinus variegatus*. Master's thesis, Georgia Institute of Technology, Atlanta, GA.

Plytycz, B. and Seljelid, R. (1993) Bacterial clearance by the sea urchin, *Strongylocentrotus droebachiensis*. *Dev Comp Immuno*., 17, p.283-289.

Podolsky, R.D., Virtue, P., Hamilton, T., Vavra, J., Manahan, D.T. (1994) Energy metabolism during development of the Antarctic sea urchin *Sterechinus neumayeri*. *Antarct J US*, 29, p.157-158.

Prim, P. and Lawrence, J.M. (1975) Utilization of marine plants and their constituents by bacteria isolated from the gut of echinoids (Echinodermata). *Mar Biol*, 33, p.167–173.

Prowse, T.A.A., Sewell, M.A., Byrne, M. (2008) Fuels for development: evolution of maternal provisioning in asterinid sea stars. *Mar Biol*, 153, p.337-349.

Rahim, S., Li, J.Y., Kitamura, H. (2004) Larval metamorphosis of the sea urchins, *Pseudocentrotus depressus* and *Anthocidaris crassispina* in response to microbial films. *Mar Biol*, 144, p.71–78.

Rassoulzadegean, F. and Fenaux, L. (1979) Grazing of echinoderm larvae (*Paracentrotus lividus* and *Arbacia lixula*) on naturally occurring particulate matter. *J Plank Res*, 1, p.215-223.

Rassoulzadegean, F., Fenaux, L., Strathmann, R.R. (1984) Effect of flavour and size on selection of food by suspension-feeding plutei. *Linoml Oceanogr*, 29, p.357-361.

Reinisch, C.L. and Bang, F.B. (1971) Cell recognition: Reactions of the sea star (*Asterias vulgaris*) to the injection of amebocytes of sea urchin (*Arbacia punctulata*). *Cell Immunol*, 2, p.496-503.

Richter, H.P., Scheurich, P., Zimmermann, U. (1981) Electric field-induced fusion of sea urchin eggs. *Dev Growth Differ*, 23, p.479–486.

Robinson, S.M.C., Castell, J.D., Kennedy, E.J. (2002) Developing suitable colour in the gonads of cultured green sea urchins (*Strongylocentrotus droebachiensis*). *Aquaculture*, 206, p.289–303.

Rodríguez, S.R. and Fariña, J.M. (2001) Effect of drift kelp on the spatial distribution pattern of the sea urchin *Tetrapygus niger*: a geostatistical approach. *J Mar Ass UK*, 81, p.179–180.

Rodríguez, S.R. and Ojeda, F.P. (1998) Behavioral responses of the sea urchin *Tetrapygus niger* to predators and food. *Mar Fresh Behav Physiol*, 31, p.21–37.

Ross M., Tocher D. R., Carboni S., Brown J. (2010) *Influence of broodstock diet on fatty acid composition of broodstock and larvae of the native oyster, Ostrea edulis*. MSc Thesis Institute of Aquaculture, Stirling University, UK.

Rumrill, S.S. (1990) Natural mortality of marine invertebrate larvae. *Ophelia*, 32, p.163-198.

Rumrill, S.S. and Chia, F.S. (1985) Differential mortality during the embryonic and larval lives of northeast pacific echinoids. Keegan, B.F. and O'Connor, B.D.S. (ed.) Echinodermata. Rotterdam, Netherlands: A. A. Balkema, p.333-338.

Russell, M. (2000) A tank-system design for the hatchery production of sea-urchin larvae. In Proceedings of the Workshop on the Co-ordination of Green Sea Urchin Research in Atlantic Canada, Université de Moncton.

Russell, M.P. (1998) Resource allocation plasticity in sea urchins: rapid, diet induced, phenotypic changes in the green sea urchin, *Strongylocentrotus droebachiensis*. *J Exp Mar Biol Eco*, 220, p.1–14.

Russell, M.P., Ebert, T.A., Petraitis, P.S. (1998) Field estimates of growth and

mortality of the green sea urchin, *Strongylocentrotus droebachiensis. Ophelia*, 48 (2), p.137-153.

Saint Martin, G.A. (1995) *Contribution a la gestion des stocks d'oursin: etude des population et transplantation de Paracentrotus lividus a Marseille (France, Mediterranee) et production de Loxechinus albus a Chiloe (Chili, Pacifique).* These Doct Univ Aix-Marseille II.

Sakai, Y., Tajima K.I., Agatsuma Y. (2004) Stock en hance ment of the short-spined sea urchin *Strongylocentrotus intermedius* in Hokkaido, Japan. In, Stock Enhancement and Sea Ranching. Development, Pitfalls and Opportunities (KM Leber, S Kitada, HL Blankenship, T Svåsand, eds), p. 465-476. Blackwell, Oxford.

Sakata, K., Kato, K., Ina, K., Machiguchi, Y. (1989) Glycerolipids as potent feeding stimulants for the sea urchin, *Strongylocentrotus intermedius*. *Agric Biol Chem*, 53, p.1457–1459.

Sala, E. (1996) The role of fishes in the organisation of a Mediterranean sub-littoral community. These Doct. Univ Aix-Marseille II.

Sala, E. and Zabala, M. (1996) Fish predation and the structure of the sea urchin *Paracentrotus lividus* populations in the NW Mediterranean. *Mar Ecol-Prog Ser*, 140, p.71-81.

Sala, E., Boudouresque, C.F., Harmelin-Vivien, M. (1998a) Fishing, trophic cascades, and the structure of algal assemblages: evaluation of an old but untested paradigm. *Oikos*, 82, p.425–439.

Sala, E., Ribes, M., Hereu, B., Zabala, M., Alva, V., Coma, R., Garrabou, J. (1998b) Temporal variability in abundance of the sea urchin *Paracentrotus lividus* and *Arbacia lixula* in the North-Western Mediterranean: comparison between a marine reserve and an unprotected area. *Mar Ecol-Prog Ser*, 168, p.135-145.

Sánchez-España, A.I., Martínez-Pita, I., García, F.J. (2004) Gonadal growth and reproduction in the commercial sea urchin *Paracentrotus lividus* (Lamarck, 1816) (Echinodermata: Echinoidea) from southern Spain. *Hydrobiologia*, 519 (1-3), p.61-72.

Santella, L.,Vasilev, F., Chun, J.T. (2012) *Fertilization in echinoderms Biochemical and Biophysical Research Communications*, 425 (3), p.588-594.

Sargent, J.R., Tocher, D.R., Bell, J.G. (2002) The lipids. In: Halver, H.E. and Hardy, R.W. (ed.) *Fish Nutrition*, 3rd edition. New York: Elsevier, p.181-257.

Sawabe, O., Oda, Y., Shiomi, Y., Ezura, Y. (1995) Alginate degradation by bacteria from the gut of sea urchins and abalones. *Microb Ecol*, 30, p.193–202.

Schatt, P., Féral, J.P. (1996) Completely direct development of *Abatus cordatus*, a brooding schizasterid (Echinodermata: Echinoidea) from Kerguelen, with description of perigastrulation, a hypothetical new mode of gastrulation. *Biol Bull*, 190, p.24–44.

Scheibling, R.E. and Hamm, J. (1991) Interactions between sea urchins (*Strongylocentrotus droebachiensis*) and their predators in field and laboratory experiments. *Mar Biol*, 110, p.105–116.

Schinner, G.O. and McClintock, J.B. (1993) Structural characteristics of marsupial brood pouches of the antarctic sea urchins *Abatus nimrodi* and *Abatus shackletoni* (Echinoidea: Spatangoida). *Journal of Morphology* , 216, p.79-93.

Schiopu, D., George, S.B., Castell, J. (2006) Ingestion rates and dietary lipids affect growth and fatty acid composition of *Dendraster excentricus* larvae. *J Exper Mar Biol Ecol*, 328, p.47-75.

Schlosser, S.C., Lupatsch, I., Lawrence, J.M., Lawrence, A.L., Shpigel, M. (2005) Protein and energy digestibility and gonad development of the European sea urchin

Paracentrotus lividus (Lamarck) fed algal and prepared diets during spring and fall. *Aquacult Res*, 36, p.972-982.

Schroeter, S.C., Barilotti, D.C., Dixon, J.D., Ebert, T.A., Kalvass, P.E. (1998) A proposed method for red sea urchin (*Strongylocentrotus franciscanus*) resource assessment in California. In: Magoon, O.T., Converse, H., Baird, B., Miller Henson, M. (ed.) *California and the World Ocean '97: Taking a Look at California's Ocean Resources: an Agenda for the Futur*e: *Conference Proceedings*, 1-2, p.437-437.

Sekirina, G.G., Skoblina, M.N., Vassetzky, S.G., Bilinkis, A.A. (1983) The fusion of oocytes of the starfish *Aphelasteris japonica*. I. Formation cell hybrids. *Cell Diff*., 12, p.67–71.

Sewell, M.A. (2005) Utilization of lipids during early development of the sea urchin Evechinus *chloroticus*. *Mar Ecol-Prog Ser*, 304, p.133-142.

Sewell, M.A. and Manahan, D.T. (2001) Echinoderm eggs: biochemistry and larval biology. In: Barker, M. (ed.) *Echinoderms 2000*. Lisse: Swets & Zeitlinger, p.55–58

Shpigel, M., McBride, S.C., Marciano, S., Lupatsch, I. (2004) The effect of photoperiod and temperature on the reproduction of European sea urchin *Paracentrotus lividus*. *Aquaculture*, 232, p,343-355.

Shpigel, M., McBride, S.C., Marciano, S., Shiri, R., Ben-Amotz, A. (2005) Improving gonad colour and somatic index in the European sea urchin, *Paracentrotus lividus*. *Aquaculture*, 245, p.101–109.

Shpigel, M., Schlosser, S.S., Ben-Amotz, A., Lawrence, A.L., Lawrence, J.M. (2006) Effects of dietary carotenoid on the gut and the gonad of the sea urchin *Paracentrotus lividus*. *Aquaculture*, 261, p.1269-1280.

Shyu, A., Raff, R.A., Blumenthal, T. (1986) Expression of the vitellogenin gene in female and male sea urchins. *Proc Natl Acad Sci*, 83, p.3865–3869.

Siikavuopio, S.I., Dale, T., Carleh¨og, M. (2007) Sensory quality of gonads from the green sea urchin, *Strongylocentrotus droebachiensis*, fed different diets. *J Shellfish Res*, 26 (2), p.637–643.

Silva, J.R. (2000) The onset of phagocytosis and identity in the embryo of *Lytechinus variegatus*. *Dev Comp Immunol*, 24, p.733-739.

Smith, L.C. (2005) *Host responses to bacteria: Innate immunity in invertebrates*. Cambridge: Cambridge University Press.

Smith, L.C., Rast, J.P., Brockton, V., Terwilliger, D.P., Nair, S.V., Buckley, K.M., Majeske, A.J. (2006) The sea urchin immune system. *ISJ*, 3, p.25-39.

Smith, M.M., Smith, L.C., Cameron, R.A., Urry, L.A. (2008) The larval stages of the sea urchin, *Strongylocentrotus purpuratus*. *J Morphol*, 269, 6, p.713–733.

Southgate, P.C. and Ito, M. (1998) Evaluation of a partial flow-through culture technique for pearl oyster (*Pinctada margaritifera* L.) larvae. *Aquacult Eng*, 18, p.1-7.

Spirlet, C., Gosjean, P., Jangoux, M. (1998) Reproductive cycle of the echinoid *Paracentrotus lividus*: analysis by means of maturity index. *Invert Reprod Develop*, 34, p.69-81.

Spirlet, C., Grosjean, P., Jangoux, M. (2000) Optimization of gonad growth by manipulation of temperature and photoperiod in cultivated sea urchins, *Paracentrotus lividus* (Lamarck) (Echinodermata). *Aquaculture*, 185, p.85–99.

Spirlet, C., Grosjean, P., Jangoux, M. (2001) Cultivation of *Paracentrotus lividus* (Echinodermata: Echinoidea) on extruded feeds: digestive efficiency, somatic and gonadal growth. *Aquacult Nutr*, 7, p.91-99.

Steinberg, P.D. and van Altena, I. (1992) Tolerance of marine invertebrate herbivores to brown algal phlorotannins in temperate Australia. *Ecol Monogr*, 62, p.189–222.

Steinhardt, R., Zucker, R., Schatten, G. (1977) Intracellular calcium release at fertilization in the sea urchin egg. *Dev Biol*, 58, p.185–196.

Stotz., W.B. (2009) The history of the Chilean urchin fishery: Chronicle of an announced death. Echinoderms: Durham. Proceedings of the 12th International Echinoderm Conference, 7-11 August 2006, Durham, New Hampshire, U.S.A. Edited by Larry G., Harris, S., Böttger, A., Walker, C.V., Lesser, M.P., CRC Press 2009, p.561–570.

Strathmann, R.R. (1971) The feeding behaviour of the planktotrophic echinoderm larvae: mechanisms, regulation, and rates of suspension feeding*. J Exp Mar Biol Ecol*, 6, p.109-160.

Strathmann, R.R. (1978) Evolution and loss of feeding larval stages of marine invertebrates. *Evolution*, 32, p.894-906.

Strathmann, R.R. (1989) Existence and function of a gel filled primary body cavity in development of echinoderms and hemichordates. *Biol Bull*, 176, p.25-31.

Strathmann, R.R. (1996) Are planktonic larvae of marine benthic invertebrates too scarce to compete within species*? Oceanol Acta*, 19, p.399-407.

Strathmann, R.R., Fenaux, L., Strathmann, M.F. (1992) Heterochronic developmental plasticity in larval sea-urchins and its implications for evolution of non-feeding larvae. *Evolution*, 46, p.972-986.

Suckling, C.C., Symonds, R.C., Kelly, M.S., Young, A.J. (2011) The effect of artificial diet on gonad color and biomass in the edible sea urchin *Psammechinus miliaris*. *Aquaculture*, 318 (3-4), p.335-342.

Suzuki, M., Kikuchi, R., Ohnishi, T. (1984) The polysaccharide degradation activity in digestive tract of sea urchin *Strongylocentrotus nudus*. *Bull Jap Soc Sci Fish*, 50, p.1255–1260.

Swalla, B.J. (2001) Phylogeny of the urochordates: implications for chordate evolution. In: Sawada, H. and Lambert, C. (ed.) *Biology of ascidians* (Proceedings of the First International Symposium on the Biology of Ascidians). Tokyo, Japan: Springer Verlag, p.219–224.

Swanson, R.L., Byrne, M., Prowse, T.A.A., Mos, B., Dworjanyn, S.A., Steinberg, P.D. (2012) Dissolved histamine: a potential habitat marker promoting settlement and metamorphosis in sea urchin larvae. *Mar Bio*, 159, p.915-925.

Sweijd, N.A. (1990) The digestive mechanisms of an intertidal grazer, the sea urchin *Parechinus angulosus*. MSc thesis. Rhodes University, Grahamstown.

Symonds, R.C., Kelly, M.S., Caris-Veyrat, C., Young, A.J. (2007) Carotenoids in the sea urchin *Paracentrotus lividus*: Occurrence of 9′-cis-echinenone as the dominant carotenoid in gonad color determination. *Comp Biochem Physiol Part B*, 148, p.432-444.

Takeuchi, K., Tajima, K., Iqbal, M.M., Sawabe, T., Ezura, Y. (1999) Studies on the taxonomy and serology of the causative bacteria of the disease of sea urchin *Strongylocentrotus intermedius* occurring at low water temperatures. *Fisheries Sci*, 65, p.264–268.

Taki, J. and Higashida, I. (1964) Investigation and problem on introduction of rocks to fishing grounds to enhance the sea urchin *Hemicentrotus pulcherrimus* in Fukui Prefecture. *Aquaculture*, 12, p.37-47.

Tegner, M.J. (1989) The feasibility of enhancing red sea urchin *Strongylocentrotus franciscanus* stocks in California: an analysis of the options. *Mar Fish Rev*, 51,

p.1-22.

Tegner, M.J. and Dayton, P.K. (1981) Population-structure, recruitment and mortality of 2 sea-urchins (*Strongylocentrotus franciscanus* and *S. purpuratus*) in a kelp forest. *Mar Ecol-Prog Ser*, 5 (3), p.255-268.

Tegner, M.J. and Dayton, P.K. (2000) Ecosystem effects of fishing in kelp forest communities. *ICES J Mar Sci*, 57, p.579-589.

Tocher, D.R. (2003) Metabolism and functions of lipids and fatty acids in teleost fish. *Rev Fish Sci*, 11, p.107-184.

Tocher, D.R. (2010) Fatty acid requirements in ontogeny of marine and freshwater fish. *Aquac Res*, 41, p.717–732.

Tocher, D.R. and Harvie, D.G. (1988) Fatty acid compositions of the major phosphoglycerides from fish neural tissues; (n-3) and (n-6) polyunsaturated fatty acids in rainbow trout (Salmo gairdneri, L.) and cod (Gadus morhua) brains and retinas. *Fish Physiol Biochem*, 5, p.229-239.

Tokin, T.B. and Filimonova, F.G. (1977) Electron microscope study of the digestive system of *Strongylocentrotus droebachiensis* (Echinodermata: Echinoidea). *Mar Biol*, 44, p.143–155.

Tortonese, E. (1965) *Fauna d'Italia, Echinodermata*. Bologna: Edizioni Calderini.

Torunski, H. (1979) Biological erosion and its significance for the morphogenesis of limestone coasts and for nearshore sedimentation (northern Adriatic). *Senckenberg Marit*, 11 (3–6), p.193–265.

Trenzado, C. E., Hidalgo, F., Villanueva, D., Furne, M., Diaz-Casado, M. E., Merino, R., Sanz, A. (2012) Study of the enzymatic digestive profile in three species of Mediterranean sea urchins. *Aquaculture*, 344, p.174-180.

Trimmer, J.S. and Vacquier, V.D. (1986) Activation of sea urchin gametes, *Annu Rev*

Cell Biol, 2, p.1–26.

Tsushima, M. (2007) Carotenoids in sea urchins. In: Lawrence, J.M. (ed.) *Edible sea urchins: biology and ecology.* Amsterdam: Elsevier Science B.V., p.159–166.

Tunnicliffe, V.A., McArthur, A.G., Mchugh, D., (1998) Biogeographical perspective of the deep-sea hydrothermal vent fauna. *Adv Mar Biol*, 34, p.353–442.

Turon, X., Giribet, G., Lopez, S., Placin, C. (1995) Growth and population structure of *Paracentrotus lividus* (Echindermata: Echinoidea in two contrasting habitats. *Mar Ecol Prog Ser,* 122, p.193-204.

Ullrich-Lüter, E.M., Dupontb, S., Arboleda, E., Hausen, H., Arnone, M.I. (2011) Unique system of photoreceptors in sea urchin tube feet. *PNAS*, 108 (20), p.8367-8372.

Unuma, T., Suzuki, T., Kurokawa, T., Yamamoto, T., Akiyama, T. (1998) A protein identical to the yolk protein is stored in the testis in male red sea urchin, *Pseudocentrotus depressus*. *Biol Bull*, 194, p.92–97.

Unuma, T., Walker, C.W. (2010) The roleof themajor yolk protein in sea urchin reproduction and its relevance to aquaculture. In: Harris, L.G., Böttger, S.A., Walker, C.W., Lesser, M.P. (ed.) *Echinoderms: Durham*. The Netherlands: Balkema, p.437–444.

Unuma, T., Yamamoto, T., Akiyama, T., Shiraishi, M., Ohta, H. (2003) Quantitative changes in yolk protein and other components in the ovary and testis of the sea urchin *Pseudocentrotus depressus*. *J Exp Biol* 206: 365–372

Vadas, R.L. (1977) Preferential feeding: an optimization strategy in sea urchins. *Ecol Mongr*, 47, p.337–371.

Vadas, R.L. and Sr Elner, R.W. (2003) Responses to predation cues and food in two species of sympatric, tropical sea urchins. *PSZN Mar Ecol*, 24, p.101–121.

Vadas, R.L., Elner, R.W., Garwood, P.E., Babb, L.G. (1986) Experimental evaluation of

aggregation behavior in the sea urchin *Strongylocentrotus droebachiensis*. *Mar Biol*, 90, p.433–448.

Vaïtilingon, D., Morgan, R., Grosjean, P., Gosselin, P., Jangoux, M. (2001) Effects of delayed metamorphosis and food rations on the perimetamorphic events in the echinoid *Paracentrotus lividus* (Lamarck, 1816) (Echinodermata). *J Exp Mar Biol Ecol*, 262, p.41-60.

Vaïtilingon, D., Rasolofonirina, R., Jangoux, M. (2003) Consumption preferences, seasonal gut repletion indices, and diel consumption patterns of the sea urchin *Tripneustes gratilla* (Echinodermata: Echinoidea) on a coastal habitat off Toliara (Madagascar). *Mar Biol*, 143, p.451–458.

Varaksina, G.S. and Varaksin, A.A. (1991) Localization of steroid dehydrogenase in testes and ovaries of sea urchins. *Biol Morya*, 2, p.77–82.

Vassetzky, S.G., Sekirina, G.G. (1985) Induced fusion of female gametes and embryonic cells. *Cell Diff*, 16, p.77–82.

Vassetzky, S.G., Skoblina, M.N., Sekirina, G.G. (1986) Induced fusion of echinoderm oocytes and eggs. *Meth Cell Biol*, 27, p.359–378.

Ventrella, V., Pirini, M., Pagliarani, A., Trombetti, F., Manuzzi, M.P., Borgatti, A.R. (2008). Effect of temporal and geographical factors on fatty acid composition of *Mytilus galloprovincialis* from the Adriatic Sea. *Comp Biochem Physiol*, B, 149, p.241–250.

Verlaque, M. (1987) Relations entre *Paracentrotus lividus* (Lamarck) et le phytobenthos de Méditerranée occidentale. In: Boudouresque, C.F. (ed.) *Colloque international sur Paracentrotus lividus et les oursins comestibles*. Marseilles: GIS Posidonie, p.5–36.

Verlaque, M. (1990) Relations entre *Sarpa salpa* (Linneus, 1758), les autres poisons

brouteurs et le phytobenthos algal mediterraneen. *Oceanol Acta*, 13, p.373-388.

Vienne, A. and Pontarotti P. (2006) Metaphylogeny of 82 gene families sheds a new light on chordate evolution. *Int J Biol Sci*, 2, p.32-37.

Villinski, J.T., Villinski, J.C., Byrne, M., Raff, R.A. (2002) Convergent maternal provisioning and life history evolution in echinoderms. *Evolution*, 56, p.1764-1775.

von Ubisch, L. (1913) Die entwicklung von *Strongylocentrotus lividus*. *Z Wiss Zool*, 106, p.409–448.

Walker, C.W. (1979) Ultrastructure of the somatic portion of the gonads in asteroids, with emphasis on flagellatedcollar cells and nutrient transport. *J Morph*, 166, p.81–107.

Walker, C.W. (1982) Nutrition of gametes. In: Jangoux, M., Lawrence, J. (ed.) *Nutrition of Echinoderms*. Rotterdam: Balkema, p.449–468.

Walker, C.W., Harrington, L.M., Lesser, M.P. and Fagerberg, W.R. (2005) Nutritive phagocyte incubation chambers provide a structural and nutritive microenvironment for germ cells of *Strongylocentrotus droebachiensis*, the Green Sea Urchin. *Biol Bull*, 209 (1), p.31-48.

Walker, C.W., Unuma, T., Lesser, M.P. (2007) Gametogenesis and Reproduction of Sea Urchins. In: Lawrence, J.M. (ed.), *Edible Sea Urchins: Biology and Ecology*. Amsterdam – London - New York – Oxford- Paris- Shannon-Tokyo, Elsevier Science, p.11-33.

Wasson, K.M. and Watts, S.A. (2007) Endocrine Regulation of Sea Urchin Reproduction. In: Lawrence, J.M. (ed.) *Edible sea urchin biology and ecology*. Amsterdam: Elsevier Science, p.55-69.

Wasson, K.M., Gower, B.A., Hines, G.A., Watts, S.A. (2000a) Levels of progesterone,

testosterone, and estradiol and the androstenedione metabolism in the gonads of *Lytechinus variegaus* (Echinodermata: Echinoidea). *Comp Biochem Physiol*, 126 (C), p.153–165.

Wasson, K.M., Gower, B.A., Watts, S.A. (2000b) Responses of ovaries and testes of *Lytechinus variegatus* (Echinodermata: Echinoidea) to dietary administration of estradiol, progesterone and testosterone. *Mar Biol*, 137, p.245–255.

Watanabe, T., Verakunpiriya, V., Watanabe, K., Viswanath, K., Satoh, S. (1998) Feeding rainbow trout with non-fishmeal diets. *Fisheries Sci*, 63, p.258–266.

Watts, S.A., Hines, G.A., Byrum, C.A., McClintock, J.B., Marion, K.R. (1994) Tissue- and species-specific variations in androgen metabolism. In: David, B., Guille, A., Féral, J-P., Roux, M. (ed.) *Echinoderms through time*. Rotterdam: Balkema, p.155–164.

Watts, S.A., Hofer, S.C., Desmond, R.A., Lawrence, A.L., Lawrence, J.M. (2011) The effect of temperature on feeding and growth characteristics of the sea urchin *Lytechinus variegatus* fed a formulated feed. *J Exp Mar Biol Ecol*, 397 (2), p.188-195.

Wilson, E.B. (1953) *The Cell in Development and Heredity*. New York: Macmillan, p.1–1232.

Wray, G.A. (1992) The evolution of larval morphology during the post-Paleozoic radiation of Echinoids. *Paleobiol*, 18, p.258-287.

Wray, G.A. (1995) Evolution of larvae and developmental modes. In: McEdward, L.R. (ed.) *Ecology of marine invertebrate larvae*. Boca Raton, FL: CRC Press, p.413-447.

Wright, J.T., Dworjanyn, S.A., Rogers, C.N., Steinberg, P.D., Williamson, J.E., Poore, A.G.B. (2005) Density-dependent sea urchin grazing: differential removal of

species, changes in community composition and alternative community states. *Mar Ecol, Prog Ser*, 298, p.143–156.

Yamaguchi, K., Araki, I., Aoki, T., Tseng, C-H, Kitamikado, M. (1989) Algal cell wall-degrading enzymes from viscera of marine animals. *Nip Suisan Gak*, 55, p.105–110.

Young, C.M. and Chia, F.S. (1987) Abundance and distribution of pelagic larvae as influenced by predation, behaviour, and hydrographic factors. In: Giese, A.C., Pearse, J.S., Pearse, V.B. (ed.) *Reproduction of marine invertebrates IX. General aspects: seeking unity in diversity.* Palo Alto, CA: Blackwell Scientific Publications, p.385-463.

Young, C.M., Tyler, P.A., Fenaux, L. (1997) Potential for deep-sea invasion by Mediterranean shallow water echinoids: pressure and temperature as stage-specific dispersal barriers. *Mar Ecol-Progr Ser*, 154, p.197–209.

Young, M.A.L. and Bellwood, D.R. (2011) Diel patterns in sea urchin activity and predation on sea urchins on the Great Barrier Reef. *Coral Reefs*, 30 (3), p.729-736.

Yui, M. and Bayne, C. (1983) Echinoderm immunity: Bacterial clearance by the sea urchin *Strongylocentrotus purpuratus*. *Biol Bull*, 165, p.473-485.

Zakhartsev, M.V., Naumenko, N.V., Chelomin, V.P. (1998) Non-methylene interrupted fatty acids in phospholipids of the membranes of the mussel *Crenomytilus grayanus*. *Rus J Mar Biol*, 24, p.183-186.

Zhong, J., Zhang, J., Mukwaya, E., Wang, Y. (2009) Revaluation of deuterostome phylogeny and evolutionary relationships among chordate subphyla using mitogenome data. *JGG*, 36 (3), p.151–160.

Zhukova, N. (1986) Biosynthesis of Non-Methylene-Interrupted Dienoic fatty acids from carbon^{-14} acetate in Mollusks. *BBA,* 878 (1), p.131-134.

Zhukova, N. (1991) The pathway of the biosynthese of Non-Methylene-Interrupted Dienoic fatty acids in mollusks. *Comp Biochem Physiol Part B*, 100 (4), p.801-804.

Publications and conferences

Carboni S., Hughes A. D., Tocher D.R., Migaud H. (accepted). Influence of broodstock diet on somatic growth, fecundity, egg carotenoids and larval survival of sea urchin (*Paracentrotus lividus*). Aquaculture Research.

Carboni S., Hughes A.D., Atack T., Tocher D.R., Migaud H. 2012. Fatty acid profiles during gametogenesis in sea urchin (*Paracentrotus lividus*): Effects of dietary inputs on gonad, egg and embryo profiles. Comparative Physiology and Biochemistry, Part A. 164 (2):376-382.

Carboni S., Hughes A.D., Vignier J., Kelly M., Migaud H. 2012. Evaluation of flow through culture technique for sea urchin (*Paracentrotus lividus*) larvae commercial production. Aquaculture Research. 43: 11.

Carboni S., Vignier J., Chiantore M., Tocher D.R., Migaud H. 2012. Effects of dietary microalgae on growth, survival and fatty acid composition of sea urchin *Paracentrotus lividus* throughout larval development. Aquaculture. 324-325: 250-258.

Carboni S., Chiantore M., Migaud H., Tocher R.D. 2011. Effects of Broodstock Diets on: *Paracentrotus lividus* Growth; Reproduction; Lipid & Fatty Acids; Carotenoids; Off-spring performances. European Aquaculture Society Conference, Greece

Carboni S., Tocher R.D. 2011. New evidence for LC-PUFAs synthesis in sea urchin larvae. Scottish Lipid Discussion Group. Aberdeen, Scotland, UK.

Carboni S., Chiantore M., Vignier J., Migaud H., Tocher R.D. 2010. *Paracentrotus lividus* aquaculture, optimization of larval rearing protocols and broodstock nutrition: Project overview. European Aquaculture Society Conference. Porto, Portugal.

Carboni S., Vignier J. 2010. Research an Development at the Ardtoe Marine Lab. Association of Scottish Shellfish Grower Meeting, Oban, Scotland (UK)

Carboni S., Vignier J., Chiantore M., Migaud H., Tocher R.D. 2010. Effects of different microalgae diets on edible sea urchin (*Paracentrotus lividus*) larval development, survival and fatty acid composition. European Aquaculture Society Conference Porto, Portugal.

Chiantore M., Shpiegel M., Kelly M., Carboni S., et al. 2010. ENRICH PROJECT: Optimization of an integrated system for sea urchin rearing and commercialization. European Aquaculture Society Conference, Porto, Portugal.