Nutritional Requirements and Digestive Competence of Postlarval Scallops (*Argopecten irradians* and *Placopecten magellanicus*), With Emphasis on the Role of Lipids and Fatty Acids

by

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Abstract

Postlarval bay and sea scallops (*Argopecten irradians* and *Placopecten magellanicus*) undergo extreme morphogenesis of feeding organs and may be particularly vulnerable to nutritional deficiencies which, to date, remain largely undefined. The goals of this study were to 1) identify diets of few components to optimize scallop growth and survival, 2) identify the nutritional requirements of postlarval and juvenile scallops with special focus on polyunsaturated fatty acids (PUFA) and 3) identify differences in digestive competence over development and between scallop species. To this end, five growth trials (3-4 wks) were conducted offering scallops unialgal or binary diets consisting of one of three diatoms and one of five flagellates. Two additional trials were conducted (7-9 wks) to examine ontogenetic changes in enzymatic activity. Two binary diet combinations, *Chaetoceros muelleri* (CHGRA) in combination with either *Pavlova* spp. (CCMP strain 459; Pav 459) or *Pavlova pinguis,* consistently ranked among the highest diets tested, yielding postlarval growth rates as high as 28 and 59 μm day^{-1} for bay and sea scallops, respectively. Growth response appears related to two n-6 polyunsaturated fatty acids (PUFA): arachidonic (AA) found in CHGRA and docosapentaenoic acid (DPA) provided by Pav 459 and *P. pinguis*. Enrichments (relative to diet) of these individual fatty acids, as well as Σn-6 fatty acids, were observed in tissues of both scallop species regardless of dietary treatment, suggesting a requirement for n-6 fatty acids in pectinids that has been largely overlooked. By contrast, the docosahexaenoic (DHA)-deficient diet of *Tetraselmis striata* / CHGRA ranked lowest for postlarval sea scallops but second highest for bay scallops, suggesting a more stringent DHA requirement in sea scallops, possibly to maintain membrane fluidity at the colder temperatures to which they are adapted. Species- and stage-specific differences in enzymatic activity were also observed with the most dramatic changes occurring before ~1.2 mm in shell height. Mean activities of the three carbohydrases tested differed between scallop species, possibly an adaptation to the natural food supply. The nutritional requirements identified in this study should aid in the development of targeted diets to optimize scallop growth and increase the understanding of natural bivalve-seston interactions.

List of Abbreviations and Symbols Used

20:4n-6 : structural abbreviation of arachidonic acid (also abbreviated as AA)

20:5n3 : structural abbreviation of eicosapentaenoic acid (also abbreviated as EPA)

22:5n-6 : structural abbreviation of docosapentaenoic acid (also abbreviated as DPA)

22:6n-3 : structural abbreviation of docosahexaenoic acid (also abbreviated as DHA)

AA: arachidonic acid (also reported as 20:4n-6)

ACTIN: *Thalassiosira weissflogii*

AFDW: ash-free dry weight

AMPL: acetone mobile polar lipids

CCMP: Center for the Culture of Marine Phytoplankton, West Boothbay Harbor, Maine

CHGRA: *Chaetoceros muelleri*

DHA: docosahexaenoic acid (also reported as 22:6n-3)

DPA: docosapentaenoic acid (also reported as 22:5n-6)

DW: dry weight

EPA: eicosapentaenoic acid (also reported as 20:5n-3)

MONO: *Pavlova lutheri*

MUFA: monounsaturated fatty acid

MRS: Marine Research Station

OD: optical density

Pav 459: *Pavlova* sp. (CCMP strain # 459)

PUFA: polyunsaturated fatty acid

SAT: saturated fatty acid

TAG: triacylglycerol

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Chapter 1: Introduction

1.1 Background

The giant scallop, *Placopecten magellanicus*, is native to cold waters ($\leq 18^{o}C$) extending from Labrador, Canada, to Cape Hatteras, North Carolina, USA (Couturier *et al.*, 1995). This is a commercially-prized species, but growth rate is relatively slow, and it takes 2-3 years to attain market size (Dadswell and Parsons, 1992). The northern bay scallop, *Argopecten irradians irradians*, is a temperate subspecies with natural populations occurring from New Jersey to Maine. Bay scallops were introduced to Canada in 1979 for culture purposes, and have subsequently established themselves in local waters in Prince Edward Island and Nova Scotia (Couturier *et al.*, 1995). *A. irradians* is a much faster-growing species than the sea scallop and is capable of attaining market size within ~6 months under favorable conditions (Castagna and Duggan, 1971). There is a need, however, to accelerate growth of bay scallops in this region so that hatchery produced postlarvae deployed in the field in the summer can attain market size before their first winter.

The scallop aquaculture industry in Atlantic Canada is largely constrained by the availability and affordability of high quality spat. Natural spatfalls of sea scallops are often limited and unreliable (Dadswell and Parsons, 1992), and hatchery raised spat are not only expensive, but also experience unexplained mortalities in larval and post-settlement stages (Couturier *et al.*, 1995). These observed mortalities may be a result of nutritional deficiencies, as little is known about the dietary requirements of these early developmental stages. Typically, diets used in scallop hatcheries have been adopted from other bivalves with more established culture practices such as clams and oysters, yet the performance of algal diets is often highly species-specific. Previous nutritional work conducted on scallops has focused mostly on diets for broodstock conditioning and larval rearing with little attention to vulnerable post-settlement stages (e.g. Soudant *et al.*, 1996b; Caers *et al.*, 1999; Pernet *et al.*, 2005).

Bay and sea scallops differ in many characteristics, such as the range of temperature tolerance, life span, development time and habitat, and may therefore have different nutritional needs. Sea scallops have separate sexes and are iteroparous, whereas

bay scallops are hermaphroditic and semelparous. As there is an inverse relationship between the degree of unsaturation of membrane lipids and temperature (Spector, 1985), the cold-water adapted sea scallop may have more stringent polyunsaturated fatty acid (PUFA) requirements than the bay scallop. This is supported by recent work demonstrating that the maintenance of membrane fluidity in sea scallops is correlated with the concentration of a specific PUFA, eicosapentaenoic acid (EPA), in gill phospholipids (Hall *et al.*, 2002). As most bivalves are unable to synthesize significant amounts of PUFAs via elongation and/or desaturation, they are reliant on dietary sources to meet particular fatty acid requirements (Ackman and Kean-Howie, 1995). Thus, sea scallops are expected to be particularly vulnerable to nutritional deficiencies.

The importance of n-3 PUFAs, such as EPA and docosahexaenoic acid (DHA), and to a much lesser extent n-6 fatty acids in dietary lipids, has been identified in several bivalve species (Langdon and Waldock, 1981; Enright *et al.*, 1986; Berntsson *et al.*, 1997). These fatty acids may play a role in membrane function, as precursors to bioactive compounds such as prostaglandins and as energy substrates. To date, no work has focused on the specific PUFA requirements of postlarval scallops and therefore, hatcheries often feed multi-component diets in the hope that nutritional requirements will be met. However, algal culture costs are high, often 30-40% of total hatchery costs (Coutteau and Sorgeloos, 1993); thus it is advantageous to limit the number of algal species cultured yet provide a nutritionally complete diet.

Morphological properties such as cell size and digestibility (related to cell wall structure) also influence the utilization and performance of microalgal diets. Several studies have investigated digestive enzymes of bivalves (Reid, 1968; Trainer and Tillinghast, 1982; Ballantyne and Berges, 1991; Ibarrola *et al.*, 1998b), and some work has focused on postlarval stages of molluscs such as abalone (Takami *et al.*, 1998) but no work has been conducted on scallop larval and postlarval stages. Furthermore, although the role of digestive enzymes over ontogeny has been studied in finfish (Douglas *et al.*, 1999; Kim *et al.*, 2001), and oysters (Luna-Gonzalez *et al.*, 2004) no work to date has characterized digestive enzymes during development in scallops. As sea scallops experience prolonged morphogenesis following settlement (Veniot *et al.*, 2003), they provide an ideal model for correlating the expression and/or activities of digestive

enzymes with major morphological changes during development. Clearly, the presence or absence (or changes in gene expression during ontogeny) as well as the activities of various intra- and extracellular digestive enzymes will influence the success of a microalgal diet at a given developmental stage.

1.2 Lipids

Marine systems are generally rich in the lipids and fatty acids necessary for growth of marine organisms. However, in an aquaculture setting it becomes imperative to both understand and provide for the specific nutritional requirements of the target species.

Lipid classes can be divided into two main groups: neutral lipids such as triacylglycerols (TAG), wax esters (WE) and sterols (ST) and polar lipids largely represented by the phospholipids (PL). While all lipid classes can be oxidized to yield caloric energy, TAG are commonly utilized for this purpose as they often contain more than one saturated fatty acid, and have a high number of reduced CH groups available for oxidation. TAG also play a critical role as a storehouse for long chain polyunsaturated fatty acids (PUFAs) required for a variety of specific metabolic functions and the synthesis of other lipid molecules.

Other lipid classes are less involved in energetic processes, but are necessary for the maintenance of cellular structural integrity and specific metabolic pathways. Sterols are associated with vitamin D synthesis, bile acid synthesis, and hormone regulation in finfish (Rust, 2002; Baker, 2003), and both sterols and phospholipids are essential for the maintenance of membrane structure and fluidity. As phospholipids are the primary constituents of the cellular lipid bilayer, their composition greatly influences membrane fluidity and function.

Fatty acids in marine systems can either be synthesized *de novo* or acquired from dietary sources, and those that cannot be synthesized in quantities capable of sustaining growth are considered "essential". Several long chain PUFAs from the n-3 and n-6 families appear to be essential to sustain growth of marine organisms and are involved in the maintenance of membrane fluidity and as precursors for bioactive molecules involved in immune response, osmoregulation and reproduction. Individual PUFAs cited as being particularly important include the n-3 fatty acids eicosapentaenoic acid (EPA; 20:5n-3)

and docosahexaenoic acid (DHA; 22:6n-3), as well as the n-6 arachidonic acid (AA; 20:4n-6) (Langdon and Waldock, 1981; Sargent *et al.*, 1999a). Although eukaryotes possess the Δ5 and Δ6 desaturation enzymes that are capable of synthesizing these PUFAs from shorter-chain, dietary precursors such as 18:3n-3 and 18:2n-6 (Cook and McMaster, 2002), marine animals are generally not capable of elongating and/or desaturating them in quantities capable of sustaining normal growth rates.

This inability to produce long-chain PUFAs in sufficient quantity has been reported in adult clams, *Mesodesma mactroides* (De Moreno *et al.*, 1976), Pacific oyster spat, *Crassostrea gigas* (Langdon and Waldock, 1981; Waldock and Holland, 1984), scallop larvae, *Pecten maximus* (Delaunay *et al.*, 1993), and adult Eastern oysters, *C. virginica,* for which Chu and Greaves (1991) were unable to detect desaturase activity using radiolabeled 16:0, 18:2n-6 and 18:3n-3. Frequent citations (Knauer and Southgate, 1999; Nevejan *et al.*, 2003b) have indicated that larvae of the Manila clam are the exception to this rule. *Tapes philippinarum,* fed a spray-dried diet of *Nannochloris sp.,* containing no fatty acids longer than 18C, were able to convert 18C precursors to C20 and 22 PUFAs (Laing *et al.*, 1990), yielding 24% DHA in larval tissues after 22 days. However, growth rate attained on this diet was substantially lower than that attained on a mixed algal diet of *Isochrysis sp.*, T-Iso, and *Chaetoceros calcitrans* (~ 3.2 *vs.* 9.0 μm d^{-1}), indicating that rates of EPA and DHA synthesis from 18C precursors were not sufficient to optimize growth.

1.2.1 Energetic Storage and Substrate Utilization

Lipids are recognized as possessing high energy content, as lipid oxidation yields ~2.5 times the energy of carbohydrate and 1.8 times that of protein (Corraze, 2001). Triacylglycerols, or TAG, constitute the common storage lipid class across most animal species. Structurally, a TAG consists of a glycerol backbone attached to three acyl or fatty acid chains. TAG generally have a lower concentration of PUFAs than phospholipids and are predominantly composed of saturated and monounsaturated fatty acids, which provide an easily-catabolized energy source. In the absence of food, clam and oyster larvae catabolized TAG preferentially during starvation, accounting for 80% of total lipid loss (Gallager and Mann, 1986; Gallager *et al.*, 1986).

TAG also play a significant energetic role in reproduction and larval development. Elevated levels accumulate in the bivalve gonad prior to spawning (Allen and Conley, 1982; Napolitano and Ackman, 1992), and in the eggs (~ 60%; Pernet *et al.*, 2003a), presumably providing energy to ensure larval viability, which has also been linked to neutral lipid concentrations in eggs (Helm *et al.*, 1973; Gallager and Mann, 1986).

Fatty acids are often oxidized to provide metabolic energy in a variety of marine organisms (Sargent *et al.*, 2002), and in many cases, short-chain, saturated fatty acids are used for this purpose. Catabolism of saturated fatty acids can release more energy than unsaturated fatty acids of the same chain length, and saturated fatty acid concentrations have been correlated with growth performance in several species of bivalves, such as the oyster *Crassostrea gigas* (Thompson and Harrison, 1992) and the scallop *Argopecten purpuratus* (Nevejan *et al.*, 2003a). Furthermore, saturated fatty acids are commonly stored in TAG of European oyster, *O. edulis,* larvae (Napolitano *et al.*, 1988), where they function primarily as an energetic substrate. PUFAs are also oxidized as an energy source, although this appears limited to specific PUFA. For example, EPA appears to be preferentially catabolized, while DHA is conserved in scallop larvae (*Patinopecten yessoensis,* Whyte *et al.*, 1991; *Pecten maximus*, Delaunay *et al.*, 1993; *Placopecten magellanicus*, Pernet and Tremblay, 2004), even when offered diets limited in DHA concentration (*Argopecten purpuratus*; Nevejan *et al.*, 2003b). Thus, for reasons of energy content and competing requirements in cellular metabolism, saturated and unsaturated fatty acids are catabolized or conserved preferentially.

While lipids play a critical role in bivalve energetics, other biological compounds contribute to these metabolic needs as well. For example, protein as well as lipid is often catabolized in larvae of the oyster *C. gigas* during metamorphosis which was also correlated with postlarval survival (García-Esquivel *et al.*, 2001). While postlarval *O. edulis* utilize glycogen as the primary energy substrate (Holland and Hannant, 1974) in the scallop *A. purpuratus*, there appears to be a switch from carbohydrate based metabolism immediately post-metamorphosis, to one that is more protein based approximately a month post-settlement (Uriarte and Farias, 1999).

1.2.2 Structural Lipids

Phospholipids are the primary structural constituent of cellular membranes forming the bilayer structure. These lipids consist of two acyl chains and a phosphoric acid group bound to an alcohol, commonly ethanalomine, choline or insitol. The fatty acid composition of membrane lipids greatly influences fluidity, as the addition of cis double bonds occupies more horizontal space reducing the tightness between membrane molecules, and organisms are able to alter membrane structure in response to temperature fluctuations (Hazel, 1988). Membrane fluidity can impact protein function (Dowhan and Bogdanov, 2002), and thus the functionality of the cell.

In the scallop *P. maximus*, phosphatidylcholine and plasmologens constitute about 70% of the polar lipids, and DHA was preferentially incorporated into these two polar lipid classes, suggesting a DHA requirement to maintain membrane function. PUFA concentrations in polar lipids are inversely related to temperature (Cossins and Lee, 1985). DHA remains stable over a wide range of temperatures and pressures, thereby enabling proper membrane function regardless of environmental conditions (Rabinovich and Ripatti, 1990). The freshwater bivalve *Anodonta* demonstrated seasonal fluctuations in both EPA and DHA levels, with higher fatty acid concentrations of these two PUFA observed in colder months (Gardner and Riley, 1972) and DHA was found superior to EPA in supporting mud crab carapace growth and survival (Suprayudi *et al.*, 2004). Although, in the event of a dietary deficiency, EPA may be able to replace DHA, as has been observed in *P. maximus* larvae (Delaunay *et al.*, 1991 as cited in Ackman and Kean-Howie, 1995).

Arachidonic acid (AA) also plays a role in the maintenance of membrane structure, although this is often overshadowed by the role of DHA (Sargent *et al.*, 1999b), and it has also been proposed that DPA (22:5n-6) may be able to substitute for DHA in membranes (Feindel, 2000), as membrane DHA was replaced by DPA in rats fed n-6 rich diets (Stubbs and Smith, 1984). Likewise, in bivalves, there may be a structural requirement for 18:1n-7 and 18:1n9 which serve as precursors to 20 and 22C non-methylene-interrupted, dienoic (NMID) fatty acids. The latter are believed to be of structural significance rather than precursors of bioactive compounds as is the case with many of the long chain PUFAs (Ackman and Kean-Howie, 1995).

Sterols also play a role in the regulation of membrane fluidity, enhancing fluidity at low temperatures, and reducing fluidity at high temperatures (Lehninger *et al.*, 1993) as well being precursors for steroid hormone production. Growth performance has been correlated with dietary sterol concentration in sea scallop postlarvae (Milke *et al.*, 2004), American lobster, *Homarus americanus* (Teshima, 1997), and with 24-methylene cholesterol during post-settlement oyster growth (Wikfors *et al.*, 1996). Cholesterol was shown to be preferentially incorporated by *P. maximus* larvae (Soudant *et al.*, 1998b), *P. magellanicus* larvae (Milke *et al.*, 2004) and adult *P. magellanicus* (Napolitano *et al.*, 1993), and has been identified as the major sterol in marine fish (Morris *et al.*, 1982). Bivalves have a limited ability to synthesize sterols (Walton and Pennock, 1972; Holden and Patterson, 1991; Wikfors *et al.*, 2005), and are reliant on dietary inputs to obtain these compounds (Knauer and Southgate, 1999).

1.2.3 Bioactive Molecules

A variety of bioactive molecules are derived from fatty acids. These compounds, such as prostaglandins and leukotrienes, are responsible for a variety of regulatory activities, such as stress response, osmoregulation, reproduction and immune function. In bivalves, prostaglandins were released by gill tissues of *Modiolus demissus* in response to a 25% decrease in osmotic pressure (Freas and Grollman, 1980), and levels of several prostaglandins synthesized from AA, have been linked with gametogenesis and spawning in pectinids (Osada *et al.*, 1989). Prostaglandins have been found in the testis and ovaries of *Patinopecten yessoensis* (reviewed by Deridovich and Reunova, 1993), and *C. gigas* (Ono *et al.*, 1982), with concentrations generally higher in the female than male gonad during gametogenesis (~4x in *P. yessoensis*; Osada *et al.*, 1989).

In molluscs, cells primarily responsible for fighting off infection are hemocytes, single cells able to wander freely through the open vascular system and across tissue boundaries. Hemocyte membranes consist of a phospholipid bilayer largely reliant on dietary inputs for maintaining its structure and function; therefore, altering dietary inputs can impact the immune capacity of these cells (Delaporte *et al.*, 2003; Hegeret *et al.*, 2004). Higher levels of EPA and AA in haemocytes appeared to be important in optimizing their cellular health in *Ruditapes philippinarum*, especially by increasing

hemocyte oxidative-burst strength and phagocytosis. Arachidonic acid may play a role as a precursor to leukotrienes, which likely exert a positive effect on the oxidative activity of these cells. Arachidonic acid has been correlated with growth rate in larval *P. magellanicus* (Pernet and Tremblay, 2004) and it has been proposed that accumulations of AA in larval sea scallop tissues during metamorphosis may be related to the stimulation of immune function (Pernet *et al.*, 2005).

1.2.4 Reproduction and Larval Development

In bivalves, lipids play a particularly important role in reproduction and larval development, which was evidenced by a strong relationship between tissue lipid concentrations and the gametogenic cycle (i.e. gonadal growth was associated with an increase in lipid reserves; Robinson *et al.*, 1981). Broodstock management and conditioning largely determine early larval performance (Lannan *et al.*, 1980) as the lipid compositions of eggs and larvae often directly reflect the broodstock diet (Helm *et al.*, 1991; Caers *et al.*, 1999). The digestive gland of bivalves plays a critical role in energy storage prior to gametogenesis (Thompson *et al.*, 1974), during which lipids are transported from the digestive gland into the developing female gonad (Vassallo, 1973) and adductor muscle carbohydrate in scallops is converted into TAG (Barber and Blake, 1985). Female gonads accumulate TAG, but male gonads do not as lipid levels necessary for sperm production are significantly lower than those required for egg production (Gabbott *et al.*, 1975; Napolitano *et al.*, 1993; Caers *et al.*, 1999).

TAG is particularly important for bivalve health as dietary concentrations have been associated with larval (Pernet *et al.*, 2003b) and juvenile sea scallop growth (Parrish *et al.*, 1993). Bivalve larvae rely on their endogenous lipid supply for energy prior to the beginning of particulate feeding at the D-stage (Gallager *et al.*, 1986; Holland, 1978), and several studies have described a positive relationship between bivalve egg lipid content and larval performance (Gallager and Mann, 1986; Le Pennec *et al.*, 1991). Approximately 50% of TAG reserves are used during embryogenesis of *P. magellanicus* and *P. yessoensis* larvae (Pernet *et al.*, 2003a; Whyte *et al.*, 1991), and an even greater proportion is used in clams and oysters, i.e. 69% and 71% in *Merceneria mercenaria* and *Crassostrea virginica*, respectively (Gallager *et al.*, 1986). Growth of *Argopecten*

purpuratus larvae appears to be correlated with the proportions of saturated fatty acids, not specific PUFAs (Nevejan *et al.*, 2003a), as an algal diet supplemented with an emulsion rich in PUFAs did not increase growth rates over an emulsion of saturated fatty acids. Protein and lipid are heavily utilized during bivalve metamorphosis (Haws *et al.*, 1993; García-Esquivel *et al.*, 2001), and TAG is the lipid class most heavily catabolized (Holland and Spencer, 1973).

Fatty acid composition, as well as lipid class composition, are pivotal to the success of molluscan reproduction. For example, DHA and AA were selectively incorporated over EPA in the polar lipid fraction of the female gonad of *P. maximus* (Soudant *et al.*, 1996b), and a higher DHA/EPA ratio was associated with hatching success and larval quality (Soudant *et al.*, 1996a). DHA was also retained during embryogenesis of the scallops *P. yessoensis* and *Crassadoma gigantea*, while EPA was depleted for energetic purposes (Whyte *et al.*, 1989; Whyte *et al.*, 1990a).

1.2.5 Indices of Nutritional Value

A variety of indices of nutritional value have been proposed to define food quality in marine organisms. By examining the dietary n-6/n-3 ratios of several different algal diets, and relating them to diet performance of *C. virginica* larvae, Webb and Chu (1983) suggested that a value of 1:2-1:3 was characteristic of good diets, but a value of 1:5 characterizes a diet of only moderate food quality. For bivalve broodstock conditioning, however, optimal dietary n-6/n-3 ratios of 1:5-1:15 have been proposed (Budge *et al.*, 1998), similar to the optimal values identified for adult finfish diets (1:5-1:10; Sargent *et al.*, 1995), indicating that adults may have lower n-6 requirements than larvae.

The relationship between DHA and EPA is considered important for growth optimization in a variety of animals. It has been proposed that adult bivalve diets should have a DHA/EPA ratio of 1:1; whereas, larvae may require a ratio of only 1:2, and finfish apparently have higher requirements where a ratio of 2:1 has been proposed for broodstock diets (reviewed by Budge *et al.*, 1998). Higher DHA levels may be required to maintain the complex neurological systems found in fish that are absent in molluscs. Although the relative composition of fatty acids can influence animal growth and health, these ratios are of little value if absolute concentrations are below a necessary threshold

(Brown and Jeffrey, 1992). Brown et al. (1989), based on a compilation of studies, determined that 1-20 fg μm^{-3} of DHA and EPA in an algal diet was satisfactory to sustain bivalve growth.

As the functional role of AA is becoming increasingly understood, it is important to examine ratios that include this fatty acid, especially as it is a competitive substrate with EPA for a variety of enzymes. For example, an EPA/AA ratio of 1.5-0.4 has resulted in impaired pigmentation and metamorphosis in turbot larvae; whereas, a ratio higher than 4.2 is suggested as a requirement to support normal growth (Sargent *et al.*, 1999b). Increased ratios of AA/EPA have also been correlated with increased growth and survival, as well as resistance to handling stress (reviewed by Bell and Sargent, 2003) in larval gilthead sea bream. The relationship between AA and EPA in the diet and its fitness consequences has to be further examined in molluscs and crustaceans.

Total lipid content in animal tissues has been used as an indicator of animal quality. Gallager et al. (1986) examined the total lipid content of several species of bivalve larvae using Oil Red O, and concluded that high lipid levels are necessary to ensure transition to the benthic stage. The concentration of neutral lipid in tissues has been correlated with survival and growth of oyster and scallop larvae (Waldock and Nascimento, 1979; Delaunay *et al.*, 1993), and the percentage of 14:0 and 16:0 has been correlated with growth of *C. gigas* larvae, perhaps because these are common components of TAG (Thompson and Harrison, 1992). TAG levels are correlated with ovarian maturity in shellfish (Pazos *et al.*, 1997), and high TAG/Sterol and TAG/PL ratios provide a size–independent measure of larval quality, with high values reflecting high larval quality in bivalves (Gallager and Mann, 1986; Delaunay *et al.*, 1992).

1.3 Digestive Enzymes

Enzymatic degradation of ingested material in bivalves primarily occurs in one of two places: extracellularly within the stomach and in association with the crystalline style, and intracellularly in the cells of the digestive diverticula. Carbohydrases are generally extracellular, which high activities of α-amylase and laminarinase frequently reported in association with the crystalline style (Wojtowicz, 1972; Fernandez-Reiriz *et al.*, 2001). Protease activity is generally intracellular in bivalves, predominantly

occurring in the tubule epithelial cells of the digestive diverticula (Ibarrola *et al.*, 1996). Lipolytic activity has been identified in the stomach, crystalline style, digestive diverticula and amoebocytes (hemocytes) of various bivalve species (Morton, 1983). The major factors that have shown to influence enzymatic activity in bivalves include: thermal acclimation / stress (Moore, 1976; Seiderer and Newell, 1979), seston concentration (Fernandez-Reiriz *et al.*, 2004), dietary organic content (Ibarrola *et al.*, 1996; Fernandez-Reiriz *et al.*, 2001) exposure to substrate (Crosby and Reid, 1971), assay pH (Trainer and Tillinghast, 1982; Brock *et al.*, 1986; Areekijseree *et al.*, 2004), and assay temperature (Trainer and Tillinghast, 1982; Brock *et al.*, 1986).

1.3.1 Lipolytic Activity

Lipolytic activity is attributable to reactions by two different groups of enzymes: esterases and lipases, and studies have shown lipolytic activity to be widely-distributed throughout digestive organs in bivalves (Morton, 1983). Esterases and lipases have specific affinities for the same bonds, but esterases generally cleave shorter-chained fatty acids. Two different esterases have been identified in the digestive diverticula of *Pecten maximus* (Reid, 1968), and there appear to be two different expressions of lipolytic activity in the clam *Scrobicularia plana*, each with different temperature and pH optima. Higher esterase activity was associated with the digestive diverticula whereas lipase activity was associated with the crystalline style. (Payne, 1978).

1.3.2 Protease Activity

Most bivalves have a complement of proteases (Reid, 1968). Levels of protease activity in bivalves are low in comparison to those found in finfish (Stark and Walker, 1983) and carnivorous invertebrates (Kozlovsakay and Vaskavsky, 1970, as cited in Reid and Rauchert, 1976), presumably because bivalves consume a relatively low-protein, high-carbohydrate diet. Protease levels are generally lower in the crystalline style than in the digestive diverticula (Brock and Kennedy, 1992; Fernandez-Reiriz *et al.*, 2001); in fact, protein digestion in the clam *Tresus capax* was approximately 4 times lower in the stomach than in the digestive diverticula (Reid and Rauchert, 1976). Protein digestion in the bivalve *Lima hians* was intracellular and dominated by trypsins, but active lipid digestion was observed both intracellularly and extracellularly in *Mya arenaria,* the

softshell clam, where intracellular digestion was dominated by cathepsins (Reid, 1966). Protease levels in the mussel *Modiolus striatulus* were much lower than carbohydrase levels; however, these proteases were able to act on casein as a substrate (Pal *et al.*, 1980), which was not the case in larvae of the scallop *Pecten maximus*. However, relatively high levels of chymotrypsin activity have been reported for some members of the Pectinidae (Reid, 1968), suggesting that the absence of protease activity in *P. maximus* larvae could be stage-specific.

1.3.3 Carbohydrase Activity

Alpha amylase is the most active enzyme in extracellular digestion, breaking down starch, glycogen and related polysaccharides in a random manner and yielding maltose and maltrotriose as the primary products (Stark and Walker, 1983). Exceptionally high levels of α-amylase activity are found in the crystalline style of bivalves; in fact, in *Placopecten magellanicus,* activities were 40x higher than those identified in the digestive gland (Wojtowicz, 1972). Alpha amylase appears to have several different isoenzymes with different molecular weight (Seiderer *et al.*, 1982) which have been reported in *M. edulis*, and *Choromytilus meridionalis* (Newell *et al.*, 1980). In *C. gigas* two α-amylase genes have been described, where gene A is found primarily in the digestive gland and is regulated with respect to variation if food quantity, whereas gene B is also found in the digestive gland, however, it is expressed at a lower level than gene A and is not regulated by changes in food quantity (Huvet *et al.*, 2003).

The term "cellulase" applies to several different enzymes including isoenzymes of true cellulose and cellobiose (Crosby and Reid, 1971), and the breakdown of cellulose is a three-phase process involving several different enzyme types. The first type includes true cellulase which acts directly on cellulose. The second enzyme is a poly-ß-glucosidase, which acts on the products of cellulase activity (cellotriose and cellobiose), and finally a ß-glucosidase (i.e., cellobiase) acts upon these reaction products to form glucose (Morton, 1983). Cellulose is not water-soluble; therefore, the majority of tests examining cellulase activity in bivalves utilize carboxymethyl-cellulose as substrate. This compound is considerably less refractory than detrital cellulose encountered by

bivalves in natural environments, and may therefore overestimate and/or misrepresent natural digestive capacity.

Cellulase activity occurs both extra- and intra-cellularly in bivalves (Crosby and Reid, 1971) and may be substrate-inducible, as higher specific activities were observed in the cockle *Cerastoderma edule* three days after an increase in diet quantity and organic content (Ibarrola *et al.*, 1996). Although it has been proposed that gut bacteria may aid in cellulose assimilation (Crosby and Reid, 1971; Crosby and Peele, 1987), this appears not to be the case in the deposit-feeding clam *Scrobicularia plana*, in which 17 isolated bacterial strains showed no cellulase activity (Morton, 1983).

Laminarinase is a multi-component enzyme complex, and the substrate laminarin appears to be degraded by 4 different enzymes (Stark and Walker, 1983) which presumably include exo- and endo-hydrolytic ß1-3-glucanases and ß-glucosidases (Morton, 1983). Chrysolaminarin is the primary storage polysaccharide in diatoms and is similar in structure to laminarin, so both are hydrolyzed by the enzyme laminarinase. Laminarinase activity has been reported in a variety of bivalves, such as *Placopecten magellanicus* (Wojtowicz, 1972), *Pecten maximus* (Stark and Walker, 1983), *Argopecten irradians, Mytilus edulis*, *Mercenaria mercenaria*, and *Crassostrea virginica* (Brock *et al.*, 1986).

This wide variability in digestive capabilities across bivalve species, in nutritional requirements of different developmental stages and species of bivalves and in microalgal biochemical composition, indicate that these factors must all be considered when selecting microalgal diets for specific applications in bivalve aquaculture.

1.4 Objectives

The main objectives of the present study are:

1) To identify high-performance algal diets comprised of a minimum number of species for the culture of postlarval and juvenile bay scallops, *Argopecten irradians* and sea scallops, *Placopecten magellanicus*.

2) To determine stage-specific dietary requirements of post-settlement bay and sea scallops.

- *Test the hypothesis that PUFA requirements differ at different developmental stages (postlarval* vs. *juvenile).*

- *Test the hypothesis that digestive enzyme presence and/or specific activity differs over early life history stages and may therefore influence the ability of a diet to support growth.*

3) To compare species-specific dietary requirements of bay and sea scallop postlarvae.

- *Test the hypothesis that sea scallops have more-stringent PUFA requirements than bay scallops.*

- *Test the hypothesis that differential uptake, utilization, and/or retention of dietary fatty acids occurs in scallop tissues, and that incorporation strategies differ between scallop species.*

Chapter 2 of this study examines the growth of postlarval sea scallops offered on of five different binary diets, emphasizing the nutritional role of lipids and fatty acids. Chapter 3 examines the growth response of postlarval and juvenile bay scallops offered these same five binary diets and one additional diet. Comparisons are made between these two developmental stages as well as between postlarval bay and sea scallops. The fourth chapter addresses growth performance of sea scallop postlarvae offered diets consisting of one of the *Pavlova* spp. in combination with the diatom *Chaetoceros muelleri*, and attempts to relate dietary success to the biochemical composition of the microalgae. The fifth chapter examines changes in enzymatic activity over ontogeny (pediveliger larvae to 4-5 mm in shell height) in both bay and sea scallops.

Chapter 2: Growth of Postlarval Sea Scallops, *Placopecten magellanicus*, on Microalgal Diets, With Emphasis on the Nutritional Role of Lipids and Fatty Acids.

Reprinted with minor modifications from *Aquaculture*, Volume 234, Lisa M. Milke, V. Monica Bricelj and Christopher C. Parrish, Growth of postlarval sea scallops, *Placopecten magellanicus*, on microalgal diets, with emphasis on the nutritional role of lipids and fatty acids, pages 293-317, Copyright 2004, with permission from Elsevier.

Abstract

Culture of the sea scallop, *Placopecten magellanicus*, is constrained by a reliable supply of high quality postlarvae, yet little is known about the diets and essential nutrients required to maximize growth and survival during these vulnerable stages. Therefore post-settlement sea scallops were exposed to binary microalgal diets consisting of a flagellate: *Pavlova lutheri*, *Pavlova* sp. (Pav, CCMP 459) or *Tetraselmis striata* (Plat-P) and a diatom: *Chaetoceros muelleri*, *Thalassiosira weissflogii* or *Fragilaria familica* for 28-30 days. The combination Pav 459/*C. muelleri* provided a superior diet for sea scallop postlarvae, yielding a growth rate of up to 28 μm day^{-1}. However, when these algae were offered singly, a 32% (Pav 459) and 64% (*C. muelleri*) decrease in growth rates was observed, indicating that both species made a significant contribution to the success of the mixed diet. The two species are characterized by unique signatures of n-6 polyunsaturated fatty acids (PUFAs): *C. muelleri* has high levels of arachidonic acid (AA) and Pav 459 (in contrast to *P. lutheri*) has high levels of n-6 docosapentaenoic acid (DPA). DPA was selectively incorporated into tissues of scallops fed all binary diets except the one rich in DPA (Pav 459/*C. muelleri*). These results, coupled with a marked increase in n-6/n-3 ratios between the diet and tissues, provide evidence that n-6 PUFAs may play an important and previously underestimated role in scallop nutrition. The diet of *T. striata* and *C. muelleri*, which yielded the lowest growth rate (8.1 μm day^{-1}) contained dietary docosahexaenoic acid (DHA; 22:6n-3) concentrations 4.5-7 x lower than any other binary treatment. Low DHA levels may thus explain the poor performance of this diet. Tissue protein and triacylglycerol concentrations reflected diet performance, with higher values generally associated with more successful diets. This work suggests that DHA, as well as the n-6 PUFAs AA and DPA may be essential for optimizing growth of sea scallop postlarvae.

2.1 Introduction

The giant scallop, *Placopecten magellanicus*, is one of the most important commercial scallop species accounting for more than 50% of scallop landings worldwide (Halvorson *et al.*, 1995). There is also great potential for sea scallop culture (Dadswell and Parsons, 1991), but the industry is largely constrained by the availability and affordability of high quality spat. Natural spatfalls are often limited and unreliable (Dadswell and Parsons, 1992) and hatchery raised spat are not only expensive, but often incur high and unexplained mortalities in larval and post-settlement stages. Observed mortalities may be a result of nutritional deficiencies, as little is known about the dietary needs of these developmental stages. Diets used in scallop hatcheries have generally been adopted from those used for other bivalves with more established culture practices (i.e., clams and oysters), yet the performance of algal diets is often highly species- and stage-specific. Previous work conducted on scallops (Pectinidae) has focused largely on larval (Whyte *et al.*, 1989; Delaunay *et al.*, 1993; Thompson *et al.*, 1994; Soudant *et al.*, 1998b; Nevejan *et al.*, 2003b; Nevejan *et al.*, 2003a) and juvenile or adult stages (Pierson, 1983; Soudant *et al.*, 1996b; Parrish *et al.*, 1999; Martinez *et al.*, 2000; Navarro *et al.*, 2000) with little attention given to animals immediately post-settlement. In *P. magellanicus*, the stage from settlement to field deployment (~300 μm to 3 mm) can take ~5 months and therefore maintaining adequate growth and survival during this period is crucial to culture success.

As sea scallops are restricted to cold waters < 20 °C (Naidu, 1991), their physiological tolerances to environmental factors, especially with respect to cellular membrane structure, differ from those of scallop species found in warmer waters. An inverse relationship between temperature and degree of membrane unsaturation has been established (Sinensky, 1974), suggesting that sea scallops may have a high requirement for polyunsaturated fatty acids (PUFAs) to maintain membrane fluidity at lower temperatures. A direct relationship between one particular PUFA, eicosapentaenoic acid (EPA, 20:5n-3), and membrane fluidity has been demonstrated in sea scallops (Hall *et al.*, 2002), illustrating not only the general necessity for PUFAs, but also the need for specific fatty acids to meet various metabolic functions. As most bivalves are unable to synthesize

significant amounts of long chain PUFAs via elongation and/or desaturation of other fatty acids, they are reliant on dietary sources to meet these fatty acid requirements (Langdon and Waldock, 1981; Waldock and Holland, 1984). Thus, sea scallops are particularly vulnerable to nutritional deficiencies.

The importance of n-3 PUFAs, such as EPA and docosahexaenoic acid (DHA, 22:6n-3), has been identified in several bivalve species such as *Crassostrea gigas* (Langdon and Waldock, 1981), *Ostrea edulis* (Enright *et al.*, 1986; Berntsson *et al.*, 1997), and *Pecten maximus* (Marty *et al.*, 1992). These fatty acids play a role in membrane function, as precursors of biologically active eicosanoids such as prostaglandins and/or as an energy substrate. The specific PUFA requirements of postlarval scallops remain unknown and therefore hatcheries often feed multi-component diets in an attempt to meet nutritional requirements. However, algal culture costs are high, typically 30% of total hatchery costs (Coutteau and Sorgeloos, 1993), and it is advantageous to limit the number of algal species cultured. To this end, the objectives of this research were: 1) to identify cost-effective algal diets which maximize survival and growth of *P. magellanicus* postlarvae, 2) to relate diet characteristics to scallop growth, nutritional condition and especially lipid/fatty acid concentration and composition, and 3) to ascertain the lipid/fatty acid requirements of sea scallops at this developmental stage, and thus set the stage for subsequent more targeted algal and supplementation trials to further test our hypotheses on specific PUFA requirements in this species.

2.2 Materials and Methods

2.2.1 Scallops

Early post-settlement sea scallops, (*Placopecten magellanicus*; initial shell height 350-380 μm) were air-shipped within the same day from PecNord, Inc., Québec, to Halifax, Nova Scotia (NS), Canada in plastic containers filled with chilled seawater. Animals were immediately transported to the National Research Council (NRC) of Canada's Marine Research Station (MRS), Sambro, and placed in a 700 l recirculating

downweller system containing 0.22 μm filtered seawater at 30 ‰ and 14 °C. Scallops were maintained on a mixed microalgal diet for two days before switching to experimental diets.

2.2.2 Microalgal Diets

Six species of microalgae were tested as five binary diets in a first feeding trial (Experiment I). A subsequent trial (Experiment II) tested the top performing mixed diet from Experiment I against its two constituents in unialgal suspensions. These included the flagellates: *Pavlova lutheri*, obtained from the Center for the Culture of Marine Phytoplankton (CCMP), West Boothbay Harbor, ME, strain 1325 (MONO); *Pavlova* sp., CCMP 459 (Pav 459); and *Tetraselmis striata* (strain Plat-P, from the National Marine Fisheries Service, Milford, CT) and the diatoms: *Chaetoceros muelleri*, CCMP 1316 (CHGRA); *Fragilaria familica*, a local isolate from Mahone Bay, NS which has not been previously tested as a bivalve diet; and *Thalassiosira weissflogii*, CCMP 1336 (ACTIN). *Chaetoceros muelleri* and *P. lutheri* are both commonly used in bivalve hatcheries. A monospecific diet of *Thalassiosira weissflogii* was shown to be an excellent diet for sea scallops 0.75-1.4 mm (Ryan, 1999) and is larger than the commonly used *Thalassiosira pseudonana* (3H) and thus above the size threshold for 100% gill retention efficiency by adult pectinids (Bricelj and Shumway, 1991). A unialgal diet of Pav 459 was shown to support higher growth rates of sea scallop veliger larvae than three other commonly used microalgae, *P. lutheri* included (Feindel, 2000), and *T. striata*, although deficient in DHA, has proven an excellent diet for post-settlement Eastern oysters, *Crassostrea virginica* (Wikfors *et al.*, 1996).

In experiment I, all five diets consisted of one diatom and one flagellate. The three flagellates were tested in combination with CHGRA and each of the three diatoms was combined with *P. lutheri*. *Pavlova lutheri*/CHGRA served as a reference diet, as both species are commonly used in bivalve hatcheries. This experimental design further allowed for within-flagellate and within-diatom species comparisons. The biochemical composition of the mixed diet was calculated from that of its individual constituents based on an equal, 50:50 contribution to total particle volume.

For experiment I, microalgae were grown either in batch culture in 20 l carboys (Pav 459, 20°C; *T. striata*, 16°C; CHGRA, 20°C; *F. familica*, 16°C) or in temperature-controlled 200 l photobioreactors in semi-continuous culture (*P. lutheri*, 21°C; ACTIN, 18°C) on f/2 media (- Si for flagellates; Guillard, 1975). In experiment II, Pav 459 was grown in batch culture as above, and CHGRA was grown in the photobioreactor at 20 °C. Mass culture of Pav 459, at scales larger than 20 l carboys, has proved unreliable to date and requires further investigation. Photobioreactors were harvested at 80% of their volume twice weekly, refilled with pasteurized seawater and fresh f/2 medium including Si for diatoms. All species were grown on 24 h light with CO_2 with the exception of *T. striata* which was grown on 14:10 L:D without the addition of CO_2. Delivery of CO_2 was made directly into the air line and regulated by a pH controller (Cole-Parmer model P-05656-00) as needed to maintain culture pH between 7.8 and 8.3. All algal cultures were harvested in late exponential growth phase.

2.2.3 Experimental Systems

For Experiment I, ~150,000 postlarval scallops were divided equally by volume into five 400 l recirculating downweller systems; for Experiment II, ~25,000 scallops were divided equally into three systems. Each system consisted of a 350 l reservoir tank from which water was pumped at a flow rate of 10-12 l min^{-1} (Aquatic Ecosystems Mag Drive MD 2) into a "basket" located on the exterior of a 50 l tank held above the reservoir. The upper tank contained two identical downwellers (40.6 cm in height, 15.2 cm in diameter) containing a base of 140 μm Nitex square mesh. Water from the "basket" flowed through the two downwellers and into the upper tank. An overflow pipe was located 30.5 cm above the floor of the upper tank, and excess water was allowed to flow back to the reservoir tank. The stocking density of each downweller was ~83 and ~14 animals cm^{-2}, for Experiments I and II, respectively. All experimental systems were held in a temperature controlled environmental chamber. Replication of tanks was not possible due to the scale of these experiments, however identical maintenance and sampling protocols were followed for all treatments and algal concentration, light and ambient temperature were kept under tight control. Constant experimental conditions are

evidenced by the comparable growth rates attained on the Pav 459/CHGRA diet in Experiments I and II (see results).

Scallops were exposed to one of five experimental diets in Experiment I, and one of three diets in Experiment II at ca. 2.15 x 10^6 μm^3 ml^{-1} for 28 days (Expt I; August 17th to September 13th, 2001) or 30 days (Expt II; August 21st to September 19th, 2002). Diets were offered at total cell concentrations equivalent in volume to 50 cells μl^{-1} of *Isochrysis galbana* clone T-Iso, which provides an optimal food level for growth of juvenile (0.5 to 5mm) bay scallops (Lu and Blake, 1996). Algal concentrations were maintained within $\leq$ 20% of the desired level in suspension by daily batch addition, and seawater in the experimental tanks was replaced twice a week to minimize fouling. All cell counts were determined on a Coulter Multisizer II fitted with a 100 μm aperture (Beckman Coulter, Fullerton, CA). Weekly sampling was conducted for scallop shell height (umbo to the distal shell edge) and % mortality, whereas sampling of scallop tissues for lipids, proteins, dry weight (DW) and ash-free dry weight (AFDW) was conducted only at the start and end of the experiment. Prior to sampling, scallops were starved overnight in 0.22 μm filtered seawater to purge gut contents, and for Experiment I, scallop samples were sieved prior to final sampling to remove empty shell debris and biodeposits. The material that passed through the sieve was examined to confirm that it contained no live scallops, and samples of shell debris were taken for DW and AFDW analysis to determine their potential contribution to final samples. Algal sampling for proteins, lipid, DW and AFDW was conducted weekly. Only growth trajectories from Experiment II will be presented and discussed as a more detailed analysis will be presented in a subsequent paper including a comparison of other diets containing *Pavlova* spp.

2.2.4 Growth and Survival

For percent survival and shell height determinations, a subsample of scallops was removed from each downweller, placed on a graduated petri dish, and scanned at 10-25x magnification using an analog camera (Pulnix model TMC7-DSP) coupled with a dissecting microscope. All video was recorded on a Sony DVCAM (DSR-V10), and calibrated using a stage micrometer. Fifty live scallops per downweller were measured from each tank at each sampling date and averaged for mean shell height determination.

Shell heights were determined using Optimas 6.5 image analysis software (Media Cybernetics, Silver Spring, MD). Tissue dry weights were obtained by filtering triplicate scallop samples of 0.1 ml (~130-2200 scallops, depending on time and treatment), measured in a graduated Eppendorf tube, onto pre-combusted, pre-weighed Whatman GF/C filters, rinsed with 2 ml of 3.4% ammonium formate to remove salts and oven dried at 80°C for 24 h. Ash weight was determined after combustion at 480°C for 24 h. The number of live individuals in each triplicate sample of 0.1 ml was determined microscopically to calculate the biomass (DW and AFDW) per individual, and to provide a number to volume conversion factor for other analyses where scallops were sampled on a volume basis. Mortalities were determined by recording the number of live, "new" dead, and "old" dead from each downweller based on video observations. "New" dead scallops were the approximate size of live scallops but showed no movement or presence of tissues. "Old" dead scallops were smaller, showed no movement, gut contents or internal tissues. Duplicate counts of 200 total scallops (dead and live) per downweller were taken for mortality estimates, converted to percent data, and then averaged.

2.2.5 Lipid Analysis

For lipid analysis, scallop samples of 0.3-0.5 ml were placed into glass tubes pre-rinsed with methanol and chloroform. Tubes were immediately placed into liquid nitrogen, and then purged with N_2 gas. Samples were stored in 3 ml of chloroform, under nitrogen at –80 °C until analysis. The volume of algae sampled varied with culture concentration and corresponded to a minimum of ~ 0.6 mg lipid. Samples for algal lipids were filtered onto pre-combusted Whatman GF/C filters, and treated as above. Multiple blanks were prepared for both scallop tissue and algal samples.

Lipid samples were shipped overnight, on dry ice, to Memorial University of Newfoundland for lipid analysis. Both algae and scallop lipids were extracted in chloroform-methanol (2:1) following a modified Folch procedure (Folch *et al.*, 1957) as described by Parrish (1999). Lipid classes were determined using the Chromarod-Iatroscan TLC/FID system. They were quantified in a MARK V Iatroscan (Iatron Laboratories, Tokyo, Japan) using lipid standards (Sigma), and summed to determine total lipid concentrations. Fatty acids were quantified from bulk lipid extracts following

derivatization for 1.5 h at 85 °C with 1.5 ml 10% BF_3/MeOH. Fatty acid methyl esters (FAME) were run on a Varian 3400 Gas Chromatograph with an Omegawax 320 column (Supelco, Bellefonte, PA). Peak detection was done at 220 °C and identified with Varian Star Chromatography Software 4.02 with known standards (PUFA 1 and 37 Component FAME Mix, Supleco Canada). A Varian Saturn mass spectrometer was used for the confirmation of fatty acids for which standards were not available (e.g. 22:5n-6).

2.2.6 Protein Analysis

Triplicate samples of 0.15 ml of scallops were placed into cryovials, and immediately frozen in liquid nitrogen after excess water was removed. They were stored at –80 °C until analysis. For organic carbon (C) and nitrogen (N) determinations, tissues were lyophilized, powdered, weighed on a Cahn electrobalance and run on a Carlo-Erba 1108 CHN analyzer (Rodano, Italy) using acetanilide as the standard. For determination of algal C/N, 2-10 ml of algal cultures were filtered onto pre-combusted GF/C filters, dried at 80 °C for 24 h and stored in desiccant. Nitrogen values were converted to mg protein using a conversion factor of 5.8 (Gnaiger and Bitterlich, 1984).

2.2.7 Statistical Analysis

One-way analysis of variance (ANOVA) was used to investigate differences in final shell height (n=100) and scallop biomass (n=3). Linear regressions were fitted to shell height data to determine scallop growth rates, and differences among growth rates were tested using analysis of covariance (ANCOVA). One-way ANOVAs were conducted to determine differences in bulk biochemical properties of algae (n=5) and scallop tissues (n=3). A multivariate analysis of variance (MANOVA) was conducted to determine differences in lipid and fatty acid profiles among algal species and scallop tissues prior to conducting any multiple comparison tests. A two-way ANOVA was used to examine mortality data with diet type and time as factors. Other two-way ANOVAs were conducted to examine fatty acid ratios with diet type and organism (algae or scallop tissues) as factors. All percent and ratio data were arcsine transformed prior to analysis to normalize the data, and all multiple comparisons were carried out using Tukey's multiple comparison test. All analyses were carried out using SYSTAT 9.0 (SPSS Inc., Chicago IL). The significance value of all analyses was set at $P<0.01$, and where no

significant difference was detected they were re-tested at $P<0.05$. Unless otherwise noted, results are reported at $P<0.01$.

2.3 Results

2.3.1 Scallop Growth and Mortalities

After 4 weeks of feeding, growth rates were clearly different between the five treatments in Experiment I, with the Pav 459/CHGRA combination yielding a superior mean growth rate of 28.1 μm day^{-1} (ANCOVA; $F_{4,1994} = 4448.3$; $P<0.001$; Fig. 2.1a) and mean scallop dry and organic weights ~2 times greater than the reference diet of *P. lutheri*/CHGRA (Table 2.1), which resulted in a mean growth rate of 19.4 μm day^{-1}. The second highest performing diet was the combination of *P. lutheri* and the diatom *F. familica*, a species previously untested as food for bivalves. The combined diet of *T. striata* and CHGRA yielded poor performance, with the lowest growth rate (8.1 μm day^{-1}), and a final mean shell height half that of the Pav 459/CHGRA diet (Fig. 2.2), and dry and organic weights 5 and 8 x lower, respectively, than the Pav 459/CHGRA diet. Furthermore, weekly instantaneous growth rates on the *T. striata*/CHGRA diet showed a marked decline over the 4 week experiment (Table 2.1). The range among growth rates was much greater in scallops exposed to diets composed of differing flagellate species (*P. lutheri*/CHGRA, Pav 459/CHGRA, *T. striata*/CHGRA) than among diets containing *P. lutheri* and differing in diatom species (Fig. 2.1a).

After sieving, the contribution of dead shell to final sample AFDW was low, ranging from 5-12%, except for the *T. striata*/CHGRA treatment that yielded a dead shell contribution of 23%. In Experiment II, the mixed diet of Pav 459/CHGRA was also the highest performing diet yielding a growth rate of 24.9 μm day^{-1} (Fig. 2.1b), comparable to that obtained in Experiment I, and a 31 and 64% decrease in growth rate was observed for scallops offered unialgal diets of Pav 459 and CHGRA, respectively.

Substantial new mortalities (20-31%) were observed during the first week of the feeding trial in all treatments (attributable to shipping stress), which decreased during the final three weeks, ranging from 3.4-9.4% per week. A two-way ANOVA conducted on the final three weeks of the feeding trial indicated both a significant effect of diet

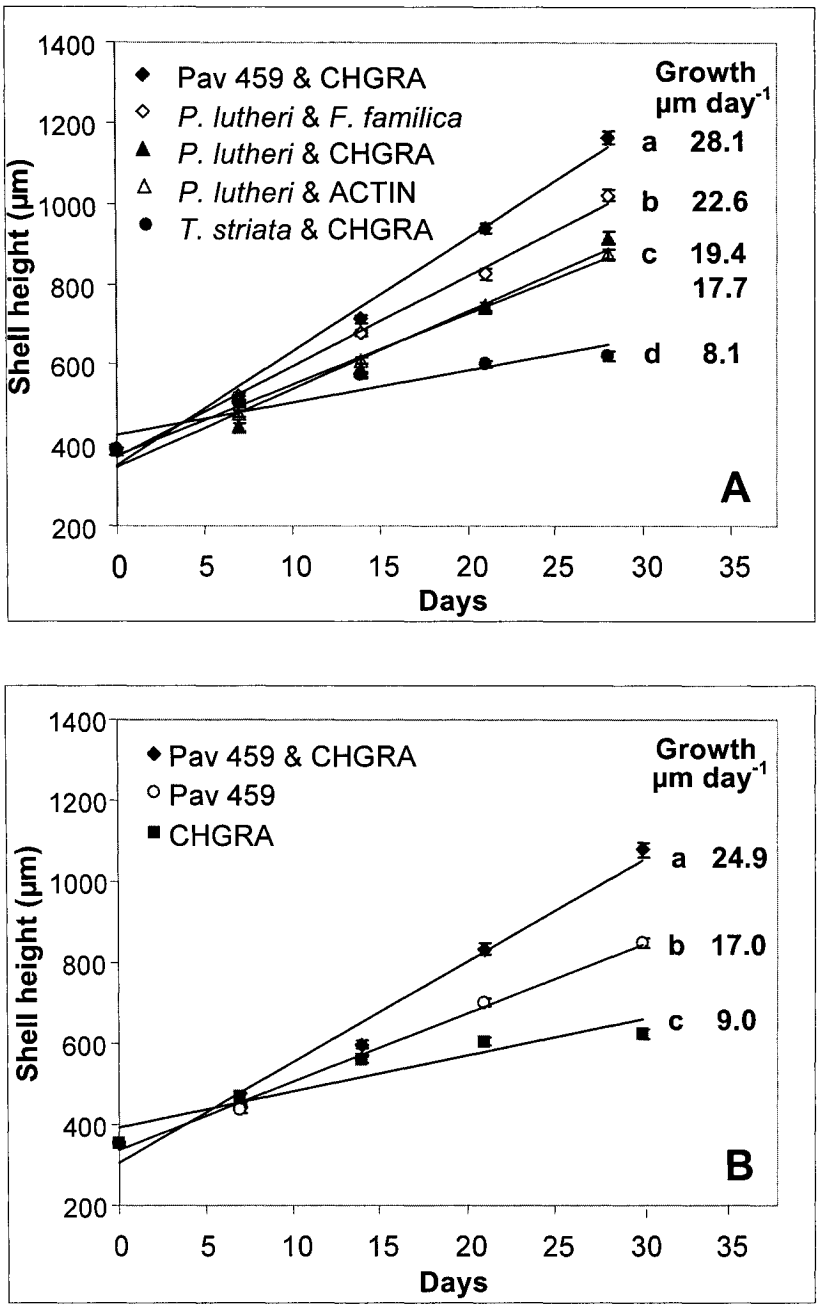

Figure 2.1: Shell growth trajectories of *P. magellanicus* postlarvae fed different algal diets. Values represent mean shell height ± standard error. Linear regression equations (calculated from individual shell height data) are as follows: A) Experiment I: Pav 459 & *C. muelleri* (CHGRA) ($y=28.13x + 371.1$, $R^2=0.990$); *P. lutheri* & *F. familica* ($y=22.59x+366.7$, $R^2=0.994$); *P. lutheri* & CHGRA ($y=19.39x+344.0$, $R^2=0.973$); *P. lutheri* & *T. weissflogii* (ACTIN) ($y=17.68x+371.1$, $R^2=0.995$) and *T. striata* & CHGRA ($y=8.11x+424.2$, $R^2=0.891$); B) Experiment II: Pav 459 & CHGRA ($y=24.92x+303.6$, $R^2=0.98$); Pav 459 ($y=16.97+334.9$, $R^2=0.99$) and CHGRA ($y=8.98+392.6$, $R^2=0.89$). Letters represent significant differences in growth rate determined by ANCOVA ($P<0.001$).

Table 2.1: Growth characteristics for scallop postlarvae including initial and final dry and organic weights (mean ± SE), and weekly instantaneous growth rates, k (based on shell height) for Experiment I. Different letters represent differences between final weights, $P<0.01$ and mean instantaneous growth rates, $P<0.05$.

	Initial	Pav 459 & CHGRA	*P. lutheri* & *F. familica*	*P. lutheri* & CHGRA	*P. lutheri* & ACTIN	*T. striata* & CHGRA
Individual scallop biomass (µg scallop $^{-1}$)						
Dry wt	8.2 ± 0.4	135.6 ± 6.0^{a}	114.0 ± 1.2^{b}	75.8 ± 2.0^{c}	80.2 ± 4.1^{c}	26.4 ± 0.2^{d}
Organic wt	2.0 ± 0.1	33.6 ± 1.3^{a}	24.1 ± 0.7^{b}	16.0 ± 0.3^{c}	16.2 ± 1.2^{c}	4.4 ±0.1^{d}
Instantaneous growth rates (k)						
Week 1	--	4.17	3.82	1.92	2.99	3.73
Week 2	--	4.52	4.11	3.97	3.48	1.85
Week 3	--	3.92	2.84	3.42	2.81	0.68
Week 4	--	3.08	3.04	2.97	2.34	0.51
Mean ± SE		3.93 ± 0.31^{a}	3.45 ± 0.31^{ab}	3.07 ± 0.44^{ab}	2.91 ± 0.24^{ab}	1.69 ± 0.74^{b}

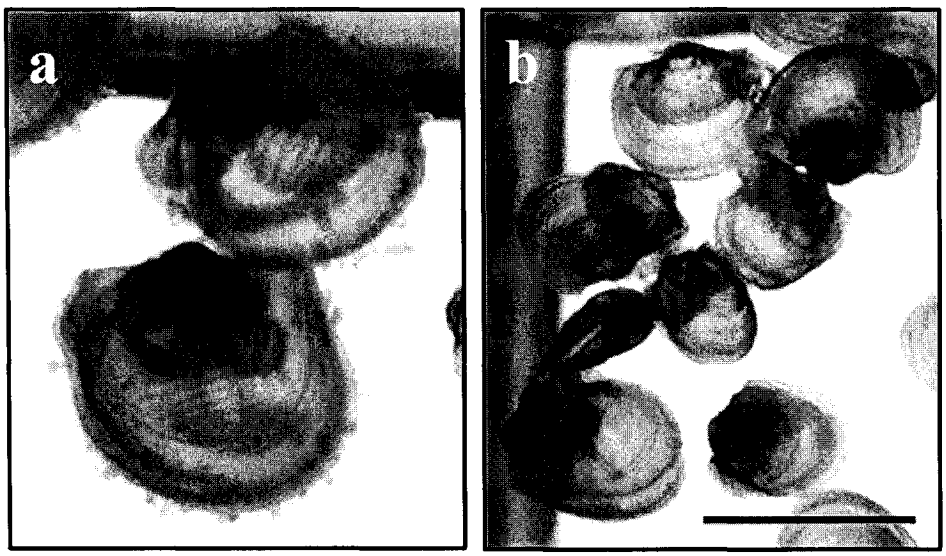

Figure 2.2: Video micrographs of scallops at the end of the 4-wk Experiment I exposed to the a) highest Pav 459/CHGRA and b) lowest *T. striata*/CHGRA performing diets. Scale bar = 1mm.

($P<0.05$) and time ($P<0.001$) on new mortalities, as well as a significant interaction ($P<0.01$), with an overall decrease in new mortalities during week four. The mean weekly new mortalities of scallops exposed to the *P. lutheri*/*F. familica* and *T. striata*/*C. muelleri* diets were higher (7.2 and 6.9%) than those offered the *P. lutheri*/*C. muelleri* treatment (4.6%). No other differential mortalities among treatments were detected.

2.3.2 Microalgal Biochemical Composition

Experimental diets were offered to scallops in equivalent biovolume concentrations (see section 2.2.2) and no differential settlement of the larger *T. weissflogii* was observed, although there was some adhesion of *F. familica* and *T. striata* to the downwellers. Total diet DW and AFDW were also comparable, varying by <25% among treatments. *Pavlova lutheri*/CHGRA and *T. striata*/CHGRA, yielded the highest biomass concentrations with values of 0.710 / 0.946 mg DW l^{-1} and 0.637 / 0.805 mg AFDW l^{-1}, respectively. There were no significant differences in protein concentrations between the six algal species tested (ANOVA; $P> 0.05$; Table 2.2). There was, however, a significant difference in the total lipid

Table 2.2: Cell size (equivalent spherical diameter, ESD), dry weight, ash-free dry weight and biochemical characteristics (lipid and protein concentration, lipid class and fatty acid composition) of microalgal species used in diets for Experiment I. All measurements expressed as mean ± SE, n=5. Different letters indicate significant differences ($P<0.01$), there was no significant difference in protein concentration ($P>0.05$). Differences in algal biomass between species were not tested.

	Pav 459	***P. lutheri***	***T. striata***	**CHGRA**	**ACTIN**	***F. familica***
Size and biomass						
ESD (μm)	3.8 ± 0.1	4.0 ± 0.1	9.2 ± 0.2	5.1 ± 0.1	11.7 ± 0.1	5.2
Dry weight (pg cell^{-1})	13.7 ± 0.9	14.7 ± 1.4	273.8 ± 51.3	30.7 ± 2.4	401.4 ± 62.1	24.0 ± 2.7
AFDW (pg cell^{-1})	11.9 ± 0.7	13.4 ± 1.0	229.2 ± 37.9	26.9 ± 1.4	317.5 ± 52.9	18.4 ± 2.1
% ash	13.1	8.8	16.3	12.4	20.9	23.3
Bulk biochemical properties (mg g AFDW^{-1})						
Total protein	611.4 ± 19.6	504.5 ± 10.8	480.4 ± 61.7	499.9 ± 38.3	544.4 ± 55.4	423.6 ± 29.7
Total lipid	240.2 ± 16.5^{ab}	234.2 ± 32.2 ^{ab}	195.7 ± 21.8^{b}	295.4 ± 25.4^{a}	176.4 ± 23.1^{ab}	116.7 ± 17.1^{b}
Total fatty acid	121.9 ± 7.3^{b}	124.9 ± 15.7^{b}	92.7 ± 11.3^{b}	224.1 ± 24.4^{a}	104.5 ± 9.7^{b}	72.7 ± 9.8^{b}
Lipid classes (% of total lipid)						
Triacylglycerols	0.6 ± 0.2^{b}	0.5 ± 0.2^{b}	0.2 ± 0.1^{b}	54.1 ± 7.2^{a}	18.8 ± 8.4^{b}	7.2 ± 0.7^{b}
Free Fatty Acids	2.8 ± 1.7^{b}	4.2 ± 1.2^{ab}	0.9 ± 0.4^{b}	3.5 ± 1.3^{b}	5.3 ± 2.4^{ab}	14.1 ± 2.2^{a}
Alcohols	2.6 ± 0.3^{ab}	5.2 ± 0.5^{a}	0.4 ± 0.2^{c}	0.1 ± 0.0^{c}	0.2 ± 0.1^{c}	1.6 ± 0.7^{bc}
Sterols	7.3 ± 0.3^{a}	5.5 ± 0.7^{ab}	1.8 ± 0.1^{c}	2.7 ± 0.4^{c}	1.8 ± 0.8^{c}	4.2 ± 0.8^{bc}
Acetone Mobile Polar Lipids	40.4 ± 1.3^{ab}	31.1 ± 4.1^{b}	66.2 ± 1.2^{a}	16.8 ± 4.9^{b}	39.3 ± 17.6^{b}	23.7 ± 1.0^{b}
Phospholipids	44.6 ± 1.8^{ab}	52.0 ± 2.2^{a}	29.7 ± 1.1^{bc}	19.8 ± 3.2^{c}	32.4 ± 14.5^{bc}	45.9 ± 2.6^{ab}
Fatty acid composition (% of total fatty acid)						
14:0	16.7 ± 0.4	12.6 ± 0.4	2.5 ± 0.3	12.7 ± 0.8	6.6 ± 0.4	15.6 ± 0.5
16:0	8.2 ± 0.3	10.2 ± 1.1	14.0 ± 0.4	14.7 ± 0.9	11.2 ± 2.0	4.5 ± 0.5

	Pav 459	*P. lutheri*	*T. striata*	CHGRA	ACTIN	*F. familica*
17:0 i	0.6 ± 0.0	0.4 ± 0.0	2.7 ± 0.3	0.4 ± 0.1	0.3 ± 0.2	0.3 ± 0.1
17:0 ai	0.2 ± 0.1	0.9 ± 0.1	0.5 ± 0.1	1.9 ± 0.3	2.4 ± 0.6	0.5 ± 0.1
Sum SAT	27.9 ± 0.8^{ab}	26.8 ± 0.8^{b}	25.6 ± 0.8^{b}	32.8 ± 1.3^{a}	23.86 ± 1.1^{b}	24.1 ± 0.5^{b}
16:1n-7	7.2 ± 0.4	14.9 ± 1.3	1.3 ± 0.5	31.1 ± 2.0	26.4 ± 7.5	21.0 ± 1.4
16:1n-5	3.4 ± 0.2	0.2 ± 0.1	--	0.4 ± 0.0	0.5 ± 0.1	0.7 ± 0.0
18:1n-9	0.2 ± 0.0	0.5 ± 0.0	6.9 ± 0.5	0.5 ± 0.0	0.7 ± 0.4	1.0 ± 0.7
18:1n-7	0.3 ± 0.0	3.1 ± 0.6	1.3 ± 0.1	0.8 ± 0.2	1.8 ± 0.6	6.7 ± 0.4
20:1n-11	--	--	4.5 ± 0.5	--	--	--
Sum MUFA	12.8 ± 0.4^{b}	20.7 ± 2.3^{ab}	18.9 ± 0.9^{ab}	34.2 ± 1.9^{a}	32.6 ± 6.8^{a}	32.1 ± 1.1^{a}
16:2n-4	1.6 ± 0.1	1.6 ± 0.1	0.1 ± 0.1	2.5 ± 0.1	2.8 ± 0.2	2.4 ± 0.1
16:3n-4	0.3 ± 0.0	1.8 ± 1.0	--	5.3 ± 1.4	17.9 ± 4.7	8.5 ± 0.4
16:4n-3	--	--	18.4 ± 0.6	--	--	0.1 ± 0.0
18:2n-6	1.1 ± 0.4	0.5 ± 0.3	7.0 ± 0.9	0.7 ± 0.1	0.6 ± 0.1	2.9 ± 0.8
18:3n-3	2.7 ± 0.5	1.0 ± 0.2	14.0 ± 1.5	0.1 ± 0.1	--	--
18:4n-3	5.0 ± 2.4	5.7 ± 1.9	7.7 ± 0.8	0.8 ± 0.7	0.2 ± 0.1	0.8 ± 0.1
20:4n-6 (AA)	1.8 ± 0.2^{b}	0.3 ± 0.2^{c}	0.2 ± 0.1^{c}	7.4 ± 1.0^{a}	0.9 ± 0.2^{bc}	1.4 ± 0.2^{b}
20:5n-3 (EPA)	30.0 ± 0.8^{a}	28.3 ± 1.3^{a}	6.6 ± 0.2^{c}	12.0 ± 1.4^{c}	16.2 ± 2.5^{bc}	22.9 ± 1.2^{ab}
22:5n-6 (DPA)	7.8 ± 0.4^{a}	0.6 ± 0.1^{b}	--	0.1 ± 0.0^{c}	--	--
22:6n-3 (DHA)	8.0 ± 0.4^{ab}	10.6 ± 1.6^{a}	0.2 ± 0.1^{d}	1.9 ± 0.7^{cd}	3.4 ± 0.7^{bc}	1.4 ± 0.8^{cd}
Sum PUFA	59.3 ± 0.6^{a}	52.6 ± 1.8^{a}	55.5 ± 1.5^{a}	33.0 ± 2.7^{b}	43.6 ± 7.8^{ab}	43.8 ± 1.4^{ab}
Sum n-3	46.2 ± 1.3	45.7 ± 3.4	47.5± 2.4	15.0 ± 2.6	20.0 ± 3.1	25.7 ± 1.2
Sum n-6	11.1 ± 1.1	2.1 ± 0.6	7.7 ± 1.0	9.4 ± 1.1	1.9 ± 0.3	5.9 ± 0.7
n-3/n-6	4.4 ± 0.5^{bc}	28.3 ± 6.0^{a}	6.6 ± 0.9^{bc}	1.7 ± 0.4^{c}	12.7 ± 3.4^{ab}	4.7 ± 0.7^{bc}
DHA/EPA	0.27 ± 0.01^{a}	0.11 ± 0.05^{a}	0.02 ± 0.02^{c}	0.15 ± 0.04^{ab}	0.20 ± 0.02^{ab}	0.06 ± 0.04^{bc}
DPA/AA	4.57 ± 0.35	2.99 ± 0.69	0.09 ± 0.09	0.02 ± 0.01	0.00 ± 0.00	0.02 ± 0.01
EPA/AA	17.9 ± 2.1	144.2 ± 28.6	38.3 ± 10.7	1.6 ± 0.2	26.2 ± 8.2	17.3 ± 2.0

Notes for Table 2.2:
* only lipid classes and fatty acids contributing >2% in at least one species are reported.
** Other identified lipid classes were: hydrocarbons, steryl/wax esters, methyl esters, ethyl ketones, methyl ketones, glyceryl ethers and diacylglycerols.
*** Other identified fatty acids were: 15:0i, 15:0, 16:0i, 16:0ai, 17:0i, 17:0, 18:0, 19:0, 20:0, 22:0, 23:0, 24:0, 14:1, 15:1, 16:1n-9, 17:1, 18:1n-11, 18:1n-5, 20:1n-9, 20:1n-7, 22:1n-11(13), 22:1n-9, 24:1, 16: 4n-1, 18:2n-4, 18:3n-6, 18:3n-4, 18:4n-1, 20:2a, 20:2b, 20:2n-6, 20:3n-6, 20:3n-3, 20:4n-3, 21:5n-3, 22:4n-6, and 22:5n-3.
**** For fatty acids, multiple comparison tests were only conducted on Σ Saturates (SAT), Σ Monoenes (MUFA), Σ PUFA, AA, EPA, DPA, DHA, n-3/n-6 and DHA/EPA as additional comparisons were constrained by the MANOVA.
--non-detectable

concentration (ANOVA; $F_{5,24}$ = 13.9; $p<0.001$; Table 2.2), most notably CHGRA contained 2.5 times more lipid than *F. familica* (295 vs. 117 mg lipid g AFDW $^{-1}$).

Pavlova lutheri contained the highest levels of phospholipids, which accounted for more than half of the total lipid in this species, yet high levels (~45%) were also found in Pav 459 and *F. familica* (Table 2.2). A significant difference was detected in the calculated phospholipid concentrations of the mixed algal diets ($P=0.037$) which ranged from 58.1-90.0 mg g AFDW^{-1}, although a Tukey's multiple comparison test failed to show individual diet differences. *Chaetoceros muelleri* was characterized by triacylglycerols (TAG) levels exceeding those of all other algae tested (54% vs. 0.2-18.8%), which was reflected in all CHGRA-containing diets (20-26% vs. 4-9%). *Tetraselmis striata* and Pav 459 contained the highest proportions of acetone mobile polar lipids (AMPL) found in any of the species tested (66.2% and 40.4%, respectively; Table 2.2), *F. familica* was the only alga characterized by high levels of free fatty acids (14% vs. <5.3%) and the two *Pavlova* species contained the highest levels of sterols (5.5-7.3%). Sterol concentrations of algal diets showed a significant positive linear relationship with scallop growth rate (R^2 = 0.82; $P<0.05$; Fig. 2.3), as the Pav 459/CHGRA and *T. striata*/CHGRA diets had the highest (1.56 mg g AFDW^{-1}) and lowest (0.22 mg g AFDW^{-1}) concentrations, respectively.

Particular emphasis is given in this study to the four long chain PUFAs 20:5n-3, 22:6n-3, 20:4n-6 and 22:5n-6 in both algal diets and scallop tissues. Both DHA and EPA have been shown to be essential fatty acids in bivalves, and AA plays a role as a

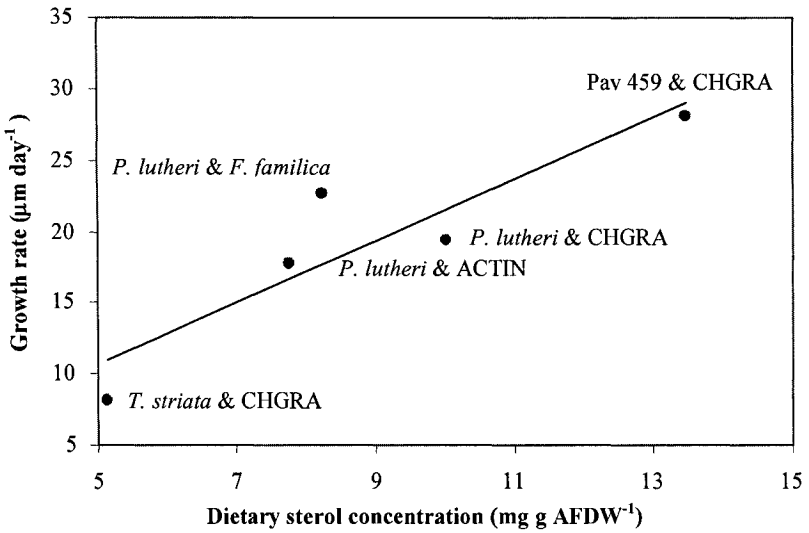

Figure 2.3: Relationship between dietary sterol concentration and mean scallop growth rate; y=2.17x-0.16, R^2=0.82, P<0.05). Removal of the *P. lutheri /F. familica* diet from this relationship increased the coefficient of determination to 0.96, P<0.05.

precursor of bioactive molecules such as prostaglandins. Docosapentaenoic acid, n-6 DPA is the only other long chain PUFA contributing >2% to either diets or tissues (Tables 2.2 and 2.3), and together these four fatty acids contribute 69-82% of the total PUFA concentrations found in scallop tissues (Table 2.3), thus necessitating their examination in algal diets. Ratios of these four PUFAs were examined to determine enrichment patterns between diets and tissues, and to identify biochemical indices of dietary nutritional value and/or scallop condition.

Both CHGRA and Pav 459, which yielded the highest scallop growth in combination, possess a unique profile of these four PUFAs. *Chaetoceros muelleri* was characterized by high levels (7.4%) of arachidonic acid (AA, 20:4n-6), and absolute concentrations of this PUFA (16.7 mg g $AFDW^{-1}$) were ~ 8-80 times higher than in any of the other species tested, as was reflected in the fatty acid concentration of all three diets containing CHGRA (Fig. 2.4a). Pavlova sp. 459 contained relatively high levels (7.8%) of docosapentaenoic acid (DPA, 22:5n-6), which accounted for <1% of the fatty acid composition of all other algal species and provided a unique fatty acid signature for this strain. The absolute concentrations of DPA in Pav 459 (9.7 mg g $AFDW^{-1}$) were one

Table 2.3: Biochemical composition of scallop tissues (protein, lipid and fatty acid concentration as well as lipid class and fatty acid composition), both initial and after 28 days exposure to different algal diets in Experiment I. All values expressed as mean ± SE, n=3. Different letters represent statistical differences among final treatments, bulk biochemical properties and lipid classes $P<0.05$ (except for TAG which was significant at $P<0.01$) and fatty acid composition $P<0.01$.

	Initial	Pav 459 & CHGRA	*P. lutheri* & *F. familica*	*P. lutheri* & CHGRA	*P. lutheri* & ACTIN	*T. striata* & CHGRA
Bulk biochemical properties (mg g AFDW^{-1})						
Total protein	406.0 ± 49.9	538.7 ± 10.1^{a}	474.8 ± 15.1^{ab}	381.2 ± 9.0^{bc}	464.3 ± 18.9^{b}	349.9 ± 22.4^{c}
Total lipid	59.8 ± 16.2	53.8 ± 6.5^{a}	35.5 ± 5.7^{ab}	63.1 ± 13.1^{a}	51.9 ± 9.6^{ab}	13.0 ± 5.1^{b}
	47.9 ± 11.3	37.8 ± 5.2^{ab}	21.3 ± 3.9^{ab}	50.7 ± 15.1^{a}	32.5 ± 6.3^{ab}	6.7 ± 2.9^{b}
Lipid classes (% of total lipid)						
Triacylglycerols	49.4 ± 4.6	31.6 ± 3.0^{a}	6.7 ± 1.1^{bc}	26.9 ± 1.5^{a}	15.5 ± 1.2^{ab}	1.6 ± 1.6^{c}
Free Fatty Acids	2.7 ± 1.2	1.7 ± 1.1	3.8 ± 2.0	2.2 ± 0.3	0.3 ± 0.3	3.2 ± 1.6
Sterols	--	7.2 ± 0.4	11.9 ± 0.7	7.7 ± 0.9	9.3 ± 0.9	14.2 ± 5.6
Acetone Mobile Polar Lipids	5.2 ± 3.8	11.1 ± 2.81	16.3 ± 3.0	14.1 ± 1.9	16.3 ± 1.5	17.6 ± 8.0
Phospholipids	39.3 ± 3.1	45.7 ± 1.2^{ab}	59.1 ± 6.6^{a}	45.1 ± 0.9^{ab}	56.3 ± 1.0^{ab}	32.8 ± 16.7^{b}
Fatty acid composition (% of total fatty acid)						
14:0	7.7 ± 0.5	5.3 ± 0.1	5.5 ± 0.1	5.0± 0.3	4.6 ± 0.1	2.4 ± 0.3
16:0	13.8 ± 0.2	11.4 ± 0.2	11.3 ± 0.2	12.5 ± 0.3	12.5 ± 0.3	12.1 ± 0.3
18:0	2.9 ± 0.1	3.3 ± 0.1	5.2 ± 0.1	3.9 ± 0.3	4.6 ± 0.2	6.8 ± 0.2
Sum SAT	26.7 ± 0.7	22.0 ± 0.4	23.9 ± 0.1	23.7 ± 0.3	24.4 ± 0.4	23.7 ± 1.8
16:1n-7	7.4 ± 0.1	8.8 ± 0.1	4.6 ± 0.1	10.6 ± 0.8	5.1 ± 0.1	4.6 ± 0.5
17:1	0.2 ± 0.2	0.6 ± 0.5	3.2 ± 0.3	2.2 ± 0.6	1.0 ± 1.0	2.5 ± 1.3
18:1n-9	5.4 ± 0.7	0.7 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	1.6 ± 0.7	2.3 ± 0.0

	Initial	Pav 459 & CHGRA	*P. lutheri* & *F. familica*	*P. lutheri* & CHGRA	*P. lutheri* & ACTIN	*T. striata* & CHGRA
18:1n-7	5.8 ± 0.7	5.1 ± 0.0	8.7 ± 0.2	8.7 ± 0.2	8.3 ± 0.5	7.1 ± 0.3
20:1n-11	--	1.3 ± 0.1	2.8 ± 0.1	1.2 ± 0.6	1.7 ± 0.9	2.9 ± 1.5
Sum MUFA	20.6 ± 0.7	18.9 ± 0.6^{b}	22.8 ± 0.2^{ab}	26.2 ± 1.1^{a}	20.4 ± 1.3^{ab}	23.6 ± 1.1^{ab}
18:2n-6	4.2 ± 0.2	0.8 ± 0.1	0.7 ± 0.2	0.4 ± 0.1	0.6 ± 0.3	1.1 ± 0.1
18:3n-3	3.3 ± 0.1	1.0 ± 0.0	0.4 ± 0.1	0.2 ± 0.0	0.3 ± 0.1	1.6 ± 0.1
18:4n-3	7.1 ± 1.1	2.9 ± 0.1	6.4 ± 0.5	4.1 ± 0.7	5.4 ± 1.6	6.4 ± 0.4
20:4n-6 (AA)	2.3 ± 0.1	8.8 ± 0.1^{b}	2.8 ± 0.0^{c}	8.1 ± 0.1^{b}	1.9 ± 0.1^{d}	10.5 ± 0.4^{a}
20:5n-3 (EPA)	9.8 ± 0.1	19.4 ± 0.2^{a}	14.6 ± 0.2^{b}	15.4 ± 0.2^{b}	18.1 ± 0.1^{a}	9.2 ± 0.3^{c}
22:5n-6 (DPA)	4.5 ± 0.3	10.1 ± 0.1^{a}	4.5 ± 0.2^{b}	3.0 ± 0.3^{c}	4.5 ± 0.2^{b}	2.9 ± 0.1^{c}
22:6n-3 (DHA)	17.1 ± 0.3	10.4 ± 0.2^{c}	20.9 ± 0.4^{a}	14.2 ± 0.8^{b}	19.2 ± 0.6^{a}	13.8 ± 0.4^{b}
Sum PUFA	52.7 ± 1.1	59.3 ± 0.5^{a}	53.3 ± 0.1^{ab}	50.1 ± 1.0^{b}	55.2 ± 1.2^{ab}	52.7 ± 1.7^{ab}
Sum n-3	39.8 ± 0.8	36.0 ± 0.3	43.9 ± 0.1	35.7 ± 0.7	45.3 ± 0.1	34.1 ± 1.0
Sum n-6	12.8 ± 0.5	21.0 ± 0.2	8.6 ± 0.1	12.4 ± 0.3	7.3 ± 0.4	16.3 ± 0.1
n-3/n-6	3.1 ± 0.1	1.7 ± 0.0^{d}	5.1 ± 0.1^{b}	2.9 ±0.0^{c}	6.2 ± 0.3^{a}	2.1 ± 0.1^{d}
DHA/EPA	1.7 ± 0.0	0.5 ± 0.0	1.4 ± 0.0	0.9 ± 0.1	1.1 ± 0.0	1.5 ± 0.0
DPA/AA	2.0 ± 0.1	1.2 ± 0.0	1.6 ± 0.1	0.4 ± 0.0	2.3 ± 0.0	0.3 ± 0.0
EPA/AA	4.3 ± 0.0	2.2 ± 0.0	5.3 ± 0.1	1.9 ± 0.0	9.4 ± 0.2	0.9 ± 0.0
DHA+DPA	21.6 ± 0.5	20.6 ± 0.3	25.3 ± 0.6	17.2 ± 1.1	23.7 ± 0.5	16.7 ± 0.5
EPA+AA	12.1 ± 0.2	28.0 ± 0.2	17.3 ± 0.2	23.5 ± 0.2	20.0 ± 0.3	19.7 ± 0.7

Notes for Table 2.3:

* Only lipid classes and fatty acids contributing >2% in at least one experimental treatment are reported.
** Other identified lipid classes were: hydrocarbons, steryl/wax esters, methyl esters, ethyl ketones, methyl ketones, glyceryl ethers, alcohols, and diacylglycerols.
*** Other identified fatty acids were: 15:0i, 15:0 ai, 15:0, 16:0i, 16:0ai, 17:0i, 17:0ai, 17:0, 20:0, 23:0, 14:1, 15:1, 16:1n-9, 16:1n-5, 18:1n-11, 18:1n-5, 20:1n-9, 20:1n-7, 22:1n-9, 16:2n-4, 16:3n-4, 16:4n-3, 16:4n-1, 18:2n-4, 18:3n-6, 18:3n-4, 18:4n-1, 20:2a, 20:2n-6, 20:3n-6, 20:3n-3, 20:4n-3, 21:5n-3 and 22:5n-3.
**** For fatty acids, multiple comparison tests were only run on Σ Saturates (SAT), Σ Monoenes (MUFA), Σ PUFA, AA, EPA, DPA, DHA and n-3/n-6 as additional comparisons were constrained by the MANOVA.
--non-detectable

to two orders of magnitude higher than those of the four other algal species tested (≤0.7 mg g $AFDW^{-1}$; Fig. 2.4a).

Tetraselmis striata was not only low in the total concentration of long-chain (≥C20) PUFAs (6.5 mg g $AFDW^{-1}$ vs. 19-58 mg g $AFDW^{-1}$ for all other algal species) but was also deficient in DHA, containing only 0.2% of this essential PUFA (Table 2.2) and DHA concentrations below those of all other algal species tested (0.20 mg g $AFDW^{-1}$ vs. 1.05-13.42 mg g $AFDW^{-1}$). The EPA content was also relatively low in *T. striata* (6.6% vs. 12.0-30.0%), with concentrations 2.7-6.0 x lower than those in the other microalgae (6.1 vs. 16.5-36.5 mg g $AFDW^{-1}$). DHA and EPA levels were highest in the two *Pavlova* species both on a concentration and % composition basis, but Pav 459 had significantly higher levels of DPA and AA than *P. lutheri* (Table 2.2). The two *Pavlova* species also differ in their n-3/n-6 ratios, with *P. lutheri* having the highest and Pav 459 having one of the lowest ratios (28.3 and 4.4, respectively; Table 2.2). The PUFA profiles of all five diets exhibited similar patterns when calculated on both an AFDW and per volume basis.

2.3.3 Scallop Biochemical Composition

Protein concentration in scallop tissues reflected diet ranking in terms of scallop growth: postlarvae on the Pav 459/CHGRA diet exhibited the highest protein concentration (538.7 μg g $AFDW^{-1}$) while those exposed to *T. striata*/CHGRA had the

lowest protein concentration (by 35%) and all other diets tested yielded intermediate concentrations ranging from 381.2-474.8 μg g $AFDW^{-1}$ (Table 2.3). Total lipid concentrations of scallop tissues offered the *T. striata*/CHGRA diet were significantly lower than those of any other CHGRA-containing dietary treatment, although no difference was observed among the other four diets (ANOVA; $F_{4,10}=5.3$; $P<0.05$; Table 2.3). A difference in total fatty acid concentration was only observed between the highest (Pav 459/CHGRA; 37.8 mg g $AFDW^{-1}$) and lowest (*T. striata*/CHGRA; 6.7 mg g $AFDW^{-1}$) performing diets (ANOVA; $F_{4,10}=4.4$; $P<0.05$; Table 2.3).

Scallops exposed to diets containing either of the two *Pavlova* species had the highest percentage of TAG (26.9-31.6%), whereas animals exposed to the *T. striata*/CHGRA diet showed significantly lower levels (1.6%) of this lipid class. There was a slight but significant difference in tissue phospholipid levels ($P<0.05$); scallops fed the *F. familica*-containing diet had higher levels than those fed the *T. striata/C. muelleri* diet (59.1 vs. 32.8%). Ratios of TAG/Sterol and TAG/Phospholipid largely reflect diet performance, with higher tissue values associated with faster growing animals, with the exception of the *P. lutheri/F. familica* diet (calculated from Table 2.3; $P<0.05$). There were no significant differences among the relative proportions of any other lipid class in scallop tissues ($P< 0.05$; Table 2.3).

2.3.4 Comparison of Dietary and Tissue Fatty Acids

Fatty acid profiles of scallop tissues largely reflected those of the diets. For example, high levels of AA were observed in the animals exposed to diets containing the AA-rich CHGRA (Table 2.3), and high levels of DPA were reflected in scallops exposed to the DPA-rich Pav 459 (Fig. 2.4b). The *T. striata/C. muelleri* diet was characterized by low concentrations of both DHA and EPA (Fig. 2.4a), and the Σn-3 concentrations in the tissues of scallops fed this diet were greatly reduced relative to those scallops fed other diets (2.3 vs. 9.4-18.2 mg g $AFDW^{-1}$).

Although scallop tissues reflected some of the fatty acid characteristics of their diet, there were also pronounced shifts in fatty acid composition indicative of selective incorporation/catabolism. In all treatments there was a highly significant increase in the

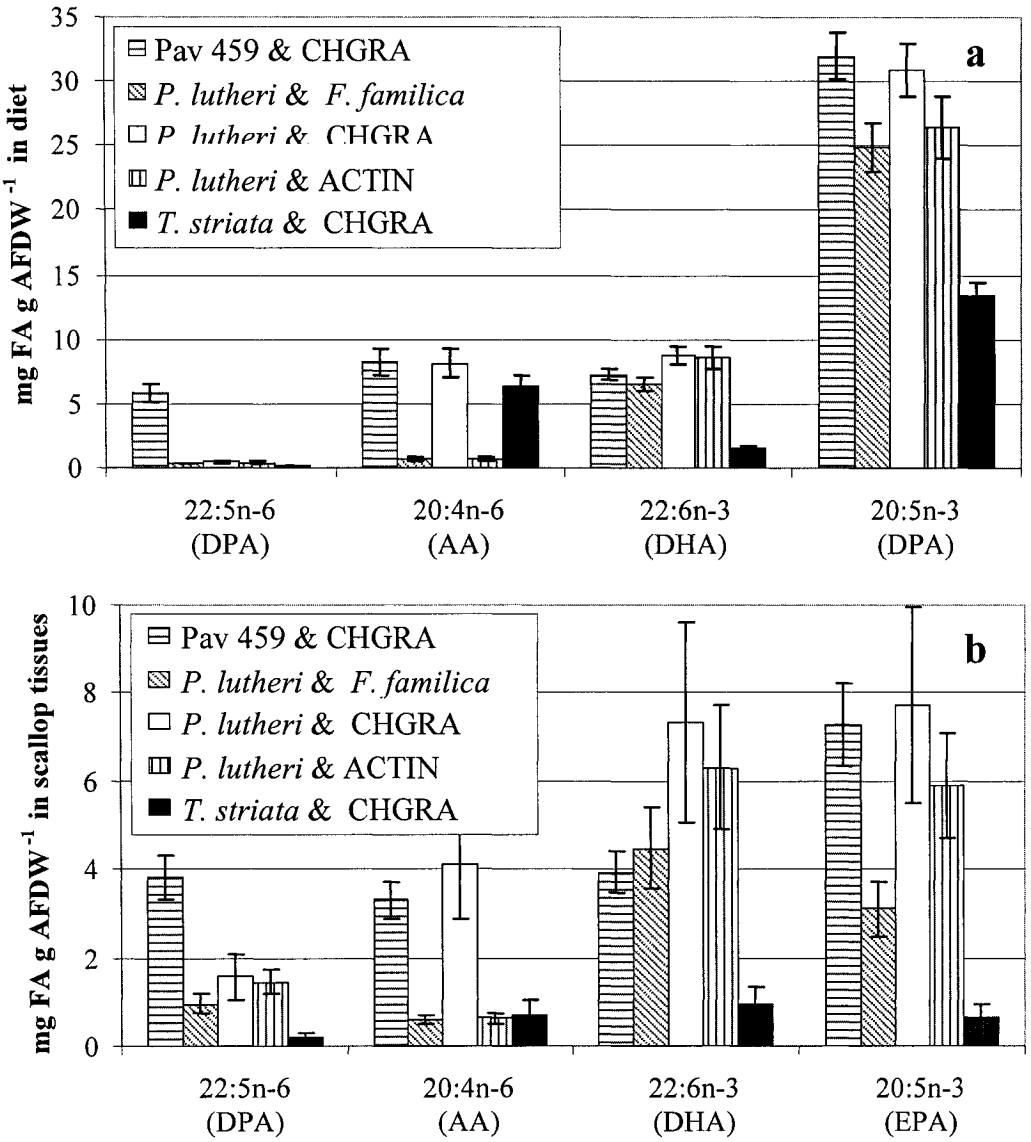

Figure 2.4: Concentrations (mean ± SE) of four selected polyunsaturated fatty acids (PUFAs) on a per ash-free dry weight (AFDW) basis from Experiment I in a) five experimental diets offered to scallops; b) scallop tissues after 4 wks exposure to experimental diets.

DHA/EPA ratio in scallop tissues relative to the diets (two-way ANOVA; $p<0.001$; Fig. 2.5a). This apparent enrichment was greatest in the *T. striata*/CHGRA diet (14x), which had the lowest DHA/EPA ratio. Lower n-3/n-6 ratios were observed both in diets and tissues of experimental treatments containing CHGRA (Fig. 2.5b), but overall n-3/n-6 ratios were higher in diets than in corresponding tissues, indicating either selective

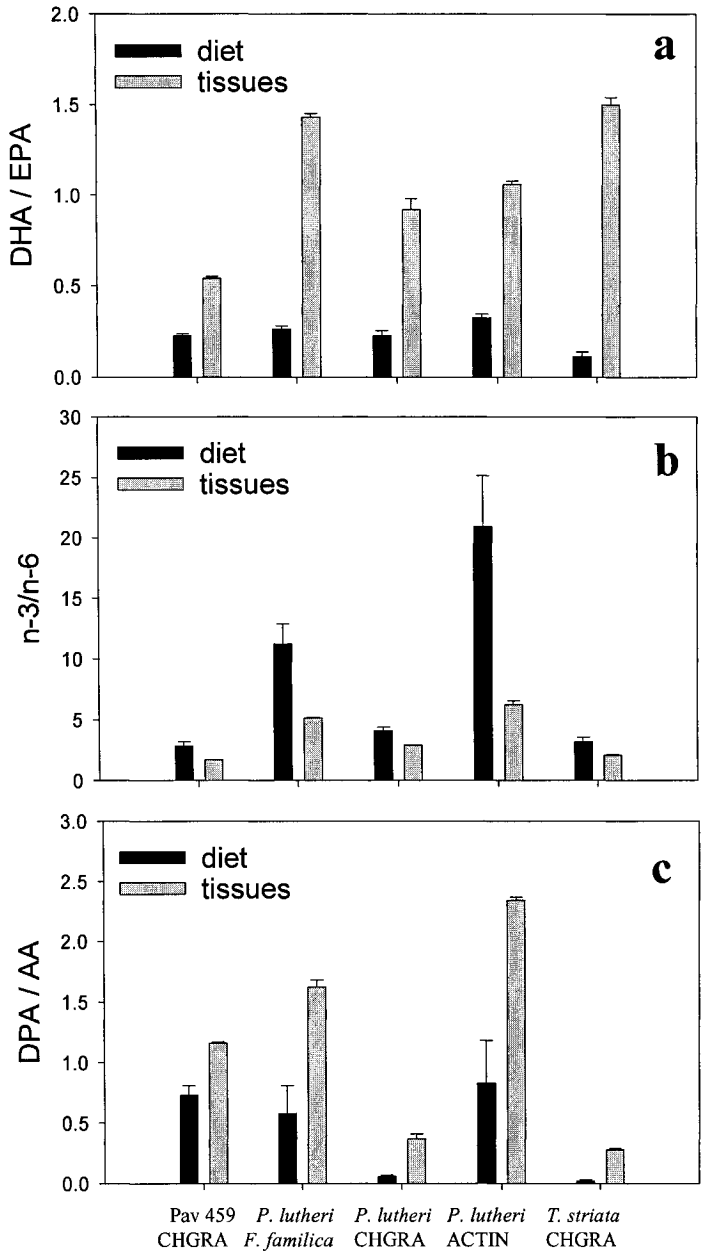

Figure 2.5: Comparison of proportions of a) docosahexaenoic acid (DHA; 22:6n-3) / eicosapentaenoic acid (EPA; 20:5n-3), b) Σ n-3 / Σ n-6 fatty acids and c) n-6 DPA docosapentaenoic acid (22:5n-6) / arachidonic acid (AA; 20:4n-6) in diets and scallop tissues from Experiment I. Dietary values presented are calculated from the mean concentration of weekly algal samples and data for scallop tissues are based on PUFA concentrations after feeding on experimental diets for 4 wks. Error bars as in Fig. 2.3.

utilization/elimination of n-3 fatty acids or selective incorporation/retention of n-6 fatty acids in scallop tissues (Fig. 2.5b). Higher DPA/AA ratios also occurred in tissues relative to the algal diet in all treatments (Fig. 2.5c).

A marked decrease in EPA concentrations in tissues relative to the diet was observed in all cases; a decrease in AA levels in tissues relative to the diet was only observed in diets containing the AA-rich CHGRA (Fig. 2.6). A substantial enrichment of DPA (up to 4-fold) was observed in scallop tissues in all treatments with the exception of scallops fed the high DPA Pav 459/CHGRA diet.

2.4. Discussion:

2.4.1 Effect of Microalgal Diets on Scallop Growth

This study has identified the Pav 459/CHGRA combination as a superior diet for sea scallop postlarvae, achieving growth rates not unlike those reported for postlarvae in the wild (30-45 μm day^{-1}; Parsons *et al.*, 1993) and comparable or superior to the highest values obtained in hatcheries: a maximum of 9 μm day^{-1} for postlarvae fed diets of 1-3 algal species (Gillis, 1993) and 3-37 μm day^{-1} for 4-6 component diets (Ryan, 1999). This study also suggests that the high nutritional value of the Pav 459/CHGRA diet is largely related to high concentrations of specific long-chain PUFAs (see sections 4.3 and 4.4). *Chaetoceros muelleri* also contains high proportions of carbohydrate (49% of organic weight; Ben-Amotz *et al.*, 1987), which has been positively related to bivalve growth and condition (Enright *et al.*, 1986; Whyte *et al.*, 1990b). Other studies, however, have shown that carbohydrate plays a negligible role as an energy substrate in postlarval stages, as it comprises a minor (<2%) fraction of total organics (García-Esquivel *et al.*, 2001), and in contrast to protein and lipid, was not significantly correlated with growth (Wikfors *et al.*, 1992). Thus carbohydrate was not included in biochemical analysis conducted in the present study and cannot be ruled out as a contributing factor explaining the elevated growth rates attained on the Pav 459/CHGRA combination, versus a diet of Pav 459 alone. Dietary digestibility and amino acid composition are two other factors potentially influencing growth which were not measured in this study. However,

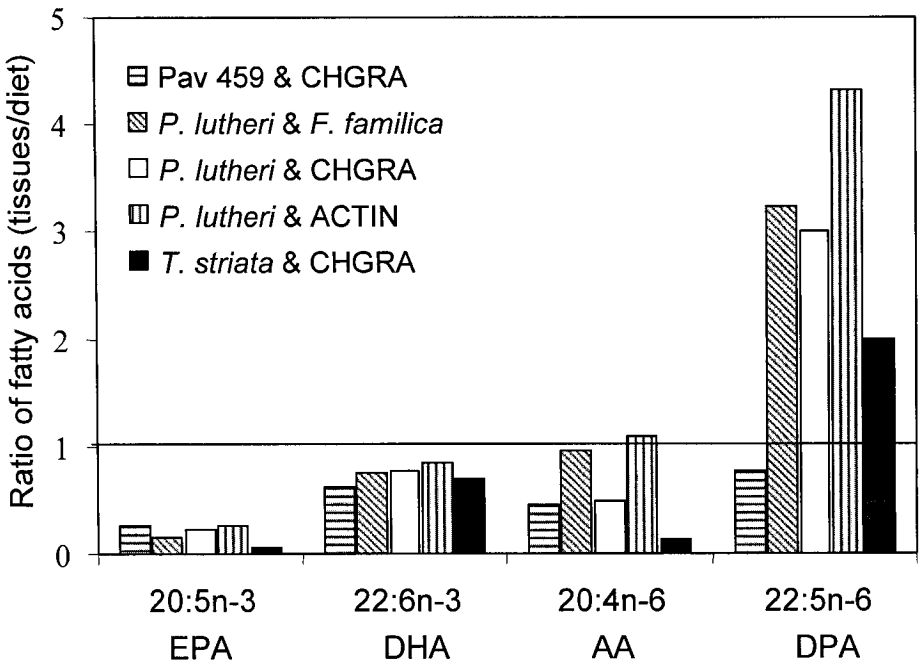

Figure 2.6: Relative proportions (based on mg g $AFDW^{-1}$) of four major polyunsaturated fatty acids found in scallop tissues compared to experimental diets after 4 wks of feeding during Experiment I. Values greater than 1 represent enrichment within the tissues compared to the diet offered.

previous studies have shown only minor variation in amino acid composition between algal species, leading Knauer and Southgate (1999) to conclude that amino acid composition is likely not a major factor in determining bivalve growth on different diets. Potential digestibility differences among algal species are discussed below and require additional research.

Despite differences in culture methods, the long chain PUFA profiles of the microalgal species tested were generally comparable to those previously reported for the diatom and flagellate species used in this study (Volkman *et al.*, 1989 ;Volkman *et al.*, 1991; Napolitano *et al.*, 1990; Feindel, 2000; J. Craigie, NRC/IMB unpub. data for *T. weissflogii* and *F. familica*). Scallop growth was not related to either total lipid or total fatty acid concentration of the microalgal diets. Algal protein concentration did not significantly differ across diet treatments ($P > 0.05$) and was therefore not considered a

factor contributing to observed differences in scallop growth, although dietary protein content was positively related to growth performance in *Argopecten purpuratus* postlarvae (1.8 mm SH) (Uriarte and Farias, 1999).

Dietary biochemical composition does not explain the high growth performance of animals exposed to the *F. familica/P. lutheri* combination. *Fragilaria familica* was characterized by the lowest total lipid and fatty acid concentrations, low levels of DHA, an undetectable level of DPA, and only moderate proportions of EPA and AA. However, lipid extracts from *F. familica* cultures also contained elevated levels of free fatty acids (14% vs. 0.9-5%), and when lipolytic degradation is high it can alter both lipid class and fatty acid composition of algae (Berge *et al.*, 1995; Budge and Parrish, 1999), and may partially confound attempts to relate lipid class or fatty acid composition to growth.

Dietary success of the *P. lutheri/F. familica* diet may also be related to the digestibility of *F. familica*, as differential digestibility of diatoms has been reported in postlarval abalone (Kawamura *et al.*, 1995), or to differences in food capture related to morphological constraints of sea scallop postlarvae at these early developmental stages. Prior to gill reflection (~1 mm SH in *P. magellanicus*), suspension feeding is unlikely to be very effective, and alternate feeding methods, such as pedal feeding may be employed until the gill is fully functional (Veniot *et al.*, 2003). The *F. familica* diet was particularly "sticky", adhering to the mesh downweller base, and the resulting microalgal film may have provided a superior algal delivery method for scallops of this particular size range.

One of the major assumptions for comparing the biochemical composition of the diet and scallop tissues in this study is that both algal species are being incorporated at equal rates, which assumes equal capture, ingestion, and digestion and thus no particle selectivity based on algal size or food quality. Although, adult scallops only capture particles greater than ~6 μm with 100% efficiency (reviewed by Bricelj and Shumway, 1991), previous studies have indicated that postlarval scallops can efficiently capture particles in the 2-10 μm range, (Kingzett, 1993; Anderson, 2003) and flow cytometric analysis has shown no selection between particles of 2 and 10 μm in postlarval (1.2-1.6 mm) *A. irradians* (Shumway *et al.*, 1997).

2.4.2 Lipid Classes

There was a positive relationship between dietary sterol concentration and scallop growth rate (Fig. 2.3). As sterols play a structural role in cellular membranes (Nes, 1974), and bivalves are generally considered to have a limited ability to synthesize sterols (Walton and Pennock, 1972; Holden and Patterson, 1991) dietary sterol concentrations and composition may be particularly important to sea scallops. *Chaetoceros muelleri* is characterized by high levels of cholesterol (46-48 % of total sterol; Copeman et al., unpubl. data; Tsita-Tzardis *et al.*, 1993), and although only binary diets including CHGRA contained significant levels of cholesterol, it was present in the scallop tissues of all treatments in this study. The *F. familica/P. lutheri* diet was characterized by the highest percentages (~18%) of 24-methylenecholesterol, which has been positively related to post-settlement oyster growth (Wikfors *et al.*, 1996), and could play a role in explaining the high growth rates attained by scallops on this diet. Preferential accumulation/retention of cholesterol and stigmasterol has been previously noted in the tissues of larval *P. maximus* (Soudant *et al.*, 1998a), whereas in this study marked enrichment of cholesterol, brassicasterol and campesterol was observed in scallop tissues relative to the diets (Copeman et al., unpubl. data). Additional research on the nutritional role of sterols in bivalves is required using diets specifically selected for this purpose.

Dietary TAG concentrations did not appear to influence diet performance, but rather reflected elevated levels in diets containing the fatty acid-rich CHGRA. There was, however, a positive relationship between the TAG composition in scallop tissues and growth rate, except for the *P. lutheri/F. familica* diet, indicating that the fastest growing scallops were able to convert proportionally more energy into storage material as neutral lipids which generally provide the main energy reserve. Excluding the *P. lutheri/F. familica* diet, TAG/sterol and TAG/phospholipid ratios in scallop tissues were an indicator of dietary success, with higher values corresponding to faster growing animals, as has been demonstrated for *P. magellanicus* larvae (Pernet *et al.*, 2003b).

2.4.3 n-3 Fatty Acids

In the present study, the *T. striata*/CHGRA diet proved unsuitable for sea scallop postlarvae, yielding growth rates almost 4 times lower than those of the highest

performing diet. However, a unialgal diet of *T. striata* (Plat-P) supported good growth of *C. virginica* postlarvae, relative to a unialgal diet of *Isochrysis* sp. (T-Iso), which was attributed to the high EPA concentration of *T. striata*, even though this species contained non-detectable levels of DHA (Wikfors *et al.*, 1996). Langdon and Waldock (1981) hypothesized that either DHA or EPA was necessary for good oyster growth, but not necessarily the presence of both, which may explain why *T. striata* is a successful species for oyster culture. However, it is possible that sea scallops have more stringent DHA requirements than oysters to ensure high growth performance. Poor success of this diet could also be attributed to poor cell digestibility of *T. striata*. *Tetraselmis suecica* was not readily digested by *P. maximus* larvae, as determined by epifluorescence of gut contents (Le Pennec and Rangel-Davalos, 1985). Thus, low digestibility coupled with lower essential PUFA concentrations, especially of DHA, may explain the depressed growth and poor condition observed in sea scallops fed this diet. However, a monospecific diet of *C. muelleri*, which is also DHA-deficient, supported a low growth rate (9.0 μm day-1) comparable to that of the *T. striata*/CHGRA diet (8.1 μm day^{-1}) suggesting that DHA-deficiency is likely responsible for the poor performance of sea scallops on both diets.

Although DHA and EPA are considered important for bivalve nutrition (Trider and Castell, 1980; Langdon and Waldock, 1981; Enright *et al.*, 1986; Marty *et al.*, 1992), it is possible that the DHA requirements of sea scallops outweigh those of EPA. Soudant et al. (1998b) proposed that DHA plays a structural role in *P. maximus* larvae, and it has been suggested that this role cannot be fulfilled by EPA (Feindel, 2000). However, an interchange between DHA and DPA, and also between EPA and AA, in scallop lipids is suggested by results of the present study. The sums of DHA+EPA, AA+EPA, and DPA+DHA were quite similar across diets (18-19% coefficient of variation, CV), whereas the sum of the proportions of AA+DPA remained relatively variable (44% CV) suggesting less homology between this fatty acid pair. Additionally, the CV for the percent fatty acid of DHA+DPA in scallop tissues (18.5%) was lower than that of either DHA (27.1%) or DPA (59.4%) across diet treatments. Similarly, the combined CV of EPA+AA was higher than that of either PUFA constituent (19.2% vs.

25.5 and 59.7%). Therefore, these two C_{20} (EPA and AA) and C_{22} (DHA and DPA) PUFAs may be substituting for each other in the same phospholipids.

2.4.4 n-6 Fatty Acids

The mixed diet of Pav 459/CHGRA yielded the highest growth rates of sea scallop postlarvae in both experiments and outperformed unialgal diets of both Pav 459 and CHGRA. Pav 459 alone supported higher growth rates of *P. magellanicus* veliger larvae than other unialgal diets, including *P. lutheri* (Feindel, 2000). Pavlova sp. 459 is characterized by an exceptionally high percentage of n-6 DPA, compared to other algae tested in this study. Marked enrichment of DPA (2-4.3x) was observed in the tissues of scallops exposed to all diets except for the DPA rich Pav 459/CHGRA combination (Fig. 2.6). Likewise, enrichment of DPA was reported in *P. maximus* female gonads (Soudant *et al.*, 1996b), and in the polar lipids of *P. maximus* larvae (Delaunay *et al.*, 1993). DPA may serve to maintain membrane fluidity or structural integrity (Soudant *et al.*, 1996b), which would make it particularly important in species adapted to cold water. Since elongation and desaturation activities for long chain PUFAs are low or absent in bivalve species (De Moreno *et al.*, 1976; Waldock and Holland, 1984; Delaunay *et al.*, 1993), selective retention of DHA and DPA could be facilitated by other mechanisms, such as acyltransferases, associated with TAG and phospholipid production in scallops (Marty *et al.*, 1992).

Growth rates for sea scallop postlarvae fed a unialgal diet of Pav 459 were reduced by 31% from those attained on the mixed Pav 459/CHGRA diet (Fig. 2.1b). As CHGRA is characterized by high concentrations of AA, and Pav 459 contains high concentrations of the other three long chain PUFAs, this decreased growth rate may be related to AA deficiency in the unialgal Pav 459 diet. Thus AA, in addition to DPA, may be necessary to sustain high growth rates of sea scallop postlarvae. Arachidonic acid is a known precursor in prostaglandin production and can increase prostaglandin release in mussels, *Modiolus demissus* (Freas and Grollman, 1980). Prostaglandins in molluscs are known to influence important functions such as ion regulation, egg production, and spawing (reviewed by Stanley-Samuelson, 1987; Deridovich and Reunova, 1993).

Therefore eicosanoids derived from AA may also play an important functional role in sea scallop postlarvae.

2.4.5 Fatty Acid Ratios

A large relative increase in DHA/EPA values between diet and scallop tissues was observed, indicating either selective DHA incorporation/retention or selective elimination/utilization of EPA in the tissues (Fig. 2.5a). Preferential incorporation of DHA at the expense of EPA has been previously observed in the polar lipids of *P. maximus* larvae, and it has been suggested that DHA may be an integral structural component of cell membranes (Soudant *et al.*, 1998b), which may therefore explain the preferential retention of DHA (Delaunay et al., 1993). However, Hall et al. (2002) described a positive relationship between the proportion of EPA in the gills of adult sea scallops and membrane fluidity, but no correlation between DHA and membrane fluidity. Although finfish rely on DHA to regulate membrane fluidity, this might not be the case in bivalves, and the specific role of DHA in scallops remains poorly understood. Furthermore, DHA enrichment relative to the diet does not occur in all marine organisms, as observed in turbot (Rainuzzo, 1993), indicating that relative enrichment of DHA in scallop tissues may be a response to species-specific needs.

In the n-6 polar fraction of *P. maximus* larvae, there was a decrease of AA associated with an increase in DPA, which reflected the dietary proportions of these two fatty acids (Soudant *et al.*, 1998b). In this study, high DPA/AA ratios were also observed in bulk lipids of scallop tissues relative to the diet (Fig. 2.5c), but this is likely more than a simple reflection of dietary levels. Active incorporation of DPA into scallop tissues (Fig. 2.6), could contribute to the increase in the DPA/AA ratio found in tissues relative to the diet. It is also possible that decreasing levels of AA could be associated with eicosanoid production in scallops, as AA is converted into other bioactive compounds (Delaunay *et al.*, 1993), although the reductions in AA concentrations involved may not have been detectable.

Eicosanoids produced from AA are more active than those formed from EPA, and compete for the same enzymes in marine fish (Sargent *et al.*, 1999b), and presumably shellfish, although this has never been directly addressed. A minimum dietary EPA/AA

ratio of 4.2 was suggested for turbot larvae, and a level of 20 had no deleterious effect, although higher values were not tested. The authors contend that excess dietary EPA relative to AA is not deleterious, whereas excess AA can be detrimental, inducing stress as a result of elevated eicosanoid production. Dietary ratios in the present study ranged from 2.2 to 55.6 (calculated from Table 2.2), such that the lowest performing diet, *T. striata*/CHGRA, had an EPA/AA ratio (2.2) lower than the minimum suggested above. This low ratio is not a result of excessively high AA concentrations, as all diets containing CHGRA have elevated concentrations of AA, but a relatively low concentration of EPA (Table 2.2). If the range of EPA/AA ratios determined to be critical for turbot larvae has any relevance to postlarval sea scallops, the low EPA/AA ratio of the *T. striata*/CHGRA diet might offer another explanation for its poor performance.

Algal diets with low n-3/n-6 ratios have been considered "good" diets for oyster larvae (Webb and Chu, 1983) and Feindel (2000) found that the n-6 long chain PUFAs, DPA and AA, were most strongly correlated with growth of larval *P. magellanicus*. Marty et al. (1992) found an increase in the ratio of n-6/n-3 fatty acids between diets and tissues in the polar lipids of *P. maximus* larvae, and this therefore suggests that n-6 fatty acids play an important role in this organism. Evidence of a requirement for n-6 fatty acids in scallops is further supported in the present study by: 1) the superior growth rates of *P. magellanicus* postlarvae possibly associated with high dietary concentrations of DPA and AA in the Pav 459/CHGRA diet, and 2) the increase of n-6/n-3 fatty acids found in scallop tissues relative to the diet (Fig. 2.5b). Therefore, we propose that the importance of n-6 fatty acids in bivalve nutrition has been largely overlooked. Future lipid supplementation trials, including partitioning of PUFAs between polar and neutral lipid fractions, will be conducted to more accurately define the FA requirements of sea scallop postlarvae.

2.5 Conclusions:

The binary Pav 459/CHGRA diet yielded scallop growth rates superior to any other binary diet tested and comparable to the mid-upper range of sea scallops offered 4-6

component hatchery diets. Therefore, the Pav 459/CHGRA diet provides an excellent diet for low-cost implementation in sea scallop hatcheries. Our results suggest that the high nutritional value of this diet may be linked to high concentrations of n-6 fatty acids DPA and AA, contributed by Pav 459 and CHGRA, respectively. Marked enrichment of DPA in scallop tissues relative to the available diet indicates that this fatty acid may play an essential role in these organisms, and that the importance of n-6 fatty acids in pectinids, and bivalves in general, requires further investigation. Although *T. striata* was shown to be an excellent diet for juvenile oysters, the *T. striata/C. muelleri* diet was unsatisfactory for sea scallop growth, potentially due to its DHA-deficiency. The inability to grow on diets low in DHA in this study suggests that sea scallops may have a more stringent DHA requirement than Eastern oysters, although the specific role of DHA remains largely unknown.

2.6 Acknowledgements:

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Chapter 3: Comparison of Early Life Stages of the Bay Scallop, *Argopecten irradians*: Effects of Microalgal Diets on Scallop Growth and Biochemical Composition.

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Abstract

The culture of bay scallops, *Argopecten irradians*, is limited by a reliable and affordable supply of spat and the ability to ensure that animals attain market size within a single growing season. The main goals of our study were thus: 1) to develop growth-optimizing algal diets for implementation in hatcheries, and 2) to identify and compare bay scallop postlarval and juvenile dietary requirements, especially of lipids and fatty acids, which if met may enhance production. Nutritional needs of postlarval bay scallops (present study) are compared with those of sea scallops, *Placopecten magellanicus*, offered the same diets in a previous companion study. To this end, postlarval (initial shell height, SH = 240 μm) and juvenile (initial SH = 10 mm) bay scallops were offered 6-7 microalgal diet combinations at 20°C, for three weeks. A similar growth ranking among diets was observed between the two developmental stages. A combination diet of *Pavlova* sp. (CCMP 459) and *Chaetoceros muelleri* was far superior to any other diet tested, yielding growth rates of 58 and 357 μm day^{-1} which were 65% and 25% higher than the next highest performing diet of *Tetraselmis striata* / *C. muelleri* in postlarvae and juveniles, respectively. The *T. striata* / *C. muelleri* diet, which is limited in the n-3 fatty acid docosahexaenoic acid (DHA), yielded very poor growth of sea scallop postlarvae in a prior study, indicating that bay scallops may have less stringent requirements for DHA than sea scallops. The Pav 459 / *C. muelleri* diet, which also supported the highest growth of sea scallop postlarvae, is characterized by elevated levels of the n-6 fatty acids, arachidonic (AA) in *C. muelleri* and 4,7,10,13,16-docosapentaenoic (DPA) in Pav 459. The two diets deficient in AA and n-6 DPA, *Pavlova lutheri* / *Thalassiosira weissflogii* and *P. lutheri* / *Fragilaria familica*, yielded the lowest growth rates in both bay scallop postlarvae and juveniles. Tissue enrichment of these two fatty acids relative to the diet, as well as overall enrichment in Σn-6 fatty acids were observed across developmental stages and dietary treatments. A similar pattern has previously been observed in sea scallop postlarvae, suggesting the possibility of a dietary requirement for n-6 fatty acids in pectinids that has often been overlooked in the past.

3.1 Introduction

The northern bay scallop, *Argopecten irradians irradians*, occurs on the Atlantic U.S. coast from Maine to New Jersey. Bay scallops are currently cultivated for the commercial market and for stock-enhancement programs as natural populations have been severely reduced as a consequence of fishing pressure, habitat loss and harmful algal events (Bricelj *et al.*, 1987; Tettelbach and Wenczel, 1993; Goldberg *et al.*, 2000). Under favorable conditions *A. irradians* can attain market size in less than a year, making this species an excellent candidate for aquaculture; however, the industry is smaller and less established than that for other bivalves, such as hard clams and oysters.

Bay scallop aquaculture is limited by the need for a reliable and fast-growing supply of postlarvae/juveniles and the availability of technology to ensure that animals reach market size within a single growing season; however, there is little information on nutritional requirements that, if met, would address these limitations. Prior work with scallops has focused largely on broodstock conditioning (O'Connor *et al.*, 2000; Caers *et al.*, 2003) and larval stages (Whyte *et al.*, 1990b; Nevejan *et al.*, 2003a; Pernet *et al.*, 2003a). Little attention has been given to early postlarval stages, or to ontogenetic changes in nutritional requirements which may correspond to transitions in the morphogenesis of feeding and digestive organs, as described in the sea scallop, *Placopecten magellanicus* (Veniot *et al.*, 2003).

Numerous studies have highlighted the importance of fatty acids, especially polyunsaturated fatty acids (PUFA), to bivalve nutrition and health (Brown *et al.*, 1989 (review); Delaunay *et al.*, 1993; Soudant *et al.*, 1996b; Caers *et al.*, 1998; Caers *et al.*, 2000). Fatty acids play a structural role in maintaining membrane fluidity and function, and act as precursors of bioactive molecules (e.g., prostaglandins) influencing processes such a reproduction, osmoregulation and stress response (Freas and Grollman, 1980; Osada *et al.*, 1989). The importance of the n-3 fatty acids DHA (docosahexaenoic acid) and EPA (eicosapentaenoic acid) has been emphasized in the molluscan literature (Langdon and Waldock, 1981; Marty *et al.*, 1992; Hall *et al.*, 2002). Less is known about the dietary requirement for n-6 fatty acids; however, conservation and/or enrichment of n-6 fatty acids in scallop tissues [particularly arachidonic (AA) and 4,7,10,13,16-

docosapentaenoic acid (DPA)] indicate that these compounds may also be critical for larval (Feindel, 2000; Pernet *et al.*, 2005) and postlarval (Milke *et al.*, 2004) scallop growth and survival.

Animals are unable to synthesize PUFA of either the n-3 and n-6 families *de novo*, and bivalves have a limited ability to elongate shorter-chained dietary PUFAs into essential long chain (20-22 carbon) PUFA such as EPA and DHA. This limitation has been reported in adult clams, *Mesodesma mactroides* (De Moreno *et al.*, 1976), Pacific oyster, *Crassostrea gigas*, juveniles (Langdon and Waldock, 1981; Waldock and Holland, 1984), adult Eastern oysters, *C. virginica,* (Chu and Greaves, 1991), and scallop larvae, *Pecten maximus* (Delaunay *et al.*, 1993). Although n-3 and n-6 fatty acids are common in the marine environment, animals in a hatchery setting rely on cultured or artificial diets to provide these nutrients. It is therefore advantageous to identify specific dietary requirements so they may be efficiently met in culture.

This study focuses on the growth performance of postlarval (~240 μm initial shell height, SH) and juvenile (~10 mm SH) bay scallops offered selected binary and unialgal diets in attempts to identify: 1) a minimal-component diet that will maximize growth rates, 2) potential differences in nutritional needs (especially of essential PUFA) of scallops at different developmental stages. Results of this study allow comparison with previous work on postlarval sea scallops, *P. magellanicus* (Milke *et al.*, 2004), a cold-adapted species characterized by a slower rate of development. Special attention was paid to fatty acids present in diets and tissues, especially long-chain PUFA.

3.2 Materials and Methods

3.2.1 Experimental Animals

Postlarval bay scallops, *Argopecten irradians irradians*, were obtained from commercial hatcheries [Lunenburg Shellfish Inc., Nova Scotia, (NS), and Atlantic Marine Seed Inc., Prince Edward Island, Canada, for postlarvae and juveniles respectively]. Scallops were transported the same day to the National Research Council's Marine Research Station (NRC/MRS), Ketch Harbour, NS, in 2-l containers of 20°C seawater.

On arrival, they were placed in 700-l recirculating downweller systems and held on a mixed algal diet at 20°C and 30‰ for at least three days prior to initiating growth trials.

3.2.2 Experimental Setup

Postlarval or juvenile scallops were held in 400-l recirculating downweller systems for 21 (May 17th to June 7th, 2002) and 22 (October 2nd to 24th, 2001) days, respectively, in a temperature-controlled, walk-in chamber maintained at 20°C. A recirculating system consisting of a 350-l reservoir tank and a 50-l upper tank containing three identical downwellers, each 15.2 cm in diameter and 40.6 cm in height filled with 0.22-µm, cartridge-filtered seawater was used for each dietary treatment as detailed in Milke et al. (2004). Tanks were cleaned twice weekly with hot freshwater, and downwellers were rinsed with filtered seawater. The large scale of the experiments and number of diets tested precluded tank replication; however identical maintenance and sampling protocols were followed for all treatments, and algal concentration, light and ambient temperature were kept under tight control within the common environmental chamber. Previous trials have shown excellent reproducibility of diet rankings among sequential experiments using the same diets in these systems (Bricelj *et al.*, 2004; Milke *et al.*, 2004).

Approximately 7,300 postlarval scallops (initial SH = 240 µm) were added to each of three downwellers, with a 140 µm square Nitex mesh base, yielding a stocking density of 40 scallops cm^{-2} per downweller, and ~ 22,000 scallops per tank. Juvenile scallops (initial SH=10 mm), were stocked at an initial density of 150 scallops per downweller (1080 µm Nitex base), or 0.8 scallops cm^{-2}. In addition to that associated with weekly sampling, thinning of juvenile scallops across all downwellers was conducted on days 12 and 15 to reduce algal consumption, yielding a stocking density of 25 scallops per downweller for the final week.

3.2.3 Microalgal Diets

Postlarval and juvenile scallops were offered one of 6 binary diets consisting of one diatom (*Chaetoceros muelleri* (CHGRA), CCMP (Center for the Culture of Marine Phytoplankton, West Boothbay Harbor, ME) strain 1316, *Thalassiosira weissflogii*, CCMP 1336, or *Fragilaria familica*, a local isolate from Mahone Bay, NS) and one

flagellate species (*Pavlova lutheri*, CCMP 1325; *Pavlova* spp., CCMP 459; *Rhodomonas lens*, CCMP 739, or *Tetraselmis striata,* strain Plat-P, from the National Marine Fisheries Service, Milford CT). In both feeding trials, the four flagellate species were offered in combination with *C. muelleri*, and each of the three diatom species were combined with *P. lutheri* allowing for among-flagellate and among-diatom comparisons. The *P. lutheri* / CHGRA diet was considered our 'reference' diet as it is commonly used in scallop hatcheries. An additional unialgal diet treatment of *Pavlova* sp. 459 (Pav 459) was included in the postlarval feeding trial. Diets were offered at a total concentration equivalent in volume to 50 cells μl^{-1} of *Isochrysis galbana*, strain T-iso (2.15 $x10^6$ μm^3 ml^{-1}), divided equally by biovolume between the two constituents of a binary diet.

Algal species, except *R. lens*, were chosen for comparison with a prior study conducted on sea scallop postlarvae (Milke *et al.*, 2004). Pav 459 and *T. weissflogii* were high-ranking unialgal diets in *P. magellanicus* larval and postlarval growth trials respectively (Ryan, 1999; Feindel, 2000), while a unialgal diet of *Rhodomonas spp.* (Cryptophyceae) supported good growth of juvenile *Ostrea edulis* (Enright *et al.*, 1986). *Fragilaria familica* was an untested local isolate, and *T. striata*, which supported excellent growth in juvenile *C. virginica* (Wikfors *et al.*, 1996), provided a test of the scallops' tolerance to DHA dietary deficiency. *Chaetoceros* spp. are rich in AA and are commonly used diatoms in scallop hatcheries. *Isochrysis galbana* (T-iso) was not included as it supported negligible growth of postlarval (2-4 mm) *P. magellanicus* as a unialgal diet (Gillis and Dabinett, 1989).

Microalgae were grown in batch (20-l carboys), or in semi-continuous culture (200-l photobioreactors) on f/2 media (-Si for flagellates; Guillard, 1975), and harvested in the late exponential growth phase. Cultures were exposed to 24-h light, and pH was maintained between 7.8 and 8.3 by CO_2 addition, except for *T. striata* which was cultured on a 14:10 light:dark cycle and received no supplemental CO_2. Culture temperatures were as follows: *F. familica* and *T. striata*, 16°C; *T. weissflogii*, 18°C; *C. muelleri*, 20°C; *P. lutheri*, 21-22°C. Temperatures for Pav 459 (20 & 23°C) and *R. lens* (20 & 22°C) varied with culture method; higher values corresponding to semi-continuous culture in both species. Pav 459 in the Pav 459 / CHGRA diet was briefly replaced with *P. lutheri*

(days 9-11) of the juvenile growth trial due to insufficient culture volumes. Therefore, growth rates on this diet are also reported for each week interval.

Particle concentrations were monitored daily using a Coulter Multisizer II (Beckman Coulter, Fullerton, California) equipped with a 100 μm aperture tube and maintained within ≤20% of the desired concentration. Scallops were drip-fed diets via a peristaltic pump (Cole Parmer Masterflex 7554-80) at a rate sufficient to meet animal consumption and maintain suspended algal concentrations in each tank system (~ 1-10 l d^{-1}).

3.2.4 Scallop Growth and Mortality

Scallops were sampled weekly for shell height and mortality. Subsamples of at least 100 live postlarvae, from each of three downwellers per treatment, were placed on a graduated Petri dish, scanned with a dissecting scope coupled with an analog camera (Pulnix TMC7-DSP), and recorded on a Sony DVCAM (DSR-V10) digital recorder. Images (30 live scallops per downweller) were analyzed for shell height (SH, umbo to distal shell margin), using Optimas 6.5 (Media Cybernetics, Silver Spring, MD) image analysis software. Mean shell heights were calculated for each downweller, then averaged for each treatment. Percent survival was calculated from the number of live, 'new' dead and 'old' dead out of 100 scallops from each of three downwellers, and then averaging for treatment mortality. 'New' dead scallops were those similar in size to live animals, but with evidence of partial tissue degradation, while 'old' dead scallops were smaller in size and contained no tissue. Weekly instantaneous growth rate (k) was calculated as: $[(\ln_F - \ln_I)/t)]*100$ where F=final SH, I=initial SH, and t=time interval in days. Ten juvenile scallops were removed weekly from each of three downwellers and SH was measured with digital calipers to the nearest 0.1 mm to assess shell growth. Juvenile scallop mortality was defined as the percent of dead animals found weekly per downweller during growth and biochemical sampling (n=19-38, depending upon week), then averaged to obtain the weekly treatment mortality. Since no dead animals were removed other than via sampling, these values represent cumulative mortalities over time.

Prior to sampling for biochemical analysis, scallops were placed overnight in 0.22 μm filtered seawater to purge gut contents. For weight determination of postlarval

samples, 0.1 ml of scallops from each downweller were measured in a 0.5 ml Eppendorf micro-centrifuge tube, counted and filtered onto pre-combusted, pre-weighed Whatman GF/C filters. Filters were rinsed twice with 2 ml of 3.4% ammonium formate solution to remove salts and stored at -20°C. Postlarval scallops were sampled for weights only at the start and end of the experiment. Ten juvenile scallops were removed weekly from each downweller for individual weight determinations and stored at -20°C until analysis, when scallops were rinsed with distilled water, tissues dissected from shells, and both fractions weighed separately. Postlarvae and juveniles were dried to constant weight at 80°C for 24-h and 48-h, respectively. Following determination of dry weight (DW), samples were combusted at 480°C for 24-h and re-weighed to determine ash content and ash-free dry weight (AFDW).

3.2.5 Biochemical Analysis

Triplicate samples (one from each of three downwellers) of 0.2 ml of postlarvae or pooled tissues from 3-6 juveniles were sampled at the start and end of the experiments for protein analysis. Samples were placed in cryovials, and frozen in liquid nitrogen following removal of excess water. They were stored at -80°C, lyophilized and powdered prior to analysis. Algal samples (~500 µg organic carbon) were filtered onto pre-combusted GF/C filters, and dried at 80°C for 24-h before storage at -20°C in desiccant. Algae were simultaneously sampled for weight by filtering a known culture volume (5-45 ml) onto pre-combusted, pre-weighed GF/C filters. Filters were treated as postlarval samples above, and algal weights allowed for the calculation of protein and lipid concentration (see below). Samples were analyzed with a CHN elemental analyzer using acetanilide as standard and nitrogen values were multiplied by 5.8 to obtain protein concentrations (Gnaiger and Bitterlich, 1984).

Samples of 0.45-3.5 ml packed postlarval volume (1,700-67,000 scallops depending upon animal size and treatment) or dissected, pooled tissues from 3-5 juvenile scallops, were taken from each of three downwellers per experimental tank at the beginning and end of the experiment. All samples were transferred into glass tubes pre-rinsed with chloroform and methanol, immediately frozen in liquid nitrogen and stored in chloroform under nitrogen gas at -80°C. A known concentration of algae (minimum 0.6

mg lipid) was filtered (juvenile trial) or collected by centrifugation followed by filtration of the supernatant (postlarval trial) onto pre-combusted Whatman GF/C filters and treated as above.

Lipid samples were shipped overnight on dry ice to Memorial University of Newfoundland (MUN), for lipid and fatty acid analyses. Samples were extracted in 2:1 chloroform : methanol following a modified Folch procedure (Parrish, 1999). Bulk lipid samples were analyzed for lipid class composition using a MARK V Iatroscan (Iatron Laboratories, Tokyo, Japan), and values were summed to determine total lipid concentrations. Samples were subsequently transmethylated with 10% BF_3/MeOH for 1.5 hours at 85°C. Resultant fatty acid methyl esters (FAMEs) were analyzed on a Varian 3400 gas chromatograph containing an Omegawax column (Supelco, Bellefonte, PA), with peak detection at 220°C. Peak identification was conducted with a Varian Star Chromatography Software 4.02, using authentic standards (PUFA 1 and 37 Component Fame Mix, Supelco Canada); identification of fatty acids for which standards were not available (e.g. 22:5n-6) was conducted on a Varian Saturn mass spectrophotometer. The identity of 22:5n-6 was confirmed by careful reference to retention data in Ackman (1986) and to mass spectral patterns in Christie (2006). The former shows chromatograms and gives retention times for GC of FAME, including those found in one of the standards (PUFA-1, Supelco Inc.) used here; Christie (2006) provides a comprehensive library of mass spectra for FAME including the two DPA isomers and an adjacent n-6 peak. In addition to the normal characteristic ions e.g. the McLafferty rearrangement ion at m/z 74 and the molecular ion at m/z 344, the key identifier in the FAME mass spectrum for the n-6 isomer of DPA is the ion at m/z 150 which ranged from 12-20% of the base peak.

3.2.6 Statistical Analysis

Differences in final scallop weights were determined with a one-way analysis of variance (ANOVA). Linear regression lines were fitted to SH data to determine scallop growth rates for both developmental stages, and differences among treatments were tested using analysis of covariance (ANCOVA); a two-way ANOVA was conducted to determine the effects of both time and diet on weekly mortalities. Biochemical properties

reported as concentration data (i.e. total lipid, protein, and fatty acid) were tested individually using a one-way ANOVA. A series of multivariate analysis of variance (MANOVA) models were used to determine differences among biochemical properties of algal diets and scallop tissues reported as percent data. The MANOVA models tested included: 1) individual lipid classes, 2) individual PUFAs, 3) Sum of SAT, MUFA, PUFA and 4) Sum of n-3 and n-6 fatty acids. When the MANOVA model was significant, subsequent one-way ANOVAs were conducted. For all ANOVA tests that yielded significant results, a Tukey's multiple comparison test was conducted to identify treatment differences. Percent data were arcsine-transformed prior to analysis to normalize the data, and a significance level of $P<0.05$ was applied unless otherwise noted. All analyses were conducted using SYSTAT 10.0 (SPSS, Chicago, IL).

3.3 Results

3.3.1 Scallop Growth and Mortalities

After three weeks of feeding, overall diet ranking based on scallop growth rates was very similar for postlarval and juvenile stages (Fig. 3.1). The Pav459 / CHGRA diet yielded the highest growth rates: 59 and 392 μm day $^{-1}$ in postlarvae and juveniles, respectively. Weekly growth rates for juvenile scallops that experienced transient substitution of Pav 459 by *P. lutheri* in the Pav 459 / CHGRA diet during week two were 286, 343, and 538 μm day^{-1} for weeks 1-3, respectively. Growth rates attained on the Pav 459 / CHGRA diet were significantly higher than on all other dietary treatments for postlarvae (ANCOVA, Tukey's Test, $P<0.001$, Fig. 3.1A), and higher than all but two treatments for juveniles (ANCOVA, Tukey's Test, $P<0.05$, Fig. 3.1B). This diet yielded growth rates 65% and 22% higher than the next highest performing diet: *T. striata* / CHGRA (Fig. 3.1A,B). Total animal organic weights were also 330% and 44% greater (Table 3.1). However, a unialgal diet of Pav 459 yielded growth rates 42% lower for postlarvae than when combined with the diatom (Fig. 3.1A; diet not tested for juveniles), indicating a strong contribution of CHGRA to the high growth rate on the binary diet.

The reference diet of *P. lutheri* / CHGRA, that differs from the highest growth-promoting diet of Pav 459 / CHGRA by the substitution of only one species within the

same genus, yielded moderate growth rates for both life history stages, 40-50% lower than those observed for the Pav 459 / CHGRA diet. Diets of *R. lens* / CHGRA and *P. lutheri* / *F. familica* resulted in moderate- to low-growth rates for both developmental

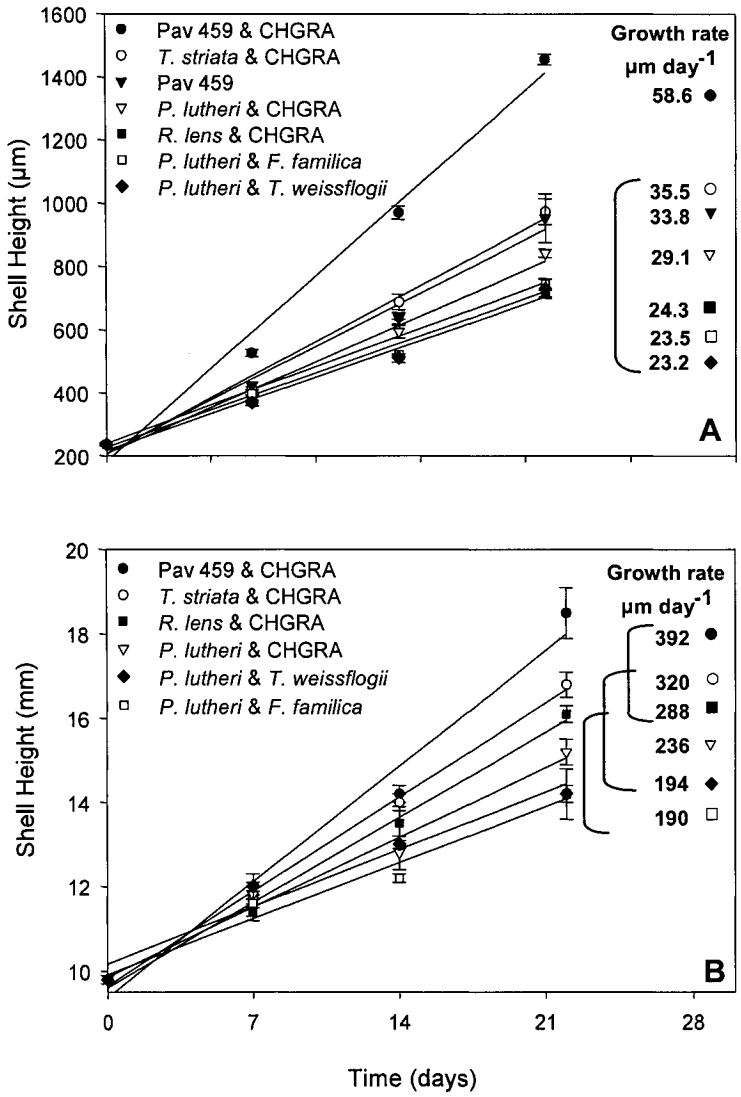

Figure 3.1: Shell growth trajectories of *A. irradians* postlarvae (A) and juveniles (B); values represent the mean shell height (SH) ± standard error (SE). Linear regressions were determined from SH data (all R^2 values were ≥0.94), and brackets represent statistically similar growth rates as determined by ANCOVA ($P<0.05$) within each developmental stage.

stages, and the *P. lutheri* / *T. weissflogii* combination was the lowest-performing diet for both postlarvae and juveniles, supporting scallop growth rates 40-48% of the best diet (Fig. 3.1A). Dry weight and AFDW of both postlarvae and juveniles yielded the same

diet ranking observed using the shell-growth trajectories (Table 3.1). Organic matter found in the shell of juveniles contributed 19-26% of the total body AFDW.

Postlarval instantaneous daily growth rates (k), based upon SH, ranged from 5.3-8.7% per day (Table 3.1), although a decreasing trend was generally observed each successive week regardless of dietary treatment. Juvenile instantaneous growth rates showed no particular trend over time, and ranged from 1.7-2.9% per day.

A two-way ANOVA on weekly new mortalities of postlarvae identified significant effects of both diet ($P<0.001$) and time ($P<0.01$), as well as an interaction between these two factors ($P<0.01$). It was not possible to run one-way ANOVAs examining factors individually (i.e. diet only and time only) when each diet and time combination was considered independently due to the limited sample size; however, one-way ANOVAs examining these two factors on pooled data (i.e. all diet data combined to examine effect of time) identified significantly higher new mortalities were observed for postlarvae fed the *P. lutheri* / *T. weissflogii* diet (4.9% vs. 0.9-2.6% for all other diets), and during the first week of the feeding trial (3.4% vs. 2.0 and 1.8% for weeks 2 and 3, respectively). Cumulative mortalities of postlarvae remained relatively low (< 13%) and did not differ significantly among treatments ($P>0.05$). For juvenile scallops, no significant effect of either diet or time was observed for new mortalities, and there was no difference in cumulative mortalities among dietary treatments (maximum 4%; $P>0.05$).

3.3.2 Microalgal Biochemical Composition

Microalgae ranged in equivalent spherical diameter (ESD) from 3.8-11.8 μm, and in AFDW from 12 to 419 pg cell^{-1} (Table 3.2). Biochemical composition is reported for the diet rather than individual constituents (except for the Pav 459 unialgal diet and for *R. lens*) (Table 3.3), as the biochemical composition of individual algal species other than *R. lens* was reported in Milke et al. (2004). Dietary protein concentrations ranged from 361-650 mg g AFDW^{-1}, although they did not vary significantly (ANOVA, $P<0.05$; Table 3.3). Total lipid concentrations, however, differed significantly among diets, with highest levels observed in the Pav 459 / CHGRA and *P. lutheri* / CHGRA combinations (296 and 306 mg g AFDW^{-1}, respectively); the lowest levels observed were for the *T. striata* / CHGRA diet (179 mg g AFDW^{-1}; ANOVA, $P<0.05$; Table 3.3). Lipid-to-protein ratios

Table 3.1: Individual scallop dry (DW) and ash-free dry weights (AFDW) at the beginning and end of 3 week-exposure to experimental diets and instantaneous daily growth rates (k) calculated from shell heights. Data reported as mean ± SE (mean of weekly values for instantaneous growth rates). Within rows, values that do not have a common superscript letter differ significantly, $P<0.05$; no difference in k was observed.

	Initial	Pav 459 & CHGRA	*T. striata* & CHGRA	Pav 459	*P. lutheri* & CHGRA	*R. lens* & CHGRA	*P. lutheri* & *F. familica*	*P. lutheri* & *T. weissflogii*
Individual scallop biomass								
Postlarvae (µg scallop^{-1})								
DW	2.4±0.1	242.1±12.6^{a}	56.0±2.9^{b}	49.5±1.7^{b}	36.9±1.6^{b}	38.9±1.3^{b}	31.8 ± 1.0^{b}	33.8±2.7^{b}
AFDW	0.6±0.0	61.3±2.6^{a}	14.2±0.5^{b}	12.8±0.2^{bc}	9.0±0.3^{c}	9.7±0.3^{bc}	8.0 ± 0.4^{c}	9.8±0.9^{bc}
Juveniles (mg scallop^{-1})								
Shell DW	109.6±2.3	598.0±50.7^{a}	500.5±29.1^{a}	--	362.7±22.3^{bc}	443.4±14.6	299.0±9.1^{c}	297.9±21.2^{c}
Shell AFDW	2.4±0.1	11.9±0.9^{a}	9.2±0.5^{b}	--	7.5±0.3^{bc}	8.5±0.2^{b}	6.2±0.1^{c}	5.5±0.4^{c}
Tissue DW	8.8±0.6	65.9±6.8^{a}	44.4±3.6^{b}	--	36.2±1.7^{bc}	42.7±2.0^{b}	29.6±1.7^{bc}	23.5±2.0^{c}
Tissue AFDW	6.7±0.3	51.5±5.4^{a}	34.8 ±2.8^{b}	--	27.1±1.2^{bc}	33.5±1.7^{b}	22.3±1.3^{bc}	17.7±1.3^{c}
Instantaneous growth rates (k)								
Postlarvae								
Week 1	--	11.4	8.0	8.3	6.6	6.9	7.4	6.3
Week 2	--	8.8	7.3	5.9	6.5	7.4	3.9	4.7
Week 3	--	5.8	5.0	5.8	5.0	1.6	5.1	5.1
Mean	--	8.7±1.6	6.7±0.9	6.6±0.8	6.1±0.5	5.3±1.9	5.5±1.0	5.4±0.5
Juveniles								
Week 1	--	2.7	2.5	--	2.7	2.2	2.4	2.9
Week 2	--	2.6	2.6	--	1.2	2.4	0.7	1.1
Week 3	--	3.3	2.3	--	2.1	2.2	1.9	1.1
Mean	--	2.9±0.2	2.5±0.1	--	2.0±0.4	2.3±0.1	1.7±0.5	1.7±0.6

Table 3.2: Size (equivalent spherical diameter, ESD), dry weight (DW) and ash-free dry weight (AFDW) of microalgae used in postlarval scallop growth trials (values for juvenile experiment not reported). Size and biomass values are reported as mean ± SE, n=3.

	CHGRA	***F. familica***	***T. weissflogii***	***P. lutheri***	**Pav 459**	***R. lens***	***T. striata***
Cell size and biomass							
ESD (μm^3)	5.5±0.3	5.5±1.4	11.8±0.2	4.3±0.1	3.8±0.1	7.3±0.6	9.1±0.1
DW (pg $cell^{-1}$)	42±18	26±7	616±237	26±3	15±2	130±29	283±6
AFDW (pg $cell^{-1}$)	32±14	18±5	419±145	20±2	12±1	93±18	221±6

Table 3.3: Bulk biochemical properties, lipid classes and fatty acid composition of mixed microalgal diets used in postlarval feeding trials and unialgal *R. lens* (see text). Data indicate means ± SE, n=3; means calculated from weekly sampling. Within rows, values that do not have a common superscript letter differ significantly, $P<0.05$; constituents marked with * were not tested, constituents marked with ** were tested, but no difference was observed. *R. lens* not included in statistical models as it was not tested as a unialgal diet.

	Pav 459 & CHGRA	***T. striata* & CHGRA**	**Pav 459**	***P. lutheri* & CHGRA**	***R. lens* & CHGRA**	***P. lutheri* & *F. familica***	***P. lutheri* & *T. weissflogii***	***R. lens***
Bulk biochemical properties (mg g AFDW^{-1})								
Total protein **	555±89	361±113	577±85	462±22	650±63	435±27	528±102	707±41
Total lipid	296±7^{a}	179±28^{b}	231±18^{ab}	306±17^{a}	213±18^{ab}	229±15^{ab}	251±18^{ab}	133±7
Total fatty acid	180±21	101±8	115±10	187±33	124±22	129±9	136±21	57±10
Lipid classes (% of total lipid)								
Triacylglycerols	23.7±14.2	26.4±17.6	4.9±2.6	19.8±11.1	21.2±12.7	4.1±3.5	9.3±4.7	0.1±0.1
Sterols	7.0±1.4^{ab}	2.6±0.6^{bc}	11.4±1.6^{a}	3.4±0.7^{bc}	5.0±1.3^{bc}	2.9±0.2^{bc}	2.4±0.5^{c}	6.1±1.1
Phospholipids **	38.9±5.2	33.4±5.8	48.5±1.6	41.7±4.7	41.1±5.9	49.6±3.2	47.4±2.3	52.0±1.6
Fatty acid composition (% of total fatty acid)								
14:0*	13.4±0.6	8.1±2.0	16.2±0.6	11.0±0.4	8.8±1.3	12.6±0.7	8.9±0.5	4.0±0.3
16:0*	12.7±2.8	13.6±3.0	11.7±1.0	10.7±2.5	12.8±2.6	6.0±0.6	9.1±0.4	13.7±0.8
18:0*	0.4±0.1	0.4±0.1	0.3±0.1	0.3±0.0	0.6±0.1	0.2±0.0	0.2±0.0	0.7±0.1
16:1n-7*	18.8±4.3	18.0±6.4	8.2±0.6	19.7±3.1	16.7±3.9	14.2±0.5	16.5±1.5	0.4±0.1
18:1n-9*	0.4±0.1	2.1±0.9	0.2±0.0	0.5±0.1	0.5±0.1	0.4±0.1	0.6±0.3	0.3±0.1
18:1n-7*	0.7±0.1	1.1±0.3	0.8±0.2	1.6±0.3	1.4±0.3	2.6±0.2	1.6±0.3	2.5±0.3
20:1n-9*	--	0.5±0.3	--	--	0.1±0.1	tr	--	0.2±0.2
16:3n-4	3.6±0.6^{b}	4.1±0.5^{b}	0.2±0.1^{c}	3.4±0.3^{b}	4.1±0.8^{b}	4.7±1.3^{b}	8.9±1.1^{a}	tr
16:4n-3	--	6.0±3.2^{a}	--	--	0.1±0.0^{b}	--	--	0.1±0.1
18:2n-6	1.7±0.4^{ab}	2.7±0.9^{ab}	3.7±1.3^{a}	0.7±0.1^{b}	1.0±0.1^{ab}	0.5±0.0^{b}	0.4±0.0^{b}	1.1±0.0
18:3n-3	1.1±0.1^{b}	6.1±3.3^{ab}	3.2±0.8^{ab}	1.0±0.3^{b}	9.7±3.0^{a}	1.2±0.1^{b}	1.2±0.3^{b}	28.1±0.8

	Pav 459 & CHGRA	*T. striata* & CHGRA	Pav 459	*P. lutheri* & CHGRA	*R. lens* & CHGRA	*P. lutheri* & *F. familica*	*P. lutheri* & *T. weissflogii*	*R. lens*
18:4n-3 **	3.3±1.8	2.2±0.9	5.8±2.0	4.3±0.8	5.6±1.5	4.8±0.7	5.3±0.5	15.3±0.9
20:4n-6 (AA)	6.3±1.4^{a}	5.8±1.8^{a}	3.5±1.1^{ab}	4.5±1.3^{ab}	5.6±2.1^{ab}	1.8±0.2^{ab}	0.5±0.1^{b}	0.1±0.0
20:5n-3 (EPA)	18.5±3.5^{bc}	12.3±1.2^{d}	21.7±2.0^{a}	23.6±3.4^{abc}	14.3±1.8^{cd}	30.5±0.5^{a}	26.3±1.4^{ab}	10.9±0.6
22:5n-6 (DPA)	3.5±1.0^{a}	0.2±0.1^{c}	8.2±0.6^{b}	0.4±0.0^{c}	0.4±0.0^{c}	0.4±0.1^{c}	0.4±0.0^{c}	0.6±0.1
22:6n-3 (DHA)	3.8±0.8^{bc}	1.6±0.3^{c}	5.6±0.9^{ab}	6.2±1.0^{ab}	4.7±0.7^{ab}	6.7±0.9^{ab}	7.7±0.6^{a}	9.0±0.5
Σ SAT **	30.6±2.2	27.9±3.8	30.9±0.5	26.6±2.4	27.7±2.5	23.3±0.9	22.9±0.8	23.9±0.5
Σ MUFA	23.3±3.9^{ab}	27.2±3.7^{a}	13.7±0.5^{b}	24.7±2.8^{ab}	22.5±3.0^{ab}	20.1±0.2^{ab}	21.8±1.5^{ab}	9.0±0.6
Σ PUFA **	46.1±6.1	44.9±7.4	55.4±0.9	48.7±5.2	49.9±5.6	56.5±1.1	55.3±1.3	67.1±1.0
Σ n-3 **	27.1±5.9	28.8±7.6	36.5±3.7	35.5±5.4	35.2±6.1	44.5±2.2	41.0±2.6	64.6±1.6
Σ n-6	13.1±1.6^{ab}	10.1±2.1^{abc}	16.6±2.7^{a}	6.9±1.7^{bcd}	8.6±2.6^{abc}	4.4±0.5^{cd}	1.9±0.1^{d}	2.1±0.4

Notes for Table 3.3:

[1]SAT = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA= polyunsaturated fatty acids; -- = non-detectable; tr = trace; nd = not determined

[2] Other lipid classes identified at <1% include: steryl/wax esters, glyceryl ethers; <3%: methyl esters, methyl ketones; <4%: hydrocarbons, ethyl ketones, alcohols; <7% diacylglycerols; <8.4% free fatty acids; and at 4.7-31.7% includes acetone mobile polar lipids.

[3] Other fatty acids identified at <1%: *ai*15:0, *i*16:0, *ai*16:0, *i*17:0, 17:0, 19:0, 20:0, 22:0, 24:0, 14:1, 15:1, 18:1n-11, 18:1n-5, 22:1n-11(13), 22:1n-7, 16:4n-1, 18:2n-4, 18:3n-4, 18:4n-1, 18:5n-3, 20:2α, 20:3n-3, 20:4n-3, 22:4n-3; <2%: *i*15:0, 15:0, 16:1n-9, 18:1n-6, 20:1n-11, 24:1, 18:3n-6, 20:3n-6, 22:5n-3; <3%: 17:0ai, 23:0, 16:1n-5, 20:1n-7, 16:2n-4, 20:2β, 21:5n-3, <4%: 17:1, 22:1n-9, 20:2n-6, 22:4n-6.

do not appear to have any relationship with the ability of the diet to support growth, as the highest (3.2) and lowest (1.5) ratios were found in the mid-performing diets of *R. lens* / CHGRA and *P. lutheri* / CHGRA, respectively; whereas, both high- and low-performing diets fell in a mid-range of 1.9-2.5 (calculated from Table 3.3).

The two highest-performing diets, Pav 459 / CHGRA and *T. striata* / CHGRA, had the highest triacylglycerol (TAG) levels (24-26%), while the diets that supported poor growth were characterized by lower, though not significantly different (ANOVA, $P>0.05$), proportions (4-9%; Table 3.3). The *P. lutheri* / CHGRA diet, that supported only moderate growth, however, was also characterized by a relatively high proportion of TAG (20%), indicating that there was no clear relationship between dietary TAG levels and scallop growth rates. Sterol levels were highest in Pav 459-containing diets and lowest in the poor-performing *P. lutheri* / *T. weissflogii* combination (7-11% vs. 2%); although no clear relationship between scallop growth rate and sterol proportion or concentration was observed for either developmental stage (R^2 values of linear regressions = 0.01-0.32). Proportions of phospholipids did not differ significantly among diet treatments and ranged from 33-50% of total lipid (ANOVA, $P>0.05$; Table 3.3). Free fatty acids comprised ≤8.5% of the total lipid for all combination diets; ≤6% for all individual algal species, except for *F. familica* in which they accounted for 14.3%.

No significant difference in total saturated fatty acids (SAT), PUFA or n-3 fatty acids was observed among dietary treatments, although the *T. striata* / CHGRA diet had total monounsaturated fatty acid (MUFA) levels higher than that of the unialgal Pav 459 diet (ANOVA, $P<0.05$; Table 3.3). The three diets supporting highest growth rates, Pav 459 / CHGRA, *T. striata* / CHGRA and Pav 459, had total n-6 levels significantly higher than those found in the lowest-performing diet (10-17% vs. 2%; ANOVA; $P<0.05$), and the two Pav 459-containing diets had high levels of n-6 DPA (4-8% vs. 0.2-0.4%). It is interesting that the only salient difference between the Pav 459 / CHGRA and *P. lutheri* / CHGRA diets, which yielded significantly different growth rates, is the marked difference in n-6 DPA (3.5 and 0.4%, respectively; Table 3.3). *P. lutheri* / *F. familica* and *P. lutheri* / *T. weissflogii*, the diets that yielded the lowest growth rates, contained the lowest levels of AA (0.5-1.8%) compared to all other binary diets (4.5-6.3%; Table 3.3), which reflects the fact that CHGRA, characterized by relatively high AA proportions, is

not included in these diets. These two inferior diets also contained the highest levels of n-3 fatty acids (41-44%) especially when compared with the two diets that yielded the highest growth rates (27-29%; Table 3.3). The two inferior diets also have the highest n-3/n-6 ratios (11-22%); in contrast, markedly lower values of this ratio are found in the two best diets of Pav 459 / CHGRA and *T. striata* / CHGRA (2-3%; Fig. 3.3A). A similar trend was observed for the EPA/AA ratio; DHA/EPA ratios remain low (~0.2) regardless of dietary treatment (Fig. 3.3 B & C). Overall, growth rates of scallops (Y, in $\mu m\ day^{-1}$) declined with increasing n-3/n-6 ratios (X) in the diet, a relationship best described by a power function (postlarvae: $Y = 52.44x^{-0.316}$, $R^2 = 0.64$, $P<0.05$; juveniles: $Y = 468.97x^{-0.326}$, $R^2 = 0.89$, $P<0.01$).

Biochemical properties are only presented for postlarval diets (Table 3.3) as overall these values did not differ greatly from those for diets used in the juvenile experiment. Some differences, however, were observed: most notably, TAG proportions were much higher in CHGRA-containing postlarval diets (20-26%) than in the same juvenile diets (3-7%), while phospholipid values were lower in postlarval (33-49%) relative to juvenile diets (48-55%). Additionally, Σn-3 was 15-18% higher in the juvenile diets of Pav 459 / CHGRA and *R. lens* / CHGRA, but a 15% decrease was noted for the juvenile diet of *P. lutheri* / *F. familica*. All differences in individual fatty acids between postlarval and juvenile diets were <10%.

3.3.3 Scallop Biochemical Composition

The lowest protein concentrations in postlarvae (390 mg g $AFDW^{-1}$) were observed in the slowest-growing scallops fed a diet of *P. lutheri* / *T. weissflogii*; whereas, the highest concentrations (570-577 mg g $AFDW^{-1}$) were associated with the faster-growing postlarvae offered diets of Pav 459 / CHGRA and *T. striata* / CHGRA (ANOVA; $P<0.05$; Table 3.4). Protein samples for scallops fed the *R. lens*-containing diet were unavailable for analysis, and thus were not included in Table 3.4 or the ANOVA model. No significant difference in protein or lipid concentration of juvenile tissues was observed among diet treatments (ANOVA; $P = 0.465$; Table 3.5). There was, however, a significant difference in TAG juvenile tissue levels among diet treatments, with significantly higher values found in the two highest- vs. the lowest- performing diets

Table 3.4: Biochemical composition of postlarval scallops initially and after 3 weeks of feeding on experimental diets. Values are mean ± SE, n=3. Within rows, values that do not have a common superscript letter differ significantly, $P<0.05$; constituents marked with * were not tested, constituents marked with ** were tested, but no difference was observed. Notes as in Table 3.

	Initial	Pav 459 & CHGRA	*T. striata* & CHGRA	Pav 459	*P. lutheri* & CHGRA	*R. lens* & CHGRA	*P. lutheri* & *F. familica*	*P. lutheri* & *T. weissflogii*
Bulk biochemical properties (mg g AFDW^{-1})								
Total protein	503.6±16.6	576.9±12.3^{a}	570.1±13.4^{a}	487.1±38.3^{ab}	542.0±6.3^{a}	nd	534.7±21.4^{a}	390.7±30.5^{b}
Total lipid	77.2±13.6	33.7±6.0^{ab}	37.0±6.1^{ab}	14.9±3.0^{b}	36.8±9.5^{ab}	36.6±13.2^{ab}	50.1±17.9^{ab}	74.9±12.7^{a}
Total fatty acid	46.9±10.0	19.7±2.8^{bc}	20.7±4.0^{bc}	7.4±1.5^{c}	21.8±6.4^{bc}	21.9±8.8^{b}	29.5±12.2^{bc}	47.5±9.5^{a}
Lipid classes (% of total lipid)								
Triacylglycerols*	9.0±1.2	2.4±0.5	4.3±0.8	4.3±1.7	1.8±0.7	6.2±2.0	2.3±0.5	2.5±1.6
Sterols*	7.3±1.2	14.0±3.9	14.2±0.4	21.4±2.3	8.7±1.9	11.5±3.5	8.9±0.5	6.7±1.8
Phospholipids*	40.4±7.4	60.9±7.1	49.0±2.8	42.1±7.8	58.2±10.5	48.6±12.3	49.4±10.9	65.3±4.7
Fatty acid composition (% of total fatty acid)								
14:0*	4.6±0.2	4.5±0.1	3.0±0.2	3.7±0.2	4.1±0.1	2.9±0.1	4.0±0.5	4.0±0.2
16:0*	12.9±0.2	11.3±0.1	21.4±2.2	10.8±0.1	12.9±0.3	11.2±0.2	11.3±0.2	11.9±0.4
18:0*	5.8±0.2	7.9±0.2	9.8±1.0	6.3±0.1	7.0±0.2	5.9±0.1	6.8±0.2	6.7±0.0
16:1n-7*	8.1±0.0	4.2±0.1	2.8±0.3	3.8±0.3	6.6±0.1	3.7±0.1	5.9±0.3	5.4±0.5
18:1n-9*	4.2±0.1	0.8±0.1	4.6±0.4	0.9±0.1	2.5±0.2	2.9±0.1	1.9±0.1	8.4±3.6
18:1n-7*	5.6±0.1	4.0±0.1	3.7±0.1	5.2±0.5	7.8±0.2	4.6±0.2	7.5±0.3	7.0±0.4
20:1n-9*	2.2±0.0	0.7±0.0	7.1±0.8	0.8±0.0	0.8±0.8	3.2±1.6	0.8±0.8	2.3±0.1
16:3n-4	5.4±0.2	4.1±1.8^{ab}	1.1±0.3^{b}	5.9±0.6^{a}	4.4±0.4^{ab}	7.6±0.2^{a}	5.4±0.1^{a}	3.5±0.7^{ab}
16:4n-3 **	0.2±0.2	3.3±1.9	0.1±0.1	1.1±0.3	0.8±0.5	0.3±0.2	--	1.0±0.4
18:2n-6	2.3±0.1	0.9±0.2^{b}	2.0±0.3^{ab}	0.8±0.1^{b}	0.8±0.1^{b}	0.6±0.0^{b}	0.6±0.1^{b}	3.2±1.4^{a}
18:3n-3	1.3±0.1	0.3±0.0^{b}	1.5±0.5^{a}	0.4±0.0^{b}	0.7±0.2^{b}	2.6±0.1^{a}	0.5±0.1^{b}	0.5±0.1^{b}
18:4n-3	2.4±0.1	2.2±0.7^{bc}	0.8±0.2^{c}	2.2±0.2^{abc}	3.0±1.0^{abc}	5.4±0.3^{ab}	5.7±0.4^{a}	4.7±1.0^{ab}

	Initial	Pav 459 & CHGRA	*T. striata* & CHGRA	Pav 459	*P. lutheri* & CHGRA	*R. lens* & CHGRA	*P. lutheri* & *F. familica*	*P. lutheri* & *T. weissflogii*
20:4n-6 (AA)	3.4±0.1	7.5±0.2^{ab}	7.5±1.1^{a}	6.1±0.2^{ab}	5.2±0.1^{b}	7.1±0.1^{ab}	3.0±0.1^{c}	1.5±0.1^{d}
20:5n-3 (EPA)	8.2±0.2	6.4±0.1^{c}	7.8±1.1^{bc}	4.1±0.2^{d}	9.3±0.2^{ab}	6.9±0.2^{bc}	10.4±0.5^{a}	9.4±0.6^{ab}
22:5n-6 (DPA)	6.6±0.1	20.2±0.4^{b}	1.9±0.4^{de}	27.6±1.1^{a}	3.2±0.1^{c}	1.3±0.1^{e}	3.7±0.2^{c}	3.1±0.3^{cd}
22:6n-3 (DHA)	15.7±0.2	12.1±0.1^{b}	4.0±0.4^{c}	9.6±0.4^{b}	18.8±1.2^{a}	17.8±0.4^{a}	18.9±0.4^{a}	17.5±1.3^{a}
Σ SAT	27.4±0.5	26.2±0.3^{b}	40.0±4.2^{a}	24.5±0.3^{b}	26.3±0.1^{b}	23.4±0.4^{b}	24.8±0.4^{b}	25.1±1.1^{b}
Σ MUFA	23.9±0.3	13.7±0.4^{b}	24.2±1.0^{a}	14.3±1.0^{b}	23.9±1.9^{a}	22.8±0.4^{a}	23.3±1.2^{a}	27.4±3.1^{a}
Σ PUFA	48.6±0.5	60.1±0.5^{a}	35.8±3.6^{c}	61.2±1.3^{a}	49.8±1.9^{b}	53.2±0.6^{ab}	51.9±1.2^{ab}	47.6±2.3^{b}
Σ n-3	29.2±0.4	25.1±1.2^{b}	18.6±2.4^{b}	18.1±0.2^{b}	33.9±2.1^{a}	35.2±0.3^{a}	36.4±1.3^{a}	34.5±2.6^{a}
Σ n-6	13.8±0.1	30.5±0.7^{b}	16.9±1.1^{c}	36.3±0.8^{a}	10.6±0.2^{d}	10.3±0.1^{d}	8.8±0.1^{d}	8.7±1.0^{d}

Table 3.5: Biochemical composition of juvenile scallop tissues initially and after 3-week exposure to experimental diets. Values are mean ± SE, n=3. Within rows, values that do not have a common superscript letter differ significantly, $P<0.05$; constituents marked with * were not tested, constituents marked with ** were tested, but no difference was observed. Notes as in Table 3.

	Initial	Pav 459 & CHGRA	*T. striata* & CHGRA	*P. lutheri* & CHGRA	*R. lens* & CHGRA	*P. lutheri* & *F. familica*	*P. lutheri* & *T. weissflogii*
Bulk biochemical properties (mg g AFDW^{-1})							
Total protein **	753.4±30.2	800.0±27.5	806.4±17.5	779.7±19.1	808.3±28.0	828.7±38.4	755.0±18.1
Total lipid **	90.6±7.8	37.9±7.4	43.5±12.4	57.8±24.2	57.4±7.3	61.6±12.6	72.9±13.5
Total fatty acid **	55.2±3.4	22.2±3.3	25.7±7.6	33.1±13.6	36.9±4.8	34.8±7.6	42.8±9.1
Lipid classes (% of total lipid)							
Triacylglycerols	0.4±0.3	7.7±0.5^{a}	6.0±1.1^{a}	1.2±1.0^{ab}	4.5±2.1^{ab}	1.1±1.1^{ab}	0.2±0.2^{b}
Sterols **	12.5±1.6	13.0±3.4	14.4±1.6	14.5±2.0	10.3±1.4	16.4±1.4	13.5±1.7
Phospholipids **	69.9±2.1	51.5±7.7	57.6±3.8	60.2±5.2	70.8±2.5	58.9±2.0	65.7±3.0
Fatty acid composition (% of total fatty acid)							
14:0*	2.8±0.0	4.9±0.2	2.0±0.4	2.8±0.4	2.5±0.2	3.6±0.2	2.3±0.1
16:0*	11.0±0.2	11.4±0.2	11.3±0.6	11.6±0.5	10.7±0.4	11.8±0.5	11.1±0.3
18:0*	7.6±0.2	7.2±0.1	6.5±0.1	7.4±0.2	7.4±0.3	7.3±0.1	7.0±0.1
16:1n-7*	4.4±0.2	5.7±1.1	3.3±1.1	3.6±0.4	2.4±0.3	3.2±0.2	3.0±0.1
18:1n-9*	1.6±0.1	1.1±0.2	3.3±0.5	1.5±0.3	1.8±0.2	1.5±0.3	1.4±0.1
18:1n-7*	3.6±0.1	2.6±0.3	4.0±1.4	4.4±0.1	2.7±0.1	5.0±0.1	4.3±0.1
20:1n-9*	3.1±0.1	1.3±0.0	6.8±0.6	2.4±0.1	3.5±0.1	1.4±0.7	2.8±0.2
16:3n-4 **	5.0±2.1	0.2±0.1	0.3±0.1	2.4±2.0	2.7±2.0	3.5±1.7	5.8±0.2
16:4n-3 **	2.8±2.4	2.3±1.2	4.0±2.2	4.7±2.1	4.1±2.0	2.9±1.9	0.5±0.0
18:2n-6	0.8±0.1	0.6±0.1^{b}	2.4±0.5^{a}	0.6±0.1^{b}	0.9±0.1^{b}	0.6±0.1^{b}	1.2±0.3^{ab}
18:3n-3	0.5±0.0	0.2±0.1^{b}	2.3±0.4^{a}	0.2±0.1^{b}	2.0±0.3^{a}	0.1±0.1^{b}	0.3±0.1^{b}
18:4n-3 **	4.6±2.0	2.3±0.7	2.7±0.6	3.3±2.7	3.6±2.1	4.7±2.3	8.6±0.4
20:4n-6 (AA)	3.4±0.1	5.6±0.3^{a}	6.4±0.8^{a}	5.4±0.2^{a}	6.8±0.3^{a}	3.1±0.1^{b}	2.7±0.2^{b}

	Initial	Pav 459 & CHGRA	*T. striata* & CHGRA	*P. lutheri* & CHGRA	*R. lens* & CHGRA	*P. lutheri* & *F. familica*	*P. lutheri* & *T. weissflogii*
20:5n-3 (EPA) **	10.1±0.3	9.5±0.5^{a}	13.1±2.1^{a}	8.3±1.1^{a}	9.7±0.3^{a}	8.6±0.3^{a}	8.3±0.7^{a}
22:5n-6 (DPA)	3.1±0.0	15.1±1.0^{a}	0.8±0.0^{c}	3.6±0.1^{b}	0.9±0.1^{c}	4.8±0.2^{b}	4.2±0.2^{b}
22:6n-3 (DHA)	21.2±0.7	14.0±0.7^{b}	6.9±2.0^{c}	22.7±0.6^{a}	23.3±0.7^{a}	27.1±0.8^{a}	25.2±0.6^{a}
Σ SAT	24.7±0.3	27.2±0.7^{a}	23.0±0.6^{b}	24.3±1.2^{ab}	23.2±0.6^{b}	24.5±0.5^{ab}	22.5±0.2^{b}
Σ MUFA	16.4±0.2	18.2±1.2^{ab}	26.4±4.1^{a}	16.9±1.1^{b}	17.6±0.5^{b}	16.5±0.3^{b}	16.9±0.7^{b}
Σ PUFA	58.9±0.5	54.6±1.9^{ab}	50.7±3.7^{b}	58.8±2.1^{ab}	59.3±1.1^{ab}	59.0±0.7^{ab}	60.5±0.6^{a}
Σ n-3	41.2±0.6	29.9±1.6^{b}	33.4±4.0^{b}	41.2±1.2^{ab}	45.5±0.8^{a}	44.8±0.5^{a}	44.7±0.6^{a}
Σ n-6	11.8±2.8	24.3±0.9^{a}	14.7±1.6^{b}	14.3±2.8^{b}	10.4±0.3^{b}	10.0±0.5^{b}	9.1±0.6^{b}

(6-8% vs. 0.2%; P< 0.01; Table 3.5). The MANOVA model examining lipid classes in postlarvae was not significant (P=0.07); therefore differences among treatments were not tested.

Levels of SAT found in scallop tissues of both developmental stages are fairly constant (~25%), except for postlarvae fed *T. striata* / CHGRA, which had elevated levels of total SAT (40%), and also ~2x the level of 16:0 found in any other sample (10.8-12.9% vs. 21.4%; Table 3.4). Total MUFA levels were generally higher in postlarvae than in juveniles fed the same diets (Tables 3.4 & 3.5). PUFA represented 35-62% of total fatty acids in scallop tissues, and unlike the MUFA, generally higher proportions were found in juveniles than postlarvae (57.2 vs. 49.7%). There was no clear association between PUFA levels in scallop tissues and growth performance. Notably, the highest tissue PUFA levels in postlarvae (~60%) were associated with Pav 459-containing diets but were significantly lower in scallops fed the second best diet, while in juveniles, the highest PUFA levels were found in the low-performing *P. lutheri* / *T. weissflogii* diet. The lowest PUFA levels were observed in both postlarvae and juveniles fed T. striata / CHGRA (36 and 51%, respectively; ANOVA; P < 0.05; Tables 3.4 & 3.5).

Both stages of scallops fed the two poorest-performing diets of *P. lutheri* / *F. familica* and *P. lutheri* / *T. weissflogii* contained the lowest AA levels (1.5-3.1%), but the highest DHA levels (18-25%; ANOVA; P<0.05; Table 3.4). There was no difference in EPA levels of juveniles (Table 3.5), but DHA levels were generally higher in juveniles than in postlarvae (19.9 vs. 14.1%). Levels of the n-6 DPA were particularly high in animals fed diets containing Pav 459; in postlarvae this value reached 28% for scallops feeding on Pav 459 alone (Table 3.4).

3.3.4 Comparison of Dietary and Tissue Fatty Acids

Little or no enrichment of total n-3 fatty acids was observed in the tissues of juvenile or postlarval scallops relative to their diet regardless of dietary treatment (Fig. 3.2A); however, there was a marked increase in the proportion of DHA (~2-10x; Fig. 3.2B) in the scallop tissues of both developmental stages. This was especially pronounced in juveniles fed the *T. striata* / CHGRA diet low in DHA and those fed *P. lutheri* / *F. familica*. This enrichment was not observed for the other long-chain n-3

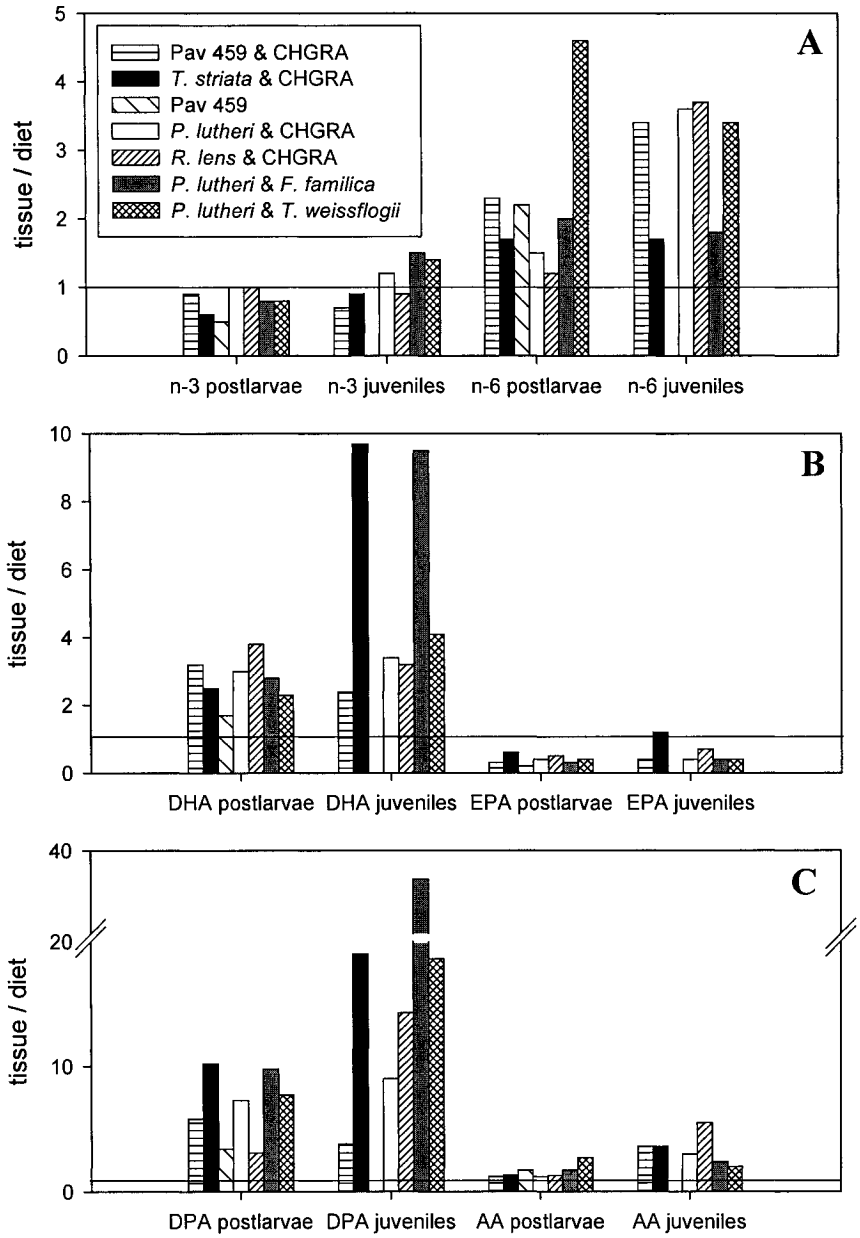

Figure 3.2: Relative proportions of fatty acids found in postlarval and juvenile bay scallop (*A. irradians*) tissues relative to those provided by the diet. Data are presented as a % of total fatty acids, values >1 indicate enrichment in the tissues relative to the diet. (A) Total n-3 and Total n-6; (B) individual n-3 fatty acids DHA (22:6n-3) and EPA (20:5n-3); (C) individual n-6 fatty acids DPA (22:5n-6) and AA (20:4n-6). Juvenile relationships based on corresponding juvenile diet data (not presented).

PUFA, EPA, which showed a relative decrease in relation to the diets provided. As a result, the DHA/EPA ratios are much higher in scallop tissues than what was provided by any of the diets, regardless of developmental stage (Fig. 3.3B). Enrichment of Σn-6 fatty acids in scallop tissues was observed across diet treatments and ontogenetic stages, with tissue values of Σn-6 fatty acids ~1.2-4.6x greater than what was provided by the diet (Fig. 3.2A). Proportions of the n-6 AA and DPA in tissues of both postlarvae and juveniles increased dramatically from those in the diet, especially DPA that increased ~35x in postlarvae fed *P. lutheri* / *F. familica* (Fig. 3.2C).

A comparison of slopes from linear regressions fitted to percent fatty acids of scallop tissues vs. microalgal diets for each of the four key PUFA indicated that in both postlarvae and juveniles, DHA and n-6 DPA increased with a steeper slope than either AA or EPA (illustrated for postlarvae in Fig. 3.4). In fact, EPA in juvenile tissues showed a negative slope relative to its proportion in the diet. The same pattern was observed when absolute PUFA concentrations (mg g $AFDW^{-1}$) were plotted for both postlarvae and juveniles.

3.4 Discussion

3.4.1 Effect of Microalgal Diets on Scallop Growth

Little difference in diet ranking was observed between postlarval and juvenile scallops, suggesting that these two developmental stages have comparable nutritional requirements. This similarity in growth performance also suggests that particles were handled, ingested and assimilated in the same manner at these two stages, as preferential selection for one algal species over another would likely elicit a difference in growth response. This is somewhat unexpected, as the two scallop developmental stages have markedly different gill morphologies which would be expected to influence particle handling (Barré, 2001). Adult and large juvenile scallops only capture particles >5-7 μm with 100% efficiency (MacDonald *et al.*, 2006). Several of the algal species used in this study averaged ~ 4-5 μm, falling below this size threshold, yet *A. irradians* was shown to capture 4 μm particles with relatively high (~ 82%) efficiency (Palmer and Williams, 1980). Postlarval scallops (<1 mm) have been shown to readily ingest smaller (~2 to 6

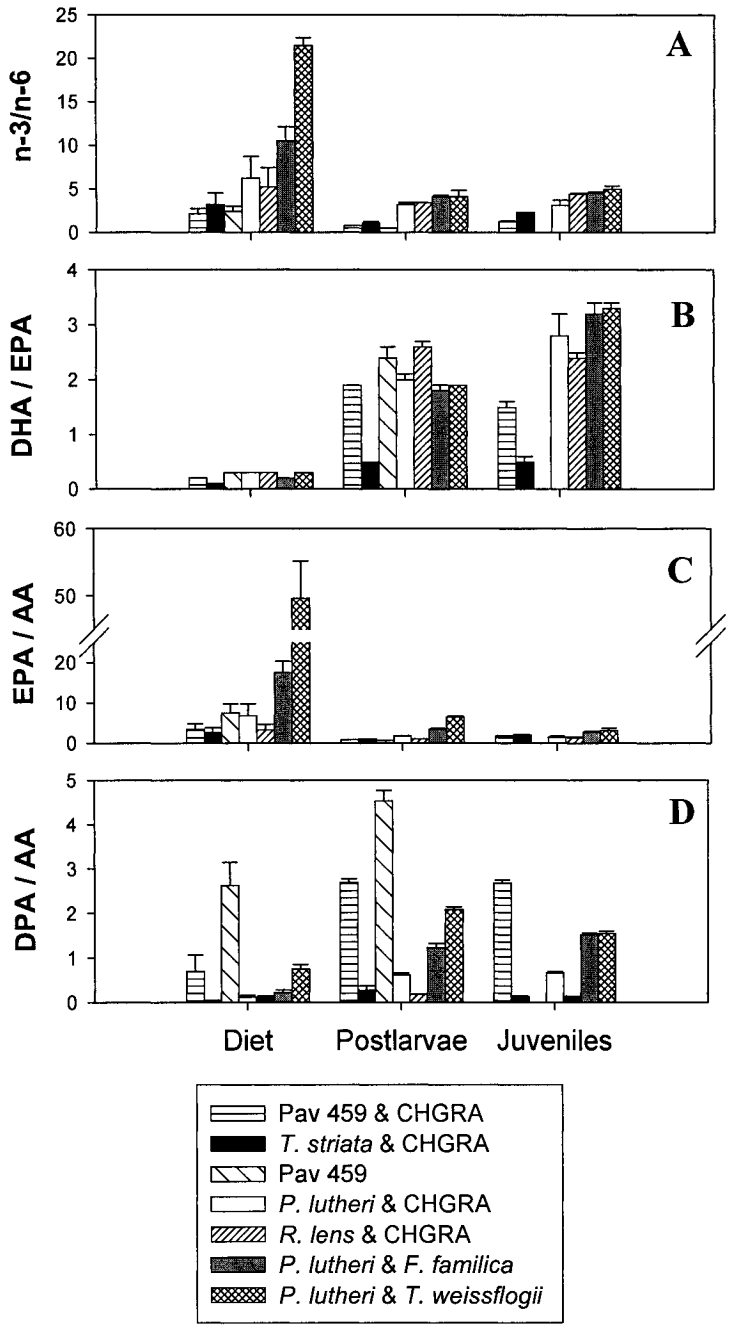

Figure 3.3: Comparison of ratios of (A) Total n-3 / Total n-6 fatty acids, (B) docosahexaenoic acid (DHA; 22:6n-3) / eicosapentaenoic acid (EPA; 20:5n-3), (C) EPA/ arachidonic acid (AA; 20:4n-6) and (D) 4,7,10.13,16-docosapentaenoic acid (n-6 DPA; 22:5n-6) / AA in experimental diets, postlarval and juvenile bay scallop (*A. irradians*) tissues. Values for diets represent a mean of weekly samplings, and tissue values are from scallops after 3 weeks of exposure to experimental diets. Error bars represent mean ± SE.

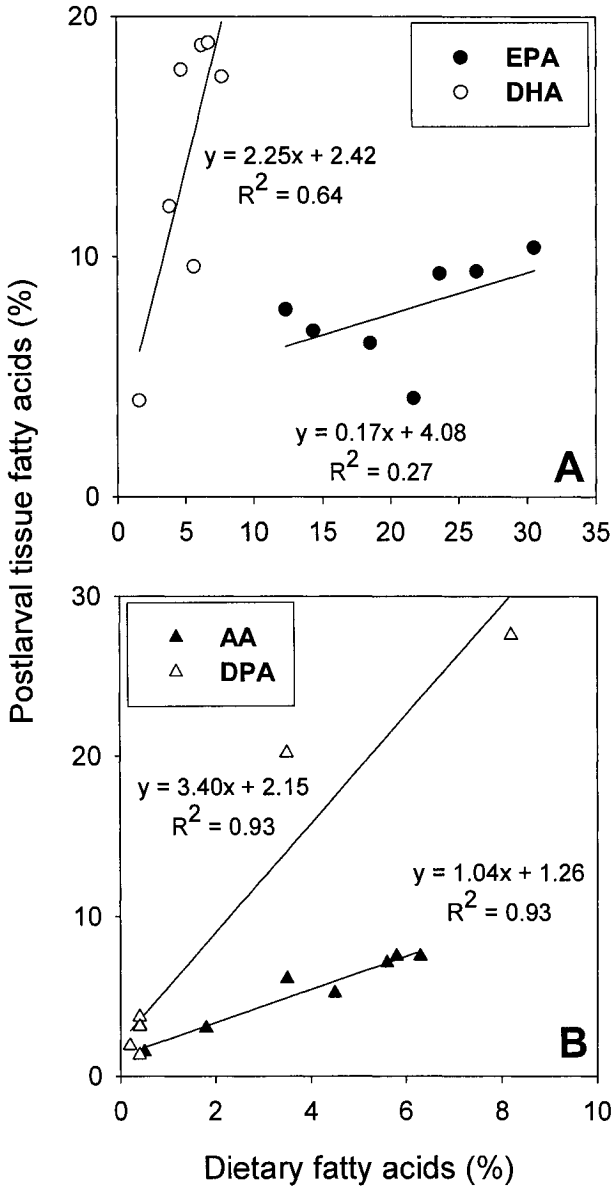

Figure 3.4: Linear relationships between mean proportions of selected PUFA in postlarval tissues relative to that provided in the diet for (A) n-3 fatty acids EPA (20:5n-3) and DHA (22:6n-3) and (B) n-6 fatty acids AA (20:4n-6) and DPA (22:5n-6) ; all correlations are significant ($P<0.05$) except for EPA where $P=0.23$. Error bars are not shown but are included in Tables 3 and 4. A similar pattern was observed for juveniles (not shown).

μm) particles, but ineffectively capture ≥20 μm particles (Kingzett, 1993; Anderson, 2003). Flow cytometric analysis indicated that *A. irradians* (1.2-1.6 mm) showed no differential retention for algal species ranging between 2 and ~10 μm in a mixed suspension (Shumway *et al.*, 1997). Therefore, although size-selectivity was not directly measured in this study and cannot be excluded, it is not expected to play an important role in explaining the differences in growth observed over the size range of algal species tested (mean ESD: 4 to 12 μm).

The present study identifies the Pav 459 / CHGRA diet as a superior binary diet for bay scallops, yielding postlarval and juvenile growth rates at 20°C similar to those of *A. irradians concentricus* fed unialgal *I. galbana* (T-iso) at 25°C and 50 cells μl^{-1} (Lu and Blake, 1996), and greater than growth rates of field-deployed *A. irradians* juveniles ~34 mm in SH (Shriver *et al.*, 2002). Superior growth has also been reported for *P. magellanicus* larvae (Pernet *et al.*, 2005) and postlarvae (Milke *et al.*, 2004) fed the Pav 459 / CHGRA diet, and a unialgal Pav 459 diet has yielded higher growth rates than unialgal diets of *P. lutheri* and *Isochrysis* sp. (strain T- iso) in *P. magellanicus* larvae (Feindel, 2000). Both components of the Pav 459 / CHGRA diet likely contribute substantially to the high growth rates of scallops on this diet, as removal of CHGRA from this diet reduced sea scallop postlarval growth rates by 31%; whereas, a unialgal diet of CHGRA was suboptimal, resulting in a dramatic drop in shell growth after ~2 weeks of exposure (Milke *et al.*, 2004). The success of the Pav 459 / CHGRA combination diet for both sea scallops and bay scallops may be attributable to the high content of n-6 PUFAs, specifically DPA and AA provided primarily by Pav 459 and CHGRA, respectively.

CHGRA was absent from the two diets that yielded lowest growth rates, *P. lutheri* / *F. familica* and *P. lutheri* / *T. weissflogii*, and is characterized by particularly high proportions of carbohydrate (Ben-Amotz *et al.*, 1987; Milke, unpub. data) and cholesterol (Tsita-Tzardis *et al.*, 1993), both of which may be positively linked with bivalve growth and nutritional condition (Enright *et al.*, 1986; Whyte *et al.*, 1990b; Soudant *et al.*, 1998a). Other studies, however, have shown no correlation between dietary carbohydrate and growth performance of bivalves such as *Mercenaria mercenaria* (Wikfors *et al.*, 1992). As carbohydrate comprises a relatively minor proportion of total organic matter in postlarval scallops (7-14% of organic weight in *A. purpuratus*; Farias *et al.*, 1998) and

was shown to be of limited importance as an energy reserve in adult *A. irradians* (Epp *et al.*, 1988), it was not investigated in this study, although it cannot be ruled out as a contributing factor to scallop growth performance. Although a positive correlation between dietary sterol concentrations and shell growth rate was found in postlarval sea scallops (Milke *et al.*, 2004), this relationship was not observed with bay scallops in the current study. Sterols play a structural role in membranes (Nes, 1974), and sea scallops may have more stringent requirements in order to maintain membrane fluidity in the colder waters to which they are adapted.

The *P. lutheri* / *F. familica* diet yielded low growth rates in bay scallops, although it yielded the second best growth in sea scallop postlarvae (Milke *et al.*, 2004). We speculate that the success of this diet for *P. magellanicus* may be related to the sticky nature of *F. familica* cells and the sea scallops' protracted development (Veniot *et al.*, 2003), such that postlarvae may utilize this alga retained on the Nitex screen of downwellers using pedal feeding until gills are fully functional. As development is much faster in *A. irradians* (Barré, 2001), food supplementation by pedal feeding on settled algae may only play a limited role in this species. In contrast, *T. striata* / CHGRA supported the lowest growth rates in sea scallops (Milke *et al.*, 2004), yet was the second best diet for bay scallops. Scallop larvae (*Pecten maximus*) are unable to digest *Tetraselmis suecica* (Le Pennec and Rangel-Davalos, 1985) and this may be responsible for the diet's poor performance in sea scallops. However, digestibility of *T. striata* may vary among scallop species, as it was associated with the second highest growth rate in bay scallops in the present study. Differential digestibility, although not investigated here, may be a contributing factor to the variable success of the diets tested. A differential need for long chain PUFA, associated with the species' different temperature environments, may also be responsible for the disparity in growth responses to *Tetraselmis* spp. between the two scallop species.

3.4.2 n-3 Fatty Acids

The nutritional importance of n-3 fatty acids, especially EPA and DHA, has been well established for bivalves (Langdon and Waldock, 1981; Marty *et al.*, 1992). In the present study, we observed substantial enrichment of DHA, but a decrease in EPA levels

in scallop tissues relative to what was provided by the diets, regardless of developmental stage or dietary treatment. This enrichment pattern was also observed in *P. magellanicus* larvae (Pernet and Tremblay, 2004; Pernet *et al.*, 2005) and postlarvae (Milke *et al.*, 2004) and in *A. purpuratus* larvae (Nevejan *et al.*, 2003b), and suggests a high requirement for DHA over EPA in scallops. It appears that in scallop larvae, EPA is used as an energetic substrate, while DHA is conserved in tissues (Whyte *et al.*, 1990a; Whyte *et al.*, 1991; Marty *et al.*, 1992).

In *P. magellanicus* postlarvae, the need for DHA may outweigh that for EPA, thus contributing to the poor performance of sea scallops fed the DHA-limited *T. striata* / CHGRA diet (Milke *et al.*, 2004). However, postlarval and juvenile bay scallops fed this diet in the present study attained growth rates second only to the highest ranking Pav 459 / CHGRA combination. These two scallop species fed the *T. striata* / CHGRA diet also incorporated DHA differently. Tissue levels of DHA in bay scallop postlarvae were 4% of total fatty acids, but sea scallops contained much higher levels of DHA (14%), even though the DHA dietary inputs were comparable (1.6 vs. 1.1%), while DHA levels remained similar between bay and sea scallops for all other dietary treatments. In *Crassostrea gigas* spat, it has been proposed that either EPA or DHA, but not necessarily both, is required to support growth (Langdon and Waldock, 1981), and a unialgal diet of *T. striata* (Plat-P) supported high growth of postlarval oysters, *C. virginica* (Wikfors *et al.*, 1996), a result attributed to the alga's high level of EPA as it contains non-detectable or low levels of DHA. Our findings thus support the theory that dietary requirements for DHA and EPA are species-specific, and differential growth and DHA incorporation between bay and sea scallops indicate a much greater DHA requirement in sea scallops, perhaps to maintain membrane fluidity at lower ambient temperatures. In the gill tissue of adult sea scallops, however, it has been demonstrated that EPA, not DHA, is correlated with temperature-induced restructuring of membranes (Hall *et al.*, 2002).

Interestingly, the Pav 459 / CHGRA and *T. striata* / CHGRA diets that promoted highest growth contained the lowest levels of DHA (1.6-3.8%), but all other diets contained higher levels (5-8%), that were subsequently reflected in tissues of animals fed these diets, yielding levels of ~18 and 23-27% in postlarval and juvenile tissues, respectively. In the prawn, *Penaeus monodon*, levels of both DHA and EPA exceeding a

certain threshold, were detrimental to growth performance, and an optimal combination of 4% each DHA and EPA, when both are offered, was proposed (Glencross and Smith, 2001). These authors suggest that this may not be a direct result of either dietary DHA or EPA, but rather their contribution to the Σn-3 pool may be detrimental, as a negative growth effect was observed for diets with Σn-3 fatty acids greater than 35% of total fatty acids (Glencross *et al.*, 2002), a level which our two lowest performing diets, *P. lutheri* / *F. familica* and *P. lutheri* / *T. weissflogii*, exceed.

3.4.3 n-6 Fatty Acids

The Pav 459 / CHGRA diet was, by far, the most successful diet for both stages of bay scallops in this study, as well as for sea scallop postlarvae (Milke *et al.*, 2004). In all cases this diet yielded growth rates 24-65% greater than the next highest-performing diet. The most striking biochemical characteristics of this binary diet are the high levels of Σn-6 fatty acids, particularly DPA and AA provided by Pav 459 (8.2%) and CHGRA (8.2%), respectively. Furthermore, the two lowest-performing diets, *P. lutheri* / *F. familica* and *P. lutheri* / *T. weissflogii*, contained the lowest levels of Σn-6 fatty acids, suggesting a requirement for n-6 fatty acids to support high growth rates. A ~2-4 fold increase in Σn-6 fatty acids was observed in bay scallop tissues relative to what was provided by the diet, regardless of developmental stage and dietary treatment (Fig. 3.2A), whereas Σn-3 levels in tissues either decreased or remained unchanged relative to the diet. A relative increase of n-6 DPA and AA levels in bay scallop tissues was also observed, with exceptionally high enrichment (3-38x) observed for DPA. Enrichment of this PUFA has also been reported for sea scallop larvae (Pernet *et al.*, 2005) and postlarvae (Milke *et al.*, 2004), in polar lipids of *Pecten maximus* larvae (Delaunay *et al.*, 1993), and gonads of female *P. maximus* (Soudant *et al.*, 1996a), and it has been proposed that n-6 DPA may play a structural role in maintaining membrane structure and fluidity (Soudant *et al.*, 1996a). Compared with EPA and AA, DHA and DPA absolute concentrations and relative proportions increased with a steeper slope in scallop tissues relative to their values in the diets. Thus, both larvae and postlarvae preferentially incorporated DHA and n-6 DPA from the diet relative to EPA and AA across all levels supplied, suggesting a greater nutritional requirement for the former two PUFA in both scallop developmental stages.

As this study analyzed fatty acids in bulk lipid samples, future work is necessary to ascertain whether these PUFA are being selectively incorporated into neutral (storage) or polar (structural) lipid fractions, as this would help further elucidate their physiological role.

Although properties such as carbohydrate and sterol composition may be contributing to dietary success (discussed above), high proportions of AA (~6%) occur in the two diets that resulted in highest growth, whereas low proportions (0.5%) characterize the lowest-ranking diet, indicating that dietary AA levels may also influence growth performance. Arachidonic acid influences immune function in bivalves (Delaporte *et al.*, 2003), is a precursor to prostaglandin production influencing functions such as egg production, spawning, and ion regulation in molluscs (Freas and Grollman, 1980, Stanley-Samuelson, 1987, Deridovich and Reunova, 1993), and therefore may play an integral role in molluscan health. While both EPA and AA are precursors to eicosanoids, those produced from AA are more biologically active. Excess levels of AA relative to EPA were deleterious to finfish larvae, leading to the suggestion of a minimum recommended EPA/AA ratio of 4.2 (Sargent *et al.*, 1999b). This ratio was greatly exceeded by our two lowest-ranking diets (17.6 and 49.7), whereas it fell below the recommended value in our two highest-ranking diets (2.7 and 3.5).

3.4.4 Fatty Acid Ratios

Total dietary n-3/n-6 ratios varied from 2 to 22, with higher values corresponding to the diets that supported lowest growth rates (Fig. 3.3A). Values in bay scallop tissues however, exhibited a much lower and narrower range across dietary treatments, 0.5-4 in postlarvae and 1.2-5 in juveniles. *Pecten maximus* larvae showed a similar trend, wherein there was an increase in the n-6/n-3 ratio of polar lipids related to an increase in the diet (Marty *et al.*, 1992). Our three highest-performing diets had n-3/n-6 ratios ranging from 2.1-3.2, and algal diets that were low (2-3) in this ratio have been considered 'good' for oyster larvae (Webb and Chu, 1983). Furthermore, the optimum dietary ratio for the prawn *P. monodon* was 2.5 (Glencross *et al.*, 2002), similar to the values of our highest-performing diets. The high n-3/n-6 ratios of the two lowest

performing diets are related to the absence of CHGRA, which provided a large proportion of AA in the other binary diets.

In all cases we observe a multi-fold increase in the DHA/EPA ratio of bay scallop tissues relative to that provided in the diet, again indicating preferential sequestering (DHA) and/or utilization (EPA) of these compounds. As DHA may play a structural role in the membranes of bay scallops, EPA might be preferentially catabolized, which would account for the increase in the DHA/EPA ratios of bay scallop tissues. Alternatively, the n-6 DPA/AA ratios largely reflect dietary proportions, with DPA-containing diets yielding the highest DPA/AA ratios in scallop tissues. DPA/AA ratios, however, are generally higher in scallop tissues than their corresponding diets. Although the functional role of n-6 DPA remains largely unknown, and it is not a PUFA commonly reported in natural phytoplankton assemblages, it appears to have a positive effect on growth performance of early life history stages of bay scallops and sea scallops, suggesting a need for n-6 fatty acids in pectinids, that has been frequently overlooked in studies of bivalve nutrition. More targeted supplementation trials are necessary to elucidate both the relative influence, and physiological role of n-6 fatty acids on scallop growth.

3.5 Conclusions

A superior diet of Pav 459 / CHGRA has been identified that promotes excellent growth of bay scallops in both postlarval and juvenile stages. In contrast, *P. lutheri* / *F. familica* and *P. lutheri* / *T. weissflogii* diets were unable to maintain high growth rates in bay scallops. There was an overall enrichment of total n-6 fatty acids, as well as of the individual n-6 fatty acids AA and DPA, in scallop tissues relative to the diet irrespective of developmental stage or dietary treatment. Although no overall enrichment of Σn-3 fatty acids was observed, there was an enrichment of DHA relative to the diet. This points to selective retention of these particular fatty acids, or selective catabolism of other dietary fatty acids leaving these in excess in scallop tissues. Our results suggest that bay scallops have less stringent requirements for DHA than sea scallops and that n-6 fatty

acids, particularly AA and DPA, may be essential to support high growth rates in pectinids.

3.6 Acknowledgments

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Chapter 4: Evaluation of Three *Pavlova* spp. and *Chaetoceros muelleri* for the Nutrition of Postlarval Sea Scallops, *Placopecten magellanicus*

Abstract

Low growth rates and survivorship of post-metamorphic sea scallops, *Placopecten magellanicus*, are often observed in hatcheries as scallops undergo a period of substantial morphogenesis and thus may be particularly susceptible to nutritional deficiencies. Diets containing flagellates from the genus *Pavlova* (especially *Pavlova* sp. CCMP 459) have proven successful for scallop rearing in previous studies. To further examine their contribution to growth performance, *Pavlova sp.* (CCMP 459) and *Pavlova pinguis*, both as unialgal diets and in combination with the diatom *Chaetoceros muelleri* (CHGRA), were tested as diets for postlarval (~350 μm and 1.6 mm) sea scallops in two growth trials each lasting ~4 weeks. No difference in growth performance was observed among scallops fed the Pav 459- and *P. pinguis*-CHGRA combination diets, although previous work has highlighted substantial decreases in growth rate (31%) when another algal species of the same genus, *Pavlova lutheri*, was substituted for Pav 459 in the combination diet. A unialgal diet of *P. pinguis* yielded growth rates 32% lower than the combination, and unialgal diets of CHGRA consistently ranked lowest of all diets tested (54 - 65% lower than combination), while unialgal Pav 459 showed variable results between the two trials. Algal sterol composition may influence growth performance, as CHGRA contains high levels of cholesterol, also present although to a lesser extent in Pav 459 and *P. pinguis*, but only at trace levels in *P. lutheri*. *Pavlova pinguis*, Pav 459, and CHGRA are also all characterized by relatively high levels of n-6 polyunsaturated fatty acids (PUFAs). Whereas Pav 459 and *P. pinguis* (in contrast to *P. lutheri*) are characterized by high levels of docosapentaenoic acid (DPA; 22:5n-6), CHGRA contains high levels of arachidonic acid (AA; 20:4n-6) which may account for high growth rates obtained on this mixed diet. Enrichment of Σn-6 fatty acids, as well as the individual n-6 fatty acids AA and DPA, was observed in tissues relative to the diet in both scallop species and across dietary treatments whereas no enrichment of Σn-3 fatty acids was observed. This provides evidence of selective uptake, retention and/or metabolism of n-6 fatty acids and suggests an important role for n-6 fatty acids in young pectinids.

4.1 Introduction

Placopecten magellanicus, commonly known as the sea or giant scallop, sustains a substantial fishery accounting for more than 50% of all scallop landings worldwide (Halvorson *et al.*, 1995). As this species commands a high market value, a successful culture industry is possible, although it is largely limited by a reliable and affordable supply of postlarvae (spat). Field collections relying on natural settlement are often unpredictable (Dadswell and Parsons, 1992) and sea scallops can be difficult to rear in a hatchery setting, with the highest mortalities observed in postlarval stages (Couturier *et al.*, 1995). These stages are marked by profound and protracted morphogenesis of feeding organs (Veniot *et al.*, 2003), and as such, sea scallops may have stage-specific nutritional requirements, that make them particularly vulnerable if these requirements are not met. The nutritional needs of postlarval scallops are poorly understood, and commercial hatcheries often feed multi-species microalgal diets to ensure adequate survival and growth performance. However, the cost of algal culture is substantial (~30% of total operational hatchery costs; Coutteau and Sorgeloos, 1993) and maintaining multiple algal species may be prohibitively expensive, especially for a species such as *P. magellanicus*, which due to slow growth has a prolonged nursery phase prior to field deployment.

While recent work has examined the dietary requirements of pectinids for broodstock conditioning and larval rearing (Caers *et al.*, 2003; Nevejan *et al.*, 2003b; Pernet *et al.*, 2006), cost-effective diets that yield high growth and survival for commercial rearing postlarvae remain to be established. However, diets including *Pavlova* sp. (CCMP strain 459; Center for the Culture of Marine Phytoplankton strain 459; referred to as Pav 459) have shown promise for *P. magellanicus* larvae (Feindel, 2000; Pernet *et al.*, 2005) and postlarvae (Milke *et al.*, 2004), as well as *Argopecten irradians* larvae (Alix *et al.*, 1997), postlarvae and juveniles (Milke *et al.*, 2006). Pav 459 has outperformed the more commonly used *Pavlova lutheri* in several of these studies, and the addition of the diatom *Chaetoceros muelleri* to a diet of Pav 459 substantially improved growth performance (Milke *et al.*, 2004; Pernet *et al.*, 2005; Milke *et al.*, 2006). Both Pav 459 and CHGRA are characterized by high levels of specific n-6 polyunsaturated fatty acids (PUFA), which may contribute to the success of this

combination diet. Unfortunately, Pav 459 has proved difficult to culture in large scale (unpublished results), which compromises its suitability for commercial applications.

Dietary success is dependant on algal biochemical composition, although it is unlikely that gross biochemical or amino acid composition are responsible for differential growth performance in bivalves (reviewed by Brown and Jeffrey, 1992). However, numerous studies have highlighted the importance of sterols, such as cholesterol (Wikfors *et al.*, 1991; Wikfors *et al.*, 1996), and fatty acid composition, especially of polyunsaturated fatty acids (PUFAs), in supporting bivalve growth (Brown *et al.*, 1989; Delaunay *et al.*, 1993; Caers *et al.*, 2000). The requirement for n-3 fatty acids, particularly docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), has been demonstrated for several bivalve species (Langdon and Waldock, 1981; Marty *et al.*, 1992; Hall *et al.*, 2002). Although n-6 fatty acids have received much less attention, it appears that they may also be beneficial for Pectinids (Marty *et al.*, 1992; Feindel, 2000; Pernet *et al.*, 2005; Milke *et al.*, 2006). Bivalves have a limited ability to synthesize long chain (20-22 carbon) PUFAs from shorter chain precursors (Langdon and Waldock, 1981; Chu and Greaves, 1991; Delaunay *et al.*, 1993), and are thus reliant on dietary inputs to meet their requirements for these fatty acids. Likewise, bivalves are generally considered unable to synthesize cholesterol from simple precursors (Holden and Patterson, 1991), suggesting that dietary sterol composition can markedly influence bivalve growth performance.

The superior growth performance of sea scallops fed diets containing Pav 459 suggests that other *Pavlova* species (Class Prymnesiophyceae, Pavlovophyceae) may elicit a similar growth response, but be more suitable for large-scale culture. Therefore, the goals of this study were to 1) test the suitability of an alternate *Pavlova* sp., *Pavlova pinguis,* to support postlarval scallop growth 2) examine the nutritional value of *Pavlova* spp. in both unialgal and binary diets with *Chaetoceros muelleri* to help identify the contribution of each algal species to scallop growth, and 3) compare with previous work on growth of sea scallop postlarvae offered two of these *Pavlova* species (Milke *et al.*, 2004). Special attention was paid to n-3 and n-6 PUFAs both in the diets and scallop tissues, given their previously demonstrated significance in bivalve nutrition.

4.2 Materials and Methods

4.2.1 Experimental Animals

Scallops for Trial I were obtained from the Massachusetts Maritime Academy, Buzzards Bay, MA, and were transported to the National Research Council's (NRC) Marine Research Station (MRS) where they were transferred to a 700-l recirculating seawater system at 14 °C. They were reared on a mixed algal diet for ~2.5 months prior to the start of the experiment. Mean initial shell height (SH) for scallops in Trial I was 1.6 mm. Scallops for Trial II (mean initial SH 350 μm) were air-shipped within the same day from PecNord, Québec, to Halifax, Nova Scotia (NS), Canada, in plastic containers filled with chilled seawater. Animals were transferred to recirculating systems and were provided a mixed algal diet for two days before transferring to experimental diets.

4.2.2 Experimental Systems

Scallops were held for 29 (Trial I; March 12th to April 9th, 2003) or 30 (Trial II; August 21st to September 19th, 2002) days in 400-l recirculating downweller systems filled with 0.22 μm-cartridge filtered seawater. Experimental systems were located in a temperature-controlled chamber maintained at 14 °C and consisted of a 50-l upper tank containing 2 (Trial II) or 3 (Trial I) identical downwellers 15.2 cm in diameter and 40.6 cm in height with a Nitex mesh base of 140 or 600 μm (Trials II and I, respectively), and a lower reservoir of 350-l. A detailed description of these systems can be found in Milke *et al.* (2004).

In Trial I ~130,000 scallops were divided equally by volume among 6 tanks and in Trial II, ~32,000 scallops were divided among 4 tanks yielding initial stocking densities of ~ 40 and 22 scallops cm^{-2} respectively. Tanks were cleaned twice weekly with hot fresh water and downwellers were rinsed with 14 °C filtered seawater. Tank replication was not possible due to the limited number of animals available and scale of experiments, although identical conditions were maintained between both experiments and tanks have shown excellent reproducibility between previous sequential growth trials (Milke *et al.*, 2004).

4.2.3 Microalgal Diets

Stock cultures were obtained from the Center for the Culture of Marine Phytoplankton (CCMP), West Boothbay Harbor, ME and included the flagellates *Pavlova* sp. (Pav 459; CCMP 459) and *Pavlova pinguis* (CCMP 609) as well as the diatom *Chaetoceros muelleri* (CHGRA; CCMP 1316). Microalgae were grown on 24 h light with f/2 media (-Si for flagellates; Guillard, 1975) either in batch culture in 20-l carboys (Pav 459, *P. pinguis* Trial II), or in semi-continuous culture in 200-l bioreactors (*P. pinguis* Trial I, CHGRA) at 21 °C for both *Pavlova* species and 18 °C for CHGRA. CO_2 was delivered to all cultures through the air line and was regulated by a pH controller (Cole-Parmer model P-05656-00) to maintain culture pH between 7.8 and 8.3. Cultures were harvested during the late exponential phase.

Scallops were offered diets at a concentration equivalent in volume to 50 cells μl^{-1} of *Isochrysis galbana* strain T-iso (2.15 x 10^6 μm^3 ml^{-1}), which was found to be optimal for growth of juvenile (0.5-5 mm) bay scallops, *Argopecten irradians* (Lu and Blake, 1996). In Trial II, four different diets were offered: binary diets (50% / 50% by volume) of Pav 459/CHGRA and *P. pinguis*/CHGRA and unialgal diets of Pav 459 and CHGRA. In Trial I, the same 4 diets were offered as well as a 25% / 75% binary diet of Pav 459/CHGRA and a unialgal diet of *P. pinguis*. The Pav 459 / CHGRA combination served as a 'reference diet' to which all other diets were compared. Algal concentrations were monitored daily using a Multisizer 3 Coulter Counter (Beckman Coulter, Fullerton California) equipped with a 100 μm aperture and maintained within 20% of the desired suspended concentration by daily batch addition, or continuous supplementation via a peristaltic pump (Cole Parmer Masterflex 7554-80) when necessary.

4.2.4 Growth and Survival

A subsample of animals was removed weekly from each downweller for shell height and survival determinations, placed on a graduated Petri dish and scanned under a dissecting microscope coupled with an analog camera (Pulnix model TMC7-DSP). Video was recorded using a Sony DVCAM (DSR-V10) calibrated with a stage micrometer. Shell heights were determined digitally using Optimas 6.5 image analysis software (Media Cybernetics, Silver Spring, MD). Forty (Trial I) or fifty (Trial II) live

scallops were measured per downweller (triplicate samples in Trial I, duplicate samples in Trial II). Means from each downweller were averaged to obtain a treatment shell height for each sampling date. Cumulative mortalities were calculated by recording the number of live, 'new' and 'old' dead scallops from video observation. No dead animals were removed or culled during the experiment. A total of 100 scallops per downweller were counted; % survival per downweller was calculated and averaged to obtain treatment mortality (in Trial II, two counts of 100 were conducted per downweller, then averaged). 'New' dead scallops were similar in size to live scallops but showed partial degradation of tissues while 'old' dead scallops were clearly smaller in size and contained no residual tissue.

Initial and final scallop biomass was determined only for Trial I due to the limited biomass of animals available in Trial II. Prior to sampling for weights and biochemical composition, scallops were placed overnight in 0.22 μm filtered seawater to purge gut contents. Scallops were then filtered (0.1 ml; 19-66 live individuals, depending on sampling date and treatment) onto pre-combusted, pre-weighed Whatman GF/C filters. Filters were rinsed with 2 ml of 3.4% ammonium formate to remove salts, and oven-dried at 80 °C for 24 h. Ash weight was determined after combustion at 480 °C for 24 h. The number of animals per sample was determined microscopically in order to calculate individual dry and organic weights (ash-free dry weight, AFDW).

4.2.5 Lipid Analysis

Triplicate initial and final scallop samples of 2-4.5 ml from Trial I (one sample per downweller, per treatment for final sampling) and all remaining animals at the end of Trial II (67-283 per downweller) were placed into 15 ml glass tubes pre-rinsed with methanol and chloroform. Excess water was removed with a Pasteur pipette and 3 ml chloroform was added to each tube which was then purged with N_2 gas, immediately frozen in liquid nitrogen and stored at -80 °C until analysis. Algal samples (30-100 ml) were sampled weekly, centrifuged, and the supernatant was filtered onto pre-combusted Whatman GF/C filters, and both filter and algal paste were placed into a glass tube and treated as above. Multiple blanks were generated for both tissues and algae.

Algae and scallops were extracted following a modified Folch procedure (Folch *et al.*, 1957) using 2:1 chloroform-methanol as described by Parrish (1999). Extracts were stored at -80 °C under N_2 atmosphere until overnight shipment on dry ice to Memorial University of Newfoundland (MUN) for lipid analysis. Lipid classes were identified using a MARK V Iatroscan (Iatron Laboratories, Tokyo, Japan) using lipid standards (Sigma), and summed to calculate total lipid concentrations.

Bulk lipid extracts were derivatized for 1.5 h at 85 °C with 1.5 ml BF_3/MeOH, and resultant fatty acid methyl esters (FAME) were run on a Varian 3400 Gas Chromatograph with an Omagawax 320 column (Supelco, Bellefonte, PA). Peak detection was conducted at 200 °C and peaks were quantified with Varian Star Chromatography Software 4.02 using known standards (PUFA 1 and 37, Supelco Canada). Fatty acids for which standards were not available (e.g., 22:5n-6) were confirmed with a Varian Saturn mass spectrometer. The identity of 22:5n-6 was confirmed by careful reference to retention data in Ackman (1986) and to mass spectral patterns in Christie (2006).

For sterol analysis, methylated lipid extracts were trimethylsilylated with N,O-bis(trimethylsilyl)acetamide (BSA) and N,O-bis(trimethylsilyl)trifluoroacetamide (BSFA) for 15 min at 85°C. Peak identification was conducted on a Varian Saturn 3800 GC connected to a Varian 2000 MS with a Varian low-bleed CP sil-8 column. Peak identification was conducted with a combination of standards (Sigma) and published mass spectral data (Jones *et al.*, 1994). Peaks were quantified on a Hewlett Packard 6890 GC with FID using a SPB-5 silica capillary column.

4.2.6 Protein Analysis

Triplicate scallop samples of 1-2 ml packed volume (one sample per downweller during final sampling) were taken at the beginning and end of the growth trial; they were placed in cryovials, excess water was removed and scallops were frozen in liquid nitrogen. Samples were stored at -80 °C until analysis when they were lyophilized, powdered by crushing with a metal rod and weighed on a Cahn electrobalance. Samples were analyzed for total organic nitrogen on a CHN analyzer using acetanilide as standard. No scallop protein samples were taken in Trial II, due to insufficient final biomass. For

algal samples in both trials, 2-10 ml of culture were sampled weekly, filtered onto pre-combusted Whatman GF/C filters, dried at 80 °C for 24 h and stored in desiccant in a -20°C freezer. In all cases nitrogen values were converted to mg protein using a conversion factor of 5.8 (Gnaiger and Bitterlich, 1984).

4.2.7 Carbohydrate Analysis

Carbohydrate analysis was only conducted for algae and scallops in Trial I. Triplicate scallop samples of 2 ml packed volume were taken at the beginning and end of the growth trial, and 30-50 ml aliquots of each algal species were taken weekly, centrifuged and filtered onto Whatman GF/C filters. Samples were placed into glass tubes, and then frozen and lyophilized. Five ml of 1M sulfuric acid were added to all samples, which were then boiled 30 min for tissues or 3 h for algae (J. Craigie, pers. comm.). The homogenate was analyzed according to the phenol-sulfuric acid method (Dubois *et al.*, 1956) and read spectrophotometrically at 490 nm using D-glucose as standard.

4.2.8 Statistical Analysis

Differences in final shell heights and scallop biomass (DW and AFDW) were investigated using a one-way analysis of variance (ANOVA). Linear regressions were fitted to shell height data over time to determine scallop growth rates, and an analysis of covariance (ANCOVA) was conducted to examine growth rate differences among dietary treatments. A two-way ANOVA was used to examine weekly new mortality data with time and treatment as factors, and a one-way ANOVA was employed to examine cumulative mortalities at the end of the experiment. Biochemical properties reported as concentration data (i.e. total lipid, total protein, total fatty acid) were tested individually using one-way ANOVAs. A series of multivariate analysis of variance (MANOVA) models were used to determine differences among biochemical properties of algae (including *P. lutheri* data from Milke *et al.*, 2004) and scallop tissues reported as percent data. The MANOVA models tested included: 1) individual lipid classes, 2) individual PUFAs, 3) Sum of SAT, MUFA, PUFA 4) Sum of n-3 and n-6 fatty acids and 5) sterol data of algae only, including *P. lutheri* (unpub. data). When the MANOVA model was significant, subsequent one-way ANOVAs were conducted. All percent data were

arcsine transformed prior to analysis to normalize data, and all multiple comparisons were carried out using Tukey's multiple comparison test. A significance value of $P<0.05$ was accepted for all analyses unless otherwise noted. All tests were conducted using SYSTAT 10.0 (SPSS, Chicago, IL).

4.3 Results

4.3.1 Scallop Growth and Mortalities

After 4 weeks of feeding, the *P. pinguis* / CHGRA combination supported the highest growth in both experiments, yielding shell growth rates of 13.8 and 25.5 μm day^{-1} (Fig. 4.1A) and final shell heights of 1992 and 1092 μm (Table 4.1) in Trials I and II, respectively. These growth rates were not significantly different from those obtained for scallops offered the reference diet of Pav 459 / CHGRA in either Trial I or II (ANCOVA; $P<0.05$); yet a combination diet of *Pavlova lutheri* / CHGRA, a diet that differs from the Pav 459 / CHGRA reference diet by the substitution of only one species within the same genus, yielded growth rates significantly lower (31%) than this reference diet in a previous experiment (ANCOVA and Tukey's Test; $P<0.001$; Fig. 4.1B; Milke *et al.*, 2004).

A 100% Pav 459 diet yielded different results between the two trials as it resulted in the second highest growth rate, slightly higher than the Pav 459 / CHGRA diet in Trial I, but in Trial II it yielded a growth rate 32% lower than this reference diet (Fig. 4.1B). A reduction in the proportion of Pav 459 in the combination diet from 50% to 25% in Trial I lowered, though not significantly, the growth rate and final shell height of scallops (10.4 μm day^{-1} and 1879 μm, respectively), although interestingly these animals had the highest DW and AFDW (813.4 and 143.3 μg, respectively; Table 4.1). The unialgal *P. pinguis* diet yielded a lower growth rate and significantly lower final scallop SH than the binary *P. pinguis* / CHGRA combination. The 100% CHGRA diet was consistently the lowest performing diet tested, yielding growth rates 36-52% lower than the Pav 459 / CHGRA reference diet (Fig. 4.1B), the lowest final shell heights in both trials, and the lowest AFDW in Trial I (not tested in Trial II; Table 4.1). Overall, growth rates in Trial I

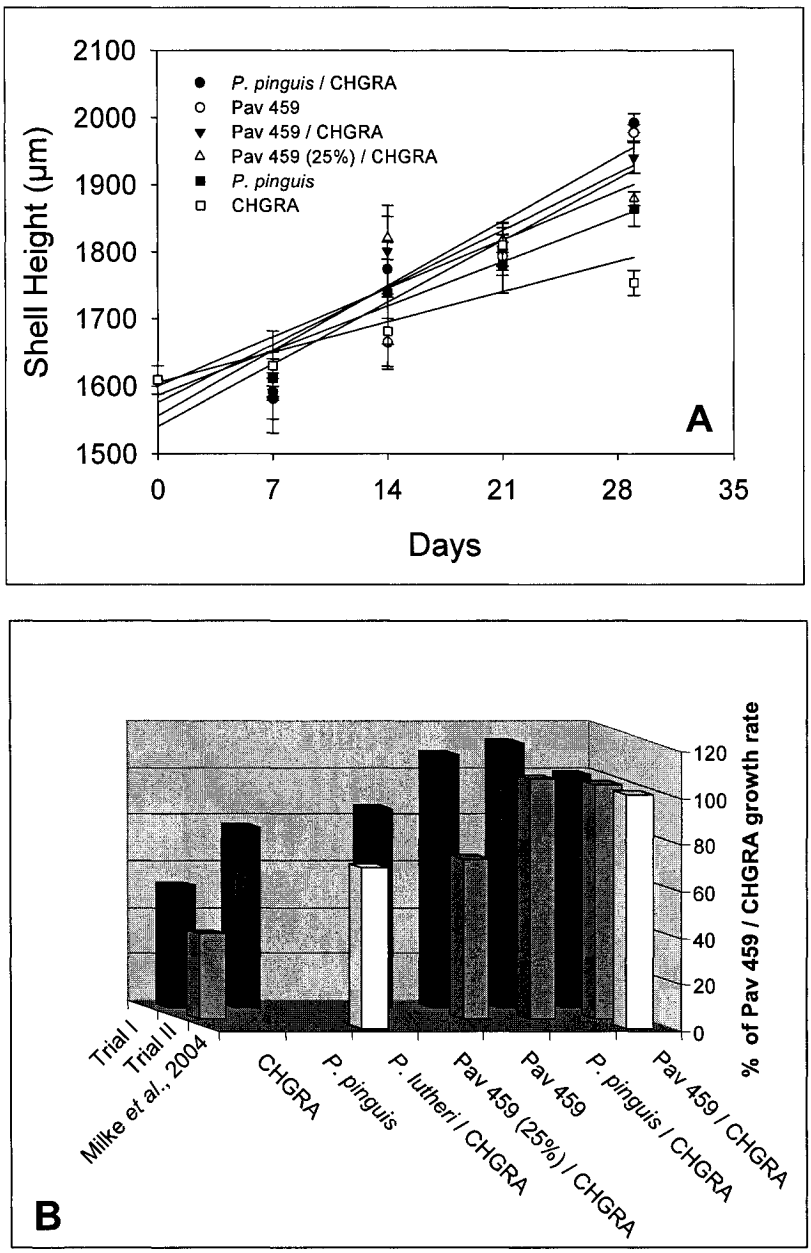

Figure 4.1: A) Growth trajectories of *P. magellanicus* exposed to different microalgal diets in Trial I. Values correspond to mean shell height ± SE; regression parameters are shown in Table 1. B) Relative shell growth rate performance of sea scallops fed *Pavlova* sp. and CHGRA containing diets using Pav 459/CHGRA as the reference. Trials I and II (present study) compared with published date (Milke et al., 2004).

(6.4-13.8μm day^{-1}) were much lower than those recorded for the same diets in Trial II (9.0-25.5 μm day^{-1}), especially for the higher-ranking diets (Table 4.1).

A two-way ANOVA on weekly new mortalities in Trial I identified a significant effect of diet ($P<0.05$); the multiple comparison test, however, was unable to detect individual differences. No effect of time or interaction between time and diet was observed, however, when examining cumulative mortalities. The unialgal diet of *P. pinguis* had significantly lower mortalities (11%) than the unialgal CHGRA diet (25%); all other diets were statistically similar (ANOVA; $P<0.05$). No difference in cumulative mortalities (9-15%; ANOVA; $P>0.05$) or weekly new mortalities due to diet (2.3-4.5%; $P>0.05$) were identified in Trial II, but a two-way ANOVA identified differences in weekly mortality ($P<0.001$), with highest losses incurred during the first week (8.2%) and the lowest during the final week (0.9%), and weeks two and three showing intermediate values (3.7 and 2.4%, respectively).

4.3.2 Microalgal Biochemical Composition

Pavlova pinguis and Pav 459 were similar in both cell size and biomass, but smaller than CHGRA, with values for equivalent spherical diameter (ESD), DW and AFDW as follows: Pav 459 (3.7 μm^3; 17.1 and 11.9 pg $cell^{-1}$), *P. pinguis* (3.7 μm^3; 18.2 and 12.8 pg $cell^{-1}$) and CHGRA (5.0 μm^3; 35.8 and 25.2 pg $cell^{-1}$). Mean densities obtained during the log phase in 20-l carboy cultures were 7.6 and 9.7 x 10^6 cells ml^{-1} for Pav 459 and *P. pinguis* in Trial II, respectively and densities of *P. lutheri* from 200-l bioreactors were 8.9 x10^6 cells ml^{-1}. There was no statistically significant difference in the gross biochemical composition of any of the tested algal species (lipid 195.4-223.6 mg g $AFDW^{-1}$; protein 449.8-487.0 mg g $AFDW^{-1}$; carbohydrate 107.1-141.0 mg g $AFDW^{-1}$; ANOVA; $P<0.05$). Levels of free fatty acids were low in all samples and ranged from 0.3-3.1%.

No difference was observed among phospholipid composition of the four algal species tested, although the proportion of triacylglycerol (TAG) was much higher in CHGRA than in *P. lutheri*, and sterol was significantly higher in the two *Pavlova* species used in Trials I and II (12.5 and 13.4% of total lipid; Fig. 4.2) than in CHGRA or *P. lutheri* (3.4 and 5.5 %; MANOVA; $P<0.05$). Marked differences in sterol class

Table 4.1: Scallop dry weight (DW), ash-free dry weight (AFDW) and mean shell height (SH) ± SE initially and after feeding on experimental diets for 29-30 days. Parameters for linear regression equations fitted to mean SH (y) vs. time (x) are also reported (y=bx+a); data for Trial I correspond to growth trajectories illustrated in Figure 1A. Different letters represent differences among final biomass (ANOVA), final SH (ANOVA) and regression slope (b; ANCOVA); $P<0.05$; no significant difference in slopes (b) was observed in Trial I.

Growth Parameters	Initial	Pav 459 (50%) CHGRA (50%)	*P. pinguis*(50%) CHGRA (50%)	Pav 459	Pav 459 (25%) CHGRA	*P. pinguis*	CHGRA
Trial I							
SH (μm)	1609±20	1940±71^{a}	1992±29^{a}	1977±27^{a}	1879±22^{ab}	1864±35^{ab}	1753±9^{b}
DW (μg scallop^{-1})	330.0±7.4	644.5±28.3^{ab}	600.4±19.5^{ab}	680.0±18.2^{ab}	813.4±91.5^{a}	555.2±17.1^{b}	710.2±20.4^{ab}
AFDW (μg scallop^{-1})	65.9±9.9	121.5±5.6^{ab}	112.4±5.4^{ab}	133.4±3.4^{ab}	143.3±16.8^{a}	102.0±5.3^{ab}	91.3±1.2^{b}
b	--	12.2	13.8	13.2	10.4	9.4	6.4
a	--	1575.1	1556.2	1540.4	1600.3	1587	1605.6
R^2	--	0.89	0.89	0.87	0.85	0.95	0.76
Trial II							
SH (μm)	353±9	1079±14^{a}	1092±62^{a}	848±1^{b}	--	--	623±20^{c}
b	--	24.9^{ab}	25.5^{a}	17.0^{bc}	--	--	9.0^{c}
a	--	303.6	317.9	334.9	--	--	392.6
R^2	--	0.98	0.99	0.99	--	--	0.88

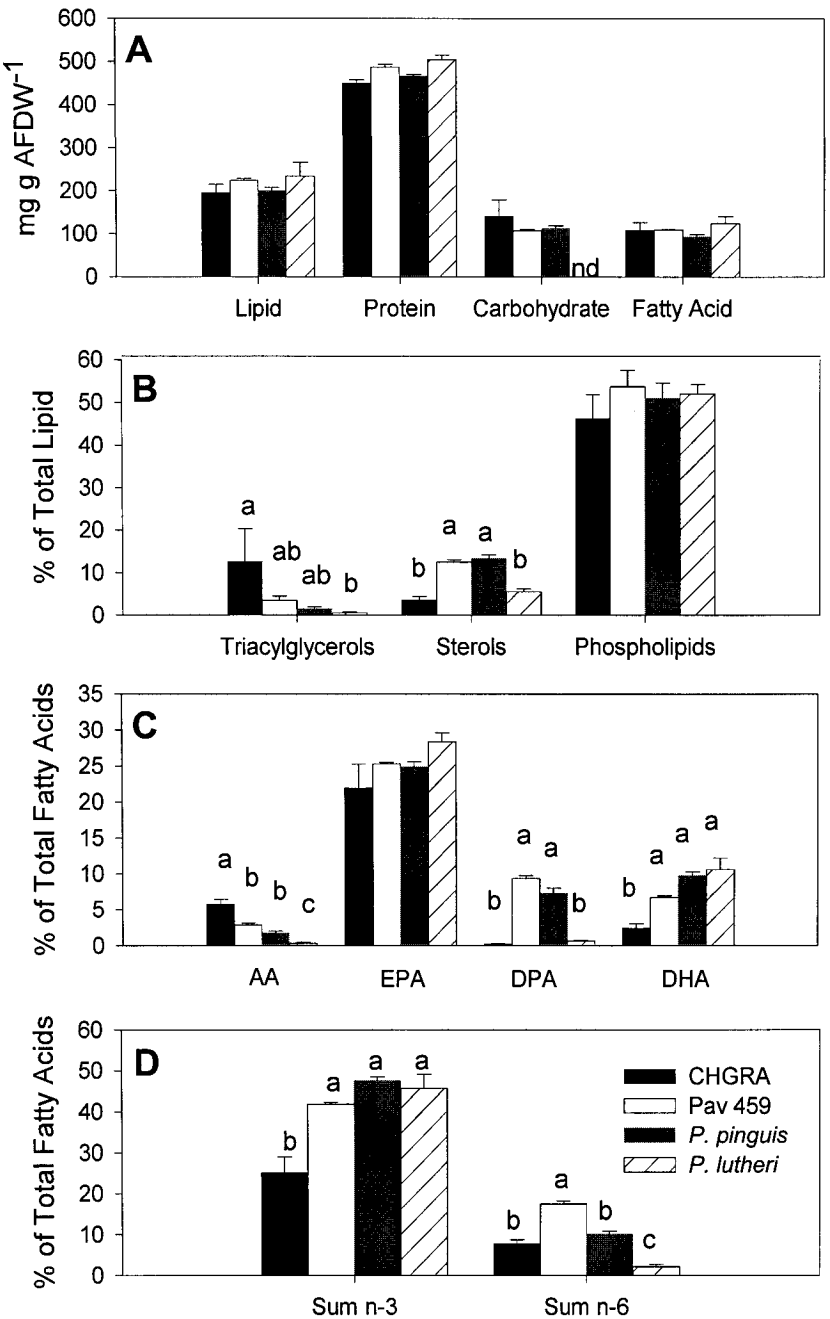

Figure 4.2: Biochemical composition of microalgae. A) gross biochemical composition, B) lipid classes, C) polyunsaturated fatty acids (AA, 20:4n-6, arachidonic acid; EPA, 20:5n-3, eicosapentaenoic acid; DPA, 22:5n-6, DHA, docosapentaenoic acid; 22:6n-3, docosahexaenoic acid), D) Total n-3 and n-6 fatty acids. Data for Pav 459, *P. pinguis* and CHGRA from Trial I, data for *P. lutheri* from Milke *et al.* (2004). Means ± SE calculated from weekly sampling (n=4). nd = not determined. Different letters indicate significant differences in the means; $P<0.05$.

composition were also identified among the algal species including *P. lutheri*. CHGRA was characterized by significantly higher proportions of cholesterol (35.6%) and isofucosterol (13.6%) as well as 24-methylenecholesterol (6.8%; MANOVA; $P<0.05$) while the *Pavlova* species, including *P. lutheri*, contained significantly higher levels of stigmasterol (27.9-37.5%) and dinosterol (9.5-22.5%; Fig. 4.3). Interestingly, significant differences in sterol composition (MANOVA; $p<0.05$) were also observed among the three *Pavlova* species. Levels of stigmastanol and cholesterol ranged from 19.5-22.4 and 3.3-4.5% in Pav 459 and *P. pinguis* respectively, but were either absent (stigmastanol) or identified only in trace amounts (cholesterol) in *P. lutheri* (Fig. 4.3) whereas none of the *Pavlova* species contained 24-methylenecholesterol.

No significant difference in saturated fatty acids (SAT) was observed among the four algal species, although CHGRA and *P. lutheri* had a higher proportions of monounsaturated fatty acids (MUFA; 24.4 and 20.7 % vs. ~11.5%) and lower proportion of PUFA (49.0 and 10.6 % vs. ~61%) than either Pav 459 or *P. pinguis* (MANOVA; $P<0.05$). CHGRA contained significantly higher levels of the n-6 arachidonic acid (AA; MANOVA; $P<0.05$; Fig. 4.2C), which was reflected in the CHGRA-containing diets, but much lower levels of the n-6 fatty acid DPA (4,7,10,13,16-docosapentaenoic acid) than either *P. pinguis* or Pav 459 (0.2% vs. 7.3-9.4%; MANOVA; $P<0.05$; Fig. 4.2C). Levels of both of these long-chained (>20C) n-6 PUFAs (AA and DPA) are also significantly lower in *Pavlova lutheri* than in either *Pavlova* species used in this study (MANOVA; $P<0.05$; Fig. 4.2C). No difference in eicosapentaenoic acid (EPA) was identified among algal species (MANOVA; $P>0.05$); however, levels of docosahexaenoic acid (DHA) varied among the algal species tested with the lowest values identified in CHGRA (2.4%), which also had the lowest values for Σn-3 fatty acids. Pav 459 offered the highest proportions of Σn-6 fatty acids (MANOVA; $P<0.05$; Fig. 4.2D), while Σn-6 values in *P. lutheri* were significantly lower than any of the algae used in Trials I and II (MANOVA; $P<0.05$; Fig. 4.2D).

Biochemical properties of algal species are only reported for Trial I as they were generally comparable to those determined for Trial II; however, there were a few

exceptions. In Trial II levels of total lipid were 19% lower and 31% higher in *P. pinguis* and CHGRA cultures, respectively, while protein concentrations in the Pav 459 cultures

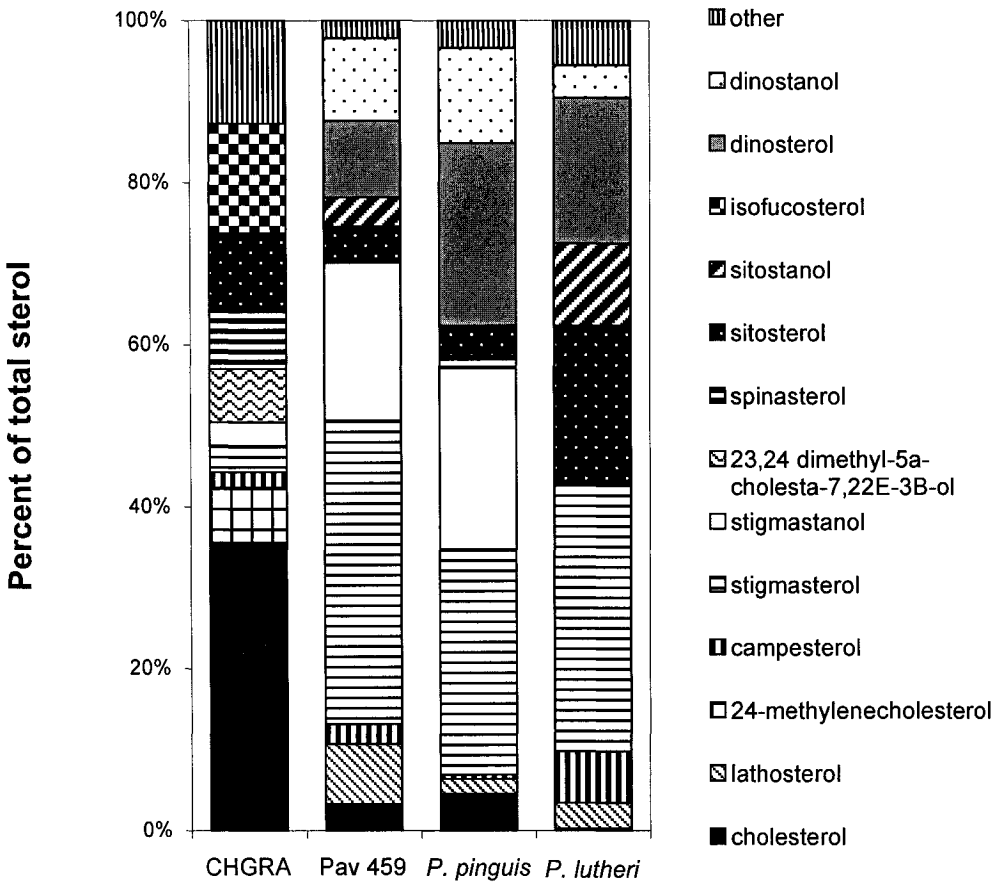

Figure 4.3: Sterol composition of three *Pavlova* spp. and *Chaetoceros muelleri*. Values for *C. muelleri* (CHGRA), Pav 459, *P. pinguis* from Trial I; values for *P. lutheri* from a previous study (Milke *et al.*, 2004). Only values >5% are reported.

from Trial II were 20% lower than those observed in Trial I. All other variation in gross biochemical composition between trials was <7%. Acetone mobile polar lipids (AMPL) were 40% higher in Trial II CHGRA cultures, while levels of phospholipid were somewhat lower for both CHGRA and Pav 459 (12 and 20%, respectively). Fatty acid composition was consistent between trials. There was little temporal variation in algal composition despite the fact that CHGRA culture was switched from semi-continuous to carboy culture for the final week of the experiment. This led to an increase in cellular

size (4.6 to 6.2 μm^3), AFDW (19.4 to 42.7 pg), carbohydrate (~10 to 25% of AFDW), and TAG (1.2-9.4 vs. 35% of total lipid) while levels of EPA decreased by ~50%.

4.3.3 Scallop Biochemical Composition

There were no differences in lipid or protein concentration of scallops from Trial I which ranged from 43.7-63.7 and 530.5-646.2 mg g $AFDW^{-1}$ respectively, however, significant differences in carbohydrate concentration were observed (Table 4.2). Scallops exposed to the poor-performing, unialgal CHGRA diet contained the lowest carbohydrate concentrations (22.5 mg g $AFDW^{-1}$; MANOVA; $P<0.05$; Table 4.2) while there was no statistical difference among the scallops offered the other diets (33.0-37.5 mg g $AFDW^{-1}$). No difference in total fatty acids was observed.

Scallops reared on the higher ranking binary diets of *P. pinguis* / CHGRA and Pav 459 / CHGRA in Trial I had statistically lower levels of TAG (0.1-0.3%) than the lower performing diets (1.4-2.9%), although levels in the slowest growing CHGRA-fed animals were undetectable (Table 4.2). This was not the case in Trial II, where there was a strong positive relationship between the proportion of TAG in tissues and growth rate (linear regression; $R^2=0.85$), as well as higher percentages of TAG (1.3-17.0%; Table 4.3) than were observed in Trial I (0.1-3.6%; Table 4.2). Sterol levels were twice as high in Trial I than in Trial II, and in Trial I they were highest in the CHGRA treatment (19.7 vs. 10.9-13.5%), while no difference was observed among treatments in Trial II (MANOVA; $P<0.05$; Table 4.2). No treatment effect upon phospholipid levels was detected in either trial.

Scallops fed 100% CHGRA were characterized by the highest proportions of Σ SAT and Σ MUFA in both feeding trials, but levels of Σ PUFA lower than other treatments (Tables 4.3 and 4.4). The highest PUFA levels were identified in animals fed the high performing Pav 459 / CHGRA and *P. pinguis* / CHGRA combinations (Trial II; 61-63%) and unialgal *Pavlova* diets (both trials; ~61%); scallops exposed to unialgal diets of Pav 459 also contained the highest proportions of n-6 fatty acids (both trials; ~32%). Levels of arachidonic acid (AA) were twice as high in scallops from Trial I vs.

Table 4.2: Biochemical composition (proximate analysis, selected lipid classes and fatty acids) of scallop tissues initially and after 4 weeks feeding on experimental diets in Trial I. Data expressed as mean ± SE, n=3. Different superscript letters represent statistically significant differences among final means, $P<0.05$; no difference was observed for variables marked with **.

	Initial	Pav 459 (50%) CHGRA (50%)	*P. pinguis* (50%) CHGRA (50%)	Pav 459	Pav 459 (25%) CHGRA (75%)	*P. pinguis*	CHGRA
Proximate analysis (mg g AFDW^{-1})							
Lipid **	53.0±5.5	58.2±2.8	63.7±0.5	54.8±7.7	54.7±3.4	54.3±3.7	43.7±0.0
Protein **	556.8±11.0	611.8±15.3	610.0±17.9	631.3±13.9	626.1±34.9	646.2±31.7	530.5±19.6
Carbohydrate	38.3±0.6	38.7±1.6^{a}	44.1±1.2^{a}	43.9±1.9^{a}	37.2±2.8^{a}	43.0±1.6^{a}	22.5±1.0^{b}
Sum FA **	32.9±3.4	35.1±1.5	37.5±1.0	33.5±4.9	34.2±2.5	33.0±2.6	23.8±2.4
Lipid classes (% of total lipid)							
Triacylglycerols	3.6±0.4	0.3±0.3^{b}	0.1±0.1^{b}	2.9±0.4^{a}	2.9±1.0^{a}	1.4±0.7^{ab}	--
Sterols	12.0±0.5	10.9±0.5^{b}	13.5±0.7^{b}	11.1±0.9^{b}	12.1±0.6^{b}	11.0±0.2^{b}	19.7±1.7^{a}
Phospholipids **	72.5±1.8	75.3±1.4	75.4±0.5	70.9±0.9	72.8±1.5	70.7±0.6	71.0±0.6
Fatty acid composition (% of total fatty acid)							
16:0	12.0±0.0	11.8±0.2^{ab}	12.4±0.1^{a}	11.3±0.0^{c}	12.2±0.0^{ab}	11.8±0.1^{ab}	11.7±0.2^{bc}
18:0	5.8±0.0	5.9±0.2^{b}	6.6±0.3^{ab}	5.2±0.1^{c}	6.2±0.1^{ab}	5.7±0.0^{bc}	6.8±0.3^{a}
16:1n-7	5.3±0.1	3.5±0.1^{b}	3.5±0.2^{b}	3.3±0.1^{b}	5.0±0.1^{a}	3.2±0.0^{b}	5.2±0.2^{a}
18:1n-9	6.0±0.0	5.5±0.0^{b}	5.5±0.1^{b}	3.8±0.1^{c}	6.4±0.1^{a}	3.8±0.1^{c}	6.4±0.1^{a}
16:4n-3 **	5.4±0.1	4.3±0.8	4.7±0.3	4.0±0.6	4.8±0.1	5.4±0.0	4.3±0.4
20:4n-6 (AA)	11.4±0.0	11.0±0.2^{b}	11.1±0.3^{bc}	10.1±0.1^{cd}	12.2±0.0^{a}	9.3±0.1^{d}	12.6±0.4^{a}
20:5n-3 (EPA)	9.6±0.1	7.8±0.1^{c}	8.6±0.1^{b}	6.3±0.1^{e}	9.5±0.0^{a}	6.9±0.1^{d}	7.8±0.1^{d}
22:5n-6 (DPA)	9.5±0.0	13.0±0.2^{c}	11.1±0.1^{d}	18.8±0.1^{a}	8.7±0.2^{e}	15.1±0.1^{b}	7.2±0.3^{f}
22:6n-3 (DHA)	14.4±0.0	12.5±0.1^{cd}	15.1±0.1^{ab}	13.6±0.1^{bc}	11.8±0.0^{d}	16.7±0.1^{a}	14.2±0.6^{b}
Sum SAT	23.4±0.0	23.7±0.4^{b}	25.3±0.2^{a}	22.8±0.3^{b}	25.0±0.1^{a}	24.6±0.2^{ab}	25.1±0.1^{a}
Sum MUFA	17.5±0.3	17.1±1.0^{abc}	15.8±0.1^{bcd}	14.5±0.9^{cd}	18.6±0.4^{ab}	13.6±0.1^{d}	19.8±0.5^{a}
Sum PUFA	58.3±0.3	58.4±0.6^{b}	58.0±0.4^{bc}	62.2±0.7^{a}	55.7±0.4^{cd}	61.2±0.1^{a}	54.5±0.5^{d}
Sum n-3	32.9±0.3	29.2±0.1^{c}	31.7±0.6^{ab}	28.2±0.7^{c}	29.4±0.4^{bc}	33.4±0.2^{a}	30.0±0.5^{bc}
Sum n-6	23.7±0.0	27.3±0.5^{b}	24.5±0.3^{c}	32.7±0.3^{a}	23.9±0.2^{cd}	26.5±0.1^{b}	22.2±0.6^{d}

Notes for Table 4.2:

[1] SAT= saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; -- = non-detectable.
[2] Other identified lipid classes identified at <1% included: steryl/wax esters, ethyl esters, methyl esters, methyl ketones, alcohols; at < 2% included: hydrocarbons, ethyl ketones, glyceryl ethers, diacylglycerols; at <4% included free fatty acids, and acetone mobile polar lipids ranged from 7-12%.
[3] Only fatty acids identified at >5% of total fatty acids are reported. Fatty acids with non-detectable levels included: 21:0, 22:0, 18:1n-7, 18:1n-6, 20:1n-9, 24:1, 18:4n-1, , 20:2α, 20:2β, 22:2NMIDa, 22:4n-3; fatty acids identified at ≤0.5% include: 15:0, 15:0ai, 16:0i, 17:0i, 19:0, 20:0, 23:0, 24:0, 14:1, 15:1, 16:1n-11, 16:1n-9, 18:1n-5, 22:1n-11(13), 22:1n-7, 16:2n-4, 16:3n-4, 16:4n-1, 18:2n-4, 18:3n-3, 20:3n-6, 20:3n-3, 20:4n-3; at ≤ 1% include: 15:0i, 16:0ai, 17:0, 18:1n-11, 18:2n-6, 18:3n-6, 18:3n-4, 20:2n-6, 21:5n-3; at ≤ 2% include: 17:0ai, 16:1n-5, 17:1, 20:1n-7, 22:1n-9, 18:4n-3, 18:5n-3, 22:2NMIDb, 22:4n-6, 22:5n-3; at ≤ 3.6% include: 14:0, 20:1n-11.

Trial II, and although significant differences among treatments were identified in Trial I, AA levels did not vary greatly, ranging from 9.3-12.6% (Table 4.2). Levels of n-6 DPA in scallop tissues reflected proportions present in the diet: scallops exposed to unialgal diets of Pav 459 contained the highest DPA levels in both trials (19-21%). There is no apparent relationship between levels of either DHA or EPA and dietary ranking, although levels of EPA in the Pav 459 / CHGRA and *P. pinguis* / CHGRA diets are both 2.4x higher in Trial II than Trial I (Tables 4.3 and 4.4).

4.3.4 Comparison of Dietary and Tissue Fatty Acids

Similar patterns among proportions of tissue fatty acids relative to that provided by the diet were observed in both feeding trials; thus only data for Trial I are presented (Fig. 4.4; see Appendix 1 for comparison). There was generally no tissue enrichment of Σn-3 fatty acids in scallop tissues relative to the diet (Fig. 4.4A). Although the relative proportion of the n-3 fatty acid EPA decreased across diet treatments, there was substantial enrichment in the n-3 DHA relative to the diet, especially pronounced in scallops fed 100% CHGRA, the DHA-deficient diet (1.7-5.9x; Fig. 4.4B). While little enrichment of Σn-3 was noted, there was a ubiquitous 2-3x enrichment in Σn-6 fatty acids. This was observed for both n-6 AA (~2-5x) and DPA (2.0-40.9x; Fig. 4.4C), with the highest DPA enrichment again observed in animals offered the unialgal CHGRA diet (~40x), which contained only 0.2%

Table 4.3: Lipid class and fatty acid composition of *P. magellanicus* tissues after 4 weeks feeding on experimental diets in Trial II. Data expressed as mean ± SE, n=3. Different superscript letters represent statistically significant differences among final means, P<0.05; constituents not included in the MANOVA models marked with *; no difference was observed for variables marked with **.

	Pav 459 (50%) CHGRA (50%)	*P. pinguis* (50%) CHGRA (50%)	Pav 459	CHGRA
Lipid classes (% of total lipid)				
Triacylglycerols**	10.4±4.4	17.0±0.8	6.5±0.9	1.3±1.3
Sterols**	3.4±0.9	6.7±0.8	7.9±0.2	6.1±1.9
Phospholipids **	61.5±9.8	60.8±0.8	56.6±2.1	37.8±4.4
Fatty acid composition (% of total fatty acid)				
16:0*	11.3±0.1	11.3±0.3	11.5±0.7	12.2±1.1
18:0*	4.6±0.4	4.3±0.3	4.4±0.2	8.2±0.6
16:1n-7*	4.6±0.1	5.4±0.2	4.7±0.3	2.9±0.1
18:1n-9*	4.0±0.0	5.0±0.0	3.2±0.1	4.9±0.4
16:4n-3 *	1.5±0.2	0.9±0.5	0.3±0.3	0.3±0.3
20:4n-6 (AA)**	5.5±0.0	5.1±0.1	6.9±0.2	4.8±1.0
20:5n-3 (EPA)	18.7±0.4^{a}	20.6±0.5^{a}	9.1±1.2^{b}	7.6±1.0^{b}
22:5n-6 (DPA)	13.3±0.1^{b}	11.7±0.2^{b}	21.0±0.2^{a}	3.2±0.6^{c}
22:6n-3 (DHA)	12.0±0.1^{a}	12.7±0.1^{a}	12.5±0.3^{a}	7.9±1.5^{b}
Sum SAT	22.1±0.2^{b}	22.4±0.4^{b}	23.0±0.5^{b}	33.0±0.2^{a}
Sum MUFA	14.0±0.8^{b}	15.8±0.6^{b}	15.2±1.9^{b}	23.7±0.0^{a}
Sum PUFA	63.0±0.6^{a}	61.0±0.3^{a}	60.9±1.1^{a}	40.1±0.6^{b}
Sum n-3	37.6±0.2^{a}	39.5±0.0^{a}	26.1±1.8^{b}	19.3±1.3^{c}
Sum n-6	22.4±0.0^{b}	18.5±0.4^{c}	32.1±0.3^{a}	11.4±0.6^{d}

Notes for Table 4.3:

[1] SAT= saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.

[2] Lipids with non-detectable levels included: ethyl esters, glyceryl ethers; other identified lipid classes identified at <1% included: ethyl ketones, steryl/wax esters, methyl esters, methyl ketones; at < 2.5% included: diacylglycerols; at >2.5% included: hydrocarbons (2-28%), free fatty acids (0.2-12%),alcohols (0-10%), and acetone mobile polar lipids (8-18%).

[3] Only fatty acids identified at ≤5.5% of total fatty acids are reported. Fatty acids with non-detectable levels included: 19:0, 21:0, 23:0, 16:1n-9, 18:1n-7, 18:1n-6, 20:1n-9, 22:1n-7, 18:4n-1, 22:2NMIDa, 22:4n-3; fatty acids identified at <0.5% include: 16:0i, 16:0ai, 20:0, 22:0, 24:0, 16:1n-11, 18:1n-5, 22:1n-11(13), 24:1, 22:1n-9, 20:3n-6, 20:3n-3, 20:4n-3; at ≤ 1% include: 15:0, 17:0, 15:1, 20:1n-11, 16:3n-4, 18:2n-4, 18:3n-6, 18:3n-4, 20:2α, 20:2β, 22:2NMIDb, 21:5n-3; 22:5n-3; at ≤ 2% include: 15:0i, 17:0i, 20:1n-7, 16:4n-1, 18:3n-3, 18:5n-3, 20:2n-6, 22:4n-6; at ≤ 5.5% include: 14:0, 15:0ai, 17:0ai, 14:1, 16:1n-5, 17:1, 18:1n-11, 16:2n-4, 18:2n-6, 18:4n-3

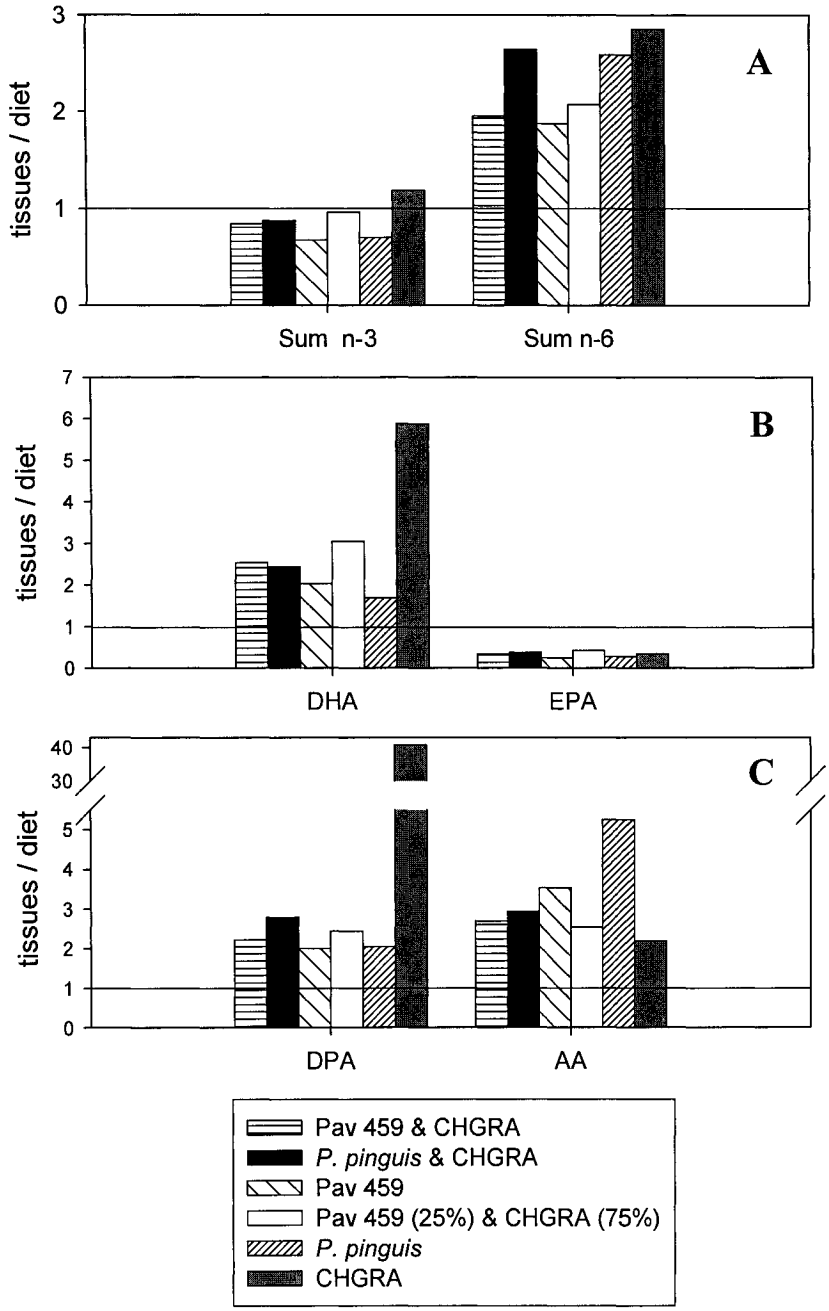

Figure 4.4: Relative proportions of fatty acids found in postlarval scallop (*P. magellanicus*) tissues relative to those provided by the diet in Trial I. Data are presented as % of total fatty acids; values >1 indicate enrichment in the tissues relative to the diet. (A) Total n-3 and Total n-6 fatty acids; (B) n-3 fatty acids DHA (22:6n-3) and EPA (20:5n-3); (C) n-6 fatty acids DPA (22:5n-6) and AA (20:4n-6).

of this fatty acid. Although levels of DPA in scallop tissues show enrichment relative to dietary inputs in both trials and across treatments, this enrichment appears proportional to the amount of DPA provided by the diet (Fig. 4.5), a trend that was not observed consistently (in both trials) for the other three long chain PUFA examined.

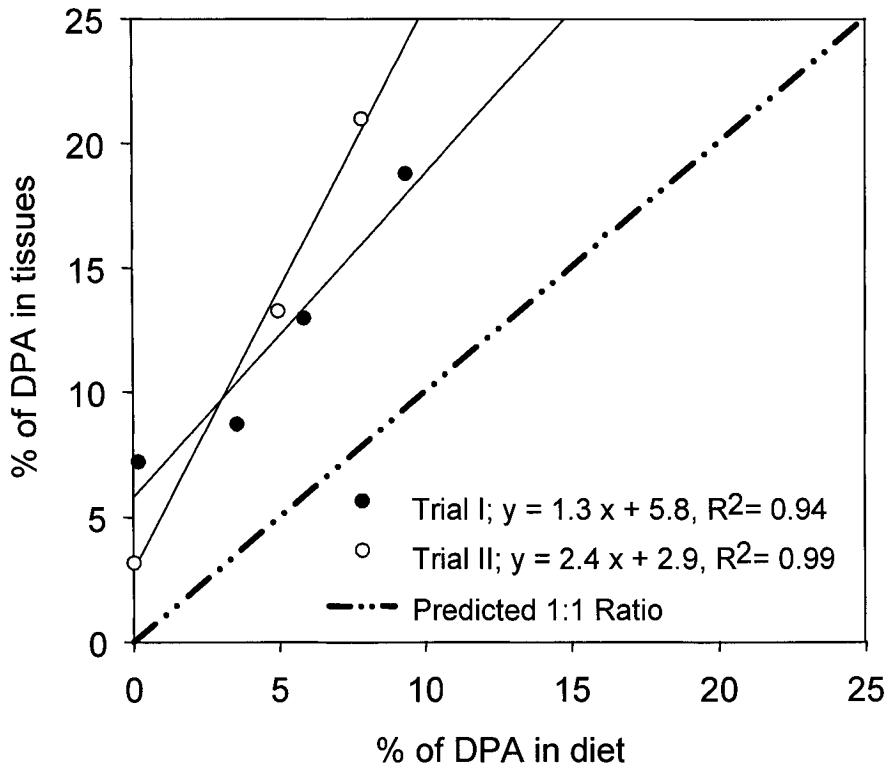

Figure 4.5: Relationship between dietary percentages of n-6 DPA (22:5n-6) provided by the Pav 459- and CHGRA-containing diets and determined in corresponding sea scallop tissues after 4 weeks of feeding in Trials I and II. Dashed line represents theoretical relationship where DPA in diets = DPA in tissues.

4.4 Discussion

4.4.1 Scallop Growth

While the *P. pinguis* / CHGRA combination supported the highest growth rate for sea scallop postlarvae in both Trials I and II, growth rates were not significantly different from animals fed the Pav 459 / CHGRA combination, previously identified as a superior diet when compared to other binary diets (Milke *et al.*, 2004). Yet *P. pinguis* grows very

readily and reliably in mass, batch or semi-continuous culture in 200-l bioreactors, whereas Pav 459 is more difficult to grow in this large scale. The growth performance of scallops in Trial II was similar to that previously reported for sea scallop postlarvae feeding upon the same diets (Milke *et al.*, 2004; i.e. Pav 459 / CHGRA; 28.1 vs. 24.9 μm day^{-1}). These growth rates generally meet or exceed growth rates of hatchery-raised postlarval sea scallops offered 1-3 (≤9μm day^{-1}; Gillis, 1993) and 4-6 species diets (3-37 μm day^{-1}; Ryan, 1999), although they are somewhat lower than growth rates reported for sea scallops in the wild (~30-60 μm day^{-1} for scallops 300-1400 μm in SH; Parsons *et al.*, 1993). Although a similar dietary ranking was observed among common diets used, scallops from Trial I exhibited much slower growth rates than those observed in Trial II, which may have resulted from the presence of a *Vorticella*-like ciliate prior to the feeding trial. The absence of statistical differences among growth trajectories in Trial I is likely an artifact of these relatively slow growth rates (approximately half of what was observed in Trial II) which may be insufficient to allow for differentiation among treatments within the 4 week duration of the experiment.

There was no statistical difference in gross biochemical composition among the algal species used within Trials I and II or *P. lutheri* (ANOVA; $P<0.05$). Thus, it is unlikely that observed differences in scallop growth performance are related to dietary protein or carbohydrate concentrations as has been previously reported for scallop (*Crassadoma gigantea* and *Argopecten purpuratus*) larvae and postlarvae (Whyte *et al.*, 1990b; Uriarte and Farias, 1999). Amino acid composition generally varies little among algal species and is therefore unlikely to have significant influence on bivalve growth (Knauer and Southgate, 1999). Differential particle selection is also unlikely to explain the difference observed in the present study. All algal species used in these trials were similar in size (3.7-5.0 μm^3 ESD) and in the size range that can be effectively retained by postlarval sea scallops (Anderson, 2003), even though this size range is below the size threshold of 100% retention efficiency in adult pectinids (~6 μm; reviewed by MacDonald *et al.*, 2006).

However, marked differences among dietary treatments were observed, with significant contributions from each algal constituent of the binary diets, in the ability to support scallop growth as evidenced by the 32 and 64% decrease in growth rate when

CHGRA and Pav 459 respectively were removed from the combination diet. This Pav 459 / CHGRA combination has previously been successful for rearing sea scallop larvae (Pernet *et al.*, 2005) and postlarvae (Milke *et al.*, 2004), and unialgal Pav 459 has out-ranked unialgal diets of *P. lutheri* and *Isochrysis* sp. (strain T- iso) when fed to *P. magellanicus* larvae (Feindel, 2000). The high nutritional value of this diet may result from its high content of n-6 PUFAs, specifically DPA and AA as well as sterol composition, especially high levels of cholesterol contributed by CHGRA. Striking similarities in fatty acid composition (most notably the relatively high level of n-6 DPA), and sterol content between Pav 459 and *P. pinguis* may explain the high (and similar) growth rates promoted by diets containing these two flagellates. In turn, the disparity in growth performance among combination diets in which Pav 459 is substituted with *P. lutheri* (31% decrease in growth rate; Milke *et al.*, 2004) is likely attributable to low levels of n-6 DPA given that CHGRA contributes high levels of both AA and cholesterol (see sections 4.2 and 4.3).

4.4.2 Lipid Classes

While algal proportions of TAG remained constant and were unlikely to influence the observed differences in growth response, a positive relationship between tissue TAG levels, as well as TAG/sterol levels, and growth performance was apparent for animals in Trial II, which may reflect the ability of healthier, faster growing, animals to convert more energy into storage compounds, as has been seen in *P. magellanicus* larvae (Pernet *et al.*, 2003b; Pernet *et al.*, 2006). This was not the case in Trial I where the highest proportions of TAG were observed in intermediately-ranked diets, while the two highest-performing combination diets reflected statistically lower values. TAG and TAG/sterol levels in this trial, as well as growth rates, were also much lower than those observed in Trial II, and may reflect stress-induced reduction in physiological condition for scallops in Trial I, as has been reported for fish, crustaceans and bivalve larvae (reviewed by Fraser, 1989). However, even TAG values for the faster-growing Trial II scallops were lower than those previously reported for sea scallop postlarvae (i.e. 10.4 vs. 31.6 % for the Pav 459 / CHGRA combination diet; Milke *et al.*, 2004).

There was no significant relationship between scallop growth rate and dietary sterol concentration in the present study (linear regression; R^2=0.36 and 0.37, respectively; $P<0.05$) while a strong positive relationship was previously described for sea scallop postlarvae (linear regression; R^2= 0.82; Milke *et al.,* 2004). Dietary sterol concentrations, however, were higher in these two current growth trials (mean 8.9 vs. 12.2-19.1 mg g $AFDW^{-1}$) and may be above threshold levels where a concentration-dependent growth response would be elicited. Alternatively, the inclusion of unialgal diets in these two growth trials (which were not tested in Milke *et al.*, 2004) may introduce dietary treatments to the regression model that support lower growth rates due to other biochemical deficiencies (i.e. cholesterol, PUFAs) provided by the second component of the binary diet, thus reducing the strength of the dietary sterol / growth rate relationship.

Sterols are required for the maintenance of cellular membrane fluidity (Nes, 1974; Dowhan and Bogdanov, 2002) especially at temperature extremes, and as such, may be particularly important for animals adapted to colder temperatures, such as *P. magellanicus*. Sea scallops are unable to tolerate temperatures much above 20°C (Naidu, 1991), and can be found in sub-zero waters at their northern limit of distribution. Many bivalve species are believed to be limited in their capacity to synthesize sterols from simple precursors such as acetate or mevalonate (Walton and Pennock, 1972; Holden and Patterson, 1991) leaving them reliant on dietary inputs to meet these requirements.

Sterols with a double bond at carbon 5, such as cholesterol, have shown positive effects on growth of the oyster *Crassostrea virginica* (Wikfors *et al.*, 1991), and selective enrichment and/or maintenance of tissue cholesterol levels have been observed in adult *C. virginica* (Berenberg and Patterson, 1981), *P. maximus* larvae (Soudant *et al.*, 1998a) and *P. magellanicus* postlarvae (Milke *et al.*, 2004) even when feeding on diets limited in cholesterol, suggesting an essential requirement of this sterol for bivalve health. CHGRA is the only alga tested in this study that contains both cholesterol and 24-methylenecholesterol which has also been positively related to post-settlement growth of oysters, *C. virginica* (Wikfors *et al.*, 1996), and preliminary trials with stable-isotope-labeled 24-methylenecholeesterol indicate conversion to cholesterol in juvenile *A. irradians* (G. Wikfors, pers. comm.). The presence of these two sterols may account for

the positive effect of CHGRA on scallop growth that is observed when this diatom is used to supplement unialgal diets of Pav 459 and *P. pinguis*. Accumulation of β-sitosterol, stigmasterol, brassicasterol, and/or campesterol, all of which were identified in at least one algal species in Trial I (Fig. 4.3), have also been observed in different developmental stages of *P. maximus* and *P. magellanicus* (Napolitano *et al.*, 1993; Soudant *et al.*, 1998a; Milke *et al.*, 2004). These four sterols have been identified in the freshwater bivalve *Diplodon delodonuts*, where due to their differing structures they were able to modify the fluidity of artificial membranes prepared from *D. delodonuts* lipids (Irazu *et al.*, 1988). Similarly, preferential accumulation and/or retention of these four sterols, in addition to cholesterol, may serve to facilitate maintenance of membrane function in pectinids. Sterol levels in tissues of sea scallop postlarvae will be reported in a subsequent paper.

4.4.3 Fatty Acids

Evidence of the importance of n-3 fatty acids has been provided in many different marine animals (Trider and Castell, 1980; D'Abramo, 1989; Marty *et al.*, 1992; Sargent *et al.*, 1999b); specifically, the long-chain PUFAs, DHA and EPA, are generally considered 'essential fatty acids'. However, in spat of the oyster *C. virginica* it appears that either EPA or DHA, but not necessarily both, was required to sustain growth (Langdon and Waldock, 1981). This also appears to be the case for bay scallop, *Argopecten irradians*, postlarvae and juveniles where a low-DHA diet of *Tetraselmis striata* / CHGRA yielded moderately high growth rates (Milke *et al.*, 2006). However, fatty acid requirements are likely species-specific as some animals, including postlarval *P. magellanicus*, appear to have a requirement for DHA (Milke *et al.*, 2004).

While we observed no relative enrichment of Σn-3 fatty acids or EPA in scallop tissues relative to the diet in this study, there was a substantial (1.7-5.9x) enrichment of DHA. The apparent lack of EPA incorporation may be an artifact of excess dietary EPA levels (22-25% of total fatty acids), selective catabolism of EPA as an energy source, or a combination of both. In scallop larvae it appears that EPA is catabolized, while DHA is conserved in tissues (Whyte *et al.*, 1990a; Marty *et al.*, 1992) even when offered diets limited in DHA (Nevejan *et al.*, 2003b). Enrichment/retention of DHA has been

described in larvae of *P. magellanicus* (Pernet and Tremblay, 2004; Pernet *et al.*, 2005), suggesting a requirement of DHA over EPA in sea scallops, which may explain the poor-performance of the DHA-deficient *T. striata* / CHGRA diet for sea scallops in contrast to findings for bay scallops. DHA appears superior to EPA in supporting growth of a variety of crustaceans and finfish (Kanazawa, 1991; Watanabe, 1993; Suprayudi *et al.*, 2004), and DHA may be required to maintain membrane structure and function as has been postulated in *P. maximus* larvae (Soudant *et al.*, 1998b).

While we observed no enrichment in Σn-3 fatty acids, there was substantial enrichment of Σn-6 fatty acids across dietary treatments in both feeding trials. Furthermore, there was substantial enrichment of two n-6 fatty acids AA and DPA primarily provided by CHGRA and Pav 459 / *P. pinguis*, respectively. It is clear that CHGRA contributes positively to the higher growth rates obtained with combination diets, as its removal from the Pav 459 / CHGRA combination led to a 32% decrease in growth rate in Trial II, and a 43% decrease in growth rate of *A. irradians* postlarvae (Milke *et al.*, 2006). While the sterol composition of CHGRA likely enhances growth performance (discussed above), the high proportion of AA found in this alga may also contribute a beneficial effect. Arachidonic acid is a precursor to bioactive eicosanoids influencing processes such as spawning and iron regulation in molluscs (Freas and Grollman, 1980; Stanley-Samuelson, 1987; Deridovich and Reunova, 1993), and helps maintain membrane structure in finfish (Sargent *et al.*, 1999a). Arachidonic acid has also been shown to influence immune response in bivalves (Delaporte *et al.*, 2003), and increased stress resistance in larval finfish (Koven *et al.*, 2001), which may explain the higher levels of AA observed in the slower-growing, ciliate-stressed scallops in Trial I vs. those from Trial II.

However, even though the addition of CHGRA to mixed diets enhanced growth, alone this alga is inadequate, yielding not only growth rates 53-65 % lower than the highest performing P. *pinguis* / CHGRA combination diets in Trial I and II, respectively, but also 32-52% lower than unialgal diets of Pav 459 and *P. pinguis*. In *P. maximus* larvae, favorable growth rates attained on a unialgal diet of *Chaetoceros calcitrans* were attributed to high dietary cholesterol and EPA levels; however, metamorphosis failed, possibly due to a lack of 22C DHA (Soudant *et al.*, 1998a). Low DHA levels are

characteristic of diatoms (Bacillariophyceae). In this study, the diatom CHGRA contained not only the lowest proportions of DHA (2.4 vs. 7.3-9.4%) of any algal species tested in these trials, but also of 22C n-6 DPA (0.2 %). This is a relatively rare fatty acid that is found at exceptionally high levels in *P. pinguis* and Pav 459 (7.3-9.4%), but not in *P. lutheri* (0.6%; Milke *et al.*, 2004). DHA may play a structural role in the cellular membranes of molluscs (Soudant *et al.*, 1998b), and although its physiological role remains largely undefined, it has been proposed that n-6 DPA may do the same (Soudant *et al.*, 1996a). A diet such as CHGRA, which is limited in these two 22C fatty acids (DHA+DPA= 2.6% vs. 16-17% for *P. pinguis* and Pav 459, respectively), may severely limit the growth performance of animals offered this diet.

A 31% reduction in growth rate has been previously observed for postlarval *P. magellanicus* offered a combination diet of *P. lutheri* / CHGRA, relative to those offered the Pav 459 / CHGRA combination (Milke *et al.*, 2004). This ranking has also been observed for postlarval and juvenile *A. irradians* for which growth rates were reduced by 50 and 40%, respectively (Milke *et al.*, 2006). *P. lutheri* had the highest, though not significantly different, levels of DHA of the three *Pavlova* species (Fig. 4.2C), so this PUFA is unlikely to be limiting growth as may be the case with a unialgal diet of CHGRA, and all three combination diets (CHGRA with *P. pinguis*, Pav 459 or *P. lutheri*), have sufficient levels of AA provided by CHGRA. This provides strong evidence that n-6 DPA is of critical importance in enhancing scallop growth. In fact, a multiple regression conducted on scallops from Trial I indicates that the only long chain PUFA (AA, EPA, DHA, DPA) to be correlated with scallop growth is DPA ($P<0.001$; $R^2=0.416$). Furthermore, marked enrichment of n-6 DPA (2-41x in Trial I; Fig. 4.4C) was observed across dietary treatments and feeding trials, and has likewise been reported in sea scallop larvae (Pernet *et al.*, 2005), the polar lipid fraction of *P. maximus* larvae (Delaunay *et al.*, 1993), the female gonad of *P. maximus* (Soudant *et al.*, 1996a) and in postlarval and juvenile *A. irradians* (Milke *et al.*, 2006). We also observed that DPA enrichment in scallop tissues increased proportionally to DPA levels provided by the diet (Fig. 4.5), a finding that was not observed consistently between trials for any other long chain PUFA (although it was observed for AA in Trial I). Although the physiological role of n-6 DPA remains unknown, it appears to positively affect the growth rate of

pectinids. The amount of DPA required and its metabolic function in pectinids and other marine organisms warrant further investigation via supplementation feeding trials that use a purified source of this fatty acid.

4.5 Conclusions

There was no growth difference between postlarval sea scallops fed the Pav 459 / CHGRA and *P. pinguis* / CHGRA combination diets, both of which promoted growth rates rivaling those reported for sea scallops feeding on diets composed of 4-6 microalgal species. Growth rates on these diets were also much higher than those previously reported for combination diets including *P. lutheri*, a diet differing by only one algal species of the same genus. Superior growth performance on either the Pav 459 / CHGRA of P. pinguis / CHGRA diet is likely due to the content of n-6 fatty acids, specifically DPA and AA provided by Pav 459/*P. pinguis* and CHGRA, respectively. Enrichment of Σn-6 fatty acids, and both n-6 DPA and AA were observed when comparing experimental diets and scallop tissues suggesting that these n-6 fatty acids may plan an important role as essential nutrients in pectinids. Dietary sterol composition also appears to influence sea scallop growth performance, especially the high levels of cholesterol provided by CHGRA.

4.6 Acknowledgments

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Chapter 5: Changes in Enzymatic Activity During Early Development of Bay and Sea Scallops, *Argopecten irradians* and *Placopecten magellanicus.*

Abstract

Poor growth and survival of scallops is often reported during early developmental stages, a period of pronounced morphogenetic change. Stage- and species-specific dietary requirements have been identified in the past, but the mechanisms responsible for observed differences remain largely unknown. Although biochemical composition is critical in determining the nutritional quality of a diet, an animal's digestive and assimilation capacity are equally important. The digestive competency of bay scallops (*Argopecten irradians*) and sea scallops (*Placopecten magellanicus*) may be expected to differ 1) over development while undergoing morphological and biochemical changes and 2) between species, as a result of adaptation to different environments and food supply. To test these hypotheses, larval scallops of the two species were raised on a mixed algal diet of *Pavlova* spp. (CCMP 459) or *Pavlova pinguis* in combination with *Chaetoceros muelleri* until they attained ~4-5 mm in shell height (SH), and were sampled for digestive enzyme activities at intervals encompassing major transitions in the development of feeding organs including the gill. Colorimetric assays were conducted to measure protease (azocasein), lipase (esterase) and carbohydrase (α-amylase, cellulase and laminarinase) activity. The most pronounced changes in enzymatic activity occurred prior to scallops attaining ~1.2 mm in SH in both scallop species. The esterase:protease ratio exhibits a similar ontogenetic pattern in both scallop species, decreasing sharply between larval and immediate postlarval stages before increasing to an intermediate level and stabilizing around 1.2 mm in SH. In contrast, carbohydrase activities differed between the species with bay scallops exhibiting higher specific activities of α-amylase and cellulase but much lower activities of laminarinase than sea scallops, which may be related to different food sources encountered in shallow estuarine vs. open water environments to which these two species are adapted. These findings have implications for developing targeted diets that could increase growth and survival of larval and postlarval scallops in commercial hatcheries as well as increasing our understanding of bivalve-seston interactions in the field.

5.1 Introduction

Scallop larval and early-postlarval development is marked by pronounced changes in behavior, physiology, biochemical composition and habitat, accompanied by major morphogenesis of feeding organs (Sastry, 1965; Beninger *et al.*, 1994; Barré, 2001; Veniot *et al.*, 2003). Therefore, concurrent changes in the food supply, dietary requirements and food utilization might be expected. In fact, stage- and species-specific differences in dietary needs have been identified for scallops (Uriarte and Farias, 1999; Milke *et al.*, 2006), although the mechanisms responsible for these differences remain largely unknown. While biochemical makeup of the diet is undoubtedly important, the ability of an animal to digest and assimilate dietary compounds is also critically important to ensure optimal growth and survival. Digestive competence may develop in parallel with morphogenesis of feeding organs, and the activities of various intra- and extra-cellular digestive enzymes will influence dietary utilization at various developmental stages.

Digestive capacity, as measured by the activity of digestive enzymes, may also be expected to differ between species with differing life histories or those that are adapted to life in different environments. The sea scallop, *Placopecten magellanicus*, is restricted to cold waters ($\leq 20^{\circ}C$; Naidu, 1991) extending from Labrador, Canada, to Cape Hatteras, North Carolina (Couturier *et al.*, 1995). Adults of this species exhibit submergence and are limited to habitats that are deeper and therefore further offshore at southerly limits of their distribution (Mann, 1989). Sea scallop postlarvae attach to a variety of substrates including bryozoans, red algae, and sand grains (Couturier *et al.*, 1995), and growth rate of sea scallops is relatively slow, attaining a market size of 90 mm shell height (SH) in ~3 years (Dadswell and Parsons, 1991). The northern bay scallop, *Argopecten irradians irradians*, is a temperate subspecies with natural populations occurring in subtidal estuarine environments from New Jersey to Maine. Bay scallops are generally found in estuarine waters less than 10m in depth (Couturier *et al.*, 1995), are associated with submerged aquatic vegetation especially during postlarval and juvenile stages (Pohle *et al.*, 1990) and grow much faster than sea scallops, attaining market size in less than a year under favorable conditions. As these two scallop species inhabit different

environments, they are exposed to a different food supply, and enzymatic activity may vary accordingly.

A number of studies have examined the digestive enzymes of adult bivalves (Reid, 1968; Trainer and Tillinghast, 1982; Ballantyne and Berges, 1991; Ibarrola *et al.*, 1998a) including bay scallops (Brock *et al.*, 1986) and sea scallops (Wojtowicz, 1972). Some work has focused on postlarval stages of commercially important molluscs such as abalone (Takami *et al.*, 1998) but little work has been conducted on bivalve larval and postlarval stages. Furthermore, although the role of digestive enzymes over ontogeny has been examined in finfish (Baglole *et al.*, 1998; Gawlicka *et al.*, 2000; Pérez-Casanova *et al.*, 2004) and crustaceans (Lovett and Felder, 1990; Kamarudin *et al.*, 1994; Ribeiro and Jones, 2000) we are aware of only two studies that have characterized digestive enzymes during early developmental stages in bivalves: larval *Pecten maximus* (Samain *et al.*, 1995) and larval/postlarval *Crassostrea gigas* (Luna-Gonzalez *et al.*, 2004).

The objectives of this study were to 1) identify changes in digestive competence as measured by *in vitro* activity of digestive enzymes over early scallop development from pediveliger larvae (250 μm) to postlarvae (4-5 mm) and 2) compare activity of key enzymes between bay and sea scallops. This information could lead to the development of targeted stage- and species-specific diets for implementation in a commercial setting, thus reducing the substantial costs of algal culture (~30% of total hatchery costs (Coutteau and Sorgeloos, 1993), and may also increase understanding of bivalve-seston interactions in the natural environment.

5.2 Materials and Methods

5.2.1 Experimental Animals

Larval bay scallops were obtained from Lunenburg Shellfish Inc., Nova Scotia (NS), Canada and transferred to the National Research Council's Marine Research Station (NRC/MRS), Ketch Harbour, NS where they were held on a mixed diet of *Pavlova* sp. (CCMP strain 459) / *Chaetoceros muelleri* (CHGRA) at 20°C for 4 days prior to initial sampling at the pediveliger stage. Post-metamorphic bay scallops from the same cohort were received ten days later and treated as above. Sea scallop larvae were spawned and reared at MRS and held on a diet of *Pavlova pinguis* / CHGRA at 14°C for

three days prior to initial sampling of pediveligers; sea scallops from the same cohort were used for post-metamorphic samplings and treated as above.

5.2.2 Experimental Systems

Larval scallops were held in aerated, 0.22 μm filtered sea water on a mixed algal diet in 60-l (bay scallop) or 700-l (sea scallop) tanks prior to initial sampling. Approximately 200,000 bay scallops [initial shell height (SH) = 310 μm] and 120,000 sea scallops (initial SH = 530 μm) were divided equally among four downwellers, two per each duplicate 400-l (bay scallops; 125 μm initial mesh size; 20°C) or 700-l (sea scallops; 130 μm initial mesh size; 14°C) recirculating seawater system. Downweller mesh sizes were increased commensurate with shell growth, reaching a maximum of 1180 μm and 1000 μm for bay and sea scallops, respectively.

Scallops were offered mixed microalgal diets consisting of one diatom and one flagellate at 2.16 x 10^6 μm^3 ml^{-1}; equivalent in bio-volume to 50 cells μl^{-1} of *Isochrysis galbana* strain T-iso, which provided an optimum dietary level for growth of bay scallop juveniles (0.5-5 mm; Lu and Blake, 1996). Microalgal stock cultures were obtained from the Center for the Culture of Marine Phytoplankton (CCMP), West Boothbay Harbor, ME and consisted of *Pavlova* sp. (CCMP 459; Pav 459), *Pavlova pinguis* (CCMP 609), and *Chaetoceros muelleri* (CCMP 1316; CHGRA). CHGRA and Pav 459 were grown in batch culture in 20-l carboys at 20°C, while *P. pinguis* was grown in semi-continuous culture in a 200-l bioreactor at 21°C. All species were grown with 24-h light on f/2 (-Si for flagellates; Guillard, 1975) with supplemental CO_2 to maintain pH and were harvested during the late exponential growth phase. Each algal species contributed to the binary diet equally (50:50) by volume, and cell concentrations were monitored by a Multisizer 3 Coulter Counter (Beckman Coulter, Fullerton California) equipped with a 100 μm aperture. Diets were maintained within ≤20% of the target concentration by daily batch additions and drip supplementation via peristaltic pump (Cole Parmer Masterflex 7554-80) when necessary.

Scallops were fed a binary diet of either Pav 459 or *Pavlova pinguis* in combination with CHGRA for 47 (bay scallop; April 24th to June 9th, 2004) or 65 (sea scallop; July 21st to September 23rd, 2004) days. *P. pinguis* and Pav 459 are comparable

in biochemical composition and have been shown to support similar growth rates in postlarval sea scallops when in combination with CHGRA (Chapter 4). Due to high mortalities, the initial cohort of bay scallops was replaced at day 19 by a second, 7 day younger cohort (initial SH= 700 μm) originating from the same broodstock. No sampling was conducted during this period of mortality in cohort 1, and cohort 2 was acclimated to the experimental diet for 6 days prior to the first sampling. Weekly samplings were conducted to measure scallop growth and survival, while seven to nine samplings, encompassing major periods of gill development, were conducted for analysis of enzyme activities up to a maximum size of 3.9 and 4.9 mm in bay and sea scallops, respectively.

5.2.3 Scallop Growth and Survival

Growth and survival was measured weekly by removal of at least 50 live animals from each downweller which were then placed on a graduated petri dish and scanned using an analog camera (Pulnix TMC7-DSP) coupled with either a dissecting microscope, or camera lens (Navitar Zoom 7000) as necessary for larger scallops (>4mm). Video was recorded on a Sony DVCAM (DSR-V10) calibrated with either a stage micrometer or ruler as appropriate. Shell height determinations (umbo to distal shell edge) were conducted by measuring either thirty (bay scallop) or fifty (sea scallop) live scallops using Optimas 6.5 image analysis software (Media Cybernetics, Silver Spring, MD). For mortality estimates, a sample of 100 scallops per downweller were counted as live, 'new' dead and 'old' dead, converted to percent data, then averaged to obtain tank and overall mortality for each sampling date. 'New' dead scallops were similar in size to live animals but showed no movement and/or partial tissue degradation, while 'old' dead scallops were smaller, showed no movement and showed substantial or complete degradation of tissues.

5.2.4 Enzymatic Assays

Preliminary experiments were conducted to assess the effect of 24-h gut evacuation vs. full gut contents on enzymatic activity of postlarval *A. irradians.* For the present study all scallops were purged of gut contents for 24h prior to sampling. Scallops were removed from each tank at each sampling date (one subset from each downweller

for postlarvae) and moved to aerated tanks (larvae) or recirculating downweller (postlarvae) systems containing 0.22 μm (bay scallop) and 0.22 or 1 μm (sea scallop) filtered sea water overnight. The number of scallops in each sample was then recorded, whole scallops (including shell) were placed into cryovials and frozen in liquid N_2 after removal of excess water. Samples were stored at -80°C until analysis.

All samples containing pooled groups of scallops were lyophilized and homogenized in 100 mM ammonium bicarbonate buffer, pH 7.8 on ice (1 g tissue wet weight: 5 ml buffer), aliquoted into 0.5 μl centrifuge tubes and stored at -80°C. Before analyses, samples were thawed on ice and centrifuged at 5,100 g at 4°C for 2 min; only supernatant was used in assays. All protein concentration and enzymatic activities were determined colorimetrically on a SpectraMax Plus or VMax 96-well microplate reader (Molecular Devices; Sunnyvale, California) and analyzed using SoftMax Pro software version 4.7.1 (Molecular Devices). Soluble protein was determined using a modified Lowry protocol (Bio-Rad, Hercules, California; Lowry *et al.*, 1951) using γ-globulin as standard and detection at 650 nm. Preliminary trials determined the optimum incubation time and pH for each enzyme assay. All analyses were conducted at 30°C allowing for direct comparisons between different enzymes and scallop species.

Digestive competence of bay and sea scallops was examined by determining specific activities of five different digestive enzymes. General protease and lipase activity were determined using azocasein and esterase hydrolysis assays respectively. Three different carbohydrate-degrading enzymes (α-amylase, cellulase and laminarinase) were tested to determine the ability of bay and sea scallops to digest common storage polysaccharides; green algae contain starch and cellulose, detrital particles are largely comprised of cellulose and diatoms contain laminarin (Brock *et al.*, 1986).

Protease activity was determined using the azocasein hydrolysis assay (Charney and Tomarelli, 1947) as modified by Ross *et al.* (2000) for microplate analysis. Homogenate (10 μl) was incubated with 5 mg ml^{-1} azocasein (Sigma A2765, St. Louis, MO; dissolved in 100 mM ammonium bicarbonate buffer; pH 7.8) for 19 h on a rotator at 60 rpm. The reaction was stopped with the addition of 50 μl of 20% trichloroacetic acid, samples were cooled on ice and tubes were centrifuged at 17,000 g for 5 min after which 100 μl of homogenate and 100 μl of 0.5 M NaOH were added to microplate wells.

Optical density was measured at 450 nm, and one unit of activity was defined as the change in optical density h^{-1} *1000.

For determination of esterase activity 25 µl tissue homogenate was incubated with 4 µl substrate (10 mM p-nitrophenyl myristate dissolved in ethanol; Sigma; Huggins and Lapides, 1947), 75 µl of 100 mM ammonium bicarbonate buffer (pH 7.8) with 0.66% Triton-x. The change in optical density was monitored continuously for 1 h at 405 nm, and activity was defined as pmol of p-nitrophenol liberated min^{-1}. The extinction coefficient of p-nitrophenol in microplate wells was determined empirically.

A p-hydroxybenzoic acid hydrazide (PAHBAH) reducing sugar assay was adopted for all carbohydrase assays (Lever *et al.*, 1973; Lever, 1977), where an initial sample (allowing for no incubation time of substrate and tissue homogenate) was adopted as the sample blank and 1 mg ml^{-1} D-glucose (AnalaR) was used as standard. Briefly, 50 µl of substrate, 10 µl of buffer, 30 µl of ddH_2O and 10 µl of tissue homogenate were added to 1.6 ml centrifuge tubes and incubated at 30°C for 30 min after which 1.5 ml of the PAHBAH reagent was added to stop the reaction. Samples were heated at 70°C for 10 min to facilitate color development. After cooling, samples were added to microplate wells and optical density was read at 410 nm. For determination of specific activities, substrates and buffers were as follows: α-amylase, 1% soluble starch (Sigma, S-2630) and 0.8 M sodium citrate buffer pH 6.5; cellulase, 2% carboxymethyl cellulose (Sigma C-5678) and 0.5 M phosphate buffer pH 6.0; laminarinase, 0.4% laminarin (Sigma L-9634) and 0.8 M sodium citrate buffer pH 5.5. Due to the high activity of laminarinase in the samples, tissue homogenates were diluted 1:1 with buffer prior to incubation.

5.2.5 Statistical Analysis

Individual t-tests (for each of the 4 enzymes examined) were conducted to determine differences in specific activity between fed scallops and those purged of gut contents in the preliminary trial. Differences among specific activities of various enzymes over ontogeny were tested using a two-way ANOVA with scallop species and size as factors (scallops were grouped into one of 11 size classes to allow comparison between species as scallops from the two experiments were not sampled at exactly the same shell heights). When one of these factors was found to be significant, post-hoc

comparisons were conducted using Tukey's multiple comparison test. Analyses were conducted on SigmaStat 5.1 or SYSTAT 10.0 (SPSS, Chicago, IL); $P<0.05$.

5.3 Results

5.3.1 Scallop Growth and Mortality

Growth of scallops fed the *Pavlova spp.* / CHGRA diets throughout the experiment (Fig. 5.1) was fitted by exponential equations and yielded predicted daily growth rates as high as 157 and 107 μm day^{-1} for bay and sea scallops, respectively. Total mortalities of bay scallop cohort 1 were fairly low (~15%) during the first 11 days of the experiment when sampling for enzymatic analysis was conducted. Scallops from cohort 2 had 76% survival by the end of the experiment at day 47. Exceptionally low mortalities were encountered during the sea scallop trial, with a final survivorship of 95% at day 65.

5.3.2 Effects of Gut Evacuation on Enzymatic Activity

A 24 h-starvation trial to assess the contribution of full vs. purged gut contents on the enzymatic activity of whole animal homogenates of postlarval (~2 mm) *A. irradians* indicated no significant difference in specific activities of esterase, protease, or laminarinase (Table 5.1; Students t-test; $P\geq0.10$). A slight, but marginally significant difference was observed in cellulase activity between these two treatments ($P=0.046$). Differences in α-amylase activity were not examined. Starvation trials were not conducted on postlarval sea scallops, *Placopecten magellanicus*, due to lack of animal availability.

5.3.3 Enzymatic Activity Over Development

The specific activity of esterase exhibited a marked decline between larval and immediate postlarval stages in both scallop species (Fig. 5.2; significant in sea scallops; ANOVA; $P<0.05$). After this initial decrease in bay scallops, values remained relatively constant ranging from 0.24 to 0.50 μmol p-nitrophenol min^{-1} g protein. In sea scallop postlarvae, values for esterase activity dramatically increased from 0.14 to 0.51 μmol p-nitrophenol min^{-1} g protein between ~525 and ~1200 μm in SH and remained constant

thereafter (Table 5.2). Values for specific activity of protease (azocasein) in larvae, on the other hand, are generally lower than those observed in postlarval sizes for both species (Fig. 5.2). In bay scallops, a steep but non-significant increase in activity is observed between larval (0.10 units of protease activity μg protein^{-1}) and 320 μm postlarvae (0.32 units of protease activity μg protein^{-1}), with a subsequent steady

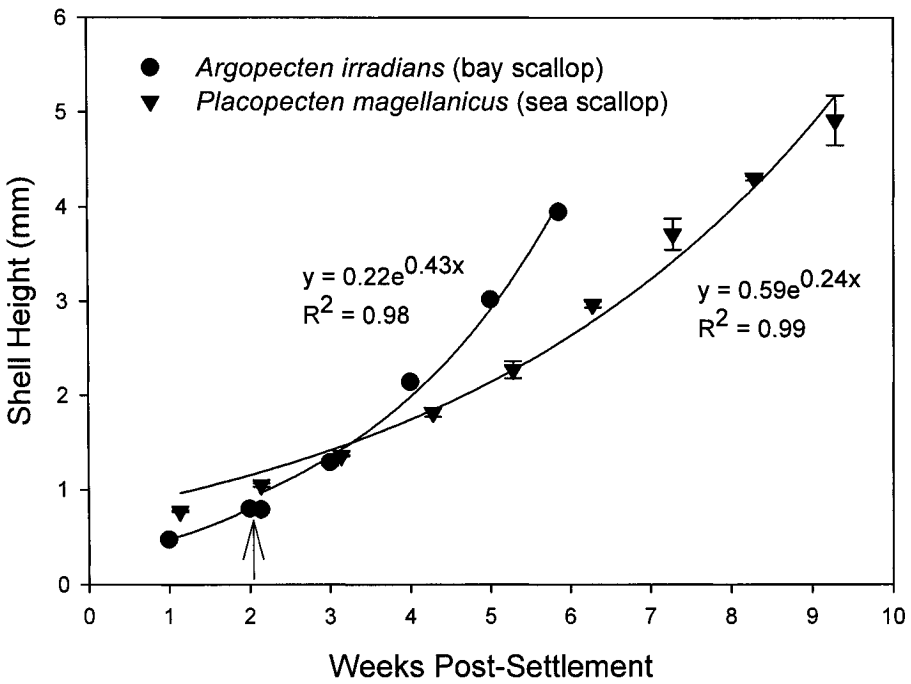

Figure 5.1: Post-metamorphic shell growth trajectories of bay scallops (*Argopecten irradians*) and sea scallops (*Placopecten magellanicus*). Values represent mean shell height ± SE, arrow indicates size at which the initial bay scallop cohort was replaced with a second batch of animals (see text).

decrease from 320-1300 μm in SH. Protease activity in sea scallops however, increases consistently from the larval stage until they attain ~1000 μm in SH and then stabilized at ~0.12 units of protease activity μg protein^{-1} (Table 5.2).

While different patterns in esterase and protease activity are exhibited over development by these two species, the ontogenetic changes in esterase/azocasein ratio are strikingly similar between them (Fig. 5.3). This ratio exhibited a ~5-7x decrease between larvae and immediate postlarvae in both species (Table 5.2; ANOVA; $P<0.05$) with values of 6.5-8.3 decreasing to 1.1-1.2. This is followed by a period of relative increase

Table 5.1: Effects of full gut contents versus 24-h gut evacuation on specific activities of selected digestive enzymes. All measurements expressed as mean ± SE, n=3. No significant differences between treatments were identified, $P<0.05$ except for cellulase, $P=0.046$. Tests for α-amylase were not included in these trials.

	non-purged	Purged
Protease (units of protease activity μg protein $^{-1}$		
Azocasein	0.46±0.00	0.47±0.03
Lipase (μmol p-nitrophenol min^{-1} g protein^{-1})		
Esterase	0.22±0.01	0.34±0.05
Carbohydrase (μmol glucose min^{-1} g protein^{-1})		
Cellulase	10.94±0.41	9.17±0.48
Laminarinase	410.45±7.10	364.75±28.68

in esterase activity from sizes of ~320-525 until scallops attained ~1200 μm in SH; this ratio remains relatively constant thereafter.

Specific activities of the three carbohydrases examined vary over development; however, most changes occur before scallops attain ~1.2mm in SH (Fig. 5.4). In bay scallops, activities of α-amylase in larvae are relatively high (~48.9 μmol glucose min^{-1} g protein^{-1}), while low values are identified at the larval stage in sea scallops (7.1 μmol glucose min^{-1} g protein^{-1}; Table 5.2), although activities in both species gradually approach a similar value (25-31 μmol glucose min^{-1} g protein^{-1}) at ~700 μm in SH (Table 5.2). While cellulase specific activity remains constant over development in sea scallops, a gradual decrease from 92.5 to ~40 μmol glucose min^{-1} g protein^{-1} is observed in bay scallops between 250 and 1000 μm in SH. Both species exhibit an increase in laminarinase activity between the larval stage and ~800-1000 μm in SH, although the increase is more pronounced in sea scallops (Fig. 5.4).

5.3.4 Comparison of Specific Activity Between Species

A two-way ANOVA failed to detect a significant difference in mean esterase specific activity or the esterase/azocasein ratio between postlarval stages of the scallop species ($P>0.05$). However, there was a significant difference in protease (azocasein) activity between species with higher values identified in bay scallop postlarvae

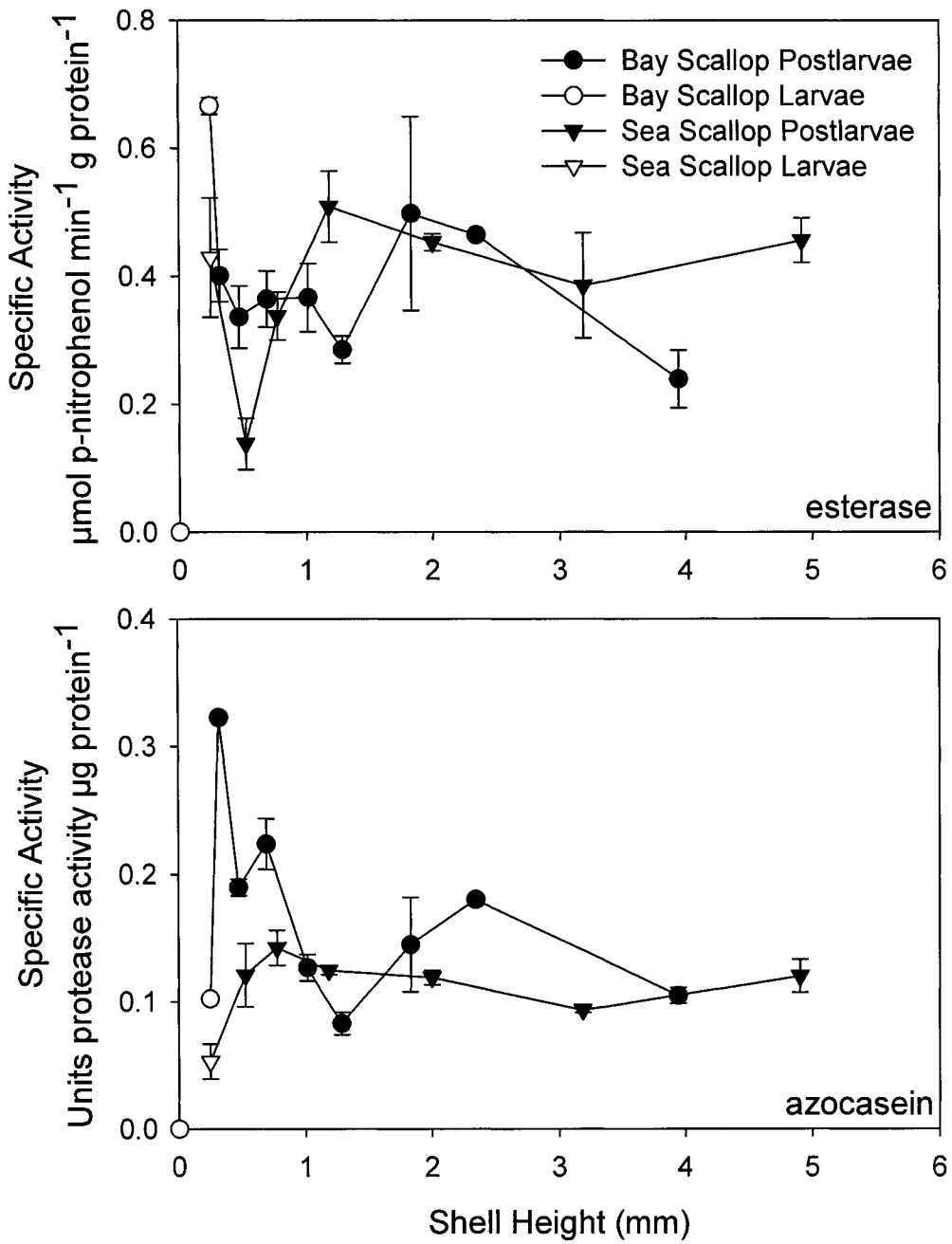

Figure 5.2: Specific activities of esterase and protease (azocasein) over development for bay and sea scallops (*Argopecten irradians* and *Placopecten magellanicus*, respectively). Values represent mean ± SE, n=2 tanks for each sampling size. Numerical values and results of statistical analysis can be found in Table 5.2.

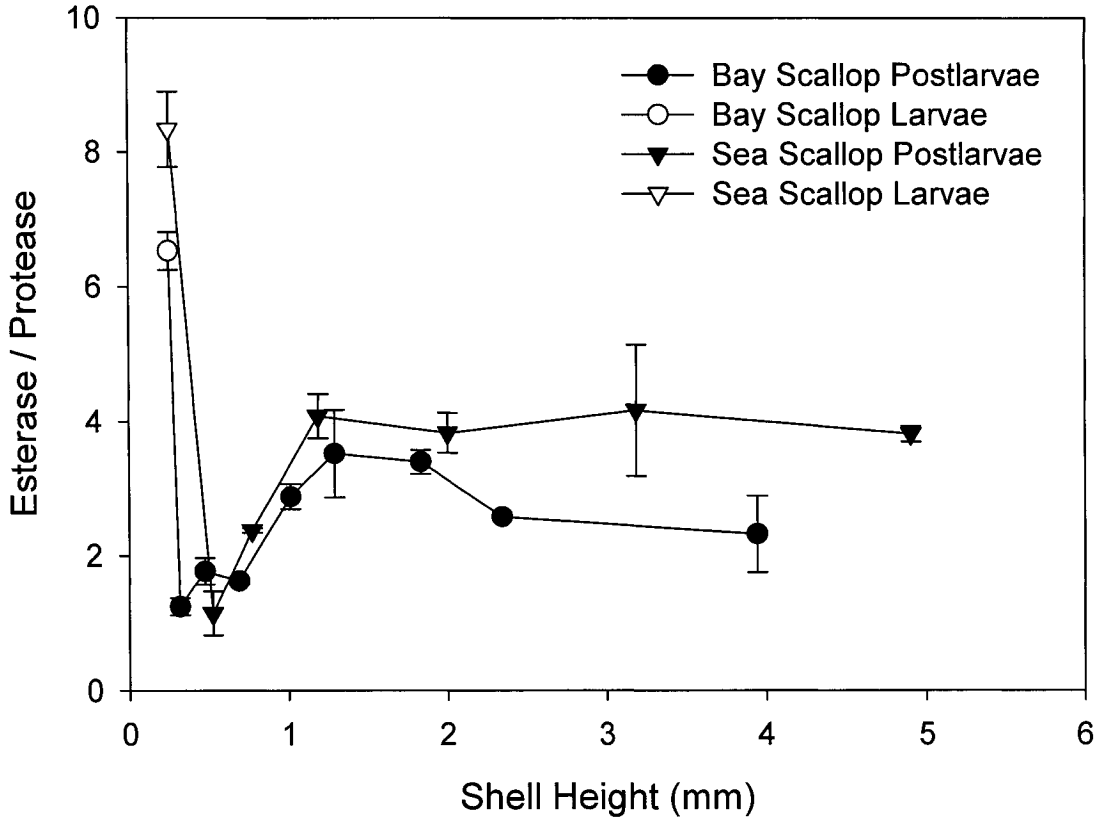

Figure 5.3: Ratio of esterase to protease activity in bay and sea scallops (*Argopecten irradians* and *Placopecten magellanicus*) over development. Values represent mean value ± SE, n=2 tanks. Statistical differences among values at different shell heights are shown in Table 5.2.

(0.17±0.02 vs. 0.12±0.01 units protease activity μg protein^{-1}; $P<0.01$). Alpha amylase and laminarinase showed highly significant differences in activity between the postlarval stages of the two scallop species ($P<0.001$), while carbohydrase activity showed a significant difference ($P<0.05$). Interestingly, bay scallop postlarvae exhibited specific activities of α-amylase and cellulase almost twice as high (37.5 and 52.9 μmol glucose min^{-1} g protein^{-1}) as those determined for sea scallops (21.1 and 27.5 μmol glucose min^{-1} g protein^{-1}), while the mean laminarinase activity of sea scallops was approximately twice as high as that found in bay scallops (Fig. 5.5; 283.2 vs. 151.7 μmol glucose min^{-1} g protein^{-1}).

Table 5.2: Specific activity over development for bay and sea scallops. Values are mean, n=2 tanks; standard error (SE) is shown in Figures 2-5. Within rows, values that do not have a common superscript letter differ significantly, $P<0.05$. Specific activities are defined as follows: esterase, μmol p-nitrophenol liberated min^{-1} g protein $^{-1}$; protease, units of protease activity μg $protein^{-1}$; carbohydrases (α-amylase, cellulase, laminarinase), μmol glucose liberated min^{-1} g $protein^{-1}$. Reported shell heights are the mean of each size class.

Size Class	1	2	3	4	5	6	7	8	9	10	11
Bay Scallops (Argopecten irradians)											
Shell Height (μm)	*250*	*320*	*474*	*690*	*1013*	*1287*	*1831*	*2343*	--	*3945*	--
esterase	0.67^{a}	0.40^{ab}	0.34^{ab}	0.36^{ab}	0.37^{ab}	0.29^{b}	0.50^{ab}	0.46^{ab}	--	0.24^{b}	--
protease	0.10^{ab}	0.32^{a}	0.19^{ab}	0.22^{b}	0.13^{ab}	0.08^{ab}	0.14^{ab}	0.18^{ab}	--	0.10^{ab}	--
esterase/protease	6.53^{a}	1.24^{c}	1.77^{bc}	1.63^{bc}	2.88^{bc}	3.52^{b}	3.40^{b}	2.58^{bc}	--	2.32^{bc}	--
α-amylase **	48.87	47.22	44.07	30.77	36.82	37.38	32.77	33.15	--	35.46	--
cellulase	92.48^{a}	70.03^{b}	71.95^{b}	67.92^{b}	43.69^{c}	38.39^{c}	32.62^{c}	39.09^{c}	--	46.06^{c}	--
laminarinase	73.87^{d}	91.75^{cd}	103.91^{cd}	112.56^{c}	171.76^{b}	191.80^{ab}	169.01^{b}	177.54^{ab}	--	208.36^{a}	--
Sea Scallops (Placopecten magellanicus)											
Shell Height (μm)	*251*	--	*526*	*773*	--	*1185*	*2000*	--	*3186*	--	*4903*
esterase	0.43^{ab}	--	0.14^{b}	0.34^{ab}	--	0.51^{a}	0.45^{ab}	--	0.39^{ab}	--	0.46^{ab}
protease	0.05^{b}	--	0.12^{ab}	0.14^{a}	--	0.12^{ab}	0.12^{ab}	--	0.09^{ab}	--	0.12^{ab}
esterase/protease	8.34^{a}	--	1.14^{c}	2.37^{bc}	--	4.08^{b}	3.83^{b}	--	4.17^{b}	--	3.82^{b}
α-amylase **	7.15	--	18.72	24.82	--	19.67	19.97	--	13.20	--	31.23
cellulase *	25.39	--	21.21	31.47	--	22.03	26.31	--	29.23	--	37.81
laminarinase	109.28^{b}	--	287.47^{a}	318.76^{a}	--	262.40^{a}	278.17^{a}	--	251.02^{a}	--	299.11^{a}

* no significant difference in specific activity over development as determined by a one-way ANOVA; P=0.42.

** no significant differences in specific activity over development as determined by the two-way ANOVA; P=0.28.

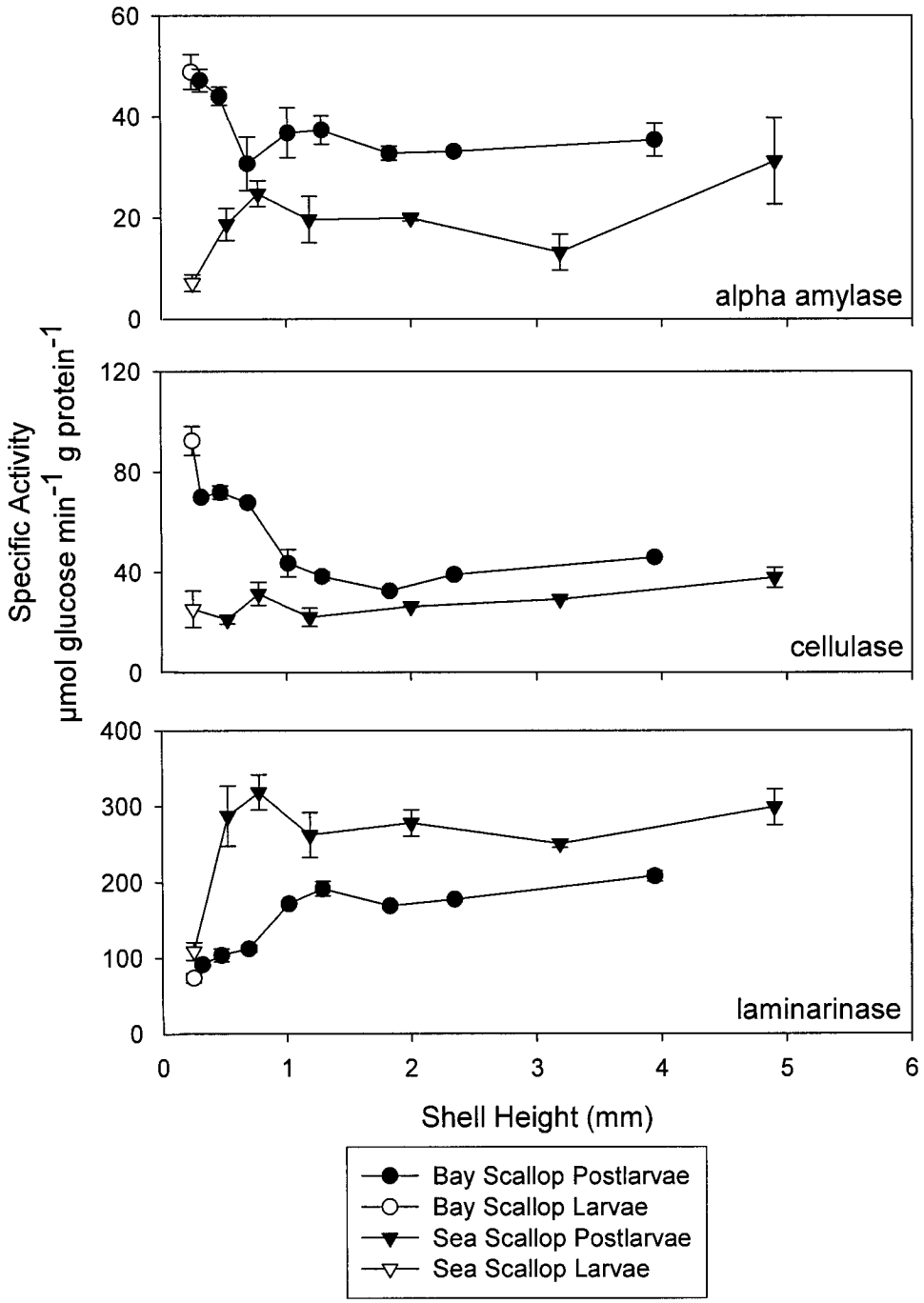

Figure 5.4: Specific activities of the carbohydrases α-amylase, cellulase and laminarinase over development in bay and sea scallops (*Argopecten irradians* and *Placopecten magellanicus*). Values represent mean ± SE, n=2 tanks for each sampling size. Numerical values for specific activity and results of statistical analysis can be found in Table 5.2.

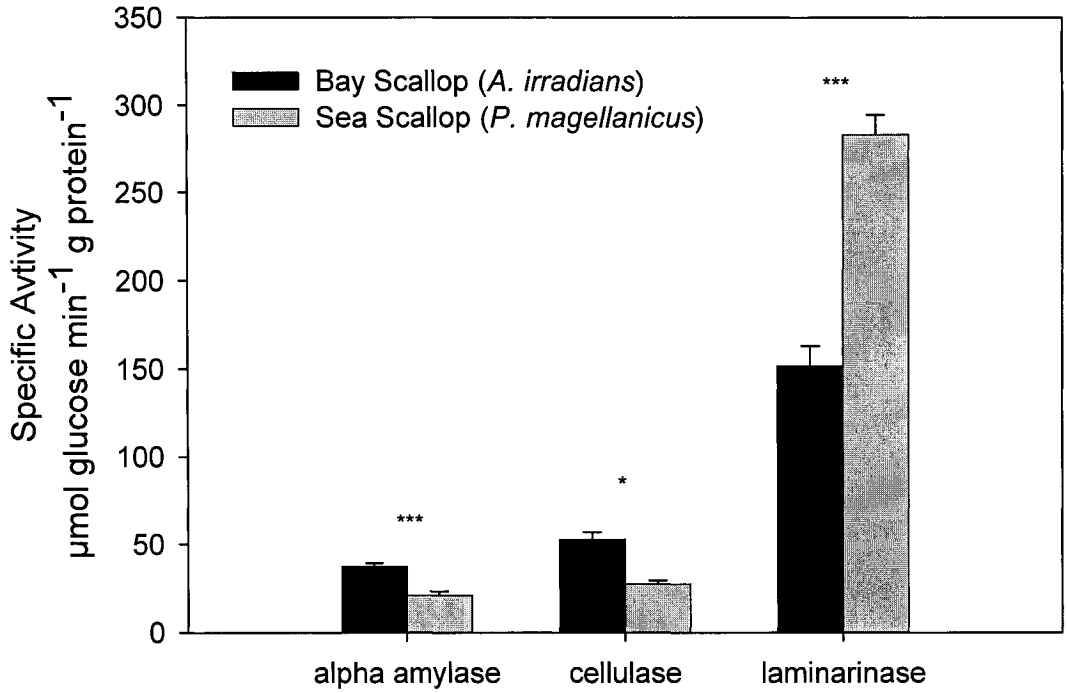

Figure 5.5: Between species differences in specific activity of the three carbohydrases (α-amylase, cellulase and laminarinase) from whole body homogenates of postlarvae. Values represent means of all postlarval sizes, ± S.E differences determined by a two-way ANOVA. *= P<0.05, *** = P<0.001

5.4 Discussion

To the best of our knowledge this is the first study to examine ontogenetic changes of enzymatic activity in scallops and to compare these between bivalve species. Sampling times were chosen to encompass previously described major periods in gill development, which may also be associated with changes in digestive competence.

Both scallop species grew exponentially over the duration of the experiment indicating a normal pattern of growth and development during these early stages. Daily growth rates met or exceeded growth rates previously recorded for *A. irradians* (Lu and Blake, 1996; Milke *et al.*, 2006) and *P. magellanicus* (Parsons *et al.*, 1993; Milke *et al.*, 2004) of similar size. Bay scallop cohorts 1 and 2 suffered mortalities early on during the

experiment, but were not sampled for enzymatic activity during this period, and thus the enzymatic activities reported should not be adversely affected.

Bivalve digestive enzyme pH optima have been extensively described and are similar across species. Values determined in this study for postlarval bay and sea scallops were similar to those previously reported for other bivalves including adult *A. irradians* and *P. magellanicus*: esterase, 6.5-8.7; protease, 5.5-7.5; α-amylase, 4.9-6.0; cellulase, 4.9-6.0 and laminarinase, 4.6-6.0. (Black and Pengelley, 1964; Wojtowicz, 1972; Payne, 1978; Brock *et al.*, 1986; Brock and Kennedy, 1992; Ibarrola *et al.*, 1996; Fernandez-Reiriz *et al.*, 2001). While reported pH optima are tissue-specific and generally reflect higher activities at higher pH in the crystalline style and lower pH in the digestive diverticula, whole body homogenates were used in this study and therefore only one pH optimum value was determined.

As pH optima are tissue-specific, substantial localization of digestive enzymes is also observed in bivalves. In the scallop, *Pecten maximus*, the majority of extracellular digestion occurs in the stomach and lumen of the digestive diverticula and is dominated by carbohydrases (Boucaud-Camou and Henry, 1995). High activities of α-amylase and laminarinase have also been reported for the crystalline style of adult *P. magellanicus* and *Mytilus chilensis* (Wojtowicz, 1972; Fernandez-Reiriz *et al.*, 2001). Protease activity is generally intracellular in bivalves, predominantly occurring in the tubule epithelial cells of the digestive diverticula (Ibarrola *et al.*, 1996), whereas lipolytic activity has been identified in the stomach, crystalline style, digestive diverticula and amoebocytes (hemocytes) of various bivalve species (Morton, 1983).

The effects of 24h-gut evacuation on enzymatic activity of postlarval *A. irradians* were negligible, with marginally significant differences only observed for cellulase. The purging of gut contents adopted for this study allowed for comparison with biochemical profiles determined from previous studies using whole animal homogenates of postlarval bay and sea scallops (Milke *et al.*, 2004; Milke *et al.*, 2006). Samain *et al.*, (1995) report no change in enzymatic activity prior to 48h during a starvation trial for *P. maximus* larvae, although they did observe some differences at 48h and suggest a need for examining starvation periods greater than 48h. In the copepod, *Calanus pacificus*, activities of laminarinase decreased after 4 days starvation, but no further decline in

activity was observed indicating the maintenance of some enzymatic activity irrespective of food availability (Cox and Willason, 1981).

Significant changes in enzymatic activity were observed over development, with the most substantial changes occurring before both scallop species attained ~1-1.2 mm in SH. Interestingly, different ontogenetic patterns of enzymatic activity were observed both among different enzymes tested and between scallop species. This contrasts with the steady increase in specific activities over ontogeny reported for amylase and protease activity in postlarval prawn, *Fennero Penaeus indicus* (Ribeiro and Jones, 2000) and early stages (gold embryo to intermolt juvenile stage V) of the lobster, *Homarus americanus* (Biesiot and McDowell Capuzzo, 1990), which might be expected as the result of increased digestive competence associated with the maturation of digestive organs. Alpha-amylase activity in larval haddock (*Melanogrammus aeglefinus*) and cod (*Gadus morhua*) were only detected on certain days when large number of live prey (that had been enriched with the flagellate *Pavlova lutheri*) were present in the gut (Perez-Casanova *et al.*, 2006).

The larval and postlarval scallop stages examined in this study encompass a period of pronounced morphogenesis in both bay and sea scallops. At sizes <500 μm, postlarval bay scallops have a rudimentary gill consisting of only one homorhabdic, inner demibranch, while at ~600 μm SH reflection of the inner demibranch is apparent which allows more effective suspension-feeding, and at 3.3 mm bay scallops possess a plicated and heterorhabdic gill resembling that of the adult (Barré, 2001). A similar pattern is observed for the gill development of sea scallops, although the period over which this occurs is more protracted, with gill reflection occurring around 1 mm and a heterorhabdic plicated gill structure evident around 7 mm in SH (Veniot *et al.*, 2003). While postlarval bay and sea scallops are capable of capturing particles within the size range our diet provided (~3.5-5.5 μm equivalent spherical diameter; Shumway *et al.*, 1997), capture efficiency may change over gill development as has been shown in particle selection trials using mixtures of 2 and 10 μm polystyrene beads in *P. magellanicus* postlarvae (Anderson, 2003). Furthermore, a sharp increase in particle clearance rate was observed at ~600 μm SH in the scallop *Patinopecten yessoensis*, the size at which gill refelction occurs (Kingzett, 1993). It is possible that the change in enzymatic activities observed

over ontogeny in the two scallop species compared in the present study, especially at smaller sizes (<1.2 mm SH), reflects changes in morphological or digestive competence over development. Pedal feeding may also occur during early life history stages of bay and sea scallops and has been described in *P. yessoensis* (Kingzett, 1993). Close association of the foot and labial palps at sizes where the gill is still very rudimentary (V.M. Bricelj, unpub. data; Barré, 2001) may allow these early stages to feed on benthic algal and microbial films in the natural environment as has been demonstrated in laboratory experiments with juvenile geoduck *Panope abrupta* (King, 1986) and Japanese scallop *Patinopecten yessoensis* (Reid *et al.*, 1992). Therefore, it is possible that ontogenetic changes in digestive enzyme activity may also reflect changing availability of the natural food supply resulting from different feeding strategies over development.

In the oyster, *Crassostrea virginica*, a ~12x increase in esterase activity was identified between larval and postlarval stages (Luna-Gonzalez *et al.*, 2004); however, the opposite trend was observed in the current study where specific activities of esterase were considerably higher in larvae than postlarvae. Lipid reserves, especially triacylglycerols (TAG), are considered essential for the metamorphic success of bivalve larvae (Gallager and Mann, 1986; Gallager *et al.*, 1986), including sea scallops (Pernet *et al.*, 2006), contributing ~ 60% of the energy necessary for metamorphosis in the rock scallop, *Crassadoma gigantea* (Whyte *et al.*, 1992). Therefore, high levels of esterase may be expected prior to and during metamorphosis to facilitate the catabolism of this energetic substrate. Protein catabolism provides a smaller proportion of energy during metamorphosis (~38% in *C. gigantea*) than that of lipids, but may become increasingly important during postlarval stages for growth and tissue restructuring.

Suspension-feeding bivalves feed on microalgae and are thus exposed to relatively low protein diets; therefore, although bivalves contain proteases, activities are generally low in comparison to those reported for finfish (Reid, 1978). Furthermore, there appears to be no dietary induction of protease activity in *Argopecten purpuratus*, whereas dietary induction of carbohydrase activity was apparent (Fernandez-Reiriz *et al.*, 2004). A relatively high protein diet [(7.84 μg protein (10^6 cells T-iso)$^{-1}$] increased growth rates in postlarval (~1.8 mm), but not juvenile (6.3 mm) *A. purpuratus* (Uriarte

and Farias, 1999), suggesting a greater requirement for protease activity in immediate post-metamorphic stages, and possibly explaining the increase in protease activity observed between larval and postlarval stages in this study. Furthermore, bay and sea scallops exhibited remarkably similar ontogenetic patterns in the ratio of esterase:protease activity, indicating a switch from lipid based metabolism in pediveliger larvae to a more protein-based metabolism in postlarvae that may be characteristic of pectinid development.

Alpha-amylase activity is commonly reported in a variety of bivalves species, including bay (Brock and Kennedy, 1992) and sea scallops (Wojtowicz, 1972), and has been associated with two separate genes in the oyster, *Crassostrea gigas*, one of which shows increased expression in the presence of elevated food concentrations (Huvet *et al.*, 2003). Alpha amylase is responsible for the degradation of starch, a common storage product in dinoflagellates and chlorophytes (Brock *et al.*, 1986). While α-amylase activity was detected in all developmental stages of both bay and sea scallops, a two-way ANOVA failed to detect a difference in α-amylase activity over development, although it is clear that the two species are exhibiting different ontogenetic patterns in the activity of this enzyme, especially at sizes <1.2 mm SH. A higher mean cellulase activity was also observed in bay scallops compared to sea scallops, which may be required to utilize the energy available in detrital material frequently encountered in their estuarine habitat, as has been described in the oyster *C. virginica* and the ribbed mussel *Geukensia demissa* (Lucas and Newell, 1984). While it is also possible that the higher activities of α-amylase and cellulase observed in bay scallops are associated with the higher temperature regime to which these animals are adapted, this is unlikely as the laminarinase activity was approximately half that observed in sea scallops, and a temperature effect would be expected across all enzymes tested.

Laminarinase activity has been widely reported in bivalves such as *Mytilus edulis*, *Mya arenaria*, *Macoma balthica* (Kristensen, 1972), *A. irradians* (Brock *et al.*, 1986), *P. magellanicus*, (Wojtowicz, 1972), and *Pecten maximus* (Stark and Walker, 1983). Although laminarin and chrysolaminarin are the main storage products of brown macroalgae, they are also storage products found in diatoms (Beattie *et al.*, 1961). Sea scallops are restricted to colder, and therefore often deeper, waters than bay scallops

found at the same latitude. Thus, it is likely that sea scallops are less exposed to vascular plant detritus common in estuarine systems, but may have more exposure to macroalgal detritus than bay scallops. Brown macroalgae are common in nearshore coastal systems and associated detrital particles are able to enhance growth of bivalves (Duggins *et al.*, 1989). Diatoms could potentially also make a greater contribution to the natural phytoplankton diet of sea scallops than bay scallops. Thus, the disparity in laminarinase activities detected between bay and sea scallops in this study may reflect an adaptation to different food supplies and requires further investigation. In fact, differential laminarinase activities have been reported for different species of amphipods adapted to different food sources, where the highest specific activities were reported in tissues of animals adapted to diets of diatoms and brown seaweeds (Johnston *et al.*, 2005). However, in both bay and sea scallops an ontogenetic increase in laminarinase activity is apparent up to sizes of ~1 mm in SH, as was also reported for post-settlement abalone, *Haliotis discus hannai* (Takami *et al.*, 1998). Though speculative, it is possible that the observed increase in laminarinase activity during early development is correlated with the increased ability of scallops, irrespective of species or the environment to which they are adapted, to capture and ingest diatoms. However, there is conflicting information as to whether digestive enzyme activity is induced by the presence of substrate in molluscs. Enzymatic activity during postlarval development of abalone, *H. discus hannai*, did not require the presence of the corresponding substrate (Takami *et al.*, 1998), but work conducted on juvenile cockles, *Cerastoderma edule,* and the scallop *A. purpuratus*, has shown a positive correlation between increased food quantity and/or organic content and enzymatic activity (Ibarrola *et al.*, 1996; Ibarrola *et al.*, 1998b; Fernandez-Reiriz *et al.*, 2004). Further work is necessary to determine if substrate availability induces an increase in enzymatic activity in bay and sea scallops, and if this may be responsible for the increased levels of laminarinase observed during early ontogeny of postlarval scallops.

Future studies should examine the activity of cellulase using natural detrital particles as substrate. This would allow a comparison with carboxymethyl cellulose as substrate that may better approximate enzymatic activities occurring in natural conditions. The examination of lysozymes, which hydrolyze the β1,4 glycoside bonds of

bacterial walls, would indicate whether bacteria is being utilized as a food source, as well as indicate the ability to defend against pathogenic bacteria.

5.5 Conclusions

Although the diet was held constant, major ontogenetic changes in enzymatic activity were determined in both scallops species, with the most pronounced differences occurring before postlarvae attained ~1-1.2 mm in SH. While the ontogenetic patterns of esterase and protease activity differed between bay and sea scallops, the esterase:protease ratio was strikingly similar between the two species. This possibly reflects a transition from a more lipid-based metabolism and dependence on lipid reserves during planktonic stages to increased proteolytic activity and high protein turnover associated with post-metamorphic growth. Carbohydrase activities differed between the two scallop species, with bay scallops exhibiting levels of α-amylase and cellulase activity approximately twice as high as sea scallops, while laminarinase activities were ~2x higher in sea scallops. This may be a result of adaptation to different particle assemblages associated with the different environments or habitats in which these two species are found.

5.6 Acknowledgements

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Chapter 6: Conclusions

Two binary, microalgal diet combinations have been identified that support excellent scallop growth across developmental stages and in both scallop species targeted by this study, thus fulfilling the first objective of this study. Growth rates observed for scallops offered Pav 459 / CHGRA and *P. pinguis* / CHGRA, rivaled those for 4-6 species diets in sea scallops, and were greater than those reported for field-deployed bay scallop juveniles. Previous studies on bivalve nutrition have attempted to identify algal species (Enright *et al.*, 1986; Wikfors *et al.*, 1992; Wikfors *et al.*, 1996; Feindel, 2000), or algae of modified biochemical composition (Parrish *et al.*, 1993; Parrish *et al.*, 1999) which may optimize growth and survival; however, growth rates attained on unialgal diets are frequently insufficient for commercial application. Supplementation of live algal diets with emulsions or microcapsules has also proved ineffective for commercial implementation due to cost, difficulty of storage, preparation and delivery, and/or bacterial contamination (reviewed by Brown *et al.*, 1989). Therefore, at present, most commercial bivalve hatcheries feed a variety of microalgal species to ensure that nutritional requirements are met. However, the associated monetary cost is high (Coutteau and Sorgeloos, 1993), and it is therefore advantageous to limit the number of microalgal species used while maintaining a high level of growth and survival. The Pav 459- and *P. pinguis*-CHGRA combination diets identified in this study meet these requirements for the culture of postlarval bay and sea scallops.

This study also identified *Pavlova pinguis* as an excellent substitute for Pav 459 in scallop diets, important given that Pav 459 has so far proved difficult to mass culture. Diets containing *P. lutheri*, commonly used in scallop hatcheries, yielded lower growth rates than the two other *Pavlova* species for both bay and sea scallops. A direct comparison of the biochemical composition of the three *Pavlova* species indicated strikingly similar biochemical profiles between Pav 459 and *P. pinguis*. However, the lack of detectable levels of n-6 DPA in *P. lutheri,* present at high levels in both *P. pinguis* and Pav 459, is the likely cause of the differential growth rates observed between diets containing these *Pavlova* species. Adoption of *P. pinguis* as a commercial diet is strongly suggested by the results of this study.

CHGRA also contributed significantly to dietary success of the Pav 459 and *P. pinguis* combinations (growth rates of sea scallops decreased 47-65% with the removal of CHGRA), likely through the contribution of AA and cholesterol. These compounds are both found at high levels in CHGRA, as well as in *Chaetoceros calcitrans* (Volkman *et al.*, 1989; Soudant *et al.*, 1998a), and may be common to *Chaetoceros* spp. Therefore, other species of this genus should be examined for inclusion in bivalve diets as they may also positively affect growth performance.

Both scallop species, regardless of dietary treatments or developmental stages, preferentially incorporated the n-6 PUFAs, AA and DPA, suggesting a nutritional requirement for these compounds that has not been previously addressed in bivalve nutrition studies. Arachidonic acid is involved in a variety of biochemical processes in molluscs such as immune response, reproduction and ion regulation (Freas and Grollman, 1980; Stanley-Samuelson, 1987; Delaporte *et al.*, 2003), and this may explain the positive contribution of AA-rich CHGRA to scallop growth. While enrichment of DPA in scallop tissues has been previously reported (Delaunay *et al.*, 1993), and it has been suggested that it may play a structural role similar to DHA (Soudant *et al.*, 1996a), its physiological role has yet to be elucidated. The present study contributes strong evidence that n-6 DPA, typically not quantified in previous shellfish and finfish studies, may play a key role in the nutrition of scallops. This may in future be found to extend to other bivalve species and finfish.

While we observed a relative enrichment of AA and DPA in both scallop species, this work was conducted on bulk lipid samples, and therefore it was not possible to determine if these compounds were being incorporated to serve primarily an energetic (neutral lipid) or structural (polar lipid) function. Furthermore, with animals of this size it was not possible to conduct tissue-specific sampling which would also give some insight into the physiological role these n-6 PUFA may play in postlarval scallops.

For this dissertation, a feeding trial was planned to supplement a unialgal CHGRA diet with lipid emulsions created from *P. pinguis* extracts, to test the hypothesis that positive growth supported by Pav 459 and *P. pinguis* combination diets was in fact due to algal lipid composition. Unfortunately, the growth trial was not successful; therefore, data is not included as a chapter in this dissertation, although it can be found in

appendix 2. No difference in growth rate was observed among different emulsion treatments (i.e. *P. pinguis*-based emulsion vs. a control emulsion prepared from olive oil). Growth rates of emulsion-fed scallops were identical to scallops fed a unialgal CHGRA diet, and much lower (~75%) than those offered a control microalgal diet of *P. pinguis* / CHGRA, suggesting that lipid emulsions were not ingested or assimilated by the sea scallops. A binary diet of *Isochrysis galbana* T-iso and CHGRA also yielded growth rates 73% lower than the *P. pinguis* / CHGRA diet (32 vs. 8 μm day^{-1}). This is an, interesting finding as *I. galbana* T-iso is commonly used in bivalve hatcheries, and a unialgal diet of *I. galbana* T-iso supported fairly high (125 μm day^{-1}) growth of 2 mm *A. irradians,* the same size as sea scallops used in this study (Lu and Blake, 1996). Further and more extensive work is necessary to examine the incorporation of n-6 PUFA into various scallop tissues and lipid fractions in order to elucidate their physiological function in postlarval bay and sea scallops. However, n-6 DPA is not available commercially in pure form (in contrast to EPA and DHA). Therefore, direct supplementation with this fatty acid is not feasible without bulk lipid extraction and purification from an n-6 DPA-rich source.

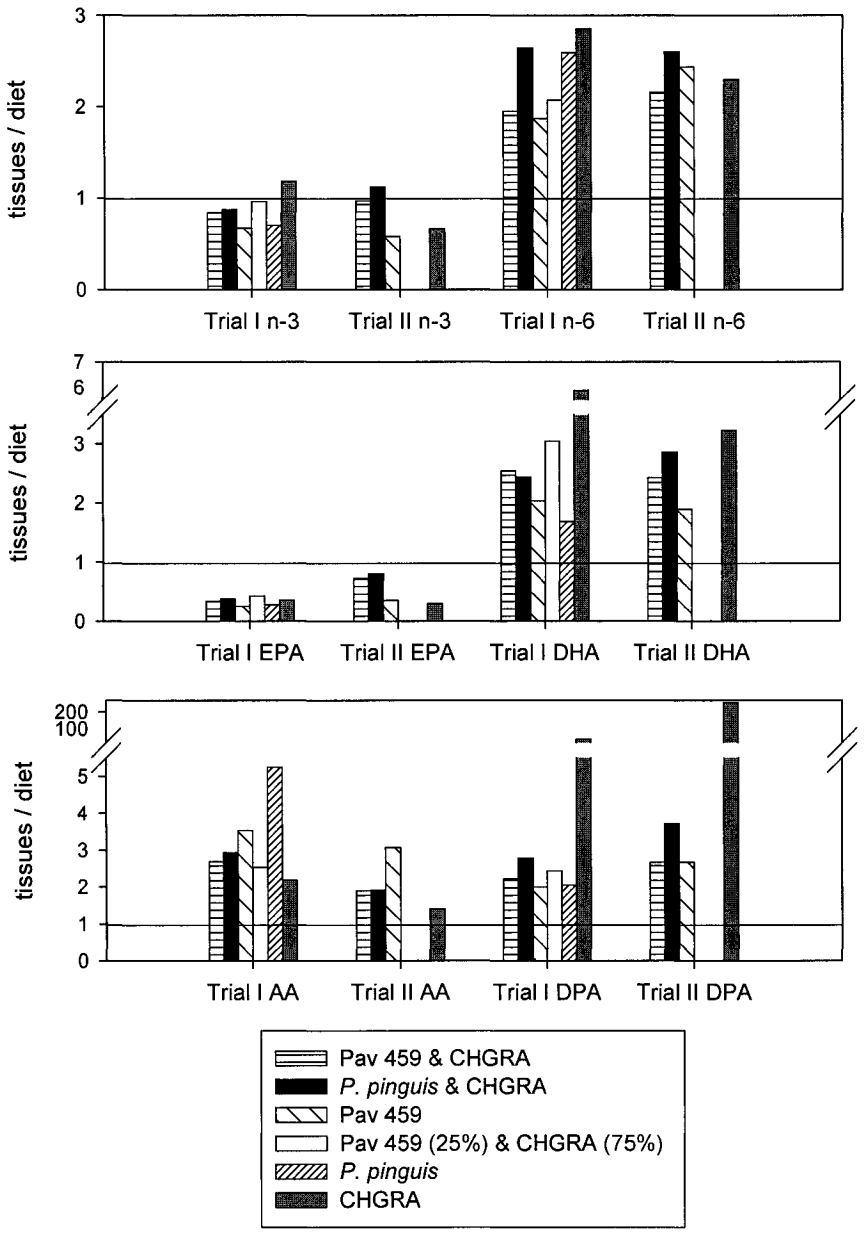
The second objective of this study was to examine stage-specific differences in dietary requirements of bay and sea scallops. Rankings among diets were similar for postlarval and juvenile bay scallops, indicating no stage-specific difference in dietary requirements for this species. However, substantial differences in enzymatic activity were observed over development in both bay and sea scallops, especially prior to ~1.2 mm in SH, and may be associated with morphogenesis of feeding organs. Species-specific differences in carbohydrase activity were also detected, possibly a result of the differing food supplies to which these two scallop species are adapted in their natural habitat. There is conflicting information in the literature as to whether enzymatic activity is inducible by the presence of substrate in molluscs (Ibarrola *et al.*, 1996; Ibarrola *et al.*, 1998b; Takami *et al.*, 1998; Fernandez-Reiriz *et al.*, 2004), and this requires further examination in postlarval scallops.

The third objective of this study was to examine species-specific differences, and some differences in dietary performance were observed, although the majority of dietary treatments exhibited a similar ranking between bay and sea scallops. The *T. striata* /

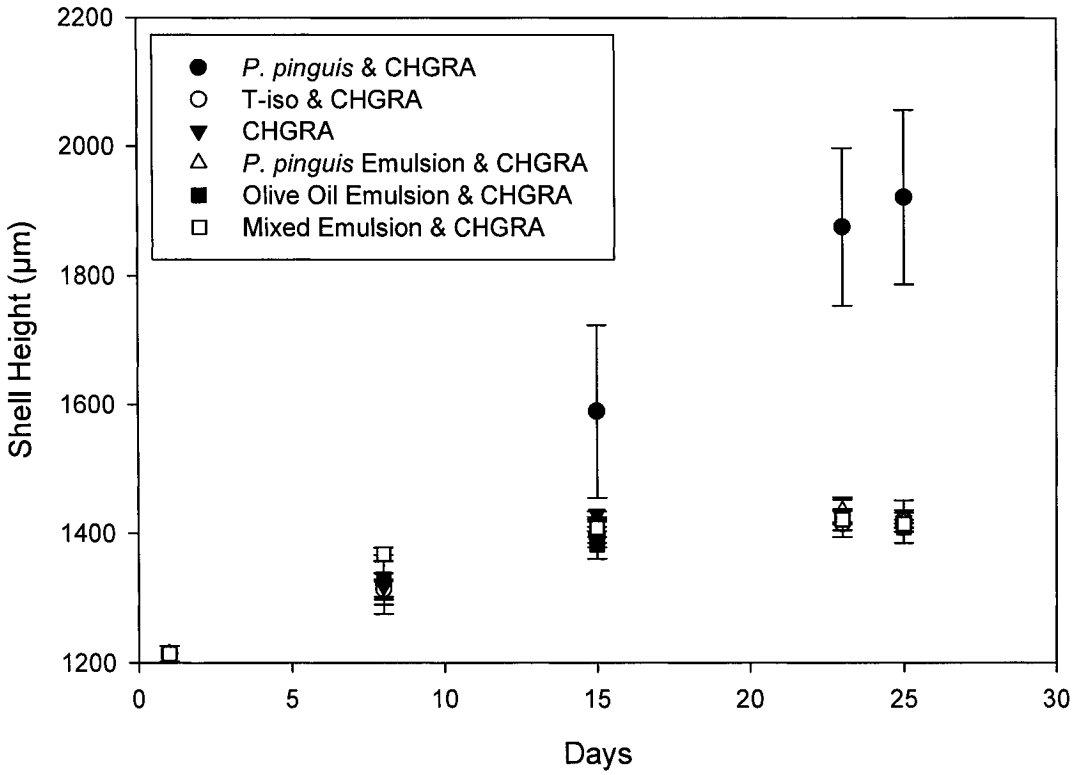
CHGRA combination was the second highest ranking diet in bay scallops, but supported sub-optimal growth rates in sea scallops, yielding weekly growth rates that decreased over time. This disparity in dietary ranking may be due to differential digestibility of algae between the two species (Le Pennec and Rangel-Davalos, 1985), but it is also possible that the observed differences result from species-specific PUFA requirements. The *T. striata* / CHGRA combination diet was limited in DHA, and while it has been suggested that bivalves require either EPA or DHA, but not necessarily both to sustain growth (Langdon and Waldock, 1981), this may not be true for sea scallops. This is further supported by the fact that DHA levels in sea scallop tissues were 3x higher than those found in bay scallops after exposure to the *T. striata* / CHGRA diet, suggesting a greater physiological need for this particular fatty acid in sea scallops, perhaps to aid in the maintenance of membrane fluidity at colder temperatures to which they are adapted.

In summary, strong evidence is provided by this study that n-6 PUFA, especially AA and DPA are required for optimal scallop growth although additional research is required to evaluate their role as essential fatty acids. Results of this study suggest that diets designed for pectinids, especially *A. irradians* and *P. magellanicus*, should include AA and DPA at levels of at least ~4-6% of total fatty acid, based on the algal concentrations employed in this study. DHA requirements, on the other hand, appear to be species-specific. Dietary levels of 1.5% appear insufficient to support growth of sea scallop postlarvae, and should therefore be increased to enhance growth performance of this species. Future work is required to determine if enzymatic activity is substrate-inducible in postlarval scallops, and whether or not this may influence dietary success at these early developmental stages.

Appendix 1: Comparison of PUFA tissue to diet ratios between Trials I and II in Chapter 4.



Appendix 2: Emulsion Growth Trial Data



Appendix 2 Figure 1: Growth trajectories of sea scallop, *Placopecten magellanicus*, postlarvae offered one of three microalgal diets consisting of *Pavlova pinguis*, *Isochrysis galbana* strain T-iso and *Chaetoceros muelleri* (CHGRA), and one of three different lipid emulsions presented in combination with CHGRA. Emulsions tested included an: 1) emulsion prepared from total lipid extract of *Pavlova pinguis* 2) emulsion prepared from an extract of olive oil and 3) a 50:50 mix of the *P. pinguis* and olive oil emulsions. Algal diets were offered at a biovolume concentration equal to 50 cells μl^{-1} T-iso, split 50:50 by volume in the case of two-species diets. Emulsion treatments were offered CHGRA at a concentration of 11.5 cells μl^{-1} (equivalent to 25 cells μl^{-1} T-iso) and lipid emulsions at a concentration of 6.1 mg (=2.3 ml) lipid per 110 l tank which is equivalent to the lipid concentration of *P. pinguis* in the *P. pinguis* & CHGRA combination diet. Scallops were reared in 110 l tanks in two identical downwellers for 25 days.

Appendix 2 Table 1: Mass and lipid composition of microalgae and emulsions used in emulsion feeding trial. nd=not detected

	P. pinguis	*I. galbana* T-iso	CHGRA	*P. pinguis* emulsion	Olive Oil emulsion
Dry Weight (pg cell^{-1})	19.53±2.31	37.40±4.01	54.82±2.93	--	--
Ash-Free Dry Weight (pg cell^{-1})	14.30±0.84	28.79±3.03	36.52±1.21	--	--
Total Lipid (mg g AFDW^{-1})	202.4±29.9	160.9±15.9	199.4±26.9	--	--
Total Fatty Acid (mg g AFDW^{-1})	97.7±18.2	85.3±10.4	129.3±26.9	--	--
Lipid Classes (% of total lipid)					
Hydrocarbons	0.38±0.19	0.48±0.45	0.78±0.42	1.29±0.59	1.83±0.64
Steryl/Wax Esters	0.27±0.08	0.28±0.20	0.07±0.07	nd	0.49±0.49
Ethyl Esters	nd	nd	nd	nd	nd
Methyl Esters	0.05±0.05	0.13±0.13	0.23±0.13	0.02±0.02	nd
Ethyl Ketones	0.13±0.07	4.92±1.20	0.20±0.10	nd	0.42±0.33
Methyl Ketones	0.11±0.06	0.64±0.57	0.29±0.21	0.39±0.24	nd
Glycerol Ethers	nd	nd	nd	nd	nd
Triacylglycerols	1.58±1.36	9.33±2.84	27.11±5.94	4.45±0.19	87.11±2.04
Free Fatty Acids	1.61±0.41	1.56±0.93	7.98±1.33	0.39±0.20	2.88±1.13
Alcohols	1.11±0.22	0.19±0.14	0.14±0.08	1.46±0.04	nd
Sterols	9.92±0.92	1.04±0.16	4.30±1.50	7.08±0.21	2.79±2.19
Diacylglycerols	0.54±0.13	0.10±0.05	0.38±0.31	3.96±1.38	0.29±0.29
Acetone Mobile Polar Lipid	31.48±1.24	30.89±1.41	21.39±1.88	33.87±0.31	0.43±0.43
Phospholipids	52.82±1.01	50.45±4.39	37.12±3.48	47.09±1.03	3.77±0.51
Fatty Acids (% of total fatty acid)					
14:0	17.95±1.72	16.49±0.36	10.75±0.59	15.81±0.12	0.01±0.00
TMTD	0.25±0.04	0.24±0.01	0.17±0.03	nd	nd
14:1	0.24±0.07	0.45±0.00	0.13±0.01	0.28±0.00	nd
15:0i	0.26±0.04	0.21±0.02	0.29±0.04	0.68±0.03	0.03±0.00
15:0ai	0.03±0.02	0.02±0.02	0.01±0.01	nd	nd
15:0	0.17±0.01	0.42±0.03	0.58±0.03	0.15±0.00	0.04±0.04
15:1	0.11±0.04	0.09±0.01	0.12±0.01	0.26±0.01	nd
16:0i	0.15±0.10	0.17±0.12	0.18±0.09	0.50±0.02	nd
16:0ai	0.31±0.07	0.32±0.01	0.25±0.03	0.40±0.04	nd
pristanic	nd	nd	0.01±0.01	nd	nd
16:0	4.30±0.64	9.64±0.42	12.92±1.94	4.36±0.01	12.04±0.06
16:1n11	0.60±0.14	0.63±0.01	0.33±0.22	0.71±0.09	nd
16:1n9	0.11±0.03	0.28±0.02	0.42±0.25	0.03±0.03	0.13±0.00

	P. pinguis	*I. galbana* T-iso	CHGRA	*P. pinguis* emulsion	Olive Oil emulsion
16:1n7	5.10±0.22	4.30±0.37	29.58±0.28	5.44±0.01	1.12±0.02
16:1n5	2.92±0.39	0.03±0.02	0.34±0.02	2.94±0.01	0.06±0.01
17:0i	0.03±0.02	0.04±0.03	0.53±0.08	0.32±0.02	0.01±0.01
17:0ai	0.12±0.01	0.73±0.14	4.84±0.30	0.18±0.05	0.02±0.02
16:2n4	1.93±0.03	0.47±0.05	2.57±0.18	1.84±0.01	nd
phytanic	0.01±0.01	nd	nd	nd	nd
17:0	0.01±0.01	0.05±0.01	0.06±0.01	nd	0.05±0.03
16:3n4	0.35+0.04	0.22±0.06	6.53±0.36	0.30±0.02	0.17±0.02
17:1	0.12±0.01	0.65±0.33	0.08±0.02	1.06±0.49	nd
16:4n3	nd	0.19±0.04	0.02±0.00	nd	nd
16:4n1	0.12±0.00	0.22±0.03	0.08±0.01	0.08±0.00	nd
18:0	0.12±0.01	0.17±0.05	1.08±0.10	0.08±0.01	3.16±0.02
18:1n11	0.01±0.01	0.16±0.03	0.01±0.01	nd	nd
18:1n9	0.16±0.03	10.02±1.30	0.52±0.04	0.12±0.01	70.61±0.56
18:1n7	0.59+0.21	1.76±0.14	0.46±0.07	0.21±0.00	3.68±0.36
18:1n6	nd	nd	nd	nd	nd
18:1n5	0.13±0.01	0.03±0.01	0.13±0.05	0.15±0.00	0.21±0.15
18:2n6	0.40±0.09	4.36±1.48	0.82±0.04	0.68±0.02	5.84±0.02
18:2n4	0.08±0.02	0.10±0.01	0.23±0.03	0.11±0.01	0.06±0.01
18:3n6	0.22±0.03	2.35±0.87	1.32±0.10	0.29±0.00	nd
19:0	0.02±0.02	0.05±0.03	nd	nd	nd
18:3n4	0.07±0.00	0.03±0.03	nd	0.08±0.01	0.03±0.01
18:3n3	1.98±0.28	4.24±0.47	0.02±0.02	1.94±0.01	0.61±0.11
18:4n3	13.31±0.98	21.37±2.67	0.40±0.01	11.27±0.12	0.20±0.14
18:4n1	nd	0.01±0.01	0.01±0.01	0.07±0.01	nd
20:0	0.04±0.01	nd	0.08±0.00	0.03±0.03	0.42±0.03
18:5n3	nd	0.29±0.29	nd	0.07±0.05	nd
20:1n11	nd	1.27±0.35	nd	nd	nd
20:1n9	nd	nd	nd	nd	0.27±0.02
20:1n7	nd	nd	0.06±0.01	0.03±0.03	0.08±0.08
20:2α	nd	nd	nd	nd	0.03±0.03
20:2β	nd	nd	nd	nd	0.02±0.02
20:2n6	nd	0.08±0.07	0.02±0.01	0.03±0.03	0.01±0.01
20:3n6	0.02±0.01	0.03±0.02	0.08±0.01	0.05±0.05	nd
21:0	nd	nd	nd	nd	0.13±0.09
20:4n6	0.81±0.27	0.15±0.02	5.89±0.45	0.51±0.02	0.01±0.01
20:3n3	nd	0.14±0.02	nd	0.02±0.02	nd
20:4n3	0.02±0.02	0.02±0.02	0.05±0.02	0.65±0.09	nd
20:5n3	21.17±0.89	0.62±0.02	15.38±0.83	29.66±0.25	0.01±0.01
22:0	0.02±0.00	0.03±0.03	0.24±0.03	nd	0.09±0.02
22:1n11(13)	0.01±0.01	nd	nd	nd	0.01±0.01
22:1n9	0.02±0.02	0.27±0.07	0.02±0.02	nd	0.13±0.12
22:1n7	0.01±0.01	0.08±0.04	0.02±0.02	nd	0.06±0.06

	P. pinguis	*I. galbana* T-iso	CHGRA	*P. pinguis* emulsion	Olive Oil emulsion
22:2NMIDa	0.02±0.01	nd	nd	nd	nd
22:2NMIDb	0.01±0.01	nd	nd	nd	nd
21:5n3	nd	0.13±0.02	nd	0.02±0.02	0.01±0.01
23:0	0.38±0.03	0.26±0.07	0.22±0.20	0.04±0.04	0.31±0.08
22:4n6	nd	0.05±0.05	nd	0.20±0.08	0.02±0.02
22:5n6	5.64±0.34	1.97±0.11	0.04±0.01	6.53±0.03	0.16±0.04
22:4n3	0.02±0.02	nd	nd	nd	nd
22:5n3	0.02±0.02	0.10±0.03	0.06±0.01	0.20±0.08	0.02±0.02
24:0	0.02±0.01	0.05±0.01	0.42±0.03	0.04±0.02	0.08±0.05
22:6n3	9.24±1.39	13.96±0.07	1.60±0.07	11.48±0.05	nd
24:1	nd	nd	0.02±0.02	0.19±0.02	0.05±0.02
Sum SAT	24.20±0.95	28.64±0.57	32.46±1.44	22.58±0.10	16.38±0.18
Sum MUFA	10.13±0.19	20.00±0.47	32.24±0.41	11.42±0.49	76.43±0.45
Sum PUFA	65.41±0.73	51.12±1.01	35.12±1.52	66.00±0.40	7.19±0.29
Sum n-3	55.75±0.39	41.07±3.41	17.52±0.92	55.22±0.41	0.86±0.23
Sum n-6	7.08±0.53	8.99±2.61	8.18±0.41	8.30±0.04	6.04±0.05

Appendix 2 Table 2: Mass and lipid composition of postlarval sea scallops from the emulsion growth trials. nd= not detected

	Initial	*P. pinguis* and CHGRA	*I. galbana* T-iso and CHGRA	CHGRA	*P. pinguis* emulsion and CHGRA	Olive Oil emulsion and CHGRA	Mix emulsion and CHGRA
Mass and bulk biochemical propoerties							
Dry Weight (μg scallop^{-1})	121.9±1.9	375.4±89.8	149.7±3.3	178.1±7.9	151.3±7.1	152.2±0.3	168.4±4.1
Ash-Free Dry Weight (μg scallop^{-1})	30.3±0.7	102.8±30.7	30.3±0.8	42.8±3.8	33.0±1.4	33.2±2.1	36.2±1.9
Total Lipid (mg g AFDW^{-1})	95.4±7.9	132.3±6.5	89.5±22.2	37.5±0.4	37.1±12.0	45.8±5.8	49.1±7.2
Total Fatty Acid (mg g AFDW^{-1})	66.9±5.6	87.8±4.1	46.6±12.3	18.1±1.1	16.8±6.3	26.7±2.3	24.1±4.0
Lipid Class Composition (% of total lipid)							
Hydrocarbons	0.14±0.06	0.90±0.18	2.67±0.80	1.22±0.24	1.87±1.03	1.53±0.36	0.66±0.36
Steryl/Wax Esters	nd	0.14±0.14	nd	0.51±0.51	1.09±1.09	0.07±0.07	0.78±0.52
Ethyl Esters	nd	nd	nd	nd	nd	nd	nd
Methyl Esters	nd	nd	nd	0.50±0.50	0.53±0.53	1.08±0.46	nd
Ethyl Ketones	0.81±0.64	0.09±0.04	0.13±0.09	0.29±0.29	4.75±4.75	0.22±0.22	1.64±1.64
Methyl Ketones	0.44±0.44	0.63±0.51	0.47±0.47	0.30±0.30	nd	1.22±0.44	nd
Glycerol Ethers	0.87±0.08	0.46±0.00	nd	nd	nd	nd	0.05±0.05
Triacylglycerols	33.23±0.79	27.89±1.80	7.31±0.26	1.81±0.57	0.37±0.37	1.50±0.19	0.74±0.46
Free Fatty Acids	0.63±0.63	2.09±1.25	2.03±0.68	0.34±0.13	2.18±0.21	3.57±0.48	1.69±1.43
Alcohols	nd	0.03±0.03	0.57±0.47	0.47±0.47	0.60±0.13	0.79±0.73	1.11±0.44
Sterols	9.44±0.27	12.22±1.29	21.28±0.28	27.22±4.67	30.62±2.34	16.33±2.71	25.97±3.98
Diacylglycerols	1.06±0.56	0.80±0.55	nd	0.27±0.19	0.38±0.38	0.20±0.20	0.16±0.16
Acetone Mobile Polar Lipid	6.74±0.79	9.00±3.04	14.00±3.25	9.73±2.88	9.78±3.00	1.02±0.71	6.74±4.29
Phospholipids	46.64±1.84	45.74±0.08	51.54±2.39	57.35±1.60	47.83±4.49	72.49±2.51	60.45±0.13

	Initial	*P. pinguis* and CHGRA	*I. galbana* T-iso and CHGRA	CHGRA	*P. pinguis* emulsion and CHGRA	Olive Oil emulsion and CHGRA	Mix emulsion and CHGRA
Fatty Acid Composition (% of total fatty							
14:0	4.29±0.00	4.80±0.06	2.08±0.17	1.47±0.13	1.39±0.14	1.14±0.05	1.16±0.05
TMTD	0.62±0.06	0.92±0.06	0.84±0.20	1.24±0.02	1.06±0.43	1.21±0.07	1.41±0.11
14:1	nd	0.10±0.01	0.59±0.53	0.13±0.06	nd	nd	nd
15:0i	0.18±0.02	0.14±0.03	0.08±0.01	0.30±0.12	0.21±0.00	0.21±0.02	0.11±0.11
15:0ai	nd	0.03±0.03	nd	0.11±0.11	0.04±0.04	0.11±0.11	0.08±0.08
15:0	0.31±0.00	0.21±0.07	0.40±0.01	0.52±0.05	0.49±0.01	0.46±0.02	0.47±0.01
15:1	0.20±0.03	0.13±0.01	0.27±0.02	0.36±0.13	0.36±0.09	0.32±0.06	0.28±0.05
16:0i	nd	nd	0.06±0.00	nd	nd	nd	nd
16:0ai	nd	nd	nd	0.05±0.05	0.03±0.03	0.08±0.00	0.07±0.01
pristanic	0.06±0.06	nd	0.03±0.03	0.08±0.00	0.05±0.05	nd	nd
16:0	14.88±0.11	11.43±0.07	13.36±0.54	13.43±0.28	13.57±0.22	13.87±0.25	13.88±0.32
16:1n11	0.10±0.10	nd	nd	nd	nd	nd	nd
16:1n9	0.12±0.02	nd	0.09±0.00	0.11±0.02	0.13±0.01	0.10±0.10	0.21±0.02
16:1n7	13.55±0.09	7.95±0.20	3.93±0.48	3.64±0.11	3.75±0.22	3.61±0.06	3.25±0.23
16:1n5	0.37±0.01	0.71±0.03	0.27±0.00	0.29±0.04	0.39±0.02	0.29±0.07	0.28±0.02
17:0i	0.25±0.01	0.09±0.02	0.26±0.03	0.33±0.02	0.43±0.04	0.50±0.10	0.43±0.01
17:0ai	1.14±0.05	1.27±0.05	0.35±0.05	0.32±0.07	0.34±0.02	0.30±0.03	0.24±0.00
16:2n4	0.76±0.02	0.63±0.02	0.11±0.01	0.11±0.03	0.19±0.03	0.09±0.09	0.20±0.08
phytanic	nd	nd	nd	nd	nd	nd	nd
17:0	0.33±0.00	0.42±0.00	0.57±0.00	0.65±0.01	0.68±0.01	0.79±0.01	0.79±0.03
16:3n4	0.83±0.03	0.68±0.01	0.04±0.04	0.03±0.03	0.03±0.03	nd	nd
17:1	2.40±0.46	2.11±0.02	3.37±0.60	2.85±0.12	2.42±0.50	3.48±0.30	2.78±0.32
16:4n3	0.55±0.39	0.28±0.00	0.18±0.05	0.68±0.48	0.57±0.39	0.16±0.03	0.34±0.19
16:4n1	nd	nd	nd	0.04±0.04	nd	nd	nd

	Initial	*P. pinguis* and CHGRA	*I. galbana* T-iso and CHGRA	CHGRA	*P. pinguis* emulsion and CHGRA	Olive Oil emulsion and CHGRA	Mix emulsion and CHGRA
18:0	3.05±0.00	2.96±0.14	5.46±0.17	6.04±0.32	5.75±0.42	6.72±0.09	6.22±0.45
18:1n11	0.13±0.01	0.11±0.04	0.09±0.01	0.18±0.04	0.13±0.03	0.17±0.01	0.15±0.01
18:1n9	2.22±0.06	0.60±0.03	2.07±0.20	0.94±0.24	1.20±0.24	1.98±0.01	1.28±0.04
18:1n7	7.47±0.10	6.12±0.01	6.03±0.19	6.00±0.28	5.36±0.19	5.86±0.22	5.51±0.05
18:1n6	nd	nd	nd	nd	nd	nd	nd
18:1n5	0.21±0.01	0.39±0.02	0.26±0.03	0.26±0.01	0.29±0.01	0.32±0.01	0.30±0.02
18:2n6	1.78±0.10	0.38±0.02	0.81±0.10	0.49±0.18	0.54±0.11	0.51±0.03	0.41±0.01
18:2n4	0.59±0.00	0.73±0.02	0.27±0.01	0.26±0.00	0.29±0.03	0.24±0.03	0.24±0.02
18:3n6	0.71±0.01	0.39±0.02	0.29±0.03	0.17±0.02	0.13±0.05	0.23±0.05	0.16±0.04
19:0	nd	nd	nd	nd	nd	nd	nd
18:3n4	nd	0.13±0.01	nd	nd	nd	nd	nd
18:3n3	1.23±0.02	0.66±0.04	0.24±0.01	0.25±0.04	0.41±0.05	0.42±0.01	0.42±0.03
18:4n3	2.63±0.23	3.31±0.14	1.29±0.11	3.87±0.08	3.07±0.64	4.31±0.24	3.45±0.42
18:4n1	nd	nd	nd	nd	nd	nd	nd
20:0	nd	nd	0.08±0.01	0.06±0.00	0.12±0.01	0.16±0.08	0.04±0.04
18:5n3	nd	nd	nd	nd	nd	0.74±0.74	nd
20:1n11	1.19±0.02	1.07±0.00	2.05±0.17	2.06±0.14	2.54±0.01	1.86±1.31	2.81±0.21
20:1n9	0.25±0.01	0.41±0.00	1.24±0.04	1.01±0.10	0.52±0.17	0.34±0.34	0.49±0.02
20:1n7	0.94±0.01	1.16±0.05	2.24±0.28	1.44±0.23	1.13±0.12	1.19±0.01	1.11±0.11
20:2α	0.35±0.00	0.16±0.00	0.29±0.04	0.96±0.01	0.57±0.48	0.28±0.04	0.42±0.00
20:2β	0.20±0.01	0.24±0.01	0.51±0.06	0.57±0.04	1.70±0.50	1.54±0.07	1.85±0.16
20:2n6	0.47±0.01	0.13±0.05	0.57±0.02	0.26±0.01	0.26±0.01	0.34±0.00	0.31±0.03
20:3n6	0.24±0.00	0.17±0.01	0.19±0.02	0.07±0.02	0.42±0.42	0.08±0.08	0.09±0.03
21:0	nd	nd	0.06±0.06	0.07±0.07	0.14±0.08	0.04±0.04	0.10±0.03
20:4n6	7.18±0.00	5.85±0.16	10.36±0.44	11.14±0.46	11.24±0.79	11.62±0.38	12.30±0.44

	Initial	*P. pinguis* and CHGRA	*I. galbana* T-iso and CHGRA	CHGRA	*P. pinguis* emulsion and CHGRA	Olive Oil emulsion and CHGRA	Mix emulsion and CHGRA
20:3n3	0.18±0.01	0.24±0.05	0.36±0.12	0.62±0.15	1.06±0.17	0.66±0.30	0.62±0.26
20:4n3	nd	0.10±0.03	0.05±0.05	0.09±0.09	0.32±0.03	0.32±0.03	0.34±0.07
20:5n3	12.58±0.02	20.69±0.02	9.88±0.43	11.11±1.56	10.66±0.54	8.16±1.00	8.43±1.01
22:0	0.07±0.07	0.09±0.01	0.20±0.07	0.24±0.06	0.28±0.09	0.37±0.05	0.22±0.06
22:1n11(13)	nd	nd	0.04±0.04	0.31±0.26	0.59±0.19	0.72±0.27	0.91±0.02
22:1n9	0.49±0.01	0.52±0.01	0.57±0.06	0.77±0.05	0.14±0..14	0.24±0.24	nd
22:1n7	0.05±0.05	0.14±0.02	0.24±0.24	0.08±0.01	0.38±0.12	0.30±0.09	0.29±0.02
22:2NMIDa	0.14±0.00	0.07±0.01	0.39±0.14	0.13±0.05	0.35±0.08	0.29±0.03	0.27±0.02
22:2NMIDb	0.45±0.00	0.59±0.01	0.90±0.19	1.01±0.07	0.91±0.05	1.02±0.01	0.99±0.07
21:5n3	0.34±0.05	0.39±0.03	0.30±0.02	0.29±0.12	0.27±0.09	0.42±0.06	0.39±0.04
23:0	0.06±0.06	0.23±0.01	0.33±0.33	0.85±0.81	0.52±0.26	0.64±0.38	0.26±0.26
22:4n6	0.44±0.07	0.67±0.05	1.67±0.11	1.99±0.20	2.37±0.07	1.68±0.00	2.04±0.14
22:5n6	3.41±0.03	6.57±0.14	5.58±0.24	4.82±0.01	4.38±0.30	4.86±0.07	5.29±0.01
22:4n3	nd	nd	0.17±0.06	nd	nd	nd	0.21±0.21
22:5n3	0.39±0.01	0.87±0.04	0.96±0.23	1.78±0.13	1.91±0.35	1.31±0.17	1.22±0.11
24:0	nd	0.14±0.01	0.50±0.37	0.20±0.00	0.73±0.44	0.18±0.01	0.13±0.02
22:6n3	9.61±0.04	11.83±0.01	16.59±0.58	12.86±0.49	12.91±0.63	13.17±0.33	14.72±0.38
24:1	nd	nd	nd	nd	0.26±0.03	nd	nd
Sum SAT	24.56±0.14	21.82±0.06	23.80±0.23	24.64±0.53	24.72±0.49	25.58±0.31	24.21±0.27
Sum MUFA	29.70±0.42	21.50±0.20	23.34±0.51	20.43±0.68	19.59±0.25	20.76±1.96	19.67±0.35
Sum PUFA	45.06±0.28	55.76±0.20	51.98±0.52	53.60±0.17	54.59±0.72	52.45±2.20	54.72±0.51
Sum n-3	27.52±0.17	38.36±0.14	30.02±0.46	31.54±0.78	31.18±0.02	29.67±1.71	30.13±0.26
Sum n-6	14.23±0.05	14.17±0.09	19.46±0.22	18.95±0.43	19.35±0.65	19.32±0.61	20.61±0.55

Appendix 5: Methods for Carbohydrate Sampling and Determination.

Carbohydrate determination modified from Dubois *et al.*, 1956

Prepared by Melissa Anderson, Institute for Marine Biosciences
Amended by Lisa Milke (4/2006) for samples on filters and use of acid digestion

Materials

- Dextrose, D-Glucose standard solution, 120 mg/100 mL
- Phenol, 5% w/v in dH_20
- Concentrated sulfuric acid
- 1M sulfuric acid
- Acid resistant cuvettes
- Spectrophotometer
- Vortex mixer
- Water bath, 30°C
- Volumetric flasks to prepare dilution series
- Graduated glass pipettes
- Micropipettes
- 20 mm OD glass test tubes and rack
- Lab coat, safety glasses, gloves
- 15 ml glass tubes with Teflon lined caps
- pre-ashed GF/C filters
- boiling water bath with test tube holder

Sampling for carbohydrate analysis

- For sampling small animals. Filter a known quantity of animals into pre-ashed GF/C filters. Make sure that 1) filters are pre-weighed so that animal weight determinations can be made after samples are lyophilized or 2) that a known number/volume of animals are sampled in association with an independent sampling of animal weights so that total sample weight can be determined.

- For sampling larger animals. Place known quantity (and ultimately dry weight) of animals in 15ml glass tube with Teflon cap. Remove any water with Pasteur pipette.

- For algal sampling. Take a known volume of algal sample and filter onto pre-ashed GF/C filter under gentle vacuum. If larger sample volumes are required, centrifuge to pellet algal cells, filter supernatant onto GF/C filter and place filter and algal pellet into 15 ml glass tube. Again, arrange for some method of sample weight determination.

- For samples in tubes, place into liquid nitrogen if available. Transfer and store at -80C until processing. Samples on filters can be folded in foil and immediately placed into -80C freezer to reduce space required by glass tubes.

Calculations for quantities to use

- Based on the assumption that shell weight consists of ~95% of the dry weight (DW) of a whole clam (shell weight of a juvenile bay scallop, 10 mm, was 93% DW), then dry tissue content of a 50 mg clam is 2.5 mg DW. Carbohydrate content consists of approximately 8-20% of tissue dry weight in oysters and scallops, respectively. Taking into account that the spring experimental clams were in poor health, carbohydrate content could be assumed to be as low at 5%. Carbohydrate compositions as mg of DW for these three percentages are listed in the table below along with the concentrations achieved with the extraction in 1 mL of sulfuric acid.

	5%	**10%**	**20%**
mg DW	0.125	0.25	0.50
µg/mL	125	250	500

- If carbohydrate content is expected to be as low as 5% DW then a minimum of 3 clams should be pooled together to ensure sufficient amounts of carbohydrates are available for triplicate solutions. Between 10 and 70 µg of carbohydrate should be present in each triplicate sample ready for phenol-sulfuric acid addition.

  -When experiments were run using postlarval (~2mm) scallops of marginal condition, 2ml packed volume of scallops was sampled (500-1200 scallops). This was far in excess (at least 20x) of what was necessary for detection, and carbohydrate concentrations ranged from 23-44 mg g AFDW^{-1} (~4% of organic weight).

  - During the postlarval scallop experiment, when algal samples were taken, 30-50 ml of algal culture (concentrations ranged from 4.9-25.2 x10^{6} cells ml^{-1}) were sampled. This was in excess (at least 5x) of what was required for detection. Carbohydrate concentration of algae (mg g AFDW^{-1}) was as follows: Pav 459 (107), P. pinguis (112) and CHGRA (141).

Lyophilization

- Always ensure that the samples remain on dry ice during this period. If not already done, place filters or postlarval animals into 15 ml glass tubes. Cover each tube with a kimwipe secured with a rubber band. Place tube in –80C freezer until ready for lyophilization.

- Plug in vacuum pump and turn on refrigeration unit and top shelf.

- Set the manual temperature control to –20°C and push 'run'.

- Once full vacuum and –20°C temperature have been reached, retrieve samples from the –80°C freezer.

- Release vacuum and place samples on top shelf

- Stop the run and switch from control to auto with the following settings:

  Segment 1, Ramp speed 0.1 °C/min, Hold 0°C, Time 99.9 min
  Segment 2, Ramp speed 0 °C/min, Hold 0°C, Time 99.9 min

- Press 'run'.

- After 24 hrs, release vacuum, remove samples, replace kimwipes with original caps, place tube in a container containing dessicant, place in the –80°C freezer, and turn off freeze dryer

Reagent Preparation

1. 5% (w/v) Phenol
   - Weigh 10 g of solid phenol into a 200 mL volumetric flask
   - Add 150 mL of dH_2O
   - Swirl until phenol is dissolved and bring to 200 mL with dH_2O
   - Store in an amber bottle with a teflon cap at 4°C

2. Standard glucose solution, 120 mg/100 mL
   - Weigh 120 mg of solid glucose into a 100 mL volumetric flask
   - Add 75 mL of dH_2O
   - Add a small amount of benzoic acid crystals
   - Swirl until glucose and benzoic acid are dissolved and bring to 100 mL with dH_2O
   - Store in glass bottle at 4°C

Sample Processing

- Retrieve sample from -80°C freezer and always keep on ice.

- Weigh sample by placing them on a tared weigh paper if sample weight is not already known.

- Calculate the volume of 1M H_2SO_4 needed, based on lyophilized weight of sample, to obtain a specific percent homogenate (w/v). For example, 1 g lyophilized weight in 5 mL buffer gives a 20% homogenate.

-For all samples processed from the sea scallop postlarval experiment (both tissues and algae) a volume of 5ml was used.

Ex: A 10% (1 g in 10 mL) homogenate is needed for a sample that weighs 0.3624 g.

$$\frac{1\text{ g}}{5\text{ mL}} = \frac{0.3624\text{ g}}{x}$$

$$x = 1.812\text{ mL of buffer}$$

- Add the homogenizing volume of sulfuric acid to the glass tube containing sample. Take care when adding acid to tissues (violent bubbling is often observed and acid has to be added gradually or sample will overflow).

- Use permanent marker to mark the level of sulfuric acid and sample in glass tube. Cover this marking with cellophane tape to make sure it doesn't rub off during boiling and handling.

- Cap tubes at a tightness that allows for some gas release, but minimizes loss.

- Place tubes into boiling water bath located in the fume hood. Boil until material appears well digested. If possible, run trials prior to sample analysis to determine the boiling time that facilitates maximum carbohydrate determination.
  -For sea scallop postlarval experiment, scallops were boiled for 30 minutes, and algal samples were boiled for 3 hours.

- If there was excessive loss of liquid, milli-Q water can be added to return sample to its pre-boil volume as marked on the tube wall.

- Remove between 10 and 70 µg of sugar to a straight walled test tube (20 mm OD) and bring up to 1 mL with dH_2O, vortex. Prepare this solution in triplicate and follow the phenol-sulfuric acid addition steps for each tube.
  -For all postlarval scallop samples run this volume was 50µl, for algal samples the volume was 200 µl.

- Prepare a blank by substituting dH_2O for the sugar solution (Can use the 0 mg/mL glucose solution from the standard curve as the blank) and follow the phenol-sulfuric acid addition steps.

Phenol-Sulfuric Acid Addition

- Add 1 mL of 5% phenol to sugar solution, vortex

- Rapidly add 5 mL of sulfuric acid to the solution by directing the stream against the surface of the liquid to ensure good mixing (do not direct the stream against the side of the test tube)

- Vortex

- Let tube stand for 30 min in a test tube rack and cover entire rack with a sheet of aluminium foil (prevents any dust from entering tubes)

- Vortex

- Place in a 25°C to 30°C water bath for 10 to 20 min

- Turn on spectrophotometer and let it warm up for at least 10 min. Set wavelength to 490 nm

- Reference the blank using the spectrophotometer (use quartz cuvettes)

- Pour the unknown carbohydrate solution into a cuvette

- Read absorbance in triplicate for each unknown sugar solution using the spectrophotometer in reference to the blank solution

- The colour created by the furan derivatives is stable for several hours and readings may be performed later if necessary

- Compare absorbance reading to a standard curve created for D-glucose

Standard Curve

The standard curve is used to determine the carbohydrate concentration of the unknown sample. The phenol-sulfuric acid assay is a colorimetric assay that uses the Beers law which states that if a compound absorbs light, then the concentration of this compound can be determined by how much light it absorbs. Serial dilutions of D-glucose are performed and assayed with the same phenol-sulfuric acid method to establish a linear regression between absorbance and concentration from which unknown carbohydrate concentrations can be interpolated.

- Create the standard curve by preparing the following dilutions of standard 120 mg/100 mL glucose solution into separate test tubes as listed for each concentration in the following table.

	µg glucose/1 mL					
	0	**10**	**30**	**60**	**90**	**120**
Glucose solution (µL)	0	8.33	25	50	75	100
Distilled water (µL)	1000	991.67	975	950	925	900

- Follow the steps for phenol-sulfuric acid addition as used for the unknown carbohydrate samples

- The standard curve remains valid providing the same solutions are always used. If new solutions are incorporated into the method then a new standard curve should be created

- It is not necessary to create a new standard curve for each subsequent analysis, however it is best to check that the standards remain constant over time. This is achieved by reading a couple of standard concentrations, i.e. 10 and 90 µg/mL, and checking whether the readings match those of the original standard curve

Unknown carbohydrate calculations

Calculate the concentration of the unknown sample by using the regression equation obtained from the standard curve.

EX: The average OD for the unknown sample is 0.521. The standard curve regression equation is:

$$y = 0.0086x + 0.0061$$

By solving for x, the unknown carbohydrate concentration is determined.

$$x = \frac{0.521 - 0.0061}{0.0086}$$

$$x = 59.87 \text{ µg/mL}$$

Appendix 6: Methods for Lipid Sampling and Extraction.

Lipid sampling

Rinse glass tubes with methanol and then chloroform, dry upside down in drying rack lined with tin foil and then recap. Caps MUST be Teflon lined (We use 16mm Kimble Glass Inc 73808-15415, Fisher number 3-340-77B)

Add tissue or algal sample on filters to tubes making sure that all forceps, scalpels, etc that are used are rinsed with chloroform. Make sure that tissue is pushed to the bottom of the tube (chloroform rinsed Pasteur pipette works well for this).

Re-cap tube and immediately place in liquid nitrogen.

Remove tube from liquid nitrogen, and add 1-2 ml of HPLC grade chloroform to tubes using a bottle top dispenser set to appropriate volume. Use minimum volume necessary to cover samples, and record the volume used. HPLC grade chloroform is marked with "Distilled in Glass" on the label

Strip tubes with nitrogen, making sure that the needle used is cleaned with chloroform to avoid any contamination.

Tightly cap tube and wrap with Teflon tape.

Mark level of chloroform with a Sharpee to make sure that there is no loss during shipment.

Store upright in –80C freezer until analysis.

Lipid extraction

This lipid extraction protocol is adopted from Parrish, C.C., 1999. Determination of total lipid, lipid classes and fatty acids in aquatic samples. In: Arts, M.T., B.C. Wainman (Ed.), Lipids in freshwater ecosystems. Springer Verlag, New York, pp. 4-12.

Protocol is based on initial sample containing 2ml of chloroform.

Add approximately 1 ml of ice cold methanol.

Grind the tissue or filter into a pulp quickly with a Teflon or metal ended rod. Wash the rod with approximately 1ml of 2:1 chloroform:methanol and then with 0.5ml of chloroform-extracted water. (The amount for the methanol, chloroform and water vary depending on the size of the sample. In a large sample the amounts may be doubled. The important thing is that the ratio of the methanol:chloroform:water is about 1:2:1). Recap the tube and sonicate the mixture for 4 minutes.

If the sample is in a test tube that can be centrifuged, then it is centrifuged for 2-3 minutes at 3000-5000 RPM (2000 RPM for 15 ml glass tubes). If the test tube is too large to be centrifuged, then flush the sample with nitrogen, cap and place the sample in a -20°C freezer until the sample separates.

Remove the organic later (bottom layer) using the double pipetting technique involving placing two pipettes inside one another. Remove all of the organic layer. Transfer the removed organic layer into a pre-cleaned vial (pre-cleaned 3 times with methanol and then 3 times with chloroform).

Wash the inside pipette into the vial containing the organic layer with 3ml of ice cold chloroform (1.5 ml washing down the outside and 1.5 ml washing down the inside). Wash the outside pipette into the tube containing the aqueous layer with a second 3 ml (again 2 washes for each the inside and the outside of the pipette) of ice cold chloroform. Re-sonicate and centrifuge the samples and double pipette when separated, using new pipettes each time. Repeat at least three times and pool all organic layers. After removing the organic layer for the third time, wash both pipettes into the vial containing the organic layer.

Concentrate down to volume under a gentle stream of nitrogen, seal with Teflon tape and store at -80°C.

If there is any color left to the organic layer, or if the filter is not sufficiently ground, the entire procedure may be repeated.

Appendix 7: Methods for Sample Preparation and Soluble Protein Determination

Sample processing and DC Protein Assay for determination of soluble protein

Prepared by Michel Goguen, Institute for Marine Biosciences
Modified by Lisa Milke (6/2006)

Sample homogenization

Shellfish samples are processed using the Polytron homogenizer. This mechanical procedure permits us to completely grind up the shell as we will be looking at the total protein concentration which includes the small percentage that comes from the shell.

Prepare ammonium bicarbonate buffer in which samples will be homogenized. It is volatile so we can easily do a buffer exchange if we need to concentrate our samples.

100mM ammonium bicarbonate

volume	Ammonium bicarbonate
75 ml	0.49375g
125 ml	0.9875g
250 ml	1.975g
500 ml	3.95g

- Put about 70% of the desired volume (ddH_2O) in flask
- pH to 7.8 with HCl
- Top up to the desired volume with ddH_2O
- Store in fridge immediately ($4^{\circ}C$). Must be made fresh each day!

Calculate the percentage of your homogenate.

Ex: 1g in 10 ml gives a 10% homogenate. So, subtract the weight of your pre-weighed tube from the weight of the same tube now containing your sample. This gives you the wet weight.

Ex: We want a 20% homogenate of a sample that weighs 0.3624g
20% = 1g in 5 ml so the volume will be 1.812 ml

*Turn on centrifuge at $4^{\circ}C$ before homogenizing as it needs to cool down.

Material needed for homogenizing

- Ice bucket
- 2 medium 250 ml beakers
- Fresh buffer (put in ice)
- 1 ml pipette with 1 ml tips

Retrieve lyophilized samples from $-80^{O}C$ freezer and always keep it on ice.

Bring sample and all material in oscilloscope room.

First check if the Polytron has the right size probe. If the sample is in cryovials, use the small probe. If you have 15 ml or 50 ml falcon tubes, use the large probe. You must also anticipate overflow. If the sample +buffer volume is already close to the top, putting in the probe may cause overflow as it needs to go all the way down to properly pulverize the shell. If this is the case, transfer all sample to a larger tube (preferably before adding the buffer volume so use your judgment!!). Often the sample will slide out as it begins to thaw on the sides of the tube. Then use a small volume of buffer to rinse out any debris left over.**Keep track of this volume as it will be part of the total final volume of the homogenate. (In the example we said 1.812 ml added to 0.3624g for 20%). The volume you use to wash out debris is part of this 1.812 ml so keep track!

Changing the probe on the Polytron

- Take the probe wrench and loosen the probe screw while holding it so it doesn't drop on the counter.
- Store probe in the appropriate box (drawer under Polytron unit)
- When putting another probe, align the bump on the top of it with the hole in the Polytron unit. Hold in place and screw it somewhat tight.

Before homogenizing, put ice in one of the beakers (keeps the sample cool while grinding) and put ddH2O in the other. (To wash the Polytron after all homogenizing is done or also between samples if you have many).

Because our samples have hard shell, take a glass rod and homogenize manually until everything becomes a pulp. The broken shell pieces can now pass through the Polytron. **If you need to wash off bits from the glass rod keep track of the volume you use!

Add the appropriate buffer volume the sample with the 1 ml pipette. (Or add the extra to the volume you already put in to get your final calculated volume).

Put the sample in the ice beaker and make sure it is tightly packed.

Take the ice beaker and insert the probe as far as you can. (overflow?)

Turn 'on' the Polytron. (Green switch on top)

With your other hand, turn the knob somewhat slowly from zero to 10-11 (full force) for about 5 seconds.

Turn the knob back to zero and pull out the probe from the sample. Always keep the sample under the probe as some homogenate drips out. Wait for around 20-30 seconds and the sample will cool down it the ice.

Repeat this for as many times as is necessary which is usually the shell is becomes an obvious uniform fine powder (no large shell pieces evident). Often 6 to 8 times is fine. Surely no more if the animals being processed have a very thin shell.

When the homogenate seems acceptable, Take 500ul-1ml of buffer with the pipette. Then, while holding the sample under the probe, squirt buffer in the grinding slots to wash out any homogenate left in the hollow probe. **Again, this is fine because we will take note of the final volume indicated on the sample tube.

Take note of the final volume on the tube.

Place the homogenized sample in the ice bucket.

Take the beaker full of ddH2O and insert the probe. Turn the Polytron to full speed for 30 seconds to wash it thoroughly. This makes sure that it will not rust. If you have more samples, take a kimwipe and simply dab the bottom of the probe to remove excess water. Repeat the grinding steps for all your samples.

After the last water wash, discard the water and turn off the Polytron.

Aliquoting samples

Before centrifuging, take samples of a uniform mix of homogenate and shell to determine the total protein.

Take a 100 ul pipette and, with gloves on, close the top of the tube with your thumb and vortex for 5 seconds. Remove from vortex, remove your thumb from the opening and immediately pipette the mix. (Take at least 2-3 samples of the mix and put them on ice)

Centrifuge your processed sample at $4^{O}C$ as fast as the rotor allows for 10 minutes and then return the sample in the ice bucket.

Aliquot a series of 50 and 100 ul samples of the supernatant into labeled 0.6 ml microcentrifuge tubes. Place these into a sample storage box and store at $-80^{O}C$. These aliquots will be used for soluble protein determination and various enzyme assays.

Soluble Protein Determination

The DC protein assay is a modified Lowry assay with reagents commercially available from BioRad (Hercules, California). It is based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent. Proteins cause a reduction of the Folin reagent, producing a blue color with maximum absorbance at 650 nm.

A standard curve should be prepared using γ-globulin as standard (stock solution of 1.39 mg ml^{-1}).

γ-globulin concentration mg ml^{-1}	µl buffer	µl γ-globulin
1.26	10	90
0.98	30	70
0.70	50	50
0.42	70	30
0.14	90	10
0	100	--

Dilute sample as appropriate to be within range of the standard curve. For postlarval scallops, samples were diluted 1:2 in buffer.

Pipette 5µl of homogenate into each well of microtitre plate (do this in triplicate). Make sure to use buffer blank as well.

Add 25µl of Reagent A (Alkaline copper tartrate). Mix in microplate reader for 5 seconds.

Add 200 µl of Reagent B (Folin reagent).

Mix in microplate reader, and read at 750nm.

To determine soluble protein divide mean OD for each sample (averaged over triplicate wells) and divide by slope of the standard curve. Then multiply by the dilution factor used in the assay. Units will be ug protein ul homogenate $^{-1}$.

Appendix 8: Methods for Determination of Esterase Activity

Esterase assay

Prepared my Michel Goguen, Institute for Marine Biosciences
Amended by Lisa Milke (6/2006)

The esterase assay is a kinetic reaction. The enzyme activity (color development) is monitored over a determined period of time opposed to developing the color and then reading the OD.

Prepare the ammonium bicarbonate buffer. It must be made up fresh each day as it is volatile. The pH used is 7.8 which is standard for the buffer.

100mM ammonium bicarbonate

volume	Ammonium bicarbonate
75 ml	0.49375g
125 ml	0.9875g
250 ml	1.975g
500 ml	3.95g

- Put about 70% of the desired volume (ddH_2O) in flask
- pH to 7.8 with HCL
- Top up to the desired volume with ddH_2O
- Store in fridge immediately ($4^{O}C$). Must be made fresh each day!

Prepare the buffer + detergent; Add 1 ml of 10 % Triton-x (stock solution in Neil's lab) to 9 ml of 100mM ammonium bicarbonate and the Triton-x will have a final 1% concentration.

Substrate; 10mM p-nitrophenyl myristate in 100% ethanol (10 mg of p-nitrophenyl myristate dissolved in 2.86ml of 100 ethanol). Aliquot in 250 ul centrifuge tubes and store at $-20^{o}C$. When the esterase attacks the substrate, the nitrophenyl group is cleaved and gives a yellow color as it is no longer linked to another compound.

Retrieve the samples to be run from the $-80^{o}C$ freezer and centrifuge them at $4^{o}C$ to pellet any debris present. Put the samples in ice until you are ready to run the assay. It is suggested to prepare the samples last to minimize any degradation.

In a microplate, first add the buffer and the samples to the wells (**In triplicate) with a micropipette. Then add the buffer + detergent with a multi-tip dispenser to avoid any bubble formation. Don't forget the blank (no sample).

	Negative control	Sample
Buffer (ul)	**50**	**25**
Buffer + detergent (ul)	**50**	**50**
Sample (ul)	**0**	**25**
Substrate (ul)	**4**	**4**
Total (ul)	**104**	**104**

Incubate the microplate in the microplate reader at 30°C for 15 minutes.

(Click on 'control' then 'incubator', set it at 30 degrees and then click on 'On'. Click again on 'control' and then 'close drawer'. The microplate will remain inside the reader at 30°C until you are ready to add the substrate)

At the same time, put the substrate tube in a 30°C water bath for 15 minutes.

During the 15 minutes, prepare the software to be able to immediately begin the assay after the substrate is added.

- Turn on the plate reader.
- Click on the soft pro max software.
- Click on 'setup', then 'kinetic', then set OD at 405 nm.
- Click on 'template' then choose 'unknown'.
- Highlight the sample or samples and name them individually.
- Set program to shake prior to reading and between readings
- Set OD max =1
- Set to Vmax (milli-units per minute) which then becomes values in the output

In this case the blank is a 'negative control' and will be read along with the other samples. So **do not set it as a blank.** Just name it 'negative control' and set it as an 'unknown'.

- Click on 'OK'

Take out the microplate and add the substrate using a multi-tip dispenser. Go then immediately to the microplate reader, insert the plate and click on 'read' to begin the assay. The incubator is left on to maintain the temperature during the assay, and the assay was allowed to run for 60 minutes.

Appendix 9: Methods for Determination of Proteolytic Activity

Azocasein hydrolysis assay

Prepared my Michel Goguen, Institute for Marine Biosciences
Amended by Lisa Milke (6/2006)

The azocasein assay is a two-step process, which takes two days to complete. It is a good idea to reserve a 37°C incubator before starting. The samples to use are the supernatant aliquots of the homogenized samples. (See sample processing protocol). You cannot use the samples having the uniform mix of shell. Any debris will interfere with the assays. Each sample should be done in triplicate if possible.

Prepare ammonium bicarbonate buffer. It must be made up fresh each day as it is volatile. The pH used is 7.8 which is standard for the buffer. pH trials were conducted with citrate buffers of lower pH, down to 3.5, but lower activity was observed. Therefore, assay was conducted in the same buffer that samples were originally homogenized in.

100mM ammonium bicarbonate

volume	Ammonium bicarbonate
75 ml	0.49375g
125 ml	0.9875g
250 ml	1.975g
500 ml	3.95g

- Put about 70% of the desired volume (ddH_2O) in flask
- pH to 7.8 with HCl
- Top up to the desired volume with ddH_2O
- Store in fridge immediately (4°C). Must be made fresh each day!

Next make up the substrate: Add 25 mg of azocasein (kept at 4°C in Neil's lab) to 5ml of 100mM Ammonium bicarbonate. Place this in a small beaker covered in glass. Heat on low heat with stirring. May take upwards of 6 hours for complete dissolution. The azocasein is dissolved when the red flakes are no longer visible.

1. In three 500 ul tubes, add 10 ul of sample + 40 ul of buffer. Make sure the three tubes are labeled properly.

2. As a blank, add 50 ul of buffer in three 500 ul tubes (NO SAMPLE)
   (The 10 ul of sample is replaced by all buffer)

3. To ALL of these tubes, add 125 ul of substrate. Then, place in the incubator for 19 hours (overnight) at 30°C and rotate at 60-70 rpm. Make sure the tubes are in a secure tube tray.

4. The next day, add 50 ul 20% TCA (Trichloroacetic acid) which is a stop solution for the reaction. Then place the samples on ice for 15 minutes. Try to prepare your 20% TCA before the 19 hours are up to be consistent.

   20% TCA:

   In Neil's lab there is a 50% stock, and we want 20%.

   C1 x V1 = C2 x V2

   So, (50%) x V2? = (20%) x (10ml Total)

   V2 = 20 x 10 / 50 = 4 ml of 50% + 6 ml of water = 10 ml total

5. Centrifuge the samples at 13500 rpm for 5 minutes.

6. Carefully transfer 100 ul of the supernatant in microplate wells. Each tube done in triplicate goes in one well. Make sure to remember which samples go in which well.

7. Add 100 ul of 0.5 M NaOH to the wells containing your samples

0.5 M NaOH

X = 0.5 M x 40 (mol. Weight) x 0.5 liters = 10g in 500 ml

The low pH of the TCA precipitates the proteins and also stops the reaction. Now the high pH of the NaOH will enhance the color of the assay.

8. In a microplate reader, read samples at 450 nm. **Don't forget to set the blank as 'blank' and not as a sample.

    - Turn on the plate reader.
    - Click on the soft pro max software.
    - Click on 'setup', then 'endpoint', then set OD at 450 nm.
    - Click on 'template' then choose 'unknown' instead of 'standards'.
    - Highlight the sample or samples and name them individually.
    - Highlight the blank then choose 'blank' instead of 'unknown'. This will subtract the blank color from our sample. Then click on 'done'.
    - Click on 'Read'

9. To calculate the 'units of protease activity', divide the 'Mean result' (OD with the blank subtracted) by the incubation time. In our case, it is 19 hours. Then multiply this result by 1000 because 1 unit of activity is equal to a change of 0.001 O.D. per hour.

Ex; 0.097 / 19 hours = 0.0051 and then x 1000 = 5.1

This is the activity for the amount of protein in the assay. Now if we take the protein concentration and multiply it by the volume of sample loaded we get;

3,6514ug/ul x 5 = 18.257 ug protein in the assay

Now divide the units of protease activity by this total amount of protein and get 'units protease activity/ug protein'

Ex; 5.1 'units of protease activity' / 18.257 ug 'total' protein

= 0.2793 units of protease activity / ug protein

Appendix 10: Methods for Determination of α-amylase Activity

α- amylase assay using the p-hydroxybenzoic acid hydrazide reducing sugar assay (PAHBAH)

Prepared my Michel Goguen, Institute for Marine Biosciences
Amended by Lisa Milke (6/2006)

The determination of alpha-amylase activity is based on measuring reducing sugars released by enzymes under optimum pH, temperature and duration developed in the laboratory. Alpha-amylase reduces starch, glycogen, poly- and oligosaccharides into glucose units.

Prepare the following reagents:

1. NaOH (1 M stock) calculate: 1 mol/l x 40.00 g/mol x 0.250 l = 10 g
   (MW of NaOH = 40 g/mol)
   (10 g in ddH_2O made up to 250 ml)

2. 1 M Bismuth nitrate stock. This is also a weight /volume solution so it needs to be toped up to 25 ml after reagents are added.

In about 20 ml add:

- 1 M Bismuth nitrate = 1.212 g
- 1 M sodium potassium tartarate = 7.055 g
- 3 M NaOH = 1 g

*top up to 25 ml

The chemicals are better dissolved with slight heating for a few minutes. The solution is then stable indefinitely at room temperature.

The day of the assay, prepare the PAHBAH reagent.
Weigh 0.761 g of PAHBAH (p-hydroxybenzoic acid hydrazide) in 100 ml of 0.5 M NaOH. Then add 100 ul of Bismuth nitrate stock. This reagent cannot be stored and must be made fresh each day.

**Do not forget to add the bismuth nitrate because it is needed for color change. The first thing to do if the assay gives no color is to verify if the Bismuth nitrate was added.

Standard curve

As this is an endpoint colorimetric assay, a standard curve is needed to calculate the amount of reducing sugar (as glucose equivalents) in our assay. A 1 mg/ml glucose solution is used as a standard.

Standard 1mg/ml glucose solution (ul)	ddH_2O (ul)	0.8 M Sodium citrate buffer pH 6.2 (ul)	[Glucose] Values entered in standard curve (ug)	PAHBAH reagent Stop sol. (ul)
0	90	10	0	1500
5	85	10	5	1500
10	80	10	10	1500
15	75	10	15	1500
20	70	10	20	1500

For the glucose standard, measure 100 mg in 100 ml of ddH_2O.

The buffer used is 0.8 m sodium citrate buffer, pH 6.2.

*** buffer used was 0.8M citrate buffer to obtain final reaction pH of 6.5**

A O.8 M (tri)sodium citrate = 0.8 M x 294.1 (MW) x 0.250 (vol.)

= 58.82 g make up to 250 ml with ddH_2O

B O.8 M citric acid = 0.8 M x 210.14 (MW) x 0.250 (vol.)

= 42.03 g make up to 250 ml with ddH_2O

Place about 100 ml of **A** in a beaker and add **B** to give a pH of 6.2
*(no need to adjust final volume as both **A** and **B** are 0.8 M)*

For the PAHBAH stop reagent, 1.5 ml is added to the sample when we want to stop the incubation. For the alpha-amylase assay for tissues of bay and sea scallop postlarvae, it has been determined that 30 minutes is optimal.

Preparing the standard curve

In five 1.6 ml aliquots, labeled 0, 5, 10, 15 and 20 ug, add the appropriate volumes of water, buffer and glucose standard.

Add 1.5 ml of PAHBAH to the tubes.
(*Once the PAHBAH has been added, you don't have to read it immediately. It can remain on the bench for a few hours*)

To develop the color, put samples in a second water bath and heat at $70^{O}C$ for 10 minutes. There should be a gradient of yellow from 0 ug glucose to 20 ug glucose.

Pipette 100 ul of the solution in 3 microplate wells. Do this for each of the five tubes.

Bring the microplate to the microplate reader.

- Turn the Microplate reader 'on'.
- Click on the soft pro max icon
- Click on 'assay' and then 'default protocol'
- Click on 'setup' on the assay task bar and then on 'endpoint"
  Then change the absorbance to 410 nm.
- Click on 'template' then click on 'standards'
- Highlight the appropriate wells and enter the glucose amounts in the concentration box (with edit, change ug/ul to ug).
- when all data is entered, click on 'done'
- Click on 'Read'

**After the readings are done, the plate will eject and the ODs for each well will appear on the screen. Go in 'view' and click on 'graph'. This will give the graph of our standard curve. The linearity is given on the bottom right and is represented by R^. A 1.000 is perfect but this rarely happens. Usually a good curve will give at least 0.990 or more. Also keep track of the slope which is represented by B. We'll use this to determine the amount of reducing sugar in our sample now that we have a linear standard curve. This is done using the following formula: $Y = mx + b$

Where y = OD, m = slope, x = concentration and b = y axis intercept

So, if we isolate x (unknown) we get; $x = \frac{y - b}{m}$

When we read our unknown samples, we'll prepare a blank which will be set at zero. So the formula then becomes OD/slope = x concentration.

Unknown samples

For the substrate we use soluble starch (Sigma, S-2630). A 1% concentration is standard and is used throughout the literature. (Several different sources of starch were tested and Sigma, S-2630 provided by far the best response).

Ex: 0.25 g in 25 ml = 1 % Mix with ddH2O, aliquot 500 ul in 500 ul tubes and freeze at $-20^{O}C$ until use.

**Boil for 5 minutes with mixing to dissolve starch and have a clear solution. (Adjust the volume if there is evaporation) Let solution cool. As it has been boiled, it is sterile and can be kept for up to 3 weeks on the bench. (in either an opaque bottle or wrap in foil). Always pour out a working solution each time in a beaker as to not contaminate the starch stock. Freezing sometimes causes the starch to clump (re-bind together) and you would need to boil again.

The samples to use are the supernatant aliquots of the homogenized samples.

(See sample processing protocol). You cannot use the samples having the uniform mix of shell. Any debris will interfere with the assays. The protein concentration must eventually be done on this sample also to then correlate the enzyme activity to the total protein.

'Heat Killed' enzymes (boiled for at least 30 minutes) are typically used as a blank. Unfortunately in our case even after several hours of boiling, we were getting some evidence of activity. Therefore, as our blank we adopted a 'time zero' blank. Samples were added to substrate solution, but the reaction was immediately stopped with the addition of PAHBAH reagent.

The samples are prepared as follows;

	Enzyme control (blank)	Enzyme assay
1% starch (substrate)	50 ul	50 ul
0.8 M sodium citrate buffer pH 6.2	10 ul	10 ul
ddH_2O	30 ul	30 ul
Appropriately diluted enzyme	____	10 ul
Appropriately diluted control enzyme	10 ul	____
Total volume	100 ul	100 ul

In 1.6 ml tubes, pipette the appropriate amounts of water, buffer and substrate. **Always prepare each sample in triplicate!!**

Pre-incubate these tubes at the assay temperature ($30^{O}C$) for 5 minutes in a water bath.

Add the enzyme (sample) to the appropriate tubes and incubate for the determined duration (30 minutes).

After 30 minutes, add 1500 ul (1.5 ml) of PAHBAH to each of the tubes. This will stop the reaction.

To develop the color, heat the samples in a water bath at approximately 70^{O}C for 10 minutes.

Remove and allow 2-3 minutes of cooling.

Pipette 100 ul of each tube in one microplate well. Keep track of the wells as you need to enter them in the software.

Bring the microplate to the microplate reader

- Turn on the plate reader.
- Click on the soft pro max software.
- Click on 'setup', then 'endpoint', then set OD at 410 nm.
- Click on 'template' then choose 'unknown' instead of 'standards'.
- Highlight the sample or samples and name them individually.
- Highlight the blank (the heat killed enzyme) then choose 'blank' instead of 'unknow'. This will subtract the blank color from our sample. Then click on 'done'.
- Click on 'Read'

Once the readings are done, click on 'view' and then 'unknowns'.
A table will appear as the following (example);

Sample	wells	values	outliers	result	Mean result	Std. Dev.	[sugar]
Sample 1	A1 A2 A3	0.105 0.116 0.110					

To get the mean result (average of the 3 wells) click on the 'mean result' title (it will turn blue) and then press enter. A new window will appear where we replace (results) with (values). The mean result will appear.

Sample	wells	values	outliers	result	Mean result	Std. Dev.	[sugar]
Sample 1	A1 A2 A3	0.105 0.116 0.110			0.110		

Now click on [protein] where you erase (results) and you enter the formula to calculate the protein concentration. Example:

(the following is typed) Meanresult/0.024

0.024 is the slope in the standard curve (example)

The following result will appear ****

Sample	wells	values	outliers	result	Mean result	Std. Dev.	[sugar]
Sample 1	A1 A2 A3	0.105 0.116 0.110			**0.111**		**4.5833**

***this is the amount of reducing sugar in our sample in ug.

Calculating specific activity (SA)

The first thing is to go back and take note of the protein concentration previously determined. This is the amount of protein per microliter of homogenate.

Example: 3.6514 ug/ul

Multiply this by the amount of sample used in the amylase enzyme assay which is 10 ul. This gives us the total amount of protein (in ug) in the assay.

3.6514 ug/ul x 10 = 36.514 ug protein in the assay

Now go to the standard curve to determine how much reducing sugar (glucose) you have. **Done with softpro max (See previous page).

Again, using the formula y = mx + b, where y = OD, m = slope and b = the y axis intercept, we isolate x (concentration of compound x) which results in; $x = \frac{y - b}{m}$

If b (the blank) is set at zero then it becomes $x = \frac{OD}{\text{Slope}}$

This gives the amount of reducing sugar in ug. If we now divide this by 180 which is the molecular weight of glucose, we get u moles. This divided by the time of incubation (min) gives us the activity expressed in u mole/minute

So, 4.5833 ug divided by 180 (glucose MW) = 0.02546 u moles

Then, 0.02546 u mole divided by 30 minutes (time of assay)

= 2.828×10^{-4} u moles/minute

Finally, divide this by the total ug of protein in the assay. This is the specific activity.

$\frac{2.828 \text{ x } 10\text{-}4 \text{ u moles/minute}}{36.514 \text{ ug protein}} = 7.745 \times 10^{-6}$ u mole/minute/ug protein

This our specific activity expressed as SA

Appendix 11: Methods for Determination of Cellulase Activity

Cellulase assay using the p-hydroxybenzoic acid hydrazide reducing sugar assay (PAHBAH)

Prepared my Michel Goguen, Institute for Marine Biosciences
Amended by Lisa Milke (6/2006)

The determination of cellulase activity is based on measuring reducing sugars released by enzymes under optimum pH, temperature and duration developed in the laboratory.

Cellulose is made of linear β (1→4) anhydrous glucose units.

Prepare the following reagents:

1. NaOH (1M stock) calculate: 1M x 40.00(MW) x 0.250(vol.) =

10 g in ddH_2O up to a volume of 250 ml

2. 1M Bismuth nitrate stock. This is also a weight /volume solution so it needs to be toped up to 25 ml after reagents are added.

In about 20 ml add:

- 1M Bismuth nitrate = 1.212 g
- 1M sodium potassium tartarate = 7.055 g
- 3M NaOH = 1 g

*Top up to 25 ml

The chemicals are better dissolved with slight heating for a few minutes. The solution is then stable indefinitely at room temperature.

The day of the assay, prepare the PAHBAH reagent.
Weigh 0.761 g of PAHBAH (p-hydroxybenzoic acid hydrazide) in 100 ml of 0.5 M NaOH. Then add 100 ul of Bismuth nitrate stock. This reagent cannot be stored and must be made fresh each day.

**Do not forget to add the bismuth nitrate because it is needed for color change. The first thing to do if the assay gives no color is to verify if the Bismuth nitrate was added.

Standard curve

As this is an endpoint colorimetric assay. A standard curve is needed to calculate the amount of reducing sugar in our assay. A 1 mg/ml glucose solution is used as a standard.

Standard 1mg/ml glucose solution (ul)	ddH_2O (ul)	0.5 M phosphate buffer pH 4.3 (ul)	[Glucose] Values entered in standard curve (ug)	PAHBAH reagent Stop sol. (ul)
0	90	10	0	1500
5	85	10	5	1500
10	80	10	10	1500
15	75	10	15	1500
20	70	10	20	1500

*Standard curve can be made up in the absence of substrate. (Substituted water for substrate)

For the glucose standard, measure 100 mg in 100 ml of ddH_2O. Aliquot 500 ul in 500 ul tubes and freeze at $-20^{O}C$ until use.

- For the substrate we use CMC (carboxymethyl cellulose), as only cellulases are capable of liberating reducing sugars from it. A 2% concentration is standard and is used throughout the literature.

-Ex: 0.5 g in 25 ml = 2% Mix with ddH2O, aliquot 500 ul in 500 ul tubes and freeze at $-20^{O}C$ until use.

*** buffer used is 0.5M phosphate buffer, pH 4.3 to obtain a final reaction pH of 6.0.**

O.5M phosphate buffer = 0.5M x 137.99 (MW) x 0.250 (vol.)
(NaH_2PO_4)
= 17.24g in about 200 ml ddH_2O

adjust pH with NaOH

For the PAHBAH stop reagent, 1.5 ml is added to the sample when we want to stop the incubation. For the cellulase assay for tissues of bay and sea scallop postlarvae, it has been determined that 30 minutes is optimal.

Preparing the standard curve

In five 1.6 ml aliquots, labeled 0, 5, 10, 15 and 20 ug, add the appropriate volumes of water, buffer and glucose standard. Once all these have been added, add the substrate.

Add 1.5 ml of PAHBAH to the tubes.
(Once the reaction has been stopped, you don't have to read it immediately. It can remain on the bench for a few hours)

To develop the color, put samples in a second water bath and heat at $70^{O}C$ for 10 minutes. There should be a gradient of yellow from 0 ug glucose to 20 ug glucose.

Pipette 100 ul of the solution in 3 microplate wells. Do this for each of the five tubes.

Bring the microplate to the microplate reader.

- Turn the Microplate reader 'on'.
- Click on the soft pro max icon
- Click on 'assay' and then 'default protocol'
- Click on 'setup' on the assay task bar and then on 'endpoint"
  Then change the absorbance to 410 nm.
- Click on 'template' then click on 'standards'
- Highlight the appropriate wells and enter the glucose amounts in the concentration box (with edit, change ug/ul to ug).
- when all data is entered, click on 'done'
- Click on 'Read'

**After the readings are done, the plate will eject and the ODs for each well will appear on the screen. Go in 'view' and click on 'graph'. This will give the graph of our standard curve. The linearity is given on the bottom right and is represented by R^. A 1.000 is perfect but this rarely happens. Usually a good curve will give at least 0.990 or more. Also keep track of the slope which is represented by B. We'll use this to determine the amount of reducing sugar in our sample now that we have a linear standard curve. This is done using the following formula: $Y = mx + b$

Where y = OD, m = slope, x = concentration and b = y axis intercept

So, if we isolate x (unknown) we get; $x = \frac{y - b}{m}$

When we read our unknown samples, we'll prepare a blank which will be set at zero. So the formula then becomes OD/slope = x concentration.

Unknown samples

The samples to use are the supernatant aliquots of the homogenized samples.

(See sample processing protocol). You cannot use the samples having uniform mix of shell. Any debris will interfere with the assays. The protein concentration must eventually be done on this sample also to then correlate the enzyme activity to the total protein.

'Heat Killed' enzymes (boiled for at least 30 minutes) are typically used as a blank. Unfortunately in our case even after several hours of boiling, we were getting some evidence of activity. Therefore, as our blank we adopted a 'time zero' blank. Samples were added to substrate solution, but the reaction was immediately stopped with the addition of PAHBAH reagent.

The samples are prepared as follows;

	Enzyme control (blank)	Enzyme assay
2% CMC (substrate)	50 ul	50 ul
0.5M phosphate buffer pH 4.3	10 ul	10 ul
ddH_2O	30 ul	30 ul
Appropriately diluted enzyme	____	10 ul
Appropriately diluted control enzyme	10 ul	____
Total volume	100 ul	100 ul

In 1.6 ml tubes, pipette the appropriate amounts of water, buffer and substrate. **Always prepare each sample in triplicate!!**

Pre-incubate these tubes at the assay temperature ($30^{O}C$) for 5 minutes in a water bath.

Add the enzyme (sample) to the appropriate tubes and incubate for the determined duration (30 minutes).

After 30 minutes, add 1500 ul (1.5 ml) of PAHBAH to each of the tubes. This will stop the reaction.

To develop the color, heat the samples in a water bath at approximately $70^{O}C$ for 10 minutes.

Remove and allow 2-3 minutes of cooling.

Pipette 100 ul of each tube in one microplate well. Keep track of the wells as you need to enter them in the software.

Bring the microplate to the microplate reader

- Turn on the plate reader.
- Click on the soft pro max software.
- Click on 'setup', then 'endpoint', then set OD at 410 nm.
- Click on 'template' then choose 'unknown' instead of 'standards'.
- Highlight the sample or samples and name them individually.
- Highlight the blank (the heat killed enzyme) then choose 'blank' instead of 'unknown'. This will subtract the blank color from our sample. Then click on 'done'.
- Click on 'Read'

Once the readings are done, click on 'view' and then 'unknowns'.
A table will appear as the following (example);

Sample	wells	values	outliers	result	Mean result	Std. Dev.	[sugar]
Sample 1	A1 A2 A3	0.105 0.116 0.110					

To get the mean result (average of the 3 wells) click on the 'mean result' title (it will turn blue) and then press enter. A new window will appear where we replace (results) with (values). The mean result will appear.

Sample	wells	values	outliers	result	Mean result	Std. Dev.	[sugar]
Sample 1	A1 A2 A3	0.105 0.116 0.110			0.110		

Now click on [protein] where you erase (results) and you enter the formula to calculate the protein concentration. Example:

(the following is typed) Meanresult/0.024

0.024 is the slope in the standard curve (example)

The following result will appear

Sample	wells	values	outliers	result	Mean result	Std. Dev.	[sugar]
Sample 1	A1 A2 A3	0.105 0.116 0.110			0.111		4.5833

***this is the amount of reducing sugar in our sample in ug.

Calculating specific activity (SA)

The first thing is to go back and take note of the protein concentration previously determined. This is the amount of protein per microliter of homogenate.

Example: 3.6514 ug/ul

Multiply this by the amount of sample used in the cellulase enzyme assay which is 10 ul. This gives us the total amount of protein (in ug) in the assay.

3.6514 ug/ul x 10 = 36.514 ug protein in the assay

Now go to the standard curve to determine how much reducing sugar (glucose) you have. **Done with softpro max (See previous page).

Again, using the formula $y = mx + b$, where y = OD, m = slope and b = the y axis intercept, we isolate x (concentration of compound x) which results in; $x = \frac{y - b}{m}$

If b (the blank) is set at zero then it becomes $x = \frac{OD}{\text{Slope}}$

This gives the amount of reducing sugar in ug. If we now divide this by 180 which is the molecular weight of glucose, we get u moles. This divided by the time of incubation (min) gives us the activity expressed in u mole/minute

So, 4.5833 ug divided by 180 (glucose MW) = 0.02546 u moles

Then, 0.02546 u mole divided by 30 minutes (time of assay)

$= 2.828 \times 10^{-4}$ u moles/minute

Finally, divide this by the total ug of protein in the assay. This is the specific activity.

$\frac{2.828 \times 10\text{-}4 \text{ u moles/minute}}{36.514 \text{ ug protein}} = 7.745 \times 10^{-6}$ u mole/minute/ug protein

This our specific activity expressed as SA

Appendix 12: Methods for Determination of Laminarinase Activity

Laminarinase assay using the p-hydroxybenzoic acid hydrazide reducing sugar assay (PAHBAH)

Prepared my Michel Goguen, Institute for Marine Biosciences
Amended by Lisa Milke (6/2006)

The determination of laminarinase activity is based on measuring reducing sugars released by enzymes under optimum pH, temperature and reaction time determined emperically. Laminarin consists of linear manitol- or glucose ended chains of ß (1→3) linked sugar residues, with occasional ß (1→6) linked branches.

Prepare the following reagents:

1. NaOH (1M stock) calculate: 1M x 40.00(MW) x 0.250(vol.) =

10 g in 250 ml ddH_2O (toped up)

2. 1M Bismuth nitrate stock. This is also a weight /volume solution so it needs to be toped up to 25 ml after reagents are added.

In about 20 ml add:

- 1M Bismuth nitrate = 1.212 g
- 1M sodium potassium tartarate = 7.055 g
- 3M NaOH = 1 g

*top up to 25 ml

The chemicals are better dissolved with slight heating for a few minutes. The solution is then stable indefinitely at room temperature.

The day of the assay, prepare the PAHBAH reagent.
Weigh 0.761 g of PAHBAH (p-hydroxybenzoic acid hydrazide) in 100 ml of 0.5 M NaOH. Then add 100 ul of Bismuth nitrate stock. This reagent cannot be stored and must be made fresh each day.

**Do not forget to add the bismuth nitrate because it is needed for color change. The first thing to do if the assay gives no color is to verify if the Bismuth nitrate was added.

Standard curve

As this is an endpoint colorimetric assay. A standard curve is needed to calculate the amount of reducing sugar in our assay. A 1 mg/ml glucose solution is used as a standard.

Standard 1mg/ml glucose solution (ul)	ddH_2O (ul)	0.8 M citrate buffer pH 5.0 (ul)	[Glucose] Values entered in standard curve (ug)	PAHBAH reagent Stop sol. (ul)
0	90	10	0	1500
5	85	10	5	1500
10	80	10	10	1500
15	75	10	15	1500
20	70	10	20	1500

*Standard curve can be made up in the absence of substrate. (Substitute water for substrate)

For the glucose standard, measure 100mg in 100 ml of ddH_2O. Aliquot 500 ul in 500 ul tubes and freeze at $-20^{\circ}C$ until use. For the substrate we use laminarin which is hydrolyzed by laminarinase. A 0.4% concentration is standard and is used throughout the literature.

-Ex: 0.1 g in 25 ml = 0.4% Mix with ddH2O, aliquot 500 ul in 500 ul tubes and freeze at $-20^{\circ}C$ until use.

*** buffer used was 0.8M citrate buffer pH 5.0 to obtain final reaction pH of 5.5**

A O.8 M (tri)sodium citrate = 0.8 M x 294.1 (MW) x 0.250 (vol.)

= 58.82 g make up to 250 ml with ddH_2O

B O.8 M citric acid = 0.8 M x 210.14 (MW) x 0.250 (vol.)

= 42.03 g make up to 250 ml with ddH_2O

Place about 100 ml of **A** in a beaker and add **B** to give a pH of 5.0
*(no need to adjust final volume as both **A** and **B** are 0.8 M)*

For the PAHBAH stop reagent, 1.5 ml is added to the sample when we want to stop the incubation. For the laminarinase assay for tissues of bay and sea scallop postlarvae, it has been determined that 30 minutes is optimal.

Preparing the standard curve

In five 1.6 ml aliquots, labeled 0, 5, 10, 15 and 20 ug, add the appropriate volumes of water, buffer and glucose standard. Once all these have been added, add the substrate.

Add 1.5 ml of PAHBAH to the tubes to stop the reaction.
(Once the reaction has been stopped, you don't have to read it immediately. It can remain on the bench for a few hours)

To develop the color, put samples in a second water bath and heat at $70^{O}C$ for 10 minutes. There should be a gradient of yellow from 0 ug glucose to 20 ug glucose.

Pipette 100 ul of the solution in 3 microplate wells. Do this for each of the five tubes.

Bring the microplate to the microplate reader.

- Turn the Microplate reader 'on'.
- Click on the soft pro max icon
- Click on 'assay' and then 'default protocol'
- Click on 'setup' on the assay task bar and then on 'endpoint"
  Then change the absorbance to 410 nm.
- Click on 'template' then click on 'standards'
- Highlight the appropriate wells and enter the glucose amounts in the concentration box (with edit, change ug/ul to ug).
- when all data is entered, click on 'done'
- Click on 'Read'

**After the readings are done, the plate will eject and the ODs for each well will appear on the screen. Go in 'view' and click on 'graph'. This will give the graph of our standard curve. The linearity is given on the bottom right and is represented by R^. A 1.000 is perfect but this rarely happens. Usually a good curve will give at least 0.990 or more. Also keep track of the slope which is represented by B. We'll use this to determine the amount of reducing sugar in our sample now that we have a linear standard curve. This is done using the following formula: $Y = mx + b$

Where y = OD, m = slope, x = concentration and b = y axis intercept

So, if we isolate x (unknown) we get; $x = \frac{y - b}{m}$

When we read our unknown samples, we'll prepare a blank which will be set at zero. So the formula then becomes OD/slope = x concentration.

Unknown samples

The samples to use are the supernatant aliquots of the homogenized samples.

(See sample processing protocol). You cannot use the samples having the uniform mix of shell. Any debris will interfere with the assays. The protein concentration must eventually be done on this sample also to then correlate the enzyme activity to the total protein.

'Heat Killed' enzymes (boiled for at least 30 minutes) are typically used as a blank. Unfortunately in our case even after several hours of boiling, we were getting some evidence of activity. Therefore, as our blank we adopted a 'time zero' blank. Samples were added to substrate solution, but the reaction was immediately stopped with the addition of PAHBAH reagent.

Due to the high activity of laminarinase activity in postlarval scallop samples, volumes of tissue homogenate were reduced in comparison to that used in α-amylase and cellulase assays.

The samples are prepared as follows;

	Enzyme control (blank)	Enzyme assay
0.4% Laminarin (substrate)	50 ul	50 ul
0.8M citrate buffer pH 5.0	15 ul	15 ul
ddH_2O	30 ul	30 ul
Appropriately diluted enzyme	____	5 ul
Appropriately diluted control enzyme	5 ul	____
Total volume	100 ul	100 ul

In 1.6 ml tubes, pipette the appropriate amounts of water, buffer and substrate. **Always prepare each sample in triplicate!!**

Pre-incubate these tubes at the assay temperature ($30^{O}C$) for 5 minutes in a water bath.

Add the enzyme (sample) to the appropriate tubes and incubate for the determined duration (30 minutes).

After 30 minutes, add 1500 ul (1.5 ml) of PAHBAH to each of the tubes. This will stop the reaction.

To develop the color, heat the samples in a water bath at approximately $70^{O}C$ for 10 minutes.

Remove and allow 2-3 minutes of cooling.

Pipette 100 ul of each tube in one microplate well. Keep track of the wells as you need to enter them in the software.

Bring the microplate to the microplate reader

- Turn on the plate reader.
- Click on the soft pro max software.
- Click on 'setup', then 'endpoint', then set OD at 410 nm.
- Click on 'template' then choose 'unknown' instead of 'standards'.
- Highlight the sample or samples and name them individually.
- Highlight the blank (the heat killed enzyme) then choose 'blank' instead of 'unknown'. This will subtract the blank color from our sample. Then click on 'done'.
- Click on 'Read'

Once the readings are done, click on 'view' and then 'unknowns'.
A table will appear as the following (example);

Sample	wells	values	outliers	result	Mean result	Std. Dev.	[sugar]
Sample 1	A1 A2 A3	0.105 0.116 0.110					

To get the mean result (average of the 3 wells) click on the 'mean result' title (it will turn blue) and then press enter. A new window will appear where we replace (results) with (values). The mean result will appear.

Sample	wells	values	outliers	result	Mean result	Std. Dev.	[sugar]
Sample 1	A1 A2 A3	0.105 0.116 0.110			0.110		

Now click on [protein] where you erase (results) and you enter the formula to calculate the protein concentration. Example:

(the following is typed) Meanresult/0.024
0.024 is the slope in the standard curve (example)

The following result will appear

Sample	wells	values	outliers	result	Mean result	Std. Dev.	[sugar]
Sample 1	A1 A2 A3	0.105 0.116 0.110			0.111		4.5833

***this is the amount of reducing sugar in our sample in ug.

Calculating specific activity (SA)

The first thing is to go back and take note of the protein concentration previously determined. This is the amount of protein per microliter of homogenate.

Example: 3.6514 ug/ul

Multiply this by the amount of sample used in the cellulase enzyme assay which is 10 ul. This gives us the total amount of protein (in ug) in the assay.

3.6514 ug/ul x 10 = 36.514 ug protein in the assay

Now go to the standard curve to determine how much reducing sugar (glucose) you have. **Done with softpro max (See previous page).

Again, using the formula y = mx + b, where y = OD, m = slope and b = the y axis intercept, we isolate x (concentration of compound x) which results in; $x = \frac{y - b}{m}$

If b (the blank) is set at zero then it becomes $x = \frac{OD}{\text{Slope}}$

This gives the amount of reducing sugar in ug. If we now divide this by 180 which is the molecular weight of glucose, we get u moles. This divided by the time of incubation (min) gives us the activity expressed in u mole/minute

So, 4.5833 ug divided by 180 (glucose MW) = 0.02546 u moles

Then, 0.02546 u mole divided by 30 minutes (time of assay)

= 2.828×10^{-4} u moles/minute

Finally, divide this by the total ug of protein in the assay. This is the specific activity.

$\frac{2.828 \text{ x } 10\text{-}4 \text{ u moles/minute}}{36.514 \text{ ug protein}} = 7.745 \times 10^{-6}$ u mole/minute/ug protein

This our specific activity expressed as SA

Appendix 13: Expanded List of Fatty Acids Corresponding to Table 2.2; Microalgae Used in Sea Scallop Growth Trials.

Percent of total fatty acids of microalgae used in sea scallop, *Placopecten magellanicus*, postlarval feeding trials. nd= not detected

	Pav 459	***P. lutheri***	***T. striata***	**CHGRA**	***T. weissflogii***	***F. familica***
14:0	16.74±0.36	12.60±0.43	2.48±0.25	12.70±0.77	6.64±0.40	15.63±0.46
14:1	0.10±0.02	0.15±0.04	0.14±0.04	0.20±0.05	0.13±0.02	0.06±0.02
15:0i	0.58±0.06	0.71±0.14	1.42±0.10	0.31±0.07	0.71±0.21	0.31±0.02
15:0ai	0.04±0.02	0.11±0.08	0.33±0.28	0.04±0.02	0.07±0.02	0.09±0.05
15:0	0.24±0.01	0.24±0.01	nd	0.85±0.11	0.77±0.02	0.18±0.03
15:1	0.23±0.02	0.30±0.04	0.52±0.11	0.21±0.01	0.40±0.06	0.17±0.03
16:0i	0.34±0.07	0.30±0.09	0.89±0.19	0.13±0.07	0.43±0.19	0.31±0.05
16:0ai	0.66±0.07	0.70±0.09	1.45±0.24	0.32±0.07	0.67±0.19	0.33±0.05
16:0	8.19±0.31	10.19±1.10	13.99±0.35	14.66±0.94	11.16±1.99	4.46±0.49
16:1n9	0.96±0.15	0.90±0.34	1.42±0.19	0.62±0.17	1.09±0.36	0.56±0.07
16:1n7	7.17±0.39	14.88±1.34	1.31±0.50	31.11±1.96	26.35±7.53	20.99±1.44
16:1n5	3.37±0.13	0.22±0.07	nd	0.41±0.04	0.54±0.06	0.65±0.04
17:0i	0.61±0.02	0.39±0.04	2.71±0.27	0.42±0.06	0.33±0.09	0.25±0.08
17:0ai	0.20±0.09	0.77±0.07	0.51±0.08	1.92±0.30	2.39±0.57	0.49±0.07
16:2n4	1.55±0.09	1.59±0.12	0.08±0.05	2.52±0.14	2.79±0.23	2.35±0.09
17:0	nd	0.01±0.01	1.56±0.24	0.07±0.01	0.02±0.01	0.03±0.02
16:3n4	0.29±0.03	1.81±1.04	0.01±0.01	5.32±1.44	17.89±4.70	8.52±0.38
17:1	0.40±0.12	0.46±0.15	1.64±0.13	0.21±0.10	1.28±0.31	1.13±0.23
16:4n3	0.01±0.01	0.03±0.02	18.40±0.57	0.03±0.01	0.03±0.01	0.06±0.04
16:4n1	0.15±0.01	0.14±0.04	0.13±0.04	0.09±0.02	0.20±0.05	0.47±0.05
18:0	0.22±0.01	0.39±0.09	0.16±0.04	0.82±0.07	0.32±0.04	0.78±0.09

	Pav 459	*P. lutheri*	*T. striata*	CHGRA	*T. weissflogii*	*F. familica*
18:1n11	nd	0.01±0.00	nd	0.02±0.01	0.06±0.04	0.02±0.01
18:1n9	0.15±0.03	0.46±0.04	6.89±0.52	0.53±0.03	0.68±0.29	0.98±0.69
18:1n7	0.33±0.04	3.14±0.63	1.34±0.13	0.78±0.18	1.75±0.55	6.68±0.44
18:1n6	nd	nd	nd	nd	nd	nd
18:1n5	0.09±0.01	0.10±0.06	nd	0.06±0.01	0.12±0.06	0.48±0.09
18:2n6	1.07±0.38	0.54±0.30	6.99±0.86	0.70±0.05	0.58±0.09	2.90±0.83
18:2n4	0.04±0.01	0.96±0.23	nd	0.12±0.08	0.07±0.01	0.22±0.02
19:0	nd	0.04±0.04	nd	nd	nd	0.34±0.16
18:3n6	0.19±0.06	0.60±0.22	0.37±0.05	0.94±0.22	0.18±0.03	1.49±0.37
18:3n4	0.03±0.02	0.10±0.03	0.06±0.06	0.43±0.21	0.06±0.02	0.50±0.25
18:3n3	2.70±0.53	0.98±0.15	13.99±1.47	0.08±0.07	0.04±0.01	0.02±0.02
18:4n3	5.04±2.36	5.66±1.90	7.68±0.83	0.84±0.72	0.22±0.05	0.76±0.12
18:4n1	0.02±0.01	0.15±0.04	0.03±0.01	0.15±0.09	0.59±0.21	0.11±0.04
20:0	0.01±0.00	0.19±0.03	nd	0.09±0.01	0.10±0.04	0.07±0.01
18:5n3	nd	nd	nd	nd	nd	nd
20:1n11	0.01±0.01	nd	4.52±0.52	nd	nd	nd
20:1n9	nd	0.01±0.01	0.99±0.18	nd	nd	0.13±0.13
20:1n7	nd	0.02±0.02	nd	0.05±0.01	0.09±0.03	0.04±0.02
20:2α	0.01±0.01	0.01±0.01	nd	nd	nd	0.03±0.03
20:2β	0.01±0.00	nd	0.03±0.02	nd	0.02±0.02	0.01±0.01
20:2n6	0.07±0.02	0.04±0.01	0.05±0.03	0.04±0.01	0.10±0.06	0.02±0.01
20:3n6	0.10±0.04	0.02±0.01	0.03±0.03	0.21±0.03	0.13±0.03	0.08±0.05
21:0	nd	nd	nd	nd	nd	nd
20:4n6	1.78±0.24	0.33±0.16	0.23±0.06	7.40±0.96	0.91±0.24	1.39±0.16
20:3n3	nd	0.02±0.01	0.02±0.01	nd	0.07±0.07	0.01±0.01
20:4n3	0.09±0.03	nd	0.47±0.07	0.06±0.01	0.08±0.04	0.12±0.12
20:5n3	30.01±0.75	28.34±1.33	6.58±0.24	11.96±1.35	16.22±2.48	22.93±1.21
22:0	0.01±0.00	0.01±0.01	nd	0.16±0.03	0.02±0.01	0.06±0.01

	Pav 459	*P. lutheri*	*T. striata*	CHGRA	*T. weissflogii*	*F. familica*
22:1n11(13)	nd	nd	0.02±0.02	nd	nd	nd
22:1n9	nd	nd	0.01±0.01	nd	0.04±0.04	0.01±0.01
22:1n7	nd	nd	nd	nd	nd	nd
21:5n3	0.26±0.06	0.01±0.01	0.05±0.04	nd	0.01±0.01	0.08±0.05
23:0	0.01±0.01	0.02±0.01	0.05±0.03	nd	nd	0.01±0.01
22:4n6	nd	nd	0.01±0.01	nd	0.02±0.01	nd
22:4n3	nd	nd	nd	nd	nd	nd
22:5n6	7.84±0.44	0.55±0.11	0.04±0.04	0.12±0.04	nd	0.04±0.03
22:5n3	nd	0.02±0.01	0.12±0.05	0.09±0.01	0.07±0.05	0.28±0.21
24:0	0.03±0.01	0.12±0.10	0.07±0.06	0.30±0.03	0.24±0.05	0.78±0.18
22:6n3	8.04±0.37	10.63±1.57	0.17±0.12	1.89±0.71	3.29±0.68	1.40±0.83
24:1	0.01±0.01	0.02±0.01	0.06±0.03	0.03±0.01	0.04±0.01	0.18±0.03

Appendix 14: Expanded List of Fatty Acids Corresponding to Table 2.3; Sea Scallop Postlarval Tissues.

Percent of total fatty acids in sea scallop, *Placopecten magellanicus*, postlarval tissues. nd= not detected

	Initial	**Pav 459 & CHGRA**	***P. lutheri* & *F. familica***	***P. lutheri* & CHGRA**	***P. lutheri* & CHGRA**	***T. straiata* & CHGRA**
14:0	7.72±0.47	5.33±0.10	5.45±0.12	4.96±0.32	4.56±0.13	2.36±0.28
14:1	nd	nd	nd	0.06±0.06	nd	nd
15:0i	0.30±0.16	0.19±0.01	0.45±0.33	0.33±0.07	0.66±0.05	0.28±0.06
15:0ai	nd	nd	0.05±0.05	0.08±0.05	nd	0.08±0.08
15:0	0.42±0.03	0.32±0.01	0.32±0.03	0.31±0.01	0.43±0.01	0.49±0.01
15:1	0.06±0.06	0.17±0.02	0.29±0.01	0.20±0.03	0.12±0.12	0.23±0.13
16:0i	0.07±0.07	0.20±0.00	0.07±0.07	0.05±0.05	0.08±0.08	nd
16:0ai	0.12±0.12	0.15±0.02	0.31±0.06	0.34±0.10	0.38±0.25	0.05±0.05
16:0	13.82±0.22	11.40±0.18	11.29±0.18	12.51±0.29	12.51±0.26	12.09±0.30
16:1n9	nd	0.15±0.00	0.33±0.17	0.46±0.23	0.40±0.23	nd
16:1n7	7.44±0.05	8.84±0.14	4.60±0.10	10.63±0.82	5.13±0.06	4.62±0.51
16:1n5	0.10±0.10	0.79±0.02	0.27±0.02	0.30±0.00	0.28±0.00	0.41±0.10
17:0i	0.29±0.10	nd	0.16±0.08	0.16±0.03	nd	0.08±0.08
17:0ai	0.42±0.21	0.67±0.05	0.25±0.03	0.79±0.05	0.77±0.04	0.13±0.07
16:2n4	0.50±0.05	0.65±0.03	0.06±0.06	0.66±0.03	0.28±0.02	0.23±0.05
17:0	0.35±0.02	0.44±0.01	0.31±0.02	0.27±0.01	0.36±0.01	0.62±0.03
16:3n4	0.13±0.13	0.66±0.04	0.35±0.01	0.83±0.05	1.62±0.76	1.52±1.44
17:1	0.18±0.18	0.61±0.48	3.15±0.25	2.15±0.59	0.95±0.95	2.51±1.26
16:4n3	0.52±0.52	1.24±0.53	0.21±0.13	0.75±0.64	1.01±0.93	0.58±0.52
16:4n1	nd	0.03±0.03	nd	nd	0.06±0.06	0.13±0.13

	Initial	Pav 459 & CHGRA	*P. lutheri* & *F. familica*	*P. lutheri* & CHGRA	*P. lutheri* & CHGRA	*T. straiata* & CHGRA
18:0	2.92±0.14	3.28±0.08	5.19±0.09	3.91±0.25	4.55±0.24	6.84±0.24
18:1n11	nd	0.08±0.04	0.36±0.01	0.28±0.02	0.11±0.11	0.12±0.12
18:1n9	5.37±0.68	0.72±0.10	0.73±0.05	0.82±0.06	1.57±0.72	2.34±0.03
18:1n7	5.80±0.73	5.05±0.02	8.72±0.17	8.65±0.21	8.25±0.54	7.11±0.30
18:1n6	nd	nd	nd	nd	nd	nd
18:1n5	0.06±0.06	0.31±0.00	0.31±0.01	0.10±0.05	0.29±0.02	0.56±0.18
18:2n6	4.15±0.22	0.81±0.06	0.69±0.23	0.40±0.07	0.60±0.32	1.07±0.09
18:2n4	0.36±0.10	0.61±0.01	0.32±0.08	0.45±0.12	0.57±0.14	0.31±0.05
19:0	nd	nd	nd	nd	nd	nd
18:3n6	0.68±0.02	0.72±0.01	0.35±0.01	0.62±0.03	nd	0.30±0.04
18:3n4	nd	nd	0.09±0.09	0.05±0.05	0.10±0.10	nd
18:3n3	3.32±0.08	0.99±0.01	0.35±0.06	0.22±0.02	0.32±0.05	1.56±0.12
18:4n3	7.10±1.10	2.85±0.13	6.39±0.47	4.07±0.73	5.37±1.64	6.38±0.38
18:4n1	0.06±0.06	0.05±0.05	nd	nd	nd	nd
20:0	0.05±0.05	nd	0.10±0.10	nd	nd	0.17±0.09
18:5n3	nd	nd	nd	nd	nd	nd
20:1n11	nd	1.27±0.10	2.81±0.05	1.20±0.60	1.73±0.86	2.90±1.45
20:1n9	1.48±0.20	nd	nd	0.51±0.51	0.88±0.88	1.83±1.42
20:1n7	0.07±0.07	0.57±0.01	1.19±0.14	0.65±0.08	0.70±0.35	0.49±0.26
20:2α	nd	0.17±0.02	nd	0.06±0.06	nd	0.08±0.08
20:2β	nd	nd	nd	nd	nd	nd
20:2n6	0.89±0.45	0.16±0.02	0.28±0.03	0.03±0.03	0.24±0.04	1.31±0.58
20:3n6	0.28±0.03	0.38±0.00	0.05±0.05	0.23±0.04	0.08±0.08	0.24±0.04
21:0	nd	nd	nd	nd	nd	nd
20:4n6	2.28±0.06	8.76±0.07	2.75±0.03	8.09±0.05	1.92±0.06	10.46±0.41
20:3n3	0.34±0.02	0.11±0.00	nd	nd	nd	0.45±0.06
20:4n3	0.13±0.06	0.16±0.01	0.05±0.05	0.04±0.04	0.05±0.05	0.31±0.09

	Initial	Pav 459 & CHGRA	*P. lutheri* & *F. familica*	*P. lutheri* & CHGRA	*P. lutheri* & CHGRA	*T. straiata* & CHGRA
20:5n3	9.82±0.09	19.27±0.16	14.55±0.21	15.38±0.23	18.06±0.26	9.24±0.34
22:0	nd	nd	nd	nd	nd	nd
22:1n11(13)	nd	nd	nd	nd	nd	nd
22:1n9	0.04±0.04	0.33±0.00	nd	0.1±0.08	nd	0.48±0.24
22:1n7	nd	nd	nd	nd	nd	nd
21:5n3	0.24±0.24	0.39±0.01	0.80±0.16	0.51±0.09	0.66±0.34	0.74±0.38
23:0	0.18±0.12	nd	nd	nd	0.05±0.05	0.54±0.54
22:4n6	nd	nd	nd	nd	nd	nd
22:4n3	nd	nd	nd	nd	nd	nd
22:5n6	4.47±0.27	10.14±0.14	4.46±0.18	3.00±0.31	4.48±0.16	2.92±0.14
22:5n3	0.36±0.02	0.55±0.01	0.69±0.05	0.54±0.02	0.63±0.04	1.05±0.07
24:0	nd	nd	nd	nd	nd	nd
22:6n3	17.09±0.26	10.43±0.21	20.87±0.41	14.20±0.81	19.22±0.57	13.80±0.37

Appendix 15: Expanded List of Fatty Acids Corresponding to Table 3.3; Microalgal Diets from Bay Scallop Experiments.

Percent of total fatty acids from diets in the postlarval bay scallop, *Argopecten irradians*, feeding trial. nd=not determined

	Pav 459 & CHGRA	***T. striata* & CHGRA**	**Pav 459**	***P. lutheri* & CHGRA**	***R. lens* & CHGRA**	***P. lutheri* & *F. familica***	***P. lutheri* & *T. weissflogii***	***R. lens***
14:0	13.37±0.64	8.05±1.98	16.17±0.57	10.99±0.35	8.81±1.26	12.63±0.66	8.87±0.52	3.96±0.34
14:1	0.18±0.05	0.22±0.04	0.15±0.08	0.30±0.05	0.20±0.03	0.26±0.05	0.30±0.02	0.12±0.01
15:0i	0.56±0.13	0.70±0.22	0.71±0.17	0.65±0.10	0.61±0.07	0.77±0.04	0.70±0.05	0.96±0.15
15:0ai	0.06±0.03	0.03±0.02	0.11±0.07	0.04±0.02	0.10±0.02	0.08±0.03	0.05±0.02	0.18±0.01
15:0	0.46±0.12	0.39±0.18	0.35±0.08	0.38±0.11	0.57±0.11	0.17±0.01	0.45±0.06	0.46±0.31
15:1	0.21±0.03	0.26±0.06	0.21±0.04	0.26±0.06	0.35±0.19	0.28±0.04	0.33±0.04	0.47±0.26
16:0i	0.29±0.11	0.41±0.17	0.36±0.05	0.42±0.14	0.31±0.12	0.55±0.06	0.46±0.14	0.35±0.08
16:0ai	0.43±0.06	0.84±0.28	0.39±0.02	0.62±0.06	0.67±0.14	0.71±0.12	0.69±0.05	1.01±0.08
16:0	12.73±2.85	13.57±2.96	11.73±1.04	10.73±2.49	12.78±2.63	5.97±0.55	9.07±0.44	13.75±0.77
16:1n9	0.38±0.18	0.53±0.13	0.25±0.12	0.51±0.37	0.64±0.44	0.66±0.62	0.58±0.54	1.26±1.16
16:1n7	18.77±4.29	18.03±6.42	8.20±0.56	19.74±3.08	16.74±3.87	14.18±0.54	16.46±1.47	0.45±0.06
16:1n5	1.35±0.27	0.30±0.06	2.77±0.32	0.36±0.01	0.33±0.00	0.37±0.02	0.40±0.03	0.14±0.06
17:0i	0.48±0.10	0.92±0.35	0.45±0.11	0.45±0.08	0.67±0.20	0.35±0.01	0.38±0.04	0.83±0.13
17:0ai	1.51±0.14	1.81±0.06	0.23±0.14	1.59±0.01	2.18±0.27	1.39±0.28	1.75±0.26	1.51±0.24
16:2n4	2.23±0.23	1.74±0.22	2.11±0.44	2.39±0.14	1.74±0.23	2.18±0.30	2.92±0.23	0.13±0.03
17:0	0.04±0.01	0.45±0.17	nd	0.04±0.01	0.08±0.01	0.01±0.01	0.03±0.02	0.11±0.02
16:3n4	3.62±0.62	4.05±0.53	0.16±0.08	3.35±0.32	4.08±0.75	4.65±1.28	8.87±1.14	0.01±0.01
17:1	0.04±0.03	0.83±0.39	0.02±0.02	0.07±0.03	0.04±0.02	0.05±0.01	0.19±0.14	0.04±0.02
16:4n3	0.02±0.01	6.00±3.19	nd	0.01±0.01	0.06±0.03	0.02±0.01	0.04±0.00	0.08±0.08

	Pav 459 & CHGRA	***T. striata* & CHGRA**	**Pav 459**	***P. lutheri* & CHGRA**	***R. lens* & CHGRA**	***P. lutheri* & *F. familica***	***P. lutheri* & *T. weissflogii***	***R. lens***
16:4n1	0.07±0.00	0.09±0.01	0.04±0.02	0.08±0.01	0.13±0.06	0.34±0.08	0.09±0.01	0.17±0.08
18:0	0.40±0.06	0.36±0.07	0.33±0.09	0.33±0.04	0.55±0.05	0.23±0.02	0.19±0.01	0.72±0.06
18:1n11	0.04±0.01	0.01±0.01	0.12±0.09	0.02±0.01	0.60±0.24	0.01±0.01	0.06±0.01	1.56±0.16
18:1n9	0.42±0.09	2.14±0.85	0.23±0.04	0.47±0.09	0.45±0.14	0.39±0.09	0.59±0.25	0.29±0.10
18:1n7	0.66±0.10	1.05±0.31	0.78±0.16	1.59±0.34	1.37±0.34	2.62±0.19	1.62±0.33	2.51±0.30
18:1n6	0.01±0.00	nd	0.01±0.01	nd	nd	nd	0.04±0.03	nd
18:1n5	0.07±0.02	0.06±0.01	0.08±0.01	0.07±0.02	0.11±0.06	0.14±0.03	0.04±0.02	0.13±0.08
18:2n6	1.71±0.38	2.67±0.88	3.69±1.27	0.70±0.12	1.02±0.11	0.49±0.03	0.42±0.02	1.09±0.05
18:2n4	0.09±0.06	0.09±0.07	0.04±0.02	0.31±0.11	0.18±0.16	0.33±0.05	0.34±0.03	0.15±0.15
19:0	nd	nd	nd	nd	nd	0.02±0.00	0.01±0.00	nd
18:3n6	1.31±0.44	1.30±0.45	0.68±0.11	1.05±0.32	1.28±0.47	1.46±0.20	0.29±0.05	0.21±0.16
18:3n4	0.02±0.00	0.03±0.00	0.03±0.00	0.11±0.01	0.01±0.01	0.14±0.03	0.15±0.01	nd
18:3n3	1.09±0.13	6.06±3.28	3.16±0.83	1.02±0.32	9.72±2.99	1.18±0.13	1.22±0.28	28.06±0.83
18:4n3	3.30±1.84	2.24±0.93	5.80±2.00	4.30±0.81	5.62±1.52	5.84±0.70	5.31±0.45	15.25±0.86
18:4n1	nd	nd	0.01±0.01	0.04±0.01	nd	0.04±0.00	0.06±0.01	nd
20:0	0.03±0.01	0.03±0.01	0.02±0.00	0.09±0.01	0.02±0.01	0.12±0.03	0.13±0.01	nd
18:5n3	nd	0.19±0.19	nd	nd	nd	nd	nd	nd
20:1n11	nd	1.13±0.76	nd	nd	0.08±0.08	nd	0.01±0.01	0.16±0.16
20:1n9	nd	0.51±0.26	nd	nd	0.08±0.08	0.01±0.00	nd	0.16±0.15
20:1n7	0.04±0.00	0.05±0.01	nd	0.05±0.01	0.04±0.00	0.04±0.01	0.05±0.01	nd
20:2α	0.01±0.01	nd	0.03±0.03	nd	nd	nd	0.01±0.01	nd
20:2β	nd	nd	nd	nd	nd	nd	0.01±0.01	nd
20:2n6	0.06±0.01	0.03±0.02	0.15±0.04	0.14±0.03	0.01±0.01	0.18±0.03	0.17±0.02	0.01±0.01
20:3n6	0.18±0.03	0.12±0.05	0.19±0.04	0.11±0.04	0.11±0.06	0.07±0.02	0.04±0.00	nd
21:0	nd	nd	nd	nd	nd	nd	nd	0.01±0.01

	Pav 459 & CHGRA	***T. striata* & CHGRA**	**Pav 459**	***P. lutheri* & CHGRA**	***R. lens* & CHGRA**	***P. lutheri* & *F. familica***	***P. lutheri* & *T. weissflogii***	***R. lens***
20:4n6	6.29±1.43	5.77±1.79	3.55±1.07	4.48±1.26	5.64±2.07	1.81±0.23	0.55±0.07	0.08±0.03
20:3n3	0.01±0.00	0.02±0.01	0.01±0.01	0.08±0.02	0.05±0.01	0.10±0.01	0.10±0.01	0.11±0.01
20:4n3	0.08±0.01	0.15±0.04	0.08±0.03	0.09±0.01	0.25±0.06	0.07±0.02	0.13±0.01	0.54±0.04
20:5n3	18.53±3.46	12.30±1.23	21.70±1.97	23.55±3.45	14.30±1.76	30.47±0.52	26.33±1.36	10.92±0.62
22:0	0.11±0.03	0.13±0.04	0.02±0.02	0.10±0.03	0.12±0.03	0.06±0.03	0.02±0.02	nd
22:1n11(13)	nd	nd	0.01±0.01	0.01±0.01	nd	0.01±0.01	0.01±0.01	nd
22:1n9	nd	nd	nd	nd	nd	nd	nd	nd
22:1n7	nd	nd	nd	nd	nd	nd	nd	nd
21:5n3	0.01±0.01	0.01±0.00	0.03±0.03	0.01±0.01	0.11±0.10	0.01±0.01	0.01±0.01	0.20±0.20
23:0	nd	0.01±0.01	nd	nd	nd	nd	nd	nd
22:4n6	0.05±0.01	0.02±0.02	0.06±0.01	0.02±0.01	0.09±0.06	nd	nd	0.15±0.14
22:4n3	0.02±0.01	0.02±0.01	0.03±0.02	0.02±0.01	0.14±0.12	0.01±0.01	0.03±0.02	0.25±0.22
22:5n6	3.46±1.04	0.19±0.10	8.23±0.60	0.44±0.00	0.41±0.05	0.38±0.06	0.40±0.03	0.58±0.13
22:5n3	0.20±0.12	0.23±0.15	0.05±0.02	0.17±0.12	0.26±0.11	0.07±0.03	0.10±0.08	0.22±0.05
24:0	0.17±0.05	0.20±0.07	0.03±0.02	0.14±0.05	0.18±0.04	0.25±0.07	0.11±0.03	0.02±0.01
22:6n3	3.81±0.83	1.57±0.28	5.60±0.89	6.24±1.00	4.66±0.69	6.70±0.94	7.73±0.57	8.95±0.51
24:1	0.31±0.10	0.35±0.16	0.17±0.09	0.31±0.20	0.25±0.12	0.30±0.21	0.26±0.10	0.01±0.01

Appendix 16: Expanded List of Fatty Acids Corresponding to Table 3.4; Bay Scallop Postlarval Tissues.

Percent of total fatty acids identified in the tissues of postlarval bay scallops, *Argopecten irradians*. nd=not detected

	Initial	**Pav 459 & CHGRA**	***T. striata* & CHGRA**	**Pav 459**	***P. lutheri* & CHGRA**	***R. lens* & CHGRA**	***P. lutheri* & *F. familica***	***P. lutheri* & *T. weissflogii***
14:0	4.64±0.22	4.48±0.05	3.08±0.20	3.68±0.15	4.05±0.08	2.85±0.09	4.02±0.48	4.00±0.18
14:1	0.36±0.10	nd	nd	nd	nd	nd	0.10±0.10	nd
15:0i	1.19±0.12	0.19±0.01	nd	0.66±0.20	0.53±0.08	0.75±0.07	0.45±0.13	0.24±0.12
15:0ai	0.34±0.02	nd	nd	0.17±0.08	nd	0.29±0.02	nd	nd
15:0	0.66±0.03	0.33±0.01	0.64±0.04	0.68±0.03	0.40±0.02	0.49±0.02	0.33±0.03	0.40±0.05
15:1	0.92±0.04	0.28±0.15	nd	0.57±0.00	0.31±0.16	0.72±0.01	0.39±0.02	0.38±0.06
16:0i	nd	nd	nd	0.04±0.04	nd	0.09±0.09	nd	nd
16:0ai	0.42±0.07	0.37±0.06	nd	0.29±0.04	0.38±0.08	0.39±0.05	0.62±0.04	0.41±0.08
16:0	12.86±0.22	11.34±0.14	21.45±2.16	10.82±0.09	12.89±0.28	11.17±0.20	11.33±0.18	11.90±0.44
16:1n9	0.89±0.50	0.82±0.08	0.54±0.08	0.77±0.28	1.49±0.31	1.06±0.12	1.85±0.15	1.18±0.16
16:1n7	8.07±0.04	4.21±0.07	2.83±0.28	3.78±0.30	6.63±0.09	3.67±0.12	5.90±0.33	5.43±0.50
16:1n5	1.05±0.08	0.64±0.08	0.17±0.17	0.94±0.13	0.40±0.09	0.20±0.10	0.28±0.04	0.35±0.02
17:0i	0.40±0.01	0.22±0.03	0.64±0.18	0.44±0.02	0.07±0.07	0.38±0.03	0.41±0.05	0.36±0.04
17:0ai	0.39±0.02	0.73±0.22	0.29±0.16	0.21±0.13	0.61±0.10	0.33±0.04	0.35±0.05	0.38±0.04
16:2n4	0.21±0.11	0.12±0.06	nd	0.64±0.03	0.37±0.06	nd	0.12±0.12	nd
17:0	0.47±0.03	0.51±0.02	0.74±0.06	0.89±0.01	0.31±0.01	0.61±0.02	0.30±0.02	0.34±0.02
16:3n4	5.45±0.21	4.07±1.83	1.13±0.25	5.86±0.57	4.36±0.37	7.56±0.25	5.37±0.13	3.54±0.72
17:1	nd	0.17±0.09	nd	nd	0.43±0.26	0.15±0.07	0.59±0.25	nd
16:4n3	0.20±0.20	3.29±1.89	0.12±0.12	1.14±0.30	0.81±0.48	0.29±0.20	nd	0.96±0.41
16:4n1	nd	nd	nd	nd	nd	nd	nd	nd

	Initial	Pav 459 & CHGRA	*T. striata* & CHGRA	Pav 459	*P. lutheri* & CHGRA	*R. lens* & CHGRA	*P. lutheri* & *F. familica*	*P. lutheri* & *T. weissflogii*
18:0	5.83±0.16	7.95±0.18	9.85±0.99	6.27±0.08	7.04±0.24	5.89±0.07	6.77±0.17	6.74±0.02
18:1n11	nd	nd	nd	nd	nd	0.64±0.02	nd	nd
18:1n9	4.18±0.10	0.84±0.07	4.60±0.41	0.91±0.08	2.47±0.21	2.92±0.06	1.92±0.11	8.40±3.65
18:1n7	5.59±0.06	4.02±0.11	3.72±0.12	5.22±0.47	7.79±0.24	4.56±0.23	7.52±0.31	6.98±0.44
18:1n6	nd	nd	1.59±0.55	nd	nd	nd	nd	nd
18:1n5	0.23±0.12	0.47±0.00	0.12±0.12	0.63±0.04	0.44±0.03	0.41±0.01	0.36±0.02	0.48±0.04
18:2n6	2.32±0.10	0.92±0.19	2.03±0.32	0.75±0.09	0.83±0.07	0.55±0.01	0.64±0.05	3.19±1.37
18:2n4	nd	0.24±0.01	nd	0.10±0.10	0.38±0.01	0.08±0.08	0.35±0.03	0.47±0.04
19:0	nd	nd	0.13±0.13	0.05±0.05	nd	nd	nd	nd
18:3n6	0.26±0.14	0.72±0.00	0.13±0.13	0.52±0.26	0.38±0.06	nd	0.38±0.06	0.08±0.08
18:3n4	nd	nd	nd	nd	0.11±0.11	nd	0.37±0.19	0.15±0.15
18:3n3	1.28±0.12	0.29±0.04	2.49±0.54	0.41±0.04	0.69±0.20	2.61±0.06	0.51±0.11	0.47±0.05
18:4n3	2.36±0.06	2.18±0.73	0.83±0.20	2.24±0.15	3.04±1.01	5.36±0.25	5.69±0.38	4.70±0.95
18:4n1	nd	nd	0.19±0.19	nd	nd	nd	0.11±0.11	nd
20:0	nd	0.05±0.05	0.17±0.09	0.26±0.01	nd	nd	nd	nd
18:5n3	nd	nd	nd	nd	nd	nd	nd	nd
20:1n11	nd	nd	nd	nd	0.77±0.77	nd	1.57±00.78	0.23±0.23
20:1n9	2.24±0.01	0.71±0.03	7.13±0.75	0.80±0.01	0.78±0.78	3.16±1.58	0.78±0.78	2.32±0.07
20:1n7	0.16±0.16	0.43±0.43	0.39±0.39	0.43±0.22	2.13±0.07	2.08±1.52	1.73±0.87	1.62±0.81
20:2α	nd	0.13±0.06	0.62±0.15	nd	0.10±0.10	0.07±0.07	0.19±0.11	0.08±0.08
20:2β	nd	nd	nd	0.22±0.22	nd	nd	0.19±0.10	0.17±0.09
20:2n6	0.79±0.03	0.56±0.01	2.61±0.39	0.95±0.02	0.32±0.05	0.67±0.02	0.38±0.01	0.43±0.03
20:3n6	0.47±0.01	0.54±0.03	1.07±0.15	0.34±0.02	0.62±0.02	0.70±0.01	0.64±0.04	0.46±0.01
21:0	nd	nd	nd	nd	nd	nd	nd	nd
20:4n6	3.39±0.10	7.52±0.19	7.55±1.07	6.10±0.15	5.22±0.14	7.08±0.07	3.04±0.09	1.46±0.12

	Initial	Pav 459 & CHGRA	*T. striata* & CHGRA	Pav 459	*P. lutheri* & CHGRA	*R. lens* & CHGRA	*P. lutheri* & *F. familica*	*P. lutheri* & *T. weissflogii*
20:3n3	0.19±0.19	0.08±0.08	0.43±0.07	nd	0.18±0.18	0.41±0.04	nd	0.29±0.29
20:4n3	0.30±0.03	nd	0.25±0.14	0.05±0.05	nd	0.37±0.02	nd	0.21±0.11
20:5n3	8.15±0.17	6.35±0.11	7.77±1.11	4.07±0.18	9.33±0.20	6.95±0.17	10.38±0.50	9.41±0.62
22:0	0.25±0.13	0.06±0.06	nd	nd	nd	nd	0.11±0.11	0.08±0.08
22:1n11(13)	nd	nd	nd	nd	nd	nd	nd	nd
22:1n9	nd	0.96±0.01	1.30±0.16	0.28±0.03	0.29±0.15	3.76±0.01	nd	nd
22:1n7	nd	nd	nd	nd	nd	0.08±0.08	0.13±0.13	nd
21:5n3	nd	0.16±0.08	1.75±0.66	nd	0.28±0.14	0.29±0.03	0.32±0.16	0.42±0.04
23:0	nd	nd	2.81±2.08	nd	nd	0.16±0.10	nd	nd
22:4n6	nd	nd	nd	nd	nd	nd	nd	nd
22:4n3	nd	nd	nd	nd	nd	nd	nd	nd
22:5n6	5.67±0.11	20.23±0.40	1.90±0.44	27.62±1.09	3.20±0.12	1.26±0.05	3.71±0.17	3.06±0.28
22:5n3	0.95±0.07	0.60±0.06	0.99±0.35	0.65±0.10	0.76±0.07	1.09±0.20	0.62±0.02	0.54±0.06
24:0	nd	nd	0.18±0.18	nd	nd	nd	0.15±0.15	0.23±0.23
22:6n3	15.74±0.16	12.11±0.06	3.97±0.38	9.57±0.38	18.81±1.16	17.84±0.39	18.93±0.44	17.45±1.26
24:1	0.24±0.24	0.10±0.10	1.80±0.48	nd	nd	nd	0.15±0.15	nd

Appendix 17: Expanded List of Fatty Acids Corresponding to Table 3.5; Bay Scallop Juvenile Tissues.

Percent of total fatty acids identified in the tissues of juvenile bay scallops, *Argopecten irradians*. nd=not detected

	Initial	**Pav 459 & CHGRA**	***T. striata* & CHGRA**	***P. lutheri* & CHGRA**	***R. lens* & CHGRA**	***P. lutheri* & *F. familica***	***P. lutheri* & *T. weissflogii***
14:0	2.78±0.04	4.91±0.22	1.96±0.42	2.78±0.38	2.46±0.20	3.61±0.24	2.33±0.07
14:1	nd	0.40±0.12	0.78±0.78	nd	nd	nd	nd
15:0i	0.27±0.02	0.12±0.12	0.13±0.13	0.24±0.15	0.08±0.08	nd	0.12±0.12
15:0ai	nd	nd	nd	nd	nd	nd	nd
15:0	0.54±0.02	0.70±0.31	1.35±1.03	0.38±0.03	0.27±0.02	0.38±0.04	0.42±0.04
15:1	0.17±0.17	0.13±0.13	0.81±0.67	0.11±0.11	0.14±0.14	0.18±0.09	0.25±0.13
16:0i	nd	nd	nd	0.05±0.05	nd	nd	nd
16:0ai	0.56±0.10	0.59±0.36	0.45±0.07	0.42±0.06	0.41±0.06	0.19±0.10	0.41±0.08
16:0	10.96±0.20	11.44±0.24	11.27±0.61	11.58±0.49	10.70±0.43	11.80±0.46	11.10±0.32
16:1n9	0.43±0.04	0.55±0.28	0.47±0.31	0.81±0.37	0.38±0.13	0.52±0.13	0.90±0.09
16:1n7	4.38±0.20	5.69±1.13	3.35±1.08	3.61±0.35	2.43±0.28	3.20±0.15	3.04±0.06
16:1n5	0.24±0.06	1.47±0.14	0.42±0.08	0.04±0.04	0.18±0.09	0.51±0.25	0.55±0.08
17:0i	0.44±0.02	0.06±0.06	0.10±0.10	0.08±0.08	0.21±0.01	0.17±0.08	0.08±0.08
17:0ai	0.69±0.15	0.88±0.16	0.56±0.21	0.64±0.13	0.64±0.16	0.39±0.02	0.10±0.10
16:2n4	0.35±0.08	0.19±0.10	nd	0.28±0.03	0.15±0.08	0.27±0.01	0.21±0.10
17:0	0.51±0.00	0.63±0.01	0.31±0.08	0.34±0.03	0.66±0.01	0.33±0.03	0.24±0.12
16:3n4	4.97±2.08	0.20±0.10	0.27±0.15	2.37±1.98	2.71±1.96	3.46±1.67	5.78±0.23
17:1	0.13±0.13	2.49±0.81	3.64±2.33	0.16±0.09	nd	0.13±0.13	nd
16:4n3	2.84±2.35	2.33±1.16	4.01±2.16	4.73±2.12	4.11±2.01	2.85±1.89	0.53±0.03
16:4n1	nd	nd	nd	0.01±0.01	nd	nd	nd
18:0	7.56±0.22	7.21±0.13	6.53±0.10	7.40±0.15	7.41±0.30	7.27±0.09	7.00±0.14

	Initial	Pav 459 & CHGRA	*T. striata* & CHGRA	*P. lutheri* & CHGRA	*R. lens* & CHGRA	*P. lutheri* & *F. familica*	*P. lutheri* & *T. weissflogii*
18:1n11	nd	nd	nd	nd	0.53±0.03	0.15±0.15	nd
18:1n9	1.62±0.14	1.05±0.15	3.29±0.46	1.50±0.25	1.80±0.24	1.49±0.32	1.38±0.08
18:1n7	3.61±0.07	2.57±0.31	4.00±1.41	4.35±0.06	2.71±0.09	4.99±0.12	4.26±0.08
18:1n6	nd	0.33±0.01	0.10±0.10	0.23±0.12	0.08±0.08	0.11±0.11	0.08±0.08
18:1n5	0.09±0.09	0.39±0.01	nd	0.31±0.11	0.12±0.07	0.25±0.14	0.30±0.15
18:2n6	0.84±0.06	0.56±0.10	2.36±0.50	0.56±0.11	0.87±0.06	0.58±0.13	1.22±0.34
18:2n4	0.13±0.07	0.07±0.07	nd	0.28±0.04	0.19±0.02	0.08±0.08	0.20±0.11
19:0	0.08±0.08	0.20±0.20	nd	nd	nd	nd	nd
18:3n6	0.06±0.06	0.42±0.21	0.32±0.16	0.39±0.08	0.13±0.07	0.37±0.07	nd
18:3n4	0.06±0.06	nd	nd	0.08±0.08	0.14±0.07	0.25±0.13	0.21±0.11
18:3n3	0.45±0.03	0.16±0.08	2.30±0.44	0.19±0.11	2.03±0.27	0.08±0.08	0.34±0.06
18:4n3	4.55±2.04	2.35±0.74	2.73±0.63	3.26±2.73	3.60±2.10	4.70±2.31	8.61±0.36
18:4n1	0.07±0.07	nd	nd	nd	nd	nd	nd
20:0	0.15±0.15	nd	nd	nd	nd	nd	nd
18:5n3	0.65±0.12	0.28±0.00	nd	0.02±0.02	0.23±0.11	0.25±0.15	0.13±0.13
20:1n11	nd	nd	nd	nd	0.12±0.12	0.73±0.73	nd
20:1n9	3.15±0.09	1.28±0.01	6.84±0.62	2.37±0.07	3.48±0.08	1.36±0.68	2.77±0.15
20:1n7	2.33±0.03	1.34±0.05	1.26±0.28	2.70±0.00	2.35±0.05	2.70±0.09	2.62±0.11
20:2α	0.08±0.08	0.09±0.09	0.14±0.14	0.26±0.13	0.28±0.07	0.22±0.11	0.23±0.11
20:2β	0.26±0.16	0.21±0.21	2.24±2.10	0.27±0.16	nd	0.09±0.09	0.08±0.08
20:2n6	0.73±0.13	0.41±0.02	3.09±0.30	0.37±0.05	0.91±0.06	0.43±0.01	0.41±0.11
20:3n6	0.66±0.02	0.57±0.01	1.15±0.33	0.68±0.04	0.75±0.05	0.66±0.03	0.59±0.02
21:0	nd	nd	nd	nd	nd	nd	nd
20:4n6	3.40±0.11	5.64±0.27	6.38±0.83	5.42±0.23	6.75±0.26	3.12±0.09	2.70±0.23
20:3n3	nd	nd	0.09±0.09	nd	0.23±0.06	nd	nd

	Initial	Pav 459 & CHGRA	*T. striata* & CHGRA	*P. lutheri* & CHGRA	*R. lens* & CHGRA	*P. lutheri* & *F. familica*	*P. lutheri* & *T. weissflogii*
20:4n3	0.06±0.06	nd	0.24±0.13	nd	0.58±0.20	nd	0.24±0.14
20:5n3	10.13±0.29	9.46±0.52	13.07±2.06	8.26±1.14	9.67±0.31	8.63±0.26	8.34±0.68
22:0	0.05±0.05	0.43±0.09	0.29±0.16	0.24±0.14	0.33±0.11	0.17±0.17	0.38±0.38
22:1n11(13)	nd	nd	nd	nd	0.14±0.14	nd	0.12±0.12
22:1n9	0.23±0.01	0.53±0.07	1.31±0.67	0.70±0.06	3.06±0.17	0.07±0.07	0.32±0.32
22:1n7	nd	nd	nd	nd	0.05±0.05	nd	0.32±0.32
21:5n3	0.55±0.03	0.22±0.12	2.61±1.67	0.81±0.09	0.34±0.18	0.45±0.10	0.67±0.16
23:0	0.07±0.07	nd	nd	nd	nd	0.08±0.08	0.14±0.14
22:4n6	2.97±2.97	1.31±1.31	0.48±0.48	3.06±3.06	nd	nd	nd
22:4n3	nd	0.11±0.11	nd	nd	nd	nd	nd
22:5n6	3.13±0.05	15.10±1.00	0.85±0.05	3.55±0.11	0.91±0.05	4.76±0.21	4.15±0.21
22:5n3	0.83±0.02	0.99±0.13	1.43±0.23	1.13±0.11	1.39±0.15	0.66±0.05	0.70±0.07
24:0	nd	nd	nd	0.13±0.07	nd	0.08±0.08	0.24±0.14
22:6n3	21.19±0.68	13.97±0.75	6.92±1.95	22.65±0.57	23.30±0.74	27.14±0.78	25.19±0.60
24:1	0.02±0.02	nd	0.11±0.11	nd	nd	0.12±0.12	nd

Appendix 18: Expanded List of Fatty Acids Corresponding to Table 4.2; Sea Scallop Postlarval Tissues from Trial I.

Percent of total fatty acids identified in the tissues of postlarval sea scallops, *Placopecten magellanicus* from Trial I. nd=not detected

	Initial	**Pav 459 (50%) & CHGRA (50%)**	***P. pinguis* & CHGRA**	**Pav 459**	***Pav 459 (25%)* & CHGRA (75%)**	***P. pinguis***	**CHGRA**
14:0	2.66±0.06	2.35±0.03	2.78±0.03	2.79±0.05	2.64±0.03	3.27±0.05	2.28±0.15
14:1	nd	nd	nd	nd	nd	nd	0.10±0.10
15:0i	0.31±0.01	0.49±0.04	0.39±0.06	0.51±0.02	0.56±0.05	0.48±0.00	0.85±0.10
15:0ai	nd	nd	0.07±0.07	0.07±0.07	0.07±0.07	nd	nd
15:0	0.30±0.00	0.37±0.01	0.37±0.00	0.37±0.00	0.39±0.01	0.37±0.01	0.53±0.04
15:1	nd	0.21±0.06	0.07±0.07	0.27±0.07	nd	0.06±0.06	0.13±0.07
16:0i	0.21±0.00	0.31±0.01	0.22±0.02	0.32±0.01	0.30±0.00	0.30±0.01	0.26±0.05
16:0ai	0.38±0.02	0.42±0.10	0.42±0.09	0.51±0.17	0.56±0.03	0.74±0.02	0.35±0.03
16:0	11.98±0.04	11.79±0.15	12.41±0.08	11.33±0.01	12.18±0.01	11.77±0.13	11.72±0.21
16:1n11	0.16±0.08	0.38±0.11	0.12±0.12	0.53±0.04	0.41±0.09	0.43±0.06	0.16±0.09
16:1n9	0.19±0.01	0.07±0.07	0.15±0.01	nd	nd	0.06±0.06	0.13±0.07
16:1n7	5.25±0.11	3.55±0.07	3.53±0.17	3.31±0.12	5.05±0.08	3.18±0.03	5.21±0.18
16:1n5	0.63±0.01	0.63±0.02	0.54±0.03	1.10±0.00	0.47±0.01	1.01±0.02	0.47±0.03
17:0i	0.23±0.00	0.29±0.01	0.31±0.00	0.21±0.02	0.32±0.00	0.25±0.01	0.42±0.01
17:0ai	0.74±0.02	0.83±0.08	0.85±0.06	0.58±0.06	1.09±0.01	0.71±0.01	1.01±0.03
16:2n4	.01	0.22±0.01	0.21±0.01	0.27±0.01	0.35±0.04	0.21±0.01	0.39±0.02
17:0	0.61±0.00	0.84±0.01	0.90±0.03	0.90±0.01	0.73±0.01	0.95±0.01	0.77±0.02
16:3n4	0.10±0.05	nd	nd	nd	0.47±0.13	nd	0.33±0.05
17:1	0.42±0.21	0.98±0.84	0.90±0.32	1.31±0.59	0.09±0.09	0.35±0.05	0.45±0.16

	Initial	Pav 459 (50%) & CHGRA (50%)	*P. pinguis* & CHGRA	Pav 459	*Pav 459 (25%)* & CHGRA (75%)	*P. pinguis*	CHGRA
16:4n3	5.41±0.14	4.26±0.84	4.72±0.28	3.99±0.64	4.79±0.14	5.40±0.02	4.34±0.36
16:4n1	nd	0.08±0.08	nd	0.12±0.12	nd	nd	nd
18:0	5.84±0.03	5.92±0.15	6.56±0.27	5.16±0.08	6.18±0.08	5.75±0.02	6.80±0.27
18:1n11	0.75±0.03	0.76±0.12	0.95±0.06	0.52±0.04	0.68±0.10	0.60±0.09	0.61±0.03
18:1n9	6.03±0.04	5.48±0.04	5.47±0.07	3.80±0.14	6.41±0.08	3.79±0.06	6.43±0.06
18:1n7	nd	nd	nd	nd	nd	nd	nd
18:1n6	nd	nd	nd	nd	nd	nd	nd
18:1n5	0.31±0.02	0.41±0.01	0.43±0.02	0.46±0.03	0.44±0.01	0.51±0.03	0.47±0.03
18:2n6	0.46±0.01	0.60±0.02	0.30±0.01	0.90±0.01	0.51±0.02	0.25±0.01	0.36±0.00
18:2n4	0.32±0.01	0.42±0.01	0.43±0.01	0.30±0.01	0.50±0.01	0.23±0.01	0.39±0.02
19:0	0.05±0.05	nd	nd	nd	nd	nd	nd
18:3n6	0.62±0.03	0.42±0.02	0.32±0.02	0.45±0.01	0.46±0.02	0.27±0.01	0.38±0.04
18:3n4	nd	nd	nd	0.04±0.04	nd	nd	nd
18:3n3	0.31±0.01	0.25±0.01	0.19±0.00	0.49±0.01	0.06±0.06	0.33±0.01	nd
18:4n3	0.55±0.01	1.25±0.88	0.47±0.04	0.67±0.03	0.27±0.01	0.93±0.00	0.48±0.14
18:4n1	nd	nd	nd	nd	nd	nd	nd
20:0	0.07±0.07	nd	nd	nd	nd	nd	nd
18:5n3	1.00±0.08	1.26±0.03	1.22±0.05	1.71±0.04	0.98±0.01	1.51±0.02	1.08±0.05
20:1n11	2.85±0.08	3.32±0.04	3.56±0.15	2.78±0.04	3.18±0.01	3.20±0.08	3.18±0.09
20:1n9	nd	nd	nd	nd	nd	nd	nd
20:1n7	0.94±0.03	1.17±0.06	0.17±0.00	0.63±0.03	1.24±0.09	0.56±0.16	1.96±0.18
20:2α	nd	nd	nd	nd	nd	nd	nd
20:2β	nd	nd	nd	nd	nd	nd	nd
20:2n6	0.18±0.00	0.42±0.02	0.07±0.07	0.83±0.01	0.20±0.01	0.11±0.06	nd
20:3n6	0.20±0.00	0.11±0.06	0.14±0.01	0.10±0.0	0.20±0.00	nd	0.07±0.07

	Initial	Pav 459 (50%) & CHGRA (50%)	*P. pinguis* & CHGRA	Pav 459	*Pav 459 (25%)* & CHGRA (75%)	*P. pinguis*	CHGRA
21:0	nd	nd	nd	nd	nd	nd	nd
20:4n6	11.44±0.03	11.04±0.16	11.06±0.33	10.09±0.08	12.20±0.04	9.35±0.08	12.60±0.36
20:3n3	nd	0.05±0.05	nd	nd	nd	nd	0.30±0.15
20:4n3	0.06±0.06	0.27±0.19	nd	0.11±0.11	0.52±0.26	0.12±0.12	0.29±0.15
20:5n3	9.62±0.13	7.83±0.09	8.65±0.10	6.31±0.04	9.53±0.04	6.90±0.08	7.76±0.09
22:0	nd	nd	nd	nd	nd	nd	nd
22:1n11(13)	nd	nd	nd	nd	nd	0.10±0.10	nd
22:1n9	0.74±0.01	0.93±0.04	0.82±0.01	0.32±0.01	1.27±0.02	0.33±0.02	1.07±0.04
22:1n7	nd	nd	nd	nd	nd	nd	0.08±0.08
22:2NMIDa	nd	nd	nd	nd	nd	nd	nd
22:2NMIDb	1.03±0.12	1.28±0.18	1.23±0.02	0.56±0.05	1.14±0.09	0.90±0.20	1.19±0.03
21:5n3	0.43±0.01	0.57±0.06	0.43±0.03	0.59±0.01	0.45±0.04	0.41±0.04	0.35±0.05
23:0	nd	0.09±0.09	nd	nd	nd	nd	nd
22:4n6	1.24±0.03	1.68±0.08	1.48±0.02	1.58±0.00	1.59±0.03	1.43±0.02	1.61±0.09
22:4n3	nd	nd	nd	nd	nd	nd	nd
22:5n6	9.52±0.04	12.99±0.19	11.09±0.10	18.79±0.14	8.72±0.14	15.09±0.10	7.21±0.25
22:5n3	1.10±0.15	0.90±0.03	0.92±0.18	0.73±0.12	0.97±0.12	1.14±0.12	1.10±0.12
24:0	0.07±0.07	nd	nd	nd	nd	nd	nd
22:6n3	14.44±0.04	12.50±0.14	15.08±0.08	13.57±0.06	11.83±0.04	16.65±0.08	14.24±0.63
24:1	nd	nd	nd	nd	nd	nd	nd

Appendix 19: Expanded List of Fatty Acids Corresponding to Table 4.3; Sea Scallop Postlarval Tissues from Trial II.

Percent of total fatty acids identified in the tissues of postlarval sea scallops, *Placopecten magellanicus* from Trial II. nd=not detected

	Pav 459 (50%) & CHGRA (50%)	***P. pinguis* & CHGRA**	**Pav 459**	**CHGRA**
14:0	4.11±0.07	4.62±0.23	3.94±0.30	3.14±0.16
14:1	0.56±0.20	0.41±0.17	0.81±0.27	5.46±0.43
15:0i	0.12±0.12	0.09±0.09	0.35±0.15	1.25±0.22
15:0ai	0.11±0.11	0.23±0.08	0.48±0.11	2.36±0.27
15:0	0.31±0.00	0.26±0.01	0.46±0.09	0.91±0.03
15:1	0.11±0.11	0.17±0.02	0.31±0.04	0.70±0.01
16:0i	0.10±0.10	0.18±0.02	0.30±0.10	nd
16:0ai	0.12±0.12	nd	0.12±0.12	nd
16:0	11.27±0.08	11.30±0.29	11.48±0.66	12.20±1.06
16:1n11	0.24±0.04	0.13±0.13	0.20±0.20	nd
16:1n9	nd	nd	nd	nd
16:1n7	4.64±0.05	5.36±0.23	4.73±0.33	2.93±0.06
16:1n5	0.98±0.05	0.76±0.01	1.87±0.12	4.16±0.27
17:0i	nd	nd	0.13±0.13	1.04±0.12
17:0ai	0.86±0.11	0.92±0.00	0.46±0.05	2.41±0.68
16:2n4	1.15±0.09	0.71±0.10	1.35±0.02	4.41±0.80
17:0	0.48±0.01	0.55±0.01	0.87±0.02	0.69±0.16
16:3n4	0.32±0.32	0.82±0.03	nd	0.63±0.00
17:1	1.56±0.25	1.93±0.36	2.59±0.61	2.71±0.44
16:4n3	1.52±0.17	0.87±0.48	0.26±0.26	0.32±0.32
16:4n1	0.11±0.11	0.13±0.13	0.47±0.28	1.17±0.22
18:0	4.58±0.36	4.30±0.28	4.42±0.16	8.18±0.56
18:1n11	0.95±0.04	0.81±0.01	0.97±0.21	3.16±0.45
18:1n9	4.00±0.02	5.00±0.03	3.25±0.23	4.94±0.44
18:1n7	nd	nd	nd	nd
18:1n6	nd	nd	nd	nd
18:1n5	0.25±0.01	0.27±0.01	0.32±0.04	nd
18:2n6	1.49±0.08	0.52±0.00	1.55±0.20	2.86±0.36
18:2n4	0.85±0.02	0.87±0.01	0.49±0.01	0.55±0.10
19:0	nd	nd	nd	nd
18:3n6	0.54±0.00	0.42±0.00	0.56±0.03	0.60±0.60
18:3n4	nd	nd	nd	0.68±0.14
18:3n3	1.17±0.04	0.54±0.03	0.91±0.13	0.25±0.25
18:4n3	1.69±0.04	2.29±0.15	0.96±0.16	1.76±1.00

	Pav 459 (50%) & CHGRA (50%)	***P. pinguis* & CHGRA**	**Pav 459**	**CHGRA**
18:4n1	nd	nd	nd	nd
20:0	nd	nd	nd	0.22±0.22
18:5n3	0.82±0.03	0.67±0.04	1.36±0.08	0.26±0.26
20:1n11	0.44±0.02	0.54±0.04	0.49±0.08	0.77±0.22
20:1n9	nd	nd	nd	nd
20:1n7	0.80±0.05	0.85±0.00	0.62±0.03	1.64±0.29
20:2α	nd	nd	nd	0.71±0.13
20:2β	nd	nd	nd	0.91±0.28
20:2n6	0.65±0.00	0.18±0.02	1.00±0.09	nd
20:3n6	0.21±0.00	0.07±0.07	nd	nd
21:0	nd	nd	nd	nd
20:4n6	5.54±0.04	5.07±0.07	6.90±0.17	4.82±0.93
20:3n3	0.21±0.00	0.07±0.07	nd	0.32±0.32
20:4n3	0.18±0.01	0.24±0.02	0.26±0.01	nd
20:5n3	18.71±0.35	20.55±0.52	9.06±1.17	7.57±0.96
22:0	nd	nd	nd	0.23±0.23
22:1n11(13)	0.08±0.08	nd	nd	nd
22:1n9	0.30±0.02	0.34±0.01	nd	nd
22:1n7	nd	nd	nd	nd
22:2NMIDa	nd	nd	nd	nd
22:2NMIDb	0.59±0.01	0.47±0.32	0.36±0.13	0.37±0.37
21:5n3	0.60±0.04	0.66±0.00	0.34±0.12	0.57±0.57
23:0	nd	nd	nd	nd
22:4n6	0.67±0.02	0.55±0.03	1.06±0.06	nd
22:4n3	nd	nd	nd	nd
22:5n6	13.28±0.06	11.70±0.20	20.98±0.15	3.17±0.55
22:5n3	0.72±0.04	0.86±0.03	0.45±0.03	0.29±0.29
24:0	nd	nd	nd	0.40±0.40
22:6n3	12.02±0.06	12.72±0.12	12.54±0.28	7.92±1.45
24:1	nd	nd	nd	0.35±0.35

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