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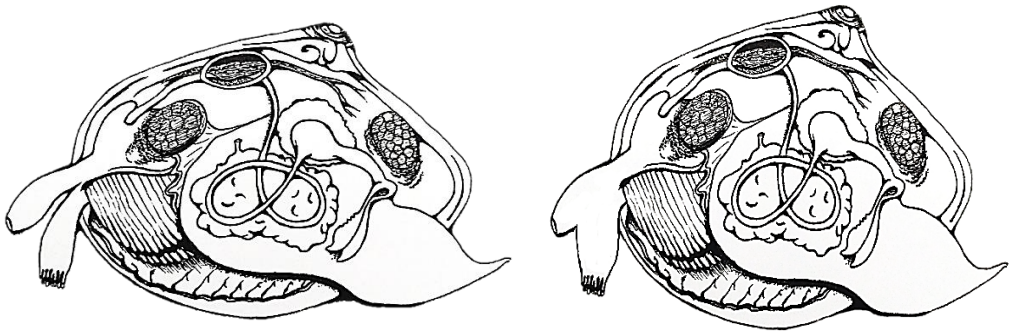
STOICHIOMETRIC CHARACTERIZATION AND PHYSIOLOGICAL BASES OF DIFFERENTIAL GROWTH IN THE CONGENERIC CLAM SPECIES *RUDITAPES DECUSSATUS* AND *R. PHILIPPINARUM*



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A mi familia,

y en especial, a Alex

~ RESUMEN ~

En la presente Tesis se plantea el estudio del crecimiento en dos especies de almejas de alto interés acuícola, pertenecientes al mismo género: la almeja japónica *Ruditapes philippinarum* (Adams and Reeve, 1850) y la almeja fina *Ruditapes decussatus* (Linnaeus, 1758). Dicho estudio se aborda desde una doble perspectiva que remite a dos importantes temas de la acuicultura relacionados con el control del crecimiento:

a) El análisis de la dependencia entre crecimiento y factores nutricionales, basado en la manipulación experimental de la composición bioquímica de las dietas y centrado principalmente en la determinación de los efectos potenciales de la alteración del balance de nutrientes y de los correspondientes mecanismos compensatorios desplegados por los bivalvos en forma de respuestas fisiológicas específicas.

b) La caracterización fisiológica de un componente endógeno del crecimiento responsable de la variabilidad inter individual del crecimiento observada al comparar diferentes fenotipos generados mediante selección de familias y de tallas individuales dentro de cada familia.

Un asunto que ha merecido especial atención en esta Tesis es el de la interacción dieta-fenotipo, en lo que se refiere al modo en que los mecanismos fisiológicos de ajuste al cambio en la calidad nutricional de la dieta resultan modulados en función de los requerimientos variables suscitados por existencia de diferentes fenotipos de crecimiento.

El estudio comprende el análisis de las respuestas agudas y crónicas de individuos juveniles de ambas especies a los mencionados cambios de composición bioquímica de la dieta, medidas en términos de tasa de crecimiento determinadas directamente y a través de sus componentes fisiológicos. En esta segunda aproximación al crecimiento se combinaron determinaciones del balance energético con las estimaciones de los balances elementales (de C, N, H) a fin de obtener una caracterización del crecimiento basada, simultáneamente, en la energía retenida al término de la cadena de procesos de ingestión, absorción, asimilación y metabolismo (el "scope for growth" o SFG) y en las relaciones estequiométricas entre elementos y su modificación a lo largo de esta cadena. Una vez caracterizados, los balances de nutrientes se combinaban con determinaciones de la composición bioquímica de los tejidos corporales a fin de identificar potenciales limitaciones nutricionales y establecer los mecanismos fisiológicos (y específicamente metabólicos) desarrollados para compensar posibles desequilibrios estequiométricos entre la dieta ingerida y los tejidos en crecimiento.

Esta Tesis consta de cinco capítulos, que incluyen una Introducción (Capítulo 1), tres capítulos (2, 3 y 4) que constituyen el cuerpo experimental y un capítulo final (Capítulo 5) donde se resumen las principales conclusiones. Los tres capítulos centrales describen la investigación realizada que se articula en torno a cuatro grandes objetivos que se describen a continuación:

La integración de datos de balances elementales en la estimación del crecimiento de los tejidos corporales basada en datos de componentes bioquímicos mayoritarios suscita una importante cuestión metodológica asociada a los requisitos de factores de conversión apropiados. El problema se plantea, específicamente, en aquellos casos en que el reducido tamaño de los individuos utilizados en los experimentos impide la disección de los tejidos blandos, ya que las muestras del animal completo no permiten el análisis elemental (a causa de la interferencia del C inorgánico de la concha en dichas determinaciones), debiendo basarse el análisis de la composición de estas muestras en la extracción y cuantificación de componentes bioquímicos. Por consiguiente, un **primer objetivo** de este trabajo (abordado en el Capítulo 3) consistió en el desarrollo procedimientos de inter conversión entre ambos tipos de determinaciones de la composición corporal, basados en análisis de correlación realizados comparando determinaciones simultáneas de composición bioquímica y elemental en muestras de distintos órganos y tejidos en especímenes adultos de ambas especies de almeja.

Un **segundo objetivo** de esta Tesis, transversal a toda la experimentación fisiológica, conste en el análisis de las bases energéticas y estequiométricas del crecimiento diferencial observado entre distintos fenotipos de crecimiento, bajo condiciones nutricionales variables. En los capítulos 2 y 4 se analizan comparativamente los mecanismos fisiológicos del crecimiento en almejas seleccionadas por su tasa (alta y baja) de crecimiento, a fin de validar las siguientes hipótesis: a) los balances energéticos permiten dar cuenta de las diferencias de crecimiento entre ambos fenotipos y b) los especímenes y/o familias de alto crecimiento presentan, por comparación con los de bajo crecimiento, atributos del comportamiento fisiológico compatibles con una más efectiva compensación de los déficits nutricionales.

Los dos objetivos finales de la Tesis remiten a la evaluación fisiológica de los efectos derivados de alterar el balance de nutriente de la dieta sobre el crecimiento, a fin de identificar potenciales limitaciones nutricionales y establecer los correspondientes

mecanismos de compensación desarrollados a través de respuestas agudas y crónicas. Así, un **tercer objetivo** se centró en explorar los efectos del contenido proteico de la dieta, comparando el comportamiento fisiológico de almejas tratadas con dos dietas iso calóricas (mismo contenido energético) pero ampliamente divergentes en la ratio proteínas/energía (P/E); dietas cuya composición se basaba en microalgas de una misma especie (*Rhodomonas lens*) cosechadas en las fases exponencial (alto contenido proteico) o estacionaria (bajo contenido proteico) del cultivo. Los resultados de esta investigación, desarrollada en juveniles de la almeja japónica (*R. philippinarum*) se presentan en el Capítulo 2, que comprende tres capítulos en los cuales el comportamiento fisiológico se aborda mediante a) métodos de energética fisiológica y comparaciones entre tratamientos (dietas y fenotipos) basadas en el balance energético o SFG (Capítulo 2.1); b) balances elementales combinados con un análisis estequiométrico de la evolución de la ratio C:N a través de la serie de procesos fisiológicos que conectan la alimentación con el crecimiento (Capítulo 2.2), a fin de identificar posibles mecanismos de regulación homeostática de los nutrientes elementales. La integración de los balances elementales en los procesos de crecimiento se contrataba por referencia a la composición elemental ponderada de los tejidos corporales, posibilitando la identificación de condiciones específicas de limitación de nutrientes mediante el cómputo de ratios umbral (*threshold element ratios*: TER) (Capítulo 2.3).

Otro procedimiento utilizado para manipular el balance de nutrientes en la dieta consistió en utilizar mezclas, en distintas proporciones, de microalgas (*R. lens*) y levadura (*Saccharomyces cerevisiae)*, con el propósito de obtener distintas dietas con un mismo contenido proteico (ratio C:N constante) pero diferentes proporciones entre lípidos y carbohidratos (L/C). El **cuarto objetivo** de la Tesis, basado en el uso de estas dietas en la alimentación de juveniles de la almeja fina (*R. decussatus*), se desarrolla en el Capítulo 4, en el que se analizan los mecanismos fisiológicos desplegados como respuesta (aguda y crónica) a las restricciones potenciales en la adquisición de nutrientes específicos, en relación con el uso de dichos nutrientes como reservas energéticas y tomando en consideración, de forma especial, los desequilibrios lipídicos derivados de los procesos digestivos intracelulares. Como en el objetivo anterior, las respuestas fisiológicas de abordan mediante una combinación de métodos de energética (determinaciones de SFG) (Capítulo 4.1) y balances elementales que, integrados con datos de composición

bioquímica de los tejidos corporales, permiten configurar una descripción estequiométrica del proceso de crecimiento (Capítulo 4.2).

Se presenta a continuación una recapitulación ordenada de los principales resultados obtenidos y conclusiones alcanzadas en los capítulos centrales de la Tesis:

Capítulo 2

a) En el tratamiento con dietas ricas en proteína (N+), los juveniles de almeja japónica obtienen mayores balances netos de energía y mayores balances elementales (C y N), con cifras de SFG que superan en un 50% como promedio los valores alcanzados por los tratados con dietas pobres en proteína (N-). Los potenciales mecanismos compensatorios del déficit de proteínas ingeridas (e.g., sobrealimentación) aparecen limitados en su eficacia por las restricciones digestivas que se observan con las dietas N-, en forma de bajos valores de la eficiencia de absorción. Las almejas tratadas con la dieta N+ también alcanzas los más elevados balances de C y N como resultado de una combinación de tasas de ingestión y eficiencias de absorción más elevadas. Sin embargo, la absorción preferente del N sobre el C, que se observa en mayor o menor grado en todos los tratamientos, resulta mucho más acusada en las almejas sometidas a un déficit crónico de N (aclimatadas a dietas N-). Este mecanismo compensatorio, que opera en los procesos de absorción, se observa en todos los fenotipos y da lugar a una regulación homeostática parcial, que se completa con la regulación postabsortiva basada en la reducción de la tasa de excreción de amonio observada en los tratamientos con la dieta pobre en N.

b) Las diferencias endógenas en el balance energético observadas entre los diferentes fenotipos responden a diferencias en los procesos de adquisición de energía, con valores de tasa de ingestión en los grupos de crecimiento rápido que duplican los correspondientes valores en los de crecimiento lento. Adicionalmente, los costes metabólicos unitarios (por unidad de energía metabolizable) eran significativamente menores en los primeros, indicando que una mayor eficiencia metabólica es también clave para explicar un mayor potencial endógeno de crecimiento. En lo que se refiere a los balances elementales, las principales diferencias se encontraron entre grupos de crecimiento intra familiares, donde los individuos de crecimiento rápido superaban en un 50% a los de crecimiento lento en relación con las tasas de absorción de C o N. Adicionalmente, los especímenes de crecimiento rápido presentan evidencias de una mayor rapidez y funcionalidad en las respuestas agudas al cambio en la composición de

la dieta, lo que revela una mayor plasticidad de este fenotipo. En cambio, los ajustes estequiométricos observados en los fenotipos de crecimiento lento dan lugar a mayores tasas de eliminación de N a través de los procesos de egestión y excreción, lo que sugiere un recambio proteico menos eficiente.

c) El factor fenotipo (familia, principalmente) es responsable de las principales diferencias en índice de condición y composición corporal por órganos, mientras que la dieta de acondicionamiento ejerce efectos menores a este respecto. Todos los fenotipos muestran sus mayores tasas de crecimiento en el tratamiento con las dietas N+, cuya ratio C:N, comparada con la de los tejidos corporales, indica buena coincidencia con las ratios umbral (TER). En cambio, la ratio C:N de las dietas N- sobrepasan ampliamente los valores umbral para este par de elementos, indicando el carácter N limitante de estas dietas. La composición de mayoría de los órganos y tejidos del cuerpo aparecen sometidos a rigurosa regulación homeostática, con la excepción de la glándula digestiva, cuya ratio C:N refleja las diferencias en la composición de las dietas suministradas debido posiblemente a la mayor tasa de recambio que experimenta este órgano. La composición elemental del cuerpo (ponderada en función del peso de cada tejido analizado) se muestra independiente de la dieta suministrada, con ratios C:N globales prácticamente constantes con los distintos tratamiento, especialmente en el caso de los fenotipos de alto crecimiento que, en general, muestran una mayor plasticidad frente al cambio en las condiciones nutricionales.

Capítulo 3

a) La aplicación de factores de conversión estándar para estimar la composición elemental a partir de los componentes bioquímicos mayoritarios revela un grado aceptable de coincidencia, tomando en cuenta las siguientes consideraciones: *1)* el uso de factores de conversión fuera del rango considerado en esta Tesis debe ser contrastado; *2)* se debe introducir un factor específico de corrección para el agua residual en muestras secas de tejidos corporales de bivalvos (y otros organismos marinos) para análisis CNH; *3)* se debe incorporar la cuantificación de sustancias ninhidina positiva (NPS) al los análisis de composición bioquímica para la estimación precisa de la composición en N, ya que este N no proteico suele representar una fracción significativa del contenido total en N. La magnitud de los contenidos en NPS en tejidos de bivalvos limita considerablemente el cómputo inverso de componentes bioquímicos mayoritarios a partir

de la composición elemental ya que no es posible la estimación exacta de proteínas a partir del contenido en N.

b) La ausencia de diferencias en composición corporal, encontrada al comparar ambas especies de almejas refleja sus grandes semejanzas a nivel morfológico, funcional y evolutivo, mientras las diferencias entre los tejidos analizados corresponden al carácter diferencial de las funciones desempeñadas por cada uno de ellos en el conjunto del organismo. Otras fuentes de variación en la composición tales como la secuencia estacional, la dieta o el sexo deben ser objeto de investigación futura.

Capítulo 4

a) Los juveniles de almeja fina (*R. decussatus*) acondicionados a dietas de alto contenido lipídico (mayor abundancia de fitoplancton) presentan mayores tasas de crecimiento y, correspondientemente, valores más elevados del balance energético. El acondicionamiento previo a estas dietas de mayor calidad, al proporcionar una óptima condición fisiológica, permite así mismo una más efectiva compensación de las condiciones nutricionales sub-óptimas, contribuyendo al mantenimiento de balances energéticos satisfactorios durante la respuesta aguda a dietas de baja calidad (ratio lípidos/carbohidratos reducida). Este resultado del acondicionamiento se basa en una combinación de factores alimentarios y digestivos que contribuyen al incremento simultaneo de la tasa de adquisición de energía y de la eficiencia de absorción a partir de la dieta rica en lípidos.

b) Los balances elementales de C y N alcanzan los valores más elevados en las almejas juveniles expuestas a la dieta rica en lípidos, lo que sugiere que este componente puede ser limitante con un régimen alimentario en el que predominen los carbohidratos. Este resultado se explica por la diferente magnitud del desequilibrio lipídico entre almejas tratadas con distintas dietas, donde, por ejemplo, el procesamiento digestivo del alimento pobre en lípidos ocurre con mayores pérdidas endógenas de este componente debido posiblemente a la baja digestibilidad relativa de las células de levadura. El ajuste, en términos estequiométricos, entre la dieta consumida y la biomasa corporal radica en los procesos postabsortivos más que en los preabsortivos, aunque la comparación entre dieta de acondicionamiento revela importantes diferencias en este aspecto. Finalmente, la composición bioquímica de los tejidos corporales refleja aproximadamente la composición de la dieta suministradas, lo que indica un bajo nivel de de regulación

homeostática de los equilibrios nutricionales inducidos experimentalmente. Los presentes resultados indican que la sustitución, por encima del 50%, de microalgas por dietas inertes ricas en carbohidratos puede comprometer el crecimiento, así como los balances de lípidos y proteínas.

c) Las principales diferencias entre especímenes de alta y baja tasa de crecimiento se explican en función de patrones diferenciales de adquisición de energía. Además, los beneficios de las dietas enriquecidas en el componente lipídico, en términos de rendimiento de crecimiento, son comparativamente mayores en los fenotipos de crecimiento rápido que en los lentos. De otro modo, las limitaciones nutricionales experimentadas por los juveniles de la almeja fina resultan más efectivamente compensadas en los seleccionados por su alta tasa endógena de crecimiento, lo que indica la importancia de un óptimo estatus energético en el mantenimiento de la regulación homeostática de los nutrientes.

Chapter 1
Introduction

1. PRESENTATION

This Thesis addresses growth of two highly valuable commercial species of clams, *Ruditapes philippinarum* (Adams and Reeve, 1850) and *Ruditapes decussatus* (Linnaeus, 1758), from a double perspective that appeals to correspondingly important topics in aquaculture:

a) The analysis of the dependency of growth on dietary factors that derive from the manipulation of biochemical composition of food, centered mainly on the assessment of the potential consequences of nutrient imbalances and the corresponding compensatory mechanisms deployed by the animals in the form of specific physiological responses.

b) The physiological characterization of an endogenous component that underlies inter individual variability in growth rate, which can be realized in a range of growth phenotypes obtained by individual size or family-selection.

One issue of particular interest in this Thesis has been the interaction diet-phenotype as regards the ways by which physiological adjustments to diet variation become modulated in order to fulfill the contrasting requirements set by the occurrence of intrinsic differences in growth performance that characterize different phenotypes. Physiological quantification and characterization of growth combined bioenergetics and elemental analysis methodologies designed to build up growth models based on both energy (SFG) and nutrient balances. Determination of elemental balances in a broad nutritional scenario was combined with the biochemical composition of body tissues to develop a stoichiometric approach centered on the analysis of potential nutrient limitations and the occurrence of physiological/metabolic mechanisms to compensate for stoichiometric imbalances between the diet and growing tissues.

An important methodological question arises as regards the integration of data concerning elemental balances in growth estimates based in proximate biochemical composition of body tissues, because of the need of reliable conversion factors. This difficulty specifically occurs when the small size of the spat used in experiments (e.g., those in Chapter 4) prevents soft body dissection from the shell, making elemental analysis of tissue samples impracticable. Thus, one **first aim** of this Thesis (accomplished in Chapter 3) was to develop inter-conversion tests for both methodologies approaching the body composition, based on correlation analysis performed on the simultaneous

determinations of both biochemical and elemental composition in samples of different organ and tissues of adult specimens of the two clam species.

One **second aim** of this Thesis, that is transversal to all physiological experimentation, consisted in the analysis of bioenergetic and stoichiometric bases of differential growth in actively growing clams under variable nutritional scenarios. Both Chapter 2 and Chapter 4 compare physiological behavior of fast and slow growing clams to test the hypothesis that *a)* energy balances account for observed differences between both phenotypes and *b)* fast growers appear better suited than their slow grower counterparts to achieve the appropriated adjustments on variable nutrient inputs so as to maintain more efficiently their elemental homeostasis.

The last two objectives of this Thesis concern to the physiological assessment of the effects on growth of changing the nutrient balances of the diets in order to identify potential nutrient limitations together with the compensatory mechanisms of such limitations deployed by clams along both the short (acute responses) and long term (chronic responses). Thus, one **third aim** was to explore the impact of isocaloric diets though presenting a wide range of variation in the protein to energy (P/E) ratios, that was accomplished by using diets based on a same species of microalgae (*Rhodomonas lens*) harvested in either the exponential or stationary phases of culture. The outcome of this research performed on juvenile Manila clams (*R. philippinarum*) is reported in Chapter 2, which comprises three subchapters where physiological behavior is approached by means of energy balances and *scope for growth* (SFG) comparisons between treatments (diets and phenotypes) (Chapter 2.1) and elemental balances combined with stoichiometric analysis based on tracking C:N ratios along the chain of physiological components connecting feeding and growth (Chapter 2.2), in order to identify possible mechanisms of homeostatic nutrient regulation. Integration of elemental balances into growth processes were referred to whole tissues elemental composition of clams and specific conditions of nutrient limitation identified by computing *threshold element ratios* (TER) (Chapter 2.3).

Similarly, nutrient balances in the diet were "manipulated" by using different proportions of mixtures of the microalgae *R. lens* and the baker's yeast *Saccharomyces cerevisiae* in order to achieve a range of lipids to carbohydrates (L/C) ratios in diets that however maintained constant C:N ratios. A **fourth aim**, based on the use of these diets to feed juvenile carpet shell clams (*R. decussatus*), was accomplished in Chapter 4 and

consisted in exploring the suit of physiological responses (both acute and chronic) to deal with potential restrictions in the income of specific nutrients, related to their use as energy reserves and, more particularly, in reference to large lipid imbalances achieved through intracellular digestive processes. These physiological responses were again approached by combining energy balances and SFG determinations (Chapter 4.1) and elemental balances that were integrated with data on biochemical composition of body tissues to configure a stoichiometric description of growth (Chapter 4.2).

The sections that follow are aimed to provide a general review of the state of the art concerning the main topics deal with in this Thesis.

2. General anatomical features and morphofunctional aspects

In this section, some general remarks on basic bivalve morphology and functioning of relevant structures will be detailed. The most characteristic feature of bivalves is the presence of two shell valves that enclose the animal, which serve a supporting function (skeleton), protection against predators and water loss (in intertidal species) and prevent from mud and sand invading the mantle cavity. The main component (> 95%) of shells is calcium carbonate ($CaCO_3$), which is formed by deposition of this salt in an organic matrix, which stands for the remaining 1-5%. Both components are arranged in three layers: the outer organic layer (periostracum), which is made up of protein (conchiolin); a middle prismatic layer of calcium carbonate (aragonite or calcite); and the inner nacreous layer, which comprises both organic (proteins) and inorganic (aragonite) fractions (Gosling, 2015). Both shell valves can be closed or opened by contraction or relaxation (respectively) of anterior and posterior adductor muscles.

As for the soft body, mantle stands for the piece of tissue next to the shell valves, which is divided into two thin layers known as lobes. This tissue is composed by connective tissue with haemolymph vessels, muscles, nerves and cilia in the inner surface. Clams possess two cylindrical tubes called siphons that are extensions of the mantle edge. The inhalant siphon allows the entry of water and suspended particles, whereas the exhalant siphon serves as an exit for waste water. Siphons of *Ruditapes spp* possess the capacity to be extended far out of the shell cavity and retracted to be enclosed inside the mantle cavity in case of shell closure. As infaunal animals, this ability enables to be burrowed in the substrate while it keeps filtering particles from the water column. The

mantle cilia are especially relevant because they direct the water entered by the inhalant siphon onto the gills, but also help rejecting material out of the mantle cavity.

Both siphons and shell characteristics are the most employed features to distinguish between *R. philippinarum* and *R. decussatus* (Hurtado et al., 2011), where siphons on the former are almost fully fused, while on the latter, siphons are separated (*Figure 1 - 1*). Both species possess a large foot, mainly used for burrowing into the substrate; this function is enabled by the occurrence of many layers of circular and longitudinal muscles adjacent to a large haemolymph space (Gosling, 2015).

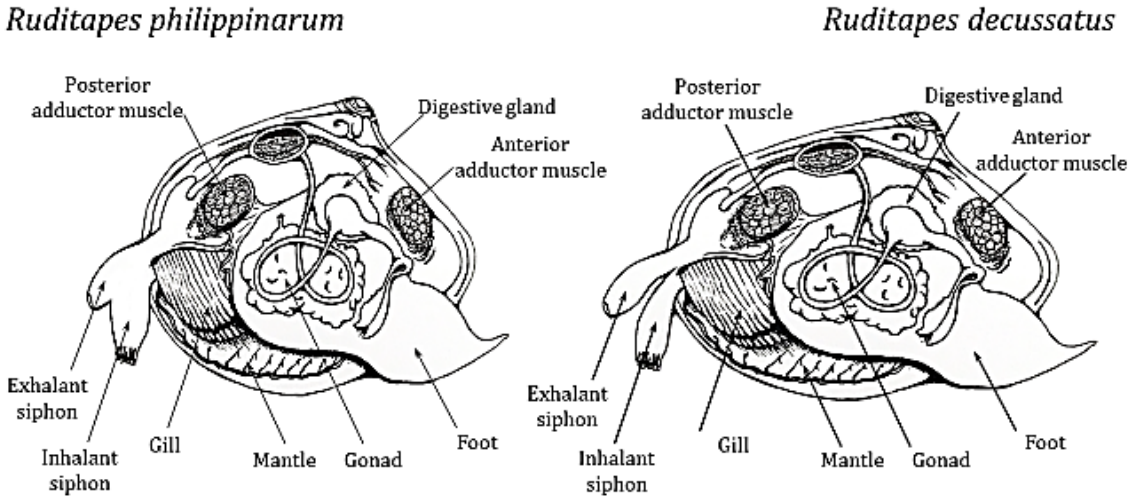

Figure 1 - 1. Morphological features of Ruditapes philippinarum (left) and R. decussatus (right). The main differences reside in shell morphology, which is more rounded in the former and more angular in the latter; and regarding siphons, these are almost fused in the former and separate in the latter species. Image drawn by M. Pérez-Cebrecos

Feeding processes are initiated in the gills, which are responsible for the entire food-gathering function (Dral, 1967; Jørgensen, 1966), and also play a role in respiratory processes. Gills consist of two structures that are divided each into two demibranchs. Each gill is fused along the dorsal margin of the mantle, and is typically expanded along the mantle cavity. Numerous ciliated filaments make up gills, which in eulamellibranch bivalves are attached to each other by interfilament junctions, that are tissue connections that leave narrow spaces between them, called ostia (Gosling, 2015). Latero-frontal cilia generate a current, which promotes the water entered by the inhalant siphon to go through the ostia and subsequently exit by the exhalant siphon; in this process the capture and transference of some particles to the frontal cilia takes place. Success on particle capture depends to a greater extent on particle size. Ward and Shumway (2004) extensively reviewed the relevant works conducted on this topic and summarized that, in general, retention efficiency (*Figure 1 - 2*) –the efficiency with which particles are captured– is

species-specific (e.g. Vahl, 1973, 1972a) and that efficiency increases, with rising particle size, to an asymptotic maximum (e.g. Haven and Morales-Alamo, 1970; Jørgensen, 1989; Langdon and Newell, 1990; Møhlenberg and Riisgård, 1978; Palmer and Williams, 1980; Riisgård, 1988; Vahl, 1972b). Considering the whole set of available data on bivalve species, critical particle size for 100% retention is above 6 µm in diameter (Møhlenberg and Riisgård, 1978), although particles above 4 µm particles are fully retained by species of mussels and clams, while oysters and scallops would reach an efficiency of 75-85% with particles of that size (Riisgård, 1988).

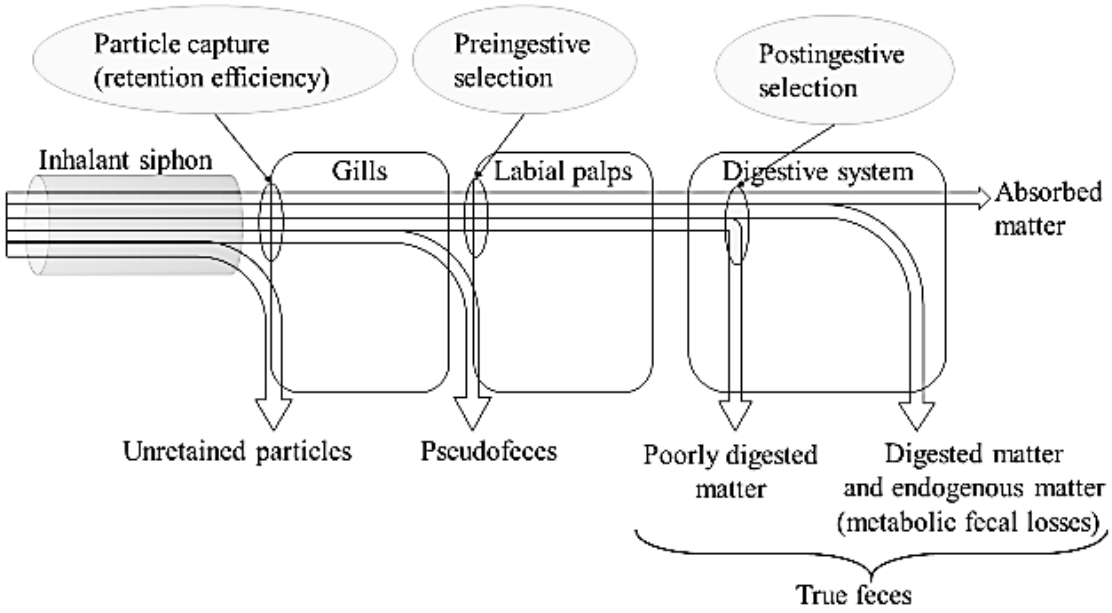

Figure 1 - 2. Diagram of the selection processes undergone by particles until absorption

The successfully retained particles are transported to the labial palps into mucous aggregates (Beninger and St-Jean, 1997a, 1997b; Foster-Smith, 1975a; Garrido et al., 2012) to subsequently enter the digestive tube via the mouth. Labial palps are selection sites prior to ingestion (*Figure 1 - 2*) designed to sort particles according to their different features such as size, specific weight or even the chemical properties of particles surface. Sorting and rejection of particles embedded in a mucous string as pseudofeces only occur above a given threshold of environmental particle concentration and is primarily considered as a way to prevent feeding processes to be collapsed due to an excess of food load (Griffiths and Griffiths, 1987). Concerning the particle features that are the subject of selective sorting during the process of pseudofeces production, the information is yet incomplete. Size effect has been particularly well documented in the two species studied in this Thesis (*R. decussatus* and *R. philippinarum*; Defossez and Hawkins, 1997). In this

study, clams were fed on differently sized particles (5 – 37 µm), but presenting the same shape, density and chemical composition to conclude that larger particles were preferentially rejected as pseudofeces. Food value of particles has been, on its side, pointed out as a relevant factor in preingestive selection since most bivalves show preferential ingestion for organic respect to inorganic suspended matter (Bayne et al., 1993; Hawkins et al., 1996; Iglesias et al., 1996, 1992; Kiørboe and Møhlenberg, 1981; Urrutia et al., 2001, 1996). Additionally, some bivalve species, such as *Crassostrea gigas*, are able to select particles not only in the labial palps, but also in the gills, which increases the success on feeding, particularly in turbid habitats (Beninger et al., 2008b).

In general, the capacity for organic enrichment of the ingested ration through pseudofeces rejection might rely on the ability of gill and palps to sort particles of different density since inorganic materials are generally heavier than organics, particularly living phytoplankton. However, a differential selection of phytoplankton in preference to organic detritus has been reported in cockles (Navarro et al., 1997; Urrutia et al., 1996) that encompasses the preferential ingestion of N over C (Urrutia et al., 1996) exhibited also by other bivalve species such as oysters (Bayne and Svensson, 2006; Beninger et al., 2008a; Pales-Espinosa et al., 2007, 2008) and mussels (Pales-Espinosa et al., 2008). Actually, the latter work reported a differential sorting efficiency even when bivalves were fed on the same microalga species, but at different growth phases of culture. For more detailed overview on preingestive selection, see also Ward and Shumway (2004).

The fraction of material finally ingested undergoes digestive process after crossing a tubular esophagus to reach the stomach. The stomach of bivalves is a complex structure that holds the crystalline style, a semi-transparent gelatinous rod, originated in the style sac, and which contains carbohydrate-splitting enzymes, that are released when this rod is dissolved as a consequence of the friction against the gastric shield (Griffiths and Griffiths, 1987; Owen, 1974; Purchon, 1971). This type of digestion combines, therefore, mechanical and chemical processes and represents the extracellular digestion phase. However, the most important digestive phase of bivalves occurs intracellularly. To this effect, a fraction of the digested matter within the stomach (*Figure 1 - 2*) is sorted by cilia and diverted into the digestive gland through the duct openings. Digestive cells possess a complex vacuolar system, including lysosomes, for the incorporation, digestion and absorption of nutrients. This process can involve a large amount of organic matter loss of

endogenous origin, since the portion of matter not digested is delivered out of digestive cells embedded in a residual digestive spherule, which implies epithelial losses in the form of apocrine secretions, including lysosomal membranes and lipid droplets (Owen, 1972). Consequently, a large amount of endogenous matter –in the range of 10 – 20% (Navarro et al., 2009)–, particularly lipids are lost in the form of metabolic fecal losses (Bradshaw et al., 1989; Hawkins and Bayne, 1985; Ibarrola et al., 2000a; Morton, 1983). Ultimately, the fraction not digested passes into the intestine, in which relevant digestive processes are not believed to occur, as a way to eliminate the generated feces. Associated to both extra and intracellular phases of digestion, two different sorts of feces have been reported: the glandular and intestinal feces, which respectively stand for matter that has been delivered to the gland, and matter that has short-circuited the digestive ducts straightforward to the intestine (Van Weel, 1961; Widdows et al., 1979). Digestive selection is believed to favor smaller (Van Weel, 1961) and organic-rich particles (Brillant and MacDonald, 2002) to be subject of intracellular digestion; but additionally, discrimination has been documented between phytoplankton species (Cucci et al., 1985; Rouillon and Navarro, 2003) or between microalgae and organic detritus (Navarro et al., 2016). Furthermore, digestive selection could also operate between morphologically identical cells but differing in their biochemical composition, as found for scallops (Brillant and MacDonald, 2003).

The circulatory system in bivalves is an open system that involves a heart with two auricles and a ventricle that delivers haemolymph to every organ in the animal. Gas exchange is produced in the gills, where oxygen diffuses from the water current into the haemolymph, although the efficiency of diffusion is relatively low (Bayne, 1976). Mantle also represents another oxygenation site, where haemolymph sinuses are broadly distributed along all the tissue (Gosling, 2015). Besides gas exchange, haemolymph plays a role as well in osmoregulation, nutrient distribution, waste elimination, internal defense and also serves as a fluid skeleton in some strategic organs, such as foot (Morton, 1967).

Excretory organs comprise the pericardial glands and the paired kidneys. The kidneys –or nephridia– consist of two spherical interconnected lobes. Each kidney possesses a glandular extreme that opens into the pericardium and a large cavity, the kidney bladder, which opens into the mantle cavity. Pericardial glands –or Keber's glands– occur at two sites, over the auricular walls of the heart and in the anterio-ventral portions (auricular pericardial gland) of the adjacent lateral mantle (mantle pericardial

gland). Keber's glands filter the haemolymph, and this filtrate is delivered to the glandular part of the kidneys, where ionic regulation through secretion and reabsorption take place. As a result, urine is accumulated in the kidney bladder prior to the released as excretory products (Kirschner, 1967; Norton and Jones, 1992). Urine comprises the end products of protein metabolism, and four nitrogen-based compounds have been identified in bivalves: ammonia, urea, amino acids and uric acid; even though ammonia represents the main compound excreted by aquatic invertebrates. Clams in fact are believed to excrete between 65 – 100% of their end product as ammonia, although these rates are species-specific and season-dependent (see review by Griffiths and Griffiths, 1987, and references therein). Besides the excretory system, some excretory products are likely to be lost by diffusion directly across the body surface and especially across the gills (Gosling, 2015).

Reproduction in bivalve mollusks depends upon both exogenous (e.g. temperature, food availability, salinity, light) and endogenous factors; and most species show a large flexibility in their reproductive strategy based on the energy available to be allocated to reproduction (Lubet, 1986). The reproductive system of clams is located at the base of the foot, and sexes are generally separate, albeit some clam species can develop hermaphrodite stages. This system consists of a pair of gonads that comprise essentially a series of branching tubules in which gametes develop to form follicles. These tubules join into ducts merging in larger ducts that form a gonoduct. Gametes are released through independent pores into the mantle cavity to be subsequently evacuated through the exhalant siphon, as fertilization is external. Reproductive cycles of commercial clams, such as *R. decussatus* and *R. philippinarum* have been widely described, since these cycles involve relevant physiological changes that also affect tissues composition and functioning. Bivalves inhabiting temperate waters are known to cope with the variability in abiotic factors using different strategies (Navarro and Iglesias, 1995): While some species develop gametes as soon as nutrient availability increases, others can start gamete production during low nutrient periods due to a previous nutrient storage process; however, most of them show intermediate strategies, making use of both stored and externally available nutrients (Sastry, 1979). Energy storage is essentially in the form of carbohydrates, and the main storage areas are muscle tissue, vesicular cells and the digestive gland (Rodríguez-Moscoso and Arnaiz, 1998). Reproductive cycles in both *R. decussatus* and *R. philippinarum* comprise a resting period in winter, where no

reproductive (or minimal) activity is shown, followed by a fast gametogenesis period in spring, and a subsequently spawning season throughout the summer season. The earliest spawning events in *R. decussatus* have been reported to occur in April (Rodríguez-Moscoso and Arnaiz, 1998), but the most frequent period and the highest intensity of spawning peaks corresponds to the period between June and August (Aníbal et al., 2011; Ojea et al., 2004; Rodríguez-Moscoso and Arnaiz, 1998; Urrutia et al., 1999). As for *R. philippinarum*, the most intense period of spawning occurs between September and October (Robert et al., 1993), although longer spawning peaks and a more intense reproductive activity have been reported (Delgado and Pérez-Camacho, 2007).

3. GROWTH

Literature on bivalve growth is overwhelmingly abundant (see review by Bayne, 2017), due to the high interest in many areas regarding the diverse goods and services provided by this zoological group to the marine coastal ecosystem. Owing to that, many approaches have been pursued, of which bioenergetics (Section 4) and stoichiometry (Section 5) principles are reviewed in the present Chapter. Factors affecting growth can be classified into endogenous (e.g. genetic) and exogenous (environment). Since growth represents the dynamical outcome of synergistic interactions between both these types of factors, only an analytical experimentally-based approach would allow discerning the contribution of each single factor on growth. In the following lines, some of the factors determining growth will be briefly listed, with a short emphasis in those that are of interest to this Thesis.

3.1 Exogenous factors: diet effects

Several environmental factors have an impact on bivalve growth, such as temperature, light, salinity, aerial exposure, wave action and tidal level, water flow and pollutants (see reviews by Bayne, 1976; Gosling, 2015; Seed, 1969), but food supply is undoubtedly the exogenous factor having the highest impact on growth, since energy to sustain growth is directly obtained from the food ingested (Seed and Suchanek, 1992).

Research conducted under controlled environmental conditions shows that growth rates increase as a positive function of food ration (Kiørboe et al., 1981; Langton et al., 1977; Walne and Spencer, 1974; Winter and Langton, 1975) until the maximum ration is reached. This limit is achieved as a result of different mechanisms -either reducing clearance rates or increasing the proportion of rejection as pseudofeces- tending to

regulate ingestion rates in order to prevent a negative effect of high food rations on growth (see e.g. Foster-Smith, 1975b; Urrutia et al., 1997; Winter, 1973).

Besides quantity of available food, composition of foodstuff is documented to limit or stimulate growth. The main source of food in bivalves is assumed to be phytoplankton, as suggested by direct correlations between chlorophyll *a* content and growth reported for both wild and cultured populations of bivalves (Figueiras et al., 2002; Pieters et al., 1980; Smaal and Van Stralen, 1990). Phytoplankton alone however does not seem to represent the entire diet, since bacteria, detritus and zooplankton are reported as well as constituents of their diet (Arapov et al., 2010; Huang et al., 2003; Langdon and Newell, 1990). Additionally, Kiørboe et al. (1981) observed enhanced growth rates of mussels when silt particles were present, suggesting that the organic matter present on silt particles could be utilized. Growth rate of scallops was also correlated with seasonal patterns of the proportions between organic and inorganic fractions of the available food (Vahl, 1980).

Proximate biochemical composition of the diet and its impact on growth has been extensively addressed due to the relevance of finding an optimum diet for aquaculture purposes. Protein levels in diets are more frequently addressed as growth drivers owing to their metabolic and structural functions. In this sense, insufficient amount of protein might be limiting to optimal growth, as indicated for both laboratory-based (Brown et al., 1997; Hawkins and Bayne, 1991; Ibarrola et al., 1996; Langton et al., 1977; Romberger and Epifanio, 1981) and field (Bayne, 2009; Gremare et al., 1997) studies. Moreover, nitrogen absorption –as an indicator of protein absorption– has been reported to be above overall absorption of organic matter in cockles (Urrutia et al., 1996) as well as absorption of proteins over lipids (Ibarrola et al., 2000b), suggesting a higher reliance on protein to sustain growth. Nevertheless, dietary needs seem to be species-dependent (Utting, 1986) and also subject to change over the lifespan of the individual, as research conducted employing larvae or spat have also pointed to a good agreement between growth and lipids (Albentosa et al., 2002, 1999; Navarro et al., 2000; Wikfors et al., 1992) or even carbohydrates (Brown et al., 1998; Whyte et al., 1989), together with proteins.

3.2 Endogenous factors: interindividual variability

Mollusks exhibit large interindividual variability in growth (Goff, 2011). Some of that variability not attributable to environmental factors can be linked to genetic

differences. In bivalve mollusks, those intraspecific differences in growth have been addressed in the past years, yet research concerning the contribution of endogenous factors to growth variability is currently a subject of interest. Pioneer studies on this topic revealed that multilocus heterozygosity is correlated with these growth differences (Bayne and Hawkins, 1997; Foltz and Chatry, 1986; Hawkins et al., 1986; Singh and Zouros, 1978; Zouros et al., 1988), and these growth differences might be partially explained by differences in acquisition of energy (Holley and Foltz, 1987). Since then, research has been mainly centered in studies based on the physiological energetics approach (e.g. Bayne, 2004, 1999; Bayne et al., 1999b, 1999a; Fernández-Reiriz et al., 2016; Hawkins and Day, 1996; Ibarrola et al., 2017; Pace et al., 2006; Toro et al., 1996; Toro and Vergara, 1998). In the last decade, our group has furthered on the physiological bases for inter individual differences in growth and differential responses to environmental factors, such as temperature or quantity and quality of available food (Prieto et al., 2020, 2019, 2018; Tamayo et al., 2016, 2015, 2014, 2013, 2011). A short review will be provided in the next section (Section 4).

4. ENERGY BALANCES: THE PHYSIOLOGICAL ENERGETICS APPROACH.

The term physiological energetic refers to the computation of physiological components of energy gains and losses of an organism. In order to experimentally test energy balance parameters, this approach makes use of the laws of thermodynamics (Kleiber, 1961) to formulate the following expression:

$$C = P + R + F + U$$

where consumption (C) represents the rate of energy acquisition through food consumption or ingested matter, production (P) is the rate of energy retained in the form of both somatic and reproductive growth, R represents the rate of metabolic energy losses, and the remaining energy losses are due to the rates of energy lost by feces (F) and excreta (U).

The most common form in which the energy balance is expressed is:

$$SFG = I - (F + U + R)$$

which results from a reorganization of the previous expression and the use, for individual production, of the term *scope for growth* (SFG), developed by Warren and Davis (1967)

an defined as the difference between the energy of the food consumed and energy losses of the animal. Consequently, SFG allows the computation of the potential energy rate that can be allocated into growth.

Regarding energy input, estimations of ingestion rates in filter-feeding mollusks start with the measurement of filtration. Filtration in bivalves is a muco-ciliary process (Ward et al., 1993; but see Jørgensen, 1990) whereby microscopic particles that are suspended in the water column are conveyed to the gill by the pumping activity of this organ, where they are retained and concentrated for feeding purposes. Methods of recording the clearance rate -the volume of water cleared from particles per unit of time- were designed to approach the more problematic direct measurement of the pumping rate, or flow rate of water provided by the biological pump (Riisgård, 2001), with both measurements becoming coincident only when suspended particles are fully retained by the gill. Thus, in concerning the use of the clearance rate as a proxy for the feeding activity, one methodological requirement is that the tracer particles attain a critical size that is compatible with retention by the gill. This has been estimated to fluctuate between 3 and 5 µm in different species of bivalves (Møhlenberg and Riisgård, 1978; Riisgård, 1988; Vahl, 1973, 1972b) depending on the development of the dorso-frontal ciliation in gill filaments (Jørgensen, 1990). It is generally assumed that for particles above these critical diameters (in the range of nanophytoplankton), the pumping rate is properly determined through the clearance rate, but this later measurement would underestimate the pumping rates for lesser sizes as a consequence of the progressive decline of gill retention efficiency. The above requirement is fully met in the present experiments, since food particles consist of 4 – 6 µm diameter cells that are above the threshold size for 100% retention efficiency reported for both clam´s species (Defossez and Hawkins, 1997; Ward and Shumway, 2004). On the other hand, since no pseudofeces production was detected in any of the present feeding conditions, all the material filtered by clams was considered to be ingested.

The estimation of the amount of material ingested that is effectively absorbed by the individual relies upon the assumption that the inorganic matter is conserved in the digestive tract; i.e., all these materials ingested with the food –particulate inorganic matter (PIM)– is evacuated in the feces (Conover, 1966). To enable absorption efficiency determinations using this Conover method, feeding experiments were designed using diets that had the same organic ration and biochemical composition as the conditioning

diets, but incorporated a constant amount (around 30% in weight) of this conservative tracer in the form of silt particles from natural sediments previously sieved (< 63 µm) and ashed at 450ºC in a muffle furnace to remove any sedimentary organic matter.

Metabolic rate (R) represents the main source of energy losses. Because of that, reduction of metabolic costs constitutes an effective mechanism to preserve energy, as these costs might account for up to 88% of the absorbed energy (Bayne and Newell, 1983). Metabolic rate summarizes the set of chemical reactions taking place in the organism and many factors, both environmental and endogenous, can alter these rates, such as temperature, salinity, oxygen availability, body size, activity, gametogenic stage and sex (see review by Bayne and Newell, 1983). Experimental determination of metabolic expenditures in bivalves is commonly assessed indirectly by computation of the oxygen consumed per unit of time. Common respirometric measurements proceed with animals placed in sealed chambers filled with oxygenated seawater and the rates of oxygen depletion over a given period of time being assumed to represent the oxygen consumption by the animal. Measurements are usually taken with the aid of oxygen probes connected to oxymeters that allows the continuous recording for a long period to obtain reliable results. Respiration rates measured in mL O_2 h^{-1} were converted to energy equivalents using an oxycaloric equivalent of 20.08 J mL O_2^{-1} (Gnaiger, 1983).

As stated in Section 2, ammonia is the main form of nitrogen excretory losses and consequently present determinations of excretion rates were based on ammonia production (Solórzano, 1969). The energy losses due to excretion (U) are frequently overlooked in energy budget determinations since its representation in SFG computation is reduced (1-10% of total metabolic expenditures: Bayne and Newell, 1983). However, this component may account for a significant proportion of energy losses under given nutritional conditions; e.g., when lipid and carbohydrate reserves are scarce and protein is oxidized to cover energy demands or when large stoichiometric imbalances between food and body tissues composition occur (see Section 5). Specifically, the O:N ratio or atomic ratio of oxygen consumed to nitrogen excreted is indicative of the proportion of energy reserves (carbohydrates and lipids) to proteins used in catabolism and has been largely used in the present Thesis to infer the nature and extent of the stoichiometric adjustments in tissue´s growth of clams fed diets differing in N content (Chapter 1).

Scope for growth (J h^{-1}) was finally estimated in the form:

$$SFG = AR - (R + U)$$

where AR represents the absorbed energy (energy ingested minus energy loss in feces) and the terms in parenthesis stand for metabolic energy loss in respiration plus N excretion.

Since scope for growth integrates the balance between several physiological parameters, this parameter is subject to fluctuate according to multiple environmental and endogenous factors. Among environmental factors, temperature and food availability have been widely studied, and the ranges of temperature and food ration to achieve optimal SFG values seem to be species-specific (Newell, 1979; Wieser, 1973). As a general rule, low salinity levels are known to limit the energy balance in marine mollusks (Stickle and Bayne, 1982), while vertical distribution on the shore also can affect SFG depending on the extent of emersion periods and the occurrence or not of compensatory responses for reduced feeding time or the highly inefficient anaerobic metabolism taking place during air exposure.

Body size –directly related to age in these species of indeterminate growth- appears as one relevant among endogenous factors affecting the SFG, together with the genetic constitution of individuals that will be considered later. Given the allometric nature of physiological rates relationships with body size, the physiological parameters involved in SFG computation need to be scaled to a power function as the basis for standardization procedures aiming to remove the size effects for comparative purposes. Scaling functions are accounting for the following equation:

$$Y = a\,X^{b}$$

where *Y* represents the physiological rate, *X* is the body size (in terms of weight or length), the intercept *a* stands for the physiological per unit of body size and *b* is the mass exponent representing the amount of increase of the physiological rate per unit of size increase. Mass exponents have been reported to vary between values of 0.3 – 0.8 for feeding rates and 0.5 – 1 for metabolic rates, depending on the species and on environmental conditions in which research is conducted (see recent references reviewed by Bayne, 2017).

Therefore, to standardize a given physiological value to the size of interest, the following expression is applied:

$$Y_{STD} = Y_{EXP} \times \left(\frac{X_{STD}}{X_{EXP}}\right)^{b}$$

where Y_{STD} and Y_{EXP} are the standardized and experimentally recorded physiological rates, respectively, and X_{STD} and X_{EXP} are the size to which standardization is desired and the experimental size, respectively.

Large inter individual variability in growth rate of bivalves reported earlier (Section 3.2) correlates with differences in the SFG between selected growth groups where components of the energy balance were recorded under constant environmental conditions and physiological rates were standardized to a common size (Tamayo et al., 2015, 2011). This endogenous component to growth variation identifies different growth phenotypes whose physiological characterization relies on the differential ability to ingest or process food within the digestive system and/or to incur in greater o lesser metabolic expenditures. In that respect, Bayne and colleagues (Bayne, 1999; Bayne et al., 1999b) put forward three models aiming to systematize the physiological mechanisms that potentially account for this inter individual variability in growth rate: *a) energy acquisition model,* according to which, higher rates of ingestion and/or absorption would result in enhanced rates of growth; *b) energy allocation model*, which expresses that differences in growth rates might arise from "altered allocation of energy between the demands of maintenance, growth, reproduction and other energy-consuming activities", and c) *metabolic efficiency model*, that implies that lower metabolic costs of growth would favor a faster growth rate.

Among the three, the *energy acquisition* is perhaps the model that has been most frequently documented. This model implies that fast growers possess an increased ability to pump water, filter, select particles, process foodstuff and/or absorb them than their slow growing counterparts; thus achieving higher absorption rates, and consequently, higher energy balances. Experiments with different genetic lines of *Crassostrea gigas* under different quality diets (Bayne et al., 1999a) showed that, irrespective of the diet supplied, F individuals attained higher feeding rates. Comparison between wild and selected lines of oysters *(Saccostrea glomerata)* also reported similar results (Bayne, 2000), including the observation that increased feeding rates of fast growers did not entail

the concomitant reduction in absorption efficiency (Bayne 2004). Similar results were obtained for other species of oysters (Bayne, 1999; Bayne et al., 1999b; Pace et al., 2006; Pernet et al., 2008; Tamayo et al., 2014; Toro et al., 1996; Toro and Vergara, 1998), mussels (Fernández-Reiriz et al., 2016; Ibarrola et al., 2017; Prieto et al., 2020, 2018; Tamayo et al., 2016) and clams (Tamayo et al., 2015, 2013, 2011); most of these studies also reported higher absorption rates per unit of food ingested. Differential physiological behavior would be a constitutive trait of growth phenotypes since, for instance, a clear relationship has been reported between higher feeding rates and larger gills of fast growers (Prieto et al., 2020, 2018; Tamayo et al., 2011). Finally, in feeding experiments above the pseudofeces threshold, preingestive selection efficiency also seems to play an important role in the differential growth of both oysters (Bayne, 2004) and mussels (Prieto et al., 2020).

The *energy allocation* model assumes that energy allocation into maintenance processes is reduced in fast growing individuals with respect to slow growers; and owing to that, a higher amount of energy remains to be allocated into other processes, such as feeding and growth. Pioneering observations concerning this model (Hawkins et al., 1986) suggested that the decreased energy requirements for maintenance were correlated with higher efficiencies of protein synthesis and lower intensities of protein metabolism; and that this surplus of energy sustain the observed increase in ingestion and absorption efficiencies. Further evidences supporting this model have been found in oysters (Bayne, 2000, 1999; Pernet et al., 2008) as well as in mussels (Bayne and Hawkins, 1997; Hawkins and Day, 1999, 1996; Prieto et al., 2018; Tamayo et al., 2016), frequently associated with other physiological traits corresponding to the acquisition model. Reduced metabolic requirements for maintenance that are implied in this model to characterize faster growth has been associated to the lower unsaturation indices of membrane lipids found in selected lines of oysters (Pernet et al. 2008).

The *metabolic efficiency* model relies upon the assumption that faster growth is sustained by lower energy expenditures per unit of tissue deposition; that is, fast growers would incur in lower costs of growth. Research supporting this model has been obtained in some species of oysters (Bayne, 2000, 1999; Bayne et al., 1999b, 1999a; Pace et al., 2006; Pernet et al., 2008; Tamayo et al., 2014; Toro and Vergara, 1998), mussels (Bayne and Hawkins, 1997; Fernández-Reiriz et al., 2016) and clams (Tamayo et al., 2013, 2011);

most of which also reported that metabolic efficiency model was coupled with the energy acquisition model, maximizing, therefore, differences in the energy budget.

5. NUTRIENT BALANCES: THE STOICHIOMETRIC APPROACH

Computation of energy balances are useful tools to explore growth, but as stated in Section 3.1, consideration of energy inputs alone might not be enough to provide a fully account of growth. For instance, juvenile oysters fed on 9 diets isocaloric but differing in biochemical composition showed great differences in terms of the energy balances that ranged from positive to negative values (Maeda-Martínez et al., 2016). From this case it follows that a complementary approach based on nutrient fluxes determination is necessary, besides the SFG approach, to identify the source of growth limitations. Hence, the integration of the stoichiometric approach (the proportion between the different nutrient elements) together with energy balance measurements can significantly improve the understanding on how bivalves grow, and also which physiological mechanisms –especially in terms of acquisition and processing of nutrients– play a role in determining differential growth. However, as noted by Bayne (2017) several limitations should be considered regarding the use of stoichiometric models: *a)* standard bioenergetics models are sufficient to describe growth when energy is the limiting resource, *b)* elemental analysis might be insufficient to provide reliable information on the dynamics of biochemical compounds in growing tissues; and *c)* growth may be limited by specific biochemical compounds that are not identifiable in terms of element ratios. Consequently, the use of stoichiometric balances should be regarded as a complementary tool, and is not intended to represent a substitute for energy balances.

Biological stoichiometry refers to the study of balances of multiple chemical elements within organisms and the proportions between them (Sterner and Elser, 2002). Research based on stoichiometric principles applied to animal growth is very scarce and mainly deals with the large differences often encountered between consumed food and consumer body tissues as regards elemental composition and how these nutrient mismatches are managed in heterotrophic organisms in order to achieve tissue homeostasis. Formally, homeostasis (in the present context) can be defined as the ability of an organism to maintain its chemical composition constant irrespective of the chemical changes in the environment, including food (Kooijman, 1995). Therefore, a strict homeostatic organism would keep the same chemical composition in the presence of whichever condition, which implies an inner physiological control; while a non-

homeostatic organism would vary proportionally its chemical composition as environmental (e.g. trophic) conditions fluctuate. Some organisms can show as well variable degrees of homeostasis, which stands for an intermediate regulation degree between the former two models. In this case, organisms are able to keep a strict homeostasis in a given range of external variation, while outside the regulation ranges, these organisms would lose regulation ability and their composition would vary proportionally according to the external conditions. Evidences of this partial regulation in heterotrophs are found in copepods (Laspoumaderes et al., 2010).

As for the homeostatic strategies that consumers can apply to keep elemental composition in their tissues when faced to variable dietary elemental composition, Sterner and Elser (2002) proposed three levels of stoichiometric regulation. A first level would rely on the preferential ingestion of specific food items, involving preingestive selection mechanisms generally based on the chemical properties of food (see Section 2). A second level relies on the alteration of assimilation patterns, involving that digestion and/or assimilation for any preferred element could be up-regulated. Alternatively, in the absence of selective feeding, overall ingestion rates could be increased to satisfy demands of the limiting nutrient, although this strategy requires the subsequent balanced release of the other elements (i.e. the elements which are in excess) to maintain homeostasis (Bayne, 2009; Darchambeau et al., 2003). Postabsorptive mechanism represents a third level of regulation and concerns to the metabolic fate of the different elements contributing to the final adjustments of the assimilated ration to meet the stoichiometric requirements of tissues biosynthesis. For instance, respiratory release of excess C may represent a significant fraction of the metabolic budget in animals feeding on relatively unbalanced foods (with high C:N ratio) that are common in both terrestrial and aquatic systems (Anderson et al., 2005), as is the case for marine bivalves relying on natural diets containing phytodetritus. Conversely, N release when N input is in excess of requirements occurs in the form of high rates of ammonia excretion reflecting the metabolic use of dietary proteins in supplying energy through deamination reactions.

Elemental balances derived from physiological measurements can be used, in combination with the elemental composition of body tissues, to compute the *threshold element ratios* (TER), a key concept for understanding nutrient deficiency in animals. It represents the critical value at which growth limitation switches from one element to another. Applied to C:N, any value above the TER would be indicative of N limitation

while values below would mean that growth is limited by C (Anderson and Hessen, 1995). A number of authors have developed different approaches to estimate TERs (Anderson et al., 2005; Anderson and Hessen, 1995; Doi et al., 2010; Frost et al., 2006; Sterner, 1997; Sterner and Elser, 2002; Urabe and Watanabe, 1992), which differ in some terms of the equations, such as the use of gross or net growth efficiencies (Sterner and Elser, 2002; Urabe and Watanabe, 1992), or the distinction between absorption efficiencies for nitrogenous and non-nitrogenous compounds (Anderson and Hessen, 1995). Nevertheless, all of the above versions are based on the same theory embracing the physiological adjustments to a given diet composition together with the specific requirements of biosynthesis in growing animal tissues.

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Chapter 2

Physiology and stoichiometry of juvenile *Ruditapes philippinarum* growth phenotypes under different protein/energy ratio dietary scenarios

~ CHAPTER 2.1 ~

Physiological processes modulate acute and chronic responses to dietary protein/energy ratio fluctuations in individuals and families of Manila clam (*Ruditapes philippinarum*) selected for variable growth rates

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ABSTRACT

A range of phenotypes differing in growth rate were designed in the Manila clam by combining separate breeding families with size segregation within each family to constitute fast and slow growing groups. Physiological components of the energy budget and scope for growth (SFG) were then compared between these different phenotypes during the acute and chronic responses to two diets that were iso-caloric but differed by 3-fold in their protein/energy (P/E) ratios. Both diets were based on the microalgae *Rhodomonas lens* obtained in either the exponential or the stationary phase of culture. The aims of the study were *1)* to test the effects of these changes in food composition on growth rate, estimated as the balance of physiological processes of energy gain and loss integrated in the SFG; and *2)* to assess the extent to which physiological adjustments to diet composition are modulated in order to fulfill the variable energy requirements posed by the occurrence of differential growth phenotypes. Growth performance improved with the high-protein (N+) diet for the different family*growth group combinations, with SFG values exceeding by 50% on average the values of the low-protein (N-) diet. Digestive constraints resulted in reduced absorption efficiency with the N- diet, which tended to cancel out the potential benefits of adjusting feeding rates in order to compensate for a low protein ration. Endogenous differences in growth rate associated with segregated phenotypes were mainly accounted for by differences in energy acquisition, with feeding rates differing by ~ 2-fold between fast and slow growers. Additionally, significant differences were recorded for the unitary metabolic costs (i.e., per unit of metabolizable energy), indicating that higher metabolic efficiency was also a component of faster growth.

Key words: growth phenotypes, protein/energy ratio, *Ruditapes philippinarum*, scope for growth.

1. INTRODUCTION

Selective breeding is one fundamental step in aquaculture practices oriented to the generation of stocks exhibiting improved traits for animal production. For commercial species of marine bivalve mollusks, faster growth has been considered of utmost interest since the variability in growth rate of bivalves ranks among the highest in the animal kingdom (Goff, 2011), and much of this variation has been reported to be genetically determined (Dégremont et al., 2005; Evans and Langdon, 2006; Toro and Paredes, 1996). In the context of a joint research project (FIGEBIV, MINECO 2013) centered around one important mariculture species (the Manila clam *Ruditapes philippinarum*), we have undertaken the analysis of this endogenous component of growth by combining physiological and genetic approaches for a) the identification of physiological components of growth variability and b) the search for candidate genes accounting for differential growth phenotypes. The desire for an experimental system appropriate to assess genotype-phenotype associations for growth traits in the context of this project has encouraged the creation of families combined with the selection of intrafamily growth groups.

Methods based on the quantification of physiological parameters liable to be subsequently integrated in an energy budget (the SFG approach) have proven to be useful in the identification of feeding and metabolic behavior traits that are mainly responsible for inherent differences in growth performance among groups of individuals conforming to differentiated growth categories of possible genetic origin (Bayne, 2000; Bayne et al., 1999b, 1999a; Fernández-Reiriz et al., 2016; Tamayo et al., 2016, 2014, 2011). As systematized by Bayne (1999), such persistent physiological differences have been reported to comprise variable capacities for both energy acquisition (feeding and digestive behavior) and energy savings associated with metabolic processes of maintenance and growth. The existence of such a strong genetic component, however, does not exclude phenotypic plasticity in the form of a flexible physiological response to ambient fluctuations, particularly food availability as the main environmental determinant of rates of growth (Bayne, 2004). Consequently, a thorough analysis of adaption capabilities to the food environment exhibited by selected groups of bivalves would require the assessment of *a)* the extent to which physiological behaviour underlying growth performance is genetically determined and *b)* how much of this behaviour can, on its own, be environmentally modulated in order to achieve the more effective exploitation

of available food resources within the limits set by the genetic constitution of individuals (Prieto et al., 2018; Tamayo et al., 2015).

In addressing the interactions between food supply and the growth rate of bivalves, several distinctive features of the food environment should be considered, especially those concerning the quantity and quality of available seston (Gosling, 2015). The main source of food in suspension feeding bivalves is assumed to be phytoplankton, and the growth of both natural and cultivated populations generally exhibits a good correlation with phytoplankton abundance, as represented by Chl *a* concentration in the water column (Figueiras et al., 2002; Pieters et al., 1980; Smaal and Van Stralen, 1990). However, different studies performed over the last few decades have emphasized the importance of other components of the seston, including mainly organic detritus together with bacteria and zooplankton (Arapov et al., 2010; Huang et al., 2003; Langdon and Newell, 1990). Concerning this point, trophic analysis of natural populations of bivalves (see Hawkins et al., 2013 for a review) has revealed that the amount of energy available in the seston (= POM) required to achieve a given growth performance increases as a function of the relative abundance of organic detritus in the diet, very likely reflecting the poor nutritional value of these materials relative to phytoplankton as a consequence of differences in biochemical composition and specifically the higher C:N ratio of detritus.

Bivalve growth is not only dependent on food density (Rico-Villa et al., 2009), but also on the balance between different constituents (e.g. Brown et al., 1998; Wikfors et al., 1992) and, in fact, gross biochemical composition indices have been extensively used in order to assess physiological condition (Lucas and Beninger, 1985). Among the major biochemical constituents (i. e. proteins, carbohydrates and lipids) in diets, proteins are more directly related to growth due to their metabolic and structural functions and may consequently become a limiting factor, as suggested by both laboratory (Brown et al., 1997; Hawkins and Bayne, 1991; Ibarrola et al., 1996; Romberger and Epifanio, 1981) and field (Bayne, 2009; Gremare et al., 1997) studies. Hence, a direct connection between protein input and growth should be appreciated (Kreeger and Langdon, 1993).

As evidence of potential N limitation in bivalves, the cockle *Cerastoderma edule* absorbs nitrogen more efficiently than overall organic matter (Urrutia et al., 1996) and absorbs proteins better than lipids (Ibarrola et al., 2000), pointing to a compensatory mechanism for strict N requirements. Similarly, protein utilization relative to energy, as measured in terms of the respective net growth efficiencies, tends to increase under conditions of food limitation (negative energy balance) in the mussel *Mytilus edulis*,

suggesting the conservation of protein deposition rates at the expense of energy (Hawkins and Bayne, 1991), while higher conversion efficiencies for protein appear on the basis of faster growth in selected oysters (*Saccostrea commercialis*) relative to controls (Bayne, 2000).

Experiments with doubly labeled (^{15}N and ^{14}C) protein in the diet (Kreeger et al., 1996, 1995) provided evidence of a noticeable feature concerning the metabolic fate of dietary protein in mussels (*Mytilus edulis*), offering a metabolically based mechanism for some of the above : the higher assimilation efficiency (90% of the absorbed ration) of the N isotope (the amino-N fraction) compared to less than 34% of the C isotope (the amino-C fraction) (Kreeger et al., 1996) suggests the intensive use of ingested proteins to fuel the N pool through transamination reactions for protein synthesis, with the consequent waste of most of the amino-C fraction, possibly as a component of metabolic fecal loss (Hawkins and Bayne, 1985). In energy terms, this poses a heavy tax on the use of dietary protein for protein deposition into the tissues, to add to the elevated metabolic costs of protein synthesis (Lee et al., 2016; Pan et al., 2018).

Given these high energy requirements involved in dietary protein utilization for the growth of bivalve tissues, two issues are relevant in the context of the present study:

1) How does changing food quality (C:N ratio), expressed as the protein to energy ratio in the diet, impact growth rate, estimated by means of the energy balance (the SFG), and which physiological components of growth are involved in that response?
2) How do physiological adjustments to diet quality become modulated in order to fulfill the contrasting energy requirements set by the occurrence of intrinsic differences in growth performance (i.e., fast *vs.* slow growing phenotypes)?

To address these questions, four differentiated growth phenotypes of Manila clam juveniles, obtained through combined interfamily and intrafamily segregation, were conditioned to two diets differing broadly in terms of their protein to energy ratios. Then, the physiological components of the energy balance, and resulting SFG, were determined and compared between these growth groups during both the acute and chronic responses to changing biochemical composition of the food.

2. MATERIALS AND METHODS

2.1 FAMILIES AND GROWTH GROUPS

Manila clam specimens used in this study belonged to the offspring of two families (1 and 8) from a set of full-sib families established for the combined characterization of growth rate, physiological parameters and SNP polymorphisms, in order to identify QTLs related to growth and growth-associated physiological components of the energy balance. Groups of sibs (families from now on) were obtained from pair matings, which were performed in May 2015 at the IRTA hatchery. Larvae from each mating were cultured in 300 L tanks at 21ºC, and fed *Isochrysis galbana* at 10000 cells mL^{-1}. Water was changed every 48h. Larvae from six matings survived until settlement. After completion of metamorphosis, spat form each family was transferred to 5 L containers with mesh bottom, which were suspended in 500 L tanks with running seawater, first at the IRTA facilities, and after they reached 3 mm, at the IATS facilities. When they reached a minimum size of 7 mm (December 2015), 85 clams were sampled randomly from each family, they were labeled, and their shell length and height were measured. Labeled animals were redistributed in five 50 L tanks provided with substrate (fine-grained sand) and kept at a density of 340 individuals per square meter until the final sampling (June 2017), while fed a diet of *Tetraselmis suecica* supplemented with *Isochrysis galbana* and *Chaetoceros* sp. Two families were chosen for this study on the basis of their growth rate (see below).

The preliminary characterization of growth performance of these families (in terms of regression of growth rate *vs.* body size) indicated a 47.6% higher growth rate in Family 1 relative to Family 8. For the specific objectives of this study, two growth groups were segregated inside each family by choosing the larger and smaller specimens to which the conditions of fast (F) and slow (S) growth, respectively, were assigned. *Table 2.1 - 1* shows the sizes and characteristics of these groups determined in order to fulfill the requirements of the experimental design: some 30 individuals per growth group presenting the highest degree of size-homogeneity possible (CV ranged from 7% in F to 16% in S groups). Size differences achieved between F and S groups were similar for both families:, i.e., ~ 2x in terms of shell length and 6x in terms of live weight.

Table 2.1 - 1. Mean (SD) size of the four groups of clams before starting the experiments

Family	Growth group	N	Length (mm)	Weight (mg)
1	F	30	23.14 (1.54)	2359.05 (468.63)
1	S	27	12.36 (1.93)	380.63 (138.36)
8	F	30	21.92 (1.91)	2095.39 (602.52)
8	S	34	11.4 (1.96)	314.07 (150.41)

2.2 MAINTENANCE AND EXPERIMENTAL DESIGN

After arrival at the laboratory of Animal Physiology (UPV/EHU, 21st June 2017), these groups were separately maintained for 10 days in a 50 L tank filled with aerated seawater (34 PPT) regulated at 17ºC and fed *Isochrysis galbana* (T-ISO clone) at a cell concentration equivalent to 1 mm^3 L^{-1} (~ 20,000 cells mL^{-1}). Water in the tank was changed daily.

In these experiments, we tested the responses of clams from different families and growth groups to diets that differed in biochemical composition and that were based on cultures of the microalgal species *Rhodomonas lens* growing in the exponential phase (Diets N+) or maintained in the stationary phase of the culture (Diets N-). A basic outline of the experimental design is presented in *Figure 2.1 - 1*: each of the aforementioned F and S groups was homogenously divided (F = 0.21, p = 0.893) into four subgroups for subsequent diet treatments, and each clam was numbered for individual determinations of growth and physiological parameters. Each of these groups was food-conditioned (acclimated) to the diets N+ or N- for 15 days (*Table 2.1 - 2*). Subsequently, each member of the pair conditioned to diets N+ or N- was exposed to one of the experimental diets based on exponential (E) or stationary (S) cultures for physiological determination, resulting in 4 experimental conditions for each growth group and family (*Figure 2.1 - 1* and *Table 2.1 - 3*). In the notation of these categories, the first letter indicates the diet used in acclimation, and the second letter indicates the experimental diets used for the acute exposure prior (3 d) and during physiological determination. That is, each group*family combination was analyzed under the four nutritional scenarios stated in *Acute exposure* in *Figure 2.1 - 1*: N+N+, N+N-, N-N+ and N-N; using different pools of clams under each condition (i.e., no repeated measurements were carried out).

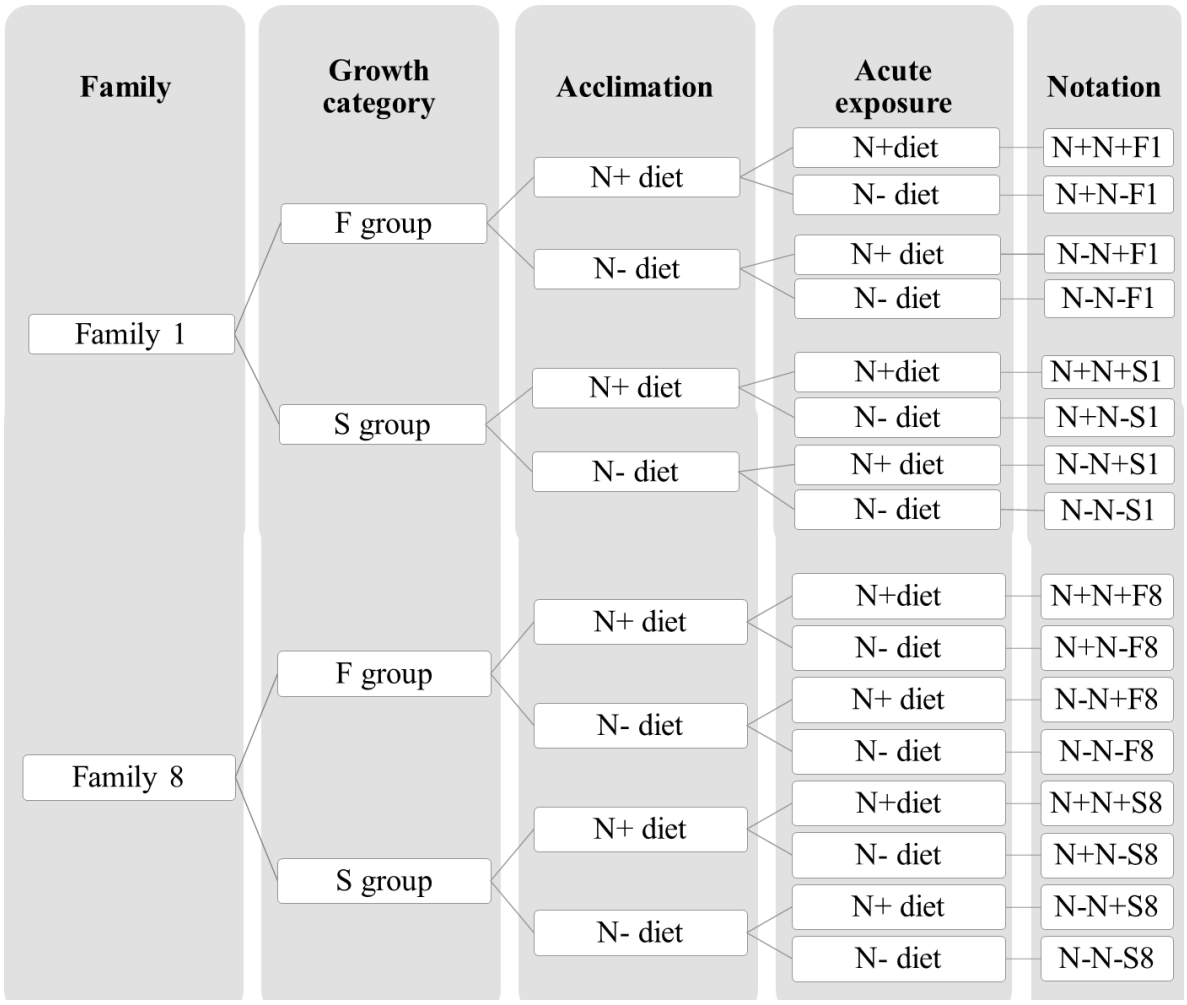

Figure 2.1 - 1. Experimental setup for the recording of physiological parameters in the fast (F) and slow (S) growing groups segregated from two families. Five individuals (n = 5) were used in each of the 16 resulting groups

2.3 Composition of diets

The basic component of diets was the microalgae *R. lens* in either exponential or stationary phase. E microalgae were obtained in a continuous culture system, in which 20% of the stock was renewed daily. To obtain S microalgae, cultures that had reached the exponential phase were maintained in a static culture system without further addition of nutrients until the stationary phase was reached. The turning point for the transition from the E to S stage of culture was identified by the color change of the culture (from red to green), indicative of N limitation.

Table 2.1 - 2. Diet composition, including the culture phase of R. lens, C:N index and protein/energy ratio

Diet	Culture phase	C:N	Protein/Energy (P/E, µg J^{-1})
N+	E (Exponential)	4.94 (0.21)	24.77 (0.53)
N-	S (Stationary)	14.54 (0.22)	8.11 (0.12)

Table 2.1 - 2 and *Table 2.1 - 3* show the composition and characteristics of two types of diets used in this study: the acclimation diets used in food conditioning of clams prior to experimentation and the experimental diets used in the acute exposure of clams during physiological measurements. The composition of the acclimation diets included only microalgae in either the E or S phase of culture. The composition of the experimental diets was based also on these microalgae as food but included 35-40% inorganic content (by weight) to fulfill the requirements of an inorganic tracer in absorption efficiency (AE) determinations by the Conover method. The inorganic component consisted of silt particles < 63 µm obtained from surficial sediment samples collected in the field that were ashed at 450ºC for organic matter combustion. Hence, experimental diets were prepared by mixing both microalgae and silt particles in the stated proportions with the aid of a magnetic stirrer and then dosed with a peristaltic pump. Both acclimation and experimental diets were dosed at approx. 1 and 1.25 mm^3 L^{-1}, respectively, in terms of particles packed volume, to achieve a POM concentration of 0.6 mg L^{-1} under each condition.

Elemental analysis of diets was conducted during the acclimation period, as well as in the course of experiments, on samples collected over preweighted glass fiber filters (GF/C) by filtering a known volume of water from the feeding tanks and washing with 50 mL of seawater. Samples were immediately frozen at -20ºC, lyophilized, and maintained at -20ºC until they were analyzed in an Euro EA Elemental Analyzer (CHNS) from EuroVector, using acetanilide as a standard. The protein/energy ratio was indirectly estimated as follows: protein content of the sample (µg) was estimated by using the equivalence P = N*5.8 (Gnaiger and Bitterlich, 1984); while energy content (J) was estimated as the product of POM and the energy equivalents (22.906 and 26.826 J mg^{-1} for E and S cultures, respectively; Platt and Irwin, 1973).

Table 2.1 - 3. Characteristics of both acclimation and exposure diets, where TPM is total particulate matter, PIM is particulate inorganic matter, POM is particulate organic matter, OC is organic content (= POM/TPM) and C:N is the carbon to nitrogen index

	Diet	TPM (mg L^{-1})	PIM (mg L^{-1})	POM (mg L^{-1})	OC	C:N
Acclimation diets	N+	0.64 (0.15)	0.09 (0.07)	0.55 (0.08)	0.87 (0.07)	4.94 (0.21)
	N-	0.56 (0.05)	0.05 (0.01)	0.51 (0.04)	0.92 (0.02)	14.54 (0.22)
Diet composition in the acute exposure of the different experimental conditions	N+N+	1.13 (0.25)	0.53 (0.19)	0.60 (0.09)	0.54 (0.08)	5.40
	N+N-	1.25 (0.24)	0.48 (0.09)	0.77 (0.16)	0.62 (0.03)	10.70
	N-N+	1.20 (0.25)	0.56 (0.19)	0.64 (0.09)	0.54 (0.08)	5.43
	N-N-	1.00 (0.15)	0.35 (0.08)	0.65 (0.11)	0.65 (0.07)	13.57

Characterization of food suspensions leading to the data in *Table 2.1 - 3* was carried out twice per week in triplicate during the acclimation period and 5-6 times in triplicate during the exposure. For this purpose, samples of water collected from the feeding tanks were filtered through preweighted glass fiber filters (GF/C), rinsed with ammonium formate (0.9% w/v) to prevent salt retention and dried for 24-48 h at 100ºC to estimate dry weight. Ash weight was computed after calcination for 6 h at 450ºC. Total particulate matter (TPM, mg L^{-1}) and particulate inorganic matter (PIM, mg L^{-1}) were calculated from the dry weight and ash weight of material retained in the filters, respectively, and the difference TPM – PIM represented the particulate organic matter (POM, mg L^{-1}).

2.4 Physiological determinations

Physiological determinations were performed individually, with five individual samples for each condition. Measurements involved in the quantification of components of the energy balance lasted 4 days for each experimental condition.

The clearance rate (CR, L h^{-1}) was measured by the flow-through chamber method (Crisp, 1971), where clams were individually placed in a 125 mL flask with a constant supply of diet. Flow rates through the flasks were regulated to produce reductions in particle concentrations in the range of 15-30%, corresponding to conditions for which CR is independent of the flow rate (Filgueira et al., 2006). Twelve to 16 such measurements were registered during the daytime (from 8 a.m. to 8 p.m.) by means of a particle counter (Beckman Z1 Counter), and the CR of each individual was estimated as the average of these measurements. The organic ingestion rate (OIR, mg h^{-1}) was then computed as the product of CR and POM.

Absorption efficiency (AE, decimal units) was estimated by the method of Conover (1966), from the organic content of food suspensions and the feces produced in the course of CR measurements. Both water samples and feces were filtered on GF/C filters and processed for total dry weight and inorganic weight determinations as described previously (Section 2.3). Organic content (OC) was computed as organic weight (= total - inorganic) divided by total weight.

The absorption rate (AR, mg h^{-1}) of organic matter was estimated as the product of OIR and AE, and the energy equivalents that were applied to the absorbed ration in SFG computation were those described in Section 2.3.

The metabolic rate was assessed as the oxygen consumption rate (VO_2, µL O_2 h^{-1}). Clams were individually placed in 150 mL chambers filled with filtered seawater at a constant temperature (17ºC) sealed with LDO oxygen probes connected to a Hatch HQ40d oximeter. Rates of oxygen consumption were computed from the decline in oxygen concentration in the chambers registered over 3-4 h. A chamber without animals was used as a control. These rates were converted to energy equivalents (J h^{-1}) by using the following oxi-caloric coefficient: 1 mL O_2 = 20.08 J (Gnaiger, 1983).

For determination of ammonia excretion rates (VNH_4-N, µg NH_4-N h^{-1}), animals were located individually in open flasks with 30 mL of filtered seawater (0.2 µm Millipore membranes) for 2-3 h, and the ammonia concentration was determined according to the phenol-hypochlorite method (Solórzano, 1969). Two flasks without animals were used as controls. Rates of ammonia excretion were converted to energy equivalents (U: J h^{-1}) by using a conversion factor of 24.853 J mg^{-1} (Elliott and Davison, 1975).

The O:N index was calculated as the proportion between atomic equivalents of oxygen consumed and nitrogen excreted by each animal.

The scope for growth (SFG, J h^{-1}) was estimated as the following difference: AR – (R + U)

After physiological determinations were concluded, clams were dissected, gill area was estimated by image analysis with Fiji software (Schindelin et al., 2012), and soft tissues were lyophilized to obtain dry weight measurements. Growth in terms of energy was indirectly estimated by the conversion factor of 23.96 J mg^{-1} (Álvarez-Jorna, 1995).

For comparative purposes, physiological rates were standardized to a common tissue dry weight of 85.95 mg (the average value), using scaling factors (*b)* obtained in a previous experiment of 0.609, 0.697 and 1.00 to scale CR, VO_2 and VNH_4-N, respectively, to soft body weight (own unpublished data). Likewise, a mass exponent of 2.00 was used to standardize gill area to a common length.

2.5 Statistical analysis

This study comprises the analysis of the effects of 4 factors on the suite of physiological traits involved in growth rate, including *a)* Two endogenous factors associated with differences in growth performance between families (family factor) and with the effects of size segregation (growth category factor). *b)* Two exogenous factors corresponding to differences in the biochemical composition of the acclimation diet prior to physiological experiments (acclimation diet factor) or the actual diet ingested during physiological determinations (exposure diet factor). Physiological measurements recorded under this experimental design were compared for significant differences through a 4-way ANOVA using R (R Core Team, 2016), after the data were tested for normality (Shapiro-Wilk) and homoscedasticity (Levene). Relationships between different components of energy balance as well as between SFG and actual growth rates were fitted through linear regression analyses (by least squares) using the same software.

3. Results

3.1 Comparison of means of pooled values corresponding to factors under analysis

Means of pooled values of the physiological components of the energy balance, gill areas and O:N indices, computed for alternative values of the 4 factors (categories) referred to above, are presented in *Table 2.1 - 4*. *Table 2.1 - 5* summarizes the results of the corresponding 4-way ANOVA, including terms for both single factors and factor interactions up to the 4^{th} degree.

Table 2.1 - 4. Means of pooled values (SE) of different parameters computed for alternative values of the factors under study: family (F; 1 vs. 8); growth group (G; F vs. S); acclimation diet (A; N+ vs. N-) and exposure diet (E;N+ vs. N-)

		CR	AE	AR	R	U	O:N	SFG	GA
A	**N+**	0.83 (0.05)	0.72 (0.02)	9.91 (0.57)	1.36 (0.08)	0.17 (0.02)	21.49 (3.28)	8.38 (0.58)	406.26 (7.14)
	N-	0.8 (0.05)	0.65 (0.02)	8.06 (0.45)	1.3 (0.11)	0.17 (0.03)	41.28 (6.87)	6.6 (0.44)	361.52 (10.2)
E	**N+**	0.89 (0.05)	0.81 (0.01)	10.28 (0.57)	1.48 (0.09)	0.27 (0.03)	12 (1.39)	8.52 (0.59)	-
	N-	0.75 (0.05)	0.56 (0.01)	7.69 (0.39)	1.17 (0.09)	0.06 (0.01)	50.77 (6.46)	6.46 (0.41)	-
G	**F**	0.95 (0.05)	0.67 (0.03)	10.3 (0.52)	1.44 (0.09)	0.12 (0.02)	36.35 (5.26)	8.74 (0.52)	370.57 (7.68)
	S	0.68 (0.04)	0.7 (0.02)	7.67 (0.46)	1.21 (0.09)	0.22 (0.03)	26.42 (5.84)	6.24 (0.47)	397.22 (10.61)
F	**1**	0.91 (0.04)	0.67 (0.02)	9.72 (0.51)	1.3 (0.11)	0.17 (0.03)	27.16 (4.73)	8.24 (0.52)	369.58 (9.38)
	8	0.73 (0.05)	0.7 (0.02)	8.25 (0.53)	1.35 (0.08)	0.16 (0.03)	35.61 (6.3)	6.74 (0.52)	398.2 (9.07)

CR= clearance rate (L h^{-1}); AE= absorption efficiency (decimal units); AR= absorption rate (J h^{-1}); R= metabolic rate (J h^{-1}); U= nitrogen excretion rate (J h^{-1}); SFG= scope for growth (J h^{-1}); O:N= oxygen:nitrogen index (atomic ratio); GA= gill area (mm^2).

CR and gill area: These two parameters are considered together on account of the functional relationship linking the filtering activity with the surface area of the filtering organ. Both endogenous factors (family and growth category) are associated with significant differences in CR and gill area, although trends are not strictly concordant: for example, offspring of Family 1 exhibit approximately 20% higher CR compared with that of Family 8, and F clams present a similar difference with respect to S clams (irrespective of family ascription). Conversely, gill areas tend to be higher in S clams and Family 8, and these differences are clearly less sharp relative to CR differences but are still significant. On the other hand, the very significant positive influence of acclimation to diets N+ on the gill area (*Table 2.1 - 5* and *Figure 2.1 - 2b*) partly supports the effect that clams tend to feed faster with this diet, especially following a period of acclimation (*Table 2.1 - 4*).

Absorption efficiency and absorption rate: Each of the factors under study exerted significant differences ($p < 0.001$) on absorption efficiency. However, even if significant, effects of endogenous factors (family and growth group) *per se* appear quantitatively irrelevant compared with the strong effect of actual dietary condition, resulting, for instance, in a 44% increase observed in the absorption efficiency of clams exposed to

Table 2.1 - 5. Summary of 4-way ANOVA testing of the significant effects of acclimation and exposure to alternative diets, growth category and family on gill area and physiological parameters. Significant differences ($p<0.05$) are highlighted in bold characters

	CR	Gill area	AE	AR	R	VNH_4-N	SFG	O:N
Acclimation (A)	$F = 0.368, p = 0.546$	**$F = 14.949, p < 0.001$**	**$F = 106.456, p < 0.001$**	**$F = 14.243, p < 0.001$**	$F = 0.202, p = 0.655$	$F = 0, p = 0.989$	**$F = 11.451, p = 0.001$**	**$F = 13.579, p < 0.001$**
Exposure (E)	**$F = 8.228, p = 0.006$**	-	**$F = 1199.494, p < 0.001$**	**$F = 57.89, p < 0.001$**	**$F = 6.594, p = 0.013$**	**$F = 88.567, p < 0.001$**	**$F = 15.122, p < 0.001$**	**$F = 52.109, p < 0.001$**
Growth category (G)	**$F = 31.335, p < 0.001$**	**$F = 5.305, p = 0.025$**	**$F = 14.949, p < 0.001$**	**$F = 25.778, p < 0.001$**	$F = 3.548, p = 0.064$	**$F = 18.561, p < 0.001$**	**$F = 22.495, p < 0.001$**	$F = 3.414, p = 0.069$
Family (F)	**$F = 13.032, p = 0.001$**	**$F = 6.12, p = 0.016$**	**$F = 15.454, p < 0.001$**	**$F = 8.536, p = 0.005$**	$F = 0.148, p = 0.701$	$F = 0.259, p = 0.613$	**$F = 8.135, p = 0.006$**	$F = 2.476, p = 0.121$
A:E	**$F = 29.94, p < 0.001$**	-	$F = 0.502, p = 0.481$	**$F = 13.532, p < 0.001$**	**$F = 6.936, p = 0.011$**	**$F = 9.952, p = 0.002$**	**$F = 16.999, p < 0.001$**	**$F = 20.479, p < 0.001$**
A:G	$F = 0.886, p = 0.35$	$F = 0.441, p = 0.509$	**$F = 12.999, p = 0.001$**	$F = 3.688, p = 0.059$	$F = 0.52, p = 0.474$	$F = 1.009, p = 0.319$	**$F = 5.347, p = 0.024$**	$F = 2.229, p = 0.14$
E:G	$F = 0.146, p = 0.703$	-	**$F = 26.049, p < 0.001$**	$F = 1.399, p = 0.241$	$F = 2.025, p = 0.16$	**$F = 15.061, p < 0.001$**	$F = 1.631, p = 0.206$	$F = 0.001, p = 0.971$
A:F	$F = 0.29, p = 0.592$	$F = 1.666, p = 0.201$	$F = 0.929, p = 0.339$	$F = 0.005, p = 0.942$	$F = 1.015, p = 0.317$	$F = 3.087, p = 0.084$	$F = 0.083, p = 0.774$	$F = 2.813, p = 0.098$
E:F	$F = 0.167, p = 0.684$	-	**$F = 19.741, p < 0.001$**	$F = 3.028, p = 0.087$	$F = 1.226, p = 0.272$	$F = 0.117, p = 0.734$	$F = 1.544, p = 0.219$	$F = 1.876, p = 0.176$
G:F	$F = 0.012, p = 0.915$	$F = 0.925, p = 0.34$	**$F = 9.759, p = 0.003$**	$F = 0.091, p = 0.764$	$F = 0.088, p = 0.767$	$F = 1.554, p = 0.217$	$F = 0.253, p = 0.617$	$F = 0.071, p = 0.79$
A:E:G	$F = 0.911, p = 0.344$	-	**$F = 30.004, p < 0.001$**	**$F = 5.319, p = 0.024$**	$F = 0.367, p = 0.547$	$F = 0.021, p = 0.885$	**$F = 5.356, p = 0.024$**	$F = 0.638, p = 0.427$
A:E:F	$F = 2.928, p = 0.092$	-	**$F = 4.146, p = 0.046$**	$F = 1.324, p = 0.254$	$F = 2.563, p = 0.114$	$F = 2.685, p = 0.106$	$F = 2.506, p = 0.118$	**$F = 4.524, p = 0.037$**
A:G:F	$F = 0.071, p = 0.79$	$F = 0.233, p = 0.631$	**$F = 13.829, p < 0.001$**	$F = 0.84, p = 0.363$	$F = 0.219, p = 0.641$	$F = 0.91, p = 0.344$	$F = 0.506, p = 0.479$	$F = 0.409, p = 0.525$
E:G:F	$F = 1.112, p = 0.296$	-	$F = 0.003, p = 0.958$	$F = 1.005, p = 0.32$	$F = 3.009, p = 0.088$	$F = 0.697, p = 0.407$	$F = 1.925, p = 0.17$	$F = 0.288, p = 0.594$
A:E: G:F	$F = 2.166, p = 0.146$	-	$F = 0.355, p = 0.553$	$F = 3.129, p = 0.082$	$F = 0.93, p = 0.338$	$F = 2.105, p = 0.152$	$F = 2.372, p = 0.128$	$F = 1.421, p = 0.238$

diets N+ relative to diets N- (*Table 2.1 - 4*). The complex behavior of this parameter in the acute *vs.* chronic response to changing diet composition in each group of clams results in a set of combined effects (interactions; *Table 2.1 - 5*) that will be the described in the next section. Absorption rate behavior combines the effects of feeding rates (CR) and absorption efficiencies. Consequently, AR values exhibited substantial significant differences (*Table 2.1 - 5*) for each factor (acclimation, exposure, growth group and family). Compared with diets N-, feeding diets N+ promoted an increase in the AR, both in the acute response (33% increase) and during acclimation (23%) (*Table 2.1 - 4*). Concerning the endogenous factors, differences in feeding rates caused greater AR values in F relative S clams or in clams from Family 1 compared with those of Family 8 (*Table 2.1 - 4*).

Metabolic expenditures (R and U) and O:N index: Both metabolic and N excretion rates increase significantly with acute exposure to N+ diets (*Table 2.1 - 4* and *Table 2.1 - 5*), but this effect is considerably higher for U (350%) than for R (26%). Consequently, values of the O:N index experienced a 4-fold decline in clams fed this high N diet. Overall, acclimation to N+ diets also promoted a significant reduction in the O:N index *Table 2.1 - 4* and *Table 2.1 - 5*), although this effect was only noticeable during acute exposure to N- diets (*Figure 2.1 - 4c*). This behavior is accounted for by the consistent significance of the exposure*acclimation interaction term for R, U and the O:N index (*Table 2.1 - 5*).

Endogenous factors (family and growth group) had no significant effects on metabolic rate or the O:N index, although F clams registered, on average, 19% more metabolic activity than S clams. Rates of ammonia excretion were significantly higher (~ 2-fold) in S than F clams, but no differences between Family 1 and 8 were recorded.

SFG: SFG integrates a diversity of effects on physiological components and was significantly affected for all tested variables, both endogenous and exogenous (dietary). Confirming their status as fast growers, F clams had significantly higher SFG than S clams, while those belonging to Family 1 had higher SFG than clams from Family 8. On the other hand, both acute and chronic exposure to N-rich diets promoted a significant increase in the SFG, with acclimation enhancing the effects of the acute change (see acclimation*exposure interaction term in *Table 2.1 - 5*).

3.2 Combined dynamics of the acute and chronic responses

Figure 2.1 - 2a to Figure 2.1 - 5*a* have been designed to represent the dynamics of the different physiological parameters combining the acute and chronic (acclimation) responses to changes in the N content of the diet. Each point (with standard deviation bars) represents the mean (n = 5) value of each group, in which different clams were used, whereas lines connecting these points for the N+N+, N+N-, N-N-, N-N+ and N+N+ sequence of experimental conditions are drawn to model the acute-chronic response to dietary change.

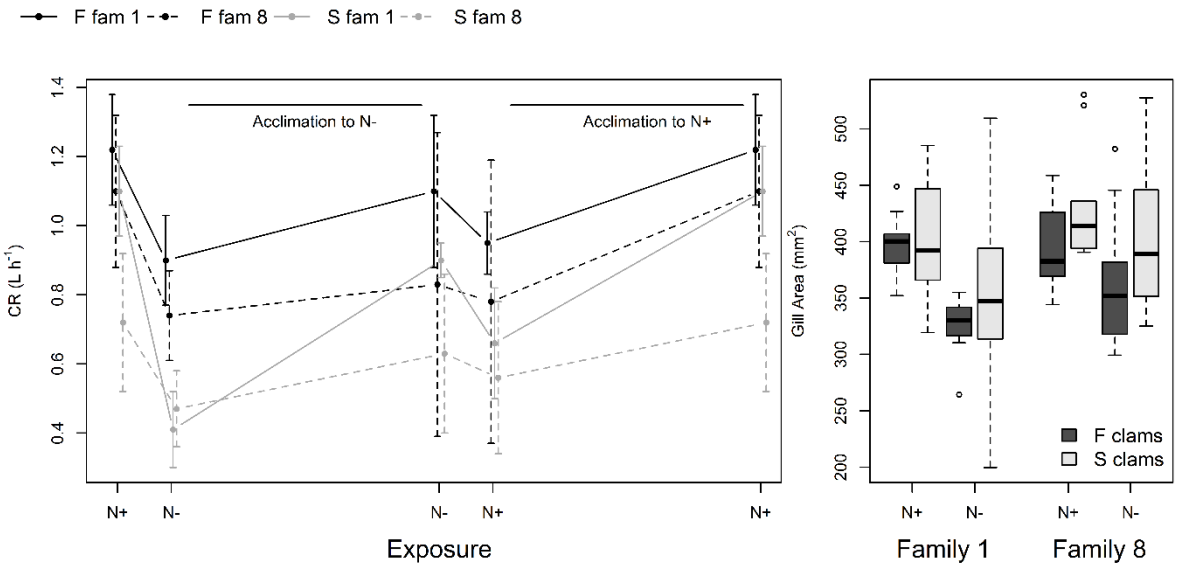

Figure 2.1 - 2 a) Size-standardized values of clearance rate in fast (black) and slow (gray) growing clams belonging to families 1 (solid lines) and 8 (dotted lines); b) size-standardized gill area values of fast (dark) and slow (light) growing clams from both families and acclimated to N+ and N- diets

The main dietary effects on CR are accounted for by the acclimation*exposure interaction (*Table 2.1 - 5*), conforming to a general pattern in which the acute change of the diet (either from N+ to N- and *vice versa*) results in a decline in feeding rate followed by a recovery along the acclimation period ("W shaped pattern"; *Figure 2.1 - 2a*). In addition, acute exposure to the low-nitrogen diet had a higher impact on CR than did the change from N- to N+, leading to bigger decreases in the feeding activity. The magnitude of these changes tends to be greater in Family 1 than Family 8, with maximal differences between the S groups of both families.

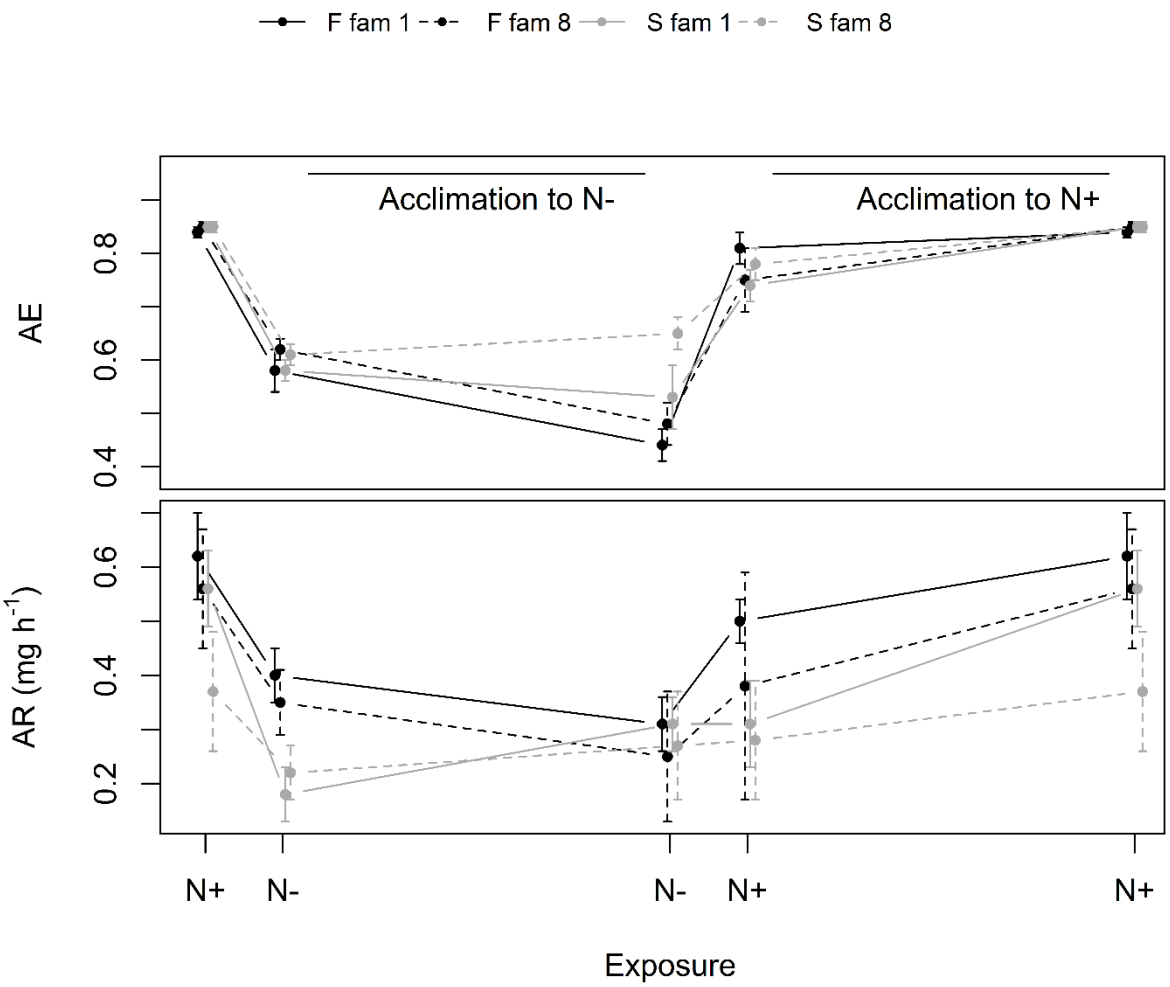

Figure 2.1 - 3 a) Absorption efficiency and b) absorption rate values of fast (black) and slow (gray) growing clams belonging to families 1 (solid lines) and 8 (dotted lines)

AE shows a positive dependence on the N content of the diet, with a general "U-shaped" pattern in which the acute response (i.e., a strong reduction in AE following the change from N+ to N- diets) is reinforced during the acclimation period (*Figure 2.1 - 3a*). However, these effects are smaller in slow growing clams (S), which are able to maintain their AE values relatively stable along the acclimation phase, resulting in higher efficiencies of S clams with the low-N diet.

Rates of absorption (AR) approximately follow this same "U-shaped" trend (*Figure 2.1 - 3b*), with some deviations from the general pattern due to the differential behavior of CR between families and growth groups: while F clams exposed to N+ diets rapidly recovered from reduced AR values achieved during chronic exposure to N-poor diets, the response of S clams required much longer acclimation periods.

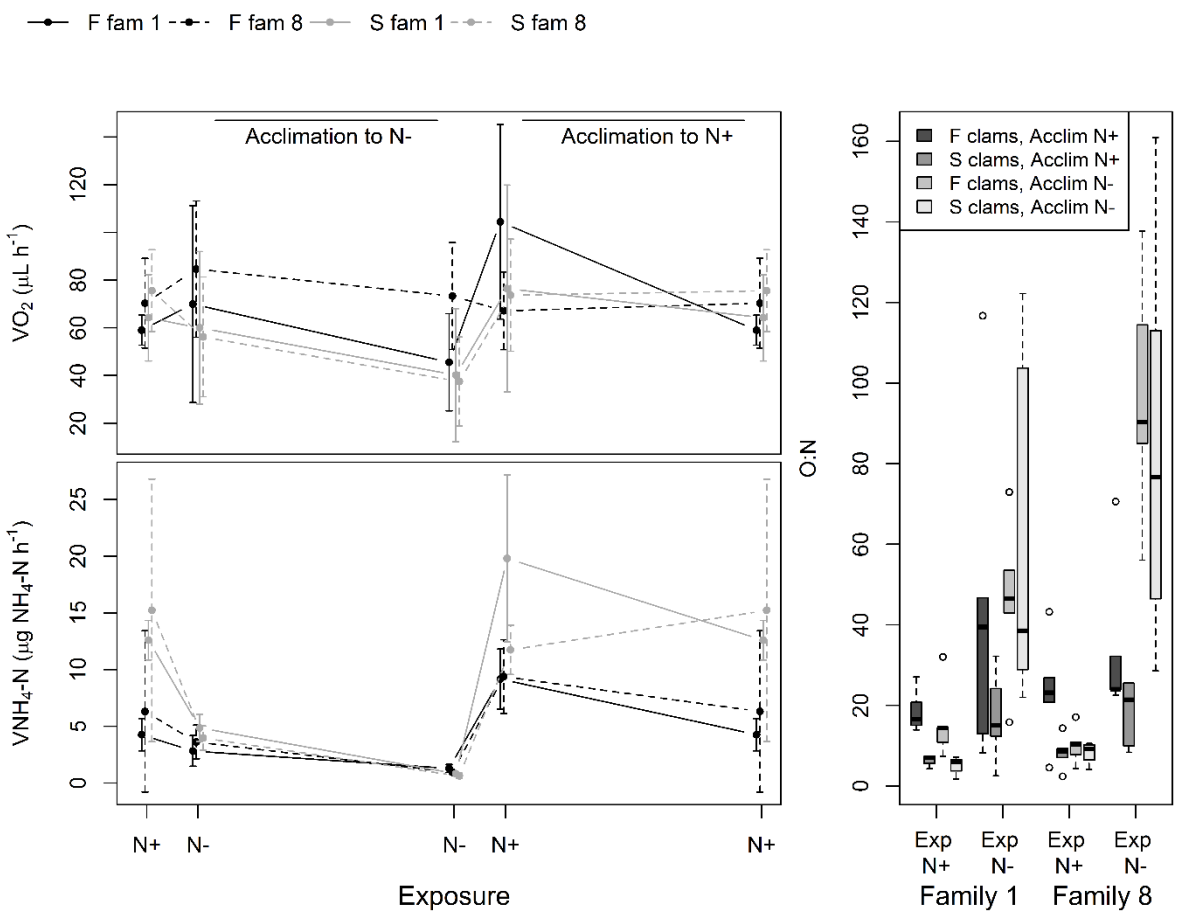

Figure 2.1 - 4. Size-standardized values of a) metabolic rate (VO_2) and b) ammonia excretion rate of fast (black) and slow (gray) growing clams belonging to families 1 (solid lines) and 8 (dotted lines); c) O:N index of each group (Acclim: acclimation diet; Exp: exposure diet)

Rates of energy expenditure (both metabolic and excretion rates) showed a rather common pattern of response to combined acute and chronic changes in N content of the diet (*Figure 2.1 - 4*). In general, acute change involving improved nutritional conditions (from N- to N+) results in a positive effect on these rates, leading to maximal values that are maintained or reduced (depending on growth group or family) during the acclimation. Following the acute decline in the N+ to N- change, acclimation to the N- diet resulted in an additional minor reduction in excretion rates, these changes being greater for S than for F clams.

The combined effects of acute exposure and acclimation to diets with different N contents on SFG fit different patterns for F and S clams (*Figure 2.1 - 5*; see also acclimation*exposure*growth group interaction in *Table 2.1 - 5*). For F clams, acute decline following the N+ to N- change is further reinforced during acclimation to the poor diet, while the increasing response to the opposite acute change is maintained along the

acclimation to the N+ diet. This "U-shaped" pattern would indicate that the SFG trend of F clams is governed by AE behavior. For S clams, any change in diet quality (either from N- to N+ or *vice versa*) resulted in a decline of SFG values in the acute response, followed by a recovery during the acclimation phase. This "W-shaped" pattern would indicate that the SFG trend in slow growing clams is governed by CR behavior.

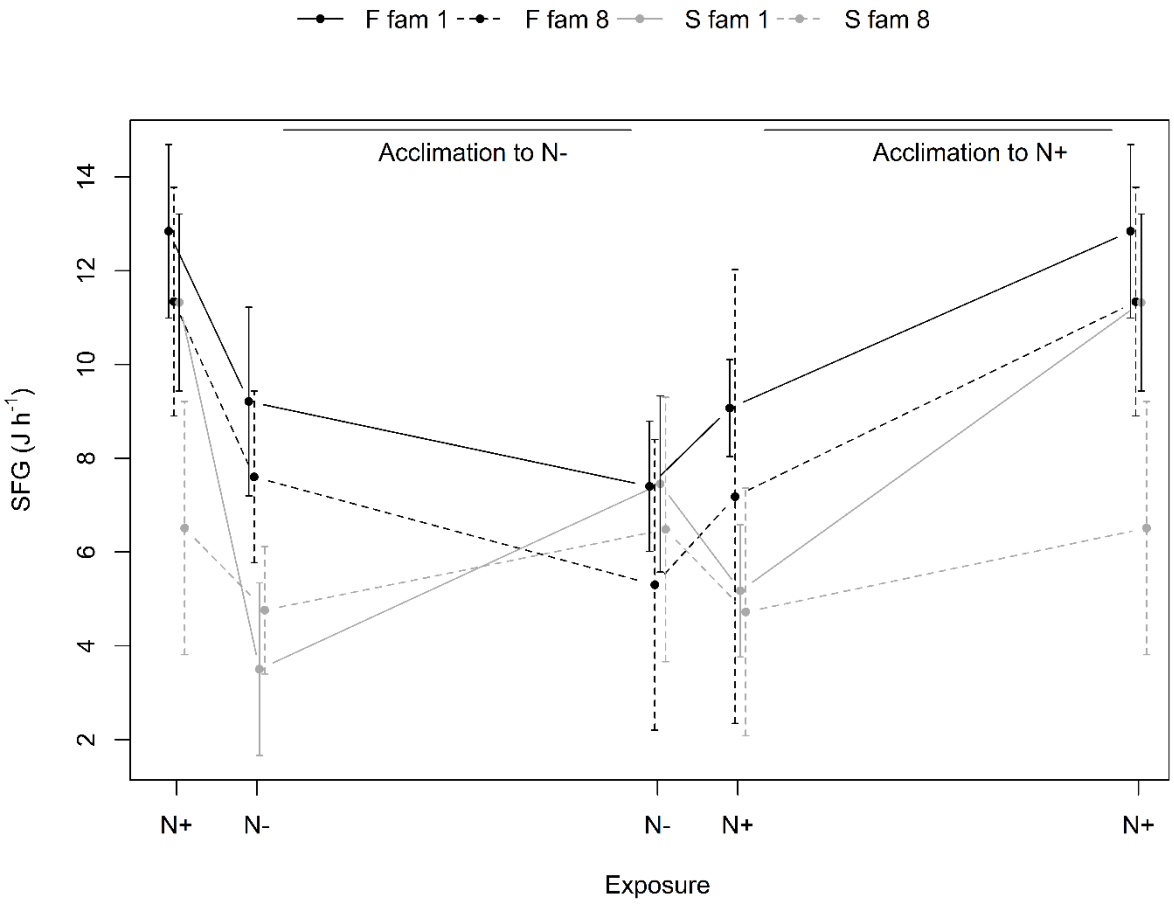

Figure 2.1 - 5. Size-standardized SFG values in fast (black) and slow (gray) growing clams belonging to families 1 (solid lines) and 8 (dotted lines)

3.3 The relationship between SFG and actual growth rate (GR)

The potential of SFG methodology to predict actual growth rates (GR) was tested by performing regression analysis of both measurements (*Figure 2.1 - 6*). For this purpose, only physiological measurements recorded under fully acclimated conditions were employed, assuming that weight changes used in actual growth measurements would reflect the stable conditions achieved in acclimated specimens. The fitted regression equation was $SFG = 1.03\ GR + 2.19$ ($F = 52.9$, $p < 0.001$), in which the slope

did not significantly differ from 1 (*F = 0.0366 p = 0.8494*), but intercept was significantly different from 0 (*F = 4.2396 p = 0.04639*), reflecting a slight overestimation of SFG over actual growth. Nevertheless, the weak significance (*p = 0.046*) concerning the deviation of the intercept from 0 is indicative of a good concordance between both measurements and would confirm the validity of SFG methodology in predicting the growth rate.

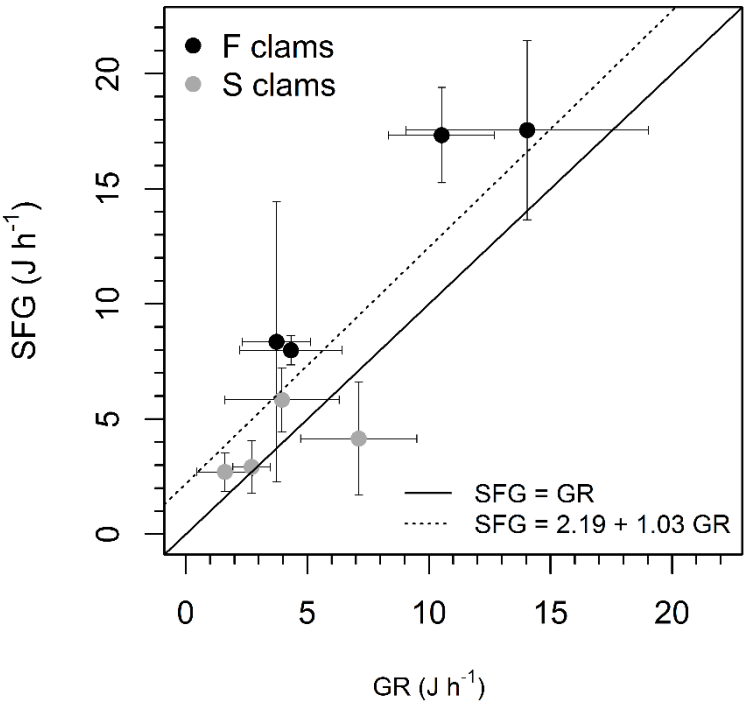

Figure 2.1 - 6. Relationship between SFG and actual growth for fast (black) and slow (gray) growing clams. The solid line represents y = x, while the dashed line is a plot of the regression equation fitted to the experimental data

4. DISCUSSION

The aims of this study were mainly to test the effect of diet quality, given as the nitrogen to energy ratio (or inversely, the C:N index), on features of the physiological behavior underlying variability among differentiated growth phenotypes. To experimentally address this question concerning the dependence of growth rate of bivalves on dietary food value, several procedures have been attempted to obtain a range of biochemical profiles. These include the use of different microalgal species (Albentosa et al., 1996; Enright et al., 1986; Epifanio, 1979; Fernández-Reiriz et al., 2015; Pettersen et al., 2010; Walne, 1970), mixtures of microalgae with inert organic particles (Albentosa et al., 2002, 1999; Maeda-Martínez et al., 2016; Pérez-Camacho et al., 1998) or manufactured microcapsules (Kreeger et al., 1996, 1995; Kreeger and Langdon, 1993) as well as the manipulation of phytoplankton cultures for the specific purpose of changing

the protein content of the cell (Kreeger and Langdon, 1994, 1993; Uriarte and Farías, 1999; Utting, 1985). This last procedure has the advantage of relying mainly on differences in biochemical composition, while other differential features related to the physical constitution of particles that might affect the rates of food processing would be virtually absent. In this study, two different diets were made up from the same species of phytoplankton (*Rhodomonas lens*) cultivated either in the exponential or stationary phase to achieve a 2.5-fold difference in the protein content (C:N ratios of 4.9 and 12.8 in diets N+ and N-, respectively). The transition from the exponential (N+) to the stationary (N-) phase of the culture was observed to result in an increase in cell size (from 44.2 to 82.5 pg $cell^{-1}$), but food supply in our experiments was not regulated to the same cell number but rather to achieve the same organic ration (mg POM L^{-1}) in both diets, and gill retention efficiency has been reported to be constant (near 100%) in that size range (Defossez and Hawkins, 1997; Ward and Shumway, 2004); hence, we generated the hypothesis that differences in physiological behavior observed between both diets respond solely to differences in their biochemical composition.

Early observations concerning limitations exerted by N availability on energy flows within coastal environments (Mann, 1982), as well as the positive relationship between protein ingestion and production exhibited by marine invertebrates (Roman, 1983), offer an appropriate reference context for the present finding that acclimation to N+ diets promoted a higher growth rate than acclimation to N- diets in juveniles of the Manila clam. This confirms previous results concerning the positive correlation reported between dietary protein content of experimental diets and growth rate in the early life stages of many different species of bivalves (Brown et al., 1998; Enright et al., 1986; Kreeger and Langdon, 1993; Maeda-Martínez et al., 2016; Uriarte and Farías, 1999; Utting, 1986; Wikfors et al., 1992), including juveniles of the Manila clam (*Ruditapes philippinarum)* (Albentosa et al., 2002; Langton et al., 1977) and the con-generic *R. decussatus* (Albentosa et al., 1999). In the specific case of Manila clams, Gallager and Mann (1981) reported a negative impact on growth for diets presenting C:N ratios above 10.5. Thus, actively growing bivalves appear to require moderate to high levels of dietary protein to optimize growth, whereas diet quality (the protein to energy P/E ratio) has been reported to be a better predictor of growth performance than the overall food ration (Kreeger and Langdon, 1993).

4.1 Acute vs. chronic response to changing dietary N content

In the present experiments, groups of clams were conditioned for 15 days to N+ or N- diets, and then physiological parameters and the resulting SFG were recorded for each acclimation group with both N+ and N- diets. The obtained set of data could thus be arranged to generate a sequence comprising the acute followed by the chronic response of physiological parameters to every change from N+ to N- and *vice versa* (see *Figure 2.1 - 2a* to *Figure 2.1 - 5a*). Growth rate differences found between N+ and N- diets were accounted for by differences in physiological behavior regarding the main components of the energy balance and features of this behavior, including both short and long-term responses to dietary change. Characteristically, the acute-chronic sequence varies for the different physiological parameters, depicting a complex pattern of food conditioning. For instance, net energy gain (the absorption rate: AR) was governed by the contrasting behavior of the feeding rate and absorption efficiency: feeding rates declined with every change in the diet (either N+ to N- or *vice versa*), and full achievement required acclimation, whereas AE increased in the acute change to the N+ diet and further improved during the acclimation to that diet. Patterns of metabolic energy expenditure were characterized by the increase in both oxygen consumption and ammonia excretion in the acute change from N- to N+, which partly declines during the acclimation to the N-rich diet.

Values of physiological parameters recorded under corresponding acclimation diets (i.e., the N+N+ and N-N- experimental sets) would be representative of stable conditions after diet acclimation, and computed SFG from these values can consequently be assumed to indicate growth performance exhibited by the different groups. Comparison of physiological behavior of clams fully acclimated to N+ and N- diets, across the different family*growth group combinations (*Figure 2.1 - 7*), indicate significantly higher rates of both energy gain and loss and resulting SFG values that were increased by 50% on average for clams fed the high-protein diet, with the only exception being the S8 (slow growers of Family 8) group.

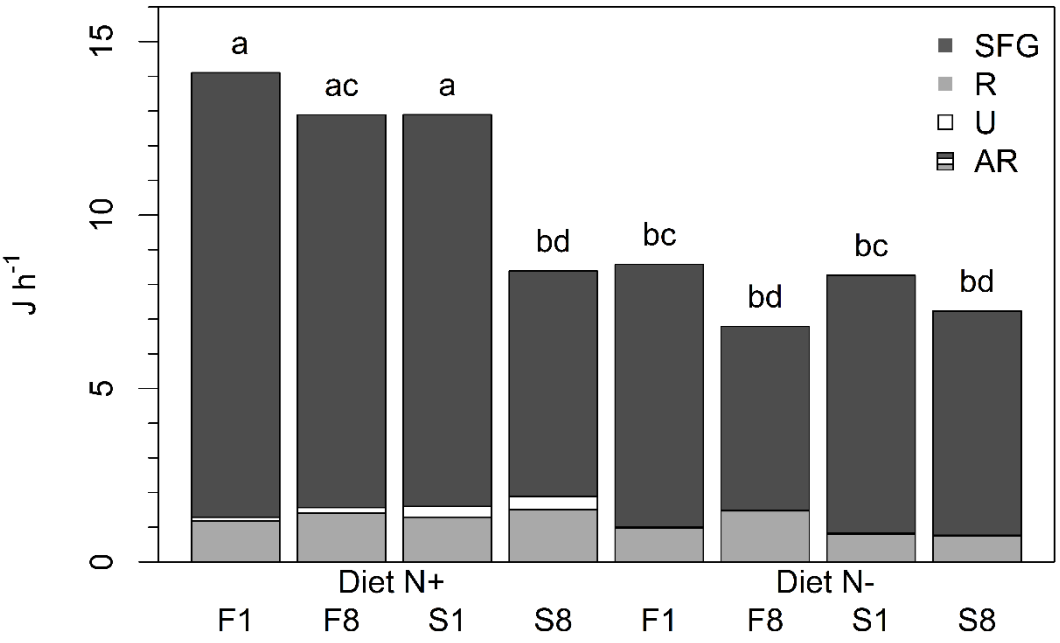

*Figure 2.1 - 7. Bar plot reporting components of net energy gain (sum of all categories) and loss (R: gray bars, U: white bars) and resulting SFG (dark gray bars) in the different family*growth group combinations fully acclimated to diets N+ and N-*

While the beneficial effect of increased protein/energy (P/E) indices of the diet on the growth rate of bivalves has been broadly documented (see references above), there is presently a noticeable lack of experimental evidence in this group concerning the concomitant effects on the energy budget and the physiological components of growth that are involved in the improvement of individual production. In this respect, commercial fish species might provide a useful reference for comparative purposes since the energetic response to variable E/P diets has been frequently tested (Bendiksen et al., 2002; Boujard and Médale, 1994; Helland and Grisdale-Helland, 1998; Morales et al., 1994): For instance, data from experiments performed on the rainbow trout fed high and low P/E diets designed on an iso-energetic basis (Saravanan et al., 2012) agree with the present results regarding the positive effects of protein-rich diets on feed intake (= OIR), digestible energy intake (= AR) and energy retention (= SFG), with nonsignificant differences in metabolic heat output associated with diet. The same results obtained in such different aquacultured animal models are indicative of common mechanisms and point to limitations of the homeostatic control of protein income, exemplified for instance by the fact that specimens exposed to low P/E diets do not resort to “overeating” to compensate for reduced dietary protein, with a resulting reduction in growth performance.

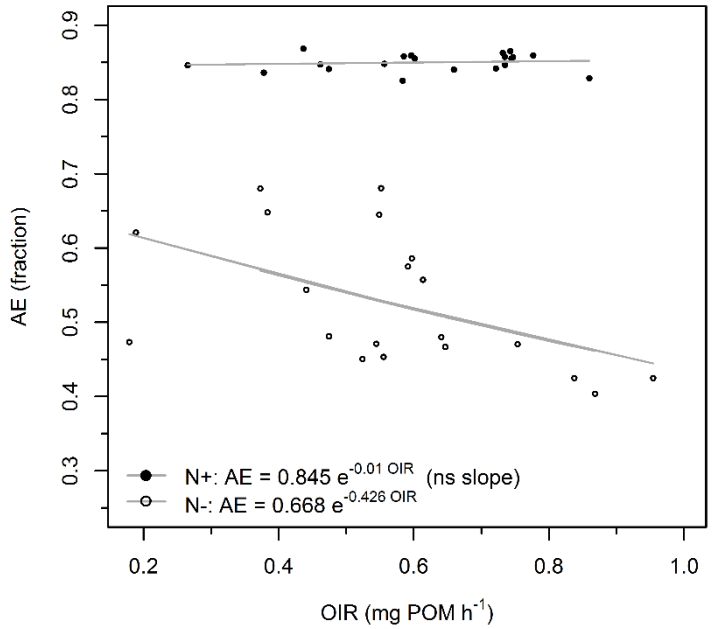

Figure 2.1 - 8. Absorption efficiency (AE) as a function of organic ingestion (OIR). Lines were fitted to mean values for the different groups of clams fully acclimated to N+ (closed circles) and N- (open circles) diets

Further analysis of the physiological components of energy gain in the present experiments suggests that the above limitations might stem from digestive constraints. Increased energy income with high-quality (high P/E) food relies partly on a behavioral response since the feeding rate has increased significantly by the term of acclimation to N+ relative to N- diets (by 12% on average). However, the outstanding effect of protein-rich diets on energy balance is mediated by a strong increase in the AE, which rises by nearly 80% (from 0.51 to 0.85) during the change from fully acclimated N- to N+. Although the AE of N (AE_N) has been reported to be higher than that of C (AE_C) under several circumstances (Bayne, 2009; Urrutia et al., 1996), consideration of this factor could not fully account for differences in AE for overall organics of the magnitude found in this study, since N absorption contributes at most 20% to the total absorption of organics. Consequently, broad differences in digestive performance (*sensu* Navarro et al., 2009) on both types of food particles should be invoked to account for a more efficient absorption of *R. lens* cells in the exponential (E) relative to the stationary (S) phase of the culture. Since most of the change (~ 80%) has already occurred in the short-term response (see *Figure 2.1 - 3a*), variable digestibility must rely on differential features of both microalgal cells (e.g., biochemical constitution or, eventually, size), rather than be based on enzyme induction processes that might take place during acclimation. This interpretation is consistent with the different behaviors exhibited by E and S cells upon

digestion (*Figure 2.1 - 8*): while the AE of E microalgae appears to be virtually independent of the ingestion rate, that of S microalgae declines with rising ingestion, revealing that digestive yield is strongly dependent on the gut residence time of food particles. This feature of the N- diet, a characteristic of poorly digestible food, would have the effect of canceling out the benefits of any potential increase in the feeding rate oriented to compensate for the low protein ration.

Feeding on different P/E diets has a neat effect on rates of energy expenditure (both oxygen consumption and ammonia excretion rates); although the energetic relevance of these dietary effects is lower, with the SFG response mainly driven by energy gain processes (as it will be discussed later). Two main points would summarize the present results concerning energy expenditure: *1)* Acclimation to a high-protein diet increases both metabolic and N excretion rates, with the stronger change being achieved in the acute response. *2)* These dietary effects are much higher for rates of excretion relative to the metabolic response, resulting in a maximum decrease of the O:N ratio by a factor of 7.5 when clams acclimated to the N- diet are fed the N+ diet. The corresponding difference in O:N ratios between clams fully acclimated to N+ and N- was a factor of 5.1.

Determination of ammonia excretion in studies regarding the scope for growth determination has been traditionally neglected in bivalves since its representation in the energy budget is considered low (1-10% of total metabolic energy expenditure in *M. edulis*; Bayne and Newell, 1983). However, this measurement gains interest in the context of studies —such as the present study— testing the effect of variable protein/energy inputs on the components of the energy balance, given that ammonia excretion represents a summary output of dietary protein metabolism. The reason, provided by studies reported in the Introduction section (see Kreeger et al., 1996, 1995), is that the preferred pathway for protein assimilation in bivalves appears to comprise incorporation to the N pool through transamination reactions, rather than the most direct incorporation to the pool of essential amino acids for protein synthesis. Consequently, Langton et al., (1977) reported in *Tapes japonica* (= *Ruditapes philippinarum*) a 2-fold increase in ammonia excretion corresponding to a 3.5-fold increase in N-protein ingestion, similar to the present results with the same species, where a 2.7-fold increase in N ingestion led to an 8.7-times increase in N excretion. A positive dependence of rates of ammonia excretion on dietary

protein ingestion generally documented in other aquatic animals, such as fishes (Brunty et al., 1997; Green and Hardy, 2008; Porter et al., 1987), pointing to a certain identity concerning the mechanisms of protein assimilation in ammoniotelic organisms. In line with the present approach, a 2 to 3-fold increase in the rate of N excretion has been reported in response to increasing dietary P/E ratios by the same factor in both shrimps (Coelho et al., 2019; Gauquelin et al., 2007) and fishes (Saravanan et al., 2012).

Consideration of the extent to which the stoichiometric C:N coupling between the diet and growing tissues occurs might provide further understanding of the observed diet-dependent behavior of N excretion. Bayne (2017) put forward a stoichiometric hypothesis fitting experimental data for *Crassostrea gigas* (Bayne, 2009; Mao et al., 2006): "When feeding behavior cannot fully compensate for an imbalance between C:N of the tissues and C:N of the diet, and nitrogen is absorbed in excess of the demand, then this excess is removed by excretion. Similarly, if insufficient N is absorbed then nitrogen excretion is reduced in order to conserve tissue nitrogen". In the present case, N surplus resulting from the elevated energy inputs achieved to sustain high growth demands with the N+ diet would account for rates of ammonia excretion that exceed 10 times the rates recorded with the N- diet, in which low protein content combines with reduced AE to doubly constrain N absorption.

4.2 Endogenous factors

Separate breeding of two families differing in growth rate and size-segregation inside each family was combined in this study to achieve a wide range of growth phenotypes for physiological determinations and SFG computation. In general, physiological differences accounting for growth variation were found to be higher for the size-segregation factor than the family factor: For instance, based on means of pooled values (*Table 2.1 - 4*), net energy gain values differed by 34% between fast (F) and slow (S) clams, while the corresponding difference between F1 and F8 amounted only to 18%; the equivalent figures for the SFG were 40 and 22%, respectively. These trends occur irrespective of the diet, since interaction terms have null or weak significance (*Table 2.1 - 5*). Energy losses, when significantly different, showed the opposite behavior. Thus, the energy balances for the different family*growth group combinations rank as follows: F.1 > F.8 > S.1 > S.8.

The relative contribution of components of energy gain and loss to SFG variation is illustrated in *Figure 2.1 - 9*. Clearly, energy balance fluctuations recorded across diets and growth phenotypes are overwhelmingly driven by the physiological processes of feeding and absorption, while the effects of metabolic energy expenditure are virtually null (regression equations for either respiration or ammonia excretion rates were only significant on their intercepts). Previous studies analyzing SFG fluctuations across size-segregated growth groups of clams (*R. philippinarum:* Tamayo et al., 2011) and mussels (*M. galloprovincialis:* Fernández-Reiriz et al., 2016) also reported that fast growth was mainly accounted (80- 90%) for by increased energy gain, while 10-20% was explained by changes in metabolism. This agrees with the general observation that limits to growth in bivalves are set primarily by functional constraints on feeding and digestion rather than by the associated metabolic costs (Bayne et al., 1989; Navarro et al., 1992), although metabolic constraints have been reported at low food concentrations (Albentosa et al., 1996; Beiras et al., 1994), i.e., when food rations approach the maintenance conditions.

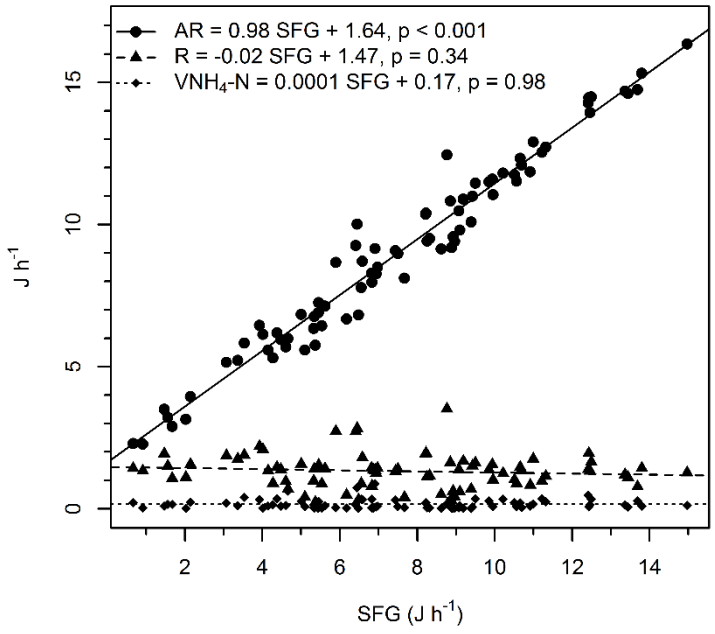

Figure 2.1 - 9. Net energy gain (AR) and loss (R and U) at different levels of the SFG. Lines fitted (minimum squares) to individual data for all experimental sets in this study

Physiological parameters accounting for SFG fluctuations were the same, irrespective of diet acclimation, and involved both rates of energy acquisition and conversion efficiencies:

1) Increased energy acquisition in faster growers was fully accounted for by the higher feeding rates found in F clams (40% increase with respect to S clams) and clams

from Family 1 (25% increase with respect to those of Family 8), since the AE was found to decline (little but significantly; *Table 2.1 - 5*) in fast growers relative to slow growers. Several studies comparing the feeding behavior of size-segregated growth groups have reported that faster feeding of F specimens correlated with larger gills in both clams (Tamayo et al., 2011) and mussels (Prieto et al., 2018). The present results preclude any generalization of this kind of relationship as gill areas were found in this case to be consistently higher in the groups of clams exhibiting lower clearance rates (i.e., in fam. 8 compared with fam. 1 and in S clams compared with F clams), suggesting that a greater pumping capacity per unit of surface area (or increased gill efficiency) would be an alternative mechanism to achieve fast feeding. On the other hand, gill area was found to differentiate during diet acclimation (higher values corresponded to the protein-rich diet), an adaptive response similar to gill and palp size adjustments to different food environments revealed in transplant experiments of different species of bivalves (Tedengren et al., 1990; Worrall and Widdows, 1983). This points to a highly plastic trait (Honkoop et al., 2003) and does not support the idea, implicit in previous studies (Prieto et al., 2019, 2018; Tamayo et al., 2011), that gill size would be a constitutive trait, liable *per se* to account for interindividual differences in feeding and growth rates. The ability to adapt the size of filtering structures was noticeably greater in F clams and clams of Family 1 (see *Figure 2.1 - 2b*), and this differential behavior might explain the prompter and more efficient feeding adjustments exhibited by fast growers during the dietary changes. Generally, F/Fam.1 clams lost less feeding and absorption capacity with diet N- and recovered earlier their previous level of activity with diet N+ than did the S/Fam.8 clams. This, combined with more restrained energy losses, resulted in fast growers achieving a better management of energy resources during nutritional fluctuations.

2) Lack of significant differences in rates of metabolic energy expenditure recorded for the different growth phenotypes implies that increased energy gain (2 to 3-fold increase in rates of absorption between fast and slow growers) does not occur at the expense of greater metabolic outputs, thus pointing to variable metabolic efficiency (Bayne, 2004, 1999). Indeed, the unitary metabolic costs (i.e., per unit of metabolizable energy or AR) were found to decline for rising SFG (*Figure 2.1 - 10*), indicating that greater metabolic efficiencies also stood out as a component of faster growth. Statistical comparison (ANOVA) of mean values for these unitary costs between the different family*growth group combinations indicates significant differences between families,

where Family 1 sibs attained 77% lower unitary costs than Family 8 sibs ($F = 6.486$, $p = 0.0159$). Similar results concerning interfamily differences in metabolic efficiency have also been reported in the mussel *Perna canaliculus* (Ibarrola et al., 2017).

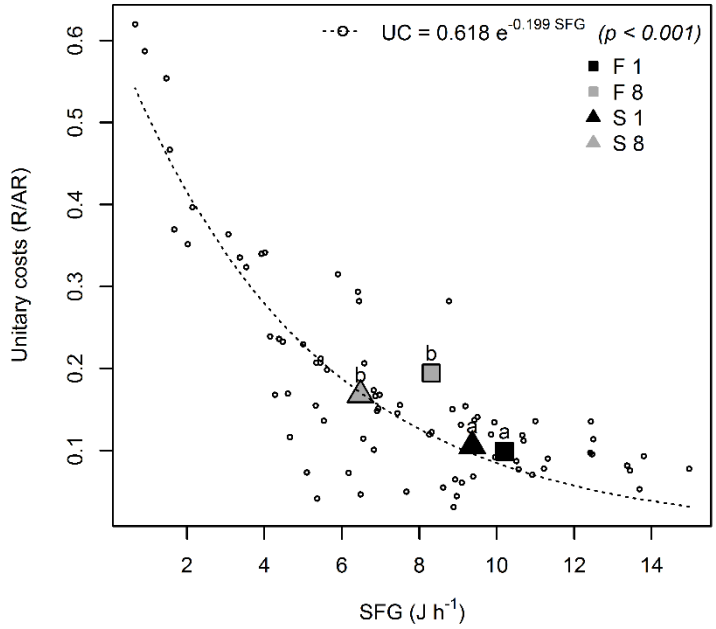

Figure 2.1 - 10. Unitary metabolic costs (computed individually) as a function of SFG (open circles), and mean values of this relationship in the different growth phenotypes (squares: F clams, triangles: S clams; black symbols: Family 1, gray symbols: Family 8), under fully acclimated conditions. Superscripts indicate significant differences ($p < 0.05$) in terms of unitary costs

Therefore, the present results confirmed most earlier studies on bivalves reporting selection for faster growth to entail faster rates of feeding and absorption (increased energy acquisition), most frequently coupled to increased metabolic efficiency represented by the reduced metabolic costs per unit of absorption (Bayne, 2000, 1999; Bayne et al., 1999b, 1999a; Fernández-Reiriz et al., 2016; Holley and Foltz, 1987; Ibarrola et al., 2017; Pernet et al., 2008; Tamayo et al., 2016, 2015, 2014, 2011; Toro and Vergara, 1998). Bayne (2000) and Pace et al. (2006) have convincingly associated these variations in costs of growth with differences in the efficiency of protein deposition in both larvae and adult oysters. In spite of very different experimental approaches used in the segregation of growth phenotypes, a noticeable uniformity regarding the complex of physiological processes underlying differential growth appears to be the rule across those studies. Moreover, this endogenous component of growth variability has been found to subsume a wide range of phenotypic plasticity for physiological traits, expressed in the form of the present feeding and digestive adjustments to a change in the biochemical

composition of food, as well as equivalent responses reported in variable nutritional (Bayne, 2000; Tamayo et al., 2015) or thermal (Tamayo et al., 2013) contexts.

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~ CHAPTER 2.2 ~

Elemental C and N balance evidence stoichiometric adjustments in growth phenotypes of the Manila clam (*Ruditapes philippinarum*) in response to diets differing in C:N ratios.

ABSTRACT

Juveniles from each of two separately bred families of the Manila clam *Ruditapes philippinarum* were used to constitute fast (F) and slow (S) growing groups by size-segregation. The 4 growth phenotypes that resulted from these family*growth group combinations were then used to measure elemental C and N balances in the acute and chronic responses to two different microalgal diets that were isocaloric but differed (x3) in their C:N ratios. Both diets were based on a same phytoplankton species (*Rhodomonas lens*) that were harvested in the exponential (low C:N ratio) or stationary (high C:N ratio) phases of an indoor culture. Aims were to assess the differential acquisition and processing of elemental nutrients according to differences in their dietary availability and the variable requirements set by growth trends exhibited by different phenotypes. Clams fed the high N diet achieved the highest C and N balances through a combination of higher feeding rates and absorption efficiencies of overall organics. However, the main differential effects were associated to increased absorption efficiencies for N relative to C, that were particularly observed in clams submitted to chronic N deficit in the diet. This occurred in all growth phenotypes and resulted in partial homeostatic regulation of nutrient imbalance operating at the preabsorptive level. Main phenotype differences were observed in the intra family (F *vs*. S growth groups) rather than in the inter family comparisons, with F clams exceeding by 50% the absorption rate of nutrients (both C and N) of S clams. Physiological responses to the acute dietary change were found faster in F clams, indicative of a higher plasticity of this phenotype. Whereas, stoichiometric adjustments by S clams resulted in higher N release through ingestion, suggesting a less efficient protein turnover.

Key words: nutrient balances, stoichiometry, growth phenotypes, *Ruditapes philippinarum*, C:N ratio, homeostasis.

1. INTRODUCTION

Growth is an important attribute of animal individuals and populations that has implications in many a field. In strictly practical terms, growth measurements in marine bivalve have been broadly considered both as a factor to be accounted of in the management and sustainability of ecosystems and as an economic target in the aquaculture industry. From an energetic point of view, growth can be viewed as "the outcome of acquisition and utilization of nutrients and energy" (Bayne, 2017) and this perspective has dominated the field since the early growth models by von Bertalanffy (1938) that considered growth as resulting from a balance between metabolic processes of assimilation (anabolism) and dissimilation (catabolism). Bioenergetic models currently in use derive from the same principles although relying on a more detailed physiological account of energy flows. In these, growth is figured out on terms of energy budgets in either the classic scope for growth (SFG) approach or according to the dynamic energy budget (DEB) theory (Kooijman, 2010). Bioenergetic models have provided with useful tools in predicting overall growth that are of general interest in aquaculture production while the experimental testing of these models has enlarged our knowledge of the mechanisms of growth as regards to the specific influence of both endogenous and exogenous (food, temperature, etc.) factors on the various components of the energy budget.

However, use of energy as the sole currency in these budgets has clear limitations stemming from fact that large differences are often encountered between consumed food and consumer body tissues as regards to elemental composition. This has special relevance in herbivorous or detritivores relying on vegetal resources, like most bivalve mollusks, where for instance rather constant C:N ratios reported for body composition (between 4.1 and 6.2) contrasts with highly variable values of C:N ratios of their diets, ranging between 4.8 and 22.5 on a seasonal/experimental basis (Bayne, 2009; Bayne and Svensson, 2006; Fielding and Davis, 1989; Grant and Cranford, 1991; Smaal and Vonck, 1997; summarized in Bayne 2017, Table 5.9 on p. 299). In the stoichiometric approach (Sterner and Elser, 2002) these mismatches are addressed in terms of nutrient homeostasis by invoking the resource to a suit of physiological/metabolic mechanism able to compensate, whenever necessary, for the imbalance between the diet and consumer´s tissues. Nutrient limitation implies that C or energy is in excess and, "to maintain overall homeostasis, consumers should release elements on food in excess of requirements while

retaining most of the limiting element"… "On the other hand, consumers may also be limited by carbon (energy), in which case it is the ingested nutrients that are subject to efficient recycling" (Anderson et al., 2005). According to this model, for the stoichiometry of growing tissues to be realized any component of the diet in excess of the appropriate ratio should be disposed off, whence important constraints on growth can be expected that would hardly be identified from the only account of energy flow measurements. This approach thus replaces reliance on energy budgeting by assessing nutrient (elemental) balances based on physiologically determined components of growth.

Several early studies emphasized the need for an evaluation of nutrients, together with energy, in the analysis of growth on account of differential demands for C and N found in bivalve species such as the scallop *Placopecten magellanicus* (Cranford, 1995; Grant and Cranford, 1991) or the mussel *Mytilus edulis* (Bayne et al., 1993; Hawkins and Bayne, 1985) This last species was incorporated in the development of the EMMY model, which computes C and N fluxes of mussels in growth estimations (Scholten and Smaal, 1999, 1998; Smaal and Scholten, 1997). Modelling efforts in the recent years have been incorporating parameters that take into account the elemental composition of diet to fit a better approach of actual growth (Bourlès et al., 2009; Brigolin et al., 2009; Emmery et al., 2011; Grangeré et al., 2009).

Mechanisms for the homeostatic nutrient regulation in bivalves can be identified at several different levels in the chain of physiological processes connecting feeding to growth. These include preingestive as well as pre and postabsorptive levels. Preingestive selection of N-rich particles has been broadly reported in feeding experiments using natural seston or mixtures of phytoplankton with suspended sediments containing organic detritus (Hawkins et al., 1996; Iglesias et al., 1996; Soletchnik et al., 1996; Urrutia et al., 1996), a selective process that would be mainly based on the ability of gill and palps to discriminate between microalgae (low C:N ratio) and detrital particles (high C:N ratio). As a matter of fact, higher selection efficiencies (SE) observed for Chlorophyll relative to overall organics were found to correlate with higher SE for N (SE_N) compared with SE for C (SE_C) in some of these studies (e.g. Iglesias et al., 1996; Urrutia et al., 1996). Above mechanisms of selective ingestion have been reported to operate in both *Cerastoderma edule* and *Mytilus edulis* to maintain a constant N-absorption from mixed diets of a diatom (*Phaeodactylum tricornutum*) and suspended sediments (Prins and Smaal, 1989), or to modulate seasonally preingestive selection for N in function of the seston composition

(C:N ratio) and growth demands in the oyster *Saccostrea glomerata* (Bayne and Svensson, 2006). Differential selection of species of phytoplankton according to their food value (Beninger et al., 2008; Cognie et al., 2001; Pales-Espinosa et al., 2008, 2007; Shumway et al., 1985) might further contribute to this enrichment. As an evidence of the potential of these selective mechanisms to contribute to nutrient homeostatic regulation, Bayne (2009) reported that the ratio of SE_N to SE_C is a positive function of the nutrient imbalance, given as potential N limitation (i.e., as the difference between C:N ratio in the seston and C:N ratio in oyster tissues).

Similar selective mechanisms at the postingestive level can also be invoked to account for N enrichment of the absorbed ration from complex diets (i.e. seston) since digestive selection (Navarro et al., 2016) has been shown to result in the preferential utilization of microalgae (low C:N ratio) vs sedimentary organic particles including phytodetritus (high C:N ratio). The expected increment in the absorption efficiency for N compared to C in such type of scenario has been certainly reported (Bayne, 2009; Cranford and Grant, 1990; Grant and Cranford, 1991; Iglesias et al., 1996; Prins and Smaal, 1989; Urrutia et al., 1996). Moreover, almost complete absorption of labile protein from detrital particles (above the AE for overall POM) found in oysters (Adams et al., 2019) might also account for preferential absorption of N relative to C.

Digestive selection of different food items present in natural diets would obviously fail to account for the differential absorption of the main elements (C and N) in the case of simple composition diets where phytoplankton was the main component (Iglesias et al., 1996). In these cases, the observed higher absorption efficiency for N compared with overall organics may result from: *1)* Higher gross absorption efficiency for proteins than for other biochemical components possibly based in specific balances of digestive enzymes. This suggestion is consistent with higher absorption efficiencies for proteins, relative to carbohydrates and lipids, found in cockles (*Cerastoderma edule*) that improved with acclimation to a diet of phytoplankton (Ibarrola et al., 2000b). Bayne (2009) has reported in oysters AE_N:AE_C ratios that range between approx. 0.8 in July to 1.0 in November and 1.3 in March suggesting that seasonally variable requirements for specific nutrients might be meet by enzyme induction. *2)* A reduced nitrogen content of digestive secretions voided with the faeces in the form of metabolic faecal losses MFL. Ibarrola et al. (2000, 1998, 1996) pointed out to the digestive imbalance of lipids in cockles caused by the faecal enrichment in this component, partly as a consequence of the cycle of intracellular digestion causing removal of the digestive cell apex. This would

tend to reduce AE for C relative to N in an extent that would be proportional to the relative magnitude of MFL in the digestive balance.

Postabsorptive mechanisms concerns to the metabolic fate of different elements contributing to final adjustments of the assimilated ration to meet the specific requirements for tissue biosynthesis. Release of excess C (or other nonlimiting nutrients) at this postabsorptive level may represent a significant fraction of the metabolic budget in animals feeding on relatively unbalanced foods that are common in both terrestrial and aquatic systems (Anderson et al., 2005). This has been broadly documented in bivalve mollusks (namely mussels) as regards several features of the protein turnover where efficiency of protein deposition was reported to increase with rates of dietary protein absorption (Hawkins, 1985). The increased ratio of protein breakdown to protein synthesis reported under conditions of reduced N input (Hawkins, 1985) probably reflects the metabolic role of dietary protein in fueling the N pool through transamination reactions for protein synthesis, with the consequent waste of most of the protein C. This would explain the disproportionately higher assimilation efficiencies for the amino-N fraction compared with the amino-C fraction reported in mussels (Kreeger et al., 1996, 1995) as the result of a N sparing mechanism at the expense of energy (C) even under conditions of negative energy balance (Hawkins and Bayne, 1991). Conversely, N release when N input is in excess of requirements occurs in the form of high rates of ammonia excretion reflecting the metabolic fate of proteins in supplying energy through deamination reactions. Resource to experimental diets providing a range of variation in the relative proportion of different nutrients, and particularly in the protein/energy ratio has thus proven useful in studying the contribution of these postabsorptive processes to the homeostatic nutrient regulation in bivalves.

In a precedent work (Arranz et al., 2020; Chapter 2.1) we have analyzed the effects of two different diets, that were isocaloric but differed broadly in the C:N ratios, on the physiological performance of juvenile clams (*Ruditapes philippinarum*) measured in terms of the energy budgets. These diets consisted of the same microalga species (*Rhodomonas lens*), cultured at different growth stages (i.e. exponential and stationary phases). Also, this study was performed on different growth phenotypes that were obtained by combining selective breeding of two families and size-group segregation inside each family, in an attempt of assessing how physiological responses to diet composition were modulated according to the variable growth demands of endogenous origin set by the occurrence of these different phenotypes. Diets of high N content were

found to greatly increase the energy acquisition of clams across phenotypes, through a combination of increasing both the feeding rates and absorption efficiencies. In spite of increased metabolic expenditures, particularly the N excretion rates, these N rich diets resulted in values of the scope for growth (SFG) that exceeded by 50% on average those of the N poor diets, which was confirmed by the corresponding actual growth data measured in clam´s groups acclimated to the two types of diets.

Endogenous differences in growth rate associated with the segregated phenotypes were mainly accounted for by differences in energy acquisition, with feeding rates differing by ~ 2-fold between fast and slow growers. Adjustments of feeding parameters to dietary changes were more efficiently performed also in fast growers. On the other hand, fast-growing phenotypes were found to exhibit increased conversion efficiencies, as represented by the reduced unitary metabolic costs (i.e., per unit of absorption) that were recorded in specimens of the family exhibiting higher rates of growth. These results were, in general, confirmatory of many previous studies analyzing the physiological basis of interindividual growth rate differences in species of bivalves including clams (Tamayo et al., 2015, 2013, 2011), mussels (Fernández-Reiriz et al., 2016; Ibarrola et al., 2017; Prieto et al., 2020b, 2020a, 2018; Tamayo et al., 2016) and oysters (Bayne, 1999; Bayne et al., 1999b, 1999a; Pace et al., 2006; Toro et al., 1996; Toro and Vergara, 1998).

In the present study, the same experimental design developed to compute energy balances (Arranz et al., 2020; Chapter 2.1) were extended to include the elemental balance of nutrients (C and N). Aims of this approach were to assess *a)* how does the differential processing of nutrients contributes to the stoichiometric adjustments to diets largely differing in C:N ratios. *b)* Which mechanisms govern differential incorporation and disposal of nutrients at the different levels in the chain of physiological processes connecting feeding and growth. *c)* How do the above physiological adjustments become modulated to meet the variable requirements set by the occurrence of different growth phenotypes.

2. MATERIAL AND METHODS

2.1 ANIMAL MAINTENANCE, DIET CHARACTERISTICS AND EXPERIMENTAL DESIGN

Features of diets, clam families, growth groups and maintenance of them, including experimental design have been previously described in Chapter 2.1 (Materials

and methods, sections 2.1-2.3); where, briefly, the fastest (mean length: 23.50 (1.67) mm) and slowest (mean length: 13.10 (1.65) mm) growing juveniles belonging to two families (1 and 8) of the Manila clam *Ruditapes philippinarum* were chosen.

Maintenance was carried out under constant ambient and feeding conditions; however, experimental conditions involved the use of the microalga species *Rhodomonas lens* at different culture stages to evaluate the acute as well as the chronic response to different N quality diets. Since stationary growth phase cultures of the microalga species *R. lens* are known to possess ca. 3 times more C:N values than exponential cultures (see Chapter 2.1, *Table 2.1 - 2*), that species was employed at either its exponential (diet N+) or stationary (diet N-) growth phase.

As specified in the previous subchapter, diet characterizations were performed twice a week in quadruplicates during the acclimation period, and 5-6 times in quadruplicates during the exposition (Chapter 2.1, *Table 2.1 - 3*). In that way, a known volume of water was sampled from the feeding tanks and was subsequently filtered through pre-weighted glass fiber filters (GF/C). Afterwards, filters for elemental (CHN) analysis were washed with 50 mL of seawater and immediately frozen at -20ºC, lyophilized, and maintained at -20ºC until being analyzed in a Euro EA Elemental Analyzer (CHNS) from EuroVector, using acetanilide as standard. A subset of samples were calcined for 6 h at 450ºC and were subsequently measured in the elemental analyzer in order to be used as a control.

For each combination of growth category and family, two groups (n = 3) were acclimated for 15 days to diets N+ and N- , respectively. After that period, physiological experiments were carried out under their acclimation diet as well as under the opposite diet, to have a full factorial experiment, as shown in Chapter 2.1, *Figure 2.1 - 1*, where the resulting 16 groups are notated according to the following order: acclimation, acute exposure, growth category and family.

2.2 ELEMENTAL BALANCES

Table 2.2 - 1. Mean (SD) results of clearance rate (CR, L h^{-1}), absorption efficiency (AE, decimal units), oxygen consumption rate (VO_2, mL h^{-1}) and ammonia excretion rate (VNH_4-N, µg h^{-1}) of Chapter 2.1 used in this subchapter to calculate elemental balances

Acclimation	Acute exposure	Growth category	Family	CR	AE	VO_2	VNH_4-N
N+	N+	F	1	1.22 (0.16)	0.84 (0.01)	0.06 (0.01)	4.26 (1.42)
			8	1.1 (0.22)	0.85 (0.01)	0.07 (0.02)	6.31 (7.1)
		S	1	1.1 (0.13)	0.85 (0.01)	0.06 (0.02)	12.58 (1.74)
			8	0.72 (0.2)	0.85 (0.01)	0.08 (0.02)	15.22 (11.56)
	N-	F	1	0.9 (0.13)	0.58 (0.04)	0.07 (0.04)	2.83 (1.35)
			8	0.74 (0.13)	0.62 (0.02)	0.08 (0.03)	3.62 (1.48)
		S	1	0.41 (0.11)	0.58 (0.02)	0.06 (0.03)	4.86 (1.21)
			8	0.47 (0.11)	0.61 (0.02)	0.06 (0.03)	3.97 (1.06)
N-	N+	F	1	0.95 (0.09)	0.81 (0.03)	0.1 (0.04)	9.16 (2.64)
			8	0.78 (0.41)	0.75 (0.06)	0.07 (0.02)	9.38 (3.25)
		S	1	0.66 (0.16)	0.74 (0.03)	0.08 (0.04)	19.79 (7.36)
			8	0.56 (0.22)	0.78 (0.03)	0.07 (0.02)	11.74 (2.18)
	N-	F	1	1.1 (0.22)	0.44 (0.03)	0.05 (0.02)	1.29 (0.35)
			8	0.83 (0.44)	0.48 (0.04)	0.07 (0.02)	0.95 (0.03)
		S	1	0.9 (0.05)	0.53 (0.06)	0.04 (0.03)	0.82 (0.06)
			8	0.63 (0.23)	0.65 (0.03)	0.04 (0.02)	0.63 (0.24)

Physiological determinations of scope for growth were described previously (Chapter 2.1), where, in short, five replicates per condition were used to determine clearance rate under the flow-through chamber method (Crisp, 1971; Filgueira et al., 2006), absorption efficiency (Conover, 1966), ammonia excretion rate (Solórzano, 1969) and metabolic rate (see *Table 2.2 - 1* containing the main results of these parameters). In the present work, these data were combined with elemental analysis of diets and feces to compute N and C balances as follows:

Ingestion rates: Particulate organic N and C (PON and POC, respectively) were calculated as the product between POM and the proportion of organic N and C present in the diet in the following way:

$$PON\ (mg\ L^{-1}) = POM \times \frac{N\%}{100}$$

$$POC\ (mg\ L^{-1}) = POM \times \frac{C\%}{100}$$

Ingestion rate of N and C (IR_N and IR_C, (mg h^{-1})) were estimated multiplying clearance rate by the particulate organic matter of each element, in these terms:

$$IR_N = PON \times CR$$

$$IR_C = POC \times CR$$

Absorption rates and absorption efficiencies: absorption rate (AR_N and AR_C, (mg h^{-1})) constitutes the difference between ingestion (IR_N and IR_C, (mg h^{-1})) and egestion rate (ER_N and ER_C, (mg h^{-1})), which is calculated through the use of organic ingestion rate (OIR), absorption efficiency (AE) and the proportion of organic N and C present in the feces, as follows:

$$ER_N = OIR \times (1 - AE) \times \frac{N\%_{(feces)}}{100}$$

$$ER_C = OIR \times (1 - AE) \times \frac{C\%_{(feces)}}{100}$$

Absorption efficiency (AE_N and AE_C, decimal units) is represented by the quotient between AR and IR of each element:

$$AE_N = \frac{AR_N}{IR_N}$$

$$AE_C = \frac{AR_C}{IR_C}$$

Carbon and nitrogen loss: carbon loss due to respiration (R_C, mg C h^{-1}) was inferred from respiration rates (mg O_2 h^{-1}), assuming a RQ = 0.9. Respiration rates were obtained with the aid of oximeters through monitoring the declining in oxygen concentration in a sealed chamber where clams had been introduced in filtered seawater. Nitrogen loss due to excretion (E_N, mg N h^{-1}) was assessed through the phenol-hypochlorite method (Solórzano, 1969), employing flasks with 30 mL of filtered seawater (0.2 µm Millipore membranes) for 2-3 h, assuming that all of the N losses are due to excretion. For both determinations, chambers without animals were employed as controls.

Elemental balances of carbon and nitrogen: Elemental balance of carbon (SFG_C, mg C h^{-1}) was calculated as the difference between the amount of C absorbed and respired:

$$SFG_C = AR_C - R_C$$

whereas elemental balance of nitrogen (SFG_N, mg N h^{-1}) was calculated as the difference between the amount of N absorbed and excreted:

$$SFG_N = AR_N - E_N$$

After concluding the experiments, clams were dissected to compute dry weight of tissues, and consequently, all rates were standardized to the common dry weight of tissue (85.95 mg) by means of the mass exponents 0.6091 (CR), 0.6967 (R) and 1.00 (VNH_4-N).

2.3 Data analysis

Relationships between two or more quantitative variables, such as AE and AR were explored through regression analysis; while comparisons between families, growth category, acclimation diet as well as exposition diet were tested through a 4-way ANOVA using R (R Core Team, 2018). These analyses were performed after having tested for normality (Shapiro-Wilk) and homoscedasticity (Levene) of the data.

3. RESULTS

3.1 DIET EFFECTS

Concerning elemental (C and N) composition, acquisition parameters (i.e., rates of food processing and digestive balances: IR, ER and AR) were mostly influenced by the composition of the diet fed the animals during physiological determinations (exposure diet) than for previous conditioning, although some effects concerning the latter nutritional variable will be considered.

In this way, ingestion rates of N were significantly higher (see *Table 2.2 - 2*) when clams were exposed to the N rich diet, doubling the values recorded in those fed the N poor diet (*Table 2.2 - 3* and *Figure 2.2 - 1*), while egestion rates of N were increased under N restricted conditions. As a consequence, absorption rates were 3 times higher when clams were fed N+ diet compared to N- diet. Acclimation did not affect physiological performance, although ingestion and absorption rates tended to increase in those individuals previously acclimated to N+ diet.

Table 2.2 - 2. Acclimation (A), exposure (E), growth category (G) and family (F) effects on 4-way ANOVA table of the parameters involved in elemental balances of nutrients (IR_N and IR_C: ingestion rates for N and C, ER_N and ER_C: egestion rates of N and C, AR_N and AE_C: absorption rates for N and C, AE_N and AE_C: absorption efficiencies for N and C, R_C: C losses by respiration, E_N: N excreted, and SFG_N and SFG_C: elemental balances for N and C). Sianificant differences ($p<0.05$) are shown in bold

	IR_N	**IR_C**	**ER_N**	**ER_C**	**AR_N**	**AR_C**
Acclimation (A)	F = 3.986, p = 0.056	F = 0.444, p = 0.511	F = 1.975, p = 0.172	**F = 4.675, p = 0.04**	F = 4.018, p = 0.056	**F = 7.449, p = 0.011**
Exposure (E)	**F = 100.671, p < 0.001**	F = 0.074, p = 0.788	**F = 7.585, p = 0.011**	**F = 72.351, p < 0.001**	**F = 169.552, p < 0.001**	**F = 37.66, p < 0.001**
Growth category (G)	**F = 23.435, p < 0.001**	**F = 30.676, p < 0.001**	**F = 31.768, p < 0.001**	**F = 26.447, p < 0.001**	**F = 17.565, p < 0.001**	**F = 23.4, p < 0.001**
Family (F)	F = 1.663, p = 0.209	F = 1.706, p = 0.203	**F = 5.492, p = 0.027**	F = 2.193, p = 0.151	F = 0.746, p = 0.396	F = 0.871, p = 0.359
A*E	**F = 4.341, p = 0.047**	**F = 6.87, p = 0.014**	F = 1.959, p = 0.173	F = 3.575, p = 0.07	**F = 8.716, p = 0.007**	**F = 7.337, p = 0.012**
A*G	F = 0.306, p = 0.585	F = 0.769, p = 0.388	F = 0.984, p = 0.33	F = 0.002, p = 0.968	F = 0.14, p = 0.711	F = 2.036, p = 0.166
E*G	F = 0.974, p = 0.333	F = 0.62, p = 0.438	**F = 5.879, p = 0.023**	**F = 9.113, p = 0.006**	F = 3.715, p = 0.065	F = 1.164, p = 0.29
A*F	F = 0.141, p = 0.711	F = 0.189, p = 0.667	F = 0.077, p = 0.784	F = 0, p = 0.99	F = 0.291, p = 0.594	F = 0.492, p = 0.489
E*F	F = 0.163, p = 0.689	F = 0.004, p = 0.951	F = 1.256, p = 0.273	F = 1.022, p = 0.321	F = 0.685, p = 0.415	F = 0.464, p = 0.502
G*F	F = 0.349, p = 0.56	F = 0.347, p = 0.561	F = 0.297, p = 0.591	F = 0.657, p = 0.425	F = 0.308, p = 0.584	F = 0.097, p = 0.758
A*E*G	F = 0.191, p = 0.666	F = 0.224, p = 0.64	F = 0.085, p = 0.773	F = 0.219, p = 0.643	F = 0.197, p = 0.661	F = 1.243, p = 0.275
A*E*F	F = 0.002, p = 0.967	F = 0.023, p = 0.88	F = 0.879, p = 0.357	F = 0.308, p = 0.584	F = 0.111, p = 0.742	F = 0.034, p = 0.854
A*G*F	F = 0, p = 0.998	F = 0.393, p = 0.536	F = 3.804, p = 0.062	F = 3.434, p = 0.075	F = 0.339, p = 0.566	F = 0.188, p = 0.668
E*G*F	F = 0.34, p = 0.565	F = 0.031, p = 0.862	F = 1.152, p = 0.293	F = 0.041, p = 0.842	F = 0.149, p = 0.703	F = 0.189, p = 0.667
A*E*G*F	F = 2.375, p = 0.135	F = 2.813, p = 0.106	F = 0.419, p = 0.523	F = 1.689, p = 0.205	F = 2.818, p = 0.105	F = 2.768, p = 0.108

Table 2.2 -2. (Cont.) Acclimation (A), exposure (E), growth category (G) and family (F) effects on 4-way ANOVA table of the parameters involved in elemental balances of nutrients (IR_N and IR_C: ingestion rates for N and C, ER_N and ER_C: egestion rates of N and C, AR_N and AE_C: absorption rates for N and C, AE_N and AE_C: absorption efficiencies for N and C, R_C: C losses by respiration, E_N: N excreted, and SFG_N and SFG_C: elemental balances for N and C). Significant differences ($p<0.05$) are shown in bold

	AE_N	AE_C	R_C	E_N	SFG_N	SFG_C	NGE_N	NGE_C
A	F = 2.819, p = 0.105	**F = 29.48, p < 0.001**	F = 0.114, p = 0.738	F = 1.313, p = 0.262	**F = 5.305, p = 0.03**	**F = 7.027, p = 0.013**	F = 0.131, p = 0.72	F = 0.93, p = 0.343
E	**F = 422.92, p < 0.001**	**F = 686.95, p < 0.001**	**F = 5.385, p = 0.028**	**F = 69.54, p < 0.001**	**F = 80.96, p < 0.001**	**F = 26.77, p < 0.001**	**F = 9.81, p = 0.004**	F = 0.36, p = 0.554
G	**F = 5.44, p = 0.028**	**F = 11.017, p = 0.003**	F = 2.963, p = 0.097	**F = 10.294, p = 0.004**	**F = 26.25, p < 0.001**	**F = 16.86, p < 0.001**	**F = 32.399, p < 0.001**	F = 1.07, p = 0.311
F	**F = 10.34, p = 0.003**	**F = 5.636, p = 0.025**	F = 0.038, p = 0.846	F = 0, p = 0.984	F = 0.637, p = 0.432	F = 0.86, p = 0.362	F = 0.108, p = 0.746	F = 0.89, p = 0.354
A*E	**F = 14.84, p = 0.001**	F = 0.203, p = 0.656	**F = 5.751, p = 0.024**	**F = 8.387, p = 0.008**	**F = 14.82, p = 0.001**	**F = 9.969, p = 0.004**	**F = 29.558, p < 0.001**	**F = 6.68, p = 0.016**
A*G	F = 2.425, p = 0.131	**F = 4.509, p = 0.043**	F = 0.013, p = 0.91	F = 0.02, p = 0.888	F = 0.162, p = 0.69	F = 1.876, p = 0.182	F = 3.07, p = 0.092	F = 0.63, p = 0.434
E*G	**F = 9.569, p = 0.005**	**F = 14.286, p = 0.001**	F = 0, p = 0.989	**F = 7.509, p = 0.011**	**F = 8.031, p = 0.009**	F = 1.035, p = 0.318	F = 3.764, p = 0.063	F = 0.06, p = 0.814
A*F	F = 0.005, p = 0.946	F = 0.832, p = 0.37	F = 0.253, p = 0.619	F = 3.653, p = 0.067	F = 0.048, p = 0.828	F = 0.282, p = 0.6	F = 0.327, p = 0.573	F = 0.60, p = 0.446
E*F	**F = 13.15, p = 0.001**	**F = 8.746, p = 0.007**	F = 1.025, p = 0.321	F = 0.005, p = 0.942	F = 0.64, p = 0.431	F = 0.146, p = 0.706	F = 0.955, p = 0.337	F = 0.001, p = 0.98
G*F	F = 0.927, p = 0.345	**F = 7.022, p = 0.014**	F = 0.557, p = 0.462	F = 1.599, p = 0.217	F = 0.002, p = 0.968	F = 0.01, p = 0.92	F = 1.526, p = 0.228	F = 0.53, p = 0.472
A*E*G	**F = 4.76, p = 0.038**	**F = 7.834, p = 0.01**	F = 0.507, p = 0.483	F = 0.207, p = 0.653	F = 0.343, p = 0.563	F = 0.75, p = 0.394	**F = 5.983, p = 0.022**	F = 0.25, p = 0.624
A*E*F	**F = 4.94, p = 0.035**	F = 3.53, p = 0.072	**F = 5.023, p = 0.034**	F = 3.674, p = 0.066	F = 0.171, p = 0.682	F = 0.158, p = 0.694	F = 0.836, p = 0.369	F = 0.83, p = 0.371
A*G*F	F = 3.87, p = 0.06	**F = 12.416, p = 0.002**	F = 0.794, p = 0.381	F = 0.661, p = 0.424	F = 0.723, p = 0.403	F = 0.403, p = 0.531	F = 0.444, p = 0.511	F = 1.18, p = 0.288
E*G*F	F = 0.946, p = 0.34	F = 0.017, p = 0.898	F = 1.988, p = 0.17	F = 0.632, p = 0.434	F = 0.004, p = 0.953	F = 0.591, p = 0.449	F = 0.125, p = 0.727	F = 1.14, p = 0.295
A*E*G*F	F = 0.61, p = 0.441	F = 0.066, p = 0.8	F = 2.998, p = 0.095	F = 1.307, p = 0.263	F = 3.993, p = 0.056	F = 1.258, p = 0.272	**F = 4.963, p = 0.035**	F = 0.38, p = 0.544

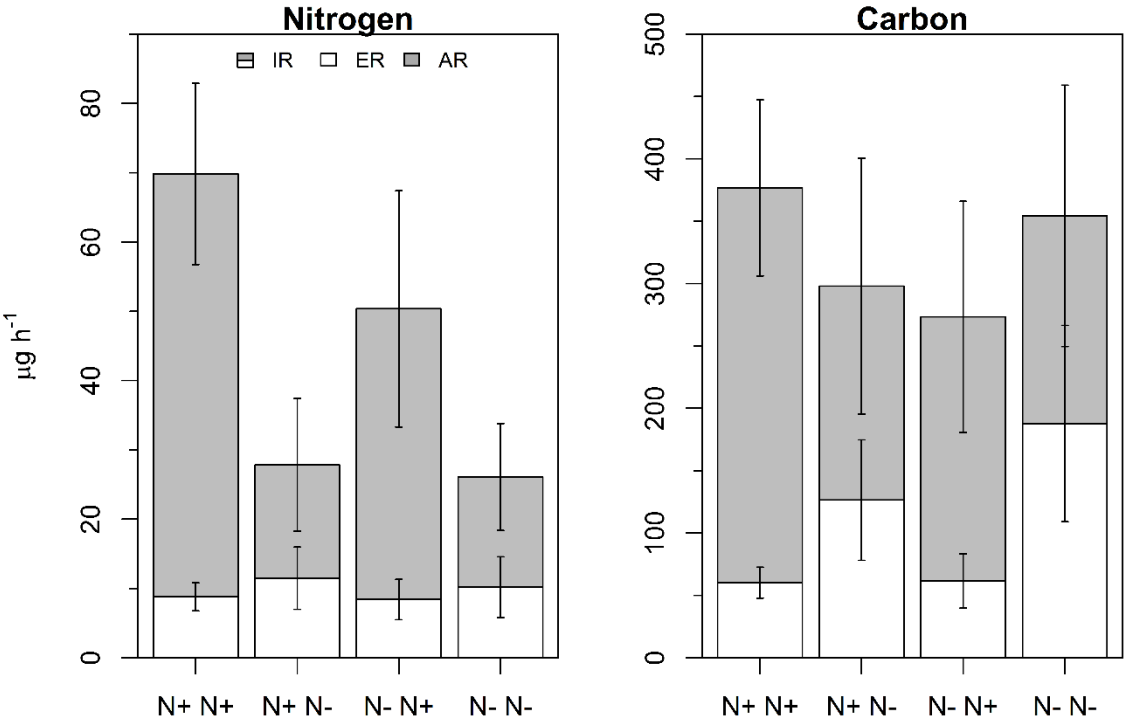

Figure 2.2 - 1. Acute and acclimated response to dietary N changes for ingestion (IR), egestion (ER) and absorption (AR) rates of N (left) and C (right)

Similarly, C acquisition showed the same differences concerning the effects of exposition diet on egestion and absorption rates, though ingestion rates did not show significant fluctuations due to exposure conditioning to different diets. Instead, a significant interaction term (*Table 2.2 - 2*) accounted for the fact that C ingestion reached the highest values when exposition and acclimation diets were coincident (*Figure 2.2 - 1*). Furthermore, acclimation also affected egestion as well as absorption rates in the same way as exposure; namely, both acute and chronic responses to N+ diets promoted decreases in egestion rates and increases in absorption rates.

Absorption efficiencies for both N and C were clearly superior ($p < 0.001$) when exposed to N+ diet; acclimation to this "high quality" diet also had a positive effect but only on AE_C. Differential performances for N and C in the different acclimation*exposition groups of clams were analyzed by plotting elemental AEs against the overall AE values for organics (Conover) obtained in Chapter 2.1 (*Figure 2.2 - 2*). While, as expected, AE_C fitted closely the Y = X line ($AE_C = 1.04\ AE - 0.003$, $R^2 = 0.997$, $p < 0.001$), AE_N clearly departed from this relationship, especially in the lower range of AE values ($AE_N = 0.88\ AE + 0.13$, $R^2 = 0.88$, $p < 0.001$). As a general rule, AE_N were above the AE for overall organics (equivalent to AE_C), but this discrepancy tended to be

higher in clams submitted to chronic N deficit (i.e., acclimates to N- diets). In fact, ANOVA analysis of the difference between AE_N and AE using acclimation and exposure diet as factors resulted in significant effects for the acute response ($F = 9.6$, $p = 0.005$) and highly significant effects for both the acclimated ($F = 114.2$, $p < 0.001$) response and the interaction term: $F = 43.7$, $p < 0.001$).

Table 2.2 - 3. Pooled mean values (SE) for acclimation, exposure, growth category and family for IR_N and IR_C (ingestion rates for N and C), ER_N and ER_C (egestion rates of N and C), AR_N and AR_C (absorption rates for N and C), AE_N and AE_C (absorption efficiencies for N and C), R_C (C losses by respiration), E_N (N excreted), and SFG_N and SFG_C (elemental balances for N and C). Values are given in terms of $\mu g\ h^{-1}$, except for AE_N and AE_C (decimal units)

	Acclimation		**Exposure**		**Growth category**		**Family**	
	N+	**N-**	**N+**	**N-**	**F**	**S**	**1**	**8**
IR_N	44.64 (5.3)	38.22 (3.82)	58.55 (4.14)	27.01 (1.79)	49.73 (4.29)	30.01 (3.47)	42.61 (4.5)	39.94 (4.7)
IR_C	329.48 (21.78)	313.78 (22.38)	316.88 (22.31)	324.86 (21.93)	377.29 (17.27)	246.53 (15.53)	337.13 (19.37)	305.38 (24.21)
ER_N	10.42 (0.87)	9.3 (0.8)	8.57 (0.58)	10.87 (0.91)	11.67 (0.77)	7.38 (0.49)	10.82 (0.82)	8.84 (0.8)
ER_C	99.96 (11.21)	124.71 (18.26)	61.07 (4.12)	155.77 (14.68)	132.67 (16.65)	86.6 (10.45)	123.88 (15.46)	101.97 (15.64)
AR_N	34.22 (5.33)	28.92 (3.64)	49.98 (3.76)	16.14 (0.98)	38.06 (4.52)	22.63 (3.38)	31.79 (4.55)	31.1 (4.5)
AR_C	229.51 (20.57)	189.07 (13.02)	255.81 (19.67)	169.1 (9.5)	244.62 (15.82)	159.94 (12.04)	213.25 (16.74)	203.41 (18.1)
AE_N	0.7 (0.03)	0.72 (0.03)	0.85 (0.01)	0.61 (0.01)	0.71 (0.03)	0.71 (0.03)	0.69 (0.03)	0.74 (0.03)
AE_C	0.68 (0.03)	0.63 (0.03)	0.8 (0.01)	0.53 (0.02)	0.65 (0.03)	0.65 (0.03)	0.64 (0.03)	0.67 (0.03)
R_C	35.81 (2.38)	37.17 (3.86)	41.7 (3.41)	32.24 (2.87)	39.93 (3.42)	31.98 (2.53)	35.93 (4.26)	37.12 (1.83)
E_N	5.97 (1.13)	7.2 (1.56)	11.57 (1.43)	2.53 (0.37)	5.56 (1.06)	8.02 (1.76)	6.4 (1.43)	6.83 (1.34)
SFG_N	28.25 (5.01)	21.72 (3.01)	38.41 (4.56)	13.61 (1.11)	32.5 (4.05)	14.6 (2.41)	25.39 (4.16)	24.27 (4.04)
SFG_C	193.7 (20.44)	151.9 (12.12)	214.11 (19.8)	136.86 (9.81)	204.69 (15.83)	127.96 (12.25)	177.32 (16.37)	166.29 (17.66)
NGE_N	0.77 (0.17)	0.78 (0.21)	0.71 (0.20)	0.82 (0.16)	0.85 (0.12)	0.67 (0.21)	0.78 (0.20)	0.76 (0.18)
NGE_C	0.82 (0.09)	0.79 (0.11)	0.81 (0.10)	0.80 (0.10)	0.82 (0.11)	0.78 (0.09)	0.82 (0.10)	0.79 (0.10)

N excretion was highly affected by diet composition, where clams exposed to N+ excreted ca. 5 times more ammonia-N than fed the N poor diet (*Figure 2.2 - 3*). This acute response varied depending on the acclimation diet, since clams acclimated to N+ transferred to N- diet reduced their rates of excretion but to a lesser extent than animals fully acclimated to N-. Conversely, when juveniles conditioned to N- diet were transferred to N+ diet, values increased up to 50% more than N+ acclimated clams. Carbon loss due to metabolic processes reflected less fluctuations between diets, where clams exposed to N+ reached approx. 20% increment of respired C.

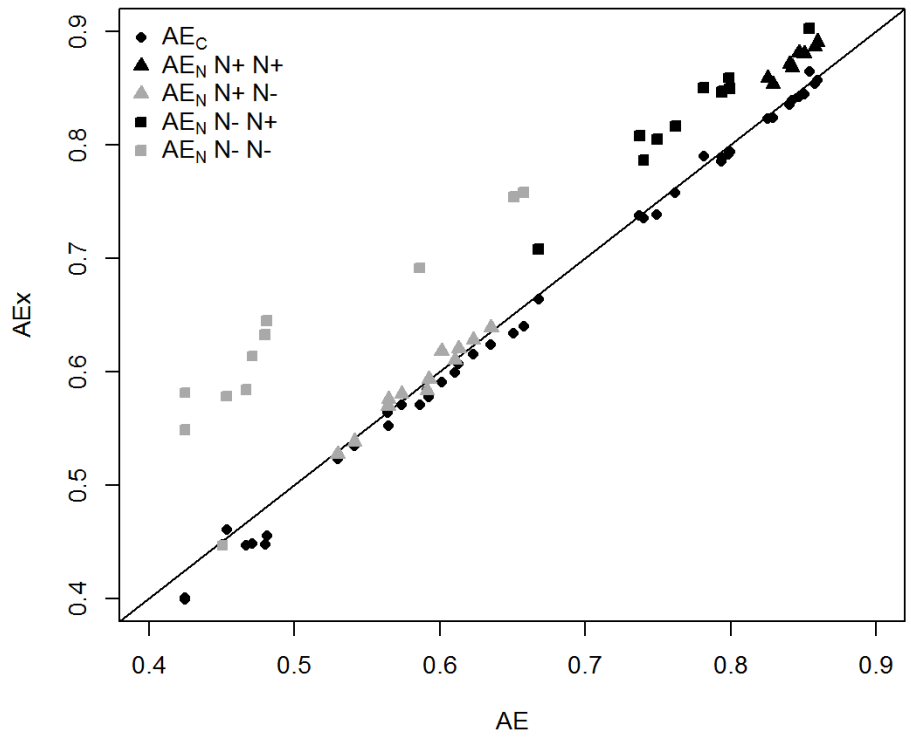

Figure 2.2 - 2. Relationship between AE_N and AE_C and Conover AE, where circles represent the AE_C relationship, triangles is AE_N from clams acclimated to N+ diet, and squares represent the same correlation for those acclimated to N- diet. In addition, for AE_N fits, black symbols represent animals exposed to N+, while grey symbols are from those exposed to N-

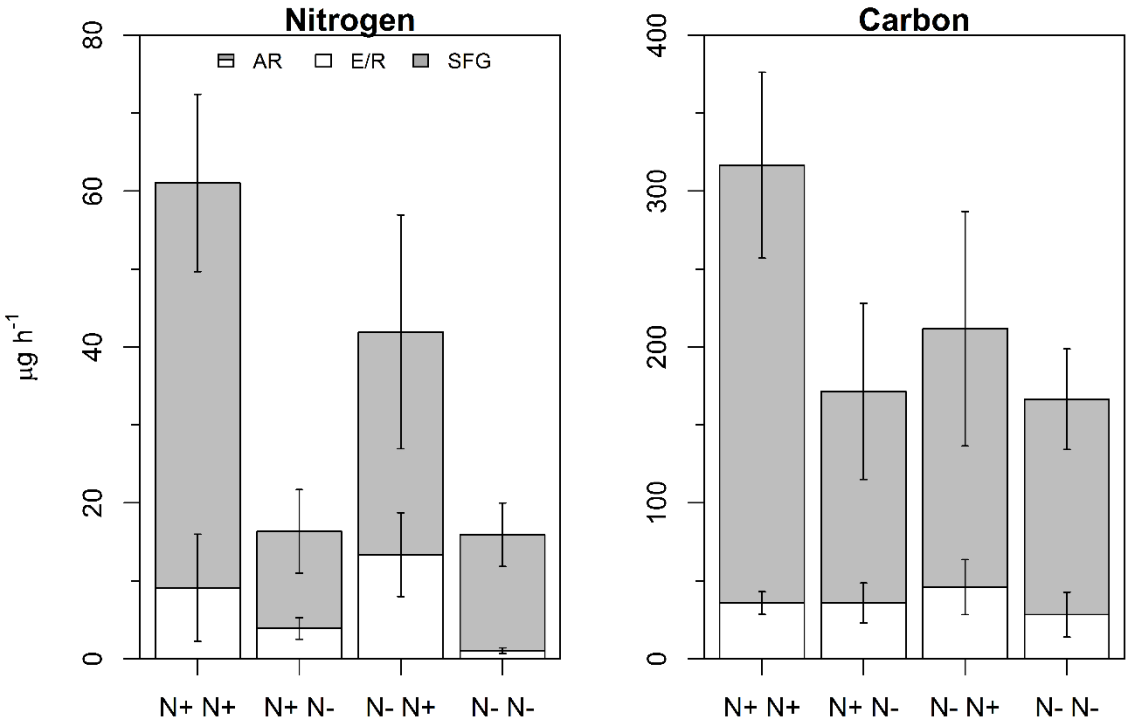

Figure 2.2 - 3. Acute and acclimated response to dietary N changes for absorption rates (AR), N excretion or respiration (left: E; right: R) and elemental balances (SFG) rates of N (left) and C (right)

As a result of the previous differences, elemental balances for N and C showed significant differences (*Table 2.2 - 2*) for acclimation, exposure and their interaction. As before, acute exposure to diets with different N content affected to a greater extent the balance of both C and N, and the best performance was accomplished in clams fed the N+ diet (*Figure 2.2 - 3* and *Table 2.2 - 3*). Conditioning diet was also important but its effects on elemental balances was only noticeable in animals fed the N+ diet, since feeding the N poor diet tended to reduce the C and N balances to the point of canceling out the potential benefits of high quality (N+) conditioning.

Net growth efficiency for C (NGE_C) was fairly constant across diets while NGR_N increased significantly in clams fed the N- diet (*Table 2.2 - 2* and *Table 2.2 - 3*). Regarding both elemental growth efficiencies, interaction terms for acclimation and exposure diets (*Table 2.2 - 2*) were significant, accounting for the fact that the highest efficiencies were achieved in the experimental groups where acclimation and exposure diets were coincident (N+N+ and N-N-).

Physiological parameters for each combination of acclimation*exposition diets were determined with a large standard deviation due to the fact that values were pooled means of different phenotypes represented by families and intrafamily segregated growth groups. Comparisons corresponding to these endogenous sources of variation will be considered next.

3.2 Growth genotype effects

Generally, significant effects involved in the parameters concerning elemental balances were mainly due to growth category (*Table 2.2 - 2*), although differences between families were also detected. Fast growing juveniles registered higher values for both ingestion and egestion rates of both N and C (*Figure 2.2 - 4*). The resulting digestive balances represented by absorption rates resulted also significantly higher. Indeed, increments of absorption in F compared to S clams ranged from 35 to 40% for N and C, respectively. Similar tendencies between families were not significant (except for N egestion rate), although results pointed out to a better performance of Family 1 compared to Family 8, whose mean absorption increments amounted only to 4% in terms of N and 8% for C. Consequently, the combination of inter- and intra-familiar effects led to a gradual decrease in processing rates from F1 to S8 groups (*Figure 2.2 - 4*).

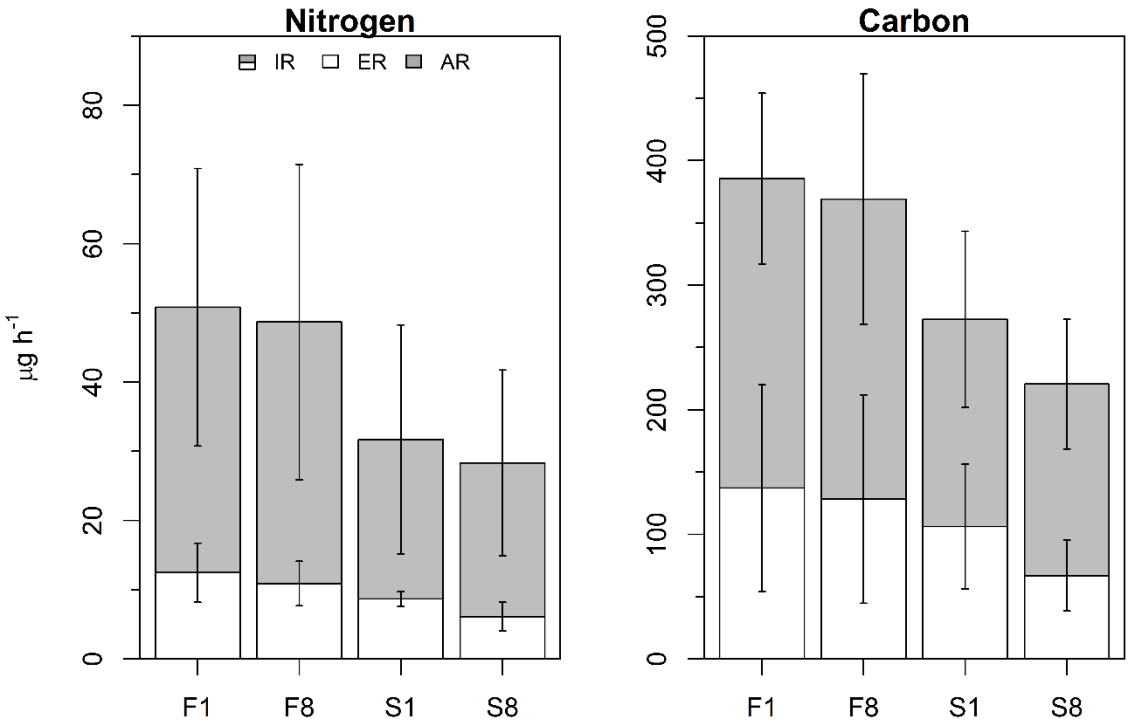

Figure 2.2 - 4. Inter-individual effects for elemental acquisition parameters (ingestion (IR), egestion (ER) and absorption (AR) rates) of N (left) and C (right)

Beside diet effects, N and C absorption efficiencies differed also between phenotypes at both inter- and intra-familiar levels (*Figure 2.2 - 5*). Both endogenous factors (Family and Growth category) exerted weak although significant effects on AEN and AEC (*Table 2.2 - 2*) and, with few exceptions, these efficiencies were higher for F8 specimens compared with those from F1 (< 10% difference between families). Diet exerted differential effects on efficiencies deployed by growth groups with the result that, together identical pooled values for F and S clams were achieved (*Table 2.2 - 3*).

Endogenous effects over C respiratory losses were negligible (as for dietary effects), with only a slight trend for a higher rate in F clams (*Table 2.2 - 3*). Instead, N excretion of F juveniles (5.56 µg h^{-1}) was significantly exceeded by S juveniles (8.02 µg h^{-1}) (*Table 2.2 - 3*), which, as shown in *Figure 2.2 - 6*, is mainly accounted for by strong phenotype (F *vs.* S) differences recorded with the N+ diet (low C:N index).

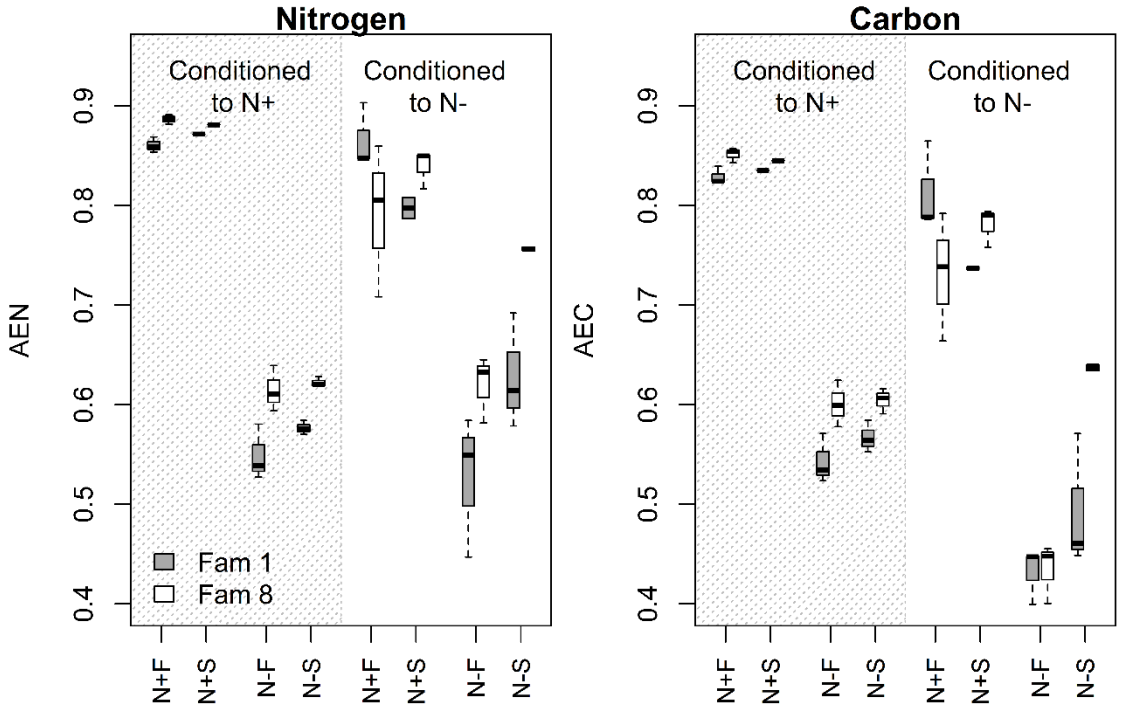

Figure 2.2 - 5. AE_N (left) and AE_C (right) of every combination of factors: acclimation (N+: dark region, N-: light region), exposure (see x axis), growth (see x axis) and family (Fam 1: dark boxes, Fam 8: white boxes)

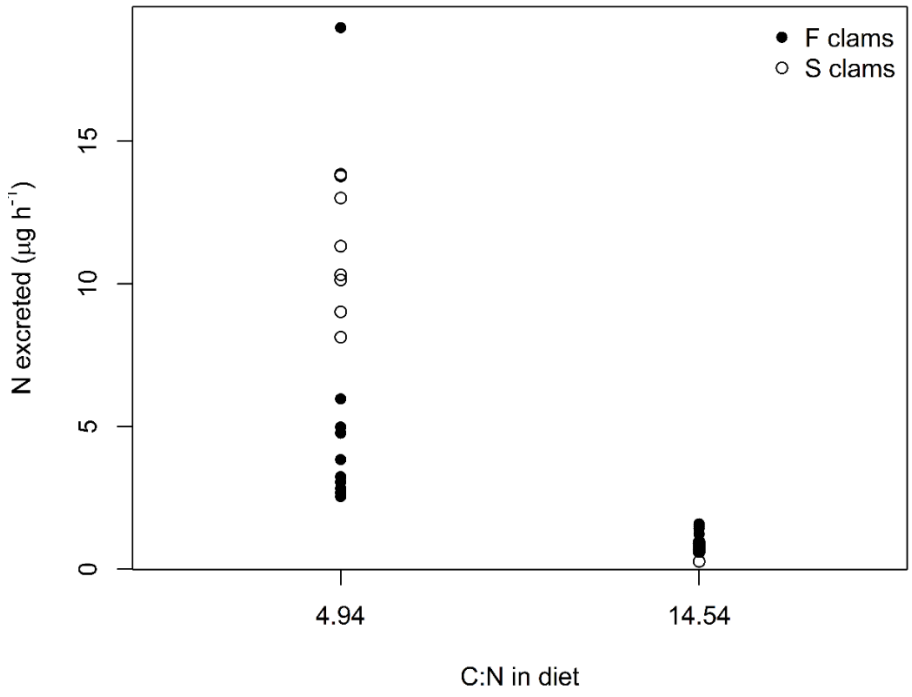

Figure 2.2 - 6. Ammonia-N excretion of fast (solid circles) and slow (open circles) growing juveniles in relation to the diet dosed

Phenotype effects on elemental balances for C and N were restricted to growth category, whereas family effects were no significant (*Table 2.2 - 2*). F vs S differences

were highly significant and resulted in elemental balances of N that were ca. 3 times higher for F than for S clams, compared with two-fold differences for C balances (*Table 2.2 - 3*). Net growth efficiencies were also higher for F compared to S clams (*Table 2.2 - 3*), but only differences in NGE_N attained the significance level (*Table 2.2 - 2*).

3.3 Acute / chronic dynamics of N and C balances and net growth efficiencies

Considering the 4 factors (acclimation, exposure, growth category and family), N and C balances were represented in *Figure 2.2 - 7* in order to account for the inter- and intra-familiar differential performances to acute and chronic dietary changes. As both (N and C) balances were subjected to the same fluctuations, yet each differed in the intensity of these changes, plots of *Figure 2.2 - 7* will be described together. When clams acclimated to N+ diet (N+N+ groups) were transferred to N- (N+N- groups), a sharp decrease of the net balances for both N and C was observed in the four phenotypes resulting from growth group*family combinations (F1, F8, S1 and S8), involving that only about one third of N and half of C was being retained with the "low quality" diet. Full acclimation to the N- diet (N-N- groups) barely affected the fast growers performance, while S clams nearly doubled their values. The acute response of clams acclimated to N- to a N enrichment (N-N+ groups) also involved differential responses in fast and slow growers: while F clams partly recovered the initial retention of C and N, S juveniles maintained or even lowered their levels, as occurred in Family 1 S specimens. These differential effects were accounted for by the diet exposure*growth phenotype interaction term (*Table 2.2 - 2*), that was only significant for SFG_N Finally, acclimation to N+ diet promoted increases in net balance values in all growth group*family phenotypes.

A similar acute / chronic dynamics was also observed for net growth efficiencies (*Figure 2.2 - 8*) with differential effects accounted for 3rd and 4th order interaction terms (*Table 2.2 - 2*), only significant for nitrogen.

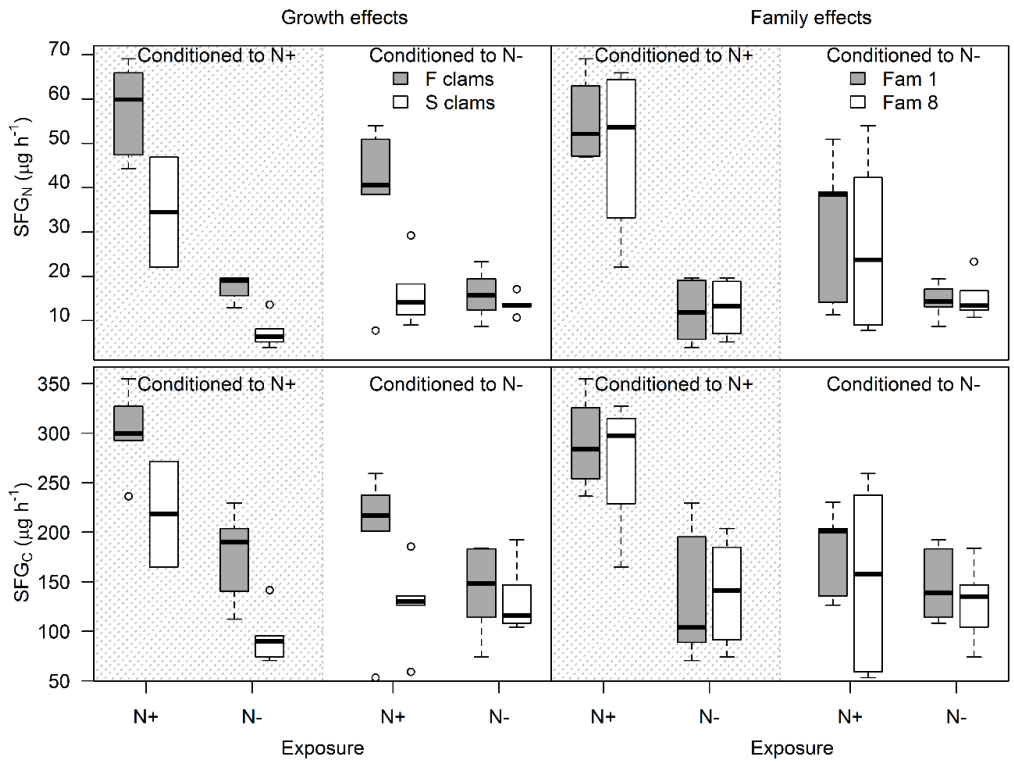

Figure 2.2 - 7. Acute and acclimated response in SFG_N (up) and SFG_C (down) for interindividual (left) and interfamiliar (right) effects, where gray boxes represent, respectively, either F or Family 1 clams; and white boxes stand for either S or Family 8 clams

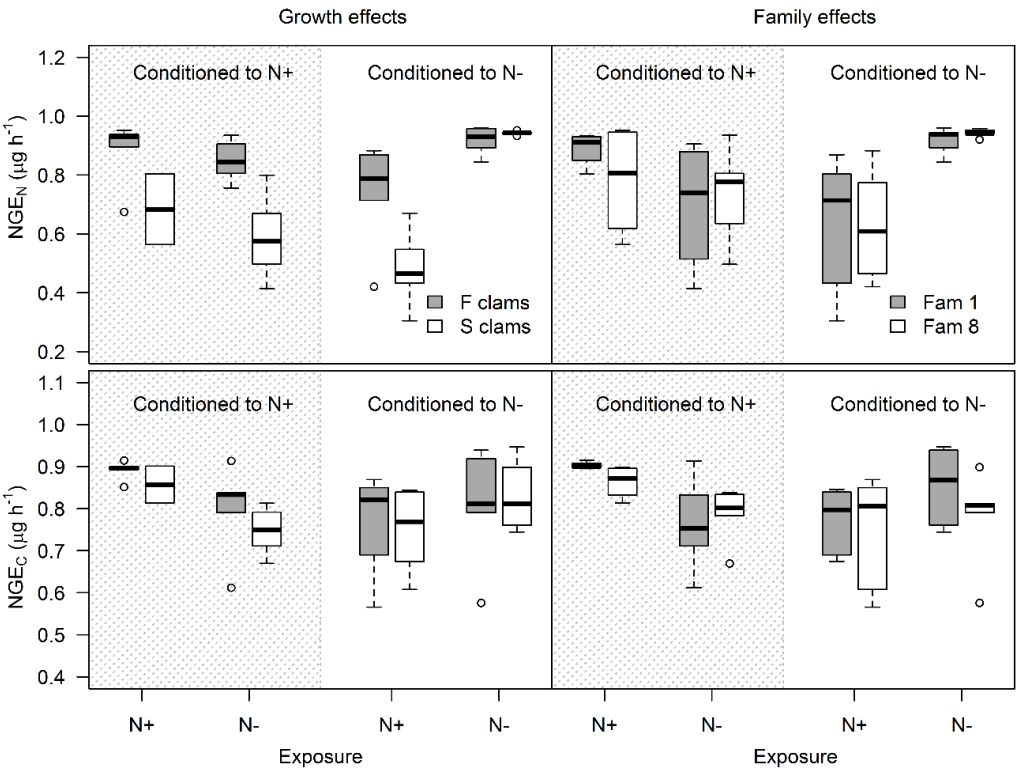

Figure 2.2 - 8. Acute and acclimated response in NGE_N (up) and NGE_C (down) for interindividual (left) and interfamiliar (right) effects, where gray boxes represent, respectively, either F or Family 1 clams; and white boxes stand for either S or Family 8 clams

4. DISCUSSION

One important issue from the stoichiometry approach concerning heterotrophic organisms is that trophic relationships might be "misled" if only energy balances are considered. In the physiological description of growth, consideration of specific nutrient balances becomes essential to account for features from feeding, digestion and metabolic behavior that appear associated to the necessary coupling between the available food and the growing tissues (Bayne, 2017). The computation of C and N balances provide for a first approach in the road to a more detailed nutritional characterization of these interactions of the organism with the food environment.

In the present study, these balances were addressed in a double experimental context: 1) The analysis of the effects of acute and chronic changes between two different diets (N+ and N-) that were isocaloric but maintained an ~ 3-fold range of variation in the C/N ratio. 2) The use of different phenotypes to provide for a broad range of growth variability of endogenous origin.

4.1 DIET EFFECTS

In this section, results of nutrient (N and C) balances reported with clams maintained with N+ and N- diets will be discussed with reference to potential mechanisms of nutrient homeostatic regulation providing a mean for matching the composition of the assimilated ration and those of the clams tissues. Since C:N ratios of food in the N- diet (14.54) largely depart from the corresponding values for body composition that closely resemble instead the composition of food in the N+ diet (C:N ratio = 4.94), an useful approach will be to analyze physiological responses in the shift from N+ to N- diets as potential mechanisms to offset dietary N limitations. As described in the Introduction section, the preferential ingestion of N based on sorting between different types of particles during pseudofeces production has proven to be an extremely effective mechanism of compensation for nutritional deficits (Bayne and Svensson, 2006), but no choice for such selection was available in the present experiments where food particles were homogeneous and feeding conditions prevented pseudofeces. In spite of that, the possibility of preingestive selection playing an important role in the homeostatic nutrient regulation in clams fed more heterogeneous diets (e.g., seston) cannot be discarded as it has been reported in different species of bivalves including oysters, mussels and cockles (Hawkins et al., 1996; Iglesias et al., 1996; Soletchnik et al., 1996; Urrutia et al., 1996).

As proposed by Sterner and Elser (2002), another level in the stoichiometric coupling between the animal and the food environment might consist in adjustments of the assimilation patterns where the ingestion/digestion/assimilation rates for a given element in default might be up-regulated. Bayne (2009) considered the possibility of an increase of ingestion rates to compensate for the low nutrient availability as a stoichiometric mechanism to maintain elemental homeostasis, without the need of selective feeding, and in fact reported results of experiments, very much alike the present, where mussels (*M. edulis*) fed phytoplankton cultures of a low N content increased the feeding rates (measured as clearance rates) compared with mussels fed phytoplankton of high N content (Bayne, 2017; see his Figure 5.38). However, no such resort to an increment of the feeding rates was observed in the present work, which resulted in ingestion rates of N being reduced by a half on average when clams were exposed to the N poor diet (see *Table 2.2 - 3*). In a previous analysis, we have reported digestive constraints in the processing of N- diets, resulting in poor AE (for overall organics), further hindered by a strong negative dependence between AE and the ingestion rate that does not occur with the N+ diets (Arranz et al., 2020; Chapter 2.1). Although we presently lack an explanation for the comparatively poor digestive performance of clams fed *R. lens* in the stationary phase (the organic component of N- diets) it becomes clear these constraints would tend to preclude the efficacy of any strategy based on "overeating" to compensate for reduced dietary N. Conversely, the higher digestibility of N+ diets can be inferred from significantly increased values of the absorption efficiency for both C and N exhibited by clams exposed to these diets of high N content (*Table 2.2 - 2* and *Table 2.2 - 3*).

However, the differential increase of AE_N over AE and AE_C when fed on N poor diet (*Figure 2.2 - 2*) was the relevant mechanism employed to compensate for the nutrient imbalance, which agrees with the previous literature described in the Introduction section (Cranford, 1995; Cranford and Grant, 1990; Grant and Cranford, 1991; Hawkins, 1985; Hawkins and Bayne, 1985; Kreeger et al., 1996; Smaal and Vonck, 1997). Quite in a same line, (Ibarrola et al., 2000a) reported that cockles (*Cerastoderma edule*) fed cultures of *Tetraselmis suecica* in the stationary phase (low N content) improved the absorption efficiency of proteins relative to carbohydrates and lipids compared with the digestive behavior in cockles fed these cultures in the exponential phase (high N content). While the perfect fit between AE_C and overall AE for organics (Conover) suggests a lack of

limitations concerning this nutrient across the different combinations of exposure*acclimation diets, nearly consistent departures of AE_N from the general relationship strongly points to a stoichiometric compensation. On the other hand, significant differential effects on AE_N are associated to acclimation rather that exposure to low N diets. For instance, clams acclimated to N- showed a clear discrepancy of AE_N with respect to overall AE that are not seen in those acclimated to N+ even when exposed to the N- diet. So, whatever the digestive mechanism involved in the preferential absorption of N it becomes clear that time is required to elicit a functional response, very likely for a noticeable N deficit to be developed. Also, acclimation time might be necessary provided that digestive adaptation proceeds through digestive enzyme induction (Ibarrola et al., 1999, 1998).

Regulation of nutrient balances can be lastly achieved in the postabsorptive phase by adjusting the proportion of C and N released from the metabolic activities according to differential income in the diet. This is seemingly achieved through a shift in the composition of the pool of substrates fueling metabolic energy production. High levels of N consumption would enhance protein degradation and consequently high amounts of N are excreted as a consequence of deamination reactions resulting from protein utilization for energy purposes; whereas, low N availability may trigger biochemical mechanisms preserving this nutrient for protein synthesis that might however be energetically expensive (e.g., high rates of protein recycling have been related to a decrease in protein absorption in mussels; Hawkins, 1985). According to the present results (see *Table 2.2 - 3*), N+ diet promoted a general increase in metabolic activities, both ammonia excretion and respired C, but the important point is that C:N ratio for metabolism changes from < 4 (fully compatible with the metabolic break down of proteins) in clams feeding on the N+ diets to 14 in those feeding the N- diets that suggests a majority use of other energy substrates.

Taking into account the observed impacts of diet quality on the parameters of the energy balance, a reasonable conclusion is that the acquisition processes contributed to a larger extent to differences in the elemental balances between N+ and N- diets Elemental balances for both C and N were increased under the low C:N conditions, despite the slight decrease of C availability under that diet. This behavior coincides with the elemental balances calculated from the data showed in Bayne (2009), where both SFG_C and SFG_N decreased proportionally as dietary C:N ratio increased. However, metabolic responses

to the diet, particularly the change in the ratio of C respired to N excreted (the metabolic C:N ratios) suggest a very relevant role of postabsorptive processes as regards to the homeostatic control of nutrient balances. Differences in the metabolic fate of nutrients dealing with this regulation are clearly shown for N, where net growth efficiency (NGE_N) was significantly increased under N- diets compared with N+ diets (*Table 2.2 - 2*), revealing the occurrence of N saving mechanisms at the metabolic level under conditions of reduced availability of this element.

4.2 Growth phenotype effects

Endogenous variability on the elemental physiology was primarily associated to intrafamiliar rather than at the interfamiliar differences. Namely, all physiological parameters dealing with elemental (C and N) acquisition (Ingestion, Egestion and Absorption) were significantly increased in F compared with S clams (by 53% and 68% for C and N, respectively, in terms of absorption). Whereas, acquisition was also higher in Family 1 with respect to Family 8 (2% and 5% for C and N) but these differences were not significant.

The above differences recorded in the elemental balances between growth groups are endorsed by the previous literature on energy balances (Arranz et al., 2020; and Chapter 2.1; Bayne, 1999; Bayne et al., 1999b, 1999a; Fernández-Reiriz et al., 2016; Ibarrola et al., 2017; Pace et al., 2006; Prieto et al., 2018; Tamayo et al., 2016, 2015, 2013, 2011; Toro et al., 1996; Toro and Vergara, 1998), where faster growth was correlated with higher acquisition ability. This seems to be a constitutive trait since it has been recorded for different species and under the variable dietary conditions provided in those studies; as occurs in the present findings where F vs S differences are supported in differences in acquisition of both energy (Arranz et al., 2020; Chapter 2.1) and nutrients irrespective of dietary C:N ratios.

Coupled with a reduced nutrient acquisition, S clams were also found to release more nitrogen in form of excretion. This N output reached a maximum when clams were fed on the N rich diet (*Figure 2.2 - 2*), in which S clams nearly exceeded by 80% the excretion of F clams. On average, C:N ratios for metabolism ranged between 7.2 for F and 4.0 for S clams (*Table 2.2 - 3*). Part of this excess of N excretion could be associated to the *stoichiometrically regulated excretion* (Anderson et al., 2005), which would be the fraction of N released to maintain mass balances and consumer homeostasis. These

stoichiometric releases would explain partially the differential performances under the different feeding scenarios; however, the endogenous fraction of this variability could correspond to differences in what Anderson et al. (2005) named *standard release,* which includes the processes of protein synthesis and breakdown. Protein turnover rates are known to be tightly related to genotype (see review by Hawkins, 1991), and associated costs can entail a significant proportion of the whole animal's metabolic costs. Precisely, some of the works of Hawkins and colleagues (Hawkins and Day, 1996; Hawkins et al., 1986, 1989a, 1989b) demonstrated that bivalve faster growth was correlated not only with faster feeding rates, but also with reduced maintenance costs due to a more efficient protein metabolism. In fact, a reduced protein turnover was presented as a basis for faster feeding and growth instead of being a consequence of the higher rates of growth. Indeed, as a result of these findings, Hawkins (1991) stated that *"genotype-dependent differences between rates of whole-body protein turnover may act as indicators of individual fitness".* Therefore, the extraordinary increase in N excretion of S clams fed on the N-rich diet could be due to a subsequent combination of higher protein turnover costs and a need of removal of the surplus of N acquired in the rich diet, which, in turn, reduced the scope for nutrient (N) and energy acquisition. Additionally, Meyer and Manahan (2010) hypothesized that metabolic inefficiency in slow growing bivalves could be partially explained by a non-uniform expression of ribosomal proteins, that might result in degradation of those proteins. As a summary expression of this reduced metabolic efficiency in slow growers as regards the nutrient balances, the net growth efficiency computed for N (NGE_N) was found to significantly decline in S (0.67) compared with F (0.85), while no differences were found between growth groups for NGE_C (*Table 2.2 - 2*). Consistently with present findings, higher conversion efficiencies for protein appear on the basis of faster growth in selected oysters (*Saccostrea commercialis*) relative to controls (Bayne, 2000).

4.3 Acute vs. chronic dynamics in N and C balances

Considering collectively the four factors (*Figure 2.2 - 6*), for each phenotype (F1, F8, S1 and S8), acute effects were different depending on the diet, showing a higher impact the change from N+ to N- than the opposite change. In addition, S clams were more sensitive and decreased their balances to a larger extent. Similarly, F clams rapidly turned a profit on the switch from N- to N+, while the others remained virtually unaltered. Both acute responses suggest a higher plasticity for fast growers, that were able to

incorporate higher nutrients at any conditions. Despite the lack of significance between families, Family 1 tended to possess higher net incorporation rates, especially for acclimated conditions and particularly in the case of S clams. The conditioning period, however, was only relevant for the low C:N conditions, where SFG_N and SFG_C responses were maximized for the combination between exposure and growth under conditioning to N+ diet.

In conclusion, the high N content diet did not only increase N balances, but also C balances, in which acquisition of energy (AR), but especially ingestion rates, were responsible for the improvements on elemental performance. AE_N was enhanced respect to organic or carbon AE, especially under chronic restricted conditions, irrespectively of the phenotype, suggesting a stoichiometric regulation in terms of alteration of absorption patterns. Endogenous differences were driven predominantly by intrafamily responses rather than by interfamiliar effects. The interaction between diet and growth type revealed a weaker performance in S clams due to a metabolic inefficiency in protein turnover, together with an increase in the N excreted to maintain elemental homeostasis. Finally, the faster response to external changes by F clams suggests a higher plasticity over S clams.

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~ CHAPTER 2.3 ~

Differential homeostatic response of body tissues to nutrient limitations among growth phenotypes of juvenile clams *Ruditapes philippinarum*

ABSTRACT

Fast and slow growing phenotypes from two separate breeding families of the Manila clam (*Ruditapes philippinarum*) were acclimated to two monoalgal diets that were isocaloric but differed in N content, ranging from full to restricted N availability (diets N+ and N-, respectively). After 35 days of food conditioning, clams were sacrificed and the soft body dissected out into different organs (gills, adductor muscle, gonad, digestive gland and remaining tissues) for the determination of the corresponding ponderal ratios (organ wt./body wt.) as well as the separate analysis of elemental (C, N, H) composition of these tissues. Previous C and N balances were integrated and compared with tissue composition of the same phenotypes in order to assess the efficacy of mechanisms brought about to compensate for N deficit as regards the homeostatic regulation of elemental nutrients during the growth process. Main differences in condition index and body organ composition among phenotypes were driven by strong family effects, while conditioning diet effects contributed to a lesser extent. All phenotypes displayed the highest growth rates under N+ diet whose C:N ratio values closely approached the threshold element ratios; whereas, C:N ratios of the N- diet clearly exceeded the ideal element ratio, suggesting a strong N limitation. Most organs showed a high degree of homeostatic regulation, with the exception of the digestive gland whose C:N ratios reflected differences in food composition, probably as a result of the higher turn-over rate of this tissue. Whole soft tissue elemental composition was independent on conditioning diet, with C:N ratios exhibiting a noticeable constancy, especially in fast growing phenotypes that were previously found to exhibit a greater capacity for faster adaption to variable nutritional scenarios.

Key words: threshold element ratios, tissues homeostasis, biometry, nutrient imbalance, *Ruditapes philippinarum*, growth phenotypes

1. INTRODUCTION

Aquaculture efforts to improve growth have been regularly focused on supplying a balanced diet that includes all the nutrients that could be otherwise limiting the optimal growth. Limits to growth can be set at different levels; that is, an animal can face scenarios where the available energy in the form of food may be insufficient. In other circumstances, even when food is enough to cover the animal's energy demands, biochemical components (frequently addressed as protein being the limiting biomolecule) or even elements (i.e. C, N or P) may be present into the composition of diet, but at inadequate levels to sustain an optimal growth, thus being limiting to the animal. That limitation is believed to take place frequently in both terrestrial and aquatic systems, being more specifically related to herbivores and detritivores (Elser et al., 2000).

In this regard, the concept of *threshold element ratios* (TER) stands for an approximation that computes, for a given elemental composition of food, the threshold at which the ratio between carbon and other element, typically nitrogen or phosphorus, would be not only enough, but also optimal for growth to be maximized. Any value above the theoretical threshold would indicate a nutrient limitation, and reciprocally, below that critical level, carbon would be limiting growth (Anderson and Hessen, 1995).A number of authors have developed different approaches to estimate TERs (Anderson et al., 2005; Anderson and Hessen, 1995; Doi et al., 2010; Frost et al., 2006; Sterner, 1997; Sterner and Elser, 2002; Urabe and Watanabe, 1992), which differ in some terms of the equations, such as the use of gross or net growth efficiencies (Sterner and Elser, 2002; Urabe and Watanabe, 1992), or the distinction between absorption efficiencies for nitrogenous and non nitrogenous compounds (Anderson and Hessen, 1995). Nevertheless, all of the above works are based on the same theory, which account for the physiological responses to a given diet and tissues elemental composition. These concepts rely on the ecological stoichiometry principles, which compute the balances and fluxes of elements between organisms, that were developed in depth by Sterner and Elser (2002).

Another concept of interest related to stoichiometry addresses the elemental homeostasis, which is defined by Kooijman (1995) as *"the ability of most organisms to keep the chemical composition of their body constant despite changes in the chemical composition of the environment including their food"*. Therefore, a lack of homeostatic regulation would result in organisms following the "you are what you eat" model (Sterner and Elser, 2002), while those animals possessing ability to regulate that process would

follow some strategies to maintain an elemental body steadiness. According to Sterner and Elser (2002, but see also the Discussion section in Chapter 2.2), animals that regulate their elemental stoichiometry can achieve their purpose by three different mechanisms: *1)* by means of preingestive selection (i.e. preferentially choosing food items that suit best their needs), *2)* through changes in their assimilation patterns, in which an element would be up- or down-regulated, and *3)* by metabolic processes, such as active N excretion when this element is in excess.

Research on bivalve mollusks growth and energetic physiology has been primarily focused on net energy gain, as well as energy fluxes mainly in response to environmental changes (see Bayne and Newell, 1983, but also the Introduction section in Chapter 2.2). However, some studies quantified the impact of different C:N ratio diets on growth and C:N composition of bivalves. For instance, the scallop *Placopecten magellanicus*, fed on either aged kelp or phytoplankton (*Chaetoceros gracilis*) did maintain its tissue C:N, despite the 3.5 fold difference in C:N between diets (Grant and Cranford, 1991). The Manila clam *Ruditapes philippinarum* showed the same output (Gallager and Mann, 1981), where the same microalga species (*Thalassiosira pseudonana*) was dosed at different C:N ratios (range ≈ 6-12). Likewise, the Pacific oyster (*Crassostrea gigas*) showed a similar pattern, in which diet C:N varied seasonally (range ≈ 4.8-12), where, as a result, no statistically significant differences were found in their tissue C:N (Bayne, 2009). As suggested by Bayne (2009), elemental homeostasis is maintained through physiological compensations to handle the nutritional imbalances and growth demands. However, the scallop *Argopecten purpuratus* displayed two different responses to varying C:N ratios in the diet, depending on the developmental stage: while postlarvae growth was improved under a high N content diet, spat increased protein content under identical feeding conditions (Uriarte and Farías, 1999).

To our knowledge, studies analyzing elemental composition on bivalves lack separate soft tissues determinations, whose composition largely differs among them. For instance, tissues composition of *R. philippinarum* have been analyzed in terms of biochemical composition of whole soft tissues (Baek et al., 2014; Beninger and Lucas, 1984; Marin et al., 2003; Robert et al., 1993), frequently associated to seasonal cycles and not specifically in response to differences in nutritional conditions; and in two occasions (Arranz et al., 2021; Chapter 3; Shiraishi et al., 1995), tissues were separated for the analysis of either seasonal biochemical composition (Shiraishi et al., 1995) or a

comparative between elemental and biochemical analysis (Arranz et al., 2021; Chapter 3). Given the well differentiated functions that each organ meets (Gosling, 2015) and, as a consequence, the differential tissue regeneration times, a differential performance of each tissue could be expected in terms of tissue homeostasis.

Other key point that deserves special attention is the large interindividual variability on growth that exists in bivalves (Goff, 2011). As previously presented for the Manila clam, faster growth has been associated to a higher acquisition ability and coupled with a higher metabolic efficiency, leading to higher net energy balances (Arranz et al., 2020; Tamayo et al., 2015, 2013, 2011; and Chapter 4.1). Faster growth was related as well to a higher elemental (N and C) absorption leading to increased net N and C balances (Chapter 2.2). Actually, the preceding contributions (Arranz et al., 2020; Chapter 2.1 and Chapter 2.2) in which fast and slow growing juveniles belonging to two selectively produced lines were analyzed under two nutritional scenarios, consisting of a high N content diet and a N restricted diet. Both works showed that under the nitrogen poor diet, all phenotypes reduced both energy and elemental balances, suggesting a strong growth limitation due to N scarcity. In addition, both inter- and intra-family differences arose, in some parameters even to a larger extent than diet effects, which led us to hypothesize that interindividual differences could be present also in terms of tissue composition and some biometrical traits.

Taking everything into account, this study aimed to *a)* determine the response of soft tissues of *R. philippinarum* to nutritional imbalances in order to establish if the previously observed (Chapter 2.2) strategies for the homeostatic regulation resulted sufficient to maintain their tissues (in terms of whole soft tissues as well as for each tissue) at a constant composition; *b)* to compare both inter- and intra-familiar responses in tissues composition in response to different N content diets in order to elucidate if the observed differential responses at the balances level apply as well in terms of tissue composition; and *c)* to analyze interindividual differences in the biometrical features of juveniles of the Manila clam.

2. MATERIAL AND METHODS

2.1 ANIMAL MAINTENANCE AND DIET CHARACTERISTICS

Juveniles belonging to two families (1 and 8) of the Manila clam *Ruditapes philippinarum* were grown for two years, and around 30 specimens characterized by fast (F) and slow (S) growth rates inside each family were chosen (see Chapter 2.1, section 2.1). These four groups were subsequently maintained under constant conditions as described in Chapter 2.1, section 2.2; whose mean growth parameters at the beginning of this experiment are described in *Table 2.3 - 1.*

Table 2.3 - 1. Mean (SD) size of the four groups of clams before dissections of tissues

Family	Growth category	Length (mm)	Live weight (mg)
1	F	23.71 (1.56)	2655.00 (515.7)
	S	13.41 (1.67)	501.23 (148.99)
8	F	22.63 (1.96)	2371.85 (653.73)
	S	12.75 (2.13)	440.66 (195.22)

Inside each of the four groups (F1, F8, S1 and S8), clams were randomly separated into two 50 L tanks, where conditions were identical with the exception of the diets supplied. In order to analyze diet effects on elemental composition of tissues, the microalga species *Rhodomonas lens* was used at either its exponential (diet N+) or stationary (diet N-) growth phase of culture, where the latter diet possesses more than 2.5 times the C/N values than the former (Chapter 2.1). In both chambers, concentration of particles was maintained constant and similar in terms of packed volume, at a concentration of 1 mm^3 L^{-1} so as to analyze solely the effect of differential N availability on diets.

Diet monitoring was carried out twice a week in quadruplicates during acclimation, where organic and inorganic content of diet as well as CHN composition was analyzed by means of the use of pre-weighted glass fiber filters (GF/C) as described in Chapter 2.2, section 2.1.

2.2 Elemental analysis of tissues and biometric parameters

After 35 days of acclimation to each diet, clams were fasted to avoid the inclusion of food particles in the elemental analyses, and subsequently dissected into 5 sets (4 in the case of S juveniles), whose groups were gill, adductor muscle, digestive gland, gonad, and remaining tissues (mantle edge, siphons and foot). Since slow growing juveniles had little or non-apparent gonad tissue, no distinction was made between gonad and remaining tissues in that group.

Immediately after separation of organs, gill was photographed over a gridded paper to subsequently estimate surface areas by image analysis through the Fiji software (Schindelin et al., 2012), and each organ was introduced into a 2 mL vial and frozen by submersion of flasks into liquid nitrogen. Afterwards, tissues were freeze-dried and weighted for dry (i.e. lyophilized) weight estimations. Finally, samples were homogenized with a mortar and pestle, and stored at -20ºC until being analyzed.

Dry weight (DW) of samples was estimated by drying at 100ºC for 24 – 48 h, while ash weight (AW) was calculated after calcination at 450ºC. Consequently, *organic content* (OC%) of tissues was obtained through the percentage of the difference of dry and ash weight (organic weight) over dry weight:

$$OC\% = \frac{DW - AW}{DW}$$

Regarding hard tissues, determinations of length and dry and ash weight were performed, thus organic fraction of shells was obtained in the same way as in soft tissues. These measurements allowed the calculation of the following biometric relationships:

Condition index (CI) can be calculated in two ways: by division of dry weight of soft tissues (DWt, mg) over shell length (L, mm) raised to the third power:

$$CI1 = 1000 \times \frac{DWt}{L^3}$$

or by computation of the dry weight of soft tissues (DWt) over the dry weight of hard tissues (DWs):

$$CI2\ (\%) = 100 \times \frac{DWt}{DWs}$$

The tissue index (TI), that is, proportion of each tissue over total flesh allows inter- and intra- familiar comparisons of differences in tissue sizes and was expressed as the dry weight of a given tissue over the total dry weight of tissues:

$$TI_i = \frac{DW_i}{TDW}$$

The ratio between dry mass and area of gills expresses the mass of gill per surface area unit (i.e. the thickness of gill):

$$Mass{:}\, surface\ area_G\ (mg\ mm^{-2}) = \frac{DW_G}{Surface\ area_G}$$

For elemental analysis purposes, two subsamples were subtracted from each sample. CHN analyses required 1 – 1.5 mg, while approximately 10 mg were needed for gravimetrical analyses. Samples for elemental composition were inserted into tin capsules in order to be analyzed in a Euro EA Elemental Analyzer (CHNS) from EuroVector, using acetanilide as standard, in the SGIker facilities (UPV/EHU). In addition, the same process was followed with ashes of every tissue to compute the inorganic fractions of N and C, that were deducted from the former samples to obtain the organic N and C fractions. In order to compute the estimate of whole soft tissues elemental composition, dry weight of every tissue was multiplied by the fraction of each element to obtain the element weight. Then, each element weight was summed and subsequently divided by total dry weight, as follows:

$$Whole\ soft\ tissue\ element\ (\%) = \frac{\sum(DWt_i \times \frac{Element_i\ (\%)}{100})}{\sum DWt_i} \times 100$$

Threshold element ratios (TER) were estimated following Frost et al. (2006), whose equation has been adapted for $TER_{C:N}$ (instead of $TER_{C:P}$), absorption efficiencies were used instead of assimilation efficiencies, and standardized (i.e. scaled to a given *b* value, see Chapter 2.2 for details) physiological rates were used instead of mass-specific rates:

$$TER_{C:N} = \frac{AE_N}{\frac{IR_C \times AE_C - R_C}{IR_C}} \times C{:}N_{tissues}$$

where AE_N and AE_C represent the absorption efficiencies for N and C, respectively, IR_C is the rate of C ingested, R_C is the C respired and $C:N_{tissues}$ is the C:N ratio of whole soft tissues.

2.3 Data analysis

Comparisons between tissues, families, growth category and effect of conditioning diets were tested through a 4-way ANOVA. When significant differences were detected on a factor containing more than two levels, Tukey's Honestly Significant Difference (HSD) tests were performed to establish which levels were responsible for those significant differences. Analyses were performed after having tested for normality (Shapiro-Wilk) and homoscedasticity (Levene) of the data. Data based on percentages was analyzed by means of nonparametric analyses (ANOVA on ranks).

Linear regression analysis was used to estimate the relationship between gill mass and surface area. In addition, ANCOVA analysis was performed to compare differences between families in terms of shell weight *vs.* shell length relationships.

All of the statistical analysis as well as elaboration of graphical material was performed by means of the R software, version 3.5.1 (R Core Team, 2018).

3. Results

3.1 Biometry

Growth of clams (in terms of live weight) was affected by diet ($F = 7.39$, $p = 0.008$), and especially by growth category ($F = 47.23$, $p < 0.001$), but family had no significant effect ($F = 0.065$, $p = 0.80$) on growth nor significant interaction terms were found. Hence, under the N rich diet, F clams grew 10.3 mg (live weight) d^{-1}, while growth of S clams only amounted up to 4.4 mg d^{-1}. However, mean growth under N- diet was 7.6 and 2.9 mg d^{-1} for F and S clams, respectively.

Furthermore, differences in terms of shell weight per unit of length were identified at the interfamily level, where ANCOVA analysis for both families in the relationship *log (shell dry weight) = b log (length) + a* showed no differences on their slopes ($F = 0.44$, $p = 0.51$), but a higher intercept ($F = 4.22$, $p = 0.04$) for Family 1 was observed (*Figure 2.3 - 1*). As for condition index (mg mm^3), differences occurred likewise only at the interfamily level ($F = 93.43$, $p < 0.001$), but being in this case clams of Family 8 those attaining higher values. Taking both results into account, Family 1 would invest on shell

thickening more than Family 8, while the latter would possess higher amount of soft tissue per unit of volume (a more detailed analysis will be provided alongside the analysis of soft tissues weights).

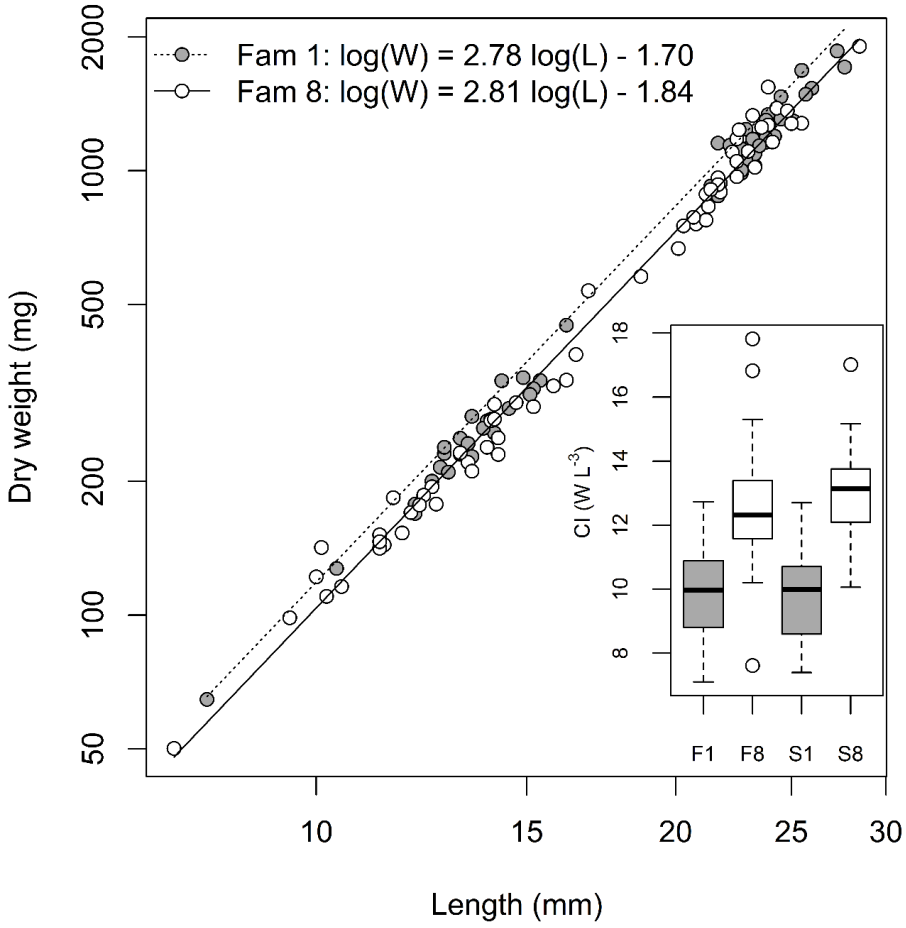

Figure 2.3 - 1. Interfamily comparisons of the relationships between shell dry weight and length, and CI (mg mm3) of the four phenotypes

The relative weight of soft tissues (*Table 2.3 - 2*) varied mostly depending on growth category, but also according to family and even diet. The latter effect only showed differences ($F = 9.23$, $p = 0.003$) in the adductor muscle, where N+ diet promoted an increase in the relative weight of that tissue. Differences between families were found in gill, where Family 1 attained a higher proportion ($F = 22.7$, $p < 0.001$), whereas Family 8 was superior ($F = 23.2$, $p < 0.001$) in terms of muscle. Finally, growth category promoted differences in all organs, where S clams had bigger gill and digestive gland proportions, while F clams contained a higher proportion in the rest of the tissues.

Table 2.3 - 2. Relative weight of every tissue (Gi: gill; AM: adductor muscle; DG: digestive gland; Go: gonad of F clams; MF: mantle and foot of F clams; GMF: gonad, mantle and foot of S clams) after acclimation (A) to either N+ or N- diet of F and S clams of both families

A	G	F	Gi	AM	DG	Go	MF	GMF
N+	F	1	15.25 (1.72)	9.68 (1.16)	18.46 (2.9)	20.19 (4.32)	36.42 (2.38)	56.61 (3.44)
		8	13.14 (1.89)	10.4 (0.96)	15.28 (1.95)	23.63 (7.19)	37.55 (4.35)	61.18 (3.2)
	S	1	19.26 (4.98)	7.91 (1.43)	18.14 (2.86)	-	-	54.69 (4.72)
		8	16.48 (4.02)	9.86 (1.2)	18.14 (3.64)	-	-	55.51 (6.22)
N-	F	1	14.9 (2.44)	9.22 (1.38)	18.06 (3.66)	22.35 (7.49)	35.46 (2.44)	57.82 (5.63)
		8	13.35 (2.27)	10.37 (1.8)	16.21 (3.34)	24.04 (7.29)	36.03 (3.16)	60.07 (5.41)
	S	1	18.48 (2.94)	7.38 (1.59)	19.08 (4.62)	-	-	55.06 (5.74)
		8	15.56 (3.56)	8.56 (0.93)	21.14 (4.38)	-	-	54.74 (5.25)

Figure 2.3 - 2 reports the relative proportion of each tissue over the total dry weight (i.e. soft and hard tissues added) according to each factor (diet, growth category and family), where the number on each chart represents the condition index (mg mg^{-1}) obtained by dividing the weight of soft tissues by the total dry weight.

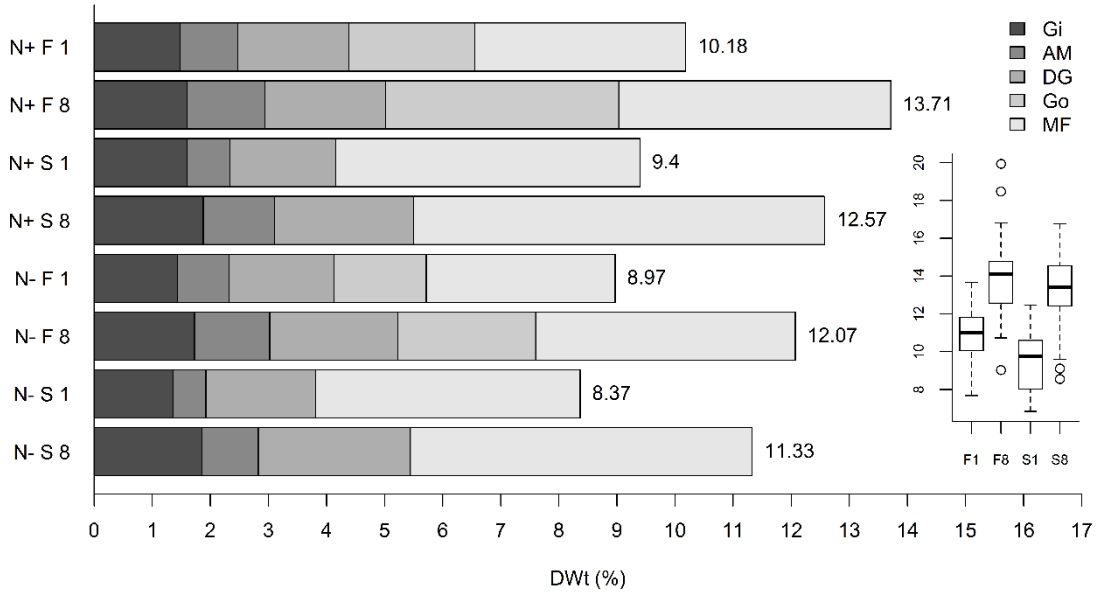

Figure 2.3 - 2. a) Relative weight of soft tissues over total dry weight of F and S clams belonging to either Family 1 or 8, and acclimated to either N+ or N- diets. b) Boxplot to illustrate condition index variation across phenotypes

Phenotype effects were specifically considered (*Figure 2.3 - 2b*) to found that condition index (mg mg^{-1}) only showed differences between families ($F = 95.15$, $p < 0.001$), where Family 8 reached values > 30% than Family 1. F clams tended to possess a higher condition index although differences did not reach the significance level ($F = 3.68$, $p = 0.058$).

Additionally, relationships between gill weight and gill surface area (*Figure 2.3 - 3*) were found to be significant in terms of diet conditioning ($F = 4.73$, $p = 0.03$) and growth category ($F = 159.70$, $p < 0.001$), but not between families ($F = 0.13$, $p = 0.72$).This ratio of mass per unit surface area is representative of gill height (i.e. thickness) and thus, in the light of these results, F clams attained ca. 50% more mean gill thickness than S clams even though S clams registered higher gill surface areas (standardized values), while clams fed N- diet would have increased by approximately 7% the thickness in their gills compared with those fed N+.

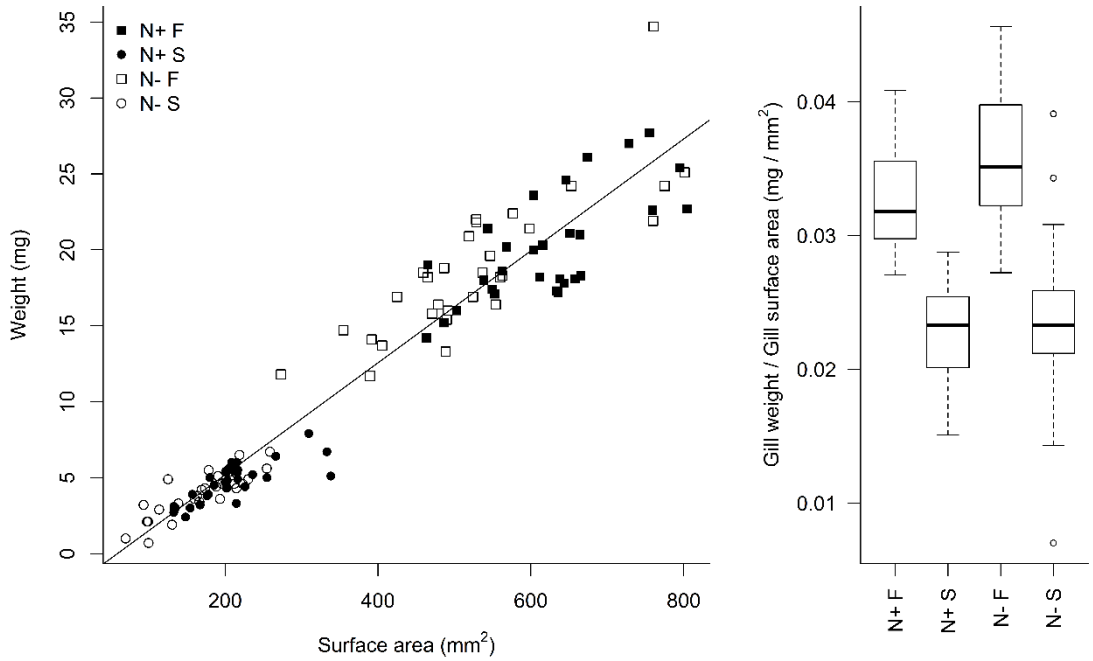

Figure 2.3 - 3. a) Regression fit and b) boxplot for weight and surface area relationships of gills for different combinations of conditioning diet and growth group (see legend)

3.2 Elemental composition of tissues

After one month of growth under either N rich or N restricted diets, overall N content (%) of tissues had reached higher values in clams acclimated to N+ diet, which resulted in lower C% and lower C:N ratios with that diet (*Table 2.3 - 3*). Growth category (F vs. S clams) showed no significant effects, although trends pointed to higher N% and lower C:N in fast growing clams. Interfamily comparisons revealed similar trends, being Family 1 the group possessing higher N%.

Table 2.3 - 3. Means of pooled values (SD) of N and C%, and the resulting C:N ratios of the four factors studied (A: diet acclimation, G: growth category, F: family, T: tissues; in which: Gill: gill, AM: adductor muscle, DG: digestive gland, Go: gonad of F juveniles, MF: mantle and foot of F juveniles, GMF: gonad, mantle and foot of S juveniles); and four way ANOVA on ranks results

		N%	C%	C:N
A	**N+**	12.8 (0.09)	49.22 (0.12)	3.87 (0.04)
	N-	12.32 (0.14)	49.64 (0.18)	4.1 (0.07)
	ANOVA	***F = 9.98, p = 0.002***	***F = 5.39, p = 0.02***	***F = 8.40, p = 0.004***
G	**F**	12.63 (0.11)	49.43 (0.14)	3.95 (0.05)
	S	12.48 (0.14)	49.43 (0.17)	4.02 (0.07)
	ANOVA	*F = 1.27, p = 0.26*	*F = 0.02, p = 0.89*	*F = 1.03, p = 0.31*
F	**1**	12.59 (0.1)	49.31 (0.14)	3.95 (0.04)
	8	12.53 (0.14)	49.55 (0.17)	4.02 (0.07)
	ANOVA	*F = 0.21, p = 0.65*	*F = 1.62, p = 0.21*	*F = 0.04, p = 0.85*
T	**Gill**	12.78 (0.08)	48.39 (0.08)	3.79 (0.03)
	AM	13.61 (0.07)	48.36 (0.1)	3.56 (0.02)
	DG	11.54 (0.2)	51.12 (0.2)	4.5 (0.1)
	Go	11.94 (0.21)	50.82 (0.23)	4.29 (0.09)
	MF	12.95 (0.18)	48.95 (0.16)	3.8 (0.06)
	GMF	12.31 (0.29)	49.36 (0.22)	4.05 (0.1)
	ANOVA	***F = 60.168, p < 0.001***	***F = 115.589, p < 0.001***	***F = 69.361, p < 0.001***
A*G	*ANOVA*	***F = 10.257, p = 0.002***	*F = 1.407, p = 0.238*	***F = 7.518, p = 0.007***
A*F		*F = 0.213, p = 0.645*	*F = 0.725, p = 0.396*	*F = 0.469, p = 0.495*
G*F		***F = 7.066, p = 0.009***	*F = 2.63, p = 0.107*	***F = 7.211, p = 0.008***
A*T		***F = 14.928, p < 0.001***	***F = 16.021, p < 0.001***	***F = 15.169, p < 0.001***
G*T		*F = 1.354, p = 0.262*	***F = 4.919, p = 0.009***	*F = 1.405, p = 0.249*
F*T		***F = 3.371, p = 0.007***	*F = 1.974, p = 0.086*	***F = 2.699, p = 0.023***
A*G*F		*F = 0.592, p = 0.443*	*F = 1.083, p = 0.3*	*F = 0.692, p = 0.407*
A*G*T		***F = 7.426, p = 0.001***	***F = 4.98, p = 0.008***	***F = 6.028, p = 0.003***
A*F*T		***F = 6.774, p < 0.001***	***F = 4.439, p = 0.001***	***F = 6.04, p < 0.001***
G*F*T		***F = 3.979, p = 0.021***	*F = 1.465, p = 0.235*	***F = 3.907, p = 0.022***
A*G*F*T		*F = 1.876, p = 0.157*	*F = 0.037, p = 0.964*	*F = 1.417, p = 0.246*

Compared with diet and phenotype (growth group and family) effects, differences among tissues regarding elemental composition are outstandingly high resulting in effects of tissue factor that are highly significant for of either N, C or C:N index (*Table 2.3 - 3*). In addition, variability in elemental composition associated to diet and phenotype was noticeably heterogeneous among the different tissues, with coefficients of variation (CV) for N contents ranging from ~ 0.5% in the gill and adductor muscle to ~ 2% in the rest of

tissues. This is accounted for by multiple significant interactions of tissue factor in ANOVA on ranks analysis (*Table 2.3 - 3*).

3.2.1 Overall effects of tissue factor and their interactions with diet

Nitrogen content was significantly higher in adductor muscle than in the rest of the tissues, while carbon contents were significantly higher in the digestive gland and gonad (*Figure 2.3 - 4a* and *4b*); consequently, minima C:N ratios (*Figure 2.3 - 4c*) were observed in the adductor muscle (3.56) and gill (3.79) and maxima in digestive gland (4.5) and gonad (4.29).

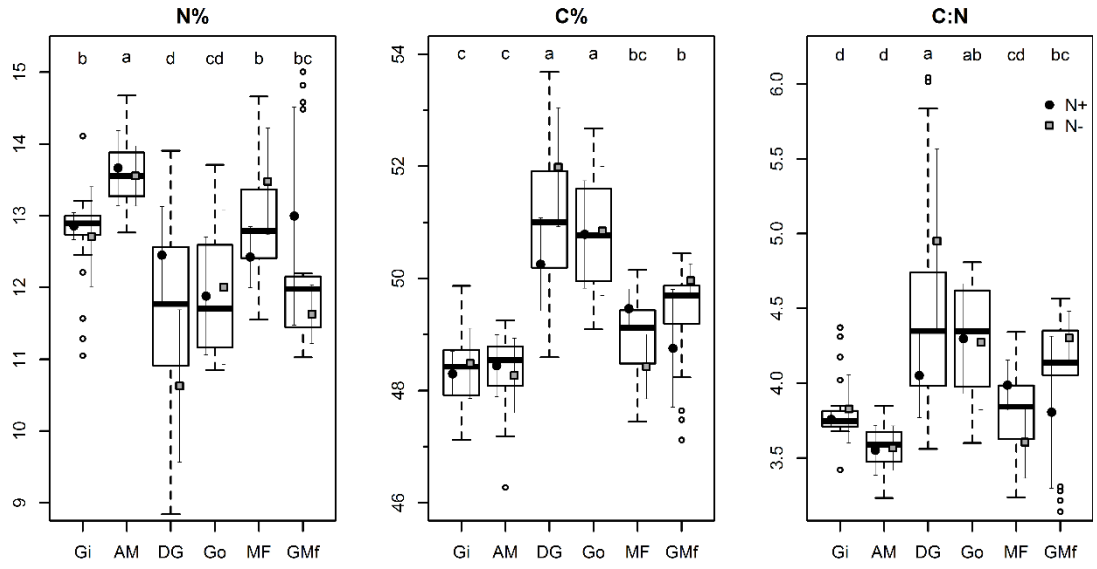

Figure 2.3 - 4. Nitrogen (left), carbon (center) and C:N (right) content for each tissue analyzed. Inside each box, black circles represent elemental composition of clams acclimated to the N+ diet, while gray squares represent composition after acclimation to the N- diet. Different letters (on top) denote significant mean differences between tissues (Tukey HSD test).

Acclimation diets affected differentially the elemental composition of the various tissues, as indicated by level differences between black circles (N+ diet) and gray squares (N- diet) inside boxes in *Figure 2.3 - 4*. In this way, both gill and adductor muscle compositions remained unaltered as no-significant differences were recorded between diets, (in spite of slight increases in C% of gills of N- acclimated clams). Indeed, homeostatic regulation of nutrient dietary changes was almost complete in the growth of gill tissues. Conversely, elemental composition of both digestive gland and remaining tissues strongly differed between clams acclimated to N+ or N- diets. This pattern of change was significant for both elements, as well as for C:N indexes (*Figure 2.3 - 4* and *Figure 2.3* - 5). Digestive glands, in particular, showed, among the different tissues, the largest degree of dietary dependence.

3.2.2. Phenotype effects

Gill and adductor muscle were the more stable organs regarding elemental composition, with only punctual effects of the diet that were phenotype-dependent. Gill tissue nitrogen kept rather constant levels of ca. 13% across groups with the exception of the S.1 group fed the diet N- (*Figure 2.3 - 5*), that showed significant differences for N% (lower) and C:N ratio (higher) (Tukey HSD test). Fast growing appears to enhance the effect of diets on elemental composition of the adductor muscle, so that higher N% (and correspondingly lower C%) were recorded in F clams fed the N+ diet as well as in S clams fed the N- diet (*Figure 2.3 - 5*). In spite of these trends, single pairwise differences were only recorded between S groups of the Fam. 8 clams fed the N+ diet and Fam. 1 fed the N- diet.

Elemental composition of the digestive gland exhibited the greatest variation, diet being the most influent factor. These effects in combination with phenotype differences resulted in C:N values ranging from above 3.5 to almost 6.0 (*Figure 2.3 - 5a*). As pointed out for the adductor muscle, growth rate variation among phenotypes combines with dietary effects so as to produce the greatest differences in composition between the groups of fast growers fed the N rich diet and low growers fed the N poor diet. Specifically, N% was higher for the group of F clams from Family 1 acclimated to N+, while C maximum level was reached in S clams from Family 8 under N- diet. C:N ratios were significantly different between phenotypes for both inter and intra-family groups, with differences between families being predominant.

The last group of tissues (gonad and remaining tissues) deserved a different treatment as the two components could be separated for differential analysis only in F clams; thus, no F vs S differences become available for testing in these organs. With regard to F clams (*Figure 2.3 - 5*), gonad registered low N% and high C% values comparable to the average composition of the digestive gland, although no significant effects of the diet were appreciated in this case. Besides, elemental composition of remaining tissues (mantle, foot and siphons) very much liked that of other structural tissues such as the gill, with relatively high N contents and low C:N values. However, diet exerted important effects on composition in this case, that were opposite to those found in the digestive gland since higher N% values were achieved with the N restricted diet specially those of the Family 1. Elemental composition of the GMF group of tissues was intermediate between gonad and remaining tissues composition recorded for F clams,

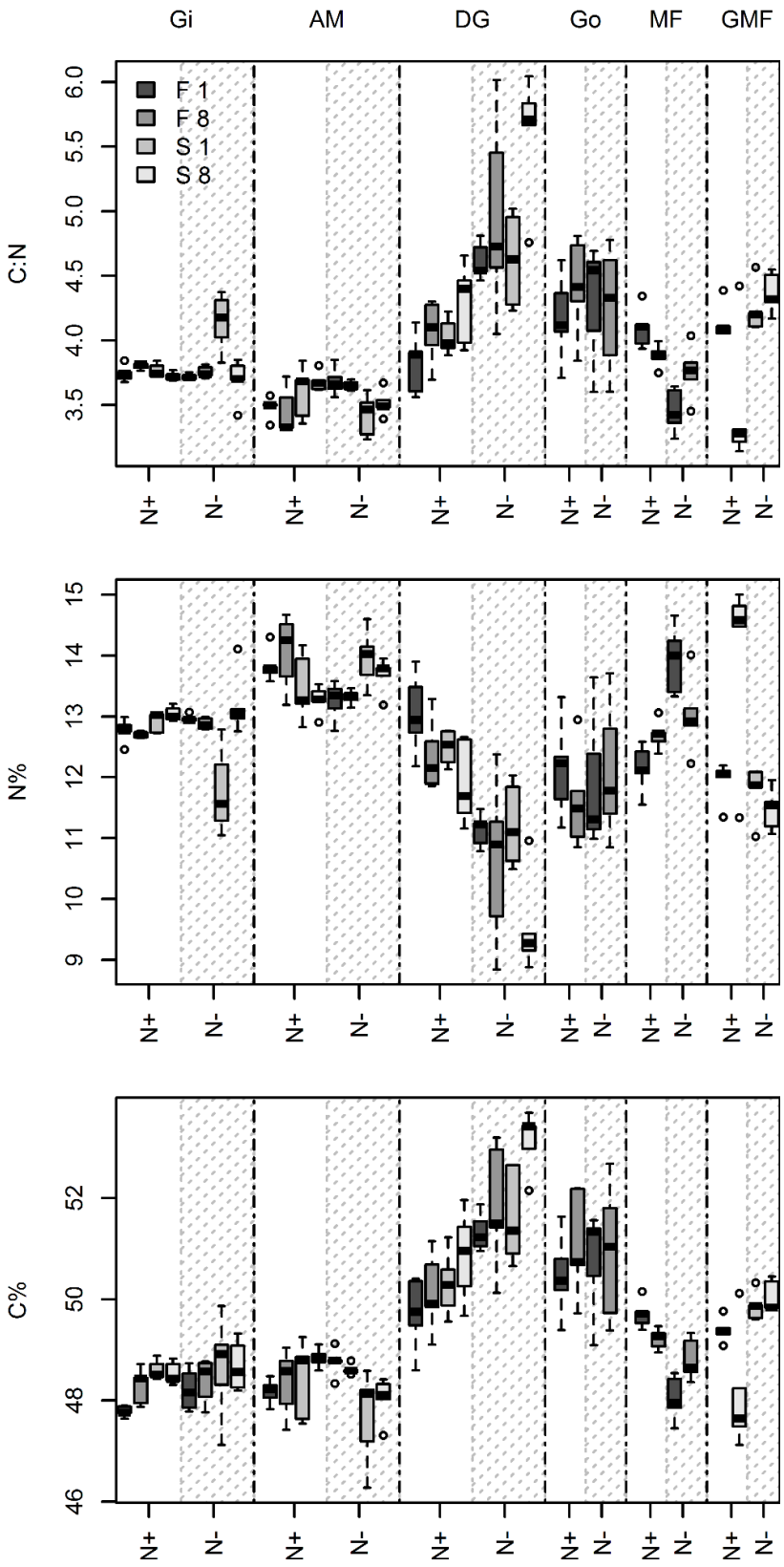

Figure 2.3 - 5. C:N (top), N% (center) and C% (bottom) content in every tissue (Gi: gill; AM: adductor muscle; DG: digestive gland; Go: gonad of F clams; MF: mantle and foot of F clams; GMF: gonad, mantle and foot of S clams) under either N+ (white background) or N- diet (shaded background) of F and S clams of both families (see legend)

with a neat positive effect of N+ diet on N% of S clams from the Family 8 (*Figure 2.3 - 5*).

3.2.3 Whole soft body composition

On account of great differences in both the elemental composition and relative size of different body components, a whole soft body composition was computed from the specific composition of each tissue weighted by the fraction represented by this tissue in the whole soft body (see Eq. in M&M). Resulting C:N ratios for the whole soft tissues in the different phenotypes (growth category*family groups) acclimated to N+ and N- diets are compared in *Figure 2.3 - 6c*) with the corresponding C:N ratios for a) the digestive gland and b) the retained ration or SFG as based on the elemental balances for C and N reported in a previous study (Chapter 2.2).

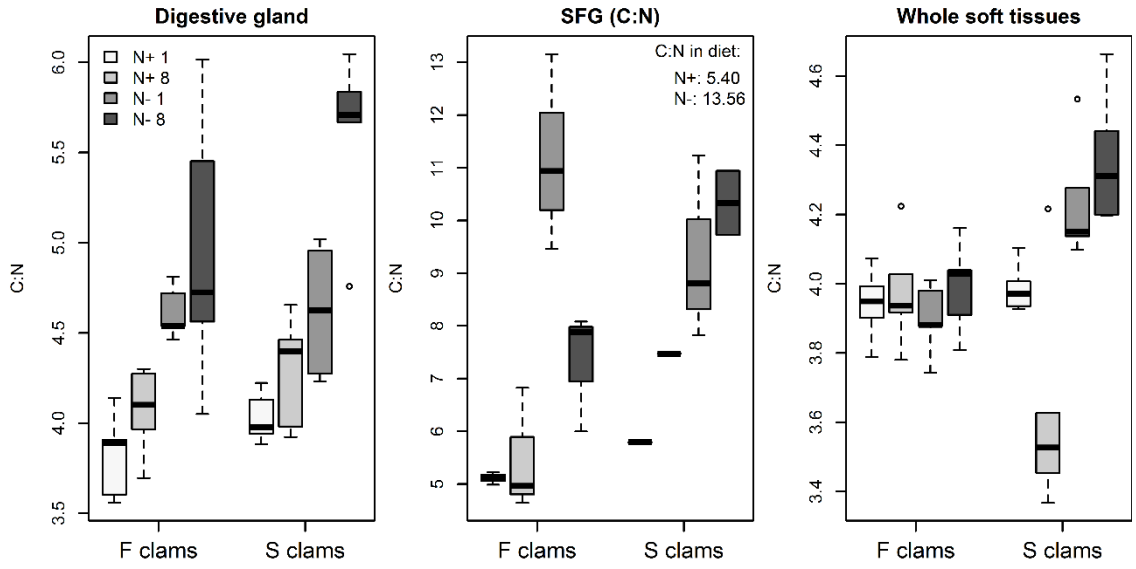

*Figure 2.3 - 6. C:N ratios of digestive gland (left), elemental balances (center) and whole soft tissues (right) for each growth category (F and S clams) and the combinations of diet*Family (see legend)*

Very significantly, patterns of C:N variation among phenotypes and conditioning diets were similar for the digestive gland and SFG, where the dietary N level and phenotype associated growth gradients combined to produce a series of increasing C:N values from N+ to N-, F clams to S clams and Family 1 to Family 8. Thus, C:N composition of the digestive gland closely approached the proportions of both components in the body retained fraction of food, suggesting a lack of homeostatic regulation in this organ. On the other hand, comparisons between C:N balances (*Figure 2.3 - 6b*) and C:N composition of whole soft tissues (*Figure 2.3 - 6c*) reflected different outputs according to the growth category: While F clams of both families maintained a

constant elemental composition irrespectively of the diet fed, whole body composition of S clams changed according to the pattern reported for the digestive gland. Furthermore, clams of the Family 8 exhibited the greatest rate of C:N change between conditioning diets.

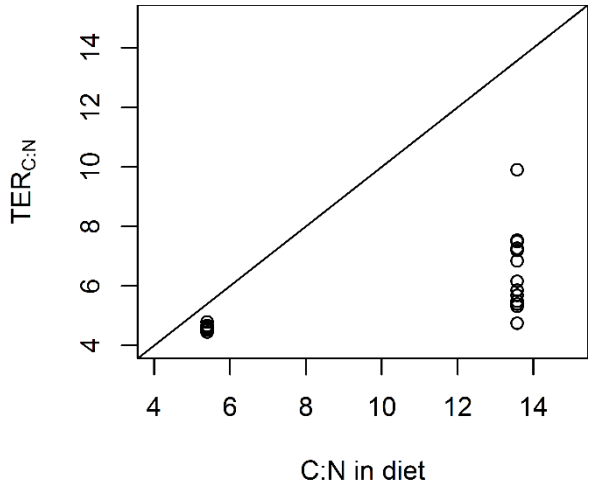

Figure 2.3 - 7. TER$_{C:N}$ of clams fed on either N+ (black circles) or N- (red circles), where nutrient (nitrogen) limitations are decreased as points are closer to the line y = x

The computation of whole soft tissues composition also allowed to calculate C:N Threshold Element Rations (TER) according to Frost et al. (2006). All TER values were below the C:N ratios of diet dosed (*Figure 2.3 - 7*), thus indicating N limitations irrespectively of the diet. However, TERs estimated for clams fed N+ fell close to the C:N ratio of the diet, involving this diet approached the ideal composition to meet nutritional needs of clams.

4. DISCUSSION

In the present work, endogenous (growth phenotype) as well as exogenous (diet) effects were responsible for different growth rates. With respect to biometrical features, inter-familiar differences were concerned mainly with condition indices, while gill thickness was the differential trait between F and S clams. Tissues composition appeared to reflect homeostatic mechanisms that proved effective in buffering the effects of dietary variation, except for the digestive gland, which varied according to the net balances achieved with the different diets. However, stability of tissues composition varied among growing phenotypes: F clams were able to maintain a constant body composition, while S clams, and especially, those from Family 8, experienced fluctuation in their C:N composition according to the diet dosed.

4.1 Biometry

A high N content diet promoted increases up to 40% in growth rates of clams after a month of exposure. As shown in previous approaches based on energy performance (Arranz et al., 2020; Chapter 2.1) and elemental balances (Chapter 2.2), maintenance under these high-quality diets promoted a strong enhancement of growth that has been confirmed in terms of actual growth in the present study. Obviously, nutrient limitations, rather than energy constraints, would be decisive in the reduced growth performances achieved with the low quality (N-) diets since energy contents (estimated according to Platt and Irwin, 1973) of these diets exceeded by 17% the energy contents of high quality (N+) diets (i.e., 26.826 vs 22.906 J mg^{-1}). A strong dependence of growth rate on dietary protein content has been generally reported in bivalves (Albentosa et al., 2002, 1999; Brown et al., 1998; Enright et al., 1986; Kreeger and Langdon, 1993; Langton et al., 1977; Uriarte and Farías, 1999; Wikfors et al., 1992) with only minor exceptions (Utting, 1986; Whyte et al., 1989). Observations regarding the preferential absorption of proteins (or N) in preference to other biochemical compounds (e.g. Hawkins, 1985; Ibarrola et al., 2000; Urrutia et al., 1996) are consistent with this pivotal role played by proteins ingrowth processes. Moreover, that the specific nutritional function of proteins as the main supplier of N takes precedence over their energy value is clearly illustrated by the fact that, compared with the amino-C fraction, the amino-N fraction of dietary proteins is absorbed and assimilated with an almost 3 times higher efficiency in *Mytilus edulis* (Kreeger et al., 1996, 1995).

Overall, biometrical parameters were found to differ mainly among phenotypes with only minor effects of the diet fed during the acclimation. This was so even for the feeding structures where a sort of diet effect would be expected similar to previously reported relationships between features of the food environment and the size of gills and palps (Compton et al., 2008; Tedengren et al., 1990; Worrall and Widdows, 1983). In this study no differences in the relative weight of gills were found between clams acclimated to the different diets although gill surface areas were significantly higher with the N+ diet (Arranz et al., 2020; Chapter 2.1). Instead, relative weight of gills showed the greatest variation between families, with Fam. 1 exhibiting a 15% increment in gill size compared with Fam. 8. Both the relative weight and surface area of the gills (Arranz et al., 2020; Chapter 2.1) were higher in S compared to F clams but the latter exhibited 50% increased values of the ratio of weight/surface area. It is tempting to consider the possibility of a

functional relationship between this ratio, representative of the gill thickness, and the gill efficiency (given as the filtration rate per unit of gill surface area), increased values of both parameters appearing as attributes of fast growers.

Other biometrical traits were also found to differ between families: For instance, Family 1, which attained the highest SFG values (Arranz et al., 2020; Chapter 2.1), invested comparatively more energy into shell thickening than Family 8 (*Figure 2.3 - 1*). In other words, the capacity to achieve higher energy balances (e.g. both F clams and clams of Family 1) would likely enable strengthening of structural tissues as reported for both shell and gill thickness. This contrasts with the growth profile of the Family 8,where comparatively reduced values of scope for growth appeared associated to a greater condition index (CI; *Figure 2.3 - 1* and *Figure 2.3* - 2) representing the proportion of soft tissues, that was increased by > 30% with respect to Family 1. These included mantle and digestive gland tissues (*Figure 2.3 - 2*) whose storage function might be indicative of a strategy of accumulation of energy reserves to face periods of energy imbalance in this family of limited growth potential. In the case of the digestive gland, the greater importance of this storage function in phenotypes of reduced energy performance would also account for the anomaly that size-specific weight of this organ, mainly involved in digestive food processing, was 13% higher in S compared with F clams ($F = 11.22$, $p = 0.001$). Since F clams are found to process twice as food as S clams, with a reduced DG size, a higher efficiency should be accorded to digestive processes met by this organ in fast growers. The suggestion in that reduced efficiency in S clams would reflect the partial occupation of this organ by storage tissues (further confirmed by the increase in digestive gland C:N ratios of slow growth phenotypes: *Figure 2.3 - 5*) in detriment of digestive tissues.

4.2 Elemental composition

Overall, elemental homeostasis of tissues appeared preserved after a month of diet exposure, but inter individual effects have been identified as strongly influential on this homeostasis. A high diversity in composition was also observed among tissues, in which differential effects of diet and phenotype have been noted.

Elemental composition of tissues (*Figure 2.3 - 4*) followed a strictly similar pattern as reported for adult *R. philippinarum* specimens (Arranz et al., 2021; Chapter 3). Therefore, very likely tissue functionality does not allow but a narrow range of variation

in elemental composition. Exceptions to this data agreement between adults and juvenile clams correspond mainly to digestive gland and gonad tissues. Composition of both these tissues is highly variable as affected by conditioning diet and phenotype, suggesting such variability might be inherent to the functionality of the corresponding organs. For instance, no effects of diet on gonadal tissue composition were evident but large deviations around the mean values would reflect different stages of gonadal maturation among both families. On the contrary, elemental composition of digestive gland exhibited a strong dependence on both conditioning diet and phenotype, and the comparison (*Figure 2.3 - 6* of trends for C:N ratios across these factors between digestive gland tissues and the corresponding trends for elemental balances computed from previous data (Chapter 2.2) revealed a noticeable similitude indicative of a very reduced degree of homeostatic regulation during the growth of this organ. It should be noted that DG composition might in no way reflect that of gut contents (ingested food) since animals were fasted for 3 days prior to the organ´s dissection, dietary effects being only representative of changes in tissue composition.

Consideration of data in *Figure 2.3 - 6* poses some additional questions that are pertinent regarding the mechanisms of nutrient homeostatic regulation during the growth of tissues. Several compensatory mechanisms for nutrient imbalances have been evaluated in a preceding study (Chapter 2.2), including increased N absorption from diets of low N content and increased N excretion with N rich diets. In spite of that, the ratio of C to N balances still keeps a strong dependence on diet composition that additionally varies among the different phenotypes. Such dependency is roughly reflected in the features of variation of C:N ratios recorded in the digestive gland while whole soft tissues exhibited a greater uniformity in composition specially in fast growers. From these comparisons, two relevant points of discussion emerge:

1. The meaning of the contrasting behavior exhibited by tissues such as the gill and adductor muscle, whose composition remained unaltered after a month exposure to diets greatly differing in C:N ratios, and the DG that closely reflected these dietary changes as affecting the elemental balances. We hypothesize that tissue growth turnover would differ between organs, digestive tissues being candidate to express higher cell turnover as a component of the adaptation to different food regimes and hence a greater dependence on food composition. Compared with short-living consumers, tissue turn-over times for most bivalve species far exceed

the one single month in which we tested the diet (Fukumori et al., 2008; Hawkins, 1985). Nonetheless, this could not apply to actively growing structures such as the digestive tissues, where significant diet-induced changes in the size of the DG have been reported to take 10-15 d. in cockles *Cerastoderma edule* (Ibarrola et al., 1999). In support of the above, tissue distribution of stable isotope signatures indicated a high turnover in the digestive gland and low in the gill of the fan shell *Pinna nobilis* (Cabanellas-Reboredo et al., 2009). Evidence on this point may however be contradictory since gills were reported to show the highest tissue cell turnover rates in the scallop *Aequipecten opercularis* as a possible response to oxidative damage characteristic of metabolically active tissues (Strahl and Abele, 2010). An alternative hypothesis has been stated before as regards to the possibility that acclimation to N poor diets might, in combination with phenotype, should induce changes in the proportion of storage tissues within the DG compatible with the increment of C:N ratios of this organ. Obviously, a more detailed analysis in terms of biochemical components would be necessary to assess this point.

2. Consideration of low turnover rates in most organs of clams would also contribute to explain rather stable C:N ratios computed for the whole soft body tissues, in contrast with the strong diet-dependent variation observed for this ratio when computed from elemental balances (*Figure 2.3 - 6*) that can be assumed to represent the composition of newly added materials during growth. In the end, DG that is the main source of variation only represents 15-20% of total soft body (*Table 2.3 - 2*). Yet, this interpretation fails to explain the crucial contradiction that the greater stability in tissue´s composition, implying lower dependence from the diet supplied, was just found in the group of fast growers, supposedly affected by higher turnover rates; whereas S clams, especially those from Family 8, closely reflected dietary composition in their tissues (*Figure 2.3 - 6c*).

An alternative interpretation would rely on the consideration that elemental balances might be biased on methodological grounds, as regards especially to limitations in the computation of some components of C loss. If that were the case, a substantial component of the homeostatic regulation of C contents, likely enough to account for discrepancies found between elemental balances and whole tissue composition, could have been missed in this study. The main bias might have occurred at the preabsorptive

level since -following the most commonly used methods- our digestive balances were based on particulate organics (POC and PON) while some evidence indicated substantial C losses in the form of dissolved organics that could not be computed in our determinations. For instance, analysis by Kreeger and Langdon (1994) of the C budget of *Mytilus trossulus* employing a ^{14}C-labeled diets identified a C component that had been digested but was not retained in the filtered feces given its soluble nature. A part of the solubilized organic materials appeared in the filtrate (around 3% of ingested ^{14}C), but the bulk (17-30% of the ingested ^{14}C) was found in the water surrounding the mussels (Kreeger and Langdon1994). This last fraction might include C solubilized from fecal materials (e.g., soluble components of MFL such as the amino C fraction wasted during protein turnover: Kreeger et al., 1996, but also other organic components of strictly metabolic origin such as short chain organic acids and volatile free fatty acids that are known to be released from animals specially during phases of anaerobiosis (Hochachka and Somero, 2002). If they occurred, the last products should have been included, together with the CO_2, in the computation of metabolic C losses as a postabsorptive component of stoichiometric adjustments.

The procedure followed in the determination of CO_2 loss could be another source of methodological bias, since respired C was determined indirectly from measurements of oxygen consumption and assuming a respiratory quotient (RQ) of 0.9. Although the assumption of this RQ seems reasonable in bivalves (e.g., Bayne, 2009 used a 0.85 value for the same purpose), the use of a direct method of determination (CO_2 -trapping techniques: Kreeger et al., 1988) would have possibly resulted in reduced figures for CO_2 release. The reason for that expectation is that a fraction of the aerobically produced CO_2 is known to be used in shell mineralization to produce $CaCO_3$. For instance, analysis of stable C isotope signatures (Marchais et al., 2015) have stablished that as much as ~10% of inorganic C deposited in the shell of different bivalve species derives from C in the food (12% in the case of Manila clams; Poulain et al., 2010). As an indirect confirmation of the above, Hawkins and Bayne (1985) identified that up to 50% of the carbon net balances corresponded to C incorporation into shell tissue in summer, whereas in winter net C incorporation was positive in shells and negative in soft tissues; these magnitudes are considered to far exceed the expected C incorporation into the organic shell matrix. On the other hand, only 20% of the N balances were devoted to new shell tissue formation (Hawkins and Bayne, 1985); therefore, the reduction in C:N ratios from elemental

balances to soft tissues composition observed in the present work seems compatible with a larger C:N ratio of materials being allocated to shell formation.

The above referred limitations in the confection of elemental balances do not invalidate our previous conclusions (Chapter 2.2), since balances are not intended for assessing absolute values, but for comparative purposes. Hence, any methodological issue that could have biased our results should be expected to be comparable among groups.

In conclusion, strong interfamiliar effects on biometrical parameters reflected constitutive traits among families. N+ diet enhanced growth in all groups, irrespectively of the phenotype, where $TER_{C:N}$ resulted similar to C:N ratio of the diet. Homeostasis of tissues was preserved differentially among tissues as well as among phenotypes, being digestive gland and slow growing clams those attaining poorer degrees of homeostatic regulation. The observed uncoupling between elemental balances and tissue composition would respond to diverse causes, although our results do not allow to distinguish which is/are the main drivers for such uncoupling.

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Chapter 3

Biochemical *vs.* elemental analysis on tissues of *Ruditapes spp.*

Methods for assessment of body tissue composition as an indication of the energetic status in bivalve populations: a comparison of biochemical and elemental analysis

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ABSTRACT

Elemental (CHN) and proximate biochemical compositions of body tissues are useful tools among the ecological indices most commonly used in evaluation of the energetic status of animal populations. Gnaiger and Bitterlich, (1984) supplied procedures for the interconversion between both these measurements based on stoichiometric relationships, that were further tested using gut contents and body tissue samples of freshwater fishes. Despite a lack of validation studies, the reported conversion factors have been broadly applied in the field of body composition analysis of marine invertebrates, especially bivalve mollusks. The aim of this study was to test the applicability of those conversion equivalents in analysis of the body tissues of two congeneric clam species (*Ruditapes decussatus* and *Ruditapes philippinarum*). To this end, proximate biochemical composition, as analyzed by fractionated extraction of tissues samples and quantification using spectrophotometric methods, was converted to elemental composition, and the resulting figures were compared with those of direct CHN analysis. The results of this comparison indicate good agreement within the ranges reported, provided that ninhydrin positive substances (NPS) are incorporated in the biochemical composition analyses. The magnitude of the nonprotein N component in bivalve tissues appears to complicate the reverse computation of biochemical components from elemental composition because no accurate estimation of proteins from N contents might be possible. Additionally, a specific correction of residual water in dried samples of bivalve tissues for CHN analysis should be applied. The absence of broad differences found between species reflects the morphological, evolutionary and functional proximity between them, whereas tissue differences display the differential role that each organ plays in the organism, although other sources of variability such as diet and sex should be addressed in future research.

Key words: body tissues, biochemical composition, elemental analysis, bivalve mollusks, genus *Ruditapes*.

1. INTRODUCTION

Many studies performed over decades in the field of ecophysiology and aquaculture of bivalves have relied on analysis of the proximate biochemical composition of body tissues (Giese, 1969). Fractionated extraction and quantification of biochemical components in bivalve tissues using conventional colorimetric methods (Bligh and Dyer, 1959; Dubois et al., 1956; Folch et al., 1957; Lowry et al., 1951; Marsh and Weinstein, 1966; Phillips and Privett, 1979) have been widely applied for different purposes (Ansell, 1974, 1972; Ansell et al., 1980; Davis and Wilson, 1983; Giese, 1969, 1967; Taylor and Venn, 1979), including computation of the caloric (energy) content using standard equivalents (Beukema and Bruin, 1979). Two different issues account for this interest:

1) Biochemical composition is assumed to reflect the nutritional status of individuals and can consequently be used to experimentally assess the effects of variable diets on the tissue-specific trends of growth. In this way, several works have focused on the food value and its impact on growth (Albentosa et al., 2003, 1999; Aranda-Burgos et al., 2014; Beukema and Cadée, 1991; Uriarte and Farías, 1999) or used biochemical composition as an indicator of nutritional and/or physiological state (Baek et al., 2014; Beukema and De Bruin, 1977; Deslous-Paoli and Héral, 1988; Okumuş and Stirling, 1998; Pogoda et al., 2013; Walne and Mann, 1975).

2) Life cycles encompass changes in biochemical composition, and the separate analysis of tissues along these cycles might aid understanding of the temporal dynamics of nutrient transference between different body systems, which is mainly associated with the specific requirements of gametogenesis. The biochemical composition of bivalves varies seasonally and depends on food availability and timing of the reproductive cycle, among other factors (Aníbal et al., 2011; Beninger and Lucas, 1984; De Zwaan and Zandee, 1972; Delgado and Pérez-Camacho, 2007; Navarro et al., 1989; Ojea et al., 2004; Robert et al., 1993; Rodríguez-Moscoso and Arnaiz, 1998; Shafee, 1981). Analysis of these changes (reviewed by Gabbott, 1976; Giese, 1969; Sastry, 1979) revealed that storage and mobilization of reserves (primarily glycogen) are closely linked to the annual reproductive cycle, suggesting a metabolic cycle of transformation of carbohydrates into lipids for the specific needs of gametogenesis in many instances (Aníbal et al., 2011; Beninger and Lucas, 1984; Dridi et al., 2006; Gabbott, 1976, 1975; Gabbott and Bayne, 1973; Ojea et al., 2004; Zandee et al., 1980). The direct transfer of nutrients from the digestive system to the gonad has also been demonstrated (Beninger et al., 2003).

A complementary approach to these subjects relies on the use of elemental (CHN) analysis of body tissues related to the composition of potential or actual food resources. Stoichiometry principles (Sterner and Elser, 2002), which refer to the balance of nutrients (elements) within and between organisms, can be applied to account for trends in body composition during growth. This multielemental approach recognizes that the nutritional demands for maintenance and growth apply to specific proportions of the same basic components (protein, carbohydrate and lipids) that are seldom present in the required ratios within the available foods and uses elemental balance analyses (Bayne, 2009; Grant and Cranford, 1991; Hawkins and Bayne, 1985; Iglesias et al., 1996; Smaal and Vonck, 1997; Tamayo et al., 2013; Urrutia et al., 1996) to supply new insight that qualifies the already existing knowledge based on energy flow models of bivalve growth.

Biochemical composition in terms of major components (protein, carbohydrate and lipids) can be inferred on a stoichiometric basis from elemental composition (Gnaiger and Bitterlich, 1984), and quantification of biochemical components in tissues using colorimetric methods allows the elemental composition to be determined using standard equivalents (Beukema and Bruin, 1979; Brody, 1945). The possibility of interconversion between biochemical composition measurements using both methods is interesting because each of these methods can meet different experimental demands. For instance, given that the preparation requirements for samples are minimal, CHN analysis yields immediate results while avoiding multiple-step processes for direct determination of biochemical components that are considerably time-consuming and involve large amounts of consumables and chemical disposal (mostly toxicants). Moreover, sample size requirements are much lower in CHN determinations (6-10 times), allowing the analysis of smaller samples and broad use of replicates. However, direct CHN analysis is not suited to addressing whole-body samples of bivalves (e.g., larvae or small individuals for which soft body cannot be readily dissected out of the shell) due to the distorting effect of the shell inorganic carbon, and body composition analysis in those cases forcibly relies on conventional biochemical methods.

Thus, intercalibration of these two methods using an empirical approach seems to be a desirable goal in studies aiming to integrate growth energetics data from different sample sources and life stages. Gnaiger and Bitterlich (1984) established a direct correspondence of this type between proximate and elemental composition using white muscle, liver, fat tissue and gut contents of the Chinese silver carp *Hypophtalmichthys*

molitrix. The equations presented in their study have been extensively applied for the purpose of calculating biochemical composition from data on CHN composition of bivalve tissues. However, and in spite of the high demand (over 50 citations in the literature referring to bivalves), we are not aware of any studies designed to validate the main results of their study using bivalve species. Indeed, Pogoda et al. (2013) combined biochemical and elemental analysis to report the body composition of oyster species (*Ostrea edulis* and *Crassostrea gigas*), but the correspondence between both methods was not approached in a quantitative manner in that study and mainly centered on the analysis of lipid classes.

Therefore, the main aim of this study was to experimentally test the accuracy of those equivalents that relate proximate biochemical to elemental composition through a comparison of direct CHN measurements and values of elemental composition derived from biochemical measurements performed in bivalve tissues. The study was designed to compare different organs and soft-body tissues in clams sampled from populations of two con-generic species (*Ruditapes decussatus* and *R. philippinarum*), so as to evaluate the applicability of this approach in a broad range of conditions. Interorgan comparison was also performed to set the foundation for assessing nutrient transference between different body systems.

2. MATERIALS AND METHODS

2.1 ANIMAL COLLECTION AND MAINTENANCE

Adult specimens (*Table 3 - 1*) of the carpet shell clam *Ruditapes decussatus* [38.03 (1.34) mm shell length] and the Manila clam *R. philippinarum* [36.30 (0.29) mm shell length], collected by hand from the same point in the muddy flats near Santoña (Cantabria, Spain), were purchased from licensed shell-fishers and transported to our facilities by the end of July 2016. In the laboratory, the clams were kept under controlled conditions of salinity (35‰), temperature (18-19ºC) and oxygen supply (9 mg/L) and fed on a *Isochrysis galbana* (T-Iso) diet at a concentration of 20000 cells mL^{-1} for 15 days until dissections were performed.

Table 3 - 1. Mean (SD) live (mg ind^{-1}) and organic weight (mg ind^{-1}) and estimated energy value of the whole animal (J g^{-1}) from both employed species

	Ruditapes decussatus	***Ruditapes philippinarum***
Live weight	11372.5 (1697.2)	11944.5 (1161.7)
Organic weight:		
Hard tissues	101.8 (11.7)	110.8 (17.3)
Soft tissues	551.5 (33.9)	536.4 (43.8)
Gill	59.6 (10.0)	50.0 (10.4)
Gonad	126.9 (31.7)	192.4 (38.3)
Digestive gland	71.9 (12.3)	49.5 (15.6)
Adductor muscle	69.9 (11.0)	66.0 (8.0)
Remaining tissues	223.1 (37.5)	178.5 (18.7)
Estimated energy value (J g^{-1})*	25.3 (0.2)	25.8 (0.3)

*Estimations based on enthalpies of combustion of biochemical components reviewed by Gnaiger and Bitterlich (1984)

After cutting off the adductor muscle, the soft tissues were excised from the shell and separated into 5 different organs: gill, adductor muscle, digestive gland, gonad and remaining tissues composed primarily of the siphons, mantle and foot. Tissues were rapidly frozen by immersion in liquid nitrogen and freeze dried. Finally, samples were ground to a powder with a mortar and pestle and stored at -20ºC until analyses were performed.

2.2 Sample analysis

Tissue samples from five individuals per species were used in both proximate biochemical composition and elemental (CHN) analysis. To that end, five subsamples were taken from each sample to determine carbohydrate (2.5 – 3 mg), protein (2 – 2.5 mg), lipid (2.5 – 3 mg), CHN (1 – 1.5 mg), and ash (~10 mg) contents. Special care was taken to keep the samples dried, particularly in those subsamples for elemental analysis, to prevent the inclusion of hydrogen from rehydration. Organic content (ash free dry weight) was estimated from the difference between the dry weight (24 h at 100ºC) and ash weight (6 h at 450ºC) of the samples.

Gross biochemical composition was determined in triplicate using colorimetric methods. Carbohydrates were extracted in TCA (5%) and quantified according to Dubois

et al. (1956) using dried glycogen from oyster as a standard. Proteins were extracted in NaOH (0.4 N) and quantified according to (Lowry et al., 1951) with a bovine sero-albumin standard. Finally, lipids were pre-extracted in acetic acid (Phillips and Privett, 1979), extracted twice in methanol:chloroform at 2:1 and 1:2 (Bligh and Dyer, 1959; Folch et al., 1957), and quantified according to Marsh and Weinstein (1966) against a tripalmitin-phosphatidylcholine 1:1 standard.

A preliminary assessment of consistent mismatches between N contents estimated from elemental analysis and those derived from protein contents in biochemical analysis drew our attention to nonprotein nitrogen. The most abundant component of this N fraction in marine invertebrates is known to be ninhydrin-positive substances (NPS), which include amino acids and taurine acting as organic osmolytes (Hochachka and Somero, 2002). Because bivalve tissues have been reported to contain at least 5% NPS over ash free dry weight (AFDW) (Navarro et al., 1989; Shumway et al., 1977), we hypothesized that these compounds could account for the observed difference between direct and protein-derived N measurements. Consequently, NPS values were determined and computed together with gross biochemical composition for a more accurate comparison of the elemental composition obtained by direct and indirect methods. Samples for NPS determinations were taken from the same 5 organs in the two species but obtained from a different pool of 5 individuals collected and processed in the same manner as for gross biochemical composition and CHN. Determinations were performed following the procedures described by Shumway et al. (1977). Approximately 10 mg of powered freeze-dried tissue from each pool was extracted in 1 mL of 80% ethanol at 95ºC, and colorimetric methods (Moore and Stein, 1954) were developed on five replicates of the extract using leucine as a standard.

Elemental analyses of carbon, nitrogen and hydrogen content were performed at the SGIker facilities (UPV/EHU) in a Euro EA Elemental Analyzer (CHNS) from EuroVector using acetanilide as standard. To compute the CHN composition of the organic fraction, three burned (450ºC) samples per tissue and species were also analyzed to correct for CHN content of ashes.

2.3 Data analysis

The biochemical elements (i.e., carbohydrates, proteins and lipids) and NPS percentage were estimated over total organic content (mean percentage of AFDW (SD):

82.56% (5.02)). The mean (SD) percent recovery of the AFDW from the added values of the four components amounted to 84.01% (14.67). Subsequently, the percentage not recovered (15.99%) was assigned to the four components according to their relative percentages. Hence, the amount of each element is expressed as the percentage of each element over the sum of carbohydrates, proteins, lipids and NPS.

Elemental components derived from biochemical composition (CHN(b)) were calculated following Gnaiger and Bitterlich (1984). CHN data from the CHN analyzer (CHN(a)) were first corrected to estimate the element percentages over AFDW (mean (SD) recovery percentage: 68.02% (3.64)) and were subsequently corrected to subtract the residual water fraction according to Gnaiger and Bitterlich (1984). Thus, the results are presented as the percentage of each element over the sum of C, H and N.

To compute an estimate of the whole soft tissues proximate composition, the dry weight of every tissue was multiplied by the fraction of each biochemical component to obtain the component weight. Each element weight was summed and subsequently divided by the total dry weight as follows:

$$Whole\ soft\ tissue\ component\ (\%) = \frac{\sum(DWt_i \times \frac{Component_i\ (\%)}{100})}{\sum DWt_i} \times 100$$

Statistical analyses were performed using nonparametric tests, which were chosen due to the nature of the data (percentages). Regression analyses, paired Wilcoxon tests, two-way ANOVA and three-way ANOVA on ranks were run using R software (R Core Team, 2018). Post hoc tests (Tukey's honestly significant difference, HSD) were run after ANOVA on ranks analyses to test differences among tissues.

3. RESULTS

Elemental composition (CHN) estimated from the proximate biochemical composition or the direct analysis of the samples yielded similar values (*Figure 3 - 1*). However, slight differences were detected. For instance, although the paired Wilcoxon tests for N values revealed an absence of differences between methods (V = 443, p-value = 0.06), C values derived from biochemical components (mean: 72.09%) were significantly higher compared with direct determinations (mean: 70.24%) (V = 1255, p-value < 0.001), and H values (mean: 9.74%) were underestimated with respect to direct

determinations (mean: 11.28%) (V = 2, p-value < 0.001). Indeed, the ranges of variation differed between direct (9.19-12.32%) and indirect (9.34-10.17%) estimates of H and the regression equation for the relationship between both sets of data was not significant (*Figure 3 - 1*). Conversely, the corresponding regression equations for C and N were highly significant.

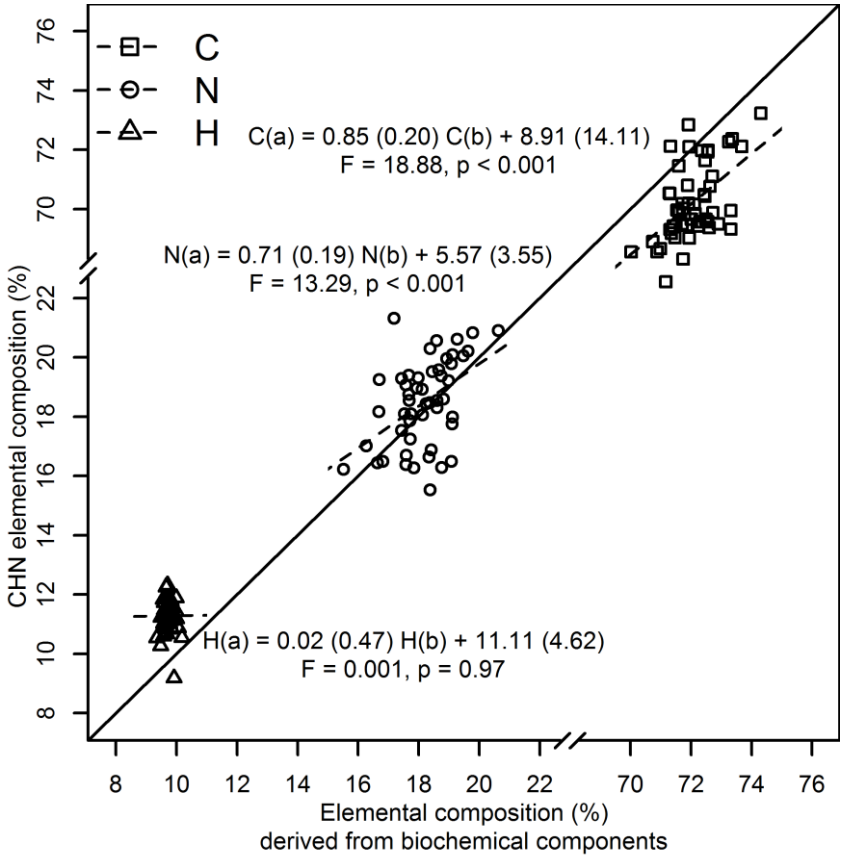

Figure 3 - 1. Relationship between direct determinations of elemental components CHN (a) and estimations based on biochemical composition CHN (b) for carbon (□) nitrogen (○), and hydrogen (Δ). Lines represent regression fits for each element between direct determinations and estimations

From these small differences, the elemental composition derived from biochemical components resulted in slight overestimations of both the C:N ratios (mean of the differences = 0.15%; paired Wilcoxon test: V = 951, p-value = 0.003) and C:H ratios (mean of the differences = 1.16%; paired Wilcoxon test: V = 1274, p-value < 0.001) compared with the direct CHN measurements.

The biochemical and elemental composition data of different tissues in both species are presented comparatively in *Table 3 - 2*. As a general outline of biochemical composition, proteins were the most abundant component (70.28%), followed by lipids (12.25%), NPS (10.83%) and carbohydrates (6.63%), irrespective of tissue and species.

Table 3 - 2. Mean (SD) values (%) by tissue and species (R. dec: R. decussatus, *R. phi:* R. philippinarum*) of biochemical composition (Cho: carbohydrates, Prot: proteins, Lip: lipids and NPS) and elemental composition (C, N and H) estimated by both methods (Direct: direct analysis, Derived: derived from biochemical composition)*

Tissue	Species	Cho	Prot	Lip	NPS	C		N		H		C:N		C:H	
						Direct	Derived	Direct	Derived	Direct	Derived	Direct	Derived	Direct	Derived
Gill	**R. dec**	4.77 (0.54)	69.94 (5.97)	14.22 (4.08)	11.07 (1.55)	70.32 (0.26)	72.1 (0.85)	17.96 (0.16)	18.15 (1.02)	11.71 (0.15)	9.75 (0.17)	3.91 (0.05)	3.98 (0.28)	6.00 (0.1)	7.39 (0.04)
	R. phi	4.08 (0.38)	73.16 (2.21)	14 (1.51)	8.75 (0.97)	70.1 (0.31)	71.92 (0.34)	18.38 (0.19)	18.36 (0.41)	11.52 (0.22)	9.72 (0.07)	3.81 (0.05)	3.92 (0.11)	6.08 (0.14)	7.4 (0.02)
Gonad	**R. dec**	5.55 (0.65)	71.92 (3.1)	12.44 (3.24)	10.09 (1.06)	70.08 (2)	71.91 (0.61)	18.25 (2.19)	18.38 (0.74)	11.67 (0.44)	9.71 (0.12)	3.89 (0.56)	3.92 (0.19)	6.01 (0.23)	7.41 (0.03)
	R. phi	5.37 (1.5)	68.78 (6.39)	17.85 (4.69)	8 (0.63)	71.83 (1.39)	72.89 (1.09)	17.43 (2.18)	17.2 (1.31)	10.74 (0.93)	9.91 (0.21)	4.17 (0.51)	4.26 (0.38)	6.72 (0.53)	7.36 (0.05)
Digestive gland	**R. dec**	7.63 (1.8)	70.01 (1.71)	11.69 (0.87)	10.67 (1.14)	70.94 (1.52)	72.17 (0.35)	17.37 (1.49)	18.08 (0.41)	11.68 (0.39)	9.75 (0.06)	4.11 (0.44)	3.99 (0.11)	6.08 (0.26)	7.4 (0.01)
	R. phi	7.2 (2.48)	63.82 (2.26)	16.11 (3.11)	12.87 (1.39)	71.76 (0.6)	72.92 (0.52)	17.04 (0.69)	17.18 (0.62)	11.2 (0.28)	9.9 (0.1)	4.22 (0.2)	4.25 (0.19)	6.41 (0.16)	7.37 (0.02)
Adductor muscle	**R. dec**	9.24 (4.24)	74.73 (3.94)	6.98 (1.33)	9.05 (1.04)	69.22 (0.37)	71.6 (0.59)	19.81 (0.31)	18.77 (0.68)	10.97 (0.24)	9.62 (0.09)	3.49 (0.07)	3.82 (0.17)	6.31 (0.16)	7.44 (0.01)
	R. phi	7.43 (2.99)	74.72 (5.01)	5.53 (0.97)	12.31 (1.49)	68.84 (0.28)	70.99 (0.72)	20.41 (0.46)	19.5 (0.84)	10.75 (0.38)	9.51 (0.12)	3.38 (0.09)	3.65 (0.19)	6.41 (0.23)	7.46 (0.02)
Remaining tissues	**R. dec**	7.7 (1.52)	66.17 (3.42)	11.78 (2.21)	14.35 (1.03)	69.65 (0.22)	72.2 (0.49)	19.05 (0.33)	18.04 (0.59)	11.3 (0.34)	9.75 (0.09)	3.66 (0.07)	4.01 (0.15)	6.17 (0.2)	7.4 (0.02)
	R. phi	7.34 (0.83)	69.56 (4.55)	11.93 (3.83)	11.17 (1.32)	69.65 (0.3)	72.16 (0.7)	19.13 (0.39)	18.09 (0.83)	11.22 (0.32)	9.75 (0.14)	3.64 (0.08)	4.00 (0.23)	6.21 (0.19)	7.4 (0.03)

No consistent tendency was found for interspecific differences in the biochemical composition (*Table 3 - 3*).

Table 3 - 3. Results of two-way ANOVA on ranks testing the effects of tissue and species (as factors) on biochemical composition (% of Cho: carbohydrates, Prot: proteins, Lip: lipids and NPS)

	Cho	Prot	Lip	NPS
Tissue	**F = 9.543, p < 0.001**	**F = 5.486, p = 0.001**	**F = 14.387, p < 0.001**	**F = 14.670, p < 0.001**
Species	F = 1.641, p = 0.208	F = 0.330, p = 0.569	F = 3.734, p = 0.060	F = 2.365, p = 0.132
Tissue*Species	F = 0.174, p = 0.950	F = 2.315, p = 0.074	F = 2.153, p = 0.092	**F = 15.400, p < 0.001**

Conversely, differences between tissues were significant for all biochemical components, including NPS (*Table 3 - 3*). Carbohydrates were especially abundant in the adductor muscle and secondarily in the digestive gland and remaining tissues, whereas the percentage of proteins was high in adductor muscle and gill (*Figure 3 - 2* and *Table 3 - 2*). Lipids showed a tissue distribution pattern approximately opposite to that of carbohydrates, with high values recorded in the gonad, gill and digestive gland and minimum contents observed in the adductor muscle. Ninhydrin-positive substances reached the highest values in the remaining tissues, followed by digestive gland and adductor muscle. Despite the lack of overall interspecific differences, certain organs such as the digestive gland and gonad exhibited noticeable differences in biochemical composition between *R. decussatus* and *R. philippinarum*. In both organs, the lipid content was higher and the protein content was lower in the former than in the latter species (*Figure 3 - 2b, c*), as shown by the trends towards significant p-values of the tissue*species interaction term for proteins and lipids (*Table 3 - 3*). This interaction term was found to be highly significant for NPS ($p < 0.001$) as a consequence of the higher content of these substances in the digestive gland and adductor muscle of Manila clam, whereas the remainder of the tissues showed higher NPS values in the carpet shell clam.

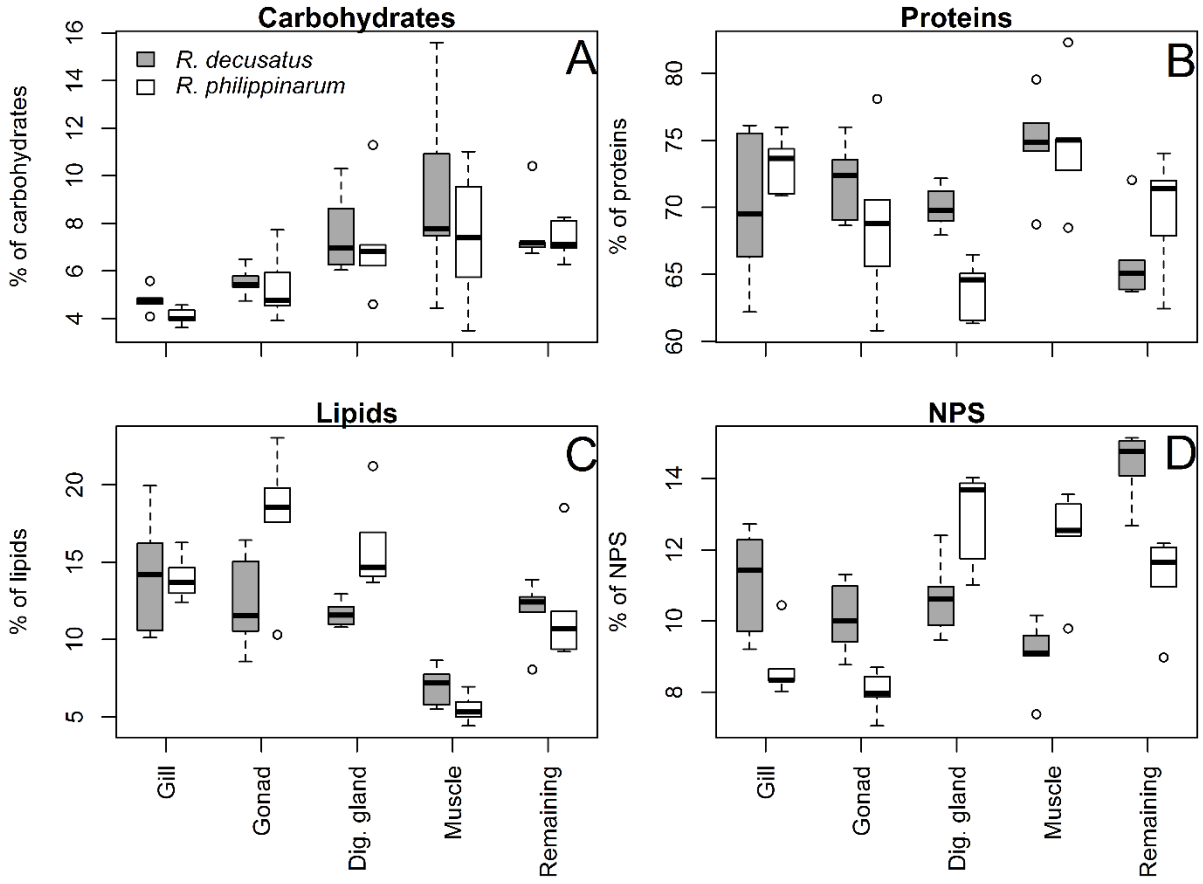

Figure 3 - 2. A) Carbohydrate, B) protein, C) lipid and D) NPS percentage in R. decussatus (gray) and R. philippinarum (white) by tissues

As reported for the biochemical composition, the elemental composition and the ratios C:N and C:H varied significantly among tissues (*Table 3 - 4*). Both methods rendered the highest C content in the digestive gland and gonad and the lowest in the remaining tissues and the adductor muscle (*Table 3 - 5*), whereas the N contents followed the opposite trend. Consequently, C:N ratios ranged between a maximum (4.11 – 4.22) in the digestive gland to a minimum (3.38 – 3.49) in the adductor muscle. Hydrogen contents, although significant among tissues, varied within a narrower range of between 11.62% (mean content of gills) and 10.86% (mean content of adductor muscle).

Table 3 - 4. Results of three-way ANOVA on ranks testing the effects of method, tissue and species (as factors) on the elemental composition (C, N and H) and C:N and C:H ratio

	C	N	H	C:N	C:H
Method	**F = 109.667, p < 0.001**	F = 3.099, p = 0.08	**F = 340.285, p < 0.001**	**F = 12.082, p < 0.001**	**F = 376.323, p < 0.001**
Tissue	**F = 15.142, p < 0.001**	**F = 17.960, p < 0.001**	**F = 9.219, p < 0.001**	**F = 16.926, p < 0.001**	**F = 8.384, p < 0.001**
Species	F = 2.682, p = 0.105	F = 0.585, p = 0.447	F = 2.411, p = 0.124	F = 0.809, p = 0.371	F = 3.577, p = 0.062
M*T	F = 1.963, p = 0.108	**F = 2.786, p = 0.032**	F = 2.258, p = 0.070	**F = 2.890, p = 0.027**	**F = 4.063, p = 0.005**
M*S	F = 0.432, p = 0.513	F = 0.053, p = 0.818	**F = 8.894, p = 0.004**	F = 0.006, p = 0.940	**F = 14.066, p < 0.001**
T*S	**F = 3.824, p = 0.007**	F = 2.394, p = 0.057	F = 0.174, p = 0.951	**F = 2.501, p = 0.049**	F = 0.490, p = 0.743
M*T*S	F = 0.148, p = 0.963	F = 0.165, p = 0.955	**F = 3.517, p = 0.011**	F = 0.172, p = 0.952	**F = 4.670, p = 0.002**

Table 3 - 5. Mean values (%) and post hoc (Tukey's HSD test) analyses for different tissues after three-way ANOVA on ranks analyses (see Table 3 - 4*) calculated for elemental components (C, N and H) and C:N and C:H ratios. Tissue elements include data from both species. Different superscripts indicate significant differences among tissues at a significance level of α = 0.05*

Tissue	Gill	Gonad	Digestive Gland	Adductor muscle	Remaining tissues
C	70.21 (0.29)^{ab}	70.95 (1.87)^{a}	71.35 (1.17)^{a}	69.03 (0.37)^{c}	69.65 (0.25)^{bc}
N	18.17 (0.27)^{bc}	17.84 (2.10)^{bc}	17.21 (1.11)^{c}	20.11 (0.48)^{a}	19.09 (0.34)^{ab}
H	11.62 (0.21)^{a}	11.20 (0.84)^{a}	11.44 (0.41)^{a}	10.86 (0.33)^{b}	11.26 (0.32)^{ab}
C:N	3.86 (0.07)^{ab}	4.03 (0.53)^{ab}	4.17 (0.33)^{a}	3.43 (0.10)^{c}	3.65 (0.07)^{bc}
C:H	6.04 (0.12)^{b}	6.37 (0.54)^{ab}	6.24 (0.27)^{ab}	6.36 (0.20)^{a}	6.19 (0.18)^{ab}

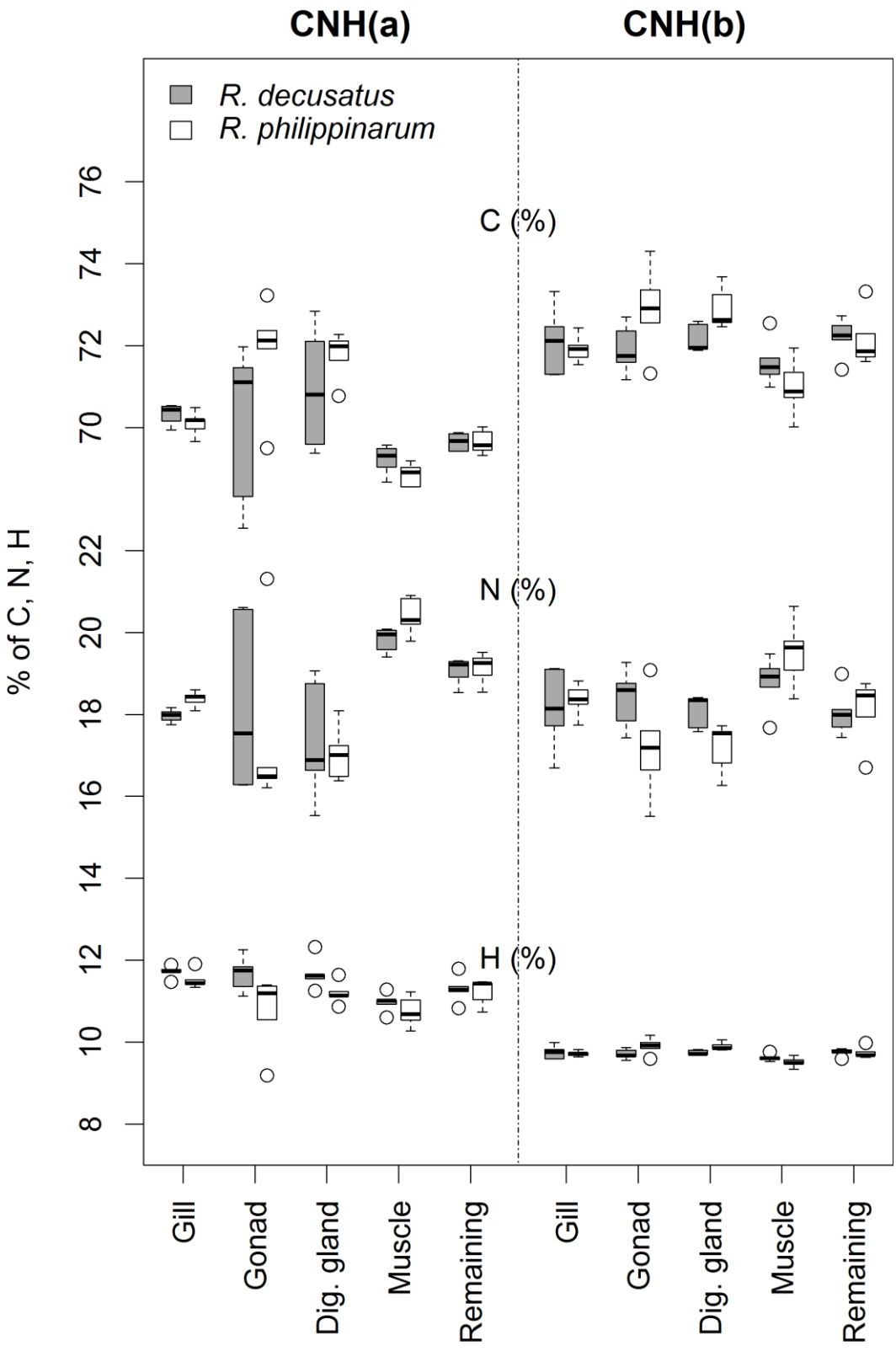

Figure 3 - 3. Carbon, nitrogen and hydrogen percentage in R. decussatus (gray boxes) and R. philippinarum (white boxes) by tissue estimated via elemental analyzer (CHN (a), left) and biochemical methods (CHN (b), right)

4. DISCUSSION

With respect to the compatibility of data on body tissue composition achieved with different analytical methods (elemental vs. biochemical analysis), this study demonstrates the suitability of the conversion factors proposed by Gnaiger and Bitterlich (1984) for application in studies with bivalves. As a consequence of the rather good level of agreement between methods, a direct correspondence was appreciated between percentages of biochemical components (*Figure 3 - 2*) and elemental components (*Figure 3 - 3*). Indeed, in a context of tissue- and species-specific variability, nitrogen matched the dynamics of protein plus NPS, whereas percentage carbon variation reflected a combination of lipid and carbohydrate content, with greater similarity to the lipid profile, given its higher proportion in most of the tissues. On the other hand, conversion equivalents used to estimate elemental composition (with the exception of H) could be the same, irrespective of species or tissues, as indicated by the virtual absence of significant interaction terms between the method and the other factors in the ANOVA (*Table 3 - 4*).

However, certain limitations of the validation procedure should be acknowledged: 1) The regression equations relating direct and biochemically estimated compositions for each element (C, H and N) (*Figure 3 - 1*) differ from the Y = X relationship expected in the case of complete correspondence, even though discrepancies between both methods are not significant in the case of N and amount to less than 3% for C. It is highly likely that this departure from strict agreement results from the rather narrow range of values fitted with the regression equations, which sets strict limits on the possibility of applying these conversion relationships for extrapolation out of the aforementioned range. Additionally, the effect of correcting for the %AFDW not accounted for by biochemical components should be considered (see Section 2: *Data analysis*) because the efficiency of extraction might differ in the different components. 2) The data for H revealed poor agreement between methods, which resulted in a nonsignificant regression equation (*Figure 3 - 1*), with direct measurements displaying a wider range of variation than estimations from biochemical components. Overestimation of %H in the direct measurement (13.7% compared with 9.74% in the indirect estimation) suggests hydration of tissue samples, not detectable in the biochemical analysis but liable to increase the H contents recorded with the elemental analyzer. It is worth noting that the above discrepancies remained after a correction for 6% residual water in dried samples was

performed following recommendations by Gnaiger and Bitterlich (1984) for samples of carp tissues. This result suggests that the amount of residual water in the dried tissues of bivalves might far exceed that of fresh water species, likely due to the hygroscopic effect of higher salt content. 3) In addition to H contents, direct and estimated measurements also differed for C contents and hence the C:N and C:H indices (see ANOVA in *Table 3 - 4*). More specifically, the C percentages derived from biochemical composition (mean of different tissues and species: 72.09%) are higher than those from direct estimation (mean: 70.24%), and this overestimation represents 2.6% of the average %C value. It is possible that standard conversion factors for C do not accurately reflect the actual composition of biochemical components in clam tissues, but most likely the reduced %C in the direct estimate is a mere consequence of increased H content caused by residual water. 4) The N percentages estimated from protein contents (mean: 17.4%) were significantly lower than those from direct estimation (mean: 18.6%), but a perfect match between direct and indirect methods of N determination was achieved after the incorporation of NPS (ninhydrin positive substances) in the biochemical composition. This outcome confirms the importance of nonprotein N in assessing the body composition of bivalves, given that current estimations have accorded as much as 10% of AFDW to the fraction represented by free amino-acids and other nitrogenous compounds serving the function of osmotic compensation (Hochachka and Somero, 2002).

The incoming discussion concerning species- or tissue-specific differences in composition is expected to rely on biochemical components, considering the good correspondence between both methods and the paucity of data reporting the elemental composition of tissues in clam species of the genus *Ruditapes*. Differences among tissues are the main source of variation in every biochemical compound (*Table 3 - 3*). Thus, proteins and lipids were abundant in supporting tissues involved in water pumping and gas exchanges, such as the gill and mantle, including the siphons. Gills in particular were essentially composed of proteins (72%) and lipids (14%) because that organ is characterized by a high surface area, including cilia, and thus a large amount of membrane is required to satisfy their functions. The remaining tissues, that comprised the foot and mantle, also included a significant amount of carbohydrates, likely reflecting the role of the mantle in storage of glycogen (De Zwaan and Zandee, 1972) as the preferential form of energy reserves in bivalves (Laing, 1993). However, the main carbohydrate depots were found in the adductor muscle, related to the energy supply for muscular control of

valve movements, although the use of this store to support gametogenesis has also been broadly documented in bivalves (Barber and Blake, 1985, 2006; Chantler, 2016; Kang et al., 2019). Previous studies in the Manila clam (Shiraishi et al., 1995) and the carpet shell clam (Ojea et al., 2004) performed in the same period of the year both revealed higher carbohydrate contents in the above group of tissues (gill, muscle and remaining tissues), with a consequent reduction in the lipid and protein levels with respect to this study. Although it is possible that differences in reproductive or energetic status might account for these discrepancies (Albentosa et al., 2007; Marin et al., 2003; Pérez-Camacho et al., 2003), the distorting effect of NPS (not quantified in the previous studies) on the proportions of the remainder of the components should also be considered.

No great interspecific differences in biochemical composition were found, although selected trends could be detected (*Table 3 - 6*). Overall, the proximate composition of each species falls within the range previously reported for *R. decussatus* (e.g., Albentosa et al., 1999; Aníbal et al., 2011; Ojea et al., 2004; Pérez-Camacho et al., 2003) and *R. philippinarum* (Baek et al., 2014; Marin et al., 2003; Robert et al., 1993), with the above-referenced exception for carbohydrates. This observation represents a minor discrepancy because this component has been reported to exhibit great seasonal and interannual fluctuations (Beninger and Lucas, 1984; Navarro et al. 1989).

Table 3 - 6. Mean (SD) values (%) of whole-flesh biochemical composition (Cho: carbohydrates, Prot: proteins, Lip: lipids, NPS: ninhydrin-positive substances) for each species

	Cho	**Prot**	**Lip**	**NPS**
R. philippinarum	6.22 (0.90)	69.42 (2.87)	14.59 (2.15)	9.77 (0.532)
R. decussatus	6.90 (1.08)	69.90 (1.09)	11.52 (1.01)	11.68 (0.47)

Lipids reached a higher level in *R. philippinarum*, which resulted in reduced values for carbohydrates and NPS, but only differences in the lipid component were statistically significant (*Table 3 - 3*). Particularly, interspecies differences in lipid contents were restricted to the gonad and digestive gland, which accounts for the significance of the tissue*species interaction term (*Table 3 - 3*). Lipid levels in both these organs are closely related to the reproductive cycle. The lipid contents of the gonad tend to peak during the spawning season (Bayne et al., 1982; Beninger and Lucas, 1984; Gabbott, 1983; Pieters et al., 1979), whereas digestive gland lipids are believed to be transferred to the gonad for gametogenesis purposes (Barber and Blake, 1985, 1981; Mori, 1975; Robinson et al., 1981). Therefore, rather than constituting interspecific variability, these

differences might reflect different reproductive stages at the time of collection (end of July) because *R. decussatus* is known to spawn between June and August (Aníbal et al., 2011; Ojea et al., 2004), whereas spawning is delayed to September-October in *R. philippinarum* (Robert et al., 1993). In addition, the latter species is characterized by a longer period of spawning and more intense reproductive activity (Delgado and Pérez-Camacho, 2007). In other words, it is highly likely that the carpet-shell clams used in this study had already spawned, whereas Manila clams were on the verge of spawning. Indeed, surplus specimens of both clams maintained in our facilities for up to four months from the end of experiments exhibited differential behavior with respect to spawning, with *R. philippinarum* clams spawning massively in the first week while *R. decussatus* showed no spawning activity at all for the entire period.

In conclusion, the equivalents reported by Gnaiger and Bitterlich (1984) were suited, within the ranges we explored, to the purpose of converting proximate biochemical composition into elemental (CHN) composition in bivalve tissues, although specific changes are required to include NPS as a highly significant component of the nonprotein N fraction. This observation sets limits for the reverse computation of biochemical components from elemental composition because no accurate estimation of proteins is possible from N contents. Additionally, in the case of bivalve tissues, a specific correction should be applied for residual water in dried samples for CHN analysis. The absence of broad differences found between species reflects the morphological, evolutionary and functional proximity between them, whereas the tissue differences displayed the differential role that each organ plays in the organism, even though other sources of variability such as diet and sex should be addressed in future research. Finally, the alleged interspecific differences in the biochemical and elemental compositions of specific tissues (digestive gland and gonad) might reflect a shift in timing of reproduction between both species, making plain the convenience of a seasonal approach in further studies linking physiological status to body tissue composition.

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Chapter 4

Physiology and stoichiometry of juvenile *Ruditapes decussatus* growth phenotypes under different carbohydrate/lipid proportion dietary scenarios

~ CHAPTER 4.1 ~

Growth and physiological performance of growth phenotypes of the carpet shell clam (*Ruditapes decussatus*) under mixed diets of different carbohydrate/lipid ratio

ABSTRACT

Fast and slow growing phenotypes, obtained by size-segregation from a same cohort of the carpet shell clam (*Ruditapes decussatus*), were acclimated to diets designed to present differences in their carbohydrate/lipid ratios and consisting in mixtures in variable proportions of live microalgae (high lipid content) and dried yeast pellets (high carbohydrate content). The aim of this study was to use physiological measurements and energy balance determinations to assess growth responses of these different phenotypes to both the acute and chronic changes in biochemical composition of the diets. Acclimation to the high lipid content diet resulted in higher scope for growth values for both phenotypes although differences with respect to the high carbohydrate content diets were greater in the fast growing group. This output was driven by both feeding and digestive responses resulting in enhanced acquisition of energy and absorption efficiency with the lipid-rich diets. Additionally, acclimation to these high quality diets, by improving physiological condition of clams, appear also to enable a better compensation of poor nutritional conditions helping to maintain energy balance levels in the acute exposure to low quality (carbohydrate-rich) diets. Main differences between fast and slow growing phenotypes are accounted for by the differential pattern of energy acquisition.

Key words: scope for growth, *Ruditapes decussatus*, growth phenotypes, carbohydrate/lipid ratio, microalgae-yeast mixed diets.

1. INTRODUCTION

Biochemical composition of suspended food has been a frequent topic in growth studies of marine bivalves particularly considering the specific benefits associated to the use of different microalgal species or combinations of species in an aquacultural context (Borowitzka, 1997; Epifanio, 1979a; Pronker et al., 2008). Regarding this subject, much less information exists however concerning the detailed analysis and quantification of growth through its physiological components integrated in an energy balance. In this experimental approach becomes essential the manipulation of food components in order to achieve a given range in biochemical composition of the food suited to explore the effects of this variable on specific physiological components of food and resulting scope for growth.

In a preceding contribution (Arranz et al., 2020; also Chapter 2.1), methods of physiological energetics were applied to address the effects of feeding on two diets, that were isocaloric but largely differed in their C:N ratios, on the growth rate of Manila clams. Both diets consisted of a same microalga species (*Rhodomonas lens*) that were harvested at different stages of the culture in order to achieve cell compositions characterized by high and low protein/energy ratios, corresponding to the exponential and stationary phases, respectively. Improved rates of growth observed with *R. lens* in the exponential phase correlated with greater scope for growth values, that resulted from increased feeding rates and absorption efficiencies recorded at high dietary protein inputs. In the present work we have pursued a complementary approach centered on exploring, using the same SFG methodology, the effects on growth of a battery of diets deploying a range of carbohydrate to lipid ratios. Thus, these diets differed in their energy contents but maintaining a same protein content and similar C:N ratios.

Specific nutritional interest of addressing the impact of carbohydrate to lipid ratio of the diet on rates of growth stems from the consideration that the energy reserves of phytoplankton, the main food component of bivalve diets in both natural or farming conditions, are in the form of lipids (Chen, 2012; Whyte, 1987), while marine bivalves store carbohydrates as a readily available reserve for maintenance (particularly under anaerobic conditions) as well as to sustain high energy demanding processes such as gametogenesis (Beninger and Lucas, 1984; Gabbott and Bayne, 1973; Rodríguez-Moscoso and Arnaiz, 1998; Zandee et al., 1980). Thus, some benefits could be expected from supplementing microalgal diets with carbohydrate-rich foodstuffs. On the other

hand, use of these supplements in the form of inert commercially available particles might reduce reliance on phytoplankton which has positive economic impact, as the production of live microalgae stocks in commercial bivalve hatcheries can account for up to 30% of the total production costs (Coutteau and Sorgeloos, 1993). Due to that, some attempts have been made to substitute live microalgal diets with less expensive sources of food, such as different types of flour or commercial concentrates of yeasts (e.g. Albentosa et al., 2003, 2002, 1999; Epifanio, 1979b; Fernández-Reiriz et al., 2006; Haven, 1965; Laing, 1987; Langdon and Siegfried, 1984; Maeda-Martínez et al., 2016; Mamat and Alfaro, 2014; Mazón-Suástegui et al., 2008; Pérez-Camacho et al., 1998). Most of those papers confirmed the feasibility of replacing, at least partially (15-50%), live monoalgal cultures by inert diets.

Experimental evidence concerning the effect of supplementing microalgae with carbohydrate-rich foodstuffs on growth and biochemical profiles of body tissues is particularly abundant in spat and juveniles (seeds) of the carpet shell clam *Ruditapes decussatus.* Reported information based on the use of mixed diets suggested a similar growth performance of clams feeding on pure microalgae suspensions *Isochrysis galbana* (T-ISO) than when cornstarch (Fernández-Reiriz et al., 1999; Pérez-Camacho et al., 1998), cornmeal (Pérez-Camacho et al., 1998) or wheatgerm (Albentosa et al., 1999; Fernández-Reiriz et al., 1998) substituted up to 50% the phytoplankton dose. The addition of cornstarch as well improved growth of *R. decussatus* respect to monospecific microalgal diets (Albentosa et al., 2003). The highest success in replacing phytoplankton was found when the same clam species was fed on a mix of microalgae species together with up to 80% of modified yeast suspensions (Albentosa et al., 1989).

Given this amount of information on the nutritional effects of formulated diets available in the carpet shell clams, the present experiments were conducted in this species (*R. decussatus*), using mixtures of the microalgae (*R. lens*) and the baker´s yeast (*Saccharomyces cerevisiae*) combined in different proportions to produce a range in the ratio of carbohydrates to lipids in the diet. These species of food were chosen on account of their similarity in cell size so as to prevent differential retention based on particle size that might interfere with the uniform utilization of both components of the diet.

Endogenous factors, possibly related with the genetic constitution of individuals, are known to greatly affect the growth rate of bivalves by modifying physiological

parameters, mainly those involved in energy acquisition (Bayne, 1999; Fernández-Reiriz et al., 2016; Prieto et al., 2018; Tamayo et al., 2011). On that basis, the analysis of interactive effects between these factors and dietary conditions is of the utmost interest, particularly in clams where previous studies performed by our lab have revealed that physiological responses to changes in both the quantity and biochemical composition of the food may be modulated differently in different growth phenotypes (Arranz et al., 2020; Tamayo et al., 2015). Consequently, present experiments were conducted in two separate groups of fast (F) and slow (S) growers that were obtained by size segregation on a single cohort of clams' spat.

These groups of F and S clams, each conditioned (acclimated) to the different diet compositions, were evaluated for differences in actual growth performance. The experimental groups that resulted from this combination between growth condition and diet of acclimation were then used in physiological experiments where the components of the energy balance and SFG were recorded while fed two diets of different biochemical composition each dosed at two different particle concentration (rations). Summarizing, aims of this study were to analyze the impact of food features related to biochemical composition and ration on the physiological components of growth, recorded in both the acute and chronic response to dietary changes and in clam's phenotypes selected for differential growth.

2. MATERIAL AND METHODS

Specimens of *Ruditapes decussatus* belonging to a same cohort were supplied by CIMA (Ribadeo, Spain) and brought to our facilities. Immediately after that, clams were classified as fast and slow growers attending to their size distribution in terms of weight and length, where percentiles 85 and 30 were, respectively, chosen to fit into those categories. Initial mean live weight representative of groups of fast and slow growing individuals were ≈ 300 and 150 mg, respectively. Before the start of experiments, clams were then monitored in the laboratory for 51 days while fed a mixture of *Rhodomonas lens* and *Isochrysis galbana*, clone T-ISO (1:1 in terms of packed volume) with a double purpose: a) to assure the viability of the stock of clam's seed in future experiments, which was achieved since that mortality rates were negligible (< 0.5%), and b) to confirm that the assigned growth categories based on size segregation maintained their condition of fast and slow growers, respectively, under a common feeding regime in laboratory conditions.

2.1 Animal maintenance and diet characteristics

Both fast and slow growing groups were distributed randomly into three different tanks that were maintained at constant and equal conditions of aeration, salinity (34‰) and temperature (17ºC). Tanks were cleaned from biodeposits and water changed on a daily basis. In all cases, the basis of the diet supplied was a mixture of the microalga species *R. lens* and the yeast *Saccharomyces cerevisiae* that differed between the three tanks in the proportion of both components. Microalgae was obtained from a lab culture freshly collected while the yeast derived from a commercial stock of dried pellets (baker's yeast Royal from Mondelez International) that were previously suspended in seawater. Yeast suspensions were tested for stability in preliminary assays, where no changes in total matter nor fluctuations in cell diameter (measured in a Coulter Counter Multisizer 3 (Beckman Coulter)) were appreciated within a 24 h period. Owing to that, stock suspensions were renewed every 24 h or less.

Although both species possess a median similar size (around 5 µm spherical diameter), percentages of mixtures were calculated based on packed volume, which was measured with the aid of a Coulter Counter Multisizer 3, fitted out with a 100 µm aperture tube. Mixed stocks of the diets were homogenized and maintained in suspension by magnetic stirrers inside beakers and dosed to the feeding tanks with a peristaltic pump at a concentration of 2 mm^3 L^{-1} (approx. 20000 cells mL^{-1}). Composition of diets is shown in *Table 4.1 - 1* where differences relied in the proportion of microalgae to yeast. In terms of biochemical composition, the main differences between diets referred to the carbohydrate/lipid proportions (*Table 4.1 - 1*). Diet characterizations were carried out twice a week, where a known volume of water was collected from the feeding tanks and filtered through pre-weighted glass fiber filters (GF/C). Salts retained in the filters were then rinsed out with ammonium formate (0.9% w/v), and dried for 24-48 h at 100ºC to estimate dry weight. Ash weight was computed after calcination for 6 h at 450ºC. Total particulate matter (TPM, mg L^{-1}) and particulate inorganic matter (PIM, mg L^{-1}) were determined through the dry and ash weight, respectively. The difference between these two parameters is known as particulate organic matter (POM, mg L^{-1}). Conversely, GF/C filters for elemental analysis (CHN) were rinsed out with 50 mL of filtered seawater and immediately frozen at -20ºC, lyophilized, and maintained at -20ºC until being analyzed. Analyses took place in the SGIker facilities (UPV/EHU), by means of a Euro EA

Elemental Analyzer (CHNS) from EuroVector, using acetanilide as standard. A subset of samples were calcined for 6 h at 450ºC and were subsequently measured in the elemental analyzer in order to subtract the inorganic C and N fraction.

Proximate composition of diets was determined in triplicate using colorimetric methods. Carbohydrates were extracted in TCA (5%) and quantified according to Dubois et al. (1956) using dried glycogen from oyster as a standard. Proteins were extracted in NaOH (0.4 N) and quantified according to (Lowry et al., 1951) with a bovine sero-albumin standard. Finally, lipids were pre-extracted in acetic acid (Phillips and Privett, 1979), extracted twice in methanol:chloroform at 2:1 and 1:2 (Bligh and Dyer, 1959; Folch et al., 1957), and quantified according to Marsh and Weinstein (1966) against a tripalmitin-phosphatidylcholine 1:1 standard.

Table 4.1 - 1. Composition, particle concentration (TPM and POM mg L^{-1}) and biochemical characteristics of experimental diets supplied. Biochemical and elemental components are expressed as the percentage of each over the sum of the components or elements, respectively

Diet	Composition	Carbo-hydrates	Proteins	Lipids	N	C	H	TPM	POM
D1	*R. lens*: 80%	15.47	50.33	34.20	14.45	68.93	16.62	1.09	1.01
	S. cerevisiae: 20%	(2.16)	(2.40)	(0.30)	(1.02)	(2.57)	(2.00)	(0.13)	(0.10)
D2	*R. lens*: 50%	24.03	49.09	26.87	14.39	65.90	19.72	1.15	1.08
	S. cerevisiae: 50%	(4.54)	(4.75)	(0.44)	(0.04)	(3.68)	(3.68)	(0.03)	(0.02)
D3	*R. lens*: 20%	32.60	47.85	19.55	13.99	67.76	18.25	1.16	1.07
	S. cerevisiae: 80%	(7.10)	(7.53)	(1.09)	(0.87)	(1.84)	(2.62)	(0.05)	(0.03)

2.2 Experimental design

Clams were maintained on the above diets for 30 days to let them acclimate before conducting the experiments. Once acclimation period was completed, the extremes in the conditioning range (D1 and D3) were selected for the analysis of physiological parameters under four feeding scenarios (see *Table 4.1 - 2*), each dosed at two different rations: the conditioning ration (2 $mm^3 L^{-1}$) and half of the former (1 $mm^3 L^{-1}$), henceforth denominated high (H) and low (L) ration, respectively. A summary of these arrangements is given in *Table 4.1 - 2*, where the 8 experimental diets tested are presented as combinations of the acclimation condition (D1 or D3) previous to the physiological experiments and the diet fed the animals during these experiments (each dosed to H or L rations). Note that that the diet of intermediary composition (D2) was solely employed for comparative growth measurements and no physiological determinations were performed under that diet. For each of the above 8 diets, physiological parameters of both

fast and slow growing clams were separately determined in groups (n = 5) of individuals (i.e., 80 experimental groups).

Table 4.1 - 2. Experimental dietary conditions obtained as crosses of acclimation diet composition (D1 or D3) and exposure diet composition (D1 or D3) and ration (H or L)

		Exposition diet			
Acclim. diet		*D1H*	*D1L*	*D3H*	*D3L*
	D1	D11H	D11L	D13H	D13L
	D3	D31H	D31L	D33H	D33L

2.3 Physiological determinations and Scope for Growth

Given that the small size of individuals prevented from performing individual determinations, all physiological measurements were carried out with animals arranged in groups of 5-7 individuals. Scope for growth measurements lasted four days for each food condition.

Clearance rate (CR, L h^{-1}) was measured by the flow-through chamber method of Crisp (1971), according to the following expression:

$$CR\ (L\ h^{-1}) = \frac{Ci - Co}{Ci} \times F$$

where Ci and Co are the particle concentrations at the inlet and outlet of the chamber, respectively, and F stands for the flow rate through the chamber. Groups of clams (n = 5) were placed in a 125 mL flask, arranged in a bath at constant temperature (17ºC), with constant supply of diet, where flow rates were regulated independently in each flask in order to produce 15-30% reductions of particle concentration. Number of measurements (12-16) were considered sufficient to characterize the filtering activity and were distributed along the daytime (from 8 a.m. to 8 p.m.) to integrate possible fluctuations in the physiological response associated to tidal rhythms (Bayne et al., 1988).

Organic ingestion rate (OIR, mg h^{-1}) was computed as the product of CR and POM.

Absorption efficiency (AE, decimal units) was estimated by the method of Conover (1966) while CR measurements were being conducted. Samples of both diet and feces

were collected, filtered on GF/C filters, and processed for TPM and POM following the same treatment described in Section 2.1. Organic content of diet and feces was then estimated as the fraction of ash free dry weight over the total dry weight of the sample. Absorption efficiency was calculated as:

$$AE = \frac{F - E}{(1 - E) \times F}$$

where F and E represent the organic content of the food and the feces, respectively.

Absorption rate (AR, mg h^{-1}) of organic matter was estimated as the product of OIR and AE. Absorption rate in energy units (*J h^{-1}*) was obtained by using the energy equivalents (26.289 and 19.960 J mg^{-1} for diets 1 and 3, respectively), based on both biochemical and elemental composition (Platt and Irwin, 1973).

Metabolic rate: was indirectly assessed as the rate of oxygen consumption (*VO_2, mL O_2 h^{-1}*). Groups of clams were placed in 150 mL sealed chambers filled with filtered seawater at constant temperature (17ºC) for 4 h. A LDO oxygen probe connected to a Hatch HQ40d oximeter registered the decline in oxygen concentration in the chambers along the time. A chamber without animals was used to correct for changes in oxygen concentration not associated to respiratory activity of animals. The rate of metabolic energy loss (R: *J h^{-1}*) was calculated using an energy equivalent or oxy-caloric coefficient of 20.08 J mL O_2^{-1} (Gnaiger, 1983).

Ammonia excretion rate (*VNH_4-N, µg NH_4-N h^{-1}*) was determined from rates of ammonia production by animals in open flasks filled with 80 mL of filtered seawater (0.2 µm Millipore membranes) at constant temperature (17ºC). Three subsamples per flask were extracted and the ammonia concentration of the water was determined according to the phenol-hypochlorite method (Solórzano, 1969), Two flasks without animals were used as a control and processed in the same way as the former. Rates of ammonia excretion were converted to energy equivalents (U: J h^{-1}) by using a conversion factor of 24.853 J mg^{-1} (Elliott and Davison, 1975).

O:N index was calculated as the ratio, in atomic equivalents, of oxygen consumed and nitrogen excreted.

Scope for Growth (SFG, J h^{-1}) was estimated according to the formula:

$$SFG = AR - (R + U)$$

All rates were divided by the number of specimens inside each flask to express the individual rates. To correct for size variation among group treatments, all physiological rates were standardized to a common dry weight of tissue (29.14 mg; obtained as the mean value of all individuals used in experiments), using the expression:

$$Y_{STD} = \frac{W_{STD}}{W_{EXP}}^{b} \times Y_{EXP}$$

where Y_{STD} and Y_{EXP} represent, respectively, the standardized and experimental rate, W_{STD} and W_{EXP} are the standard and experimental weights of individuals and b is the mass exponent scaling physiological rate to body size, according to own mass unpublished results: 0.6091, 0.6967 and 1.00, for CR, VO_2 and VNH_4, respectively.

2.4 Data analysis

Analysis of conditioning effects on growth rate were conducted through 2-way ANOVA (Factors: conditioning diet and growth group). Acute effects of diets fed during the experiments on the physiological parameters and SFG were tested (separately for each acclimation condition) in the context of a 3-way ANOVA (Factors: diet composition, ration and growth group). Analyses were performed after data were tested for normality (Shapiro-Wilk) and homoscedasticity (Levene).

All of the statistical analysis and graphical elaboration of figures was performed by means of the R software, version 3.5.1 (R Core Team, 2018).

3. Results

Among the three conditioning diets, growth reached a maximum (*Table 4.1 - 3*) in the diet containing the highest proportion of microalgae, followed by the intermediate diet, and finally, growth was drastically reduced in D3. Both (growth and diet) factors showed significant effects, where conditioning effect ($F = 3.85$, $p = 0.02$) contributed to a lesser extent than growth category ($F = 25.05$, $p < 0.001$), in spite of the high standard deviation. Summarizing, a same trend regarding the negative effect of reducing the proportion of microalgae in the diet was observed for both growth groups, that maintained a consistent difference (approximately 5x between F and S clams) except for the diet D3 where S calms presented growth rates reduced ~10x with respect to F clams. In other words, feeding on a diet that affected more negatively growth rates also increased the differences between fast and slow growing clams.

Table 4.1 - 3. Mean (SD) growth parameters (L: length, Wi: width, W: live weight) for F and S clams conditioned to the three diets (D1, D2 and D3) before and after the conditioning period

Condit.	G	Before conditioning			After conditioning			GR (mg d^{-1})	N
		L (mm)	Wi (mm)	W (mg)	L (mm)	Wi (mm)	W (mg)		
D1	F	14.41 (0.91)	9.51 (0.57)	449.97 (83.75)	14.74 (0.94)	9.98 (1.23)	484.48 (90.84)	1.4 (2.74)	122
	S	11.07 (0.89)	7.55 (0.74)	228.97 (76.59)	11.18 (0.98)	7.65 (0.82)	238.24 (86.01)	0.34 (2.49)	127
D2	F	13.65 (0.68)	9.01 (0.45)	374.8 (55.88)	13.78 (0.76)	9.1 (0.52)	399.71 (68.67)	1.02 (1.89)	147
	S	10.44 (0.55)	7.14 (0.5)	181.47 (49.07)	10.5 (0.65)	7.15 (0.58)	187.74 (55.29)	0.21 (1.47)	145
D3	F	14.57 (0.96)	9.57 (0.61)	457.23 (85.06)	14.63 (0.97)	9.61 (0.94)	471.12 (87.18)	0.57 (2.50)	122
	S	11.01 (0.91)	7.53 (0.69)	221.89 (71.72)	11.08 (0.91)	7.55 (0.7)	226.46 (71.17)	0.04 (1.94)	127

3.1 Acclimated response

Physiological responses were only recorded for the extreme diets; i.e., no physiological experiments were conducted on clams fed the D2 diet. Hence, as a reference for interpreting the growth response, effects of acclimation were compared on the basis of comparing the physiological behavior and SFG recorded in F and S clams fed the conditioning diets; i.e., D1 or D3 dosed at the high ration. Confirming the main results shown in *Table 4.1 - 3*, the scope for growth also showed significant differences associated to diet composition and growth category (*Table 4.1 - 4*), where D1 promoted higher SFG values than D3, especially in F clams. These effects on SFG, particularly as to the F *vs.* S differences, resulted from different physiological responses promoted by diets D1 and D3. For instance, D1 increased both the acquisition (due to a higher absorption efficiency) and the energy expenses of S clams. On the contrary, F clams maintained a higher acquisition when fed on D1, but without important increases in the metabolic expenses, thus being SFG increased to a higher extent in D1 respect to D3.

3.2 Acute response

In order to simplify the analysis of acute physiological responses to both quantity and quality changes in processed food, each acclimation group was considered separately. However, these responses were subsequently addressed comparatively between D1 and D3 conditioned clams.

*Table 4.1 - 4. Mean (SD) values of physiological parameters (CR: mL h^{-1}, AE: decimal units, AR: J h^{-1}, VO_2: µL h^{-1}, VNH_4-N: nL h^{-1}, and SFG: J h^{-1}) included in the energy balance of F and S clams that were conditioned to D1 and D3, and results of ANOVA analyses testing for significant effects of diet (D), growth category (G) and interaction effects between them (D*G)*

Diet	**D1**		**D3**		**Statistical differences**		
Growth	**F**	**S**	**F**	**S**	**D**	**G**	**D*G**
CR	119.98 (37.6)	96.31 (16.11)	165.12 (69.85)	125.96 (20.51)	-	-	-
AE	0.79 (0.08)	0.89 (0.02)	0.38 (0.1)	0.5 (0.08)	***	**	-
AR	3.42 (0.95)	1.96 (0.35)	1.79 (0.56)	1.25 (0.21)	***	**	-
VO_2	29.92 (4.68)	34.38 (15.07)	28.1 (15.67)	10.34 (5.83)	*	-	*
VNH_4-N	425.87 (93.13)	477.41 (118.54)	286.6 (84.79)	211.69 (74.44)	***	-	-
SFG	2.81 (1)	1.26 (0.53)	1.22 (0.7)	1.04 (0.16)	**	*	*

Clearance rate (*Figure 4.1 - 1*) showed significant differences (*Table 4.1 - 5*) in the response to exposure diets only in clams previously acclimated to D1, where a change from D1 to D3 promoted increases in their filtration activity (77% mean rise). Similarly, remarkable interindividual differences were displayed for D1 conditioned clams, F clams doubling filtration rates of S clams. Increases for those acclimated to D3 were not statistically different, although trends indicate higher CRs in F clams (27% mean increase). Overall, ration effects were not significant due to the contrasting behavior found with exposure diets D1 and D3: Rising particle concentration promoted a decline in CR with D1 composition while the opposite occurred for D3 (accounted for by the interaction term D*R, only significant for D3; *Table 4.1 - 5*).

Irrespective of conditioning diet, main effects on absorption efficiency were associated to features of food supplied during experiments (*Figure 4.1 - 2*). AE was significantly (46-47%) higher with diet D1 compared to D3 (*Table 4.1 - 5*). Increasing ration produced opposite effects on the AE recorded with diets D1 (positive effect) and D3 (negative effect) as accounted for the interaction D*R (*Table 4.1 - 5*). Differences between growth groups achieved less relevance, although S clams registered approximately 10% higher AEs than F clams.

Table 4.1 - 5. Main ANOVA results for clearance rate (CR), absorption efficiency (AE), absorption rate (AR), metabolic rate (R), ammonia excretion rate (U) and scope for growth (SFG) for clams previously conditioned to D1 and D3, for comparisons among exposure diets (D), ration (R) and growth category (G)

	CR	**AE**	**AR**	**R**	**U**	**SFG**
Acclimated to D1						
D	**F = 17.42, p < 0.001**	**F = 47.044, p < 0.001**	F = 0.659, p = 0.423	**F = 10.229, p = 0.003**	**F = 6.638, p = 0.015**	F = 2.576, p = 0.118
R	F = 0.521, p = 0.476	F = 0.101, p = 0.753	**F = 9.112, p = 0.005**	F = 2.085, p = 0.158	F = 1.74, p = 0.197	**F = 7.066, p = 0.012**
G	**F = 28.362, p < 0.001**	**F = 4.322, p = 0.046**	**F = 55.008, p < 0.001**	**F = 5.289, p = 0.028**	F = 0.134, p = 0.717	**F = 46.944, p < 0.001**
D:R	F = 0.767, p = 0.388	**F = 19.126, p < 0.001**	F = 2.444, p = 0.128	F = 2.082, p = 0.159	**F = 5.742, p = 0.023**	F = 1.438, p = 0.239
D:G	**F = 16.184, p < 0.001**	F = 0.488, p = 0.49	**F = 10.551, p = 0.003**	F = 0.139, p = 0.712	F = 1.041, p = 0.315	**F = 9.93, p = 0.004**
R:G	F = 0.019, p = 0.892	F = 2.793, p = 0.104	F = 0.277, p = 0.603	**F = 5.947, p = 0.02**	F = 1.357, p = 0.253	F = 1.271, p = 0.268
D:R:G	F = 0.024, p = 0.878	F = 3.22, p = 0.082	F = 0.864, p = 0.359	F = 0.802, p = 0.377	F = 1.842, p = 0.184	F = 1.306, p = 0.262
Acclimated to D3						
D	F = 0.462, p = 0.502	**F = 135.754, p < 0.001**	**F = 27.274, p < 0.001**	F = 2.502, p = 0.124	**F = 42.64, p < 0.001**	**F = 22.039, p < 0.001**
R	F = 0.086, p = 0.771	**F = 38.272, p < 0.001**	**F = 5.971, p = 0.02**	F = 2.356, p = 0.135	**F = 8.191, p = 0.007**	F = 3.543, p = 0.069
G	F = 2.977, p = 0.094	**F = 6.137, p = 0.019**	**F = 26.357, p < 0.001**	**F = 6.302, p = 0.017**	F = 1.66, p = 0.207	**F = 18.213, p < 0.001**
D:R	**F = 6.669, p = 0.015**	**F = 31.518, p < 0.001**	F = 0.15, p = 0.701	F = 0.737, p = 0.397	F = 0.989, p = 0.327	F = 0.003, p = 0.96
D:G	F = 0.35, p = 0.558	**F = 4.355, p = 0.045**	F = 3.244, p = 0.081	F = 2.726, p = 0.108	F = 0.182, p = 0.672	**F = 6.265, p = 0.018**
R:G	F = 0.28, p = 0.6	F = 2.692, p = 0.111	F = 0.612, p = 0.44	F = 0.106, p = 0.746	F = 3.581, p = 0.068	F = 0.47, p = 0.498
D:R:G	F = 0.137, p = 0.713	F = 0.597, p = 0.445	F = 0.004, p = 0.951	F = 0.423, p = 0.52	F = 1.622, p = 0.212	F = 0.035, p = 0.853

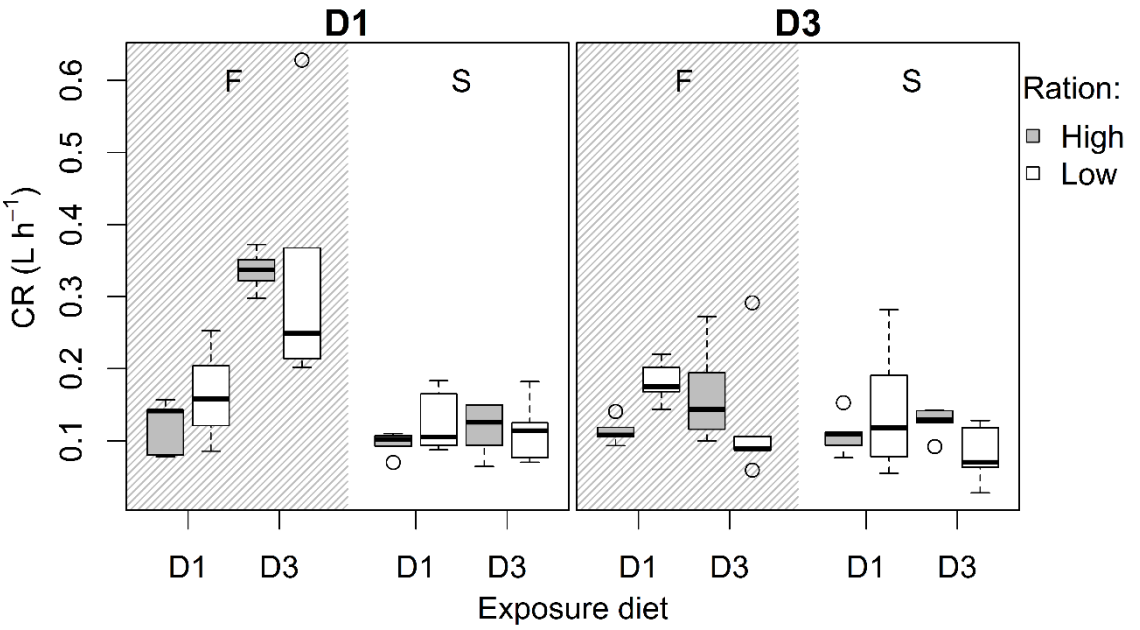

Figure 4.1 - 1. CR (standardized values) of F (shaded areas) and S (white areas) clams exposed to diets D1 and D3. Gray and white boxes stand for high and low ration, respectively. Results for specimens conditioned to diets D1 and D3 are shown in left and right panel, respectively

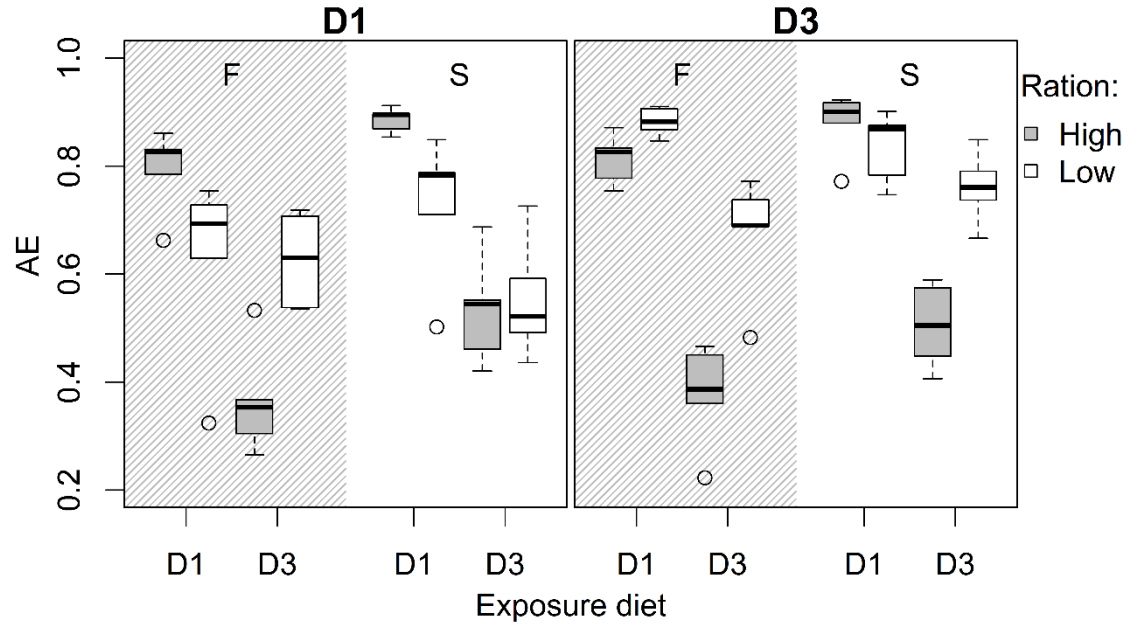

Figure 4.1 - 2. AE values of F (shaded areas) and S (white areas) clams exposed to diets D1 and D3. Gray and white boxes stand for high and low ration, respectively. Results for specimens conditioned to diets D1 and D3 are shown in left and right panel, respectively

Absorption rates reflected the effects of ration in the form of a general increment associated to high food concentration (*Table 4.1 - 5*); however, such effects were partly compensated with diets D3 due to the declining behavior of AE at high ration. Consistent differences in CR between growth groups also resulted in rates of absorption that were

significantly higher (2 – 2.5 times) in F clams compared with S clams (*Table 4.1 - 5*). *Figure 4.1 - 3* shows a general tendency for absorption rates to decrease with declining the ration, food quality (D1 vs D3) or growth performance (F vs S), with the sole exception of F clams conditioned to D1 and fed the diet D3 where a strong overfeeding response was recorded (see *Figure 4.1 - 1*).

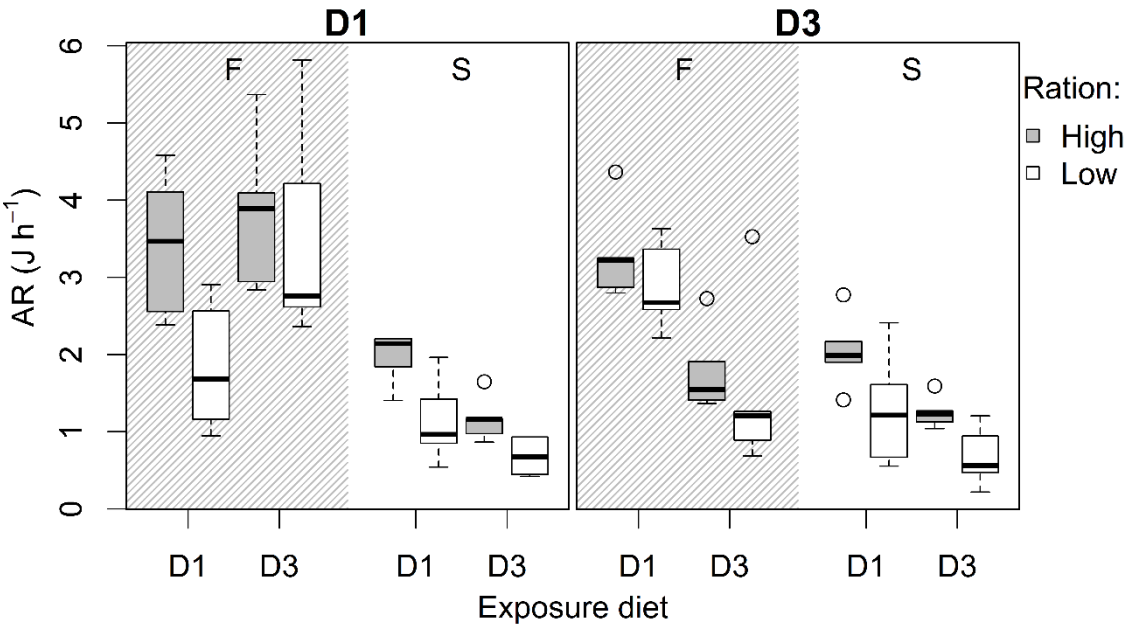

Figure 4.1 - 3. AR (standardized values) of F (shaded areas) and S (white areas) clams exposed to diets D1 and D3. Gray and white boxes stand for high and low ration, respectively. Results for specimens conditioned to diets D1 and D3 are shown in left and right panel, respectively

Main differences in metabolic rates (*Figure 4.1 - 4*) were found between growth groups, with higher rates recorded for F clams; however, large intragroup variability reduced the significance of these effects (*Table 4.1 - 5*). Also, rates of metabolism tended to decline in clams fed on D3 diet, although this effect was only significant following acclimation to the D1 diet (*Table 4.1 - 5*). No significant effects of ration were recorded except for S clams (R*G interaction) acclimated to D1 (*Table 4.1 - 5*).

Ammonia excretion rate (*Figure 4.1 - 5*) constituted a minor component in the energy budget, and indeed only around 2% of metabolic energy expenditure corresponded to ammonia releases. Yet responses were consistent as to the increased N excretion rates recorded on exposure to D1 compared with D3, irrespectively of the acclimation diet (*Table 4.1 - 5*). Feeding a high ration also increased ammonia release, with different intensity in clams acclimated to D1 (20% increase) and D3 (50% increase). No significant differences in rates of excretion were detected between growth groups.

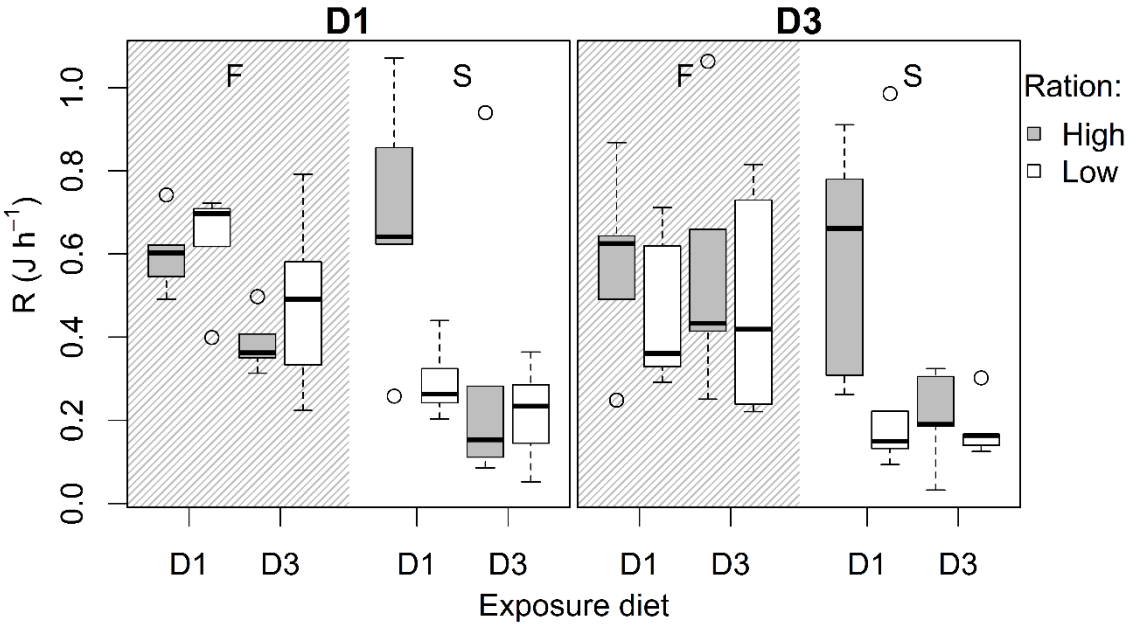

Figure 4.1 - 4. R (standardized values) of F (shaded areas) and S (white areas) clams exposed to diets D1 and D3. Gray and white boxes stand for high and low ration, respectively. Results for specimens conditioned to diets D1 and D3 are shown in left and right panel, respectively

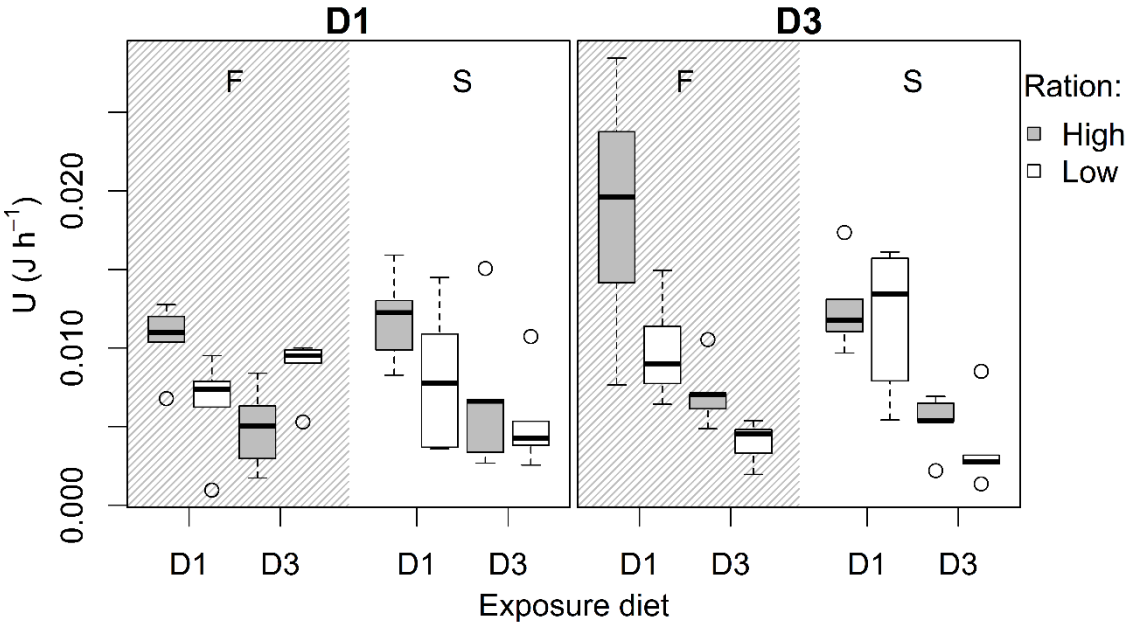

Figure 4.1 - 5. U (standardized values) of F (shaded areas) and S (white areas) clams exposed to diets D1 and D3. Gray and white boxes stand for high and low ration, respectively. Results for specimens conditioned to diets D1 and D3 are shown in left and right panel, respectively

Scope for growth (*Figure 4.1 - 6*) largely reflected the behavior of absorption rates (*Figure 4.1 - 3*), with growth condition exerting highly significant effects in both acclimation diets (*Table 4.1 - 5*). On average, values for F clams attained over twice the values of S clams (2.23 and 0.91 J h^{-1}, respectively). Also average values of SFG were

higher in clams acclimated to D1 compared to D3; although not statistically tested, these differences associated to the composition of conditioning diet were mainly associated to the behavior of fast growers (*Figure 4.1 - 6*). Compared with feeding the D1 diet, there is a general decline in the SFG values recorded with the D3 diet, which is mainly due to the reduction in AE values observed with this last diet, especially when fed the high ration (*Figure 4.1 - 2*). This effect of food composition was more intense in slow compared with fast growing clams (as accounted for the significant interactions D*G; *Table 4.1 - 5*). In contrast with the general behavior of clams acclimated to the D3 diet, F clams acclimated to the D1 diet were found able to compensate the negative effects of reduced food quality by means of a positive feeding response, resulting in the absence of significant differences in SFG between D1 and D3 diets (*Table 4.1 - 5*).

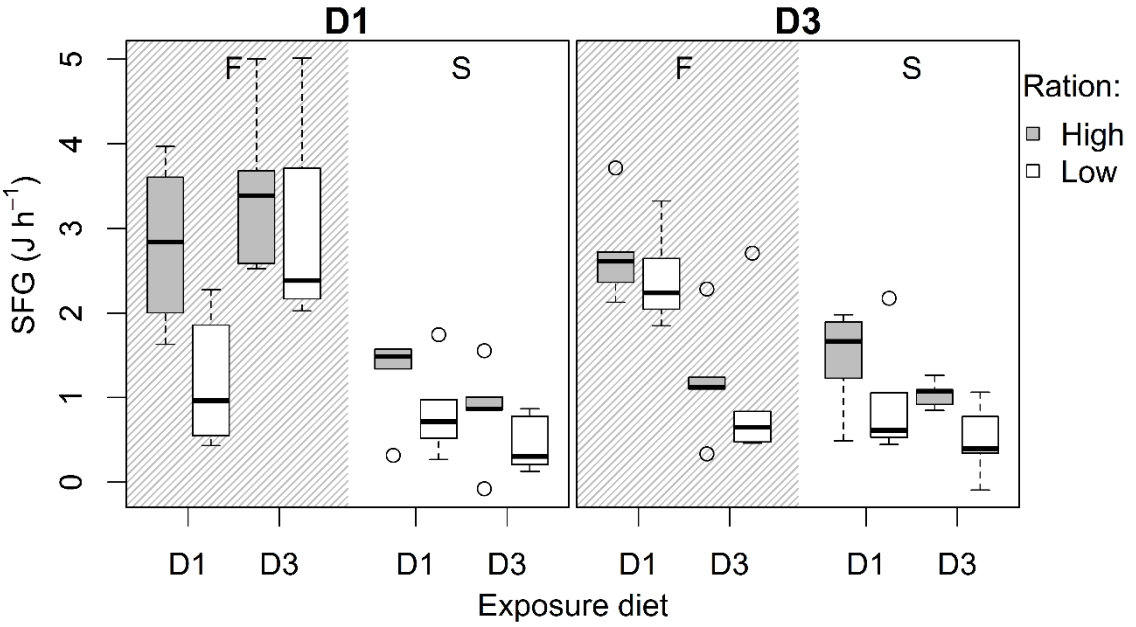

Figure 4.1 - 6. SFG (standardized values) of F (shaded areas) and S (white areas) clams exposed to diets D1 and D3. Gray and white boxes stand for high and low ration, respectively. Results for specimens conditioned to diets D1 and D3 are shown in left and right panel, respectively

4. Discussion

Actual measurements of growth and energy balances recorded in juvenile *Ruditapes decussatus* reflected a combination of effects associated to both endogenous and exogenous factors related to features of food suspensions. Overall, growth conditions improved with increasing the proportion of phytoplankton relative to yeasts in the diet that, in terms of biochemical composition, represented both a negative effect of increasing the carbohydrate to lipid ratio and a positive effect of energy content. Indeed,

physiological components of growth performed better in clams either acclimated or exposed to D1 diets relative to D3 diets and also benefited more from the increase in ration with the high quality diets. Similarly, actual growth determinations based on size increments during conditioning revealed a continuous decreasing trend from D1 to D3. Original differences in growth performance between F and S clams also increased from D1 to D3, which reflect a higher ability for F individuals to maintain a comparatively higher rate of growth than S specimens under poorer feeding conditions. Similar results were appreciated in Chapter 2.1 / Arranz et al. (2020), where interindividual differences in growth rates reached a maximum when conditioned to the N limited diet.

Much of the above dietary effects, differentially expressed in fast and slow growers, rely on the feeding response, as exemplified in D1 conditioning by the increase in clearance rates observed in F clams when fed the D3 diets at both rations (*Figure 4.1 - 1*). That increase can be interpreted to act as a compensatory mechanism for the general reduction in absorption efficiencies (*Figure 4.1 - 2*) that will be discussed later. Hence, this response resulted in regulation of absorption rates that reached similar values with both regimes of diet exposure (*Figure 4.1 - 3*). Since no such response was observed in D3 acclimation it becomes possible that composition of D1 diet had provided, along the conditioning period, the appropriate nutritional background (i.e., energy resources) required to sustain such a compensatory response. This response was also restricted to F individuals, revealing that increased endogenous capacity for food collection would be a key feature underlying better growth performance. Traits of physiological behavior fitting the energy acquisition model (Bayne, 1999) have been largely reported in the congeneric clam species *Ruditapes philippinarum* (Arranz et al., 2020; Tamayo et al., 2015, 2013, 2011) where a greater capacity for feeding and absorption characterizes fast growing phenotypes. On the contrary, conditioning to D3 diet seemed to limit not only growth, but also to cancel the potential to increase feeding rates exhibited by F clams after D1 conditioning. These contrasting effects of conditioning diet suggest that nutritional status would set limits for some effects of endogenous factors to be displayed.

Digestive behavior of food ingested with the different exposure diets is virtually the most important component of the physiological response as regards to growth determination. The reduction of absorption efficiency associated to food composition, from exposure diet D1 to D3, was highly significant in all conditions (*Table 4.1 - 5*), and

this quality-dependent effect appeared reinforced in clams conditioned to the D3; the combined acute and chronic effects of decreasing food quality resulted in an average decrease by ~ 50% in the AE (*Table 4.1 - 4*). Rates of absorption -the net energy gain- and SFG values reflected the above effects of food composition on digestive performance, that concomitantly with overall positive effects of ration and interindividual differences in feeding rates accounted for by the sequence of values shown in *Figure 4.1 - 3* and *4.1 - 6*, where F clams ranked above S clams and clams fed the D1 and high ration ranked above those fed the D3 and low ration (with the exception already discussed of F clams conditioned to D1and fed D3).

Opportunities brought about by increasing food concentration (ration) as regards energy gain are however different between D1 and D3 since absorption efficiency was virtually independent on ingestion rate with diets D1 while found to sharply decline with the increasing rates of ingestion of D3 (*Figure 4.1 - 7*), thus party canceling out the benefits of feeding at high ration of this diet. In addition to the overall reduction in AE, strict reliance of this efficiency on gut transit time of food, which is implicit in this relationship for D3, has been identified in several bivalves as a trait characteristic of gut processing of poorly digestible items such as phytodetritus, particularly of vascular plant origin (Arambalza et al., 2014; Navarro et al., 2016, 2009) or low quality phytoplankton (Arranz et al., 2020). Digestibility can be assumed to result from the substrate specificity of the digestive enzyme pool fitting the biochemical composition of food. Unlike digestive processing of phytoplankton, it is uncertain as to whether enzymes in the gut of bivalves might be fully effective in the digestion of *S. cerevisiae* cells, that is the main component (80%) of D3 diets. Digestive breakdown of glycogen, the main energy reserve in yeasts (up to 40% in weight; Deshpande et al., 2011; Lillie and Pringle, 1980), is very likely performed by α-amylases, abundantly present in both the digestive gland and crystalline style of bivalves (Ibarrola et al., 1999, 1998; Reid, 1968; Seiderer et al., 1982) but it is much more problematic to ascertain the effectivity of the cellulolytic complex, primarily designed for the cleavage of phytoplankton cell wall (Brock, 1989), against the polysaccharide composition of the yeast´s cell wall including different beta-glucans, mannans and chitin (Aguilar-Uscanga and Francois, 2003). In this respect, it is meaningful that the only case reported in the literature of an effective replacement (up to 80%) of phytoplankton by yeast in the production of *R. decussatus* spat occurred when

these yeast products had been manipulated to increase digestibility (Albentosa et al., 1989).

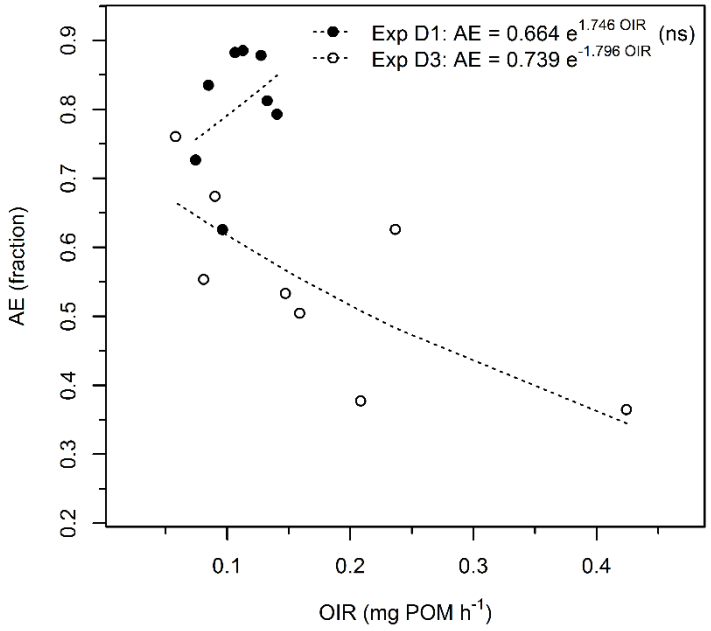

Figure 4.1 - 7. Relationship between AE and OIR for each exposure diet (D1: closed circles and D3: open circles). Fitted regressions were only significant for clams exposed to D3

Moreover, benefits of phytoplankton in the diet of clams might not only rely on its better digestibility but also on the provision of more appropriate nutrient balances. As the main structural components of tissues, proteins have been addressed as the key constituent in supporting growth (Bayne, 2009; Brown et al., 1997; Gremare et al., 1997; Hawkins and Bayne, 1991) but all diets tested in this study has a same protein content. However, increased proportion of carbohydrates at the expenses of lipids in the diet with the greatest degree of substitution of microalgae by yeasts (e.g., D3; *Table 4.1 - 1*) might result in nutrient limitation under some conditions. Digestion in bivalves comprises a great amount of intracellular processes hold by the digestive gland that results in the breaking off and subsequent release of digestive cell apices in the form of membranous "fragmentation spherules" (Morton, 1983; Purchon, 1971) which are reputed to have a high lipid content (Owen, 1972). This forms the bulk of metabolic fecal losses (MFL; Hawkins and Bayne, 1985), an organic component of endogenous origin voided with the feces that has been reported to constitute a very relevant although variable component in digestive balance of bivalves: For instance, MFL were quantified in cockles (*Cerastoderma edule*) to represent between 12% (in readily digestible diets like phytoplankton) and 26% (in diets more refractory to digestion like vascular plant detritus)

of organic ingestion (Navarro et al., 2009). Given the abundance of lipids in MFL composition, the above is consistent with the finding that the digestive balance of lipids was drastically reduced (even to negative values) under feeding conditions involving high MFL (Ibarrola et al., 1998, 1996). Those conditions are very likely represented by diets D3 in the present case, where high demand of dietary lipids to offset digestive constraints met in the form of MFL would occur concomitantly with the dietary restriction of this biochemical component. Such limitation, along with reduced enzymatic digestibility would account for most of the restrictions to net energy gain found with this diet in comparison with diet D1**.**

Metabolic expenses exerted a minor impact of energy balances, in such way that effects of factors on both AR (*Figure 4.1 - 3*) and SFG (*Figure 4.1 - 6*) were comparable. However, the amount of energy respired per unit of energy absorbed (R/AR), which approaches the metabolic costs of growth, revealed a trend ($F = 2.96$, $p = 0.09$) for F clams possessing lower unitary metabolic costs than slow growers (F: 0.23 (0.15); S: 0.32 (0.28)), which did not reached statistical significance due to the high deviance, despite a mean 27% decrease in F clams compared with S clams. Many physiological works analyzing interindividual variability on growth reported this behavior of fast growers possessing lower costs of growth than their slow growers counterparts (e.g. Bayne, 2000; Bayne et al., 1999; Bayne and Hawkins, 1997; Fernández-Reiriz et al., 2016; Hawkins and Day, 1996; Pace et al., 2006; Tamayo et al., 2016, 2015, 2014, 2013, 2011), fitting the metabolic efficiency model (Bayne, 1999).

As a conclusion, the present study reported that *Ruditapes decussatus* juveniles fed on diets differing in terms of carbohydrate/lipid proportion showed *a)* an enhanced acquisition response when the high lipid content diet (D1) was dosed, *b)* that dietary conditioning effects of D1 improved physiological condition that enabled to face acute nutritional impoverishments by physiological compensation, *c)* that main diet-related effects occurred at the feeding and absorptive level, and *d)* that interindividual physiological differences mainly relied differential acquisition ability, thus fitting to the acquisition efficiency model.

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~ CHAPTER 4.2 ~

Differential stoichiometric responses of *Ruditapes decussatus* growth phenotypes to variable carbohydrate/lipid content diets

ABSTRACT

Juveniles of the carpet shell clam (*Ruditapes decussatus*) from the same cohort were size-segregated to obtain fast and slow growing phenotypes that were subsequently acclimated to diets presenting a range of carbohydrate/lipid proportions but similar C:N ratios. Experiments were then conducted to determine elemental (C and N) balances as well as proximate composition of body tissues so as to fulfill the following aims: *a)* to identify strategies of homeostatic nutrient regulation as related to either endogenous (growth phenotype) or dietary factors; *b)* to quantify the degree to which stoichiometric adjustments (at both pre and postabsorptive levels) are accomplished throughout the successive components of elemental balances; *c)* to assess differences in the biochemical composition of body tissues associated to both conditioning diet and growth phenotype; *d)* to evaluate the level of concordance, in terms of elemental composition, between body tissues and the retained fraction of elemental balances. Elemental balances of both C and N achieved higher values under the lipid-rich diets, which points out to nutritional limitations in juvenile clams fed on high carbohydrate/lipid proportion resulting from a lower digestibility likely coupled to digestive lipid imbalance derived from limited dietary lipid income. These nutritional limitations were better dealt with by fast growing phenotype pointing to the importance of improved energetic status in maintaining homeostatic nutrient regulation. Stoichiometric coupling between consumed diets and biosynthetic requirements of growing tissues relied on postabsorptive rather than preabsorptive mechanisms although important differences in this respect were found between conditioning diets. Lastly, proximate biochemical composition of body tissues reflected that of diets, which indicates a poor level of homeostatic compensation for the nutritional imbalances induced in the present experiments, and thus suggesting that microalgal replacements by inert diets above 50% can compromise growth performance as well as lipid and protein content.

Key words: Growth phenotypes, *Ruditapes decussatus*, stoichiometric balances, proximate composition, carbohydrate/lipid proportion

1. INTRODUCTION

Bivalve growth and effects of diets on growth has attracted considerable attention (Gosling, 2015). Classical energy balance works have been enriched in the past years by the addition of analyses of nutrient acquisition performance (e.g. Bayne et al., 1993; Cranford, 1995; Hawkins and Bayne, 1985; Iglesias et al., 1996; Smaal and Vonck, 1997; Urrutia et al., 1996); which, under the viewpoint of ecological stoichiometry principles (Sterner and Elser, 2002), might provide a deeper understanding of dynamics of the physiological processes that model growth. As stated by Sterner and Elser (2002, but see also the Discussion section in Chapter 2.2), regulation of elemental stoichiometry of animals can be set at three levels: *1)* preingestive selection, in which animals preferentially select foodstuff that suit best their needs; *2)* adjustments in assimilation patterns, that implies selective up- or down-regulation of specific elements; and *3)* metabolism, which allows the elimination of a nutrient when it is in excess. These strategies, aimed to maintain animal's homeostasis, reflect that computations of exclusively energy balances might lead to incomplete conclusions, especially for analyses where different composition diets are tested. Elemental-based net budgets of fast and slow growing juveniles of *Ruditapes philippinarum* were analyzed in a previous subchapter (Chapter 2.2) to evaluate responses to isocaloric monoalgal diets differing in terms of C:N ratio, where protein content resulted the differential element on diets composition. In that work, stoichiometric regulation processes relied on differential absorption for N and stoichiometric N release; but also an endogenous decoupling between the amount of energy and net incorporation of elements was detected, underlining the relevance of the computation of nutrient fluxes.

Performance of *Ruditapes decussatus* juveniles on net energy balances feeding on mixed diets based on different mixtures of microalgae and yeast (Chapter 4.1) revealed positive growth and energy balances for both diets, although the diet containing more proportion of yeast resulted less suitable for both fast and slow growing clams. Even though replacements with yeast up to 80% had been previously described as feasible for growing *R. decussatus* specimens (Albentosa et al., 1989), previous subchapter results pointed out that growth might be restrained at high yeast contents in the diet. Diets provided in the previous experiment (Chapter 4.1) were based on a similar C:N ratio composition, where changes in biochemical profile entailed essentially differences in terms of proportions of lipids and carbohydrates. Although diet C:N composition did not

differ as in Chapter 2.2, on account of the outcomes found in Chapter 4.1, we expected some sort of nutritional limitation in clams reared at low phytoplankton and high yeast doses that would explain the differences found for both diets, especially regarding F clams; which could be related to an uncoupling between energy and elements incorporation, as happened in Chapter 2.2. Therefore, in this work we use the same experimental approach, including the selection of fast (F) and slow (S) growth phenotypes, to analyze the effects of rearing juveniles of the carpet shell clam *R. decussatus* with diets differing in biochemical composition on both the elemental balances measured under variable dietary conditions and the biochemical composition of body tissues. Aims were *a)* to identify strategies of homeostatic nutrient regulation in the form of differential processing of elemental components related to either endogenous or dietary factors; *b)* to quantify the degree to which stoichiometric adjustments are accomplished throughout the successive components of elemental balances, specifying their distribution between pre and postabsorptive processes; *c)* to assess differences in the biochemical composition of body tissues associated to both conditioning diet and growth phenotype; *d)* to evaluate the level of concordance, in terms of elemental composition, between body tissues and the retained fraction of elemental balances.

2. MATERIAL AND METHODS

Specimens (2600) of *Ruditapes decussatus* belonging to a same spat cohort were supplied by CIMA (Ribadeo, Spain) and brought to our facilities where they were divided in three different groups: *1)* A group of 49 clams, representative of the full range of sizes (~ 10 – 17 mm shell length), designed for the analysis relationships between some biometrical parameters of body size, and *2)* two other groups obtained by size segregation, based on shell lengths, of larger (percentile 85) and smaller (percentile 30) individuals designed to constitute the groups of fast and slow growing clams, respectively.

2.1. BIOMETRICAL ANALYSIS

After shell lengths were measured with Vernier calipers (range 10.1 – 16.9 mm), clams were dissected out from the shell and the dry weights (DW) and ash weights (AW) of soft body and empty shells of each individual separately recorded after drying at 100ºC for 24-48 h and combustion at 450ºC for 6 h, respectively.

Subsequently, *organic contents* (OC%) of both, soft and hard tissues, were obtained as the percentage of the difference of dry and ash weight (ash free dry weight: AFDW) over dry weight:

$$OC\% = \frac{DW - AW}{DW}$$

These measurements were also used in the computation of the *condition index* (CI), calculated as the percent ratio of soft tissues (DWt) to hard tissues (DWs) dry weights:

$$CI\ (\%) = 100 \times \frac{DWt}{DWs}$$

2.2 Animal maintenance, diet characteristics and experimental design

Detailed information on diets composition, animal maintenance and the experimental design is reported in a previous subchapter (Chapter 4.1, Materials and methods, sections 2.1-2.2); although in this section, a brief description is provided.

Fast (about 400 mg live weight) and slow (approx. 200 mg live weight) growing specimens of the carpet shell clam *Ruditapes decussatus*, obtained as described above, were fed on three diets of different composition.

Animals were maintained in three separate feeding tanks maintained in the same conditions of salinity (34‰) and temperature (17ºC), each tank receiving a different composition of food (diet) dosed at the same concentration of 2 $mm^3\ L^{-1}$ (approx. 20000 cells mL^{-1}); tanks were cleaned from biodeposits and water changed on a daily basis. Diet compositions consisted of mixtures of the microalgae species *Rhodomonas lens* (freshly collected) and the baker's yeast (commercial brand Royal from Mondelez International) *Saccharomyces cerevisiae* (dried pellets) in the following proportions *R. lens: S. cerevisiae*, in terms of packed volumes of both types of particles: 80:20 for Diet 1 (D1); 50:50 for diet 2 (D2) and 20:80 for diet 3 (D3). The most important difference among diets in terms of biochemical composition relied on the gradual decrease in the ratio of lipids to carbohydrates from D1 to D3. For further details, see *Table 4.1 - 1* (Chapter 4.1).

Diet was characterized frequently during the conditioning period as well as in the course of acute exposure experiments, by filtering a known amount of water onto pre-

weighted glass fiber filters (GF/C). Particle concentration and organic content of suspended food determinations were based on gravimetric measurements. After, salts retained in the filters were rinsed out with ammonium formate (0.9% w/v), filters were dried for 24-48 h at 100ºC to estimate dry weight and subsequently burned out for 6 h at 450ºC to estimate ash weight. Total particulate matter (TPM, mg L^{-1}) and particulate inorganic matter (PIM, mg L^{-1}) were determined through the dry and ash weight, respectively, and particulate organic matter (POM, mg L^{-1}) was obtained as the difference TPM – PIM. As for elemental analysis (CHN), GF/C filters were rinsed with 50 mL of filtered seawater and immediately frozen at -20ºC, lyophilized, and maintained at -20ºC until being analyzed. Analyses took place in the SGIker facilities (UPV/EHU), by means of a Euro EA Elemental Analyzer (CHNS) from EuroVector, using acetanilide as standard. A subset of samples were ashed for 6 h at 450ºC and were subsequently measured in the elemental analyzer in order to correct for the inorganic C and N fractions.

Clams were maintained on the above diets for 30 days to let clams to acclimate to feeding conditions before conducting the experiments, that consisted in physiological determinations required in both energy and elemental balances performed on the groups of clams acclimated to D1 and D3, each acclimation group measured during feeding the D1 and D3 diets dosed a two different rations, the acclimation ration (2 mm^3 L^{-1}) and half of that (1 mm^3 L^{-1}). For clarification purposes, see *Table 4.1 - 2* from (Chapter 4.1), where group arrangements for acclimation and exposure feeding conditions are displayed.

2.3 Determinations of elemental balances

Physiological determinations of scope for growth needed to compute the elemental balances were described previously (Chapter 4.1), where, in short, five replicates per experimental group were used to determine clearance rate (Crisp, 1971), absorption efficiency (Conover, 1966), ammonia excretion rate (Solórzano, 1969) and metabolic rate (see *Table 4.2 - 1* containing the main results of these parameters). Physiological rates were standardized to a common dry weight of tissue (29.14 mg soft tissues dry weight) before the computation of elemental balances.

Table 4.2 - 1. Mean (SD) size-standardized values of clearance rate (CR), absorption efficiency (AE), metabolic rate (VO_2) and ammonia excretion rate (VNH_4-N) obtained in Chapter 4.1 and used here to calculate the elemental balances

Acclimation	Exposure diet	Exposure ration	Growth	CR (L h^{-1})	AE	VO_2 (µL h^{-1})	VNH_4-N (µg h^{-1})
D1	D1	H	F	0.11 (0.04)	0.79 (0.08)	28.21 (4.41)	0.39 (0.09)
			S	0.09 (0.02)	0.89 (0.02)	32.41 (14.21)	0.44 (0.11)
		L	F	0.16 (0.06)	0.63 (0.18)	29.55 (6.33)	0.24 (0.12)
			S	0.12 (0.04)	0.73 (0.13)	13.84 (4.33)	0.3 (0.17)
	D3	H	F	0.32 (0.04)	0.34 (0.03)	19.07 (4.32)	0.18 (0.06)
			S	0.11 (0.04)	0.53 (0.1)	14.77 (16.79)	0.25 (0.18)
		L	F	0.39 (0.18)	0.57 (0.05)	25.3 (10.94)	0.3 (0.09)
			S	0.11 (0.04)	0.55 (0.11)	10.16 (5.7)	0.2 (0.12)
D3	D1	H	F	0.11 (0.02)	0.81 (0.05)	27.01 (10.67)	0.69 (0.3)
			S	0.1 (0.03)	0.88 (0.06)	27.45 (13.5)	0.47 (0.11)
		L	F	0.17 (0.03)	0.88 (0.03)	21.72 (8.91)	0.37 (0.12)
			S	0.14 (0.09)	0.84 (0.07)	14.87 (17.7)	0.43 (0.18)
	D3	H	F	0.14 (0.04)	0.4 (0.05)	17.19 (4.72)	0.22 (0.04)
			S	0.12 (0.02)	0.5 (0.09)	9.95 (6.32)	0.18 (0.07)
		L	F	0.12 (0.09)	0.67 (0.11)	22.76 (12.94)	0.15 (0.05)
			S	0.09 (0.03)	0.76 (0.08)	6.99 (0.92)	0.09 (0.03)

In the present work, standardized values of parameters were combined with elemental analysis of diets and feces to compute N and C balances as follows:

Ingestion rates: Particulate organic N and C (PON and POC, respectively) were calculated as the product between POM and the proportion of organic N and C present in the diet as follows:

$$PON\ (mg\ L^{-1}) = POM \times \frac{N\%}{100}$$

$$POC\ (mg\ L^{-1}) = POM \times \frac{C\%}{100}$$

Ingestion rate of N and C (IR_N and IR_C, (µg h^{-1})) were estimated multiplying clearance rate by the particulate organic matter of each element, in these terms:

$$IR_N = PON \times CR$$

$$IR_C = POC \times CR$$

Absorption rates and absorption efficiencies: absorption rate (AR_N and AR_C :µg h^{-1}) representing the difference between ingestion (IR_N and IR_C :µg h^{-1}) and egestion rates

(ER_N and ER_C :µg h^{-1}), were calculated by using the organic ingestion rate (OIR), absorption efficiency (AE) and the proportion of organic N and C present in the feces, as follows:

$$ER_N = OIR \times (1 - AE) \times \frac{N\%_{(feces)}}{100}$$

$$ER_C = OIR \times (1 - AE) \times \frac{C\%_{(feces)}}{100}$$

Absorption efficiency (AE_N and AE_C, decimal units) was then estimated as the quotient between AR and IR of each element:

$$AE_N = \frac{AR_N}{IR_N}$$

$$AE_C = \frac{AR_C}{IR_C}$$

Carbon and nitrogen loss: carbon loss due to respiration (R_C, µg C h^{-1}) was computed from respiration rates (mg O_2 h^{-1}), assuming a RQ = 0.9. Respiration rates were obtained with the aid of oximeters through monitoring the declining in oxygen concentration in sealed chambers (volume) filled with filtered seawater (0.2 µm Millipore membranes). Nitrogen loss due to excretion (E_N, µg N h^{-1}) was assessed through the determination of ammonia production for a 3h period in flasks filled with 80 mL of filtered seawater (0.2 µm Millipore membranes), using the phenol-hypochlorite method (Solórzano, 1969). The assumption was taken that all of the N losses are due to excretion. In both determinations, chambers without animals were employed as controls, and were processed in the same way as the rest of the chambers.

Elemental balances of carbon and nitrogen: Elemental balances of carbon (SFG_C: µg C h^{-1}) and nitrogen (SFG_N: µg N h^{-1}) were calculated as the difference between the amount of C or N absorbed and excreted:

$$SFG_C = AR_C - R_C$$

$$SFG_N = AR_N - E_N$$

2.4 Biochemical and elemental composition

Aims of this section were to test the effect of variables under analysis: acclimation and exposure feeding conditions, including both diet composition and ration, on body composition of clams belonging to F and S groups. To this end, different treatment groups at the end of experiments were compared with the corresponding reference groups sacrificed before the experiments that had been maintained under uniform feeding conditions (*Isochrysis galbana* T-Iso clone at 20000 cells mL^{-1}) along the 51 d period of accommodation of clams to laboratory conditions provided to complete size segregation of growth groups (see Chapter 4.1). The treatment groups chosen corresponded to each of the conditioning diets (D1, D2 and D3); in the case of D1 and D3 conditioning, sampling included the groups of clams that, during physiological measurements, were fed the same diet composition used in conditioning, dosed at high and low rations; i.e., F and S clams from D1.1H, D1.1L, D3.3H, D3.3L (see *Table 4.1 - 2* from Chapter 4.1).

Samples for biochemical analysis were processed as follows: Clams introduced in vials were frozen by immersion into liquid nitrogen and then freeze-dried and weighted for total dry (i.e. lyophilized) weight estimations. Finally, samples were homogenized with a mortar and pestle, and stored at -20ºC until being analyzed.

Biochemical composition was determined in triplicates by means of colorimetric methods and using a spectrophotometer (Shimadzu UV-160A). Additional subsamples (~ 800 mg) were subtracted for gravimetrical analysis in order to estimate ash free dry weight (AFDW) as a reference to the biochemical composition of the organic fraction.

As these analyses were conducted over the total (soft + hard tissues) animal, organic content (%) of the samples decreased due to the elevated proportion of inorganic weight of shells. Hence, the amount of tissue employed for both gravimetric determinations and biochemical analyses was largely increased respect to a conventional soft tissue analysis to minimize errors due to potential weak color signals. Before running the analyses, several preliminary assays were conducted (data not shown) to assess the minimum weight of sample to be analyzed without methodological errors, as well as to discard the occurrence of interferences in the development of chemical reactions due to the presence of inorganic materials of shells.

Biochemical components (carbohydrates, proteins and lipids) were extracted from independent samples of 20 mg for CH and proteins and 30 mg for lipids. Carbohydrates

were extracted in TCA (5%) using and quantified following the Dubois et al. (1956) method at 490 nm, using dried glycogen from oyster as a standard, Proteins were extracted in NaOH (0.4 N) and quantified according to Lowry et al., (1951) at 750 nm, using a bovine seroalbumin as standard. Samples for lipid quantification were pre-extracted in acetic acid (Phillips and Privett, 1979) and then extracted twice in methanol:chloroform 2:1 and 1:2 (Bligh and Dyer, 1959; Folch et al., 1957). Colorimetric determination was conducted according to Marsh and Weinstein (1966) at 375 nm using a tripalmitin-phosphatidylcholine 1:1 standard.

Since the presence of shells prevented the direct CHN analysis, the conversion equivalents reported by Gnaiger and Bitterlich (1984), that had been tested for the clam species *R. decussatus* and *R. philippinarum* (Arranz et al., 2021; Chapter 3), was employed to obtain the elemental composition of body tissues from the biochemical analysis.

2.5 Data analysis

Linear regression analysis was used to estimate the relationships between two or more quantitative variables in the case of biometrical parameters. Statistical significance of differences between treatments (i.e., diet composition and ration or growth category) with respect to either elemental balances parameters or tissues composition, were tested through a 2 or 3-way ANOVA, or nonparametric ANOVA on ranks for analyses of percentage values, depending on the specific case being analyzed. Analyses were performed after having tested for normality (Shapiro-Wilk) and homoscedasticity (Levene) of the data. When statistically significant differences appeared in a factor containing more than two levels, post-hoc Tukey's HSD (Honestly Significant Differences) tests were run after ANOVA on ranks analyses. All of the statistical analysis as well as elaboration of graphical material was performed by means of the R software, version 3.5.1 (R Core Team, 2018).

3. RESULTS

3.1. BIOMETRICAL RELATIONSHIPS

Figure 4.2 - 1 shows relationships between some biometrical parameters analyzed in the group of clams covering the complete size range (10 – 17 mm sell length) of the cohort used in present experiments. A highly significant agreement was found for relationships between different parameters representative of size such as live weight *vs*. shell length ($F = 1065$, $p < 0.001$) or dry weight of soft tissues *vs.* live weight ($F = 987$, $p < 0.001$). This latter relationship was extensively applied, along this study, to convert direct live weight measurements into dry weight of the soft tissues, the size parameter used for standardization purposes. Concerning organic contents, no significant regressions were found with size for either soft or hard tissues: Most clams exhibited 80 – 90% of ash free dry weight (AFDW) in soft tissues and between 2.2 – 3.4% AFDW in shell valves (*Figure 4.2 - 1*). Relationships were likewise negligible for condition index (CI) *vs*. body size (given in terms of either length or live weight, *Figure 4.2 - 1*).

3.2. ELEMENTAL BALANCES

Components of elemental C and N balances were calculated using physiological rates and efficiencies reported in *Table 4.2 - 1*, for the different experimental groups of fast and slow growing clams acclimated to diets of different composition and then fed two different composition diets dosed at high and low ration. A summary overview of the averaged effects (based on means of pooled values) of each of the above variables on those components is given in *Table 4.2 - 2.* According to data reported in this Table: *a)* Feeding rates (represented by Ingestion) increased in clams acclimated to D1 relative to D3, but at the expenses of reducing AE, resulting in minor differences in Absorption rates (AR) between both conditioning groups of clams. Similar minor effects were observed in the balances (SFG) since energy expenditure components were barely affected by diet conditioning. *b)* Irrespective of the maintenance diet, exposure of clams to D3 induced a strong positive feeding response resulting in ~ 50% increment in IRs, relative to the rates of clams fed the D1. However, benefits of this response were fully canceled by the reduced AE experienced by clams when fed the D3 relative to D1 dietary composition, which resulted in the same values of the ARs with either of the exposure diets.

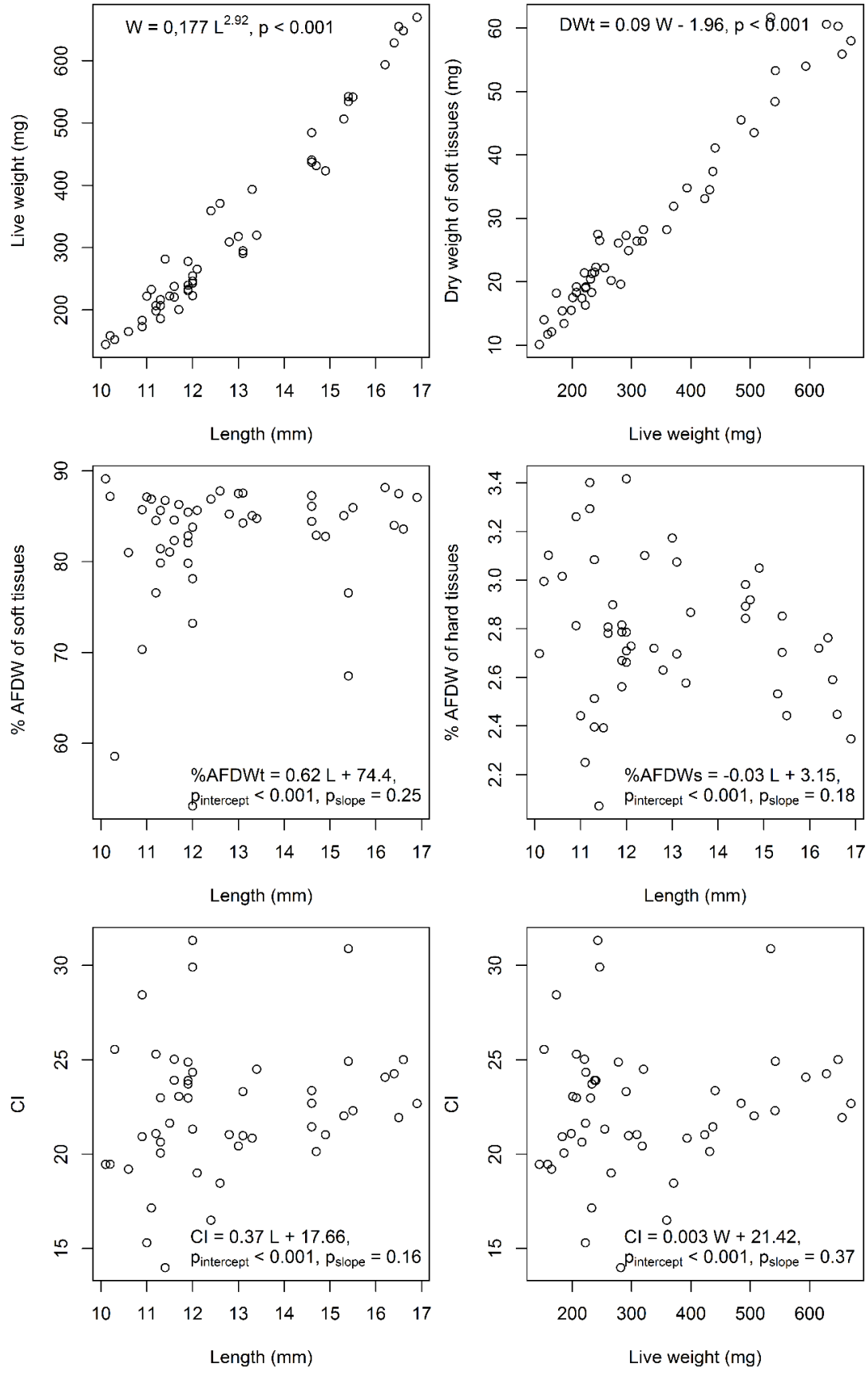

Figure 4.2 - 1. Relationships between biometric parameters in carpet shell clam in the size range of 10-17 mm shell length

SFG_C were also the same with both diets but high rates of N excretion with D1 reduced SFG_N values in some extent. c*)* A two-fold increase in food concentration (ration) produced consistent increments (~ 50%) in all components of C and N balances and resulting SFGs, as no effects of ration were observed on averaged values of AE. *d)* Compared with S, F clams exhibited an ~ 50% increment in the feeding rates; that increment declined to ~ 35% for AR and SFG due to the slightly higher AEs recorded in slow growers.

Table 4.2 - 2. Means of pooled values for the four factors (conditioning and exposure diets, ration and growth category) regarding elemental balances parameters

	Conditioning diet		**Exposure diet**		**Ration of exposure**		**Growth category**	
	D1	**D3**	**D1**	**D3**	**H**	**L**	**F**	**S**
IR_N	14.28 (1.83)	10.91 (0.82)	10.08 (0.57)	15.75 (2.05)	15.82 (1.49)	9.55 (1.19)	15.48 (1.89)	10.02 (0.71)
IR_C	73.76 (9.21)	56.8 (4.17)	53.59 (2.94)	79.89 (10.44)	81.42 (7.58)	50.02 (5.99)	80.09 (9.5)	52.03 (3.64)
ER_N	5.4 (1.16)	2.93 (0.51)	1.69 (0.17)	7.26 (1.24)	5.75 (1.17)	2.66 (0.49)	5.63 (1.24)	2.85 (0.44)
ER_C	30.59 (6.3)	16.25 (2.77)	10.16 (1.07)	39.99 (6.8)	31.77 (6.3)	15.52 (2.94)	31.85 (6.73)	15.88 (2.39)
AR_N	8.88 (0.84)	7.99 (0.54)	8.39 (0.51)	8.49 (0.94)	10.07 (0.52)	6.89 (0.76)	9.85 (0.85)	7.17 (0.49)
AR_C	43.17 (3.82)	40.55 (2.78)	43.43 (2.57)	39.89 (4.23)	49.64 (2.51)	34.5 (3.54)	48.25 (3.85)	36.15 (2.53)
AE	0.65 (0.03)	0.74 (0.03)	0.8 (0.02)	0.56 (0.03)	0.68 (0.04)	0.71 (0.02)	0.67 (0.03)	0.71 (0.03)
AE_N	0.69 (0.03)	0.76 (0.03)	0.83 (0.02)	0.59 (0.02)	0.71 (0.03)	0.74 (0.02)	0.71 (0.03)	0.74 (0.03)
AE_C	0.65 (0.03)	0.74 (0.03)	0.81 (0.02)	0.56 (0.03)	0.68 (0.04)	0.71 (0.02)	0.68 (0.03)	0.72 (0.03)
E_N	0.31 (0.03)	0.36 (0.04)	0.44 (0.03)	0.2 (0.02)	0.4 (0.04)	0.28 (0.03)	0.35 (0.04)	0.32 (0.03)
R_C	7.58 (0.7)	6.71 (0.73)	8.56 (0.66)	5.39 (0.66)	8 (0.74)	6.34 (0.67)	8.58 (0.53)	5.87 (0.78)
SFG_N	8.58 (0.84)	7.62 (0.52)	7.95 (0.49)	8.29 (0.94)	9.67 (0.52)	6.61 (0.76)	9.5 (0.85)	6.85 (0.48)
SFG_C	35.59 (3.7)	33.84 (2.51)	34.87 (2.46)	34.51 (3.98)	41.64 (2.5)	28.16 (3.3)	39.67 (3.75)	30.28 (2.35)

The above summary description gives but a limited account of the complexity of effects emerging from the combination of up to four factors affecting the physiological behavior, particularly as regards to the interaction between acute (exposure) and chronic (conditioning) effects of the change in diet composition. To deal with this complexity, both conditioning and exposure dietary factors were independently approached by means of a series of multiple-factor ANOVA testing statistical significance of *a)* effects of conditioning diet and growth category (two factors) on parameters recorded in clams fed the D1 and D3 diets (*Table 4.2 - 3*), and *b)* effects of exposure diet composition and ration together with growth category (three factors) on parameters recorded in clam groups acclimated to diet D1 (*Table 4.2 - 4*) and diet D3 (*Table 4.2 - 5*). Parameters analyzed

were of two types: *1)* rates and efficiencies involved in the elemental balances of both elements (C and N) and *2)* C:N ratios computed for these rates and efficiencies.

3.2.1 Conditioning effects

Mean values of parameters for the complete set of experimental groups are given in *Table 4.2 - 3*, together with results of two-factor ANOVAs comparing these means as a simultaneous function of conditioning diet and growth group. No significant effects associated to the composition of conditioning diet were found for any of the components of elemental balances in experiments where clams were fed the D1, but C:N ratios of feces significantly decreased in the conditioning group D3, which entailed significant differences also in the C:N ratios for both the rates and efficiencies of absorption. Conversely, significant differences were found between conditioning groups for the ingestion and egestion rates of clams fed the diet D3, but without significant changes in the C:N ratios of any of the parameters of elemental balances with this exposure diet (*Table 4.2 - 3* and *Figure 4.2 - 2*).

F clams were characterized by higher ingestion, egestion and absorption rates of both N and C than S clams, although these differences attained high significance only when fed the diet D3 (*Figure 4.2 - 2* and *Table 4.2 - 3*). The absorption efficiencies of both elements, instead, were significantly higher in S than in F clams with both exposure diets. On the other hand, effects of conditioning to either D1 or D3 diets were neatly different for growth groups, although the parameters affected differed between clams feeding the D1 and D3. The greatest differences were recorded with exposure to D3, where all the acquisition parameters experienced a ~ two-fold increase in F clams conditioned to D1 relative to D3 while no such effect was observed in S clams. This complex response is accounted by significant effects of both growth group (G) and the interaction with conditioning diet (D*G) for most parameters of the elemental balances and resulting SFG values. Instead, the main F vs S differences during exposure to D1 were recorded in the C:N ratios for egestion and absorption, as well as AEs, where significant effects of growth group (G) were recorded. However, the sign of these F vs S differences changed between D1 and D3 conditioning groups, as accounted for by significant interactions D*G (*Table 4.2 - 3*).

Table 4.2 - 3. Mean (SD) values of physiological responses of F and S clams conditioned to either D1 or D3 then fed on either D1 or D3: ingestion (IR), egestion (ER), absorption (AR), ammonia excretion (E) and respiration (R) rates, and elemental balances (SFG) for N and C (µg h^{-1}). $I_{C:N}$, $E_{C:N}$, $A_{C:N}$ and $M_{C:N}$ stand for the C:N ratio of the ingested, egested and absorbed materials, and the C:N of metabolic losses (R_C/E_N), respectively. Results of two-way ANOVA p-values for comparisons between acclimation effects are also shown; where D and G represent diet and growth group effect, respectively. Asterisks display different significance levels (: p < 0.05, **: p < 0.01; ***: p < 0.001). Superscripts (D, G and D*G) indicate differences between D1.1 and D3.3 clams*

	Diet	D1		D3		Statistical differences		
	Growth	F	S	F	S	D	G	D*G
Exposure to D1	**IR_N**	13.21 (4.14)^{D}	10.6 (1.77)	12.45 (1.96)	11.99 (3.12)	-	-	-
	IR_C	68.74 (21.54)^{D}	55.18 (9.23)	64.82 (10.19)	62.39 (16.22)	-	-	-
	ER_N	2.32 (1.34)^{D,G}	1 (0.28)	2.16 (0.64)	1.32 (0.76)	-	*	-
	ER_C	14.97 (8.61)^{D,G}	6.04 (1.71)	11.67 (3.45)	7.74 (4.43)	-	*	-
	AR_N	10.88 (3.2)	9.6 (1.57)	10.29 (1.69)	10.66 (2.81)	-	-	-
	AR_C	53.77 (15.7)	49.14 (7.99)	53.15 (8.78)	54.64 (14.51)	-	-	-
	AE_N	0.83 (0.06)^{D,G}	0.91 (0.02)	0.83 (0.04)	0.89 (0.06)	-	**	-
	AE_C	0.79 (0.08)^{D,G}	0.89 (0.02)	0.82 (0.04)	0.88 (0.06)	-	**	-
	E_N	0.41 (0.09)	0.46 (0.12)	0.73 (0.32)	0.49 (0.11)	-	-	-
	R_C	9.9 (1.55)	11.38 (4.99)	9.48 (3.75)	9.64 (4.74)	-	-	-
	SFG_N	10.47 (3.2)	9.13 (1.65)	9.56 (1.71)	10.17 (2.81)	-	-	-
	SFG_C	43.86 (16.38)	37.76 (10.45)	43.67 (8.99)	45 (15.05)	-	-	-
	$I_{C:N}$	5.21 (0.02)^{D}	5.21 (0.02)	5.21 (0.02)	5.21 (0.02)	-	-	-
	$E_{C:N}$	6.44 (0.03)^{D,G,D*G}	6.01 (0.04)	5.39 (0)	5.85 (0)	***	***	***
	$A_{C:N}$	4.95 (0.12)^{D}	5.12 (0.02)	5.17 (0.01)	5.12 (0.05)	**	*	**
	$AE_{C:N}$	0.95 (0.02)^{D}	0.98 (0.00)	0.99 (0)	0.98 (0.01)	**	*	**
	$M_{C:N}$	25.49 (8)	25.48 (12.28)	14.74 (8.08)	20.49 (11.03)	-	-	-
	$SFG_{C:N}$	4.12 (0.34)	4.08 (0.71)	4.56 (0.36)	4.37 (0.64)	-	-	-
Exposure to D3	**IR_N**	41.54 (4.71)	14.25 (4.54)	18.45 (4.85)	15.29 (2.88)	**	***	***
	IR_C	212 (24.02)	72.7 (23.19)	94.14 (24.74)	78.02 (14.71)	**	***	***
	ER_N	25.45 (1.65)	6.5 (2.97)	10.02 (1.78)	7.05 (2.19)	***	***	***
	ER_C	137.2 (10.06)	35.56 (16.26)	54.51 (11.22)	39.25 (12.19)	**	***	***
	AR_N	16.1 (3.25)	7.74 (1.87)	8.43 (3.17)	8.23 (1.57)	-	**	**
	AR_C	74.79 (15.45)	37.14 (8.77)	39.63 (13.94)	38.76 (7.93)	-	**	*
	AE_N	0.38 (0.04)	0.56 (0.1)	0.45 (0.05)	0.54 (0.08)	-	**	-
	AE_C	0.35 (0.04)	0.53 (0.1)	0.42 (0.04)	0.5 (0.09)	-	**	-
	E_N	0.19 (0.07)	0.27 (0.19)	0.24 (0.04)	0.19 (0.08)	-	-	-
	R_C	6.69 (1.52)	5.19 (5.89)	6.04 (1.66)	3.49 (2.22)	-	-	-
	SFG_N	15.91 (3.19)	7.47 (1.82)	8.19 (3.15)	8.04 (1.64)	-	**	**
	SFG_C	68.1 (14.15)	31.95 (13.45)	33.59 (12.8)	35.27 (6.86)	-	*	*
	$I_{C:N}$	5.1 (0.03)	5.1 (0.03)	5.1 (0.03)	5.1 (0.03)	-	-	-
	$E_{C:N}$	5.39 (0.05)	5.47 (0)	5.42 (0.16)	5.56 (0.10)	-	*	-
	$A_{C:N}$	4.64 (0.07)	4.81 (0.11)	4.73 (0.12)	4.7 (0.13)	-	-	-
	$AE_{C:N}$	0.91 (0.01)	0.94 (0.02)	0.93 (0.02)	0.92 (0.03)	-	-	-
	$M_{C:N}$	37.31 (6.38)	36.99 (62.14)	25.89 (4.14)	26.2 (26.51)	-	-	-
	$SFG_{C:N}$	4.27 (0.05)	4.13 (1.08)	4.11 (0.19)	4.4 (0.3)	-	-	-

As a general observation, C:N ratios decreased sequentially along the successive balance components, from average values of 5.15 for the ingested ration to 4.9 for the absorbed ration and to 4.25 for the final balance or SFG, due to the increase in the C losses relative to N losses in the feces but mainly as regards metabolic products (average

C:N ratio for metabolism = 26.5). This occurs for every experimental group considered in this study, although the magnitude of the latter stoichiometric adjustments differed between treatments according to differences in metabolic C:N values, resulting grater in clams fed the D3 compared to D1 (31.59 *vs.* 21.55) or conditioned to D1 relative to D3 (31.31 *vs.* 21.83), while remained similar for the two growth groups (26.47 and 27.29 in F and S clams, respectively).

3.2.2 Acute effects of the change in diet composition and ration

Short-term effects of changes in the quality (composition, C) and quantity (ration, R) of exposure diets on the components of the elemental balances were analyzed in F and S individuals. To facilitate interpretation of these effects, statistical testing relied on three-factor ANOVAs applied to each conditioning group (D1 and D3) separately considered (*Table 4.2 - 4* and *Table 4.2 - 5*). Both attributes of exposition diets (i.e., food composition and concentration), considered either as isolated factors or through the interaction C*R, exerted significant effects on most acquisition parameters such as ingestion, egestion and absorption rates of C and N, as well as on the absorption efficiencies. Moreover, these characteristics hold for both groups of conditioning D1 (*Table 4.2 - 4*) and D3 (*Table 4.2 - 5*). In general, rates of nutrient acquisition (both C and N) increased with increasing food concentration (H vs L rations) and were higher when feeding the diet D3 compared with D1, although this late effect of food composition was much stronger in clams acclimated to D1 compared to D3 (*Table 4.2 - 2*).

Absorption efficiencies of both N and C (*Figure 4.2 - 3*) were mainly affected by the composition of exposure diet, where clams fed the D3 exhibited a ~ 40% reduction in AEs relative to D1. As already noted (*Table 4.2 - 2*), this tends to cancel out the effects of increased feeding rates achieved with D3 so as to result in absorption rate differences being only marginally significant or no-significant (*Table 4.2 - 4* and *Table 4.2 - 5*). The behavior of AE in response to a change in food concentration (ration) was strongly dependent on diet composition, which was reflected in the high significance of the corresponding interaction term C*R (*Table 4.2 - 4* and *Table 4.2 - 5*). For instance, effects of increasing D1 ration were positive or null, while this effect was clearly negative in the case of D3 (*Figure 4.2 - 3*), possibly as a consequence of the greater increases of the ingestion rates achieved with this diet.

Table 4.2 - 4. Three-way ANOVA main results for elemental balances parameters, employing composition and ration of exposure diet, and growth category as factors for clams conditioned to D1

	Composition (C)	Ration (R)	Growth category (G)	C:R	C:G	R:G	C:R:G
IR_N	**F = 38.853, p < 0.001**	**F = 15.741, p < 0.001**	**F = 49.325, p < 0.001**	F = 2.214, p = 0.148	**F = 42.316, p < 0.001**	F = 1.014, p = 0.322	F = 0.876, p = 0.357
IR_C	**F = 34.826, p < 0.001**	**F = 15.302, p = 0.001**	**F = 49.061, p < 0.001**	F = 2.757, p = 0.108	**F = 41.168, p < 0.001**	F = 1.062, p = 0.312	F = 0.992, p = 0.328
ER_N	**F = 107.635, p < 0.001**	**F = 17.781, p < 0.001**	**F = 70.134, p < 0.001**	**F = 30.514, p < 0.001**	**F = 58.418, p < 0.001**	**F = 11.169, p = 0.002**	**F = 15.01, p = 0.001**
ER_C	**F = 88.82, p < 0.001**	**F = 13.267, p = 0.001**	**F = 66.438, p < 0.001**	**F = 23.826, p < 0.001**	**F = 50.656, p < 0.001**	**F = 7.743, p = 0.01**	**F = 10.178, p = 0.003**
AR_N	**F = 5.441, p = 0.027**	**F = 10.598, p = 0.003**	**F = 25.883, p < 0.001**	F = 2.903, p = 0.099	**F = 23.006, p < 0.001**	F = 0.737, p = 0.398	F = 1.872, p = 0.182
AR_C	F = 2.45, p = 0.129	**F = 11.755, p = 0.002**	**F = 21.565, p < 0.001**	F = 2.579, p = 0.12	**F = 20.927, p < 0.001**	F = 0.594, p = 0.447	F = 1.449, p = 0.239
AE_N	**F = 56.235, p < 0.001**	F = 1.266, p = 0.27	**F = 4.714, p = 0.039**	**F = 17.793, p < 0.001**	F = 0.034, p = 0.854	F = 2.335, p = 0.138	F = 3.251, p = 0.082
AE_C	**F = 47.218, p < 0.001**	F = 1.698, p = 0.203	**F = 6.44, p = 0.017**	**F = 13.818, p = 0.001**	F = 0.046, p = 0.832	F = 1.441, p = 0.24	F = 1.831, p = 0.187
E_N	**F = 6.543, p = 0.016**	F = 3.228, p = 0.083	F = 0.369, p = 0.548	F = 3.292, p = 0.08	F = 0.564, p = 0.459	F = 0.609, p = 0.442	F = 1.215, p = 0.28
R_C	**F = 9.172, p = 0.005**	F = 2.401, p = 0.132	**F = 5.127, p = 0.031**	F = 1.527, p = 0.227	F = 0.356, p = 0.556	**F = 5.921, p = 0.022**	F = 0.464, p = 0.501
SFG_N	**F = 6.144, p = 0.019**	**F = 10.324, p = 0.003**	**F = 26.811, p < 0.001**	F = 2.695, p = 0.112	**F = 23.242, p < 0.001**	F = 0.695, p = 0.412	F = 1.781, p = 0.193
SFG_C	**F = 5.061, p = 0.033**	**F = 9.056, p = 0.005**	**F = 16.299, p < 0.001**	F = 1.664, p = 0.208	**F = 19.01, p < 0.001**	F = 0.035, p = 0.853	F = 1.806, p = 0.19
$IR_{C:N}$	**F = 2.4 10^{29}, p < 0.001**	**F = 4.5 10^{28}, p < 0.001**	F = 1.79, p = 0.192	**F = 9.9 10^{28}, p < 0.001**	F = 0.507, p = 0.482	F = 2.275, p = 0.143	F = 0.768, p = 0.388
$ER_{C:N}$	**F = 721.218, p < 0.001**	F = 3.65, p = 0.066	**F = 131.788, p < 0.001**	**F = 21.804, p < 0.001**	F = 2.896, p = 0.1	**F = 9.28, p = 0.005**	**F = 68.206, p < 0.001**
$AR_{C:N}$	**F = 43.667, p < 0.001**	F = 0.174, p = 0.68	**F = 11.553, p = 0.002**	F = 3.205, p = 0.084	F = 0, p = 0.998	F = 0.387, p = 0.539	F = 0.005, p = 0.943
$AE_{C:N}$	**F = 5.375, p =0.0280**	F = 2.346, p = 0.14	**F = 12.216, p = 0.002**	F = 1.058, p = 0.312	F = 0.01, p = 0.92	F = 0.364, p = 0.55	F = 0.023, p = 0.881
$M_{C:N}$	F = 0.055, p = 0.816	F = 0.213, p = 0.648	F = 1.316, p = 0.261	F = 1.585, p = 0.218	F = 0.74, p = 0.397	F = 1.288, p = 0.266	F = 0.762, p = 0.39
$SFG_{C:N}$	F = 0.36, p = 0.554	F = 1.132, p = 0.296	F = 1.772, p = 0.194	F = 0.052, p = 0.821	F = 2.686, p = 0.112	F = 2.883, p = 0.101	F = 2.083, p = 0.16

Table 4.2 - 5. Three-way ANOVA main results for elemental balances parameters, employing composition and ration of exposure diet, and growth category as factors for clams conditioned to D3

	Composition (C)	**Ration (R)**	**Growth category (G)**	**C:R**	**C:G**	**R:G**	**C:R:G**
IR_N	F = 0.858, p = 0.362	**F = 22.319, p < 0.001**	F = 1.652, p = 0.209	**F = 4.714, p = 0.039**	F = 0.289, p = 0.595	F = 0.022, p = 0.883	F = 0.234, p = 0.632
IR_C	F = 0.27, p = 0.607	**F = 20.431, p < 0.001**	F = 1.638, p = 0.211	**F = 5.458, p = 0.027**	F = 0.242, p = 0.626	F = 0.029, p = 0.866	F = 0.259, p = 0.615
ER_N	**F = 61.064, p < 0.001**	**F = 53.266, p < 0.001**	F = 3.501, p = 0.072	**F = 46.005, p < 0.001**	F = 3.728, p = 0.064	F = 3.038, p = 0.092	F = 0.165, p = 0.688
ER_C	**F = 54.977, p < 0.001**	**F = 48.681, p < 0.001**	F = 3.091, p = 0.09	**F = 43.723, p < 0.001**	F = 3.523, p = 0.071	F = 1.94, p = 0.175	F = 0.106, p = 0.747
AR_N	**F = 4.922, p = 0.035**	**F = 9.061, p = 0.005**	F = 0.756, p = 0.392	F = 0.016, p = 0.901	F = 0.02, p = 0.888	F = 0.926, p = 0.344	F = 0.21, p = 0.65
AR_C	**F = 8.9, p = 0.006**	**F = 6.988, p = 0.013**	F = 0.734, p = 0.399	F = 0.016, p = 0.899	F = 0.072, p = 0.791	F = 0.85, p = 0.364	F = 0.276, p = 0.603
AE_N	**F = 112.266, p < 0.001**	**F = 25.045, p < 0.001**	F = 2.151, p = 0.154	**F = 25.346, p < 0.001**	F = 2.089, p = 0.159	F = 3.114, p = 0.089	F = 0.629, p = 0.434
AE_C	**F = 114.736, p < 0.001**	**F = 24.85, p < 0.001**	F = 2.469, p = 0.127	**F = 25.158, p < 0.001**	F = 2.484, p = 0.126	F = 1.317, p = 0.261	F = 0.873, p = 0.358
E_N	**F = 43.793, p < 0.001**	**F = 7.177, p = 0.012**	F = 1.899, p = 0.179	F = 0.862, p = 0.361	F = 0.096, p = 0.759	F = 2.483, p = 0.126	F = 2.357, p = 0.136
R_C	**F = 4.652, p = 0.04**	F = 1.012, p = 0.323	F = 3.873, p = 0.059	F = 1.971, p = 0.171	F = 1.345, p = 0.256	F = 1.06, p = 0.312	F = 0.006, p = 0.937
SFG_N	F = 3.486, p = 0.072	**F = 8.298, p = 0.008**	F = 0.638, p = 0.431	F = 0.006, p = 0.941	F = 0.016, p = 0.901	F = 1.113, p = 0.300	F = 0.298, p = 0.59
SFG_C	**F = 6.557, p = 0.016**	**F = 6.412, p = 0.017**	F = 0.118, p = 0.734	F = 0.074, p = 0.788	F = 0.388, p = 0.538	F = 0.47, p = 0.498	F = 0.342, p = 0.563
$IR_{C:N}$	**$F = 1.2\ 10^{30}$, p < 0.001**	**$F = 2.1\ 10^{29}$, p < 0.001**	**$F = 1.8\ 10^{27}$, p < 0.001**	**$F = 4.5\ 10^{29}$, p < 0.001**	F = 0.003, p = 0.958	F = 3.748, p = 0.063	F = 2.433, p = 0.13
$ER_{C:N}$	**F = 433.999, p < 0.001**	**F = 69.35, p < 0.001**	**F = 26.568, p < 0.001**	**F = 164.463, p < 0.001**	**F = 27.38, p < 0.001**	**F = 706.251, p < 0.001**	**F = 43.33, p < 0.001**
$AR_{C:N}$	**F = 340.054, p < 0.001**	**F = 70.749, p < 0.001**	F = 3.738, p = 0.063	**F = 6.17, p = 0.019**	F = 3.395, p = 0.076	**F = 8.022, p = 0.008**	F = 1.75, p = 0.197
$AE_{C:N}$	**F = 60.844, p <0.001**	**F = 14.648, p <0.001**	F = 2.290, p = 0.141	**F = 16.694, p <0.001**	F = 3.574, p = 0.069	**F = 7.840, p =0.009**	F = 1.916, p = 0.177
$M_{C:N}$	**F = 6.493, p = 0.017**	F = 1.343, p = 0.256	F = 1.702, p = 0.203	F = 1.776, p = 0.193	F = 1.082, p = 0.307	F = 2.215, p = 0.148	F = 0.33, p = 0.57
$SFG_{C:N}$	**F = 11.051, p = 0.002**	F = 0.333, p = 0.568	**F = 4.965, p = 0.034**	F = 4.188, p = 0.05	F = 2.507, p = 0.125	F = 3.442, p = 0.074	F = 0.038, p = 0.848

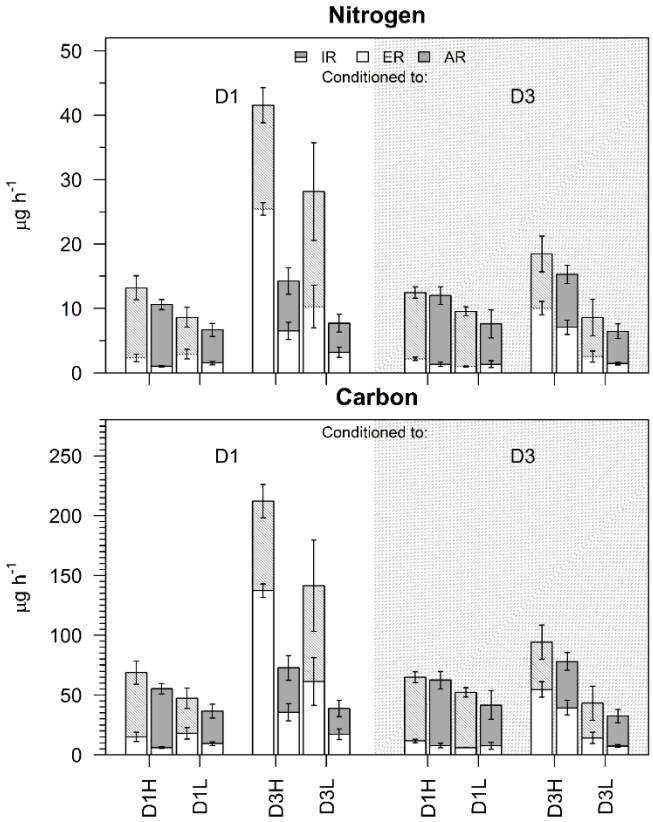

Figure 4.2 - 2. Ingestion (IR), egestion (ER) and absorption rate (AR) mean values for N (up) and C (down) of F (light bars) and S (dark bars) clams subject to all nutritional conditions .

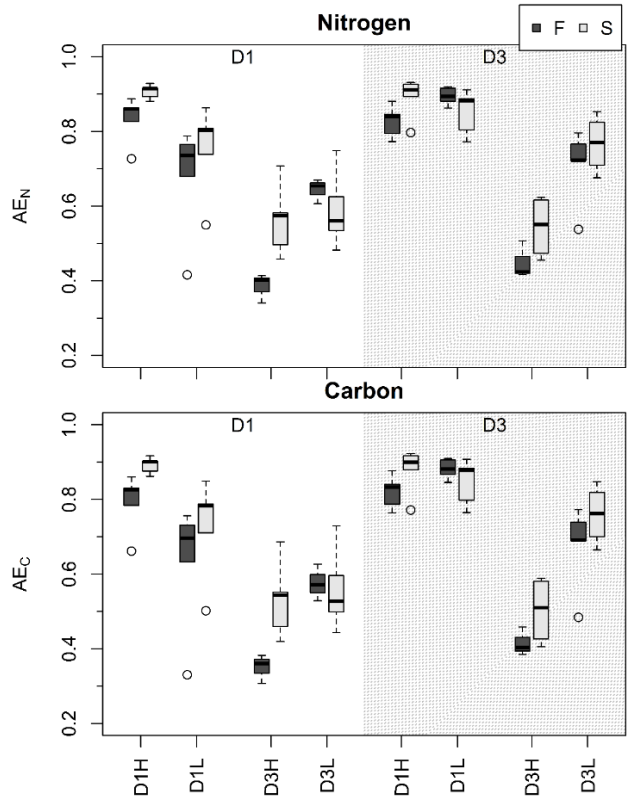

Figure 4.2 - 3. Absorption efficiencies for N (up) and C (down) of F (dark boxes) and S (light boxes) clams subject to all nutritional conditions

Main effects on metabolic processes were associated to diet composition, where the change from diet D1 to D3 promoted reductions in both the excreted N and respired C that were, respectively, 54 and 30% on average (i.e., computed for both conditioning diets and in F and S clams) (*Table 4.2 - 2*).

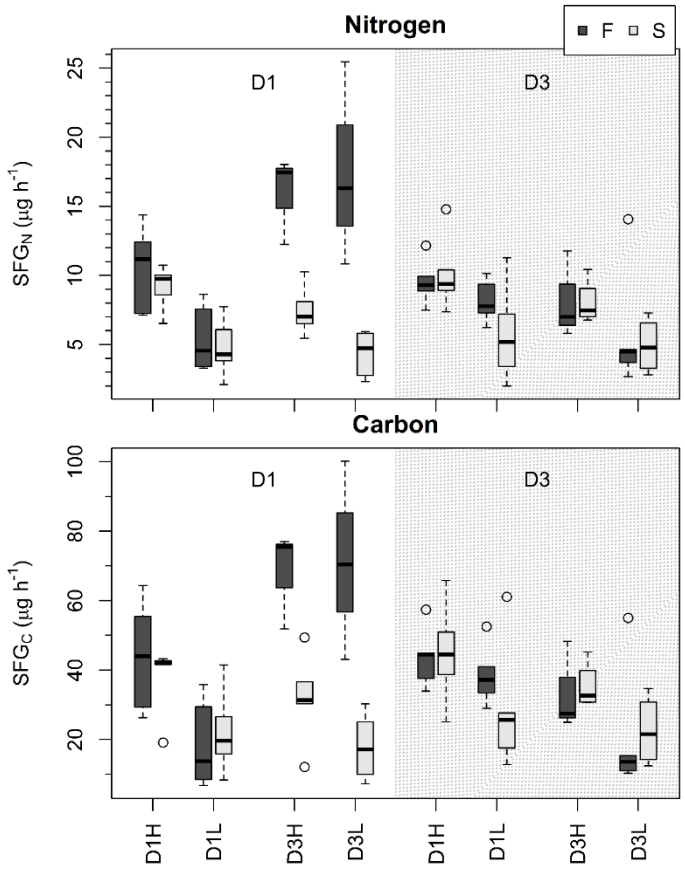

Figure 4.2 - 4. Net balances for N (up) and C (down) of F (dark boxes) and S (light boxes) clams subject to all nutritional conditions

Growth category (F *vs*. S) of clams conditioned to D1 exerted highly significant effects on all the acquisition parameters which correspond to the higher rates achieved by F compared to S clams (*Table 4.2 - 4*); however, most of these differences were observed in clams fed the D3 rather than the D1 diet (*Figure 4.2 - 2*) as accounted for by highly significant C*G interaction term for these parameters (*Table 4.2 - 4*). None of these growth category effects on the acquisition rates of nutrients were observed in clams acclimated to D3, where instead metabolic losses of C and N were significantly higher in fast growers (*Table 4.2 - 3* and *Table 4.2 - 5*).

Resulting balances (SFG) of N and C are shown in *Figure 4.2 - 4*. SFG values were consistently higher (~ 40%) in clams fed the high ration although differences with

the low ration were marginally significant (*Table 4.2 - 4* and *Table 4.2 - 5*). Also, food composition and growth condition exerted significant effects on N and C balances, but only in clams conditioned to D1, where the most relevant effect was the great difference observed between F and S clams fed the D3 (*Figure 4.2 - 4*), accounted for by high significance of the C*G interaction (*Table 4.2 - 4*).

Food composition exerted significant effects on C:N ratios for all the acquisition parameters, including AE (*Table 4.2 - 4* and *Table 4.2 - 5*). C:N ratios for metabolic products and SFG were significantly affected by food composition but only in D3 conditioned clams, where extensive effects of ration on acquisition parameters were also recorded, both as isolated factor and in combination with food composition (C*R interaction; *Table 4.2 - 5*). Significant F vs S differences were found as regards the C:N ratio of some acquisition parameters, mainly in D1 conditioning; however, C:N ratios for SFG only differed significantly between growth groups after conditioning to D3.

3.3 Whole (soft + hard) tissues composition

A subset of the groups of clams employed in elemental balances determinations was chosen for whole tissues composition determinations, as reported in Section 2.3 of the present Chapter. Mean values (SD) of each group are detailed in *Table 4.2 - 6*, in which both proximate composition and derived elemental composition were displayed. For comparison purposes, initial (pre-experimental) composition of both F and S clams was also determined.

From the combination of groups selected for biochemical and elemental composition the following statistical comparisons are possible (see *Table 4.2 - 7*): Differences in the composition of conditioning diets (D1 vs D3) had no effects on either biochemical or elemental composition when clams were fed the same diet composition during physiological experiments; however, protein (and N) contents were significantly higher in F clams while carbohydrates (and C) contents were higher in S clams, for this group comparison (*Table 4.2 - 7a*; *Figure 4.2 - 5*). Changing ration during physiological determinations produced differential effect on body composition, where no effects were recorded for the D1 diet (*Table 4.2 - 7b*), while protein content increased (< 10%) with D3 dosed at low ration at the expenses of other biochemical components, which resulted in significant differences for all elemental components (*Table 4.2 - 7c*), as protein constitutes the main component of tissues. Also, F vs S differences were recorded with

this group combination, with F clams presenting significantly higher protein and lower lipid contents (*Table 4.2 - 6* and *Table 4.2 - 7c*). Compared with D1 and D3 diets, conditioning to D2 resulted in higher protein contents and lower carbohydrates (relative to D3) or lipids (relative to D1), which resulted in significantly higher N and lower C and H contents (*Table 4.2 - 7e* and *7f*, and *Figure 4.2 - 5*).

Table 4.2 - 6. Mean (SD) values of biochemical and derived elemental composition for each group of clams attending to their feeding condition (A: acclimated; E: exposed; R: ration) and growth category (G). Values are expressed as percentage over the sum of components/elements

				Biochemical composition			**Derived elemental composition**		
A	**E**	**R**	**G**	**CHO**	**Protein**	**Lipid**	**C**	**N**	**H**
Composition before experiments (fed on T-Iso)			F	25.42 (1.35)	62.94 (1.61)	11.63 (0.6)	74.66 (0.28)	15.16 (0.33)	10.18 (0.05)
			S	18.61 (1.56)	70.2 (1.98)	11.2 (1.05)	73.51 (0.35)	16.51 (0.41)	9.98 (0.06)
D1	D1	H	F	17.44 (3.1)	64.46 (3.09)	18.11 (2)	74.82 (0.53)	14.93 (0.62)	10.25 (0.1)
			S	21.81 (6.57)	62.04 (8.03)	16.16 (1.97)	75.1 (1.37)	14.61 (1.62)	10.28 (0.24)
		L	F	15.8 (3.73)	66 (5.37)	18.19 (2.24)	74.6 (0.93)	15.19 (1.1)	10.21 (0.17)
			S	19.98 (5.09)	62.09 (4.38)	17.93 (3.39)	75.18 (0.73)	14.51 (0.86)	10.31 (0.13)
D2	-	H	F	17.79 (3.33)	68.75 (4.62)	13.46 (2.47)	73.88 (0.81)	16.06 (0.96)	10.06 (0.15)
			S	17.71 (3.44)	66.76 (4.34)	15.53 (4.03)	74.31 (0.84)	15.54 (1.01)	10.15 (0.17)
D3	D3	H	F	20.15 (2.98)	65.45 (4.28)	14.4 (3.38)	74.44 (0.83)	15.4 (0.99)	10.16 (0.16)
			S	24.51 (3.61)	58.03 (1.68)	17.46 (3.66)	75.79 (0.38)	13.8 (0.46)	10.41 (0.08)
		L	F	16.52 (7.07)	71.6 (8.73)	11.88 (2.23)	73.37 (1.43)	16.66 (1.68)	9.96 (0.25)
			S	20.63 (4.21)	63.43 (3.84)	15.95 (1.61)	74.86 (0.6)	14.9 (0.71)	10.24 (0.1)

Diet conditioning effects were additionally analyzed in comparison with the pre-experimental condition. For this purpose, and taking into account the virtual absence of acute effects on clams composition (*Table 4.2 - 7*), two-way ANOVAs were run employing as factors acclimation diet (T-Iso, D1, D2 and D3) and growth category.

Table 4.2 - 7. Main two-way ANOVA on ranks results for every biochemical component and element in which six combinations of comparisons are displayed (see heading above each comparison)

	Biochemical composition			**Derived elemental composition**		
	CHO	**Protein**	**Lipid**	**C**	**N**	**H**
***a)* D1.1H *vs.* D3.3H – Conditioning diet effect: tested in uniform conditions of food composition and ration**						
Acclim.	F = 4.339, p = 0.054	F = 1.32, p = 0.267	F = 1.358, p = 0.261	F = 1.123, p = 0.305	F = 1.116, p = 0.306	F = 0.755, p = 0.398
G	**F = 4.708, p = 0.045**	**F = 6.125, p = 0.025**	F = 0.054, p = 0.819	**F = 5.684, p = 0.03**	**F = 5.24, p = 0.036**	F = 4.347, p = 0.053
A*G	F = 0, p = 1	F = 2.531, p = 0.131	F = 2.663, p = 0.122	F = 3.119, p = 0.096	F = 3.419, p = 0.083	F = 3.992, p = 0.063
***b)* D1.1H *vs.*D1.1L – Ration effect: tested in uniform conditions of food composition (D1)**						
Ration	F = 0.4, p = 0.536	F = 0.022, p = 0.884	F = 0.582, p = 0.457	F = 0.131, p = 0.722	F = 0.131, p = 0.722	F = 0.128, p = 0.726
G	F = 4.225, p = 0.057	F = 1.601, p = 0.224	F = 2.568, p = 0.129	F = 0.887, p = 0.36	F = 0.754, p = 0.398	F = 0.327, p = 0.576
R*G	F = 0.156, p = 0.698	F = 0.798, p = 0.385	F = 0.21, p = 0.653	F = 0.425, p = 0.524	F = 0.524, p = 0.48	F = 0.51, p = 0.485
***c)* D3.3H *vs.*D3.3L – Ration effect: tested in uniform conditions of food composition (D3)**						
Ration	F = 2.676, p = 0.121	**F = 7.005, p = 0.018**	F = 2.811, p = 0.113	**F = 6.631, p = 0.02**	**F = 6.631, p = 0.02**	**F = 7.097, p = 0.017**
G	F = 3.237, p = 0.091	**F = 10.611, p = 0.005**	**F = 8.338, p = 0.011**	**F = 12.265, p = 0.003**	**F = 12.265, p = 0.003**	**F = 11.433, p = 0.004**
R*G	F = 0.328, p = 0.575	F = 0.839, p = 0.373	F = 1.701, p = 0.211	F = 0.382, p = 0.545	F = 0.382, p = 0.545	F = 0.378, p = 0.547
***e)* D1.1H *vs.*D2.1H – Conditioning diet effect: uniform vs short-term change in food composition (D2 → D1)**						
Acclim.	F = 0.024, p = 0.879	**F = 5.439, p = 0.033**	**F = 10.253, p = 0.006**	**F = 6.668, p = 0.02**	**F = 6.697, p = 0.02**	**F = 8.274, p = 0.011**
G	F = 0.024, p = 0.879	F = 0.006, p = 0.937	F = 0.035, p = 0.853	F = 0.062, p = 0.806	F = 0.028, p = 0.869	F = 0.068, p = 0.797
A*G	F = 3.715, p = 0.072	F = 0.058, p = 0.812	F = 3.202, p = 0.093	F = 0.34, p = 0.568	F = 0.446, p = 0.514	F = 0.919, p = 0.352
***f)* D2.3H*vs.*D3.3H– Conditioning diet effect: uniform vs short-term change in food composition (D2 → D3)**						
Acclim.	**F = 9.776, p = 0.007**	**F = 11.409, p = 0.004**	F = 0.53, p = 0.477	**F = 7.624, p = 0.014**	**F = 6.595, p = 0.021**	**F = 7.01, p = 0.018**
G	F = 1.658, p = 0.216	**F = 6.418, p = 0.022**	F = 0.191, p = 0.668	F = 2.353, p = 0.145	**F = 7.649, p = 0.014**	**F = 6.5, p = 0.021**
A*G	F = 0.685, p = 0.42	F = 3.22, p = 0.092	F = 0.191, p = 0.668	F = 2.353, p = 0.145	F = 2.195, p = 0.158	F = 2.462, p = 0.136

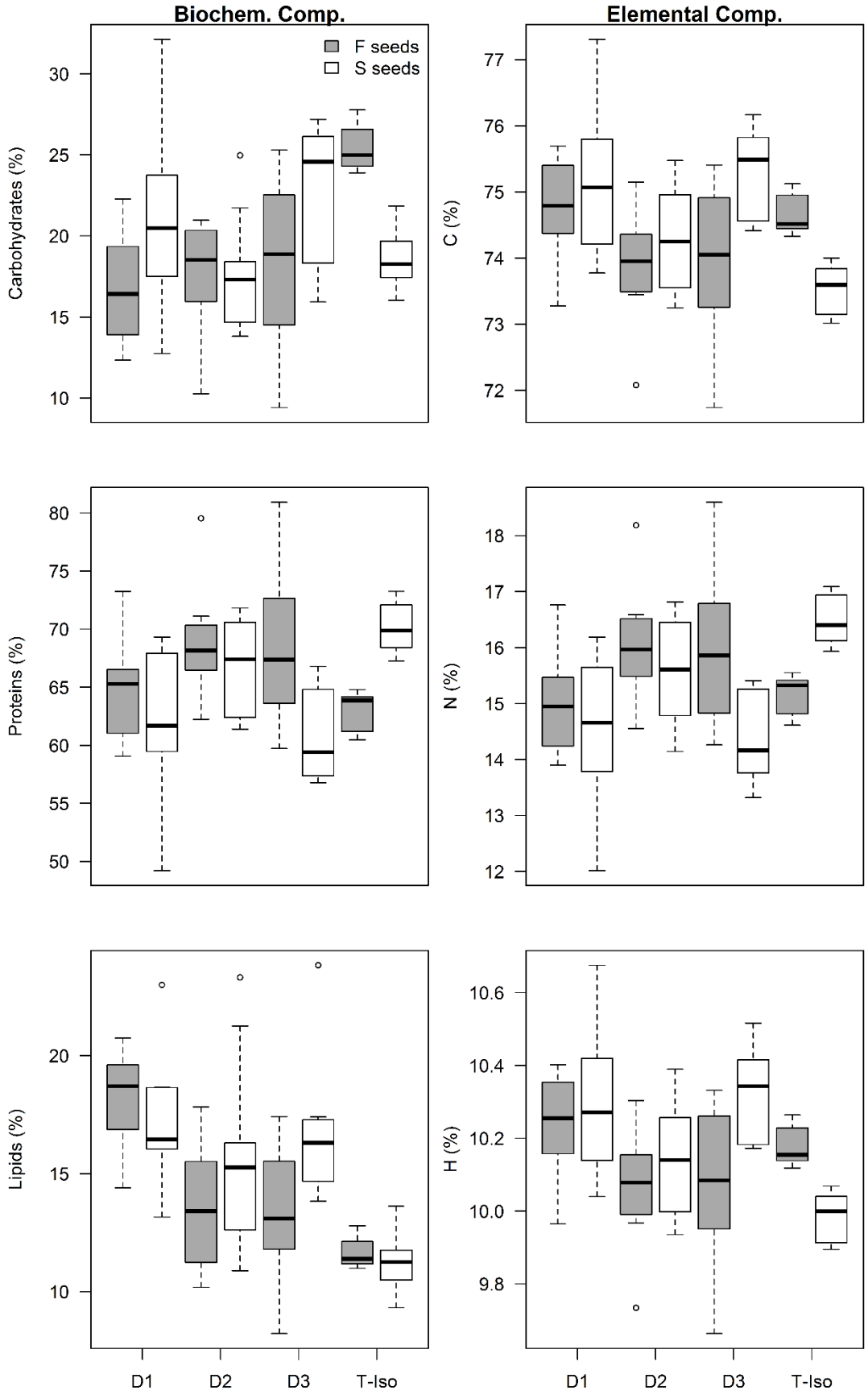

Figure 4.2 - 5. Biochemical (left) and derived elemental composition (right) of F (dark bars) and S (light bars) clams conditioned to either D1, D2 or D3, and the reference group (T-Iso)

For each biochemical component and element, significant differences were found based on the conditioning diet (*Table 4.2 - 8*). Growth category had no effects *"per se"* on body composition but significant F *vs.* S differences were recorded through all A*G interaction terms. Compared with the reference group (T-Iso), after a month of conditioning, carbohydrates percentage decreased with D2 and rested at intermediate values under D1 and D3 diets (*Figure 4.2 - 5*); protein content remained the same value with D2 conditioning while decreased significantly with D1 and D3; lipid content increased significantly in all experimental diets, especially for D1 clams (*Figure 4.2 - 5*). Differences in percentages of elemental composition reflected these diet effects on biochemical composition, especially changes in protein levels.

Table 4.2 - 8. Main two-way ANOVA on ranks results of proximate and elemental composition, including Tukey's HSD tests (including mean values) for acclimation diets

		CHO	Protein	Lipid
Acclim.		**F = 4.442, p = 0.006**	**F = 7.436, p < 0.001**	**F = 40.526, p < 0.001**
	T-Iso	20.88^{a}	67.78^{a}	11.34^{a}
	D1	18.76^{ab}	63.65^{b}	17.60^{c}
	D2	17.75^{b}	67.75^{a}	14.50^{b}
	D3	20.45^{ab}	64.63^{b}	14.92^{b}
Growth		F = 1.409, p = 0.238	F = 0.556, p = 0.458	F = 1.753, p = 0.189
A*G		**F = 11.700, p < 0.001**	**F = 17.69, p < 0.001**	**F = 4.471, p = 0.006**
		C	**N**	**H**
Acclim.		**F = 12.035, p < 0.001**	**F = 12.486, p < 0.001**	**F = 15.282, p < 0.001**
	T-Iso	73.90^{a}	16.06^{a}	10.05^{a}
	D1	74.93^{c}	14.81^{c}	10.26^{c}
	D2	74.10^{ab}	15.80^{ab}	10.10^{ab}
	D3	74.61^{bc}	15.19^{bc}	10.19^{bc}
Growth		F = 0.169, p = 0.682	F = 0.144, p = 0.705	F = 0.04, p = 0.842
A*G		**F = 16.397, p < 0.001**	**F = 16.132, p < 0.001**	**F = 14.401, p < 0.001**

F *vs.* S differences in composition varied with conditioning diet. Namely, in the reference group, F clams presented higher carbohydrate and lower protein levels than S clams, while diets D1 and D3 both promoted higher protein levels (*Figure 4.2 - 5*) as already noted. Protein levels were the same under D2, with F clams storing slightly more carbohydrates at the expense of lipids (*Figure 4.2 - 5*).

4. DISCUSSION

A combination of acute and chronic responses to diets differing in biochemical composition were found, in spite of similar dietary C:N ratios, to promote differential effects on the acquisition and use of C and N in *Ruditapes decussatus* juveniles. Additionally, these effects were dependent on the ration dosed and differed between fast

and slow growers. As governed by physiological components of growth, main trends observed for the balances of these elements corresponds to the overall energy budgets (Chapter 4.1), although comparison of both elemental and energetic approaches revealed significant differences as regards the behavior of some of these components.

4.1 Feeding responses

Since C and N availability with different diets were rather uniform, rates of ingestion of both elements (IR_N and IR_C) reflected the behavior of the clearance rate (CR) already documented in a previous chapter (see *Table 4.2 - 1*). In this respect, the main evidence of a feeding response to changing diet composition refers to the strong increment in CR of clams conditioned to D1 when transferred to D3, that was especially noticeable in F clams: As discussed earlier as regards energy acquisition (Chapter 4.1), this represents a compensation for the drastic reduction in absorption efficiencies, both AE_N and AE_C in this case, recorded on feeding D3. Thus, although high rates of N and C egestion tended to cancel out the increment in ingestion of both elements, the outcome is that rates of absorption were, on average, improved between exposure diets D1 and D3 (*Table 4.2 - 3*). In the absence of such intense feeding response, as occurred for clams conditioned to D3, or S clams of the D1 conditioning group, the exposure to low quality D3 diet was seen to result in significantly lower rates of N and C absorption (*Figure 4.2 - 2*) that translates in lower SFG values (*Table 4.2 - 3*). Increased feeding activity to compensate for the presence of poorly digestible food items (more abundant in D3 diets) is common in bivalves (Cranford and Hill, 1999; Navarro et al., 1996) but we can infer from the present results that only an adequate physiological status of individuals would enable such adjustment to be accomplished, which explains this response were restricted to the F clams from the D1 conditioning group. Clams of this same condition (F/D1) were also found to compensate for the quantitative decrease in food supply, from H to L ration, by minimizing both N and C fecal losses, confirming the potential of prior nutritional status in active physiological regulation.

4.2 Digestive responses

Digestive performances of clams fed the two diet compositions tested in physiological experiments were neatly different as the absorption efficiencies of both N and C attained with D1 were ~ 50% higher compared with D3, with only minor variability

arisen from conditioning diet and growth category superimposed to this difference. Contrarily to D1, a neatly reduced digestibility of D3 was inferred from the negative relationship found with this diet between the overall organics absorption efficiency (Conover) and the ingestion rate of organics (see Chapter 4.1), that also applies to the absorption processes of both elements ($AE_N = 0.73\ e^{-13.99\ IR_N}$ and $AE_C = 0.72\ e^{-3.34\ IR_C}$). Besides, specific digestive requirements related to the high lipid content of MFL might become constrained by the reduced dietary supply attained with D3 (see Chapter 4.1).

As a potentially limiting component, the digestive fate of lipids with the different diet compositions was approached in an indirect way by using a ratio of elements (N and C) to energy, to compare the amount (µg) of each element ingested or absorbed per Joule of energy ingested or absorbed, respectively. The main difference in these ratios was determined by differences in food composition where values attained with diets D1 were consistently lower, due to the higher energy content that resulted from higher lipid abundance characteristic of this phytoplankton rich diet, compared with the lower energy content / higher abundance of carbohydrates in the yeast rich D3 (*Figure 4.2 - 6*). Accordingly, departure of ratios computed for the absorbed ration from the corresponding values for the ingested ration (dashed lines in *Figure 4.2 - 6*) would be indicative of a reduction of the lipid content of feeds along the digestion and absorption processes, compatible with the reported lipid enrichment of fecal materials in bivalves (Bradshaw et al., 1989; Ibarrola et al., 1998, 1996), associated to endogenous MFL (Hawkins and Bayne, 1985) having a predominance of lysosomal membranes and lipid droplets residuals derived from intracellular digestion (Morton, 1983; Owen, 1972). This interpretation would account for the observation that the alleged digestive lipid imbalance was found higher under the lipid-poor D3 diet, especially when dosed at high ration and in F clams (*Figure 4.2 - 6*), both conditions characterized by elevated digestive turnover rates and consequent high MFLs.

The absorption *vs.* ingestion ratio differences were also found to be greater, in general, for N than for C (*Figure 4.2 - 6*), reflecting the preferential absorption of N relative to C. As a matter of fact, the C:N ratios computed from absorption efficiencies were always below 1 (*Figure 4.2 - 7*), although important differences were found between conditions: as a general rule, preferential N absorption (lower $AE_{C:N}$ ratios) was more intense in F clams and clams fed the low quality D3 diet dosed at high ration, although some important exceptions were found especially within the D3 conditioning group

(*Figure 4.2 - 7*). However, a relevant aspect of this trait behavior concerns to the coupling between a higher capacity for N *vs.* C absorption and reduced values of absorption efficiency of overall organics (Conover) characteristic of clams fed the D3 diet, but partly caused also by the higher feeding rates exhibited by the above experimental groups (see Chapter 4.2 and *Table 4.2 - 1*). *Figure 4.2 - 7* (insert) clearly illustrates this behavior by showing the progressive separation of AE_N from the line defined by total and C absorption efficiencies as values declined, in a process similar (albeit to a lesser extent) to that exhibited by clams of the congeneric species *R. philippinarum* fed diets differing neatly by their N content (see Chapter 2.2).

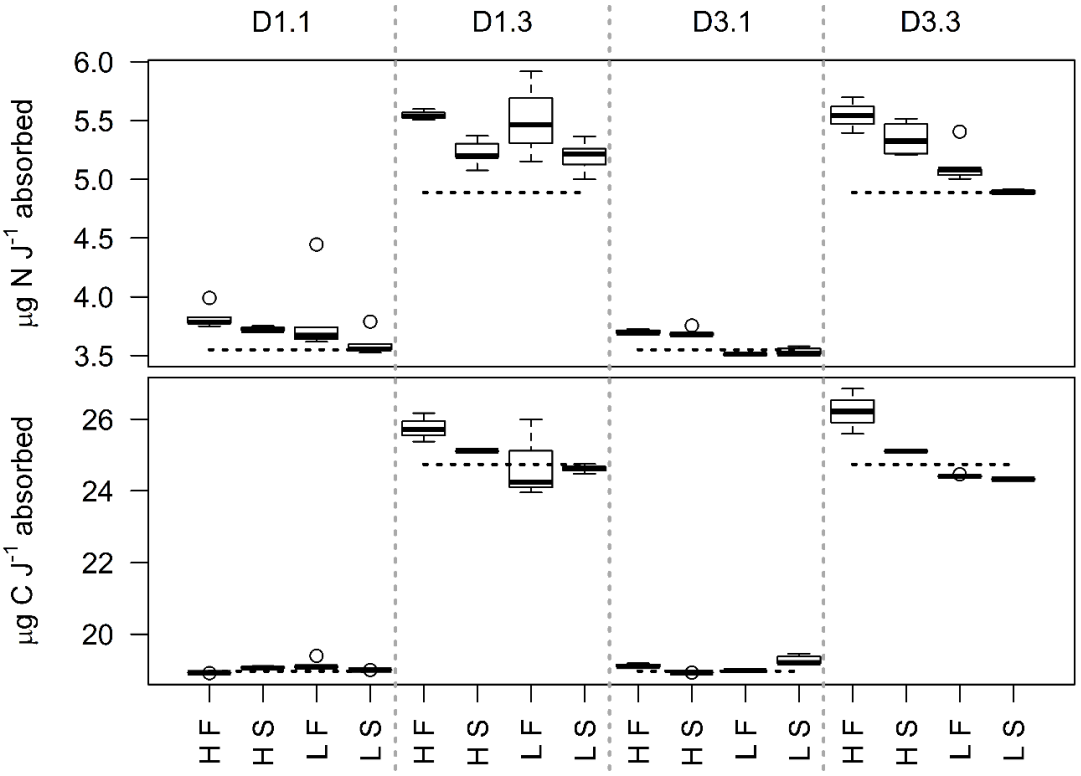

Figure 4.2 - 6. Rates of N (up) and C (down) absorption (µg) per Joule absorbed. Horizontal dotted lines represent rates of N and C (respectively) per Joule ingested

Preferential absorption of N relative to C appeared in that case associated to reduced N availability in the diets presenting reduced AE values and consequently interpreted as a compensation for nutrient imbalances in order to maintain elemental homeostasis (Cranford, 1995; Cranford and Grant, 1990; Grant and Cranford, 1991; Hawkins, 1985; Hawkins and Bayne, 1985; Kreeger et al., 1996; Smaal and Vonck, 1997). No differences in the dietary availability of N occurred in the present case and the preferential absorption of N under conditions promoting low AE values, rather than active

regulation mechanisms, might simply reflect the greater weight on digestive balances of endogenous losses (MFL) presenting an extremely high C:N ratio.

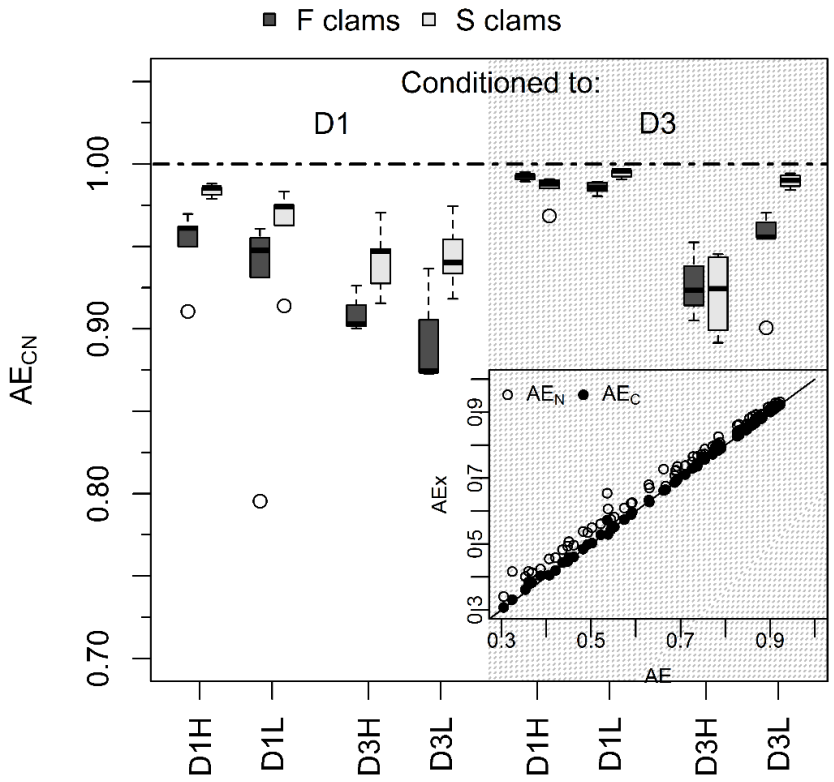

Figure 4.2 - 7. $AE_{C:N}$ ratios of F (dark boxes) and S (light boxes) clams subject to the different nutritional condition. Insert: The relationship between absorption efficiency of each element (C and N) and overall organics absorption efficiency (Conover)

4.3 Postabsorptive processes and elemental balances

Release of metabolic products through respiration and ammonia excretion showed little variation among conditions and had minor effects on energy balances (Chapter 4.1) but the combined contribution of both metabolic expenses to the net elemental balances, regarding C:N ratios, resulted crucial. When changes in these ratios are traced through the successive components of elemental balances (*Figure 4.2 - 8*) it becomes clear that the main step in the relative N enrichment achieved for the retained fraction (or SFG) occurs in the postabsorptive phase, which was essentially due to large amounts of C respired respect to the N excreted. On average, contribution of these postabsorptive processes amount to ~ 70% of total C:N change compared with ~ 30% attributable to digestive processes in the preabsorptive phase.

However, important difference, in both the magnitude of overall stoichiometric adjustments (total reduction of C:N ratios from the ingested to the retained fractions) and

its distribution between pre and postabsorptive components, can be attributed to different growth or diet conditioning groups of clams. For instance, a more complete adjustment was observed in F clams (21.5% mean reduction) compared with S clams (15.7%), as well as in clams conditioned to D1 (22.5%) compared with those conditioned to D3 (15.3%), while no differences at this respect were observed between clams fed either D1 (19.0%) or D3 (18.5%). Thus, it appear that improved physiological status associated to both endogenous and nutritional conditions, prior to the onset of physiological measurements, would have enabled a more efficient stoichiometric coupling between consumed diets and biosynthetic requirements of growing tissues. Regarding the distribution of these adjustments between pre and postabsorptive processes, no differences were observed between conditioning groups D1 and D3 departing from the mean 29% /71% distribution and only little deviations were observed for different growth groups, with metabolic processes presenting a slightly higher influence (73%) in S compared with F (69%) clams. However, great differences were observed associated to the feeding regime during physiological experimentation where, for instance, the proportional contribution changed from 12%/88% in clams fed the diet D1 to 45% / 55% un those fed the diet D3. Clearly, the improved digestive balance of N achieved with the latter diet accounts for this greater contribution of preabsorptive processes towards total stoichiometric adjustments.

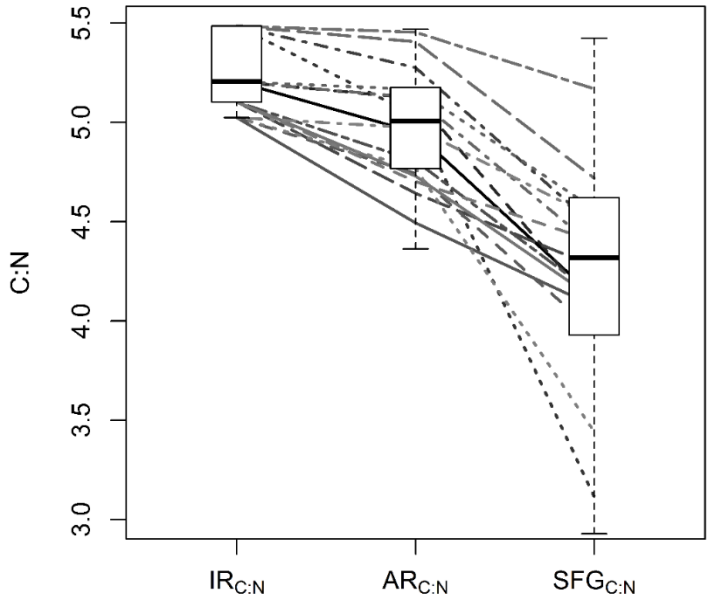

Figure 4.2 - 8. C:N ratios for IR, AR and SFG. Lines represent each group combination trajectory, while boxplots stand for means of pooled values for each variable

4.4 Whole tissues composition

Inclusion of hard tissues in present determinations must be taken into account as regards protein content, since the shell organic matrix is essentially made up of protein (Gosling, 2015). From biometrical measurements regarding the weight fractions of soft and hard fractions of body tissues and the corresponding organic contents of both fractions (*Figure 4.2 - 1*), we have determined that this organic matrix comes to represent 13-14% of the total organic body weight, irrespective of size. On that account, C:N ratios of whole body tissues computed from these biochemical composition data would be expected to be reduced with respect to the ratios based on the analysis of elemental composition of soft tissues, as those performed in previous studies with juveniles of the congeneric species *R. philippinarum* (see Chapter 2.3) or in different organs of adults in both clam species (Arranz et al., 2021; Chapter 3). Paradoxically, present C:N values (range: 4.5 -5.0) were clearly above those reported in Chapter 2.3 (range: 3.9 - 4.4) or Chapter 3 (range: 3.9 – 4.2), which very likely reflects the fact that biochemical analysis in this study did not include non-protein N compounds, such as NPS, an important biochemical component of soft tissues in both species where it may reach to represent 9 – 14% of the organic fraction (Arranz et al., 2021; Chapter 3). This same methodological limitation would also account for the apparent discrepancy, in terms of C:N ratios (*Figure 4.2 - 9*), between body tissue composition of clams conditioned to D1 and D3 diets (average: 5.0) and the fraction retained as SFG in the computation of elemental balances (4.3: mean value of all groups tested in physiological experiments); both ratios would be coincident on the assumption that tissues formed along the conditioning period should be molded according to the composition of the retained fraction. It is particularly remarkable in the case of S clams fed on the low lipid content diet, where C:N ratios of tissues were 1 unit superior respect to the incorporated ratio. Actually, this phenotype increased C:N ratios of tissues proportionally from the reference diet (~ 4.5) to D1 (~ 5) and D3 (~ 5.5); whereas F clams maintained a higher degree of tissues homeostasis.

On the other hand, fluctuations of the protein fraction associated to dietary conditioning could be expected to became attenuated on account of the inclusion of the shell matrix as a rather stable structural component; however, both conditioning diet and growth condition were found to significantly affect protein levels in different comparisons (see *Table 4.2 - 7 and Table 4.2* - 8). Significantly higher protein contents recorded on D2 conditioning, compared to D1 and D3, might be indicative of higher rates

of somatic growth promoted by increased ingestion rates from the mixture 50:50 of microalgae and yeasts. This hypothesis assumes a positive feeding response to the abundant presence of yeast in the diet, similar to that observed in several groups of clams exposed to D3 diets, but where the concurrent drawbacks of reduced digestibility of yeasts would had been accordingly reduced. In support of this interpretation, Albentosa et al. (2003) and Navarro et al. (2000) have reported that the incorporation of carbohydrate or lipid supplements to a phytoplankton diet resulted in 6-8-fold increments in the ingestion rates of clams and scallops, respectively.

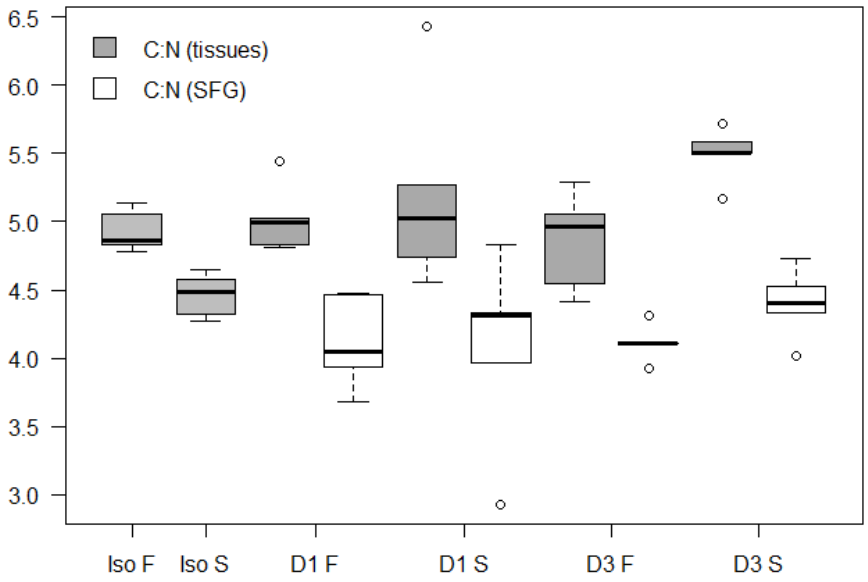

Figure 4.2 - 9. Comparison between C:N composition of tissues (gray boxes) and net incorporation of C:N (white boxes) of F and S clams fed on either the reference diet (Iso), D1 or D3

Concerning carbohydrates and lipids, body composition differences appear to reflect the composition of conditioning diets, with lipid contents appearing significantly higher with the phytoplankton rich D1 and carbohydrates reaching a peak with the yeast rich D3, although differences were no-significant for this last component. These results resemble earlier studies regarding the effects of conditioning diets on the distribution of both components of energy reserves, since higher lipid and lower carbohydrate contents were also reported in spat of the species fed on microalgae respect to those fed on wheat germ flours (Albentosa et al., 1999; Fernández-Reiriz et al., 1998) or cornstarch (Fernández-Reiriz et al., 1999), which suggests a limited ability of *R. decussatus* for maintaining nutritional homeostasis. This limitation could be aggrieved in the present

case if the processing of less digestible yeast would entail a reduced digestive balance of lipids associated to MFL.

In conclusion, results of the present work reported that *Ruditapes decussatus* juveniles fed on diets possessing a similar C:N ratio, but differing in terms of carbohydrate/lipid proportion *a)* reinforced the findings described in Chapter 4.1 that pointed to nutritional limitations of the low lipid content diet, probably due to reduced digestibility coupled to impaired digestive balances of lipids, *b)* establishes the necessity for a minimum nutritional condition to maximize growth phenotype differences, *c)* identifies the greatest impact on elemental balances at the preabsorptive and absorptive level in quantitative terms, although C:N ratios were modified essentially at the postabsorptive level and *d)* revealed differences in proximate composition among diets that suggest that microalgal replacements by inert diets above 50% can compromise growth performance as well as lipid and protein content.

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Chapter 5

Concluding remarks

~ CONCLUDING REMARKS ~

In this section, a summary of the main results of this Thesis will be provided together with the main conclusions attained. Regarding results reported in Chapter 2:

a) Manila clam juveniles were found to achieve higher rates of net energy and elemental (C and N) balances under the high protein content diet (N+), with SFG values exceeding by 50% on average the values of the low-protein (N-) diet. Potential compensatory mechanism to offset low protein inputs with the N- diets appeared limited by digestive constraints resulted in reduced absorption efficiency. Clams fed the high N diet attained the highest C and N balances through a combination of higher feeding rates and absorption efficiencies of overall organics. However, the main differential effects were associated to increased absorption efficiencies for N relative to C, that were particularly observed in clams submitted to chronic N deficit in the diet. This occurred in all growth phenotypes and resulted in partial homeostatic regulation of nutrient imbalance operating at the preabsorptive level, coupled with a postabsorptive homeostatic regulation based on the reduction of N excretion with the N poor diet.

b) Endogenous differences in energy balances associated with segregated phenotypes were mainly accounted for by differences in energy acquisition, with feeding rates differing by ~ 2-fold between fast and slow growers. Additionally, significant differences were recorded for the unitary metabolic costs (i.e., per unit of metabolizable energy), indicating that higher metabolic efficiency was also a component of faster growth. As to elemental balances, main phenotype differences were associated to the intra family comparisons (F *vs*. S growth groups), with F clams exceeding by 50% the absorption rate of nutrients (both C and N) of S clams. Also, F clams appeared best suited than S calms to adjust physiological parameters in the acute response to dietary change, indicative of a higher plasticity of this phenotype. Whereas, stoichiometric adjustments by S clams resulted in higher N release through egestion and excretion, suggesting a less efficient protein turnover.

c) Main differences in condition index and body organ composition among phenotypes were driven by strong family effects, while conditioning diet effects contributed to a lesser extent. All phenotypes displayed the highest growth rates under N+ diet whose C:N ratio values closely approached the threshold element ratios; whereas, C:N ratios of the N- diet clearly exceeded the ideal element ratio, suggesting a strong N

limitation. Most organs showed a high degree of homeostatic regulation, with the exception of the digestive gland whose C:N ratios reflected differences in food composition, probably as a result of the higher turn-over rate of this tissue. Whole soft tissue elemental composition was independent on conditioning diet, with C:N ratios exhibiting a noticeable constancy, especially in fast growing phenotypes that were previously found to exhibit a greater capacity for faster adaption to variable nutritional scenarios.

Main results attained in Chapter 3 are summarized as follows:

a) Application of standard conversion factors to estimate elemental composition from direct proximate composition analysis reveals a good agreement within the ranges reported has been obtained, provided that some considerations are taken into account: *a)* the use of conversion factors outside the ranges provided in this Thesis requires to be previously contrasted, *b)* a specific correction for residual water in dried samples of bivalve tissues for CHN analysis should be applied when analyzing body tissues of marine animals, and *c)* quantification of ninhydrin positive substances (NPS) must be incorporated in the biochemical composition analyses for the precise computation of the composition in terms of N, since these non-protein N substances can represent a significant fraction of the N content. The magnitude of NPS contents in bivalve tissues would impair the reverse computation of biochemical components from elemental composition, since no accurate estimation of proteins from N contents might be possible.

b) The absence of differences in body composition found between species as regards both elemental and proximate biochemical composition reflects the morphological, evolutionary and functional proximity between them, whereas tissue differences display the differential role that each organ plays in the organism. Other sources of variability such as diet and sex should be addressed in future research.

Regarding Chapter 4, main results are summarized as follows:

a) Carpet shell clam juveniles exhibited higher scope for growth values when conditioned to the high lipid content diet. Additionally, previous conditioning to these high quality diets, by improving physiological condition of clams, appear also to enable a better compensation of poor nutritional conditions helping to maintain energy balance levels in the acute exposure to low quality (low lipid to carbohydrate ratio) diets. This

output was driven by both feeding and digestive responses resulting in enhanced acquisition of energy and absorption efficiency with the lipid-rich diets.

b) Elemental balances of both C and N achieved higher values in juvenile clams fed the lipid-rich diets, which points out to nutritional limitations under a food regime of high carbohydrate to lipid ratios. This resulted from lower digestibility of yeast particles and the concurrent digestive imbalance of lipids derived from the limited dietary income of this component. Stoichiometric coupling between consumed diets and biosynthetic requirements of growing tissues relied on postabsorptive rather than preabsorptive mechanisms, although important differences in this respect were found between conditioning diets. Lastly, proximate biochemical composition of body tissues reflected that of diets, which indicates a rather poor level of homeostatic compensation for the nutritional imbalances induced in the present experiments, and thus suggesting that microalgal replacements by inert diets above 50% can compromise growth performance as well as lipid and protein balances.

c) Main differences between fast and slow growing phenotypes are accounted for by the differential pattern of energy acquisition, where, additionally, the increased growth performances achieved when fed on the lipid-rich foods were comparatively greater in the fast- compared to the slow-growing group. Thus, nutritional limitations undergone by the carpet shell clam juveniles were better dealt with by fast growing phenotype pointing to the importance of improved energetic status in maintaining homeostatic nutrient regulation.

In the light of the main effects observed in this Thesis, the following conclusions were drawn:

1. High quality foods represented by high protein to energy ratios or lipid-rich diets were found to account for high growth rates of either species of clams (*Ruditapes philippinarum* and *R. decussatus*), correlated with increased balances of both energy (SFG) and elemental nutrients.
2. Growth limitations due to insufficient dietary protein or lipid were primarily due to digestive constraints. In fact, main diet-related effects occurred at the feeding and absorptive level. Dietary protein content and growth of juvenile Manila clams was directly correlated, thus moderate to high levels of dietary protein seem to be required to achieve an optimal growth in juvenile bivalves.

3. Growth rate differences among phenotypes of both clam species were maintained along the experimental period, irrespective of the nutritional conditions provided; which demonstrates the endogenous origin of inter-individual differences on growth.
4. Both clam species displayed differential physiological responses among phenotypes, in which fast growing individuals achieved significantly superior values of scope for growth due to higher acquisition and food processing ability (energy acquisition model), coupled to reduced unitary metabolic costs (metabolic efficiency model).
5. Fast growing phenotypes were characterized by faster short-term physiological responses to nutritional fluctuations, which allow the optimization of energy and elemental balances and suggests a higher degree of phenotypic plasticity.
6. Improved physiological status achieved by fast growers and under high quality diet conditioning enabled physiological compensation for acute changes in diet composition regarding the energy and elemental balances. As a corollary, optimal nutritional conditions would allow to maximize growth phenotype differences.
7. Irrespective of the level of N inputs slow growing appeared associated to higher N stoichiometric release, thus suggesting a less efficient protein turnover compared to fast growers.
8. Under restrictive nutritional conditions, fast growers were able to maintain a higher degree of tissues homeostasis than their slow grower counterparts.
9. Specifically regarding lipids, conditioning to a high lipid content diet enhanced physiological compensations that allowed juveniles to face acute nutritional deficits; whereas the low lipid content diet was responsible for reduced digestibility coupled to impaired digestive balances of lipids.
10. Stoichiometric N imbalances were partially compensated by a differential increase of the absorption efficiency for N over overall organics absorption efficiency, especially when clams experienced chronic N restrictions. This occurred irrespectively of the phenotype, suggesting a stoichiometric regulation based on the alteration of absorption patterns. Reduced ammonia excretion likewise suggested N conservation as a mechanism of postabsorptive regulation.

11. Physiological adjustments in response to different dietary C:N ratio allowed the maintenance of a partial homeostasis of tissues, where fast growers achieved a higher degree of homeostasis.
12. Proximate composition of the carpet shell clam showed significant changes among diets that suggest that microalgal replacements by inert diets above 50% can compromise growth performance as well as lipid and protein balances.
13. Simultaneous analysis of elemental and proximate biochemical composition on samples of different tissues of both clam species allowed suitable factors for the inter-conversion between both analytical methods to be tested. Results showed a complete methodological agreement, provided that determinations of NPS and corrections for residual water are included.