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.

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 assessment of a) the extent to which physiological behavior

underlying growth performance is genetically determined and b) how much of this behavior 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 observations: 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 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 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 17 ºC 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 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). 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 1 and Table 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 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).

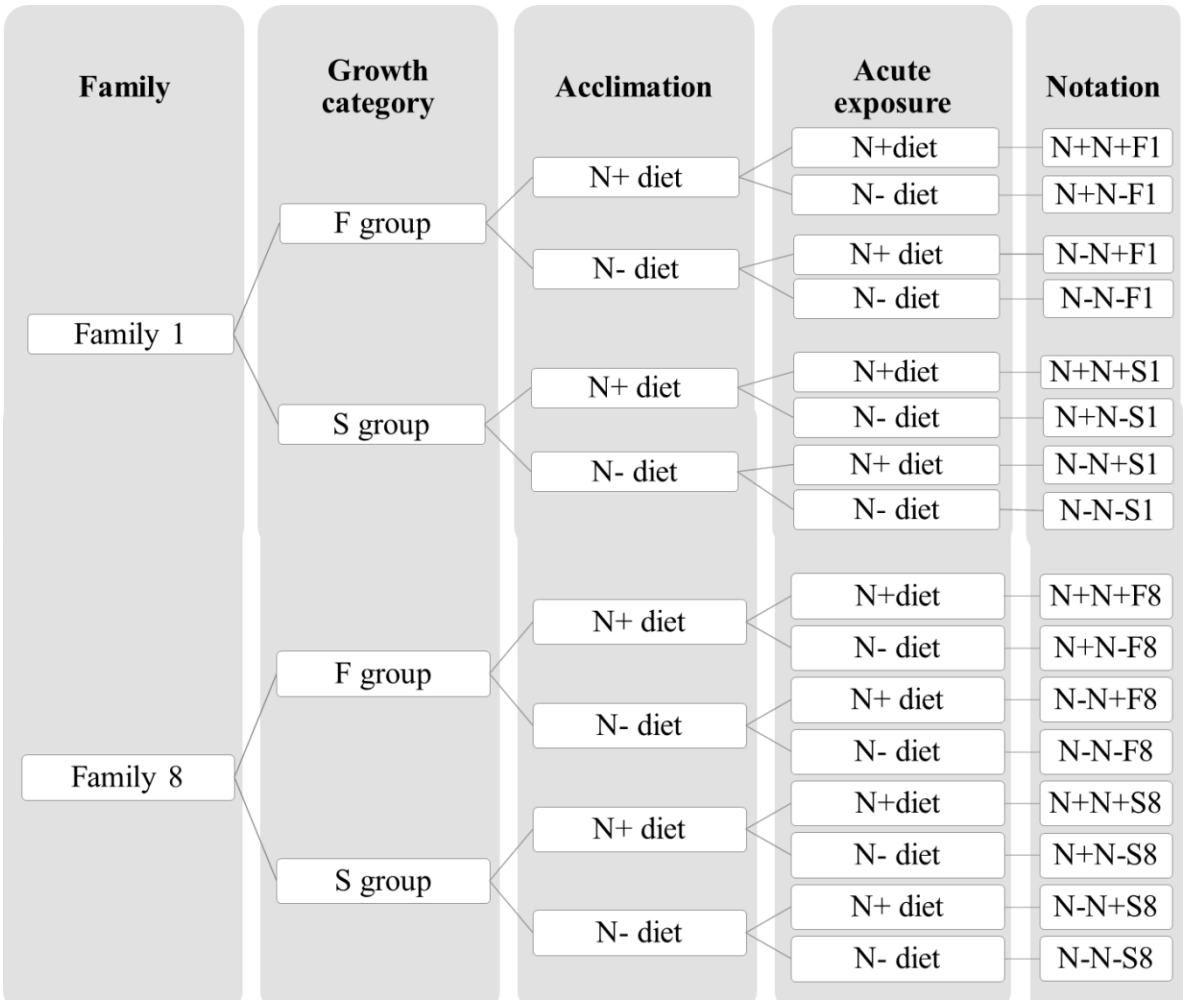

Figure 1.- Experimental setup for the recording of physiological parameters in the fast (F) and slow (S) growing groups segregated from two families. Five individuals (n=5) were used in each of the 16 resulting groups.

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. 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)

Tables 2 and 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 450 ºC 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 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 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.

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 4. Table 5 summarizes the results of the corresponding 4-way ANOVA, including terms for both single factors and factor interactions up to the 4th degree.

Table 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=clearance rate (L h^{-1}); GA=gill area (mm^2); 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).

		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 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 5 and Figure 2b) partly supports the effect that clams tend to feed faster with this diet, especially following a period of acclimation (Table 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 diets N+ relative to diets N- (Table 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 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 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 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 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 4 and 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 5), although this effect was only noticeable during acute exposure to N- diets (Figure 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 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 5).

3.2 Combined dynamics of the acute and chronic responses

Figures 2a to 5a 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.

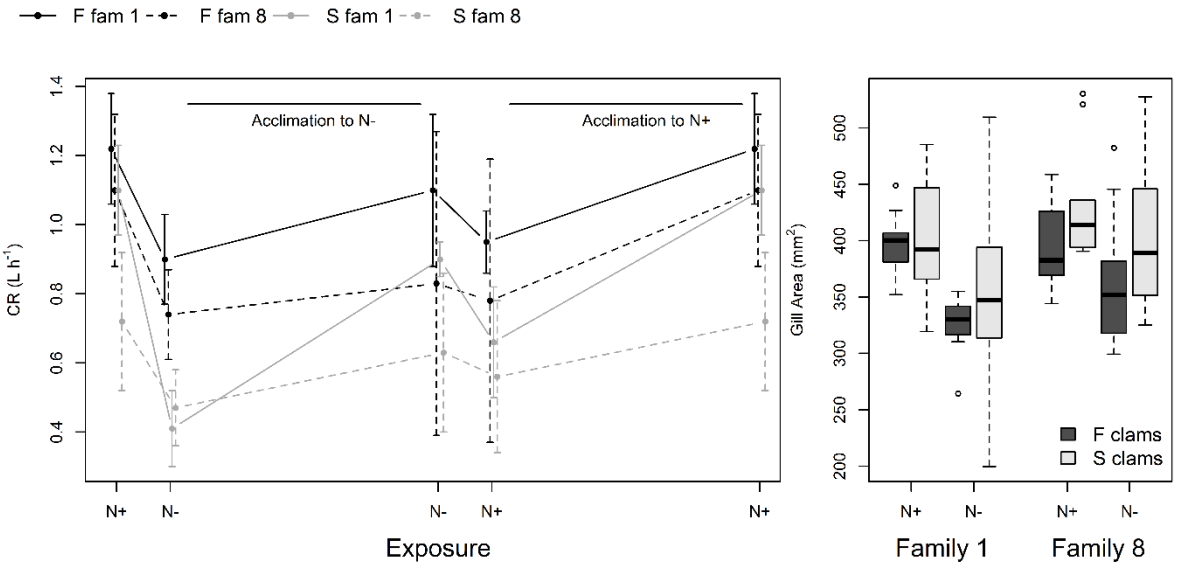

Figure 2a) Size-standardized values of clearance rate in fast (black) and slow (gray) growing clams belonging to families 1 (solid lines) and 8 (dotted lines); b) size-standardized gill area values of fast (dark) and slow (light) growing clams from both families and acclimated to N+ and N- diets.

The main dietary effects on CR are accounted for by the acclimation*exposure interaction (Table 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 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.

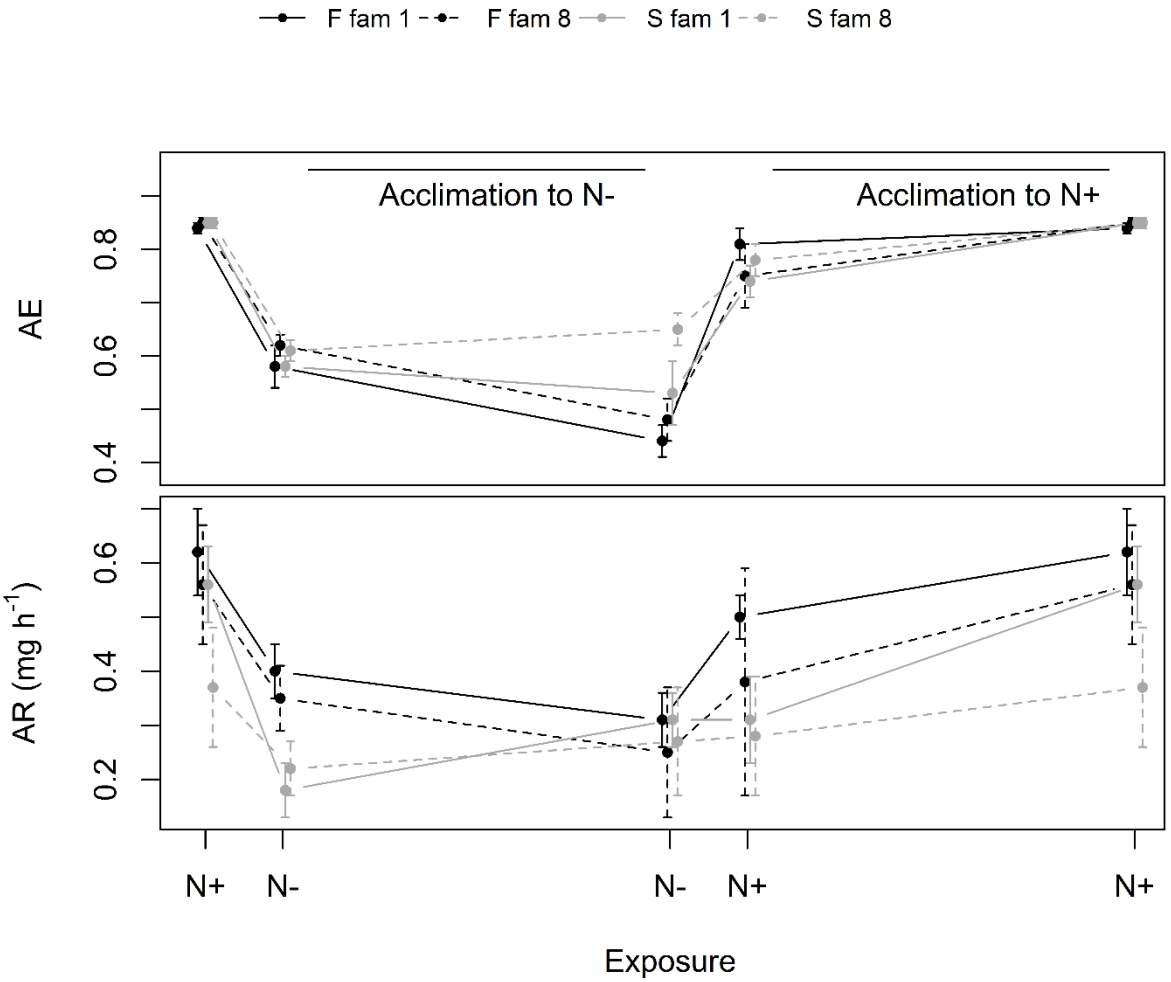

Figure 3a) Absorption efficiency and b) absorption rate values of fast (black) and slow (gray) growing clams belonging to families 1 (solid lines) and 8 (dotted lines)

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 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 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.

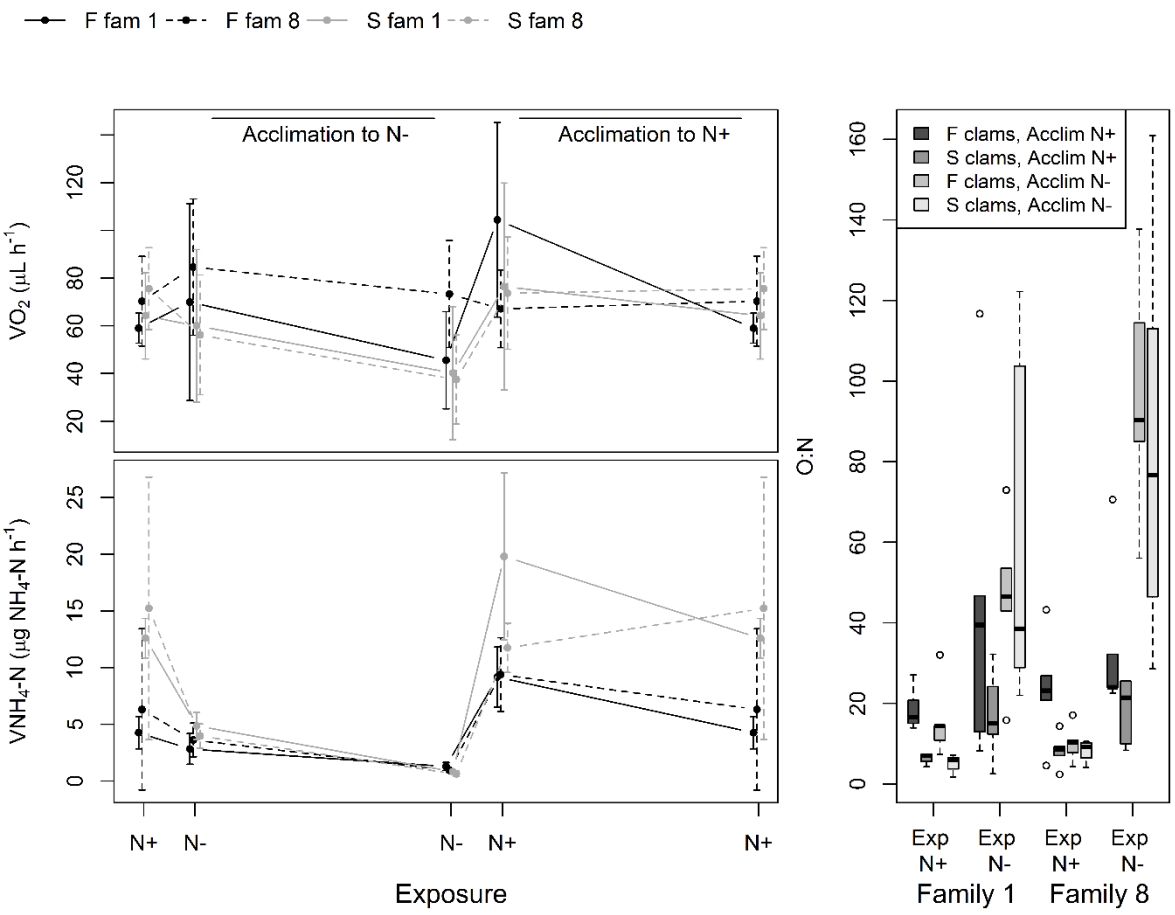

Figure 4 Size-standardized values of a) metabolic rate (VO2) and b) ammonia excretion rate of fast (black) and slow (gray) growing clams belonging to families 1 (solid lines) and 8 (dotted lines); c) O:N index of each group (Acclim: acclimation diet; Exp: exposure diet)

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 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 5; see also acclimation*exposure*growth group interaction in Table 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.

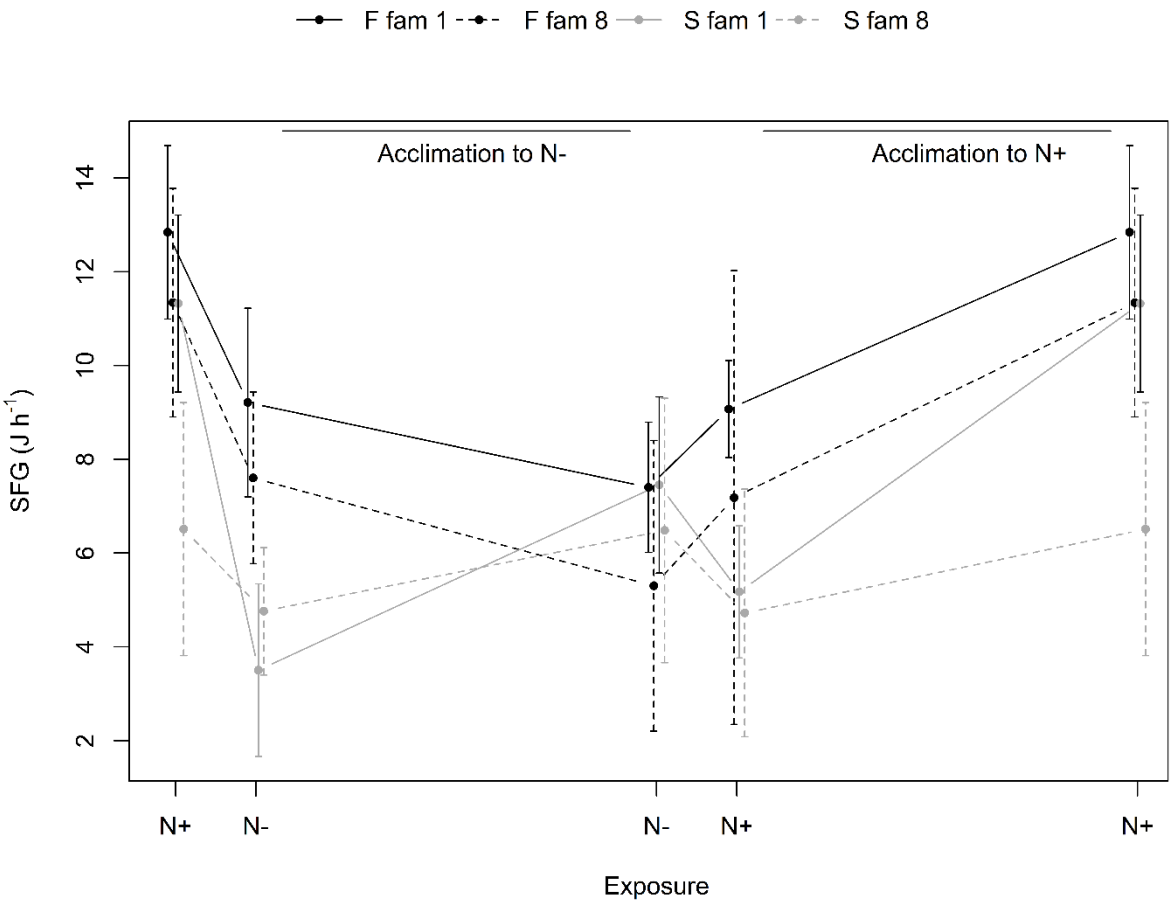

Figure 5 Size-standardized SFG values in fast (black) and slow (gray) growing clams belonging to families 1 (solid lines) and 8 (dotted lines).

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 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.

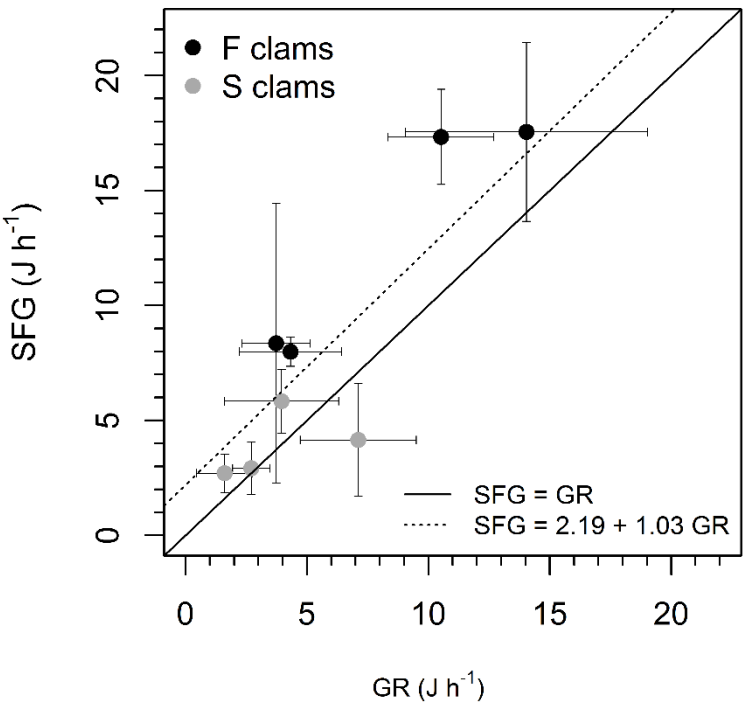

Figure 6 Relationship between SFG and actual growth for fast (black) and slow (gray) growing clams. The solid line represents y=x, while the dashed line is a plot of the regression equation fitted to the experimental data.

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

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 Figures 2a to 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 conditioned to N+ and N- diets, across the different family * growth group combinations (Figure 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.

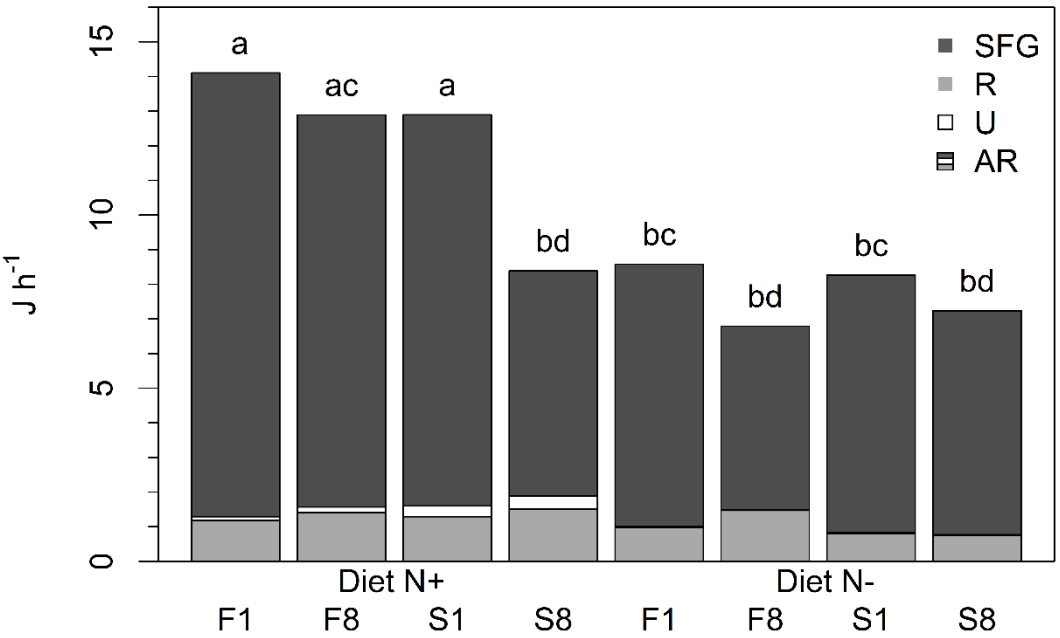

*Figure 7: Bar plot reporting components of net energy gain (sum of all categories) and loss (R: gray bars, U: white bars) and resulting SFG (dark gray bars) in the different family * growth group combinations fully acclimated to diets N+ and N-.*

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.

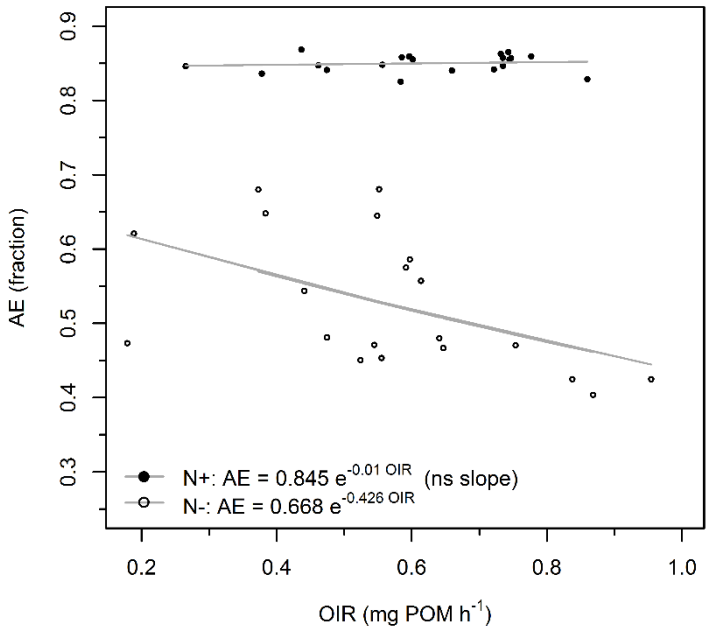

Figure 8 Absorption efficiency (AE) as a function of organic ingestion (OIR). Lines were fitted to mean values for the different groups of clams fully acclimated to N+ (closed circles) and N- (open circles) diets.

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 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 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 has been 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 behaviour 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 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 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 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.

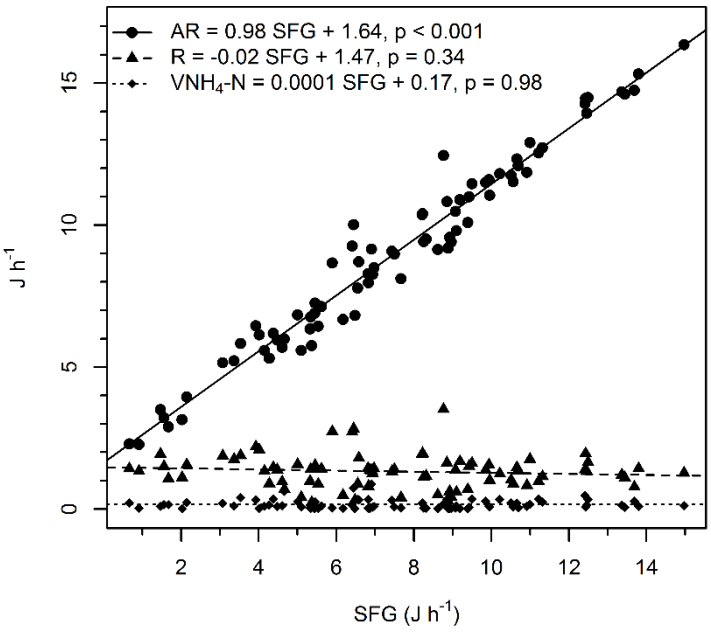

Figure 9 Net energy gain (AR) and loss (R and U) at different levels of the SFG. Lines fitted (minimum squares) to individual data for all experimental sets in this study.

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 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 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 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).

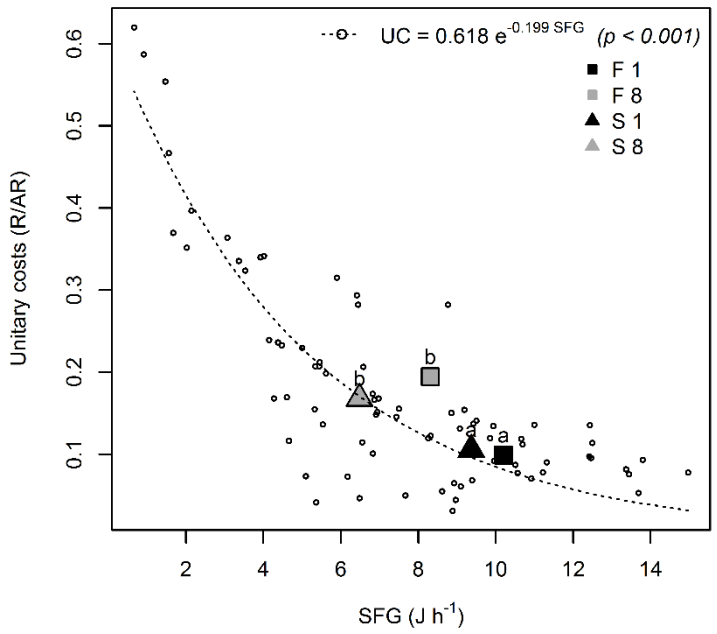

Figure 10.- Unitary metabolic costs (computed individually) as a function of SFG (open circles), and mean values of this relationship in the different growth phenotypes (squares: F clams, triangles: S clams; black symbols: Family 1, gray symbols: Family 8), under fully acclimated conditions. Superscripts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