Elemental C and N balances evidence stoichiometric adjustments to dietary protein content in growth phenotypes of the Manila clam (*Ruditapes philippinarum*)

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ABSTRACT

Juveniles from 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 growth phenotypes that resulted from these 4 family*growth category 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 protein:energy ratios. Both diets were based on the 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 the 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 with increased absorption efficiencies for N relative to C, which were particularly observed in clams subjected to chronic N deficit in the diet. This occurred in all growth phenotypes and resulted in the partial homeostatic regulation of nutrient imbalance operating at the preabsorptive level. Further adjustments occurred at the postabsorptive level with increased N disposal in the form of ammonia excretion and the resulting decrease of metabolic C:N indices recorded in clams fed the high N diets. The main phenotypic differences were observed in the intrafamily (F vs. S growth groups) rather than in the interfamily comparisons, with F clams exceeding the absorption rate of nutrients (both C and N) of S clams by 50%. Physiological responses to the acute dietary change were found to be faster in F clams, indicative of a higher plasticity of this phenotype. Whereas, stoichiometric adjustments by S clams resulted in higher N release through excretion, suggesting less efficient protein turnover.

1. Introduction

Growth is an important attribute of animal individuals and populations of marine bivalves and a factor to be accounted for in the management and sustainability of coastal ecosystems, as well 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 to result from a balance between metabolic processes of assimilation and catabolism. Bioenergetic models currently in use, either the classical scope for growth (SFG) approach or

the dynamic energy budget (DEB) theory (Kooijman, 2010), derive from the same principles, although relying on a more detailed physiological account of energy flows. These models have provided useful tools for 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 with regard to the specific influence of both endogenous and exogenous (food, temperature, etc.) factors on the various components of the energy budget.

However, the use of energy as the sole currency in these budgets has clear limitations stemming from the fact that large differences are often encountered between consumed food and consumer body tissues with regard to elemental composition. This has special relevance in

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herbivorous or detritivores relying on vegetal resources, such as most bivalve molluscs, where for instance,the rather constant C:N ratios reported for body composition (between 4.1 and 6.2) contrasts with the highly variable values of C:N ratios in 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 that involves the resource in a suit of physiological mechanisms able to compensate, whenever necessary, for the imbalance between the diet and consumer 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 description, for stoichiometry to be realized in growing tissues, any component of the diet in excess of the appropriate ratio should be disposed of, whence important constraints on growth can be expected that would hardly be identified from only the 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 (Cranford, 1995; Grant and Cranford, 1991; Bayne et al., 1993; Hawkins and Bayne, 1985), and modelling efforts have tended to incorporate parameters that take into account the elemental composition of diet to fit a better approach of actual growth (Scholten and Smaal, 1999; Scholten and Smaal, 1998; Smaal and Scholten, 1997; Bourlès et al., 2009; Brigolin et al., 2009; Emmery et al., 2011; Grangeré et al., 2009).

Mechanisms for 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. The 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 gills and palps to discriminate between microalgae (low C:N ratio) and detrital particles (high C:N ratio). In 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). Differential selection of species of phytoplankton according to their food value (Beninger et al., 2008; Cognie et al., 2001; Pales-Espinosa et al., 2008; Pales-Espinosa et al., 2007; Shumway et al., 1985) might further contribute to ingested N enrichment. As 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 (N limitation) represented by the difference between the C:N ratio in the seston and the C:N ratio in oyster tissues.

Similar selective mechanisms at the postingestive level can also be invoked to account for the 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 scenarios has been certainly reported (Bayne, 2009; Cranford and Grant, 1990; Grant and Cranford, 1991; Prins and Smaal, 1989; Iglesias et al., 1996; Urrutia et al., 1996). Even in the absence of any scope for digestive selection (e.g., more homogenous diet formulations as those used in the present study), the net N enrichment may result from the preferential absorption of dietary

proteins relative to carbohydrates and lipids reported in cockles (*Cerastoderma edule*) fed phytoplankton (Ibarrola et al., 2000b). The 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 the preferential absorption of N relative to C.

Postabsorptive mechanisms concern the metabolic fate of different elements contributing to final adjustments of the assimilated ration to meet the specific requirements for tissue biosynthesis. The 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 molluscs (namely, mussels) with regard to several features of protein turnover, where the 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 fuelling 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 efficiency of tissue deposition for the amino-N fraction compared with the amino-C fraction reported in mussels (Kreeger et al., 1996; Kreeger et al., 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. Resort to experimental diets providing a range of variation in the relative proportion of different nutrients, particularly in the protein/energy ratio, have thus proven useful in studying the contribution of these postabsorptive processes to homeostatic nutrient regulation in bivalves.

In a previous work (Arranz et al., 2020), we analysed 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). Different groups of clams were food conditioned (15 d.) with these two diets and physiological components of growth and SFG measurements were then performed in response to both the acute and chronic change in dietary composition. Additionally, the 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 to assess 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.

In the present study, the same experimental design developed to compute energy balances (Arranz et al., 2020) was extended to include the elemental balance of nutrients (C and N), aiming to test the following hypothesis concerning the occurrence of compensatory responses for differential stoichiometric imbalances between high and low N diets:

H1. Increased feeding rates acted to compensate for decreased N availability in the diet (overfeeding response).

H2. Chronic N deficit of dietary origin is compensated through the preferential absorption of N relative to C (energy): The pre-absorptive level.

H3. Metabolic activities further contributed to the stoichiometric adjustments, with metabolic C:N indexes reflecting the differential release of elements in excess: The post-absoptive level.

H4. Higher growth demands of endogenous origin are better accomplished under conditions of high N availability which resulted in the maxima balances of both N and C (energy) being achieved in fast growing phenotypes fed the N rich diet.

H5. Less efficient protein metabolism of slow growers is revealed in higher rates of ammonia excretion and reduced values of N net growth efficiency, relative to fast growers.

H6. Time course of physiological adjustments to diet quality differ between food conditioned groups and reveals differential phenotypic plasticity.

2. Material and methods

2.1. Animal maintenance, diet characteristics and experimental design

Features of diets, clam families, growth categories and the maintenance of them, including experimental design, have been previously described in Arranz et al. (2020). Briefly, specimens belonging to any of two full-sib families (1 and 8) of the Manila clam *Ruditapes philippinarum* were size-segregated to constitute F (fast growth) and S (slow growth) categories by choosing the largest and smallest clams inside each family. The mean shell lengths for the F and S categories were 23.50 (1.67) mm and 13.10 (1.65) mm, respectively. The different growth category*family combinations resulted in four growth phenotypes (F1, F8, S1 and S8) that were used in subsequent experiments.

Maintenance was carried out under constant ambient conditions in a recirculating seawater (34 PPT) system regulated at 17 °C. Two groups of clams from each of the above phenotypes were each fed the same ration (~ 0.5 mg POM L^{-1}) of the microalgal species *R. lens* reared in either the exponential (C:N ratio = 4.94) or the stationary (C:N ratio = 14.5) phases of the culture (Arranz et al., 2020). The chosen notation was N+ for diets based on microalgae in the exponential phase and N- for those in the stationary phase. These diets differed by ca. 3-fold in N content and were used to assess elemental balances of clams in the context of the acute and chronic physiological response to dietary change.

Clam groups were conditioned for 15 days to diets N+ or N-. Following that "acclimation" period, physiological measurements were carried out while subgroups of clams from each conditioning group were exposed to either the conditioning diet (chronic response) or the alternative diet (acute response). This design resulted in the 16 experimental groups reported in Table 1.

2.2. Elemental balances

Samples for diet characterization were performed twice a week in quadruplicate during the acclimation period and 5–6 times in quadruplicate during the exposure experiments (Arranz et al., 2020). Elemental analysis (CNH) of diets was conducted on samples collected over preweighed glass fibre filters (GF/C) by filtering a known volume of water from the feeding tanks and washing with 50 ml of filtered seawater. Individual samples of faeces produced over 8–10 h were similarly collected over preweighed GF/C filters and washed with 50 ml filtered seawater. Both types of samples were immediately frozen at −20 °C, lyophilized, and maintained at −20 °C until being analysed in a Euro EA Elemental Analyser (CHNS) from Euro Vector, using acetanilide as a standard. A subset of samples was calcined for 6 h at 450 °C and subsequently measured in an elemental analyser to be used as a control.

Physiological components of the scope for growth were determined as previously described (Arranz et al., 2020), where in short, five replicates per condition were used to determine the 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 mean values in Table 1). Individual values of this dataset were combined with elemental analysis of diets and faeces to compute N and C balances as follows:

2.2.1. 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:

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

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

The ingestion rates of N and C (IR$_N$ and IR$_C$: mg h^{-1}) were estimated by multiplying the clearance rate by the particulate organic matter of each element, in these terms:

$$IR_N = PON \times CR$$

$$IR_C = POC \times CR$$

2.2.2. 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 faeces, 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 then given by

Table 1

Mean (SD) values 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}), from Arranz et al. (2020), used to calculate the present elemental balances.

Food conditioning	Acute exposure	Growth category	Family	CR	AE	VO2	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)

the quotient between AR and IR of each element:

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

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

2.2.3. Carbon and nitrogen losses

Carbon loss due to respiration (R_C, mg C h^{-1}) was derived from respiration rates (ml O$_2$ h^{-1}), assuming a respiratory quotient RQ = 0.9 mol CO$_2$ mol O$_2^{-1}$. Respiration rates were obtained with the aid of oximeters by monitoring the decline in oxygen concentration in sealed chambers filled with seawater where clams had been introduced. Nitrogen loss due to excretion (E_N, mg N h^{-1}) was assessed through the determination of increments in ammonia concentration by the phenol-hypochlorite method (Solórzano, 1969), employing flasks filled with 30 ml of filtered seawater (0.2 μm Millipore membranes) for 2–3 h. For both determinations, chambers without animals were employed as controls.

2.2.4. Elemental balances and growth efficiencies of carbon and nitrogen

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

$$SFG_C = AR_C - R_C,$$

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

$$SFG_N = AR_N - E_N$$

The growth (deposition) efficiencies of both elements were computed as follows:

$$NGE_N = \frac{SFG_N}{AR_N}$$

$$NGE_C = \frac{SFG_C}{AR_C}$$

After concluding the experiments, clams were dissected to compute the dry weight of tissues, and consequently, all rates were standardized to the common dry weight of soft tissues (85.95 mg) using mass exponents of 0.6091 (CR), 0.6967 (R) and 1.00 (VNH$_4$-N).

2.3. Data analysis

Relationships between two or more quantitative variables were explored through regression analysis, while comparisons for qualitative factors, family, growth category, acclimation diet and exposure diet were tested through 4-way ANOVA using R (R Core Team, 2018). These analyses were performed after testing for the normality (Shapiro–Wilk) and homoscedasticity (Levene) of the data.

3. Results

Results of the present experiments have been disposed in order to fit the sequence of hypothesis set in the introductory section.

3.1. Diet effects

H1. Increased feeding rates acted to compensate for decreased N availability in the diet (overfeeding response)

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 supplied to the animals during physiological determinations (exposure diet) than for previous conditioning, although some effects concerning the latter variable will be considered.

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

Ingestion rates of C reached the highest values in those groups fed the same diet used in conditioning (e.g., N + N+ and N-N-; Fig. 1). However, trends were similar for C and N as regards the net acquisition (higher absorption rates of C in clams fed the N+ diet).

Thus, maximization of energy obtained from high quality foods appear to be the rule in these experiments, rather than compensatory overfeeding for N income regulation on low quality diets. An interpretation of these features based on the occurrence of digestive constraints will be discussed in the corresponding section.

H2. Chronic N deficit of dietary origin is compensated through the preferential absorption of N relative to C (or energy): The pre-absorptive level.

Absorption efficiencies for both N and C were clearly superior ($p < 0.001$) when exposed to the N+ diet; acclimation to this high quality diet also had a positive effect but only on AE_C. Differential performance for N and C in the different acclimation*exposition groups of clams were analysed by plotting elemental AEs against the overall AE values for organics (Conover) reported in Arranz et al. (Arranz et al., 2020; Fig. 2). As expected, the AE_C closely fitted the Y = X line ($AE_C = 1.04\,AE - 0.003$, $R^2 = 0.997$, $p < 0.001$), while 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 was 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., conditioned to N-diets). In fact, results of ANOVA testing the difference between AE_N and AE (Conover),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 chronic response ($F = 114.2$, $p < 0.001$) and the interaction term: ($F = 43.7$, $p < 0.001$).

H3. Metabolic activities further contributed to the stoichiometric adjustments, with metabolic C:N indices reflecting the differential release of elements in excess: The postabsoptive level.

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

As a result of the previous differences, elemental balances for N and C showed significant differences (Table 3) for conditioning, exposure and their interaction. As before, acute exposure to diets with different N contents affected the balance of both C and N to a greater extent, and the best performance was accomplished in clams fed the N+ diet (Fig. 3 and Table 2). Conditioning diet was also important, but its effects on elemental balances were 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 cancelling out the potential benefits of high-quality (N+) conditioning.

Overall, metabolic C:N indices (Table 2) reflected the differential stoichiometric release of both elements according to the diet supplied: Both chronic and acute exposure to diets of high N content promoted a 2 to 4-fold reduction of this index (highly significant; Table 3), that was mainly based on increased rates of ammonia excretion.

Table 2

Pooled mean values (SE) for conditioning, 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), $M_{C:N}$ (metabolic C:N indices), SFG_N and SFG_C (elemental balances for N and C) and NGE_N and NGE_C (net growth efficiencies for N and C): Units are µg h^{-1} for rates and SFG and decimal for efficiencies.

	Conditioning		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)
$M_{C:N}$	9.94 (2.16)	17.01 (3.68)	5.04 (0.88)	20.75 (3.36)	14.79 (2.91)	12.12 (3.52)	12.56 (2.94)	14.72 (3.41)
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 (0.04)	0.78 (0.04)	0.71 (0.05)	0.82 (0.03)	0.85 (0.02)	0.67 (0.05)	0.78 (0.04)	0.76 (0.04)
NGE_C	0.82 (0.02)	0.79 (0.02)	0.81 (0.02)	0.80 (0.02)	0.82 (0.02)	0.78 (0.02)	0.82 (0.02)	0.79 (0.02)

The above was clearly reflected in the net growth efficiencies for C and N. The efficiency for C (NGE_C) was fairly constant across diets, while NGE_N increased significantly in clams fed the N- diet (Tables 2 and 3) pointing to compensation for N deficit. Regarding both elemental growth efficiencies, interaction terms for acclimation and exposure diets (Table 3) were significant, accounting for the fact that the highest efficiencies were achieved in the experimental groups where conditioning 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 because 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

H4. Higher growth demands of endogenous origin are better accomplished under conditions of high N availability, which resulted in maxima balances of both N and C (energy) being achieved in fast growing phenotypes fed the N rich diet.

Generally, significant effects on physiological parameters involved in elemental balances were mainly due to growth category (Table 3), although differences between families were also detected. Fast-growing juveniles registered higher values for both ingestion and egestion rates of both N and C (Fig. 4). The resulting digestive balances represented by absorption rates were 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 the 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 intrafamiliar effects led to a gradual decrease in processing rates from the F1 to S8 groups (Fig. 4).

Fig. 5 shows the distribution of AE values (both C and N) across dietary conditions and phenotypes. The mains effect on these parameters consists in AEs increasing their values by ~70% in the change from N- to N+ diets. This combines with the ranking distribution of food processing rates among phenotypes (Fig. 4) to result in fast growers fed the high quality diet achieving the highest values for both C and N balances (Tables 2; Fig. 7). In addition, both endogenous factors (family and growth category) exerted weak although significant effects on AE_N and AE_C (Table 3), and with few exceptions, these efficiencies were higher for F8 specimens than for F1 specimens (<10% difference between families).

H5. Less efficient protein metabolism in slow growers is revealed in higher rates of ammonia excretion and reduced values of net growth efficiency for N.

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

Phenotype effects on elemental balances for C and N were restricted to growth category, whereas family effects were not significant (Fig. 7; Table 3). F vs. S differences were highly significant (Tables 2 and 3) 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; Fig. 7). Net growth efficiencies were, in general; higher for F clams than for S clams (Table 2; Fig. 8), but only differences in NGE_N attained the significance level (Table 3).

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

H6. Time course of physiological adjustments to diet quality differed between food conditioned groups and revealed differential phenotypic plasticity.

Considering the 4 factors (conditioning, exposure, growth category and family), N and C balances are represented in Fig. 7 to account for the inter- and intrafamiliar 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, the plots of Fig. 7 will be described together. When clams conditioned 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 the fact that only about one third of N and half of C was being retained following this change from high to low quality diet. Full conditioning to the N- diet (N-N- groups) barely affected the fast growers performance, while S clams nearly doubled their values. The acute response of N- conditioned clams to 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 clams 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 3), which was only significant for SFG_N. Finally, compared to low N conditioning, high N conditioning promoted a neat increase in the net C and N balances for all growth

Table 3

Diet conditioning (C) and 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, $M_{C:N}$ (metabolic C:N indices), and SFG_N and SFG_C: elemental balances for N and C).

	IR_N	IR_C	ER_N	ER_C	AR_N	AR_C	AE_N	AE_C
C	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**	F = 2.819, p = 0.105	**F = 29.48, p < 0.001**
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**	**F = 422.92, p < 0.001**	**F = 686.95, p < 0.001**
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**	**F = 5.44, p = 0.028**	**F = 11.017, p = 0.003**
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	**F = 10.34, p = 0.003**	**F = 5.636, p = 0.025**
C*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**	**F = 14.84, p = 0.001**	F = 0.203, p = 0.656
C*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	F = 2.425, p = 0.131	**F = 4.509, p = 0.043**
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	**F = 9.569, p = 0.005**	**F = 14.286, p = 0.001**
C*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	F = 0.005, p = 0.946	F = 0.832, p = 0.37
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	**F = 13.15, p = 0.001**	**F = 8.746, p = 0.007**
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	F = 0.927, p = 0.345	**F = 7.022, p = 0.014**
C*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 = 0.219, p = 0.661	F = 1.243, p = 0.275	**F = 4.76, p = 0.038**	**F = 7.834, p = 0.01**
C*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	**F = 4.94, p = 0.035**	F = 3.53, p = 0.072
C*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	F = 3.87, p = 0.06	**F = 12.416, p = 0.002**
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	F = 0.946, p = 0.34	F = 0.017, p = 0.898
C*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	F = 0.61, p = 0.441	F = 0.066, p = 0.8

	R_C	E_N	$M_{C:N}$	SFG_N	SFG_C	NGE_N	NGE_C
C	F = 0.114, p = 0.738	F = 1.313, p = 0.262	**F = 5.596, p = 0.026**	**F = 5.305, p = 0.03**	**F = 7.027, p = 0.013**	F = 0.131, p = 0.72	F = 0.932, p = 0.343
E	**F = 5.385, p = 0.028**	**F = 69.54, p < 0.001**	**F = 30.271, p < 0.001**	**F = 80.96, p < 0.001**	**F = 26.77, p < 0.001**	**F = 9.81, p = 0.004**	F = 0.359, p = 0.554
G	F = 2.963, p = 0.097	**F = 10.294, p = 0.004**	F = 2.79, p = 0.107	**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 = 0.038, p = 0.846	F = 0, p = 0.984	F = 1.001, p = 0.326	F = 0.637, p = 0.432	F = 0.86, p = 0.362	F = 0.108, p = 0.746	F = 0.89, p = 0.354
C*E	**F = 5.751, p = 0.024**	**F = 8.387, p = 0.008**	**F = 9.702, p = 0.004**	**F = 14.82, p = 0.001**	**F = 9.969, p = 0.004**	**F = 29.558, p < 0.001**	**F = 6.68, p = 0.016**
C*G	F = 0.013, p = 0.91	F = 0.02, p = 0.888	F = 2.305, p = 0.141	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 = 0, p = 0.989	**F = 7.509, p = 0.011**	F = 0.083, p = 0.776	**F = 8.031, p = 0.009**	F = 1.035, p = 0.318	F = 3.764, p = 0.063	F = 0.057, p = 0.814
C*F	F = 0.253, p = 0.619	F = 3.653, p = 0.067	F = 1.856, p = 0.185	F = 0.048, p = 0.828	F = 0.282, p = 0.6	F = 0.327, p = 0.573	F = 0.598, p = 0.446
E*F	F = 1.025, p = 0.321	F = 0.005, p = 0.942	F = 1.346, p = 0.257	F = 0.64, p = 0.431	F = 0.146, p = 0.706	F = 0.955, p = 0.337	F = 0.001, p = 0.981
G*F	F = 0.557, p = 0.462	F = 1.599, p = 0.217	F = 1.098, p = 0.304	F = 0.002, p = 0.968	F = 0.01, p = 0.92	F = 1.526, p = 0.228	F = 0.532, p = 0.472
C*E*G	F = 0.507, p = 0.483	F = 0.207, p = 0.653	F = 1.09, p = 0.306	F = 0.343, p = 0.563	F = 0.75, p = 0.394	**F = 5.983, p = 0.022**	F = 0.246, p = 0.624
C*E*F	**F = 5.023, p = 0.034**	F = 3.674, p = 0.066	F = 2.68, p = 0.114	F = 0.171, p = 0.682	F = 0.158, p = 0.694	F = 0.836, p = 0.369	F = 0.829, p = 0.371
C*G*F	F = 0.794, p = 0.381	F = 0.661, p = 0.424	F = 1.751, p = 0.197	F = 0.723, p = 0.403	F = 0.403, p = 0.531	F = 0.444, p = 0.511	F = 1.175, p = 0.288
E*G*F	F = 1.988, p = 0.17	F = 0.632, p = 0.434	F = 1.113, p = 0.301	F = 0.004, p = 0.953	F = 0.591, p = 0.449	F = 0.125, p = 0.727	F = 1.14, p = 0.295
C*E*G*F	F = 2.998, p = 0.095	F = 1.307, p = 0.263	F = 2.644, p = 0.116	F = 3.993, p = 0.056	F = 1.258, p = 0.272	**F = 4.963, p = 0.035**	F = 0.378, p = 0.544

Significant differences ($p < 0.05$) are shown in bold.

group*family phenotypes.

Similar acute vs. chronic dynamics were also observed for net growth efficiencies (Fig. 8), with differential effects accounting for 3rd- and 4th-order interaction terms (Table 3), which were only significant for nitrogen.

4. Discussion

One important issue from the stoichiometric approach concerning heterotrophic organisms is that trophic relationships might be "misleading" if only energy balances are considered. In the physiological
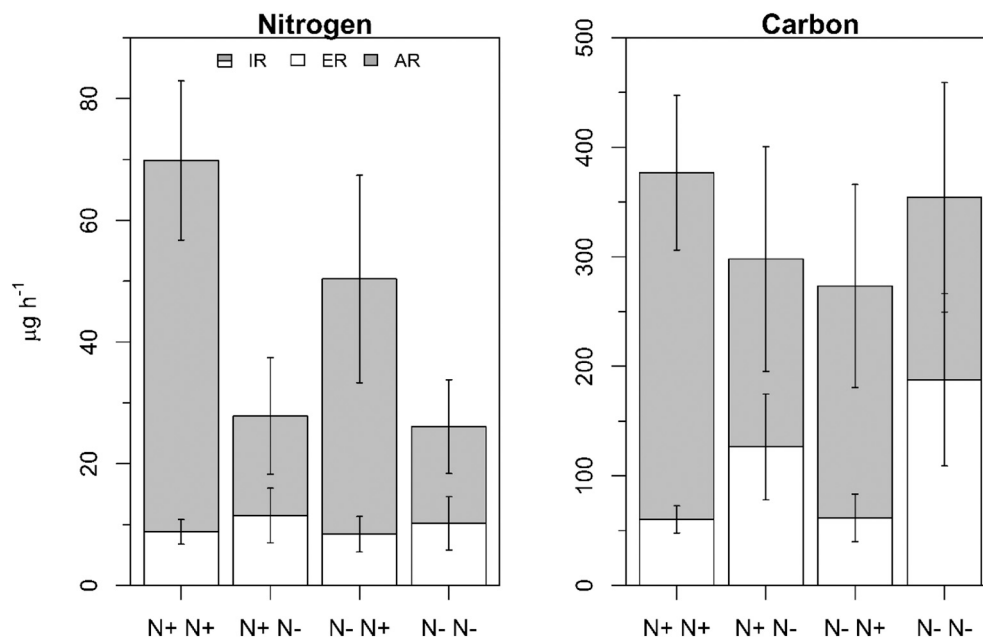
Fig. 1. Acute and acclimated response to dietary N changes for ingestion (IR), egestion (ER) and absorption (AR) rates of N (left) and C (right).
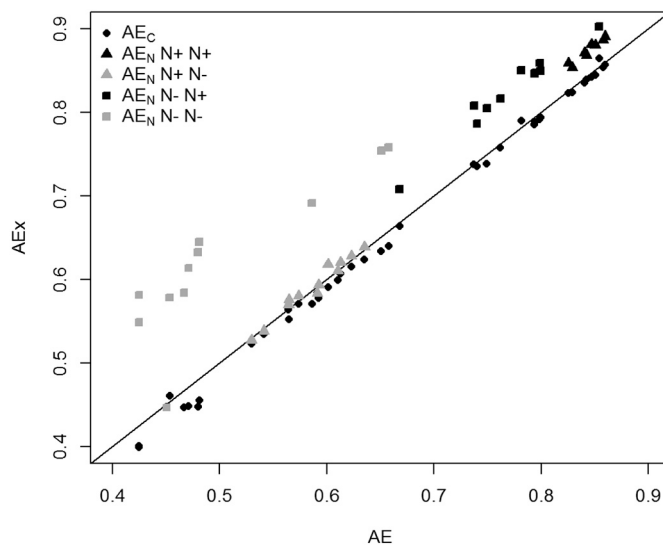

Fig. 2. Relationship between AE_N and AE_C and Conover AE, wherein circles represent the AE_C values, triangles represent AE_N from clams conditioned on the N+ diet, and squares represent AE_N from clams conditioned on the N- diet. For AE_N data points, black symbols represent animals exposed to N+, while grey symbols are from those exposed to N-.

description of growth, consideration of specific nutrient balances becomes essential to account for features from feeding, digestion and metabolic behaviour that appear to be associated with the necessary coupling between the available food and the growing tissues (Bayne, 2017). The computation of C and N balances provides 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 a ~ 3-fold range of variation in the C:N ratio and 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, the 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 means for matching the composition of the assimilated ration and those of the clam tissues. Since the 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), a useful approach will be to analyse 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 pseudofaecal production has proven to be an extremely effective mechanism for the compensation for nutritional deficits (Bayne and Svensson, 2006), but no choice for such selection was available in the present experiments wherein food particles were homogeneous and feeding conditions prevented pseudofaeces. However, the possibility of preingestive selection playing an important role in 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 of adjustments of the assimilation patterns wherein the ingestion/digestion/assimilation rates for a given element in default might be upregulated. Bayne (2009) considered the possibility of an increase in ingestion rates to compensate for low nutrient availability as a stoichiometric mechanism to maintain elemental homeostasis without the need for selective feeding. In fact, the results of experiments, very similar to the present study, showed that mussels (*M. edulis*) fed phytoplankton cultures with a low N content increased feeding rates compared with mussels fed phytoplankton with a high N content (Bayne, 2017; see his Fig. 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 half on average when clams were exposed to the N-poor diet (see Table 2). In a previous analysis, we reported digestive constraints in the processing of N- diets, resulting in
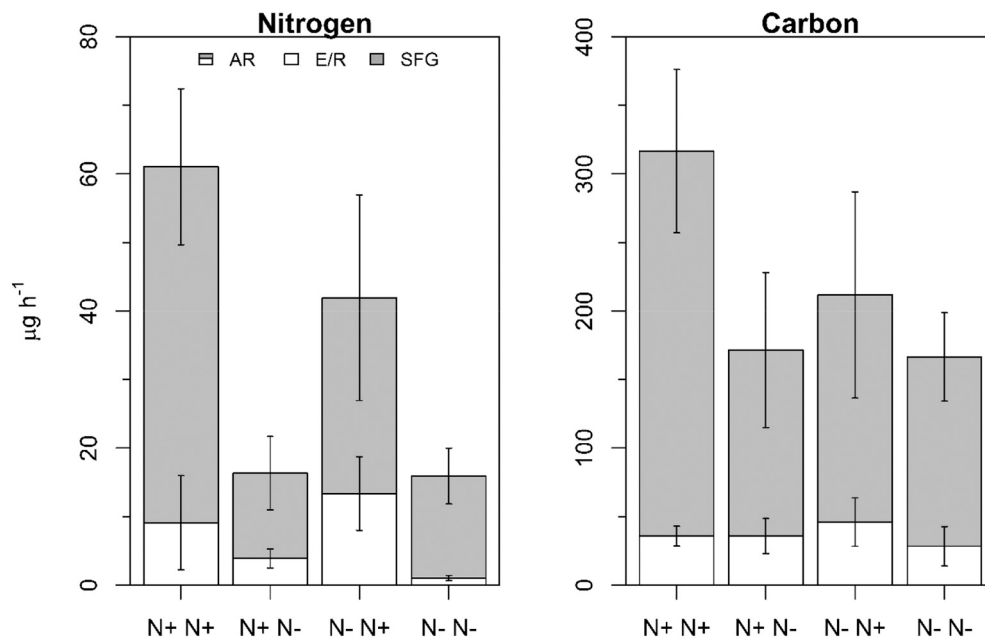
Fig. 3. Acute and acclimated response to dietary N changes for absorption rates (AR), N excretion (E; left) or C respiration (R; right) and elemental balance (SFG) rates of N (left) and C (right).
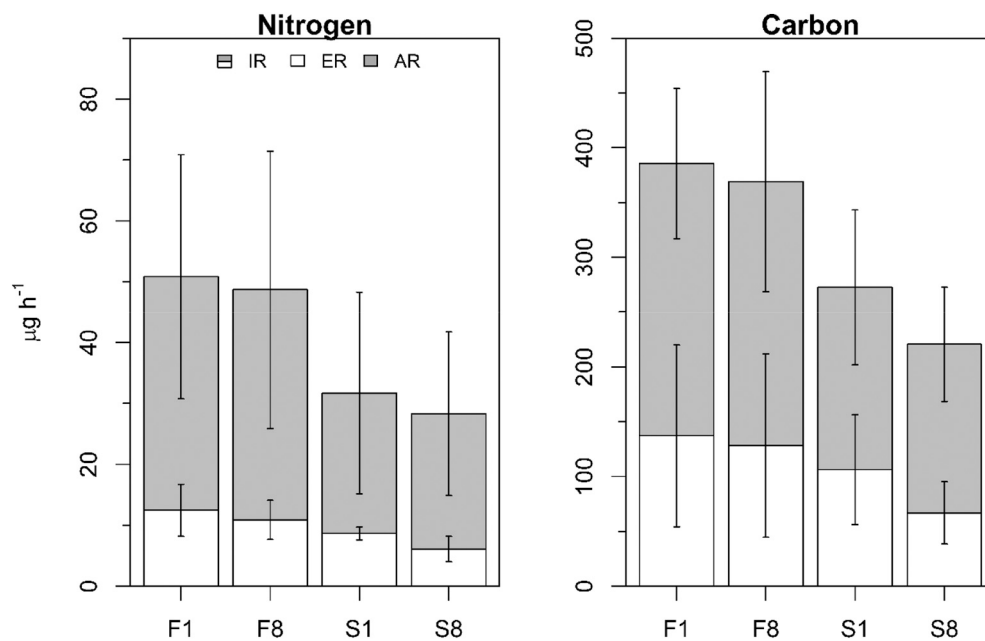

Fig. 4. Growth phenotype comparison for elemental acquisition parameters (ingestion (IR), egestion (ER) and absorption (AR) rates) of N (left) and C (right).

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). 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 that 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 (Tables 2 and 3).

However, the differential increase in AE_N over AE and AE_C when fed a N-poor diet (Fig. 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). Similarly, Ibarrola et al. (2000a) reported that cockles (*C. 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 behaviour 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 point to stoichiometric compensation.
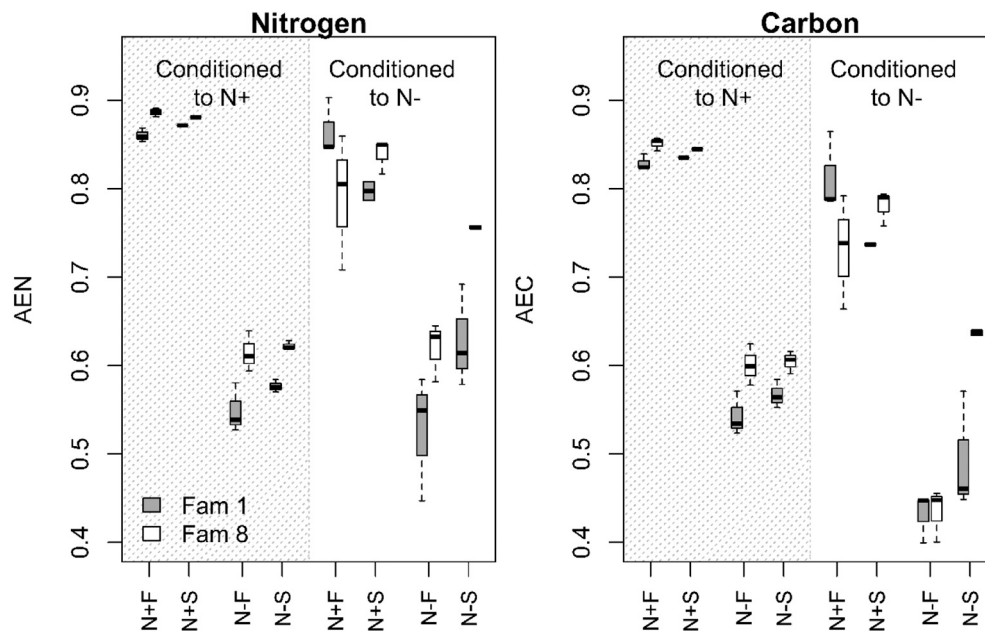
Nitrogen   **Carbon**

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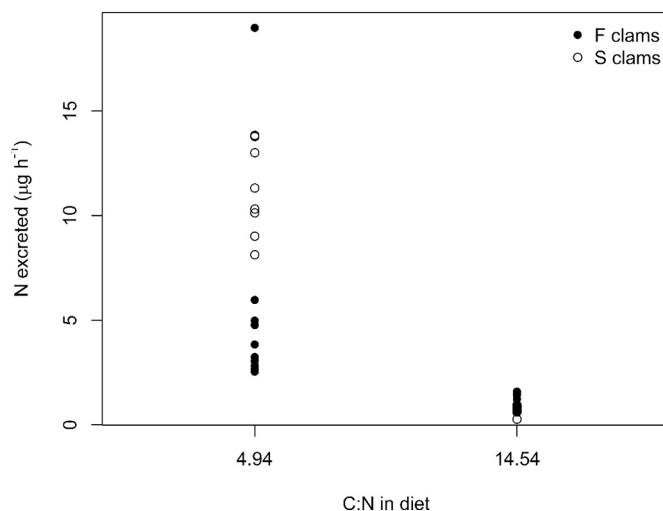

Fig. 6. Ammonia-N excretion of fast (solid circles) and slow (open circles) growing juveniles in relation to the diet dosed.

On the other hand, significant differential effects on AE_N are associated with conditioning rather than exposure to low N diets. For instance, clams conditioned to N- showed a clear discrepancy of AE_N with respect to overall AE that is not seen in those conditioned to N+, even when exposed to the N- diet. Therefore, regardless of 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. Additionally, acclimation time might be necessary provided that digestive adaptation proceeds through digestive enzyme induction (Ibarrola et al., 1999; Ibarrola et al., 1998). Bayne (2009) reported AE_N:AE_C ratios in oysters 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 met by enzyme induction. When considering digestive balances (net absorption), one alternative/complementary explanation for the preferential absorption of N may be the C enrichment of faecal materials contributed by endogenous materials in the form of metabolic faecal losses (MFL;

Hawkins and Bayne, 1985) that were found to consist mainly of lipids in cockles (e.g., up to 66% compared with 25%of proteins and 6% carbohydrates; Ibarrola et al., 2000a).

Finally, the regulation of nutrient balances can be achieved in the postabsorptive phase by adjusting the proportion of C and N released in metabolic activities according to differential income in the diet. This is seemingly achieved through a shift in the composition of the pool of substrates fuelling 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), the N+ diet promoted a general increase in metabolic activities, both ammonia excretion and respired C, but the important point is that the C:N ratio for metabolism (Table 2) changes from 5 (fully compatible with the metabolic breakdown of proteins) in clams feeding on the N+ diets to 20 in those feeding the N- diets, which suggests the majority use of other energy substrates. These differences could become even larger if a more precise RQ (respiratory quotient) value reflecting protein-based catabolism (i.e., RQ = 0.8) was applied to N+ diets instead of the common RQ = 0.9. A further source of energy expenditure associated with N-preserving mechanisms, more prominent with the N- diets, could have been disregarded in the present postabsorptive measurements, since C disposal corresponding to protein metabolism in the form of the amino-C fraction was found to occur in mussels mainly by the excretion of dissolved organic matter (DOM; not determined here) rather than in the form of respired CO_2 (i.e., 60 and 10%, respectively, of the amino-C assimilated) (Kreeger et al., 1996).

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 in C availability under the N+ diet. This behaviour coincides with the elemental balances calculated from the data shown in Bayne (2009), where both SFG_C and SFG_N decreased proportionally as
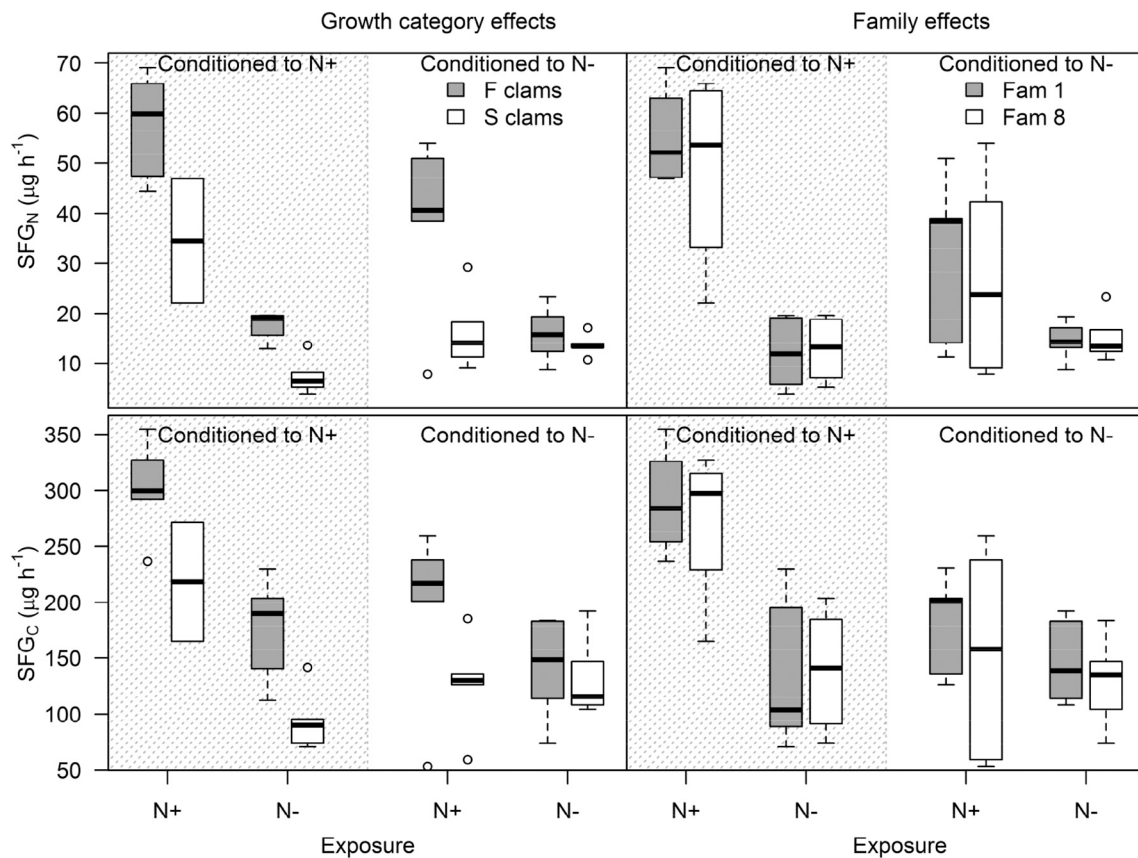
Growth category effects

Family effects

Fig. 7. Acute and chronic responses in SFG$_N$ (up) and SFG$_C$ (down) for interindividual (left) and interfamiliar (right) effects, where grey boxes represent, respectively, either F or Family 1 clams; and white boxes represent either S or Family 8 clams.

the 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 ratio), suggest a very relevant role of postabsorptive processes with regard to the homeostatic control of nutrient balances. Differences in the metabolic fate of nutrients dealing with this regulation are clearly shown for N, such that net growth efficiency (NGE$_N$) was significantly increased under N- diets compared with N+ diets (Table 3), 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 in elemental balance was primarily associated with intrafamiliar rather than 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). However, acquisition was also higher in Family 1 than in 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 phenotypes are endorsed by the previous literature on energy balances (Arranz et al., 2020; Bayne, 1999; Bayne et al., 1999b; Bayne et al., 1999a; Fernández-Reiriz et al., 2016; Ibarrola et al., 2017; Pace et al., 2006; Prieto et al., 2018, 2020; Tamayo et al., 2016; Tamayo et al., 2015; Tamayo et al., 2013; Tamayo et al., 2011; Toro et al., 1996; Toro and Vergara, 1998), wherein 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 wherein F vs. S differences are supported in differences in acquisition of both energy

(Arranz et al., 2020) and nutrients, irrespective of dietary C:N ratios.

Coupled with reduced nutrient acquisition, S clams were also found to release more nitrogen in the form of excretion. This N output reached a maximum when clams were fed the N-rich diet (Fig. 2), in which S clams nearly exceeded the excretion of F clams by 80%. On average, C:N ratios for metabolism ranged between 7.2 for F and 4.0 for S clams (Table 2). Part of this excess N excretion could be associated with *stoichiometrically regulated excretion* (Anderson et al., 2005), which would represent the fraction of N released to maintain mass balances and consumer homeostasis. These stoichiometric releases would partially explain 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. Specifically, some of the works of Hawkins and colleagues (Hawkins and Day, 1996; Hawkins et al., 1989a, 1989b; Hawkins et al., 1986) demonstrated that faster bivalve growth was correlated not only with faster feeding rates but also with reduced maintenance costs due to more efficient protein metabolism. In fact, reduced protein turnover was presented as a requirement 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 the N excretion of S clams fed the N-rich diet could result from a combination of higher protein turnover costs and a need for the removal of the N surplus acquired from the rich diet, which, in turn, reduced the scope for nutrient (N) and energy acquisition. Additionally, Meyer and Manahan (2010) hypothesized that
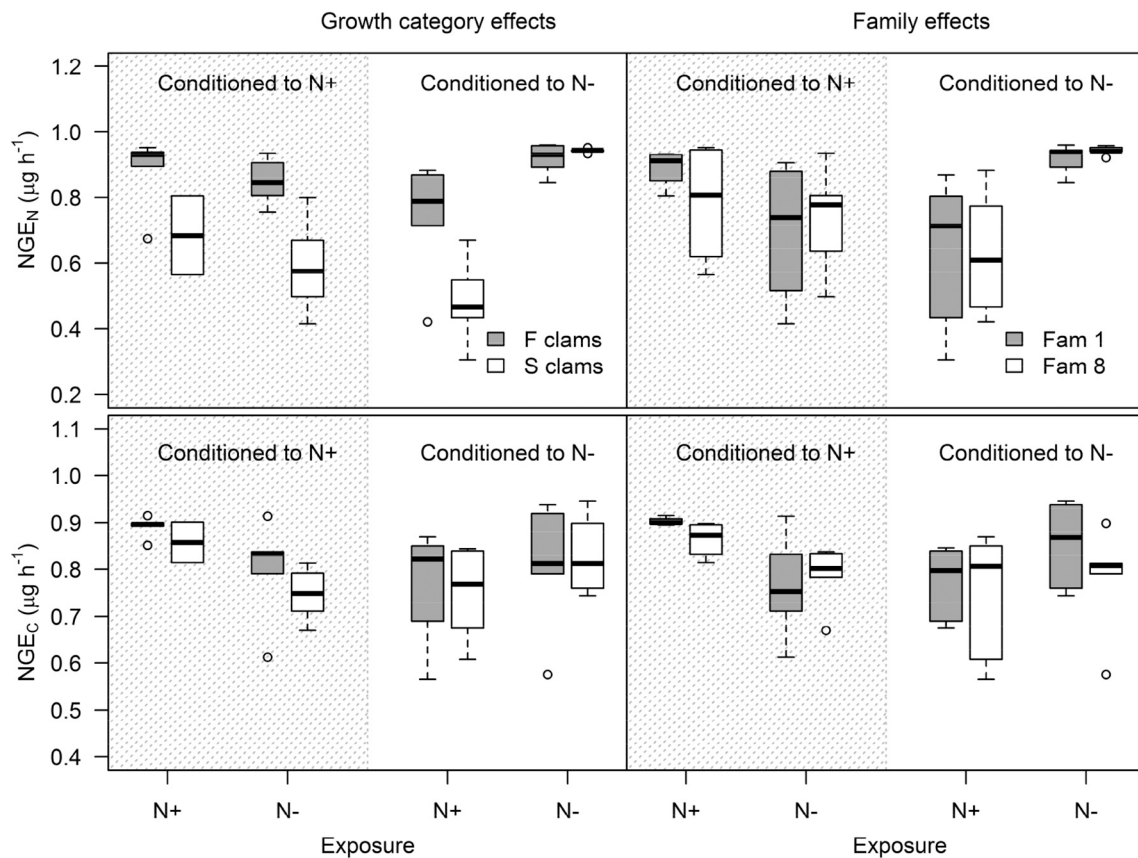
Fig. 8. Acute and chronic responses in NGE_N (top) and NGE_C (down) for interindividual (left) and interfamiliar (right) effects, where grey boxes represent, respectively, either F or Family 1 clams, and white boxes represent either S or Family 8 clams.

metabolic inefficiency in slow-growing bivalves could be partially explained by a nonuniform expression of ribosomal proteins, which might result in the extensive degradation of those proteins. As a summary expression of this reduced metabolic efficiency regarding the nutrient balances in slow growers, 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 3). Consistent with the 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

The time course of physiological adjustments is an important point in the adaptive response to changing diet quality that can eventually be assessed by considering C and N balances (SFG_C and SFG_N; Fig. 7). Irrespective of some differences in the behaviour between growth phenotypes, acute responses were more intense in the change from N+ to N- than in the opposite change. This is evidenced by the strong reduction observed in both C and N balances for clams conditioned to N+ when exposed to N- diets, while N- conditioned clams did not experience a neat recovery of these balances when exposed to the N+ diet. In addition, S clams were more sensitive to nutritional deterioration and decreased their balances to a larger extent than F clams when changed from N+ to N-. In contrast, F clams rapidly turned a profit on the switch from N- to N+, while balance performances remained virtually unaltered in S clams. Therefore, the consideration of acute responses suggests a higher plasticity for fast growers that were able to improve nutrient incorporation under any conditions. Despite the lack of significance between families, clams of Family 1 tended to possess higher net

incorporation rates, especially under acclimated conditions and particularly in the case of S clams.

In conclusion, the high N diet not only increased N balances but also C balances, in which gross energy acquisition (the ingestion rates) was the main factor responsible for the observed improvements in growth performance. Irrespective of growth phenotypes, AE_N was enhanced with respect to overall organics or carbon absorption efficiencies, especially under chronic N restricted conditions, suggesting stoichiometric regulation based on adjustments of absorption patterns. Endogenous differences were driven predominantly by intrafamily rather than by interfamily effects. The interaction between diet and growth category accounted for a weaker performance in S clams due to metabolic inefficiency (high metabolic C:N indices) associated with an increase in the N excreted to maintain elemental homeostasis. Finally, the more effective response of F clams to dietary changes suggests a higher degree of phenotypic plasticity with respect to S clams.

CRediT authorship contribution statement

Kristina Arranz: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Visualization. **Iñaki Urrutxurtu:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – review & editing. **Enrique Navarro:** Conceptualization,

Methodology, Formal analysis, Investigation, Resources, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Adams, C.M., Mayer, L.M., Rawson, P., Brady, D.C., Newell, C., 2019. Detrital protein contributes to oyster nutrition and growth in the Damariscotta estuary, Maine, USA. Aquac. Environ. Interact. 11, 521–536.

Anderson, T.R., Hessen, D.O., Elser, J.J., Urabe, J., 2005. Metabolic stoichiometry and the fate of excess carbon and nutrients in consumers. Am. Nat. 165, 1–15.

Arranz, K., Urrutxurtu, I., Ibarrola, I., Urrutia, M.B., Saavedra, C., Cordero, D., Navarro, E., 2020. 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. Aquaculture 521, 735056. https://doi.org/10.1016/j.aquaculture.2020.735056.

Bayne, B.L., 1999. Physiological components of growth differences between individual oysters (*Crassostrea gigas*) and a comparison with *Saccostrea commercialis*. Physiol. Biochem. Zool. 72, 705–713. https://doi.org/10.1086/316714.

Bayne, B.L., 2000. Relations between variable rates of growth, metabolic costs and growth efficiencies in individual Sydney rock oysters (*Saccostrea commercialis*). J. Exp. Mar. Biol. Ecol. 251, 185–203. https://doi.org/10.1016/S0022-0981(00)00211-2.

Bayne, B.L., 2009. Carbon and nitrogen relationships in the feeding and growth of the Pacific oyster, *Crassostrea gigas* (Thunberg). J. Exp. Mar. Biol. Ecol. 374, 19–30.

Bayne, B., 2017. Biology of Oysters, 1st ed. Academic Press.

Bayne, B.L., Svensson, S., 2006. Seasonal variability in feeding behaviour, metabolic rates and carbon and nitrogen balances in the Sydney oyster, *Saccostrea glomerata* (Gould). J. Exp. Mar. Biol. Ecol. 332, 12–26. https://doi.org/10.1016/j.jembe.2005.10.019.

Bayne, B.L., Iglesias, J.I.P., Hawkins, A.J.S., Navarro, E., Heral, M., Deslous-Paoli, J.-M., 1993. Feeding behaviour of the mussel, *Mytilus edulis*: responses to variations in quantity and organic content of the seston. J. Mar. Biol. Assoc. U. K. 73, 813–829.

Bayne, B.L., Hedgecock, D., McGoldrick, D., Rees, R., 1999a. Feeding behaviour and metabolic efficiency contribute to growth heterosis in Pacific oysters [*Crassostrea gigas* (Thunberg)]. J. Exp. Mar. Biol. Ecol. 233, 115–130. https://doi.org/10.1016/S0022-0981(98)00125-7.

Bayne, B.L., Svensson, S., Nell, J.A., 1999b. The physiological basis for faster growth in the Sydney rock oyster, *Saccostrea commercialis*. Biol. Bull. 197, 377–387. https://doi.org/10.2307/1542792.

Beninger, P.G., Valdizan, A., Cognie, B., Guiheneuf, F., Decottignies, P., 2008. Wanted: alive and not dead: functioning diatom status is a quality cue for the suspension-feeder *Crassostrea gigas*. J. Plankton Res. 30, 689–697.

Bourlès, Y., Alunno-Bruscia, M., Pouvreau, S., Tollu, G., Leguay, D., Arnaud, C., Kooijman, S., 2009. Modelling growth and reproduction of the Pacific oyster *Crassostrea gigas*: advances in the oyster-DEB model through application to a coastal pond. J. Sea Res. 62, 62–71.

Brigolin, D., Dal Maschio, G., Rampazzo, F., Giani, M., Pastres, R., 2009. An individual-based population dynamic model for estimating biomass yield and nutrient fluxes through an off-shore mussel (*Mytilus galloprovincialis*) farm. Estuar. Coast. Shelf Sci. 82, 365–376.

Cognie, B., Barillé, L., Rincé, Y., 2001. Selective feeding of the oyster *Crassostrea gigas* fed on a natural microphytobenthos assemblage. Estuaries 24, 126–134.

Conover, R.J., 1966. Assimilation of organic matter by zooplankton. Limnol. Oceanogr. 11, 338–345.

Cranford, P.J., 1995. Relationships between food quantity and quality and absorption efficiency in sea scallops *Placopecten magellanicus* (Gmelin). J. Exp. Mar. Biol. Ecol. 189, 123–142.

Cranford, P.J., Grant, J., 1990. Particle clearance and absorption of phytoplankton and detritus by the sea scallop *Placopecten magellanicus* (Gmelin). J. Exp. Mar. Biol. Ecol. 137, 105–121. https://doi.org/10.1016/0022-0981(90)90064-J.

Crisp, D.J., 1971. Energy flow measurements. In: Holme, N.A., Mc Inture, A.D. (Eds.), Methods for the Study of Marine Benthos. Blackwell, Oxford, pp. 197–323.

Emmery, A., Lefebvre, S., Alunno-Bruscia, M., Kooijman, S., 2011. Understanding the dynamics of δ13C and δ15N in soft tissues of the bivalve *Crassostrea gigas* facing environmental fluctuations in the context of dynamic energy budgets (DEB). J. Sea Res. 66, 361–371.

Fernández-Reiriz, M.J., Irisarri, J., Labarta, U., 2016. Flexibility of physiological traits underlying inter-individual growth differences in intertidal and subtidal mussels *Mytilus galloprovincialis*. PLoS One 11, e0148245.

Fielding, P.J., Davis, C.L., 1989. Carbon and nitrogen resources available to kelp bed filter feeders in an upwelling environment. Mar. Ecol. Prog. Ser. 181–189.

Filgueira, R., Labarta, U., Fernandez-Reiriz, M.J., 2006. Flow-through chamber method for clearance rate measurements in bivalves: design and validation of individual chambers and mesocosm. Limnol. Oceanogr. Methods 4, 284–292.

Grangeré, K., Ménesguen, A., Lefebvre, S., Bacher, C., Pouvreau, S., 2009. Modelling the influence of environmental factors on the physiological status of the Pacific oyster

Crassostrea gigas in an estuarine embayment; the Baie des Veys (France). J. Sea Res. 62, 147–158.

Grant, J., Cranford, P.J., 1991. Carbon and nitrogen scope for growth as a function of diet in the sea scallop *Placopecten magellanicus*. J. Mar. Biol. Assoc. U. K. 71, 437–450.

Hawkins, A.J.S., 1985. Relationships between the synthesis and breakdown of protein, dietary absorption and turnovers of nitrogen and carbon in the blue mussel, *Mytilus edulis* L. Oecologia 66, 42–49.

Hawkins, A.J.S., 1991. Protein turnover: a functional appraisal. Funct. Ecol. 5, 222–233.

Hawkins, A.J.S., Bayne, B.L., 1985. Seasonal variation in the relative utilization of carbon and nitrogen by the mussel *Mytilus edulis*: budgets, conversion efficiencies and maintenance requirements. Marine ecology progress series. Oldendorf 25, 181–188.

Hawkins, A.J.S., Bayne, B.L., 1991. Nutrition of marine mussels: factors influencing the relative utilizations of protein and energy. Aquac. Biol. Cultivat. Mussels 94, 177–196. https://doi.org/10.1016/0044-8486(91)90117-P.

Hawkins, A.J., Day, A.J., 1996. The metabolic basis of genetic differences in growth efficiency among marine animals. J. Exp. Mar. Biol. Ecol. 203, 93–115.

Hawkins, A.J.S., Bayne, B.L., Day, A.J., Denton, E.J., 1986. Protein turnover, physiological energetics and heterozygosity in the blue mussel, *Mytilus edulis*: the basis of variable age-specific growth. Proc. Royal Soc. Lond. Ser. B Biol. Sci. 229, 161–176.

Hawkins, A.J.S., Bayne, B.L., Day, A.J., Rusin, J., Worrall, C.M., 1989a. Genotype-Dependent Interrelations between Energy Metabolism, Protein Metabolism and Fitness.

Hawkins, A.J.S., Widdows, J., Bayne, B.L., 1989b. The relevance of whole-body protein metabolism to measured costs of maintenance and growth in *Mytilus edulis*. Physiol. Zool. 62, 745–763.

Hawkins, A.J.S., Smith, R.F.M., Bayne, B.L., Heral, M., 1996. Novel observations underlying the fast growth of suspension-feeding shellfish in turbid environments: *Mytilus edulis*. Mar. Ecol. Prog. Ser. 179–190.

Ibarrola, I., Navarro, E., Iglesias, J.I.P., 1998. Short-term adaptation of digestive processes in the cockle *Cerastoderma edule* exposed to different food quantity and quality. J. Comp. Physiol. B 168, 32–40.

Ibarrola, I., Navarro, E., Iglesias, J.I.P., Urrutia, M.B., 1999. Time-course of digestive-enzyme acclimation in the cockle *Cerastoderma edule*. Mar. Biol. 135, 47–56.

Ibarrola, I., Navarro, E., Iglesias, J.I.P., Urrutia, M.B., 2000a. Temporal changes of feeding and absorption of biochemical components in *Cerastoderma edule* fed an algal diet. J. Mar. Biol. Assoc. U. K. 80, 119–125.

Ibarrola, I., Navarro, E., Urrutia, M.B., 2000b. Acute and acclimated digestive responses of the cockle *Cerastoderma edule* (L.) to changes in food quality and quantity: I. feeding and absorption of biochemical components. J. Exp. Mar. Biol. Ecol. 252, 181–198. https://doi.org/10.1016/S0022-0981(00)00233-1.

Ibarrola, I., Hilton, Z., Ragg, N.L., 2017. Physiological basis of inter-population, inter-familiar and intra-familiar differences in growth rate in the green-lipped mussel *Perna canaliculus*. Aquaculture 479, 544–555.

Iglesias, J.I.P., Urrutia, M.B., Navarro, E., Alvarez-Jorna, P., Larretxea, X., Bougrier, S., Heral, M., 1996. Variability of feeding processes in the cockle *Cerastoderma edule* (L.) in response to changes in seston concentration and composition. J. Exp. Mar. Biol. Ecol. 197, 121–143.

Kooijman, S.A.L.M., 2010. Dynamic Energy Budget Theory for Metabolic Organisation. Cambridge University Press, United Kingdom.

Kreeger, D.A., Hawkins, A.J.S., Bayne, B.L., Lowe, D.M., 1995. Seasonal variation in the relative utilization of dietary protein for energy and biosynthesis by the mussel *Mytilus edulis*. Mar. Ecol. Prog. Ser. 177–184.

Kreeger, D.A., Hawkins, A.J.S., Bayne, B.L., 1996. Use of dual-labeled microcapsules to discern the physiological fates of assimilated carbohydrate, protein carbon, and protein nitrogen in suspension-feeding organisms. Limnol. Oceanogr. 41, 208–215.

Meyer, E., Manahan, D.T., 2010. Gene expression profiling of genetically determined growth variation in bivalve larvae (*Crassostrea gigas*). J. Exp. Biol. 213, 749–758.

Navarro, E., Méndez, S., Urrutia, M.B., Arambalza, U., Ibarrola, I., 2016. Digestive selection underlies differential utilization of phytoplankton and sedimentary organics by infaunal bivalves: experiments with cockles (*Cerastoderma edule*) using cross-labelled mixed diets. Mar. Environ. Res. 120, 111–121.

Pace, D.A., Marsh, A.G., Leong, P.K., Green, A.J., Hedgecock, D., Manahan, D.T., 2006. Physiological bases of genetically determined variation in growth of marine invertebrate larvae: a study of growth heterosis in the bivalve *Crassostrea gigas*. J. Exp. Mar. Biol. Ecol. 335, 188–209. https://doi.org/10.1016/j.jembe.2006.03.005.

Pales-Espinosa, E., Barillé, L., Allam, B., 2007. Use of encapsulated live microalgae to investigate pre-ingestive selection in the oyster *Crassostrea gigas*. J. Exp. Mar. Biol. Ecol. 343, 118–126.

Pales-Espinosa, E., Allam, B., Ford, S.E., 2008. Particle selection in the ribbed mussel *Geukensia demissa* and the eastern oyster *Crassostrea virginica*: effect of microalgae growth stage. Estuar. Coast. Shelf Sci. 79, 1–6. https://doi.org/10.1016/j.ecss.2008.02.022.

Prieto, D., Urrutxurtu, I., Navarro, E., Urrutia, M.B., Ibarrola, I., 2018. *Mytilus galloprovincialis* fast growing phenotypes under different restrictive feeding conditions: fast feeders and energy savers. Mar. Environ. Res. 140, 114–125. https://doi.org/10.1016/j.marenvres.2018.05.007.

Prieto, D., Tamayo, D., Urrutxurtu, I., Navarro, E., Ibarrola, I., Urrutia, M.B., 2020. Nature more than nurture affects the growth rate of mussels. Sci. Rep. 10, 1–13.

Prins, T.C., Smaal, A.C., 1989. Carbon and nitrogen budgets of the mussel *Mytilus edulis* L. and the cockle *Cerastoderma edule* (L.) in relation to food quality. Sci. Mar. 53, 477–482.

R Core Team, 2018. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Viena-, Austria.

Scholten, H., Smaal, A.C., 1998. Responses of *Mytilus edulis* L. to varying food concentrations: testing EMMY, an ecophysiological model. J. Exp. Mar. Biol. Ecol. 219, 217–239. https://doi.org/10.1016/S0022-0981(97)00182-2.

Scholten, H., Smaal, A.C., 1999. The ecophysiological response of mussels (*Mytilus edulis*) in mesocosms to a range of inorganic nutrient loads: simulations with the model EMMY. Aquat. Ecol. 33, 83–100.

Shumway, S.E., Cucci, T.L., Newell, R.C., Yentsch, C.M., 1985. Particle selection, ingestion, and absorption in filter-feeding bivalves. J. Exp. Mar. Biol. Ecol. 91, 77–92.

Smaal, A.C., Scholten, H., 1997. EMMY: an ecophysiological model of *Mytilus edulis* L. In: Food Supply and Demand of Bivalve Suspension Feeders in a Tidal System, pp. 147–190.

Smaal, A.C., Vonck, A., 1997. Seasonal variation in C, N and P budgets and tissue composition of the mussel *Mytilus edulis*. Mar. Ecol. Prog. Ser. 153, 167–179.

Soletchnik, P., Goulletquer, P., Héral, M., Razet, D., Geairon, P., 1996. Evaluation du bilan énergétique de l'huître creuse, *Crassostrea gigas*, en baie de Marennes-Oléron (France). Aquat. Living Resour. 9, 65–73.

Solórzano, L., 1969. Determination of ammonia in natural waters by the phenolhypochlorite method. Limnol. Oceanogr. 14, 799–801.

Sterner, R.W., Elser, J.J., 2002. Ecological Stoichiometry: The Biology of Elements from Molecules to the Biosphere. Princeton University Press.

Tamayo, D., Ibarrola, I., Urrutia, M.B., Navarro, E., 2011. The physiological basis for inter-individual growth variability in the spat of clams (*Ruditapes philippinarum*). Aquaculture 321, 113–120.

Tamayo, D., Ibarrola, I., Navarro, E., 2013. Thermal dependence of clearance and metabolic rates in slow-and fast-growing spats of manila clam *Ruditapes philippinarum*. J. Comp. Physiol. B. 183, 893–904.

Tamayo, D., Ibarrola, I., Cigarría, J., Navarro, E., 2015. The effect of food conditioning on feeding and growth responses to variable rations in fast and slow growing spat of the Manila clam (*Ruditapes philippinarum*). J. Exp. Mar. Biol. Ecol. 471, 92–103.

Tamayo, D., Azpeitia, K., Markaide, P., Navarro, E., Ibarrola, I., 2016. Food regime modulates physiological processes underlying size differentiation in juvenile intertidal mussels *Mytilus galloprovincialis*. Mar. Biol. 163, 131.

Toro, J.E., Vergara, A.M., 1998. Growth and heterozygosity in a 12-month-old cohort of *Ostrea chilensis* obtained by mass spawning in the laboratory. Mar. Ecol. 19, 311–323.

Toro, J.E., Vergara, A.M., Gallegillos, R., 1996. Multiple-locus heterozygosity, physiology and growth at two different stages in the life cycle of the Chilean oyster *Ostrea chilensis*. Mar. Ecol. Prog. Ser. 134, 151–158.

Urrutia, M.B., Iglesias, J.I.P., Navarro, E., Prou, J., 1996. Feeding and absorption in *Cerastoderma edule* under environmental conditions in the bay of Marennes-Oleron (Western France). J. Mar. Biol. Assoc. U. K. 76, 431–450.

von Bertalanffy, L., 1938. A quantitative theory of organic growth (inquiries on growth laws. II). Hum. Biol. 10, 181–213.