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Stoichiometry of growth under variable scenarios of nutrient limitation: Differential homeostasis of body composition among growth phenotypes of the Manila clam

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ABSTRACT

Fast- and slow-growing phenotypes from two separate breeding families of the Manila clam (*Ruditapes philippinarum*) were alternatively fed two monoalgal diets with high and low N content (C:N ratios of 4.9 and 13.5, respectively). After 35 days of food conditioning, clams were sacrificed, and the soft body was dissected out into five different tissue fractions to determine the corresponding ponderal ratios (tissue wt./body wt.) and a separate analysis of the elemental composition of these tissues. Previously reported C and N balances performed with the same conditioning diets were integrated and compared with tissue composition of the same phenotypes in order to assess the efficacy of mechanisms elicited to compensate for N deficit. Broad differences in dietary N content resulted in only minor changes in whole-body C:N composition which suggests a noticeable degree of homeostatic regulation of nutrient balances. This regulation was found to be stricter in fast-compared to slow-growing phenotypes and differed among the various body tissues. Using the threshold element ratio approach, physiological mechanisms were identified that partly compensate for large stoichiometric mismatches between low-N food and body tissues.

1. Introduction

Aquaculture efforts to improve growth have regularly focused on supplying a balanced diet that includes the required nutrients that could be otherwise limiting optimal growth. Food limitations can occur at quantitative or qualitative levels; that is, available energy in the form of food may be insufficient to sustain optimal growth or either food composition may lack the appropriated balance of components required for tissue biosynthesis, resulting in specific nutrient deficiencies. This last constraint has been reported to occur most frequently in herbivores and detritivores in both terrestrial and aquatic systems where food composition (particularly the C:N ratio) may differ broadly from that of animal tissues (Elser et al., 2000).

Regarding eventual applications in aquaculture, one useful approach to this issue is to experimentally assess how the variable composition of food used in conditioning impacts the composition of growing tissues, evidencing variable levels of nutrient homeostasis. This is defined (Kooijman, 1995) as *"the ability of most organisms to keep the chemical composition of their body constant despite changes in the chemical composition of the environment including their food"*. Therefore, a lack of homeostatic regulation would result in organisms following the "you are what you eat" model (Sterner and Elser, 2002), while those animals possessing the ability to regulate nutrient fluxes would follow some strategies to maintain an elemental body steadiness. According to Sterner and Elser (2002, see also Arranz et al., 2022), animals able to regulate their elemental stoichiometry can achieve their purpose by following three mechanisms: *1)* by means of pre-ingestive selection (i.e. preferentially choosing food items that suit best their needs), *2)* assimilation pattern modifications, in which an element uptake would be up- or down-regulated, and *3)* by metabolic processes involving the active excretion of any element that is in excess.

Although energy-budget-dependent physiological models of growth are abundant in marine bivalves, comparatively few studies have included a stoichiometric approach aimed at exploring the relationships between food composition and that of the growing tissues (Bayne, 2017). For example, the scallop *Placopecten magellanicus*, fed on either aged kelp or phytoplankton (*Chaetoceros gracilis*) maintained its tissue C:N ratio despite of a 3.5 fold difference in C:N ratio between diets (Grant

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and Cranford, 1991), and similar tissue C:N ratio homeostasis was observed in juveniles of the Manila clam *Ruditapes philippinarum* fed *Thalassiosira pseudonana* microalgae manipulated to provide a 6 to 12 range in C:N values (Gallager and Mann, 1981). Likewise, large seasonal variations in the composition of natural diets reported for *Mytilus edulis* (C:N range = 7.3–44.3; Smaal and Vonck, 1997) and *Crassostrea gigas* (C:N range = 4.8–12; Bayne, 2009), resulted in minor or null variation in the composition of body tissues (C:N ratios of 5.9–7 and 5.7, respectively). In both studies, the predicted tissue composition based on C and N elemental balances resembled the actual composition, indicating that elemental homeostasis was maintained through physiological mechanisms of compensation (both pre- and post-absorptive) suited to meet nutritional imbalances and growth demands.

Ecological stoichiometry principles (Sterner and Elser, 2002) have provided some useful tools for the quantitative assessment of coupling between the composition of the resources and their consumers. The concept of ideal food ratio (Urabe and Watanabe, 1992; Anderson and Hessen, 1995) combines elemental balances derived from bioenergetic models and elemental body composition, and stands out for the composition of food that enables balancing nutrient acquisition against its deposition in tissues, leading to optimal production. The ideal ratio is otherwise known as the *threshold element ratios* (TER) because it specifies the dietary combination at which limitation switches from one element to the other: For example, in the present case, $TER_{C:N}$ represents the turning point for N to C limitation (Anderson et al., 2005; Frost et al., 2006). Several authors have developed different approaches for estimating TERs (Urabe and Watanabe, 1992; Anderson and Hessen, 1995; Sterner, 1997; Sterner and Elser, 2002; Anderson et al., 2005; Frost et al., 2006; Doi et al., 2010), which differ in some terms of the equations, such as the use of gross or net growth efficiencies (Urabe and Watanabe, 1992; Sterner and Elser, 2002), or the distinction between absorption efficiencies for nitrogenous and non-nitrogenous compounds (Anderson and Hessen, 1995). Interestingly, one useful criterion to identify compensatory physiological traits in the case of food-consumer nutritional mismatches is to assess how specific physiological responses would affect TER values, approaching them to actual food composition.

Regarding a detailed analysis of stoichiometric coupling, much insight can be gained from the fractionated characterization of different body constituents, including the different organs and tissues involved in growth-related functions, such as feeding, assimilation, and reserves storage, on the assumption that variable nutrient assignation to these different systems may be inferred from this approach. Although large inter-organ differences in biochemical composition are known to occur, to our knowledge, studies analyzing the elemental composition of bivalves lack separate determination of different tissues. For instance, the composition of *R. philippinarum* has been analyzed in terms of biochemical composition of whole soft tissues (Beninger and Lucas, 1984; Robert et al., 1993; Marin et al., 2003; Baek et al., 2014), commonly in association with seasonal cycles and not specifically in response to differences in nutritional conditions. On two occasions, tissues were separated for the analysis of either seasonal biochemical composition (Shiraishi et al., 1995) or a comparison between elemental and biochemical analyses (Arranz et al., 2021). Given the well differentiated functions accomplished by each type of tissue involving a diversity of turnover rates (Gosling, 2015), differential performance of each tissue could be expected in terms of nutrient homeostasis.

On the other hand, the occurrence of differentiated growth phenotypes exhibiting large endogenous differences in growth performance poses the question of how these variable demands for growth would affect the capacity to compensate for nutritional mismatches between consumed food and growing tissues. According to previous reports in Manila clams, phenotypes selected for faster growth exhibited both higher energy acquisition and metabolic efficiency, resulting in increased energy balances accounting for improved growth (Tamayo et al., 2011, 2013, 2015; Arranz et al., 2020). Moreover, these increased energy demands were better accomplished under dietary conditions of high N availability compared with N deficit-conditions (Arranz et al., 2020) and one outstanding aspect of such a phenotype–diet quality interaction was that growth efficiency for N decreased between fast- and slow-growing juveniles belonging to two selectively produced lines, which did not occur for C or energy (Arranz et al., 2022). This highlights the constitutive differences in the ability for the homeostatic regulation of nutrient deficit, which might eventually be reflected as interindividual differences in both biometrical relationships between different body constituents and whole-body elemental composition.

Considering everything, this study was designed to assess biometrical relationships and compare elemental composition between different body tissues of groups of juvenile clams (*R. philippinarum*) selected for variable growth rates following food conditioning to diets representing high and low N availability. This approach was performed in the context of energy and elemental balances (Arranz et al., 2020, 2022) and aimed to test the following hypotheses: 1) Whole body composition reflected differences between high/low N conditioned groups, revealing specific nutrient limitations that can be tested using the TER approach. 2) Physiological mechanisms of homeostatic nutrient regulation partially compensate for nutritional deficits, approaching ideal food composition to the composition of the actual diets provided. 3) Different body organs exhibit variable capacities for these compensations associated with different functions and tissue turnover rates. 4) Variable demands for biosynthesis modulate physiological responses of fast- and slow-growing phenotypes, resulting in different degrees of homeostatic nutrient regulation.

2. Material and methods

2.1. Animal maintenance and diet characteristics

Two families (1 and 8) were randomly chosen for this study from a set of several clam pair-mattings. Larvae and postlarvae were raised in standard bivalve hatchery conditions, in an open seawater circuit maintained at 20–22 °C temperature, 36–38 ‰ salinity, 7.8–8.2 pH and 85–100% oxygen saturation. Juveniles were grown for two years, and two differentiated groups (n = 30) of fast (F) and slow (S) growth specimens were selected within each family by size segregation to constitute four different growth phenotypes: F1, F8, S1 and S8 (see Arranz et al., 2020 for details).

Each of these groups was subsequently split into two homogenous subgroups of clams for conditioning to diets with high or low N content (N+ and N- diets, respectively). These diets consisted of microalgal cells of the species *Rhodomonas lens* cultivated either in the exponential phase (C:N ratio = 4.94) or the stationary phase (C:N ratio = 14.5), that were dosed to the conditioning tanks (50 L) at a common particle concentration of 1 $mm^3\ L^{-1}$ (approximately 0.5 mg POM L^{-1}). Diet monitoring was carried out twice a week in quadruplicates during the conditioning period (35 d), where organic and inorganic fractions and CHN composition of suspended particles were determined after filtering through pre-weighted glass fiber filters (GF/C), as described in Arranz et al. (2022).

2.2. Biometric parameters and elemental analysis of tissues

Shell length (mm) and live weight (mg) were monitored during the food conditioning period, and individual growth rates (mg d^{-1}) were estimated as the slopes of linear regressions relating the live weight (mg) to the time (d).

After 35 d of food conditioning to each diet, clams were fasted for 3 d, to avoid the inclusion of food particles in the tissue sample for elemental analysis, and subsequently dissected out from the shell and the soft body was divided into 5 sets (4 in the case of S juveniles) that consisted of gills, adductor muscles, digestive gland, gonad, and remaining tissues (mantle, siphons, and foot). Owing to the absence of gonadal tissue in slow-growing clams, no distinction was made between

the gonad and the remaining tissues in these groups.

Following tissue dissection, individual tissue fractions were placed into a 2 mL vials and frozen by immersion into liquid nitrogen. The dissected gill was previously suspended in seawater within a Petri dish and photographed over a gridded paper to subsequently estimate surface areas by image analysis using Fiji software (Schindelin et al., 2012). The tissue fractions were freeze-dried and individually weighted for dry weight estimations. Finally, the samples were homogenized with a mortar and pestle, and stored at −20 °C until being analized.

The *organic content* (*OC*) of the hard (shell) and soft tissues was estimated by dividing the ash-free dry weight (AFDW) by the dry weight (DW). (AFDW = DW of samples recorded after drying at 60 °C for 48 h, minus the ash weight (AW) recorded after calcination at 450 °C)

$$OC(\%) = 100\,\frac{DW - AW}{DW}$$

These measurements allowed the calculation of the following biometric relationships:

The *condition index* (*CI*) was calculated in two ways:

1) Dry weight of soft tissues (DWt, mg) divided by the cube of shell length (L, mm):

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

2) Dry weight of soft tissues (DWt) divided by the dry weight of hard tissues (DWs):

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

A tissue index (*TI*) for each body fraction was computed as the proportion of dry weights of each tissue to the total soft body dry weight:

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

Finally, an index of gill thickness was calculated as the mass of gill tissue per unit of surface area:

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

For CHN quantification, two subsamples were obtained from each dry tissue sample, for: *1)* elemental analyses (1–1.5 mg) and *2)* gravimetrical determination of the organic fraction (10 mg). Samples for elemental composition were inserted into tin capsules for analysis in a Euro EA Elemental Analyzer (CHNS; EuroVector), using acetanilide as a standard, in the SGIker facilities (UPV/EHU). The same process was followed with ashes from every tissue to compute the inorganic fractions of N and C, which were subtracted from the former samples to obtain the organic N and C fractions.

No specific elemental analysis of shell tissue to compute C:N ratios was feasible because of the elevated inorganic C content of this organ (>90 %). Instead, the organic content of the shell, measured as the AFDW (see above), was converted into the corresponding C and N contents according to the estimated biochemical composition of the organic matrix using the appropriated equivalents (Arranz et al., 2021). For this purpose, we used values of 88.7% protein, 11.3% lipid and no trace of carbohydrates that were determined in the extracted (acid soluble) fraction (64% of AFDW) of shells of the related species *Ruditapes decussatus* (own unpublished results) and assumed a mixed proportion of structural carbohydrates (mainly chitin) and proteins (Marin et al., 2012) for the remaining 36% of the shell AFDW. C:N ratio estimated in this way for the organic fraction of the shell was 4.03.

The dry weight of each tissue sample was multiplied by the fraction of each element to obtain the element weight. Then, in order to compute the elemental composition of the entire soft tissue, each element weight was summed and subsequently divided by the total dry weight, as follows:

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

Two subgroups (n = 5) from each group conditioned to N+ and N- diets were created for elemental balance (C and N) determinations under the following experimental instances (Arranz et al., 2022): a) conditioned to N+ and fed the N+ diet during balance determinations (N+N+ group); b) conditioned to N+ and fed the N- diet (N + N-); c) conditioned to N- and fed the N+ diet (N-N+) and d) conditioned to N- and fed the N-diet (N-N-). The amount of retained element (SFG) was computed from these balances as follows:

$$SFG_C = IR_C \times AE_C - R_C$$

$$SFG_N = IR_N \times AE_N - E_N$$

Where *IR* is ingestion rate, *AE* is absorption efficiency, R_C is respired carbon (CO_2) estimated from the rates of oxygen consumption by applying a standard respiratory quotient ($RQ = 0.9$) and E_N is excreted nitrogen (ammonia).

The ideal C:N ratios of food or threshold element ratios ($TER_{C:N}$) were then estimated following Anderson et al. (2005) by using the following equation:

$$TER_{C:N} = \frac{max.\ GGE_{\ N}}{max.\ GGE_{\ C}} \times C : N_{tissues}$$

where maximum gross growth efficiency (*max.GGE*) of both elements was computed for the different growth phenotypes conditioned to N+ and N- diets (Arranz et al., 2022) by dividing the amount of retained element (SFG_N *or* SFG_C) by the corresponding elemental ingestion rates (IR_N *or* IR_C), and $C{:}N_{tissues}$ is the C:N ratio of whole tissues, including the organic fraction of the shell. GGEs are assumed to present maximum values because $TER_{C:N}$ represents the food C:N ratio at which both elements are limiting (Doi et al., 2010) and thus constitutes the threshold for change from energy (C) to nutrient (N) limitation.

2.3. Data analysis

Significant effects of different factors (conditioning diet, family, growth category, and body tissue) on elemental composition and TERs were tested using a 4-way ANOVA. When significant differences were detected for a factor containing more than two levels, Tukey's honestly significant difference (HSD) tests were performed to establish which levels were responsible for those significant differences. Analyses were performed after testing for normality (Shapiro-Wilk) and homoscedasticity (Levene) of the data. Data based on percentages were analyzed using nonparametric analyses (ANOVA on ranks).

Linear regression analysis was used to estimate the relationship between the gill mass and surface area. Similarly, linear regression equations were fitted to log-log transformed data of shell weight versus shell length, and interfamily differences were compared using ANCOVA.

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

3. Results

3.1. Growth rate and biometric parameters

The growth of clams (in terms of live weight) was affected by diet ($F = 7.39$, $p = 0.008$), and especially by growth category ($F = 47.23$, $p < 0.001$), but the comparison between families revealed no significant effect ($F = 0.065$, $p = 0.80$) on growth nor significant interaction terms

were found. Hence, under the N+ diet, F clams grew by 10.3 mg (live weight) d^{-1} compared with 4.4 mg d^{-1} in S clams. Whereas, mean growth rates under N- diet were 7.6 and 2.9 mg d^{-1} for F and S clams, respectively.

Furthermore, differences in shell weight per unit of length were identified at the interfamily level, where ANCOVA analysis for both families in the relationship log *(shell dry weight)* = *b* log *(length)* + *a* showed no differences in their slopes ($F = 0.44$, $p = 0.51$), but a higher intercept ($F = 4.22$, $p = 0.04$) for Family 1 was observed (Fig. 1). Likewise, interfamily differences occurred for *CI* (mg mm^{-3}), ($F = 93.43$, $p < 0.001$), but in this case clams of Family 8 had higher values. Taking both results into account, Family 1 would invest more in shell thickening than Family 8, while the latter would possess a higher amount of soft tissue per unit of volume (a more detailed analysis will be provided alongside the analysis of soft tissues weights).

The percentage of soft body weight over total dry weight (Fig. 2a) was clearly associated with the growth phenotypes. These differences were specifically tested using computed condition indices (Fig. 2b) to find that *CI* (mg mg^{-1}) only showed differences between families ($F = 95.15$, $p < 0.001$), where Family 8 reached values > 30% than Family 1. F clams tended to possess a higher condition index, although the differences did not reach significance ($F = 3.68$, $p = 0.058$). In contrast, the relative weight of each tissue over soft body dry weight (Fig. 2a) varied mostly depending on the growth category, but also on the family and diet. The growth category promoted differences in all organs, where S clams had larger gill and digestive gland proportions, whereas F clams contained a higher proportion in the rest of the tissues. Differences between families were found in the gills, where Family 1 had a higher proportion ($F = 22.7$, $p < 0.001$), whereas Family 8 presented a higher proportion of adductor muscle ($F = 23.2$, $p < 0.001$). Conditioning to different diets had minor effects on the adductor muscle, where the N+ diet promoted an increase in its relative tissue weight ($F = 9.23$, $p = 0.003$).

The relationships between gill weight and gill surface area (Fig. 3) were significant in terms of diet conditioning ($F = 4.73$, $p = 0.03$) and growth category ($F = 159.70$, $p < 0.001$), but not between families ($F = 0.13$, $p = 0.72$). This ratio of mass per unit surface area would be

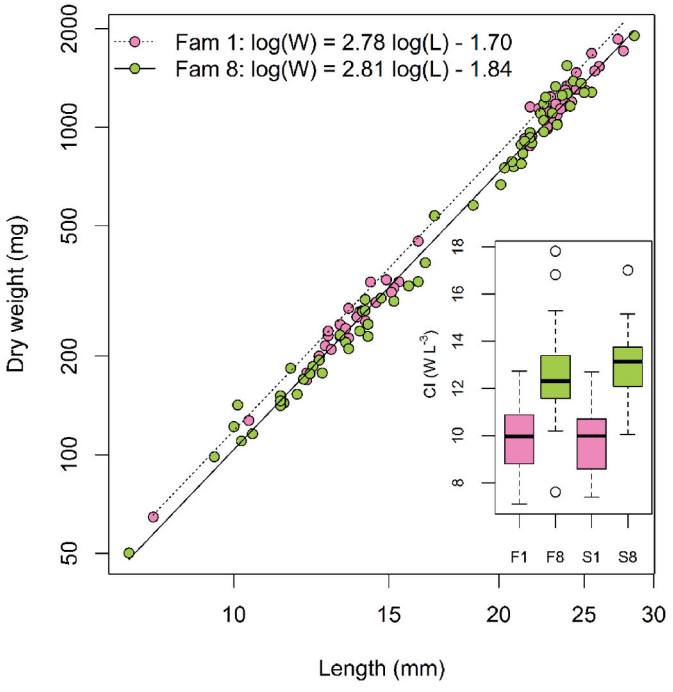

Fig. 1. Interfamily comparisons of the relationships between shell dry weight and length. Boxplot: Comparison of CI (mg mm^{-3}) among growth phenotypes.

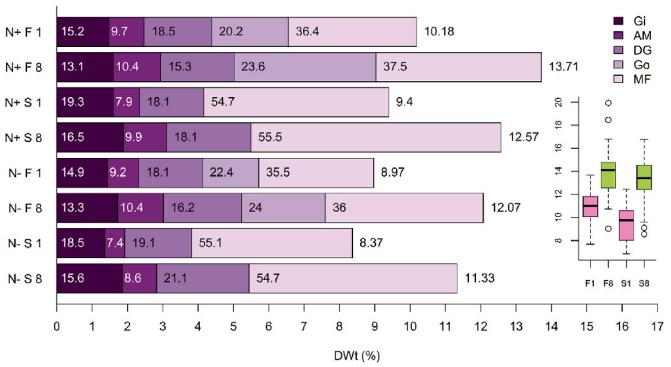

Fig. 2. a) Percentage of soft tissues over total dry weight (soft + hard tissues) in the different combinations of diet and growth phenotype. Inserted figures represent percent values of each tissue weight over total soft body weight. GI: Gill; AM: Adductor muscle; DG: Digestive gland; Go: Gonad; MF: Remaining (Mantle-foot) b) Inserted boxplot illustrating condition index (CI: mg mg^{-1}) variation across phenotypes.

proportional to gill height (i.e., thickness); thus, F clams attained approximately 50% more mean gill thickness than S clams, even though S clams registered higher gill surface areas (standardized values), whereas gill thickness of clams conditioned to N- diet would have increased by approximately 7% compared with of those fed N+.

3.2. Elemental composition of tissues

After one month of growth under either N+ or N- diets, the overall N content (%) of tissues had reached higher values in clams acclimated to the N+ diet, which resulted in lower C% and lower C:N ratios with that diet (Table 1). Growth category (F versus S clams) showed no significant effects, although trends indicated higher N% and lower C:N in fast-growing clams. Interfamily comparisons revealed similar trends, with Family1 being the group with the highest N%.

Compared with diet and phenotype (growth group and family) effects, differences among tissues regarding elemental composition were outstandingly high, resulting in tissue factor effects that were highly significant for either N, C, or the C:N ratio (Table 1). In addition, variability in elemental composition associated with diet and phenotype was noticeably heterogeneous among the different tissues, with coefficients of variation (CV) for N contents ranging from ~0.5% in the gill and adductor muscle to ~ 2% in the rest of tissues. This is accounted for by multiple significant interactions of the tissue factor in the ANOVA on ranks analysis (Table 1).

3.2.1. Overall effects of tissue factor and their interaction with diet

Nitrogen content was significantly higher in the adductor muscle than in the rest of the tissues, whereas carbon content was significantly higher in the digestive gland and gonad (Fig. 4a); consequently, minima C:N ratios were observed in the adductor muscle (3.56) and gill (3.79) and maxima in the digestive gland (4.5) and gonad (4.29).

Conditioning diets differentially affected the elemental composition of the various tissues, as indicated by the level differences between black circles (N+ diet) and gray squares (N- diet) inside boxes in Fig. 4a. Both gill and adductor muscle compositions remained unaltered, as no-significant differences were recorded between diets, (despite a slight increase in the C% of gills of N- acclimated clams). Indeed, homeostatic nutrient regulation during dietary changes was almost complete in the growth of gill tissues. Conversely, the elemental composition of both the digestive gland and the remaining tissues strongly differed between clams conditioned to N+ and N- diets. This pattern of change was significant for both elements, as well as for the C:N ratio (Fig. 4a and b). Among the different tissues, the digestive glands showed the highest degree of dietary dependence.

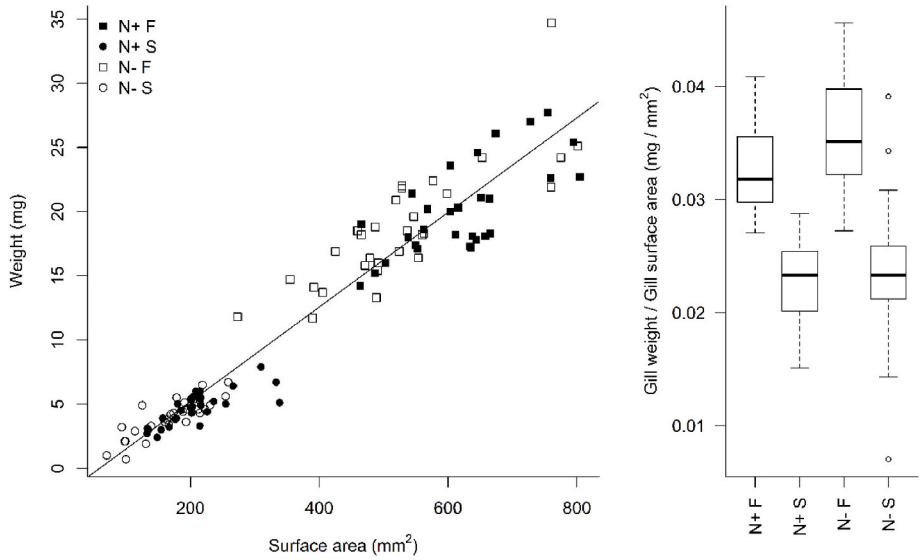

Fig. 3. a) Regression fit and b) boxplot for weight and surface area relationships of gills for different combinations of conditioning diet and growth group (see legend).

3.2.2. *Phenotype effects*

The gill and adductor muscle were the most stable organs with respect to elemental composition, with only limited effects of the diet that were phenotype-dependent. Gill tissue nitrogen maintained rather constant levels of approximately 13% across groups with the exception of N- diet-fed S.1 group (Fig. 4b), which showed significant differences in N% (lower) and C:N ratio (higher) (Tukey's HSD test). Fast growth appears to enhance the effect of diets on the elemental composition of the adductor muscle, so that higher N% (and correspondingly lower C:N) was recorded in the N+ diet-fed F clams and N- diet-fed S clams (Fig. 4b). Despite these trends, single pairwise differences were only recorded between the S groups of the N+ diet-fed clams of the Family 8 and the N- diet-fed clams of the Family 1.

The elemental composition of the digestive gland exhibited the greatest variation, with diet being the most influential. These effects, in combination with phenotypic differences, resulted in C:N values ranging from between 3.5 and 6.0 (Fig. 4b). As noted for the adductor muscle, growth rate variation among phenotypes combined with dietary effects produced the greatest differences in composition between the groups of N+ diet-fed fast growers and the N- diet-fed slow growers. Specifically, N% was higher for the group of N+ diet-fed F clams from Family 1, while the maximum C level was reached in N- diet-fed S clams from Family 8. C:N ratios were significantly different between phenotypes for both inter- and intra-familial groups, with differences between families being predominant.

The last group of tissues (gonad and remaining tissues) underwent a different treatment, as the two components could be separated for differential analysis only in F clams; thus, no F versus S differences could be tested in these organs. With regard to F clams (Fig. 4b), gonad registered low C:N values comparable to the average composition of the digestive gland, although no significant effects of the diet were observed in this case. In addition, the elemental composition of the remaining tissues (mantle, foot and siphons) resembled that of other structural tissues, such as the gill, with relatively high N contents and low C:N values. However, diet exerted important effects on composition in this case, which were opposite to those found in the digestive gland, since higher N% (low C:N) values were achieved with the N-restricted diet especially in Family 1. The elemental composition of the GMF tissue group was intermediate between the gonad and the remaining tissues composition recorded for F clams, with a neat positive effect of N+ diet on N% of S clams from the Family 8, which showed the lowest C:N ratio (Fig. 4b).

3.2.3. *Whole body composition*

Because of the great differences in both the elemental composition and relative size of different body components, the whole soft body composition was computed from the specific composition of each tissue weighted by the fraction represented by this tissue in the whole soft body (see section 2.2). The resulting C:N ratios for the whole tissues in the different phenotypes (growth category x family groups) conditioned to N+ and N- diets are compared in Fig. 5 with the corresponding C:N ratios for a) the digestive gland and b) the retained ration or SFG, based on the elemental balances for C and N reported in a previous study (Arranz et al., 2022).

Significantly, C:N variation patterns among phenotypes and conditioning diets were similar for the digestive gland and SFG, where the dietary N level and phenotype associated growth gradients were combined to produce a series of increasing C:N values from N+ to N-, F clams to S clams and Family 1 to Family 8. Thus, even if C:N values recorded in tissues were considerably reduced compared to those in the retained fraction of food (based on C and N balances), it is important to note that the elemental composition of the digestive gland closely reflected the trends observed in these balances, suggesting a limited capacity for homeostatic regulation of dietary imbalances in this organ compared to whole-body tissues. However, full independence of whole tissues composition relative to food composition was only achieved in F clams (Fig. 5) whereas S clams changed according to the pattern exhibited by the digestive gland, with C:N ratios of S clams of Family 8 showing the greatest dependence on food composition.

3.3. *Threshold element ratios versus physiological compensation of nutritional imbalances*

TER ratios for the pairing C:N were estimated (section 2.2) using *GGE* values computed from elemental balances reported previously for groups of clams conditioned to varied C:N ratio diets (Arranz et al., 2022) together with the whole body tissue composition presented herein. In these experiments, acute and chronic responses to dietary changes were elucidated by feeding both N+ and N- diet-conditioned groups of clams each food composition (N+ or N-) to constitute four experimental groups (Fig. 6) for the determination of elemental balances.

TER values were compared, using four way-ANOVA (Table 2), for significant differences associated with conditioning diet (C), exposure diet (E), growth condition (G), and family (F). The most significant

Table 1
Means of pooled values (SD) of N and C%, and the resulting C:N ratios in the four factors studied (A: conditioning diet, G: growth category, F: family, T: tissues; in which: Gill: gill, AM: adductor muscle, DG: digestive gland, Go: gonad of F juveniles, MF: mantle and foot of F juveniles, GMF: gonad, mantle and foot of S juveniles); and results of four-way ANOVA on ranks.

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

differences were associated with diet composition (both conditioning and exposure diets, as well as the interaction), with only minor significance accredited to phenotype (i.e., the G × F interaction) accounting for higher *TER* values in F clams of the Family 1, irrespective of the diet.

As shown in Fig. 6a, $TER_{C:N}$ values were close to the C:N ratios of the diet given to N+ diet-fed clams, suggesting that this diet approached the ideal composition to meet nutritional needs. In contrast, *TER* values were well below the C:N ratio of food in the case of N-diets, indicating strong N limitations. However, *TER* values recorded with the low-N diet increased in the group subjected to chronic N deficit (i.e., conditioned to the N-diet) compared to the N+ conditioned group. The statistical significance of this *TER* increase was accounted for by the C × E interaction term in Table 2. The physiological mechanisms of nutrient regulation that underlie this effect are illustrated in Fig. 6b and c, where the preferential N absorption (higher AE_N/AE_C ratios), together with N-sparing metabolic processes (higher metabolic C:N ratios) were found to be associated with a chronic N deficit during the feeding of clams with the low-N diet.

4. Discussion

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

A high-N content diet promoted increases up to 40% in growth rates of clams after a month of exposure. As shown in previous approaches based on energy (Arranz et al., 2020) and elemental balances (Arranz et al., 2022), maintenance under these high-quality diets promoted a strong enhancement in growth, which was confirmed in terms of actual growth in this study. Evidently, nutrient limitations, rather than energy constraints, would be decisive in the reduced growth performances displayed with the low quality (N-) diets, since energy contents (estimated according to Platt and Irwin, 1973) of these diets were 17% higher than energy contents of high quality (N+) diets (i.e., 26.826 vs 22.906 J mg^{-1}). Stoichiometric mismatches between food and body tissues have been reported to result in negative effects on the growth of natural populations of oysters and mussels, with both growth rates and efficiencies decreasing with increasing the C:N ratio of seston on either a spatial or seasonal basis (Bayne and Svensson, 2006; Bayne, 2009; Bracken, 2017). Similarly, a strong dependence of growth rate on dietary protein content has also been reported in different bivalves fed with different N content-microalgal formulations in the laboratory (Langton et al., 1977; Enright et al., 1986; Wikfors et al., 1992; Kreeger and Langdon, 1993; Brown et al., 1998; Albentosa et al., 1999, 2002; Uriarte and Fari;as, 1999), with only minor exceptions (Utting, 1986; Whyte et al., 1989). Observations regarding the preferential absorption of proteins over other biochemical compounds (or N vs C) (e.g. (Hawkins, 1985; Urrutia et al., 1996; Ibarrola et al., 2000; Bayne and Svensson, 2006),) are consistent with this pivotal role played by proteins ingrowth processes.

Overall, biometrical parameters were found to differ mainly among phenotypes with only minor effects of the diet fed during the conditioning. Even parameters concerning feeding structures such as the relative gill size, which are commonly related to features of the food environment (Worrall and Widdows, 1983; Tedengren et al., 1990; Compton et al., 2008; Capelle et al., 2021), appeared to be barely related to diet quality in this study. No differences in the relative weight of gills were found between clams conditioned to N+ and N- diets, although gill surface areas were significantly higher with the N+ diet (Arranz et al., 2020). Instead, the relative weight of gills showed the greatest variation between families, with Family 1 exhibiting a 15% increase in gill size compared with Family 8. Both the relative weight and surface area of the gills (Arranz et al., 2020) were higher in S clams, as compared to F clams, but the latter exhibited 50% increased values of the ratio of weight/surface area. The possibility of a functional relationship between this ratio, representative of the gill thickness, and the gill efficiency (given as the filtration rate per unit of gill surface area) is tempting, increased values of both parameters appearing as attributes of fast growers.

Other biometrical traits were also found to differ between families: for instance, Family 1, which attained the highest SFG values (Arranz et al., 2020), invested comparatively more energy in shell thickening than Family 8 did (Fig. 1). Alternatively, the capacity to achieve higher energy balances (e.g., both F clams and clams of Family 1) would likely enable strengthening of structural tissues as reported for both shell and gill thicknesses. This contrasts with the growth profile of the Family 8,

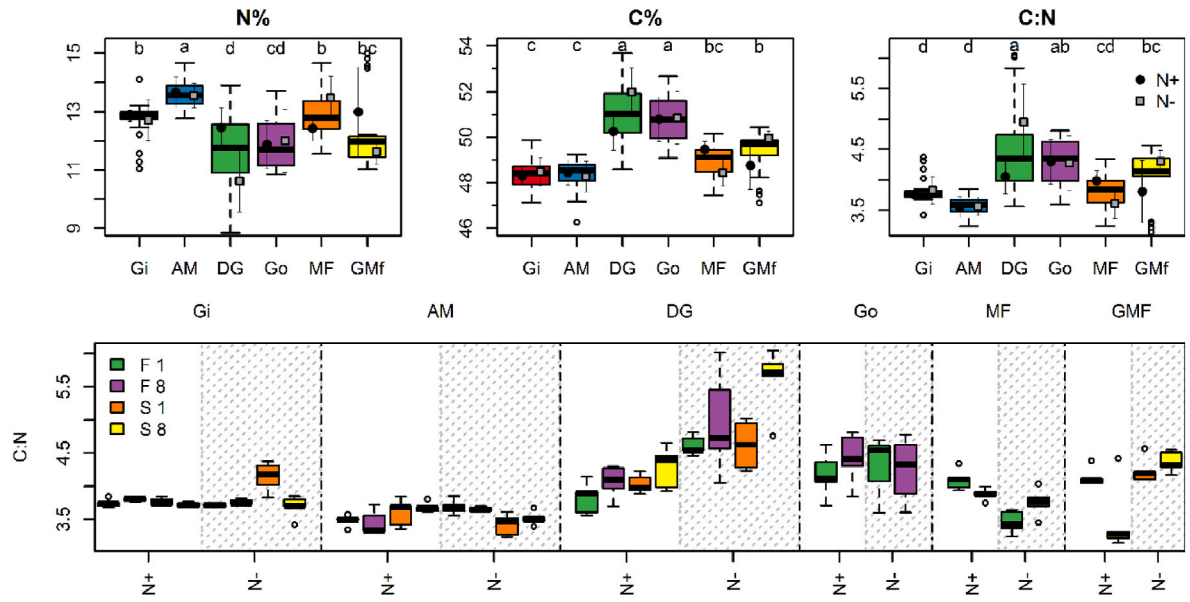

Fig. 4. a) Nitrogen (left), carbon (center) and C:N (right) content for each tissue analyzed. For each box, black circles represent elemental composition of clams acclimated to the N+ diet, while gray squares represent composition after conditioning to the N- diet. Different letters (on top) denote significant mean differences between tissues (Tukey's HSD test). b) Values of the C:N index in every tissue (Gi: gill; AM: adductor muscle; DG: digestive gland; Go: gonad and MF: mantle and foot; in S clams gonad, mantle and foot were merged in a single fraction: GMF) under either N+ (white background) or N- diet (shaded background) of F and S clams of both families (see legend).

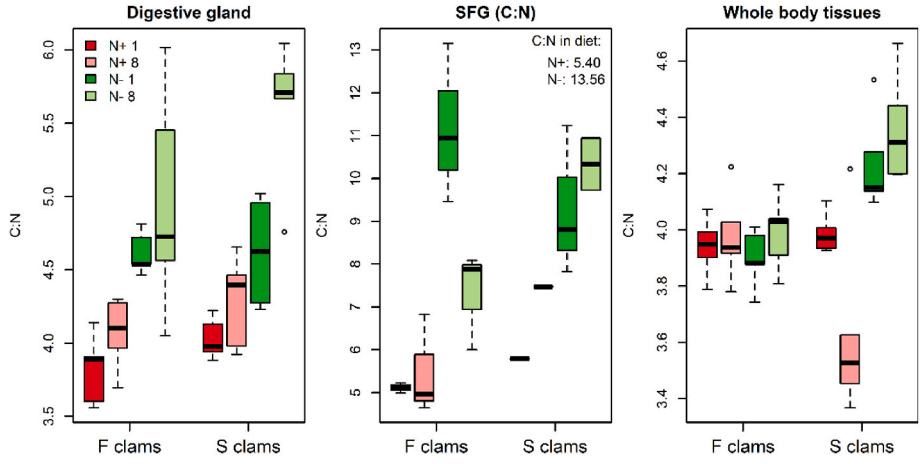

Fig. 5. C:N ratios of a) digestive gland (left), b) elemental balances (center) and c) whole tissues (right) for each growth category (F and S clams) and the combinations of diet xfamily (see legend).

where comparatively reduced values of SFG appeared to associate to a greater condition index (Figs. 1 and 2) representing the proportion of soft tissues, that was increased by > 30% with respect to Family 1. These included mantle and digestive gland tissues (Fig. 2), whose storage function might be indicative of a strategy of accumulation of energy reserves by this family of limited growth potential to face periods of energy imbalance. In the case of the digestive gland, the greater importance of this storage function in phenotypes of reduced energy performance would also account for the anomaly that size-specific weight of this organ, mainly involved in digestive food processing, was, for instance, 13% higher in S than in F clams ($F = 11.22$, $p = 0.001$). The partial occupation of this organ by storage tissues (further confirmed by the increase in digestive gland C:N ratios of S clams; Fig. 4b) in detriment of digestive tissues, would explain the reduced digestive performance reported in slow growers (Arranz et al., 2020).

The elemental composition of different tissues varied, very likely to reflect their different functions. Regarding the mean values (Fig. 4a), a clear distinction was evident between the group represented by the gonad and digestive gland (C:N index >4) and the group of remaining body components (C:N index <4). According to previous reports on the adults of this species (Arranz et al., 2021), the significantly higher C:N values in the former group are assumed to reflect the storage of energy reserves in the form of carbohydrates and lipids in the digestive gland and gonadal tissue, respectively. Consequently, the composition of both these organs is highly variable as they are affected by the conditioning diet and phenotype (Fig. 4b), suggesting the influence of these factors on either energy availability or demands associated with gametogenesis. For instance, no effects of diet on gonadal tissue composition were evident, but large deviations around mean values would reflect inter-familiar differences concerning the stages of gonadal maturation. Instead, the elemental composition of the digestive glands exhibited a strong dependence on both conditioning diet and phenotype, with trends in C:N ratios across these factors (Fig. 5a) that exhibited a noticeable similarity with C:N ratios computed for the retained fraction

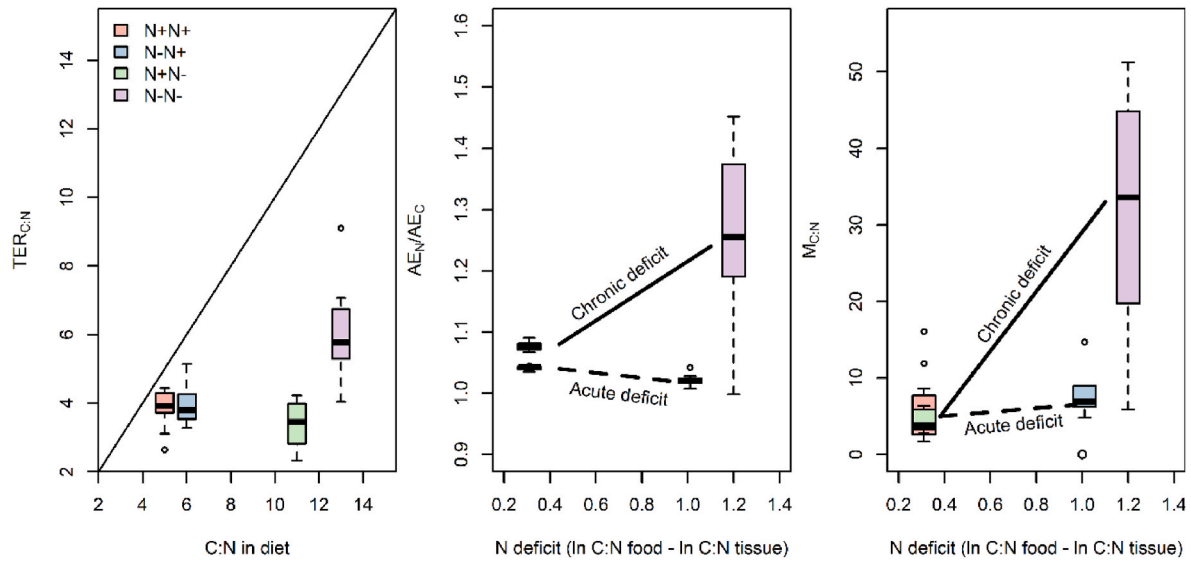

Fig. 6. a) $TER_{C:N}$ of clams fed on either N+ or N- (see legend): line y = x represents the condition for TER values coinciding with actual diet composition. b) Ratio of absorption efficiencies for N and C and c) metabolic C:N ratio (=C respired/N excreted) plotted as a function of nutritional deficit (ratio differences between food and body tissues). Experimental groups in elemental balance determinations were: N + N+: conditioned to N+, fed the N+ diet; N-N+: conditioned to N-, fed the N+ diet; N + N-: conditioned to N+, fed the N- diet; N-N-: conditioned to N-, fed the N- diet.

Table 2
Four way-ANOVA table of TER values for food conditioning (C), food exposure (E), growth condition (G) and family (F) as factors.

	F value	p value
Conditioning (C)	59.183	**4.7×10^{-8}**
Exposure (E)	11.018	**0.002**
Growth (G)	3.105	0.090
Family (F)	0.028	0.868
C*E	33.118	**5.4×10^{-6}**
C*G	0.549	0.465
E*G	0.004	0.948
C*F	2.960	0.098
E*F	4.370	**0.047**
G*F	10.562	**0.003**
C*E*G	0.443	0.512
C*E*F	2.712	0.112
C*G*F	7.566	**0.011**
E*G*F	0.212	0.649

(SFG) from elemental balances (Arranz et al., 2022) (Fig. 5b). Since the animals were fasted for 3 days prior to tissue dissection, any distorting effect of ingested food on digestive gland composition should be discarded.

As stated previously (see Introduction), stoichiometric mismatches between food and body tissues in marine bivalves are compensated through the preferential pre-ingestive selection and absorption of the deficit element (most frequently N) coupled with the preferential metabolic release of the element excess (usually C) (Urrutia et al., 1996; Smaal and Vonck, 1997; Bayne and Svensson, 2006; Bayne, 2009). The efficacy of these physiological mechanisms with regard to the strict homeostatic regulation of nutrients has seldom been tested on quantitatively (but see Smaal and Vonck, 1997). In the present experiments, stoichiometric mismatches were observed to occur mainly with N- diet, where a large relative deficit of N is evident (C:N = 13.5), whereas the composition of N+ diet (C:N = 4.9) falls close to that of body composition. Physiological compensation has been reported for the chronic deficit as the preferential absorption of N (higher AE_N/AE_C) and the preferential release of C (higher metabolic C:N ratio) were both observed in clams fed and conditioned to N- diets (Fig. 6b and c). However, despite the fact that these mechanisms produced a significant increase in $TER_{C:N}$ values (Fig. 6a; Table 2), the ideal C:N composition of food (mean: 6.1) remained below the C:N ratio of the actual composition of the N- diet (13.5). In other words, when whole-body tissue composition was considered (Fig. 5c), the physiological response to N deficit could not account for the noticeable stability and independence of dietary composition that was observed in the body composition, especially of F clams, and C:N ratios of the retained fraction from food (Fig. 5b) -assumedly to represent the composition of newly added materials during tissues growth -were observed to change with the conditioning diet, from ~6 with N+ to ~9 with N-. Considering the above discrepancies, the following two interpretations are offered that can be viewed as either alternative or complementary hypotheses.

1). *Turnover times of most body constituents would be rather long so as to buffer dietary effects on the body composition of growing tissues over the* 1 month *conditioning.* Compared to short-lived consumers, tissue turnover times for most bivalve species (Hawkins, 1985; Fukumori et al., 2008) far exceeded the conditioning time in the present experiments. Nonetheless, this could not apply to actively changing structures such as the digestive gland, where C:N composition has been shown to strongly respond to C:N ratio in the diet (Fig. 5a). In support of this, significant diet-induced restructuration of the digestive gland involving changed size and protein content have been reported to take 10-15d. in cockles *Cerastoderma edule* (Ibarrola et al., 1999), and tissue distribution of stable isotope signatures indicated a high turnover in the digestive gland and low in the gill of the fan shell *Pinna nobilis* (Cabanellas-Reboredo et al., 2009). However, evidence on this point may be contradictory since gills were reported to show the highest tissue cell turnover rates in the scallop *Aequipecten opercularis* as a possible response to oxidative damage (Strahl and Abele, 2010). An alternative interpretation has been stated previously regarding the possibility that conditioning to N-poor diets might, in combination with phenotype, induce changes in the proportion of storage tissues within the digestive gland compatible with the increment of C:N ratios of this organ. A more detailed analysis in terms of biochemical components is necessary to assess this point.

The hypothesis of a differential tissue turnover could eventually be applied to the interpretation of the stronger dependence of slow-growing phenotype composition on the diet (Fig. 5c), on the

assumption that slow growth might result from a faster renewal of tissues in this group of clams. Reduced growth efficiency of N found in slow-growing clams, which is mainly associated with increased rates of N excretion (Arranz et al., 2022), points to faster rates of protein turnover and reduced rates of protein deposition (Bayne et al., 1999) which is the main component of tissue growth.

2) *Apparent uncoupling results from methodological limitations in the computation of elemental balances, especially with respect to some components of C loss*. To avoid underestimation of metabolic C release, in the present balances, respired CO_2 was computed from the rates of oxygen consumption by applying an averaged respiratory quotient (RQ = 0.9). This estimation procedure was considered more accurate than the direct CO_2 measurement because it prevents from any confounding effect due to the possibility that a fraction of metabolically produced CO_2 can be "sequestered" in the process of shell mineralization. In fact, analysis of stable C isotope signatures (Marchais et al., 2015) have established that as much as 10% of inorganic C deposited in the shells of different bivalve species might be derived from dietary C (12% in the case of Manila clams, Poulain et al., 2010). However, some evidence in marine bivalves indicates substantial C losses in the form of dissolved organic matter (DOM), meaning that respired C does not exhaustively accounts for the release of this element regarding C balances. For instance, using ^{14}C-labelled diets, Kreeger and Langdon (1994) identified, in *Mytilus trossulus*, a labelled component of the DOM that accounts for as much as 17–30 % of the ingested ^{14}C. This component might comprise C solubilized from fecal materials (e.g., the unaccounted fraction of metabolic fecal losses including the amino C fraction wasted during protein assimilation (Kreeger et al., 1996)) but also other organic components of strictly metabolic origin such as short-chain organic acids and volatile free fatty acids, which are known to be released from animals, especially during anaerobic phases (Hochachka and Somero, 2002). If this were the case, a substantial component of the homeostatic regulation of C contents, likely enough to account for discrepancies found between elemental balances and whole tissue composition, could have been missed in this study.

In conclusion, conditioning to broadly varied-N diets for 1 month resulted in only minor changes in whole-body C:N composition which suggests a noticeable degree of homeostatic regulation of nutrient balances. This regulation was found to be stricter in fast-growing compared with slow-growing phenotypes and differed among the various body tissues. Physiological mechanisms that partly compensate for large stoichiometric mismatches between low-N food and body tissues have been identified. Different hypotheses have been proposed to account for the observed uncoupling between the elemental balances and tissue composition.

CRediT authorship contribution statement

Kristina Arranz: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Iñaki Urrutxurtu:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Ignasi Gairin:** Methodology, Investigation, Data curation. **Enrique Navarro:** Writing – review & editing, Project administration, Investigation, Funding acquisition, Conceptualization, Resources.

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.

Data availability

Data will be made available on request.

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