Variability of the total free amino acid (TFAA) pool in *Mytilus galloprovincialis* cultured on a raft system. Effect of body size

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Abstract

Variability of total free amino acid (TFAA) pool in *Mytilus galloprovincialis* was analysed following raft culture of growing mussels. With the aim to ensure the actual effect of animal's size, a mussel population was sampled in laboratory covering the field size (15-60 mm shell length). TFAA showed a constant profile regardless size increase of individuals in both field and laboratory experiments. Environmental salinity and temperature varied within a relatively narrow range in the field (34-35‰ and 14-19°C, respectively). Despite the narrow variability of TFAA, significant differences were reported for the most representative amino acids. Specifically, taurine content significantly dropped with soft tissues increase in both field and laboratory experiments. Up to 40% of taurine drop in growing individuals was counterbalanced by the increase in alanine as main protein free amino acid (PFAA) whereas glycine showed high variability and inconsistency. PFAA increased linearly with the endogenous condition of individuals and were inversely correlated with protein content. In summary, TFAA pool of *M. galloprovincialis* remained unchanged with animal's size which might ensure optimal protein

turnover rates during growth and establish a significant link between energetic status (condition) and PFAA, values that are equilibrated by a significant drop of the most abundant taurine.

Keywords: Free amino acids, *Mytilus galloprovincialis*, culture, growth

Introduction

Variability of the total free amino acid (TFAA) pool in bivalve molluscs is mainly affected by osmoregulation, counterbalancing osmolarity changes in the environment (Shumway & Youngson 1979). Specifically, taurine, glycine and alanine are the most representative amino acids in volume regulation of bivalves to maintain osmotic equilibrium (Livingstone *et al.* 1979) showing the highest amounts within the free amino acid pool. However, it has also been reported that TFAA might vary with the physiology of the animals (Helland *et al.* 2000; Rosa *et al.* 2005) and that endogenous values of the individuals (i.e. energetic status), commonly not considered, might have also an extraordinary importance (Babarro *et al.* 2006a). Pool of TFAA may also participate in several energy-yielding metabolic pathways as substrates, constituents of proteins, enzymes and hormones, and certain amino acids are also involved in reproduction and development (Livingstone 1985; Zurburg *et al.* 1989; Sokolowski *et al.* 2003). Therefore, TFAA pool represents high amounts in soft tissues of marine invertebrates (Bishop *et al.* 1983) although its value might vary with the rate at which materials either leave or enter that pool (Hawkins & Hilbish 1992). The balance between supply and biosynthesis as well as between osmotic equilibrium and catabolism would determine TFAA values (Yancey *et al.* 1982).

Studies of the spatial and/or temporal variability of TFAA in bivalve molluscs are neither abundant nor consistent. Especially when studying temporal fluctuation or seasonality of TFAA

pool, wide body size ranges have been occasionally used (Zurburg *et al.* 1989; Hummel *et al.* 1994) although the incidence of individual's size or animal condition are rather unexplored (Babarro *et al.* 2006a). No clear information is obtained from the bibliography about this particular point (see review of Livingstone 1985; Sokolowski *et al.* 1999).

One might expect little temporal variation in TFAA pool due to the fact that seasonal patterns can be considered of minor importance in locations with rather constant salinity conditions, especially above 20 PSU values (Kube *et al.* 2006; 2007). However, it is also true that spatial-temporal variation of the TFAA pool might be affected by a number of biotic and other environmental parameters (Sokolowski *et al.* 2003).

The main function of the most abundant free amino acid taurine in mussels is the intracellular osmoregulation processes in soft tissues. Therefore, it might be expected that taurine scales up with the amount of tissues of the individual (Allen & Garret 1972). However, an inverse relationship between relative taurine amounts as $\mu\text{moles per gram}$ of soft tissues and bivalve's body size was firstly reported by Allen & Garret (1972) and extended with our own studies (Babarro *et al.* 2006b). We have reported this inverse relationship in both gill epithelium and labial palp tissues of growing mussels and related it to the fact that this sulphur-containing free amino acid is important in the surface-linked processes of both organs (Babarro *et al.* 2006b). Taurine content variability in growing individuals may affect significantly the amount of the **total** free amino acid (TFAA) pool with consequences for the protein turnover. There is a significant lack of knowledge on this topic and the effect of individual's size on the total free amino acid (TFAA) pool.

In the present work, we collected wild juveniles of *M. galloprovincialis* from a source of wild mussel seed commonly used by the farmers in the Galician coastline (Miranda; NW Spain). The individuals were cultured on a raft system in the Ría de Ares-Betanzos from seed (15 mm) to harvest size (60 mm shell length). The effect of individual's growth throughout the

cultivation season on total free amino acid (TFAA) pool profiles was investigated exploring the hypothesis that TFAA pool is in equilibrium during growth of individuals. In order to study the actual effect of body size excluding the temporal component of individual's growth on the raft, a similar body size range to that obtained in the field was considered in a laboratory experiment. We have considered relevant to group amino acids in both protein and non-protein free amino acids in order to draw stronger conclusions. This was done because proteolysis or protein breakdown might cause abrupt changes in the amount of free amino acids (Viarengo et al. 1992) and that the non-protein amino acid taurine represents the major component in *M. galloprovincialis* free amino acid pool (Pranal et al. 1995; Pruski et al. 2000; Babarro et al. 2006a; b).

Materials and Methods

Mussel sampling

A mussel seed population (*Mytilus galloprovincialis*) from an intertidal location in the Ría de Ares-Betanzos (Miranda; NW Spain) was selected for raft culture. Shell length (mm/ind) and dry soft tissues (g/ind) values of Miranda population were 13.0 ± 3.5 and 0.015 ± 0.003 , respectively. Such location represents an important ground for juvenile's collection for its further use on mussel raft culture in Galicia. Mussel seed was cultured in the Ría Ares-Betanzos from February to December 2003 following commercial techniques of the Galician mussel culture. For description of growth analysis of the individuals as well as the variability of the environmental parameters obtained for the experimental location (temperature, salinity and chlorophyll-a values), we refer to a previous article of our own research group (Peteiro et al. 2006). Briefly, a number of two-rope pair was used for the cultivation of the individuals with a density value of approximately 15 kg of mussel seed per rope. Mussels were collected from

each rope and at 3-4 m depth, using two replicates of 200-300 mussels. Individual shell lengths were recorded as the maximum measure along the anterior-posterior axis to the nearest 0.1 mm, using callipers (Mitutoyo[®]), and each sample was divided into 1-mm shell length classes. Adjusted shell length was calculated by the formula $L = (C_L F) N^{-1}$ (see Peteiro *et al.* 2006), where L is the average shell length, C_L the length class, F the frequency and N is the total number of mussels considered. From each replicate sample, sub-samples of 12-15 mussels were selected from each of five length classes below and above the adjusted length in order to obtain soft tissues weight. After dissecting the tissues from the shell valves, both soft tissues and shell were dried at 110°C until constant weight was obtained. Condition index (CI) was calculated for each replicate sample using the formula $CI = (DW_{\text{tissue}} / DW_{\text{shell}}) \times 100$ (Freeman 1974).

Values of the environmental parameters that corresponded to this field experiment (Peteiro *et al.* 2006), are presented in Figure 1A showing a range for both salinity and temperature values of 34.5-35.3‰ and 14-19°C, respectively. Maximum chlorophyll-a value was observed in August 2003 (8.3 µg/l) compared to a lower range of 0.5-4 µg/l throughout the rest of the year (Figure 1B).

For the laboratory experiment, *M. galloprovincialis* individuals from 15 to 60 mm shell length were collected from an adjacent raft in spring 2003. The mussels were transported to the laboratory and grouped into three groups: 20, 40 and 60 mm shell length class for further analysis. Individuals were placed on the bottom of series of four 19-litre cages (45 x 40 x 14 cm, length x width x height) and maintained for a week in an open flow system (see Labarta *et al.* 1997 and Babarro & Fernández Reiriz 2010 for a description in detail). An input flow was distributed into the series of four cages with values of approx. 3 l min⁻¹. The experimental cages were of open flow design using filtered (10 µm) seawater (Cartridge CUNO Super Micro-Wynd 10 µm) with salinity and temperature values of 35.5‰ and 15°C, respectively. The filtered seawater was supplemented with a mixture of microalgae (Tahitian *Isochrysis* aff. *galbana*, T-

ISO) and sediment from the seafloor below the rafts (40:60 microalgae:sediment, by dry weight) supplied with a peristaltic pump at constant flow. Particulate material load was maintained at values of 1.2 mg l^{-1} with an organic content percentage of 50%, then simulating the mean values of food availability for the animals in their natural environment of Galician Rías. After one week of acclimation period, individuals of different sizes were sampled for TFAA analysis and the determination of other endogenous parameters.

Protein content and free amino acids

Protein content of soft tissues was determined following Lowry *et al.* (1951) after alkaline hydrolysis with 0.5N NaOH at 30°C as percentage values of organic tissue (%OM). For each sampling time in the field study and sampling size in the lab experiment, three groups of 12-15 or 2-3 individuals depending on the experimental body size were taken from mean (adjusted shell length) values of the whole size distributions for growth analyses (see before). Soft tissues of these individuals were separated, stored at -80°C and freeze-dried. Prior to the protein analysis, tissues of mussels were homogenized with an ultrasonic Branson Sonifier (250/450 USA).

For the determination of free amino acids, three replicates of a variable number of individuals depending on the experimental body size (see last paragraph) were taken from mean values of the whole size distributions for growth analyses. Soft tissues were excised, placed on a bed of broken ice and squeezed between aluminium blocks pre-cooled in liquid nitrogen. Before preparing the extracts for HPLC analyses, frozen tissues were lyophilised for 48h and the resulting were powdered and weighed. Briefly, approx. 100 mg of dry tissue was suspended in 5 mL 0.2 M perchloric acid following Krause *et al.* (1995) and Babarro *et al.* (2006a). The mixture was homogenised in an Ultra Turrax blender for 2 min and kept in an ultrasonic bath for 30 min and then centrifuged at 10000 g for 20 min. The supernatant was filtered through a

0.45µm membrane. Determination of free amino acids was performed by reversed-phase high-performance liquid chromatography of the dabsyl derivatives. Amino acids standards and dabsyl chloride were purchased from Sigma. Free amino acid separation method consisted in a slight modification of that reported by Krause *et al.* (1995). The chromatograph was a Waters Alliance HPLC System with a 2690 separations module and a Waters 996 photodiode array detector (440-480 nm). The stationary phase was a C₁₈ column (Waters Symmetry, 150 x 4.6 mm, 3.5 µm particle size, 100 Å pore size) thermostated at 50°C by an Alliance System column oven. 20 µL of the derivatized samples were injected. Dabsylated amino acids were eluted at a flow-rate of 1 mL/min using a gradient made with phase A (9 mM sodium dihydrogenphosphate, 4% dimethylformamide and 0.15% triethylamine titrated to pH 6.55 with phosphoric acid) and B (80% aqueous acetonitrile) with the elution profile proposed by Pinho *et al.* (2001). Nor-leucine was used as internal standard for quantification. Amino acids were grouped into protein free amino acids (PFAA) and non-protein free amino acids (NPFAA), the latter group represented by the sum of taurine and ornithine.

Statistical analysis

Concentrations of TFAA are presented as the mean $\pm$ standard deviations of the three replicates used and were quantified as µmoles per g of dry soft tissues and relative percentages that each amino acid represents in the TFAA pool. ANOVA was performed on TFAA, shell length, soft tissues weight, condition index and protein content variability obtained during the experiments. Correlation analyses between different groups of free amino acids (protein and non-protein free amino acids) and the endogenous factors condition index and protein content of soft tissues were performed following Pearson's coefficients and linear functions. Relationships between groups of protein and non-protein free amino acids as well as specifically taurine, glycine and alanine with increasing size (soft tissues weight) and conditioning of individuals

followed both allometric and linear relationships. All analyses were done with a statistical computer package (STATISTICA 6.0 Statsoft Inc. USA).

Results

Growth patterns and protein content of soft tissues

An increase of shell length and soft tissues dry weight was observed throughout the growth season with the exception of a significant drop in soft tissues ($P < 0.001$) at the end of the culture (December 2003; Figure 2A). Similarly, condition index of individuals increased continuously during mussel culture but also dropped significantly at the end of the culture ($P < 0.001$) (Figure 2B).

The proportion of protein (as organic grams per kg) dropped continuously in the soft tissues of the mussels from $738.6 \text{ g kg}^{-1} \text{ OM}$ at the beginning of the culture to $356.4 \text{ g kg}^{-1} \text{ OM}$ in August (Figure 2C) ($P < 0.001$). An increase in the protein content of cultured mussels was observed from the minimum values in August up to values of $572.6 \text{ g kg}^{-1} \text{ OM}$ at the end of the culture (Figure 2C) ($P < 0.001$).

Taurine, glycine and alanine

Taurine was the most abundant amino acid and showed an inverse relationship with size of individuals either for raft cultured mussels or those maintained in the laboratory (Figure 3A). Taurine is the amino acid with the highest differences between field and lab experiments considering larger animals (Figure 3A, Tables 1-2). Highest concentrations of taurine were obtained at the beginning of the culture for juveniles (62% TFAA; $209 \mu\text{moles g DW}^{-1}$ and 37% TFAA; $137 \mu\text{moles g DW}^{-1}$ for both field and laboratory experiments, respectively). Both

cultured and laboratory maintained mussels showed a significant drop in taurine with increasing size of individuals. The lowest taurine relative amounts were reported for the largest mussels (26% TFAA; 83 $\mu\text{moles g DW}^{-1}$ and 12% TFAA; 48 $\mu\text{moles g DW}^{-1}$ for field and laboratory mussels, respectively) (Figure 3A, Tables 1-2). No clear pattern was observed for glycine variation with growth of individuals (Figure 3B). Higher variability of glycine was, however, observed at the beginning of the culture for the field study with values ranging between 19-36% TFAA (65-107 $\mu\text{moles g DW}^{-1}$). Concentrations of glycine in laboratory sampled mussels represented a narrower range between 17-21% of TFAA (65-77 $\mu\text{moles g DW}^{-1}$) for the whole size range analysed (Figure 3B, Tables 1-2).

Alanine concentrations showed a significant exponential increase with size of cultured and laboratory maintained mussels (Figure 3C). Lowest concentrations of alanine were obtained at the beginning of the culture for juveniles (4-5% TFAA; 14 $\mu\text{moles g DW}^{-1}$) and the lowest size sampled in the laboratory (11.9% TFAA; 43 $\mu\text{moles g DW}^{-1}$) (Figure 3C, Tables 1-2). Both cultured and laboratory maintained mussels showed a significant increase with size up to the highest alanine concentrations of 18% TFAA; 52 $\mu\text{moles g DW}^{-1}$ and 22% TFAA; 87 $\mu\text{moles g DW}^{-1}$, respectively in the largest size animals (Figure 3C, Tables 1-2).

Fluctuations of the main free amino acids i.e. taurine, alanine and glycine were grouped as the ratio taurine / (alanine + glycine) considering their percentage values within the free pool and is illustrated in Figure 3D. This index dropped steeply from 2.6 for the lowest size class in the field experiment (0.01 g of soft tissues; 15 mm shell length) to a range values of 0.7-1.1 for the rest of the animal's size values (Figure 3D). Similarly, Tau/(Ala + Gly) ratio varied between 0.3-1.1 for the laboratory maintained mussels and as was reported for the field experiment, highest value (1.1) corresponded to the lowest size class (0.08 g of soft tissues; 20 mm shell length) (Figure 3D above).

Total free amino acid (TFAA), protein free amino acids (PFAA) and non-protein free amino acids (NPFAA) pools

Concentrations of total free amino acid (TFAA) pool in soft tissues of mussels throughout cultivation on the raft are presented in Table 1 as $\mu\text{moles g DW}^{-1}$ and relative percentage relative to the total free pool. TFAA in mussels of different body size sampled after laboratory acclimation conditions are presented in Table 2. When analysing the effect of size on TFAA in the field experiment, it can be observed a rather constant profile relative to soft tissues weight of individuals (range: 273-335 $\mu\text{moles g DW}^{-1}$; Figure 4A). The only exception to this pattern was represented by a significant drop in the amount of TFAA (195 $\mu\text{moles g DW}^{-1}$) coinciding with an abrupt drop also observed in the amount of soft tissues and condition index (Figure 2A-B) at the end of the culture. In the laboratory acclimatised mussels with different size, a similar constant profile was also obtained for TFAA with increasing soft tissues of individuals, values ranging from 350 to 401 $\mu\text{moles g DW}^{-1}$ (Figure 4B).

Nevertheless, when subgroups are established within the TFAA pool i.e. protein free amino acids (PFAA) and non-protein free amino acids (NPFAA; mainly taurine), strong differences were observed following increasing size of individuals. Both PFAA and NPFAA varied positively and negatively, respectively, with the soft tissues weight of growing individuals although more clearly for the laboratory maintained animals (Figure 5A-B). Indeed, PFAA variability with soft tissues weight for the field study presented significantly lower variance explained ($r^2=0.16$; Figure 5A). However, correlations were more significant between PFAA and NPFAA with the condition index and protein content values of the bivalves (Figure 6A-C). A highly significant and positive correlation was reported between PFAA group and condition index of individuals throughout the cultivation time on the raft (Figure 6A). Moreover, this pattern was also obtained for laboratory maintained individuals (Figure 6A). Alternatively, a negative but residual ($P \sim 0.044$) relationship between PFAA and protein content

of the soft tissues was observed considering altogether experimental mussels (both cultured and maintained in the laboratory; Figure 6B). The group of NPFAA followed an inverse relationship with condition index of individuals (Figure 6C) which together with the positive relationship reported in Figure 6A established that the balance obtained for the TFAA pool relative to size increase effect is consequence of such inverse fluctuation between taurine (main NPFAA) and PFAA.

Discussion

Variability of TFAA profiles analysed in the present study showed a rather constant pattern relative to size factor either for raft cultured individuals (temporal growth) or those animals sampled in the laboratory (Table 1-2; Figure 4A-B). Nevertheless, this constant TFAA profile with increasing size of individuals was clearly based on a balance between the two main groups of the free pool: the non-protein amino acid taurine (the most abundant amino acid of the free pool) and the whole group of protein free amino acids (PFAA), both groups being inversely correlated (Figure 5A-B; Figure 6A-C).

On one hand, taurine (and aspartic acid but in a much lower magnitude; see Tables 1-2) were the only free amino acids that showed a significant drop with increasing size of individuals (Figure 3A; Table 1-2). No clear pattern relative to size increase was observed for the amino acids glutamic acid (1.5-2.1% TFAA and 2.7-3.1% TFAA for cultured and lab maintained mussels, respectively) and glycine (19.4-36.2% TFAA and 17-21% TFAA for cultured and lab maintained mussels, respectively) (Tables 1-2). Although in a much lower magnitude, the same absence of any significant pattern with size of mussels can be also reported for cysteine, ornithine and tyrosine values ranging between 0.02-1.9% TFAA in different size animals (Tables 1-2). On the other hand, PFAA increased with size of individuals (Figure 5A-B) but

more significantly with their condition index values (Figure 6A) most likely as a consequence of the necessities for protein turnover (synthesis, deposition, breakdown) in tissues of growing individuals. Protein maintenance requirements increase allometrically with body size and the conversion of protein (and energy) have been described to be similarly dependent on mussel size (Hawkins & Bayne 1991).

Growth represents an increase in volume and mass of individuals. Considering the reported functionality of the most abundant free amino acid taurine as the main osmolyte to control the osmotic balance, an increase in its content with increasing size of the animals might be expected (Welborn & Manahan 1995). Indeed, taurine represents the largest amount in the free amino acid pool of *Mytilus galloprovincialis* (Pranal *et al.* 1995; Pruski *et al.* 2000; Babarro *et al.* 2006a, b) despite the rather slow rates of taurine synthesis reported for adults (Bishop *et al.* 1983; Welborn & Manahan 1995). This would be the reason why taurine has been suggested to be taken from dietary sources (Hosoi *et al.* 2003) and its precursors methionine and cysteine (Welborn & Manahan 1995). The fact that taurine was the amino acid with the highest differences between experimental populations in larger animals (Figure 3A) would reflect such different impact of the diet composition. The importance of the diet factor was also reflected by Kube *et al.* (2007) and it is obvious in our study when comparing the wider particle's heterogeneity in the field location of the Ría de Ares-Betanzos (NW Spain) and the single microalgae diet supplemented in the laboratory. In fact, alanine concentrations increased similarly with size in both field and laboratory experiments (Figure 3C), whereas taurine drop was more pronounced in the lab experiment for larger specimens (Figure 3A) more likely as a consequence of the fact that most of microalgae species i.e. *Isochrysis galbana* lack taurine (Welborn & Manahan 1995). The ratio tau / (ala + gly) reported in Figure 3D highlighted that taurine concentration is 2.6-fold higher than the sum of alanine + glycine in the lowest size of field cultured mussels (0.01 g soft tissues dry weight, 15 mm shell length) but a steep drop even

1 at juvenile stages caused that values ranged between 0.7-1.1 for the rest of the mussel growth
2 period. The fact that taurine requirements are more pronounced in juvenile stages than adults
3 was already reported by Welborn & Manahan (1995). Indeed, Welborn & Manahan (1995)
4 established that taurine increased by a factor of 43-fold as *Crassostrea gigas* larvae grew from
5 80 to 320 μm shell length although the mol-percentage values that taurine represents in the free
6 pool is approx. 62% for the latter stage, equal to that reported in our study for the initial size of
7 cultured mussels (Table 1).

8 Therefore, in order to equilibrate the increase of protein free amino acids in soft tissues of
9 growing individuals, taurine amount was reported here to drop with body size of mussels in both
10 experiments we have carried out. Although an inverse relationship between taurine amounts and
11 increasing size of bivalves was firstly reported by Allen & Garret (1972) when studying whole
12 tissues of *Mya arenaria*, we have extended such knowledge reporting that this inverse
13 relationship was observed for the specific organs gill epithelium and labial palps (Babarro *et al.*
14 2006b). Both gill epithelium and labial palps are well-known as mucus producers organs that in
15 turn contain high amounts of sulphate anion (Lee *et al.* 2004).

16 Osmolarity has been described as the main abiotic factor playing a role on variability of
17 the free pool of amino acids specially when analysing its spatial variability (Kube *et al.* 2006).
18 In the present study, salinity values of the experimental sampling location were observed to vary
19 within a narrow range throughout the growing season (Figure 1). Therefore, it is possible to
20 assume that no great impact on TFAA values was expected from this abiotic factor. Beside, in a
21 comparison between *Mytilus* spp. and *Macoma balthica*, Kube *et al.* (2006) have established
22 that *Mytilus* spp. may utilise other osmolytes such as betaines to balance osmolarity when is
23 needed, with the main amino acids taurine, glycine and alanine being less important for that
24 purpose.

1 The endogenous parameters that characterised the experimental growing individuals i.e.
2 condition index and protein content of soft tissues represented significant aspects to highlight
3 their high correlation with the free amino acid pool (Figure 6A-C). Therefore, it can be reported
4 a relevant influence of these endogenous parameters (commonly not considered) to analyse
5 variability of free amino acids considering size and/or temporal gradient of growing mussels.
6 The relationship TFAA – PFAA – protein content – condition index of individuals was,
7 therefore, observed to be crucial in the interpretation of the temporal patterns associated to
8 growth of individuals under study. Condition index of individuals represents an important eco-
9 physiological parameter to measure the apparent health of the animals (Orban *et al.* 2002) and
10 therefore, it would be a reflection of local trophic and abiotic conditions as well as its influence
11 on growth rates (Figure 2B).

12 Considering the main amino acids taurine, glycine and alanine, it is important to highlight
13 that taurine drop with increasing size of individuals was mainly counterbalanced by alanine
14 increase (Figure 3A, C). Glycine variability was relatively high, specially at early stages of
15 growth, to establish any significant pattern relative to size (Figure 3B). Together with taurine,
16 both alanine and glycine may be also important organic compounds playing a role in
17 osmoregulatory processes of bivalves (see before) but alanine was the free amino acid which
18 followed a positive relationship with condition and increasing size of individuals (Figure 3C).
19 Metabolic turnover and biosynthesis of glycine are rapid (Livingstone *et al.* 1979) and have
20 been closely related to the reproductive cycle of bivalves (Zurburg *et al.* 1989; Sokolowski *et al.*
21 2003). On the contrary, alanine *de novo* synthesis require less energy than other free amino
22 acids (Hosoi *et al.* 2003), with several pathways being operative for its synthesis i.e. via
23 transamination to pyruvate. Moreover, alanine has been described to present a strong seasonal
24 variation following changes in soft tissues which in turn might establish a link with the
25 physiological state of the individuals (Sokolowski *et al.* 2003).

From the present results, it was evident that the pool of free amino acids tended to remain rather constant throughout the growing season of individuals despite significant changes in the specific main components of such pool. Differences in soft tissues weight and condition index of individuals had a strong impact on the variability of the free amino acids and such influence could be established according to significant allometric and linear relationships. These relationships together with those obtained in our previous studies highlighted the effect of using different size parameters like soft tissues weight, shell length, condition index and/or protein content, commonly not considered in the scientific literature, when studying the temporal and/or spatial variability of TFAA as eco-physiological tool for bivalves (present study and Babarro *et al.* 2006a, respectively). We establish here a significant effect of the latter endogenous factors for the analyses of specific free amino acids in different animal's sizes. Despite the great spatial and seasonal variability in TFAA of *Mytilus* spp. along the European coast (Hummel *et al.* 1996; Kube *et al.* 2006; Babarro *et al.* 2006a), our data showed a rather constant profile for the free pool of amino acids relative to growth/size of *M. galloprovincialis* based on an inverse fluctuation of both taurine, as the most abundant free amino acid, and PFAA (mainly alanine) in soft tissues.

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Table 1. Composition of total free amino acid (TFAA) pool for the mussels (*Mytilus galloprovincialis*) from Miranda (NW Spain) during their cultivation on the raft. Values are expressed as μmol per g of dry weight (DW^{-1}) (SD in brackets) as well as percentage values of the total TFAA pool. Non-protein amino acids: taurine and ornithine. Alanine represents the sum alanine and β -alanine.

		Mean size, mm		13 (2.1)		18 (3.4)		28.4 (4.1)		37.9 (5.0)		59.8 (7.2)		63.3 (7.9)	
		February-2003		March-2003		June-2003		August-2003		October-2003		December-2003			
		μmol g DW ⁻¹	% TFAA	μmol g DW ⁻¹	% TFAA	μmol g DW ⁻¹	% TFAA	μmol g DW ⁻¹	% TFAA	μmol g DW ⁻¹	% TFAA	μmol g DW ⁻¹	% TFAA	μmol g DW ⁻¹	% TFAA
	Aspartic acid	18.95 (0.7)	5.66	6.53 (1.0)	2.20	6.14 (1.81)	2.24	4.86 (0.3)	1.70	4.20 (0.5)	1.32	2.67 (0.2)	1.37		
	Glutamic acid	5.99 (0.7)	1.79	4.47 (0.3)	1.51	4.87 (1.21)	1.78	6.10 (1.2)	2.14	6.18 (0.3)	1.94	4.18 (0.7)	2.14		
	Asparagine	0.67 (0.2)	0.20	0.12 (0.02)	0.04	0.48 (0.61)	0.18	1.01 (0.4)	0.35	2.68 (0.1)	0.84	0.10 (0.5)	0.05		
	Glutamine	2.45 (0.3)	0.73	1.50 (0.5)	0.51	9.83 (1.0)	3.59	17.83 (1.2)	6.25	29.45 (0.12)	9.23	10.63 (1.2)	5.44		
	Serine	2.18 (0.2)	0.65	1.22 (0.4)	0.41	5.58 (0.66)	2.24	10.66 (1.4)	3.74	14.38 (0.5)	4.51	5.06 (0.6)	2.59		
	Glycine	65.15 (6.5)	19.45	107.31 (2.7)	36.24	79.04 (6.2)	28.87	65.18 (0.9)	22.84	56.32 (2.1)	17.65	46.12 (2.2)	23.60		
	Alanine (and β-Alanine)	14.01 (0.9)	4.18	14.60 (1.4)	4.93	24.69 (3.3)	9.02	52.13 (4.6)	18.27	55.51 (4.5)	17.40	32.51 (2.8)	16.64		
	Taurine	209.0 (19)	62.37	140.75 (10.5)	47.46	114.76 (10.8)	41.92	79.03 (2.9)	27.69	83.06 (4.3)	26.03	61.53 (8.5)	31.49		
	Cysteine	0.62 (0.1)	0.18	0.50 (0.02)	0.17	0.17 (0.02)	0.06	0.24 (0.08)	0.08	0.19 (0.02)	0.06	0.23 (0.03)	0.12		
	ornithine	0.49 (0.1)	0.15	0.05 (0.0)	0.02	0.08 (0.03)	0.03	0.31 (0.10)	0.11	0.44 (0.09)	0.14	0.16 (0.03)	0.08		
	Tyrosine	2.70 (0.6)	0.81	1.13 (0.1)	0.38	0.24 (0.05)	0.09	1.11 (0.38)	0.39	1.84 (0.6)	0.58	0.19 (0.11)	0.10		
	Proline	1.18 (0.1)	0.35	1.36 (0.3)	0.46	1.71 (1.2)	0.62	6.79 (0.17)	2.38	15.49 (0.9)	4.85	3.09 (0.3)	1.58		
Essential amino acids	Valine	0.74 (0.2)	0.22	0.23 (0.05)	0.08	1.30 (0.24)	0.47	3.79 (0.1)	1.33	3.87 (0.4)	1.21	1.76 (1.0)	0.90		
	Methionine	0.42 (0.1)	0.13	0.09 (0.02)	0.03	1.16 (0.14)	0.42	4.66 (0.18)	1.63	3.98 (0.17)	1.25	0.59 (0.1)	0.30		
	Isoleucine	0.83 (0.7)	0.25	0.39 (0.10)	0.13	1.02 (0.27)	0.37	2.10 (0.22)	0.74	3.88 (0.34)	1.22	1.40 (0.23)	0.72		
	Leucine	0.18 (0.1)	0.05	0.30 (0.04)	0.10	1.36 (0.25)	0.50	3.22 (0.08)	1.13	5.66 (0.31)	1.77	1.84 (0.13)	0.94		
	Tryptophan	0.19 (0.1)	0.06	0.03 (0.01)	0.01	1.31 (0.7)	0.48	1.00 (0.35)	0.35	1.25 (0.3)	0.39	0.32 (0.2)	0.16		
	Phenylalanine	0.12 (0.1)	0.03	0.20 (0.02)	0.07	0.74 (0.04)	0.27	1.29 (0.2)	0.45	3.18 (0.1)	1.00	0.65 (0.2)	0.33		
	Arginine	4.61 (0.2)	1.38	6.70 (0.75)	2.26	5.50 (1.1)	2.01	7.10 (0.3)	2.49	5.70 (1.7)	1.79	6.13 (1.3)	3.14		
	Threonine	3.45 (0.2)	1.03	8.10 (1.9)	2.73	12.39 (3.4)	4.53	11.89 (2.02)	4.17	15.85 (2.6)	4.97	13.00 (1.7)	6.65		
	Lysine	0.43 (0.1)	0.13	0.36 (0.2)	0.12	0.81 (0.3)	0.30	4.38 (0.3)	1.53	4.69 (0.5)	1.47	1.73 (0.3)	0.89		
	Histidine	0.72 (0.2)	0.21	0.15 (0.02)	0.05	0.60 (0.13)	0.22	0.71 (0.2)	0.25	1.84 (0.5)	0.40	1.50 (0.07)	0.77		
	Σ Total	335.1 (29.6)	100	296.09 (30.2)	100	273.8 (23.3)	100	285.4 (3.4)	100	319.1 (11.9)	100	195.4 (5.6)	100		
	Σ EAA	11.7 (0.4)	3.4	16.55 (3.0)	5.58	26.19 (1.89)	9.57	40.14 (2.3)	14.06	49.3 (3.1)	15.46	28.92 (5.2)	14.80		
	T:G ratio		3.2 (0.1)		1.3 (0.1)		1.5 (0.1)		1.2 (0.1)		1.5 (0.1)		1.3 (0.3)		
Serine and Threonine	μmol g DW ⁻¹	5.6 (0.4)	1.7	9.32 (2.5)	3.14	17.97 (0.4)	6.77	22.55 (1.8)	7.91	30.23 (2.9)	9.48	18.06 (2.8)	9.24		
	Σ PTFAA	125.59	37.49	155.29	52.45	158.94	58.26	206.05	72.20	236.14	73.85	133.7	68.43		
	Σ NPFAA	209.49	62.52	140.8	47.55	114.84	41.75	79.34	27.80	83.5	26.17	61.69	31.57		

Table 2. Composition of total free amino acid (TFAA) pool for the mussel (*Mytilus galloprovincialis*) sampled after maintenance in the laboratory (see Mat and Met). Values are expressed as μmol per g of dry weight (DW^{-1}) (SD in brackets) as well as percentage values of the TFAA pool. Non-protein amino acids: taurine and ornithine. Alanine represents the sum alanine and β -alanine.

		Body size					
		20 mm		40 mm		60 mm	
		Small	SL	Medium	SL	Large	SL
		$\mu\text{mol g DW}^{-1}$	% TFAA	$\mu\text{mol g DW}^{-1}$	% TFAA	$\mu\text{mol g DW}^{-1}$	% TFAA
	Aspartic acid	6.09 (0.4)	1.68	5.51 (0.6)	1.59	3.99 (0.8)	1.01
	Glutamic acid	9.78 (1.0)	2.69	10.83 (1.0)	3.12	12.21 (1.8)	3.09
	Asparagine	4.51 (0.3)	1.24	4.90 (0.2)	1.41	4.38 (0.71)	1.11
	Glutamine	18.27 (1.6)	5.03	33.74 (1.9)	9.73	47.61 (1.6)	12.04
	Serine	15.94 (2.4)	4.39	26.32 (2.1)	7.59	29.63 (2.8)	7.49
	Glycine	77.35 (2.6)	21.29	65.67 (2.6)	18.94	68.65 (3.2)	17.36
	Alanine (and β -Alanine)	43.21 (3.8)	11.90	68.1 (2.8)	19.64	87.39 (7.3)	22.10
	Taurine	134.73 (11.0)	37.09	63.11 (9.0)	18.20	48.37 (7.9)	12.23
	Cysteine	-----	-----	-----	-----	-----	-----
	ornithine	0.78 (0.4)	0.21	0.61 (0.2)	0.18	1.24 (0.38)	0.31
	Tyrosine	4.44 (0.8)	1.22	6.49 (0.6)	1.87	5.22 (2.0)	1.32
	Proline	7.66 (0.9)	2.11	8.10 (1.2)	2.34	15.72 (3.1)	3.98
Essential amino acids	Valine	2.21 (0.09)	0.61	3.63 (0.2)	1.05	6.19 (1.5)	1.57
	Methionine	1.93 (0.3)	0.53	4.22 (0.5)	1.22	7.63 (0.72)	1.93
	Isoleucine	0.22 (0.07)	0.06	0.98 (0.17)	0.28	0.99 (0.20)	0.25
	Leucine	1.65 (0.11)	0.45	2.73 (0.15)	0.79	4.17 (0.9)	1.05
	Tryptophan	2.54 (0.03)	0.70	4.08 (0.13)	1.18	6.90 (1.3)	1.74
	Phenylalanine	1.13 (0.22)	0.31	1.82 (0.32)	0.52	2.81 (0.7)	0.71
	Arginine	12.76 (1.04)	3.51	11.05 (1.00)	3.19	12.29 (2.8)	3.11
	Threonine	10.33 (0.60)	2.84	10.92 (0.82)	3.15	13.73 (2.8)	3.47
	Lysine	5.78 (1.41)	1.59	10.84 (1.30)	3.13	10.80 (3.0)	2.73
	Histidine	1.95 (0.8)	0.54	3.06 (0.9)	0.88	5.54 (2.3)	1.40
Σ Total		363.3 (43.0)	100	346.7 (35.2)	100	395.5 (23.3)	100
Σ EAA		40.5 (1.8)	11.15	53.3 (1.3)	15.13	71.0 (1.89)	17.97
T:G ratio			1.74 (0.1)		0.96 (0.1)		0.70 (0.1)
Serine and Threonine $\mu\text{mol g DW}^{-1}$		26.3 (0.9)	7.23	37.2 (1.3)	10.56	43.36 (0.4)	10.96
Σ PFAA		227.75	62.69	282.99	81.62	345.85	87.45
Σ NPFAA		135.51	37.30	63.72	18.38	49.61	12.55

Figure Captions

Figure 1. Variability of the environmental parameters temperature (°C) and salinity (‰) (A) and chlorophyll a (µg/l) (B) for the experimental location where mussel raft culture was carried out from February to December.

Figure 2. Growth patterns of the mussel Mytilus galloprovincialis under raft culture in shell length and soft tissues dry weight (A). Variability of the condition index values throughout the cultivation time of the mussel population (B). Variability of the protein content in soft tissues of the mussel population cultured on the experimental raft from February to December (C). Values corresponded to mean and SD (standard deviation) of two replicates of 200-300 mussels for Figure A and B, and three replicates of 12-15 or 2-3 individuals depending on the experimental size of individuals (Figure C) (see Material and Methods).

Figure 3. Variability of the main free amino acids taurine (A), glycine (B) and alanine (C) relative to body size of individuals under raft culture and sampled in the laboratory. Equations shown in the text were fitted to power functions in all cases. Variability of the ratio taurine/(alanine +glycine) as percentage values of total free amino acid (TFAA) pool relative to growth/size of individuals in the field and under laboratory conditions (D). Values corresponded to mean and SD (standard deviation) of three replicates of 12-15 or 2-3 individuals depending on the experimental size of individuals (see Material and Methods).

Figure 4. Total amount of the free amino acids pool (TFAA) in the mussel population cultured on the raft (A) and maintained in the laboratory (B) as a function of the soft tissues dry weight. For mean and SD (standard deviation) values, see legend of Figure 3.

Figure 5. Protein free amino acids (PFAA) and non-protein free amino acids (NPFAA) variability with soft tissues weight of the individuals cultured on the raft (A) and sampled after laboratory maintenance (B).

Figure 6. Relationship between protein free amino acids (PFAA) and condition index of the individuals under study either cultured on the raft or sampled after laboratory maintenance (A). Negative correlation between protein free amino acids (PFAA) and the protein content of soft tissues considering all data (cultured mussels and those sampled in the laboratory) (B). Inverse relationship between the non-protein free amino acids (taurine + ornithine) and condition index of the individuals under study either cultured on the raft or sampled in the laboratory (C).

Figure 1

Figure 2

A

B

C

D

Figure 3

Figure 4

Figure 5

Figure 6