Free amino acid composition in juveniles of *Mytilus galloprovincialis*: spatial variability after *Prestige* oil Spill

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Abstract

Composition of free amino acids (FAA) in juveniles of *Mytilus galloprovincialis* was analysed along a large geographical coastline area in Galicia (NW Spain). Individuals were sampled in February 2003, three months after the *Prestige* oil spill. Pollution values at sampling time were reported as polycyclic aromatic hydrocarbons (PAHs) concentrations in soft tissues of individuals and varied between the highest amount observed in Carrumeiro mussels (502 ng/g dw) and the lowest in Pindo mussels (196 ng/g dw), both locations being close to each other in the centre of the geographical area under study. Pollution values in the other populations varied within the range of 241-347 ng/g dw. Total free amino acids (TFAA) were highest in Aguiño-Pindo-Carrumeiro juveniles at the centre of the Coastline area studied (420-462 µmol/g dw) as compared to the other populations at North and South of Galicia (312-347 µmol/g dw). TPFAA results were based on the variability observed in protein free amino acids (PFAA µmol/g dw) among populations (214-249 µmol/g dw for Aguiño-Pindo-Carrumeiro mussels and 98-149 µmol/g dw for the other populations) whereas non-protein free amino acids (NPFAA) taurine and ornithine did not show any significant spatial pattern of variation. Glycine and alanine

represented the most abundant PFAA (16-29% and 2.7-11.9% of TPFAA, respectively) and significant correlations between PFAA and both the protein content of soft tissues (r= -0.82) and the condition index of juveniles (r= 0.86) were observed. No significant relationships were detected, however, between pollution values in soft tissues as PAHs and FAA profiles with the exception of alanine concentrations as percentage of TFAA (r= 0.88; P<0.01). The latter seemed to be an "all or nothing" effect likely due to the influence of other abiotic factors at one of the sampling sites. Such relationship was found not significant when the outlier represented by Carrumeiro mussels was removed from the analysis. The most abundant free amino acid taurine (43.2-68.5 %TFAA) followed an inverse variability of that of glycine and by extension of the group PFAA most likely as a compensatory decrease in mussel populations with low protein content (and high condition index). Accordingly, taurine:glycine (t:g) ratio varied between 1 and 2 in most mussel populations but increased up to 3.2-4.2 in Miranda and Bueu mussels at both ends of the geographical interval studied with a corresponding PAHs concentrations of 261 and 304 ng/g dw, respectively. These mussel populations with the highest t:g ratios were characterised by the lowest PFAA contents (below 40%) and condition index values (below 10%).

Results of the present study established a significant link between energetic status of growing juveniles and FAA concentrations in environments with different pollution degrees. Variability of the free amino acids profiles in soft tissues were related to endogenous factors of juveniles (protein content, condition index) whereas no relationship with contamination values could be observed. The utility of t:g ratio as general condition factor for *M. galloprovincialis* is also corroborated for *in situ* growing juveniles.

Keywords: Free amino acids, *Mytilus galloprovincialis*, juveniles, spatial variability, Post-*Prestige* oil spill, animal condition

1. Introduction

Free amino acids (FAA) have been primarily related to osmoregulation processes that individuals carry out to counterbalance salinity fluctuations in the environment (Shumway and Youngson 1979). Taurine, glycine and alanine are the most representative amino acids in volume regulation of bivalves (Livingstone et al. 1979). FAA also take part in different 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). Therefore, FAA represent high amounts in tissues of marine invertebrates (Bishop et al. 1983). Size of FAA pool may be influenced by changes in the rate at which materials either leave or enter that pool (Hawkins and Hilbish 1992), the balance between supply and biosynthesis on one hand, and osmotic equilibrium and catabolism on the other hand, would determine FAA values (Yancey et al. 1982).

Contaminated environments may cause significant variations in a number of biological responses of mussels like clearance rates of the particulate matter in the seawater, tolerance to air exposure, antioxidant enzyme activities among others (Eertman et al. 1995). With regard to our current interest, pollution may cause alterations in the concentrations of the major free amino acids (Jeffries 1972; Livingstone 1985; Scholz 1987; Hummel et al. 1994; 1996) and also in enzymes involved in its metabolism i.e. aminotransferases (Narvia and Rantamäki 1997). In this context, FAA may reflect metabolic status and provide information on the physiological condition of the organisms (see review of Livingstone 1985), functioning as a convenient index of stress. Indeed, not only total FAA, but also relative proportions of certain amino acids like taurine:glycine (t:g ratio), the sum of serine and threonine or alanine have been used as general stress indicators (Zurburg et al. 1989; Pranal et al. 1995; Sokolowski et al. 2003), also when

considering metallic and organic polluted environments (Livingstone 1985; Hummel et al. 1994; 1996). Occasionally, other behavioural responses such burrowing capacity of *Macoma balthica* have been reported to be more sensitive indicator of stress than condition index and free amino acids (see Duquesne et al. 2004).

As general pattern, a decrease in total FAA and the sum of serine and threonine and an increase in t:g ratio were observed in molluscs as indication of environmental deterioration (Jeffries 1972; Roesijadi and Anderson 1979; Livingstone 1985; Scholz 1987; Hummel et al. 1996). The sum of serine and threonine was reported to be highly questionable when the effects of pollution were investigated in the mussel *Mytilus edulis* (Hummel et al. 1994). With regard to t:g ratio, marine bivalves under stress usually showed a decline in glycine and an increase in taurine, changes responsible of the general increase of this ratio in polluted environments (see before). The most common pattern, therefore, consists in a decrease of free amino acids in tissues of bivalves exposed to contaminants that might be related to the fact that uptake of amino acids is reduced as a consequence of valve closure under such circumstances (Viarengo et al. 1980) that in turn might cause a reduction of protein metabolism. Only incidentally the opposite (decline in taurine and t:g ratio) occurred in molluscs facing crude oil-contaminated sediments (Augenfeld et al. 1980). It is important to highlight here that exposure to hydrocarbons may promote protein catabolism by destabilization of lysosomal membranes (Viarengo et al. 1992), with a concomitant increase of protein amino acids.

Functional mechanisms of FAA changes in most abundant amino acids taurine, glycine and alanine with regard to contaminated environments are not completely understood (Hummel et al. 1996) and therefore, represent empirical indices of stress because fate of these compounds needs more investigation. Specific actions of these amino acids in molluscs have been already reported elsewhere (Livingstone et al. 1979; Huxtable 1992; Hummel et al. 1996; Sokolowski et al. 2003). Taurine concentrations may reflect differences in its availability with the diet (Pruski

et al. 2000), since its endogenous synthesis is low in molluscs (Bishop et al. 1983) whereas glycine might be more appropriate for osmoregulation than taurine as consequence of its rapid turnover and fast biosynthesis together with great abundance (Livingstone et al. 1979; Sokolowski et al. 2003).

The main criticism to the use of FAA as stress indicator has been the inherent risks of misinterpretation due to large temporal-spatial fluctuations (Zurburg et al. 1989). Additionally, wide body size ranges have been used for testing both temporal and spatial variability in FAA (see Zurburg et al. 1989 and Hummel et al. 1994 as examples) whereas the incidence of individual's body size or animal condition is rather unexplored. No abundant information is obtained from scientific literature on this topic and reports including both stress indices resulting from FAA analysis and animal condition are rare with no extra information (see review of Livingstone 1985; Sokolowski et al. 1999).

In the present survey, we have collected juveniles of *M. galloprovincialis* from different rocky shore areas along the Galician coastline that represent natural mussel grounds for culture purposes of this species in Spain. Sampling was carried out in February 2003, three months after the *Prestige* oil spill that affected a wide geographical area of the continental coastline. We have performed analyses of FAA composition in a number of field mussel populations with the main aim of revisiting FAA as eco-physiological tool for juveniles that have been living with different degrees of stress. Since proteolysis or protein breakdown might cause abrupt changes in amino acids of the free pool (Viarengo et al. 1992) and the fact that the non-protein amino acid taurine represents the major component in the free pool of *M. galloprovincialis* (Pranal et al. 1995; Pruski et al. 2000), we considered relevant to group amino aids in both protein and non-protein free amino acids in order to draw more accurate conclusions. Spatial variability of FAA profiles will be evaluated considering the previous information of the spill along such

geographical North-South gradient with regard to PAHs as well as the endogenous factors i.e. size, protein content of soft tissues and condition index of mussels.

2. Material and methods

2.1. Mussel Sampling

Figure 1 illustrates the Galician Coastline and locations where juveniles of *Mytilus galloprovincialis* were sampled. These locations represent important grounds for juveniles collection for its further use on raft culture. Three replicates of 300 individuals were sampled in February 2003. Individual mussel length was measured to the nearest 1 mm using calipers, and each sample was divided into 1 mm length classes. Adjusted shell length was calculated by the formula: $L= (C_L \times F)/N$ (Box et al. 1989), where L is the shell length average value, C_L is the individual length class, F is the frequency, and N is the total number of individuals considered. From each replicate sample, sub-samples of 12-15 mussels were selected from five length classes below and above the adjusted length to obtain weight values. First, the adductor muscle was cut and individuals were placed with their ventral edge on filter paper to remove the internal water. After dissecting the tissues from the shell valves, both were dried at 110ºC until constant weight, then soft tissues and shell were weighed separately. Condition index values were obtained according to the formula: $CI=(DW_{tissue}/DW_{shell}) \times 100$, where DW_{tissue} corresponds to dry weight of soft tissues and DW_{shell} to dry weight of the shell (Freeman 1974). Values of shell length, total dry weight and condition index of all mussel populations under study are presented in Table 1.

2.2. Determination of polycyclic aromatic hydrocarbons (PAHs).

Collected soft tissues for PAHs determinations were frozen at –20ºC. For analytical techniques used in the extraction procedure, fractionation and determination of PAHs, we refer to our previous article (Labarta et al. 2005). All samples contained the whole set of 2 to 6 ring PAHs and the relative proportion of Σ(2-3 ring PAHs predominant in the oil) and Σ (4-6 ring PAHs predominant in industrial areas) is illustrated as L/H ratio (from Labarta et al. 2005).

2.3. Protein content and free amino acids

Protein was determined following Lowry et al. (1951) after alkaline hydrolysis with 0.5N NaOH at 30ºC. Soft tissues of each sub-sample (three) of 12 to 15 individuals each were separated, freeze-dried and stored at –70ºC. Prior to the protein analysis, tissues of mussels were pulverised with an ultrasonic Branson Sonifier (250/450 USA).

For free amino acids determination, three replicates of a number of individuals (12-15 juveniles each) were taken at random from mean values of the whole size distributions. 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 48 h, dry tissues were then powdered and weighed. Briefly, approx. 100 mg of dry tissue was suspended in 5 mL 0.2 M perchloric acid. 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 reverse-phase high-performance liquid chromatography of the dabsyl derivatives. All 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 C_{18} column (Waters Symmetry, 150 x 4.6 mm, 3.5 µm particle size, 100 Å pore

size) thermostated at 50ºC either by an Allience System column oven. Twenty 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 0.1-0.2% triethylamine titrated to pH 6.55 with phosphoric acid) and B (80% aqueous acetonitrile) with a gradient profile that corresponds to that used by Pinho et al. (2001). For quantification, nor-leucine was used as internal standard. Amino acids were grouped in protein free amino acids (PFAA) and non-protein free amino acids (NPFAA), the latter group represented the sum of taurine and ornithine.

2.4. Statistical analysis

Concentrations of amino acids are presented as the mean ± standard deviations of three replicates in µmol per g of dry mass and relative percentages that each amino acid represents in the total free pool. Comparisons of FAA concentrations in mussel populations (in spatial scale) were done by ANOVA (one way). Percentage values of each free amino acid and different ratios used in the current study for statistical analyses were arc-sin and log transformed, respectively. Homogeneous groups could be established *a posteriori* by t-test. When variances were not homogeneous (Levene´s test), non-parametric Kolmogorov-Smirnov and Mann-Whitney tests were used. Correlation analyses between pollution values, FAA and endogenous factors of the animals were performed following Pearson's coefficient. All analyses were done with a statistical computer package (STATISTICA 6.0).

3. Results

3.1. Contamination levels in soft tissues of mussels (PAHs)

The amount of PAHs obtained in soft tissues of juveniles at sampling time are presented in Figure 2. Moreover, L/H index (see Material and Methods) is also included. Total amount of PAHs varied widely from the lowest obtained in mussels from Pindo (196 ng/g dw) and the highest in Carrumeiro (502 ng/g dw), both populations being quite close geographically (Figure 1). The amount of PAHs of the other mussel populations ranged between 304-347 ng/g dw in Bueu and Aguiño as well as between 240-270 ng/g dw for Lobeiras, Redes and Miranda (Figure 2). L/H ratio was the highest Aguiño mussels (0.91), whereas a lower range was obtained in Bueu and Pindo populations (0.64-0.66) and the lowest values corresponded to mussel tissues from Carrumeiro, Lobeiras, Redes and Miranda (0.50-0.55) (Figure 2).

3.2. Protein content of soft tissues

Protein content of soft tissues of different mussel populations under study is presented in Table 1. The proportion of protein in juveniles from Bueu, Carrumeiro and Redes represented the highest values obtained (0.78-0.79 g/g dw; $p<0.05$ ANOVA) compared to the Miranda and Pindo mussels group (0.72-0.74 g/g dw) and the lowest values reported for mussel populations from Aguiño and Lobeiras (0.61 and 0.68 g/g dw, respectively; $p<0.05$ ANOVA; Table 1).

3.3. Total free amino acids (TFAA): Protein free amino acids (PFAA) and non-protein free amino acids (NPFAA).

Concentrations of total free amino acids in soft tissues of juvenile mussels are presented in Table 2. Sum of taurine, glycine and alanine represented 80-87% of the free pool in all populations and taurine was the most abundant amino acid in all cases (43-68% TFAA; Table 2). Concentrations of TFAA in soft tissues of mussels were highest in Aguiño-Pindo-Carrumeiro populations at the centre of area studied (420-462 µmol/g dw; $P<0.01$ ANOVA) as

compared to rest of populations that in turn ranged between 312 and 347 µmol/g dw ($P>0.05$ between them; ANOVA) (Figure 3A). The latter differences in TFAA profiles were based on protein free amino acids (PFAA) variability that represented significantly higher values also in the latter group Aguiño-Carrumeiro-Pindo (214-249 µmol/g dw; $P<0.01$ ANOVA) with regard to the rest of populations (98-149 µmol/g dw; Figure 3A). Non-protein free amino acids (NPFAA; taurine and ornithine) were not observed to vary significantly among mussel populations with the exception of the highest values in Pindo mussels ($P<0.001$ ANOVA; Figure 3A). The resulting ratio PFAA/NPFAA varied between 0.9-1.3 for Aguiño-Carrumeiro-Pindo mussels (mean: 1.15 ±0.25) and 0.5-0.8 for the rest of mussel populations (mean: 0.65 ±0.15) (Figure 3A).

3.4. Taurine, glycine and alanine

Taurine was the most abundant amino acid in all mussel populations (Table 2). Taurine concentrations did not show any significant variability between populations (176-213 µmol/g dw) with the exception of Pindo mussels (247 µmol/g dw; $P<0.001$ ANOVA). However, percentage values that taurine represented in TFAA varied between the lowest in Aguiño-Carrumeiro mussels (about 43% TFAA) followed by Pindo-Lobeiras-Redes (53-56% TFAA; $P<0.05$ ANOVA) and the highest values observed in Bueu and Miranda at South and North of Galician coastline, respectively (62-68% TFAA; $P<0.05$ ANOVA; Figure 3B). Glycine varied inversely with taurine ($r= -0.82$; $P<0.05$; Pearson's coefficient), between the lowest values observed in Bueu and Miranda mussels (16-19% TFAA), compared to the rest of populations within the range of 24-29% TFAA (Figure 3B; $P<0.05$ ANOVA). Alanine also showed an inverse correlation with taurine ($r= -0.81$ $P<0.05$), but not with glycine ($r= 0.36$; $P>0.05$). Highest concentrations of alanine were observed in Carrumeiro (11.9% TFAA; $P<0.01$

ANOVA) followed by Aguiño mussels (6% TFAA) whereas the rest of mussel populations ranged between 3-5% of TFAA (Figure 3B).

3.5. Essential amino acids (EAA)

Essential amino acids (EAA) represented 3.4-6.9% of TFAA in all mussel populations (Table 2). Within EAA group, arginine and threonine were the most abundant amino acids with percentages above 1% of TFAA, whereas valine, methionine, isoleucine, leucine, tryptophan, phenylalanine, lysine and histidine always ranged below 0.5% of TFAA (Table 2). Variability of EAA followed similar patterns than that of PFAA ($r= 0.88$; $P<0.01$; Figure 3C) and ranged between the highest values observed in Carrumeiro and Aguiño mussels (6-7% TFAA; $P<0.05$ ANOVA) and 3.5-4.6% of TFAA for the rest of populations. Accordingly, mussels from Carrumeiro and Aguiño presented two-fold higher mean values of threonine ($P<0.05$ ANOVA) as most abundant EAA compared to the rest of populations (Figure 3C). Arginine values ranged between 1.1-1.8% TFAA in all mussel populations ($P>0.05$ ANOVA; Figure 3C).

3.6. Relationships between TFAA, pollution values, protein content and condition index of individuals

Analyses of correlation between amino acid profiles and pollution values in soft tissues of the animals showed that alanine (as percentage values of TFAA) was the only significant case (Figure 4A). A clearer view to the latter relationship, showed a rather “all or nothing” effect based on the fact that alanine concentrations in Carrumeiro mussels (with the highest PAHs values) deviated by far from the other mussel populations (3-fold higher values). No significant relationship was observed between alanine and pollution values when Carrumeiro mussels were excluded from the analyses (Figure 4B).

PFAA, however, varied inversely with the protein content of soft tissues ($r= 0.82$; $P<0.01$; Figure 4C), after excluding Carrumeiro mussels from the analysis (see before). Additionally, a positive relationship was also observed between PFAA and condition index of the individuals ($r= 0.86$; $P<0.01$; Figure 4C).

3.7. Taurine:Glycine (t:g) ratio and sum of serine and threonine

Variability of both t:g ratio and sum of serine and threonine were presented in Table 2. The t:g ratio was highest in Bueu and Miranda mussels (4.2 and 3.2, respectively; $P<0.05$ between them ANOVA) whereas a range of 1.5-2.3 was observed for the other mussel populations (Table 2). Sum of serine and threonine showed the highest concentrations in Aguiño and Carrumeiro mussels (23.1 and 17.9 µmol/g dw, respectively; $P<0.05$ between them ANOVA) followed by Pindo mussels (10.3 µmol/g dw; $P<0.01$ ANOVA) and a range of 4.8-8.4 µmol/g dw for the rest of the populations (Table 2). Both biochemical indices t:g ratio and sum of serine and threonine did not follow any significant pattern of variation according to pollution values reported for the soft tissues. However, significant relationships were established for both indices and PFAA as percentage of TFAA (Figure 5). Values of t:g ratio were higher than 3 in animals with PFAA below 40% of TFAA. Considering the significant relationship between PFAA and condition index illustrated in Figure 4C, it might be drawn further that t:g ratio above 3 was obtained in mussels with lower condition index than 10% of soft tissues, both variables being significantly correlated ($r= 0.77$; $P<0.05$). No significant relationship was obtained between the sum of serine and threonine and condition index of the animals ($r= 0.60$; $P>0.05$).

4. Discussion

Mussel populations sampled after *Prestige* oil spill represented a wide geographical area along the Galician Coastline where farmers collect juveniles for mussel raft culture (Figure 1). Monitoring the condition of juveniles susceptible of being cultivated after such ecological disaster is of great importance to investigate metabolic changes that might have occurred as consequence of environmental changes. Juveniles sampled in the present study were distinctly impacted by the oil spill in their locations. According to PAHs reported at sampling time, three months after the spill, a range of 196-304 ng/g dw characterised most populations with highest values being reported in Aguiño (347 ng/g dw) and Carrumeiro (502 ng/g dw) mussels (Figure 2). PAHs composition, however, was described to represent a combination of fossil and pyrolytic sources (Labarta et al. 2005) and in specific locations, the uptake of oil hydrocarbons occurred over a background load of pyrogenic PAHs that partly accounting for the variability. The latter point with regard to the background load of mussels living in the natural grounds commonly used for gathering mussel seed made difficult to ensure a proper control (non-contaminated) site along the geographical gradient studied and might also cause that mussel populations relatively close to each other in the centre of the Galician coastline were characterised with both lowest and highest PAHs concentrations i.e. Pindo and Carrumeiro (Figures 1 and 2). Labarta et al. (2005) have pointed out the convenience of studying the oil spill impact using physiological and biochemical indicators (survival potential, lipid metabolism) and extending the pollution analyses to other hydrocarbons, i.e. n-alkane series that might have an effect on metabolic routes.

Analyses of FAA in the present study showed no significant links between pollution reported in soft tissues, either as PAHs or L/H ratio (see Material and Methods), and variability of amino acids. Mussel populations that were grouped as “high FAA content” (Aguiño-Pindo-Carrumeiro) included individuals from both ends of the range reported for PAHs (Figure 2 and 3) suggesting that differences in FAA are not in direct concordance with levels of pollution in

soft tissues. Spatial variability of TFAA was linked to the group of protein FAA, which in turn was significantly correlated with protein content of the tissues and condition of the animals (Figure 4C). The latter relationships TFAA-PFAA-protein content-condition index seemed to be crucial in the interpretation of the present data collected *in situ*. These results mean that TFAA variability would be rather explained by endogenous factors as compared to pollution reported for soft tissues at sampling time and would establish a significant incidence of the energetic status of growing juveniles in their natural environments once they are able to live under a certain degree of stress. Hummel et al. (1989) also described that other factors than pollution i.e. salinity, temperature, food or wave exposure were responsible for eco-physiological variability in measurements involving amino acids of the individuals.

Condition index represents a factor of eco-physiological importance as a measure of the apparent health of individuals (Orban et al. 2002) and, therefore it would be a reflection of local trophic and abiotic conditions. Protein FAA, as responsible of the variability of total FAA, were higher in mussel populations with higher condition index (Figure 4C above) but also showed an inverse relationship with protein content of soft tissues (Figure 4C). A similar link between condition index variability of *M. galloprovincialis* and biochemical composition of soft tissues was observed by Orban et al. (2002): condition index varied according to glycogen and lipids components, but was inversely correlated with the protein content of soft tissues as a consequence of the fact that growth involves protein deposition.

Alanine was the only amino acid significantly correlated with pollution values in soft tissues (Figure 4A). This amino acid represented mean percentage values of 5.6% TFAA and the latter statistical significance was based on the highest values reported for Carrumeiro mussels (11.9% TFAA; Table 2), that in turn, showed also the highest concentrations of PAHs (Figure 2). However, the latter relationship could not be obtained when Carrumeiro mussels are omitted from the analysis (Figure 4B). From the biological point of view, this result would

represent an “all or nothing” effect that was also observed in mussels exposed to metal pollution (Hummel et al. 1994) demonstrating the ability of individuals to carry out compensation processes. Here, it is important to highlight that Carrumeiro location is situated in the centre of the spill zone and close to the mouth of the Xallas River with the consequent freshwater component playing a role (Labarta et al. 2005). The importance of alanine in the osmoregulation processes of bivalves and their anaerobic metabolism have been widely described (de Vooys 1991) and, the fact that alanine values were that high in Carrumerio mussels compared to the rest of populations might be considered a consequence of the specific abiotic factors of this intertidal location i.e. salinity fluctuation and emersion-immersion exposure more than the effect of pollution. This would also explain the significant relationship between PFAA and protein content of soft tissues obtained after excluding this population (Carrumeiro) from the analyses (Figure 4C).

Spatial variability in both alanine and glycine represented more than 50% of the differences between Aguiño-Carrumeiro-Pindo mussels, with the highest PFAA, and the other mussel populations (Figure 3B). Together with alanine, glycine is also an important organic compound playing a role in osmoregulatory processes of bivalves (Bishop et al. 1994) but while alanine showed wider variability (see above), glycine followed similar relationships than PFAA with regard to protein content and condition index of juveniles. Metabolic turnover and biosynthesis of glycine are rapid (Sokolowski et al. 2003) and although it is a metabolite closely related to reproductive cycle of bivalves (Zurburg et al. 1989), we cannot exclude the importance of factors like local environmental differences that would also reflect variability in condition index values of immature juveniles in the current study.

Interestingly, taurine followed an inverse variation of that of glycine as most representative amino acid of the PFAA group ($r= -0.82$; Figure 3B). When the effect of pollution was studied in bivalves considering variability of amino acids, a consistent increase in

taurine (and t:g ratio) is observed (see Introduction). Such a general pattern of taurine increase in mussel populations with high oil impact levels did not occur in our field survey (Figure 3B). As was the case of the present study, an inverse relationship between taurine concentrations and protein free amino acids (and condition index) was also obtained when analysing different body size classes of mussels (*M. galloprovincialis*) maintained in the laboratory with different condition values (unpublished results). Values of t:g ratio varied between 1 and 2 in all populations (including those with highest PAHs concentrations) except mussels from Bueu (4.2) and Miranda (3.2) at both ends of the geographical area studied (Table 2). Average normal values of t:g ratio for *Mytilus edulis* have been reported to be about 2 (Hummel et al. 1996) whereas chronic stress is identified by values between 3 and 5 (Hummel et al. 1996). Values of t:g ratio above 3 in the present study were related to populations with the lowest protein FAA contents (below 40%; Figure 5) and condition index (below 10%; Table 1). No significant relationship was obtained between the other index sum of serine and threonine and condition of animals (see Results) confirming that such a general stress indicator is highly questionable as described for *M. edulis* by Hummel et al. (1994).

Usually, studies focusing on FAA variability of individuals subjected to different degrees of stress did not take into account the information related to endogenous factors of experimental animals (see Introduction). From results of the current survey has resulted evident that the latter factors would help to understand spatial variability in FAA once animals are able to live with different degree of environmental stress within the range reported here. Differences obtained for FAA profiles are closely related to differences in condition index of individuals despite small differences in shell length of the juveniles which in turn establish a link with environmental factors at each sampling site. The risk of misinterpretations when considering FAA as eco-physiological tool for bivalves might be significantly reduced when considering endogenous factors of the individuals and the present study extends such idea about applicability of FAA

analyses in tissues of *M. galloprovincialis* collected *in situ* from natural grounds along the Galician rocky shore.

As a summary, TFAA and derived-indices (t:g ratio, sum of serine and threonine, alanine) did not show any direct evidence of being affected by pollution values reported for the area under study. On the contrary, TFAA profiles followed patterns of endogenous factors i.e. protein content of the soft tissues and condition index of the animals as energetic status indicator that might correspond to protein turnover processes of growing individuals at each location. These relationships are of great interest when spatial variability in FAA profiles are studied. Applicability of the general biochemical indicator t:g ratio was confirmed to vary significantly with the latter endogenous factors most likely as a consequence of the nutritional status of juveniles along the Galician Coastline at initial phases of their growth.

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Table 1. Values of shell length, total dry mass, condition index and protein content of soft tissues in mussel populations under study. Values correspond to means of three replicates (± SD). Number of pooled juveniles for adjusted shell length measurements were 300 for each mussel population and from these sampled mussels, 36-45 individuals were considered for mass, condition index and protein content determinations (see Material and Methods).

	South →						North
	Bueu	Aguiño	Pindo	Carrumeiro	Lobeiras	Redes	Miranda
L (mm)	11.9 (4.2)	17.7 (4.8)	15.5 (6.8)	15.6 (6.0)	17.5 (6.5)	19.8 (5.9)	16.0 (3.6)
DW total (g)	0.09 (0.02)	0.23 (0.04)	0.17 (0.07)	0.15 (0.01)	0.25 (0.07)	0.28 (0.01)	0.07 (0.01)
CI	8.8 (0.8)	18.6 (1.2)	14.6 (2.2)	11.8 (0.9)	17.2 (1.6)	11.8 (0.5)	7.1 (1.4)
Protein content (g per g dry tissue)	0.79 (0.02)	0.61 (0.03)	0.72 (0.01)	0.78 (0.02)	0.68 (0.03)	0.79 (0.02)	0.74 (0.02)

Table 2. Composition of free amino acids (FAA) for all mussel (*Mytilus galloprovincialis*) populations under study. Values are expressed as µmol per g of dry weight (SD in brackets) as well as percentage values of the total FAA pool. Non-protein amino acids: taurine and ornithine. Alanine represents the sum of an incomplete separation between alanine and β-alanine. Different letters (a, b, c, d, e) mean significant differences between mussel populations in the spatial component comparison (see main text).

	South →												North	
	Bueu		Aguiño		Pindo		Carrumeiro		Lobeiras		Redes		Miranda	
	µmol /g dw	% FAA	µmol /g dw	% FAA	µmol /g dw	% FAA	µmol /g dw	% FAA	µmol /g dw	% FAA	µmol /g dw	% FAA	µmol /g dw	% FAA
Aspartic acid	16.05 (2.2)	5.14	13.04 (1.1)	2.96	21.59 (0.9)	4.68	16.50 (3.6)	3.93	12.18 (0.3)	3.85	16.49 (3.4)	4.75	18.95 (0.7)	5.66
Glutamic acid	4.66 (1.0)	1.49	8.89 (0.1)	2.02	12.80 (1.1)	2.77	11.36 (1.7)	2.70	6.28 (0.1)	1.99	6.08 (1.2)	1.75	5.99 (0.7)	1.79
Asparagine	0.42 (0.1)	0.13	2.20 (0.9)	0.50	0.58 (0.7)	0.13	3.68 (0.1)	0.88	0.96 (0.1)	0.30	0.84 (0.1)	0.24	0.67 (0.2)	0.20
Glutamine	1.59 (0.3)	0.51	11.19 (0.6)	2.54	3.86 (0.2)	0.84	14.68 (1.2)	3.50	5.00 (0.1)	1.58	2.34 (0.5)	0.67	2.45 (0.3)	0.73
Serine	1.52 (0.4)	0.49	10.11 (0.3)	2.29	3.61 (0.1)	0.78	7.06 (0.8)	1.68	3.38 (0.2)	1.07	1.95 (0.2)	0.56	2.18 (0.2)	0.65
Glycine	50.83 (9.0)	16.28^{a}	128.8 (5.5)	29.21^{b}	124.8 (7.2)	27.04^{b}	102.3 (15.7)	24.35^{b}	83.05 (3.8)	26.28^{b}	84.4 (13.7)	24.33^{b}	65.15 (6.5)	19.45^{a}
Alanine (and β-Alanine)	8.46 (0.8)	2.71^{d}	28.93 (0.5)	6.56^{b}	20.38 (0.6)	4.41^{c}	50.14 (3.9)	11.93^{a}	14.08 (0.2)	4.46^{c}	17.20 (1.8)	4.96^{c}	14.01 (0.9)	4.18^{c}
Taurine	213.93 (37)	68.50^{a}	192.7 (7.9)	43.71^{c}	247.1 (14.9)	53.54^{b}	181.7 (32.8)	43.25^{c}	176.0 (12.4)	55.68^{b}	197.5 (35.9)	56.94^{b}	209.0 (19.3)	62.37^{a}
Cysteine	0.16 (0.01)	0.05	0.50 (0.3)	0.11	0.31 (0.1)	0.07	0.28 (0.1)	0.07	0.19 (0.1)	0.06	0.52 (0.3)	0.15	0.62 (0.1)	0.18
ornithine	0.25 (0.1)	0.08	0.37 (0.1)	0.08	0.48 (0.1)	0.10	0.30 (0.1)	0.07	0.30 (0.1)	0.10	0.48 (0.1)	0.14	0.49 (0.1)	0.15
Tyrosine	2.83 (0.2)	0.91	4.20 (0.4)	0.95	4.56 (0.2)	0.99	4.04 (1.1)	0.96	0.92 (0.1)	0.29	2.20 (0.2)	0.63	2.70 (0.6)	0.81
Proline	0.58 (0.1)	0.18	9.39 (0.5)	2.13	2.41 (0.2)	0.52	4.79 (0.6)	1.14	1.35 (0.2)	0.43	0.98 (0.1)	0.28	1.18 (0.1)	0.35
Essential amino acids Valine	0.72 (0.05)	0.23	1.78 (0.3)	0.40	0.99 (0.6)	0.21	0.93 (0.1)	0.22	0.40 (0.1)	0.13	1.05 (0.2)	0.30	0.74 (0.2)	0.22
Methionine	0.18 (0.1)	0.06	1.17 (0.2)	0.27	0.61 (0.1)	0.13	1.17 (0.1)	0.28	0.45 (0.1)	0.14	0.26 (0.1)	0.07	0.42 (0.1)	0.13
Isoleucine	0.47 (0.4)	0.15	0.57 (0.6)	0.13	0.90 (0.8)	0.20	0.46 (0.3)	0.11	0.37 (0.1)	0.12	0.93 (0.7)	0.27	0.83 (0.7)	0.25
Leucine	0.13 (0.02)	0.04	1.22 (0.3)	0.28	0.37 (0.1)	0.08	0.90 (0.1)	0.21	0.27 (0.1)	0.09	0.19 (0.1)	0.06	0.18 (0.1)	0.05
Tryptophan	0.15 (0.01)	0.05	1.91 (0.1)	0.43	0.53 (0.1)	0.12	0.93 (0.1)	0.22	0.39 (0.1)	0.12	0.21 (0.1)	0.06	0.19 (0.1)	0.06
Phenylalanine	0.08 (0.01)	0.02	0.75 (0.2)	0.17	0.25 (0.1)	0.05	0.41 (0.1)	0.10	0.21 (0.1)	0.07	0.07 (0.1)	0.02	0.12 (0.1)	0.03
Arginine	5.33 (0.04)	1.71	7.15 (1.2)	1.62	5.81 (0.4)	1.26	4.62 (1.5)	1.10	3.90 (0.6)	1.23	6.31 (0.4)	1.82	4.61 (0.2)	1.38
Threonine	3.33 (0.06)	1.06^{b}	13.01 (1.6)	2.95^{a}	6.65 (0.5)	1.44^{b}	10.81 (2.5)	2.57^{a}	4.99 (0.2)	1.58^{b}	5.43 (0.7)	1.56^{b}	3.45 (0.2)	1.03^{b}
Lysine	0.33 (0.03)	0.11	2.05 (0.3)	0.46	1.45 (0.1)	0.32	1.90 (0.1)	0.45	0.63 (0.1)	0.20	0.68 (0.3)	0.20	0.43 (0.1)	0.13
Histidine	0.29 (0.2)	0.09	1.00 (0.1)	0.23	1.53 (0.2)	0.33	1.19 (0.2)	0.28	0.78 (0.1)	0.25	0.81 (0.1)	0.23	0.72 (0.2)	0.21
Σ Total	312.3 (43.0)^{b}	100	441.0 (15.2)^{a}	100	461.6 (25.3)^{a}	100	420.2 (26.3)^{a}	100	316.0 (17.9)^{b}	100	346.9 (37.5)^{b}	100	335.1 (29.6)^{b}	100
Σ EAA	11.0 (0.8)	3.5^{b}	30.6 (2.5)	6.9^{a}	19.1 (1.98)	4.2^{b}	23.3 (4.8)	5.6^{a}	12.3 (0.9)	3.9^{b}	15.9 (0.8)	4.6^{b}	11.7 (0.4)	3.4^{b}
T:G ratio		4.2 (0.1)^{a}		1.5 (0.1)^{d}		2.0 (0.1)^{c}		1.8 (0.1)^{c}		2.1 (0.1)^{c}		2.3 (0.1)^{c}		3.2 (0.1)^{b}
Serine and Threonine µmol /g dw	4.8 (0.9)^{e}	1.6	23.1 (1.3)^{a}	5.2	10.3 (0.4)^{c}	2.2	17.9 (3.3)^{b}	4.3	8.4 (0.4)^{d}	2.6	7.4 (0.9)^{d}	2.1	5.6 (0.4)^{e}	1.7

Legend of Figures

Figure 1. Map of Galicia (NW Spain) representing locations where mussel juveniles were collected from North (Miranda and Redes) to South (Bueu). *Prestige* sinking is also located in front of the Galician Coastline.

Figure 2. Variation of polycyclic aromatic hydrocarbons (PAHs) concentrations in soft tissues of juveniles. L/H ratio corresponds to the ratio Σ(2-3 ring PAHs) / Σ (4-6 ring PAHs) (see Material and Methods).

Figure 3. (A) Total amount of free amino acids (TFAA), protein free amino acids (PFAA) and non-protein free amino acids (NPFAA) variability in mussels. The ratio PFAA/NPFAA is also included. (B) Variability of taurine, glycine and alanine concentrations as percentage values of TFAA (%). (C) Variability of essential amino acids (EAA) and, most abundant arginine and threonine concentrations as percentage values of TFAA. Different letters (a, b, c, d, e) mean significant differences in the spatial component comparisons (see main text). Letters to identify differences in TFAA (part A) serve also to identify significant differences in PFAA (not shown in the Figure for space limitations). For significant differences in alanine concentrations in part B, we refer to Table 2.

Figure 4. (A) Alanine concentrations as percentage of TFAA with regard to total amount of PAHs in soft tissues of all mussel populations and (B) excluding the “outlier” represented by Carrumeiro mussels. (C) Relationships between PFAA as percentage values of TFAA, protein content in soft tissues and condition index of mussels considering all populations.

Figure 5. Variability of the indices taurine:glycine ratio (t:g) and the sum serine and threonine with regard to PFAA values as percentage of TFAA.

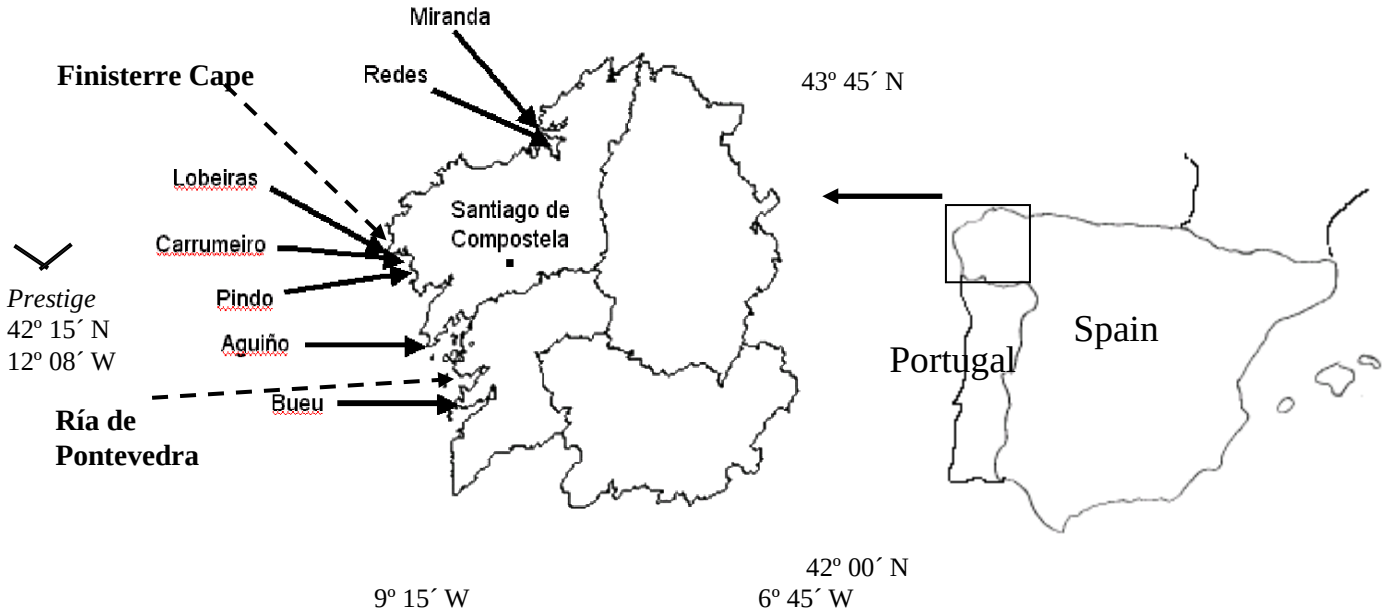
Miranda
Finisterre Cape
Redes
43º 45´ N
Lobeiras
Santiago de
Compostela
Carrumeiro
Prestige
42º 15´ N
12º 08´ W
Pindo
Aguiño
Spain
Portugal
Bueu
Ría de
Pontevedra
42º 00´ N
9º 15´ W
6º 45´ W

Figure 1

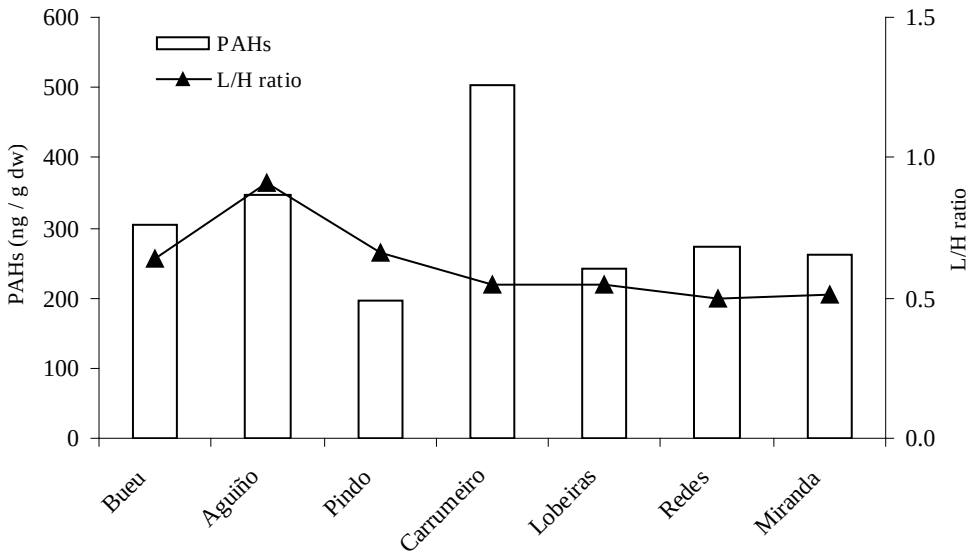
PAHs
L/H ratio
PAHs (ng / g dw)
L/H ratio
600
500
400
300
200
100
0
1.5
1.0
0.5
0.0
Bueu
Aguiño
Pindo
Carrumeiro
Lobeiras
Redes
Miranda

Figure 2

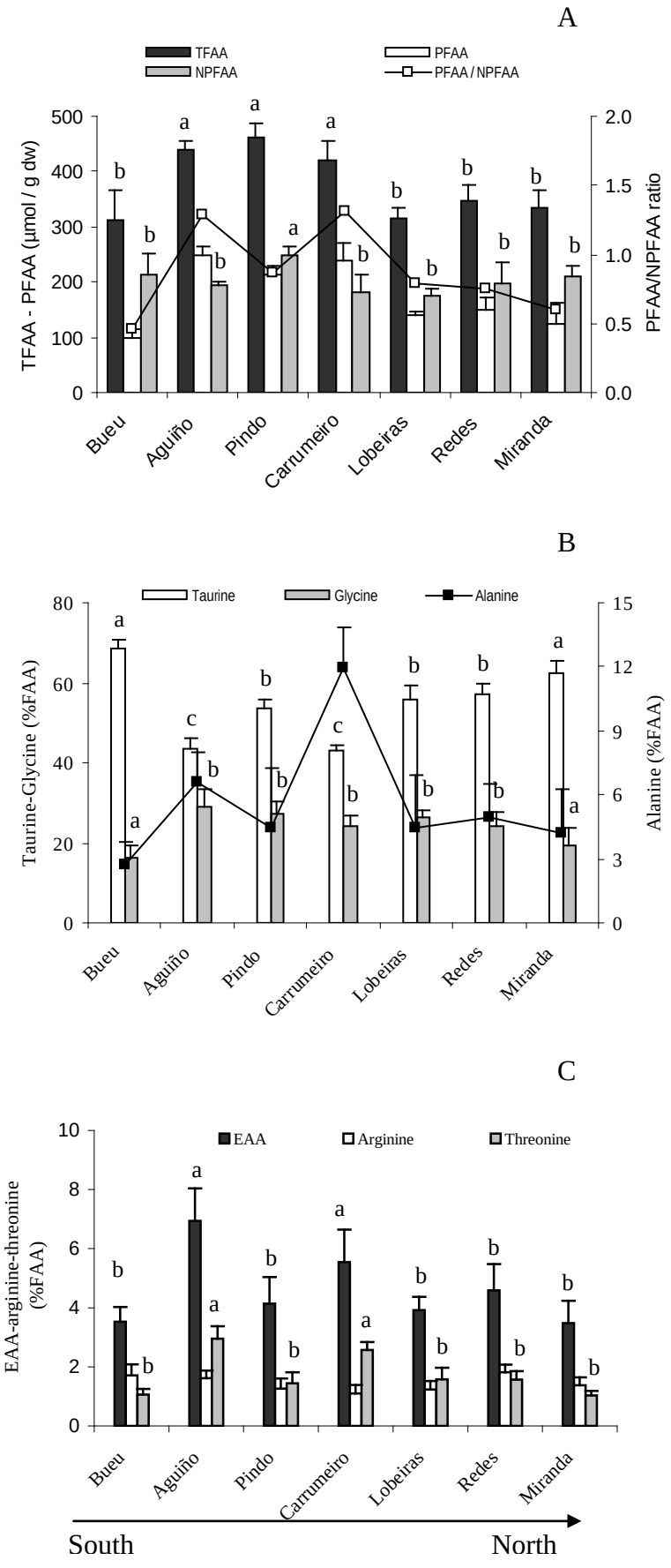
A
TFAA
NPFAA
PFAA
PFAA / NPFAA
TFAA - PFAA (µmol / g dw)
PFAA/NPFAA ratio
Bueu
Aguiño
Pindo
Carrumeiro
Lobeiras
Redes
Miranda
B
Taurine
Glycine
Alanine
Taurine-Glycine (%FAA)
Alanine (%FAA)
C
EAA
Arginine
Threonine
EAA-arginine-threonine (%FAA)
South
North

Figure 3

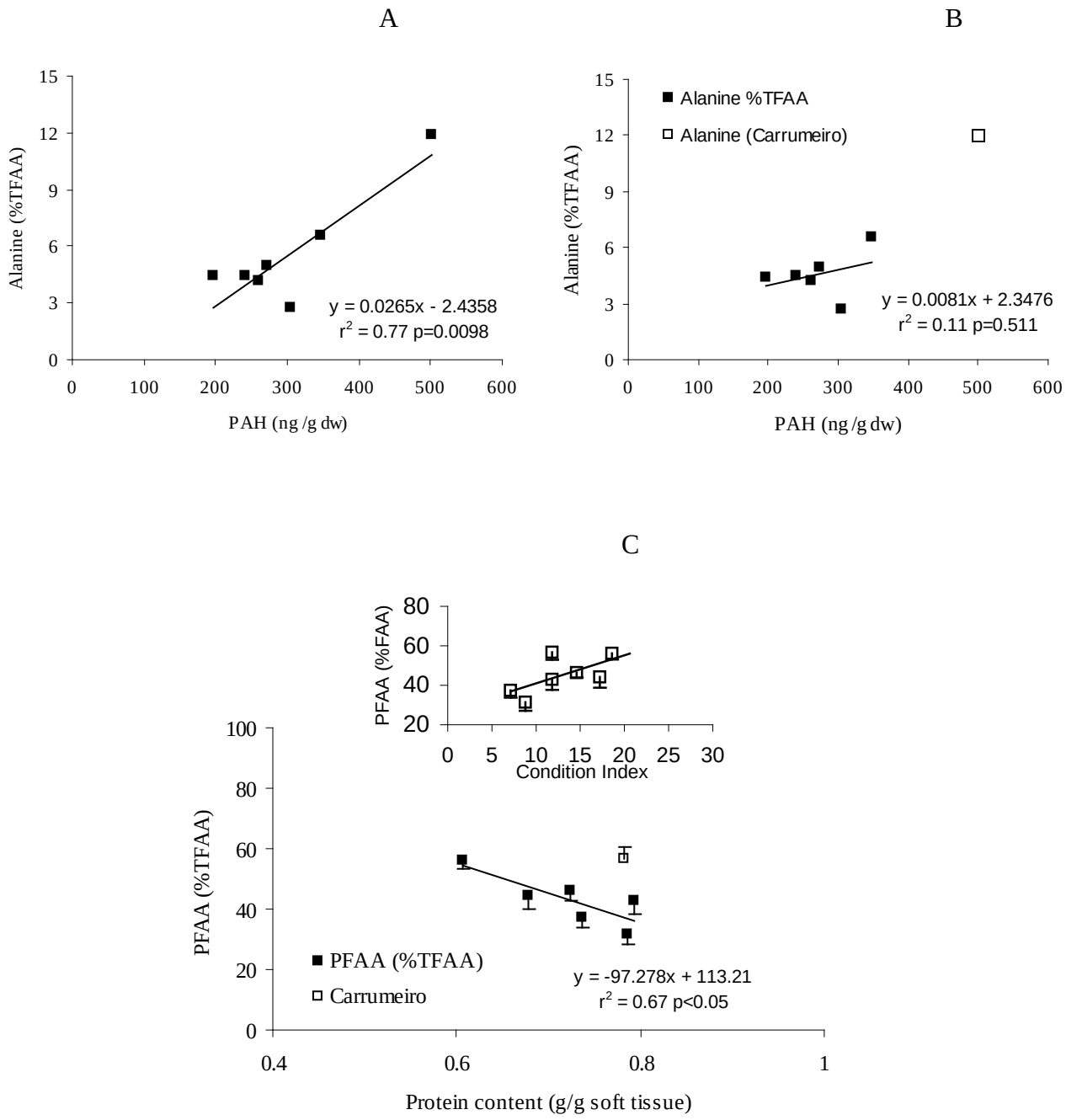
A
B
C
Alanine (%TFAA)
PAH (ng /g dw)
y = 0.0265x - 2.4358
r2 = 0.77 p=0.0098
Alanine %TFAA
Alanine (Carrumeiro)
y = 0.0081x + 2.3476
r2 = 0.11 p=0.511
PFAA (%FAA)
Condition Index
PFAA (%TFAA)
Carrumeiro
y = -97.278x + 113.21
r2 = 0.67 p<0.05
Protein content (g/g soft tissue)

Figure 4

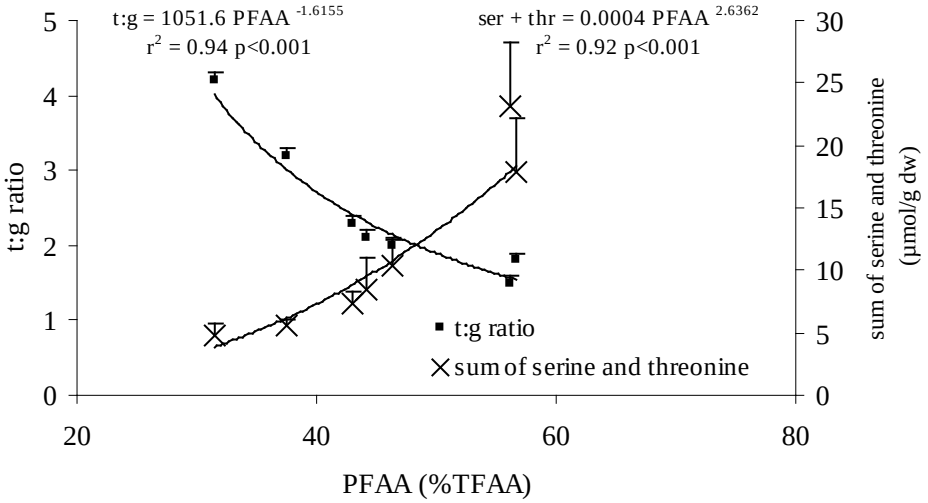
t:g = 1051.6 PFAA -1.6155
r2 = 0.94 p<0.001
ser + thr = 0.0004 PFAA 2.6362
r2 = 0.92 p<0.001
t:g ratio
sum of serine and threonine
(µmol/g dw)
t:g ratio
sum of serine and threonine
PFAA (%TFAA)
0
1
2
3
4
5
0
5
10
15
20
25
30
20
40
60
80

Figure 5