Journal of Life Sciences 5 (2011) 549-554

Does *Procambarus Clarkii* (Girard, 1852) Represent a Threat for Estuarine Brackish Ecosystems of Northeastern Adriatic Coast (Italy)?

Sandra Casellato and Luciano Masiero

Laboratory of Benthos Ecology, Biology Department, Padova University, Padova 58/B 35121, Italy

Received: December 09, 2010 / Accepted: January 31, 2011 / Published: July 30, 2011.

Abstract: The ongoing expansion of *P. clarkii* in the aquatic environments of northern Italy, where it is very common and abundant in most of the streams, lakes and reservoirs, led us to worry about the possibility of invasion into the estuarine and lagoonal brackish environments of the Adriatic coast, which is important not only for their peculiar fauna and flora and high biodiversity, but also for fishing and aquaculture. We carried out laboratory tests to investigate whether this species could live, moult and breed in water with different salinity levels. Several stocks of adult individuals, acclimated before the laboratory experiments, were maintained and monitored daily for a long period (100 days) in glass aquaria with water salinity varying from 5 to 33 ppt. They not only survived during the whole experiment period in levels up to 25 ppt, but also they regularly moulted and mated. These results confirmed that the species could invade the estuarine and brackish environments of the Adriatic coast, with all the implied consequences for the native species of both vertebrates and invertebrates and for human livelihoods.

Key words: Biological invasion, *Procambarus clarkii*, estuarine, brackish areas.

1. Introduction

The success and expansion of the crayfish *Procambarus clarkii*, a species native to northeastern Mexico and south-central United States (Louisiana), is related to its high ecological plasticity. This astacid can be defined as a freshwater species but it is known that it is able to live in most types of water bodies, including brackish ones [1-3], the only exception is mountain streams, with high flow velocity [4]. The decline of native crayfish in most part of Europe is related to the presence of this widespread astacid species, which is also a vector of the aphanomycosis, caused by the fungus *Aphanomyces astaci*, which is lethal to most European crayfish. It is a major predator of aquatic invertebrates and vertebrates, such as fishes and amphibians, and it is responsible for the decline or extinction of many native species [5-7]. Its habit of burrowing causes bank collapse and increased water turbidity in rivers and streams [8]. The species is essentially omnivorous subsisting on a diet of macrophytes and small animals, such as snails, insects, fishes, and tadpoles. Due to its grazing and predatory activity, it modifies energy pathways, reducing food web complexity and structure. Feeding also on detritus, for its benthonic habit, it opens the detritic food chain to higher trophic levels, increasing the crayfish predators [6].

The ongoing expansion of *P. clarkii* in many aquatic environments of Italy [9-15] (Fig. 1), particularly in the northeastern part, where it is very common and abundant in most of the streams, lakes and reservoirs (Fig. 2), led us to worry about the possibility that it could invade the estuarine and lagoonal brackish environments of the Adriatic coast, which is important for its particular fauna, flora and high biodiversity.

Corresponding author: Sandra Casellato, associate professor, research field: aquatic ecology. E-mail: sandra.casellato@unipd.it.

We also asked if this dangerous astacid could represent a threat to the human activities of fishing and aquaculture, which is an important part of the local economy. Therefore, the tolerance of *P. clarkii* to brackish and salt waters was investigated, with laboratory experiments, using water salinity from 5 to 33 ppt, including the range of the estuarine waters in northeastern Italy and the mean salinity of the Adriatic Sea [16].

2. Material and Methods

2.1 Investigation of Presence of P. Clarkii in the Specified Estuarine Area

The area investigated (Fig. 3) was the last part of the hydrological basin of the rivers Brenta and Bacchiglione, flowing together into the Adriatic Sea (from 45°16′02.04″N and 12°05′59.96″E to 45°10′52″N and 12°18′42″E). This area borders the Lagoon of Venice, where numerous aquaculture and fishery farms are present.

This hydrologic system consists of a great variety of water courses (small irrigation canals, shallow ponds and streams). In many of these, the presence of *P. clarkii* was verified by qualitative samples (present or not present, with different abundance) collecting the animals with a 0.6 cm mesh, 4.5 m long bag seine [17] with a chain attached to the bottom to facilitate sampling in the vegetated canals, or by verifying the presence of their burrows along the river banks. In the sampled sites water salinity was checked, using a hand refractometer model Atago S/Mill (salinity 0-100 ppt).

2.2 Laboratory Tests

Several stocks of adult individuals, males and females, caught in the canals and streams explored, were acclimated before the laboratory experiments for 2 weeks in freshwater, and then used for testing the capacity of this species to survive in brackish and salt waters. Five individuals (in triplicate) for each salinity value were maintained in glass aquaria (30 × 30 × 25 cm) (5 specimens/20L) in a climate chamber at a controlled temperature of 17 ℃, and monitored daily for 100 days. Water salinity in aquaria tests ranged from 5 to 33 ppt (5, 10, 15, 20, 25, 33). Dissolved oxygen: 8.1 ± 0.2 mg/L and pH: 7.8 ± 0.3 were controlled during the experiment, using standard oxygen and pH meters, and total hardness: 106 ± 12 mg $CaCO_3$/L was determined according to the standardized colorimetric method (American Public health Association, 1998) [18]. Animals were fed every 2 days, using commercial fish food (Tetra Wafer Mix for crustacean) and test water was completely renewed every 4-5 days.

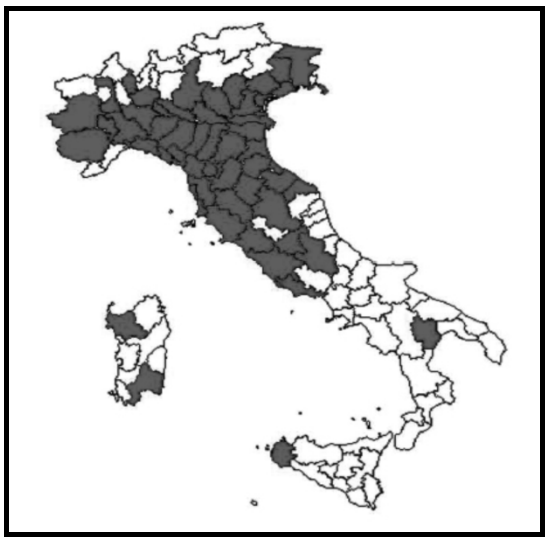

Fig. 1 Distribution of *P. clarkii* in Italy (from Aquiloni et al. 2010) [14].

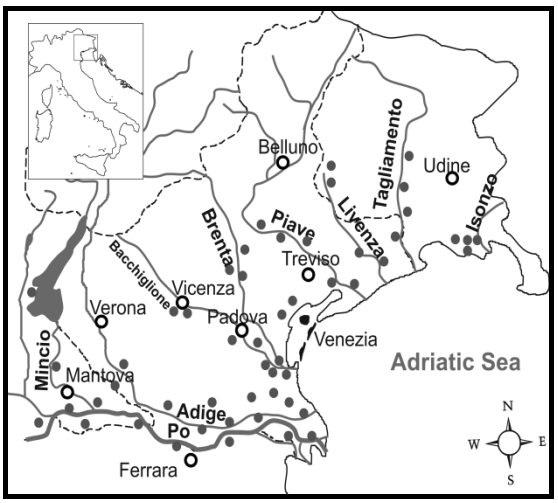

Fig. 2 Distribution of *P. clarkii* in the northeastern part of Italy.

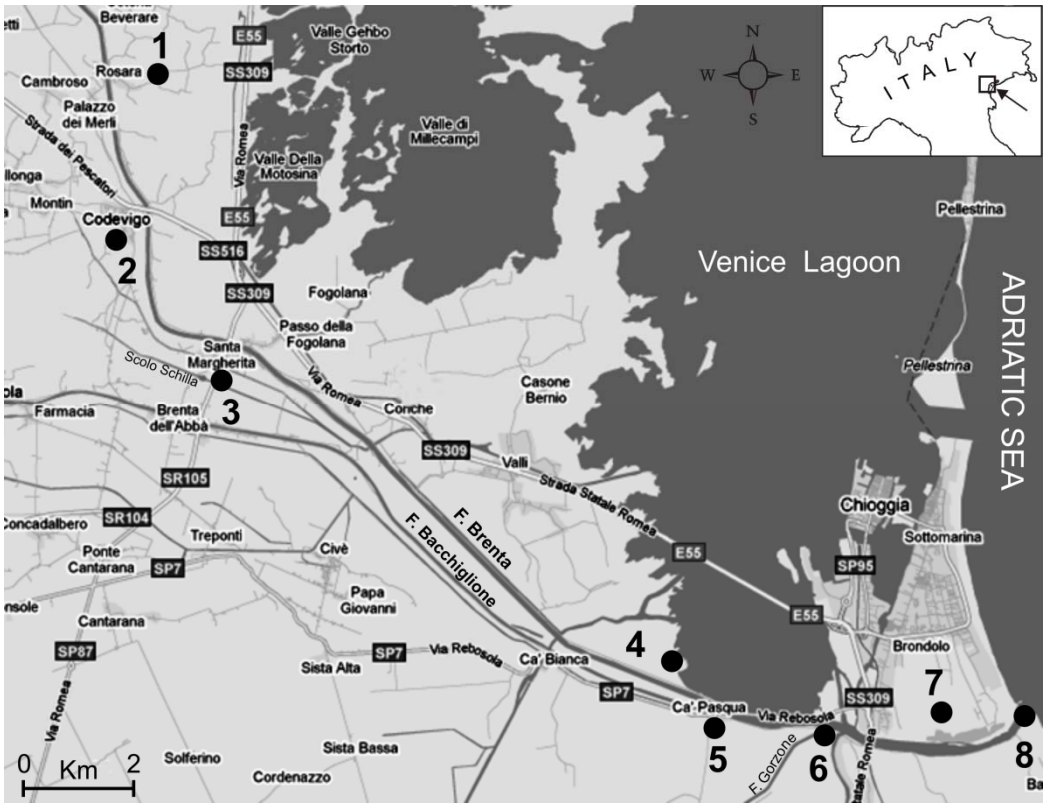

Fig. 3 The last part of the hydrological basin of the rivers Brenta and Bacchiglione, flowing into the Adriatic Sea. The sampling sites are indicated by numbers.

3. Results

3.1 Spread of P. Clarkii in the Explored Area

P. clarkii was present in all the explored sites (Table 1), except site 4, the Venetian Lagoon coast where measured salinity was 25 ± 2 ppt, and site 8, the mouth of the river Brenta where the salt wedge from the Adriatic Sea is present. The water salinity in this estuarine area varies from 5 to 15 ppt [16]. In sites 1, 2, and 3 the salinity value did not exceed 2 ppt during the exploration period, and in 5, 6, and 7 sites, the range varied between 1.5 to 3.0 ppt.

Table 1 Semiquantitative data on the presence of the invasive crayfish in the explored sites.

Explored sites	1	2	3	4	5	6	7	8
abundance	++	++	+++	-	++	+++	++	-

+ = present not abundant, ++ = abundant, +++ = very abundant, - = not present.

3.2 Laboratory Tests with Water Salinity from 5 to 33 ppt

Survival rapidly decreased with the increasing water salinity, but only at the highest salinity used (Table 2). For short exposition periods (10 days) complete survival of the specimens was observed for salinity values of 5, 10, 15 and 20 ppt. Half of the specimens utilized for the experiments not only survived for a period of 60 days in waters with salinity up to 15 ppt, but also moulted and mated. During the test period (Mar.-June 2010) we observed mating specimens and, after a short period, the emission of fertilized eggs, carried by the females under the abdomen, where they should have completed their embryonic development. These eggs, dark and meanly 5-6 mm length, however, were not laid, presumably because there wasn't a suitable environment in the glass aquaria. In natural environments, this species digs burrows down 2 feet (0.6 m) in the banks of water courses and lakes. From literature we know that the hatching needs of higher temperatures (above 20 ℃) [19].

About 70% of the exposed animals survived for 30 days

Table 2 Survival (%) of *P. clarkii* exposed, for a long time, to different water salinity levels.

Salinity (ppt)	Time of survival									
	10 days	20 days	30 days	40 days	50 days	60 days	70 days	80 days	90 days	100 days
0	100	100	100	100	97	96	96	96	96	96
5	100	100	87	75	73	67	65	63	62	62
10	100	89	78	55	53	49	49	49	49	47
15	100	89	68	50	50	50	47	46	46	45
20	100	89	67	55	44	39	37	41	44	43
25	89	78	67	41	39	37	36	34	34	33
30	78	44	22	0	0	0	0	0	0	0
33	22	0	0	0	0	0	0	0	0	0

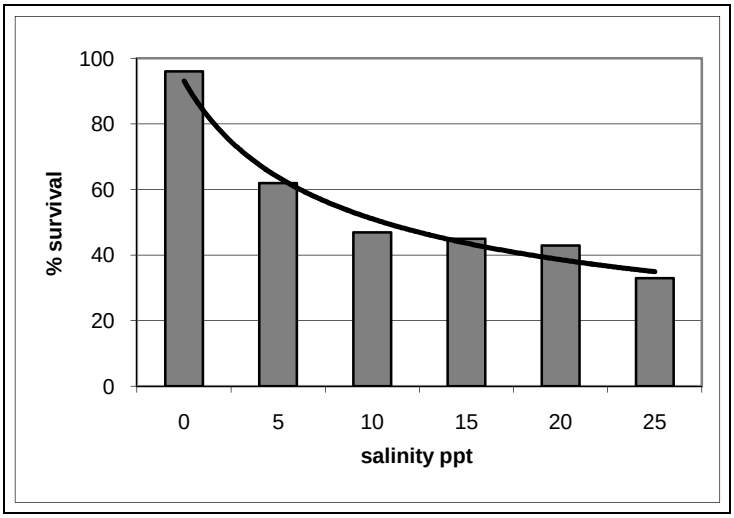

Fig. 4 Survival of *P. clarkii* exposed to different water salinity levels (vs blank) for 100 days.

at salinity up to 25 ppt, reduced to a third for the entire experimental period (100 days). At the highest salinity levels, 30 and 33 ppt, the period of survival was greatly reduced (Table 2). After one month complete mortality was observed for the specimens exposed to 33 ppt (the Adriatic Sea water salinity).

At the end of the experiments, 62% of the exposed animals had survived at 5 ppt, 47% at 10 ppt, 45% at 15 ppt, 43% at 20 ppt and only 33% at 25 ppt (Fig. 4).

Using a linear regression analysis, it is evident (at the level of $P < 0.05$) that with a 1 salinity unit increase survival decreased 2.1% (Pearson correlation = 0.89059).

4. Discussion and Conclusion

From the results of the laboratory tests, it seems evident that this species can not only survive in water with salinity up to 15 ppt (mean salinity of the brackish estuarine areas of the North Adriatic coast), for a long time, but can also moult and breed. Mortality increased with salinity, in the laboratory experiments, but only for longer exposition period (100 days). Survival was 100% for short exposition period at each salinity level.

The evidence of the possibility for *P. clarkii* to survive in brackish waters is also confirmed in the Refs. [20-23]. In particular, Sarver et al. [21] demonstrated that this crayfish is able to regulate its metabolism in relation to modified water salinity. When exposed to salinities up to 20 ppt, this species tended to hyper regulate their haemolymph osmotic pressure. At about 20 ppt haemolymph became isosmotic to external

solution. This stabilization needs 48 h.

This species has been already reported in other brackish wetlands: in Portugal: the Lagoon of Rossos Aveiro (40°40′N, 8°40′W) [1] and the Palude di Torre Flavia (Central Italy) (41°57′35″N, 12°02′56″E), a relictic coastal Mediterranean biotope, directly connected to the Tyrrhenian Sea [2]. This latter study demonstrated that *P. clarkii* can live and reproduce in waters, with salinity varying between 29.6 and 16.2 ppt, and represents a threat not only for marine ecosystem, but it can also damage human livelihoods, through its impact on fishing and aquaculture.

Our first question was: does *P. clarkii* represent a threat for estuarine and lagoonal ecosystems of northeastern Adriatic coast? It is important to take into consideration the notion that the laboratory experiments conditions were not exactly those of the natural environment, where the crayfish finds a much higher variability of biotic and abiotic factors, influencing its life history and its survival (including predators).

P. clarkii is abundant in the streams, irrigation canals of the explored area, where the water salinity did not exceed 3 ppt, but it has not been found, until now, in the lagoonal coast, where the salinity ranged up to 27 ppt, or in the real estuarine stretch of the River Brenta, where the annual range varied from 5 to 15 ppt [15]. The invasion of the water courses of the explored area by this astacid is quite recent, but we think that it will be rapid.

The lagoonal Adriatic coast is not only a fragile ecosystem, a transition area with high biodiversity, offering important refuge zones for limicolous birds and for endemic fishes, but also an area where aquaculture and fish farms are numerous and represent an important part of the local economy. Therefore, the spread of this crayfish threatens not only the native fauna and flora but also the local human population.

In every location where *P. clarkii* has colonized new habitats, the elimination of submerged vegetation, the reduction of macroinvertebrate (including indigenous crayfish) and vertebrate populations, particularly limicolous birds and endemic fishes, has been observed.

From a biological point of view, a great variety of changes could follow the establishment of *P. clarkii* in the transition zones of the northern Adriatic coast, considering the damage that *P. clarkii* caused in the different world areas, where it was introduced [24-26]. From a socio-economic point of view, crayfish represent a serious damage to human livelihoods, in an area where aquaculture and fishing are very important.

According to the results of our laboratory experiments, we can expect that the non indigenous red swamp crayfish will be able to invade the estuarine and lagoonal areas of the Adriatic coast. It is only a matter of time!

We need to conduct further studies on its population dynamics with measures of density, length and sex of the local population, to better understand their status and the evolution of this invasion.

Acknowledgment

This work was financed by MIUR (Italian Ministry of Public Instruction).

References

[1] M.L. Fidalgo, A.P. Carvalho, P. Santos, Population dynamics of the red swamp crayfish, *Procambarus clarkii* (Girard, 1852) from the Aveiro region, Portugal (Decapoda, Cambaridae), Crustaceana 74 (2001) 369-375.

[2] M. Scalici, S. Chiesa, S. Scuderi, D. Celauro, G. Gibertini, Population structure and dynamics of *Procambarus clarkii* (Girard, 1852) in a Mediterranean brackish wetland (Central Italy), Biol. Invasions 12 (2010) 1415-1425.

[3] G. Wizen, B.S. Galil, A. Shlagman, A. Gasith, First record of red swamp crayfish, *Procambarus clarkii* (Girard, 1852) (Crustacea: Decapoda: Cambaridae) in Israel - too late to eradicate?, Aquatic Invasions 3 (2) (2008) 181-185.

[4] S. Barbaresi, F. Gherardi, The invasion of the alien crayfish *Procambarus clarkii* in Europe, Italy, Biol. Invasions 2 (2000) 259-264.

[5] M.J. Cruz, R. Rebelo, Vulnerability of Southwest Iberian amphibians to an introduced crayfish, *Procambarus clarkii*, Amphibia-Reptilia 26 (2005) 293-303.

[6] W. Geiger, P. Alcorlo, A. Baltanàs, C. Montes, Impact of an introduced Crustacean on the trophic webs of

Mediterranean wetlands, Biol. Invasions 7 (2005) 49-73.

[7] C.F. Rodriguez, E. Bécares, M. Fernandez-Alaez, C. Fernandez-Alaez, Loss of diversity and degradation of wetland as a result of introducing exotic crayfish, Biol. Invasions 7 (2005) 75-85.

[8] S. Barbaresi, E. Tricarico, F. Gherardi, Factors inducing the intense burrowing activity by the red swamp crayfish, *Procambarus clarkii*, an invasive speciesw, Naturwissens-chaften 91 (2004) 342-345.

[9] A.J.M. Dörr, G. La Porta, G. Pedicillo, M. Lorenzoni, Biology of *Procambarus clarkii* (Girard, 1852) in lake Trasimeno, Bulletin Francais de la Pĕche et de la Pisciculture 380-381 (2006) 1155-1168. (in Italian)

[10] G. Fea, P.A. Nardi, D. Ghia, M. Spairani, R. Manenti, S. Rossi, et al., Dati preliminari sulla distribuzione in Lombardia dei gamberi d'acqua dolce autoctoni e alloctoni, Atti. Soc. It. Sc. Nat. Museo Civico di Storia Naturale di Milano 147 (2006) 201-210. (in Italian)

[11] D. Savini, Rinvenimento della specie aliena invasiva *Procambarus clarkii* (Astacida: Cambaridae) nella riserva naturale "Bosco Siro Negri" (Zerbolò, Pv), Studi. Trent. Sci. Nat. Acta Biol. 83 (2007) 33-37.

[12] D. Savini, A. Occhipinti-Ambrogi, Bad moon rising, the lousiana swamp red crayfish, a threat to the freshwater ecosystems in Lombardy, Mem Soc. Sc. Nat. Museo Civico di Storia Naturale Milano 36 (1) (2008) 19-20.

[13] L. Garzoli, D. Savini, A. Occhipinti Ambrogi, Analisi del contenuto stomacale di *Procambarus clarkii* (Girard, 1852), gambero invasivo nella riserva naturale "Bosco Siro Negri" (Zerbolò, Pv), Studi Trent. Sci. Nat. 86 (2009) 105-109. (in Italian)

[14] L. Aquiloni, E. Tricarico, F. Gherardi, Crayfish in Italy: Distribution, threats and management, Int. Aquat. Res. 2 (2010) 1-14.

[15] R. Rossi, M. Lanzoni, E. Rizzati, S. Mantovani, G. Castaldelli, Distibuzione del Gambero rosso della Lousiana nel Delta del Po, Il Pesce 1 (2006) 93-103. (in Italian)

[16] S. Casellato, Oligochaete fauna of estuarine areas and lagoons on the northern Adriatic coast (Italy), Boll. Zool. 61 (1994) 261-269.

[17] W. Momot, R. Romaire, Use of a seine to detect stunted crawfish populations in ponds, a preliminary report, J. World Maricult. Soc. 12 (2) (1983) 384-390.

[18] American Public Health Association, Standard Methods for the Examination of Water and Wastewater, 200th ed., APHA-AWWA-WPCF, Washington DC, 1998.

[19] C. Souty-Grosset, D.M. Holdich, P.Y. Noel, J.D. Reynolds, P. Haffner (Eds.), Atlas of Crayfish in Europe, Pubblications Scientifiques du Museum Nationel d'Histoire Naturelle, Paris, 2006. (in Italian)

[20] T. Fujimori, H. Abe, Physiological role of free D-alanine in the crayfish *Procambarus clarkii* with special references to osmotic and anoxic stress responses, Comparative Biochemistry and Physiology Part A 131 (2002) 893-900.

[21] R.G. Sarver, M.A. Flynn, C.W. Holliday, Renal Na, k-ATPase and osmoregulation in the crayfish, *Procambarus clarkia,* Comp. Biochem. Physiol. 107 (2) (1994) 349-356.

[22] J.E. Newsom, K.B. Davis, Osmotic responses of haemolimph in red swamp crayfish (*Procambarus clarkii*) and white river crayfish (*P. zonangulus*) to changes in temperature and salinity, Aquaculture 126 (1994) 373-381.

[23] F. Mu, X.G. Wu, Y.X. Cheng, Y.Q. Chen, Effects of salinity and body weight on metabolism of *Procambarus clarkii*, Mar. Fish 29 (2007) 153-158.

[24] F. Gherardi, Crayfish invading Europe: The case study of *Procambarus clarkii*, Mar. Freshw. Behav. Physiol. 39 (3) (2006) 175-191.

[25] P.M. Anastácio, A.M. correia, J.P. Menino, Processes and patterns of plant destruction by crayfish: Effects of crayfish size and developmental stage of rice, Arch. Hydrobiol. 162 (2005) 37-51.

[26] P.M. Anastácio, V.S. Parente, A.M. Correia, Crayfish effects on seeds and seedlings: Identification and quantification of damage, Freshw. Biol. 50 (2005) 697-704.

Journal of Life Sciences 5 (2011) 555-561

Metabolizable Energy and Amino Acid Bioavailability of Field Pea Seeds in Broilers Diets

Vassilios Dotas, Asterios Hatzipanagiotou and Konstantinos Papanikolaou

Department of Animal Production, Faculty of Agriculture, Aristotle University of Thessaloniki, Thessaloniki 54124, Greece

Received: November 29, 2010 / Accepted: February 17, 2011 / Published: July 30, 2011.

Abstract: The aim of this study was to determine the apparent (AME) & true (TME_n) metabolizable energy as well as the crude protein (CP) & amino acid (AA) total tract (by excreta collection) digestibility (bioavailability) of field pea seeds (FPS) of the Greek cultivar "Olympos". Forty eight broilers were placed in individual cages and randomly allocated into 4 dietary treatments. Birds consumed 80 g/d of either a typical commercial diet or the same diet in which 100, 200 or 300 g/kg had been substituted by ground FPS. The experiment lasted 15 d. Apparent and true CP bioavailability of FPS were significantly decreased ($P < 0.05$) only at the inclusion rate of 300 g/kg. AA bioavailability remained at high levels (~0.80), with the exception of methionine and valine and was similar to CP mean. The mean AME and TME_n values of FPS were estimated equal to 10.8 and 11.0 MJ ME/kg, respectively.

Key words: Field pea seeds, broilers, metabolizable energy, crude protein, amino acid, bioavailability.

1. Introduction

Since the use of animal origin proteins in poultry diets was banned in the European Union (EU) in 2000, the role of soybean meal (SBM) in poultry nutrition became of major importance [1-4]. Legume seeds are a valuable alternative to SBM energy and protein source and for that reason are used to a large extent in poultry nutrition [5]. Field pea seeds (FPS) (*Pisum sativum* L.) are one of the world's most important grain legumes [6, 7]. However, their energy value and crude protein (CP) & amino acid (AA) bioavailability varies and can be significantly affected by a number of factors. Crude fiber (CF), content in tannins and trypsin inhibitors activity among them play an important role [3, 7-9]. CP quality is affected to a large extent by its AA bioavailability. Because post-ileal fermentation contributes in variable degrees to AA excretion, it has often been argued that digestibility measured at the terminal ileum is more suitable for feed evaluation than measurements of total excretion [9-12]. However, for practical purposes, CP quality of a single feedstuff seems to be estimated satisfactorily by using the classical total tract (excreta collection) digestibility. Also, the nutritive value of a poultry feed is expressed by its energy content. The mean energy values of apparent metabolizable energy (AME) and true metabolizable energy (TME_n) of FPS were estimated for adult broilers which were 10.1-11.6 and 11.4-12.9 MJ/kg, respectively [8, 13].

FPS are used to a significant degree in poultry nutrition in EU countries [6]. However, the variability between cultivars in energy content, nutrients, antinutritional factors, as well as the limited information concerning domestic (local) cultivars, suggests the need for further investigation.

The aim of this study was to estimate the nutritive value of a Greek cultivar of FPS by determining their AME & TME_n, as well as their CP and AA bioavailability, in broilers diets at inclusion rates of 100, 200 and 300 g/kg, respectively.

Corresponding author: Vassilios Dotas, Ph.D., research field: animal nutrition and physiology of nutrition. E-mail: vdotas@agro.auth.gr.

2. Materials and Methods

2.1 Field Pea Seeds "Olympos"

Ground FPS (screen diameter < 2.0 mm) of the Greek cultivar "Olympos" were used in this study. FPS "Olympos" have a deep green color and their content in ANFs is relatively low (2.4 g/kg DM for total phenols-tannins and non tannins-and 2.90 TIU/mg DM for trypsin inhibitors).

Table 1 showed the content of FPS "Olympos" in nutrients, AA, total phenols and specific protein inhibitors.

2.2 Experimental Diets, Birds, Housing and Design

Forty eight (48) Cobb-500 broilers (24 male and 24 female) were used. Birds were placed in individual cages in vertical decks of a digestibility chamber and randomly allocated into four dietary treatments. Each group was comprised of 6 male and 6 female broilers. Birds consumed 80 g/d of either a typical commercial wheat-corn-soybean meal diet (13.2 MJ ME/kg, 224 g CP/kg) or the same diet in which 100, 200 or 300 g had been substituted by ground FPS (C, P_{100}, P_{200}, P_{300}, respectively). The experiment lasted 15 d (counting from the 28th d of age). The first 4 days needed for the adaptation of birds in cages, 7 days was the duration of preliminary period and 4 days needed for the excreta collection. Fe_2O_3 (3 g/kg) was used as indigestible marker. The procedure for the endogenous nitrogen losses estimation lasted 28 h and was divided in 4 sub-periods, as following: Allowance of diet with marker for 4 h, fasting period of 16 h, allowance of N-free diet for 4 h and finally re-allowance of diet with marker for another 4 h. The composition of N-free diet was the following: sucrose (815 g/kg), corn seed oil (100 g/kg), ground wheat straw (30 g/kg) and vitamins & trace minerals premix (55 g/kg). Table 2 showed the composition of experimental control digestibility diet. Tables 3 and 4 showed the composition of experimental C and P_{100} diets in AA and the calculated [14] and chemically determined (Weende) analysis of experimental digestibility diets, respectively.

Table 1 Content of FPS "Olympos" in nutrients, AA, tannins and trypsin inhibitors.

	Content			
	g/kg DM	% of DM	g/16g N	TIU/mg DM
Nutrients				
Crude protein	248			
Ether extract	19			
Crude fiber	66			
Amino acids				
Aspartic acid		2.8	11.6	
Glutamic acid		4.4	17.7	
Serine		1.1	4.6	
Glycine		1.1	4.3	
Threonine		0.9	3.5	
Arginine		2.3	9.3	
Valine		1.1	4.3	
Phenylalanine		1.2	4.9	
Isoleucine		0.8	3.5	
Leucine		1.8	7.3	
Lysine		1.8	7.1	
Cystine		0.4	1.6	
Methionine		0.2	0.8	
Alanine		0.9	3.9	
Other nitrogenous ingredients		3.6	14.6	
Phenols				
Total	2.39			
Non-tannins	1.62			
Tannins	0.77			
Trypsin inhibitors				2.90

2.3 Calculations and Determinations

During the experimental 4d-period the feed intake per bird was recorded daily as well as the quantity of excreta. Daily excreta of each bird were being weighted and then dried in an oven at 60 ℃. The total dried excreta quantity of each bird was being mixed, ground (screen diameter 1.00 mm) and placed in an air-seal glass vial. Then, representative samples were given for determining energy content by using a Gallenkamp, Autobomb, automatic adiabatic bomb calorimeter, as well as for CP determination by using a VELP SCIENTIFICA UDK 132, SemiAutomatic Distillation Unit. The same calculations and determinations were being performed in ground FPS as

Table 2 Composition of experimental control digestibility diet.

Ingredients	g/kg
Wheat	420.00
Corn	160.00
Soybean meal	320.00
Soybean oil	15.00
Vegetable fat	14.00
Skimmed milk	15.00
$CaCO_3$	16.00
Monocalcium phosphate (MCP)	12.50
Sodium carbonate	3.00
Salt	3.00
L-Lysine, HCl	3.50
DL-Methionine (99%)	3.00
Threonine	2.00
Formic acid	2.50
Vitamins & trace minerals premix*	2.50
Probiotic	2.00
Xylanase	2.00
Phytase	2.00
Coccidiostatic	2.00
Total	1000.00

*Composition of vitamins and trace minerals premix per kg of diet: 12,000 I.U. Vitamin A; 5,000 I.U. Vitamin D_3; 100 mg Vitamin E; 4 mg Vitamin K_3; 2.60 mg Vitamin B_1; 8 mg Vitamin B_2; 3 mg Vitamin B_6; 0.015 mg Vitamin B_{12}; 10 mg Vitamin C; 85 mg Niacin; 2 mg Folic acid; 20 mg Panthothenic acid; 0.20 mg Biotin; 500 mg Choline-Cl; 120 mg Manganese; 100 mg Zinc; 40 mg Iron; 20 mg Copper; 1 mg Iodine; 0.30 mg Selenium; 0.20 mg Cobalt.

Table 3 Composition of experimental C and P_{100} diets in AA (g/kg).

	Diets	
	Control - C	100 g FPS/kg - P_{100}
Aspartic acid	13.2	14.8
Glutamic acid	44.0	42.0
Serine	9.0	9.2
Glycine	7.0	7.2
Threonine	8.7	8.9
Arginine	10.4	11.4
Valine	7.5	7.7
Phenylalanine	9.0	9.2
Isoleucine	6.6	7.0
Leucine	15.1	14.2
Lysine	13.8	14.0
Cystine	4.9	4.9
Methionine	4.9	4.8
Alanine	9.0	9.6

Table 4 Calculated [14] and chemically determined (Weende) composition of experimental digestibility diets.

	Treatments			
	C	P_{100}	P_{200}	P_{300}
Calculated composition (g/kg)				
Crude protein	226.2	225.4	223.6	222.4
Ether extract	66.7	62.6	58.4	53.4
Crude fiber	33.2	34.8	36.7	39.6
Ash	54.6	52.4	51.2	48.1
Ca	10.0	10.5	10.2	9.8
P (total)	6.9	6.9	7.0	6.7
Lysine	14.0	14.2	14.4	14.5
Methionine + cystine	10.0	9.8	10.2	10.4
ME (MJ/kg)	13.17	12.97	12.76	12.55
Chemical analysis (g/kg) (Weende)				
Dry matter	894.0	893.0	892.0	890.0
Crude protein	225.0	224.0	223.0	222.0
Ether extract	67.0	62.0	57.0	52.0
Crude fiber	32.0	35.0	37.0	40.0
Ash	55.0	52.0	50.0	47.0
Ca	10.0	9.8	9.7	9.4
P (total)	6.9	6.6	6.8	6.6

well as in samples of the 4 experimental diets. The determination of AA in FPS, in diets C and P_{100} and in their derived excreta was performed by using an Eppendorf LC 3000 AA analyzer. Total phenols content of FPS was determined according to methods described by Terrill et al. (1990) [15] and Makkar et al. (1993) [16], whereas trypsin inhibitor activity according to analytical methods described by American Association of Cereal Chemists (1983) [17].

Metabolizable energy (ME) of FPS was estimated by the method of difference. CP and AA bioavailability were determined according to experimental procedure described by Bragg et al. (1969) [18]. Colorless excreta derived from N-free diet were used for the determination of endogenous nitrogen and AA.

The coefficient of apparent total tract digestibility of energy ($CATTD_{energy}$) of the diets was determined as:

$CATTD_{energy} = (GE_{diet} - GE_{excreta})/GE_{diet}$,

Where GE: Gross Energy.

The coefficients of apparent total tract digestibility of CP and AA of the diets ($CATTD_{CP}$ and $CATTD_{AA}$) were determined by using similar equations. The coefficient of true total tract digestibility of energy

($CTTTD_{energy}$) of the diets was determined by the method of difference and by adding 4.1 kcal per each g of endogenous CP. The coefficients of true total tract digestibility of CP and AA ($CTTTD_{CP}$ and $CTTTD_{AA}$) were calculated as following:

$CTTTD_{CP} = [CP_{diet} - (CP_{ex} - CP_{en})]/CP_{diet}$,

$CTTTD_{AA} = [AA_{diet} - (AA_{ex} - AA_{en})]/AA_{diet}$,

where: CP_{diet}: Crude protein in diet; CP_{ex}: Crude protein in excreta; CP_{en}: Endogenous crude protein; AA_{diet}: Amino acids in diet; AA_{ex}: Amino acids in excreta; AA_{en}: Endogenous amino acids.

The coefficients of apparent and true digestibility of energy, CP and AA of FPS were calculated from those of diets by the method of difference.

2.4 Statistical Analysis

All data obtained in this study were subjected into two factors factorial analysis (2 × 4, sex × diet) [19, 20]. For significant treatment effects or interactions, statistically significant differences among means were identified by using Duncan and Dunnett test. Differences between means were considered significant at $P < 0.05$.

3. Results

Final BW of birds was significantly ($P < 0.05$) reduced only in the treatment containing the highest rate of FPS inclusion (300 g/kg). Apparent and true (total tract) CP digestibility of the diets were not significantly affected by FPS inclusion up to the level of 300 g/kg (Table 5).

Endogenous N showed a significant increase ($P < 0.05$) by increasing rates of FPS inclusion (Table 6). Apparent and true (total tract) CP digestibility of FPS showed a linear trend of reduction by increasing inclusion rates of FPS and this reduction was significant ($P < 0.05$) at the level of 300 g/kg. This linear tendency of reduction was detected in both male and female chicks. In all cases the mean values of coefficients of digestibility were higher but not significantly different in male than those in female chicks. No significant interactions were detected between diet × sex concerning $CATTD_{CP}$ and $CTTTD_{CP}$ of FPS.

Table 7 showed the apparent and true total tract

Table 5 Nitrogen balance[1] (g/bird/day) of experimental broilers diets.

Parameters		Treatments				SE	P
		C	P_{100}	P_{200}	P_{300}		
Number of birds[2] (n)		12	12	12	12	-	-
Feed intake (g/bird/day)		80	80	80	80	-	-
Dietary nitrogen (N)		2.880	2.870	2.850	2.840	-	-
N in excreta	M	0.483^{a}	0.488^{a}	0.507^{ab}	0.540^{b}	0.024	*
	F	0.529^{a}	0.534^{a}	0.553^{ab}	0.584^{b}	0.036	*
	T	0.506^{a}	0.510^{a}	0.503^{a}	0.562^{b}	0.022	*
Endogenous N	M	0.057^{a}	0.063^{ab}	0.072^{b}	0.073^{b}	0.014	*
	F	0.063^{a}	0.067^{a}	0.075^{b}	0.072^{b}	0.008	*
	T	0.060^{a}	0.065^{ab}	0.073^{b}	0.072^{b}	0.007	*
$CATTD_{CP}$[3]	M	0.832	0.830	0.822	0.810	0.020	NS
	F	0.816	0.814	0.806	0.794	0.022	NS
	T	0.824	0.826	0.814	0.802	0.020	NS
$CTTTD_{CP}$[4]	M	0.852	0.851	0.847	0.835	0.018	NS
	F	0.838	0.836	0.832	0.819	0.017	NS
	T	0.845	0.843	0.839	0.827	0.016	NS

[1] Means and standard error; [2] Total number of birds per treatment (6 male, 6 female); M: Male, F: Female, T: Total;
[3] $CATTD_{CP}$: Coefficient of apparent total tract digestibility of CP; [4] $CTTTD_{CP}$: Coefficient of true total tract digestibility of CP;
[a, b] Means in the same row sharing a different superscript are significantly different ($P < 0.05$).

Table 6 Nitrogen balance[1] (g/bird/day) of FPS in broilers diets.

Parameters		FPS inclusion (g/kg)			SE	P
		100	200	300		
Number of birds[2] (n)		12	12	12	-	-
Feed intake (g/bird/day)		8.00	16.00	24.00	-	-
Dietary nitrogen (N)		0.287	0.573	0.860	-	-
N in excreta	M	0.053[a]	0.121[b]	0.202[c]	0.024	*
	F	0.058[a]	0.130[b]	0.214[c]	0.028	*
	T	0.055[a]	0.125[b]	0.208[c]	0.020	*
Endogenous N	M	0.012[a]	0.026[b]	0.032[c]	0.002	*
	F	0.011[a]	0.024[b]	0.030[c]	0.003	*
	T	0.011[a]	0.025[b]	0.031[c]	0.002	*
$CATTD_{CP}$[3]	M	0.815[a]	0.788[ab]	0.765[b]	0.028	*
	F	0.798[a]	0.772[ab]	0.751[b]	0.024	*
	T	0.806[a]	0.780[ab]	0.758[b]	0.026	*
$CTTTD_{CP}$[4]	M	0.857[a]	0.834[ab]	0.802[b]	0.027	*
	F	0.836[a]	0.815[ab]	0.786[b]	0.021	*
	T	0.846[a]	0.825[ab]	0.794[b]	0.022	*

[1] Means and standard error; [2] Total number of birds per treatment (6 male, 6 female); M: Male, F: Female, T: Total;
[3] $CATTD_{CP}$: Coefficient of apparent total tract digestibility of CP; [4] $CTTTD_{CP}$: Coefficient of true total tract digestibility of CP;
[a, b, c] Means in the same row sharing a different superscript are significantly different ($P < 0.05$).

Table 7 Amino acids bioavailability of FPS.

	Bioavailability	
	Apparent	True
Aspartic acid	85.0	89.2
Glutamic acid	88.2	91.4
Serine	83.2	87.4
Glycine	85.0	89.0
Threonine	83.2	86.5
Arginine	88.6	92.8
Valine	43.6	54.5
Phenylalanine	82.9	87.7
Isoleucine	79.8	89.5
Leucine	85.8	90.6
Lysine	83.8	87.4
Cystine	78.5	85.4
Methionine	72.6	78.5
Alanine	82.0	88.5

digestibility (bioavailability) of AA of FPS as calculated by the difference from the respective values of diets C and P_{100}. From this table it can be concluded that apparent and true bioavailability of AA remained at high levels (~0.80) with the exception of methionine and valine and was similar to CP mean.

Table 8 showed the calculations and estimations concerning AME and TME_n values of experimental diets and FPS. From this table it can be conducted that AME of the diets showed a linear decrease which was significant in diets P_{200} and P_{300} as compared to P_{100} ones. This result was detected in both sexes as well as for the total of birds. The AME of FPS was significantly ($P < 0.05$) affected by the rate of FPS inclusion and varied from 10.50 ± 0.13 to 11.21 ± 0.21 MJ/kg (mean 10.83 ± 0.17 MJ/kg). The TME_n values of FPS varied from 10.63 ± 0.23 to 11.36 ± 0.16 MJ/kg (mean 10.98 ± 0.20 MJ/kg) and were significantly affected by the rate of FPS inclusion. The mean AME and TME_n values were higher in male than female chicks. No significant interactions were observed between diet × sex.

4. Discussion

The results of this study concerning the $CATTD_{CP}$ values of FPS (mean 0.782) are in general agreement with those of other researchers who estimated values from 0.785 in adult layers [21] to 0.810 [13] or slightly higher, as 0.816 [22].

Table 8 AME and TME_n[1] (kcal/kg) of experimental diets and FPS.

Parameters		Treatments				SE	P
		C	P_{100}	P_{200}	P_{300}		
Total number of birds[2] (n)		12	12	12	12	-	-
AME of diet	M	3113[a]	3073[ab]	3010[b]	2938[c]	42	*
	F	3081[a]	3038[ab]	2975[b]	2914[c]	38	*
	T	3097[a]	3055[ab]	2993[b]	2926[c]	36	*
AME of FPS	M		2710[a]	2598[b]	2530[c]	45	*
	F		2650[a]	2551[b]	2490[c]	42	*
	T		2680[a]	2575[b]	2510[c]	40	*
			X = 2588 kcal or 10.83 MJ/kg				
TME_n of FPS	M		2748[a]	2638[b]	2564[c]	58	*
	F		2685[a]	2590[b]	2522[c]	62	*
	T		2716[a]	2614[b]	2543[c]	48	*
			X = 2624 kcal or 10.98 MJ/kg				

[1] Means and standard error; [2] Each group was consisted of 12 birds (6 male, 6 female); M: Male, F: Female, T: Total.

[a, b, c] Means in the same row sharing a different superscript are significantly different ($P < 0.05$).

The mean values of apparent and true AA bioavailability of FPS determined in this study were 0.80 and 0.85 respectively and this result is in accordance to those of Castell et al. (1996) [23] and Igbasan and Guenter (1996) [24, 25] who report mean values of AA apparent digestibility of FPS cultivars with deep green color equal to 0.79.

Our results concerning AME and TME_n values of FPS are in general agreement with those suggested in other researches [8, 13, 26-28] in which the calculated values of AME varied from 10.23 to 11.59 MJ/kg.

5. Conclusion

FPS of the Greek cultivar "Olympos" are a valuable energy and protein source for broilers and could be included in their diets up to a level of 200 g/kg without any adverse effects on the diets digestibility and broilers performance, contributing to natural feed resources exploitation and sustainable development.

References

[1] V. Christodoulou, V.A. Bampidis, B. Hučko, C. Iliadis, Z. Mudřík, Nutritional value of chickpeas in rations of broiler chickens, Archiv. Geflügelk 70 (2006) 112-118.

[2] D. Diaz, M. Morlacchini, F. Masoero, M. Moschini, G. Fusconi, G. Piva, Pea seeds (*Pisum sativum*), faba beans (*Vicia faba var. minor*) and lupin seeds (*Lupinus albus var. multitalia*) as protein sources in broiler diets: Effect of extrusion on growth performance, Ital. J. Anim. Sci. 5 (2006) 43-53.

[3] S. Palander, P. Laurinen, S. Perttilä, J. Valaja, K. Partanen, Protein and amino acid digestibility and metabolizable energy value of pea (*Pisum sativum*), faba bean (*Vicia faba*) and lupin (*Lupinus angustifolius*) seeds for turkeys of different age, Anim. Feed Sci. Technol. 127 (1-2) (2006) 89-100.

[4] A. Garsen, V. Dotas, M. Anastasiadou, I. Nikolakakis, I. Mitsopoulos, D. Dotas, Performance and carcass charactheristics of broilers as affected by the replacement of soyabean meal with chickpea (*Cicer arietinum* L), in: Proceedings of 1st Mediterranean Summit of W.P.S.A. Advances and Challenges in Poultry Science, Chalkidiki (Porto Carras), May 7-10, 2008, pp. 908-912.

[5] F. Gatel, Protein quality of legume seeds for non-ruminant animals: A literature review, Anim. Feed Sci. Technol. 45 (3-4) (1994) 317-348.

[6] FAO (Food and Agriculture Organization of the United Nations), The State of Food and Agriculture, FAO Agriculture Series, No. 26, Rome, Italy, 1993.

[7] J. Wiseman, W. Al-Mazooqi, T. Welham, C. Domoney, The apparent ileal digestibility, determined with young broilers, of amino acids in near-isogenic lines of peas (*Pisum sativum* L.) differing in trypsin inhibitor activity, J. Sci. Food Agric. 83 (2003) 644-651.

[8] F.A. Igbasan, W. Guenter, B.A. Slominski, Field peas: Chemical composition and energy and amino acid availabilities for poultry, Can. J. Anim. Sci. 77 (2) (1997) 293-300.

[9] H. Kluth, M. Mantei, C. Elwert, M. Rodehutscord, Variation in precaecal amino acid and energy digestibility between pea (*Pisum sativum*) cultivars determined using a linear regression approach, Br. Poult. Sci. 46 (3) (2005) 325-332.

[10] R.A.H.M. Ten Doeschate, C.W. Scheele, V.V.A.M. Schreurs, J.D. Van der Klis, Digestibility studies in broiler chickens: Influence of genotype, age, sex and method of determination, Br. Poult. Sci. 34 (1993) 131-146.

[11] V. Ravindran, L.I. Hew, G. Ravindran, W.L. Bryden, A comparison of ileal digesta and excreta analysis for the determination of amino acid digestibility in food ingredients for poultry, Br. Poult. Sci. 40 (1999) 266-274.

[12] I.T. Kadim, P.J. Moughan, V. Ravindran, Ileal amino acid digestibility assay for the growing meat chicken-comparison of ileal and excreta amino acid digestibility in the chicken, Br. Poult. Sci. 44 (2002) 588-597.

[13] A. Brenes, B.A. Rotter, R.R. Marquardt, W. Guenter, The nutritional value of raw, autoclaved and dehulled peas (*Pisum sativum* L.) in chicken diets as affected by enzyme supplementation, Can. J. Anim. Sci. 73 (3) (1993) 605-614.

[14] NRC (National Research Council), Nutrient Requirements of Poultry, 9th rev. ed., National Academy Press, Washington, DC, 1994.

[15] T.H. Terril, R.W. Windham, J.J. Evans, S.C. Hoveland, Condensed tannin concentration in *Sericea lespedeza* as influenced by preservation method, Crop Sci. 30 (1990) 219-224.

[16] P.S.H. Makkar, M. Blümmel, N.K. Borowy, K. Becker, Gravimetric determination of tannins and their correlations with chemical and protein precipitation methods, J. Sci. Food Agric. 61 (1993) 161-165.

[17] AACC (American Association of Cereal Chemists), Approached Methods of the AACC, Method 71-10, St. Paul, MN, 1983.

[18] D.B. Bragg, C.A. Ivy, E.L. Stephenson, Methods for determining amino acid availability of feeds, Poult. Sci. (48) (1969) 2135-2137.

[19] D.E. Hinkle, W. Wiersma, S.G. Jurs, Applied Statistics for the Behavioral Sciences, 5th ed., Houghton Mifflin, Boston, 2003.

[20] SPSS, SPSS for Windows, Rel. 15.0.0., Chicago: SPSS Inc, 2006.

[21] K. Gruhn, R. Zander, Studies of the influence of various quotas of feed peas on the digestibility of their crude nutrients in colostomized laying hens, Arch. Anim. Nutr. 40 (4) (1990) 297-303.

[22] V. Dotas, Dietary evaluation of field pea seeds (*Pisum sativum* L.) and their use in layers diets, MSc. Thesis, Faculty of Agriculture, Aristotle University of Thessaloniki, 2006.

[23] A.G. Castell, W. Guenter, F.A. Igbasan, Nutritive value of peas for nonruminant diets, Anim. Feed Sci. Technol. 60 (1996) 290-327.

[24] F.A. Igbasan, W. Guenter, The enhancement of the nutritive value of peas for broiler chickens: An evaluation of micronization and dehulling processes, Poult. Sci. 75 (10) (1996) 1243-1252.

[25] F.A. Igbasan, W. Guenter, The feeding value for broiler chickens of pea chips derived from milled peas (*Pisum sativum* L.) during air classification into starch fractions, Anim. Feed Sci. Technol. 61 (1996) 205-217.

[26] R.D. Reichert, S.L. MacKenzie, Composition of peas (*Pisum sativum*) varying widely in protein content, J. Agric. Food Chem. 30 (2) (1982) 312-317.

[27] H. Würzner, F. Lettner, J. Eder, Peas (*Pisum sativum* L.) and field beans (*Vicia faba* L.) in diets for broiler chickens, Bodenkultur 39 (3) (1988) 259-268.

[28] B. Carré, E. Beaufils, J.P. Melcion, Evaluation of protein and starch digestibilities and energy value of pelleted or unpelleted pea seeds from winter or spring cultivars in adult and young chickens, J. Agric. Food Chem. 39 (3) (1991) 468-472.

Journal of Life Sciences 5 (2011) 562-568

Chemical Composition of Meat in Castrated Male Brahman Cattle in Venezuela

José A. Miguel[1], Jesús Ciria[1], Begoña Asenjo[1], Hector Pargas[2] and David Colmenarez[2]

1. College of Agricultural Engineers, University of Valladolid, Soria 42004, Spain

2. University Centroccidental Lisandro Alvarado, Barquisimeto 87220, Venezuela

Received: October 13, 2010 / Accepted: January 24, 2011 / Published: July 30, 2011.

Abstract: Chemical composition of Brahman cattle was studied and determined in this study. Fifty males had been castrated at birth, farmed semi-intensively on the Venezuelan plain and then slaughtered at the age of 31 months with an approximate weight of 500 kilograms. The average pH value of beef was 5.84 after obtained for 24 hours after slaughtering, which matches the value found in similar studies made on the same breed in Venezuela (5.8). The moisture, crude protein and ash contents correspond to the expected values of cattle, while the intramuscular fat values are slightly higher than those of the different breeds of *Bos indicus*. The total collagen values found in this study are either similar or lower than those found by other researches from the different breeds of *Bos indicus* and their crossbreeds, while the collagen solubility value (39%) was higher than the one found in other studies on the Brahman breed.

Key words*: Bos Indicus*, Brahman, cattle castrated, collagen, pH, meat.

1. Introduction

Venezuelan beef cattle is mainly made up of Zebu animals of the Brahman, Nelore and Guzerat breeds, as well as their crossbreeds with the aforementioned races and various other European breeds [1]. The production cycle of the meat circle in Venezuela for cattle lasts from approximately three to four years, the median time it takes a producer to raise a calf from birth to 480 kg of live weight, after which the animal is ready to be taken to be slaughtered. The time it takes to achieve this weight depends on the breed that is being raised (Zebu, crossbreed, dairy cow), the type of farming (intensive, feedlot or pasture) and/or the operational purpose (milk and meat, or meat only).

Beef play a vital role in the Venezuelan economy. They are an essential part of the nation's food supply and constitute 18% of the protein of the basic Venezuelan diet. Venezuelan beef production in itself is not sufficient to meet the nation's demands and has to be supplemented by imports. The main worry of the Venezuelan state is the supply cycle, while the quality and traceability are given secondary importance. This means that no legislation exists that would regulate the necessary means and practices to guarantee the origin of the cattle, the sanitary status of the national cattle production system and the reliability of the foodstuffs it produces so that all the cattle born in, or imported to, Venezuela could be identified, registered and monitored individually. In this way the end consumer is left completely in the dark regarding the sex, weight and age of the animal. Because of this, quality does not play any kind of role in the commercial use of beef in Venezuela.

Faced with this reality, it would be necessary to improve the descriptive system of Venezuelan beef products and to educate both the producers and the consumers with its use.

The chemical composition of beef is especially important for the beef's quality because it affects the

Corresponding author: José A. Miguel, Ph.D., Engineer Agronomo, research fields: natural resources and environmental management. E-mail: jangel@agro.uva.es.

technological, hygienic, sanitary and sensory properties of the beef [2]. There are numerous factors affecting the quality of beef, the most important ones are genetic type, diet, sex, age, rearing and treatment before slaughtering [3].

The objective of this study is to determine the chemical composition of beef of males of Brahman cattle that have been castrated at birth, farmed semi-intensively on the Venezuelan plain and slaughtered at the age of 31 months.

2. Material and Methods

The research was carried out in Lara, a region of Venezuela that is characterized by its semi-arid climate, an annual mean temperature that ranges from 22 ℃ to 28 ℃, annual rainfalls of over 1,000 mm and relative moisture that changes from 77% to 85%.

Fifty males of the Brahman breed were chosen for this study. The males had been castrated at birth, weaned at the age of approximately eight months and reared until they reached the age of approximately 15 months and a weight of 350 kg. During this period their diet consisted of fresh forage, mineral salt and water *ad libitum* in a rotational grazing system where the pastures were used for two days and then left to recover for 28 days. The forage available in the pastures was *rachiaria deumbens* and *brachiaria hunmidicola*. Later the diet was changed on the period of fattening, during which a supplementary ration (Table 1) of 1% of live weight was administered. At the age of 31 months the animals were slaughtered, at which moment their weight ranged from 449 to 531 kg, with a mean of 496.6 kg. The animals were later transported to the Matadero Industrial Occidenta Ltd. of conventional Venezuelan roads.

Table 1 Ingredients of supplementary foodstuffs.

Ingredient	Composition (%)
Soy bean husk	15
Ground corn	35
Mixed minerals	15
Yacija[1]	15
Corn flour	10
Molasses	10

[1] A subproduct of the poultry industry, this fodder contains rice hulls, wood shavings, food concentrates etc..

The temperature and pH measurements were carried out after the slaughtering and before the carcasses were taken to the cold storage, approximately 30 minutes *postmortem*. The measurements were taken from the level of the *Longissimus dorsi* twelfth thoracic vertebra. This procedure will be referred to as the measuring of the pH and the temperature at starting time (pH0, Temp0). A portable pH meter (Digimed DM-2) was used for the measurements. The pH meter had a penetrating electrode (DMR-CF1) and a temperature catheter (DMF-P1X). Another measurement of pH and temperature was carried out 24 hours after the refrigeration (pH24, Temp24) at the same place.

Samples of the *Longissimus dorsi* were taken from the level of the sixth rib of the mid-left part of the carcass of every animal. This was done according to the method in order to determine their chemical composition [4]. The following methodologies were used:

(1) The moisture was measured according to the method described in ISO R-1442 [5] and the Methods of Analysis of Meat Products;

(2) The ash content was measured according to the method described in ISO R-936 [6] and Methods of Analysis of Meat Products;

(3) The fat content was measured according to the method described in ISO R-1443 [7] and Methods of Analysis of Meat Products;

(4) The protein content was measured according to the method described in ISO R-937 [8] and Methods of Analysis of Meat Products;

(5) The method [2] was used to determine the total collagen and soluble contents.

3. Results and Discussion

3.1 pH and Temperature

Table 2 shows the initial and final pH and temperature values. The numbers show that the highest

Table 2 Initial pH (pH0) and temperature (Temp0) values, final pH (pH24) and temperature (Temp24) values of male Brahmans castrated at birth and slaughtered at the age of 31 months.

	Mean	Minimum	Maximum	Std. Dev.
pH0	7.01	6.87	7.10	0.073
Temp0	34.99	33.10	36.40	0.993
pH24	5.84	5.73	5.95	0.059
Temp24	3.53	3.10	4.00	0.329

and lowest values of pH24 are between 5.73 and 5.95, with a mean of 5.84. The final and initial pH values correspond with those obtained by Vitto et al. [9] from castrated male Brahmans, which were 6.94 and 5.83 for the initial and final pH values, respectively.

The pH values at the upper limit of the expected scale could be attributed to the notably nervous temperament of the Brahman breed, a typical characteristic of the *Bos indicus.* When this nervousness is combined with the way the animals are handled before they are slaughtered, especially when they are transported since there exist only a few regulations on the transportation of animals, the conditions are hardly such that they would decrease the stress caused to the animals by the transportation procedure. Furthermore, Van De Water et al. [10] imply that transportation has a highly stressful effect on cattle.

Breed is a determining factor in the quality of beef, especially since different breeds of cattle act differently when they face stressful situations. There are large amounts of the *Bos indicus* in Venezuela, especially in highly crossbred Brahmans, and these get easily excited and stand up to the slaughterers because of their nervous character, something that makes them very sensitive to stress.

In Venezuela, the aforementioned factors constantly threaten the organoleptic, technologic and sanitary quality of the beef of livestock since there are neither laws nor regulations that would give said animals such rights to minimal conditions before they are slaughtered that do exist in the European Union and United State through such laws as “Animal Welfare” and “Humane Slaughter Act”, respectively.

The effect of the climate on the quality of beef also needs to be taken into account, for it might cause such syndromes as the PSE (pale, soft, exudative) meat or the DFD (dark, firm, dry) meat in which, among other things, the pH value is at risk. Furthermore, a study by Gallo et al. [11] on the effects of 36 hours of transportation with and without breaks on the quality of beef, states that the median pH in all animals, rested or not, was 5.8, which is the limit of being acceptable.

These negative results on the quality of beef are caused when the animals are exposed to physical stress and lack of food for large amounts of time before they are put to death. This exhausts their energy reserves, thus lowering their levels of muscle and hepatic glycogen, which prevents the fall in muscular tissue pH levels that normally happens after the animals have been slaughtered. However, there are other conditions related to transportation and the feeding of animals that may have affected the quality of the beef, such as the travelling conditions and the climate.

The diet may also affect the development of the final pH values. Even though pasture-based diets are typically rich in fibres and poor in starch, a fact that raises acetate/propionate levels, the animals that were grazing before being slaughtered generally have a normal pH value. However, this level is higher than the level of those animals that were slaughtered while on a concentrate food diet [12]. In his study on young Angus crossbreeds that included some animals on pasture-based diets and others on corn-based diets, Young et al. [13] observed a greater variability in the final pH values and lower glycolysis and glycogen residue levels of those animals that had been on a pasture-based diet.

Similar results were found by Vestergaard et al. [14] who conclude that the animals that are on pasture-based diets before being slaughtered have, under normal circumstances, enough glycogen to reach a final pH level that is acceptable. However, low glycolysis levels with a high potential for glycogen loss

as a reaction to poor handling before being slaughtered may represent a risk of high final pH values [15]. This is supported by the study of Immonen et al. [16] which establishes that high energy diets protect the cattle from possible falls in glycogen levels that could be caused by stress. Thus the animals on pasture-based diets are generally less accustomed to the presence of, and being handled by, humans, as opposed to animals fed in cow sheds. This could also affect the glycogen loss before being slaughtered [12].

A notable Pearson correlation between pH24 and Temp24 (r: -0.81; $P < 0.01$) must be noted (Fig. 1). When the linear regression of these variables is drawn (Fig. 1) it becomes apparent that the final pH values tend to be higher in carcasses that are colder. This result might be related to the fat content of the carcasses, which could affect the fall rate of both the temperature and the pH level.

3.2 Protein, Moisture and Ash

The moisture, pure protein and ash contents (Table 3) correspond with the expected contents for beef taken from the *Longissimus dorsi* (72-75% moisture, 21-24% protein and 1% ash) [17, 18]. As for the fat content, the obtained values (3.41%) were slightly higher than those found by the aforementioned authors (1-3%), but lower than those observed in castrated males by Costa et al. [19], which were 11.8 and 14.6 in every 6 out of 100 in Nelores and F1 Nelores crossbred with Sindi, respectively. However, the study does not clarify whether values for intramuscular fat are for humid or dry matter, since the Nelore breed has a protein content of 83.9%, which does imply that the protein content is for dry matter.

According to the study by Arenas et al. [20] on 77 bulls and 12 young bulls that represented seven different breeds: Brahman, a commercial Zebu crossbreed, Brahman F1 crossbreeds (F1 Romosinuano, F1 Angus, F1 Senepol, F1 Simental) and 3/4 *Bos taurus* the following content values were measured from the bulls: 21.73%; 74.23%; 1.09% and 1.38% in protein, moisture, ash and intramuscular fat, respectively. The contents of 73.86% and 1.45% were measured from young bulls in moisture and intramuscular fat, respectively.

3.3 Collagen

The values of total collagen, soluble collagen and the solubility of collagen of this study (Table 4) are higher than those found by Riley et al. [21] in 466 castrated male Brahmans that were evaluated from 1998 to 2001. They received values of 3.49% and 2.88% for total

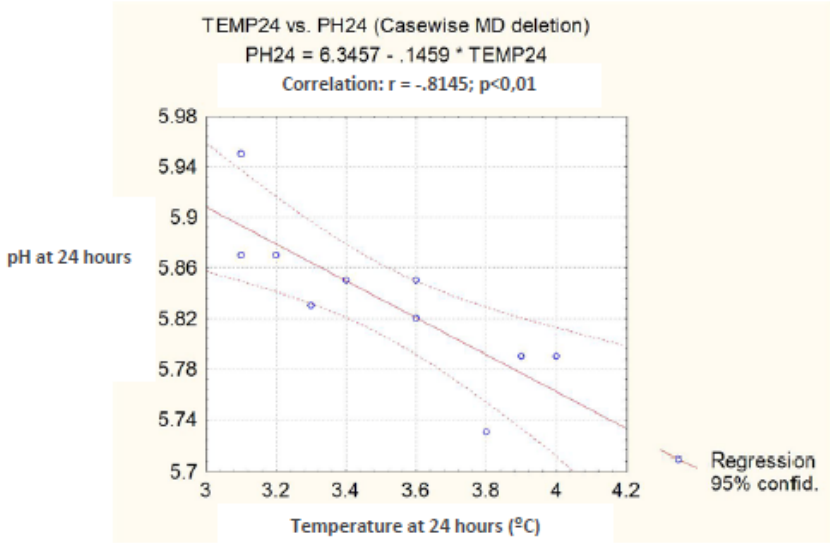

Fig. 1 Pearson correlation between pH level and temperature 24 hours after slaughtering in Brahman males castrated at birth and slaughtered at the age of 31 months.

Table 3 The estimated protein, moisture, ash and intramuscular fat contents percent taken from the *Longissimus dorsi* from the level of the sixth rib.

	Mean	Minimum	Maximum	Std. Dev.
Protein	22.30	21.37	23.71	0.8509
Moisture	73.07	72.07	73.99	0.7603
Ash	1.13	1.04	1.20	0.0494
Intramuscular fat	3.41	1.86	5.61	1.1787

Table 4 The total collagen content, the soluble collagen content percent and the solubility of the collagen of male Brahmans beef that had been castrated at birth and slaughtered at the age of 31 months.

	Mean	Min.	Max.	Std. Dev.
Total collagen	5.30	2.64	7.89	1.5613
Soluble collagen	2.14	0.91	3.86	0.8376
Solubility	39.58	33.15	49.31	5.4919

collagen and soluble collagen, respectively. This represents a 17.48% rate of collagen solubility. The differences noted in these animals, which were all castrated and of the same breed, could have been caused by the fact that the latter ones were slaughtered at a younger age (approximately 14 months), that their diet was intensive in nature and that they had been given growth promoters.

The effect of age on the values of total collagen and soluble collagen has been studied by various researchers, such as Jurie et al. [22] who studied the effect of age on the parameters of growth, carcass features and the muscles of four French cattle breeds (Charolais, Aubrac, Limousin and Salers) that had been slaughtered at 15, 19 and 24 months of age. The study found out that the amount of total collagen and insoluble collagen increases from the age of 19 months onwards, and decreases between 15 and 19 months of age. Nakamura et al. [23] explains that as the cattle grows older, age causes changes in the molecular structures of the collagen since there are intermolecular bridges between the collagen fibre that maintain them stable. Thus, when an animal grows older, these links become more and more stable, insoluble and harder to break, which causes the beef to become tougher.

A study by Li et al. [24] used young bulls of the native Chinese yellow cattle to study the effects of age on the quality of the beef and intramuscular connecting tissue. The study did not note significant differences between the total collagens of animals of maturity class A (9-30 months) and maturity class C (42-72 months). However, the study did note significant differences in solubility between the two groups even though the solubility values for cattle from maturity class C were higher than those of class A (16.59% vs. 23.22%).

Collagen solubility is also affected by hormonal effects. Boccard et al. [25] observed low hydroxyproline contents in castrated animals as a consequence of the lack of an anabolic effect of testosterone on collagen synthesis. Less collagen synthesis means less solubility. Males that had not been castrated yielded better collagen solubility results than castrated ones [26].

According to Jaturasitha et al. [27] castrated males of a crossbreed of American Brahmans with Thai *Bos indicus* had similar total collagen values (5.89%), but much lower values of collagen solubility (6.53%). These differences in collagen solubility are explained by the fact that the animals in question were more than 5 years old, and the study was made on abandoned working animals. The study of Monson et al. [28] on 60 young commercial Mexican bulls that had been fed in cow sheds for six months and later slaughtered at the age of 21 months found higher total collagen values (6.29%) but lower ones for soluble collagen and collagen solubility (1.4% and 22.2%, respectively). On the other hand, the values for total collagen and collagen solubility found in this study are lower than those encountered by Stolowski et al. [29] in male and female crossbreeds of the Angus and Brahman breeds. They had total collagen and collagen solubility values of 2.0% and 17.21%, respectively.

The values of total collagen in this study are similar to those measured from the Holstein and Brown Swiss breeds by Monson et al. [28]. The values were 5.75% and 5.47%, respectively, which are also higher than those measured from the Limousin and Blonde d'Aquitaine breeds, 4.16% and 3.40% respectively. In

the same study the collagen solubility values were found to be between 33.91% and 44.14% for the four races in question, a value that is very similar to the 39.58% found from the castrated Brahman in this study. The highest values were for the Limousin and Blonde d'Aquitaine breeds, 41.87% and 44.14%, respectively. These results were to be expected since these breeds mature slowly and grow quickly during the fattening period. Spanghero et al. [30] found that Italian Simmental males that had been under intensive care and later slaughtered at the age of 11 months had total collagen and solubility values of 5.75% and 21.6%, respectively.

In conclusion, the initial and final pH values correspond to those found in other studies on this breed in Venezuela. The final pH values correlate with the final temperatures. The moisture, protein and ash contents coincide with the expected values for beef, while the amount of intramuscular fat is a bit higher than the normal amount found from the different breeds of *Bos indicus.*

The total collagen values of the different breeds of *Bos indicus* and their crossbreeds found in this study are similar or lower than in previous studies, whereas the value of collagen solubility (39.02%) is higher than the one found on other studies on the Brahman breed. The value corresponds to the ones found from the different breeds of *Bos taurus* in which the largest values are expected to be found from the breeds that are late to mature and that grow quickly during the fattening period.

4. Conclusions

Initial pH and end values match those found in other work for this breed in Venezuela, where the final pH limit slightly higher than expected for cattle. Final pH values are correlated with the final temperature and with the percentage of fat in the tissue composition.

The values of total and soluble collagen found to compare them with results in races *Bos indicus* and their crossings are similar or lower, while the solubility of the collagen was higher than that found in other works for Brahman breed and match those found in breeds *Bos taurus* where the highest values are expected in races that are late maturation and growth speed fast during the fattening period.

References

[1] A. Atencio, Characterization and management of flocks Brahman, Guzerá and Nelore raised on the plains of Venezuela, VII Seminario Manejo y Utilización de pastos y forrajes en sistemas de producción animal. Barinas: Universidad Nacional Experimental de los Llanos Ezequiel Zamora, (VII Symposium Management and Utilization of Pasture and Fodder in Animal Production Systems, Experimental National University of the Llanos Ezequiel Zamora, Barinas), 2001, pp. 1-20. (in Spanish)

[2] M. Oliván, M.J. Martínez, M. Mocha, P. García, A. Martínez, P. Castro, et al., Chemical analysis of meat, in: Methodology for the Study of the Quality of the Carcass and Meat in Ruminants, Ganadera 1, Monografías INIA, 2000, pp.183-203.

[3] J. Ciria, B. Asenjo, Technical conditions for controlling the quality of the product, factors to consider in the preslaughter and postslaughter, in: Methodology for the Study of the Quality of the Carcass and Meat in Ruminants, Ganadera 1, Monografías INIA, 2000, pp. 21-45.

[4] C. Sañudo, J.L. Olleta, M.M. Campo, B. Panea, E. Rota, Proposal of sampling, in: Standardization of Methodologies to Assess the Quality of the Product (Live Animal, Carcass, Meat and Fat) in Ruminants, Ganadera 3, Monografías INIA, 2005, pp. 201-215.

[5] ISO R-1442, Determination of moisture content, in: International Standards Meat and Meat Products, International Organization for Standadization, Geneva, 1973.

[6] ISO R-936, Determination of inorganic compounds, in: International Standards Meat and Meat Products, International Organization for Standadization, Geneva, 1973.

[7] ISO R-1443, Determination of total fat content, in: International Standards Meat and Meat Products, International Organization for Standadization, Geneva, 1973.

[8] ISO R-937, Determination of nitrogen content, in: International Standards Meat and Meat Products, International Organization for Standadization, Geneva, 1978.

[9] R. Vitto, Evaluation of the effect of some factors on pH of cattle bos indicus in Venezuela carcass, in: XI Jornadas Sobre Producción Animal, Zaragoza, Spain, 2005, pp. 738-740.

[10] G. Van De Water, F. Verjans, R. Geers, The effect of short distance transport under commercial conditions on the

physiology of slaughter calves; pH and colour profiles of veal, Livestock Production Science 90 (2003) 458-462.

[11] C. Gallo, M.A. Espinoza, J. Gasic, Effects of transport by truck for 36 hours with no rest on live weight period and some aspects of the quality of the meat, Archivos de medicina veterinaria (Archives of Veterinary Medicine) 33 (1) (2001) 43-53.

[12] A. Priolo, D. Micol, J. Agrabiel, Effects of grass feeding system on ruminant eat colour and flavour, A review, Animal Research 50 (2001) 185-200.

[13] O. Young, C. Daly, A. Graafhuis, S. Moorhead, Effect of cattle diet on some aspect of meat quality, in: Proc. 43rd International Congress of Meat Science and Technology, Auckland, New Zealand, 1997.

[14] M. Vestergaard, N. Oksbjerg, P. Henckel, Influence of feeding intensity, grazing and finishing feeding on muscle fibre characteristics and meat colour of semitendinosus, longissimus dorsi and supraspinatus muscles in young bulls, Meat Science 54 (2000) 177-185.

[15] R. Bowling, Comparison of forage-finished and grain-finished beef carcasses, Journal of Animal Science 45 (1997) 209-221.

[16] K. Immonen, M. Ruusunen, K. Hissa, E. Puolanne, Bovine muscle concentration in relation to finishing diet, slaughter and ultimate pH, Meat Science 55 (2000) 25-31.

[17] K.O. Honikel, R. Hamm, Measurement of water-holding capacity and juiciness, in: A.M. Pearson, T.R. Dutson (Eds.), Quality Attributes and Their Measurement in Meat, Poultry and Fish Products, Advances in Meat Research Series, Vol. 9, Blackie Academic & Professional, Cap. 5, London, 1994, pp. 125-132.

[18] L. Sanchez, J.A. Carballo, B. Sanchez, L. Monserrat, Characteristics of the carcass and the meat of male animals obtained crossing Rubia Gallega breed with Nellore, Archivos de Zootecnia 54 (2005) 485-489.

[19] D. Costa, V. Rodrigues, J. Silva, O. Neto, S. Sousa, J. Sousa, et al., Meat quality of Nellore and F1 Nellore × Sind steers, Archivos de Zootecnia 57 (2008) 345-348.

[20] L. Arenas de Moreno, Effect of strategic supplementation, implant regime and gender on the proximate and mineral composition of raw and cooked bovine meat, Meat Science 18 (1) (2008) 65-72.

[21] D.G. Riley, D.D. Johnson, J.R. Chase, R.L. West, S.W. Coleman, T.A. Olson, et al., Factors influencing tenderness in steaks from Brahman cattle, Meat Science 70 (2005) 347-356.

[22] C. Jurie, J.F. Martin, A. Listrat, R. Jailler, J. Couliolli, B. Picard, Effects of age and breed of beef bulls on growth parameters, carcass and muscle characteristics, Animal Science 80 (2005) 257-263.

[23] Y.N. Nakamura, H. Iwamoto, Y. Ono, N. Shiba, S. Nishimura, S. Tabata, Relationship among collagen amount, distribution and architecture in the *longissimus thoracis* and pectoralis profundus from pigs, Meat Science 64 (2003) 43-50.

[24] C. Li, X. Xu, G. Zhou, S. Xu, J. Zhang, Effects of carcass maturity on meat quality characteristics of beef semitendinosus muscle for chinese native yellow steers, Animal 1 (2007) 780-786.

[25] R. Boccard, The influence of age, sex and breed of cattle on their muscle characteristics, Meat Science 4 (1979) 261-281.

[26] R. McCormick, The flexibility of the collagen compartment of muscle, in: Proceedings of the 38th International Congress of Meat Science and Technology, Clermont-Ferrand, France, 1992, pp. 51-60.

[27] S. Jaturasitha, P. Thirawong, V. Leangwunta, M. Kreuzer, Reducing toughness of beef from Bos indicus draught steers by injection of calcium chloride: Effect of concentration and time post-mortem, Meat Science 68 (2004) 61-69.

[28] F. Monson, C. Sañudo, I. Sierra, Influence of cattle breed and ageing time on textural meat quality, Meat Science 68 (2004) 595-602.

[29] G. Stolowski, Factors influencing the variation in tenderness of seven major beef muscles from three Angus and Brahman breed crosses, Meat Science 73 (2006) 475-483.

[30] M. Spanghero, L. Gracco, R. Valusso, E. Piasentier, In vivo performance, slaughtering traits and meat quality of bovine (Italian Simmental) and buffalo (Italian Mediterranean) bulls, Livestock Production Science 91 (2004) 129-141.

Journal of Life Sciences 5 (2011) 569-574

Alternative Distributions to Estimate Usual Intake of Nutrients for Groups

José Eduardo Corrente[1], Juliana M. Morimoto[2], Dirce Maria Lobo Marchioni[2] and Regina Mara Fisberg[2]

1. Department of Biostatistics, Biosciences Institute, UNESP, Botucatu, São Paulo 18618-900, Brasil

2. Department of Nutrition, Faculty of Public Health, University of São Paulo, São Paulo 18618-900, Brasil

Received: November 10, 2010 / Accepted: December 07, 2010 / Published: July 30, 2011.

Abstract: When assessing food intake patterns in groups of individuals, a major problem is finding usual intake distribution. This study aimed at searching for a probability distribution to estimate the usual intake of nutrients using data from a cross-sectional investigation on nutrition students from a public university in São Paulo state, Brazil. Data on 119 women aged 19 to 30 years old were used. All women answered a questionnaire about their lifestyle, diet and demographics. Food intake was evaluated from a non-consecutive three-day 24-hour food record. Different probability distributions were tested for vitamins C and E, panthotenic acid, folate, zinc, copper and calcium where data normalization was not possible. Empirical comparisons were performed, and inadequacy prevalence was calculated by comparing with the NRC method. It was concluded that if a more realistic distribution for usual intake is found, results can be more accurate as compared to those achieved by other methods.

Key words: Diet, nutrients, adequate intake, measurement error models, usual intake distribution.

1. Introduction

Food ingestion has accompanied human beings during their existence and, from simple objectives, such as appeasing one's hunger due to physiological need, it has become part of people's lifestyles, thus contributing to their health or disease conditions. Hence, studies on food intake constitute effective instruments to collect information on the characteristics of food ingestion by a great part of the population.

With this respect, nutritional assessment refers to the evaluation of the dietetic intake of a group in relation to a given pattern concerning nutritional adequacy and the prevention of chronic diseases [1]. This can be achieved by means of various methods; however, the most frequently used method nowadays is the 24-hour recall (R24h). The R24h, as its name indicates, consists in defining and quantifying all the food and drinks ingested in the 24-hour period prior to the interview [2]. Also, the quality of information will depend on the memory and cooperation of respondents, which is one of the greatest limitations to this method [2]. This occurs because the respondent may under- or overestimate the portions ingested [3]. However, the greatest limitation is the fact that one recall day alone does not represent an individual's usual intake due to the high within-person variability of nutrient intake.

Nevertheless, to evaluate the nutritional adequacy of a group or population, the Recommended Dietary Allowances (RDA), or a certain percentile of it, as well as the Dietary Reference Intakes (DRI) can be used as a cut-off point for assessing the prevalence of nutrient intake inadequacy.

RDA is the level of ingestion which is sufficient to meet nutritional requirements for almost all healthy individuals in a certain stage of their lives or according to their gender. Under the assumption of normal nutritional requirements, RDA is defined as the value corresponding to two standard deviations of the

Corresponding author: José Eduardo Corrente, Ph.D., research field: biostatistics. E-mail: jecorren@ibb.unesp.br.

estimated average requirement (EAR). Then, a method has been proposed considering EAR as a cut-off point to evaluate the prevalence of inadequacy of a given nutrient for a group of individuals [4]. But in order to apply this method, the nutrient intake distribution must be normal or at least symmetrical.

Knowledge about usual diet is necessary to evaluate nutrient intake since the effects of inadequate nutrient ingestion do not occur after few days. Thus, information referring to several days must be used, but reflecting only between-person variability and removing within-person variability, which is often high. This fact causes serious problems to the analysis of usual intake distribution, such as the lack of normality, problems related to normalizing transformation and negative variances among others.

Estimating the prevalence of nutrient intake inadequacy has been a constant challenge, since nutrient intake distribution is not always normal or symmetrical. This is due to the fact that the main characteristic of a healthy individual's or a population's dietary intake is the daily variability of their diet [5]. Even if individuals follow a stable intake pattern, there are no elements or consistency. Hence, daily food intake is considered to be a random event due to certain factors, such as day-to-day variation (weekend), food variety, seasonality, socio-economic and cultural aspects among others, which contribute to daily variability.

Therefore, the statistical analysis of food intake data aims at subduing the effects of certain limitations in the recalls by means of analytical estimation and by removing the effects of dietary intake within-person variability. Nevertheless, complex models have been used for that purpose.

A rather useful statistical model for analyzing dietary intake data is the model with measurement errors, which is used by the PC_SIDE software [6]. Again, the problem lies in data normality, which is not always present, depending on the type of nutrient under analysis. Thus, models with measurement errors were introduced in the context of generalized linear models [7]. They do not require data normality, but only that such data distribution belongs to an exponential family [8]. With this regard, the concept of instrumental variables was introduced, and routines for the STATA software were developed so as to evaluate nutrient intake distribution in a population without the need for data normalizing transformation [9].

Even so, it is observed that such models are still not sufficient due to the fact that intake distribution based on various 24-hour recalls is still different from those belonging to the exponential family.

Therefore, this study aimed at using probability distributions which do not belong to the exponential family to estimate the usual intake of nutrients by a population of female students and at obtaining the inadequate intake likelihood.

2. Methodology

A cross-sectional study was performed, and intake data were collected from 119 female students from a public university in São Paulo state, Brazil, whose ages ranged from 19 to 30 years old. Their usual intake was measured by means of three 24-hour recalls on non-consecutive days. The nutrient intake evaluated was for calories, protein, total lipids, fiber, cholesterol, retinol, vitamins C, B_1, B_2, B_6, B_{12}, D, E, iodine, niacin, folacin, pantothenic acid, calcium, manganese, zinc, magnesium, potassium, phosphorus, iron, copper and selenium [10].

Initially, normality tests were performed on the intake data in order to find the inadequacy likelihood.

For the nutrients whose distributions were not normal, the Weibull, log-normal and extreme-value distributions were utilized. Based on the fitting of such distributions' parameters, DRIs were used for calculating the inadequacy likelihood. These likelihoods were compared according to the empirical distribution of the data.

The ethical principles of Resolution 196/96 by the National Health Council were observed for data

collection, and the research project was approved by the Research Ethics Committee of the University of São Paulo Faculty of Public Health.

3. Results and Discussion

By observing the normality for the collected data, all the micro and macronutrients showed asymmetrical distributions when using the Shpiro-Wilk test on the PROC UNIVARIATE of software package SAS *for Windows*, v.9.1.3 [11].

Hence, by using EAR as a cut-off point and eliminating within-person variability, the inadequacy likelihood for these nutrients could be under or overestimated. Therefore, the Weibull, log-normal and extreme-value distributions were tested using the STATISTICA software, v.6.0 [12] since the nutrient distributions were very asymmetrical. Despite this fact, vitamin C, E, Pantothenic acid, folate, zinc, copper and calcium were fitted to such distributions. Therefore, we considered only such nutrients.

Fig. 1 shows the histogram and the fitted distribution for nutrients vitamin C, pantothenic acid and calcium. Figures for the other nutrients will not be shown since most of them were fitted to a Weibull distribution.

According to Fig. 1, it is observed that the distributions of calcium, copper, folate, pantothenic acid, vitamin C, vitamin E and zinc intake were completely asymmetrical. In these cases, a transformation cannot always normalize the data or, at least, make the distribution symmetrical. The presence of outliers that hinder the transformation can also be noted.

As these forms are very asymmetrical, attaining within-person variability is not possible since negative variance values are obtained in the analysis of variance for the effect of the recalls. This is due to the fact that the data do not have a normal distribution and are not always normalized by a transformation. Hence, the intake estimation for these nutrients may be underestimated or overestimated.

By considering EARs as a cut-off point for estimating inadequate intake likelihood, the intake inadequacy likelihoods for these nutrients were calculated by comparing with the empirical distribution. The empirical distribution was obtained only from the frequency of intake values beneath the recommended value. For the fitted distributions, on the other hand, the intake likelihood smaller than or equal to the recommended value was found by using the distribution corresponding to the respective estimated parameters, according to Fig. 1. Results are shown in Table 1.

Hence, Table 1 shows the nutrients considered, the likelihood distribution fitted to such nutrients' intake, the cut-off points and the inadequacy likelihoods by using the likelihood according to the fitted distribution and only the empirical intake distribution. It can be observed that the values obtained by the likelihood according to the fitted distribution are similar to those of the empirical distribution, with errors of approximately 0.02. The greatest errors made are for vitamin E and copper, which are of approximately 0.05. Even so, it can be stated that the distributions used provide good estimation of inadequacy prevalence for these nutrients in the group of women.

Similar data were analyzed by the National Research Council (NRC) method, in which the data are initially transformed so as to follow an approximately normal distribution [10]. It is supposed that within-person variability is the same for all individuals, and a set of intermediate values is constructed by conserving the mean and within-person variability. After such estimation, the inverse transformation is used and, with this new empirical distribution of the retransformed intermediate values, the nutrient intake inadequacy likelihood is obtained. 99% inadequate intake was found for folate, 47% for zinc, 33% for copper and 95% for calcium [10]. By considering the fitted likelihood distributions (Table 1), it was observed that the inadequacy likelihood for folate and calcium was overestimated (91.20% and 77.55%, respectively) whereas that for zinc and copper was underestimated

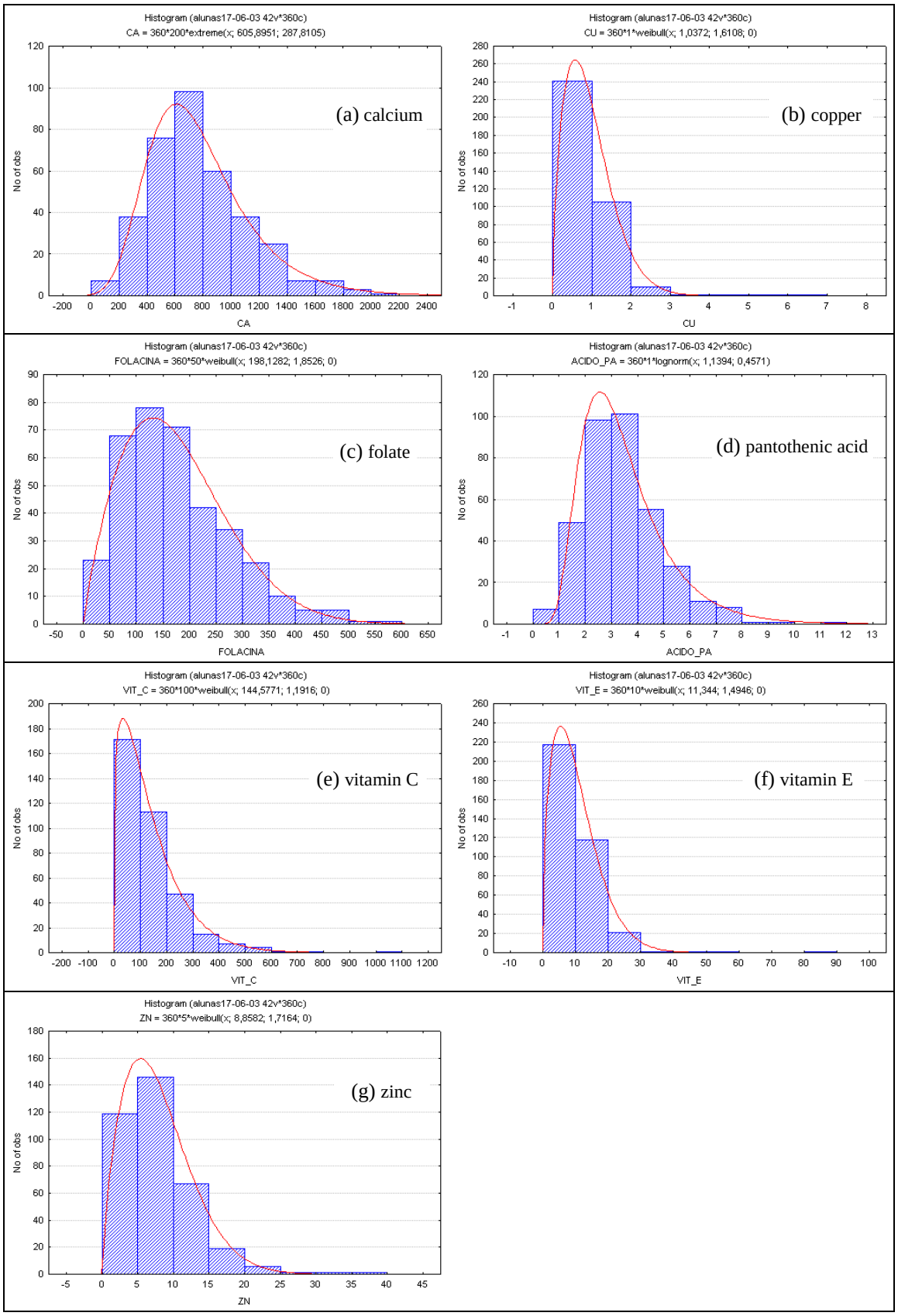

Fig. 1 Histogram and likelihood distribution fitting of intake data.

Table 1 Comparisons between values estimated for inadequacy likelihoods of vitamins C, E, B_2, and pantothenic acid, according to the fitted and empirical distributions.

Nutrient	Fitted Distribution	EAR cut-off point	Inadequacy likelihood (fitted)	Inadequacy likelihood (empirical)
Vitamin C	Weibull	73.2	0.3588	0.3722
Vitamin E	Weibull	5.32	0.2743	0.2222
Pantothenic acid	Extreme value	5.0	0.8481	0.8639
Folate	Weibull	320	0.9120	0.9028
Zinc	Weibull	8	0.5681	0.5944
Copper	Weibull	0.7	0.4119	0.4611
Calcium	Extreme value	1000	0.7755	0.7750

(56.81% and 41.19%, respectively).

There is a challenge to be faced in order to obtain nutrient inadequacy likelihoods [13-14]. One of them is that long-term nutritional assessments must be conducted for correctly estimating such likelihood, which is usually impossible due to the costs that such procedure would generate [15]. Despite this fact, a pilot study is necessary for the correct implementation of such methodologies [13].

The use of EAR as a cut-off point, as utilized in this study by applying only the distribution that best fits the data, such as a probabilistic method that considers intake and requirement variability, provides good results for inadequacy, showing that its estimation should be closer to the real prevalence of inadequacy [16].

4. Conclusions

Estimating nutrient inadequacy likelihood has been one of the greatest challenges for dieticians and statisticians. Despite the complex statistical methodologies developed for analyzing data from nutritional assessments, a lot still needs to be done so as to estimate such likelihood as accurately as possible and actually represent the population's intake. This occurs because, according to the method used, actions which would, in fact, not be necessary may be recommended, or contrarily, actions that would benefit certain members of the population may be disregarded.

Hence, a nutritional assessment to estimate the prevalence of nutrient inadequacy must be very well planned, and statistical techniques and methodologies which are compatible with the obtained data must be used for an appropriate estimation of inadequacy likelihood.

Acknowledgments

This research was supported by the FUNDUNESP (Processes no. 00112/09 and 01312/10).

References

[1] K.W. Dood, P.M. Guenther, L.S. Freedman, A.F. Subar, V. Kipnis, D. Midthune, et al., Statistical methods for estimating usual intake of nutrients and foods: A review of the theory, Journal of the American Dietetic Association 106 (10) (2006) 1640-1650.

[2] R.M. Fisberg, D.M.L. Marchioni, B. Slater, Recomendações nutricionais, Inquéritos alimentares: Métodos e bases científicos, Editora Manole, (Nutritional Recommendations, Food Surveys: Methods and Scientific Bases, Manole Editor), 2005. (in Portuguese)

[3] E. Faggiano, P. Vineis, D. Cravanzola, P. Pisani, G. Xompero, E. Riboli, et al., Validation of a method for the estimation of food portion size, Epidemiology 3 (1992) 937-962.

[4] G.H. Beaton, Approaches to analysis of dietary data: Relationship between planned analyses and choice of methodology, Am. J. Clin. Nutr. 59 (1994) 253S-261S.

[5] W.C. Willett, Nutritional Epidemiology, 2nd ed., Oxford University Press, New York, 1998.

[6] S.M. Nusser, W.A. Fuller, P.M. Guenther, Estimating usual dietary intake distributions: Adjusting for measurement error and non-normality in 24-hour food intake data, in: L. Lyberg, P. Biemer, M. Collins, E. Deleeuw, C. Dippo, N. Schwartz, et al. (Eds.), Survey Measurement and Process Quality, Wiley, New York, 1997, pp. 689-709.

[7] J.W. Hardin, R.J. Carroll, Measurement error, GLMs, and notational conventions, The Stata Journal 3 (4) (2003)

329-341.

[8] P. McCullagh, J.A. Nelder, Generalized Linear Models, 2nd ed., Chapman & Hall, London, 1989.

[9] J.W. Hardin, H. Schmiediche, Instrumental variables, bootstrapping and generalized linear models, The Stata Journal 3 (4) (2003) 351-360.

[10] J.M. Morimoto, D.M.L. Marchioni, R.M. Fisberg, Using dietary reference intake based methods to estimate prevalence of inadequate nutrient intake among female students in Brazil, JADA 106 (5) (2006) 733-736.

[11] SAS for Windows, SAS/STAT user's guide, Cary, N.C. 1996.

[12] StatSoft Inc., Statistica for windows, v.6.0, Tulsa, OK, 2001.

[13] S.P. Murphy, M.I. Poos, Dietary reference intakes: Summary of applications in dietary assessment, Public. Health. Nutr .5 (6) (2002) 843-849.

[14] S.P. Murphy, S.I. Barr, Challenges in using the dietary reference intakes to plan diets for groups, Nutrition Reviews 63 (8) (2005) 267-271.

[15] F. Verret, Methodological challenges in analyzing nutrition data from the Canadian community health survey – nutrition, in: Proceedings of Statistics Canada's Symposium, Methodological Issues in Measuring Population Health, Statistics Canada International Symposium Series, 2006.

[16] B. Lauzon, J.L. Volatier, A. Martin, A Monte Carlo simulation to validate the EAR cut-point method for assessing the prevalence of nutrient inadequacy at the population level, Public. Health. Nutrition. 7 (2004) 893-900.

Journal of Life Sciences 5 (2011) 575-579

Computer Supported Sensory Profiling Analysis of Three *Agaricus* Cultivars

András Geösel[1], László Sipos[2], Brian McGuinness[3], Júlia Győrfi[1] and Zoltán Kókai[2]

1. Department of Vegetable and Mushroom Growing, Corvinus University of Budapest, Budapest H-1118, Hungary

2. Sensory Laboratory, Corvinus University of Budapest, Budapest H-1118, Hungary

3. Teagasc Kinsealy Research and Development Centre, Dublin 13, Ireland

Received: May 04, 2010 / Accepted: July 14, 2010 / Published: July 30, 2011.

Abstract: The sensory profile analysis is a commonly used method in the evaluation of directly consumed horticultural products (fruits, vegetables, grapes etc.). The results of this type of analysis give an opportunity to distinguish the evaluated products from each other from a consumption point of view. In this paper, three *Agaricus* species ("white button mushroom", "cream type" and "almond portobello"), were characterized by sensory profile analysis. The sensory attributes can have an influence on consumer product preference therefore it is essential to describe products, for example mushrooms. This is the first report which focus on describing the full sensory profile of these fresh mushrooms.

Key words: *Agaricus blazei*, *Agaricus subrufescens*, *Agaricus bisporus*, mushroom, profile analysis.

1. Introduction

Approximately 14,000 described species of the 1.5 million fungi estimated in the world produce fruiting bodies that are large enough to be considered as mushrooms. In 2005, the entire global mushroom market was valued at over $45 billion. The industry itself can be divided into three main sectors: (1) Edible Mushrooms, (2) Medicinal Mushroom products, and (3) Wild Mushrooms [1]. Because of the presence of toxic and carcinogenic elements in wild mushrooms [2] that are likely to have a negative effect on human health, it is generally recommended to consume only mushroom products that have been safety and quality assured.

Around one third of total global mushroom production is dedicated to the production of *Agaricus bisporus* (and its hybrid) species. In some regions (usually in Western-Europe) the white button mushroom is preferred, while in other regions (Southern-Europe) the cream type (portobello or chestnut brown) is more preferred. The global production of *Agaricus* species is over two billion tons/year [3]. Previous field biological, biochemical and horticultural research has focused on the valuable and cultivated *Agaricus* species, *Agaricus blazei* (MURRILL) [4]. *A. blazei* currently has several taxonomic identities and is also known as *A. brasiliensis* and *A. subrufescens* [5, 6]. In this paper, we have opted to use Kerrigan's classification of *A. subrufescens*.

Previous research has shown the chemical composition of mushrooms but no research publication could be found that examined the complex sensory distinctions (texture, taste, flavour, and colour) of mushrooms from a consumption point of view [7]. The almond-like taste and odour of *A. subrufescens* has been mentioned previously without any empirical scientific analysis, which may have an influence on its consumption [8]. Based on chemical analysis, the taste of the mushroom depends upon the strains and

Corresponding author: Zoltán Kókai, Ph.D., research fields: sensory analysis, sensory profile, statistics. E-mail: zoltan.kokai@uni-corvinus.hu.

cultivation media [9]. Therefore in our research the very same media were used to cultivate *A. subrufescens* as for the other *Agaricus* cultivars. We consider it important to conduct this research in the hope of facilitating an improved acceptance of the three most important *Agaricus* species on the global market, by helping mushroom producers and related food and pharmaceutical industries further understand and improve their mushroom processing strategies.

The subjective element of sensory quality cannot be totally eliminated due to varying consumer tastes. However, several testing techniques may be applied to greatly improve the reliability of sensory data [10, 11]. Tests take place in a specially designed sensory laboratory with the test structure tending to be much more detailed than that used in consumer tests [12]. The objective of such laboratory evaluations is the detailed sensory description of the samples.

The sensory profile analysis method was chosen from many reliable, descriptive methods that are designed to take all of the relevant human senses into account. The method we have chosen can be used to define a production standard and to compare a product with those of similar type already on the market. The sensory profile analysis is one of the most complex food tests with the main advantage being the full description of a food product by rating its characteristics and their relative intensities on a numerical scale.

2. Material and Methods

2.1 Mushroom Samples

The *A. subrufescens* fruit bodies we examined (strain '1105') were cultivated and harvested at Corvinus University Budapest (CUB) in the Department of Vegetable and Mushroom Growing. For the cultivation of *A. subrufescens* commercial Phase II mushroom compost was used as a substrate. The conditions of cultivation (e.g. spawn-run, casing, case-run, RH%, air and compost temperature etc.) were the same as in case of the cultivation of *Agaricus bisporus*, except higher temperature during pinning [13]. White button mushrooms (*Agaricus bisporus* Sylvan 'A15', or white button champignon) and cream type ('Le Lion C9' or brown champignon) were bought from a local market. EUREPGAP and IFS standardization guarantee the traceability of the compost and the mushroom product from the composter and the farmer all the way to the market. For the experiment the very same Phase II compost were purchased from a Hungarian composter whose substrate were used for the cultivation of the white button and cream type mushrooms. Due to the following international cultivation technology standards of white and brown mushrooms, any influence on taste results related more to the species/cultivars than technology applied [14].

For the tests, the trained product assessors (who have practical experience in using the methods and in describing products, but haven't any specific information about the mushroom samples to be tested) got one full, fresh mushroom from all cultivars and also a longitudinal section from each cultivar.

2.2 Sensory Analysis

The international standard requires 8-16 people for product assessment [12]; using this standard as a guide, 14 people were selected in the trained sensory panel. The experiment was held in the sensory laboratory facilities at CUB, which fully meet all required ISO11035:1994 standards. In the first round, the product attributes were evaluated and noted by tasters. After this, descriptors were discussed and references were determined within the group, followed by a new tasting. In the second tasting, the assessors voted between 0-100 points for two descriptor attributes [15]. Every tasting was repeated three times. Statistical significance was determined by t-test depending on the equality of variances (T-Test) [16].

3. Results and Discussion

In total, 19 descriptive phrases were used in the experiments based on a consensus group decision. The

descriptors (Table 1) are the main results of this paper, because no complex phrases were found previously in references used for mushrooms. The taste and odour were characterised for both caps and stalks. Only the main attributes are demonstrated which have an influence on mushroom consumption and the industrial food market.

3.1 Colour and Structure

Cap colour showed significant differences between white champignon, cream type and *A. subrufescens*. The white champignon variety has the brightest ($P < 0.01$), while *A. subrufescens* has the darkest colour ($P < 0.05$). *A. subrufescens* was characterized as having the most blotchy cap ($P < 0.01$) while other *Agaricus* species had uniform cap colour. The thickness of caps was also scored: the cream type and white button mushroom were statistically similar ($P < 0.01$) while *A. subrufescens* had a lower cap thickness value. Stalk colour was described by assessors and results show a strong correlation between stalk texture and its tendency to discolour. Due to sample processing (washing, slicing) the white button mushroom achieved the lowest score (darkest colour) in its stalk evaluation, while *A. subrufescens* was the brightest ($P < 0.01$). Another finding which may be important from the food industry's perspective was the fragility of the mushroom fruit bodies. Based on data, *A. subrufescens* was the most fragile and cream type was the most "firm" ($P < 0.01$) while the fragility value for the white button mushroom scored between these two. Around 5-15% losses could be calculated by processing mushrooms therefore it is important to measure fragility of mushrooms [17].

3.2 Odour

The white button mushroom achieved the highest odour scores, while *A. subrufescens* had a low odour value. There were no odour differences between white button and cream type mushrooms ($P < 0.01$). The low odour intensity of *A. subrufescens* could be an advantage in the food industry, because the characteristic

Table 1 The main descriptors and means of achieved scores by mushrooms (scale: 0-100).

Attribution	Value (0)	Value (100)	White button mushroom (reference)	Cream type	*A. subrufescens*
Cap colour	dark	bright	90.0	32.78	25.71
Cap blotchiness	no blotches	blotches	0	29.21	81.0
Cap thickness	thin	thick	50.0	55.86	21.24
Cap format	flat	round	40.0	44.79	70.07
Stalk colour	dark	bright	40.0	55.36	81.86
Stalk length	short	long	20.0	19.36	85.93
Stalk thickness	thin	thick	50.0	65.29	16.93
Gills colour	dark brown	white	15.0	33.14	80.57
Fragility	firm	fragile	60.0	35.64	73.5
Hardness	soft	hard	40.0	74.29	28.72
Sliminess	dry	glutinous	70.0	48.43	53.43
Succulence	dry	juicy	90.0	42.86	42.86
Mushroom-like odour	soft	high	65.0	58.07	40.71
"Soil" odour	soft	high	25.0	33.79	61.5
"Fresh" odour	unfresh	fresh	15.0	64.36	53.29
Intesity of "mushroom-like" taste	soft	high	60.0	66.29	45.21
Intesity of "sweet" taste	soft	high	0	40.71	59.71
Intesity of "fresh" taste	unfresh	fresh	20.0	67.21	48.36
Aftertaste	no	strong	40.0	51.79	60.5
Other taste	no	strong	0	37.93	72.79

mushroom-odour may be seen as a negative trait by some consumers. The test group also found that the "soil-like" odour in the white button mushroom was low, while *A. subrufescens* had a significantly higher value. Usually, the "fresh" odour is related to storage conditions and is not considered to be a host-specific attribute. Therefore, the detailed analysis is not included. The assessors described their impressions about "other odours" but only 57% of the assessors mentioned the almond-like odour that can be found in previous studies [8].

3.3 Taste

No significant differences were found in the intensity of "mushroom-like" taste between white button and cream type mushrooms. The taste of *A. subrufescens* was clearly distinct from cream type and white button mushroom varieties. From the food industry's perspective this could be either an advantage or a disadvantage during processing of *A. subrufescens* products according to the market requirements. Mushroom sweetness could be an important factor in consumer product preference and may have effect in processing [17]. All of three examined mushroom species were distinct from each other ($P < 0.01$) with the sweetest taste observed in fresh *A. subrufescens*. Cream type mushrooms and white button mushroom were not as sweet as *A. subrufescens*.

The aftertaste can be critical to consumer food preferences. *A. subrufescens* had a strong distinctive aftertaste which many consumers may dislike. Assessors described the complex aftertaste of *A. subrufescens* as having the flavour of Daffodils or Violets. The other mushrooms tested had a significantly lower aftertaste value ($P < 0.01$).

The full sensory profiles of the fresh mushrooms assessed are present in Fig. 1. The chart demonstrates clear differences between the mushroom varieties tested. The spider chart underlines the fact that the fresh mushrooms assessed have very distinct sensory profiles. A full sensory profile assessment has never been completed for these varieties of mushroom and it may help to mushroom producers, mushroom breeders and for the mushroom industry in general.

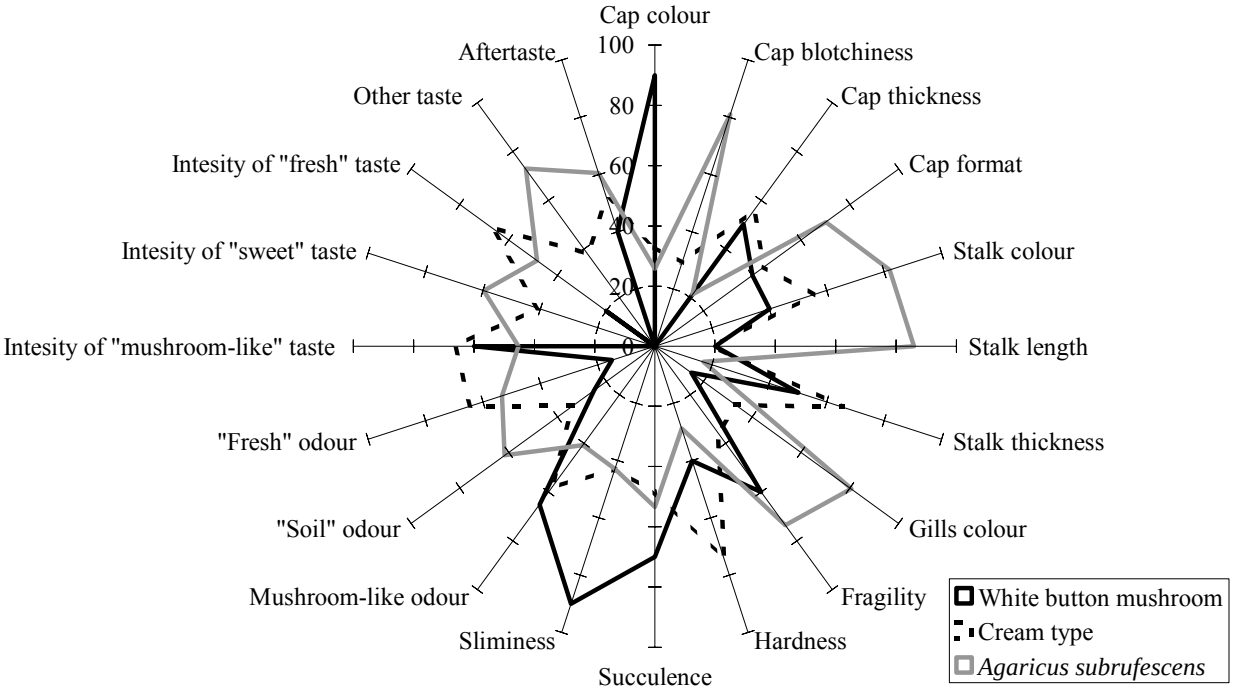

Fig. 1 The full sensory profile of the tested fresh *Agaricus subrufescens*, *Agaricus bisporus* white button ('A15') and chestnut brown ('Le Lion C9') mushrooms.
The central represents 0 values for each attribution, while 100 at the end of coordinate system. (Each value is expressed as mean).

References

[1] S.T. Chang, The world mushroom industry: Trends and technological development, International Journal of Medicinal Mushrooms (4) (2006) 297-314.

[2] O.S. Falade, O.O. Adepoju, O. Owoyomi, R.S. Adewusi, Chemical composition and toxic trace element composition of some Nigerian edible wild mushrooms, International Journal of Food Science and Technology (1) (2008) 24-29.

[3] FAOSTAT, Food and Agricultural Commodities Production, 2007, available online at: http://faostat.fao.org/site/339/default.aspx. (accessed on Jan. 27, 2010)

[4] S.Y. Tsai, H.L. Tsai, J.L. Ma, Non-volatile taste components of *Agaricus blazei, Agrocybe cylindracea* and *Boletus edulis*, Food Chemistry 107 (2008) 977-983.

[5] F. Mourão, G.A. Linde, V. Messa, P.L. da Cunha Júnior, A.V. da Silva, A.F. da Eira, et al., Antineoplasic activity of *Agaricus brasiliensis* basidiocarps on different maturation phases, Brazilian Journal of Microbiology (40) (2009) 901-905.

[6] R.W. Kerrigan, *Agaricus subrufescens*, a cultivated edible and medicinal mushroom, and its synonyms, Mycologia (1) (2005) 12-24.

[7] P.K. Ouzouni, W.D. Koller, A.V. Badeka, K.A. Riganakos, Volatile compounds from the fruiting bodies of three *Hygrophorus mushroom* species from Northern Greece, International Journal of Food Science and Technology (4) (2009) 854-859.

[8] P. Stamets, Growing Gourmet and Medicinal Mushrooms, Ten Speed Press, Berkley, Toronto, USA, 2000, pp. 208-216.

[9] A. Harada, S. Gisusi, S. Yoneyama, M. Aoyama, Effects of strain and cultivation medium on the chemical composition of the taste components in fruit-body of *Hypsizygus marmoreus*, Food Chemistry (84) (2004) 265-270.

[10] H. Stone, J.L. Sidel, Sensory Evaluation Practices, Elsevier Academic Press, California, USA, 2004, pp. 201-247.

[11] Z. Kókai, J. Heszberger, K. Kollár-Hunek, G. Kollár, A new VBA software as a tool of food sensory tests, Hungarian Journal of Industrial Chemistry (30) (2002) 239.

[12] ISO 11035:1994, Sensory analysis - Identification and selection of descriptors for establishing a sensory profile by a multidimensional approach.

[13] A. Geösel, J. Győrfi, Growing experiments with a medicinal mushroom *Agaricus blazei* (Murrill), International Journal of Horticultural Science (4) (2008) 45-49.

[14] J.H. Yang, H.S. Lin, J.L. Mau, Non-volatile taste components of several commercial mushrooms, Food Chemistry (4) (2001) 465-471.

[15] Z. Kókai, J. Heszberger, K. Kollár, E. Papp, ProfiSens - A profile analysis supporting software in food industry, related research and education, Periodica Polytechnica Chem. Eng. (1) (2004) 31-40.

[16] R.G.D. Steel, J.H. Torrie, D. Dickey, Principles and Procedures of Statistics: A Biometrical Approach, 3rd ed., The McGraw-Hill Company Inc., New York, General Papers, 1996.

[17] G.Y. Beke, Hűtőipari kézikönyv 1, Mezőgazda Kiadó, (Handbook of Cold Storage 1, Agricultural Press,) Budapest, Hungary, 2002, pp. 216-240. (in Hungarian)

Journal of Life Sciences (ISSN 1934-7391)

Call for Paper

Descriptions

The *Journal of Life Sciences*, a monthly professional academic journal, is striving to provide the best platform for researchers and scholars worldwide to exchange their latest findings and results.

Instructions for Authors

1. The manuscript should be original, and has not been published previously. Please don't submit material that is currently being considered by another journal.
2. Manuscripts may be 3,000-8,000 words or longer if approved by the editor, including abstract, text, tables, footnotes, appendixes, and references. The title should not be exceeding 15 words, and abstract should not be exceeding 200 words. 3-8 keywords or key phrases are required.
3. Authors should submit manuscripts online at http://www.davidpublishing.com or send them to Managing Editor by e-mail.
4. A manuscript file in MS Word (or some other word processing format) is required. Although there are no file size limitations, note that large files will take longer to upload and download depending on the Internet connection. Please try to keep the maximum .doc file size less than 5 MB.
5. Figures are in grayscale status in printed journals which can be colored in e-journal. So if there are color figures in your paper, you can use different lines or symbols to instead of colors if necessary.
6. The submitted paper should be in our format, you can download a file which shows the basic format and the instructions for authors from the followed website: http://www.davidpublishing.com/down_Life.php.

Peer review Policy

Journal of Life Sciences is a refereed journal. All research articles in this journal undergo rigorous peer review, based on initial editor screening and anonymised refereeing by at least two anonymous referees.

Editorial Procedures

All papers considered appropriate for this journal are reviewed anonymously by at least two outside reviewers. The review process usually takes two to three weeks. Papers are accepted for publication subject to no substantive, stylistic editing. The Editor reserves the right to make any necessary changes in the papers, or request the author to do so, or reject the paper submitted. A copy of the edited paper along with the first proofs will be sent to the author for proofreading. They should be corrected and returned to the Editor within seven days. Once the final version of the paper has been accepted, authors are requested not to make further changes to the text.

Submitting of Manuscripts

All manuscripts submitted will be considered for publication. Please visit our website at www.davidpublishing.com for our automatic paper submission systems or as an email attachment to life-sciences@davidpublishing.com and life-sciences@hotmail.com.
Welcome to visit our website at http://www.davidpublishing.com.